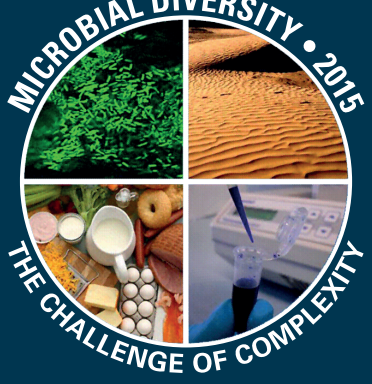
MICROBIAL DIVERSITY 2015

THE CHALLENGE OF COMPLEXITY



MD **2015**

Microbial Diversity 2015

THE CHALLENGE OF COMPLEXITY

MD2015

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Dear Attendees at the Microbial Diversity 2015 Conference,

it is my pleasure to welcome you in Perugia, a city of pre-Etruscan origin, inhabited by the Etruscans since the VIII century B.C.. This almost three millennia old city offers a wealth of natural landscapes and monuments, including the Etruscan Arch and the Etruscan well, the S. Peter Basilica funded in 1066, the Medieval downtown with the Cathedral, the monumental Fountain Major and the castle shaped Palazzo dei Priori, the home of the Perugia Municipality. This historical building, erected between 1293 and 1443, includes the superbly painted Sala dei Notari, where the Conference will take place.

On behalf of the Italian Society of Food, Agricultural and Environmental Microbiology (SIMTREA), I am glad to welcome you at the III edition of the Conference entitled: "The Challenge of Complexity", following the first two highly successful editions in Milan (MD2011) and in Turin (MD2013), which paved the way to launch the Microbial Diversity Conferences as International meetings.

The meeting aims at gathering scientists in the fields of agricultural, environmental and food microbiology, in order to promote discussion and exchange of information and experiences regarding the complexity intrinsic in microbial biodiversity. Biology has long been defined the science of complexity for the wealth of relationships among organisms and between the biotic and abiotic components of the environment. In this frame, the increasing awareness of the complexity involved in Microbial Diversity is fueled by unprecedented microbiological studies, innovative technologies in molecular biology and increasing data interpretation efficiency with bioinformatics tools. This perspective poses exciting challenges in terms of methods and substance, which constitute the two scientific standpoints of the MD2015 Conference.

Over 250 scientists, from 36 countries all over the world, will give life to this scientific event with 3 keynote lectures, 6 invited and 30 selected speeches, 18 young researchers short presentations and more than 150 posters. For the first time, the posters will be included in a Poster Book included among the participants' materials and available from the meeting website (www.md2015.org). This initiative aims at encouraging the consultation and the discussion of the meeting presentation in the months to come even beyond the limit of the MD 2015 participation. Two specialized one-day schools have been introduced at the end of the meeting in key arguments for the study of microbial Biodiversity. We hope that in these days all participants will be sustained by the awareness that only an open and transversal discussion can help microbiologists to ferry even more the microbial biodiversity studies from description to deeper understanding.

This book collects the abstracts presented to MD2015. The topics presented span from taxonomy to the metabolic complexity in agricultural and natural environment, from the complexity in food ecosystems to the genetics of complex microbial communities, from the functional interdependence of microbial associations to the challenge of exploiting the microbial diversity in industrial environments, from bioinformatics to advanced molecular biology.

A warm thanks to our patrons, particularly the administration of the Perugia municipality for granting the Sala dei Notari and the Sala della Vaccara for this Conference and to our scientific partners, the Federation of European Microbiological Societies (FEMS) and the International Committee on Food Safety and Hygene (ICFMH). Our gratitude is also to the other main sponsors, BioaWare, Bruker and the Institut Français, to the local sponsors, for the typical food products, and to the publishers Garaland and Zanichelli –CEA that providing prizes for the best posters.

Finally yet importantly, Bruker Optics and Daltonics gave support and organized one of the two schools proposed to participants as free side events after the meeting. BioAware granted the other school and developed an ad hoc web site with very friendly conditions that SIMTREA will use for the years to come.

I wish you all a fruitful and successful conference, in scientific and human terms, and a pleasant stay in Perugia!

Gianluigi Cardinali

Chair of the MD2015 Organizing Committee

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MD2015 SCIENTIFIC PROGRAMME

TUESDAY, OCTOBER 27, 2015

10:00 – 14:00 Registration (Sala della Vaccara)
14:00 – 14:15 Welcome Introduction (Sala dei Notari)
Mayor of Perugia, *Andrea Romizi*
Rector of the University of Perugia, *Franco Moriconi*
Director of the Department of Pharmaceutical Sciences, *Benedetto Natalini*
President of CEMIN Centre of Excellence, Member of the Board of Governors *Fausto Elisei*
President of the SIMTREA, *Erasmo Neviani*

14:15 – 15:00 KEYNOTE LECTURE
Chair: Gianluigi Cardinali, (Italy)

CLETUS P. KURTZMAN, USDA-ARS (USA)
Taxonomic concepts and practice with complex microbial communities

SESSION I
METABOLIC COMPLEXITY OF AGRICULTURAL AND NATURAL ENVIRONMENTS

Chairs: **Jan Dirk Van Elsas** (Netherlands)**, Daniele Daffonchio** (Italy)

15:00 – 15:30 PLENARY LECTURE: JAN DIRK VAN ELSAS,
University of Groningen (Netherlands)
The soil/plant-fungal/bacterial network – mechanisms of interaction in the soil

15:30 – 16:45 SELECTED LECTURES:
15:30 – 15:45 **Federico Rossi** (Italy)
Biological soil crusts: a microenvironment affected by the microbial secreted exopolysaccharidic matrix
15:45 – 16:00 **Pelin Yilmaz** (Germany)
Expanding the world of marine bacterial and archaeal clades
16:00 – 16:15 **Francesca Mapelli** (Italy)
Spatial pattern of soil bacterial diversity in a mixed and uneven polluted site, and assessment of rhizoremediation potential
16:15 – 16:30 **Cene Gostinčar** (Slovenia)
Specialists in everything: the black yeasts Aureobasidium *spp.*
16:30 – 16:45 **Andrea Squartini** (Italy)
Sequencing directly the ribosomal 16s rRNA pool from bacterial communities: a novel PCR-independent approach to microbial diversity analyses

16:45 – 17:15 COFFEE BREAK (Cathedral Cloister)

SPECIAL SESSION I: *Young Researchers Presentations*

Chairs: **Cletus P. Kurtzman** (USA)**, Pier Sandro Cocconcelli** (Italy)

17:15 – 18:45 SELECTED LECTURES:

17:15 – 17:25 **Rahi Praveen** (India)
Matrix-assisted laser desorption/ionization time-of-flight mass-spectrometry (MALDI-TOF MS) based identifications of microorganisms of non-clinical origin

17:25 – 17:35 **Luca Roscini** (Italy)
Bringing the ITS barcode in the NGS framework

17:35 – 17:45 **Sarath Vega Gutierrez** (USA)
Initial studies on the diversity of spalting fungi in the southern Amazon forest of Peru

17:45 – 17:55 **Ilario Ferrocino** (Italy)
Gut microbiota and metagenomic diversity of omnivore, vegetarian and vegan healthy subjects

17:55 – 18:05 **Sun Jeong Jeon** (Korea)
Mucor *sp. nov. from tangerine fruit and* Mucor *spp. from plant leaves in Korea*

18:05 – 18:15 **Eleonora Egidi** (Italy)
Temperate native grasslands with different fire histories differ in their fungal community composition

18:15 – 18:25 **Fatma Salem** (Egypt)
Anticancer metabolites of endobiotic fungus Trichothecium roseum *and its biochemical effects as a strong candidate for MCF-7 breast cancer cell-line*

18:25 – 18:35 **Giorgia Perpetuini** (Italy)
Link between Lactobacillus pentosus *adaptation to olive brine and its ability to form biofilms*

PARALLEL SESSIONS

18:40 – 19:30 **Technical Session of BRUKER OPTICS and DALTONICS** (Sala della Vaccara)

18:40 – 19:30 **Dissemination of YeSVitE project** (Sala dei Notari)

TYPICAL UMBRIAN APERITIVE 19:30 – 20:00 (Sala della Vaccara)

POSTER SESSION (Cathedral Cloister)

All posters are exhibited during the meeting at the 1st floor of the Cathedral Cloister
(one floor above the place were coffee breaks and lunches are served)

WEDNESDAY, OCTOBER 28, 2015

SESSION II

THE COMPLEXITY OF FOOD ECOSYSTEMS: PHYSIOLOGY OF SINGLE STRAINS IN PURE COLTURE VS. COMPLEX CONSORTIA

Chairs: **Sylvie Lortal** (France)**, Erasmo Neviani** (Italy)

08:30 – 09:00 PLENARY LECTURE: SYLVIE LORTAL, INRA - Rennes (France)
Wooden tools: complex reservoirs of microbial diversity for food fermentation

09:00 – 10:15 SELECTED LECTURES:

09:00 – 09:15 **Ileana Vigentini** (Italy)
Melatonin and tryptophan-derivatives in wine: the yeast contribution during alcoholic fermentation

09:15 – 09:30 **Stephane Chaillou** (France)
Ecological engineering of meat microbial ecosystems: factorial design of complex meat preservative cultures for spoilage reduction

09:30 – 09:45 **Daniela Bassi** (Italy)
Understanding the bacterial communities of hard cheese with blowing defect

09:45 – 10:00 **Eugenio Parente** (Italy)
FoodMicrobionet: a tool for the visualisation and analysis of the structure of bacterial food microbial communities

10:00 – 10:15 **Monica Agnolucci** (Italy)
Molecular and functional diversity of lactic acid bacteria and yeasts characterizing sourdough Tuscan bread

10:15 – 10:45 COFFEE BREAK (Cathedral Cloister)

10:45 – 11:30 KEYNOTE LECTURE
Chair: **Marco Gobbetti** (Italy)

RUDI VOGEL, Technische Universität München (Germany)
Microbial networks and metabolic fluxes in food fermentations

SESSION III

GENES AND FUNCTIONS IN COMPLEX MICROBIAL COMMUNITIES

Chairs: **Peter N. Golyshin** (UK)**, Giancarlo Ranalli** (Italy)

11:30 – 12:00 PLENARY LECTURE: PETER N. GOLYSHIN,
Bangor University (UK)
Mining metagenomes for novel enzymes

12:00 – 13:15 SELECTED LECTURES:

12:00 – 12:15 **Lucia Aquilanti** (Italy)
Occurrence of antibiotic resistance genes in feces and saliva of healthy omnivores, ovo-lacto vegetarians and vegans

12:15 – 12:30 **Cristophe Monnet** (France)
Metatranscriptome RNA-Seq analysis of cheese surface microbiota identifies physiological responses occurring during ripening

12:30 – 12:45 **Rosa Guarcello** (Italy)
Identification of amine-degrading non-starter lactic acid bacteria from Sicilian and Apulian typical/traditional cheeses and characterization of enzymatic activities

12:45 – 13:00 **Alessia Levante** (Italy)
Development of a method employing a metabolic gene to monitor non-starter lactic acid bacteria strains and their evolution during ripening of cheese

13:00 – 13:15 **Lorenzo Brusetti** (Italy)
Bacterial diversity and functionality in mineral soils of early successional stages in a glacier Alpine moraine

13:15 – 14:15 LUNCH (Cathedral Cloister)

SESSION IV

NEW TOOLS AND STRATEGIES TO UNRAVEL THE COMPLEXITY OF MICROBIAL DIVERSITY

Chairs: **Vincent Robert** (Netherlands)**, Gianluigi Cardinali** (Italy)

14:30 – 15:00 PLENARY LECTURE: VINCENT ROBERT CBS, Utrecht (Netherlands)
Biodiversity bioinformatics, challenges and opportunities

15:00 – 16:15 SELECTED LECTURES:

15:00 – 15:15 **Joseph Mellor** (USA)
Pooled library preparation for deep sequencing of diverse microbial samples

15:15 – 15:30 **Anna Greppi** (Italy)
16S rRNA-based HTS approach to monitor the microbiota development during storage of beefburgers in active packaging

15:30 – 15:45 **Volha Shapaval** (Norway)
Fourier transform infrared spectroscopy for exploring microbial phenotypic diversity

15:45 – 16:00 **Pasquale Filannino** (Italy)
A comprehensive snapshot of plant niche environments sensing and adaptive regulation models for Lactobacillus plantarum *C2 through whole transcriptome and phenotypic microarray*

16:00 – 16:15 **Koenraad Van Hoorde** (Belgium)
MALDI-TOF MS of microbial mixtures: impressions of its usability for culture-independent analyses of microbial diversity in food ecosystems

16:15 – 16:45 COFFEE BREAK (Cathedral Cloister)

SPECIAL SESSION II: *Young Researchers Presentations*

Chairs: **Rudi Vogel** (Germany), **Jan Dirk Van Elsas** (Netherlands)

17:00 – 18:30 SELECTED LECTURES:

17:00 – 17:10 **Canan Canal** (Turkey)
Characterization of yeast flora of "hurma" olives using molecular methods and mid-IR spectroscopy

17:10 – 17:20 **Claudia Colabella** (Italy)
The use of FT-IR spectroscopy and ITS sequencing as useful tools for strain dereplication in medical environment

17:20 – 17:30 **Amélie Rouger** (France)
Bacterial communities' dynamics and interactions during poultry meat storage to improve food quality and safety

17:30 – 17:40 **Alberoni Daniele** (Italy)
Administration of lactobacilli and bifidobacteria on Apis mellifera *L. beehives to increase health of the bee super-organism*

17:40 – 17:50 **Hye Won Lee** (Korea)
A new zygomycete species and two new recorded fungi from Dokdo, Korea

17:50 – 18:00 **Irene Aloisio** (Italy)
Influence of intrapartum antibiotic prophylaxis against group B Streptococcus *on the early newborn gastrointestinal composition*

18:00 – 18:10 **Nassima Illikoud** (France)
Genetic diversity of Brochothrix thermosphacta *and food spoilage*

18:10 – 18:20 **Paola Torres-Andrade** (USA)
Preliminary assessment to the fungal colonization on Douglas-fir, western red cedar and red alder in ground contact exposure

18:20 – 18:30 **Alessia Bani** (Italy)
The leaf microbial community degradation process and endophytic bacteria

POSTER SESSION (Cathedral Cloister)

All posters are exhibited during the meeting at the 1st floor of the Cathedral Cloister
(one floor above the place were coffee breaks and lunches are served)

20:00 GALA DINNER (Etruscan Chocohotel)

(Shuttle buses to the Gala dinner place will depart from Piazza Italia at 19.40)

THURSDAY, OCTOBER 29, 2015

SESSION V

METAORGANISMS: FUNCTIONAL INTERDEPENDENCY OF MICROBIAL ASSOCIATIONS WITH PLANTS, ANIMALS AND HUMANS

Chairs: **Benoît Foligné** (France)**, Luca Cocolin** (Italy)

08:30 – 09:00 PLENARY LECTURE: BENOÎT FOLIGNÉ,
Institute Pasteur - Lille (France)
Appraisal of microbial diversity for health: of mice, cats and men …

09:00 – 10:15 SELECTED LECTURES:
09:00 – 09:15 **Maddalena Del Gallo** (Italy)
Bacterial biodiversity on Solanum tuberosum *inoculated by endophytes*
09:15 – 09:30 **Fabio Minervini** (Italy)
Lactic acid bacteria are endophytic components of durum wheat plant following the whole life cycle from soil to flour
09:30 – 09:45 **Marco Fusi** (Italy)
Gill-bacteria association in dual-breathing animals living in mangrove ecosystem
09:45 – 10:00 **Danilo Ercolini** (Italy)
Microbiota and metabolome signatures in plant-based compared to omnivore diets
10:00 – 10:15 **Ramona Marasco** (Italy)
Bacterial diversity and functional services within the rhizosheath of a desert plant

10:15 – 10:45 COFFEE BREAK (Cathedral Cloister)

10:45 – 11:30 KEYNOTE LECTURE

Chair: Fabrizio Fatichenti (Italy)

BERNARD DUJON, Institut Pasteur (France)
Genetic bases of microbial diversity

SESSION VI

BRINGING THE MICROBIAL COMPLEXITY IN THE INDUSTRIAL ENVIRONMENT

Chairs: **Nikolas Kalogerakis** (Greece)**, Sergio Casella** (Italy)

11:30 – 12:00 PLENARY LECTURE: NIKOLAS KALOGERAKIS,
Technical University of Crete (GR)
Integrating microbiology and engineering for bioremediation

12:00 – 13:15 SELECTED LECTURES:

12:00 – 12:15 **Lorenzo Favaro** (Italy)
Exploring and FTIR-ing yeast diversity towards the development of superior strains for lignocellulosic ethanol

12:15 – 12:30 **Ramon Gonzalez** (Spain)
Diversity in the response of aerobic metabolism to environmental factors among different wine yeast species

12:30 – 12:45 **Noura Raddadi** (Italy)
Characterization of surface-active molecules produced by marine bacterial isolates

12:45 – 13:00 **Mathabatha Evodia Setati** (South Africa)
Metagenomic and ARISA profiling of the wine microbial consortium and its functional potential

13:00 – 13:15 **Fabrizio Cappa** (Italy)
Bio-hydrogen production of Clostridium acetobutylicum *DSM 792 and the pSOL1 deficient mutant MU56 in deproteinized cheese whey*

13:15 – 14:30 LUNCH (Cathedral Cloister)

14:30 – 15:00 FAREWELL – END OF THE CONFERENCE

Presentation Award winning posters
President of the MD2015 Organizing Committee
President of the SIMTREA
Announcement of the 4th International Conference on Microbial Diversity 2017
Closing remarks
End of the Conference and Departure

MD2015 SATELLITE EVENTS

a. SIMTREA meeting for steering board election

The meeting for the election of the 2016-2018 SIMTREA steering board will be held on Oct. 29th 2015 at 15.30 in the Sala dei Notari of the historical Palazzo dei Priori (P.zza IV Novembre - Perugia)
SIMTREA www.simtrea.org

b. YeSVitE project - Annual Meeting

The Second Annual Meeting of YeSVitE will take place on October 29th 2015 at 18.00 in the Sala della Vaccara of the historical Palazzo dei Priori (P.zza IV Novembre - Perugia)

c. BioAware School of Bioinformatics

Coordinator of the school: Dr. Vincent Robert (Bio-Aware)
maximum number of participants : 20
For info and registration contact Vincent Robert v.robert@bio-aware.com

d. Bruker School of FTIR spectroscopy applied to Microbiology

Coordinator of the school: Dr. Pierangelo Morini (Bruker - Italia)
maximum number of participants : 20, minimum number of participants: 4

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KEYNOTE LECTURES

TAXONOMIC CONCEPTS AND PRACTICE WITH COMPLEX MICROBIAL COMMUNITIES

KURTZMAN Cletus P. *

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Ecology is a study of relationships among organisms and habitats, and a key factor in understanding microbial ecology is detection and correct identification of the microorganisms present. For yeasts, as well as other microbial groups, the introduction of DNA-based methods provided a major turning point in the advancement of microbial ecology. Rapid, accurate identification of yeasts became possible with the introduction of a barcoding system based on nucleotide sequences of the D1/D2 domains of the nuclear large subunit ribosomal RNA gene (Kurtzman and Robnett, 1998; Fell et al., 2000) and the internal transcribed spacer (ITS), which is located between the small and large subunit rRNA genes (Scorzetti et al., 2002; Kurtzman and Robnett, 2003). Use of these and other gene sequences has resulted in a doubling of known yeast species in little over a decade and has allowed grouping of species into phylogenetically circumscribed clades. An example is the *Yarrowia* clade (Fig. 1), which consists of 13 species, but prior to DNA comparisons *Y. lipolytica* was the only known species of this clade.

What first became apparent from molecular comparisons was that the Ascomycota is comprised of three major lineages, the Saccharomycotina (typical budding yeasts such as *Saccharomyces*), the Pezizomycotina (filamentous molds such as *Aspergillus*) and the Taphrinomycotina, a basal group that includes *Schizosaccharomyces*, *Taphrina* and several other genera. Phylogenetic analysis of the Basidiomycota showed that yeasts occur in nearly all major lineages of this subphylum. Because our working definition of a yeast is a fungus of the Ascomycota or the Basidiomycota that divides by budding or fission and which produces a sexual state that is not enclosed in a fruiting body, it has become apparent that the yeast morphotype is found in many of the major lineages of the Mycota (Kurtzman et al., 2011).

Phylogenetic placement of yeasts is having a major effect on classification with many species being reassigned to other genera. In addition, the system of classification used for fungi, which was given in the *International Code of Botanical Nomenclature* (Vienna Code), specified that asexual species could not be classified in the same genus as species with a sexual state. DNA comparisons showed two things. Genera circumscribed on the morphology of sexual states were often polyphyletic and that asexual species were often closely related to sexually reproducing species. The new code of nomenclature, the

International Code of Nomenclature for algae, fungi, and plants (Melbourne Code) (McNeill et al., 2012), now permits inclusion of sexual and asexual species in the same genus as seen for *Yarrowia* (Fig. 1).

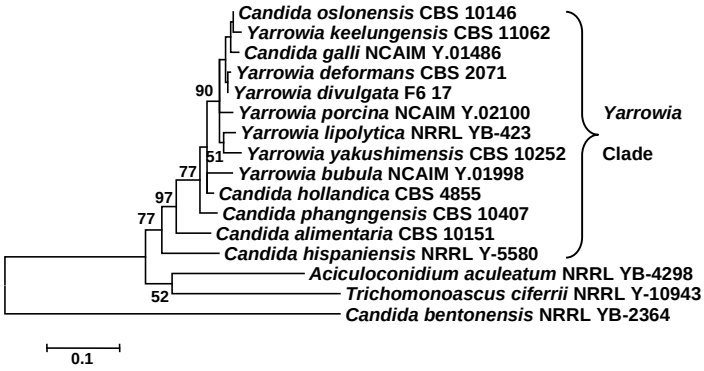

FIGURE 1. Phylogenetic placement of teleomorphic and anamorphic species of the *Yarrowia* clade determined from maximum likelihood analysis of D1/D2 LSU rRNA gene sequences. As a result of changes implemented in the Melbourne Code, the *Candida* species present in this clade can be reassigned to *Yarrowia* as new combinations.

With a system of classification based on phylogeny, we can now ask if members of a genus or larger clade have the same or similar ecological niches and whether a phylogeny-based system of classification can serve to predict which species may be of medical and biotechnological importance. For example, species of *Saccharomyces* are ethanol tolerant and widely used for fermentation of alcoholic beverages. The species may be isolated from ripe fruit, but also from tree bark, which is an unusual substrate for sugar tolerant species. With the widespread availability of DNA-based markers, this issue can be further examined to determine if tree bark is really a niche for *Saccharomyces* species or have they collected on bark following distribution by insects, rain or other vectors (Libkind et al., 2011)?

In contrast to *Saccharomyces*, species of *Pichia*, *Saturnispora* and *Kregervanrija* have much reduced ethanol tolerance and assimilate glucose, but often no other sugars (Table 1). However, organic acids such as citric and succinic acids serve as carbon sources and may reflect the success of these species in colonization of habitats such as tree fluxes and acidified food products where organic acids are common. Some yeasts are able to grow on methanol as a sole source of carbon. Methanol can be formed in metabolizing tree leaves and is also a degradation product of lignin. Yeasts that metabolize methanol are now assigned to the genera *Ogataea*, *Kuraishia* and *Komagataella*, but in pre-DNA systematics, the methanol utilizing species were scattered among many other genera, thus concealing their kinship and common metabolism. Similarly, species that can ferment D-xylose to ethanol are found only in the ascosporic genera *Pachysolen*, *Scheffersomyces* and *Spathaspora*. The latter two genera are somewhat closely related but, surprisingly, *Pachysolen* is a distant relative to these taxa (Kurtzman and Robnett, 2013), which suggests that the trait of D-xylose fermentation may have arisen twice in the ascomycete yeasts.

Genera	Characteristics
Blastobotrys (teleomorph = *Trichomonascus*)	Growth on adenine, glycine, uric acid, n-hexadecane, putrescine
Komagataella, Kuraishia, Ogataea	Growth on methanol
Pachysolen, Scheffersomyces, Spathaspora	Fermentation of D-xylose
Pichia	Growth on glucose (but none or few other sugars), ethanol, glycerol, organic acids
Saccharomyces	High ethanol tolerance
Torulaspora, Zygosaccharomyces	High sugar tolerance
Yarrowia	Production of lipases and proteases

TABLE 1. Unique metabolic characteristics of certain phylogenetically defined yeast genera.

Estimation of microbial populations is still frequently made from agar plate counts. Many microbial species require special growth conditions and plating on commonly used media at moderate temperatures with short incubation times can result in missing the presence of these taxa (Rosa and Péter, 2006). DNA probes designed for known species can be used to detect and quantify populations in particular habitats. Peptide nucleic acid (PNA) probes offer a means for detection and quantification of species in clinical samples, food products and other substrates through fluorescence *in situ* hybridization (FISH). PNA probes have a peptide backbone to which is attached nucleotides complementary to a species-specific target sequence, and a fluorescent label is added for detection by fluorescence microscopy (Stender et al., 2001). If probes are complementary to rRNA, the whole cell of the target species will fluoresce when visualized, which will also allow quantification by cell counts. A number of other methods have been developed for DNA-based detection of species, but these also require a known target species. For example, the technique of real time PCR is widely employed in food and beverage analyses and has been used for detection and quantification of spoilage yeasts in orange juice and in wine fermentations (e.g., Cocolin et al., 2001). Another useful method is denaturing gradient gel electrophoresis (DGGE), which has been used for species identification and quantification of yeast populations in foods and beverages. The technique is based on separation of DNA fragments that differ in nucleotide sequences (e.g., species-specific) through decreased electrophoretic mobility of partially melted double-stranded DNA amplicons in a polyacrylamide gel containing a linear gradient of DNA denaturants (i.e., a mixture of urea and formamide). A related technique is temperature gradient gel electrophoresis (TGGE), in which the gel gradient of DGGE is replaced by a temperature gradient (Muyzer and Smalla, 1998). Applications of DGGE have included identification and population dynamics of yeasts in sourdough bread, in coffee fermentations and on wine grapes (Prakitchaiwattana et al., 2004). Levels of detection are often around 10^3 cfu ml^{-1}, but10^2 cfu ml^{-1} have been reported, which compares favorably with standard plate count methods (Prakitchaiwattana et al., 2004).

While species-specific DNA sequences have been quite helpful for detecting known species, many species are still unknown. Genus-specific DNA probes can expand the reach of detection and have been developed for some economically important genera, such as done for *Zygosaccharomyces* (Hulin and Wheals, 2014). However, detection and quantification

of all species in a particular habitat is a daunting endeavor, but metagenomic methods have the potential to address this problem. For example, the *Tara* Oceans sampling project used metagenomics technology to determine the microbial composition of marine habitats worldwide (Sunagawa et al., 2015). Shotgun Illumina sequencing using 16S rRNA tags placed 93% of sequences in known phyla and suggested that perhaps 59% of the sequences were prokaryotic. Photosynthetic cyanobacteria, such as *Prochlorococcus* and *Synechococcus* represented about 17% of the 16S tags, but large-scale detection and quantification of individual species is yet to be done. Nonetheless, refinement of the Illumina approach should give a far better understanding of microbial ecology than is currently possible.

Key words Microbial ecology, yeasts, molecular identification, metagenomics

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MICROBIAL NETWORKS AND METABOLIC FLUXES IN FOOD FERMENTATIONS

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With the major exception of milk, most raw materials for food fermentations cannot be pasteurized prior to fermentation. Therefore, the composition and dynamics of microbiota developing from autochthonous contaminants or deliberately added starter strains in food fermentations is dictated by intrinsic (e.g. nutrients, pH, aw) and extrinsic (e.g. T, atmosphere) parameters. The metabolism and genetic background thereof has been studied for many members of such microbiota, partly in detail. However, most of these investigations were done with single strains of microorganisms in suspension, while most food fermentations harbor typical (stable) microbial consortia. Therein, lactic acid bacteria comprise the core group and have attracted most attention. In many food fermentations they are in mutual interactions with yeasts (e.g. sourdough, water and milk kefir, olive), acetic acid bacteria (water and milk kefir) or coagulase negative staphylococci (e.g. sausage fermentation).

When it comes to the selection of starter strains for food fermentation many strain specific special traits need to be considered. While some of these, like production of aroma components, homopolysaccharides or bacteriocins can be referred to defined genetic determinants, others like competitiveness over autothonous microbiota of a specific raw material or compatibility with other strains in a starter culture of mixed strains are suspected to be multifactorial. A better understanding of the interaction of natural microbial consortia in food fermentation enables deliberate combinations and exploitation of strains as synergistic pairs or even groups and thus foster food safety, process stability and reproducible quality of a product. In this communication, examples for nutrient competition, tolerance and mutual interaction are given focusing on the metabolism of consortial key members in sausage fermentation, water kefir and sourdough.

Sausage fermentation is determined by the interactive metabolism of coagulase negative staphylococci (CNS) and facultatively heterofermentative lactobacilli with clearly defined key roles (Rantsiou et al. 2005). CNS, namely *Staphylococcus (S.) carnosus* and *S. xylosus* reduce nitrate to nitrite, which subsequently impacts on color and flavor of the products and supports inhibition of unwanted bacteria in the acid environment resulting from the lactic fermentation of *Lactobacillus (L.) sakei* or *L. curvatus.* Consequently, current strain selection focuses on maximal nitrate reductase activity or pH reduction, which is the key factor in product safety. However, staphylococci compete for nutrients with the lactobacilli and suffer from low pH. This frequently results in very low numbers of staphylococci in the intermediate (acid) phase of sausage fermentation. While numbers may increase with

increasing pH in the later ripening phase, it is mostly unclear whether the staphylococci found at all are members of the starter culture or of the autothonous microbiota harboring an unknown risk potential, which is usually higher than that one of controlled starter strains (Marty et al. 2012a; Marty et al. 2012b). For the development of starter cultures typically containing CNS and lactobacilli current single strain evaluation is a limited tool to find effective strain combinations. We have therefore used co-cultivation and growth studies with CNS in media, which were prefermented by lactobacilli, to identify strains with compatible metabolic requirements. It was demonstrated that the growth and metabolism of staphylococci was not only determined by pH (Ravyts et al. 2010), but also by the availability of arginine, tyrosin or serine, and oxidative stress induced by the lactobacilli (unpublished data). Thus, effective strain combinations can be derived upon consideration of metabolic complementarity and tolerance to stressors originating from microbial metabolism and changing environmental conditions in the ripening process.

Water kefir is a mildly sour and alcoholic drink fermented by a stable microbial multispecies community. With its high sugar content and low amino acid concentration water kefir medium represents a demanding habitat. The microbial consortium forms granula consisting of insoluble exopolysaccharides, which ensure spatial proximity of the microbiota members (Figure 1).

FIGURE 1. Water kefir granula.

Water kefir granula host a microbial consortium of lactic and acetic acid bacteria, yeasts and bifidobacteria, some of which cannot yet be cultured under laboratory conditions (Gulitz et al. 2013). It is remarkable that different types of microbial consortia can form a “metabolic body” of similar overall capacities. This suggests the presence of metabolic networks and inter-genera metabolic fluxes, which can even be shared between different partners, with

differently shaped and distributed shares of the same overall metabolic turnover. Up to now, reconstitution of the granula with combinations of single isolates, have not been successful. This may be due to unknown metabolic interactions or environmental conditions needed for granula formation. We have studied the synergism between main representatives of water kefir yeasts and lactobacilli in a co-culture model system. Co-cultivation of yeasts and lactobacilli in water kefir medium significantly increased cell yield of all interaction partners, delineating the interaction of these water kefir isolates as mutualism. The support of *Zygotorulaspora (Z.) florentina* was due to the acidification of the medium by the lactobacilli, whereas the growth of lactobacilli was improved by the disposal of essential nutrients produced by yeasts. The trophic interaction between *Lactobacillus (Lb.) hordei* and yeasts is constituted by the release of amino acids and vitamin B_6 from yeasts, whereas *Lb. nagelii* is supported in growth by their production of amino acids. The interaction of *Z. florentina* and *Lb. nagelii* was further examined to reveal that co-cultivation elicited the release of arginine by the yeast, which was an essential nutrient for *Lb. nagelii (Stadie et al. 2013).*

In traditional ambient temperature, backslopped sourdoughs based on rye or wheat (often designated as Type I doughs) heterofermentative lactobacilli live in consortia with yeasts, with key organisms *L. sanfranciscensis* and *Candida (C.) humilis* (Meroth et al. 2003a; Meroth et al. 2003b). At first glance it is not at all clear why a heterofermentative bacterium should be able to take over in this habitat, because it should gain only 1 ATP from glucose and therefore run into a metabolic disadvantage as compared to any homofermenter. However, the role of glucose in sourdough and for this bacterium is different from that one in other environments or for most bacteria. This is because the main carbon source in sourdough is maltose resulting from hydrolysis of starch by cereal enzymes. Upon pmf driven import of maltose *L. sanfranciscensis* uses maltose phosphorylase to gain glucose and glucose-1-P (Ehrmann and Vogel 1998). While the latter is converted to glucose-6-P and enters glycolysis without ATP consumption, glucose is released in the abundance of maltose. This causes glucose repression in most competitors, preventing them from the use of maltose, while this repression is absent in *L. sanfranciscensis*. Furthermore, *L. sanfranciscensis* can produce acetate instead of ethanol in the heterofermentative pathway in the presence of an electron acceptor and gain additional ATP in the acetate kinase reaction (Knorr et al. 2001). In the lack of oxygen in sourdough fructose is used as electron acceptor and reduced to mannitol (Korakli and Vogel 2003). Here it comes to the role of *C. humilis* in this consortium. It hydrolyses gluco-fructans present in these flours providing fructose for reduction to mannitol by *L. sanfranciscensis* and using glucose thereof and released from *L. sanfransiscensis* upon maltose cleavage. While *Saccharomyces cerevisiae* might be able to fill this role of *Candida* in the sourdough system, most strains are not acetate tolerant and perceive acetate as a stressor eliciting sporulation. Still, other lactobacilli share these metabolic traits, so: why *L. sanfranciscensis*? Comparative transcriptomic analyses enable predictions of further responses to environmental conditions and further interactions between *L. sanfranciscensis* and *C. humilis*.

L. sanfranciscensis is a minimalistic specialist displaying the smallest *Lactobacillus* genome so far (Vogel et al. 2011). It is therefore a nice model to facilitate interpretation of gene expression changes in a limited genomic setting. Indeed a widely overlapping gene expression change can be observed when *L. sanfransiscensis* is grown under aerobic

conditions, or in the presence fructose or *C. humilis* as compared to gene expression upon anaerobic growth with respect to protein, carbohydrate and lipid metabolism. The whole *opp* operon (*oppABCDF*), the aminopeptidase *pepN*, aminotransferases A and amino acid permease were upregulated whereas genes for carbohydrate (β-phosphoglucomutase, gluconokinase, ribokinase) and lipid metabolism (enoyl-ACP reductase, acetyl-CoA carboxylase carboxyl transferase, S-malonyltransferase) were downregulated. Remarkably, the number of genes involved in stress responses was increased during co-cultivation with *C. humilis* compared to aerobic incubation. Besides upregulation of *Clp* protease and *uvrABC* as observed in the presence of oxygen, gene expressions of universal stress protein (*uspA*), molecular chaperone *GroES*, multidrug resistance protein and ABC transporter as well as thioredoxin and peptide methionine sulfoxide reductase (*msrA*) were increased. This suggests a role of *C. humilis* of adjusting the redox system/electron acceptor availability in sourdough (possibly by increased methionine oxidation) influencing the performance of *L. sanfransiscensis* to a response, which partially resembles peroxide and thiol stress of other bacteria. The visualization with *iPath2* depicted in Figure 2 revealed that besides the downregulation of lipid metabolism, the conversion and formation of the glutathione precursor peptide cysteine-glycine was upregulated.

The synthesis of glutathione is unfeasible due to the absence of enzymes like γ-glutamyl-cysteine synthetase (*gshA*), glutathione synthetase (*gshB*) or glutathione biosynthesis bifunctional fusion gene (*gshA/B/ gshF*) and proposes therefore a role of the mentioned dipeptide. However, as the reactive thiol group of cysteine is still present, a role in thiol redox homeostasis in *L. sanfranciscensis* cannot be excluded. Further work involving *tcyB* knock out mutants and comparative transcriptome sequencing corroborated the capacity of *L. sanfranciscensis* to cope with oxidative and specifically thiol stress (Stetina et al. 2014). While it is known that thiols play a decisive role in redox homeostasis and gluten network formation it is unexpected that a bacterium living in anaerobic sourdough needs such a trait to be competitive over others. The necessity for this capability may result from *C. humilis* sharing this environment. So, if *L. sanfranciscensis* wants to take advantage of *C. humilis´* metabolism it needs to be able to cope with it´s "oxidative lifestyle".

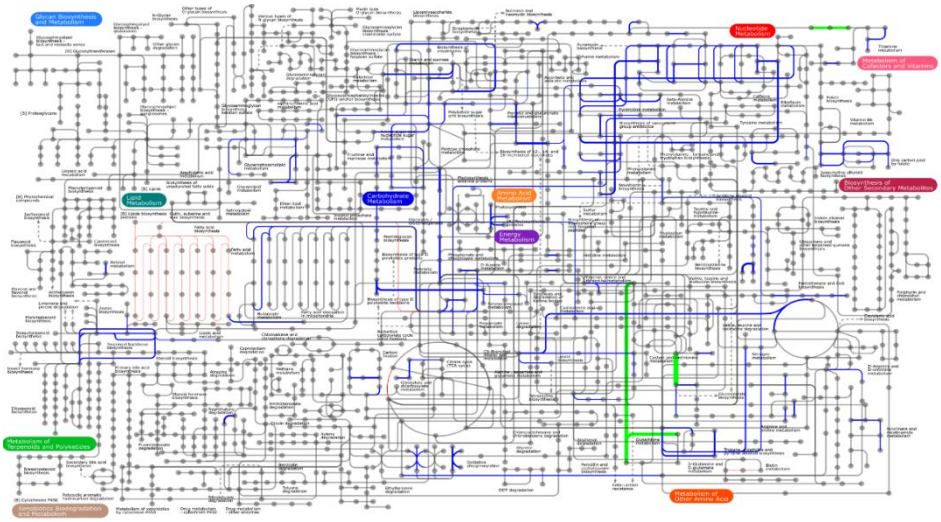

FIGURE 2. Metabolic pathway visualization of the change in gene expression of *L. sanfranciscensis* TMW 1.1304 incubated with *Candida humilis* TMW 3.191 created with *iPath2* (Letunic et al. 2008), website: http://pathways.embl.de/ Accessed: 03/28/2014. The colors of the pathways indicate the level of gene expression; red mark the underexpressed genes [expression ratios ≤ 0.5]; green are the overexpressed genes [expression ratios ≥ 2] and blue lines show no differential expressed genes [expression ratios > 0.5 < 2). The thickness of the lines represents the level of gene expression ratios, the thicker the colored lines, the higher the corresponding gene expression ratios and vice versa (colored, scaleable version in the pdf document).

Resuming the different glimpses obtained on interactive traits from three different fermentations it is a challenge to derive general principles. Also, we need to consider that these fermentations are solid state systems with spatially separated microenvironments and limited diffusion. Still, the presented data suggest that interaction goes hand in hand with competition and stress tolerance. While one partner may deliver essential or desirable nutrients those can only be used by a partner, which can cope with the stressful environment created thereby. Typical stresses are low pH and oxidative stress resulting from the formation of reactive oxygen species or thiol oxidation. Typical desirable nutrients founding mutualistic relations are amino acids, namely those involved in intracellular and microenvironmental pH maintenance. One central compound is arginine found as a key metabolite in many food fermentations, which offers alternative energy gain in addition to neutralization. Other metabolite groups including lipids or nucleic acids are less explored. In typical associations of lactobacilli and yeasts data suggest an important role for them in satisfying auxotrophies of partners. Omics technologies may be used to derive hypotheses on such metabolic networks (van Hijum et al. 2013) as demonstrated for *L. sanfransiscensis* in interaction with *C. humilis*.

Keywords Interaction, metabolism, food fermentation, transcriptomics

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GENETIC BASES OF YEAST DIVERSITY: INSTABILITY AND EVOLUTION OF GENOMES

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Yeasts offer us a very large collection of unicellular fungal species to contemplate the microbial diversity and the complexity of their interactions, in addition to their importance for basic sciences (Kurtzman et al., 2011). The baker's yeast *Saccharomyces cerevisiae*, the major player of domesticated alcoholic fermentations for centuries, entered the field of Genetics in the mid 20th century where it played a rapidly increasing role after having been instrumental at the origin of Microbiology and Enzymology roughly hundred years before (Barnett, 2003). In 1996, it marked the dawn of Genomics by being the first eukaryote entirely sequenced (Goffeau et al., 1996) and ever since has played a considerable role in its developments (Dujon, 2015a). Today, the genomes of nearly a hundred different yeast species have been sequenced and analyzed (Dujon, 2010, Dujon, 2015b), offering us an outstanding wealth of information to examine their evolutionary origin and to determine the genetic bases of their physiological diversity. A clear picture of the molecular mechanisms responsible for yeast genome evolution is now emerging from the comparative analyses complemented by experimental results. In this presentation, I will summarize the present status of yeast genomics and, using selected examples, will try to illustrate the multiple bases of their evolutionary changes.

The diversity of yeast genomes and their evolutionary history

Most yeast species presently studied at the genomic level belong to the *Saccharomycotina* subphylum of *Ascomycota* (Figure 1) and, although some sequences of *Basidiomycota* yeasts are now available as well, they remain in too small a number to be incorporated in a comprehensive view of yeast genome evolution given their large evolutionary dispersion. And even within the *Saccharomycotina* only (budding yeasts), the evolutionary distances between the distinct branches are much more important than could have been anticipated from their biological similarities (Souciet et al., 2000, Dujon et al., 2004), hence complicating the global reconstruction of their evolutionary history. The difficulty is further enhanced by the biased taxon sampling of available sequences because, beside the two favored experimental models of molecular geneticists *S. cerevisiae* and the fission yeast *Schizosaccharomyces pombe* (a member of the *Taphrinomycotina* subphylum separated from other yeasts very anciently), most yeast species selected for genomic studies have been were chosen for their metabolic properties or their pathogenic characters, and only few to examine basic evolutionary problems. The increasing efficiency of DNA sequencing methods should probably reduce this bias in the near future. The genome of S. pombe, and of the few other

Schizosaccharomyces species presently sequenced (Rhind et al. 2011), is so different from that of *S. cerevisiae* (in agreement with their enormous phylogenetic distance) that only limited conclusions can be drawn from their direct comparison. A number of their similarities (genome compactness, incomplete mitDNA) correspond to converging evolution rather than conservation from their common ancestry.

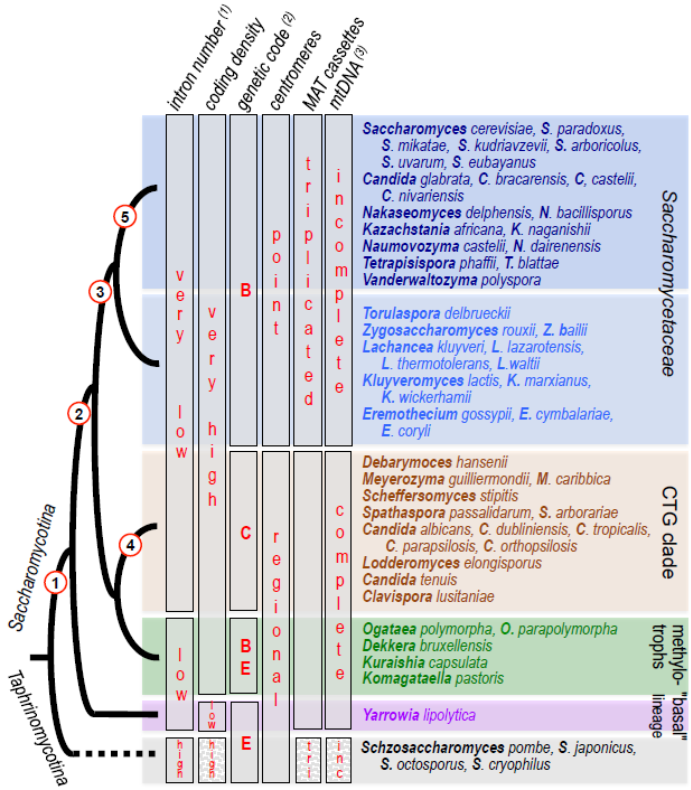

FIGURE 1. Overview of *Ascomycota* yeast genome evolution.

Major subgroups defined from proteome comparisons coincide with distinct genome architectures whose most characteristic features are listed in vertical boxes.(1): refers to spliceosomal introns only; (2): B and E refer to bacterial and eukaryotic tRNA decoding rules, respectively, C refers to yeast CUG code (Marck et al., 2006, Morales et al., 2013); (3): incomplete (inc) refers to absence of complex I genes. The similarities between *Schizosaccharomyces* and other yeasts (dotted boxes) correspond to converging evolution rather than conservation from a common ancestry. Major hypothetical evolutionary events deduced by parsimony are indicated by numbered circles on the consensus tree topology: 1: intron loss; 2: switch to bacterial decoding mode and other changes in non-coding RNAs; 3: point centromeres, triplication of mating cassettes and loss of mitochondrial genes; 4: genetic code alteration; 5: whole genome duplication. Major yeast species whose genomes served to define the subgroups are listed (published complete sequences only, hybrids and draft sequences ignored, Dujon, 2015b). Taxonomy according to (Kurtzman et al., 2011). All yeasts belong to the *Saccharomycotina* subphylum, except *Schizosaccharomyces* (Taphrinomycotina, dotted line). The *Pezizomycotina* branching (filamentous fungi) is not shown.

Perhaps the most significant difference is the fact that genes of fission yeasts harbor a normal number of spliceosomal introns for eukaryotes, as opposed to their very reduced number in other yeasts (Neuveglise et al., 2011). This suggests accelerated intron loss at the origin of the *Saccharomycotina* lineage, perhaps correlated with the loss of elements of the RNA interference machinery (the phenomenon seems to have disappeared except in one species where it may have been regained). On the opposite, fission yeast genomes show a near extinction of transposable elements which, albeit inconstantly found in budding yeasts (Bleykasten-Grosshans and Neuveglise, 2011), may have played a considerable role in generating their evolutionary diversity.

The budding yeasts sequenced so far define four major subgroups recognized by their distinct genome architectures as well as by global proteome comparisons (Figure 1). They are unequally supported by the number of species examined, and further studies of other yeasts may reveal additional ones (only few additional sequences with a sufficient degree of completion are available so far). The reduction of intron number seems to have continued in the two most extensively studied subgroups, the *Saccharomycetaceae* and the CTG clade, perhaps as a consequence of continued pressure for genome compactness. Except for the "basal" lineage, only represented so far by *Yarrowia lipolytica*, the coding density is very high (> 70 %) and the total protein-coding gene numbers in each genome fluctuate within relatively narrow limits (ca. 4500-6000 for haploid equivalents). Several evolutionary innovations are worth-mentioning such as the formation of point centromeres in the *Saccharomycetaceae* family (with two conserved short sequences specifically recognized by kinetochore proteins instead of long, variable sequence segments) or the specific alteration of the genetic code in the CTG clade (the CUG codon encodes Ser instead of Leu and very large numbers of codons have been changed accordingly in these genomes). Each subgroup alone covers a broad evolutionary range. Synteny is only exceptionally conserved between distinct members, sequence divergence is extremely high and the core proteome common to all species is relatively limited compared to their pan-proteome, indicating the importance of gene loss and gain. Nonetheless, each genome of a given subgroup harbors the characteristic features of its subgroup such that these signatures are sufficient to classify novel unknown yeasts solely from their genome sequences.

Sources of genome evolution

The broad evolutionary range covered by yeasts offers a unique opportunity to identify the mechanisms of genome instability at the source of evolution. Beside sequence divergence and loss of synteny, which are obviously not unique to yeasts, yeast species differ from one another by the presence/absence of some protein-coding gene families that represents the overall result of multiple mechanisms (Table 1). The frequent loss of genes observed in yeast genomes is consistent with the low frequency of essential genes found by systematic deletions in *S. cerevisiae* and with the preponderance of clonal reproduction versus sexual reproduction in most lineages. It primarily occurs by single gene deletions (as judged from comparisons between syntenic species, interspecies hybrids or inheritors from the whole-genome duplication in the *Saccharomycetaceae* family) but few pseudogenes are also found. If some losses only concern multiple gene families, others result in irreversible loss of function (e.g. galactose utilization) or may even confer novel adaptive functions by alteration of regulatory networks. Equivalent proportions of gene gains must, obviously, compensate

the losses over long evolutionary times, and it is now clear that this is achieved by the combination of distinct mechanisms. Next to the duplications that increase gene copy number without enlarging the repertoire of their products (at least initially), horizontal acquisition of single genes or of large chromosomal segments bearing several genes have been demonstrated in yeast genomes. Prone to subsequent duplications in the host genome after or during the transfer events, they often bear important innovative power for the recipient lineages (e.g. life under anaerobiosis, nitrate assimilation). Capture of genes or fragments from plasmids, transposable elements or viral sources has also been mentioned in yeast genomes and, in specific cases, has determined important novel function (e.g. mating type switching in *Kluyveromyces*). Similarly, the presence of mtDNA fragments (NUMTs) in yeast chromosomes is also almost universally observed and the transfer of mtDNA sequences to chromosomes has been obtained experimentally. This phenomenon has an intrinsic mutagenic potential by DNA insertion, but no evidence exists so far to my knowledge that NUMTs created novel functions. By contrast, the de novo creation of functional protein-coding genes by limited mutational alteration of ancestral non-coding sequences, for long time regarded as nearly impossible, has now been clearly demonstrated in *S. cerevisiae*. Although the number of such examples remains presently limited, the abundance of protogenes (i.e. ribosome-bound transcripts of non-coding regions) in the *S. cerevisiae* genome suggests a considerable evolutionary potential for this phenomenon. It may, indeed, explain at least in part the universal presence of orphan genes in every yeast genome.

Type of phenomenon	Mechanisms	Consequences
DNA sequence alteration	Nucleotide substitutions Indels	Silent or non-silent sequence divergence of gene products
Gene loss	Deletions Pseudogene formation	Frequently observed in yeasts possible loss of function or minor effects
Gene duplication	Repeat arrays Segmental duplications	Gene product repertoire unchanged but expansion of gene family (formation of paralogs) and increase of redundancy
Exon shuffling	Transposon-mediated retrogene formation	Ectopic duplication leading or not to gene fusion / truncation
Horizontal acquisition	Single genes Introgression of chromosomal segments	Acquisition of novel functions critical for the formation of new lineages
Inter-organellar transfer	Mitochondria: NUMTs Plasmid or virus: NUPAV	potentially mutagenic, probably mostly silent but innovative potential
De novo gene creation	Limited mutations from protogenes	probably mostly silent but important innovative potential
Whole-genome duplication	Autopolyploidy followed by gene loss	Probably critical for the formation of novel lineages but not frequently observed in yeasts.
Hybridization	Allopolyploidy followed by gene loss and/or loss of heterozygosity	Frequently observed in yeasts but uncertain role in their natural evolution
Chromosomal rearrangement	Transpositions Chromosome fusions	Loss of synteny and partial sexual isolation by reduction of meiotic fertility

TABLE 1. Sources of genome evolution identified in yeasts.

Finally, yeast genomes even challenge the classical notion of clear, tree-like phylogenies during evolution because interspecies hybrids, for long time suspected as evolutionary dead-ends, have now been found in several distinct lineages (Morales and Dujon, 2012). Originally suspected for the major brewing strains, *Saccharomyces pastorianus*, the phenomenon affects wine yeasts but also other genera of *Saccharomycetaceae* not directly involved in industrial fermentations such as *Zygosaccharomyces*. Hybrid genomes were also found in

several genera of the CTG clade (*Candida*, *Millerozyma*), the methylotroph subgroup (*Dekkera*) and even among *Basidiomycota* yeasts (*Cryptococcus*). Stable cellular fusion between distinct parental species, therefore, appears to be a universal phenomenon in yeasts, the genetic make-up of the resulting hybrid lines being very variable depending upon the ploidy of the parents and the post-hybridization events. In this respect, an interesting example was recently provided by *Millerozyma sorbitophila* (Leh-Louis et al., 2012), a member of the CTG clade, because this hybrid genome, suspected to be recently formed, contains heterozygous chromosome pairs (the two parents differed from each other by 12-15% sequence divergence) together with other chromosome pairs partly or entirely homozygous. It looks, therefore, in the process of resolution by a long-range loss of heterozygosity mechanism along chromosomes, a process also observed in other hybrids, which is prone to eventually generate normal, entirely homozygous genomes but of dual origin. In absence of sequence data about the parents, traces of this process remain essentially unnoticeable in genomes and, therefore, many natural yeast species may in reality have multiple parental origins, as was recently discovered for the *S. bayanus* type strain.

Conclusion

Despite the considerable progress made during the last two decades since the pioneer sequence of *S. cerevisiae*, yeast genomes probably hide more surprises to be discovered, but the global picture now available and the volume of available genome information provide solid bases to frame future research on the diversity of this important group of micro-organisms and to accelerate our understanding of the complexity of their interactions.

Keywords Chromosome, duplication, hybrid, orphan, transfer

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SESSION I

METABOLIC COMPLEXITY OF AGRICULTURAL AND NATURAL ENVIRONMENTS

PLENARY LECTURE

THE SOIL/PLANT-FUNGAL/BACTERIAL NETWORK – MECHANISMS OF INTERACTION IN THE SOIL

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Background

Plants are intricately linked to the soils they are growing in, in terms of the acquisition of nutrients and the tolerance of, or resistance to, soil-borne pathogens. Next to offering 'conditions', here taken as the total of physical-chemical (abiotic) conditions, soils are also important to plants by virtue of their providing a living system, in which bacteria and fungi play the major key roles. For instance, mycorrhizal fungi serve as functional extensions of plant root systems, and so furnish sources of phosphorus as well as nitrogen to the plant. In contrast, saprotrophic fungi explore the soil to suit their nutritional needs, and by doing so have major ecosystem roles as decomposers and thereby nutrient recyclers. Bacteria in soil may be looked upon in similar ways, thus exerting dual roles in dependency of bacterial type. On the one hand, they can be directly involved in the furnishing of compounds or conditions that spur plant growth (e.g. nitrogen fixers, phosphate solubilizers, plant hormone producers, ACC deaminase producers), whereas, on the other hand, they may also have nutrient cycling ecosystem roles. Importantly, the aforementioned two ecological types of fungi can create selective conditions for soil bacteria, in their so-called mycospheres.

Surprisingly, the interrelationships between these two major groups of soil microorganisms have not been extensively studied until recently. The now almost classical work performed by Jean Garbaye and co-workers in the 90-ies (later followed by that of Frey-Klett, Deveau, and others) showed early on that particular pseudomonads in the soil can act as 'mycorrhization helper bacteria', i.e. they spurred the colonization of trees by (ecto)mycorrhizal fungi. Mainly ecological mechanisms have been subsequently sought (e.g. Deveau et al, 2007). In more recent years, specific fungal-bacterial interactions have been studied to a greater extent, with the aim to better understand the mechanisms behind such interactions. The grand challenge here has been perceived to reduce the complexity of the natural systems down to one that allows specific mechanisms to be studied. In the light of the phylogenetic diversity that is present both within the bacterial and the fungal communities of most soils, it is likely that such mechanisms also vary greatly. Here, we will briefly examine the work developed in the Microbial Ecology group in Groningen, with respect to the mechanisms that underlie the interaction between soil *Burkholderia* types, in particular *B. terrae*, and a mycorrhizal / saprotrophic soil fungus (*Laccaria proxima* and *Lyophyllum* sp strain Karsten).

Case study – *Burkholderia terrae* as a prime colonizer of soil fungi

There are potentially numerous types of bacterium-fungus interactions, and one may predict that the molecular mechanisms involved in these are inherently complex. Hence, we set out, in a series of experiments, to dissect the soil system into components that were studiable, in other words, a reduction of complexity was sought. Experiments were started in the field and continued in laboratory (microcosm) studies (Warmink and van Elsas, 2008; Warmink et al, 2009).

In the field and microcosm experiments, particular *Burkholderia terrae* strains, BS001 and BS110, were found to be interactive with the ectomycorrhizal fungus *Laccaria proxima*, and with *Lyophyllum* sp. strain Karsten (Warmink and van Elsas, 2008). Strain BS001 produces a biofilm on the latter host and sequesters the carbon and energy source glycerol from it. Remarkably, strain BS001 can « castrate » its host, significantly inhibiting primordium setting (sporulation) (Nazir et al, 2013). Furthermore, it has broad comigration capacity with a suite of six out of nine fungi (encompassing different ecological types, and belonging to both the ascomycota and basidiomycota) through the soil, also forming a biofilm on *Trichoderma harzianum*. Comparative analysis of the 11.5-Mb genome of strain BS001 (Nazir et al 2012) showed the presence of a whole range of key genes for mycosphere-relevant functions. Numerous regions of genomic plasticity (RGP) were found, including a 70,422 kb long region (denoted RGP79, containing a type-4 secretion system) and other plasmid-type traits. Furthermore, biofilm formation genes and type-2, type-3 and type-6 secretion systems were present. Using mutation analysis, the type-3 secretion system was revealed to be important for the interaction with the fungus. Furthermore, *B. terrae* BS001 can take up and utilize numerous carbonaceous compounds (such as glycerol, methylglyoxal, fatty acids, sugars and amino acids). Over evolutionary time, the BS001 genome may have acted as a «collector» of habitat-relevant traits, be these bulk soil, fungi and/or plants alike. A legacy of a presumably versatile and biphasic life style. Transcriptome analyses in confrontation assays in microcosms very recently revealed that strain BS001 modulates the expression of key genetic circuits as a response to both a soil–mimicking environment and fungal hyphae (Haq et al, in rev, 2015). The stationary–phase sigma factor RpoS, as well as genes under its control, were expressed to a large extent across treatments. Strain BS001 perceived the presence of fungal hyphae at a distance of 15 mm, upregulating several chemotaxis–related genes. Later, a gene encoding a SET-domain-containing (secreted effector) protein was also upregulated. Finally, five genes potentially involved in oxidative stress responses were highly upregulated by the fungus. Strain BS001, being in a stress–dominated state, showed both early and late responses to *Lyophyllum* sp. strain Karsten, characterized by dynamically–changing chemotaxis, metabolic signalling, potential modulation of the eukaryote host and an oxidative stress response.

Further data and outlook

From the genomic information obtained, we surmised that *Burkholderia terrae* strain BS001 (next to BS110) is an organism that – from an organism living under bulk soil conditions - has specifically evolved to interact with higher organisms in the soil, be these fungi, plants or other organisms. It is likely that, in their life style, such bacteria switch from a phase of 'survival' in bulk soil under locally-determined soil conditions, to one in which interactivity with a host such as a soil fungus is the main life driver. Most of our data

point to a rapid bacterial gene expression response to fungal hyphae that emerge in the soil. It has also become increasingly clear that the interaction of *Burkholderia terrae* BS001 with the hyphae is intrinsically complex, several genetic systems playing roles, which are variable in time and possibly also with respect to space. Such inherent complexity is most likely the rule rather than the exception in fungal-interactive soil bacteria. On top of this, the soil matrix is inherently heterogeneous and also dynamic in time. The fact that *B. terrae* strain BS001 is a generalist interactor rather than a specialist is supported by recent data, in which we show that a fungal cell wall glycolipid with acronym CMH may serve as the fungal cell surface anchor for bacterial adsorption (Barreto-Bergter, unpubl). Based on our hypothesis that CMH is not the only cell wall component involved, we are currently performing experiments aimed at unravelling the overall complexity of this interaction.

Furthermore, the patterns of gene expression of *B. terrae* BS001 - upon confrontation with the fungal host L. sp. strain Karsten - revealed a highly complex pattern of up- and downregulated bacterial genes. Clearly, a plethora of gene systems get induced or arrested, much in line with a recent study performed in the Zurich group of Eberl and Weisskopf (pers. comm, 2015). In our own work, the raised expression of a gene encoding a SET domain protein was interesting, as such proteins are secreted in other systems and introduced into the nuclei of eukaryotic hosts, in which they tinker with the chromatin and modulate eukaryote gene expression.

Concluding, one can observe that the study of the molecular basis of the interactions between bacteria and fungi in the soil is still in its infancy. It is recommended that other focused systems are dissected with respect to the questions pertaining to the benefits reaped from the interactions by both the bacterial and the fungal counterparts. In the light of the fact that both bacterial and fungal populations in soil systems are heterogeneous in nature, and that the very system (soil) is heterogeneous and dynamic, it is imperative that future studies tackle such heterogeneity, for instance, by adapting sampling schemes, performing time course sof development and redoing experiments to check for reproducibility. On top of this, the working of interactive systems in the midst of a large microbial diversity also needs to be addressed.

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SELECTED LECTURE

BIOLOGICAL SOIL CRUSTS: A MICROENVIRONMENT AFFECTED BY THE MICROBIAL SECRETED EXOPOLYSACCHARIDIC MATRIX

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Introduction

Biological crusts (BSCs) are complex microbial associations constituted by cells and microbial filaments embedded in a polysaccharidic matrix (EPS) that binds them together and with soil particles. EPSs represent a huge carbon source directly available to heterotrophic organisms, affect soil characteristics, water regimes, and establish complex interactions with plants. The induction of BSCs on degraded soils is considered a feasible approach to amend and maintain land fertility, as it was reported in a number of recent studies. It was recently shown that BSC induction is beneficial in enhancing SOC (Soil Organic Carbon) and in increasing the abundance of phototrophic organisms and vegetation cover.

In this presentation, the contribution to the structuring of the soils of the polysaccharidic matrix of the crusts will be discussed moving from the different characteristics of two operationally-defined EPS fractions, the colloidal (C-EPS) and the EDTA extractable (tightly bound, TB-EPS) fractions. In BSCs, C-EPSs are loosely bound to cells and sediments while TB-EPSs are tightly bound to the crustal biotic and abiotic constituents of the crusts. The results obtained suggest that the colloidal fraction of the EPSs, which is more dispersed in the soil, is more easily degradable and thus constitute an abundant C source for the microflora residing in the crusts, while the EPS fraction tightly bound to the soil particles, which is characterized by a high molecular weight, plays a key role in giving a structural stability to the BSCs and in affecting the hydrological behavior of the soil covered by the crusts.

Material and methods

BSC samples were collected in 2011 in a research area in Hobq desert, Inner Mongolia, China (40°21’30’’-22’30’’N; 109°50’30’’-51’50’’E) constituted by moving sandy dunes, in which BSC formation had been induced by cyanobacteria inoculation performed in three different years (2003, 2005, 2007), so that induced BSCs were of different ages (respectively 8, 6 and 4 years old). Samples were collected in triplicates (experimental triplicates, N=3). Unconsolidated sand under the crusts, and unconsolidated sand from non-inoculated sites (control, CK) were also collected.

EPS extraction was performed grinding BSC samples in a mortar and roughly 100 mg of powder were treated with distilled water for 15’ at 30°C before being centrifuged at 6000 x *g* to collect the C-EPS-containing supernatants. The procedure was repeated three times for each sample. Pellets were treated for TB-EPS extraction according to a method modified

from De Brouwer and Stal (2001) and Rossi et al. (2012) using a 0.1 M Na_2EDTA solution, repeating the extraction three times. Finally, C-EPS and TB-EPS contents were determined using phenol-sulfuric acid assay(Dubois et al., 1956). The activity of sucrose and dehydrogenases, which are involved in polysaccharide degradation, were determined according to the methods of Guan (1986), by using respectively the 3,5-dinitrosalicylic acid assay and the 2,3,5-triphenyltetrazolium chloride assay. In order to determine the MW distribution of the EPS fraction, samples were analyzed using using a Varian ProStar HPLC chromatograph (Varian, USA) equipped with a refractive index detector and columns for Size Exclusion Chromatography (SEC). Analysis was conducted using deionized water as eluent, at a flow rate of 0.4 ml min^{-1}, and using dextran at different MWs (2000, 76 and 64 kDa) and saccharose as standards for the titration.

Results

Both C-EPS and TB-EPS showed to be mainly concentrated within the crust thickness ($P<0.01$) with no statistical difference between IBSC on dunes and between adjoining dunes (Figure 1). EPS were also find in the soil below the crusts, where they are in significantly higher contents compared to CKs. Roughly 25 µg g^{-1} soil of both C-EPS and TB-EPS were found in non-inoculated sites. Contents up to 10-fold higher EPS amounts were found in induced BSCs, and 4-fold higher amounts were found in the soil beneath the crusts.

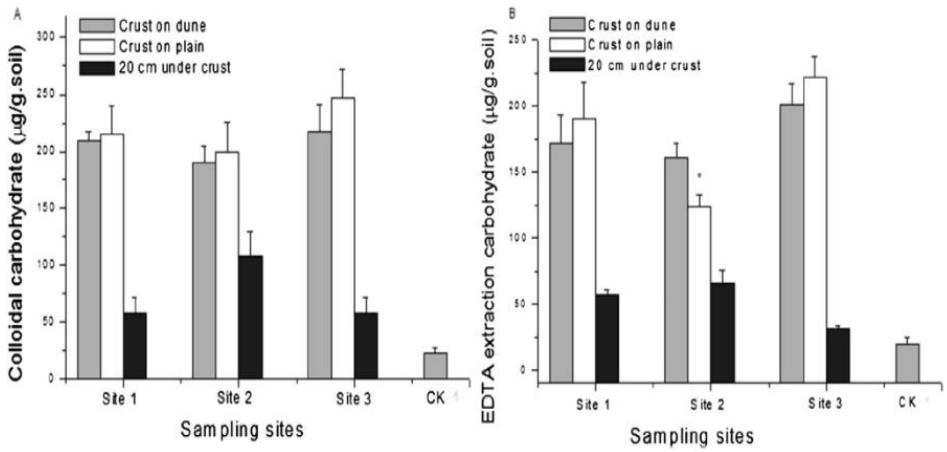

FIGURE 1. (A) C-EPS or colloidal carbohydrate contents and (B) TB-EPS contents, extracted using EDTA solution. Site 1, 8 years old crusts; site 2, 6 years old crusts; site 3, 4 years old crusts.

The activities of both sucrase and dehydrogenase increase with the age of the crusts with robust linear correlations ($r^2= 0.89$, $P<0.05$ for dehydrogenase and $r^2= 0.95$, $P<0.05$ for sucrase). Almost inconsistent activities were detected in the soil underlying the crusts and in CKs.

Results show that both EPS fraction are constituted by molecules belonging to different MW fractions (Figure 2). C-EPS are constituted by one major fraction (roughly 61% of the molecules) in the range between 2000 and 76 kDa, and a second (roughly 24% of the

molecules) in the range between 64 and 0.34 kDa. TB-EPS resulted constituted for almost the totality (roughly 80-90%) of molecules in the range 2000-76 kDa.

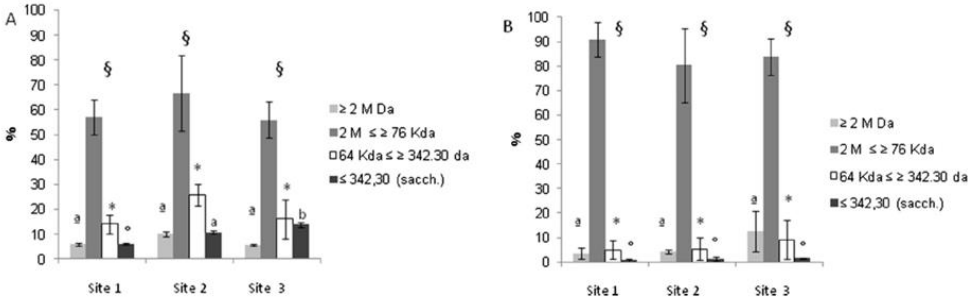

FIGURE 2. Size distribution (%) of the two operationally-defined EPS fractions in IBSC of different ages. (A) C-EPS, (B) C-EPS contents and (B) TB-EPS contents, extracted using EDTA solution. Site 1, 8 y.o. crusts; site 2, 6 y.o. crusts; site 3, 4 y.o. crusts.

Regarding C-EPS, while no statistically significative difference was found between the distribution of the three classes at higher MWs, the amount of the smaller fraction (MW<0.34 kDa) resulted inversely correlated with the age of the crusts (r^2=0.84, P<0.05).

Discussion

It emerges that EPS excretion by crust organisms (mainly cyanobacteria, microalgae and fungi) on hyper-arid soils represent a notable input of carbon available within the crust thickness and leaching in the underlying soil. C availability leads to an increase in microbial activity and changes in bacterial community structure(Rasche et al., 2011), compared to non inoculated sandy soil counterparts. For the first time, the analysis of MW distribution of the two EPS fractions was analyzed in BSC of different ages, to observe eventual differences related to the time BSC had to develop. What emerges is that the two fractions are significantly different. While TB-EPS, which is the more tightly bound to cells, is constituted mainly by large molecules for almost its entirety, C-EPS is constituted by molecules more distributed through different MW ranges. Interestingly, regarding C-EPS, the lower MW fraction amount resulted significantly correlated with the age of the crusts, diminishing with their age. It can be hypothesized that enzymatic processes are directed to higher MW fractions that are degraded to simple sugars (glucose and fructose), which are consumed preferentially by newly recruited heterotrophic organisms. This is supported by the observed increase of sucrase and hydrolase activity with the age of the crusts. Simple sugars are directly available to be respired by heterotrophs(Mager and Thomas, 2010). On the other hand, TB-EPS are stably constituted by high MW polymers. Thus, while enzymatic activity is mainly directed towards the more soluble EPS fraction, the more condensed EPS fraction remains stable, being constituted mainly by high MW sugars, and possibly plays a structural role in BSCs.

These results show that the increase of microbial associations on hyper-arid soils is key to provide available carbon sources. This work represent a first approach to study the processes leading to the decomposition of the exopolysaccharidic matrix in BSC, which represent one of the most respired carbon sources after rainfall events(Thomas et al., 2008).

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SELECTED LECTURE

EXPANDING THE WORLD OF MARINE BACTERIAL AND ARCHAEAL CLADES

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Introduction

The Planet Earth is a blue planet. Our heritage, economy and wellbeing are inextricably linked to the marine environment – the oceans play a key role in the global biogeochemical cycles of carbon, nitrogen, phosphorus, silicon, and a variety of other elements. Currently, the growing human population with its need for agriculture, industrial production and fossil fuel consumption places an immense stress on marine ecosystems. As a result, key elemental cycles of inorganic carbon, nutrients, and dissolved oxygen are being altered at an increasing speed.

In this changing and highly dynamic system, marine microbes act as the invisible "gatekeepers". They inhabit all marine ecosystems, from the tropics to the polar waters and from well-lit surface waters to the deep abyss. They harvest and transduce solar energy - with an estimated contribution to global primary productivity of between 50% to 90% (Falkowski et al., 1998). They catalyze key biogeochemical transformations of all nutrients and trace elements that sustain the organic productivity of the ocean. They produce and consume most greenhouse gases (carbon dioxide, nitrous oxide, and methane), which is of particular importance with respect to anthropogenic disturbances of marine ecosystems. Finally, they also represent a vast and dynamic reservoir of genetic variability that is yet to be tapped into.

For many years the study of marine microbes was hampered by the fact that the majority of microorganisms (90-99%) cannot be cultured under standard laboratory conditions. It was only with the development of a molecular toolbox to sequence DNA from the natural environment that information about the exceptional bacterial and archaeal diversity in the ocean began to accumulate. To date, perhaps thousands of research papers have been written on marine microbial diversity and communities. Nevertheless, determining which microbial taxa are out there, where they live, and what they are doing is still an important paradigm in marine microbial ecology. The importance of these questions is underlined by concerted, large-scale, and global ocean sampling initiatives, for example the International Census of Marine Microbes (Amaral-Zettler et al., 2010), Global Ocean Sampling expedition (Yooseph et al., 2007), Tara Oceans (Bork et al., 2015), or the Malaspina expedition (http://scientific.expedicionmalaspina.es/).

Given decades of effort, we now know that the large majority of known marine Bacteria and Archaea belong to a dozen bacterial and archaeal phyla. Furthermore, in addition to the classically culturable Bacteria and Archaea, at least 50 "clades", at different taxonomic

depths, exist. Originally, these clades were defined as clone sequences originating from one or two specific environments, and represented the "uncultivated" marine Bacteria & Archaea (Fuhrman and Hagström, 2008). With the development of improved cultivation methods, single-cell genomics, and finally genome assemblies from metagenomes, a lot more is now known about these clades in terms of their phylogeny, physiology, ecology, and metabolism. Despite the fact that, along with the cultivable Bacteria & Archaea, these marine clades account for the majority of the marine microbial diversity, there is still an underexplored, novel portion remaining. For example, a survey of GOS metagenomic reads containing 16S rRNA fragments revealed that, 4-5 % of these fragments could not be taxonomically assigned to any known orders, while 20% could not be assigned to known families (Yilmaz et al., 2011). Incidentally, it has been suggested that these unclassified sequences overlap with the "rare biosphere" (Sogin et al., 2006). Rare and low-abundant taxa are often not captured by cultivation, or by environmental sequencing, and their ecology and metabolic roles therefore remain poorly understood. While it is possible that rare taxa may just represent negligible phylogenetic novelty, there is growing evidence that they often contribute to biogeochemical cycles, and can increase in abundance with changing conditions (Lynch et al., 2012).

Results and Discussion

In this study, our aim was to characterize the *"known"* and *"unknown"* phylogenetic diversity of marine microbes, specifically focusing on the *unknown* clades. In order to achieve this, we mined the SILVA 16S rRNA datasets for sequences originating from the marine water column. New phylogenetic trees were constructed for all phyla that contained members from marine origin. Aspects such as size, phylogenetic depth, or a standard nomenclature format of environmental clades are generally not considered, and few systematic surveys exist. To overcome this issue, we applied the candidate taxonomic unit (CTU) circumscription system, along with a standardized nomenclature (Yarza et al., 2014) to the sequences in these phylogenetic trees, instead of a subjective taxa delineation and nomenclature method. Due to the exhaustive manual curation undertaken for SILVA taxonomy, with the help of domain experts, almost all major *known* marine clades have been annotated in the original SILVA guide tree. By mapping the sequences from this guide tree to the new trees, and marking all clades that contained sequences from *known* marine clades, we uncovered which clades remained as *unknown*.

With this new phylogenetic and taxonomic framework, we performed a large-scale meta-analysis of publicly available 16S rRNA amplicon datasets (specifically ICoMM marine water samples) to gain insights into the global distribution of *unknown* marine clades, their ecology, biogeography, and interaction with oceanographic variables. ICoMM, which represents a first inventory of marine microbial diversity and biogeography based on rRNA gene data, is an ideal dataset for this analysis – it is based on a single 16S rRNA region, a standardized experimental setup was used for all samples, and most importantly, it contains rich contextual metadata.

Our results showed that there are at least 92 marine clades that have been so far unrecognized. Mostly, these clades are relatively more abundant below the marine epipelagic zone and the oxygen minimum zones. These clades carry a nomenclature, a rank and a classification that is compatible with the hierarchical structure proposed by the Bacteriological Code. In addition, the inference of ecological and physiological properties reinforces their taxonomic coherence. We have further demonstrated the usefulness of the CTU approach to give

meticulous taxonomic standing to uncultured diversity and to evaluate ranks and classifications of existing taxonomies. In this regard, the remarkable abundance of high taxa (i.e. genus and above) detected, just within the marine microbes, suggests a general lack of criteria for their delineation in phyla like Actinobacteria, Planctomycetes, Lentisphaerae, Deferribacteres and Proteobacteria. Therefore, the implementation of this technique into microbiologist's routine foresees an important boost in taxonomy, making it more pragmatic. Most of the *unknown* clades we identified were interspersed by known taxa with cultivated members, whose genome sequences are available. This result encouraged us to perform metabolic predictions for the *unknown* marine clades using the PICRUSt approach (Langille et al., 2013). PICRUSt is designed to predict the functional composition of metagenomes using marker gene data (such as rRNA) and a database of reference genomes. More specifically, an ancestral-state reconstruction algorithm predicts which gene families are possibly present. We acknowledge that phylogeny and function are at best imperfectly correlated, however the original PICRUSt paper, along with several others have demonstrated that 16S rRNA based phylogenetic trees mirror functional gene clusters. We present these predictions not as the ground-truth, but as a possibility for these *unknown* clades, given their habitat and geographical distribution that we determined based on the ICoMM dataset.

Keywords
Marine microbiology; taxonomy; 16S rRNA; ecology; phylogeny

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SELECTED LECTURE

SPATIAL PATTERN OF SOIL BACTERIAL DIVERSITY IN A MIXED AND UNEVEN POLLUTED SITE, AND ASSESSMENT OF RHIZOREMEDIATION POTENTIAL

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Introduction

The use of plants to extract and modify the pollutants (phytoremediation) is a recent development in green technology aimed to remedy soils, sediments, surface water and groundwater contaminated by metals, organics and radionuclides. Furthermore, root associated microorganisms can contribute to soil bioremediation by degrading or modifying the pollutants (rhizoremediation) and/or sustaining plant growth (plant growth promotion, PGP) (Ma et al. 2011). Plant-microbe interactions in the rhizosphere also have the potential to favor microbial cometabolic degradative processes of toxic organic chemicals, such as Polychlorinated Biphenyls (PCBs), due to root exudate molecules that act as co-metabolites. In PCBs phyto- rhizo- remediation, three main mechanisms are involved: i) plant uptake from soil (phytoextraction) and accumulation in stem and leave tissues, ii) enzymatic transformation (phytodegradation) and iii) plant enhancement of the microbial activity in the root zone, improving bioremediation, by the release of secondary metabolites (SMs) in root exudates (rhizoremediation) (Uhlik et al. 2013).
The SIN Caffaro is a large polluted site of national priority located in the Northern Italy, originated by the activities of the former Caffaro s.p.a. chemical factory. The soil in the site presents a mixed contamination of halogenated Persistent Organic Pollutants, particularly PCBs, and heavy metals in variable concentrations, uneven distributed in the area and often exceeding the safety values. This study represents the first report about the structure of the bacterial communities associated to highly polluted soils of the SIN Caffaro (Italy) along a gradient of environmental selection toward the resident community potentially able to support soil remediation. The diversity of the cultivable bacteria associated to the rhizosphere of three autochthonous plants collected from the most contaminated area is moreover described, focusing on the study of PGP activities that might help plant growth during future intervention on a site-scale.

Materials and Methods

Soil samples were collected during different sampling campaigns (phases 1 to 3) in three areas within the SIN Caffaro. During phase 2, 63 samples were collected from 9 stations at 7 depths along the soil horizon, comprised between 0-100 cm. Finally, during phase 3, 64 soil samples were collected at 0-40 cm depth and an accurate homogenization procedure was

applied to obtain a homogeneous sample representative of the layer of interest. Detailed chemical analyses were performed on the collected soil samples, which were also microbiologically investigated. To depict bacterial community's structure, we used cultivation independent analyses, including Automated Ribosomal Intergenic Spacer Analysis (ARISA) fingerprinting and Illumina tag screening of the V4-V5 hypervariable regions of the 16S rRNA gene. Hydrolytic activity was estimated by means of fluorescein diacetate (FDA) test (Green et al. 2006) on the samples collected during Phases 2 and 3. Furthermore, the rhizosphere of three autochthonous plant species was collected in the most contaminated area of the site and a collection of bacterial strains was established on different media, identified and tested in vitro and in vivo for PGP potential.

Results and Discussion

Detailed chemical analyses, including the quantification of different classes of pollutants such as PCBs and heavy metals, were performed on the samples collected along the soil horizon during the phase 2. In this step, 63 samples were collected from 9 stations at 7 depths comprised between 0-100 cm. The results clearly showed that the distribution of pollutants deeply changed along the vertical profile, presenting the highest pollutants' concentration in the first layers of soil, up to 40 cm depth. The measured chemical data allowed to clarify pollutants' distribution in the three studied areas and to setup the sampling strategy of phase 3, aimed to create a map of pollutants' distribution in the SIN Caffaro.

Molecular analyses were applied to investigate the soil dwelling bacterial communities showing that their overall structure was significantly different according to area and depth of collection. Moreover, the adopted multidisciplinary approach allowed to verify that the concentration of different classes of pollutants was significantly related to the pattern of bacterial diversity in the analyzed soils.

Besides bacterial community composition, the level of pollution also influenced soil activity, by means of hydrolytic activity. The FDA analysis showed that i) the 63 soils analyzed during phase 2 were significantly different according to the depth of collection, while the soils collected at phase 3 (0-40 cm) were significantly different according to the area of collection.

Three autochthonous plants growing in the most polluted area of SIN Caffaro were collected to establish different bacteria collections on mineral medium (MM) supplemented with biphenyl and on diluted TSA medium. Bacteria collections established from *Medicago*, *Centaurea* and *Dactylis* spp. on MM supplemented with biphenyl showed high similarity from the taxonomic perspective, although the rhizobiome of *Medicago* sp. was peculiarly enriched of Actinobacteria. Overall, the main taxonomic classes detected in the established bacteria collection were Actinobacteria and Gammaproteobacteria, both comprising known degraders of recalcitrant aromatic molecules.

The bacteria collection was screened in vitro for several PGP activities and resistance to abiotic stresses. The estimated PGP traits include PO_4 solubilization and the production of siderophores, indole-3-acetic acid (IAA), protease, ammonia, exopolysaccharides (EPS). Statistical analysis of the results showed that the rhizospheric bacteria isolated from different plants could not be differentiated based on in vitro PGP ability. On the opposite, bacteria collections established on MM medium supplemented with biphenyl and rich diluted medium (TSA 1:10) were significantly different for their PGP activities. Basing on the results of the in vitro tests, few bacterial strains were selected for *in vivo* test realized under greenhouse conditions. The bacteria were inoculated to the plants at 10^8 cell/g of soil and plant growth

was evaluated by measuring the number of leaves, length and weight of roots and shoots. Four strains belonging to the *Pseudomonas* and *Arthrobacter* genera were able to promote the growth of tomato, chosen as model plant (Figure 1).

Conclusions

Chemical analyses performed during phase 2 indicated the occurrence of a sharp profile of pollutants according to depth, showing higher values of contaminants in the first 0-40 cm of the soil horizon. Molecular analyses indicated that the bacterial community's structure was significantly different according both the area and depth of collection and it was significantly related to the main classes of pollutants occurring at the SIN Caffaro. Moreover, a significant relationship occurred between the soil hydrolytic activity and the depth and area of samples' collection. Thus, we can conclude that the high level of pollution was the driving force for the selection of the soil microbiome in the SIN Caffaro. This allows to hypothesize that a degrading microbial community is present in the soil, potentially able to sustain a remediation process by biostimulation.

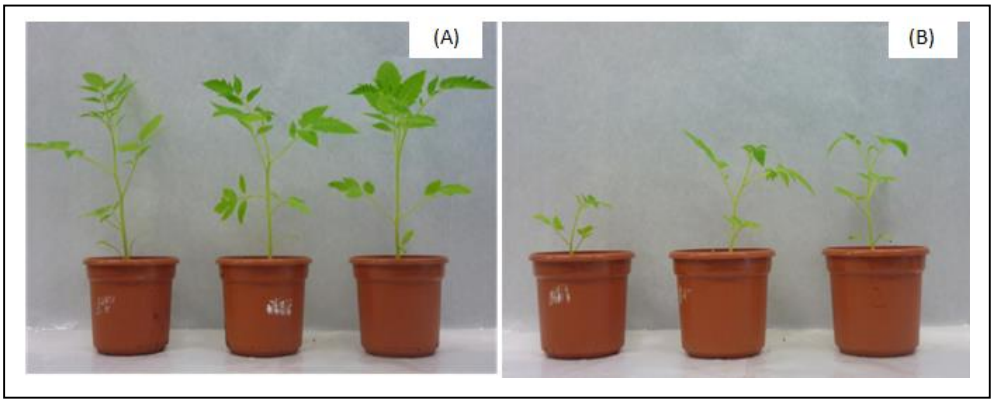

FIGURE 1. Example of *in vivo* promotion of tomato plants by (A) rhizobacteria isolated from authochthonous plants growing at SIN Caffaro compared to (B) negative, non-inoculated plants.

Accordingly, the bacterial populations associated to the roots of the studied autochthonous plants were mainly selected by the pollution profile rather than the plant species. The bacteria collection showed an overall high PGP potential, encompassing several bacteria strains potentially exploitable for sustaining plant growth under field conditions during future remediation intervention.

Acknowledgement

The authors gratefully thank the collaboration of the ERSAF and Caffaro working group.

Keywords
Rhizoremediation, polychlorinated biphenyls, plant growth promoting bacteria, molecular ecology, soil, rhizosphere

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SELECTED LECTURE

SPECIALISTS IN EVERYTHING: THE BLACK YEASTS AUREOBASIDIUM *SPP.*

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Black yeasts from the genus *Aureobasidium* (Dothideales) are studied mainly for their biotechnological applications and use as biocontrol agents in agriculture. Numerous past studies indicated the great industrial potential, which should be investigated in more detail. While doing so, possible hazards they represent should also be taken into account to ensure that their exploitation is both productive and safe.

Versatile and complex

The morphology of *Aureobasidium* spp. is exceptionally plastic (Slepecky & Starmer, 2009). This makes their taxonomy a non-trivial task, requiring a systematic application of molecular phylogeny to reliably distinguish the species. By doing so, four distinct clusters were reported in the species then known as *A. pullulans*. Although at the time they were classified as varieties (Zalar *et al.*, 2008), it was increasingly becoming clear that the diversity of the strains is too large to be accommodated in a single species. Therefore, based on the genomic sequencing and the distances between the genomes, the varieties were finally re-defined as species: *A. pullulans*, *A. melanogenum*, *A. subglaciale* and *A. namibiae*, the first two of which contain the majority of isolates from tropic and temperate climates (Gostinčar *et al.*, 2014). Apart from their morphology, the versatility of *Aureobasidium* spp. is also apparent in the large number of habitats they occupy, from plant surfaces and household dust to hypersaline water ponds, food preserved with refrigeration, salting and drying, and Arctic glaciers, along with some more exotic choices, such as aviation fuel tanks and the surface of degrading plastics (reviewed in Gostinčar *et al.* (2014)). Considering this wide range of habitats, it is perhaps not surprising that this ecological strategy is accompanied by a similarly versatile physiology. Due to their polyextremotolerant nature the yeasts can withstand a variety of stressful conditions, including extremes in temperature, pH and salinity/ water activity.

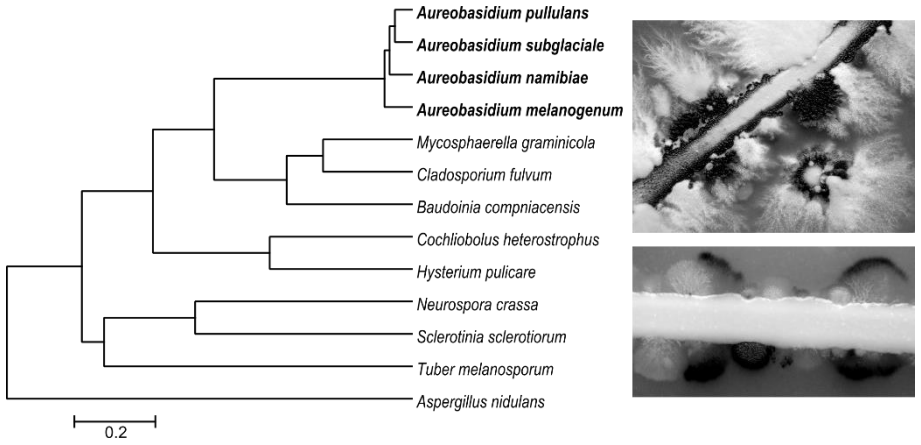

FIGURE 1. Phylogenomic tree deduced from whole proteomes of selected species from *Pezizomycotina* (left; constructed following the method described in Zajc *et al.* (2013)) and morphology of cultures of *A. subglaciale* (right top, MEA) and *A. pullulans* (right bottom, MHM).

Genomic complexity

A *de novo* genome sequencing of four *Aureobasidium* spp. revealed the genomic footprint behind their great adaptability (Gostinčar *et al.*, 2014). Although their genome sizes were smaller than most of the sequenced Dothideomycetes, the numbers of predicted proteins were comparable to related species.

	A. pullulans	*A. subglaciale*	*A. namibiae*	*A. melanogenum*
Assembly length (Mbp)	29.62	25.80	25.43	26.20
Number of scaffolds	186	75	47	150
% of assembly covered by repeats	1.45%	0.87%	0.78%	0.97%
GC content (%)	50.02%	50.78%	51.12%	49.85%
Number of genes	11866	10809	10266	10594

TABLE 1. Genomic properties of *Aureobasidium* spp.

The predicted proteome contained many enzymes, especially those involved in the degradation of the plant material, the diversity of which was comparable even to fungal plant pathogens. High numbers of sugar transporters and alkali metal cation transporters were predicted, and this abundance is possibly associated with the nutritional versatility of *Aureobasidium* spp. and their stress tolerance. Additionally, enzymes with a possible role in the degradation of plastic and aromatic compounds were identified, as well as proteins involved in the synthesis of extracellular polysaccharides and siderophores.

	A. pullulans	*A. subglaciale*	*A. namibiae*	*A. melanogenum*
Predicted secreted proteins	**869**	**813**	**734**	**725**
Carbohydrate-active enzymes	**308**	**288**	**279**	**259**
Glycoside hydrolases	235	222	219	208
Peptidases	**72**	**60**	**59**	**61**
Lipases	8	11	8	7
Peroxidases	5	7	7	7
Other functions	90	88	100	90
Unknown function	391	363	282	304

TABLE 2. *In silico* predicted secreted proteins of *Aureobasidium* spp.

Not just a pretty face: Biotechnological uses

Aureobasidium pullulans has been known for a long time as a producer of pullulan, a linear α-D-glucan composed of maltotriose units with many uses in food and pharmaceutical industry (Chi *et al.*, 2009). At least one of the strains is known to produce an antifungal compound aureobasidin A (Takesako *et al.*, 1991). Besides this, the unusually large number of enzymes produced by the species of this genus include many with suggested biotechnological uses (Chi *et al.*, 2009, Molnarova *et al.*, 2013). These enzymes are thought to be crucial in the ability of *A. pullulans* to outcompete other species, a trait used in agriculture, where spore suspensions of *A. pullulans* are marketed as a biological control of fungal post-harvest diseases as well as to protect blossoming plants against fire blight.

Between polyextremotolerance and pathogenicity

The good stress resistance of *Aureobasidium* spp. is thought to be linked to the emerging medical relevance of the genus (or at least one species within it). Its polyextremotolerant generalistic character encompasses traits that can serve as pre-adaptations for survival in the animal body, in which microorganisms encounter numerous types of stress (similar to those found in nature) (Gostinčar *et al.*, 2010, Casadevall *et al.*, 2011, Gostinčar *et al.*, 2011). Anthropogenic changes in the environment may play a role in these processes and since *Aureobasidium* species are among those that come in daily contact with humans, the evolution of our interactions should be investigated more thoroughly (Gostinčar *et al.*, 2011). When considering the safety of working with *Aureobasidium* spp. it is important to note that all strains isolated from human (opportunistic) infections belong to the species *A. melanogenum*. The division of the previous species complex *A. pullulans* into new species will thus facilitate the identification of the more problematic strains and help in limiting the possible health hazards of *Aureobasidium* spp. use in biotechnology and agriculture.

Keywords
Aureobasidium pullulans, black yeast, stress tolerance, genomics, biotechnology

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SELECTED LECTURE

SEQUENCING DIRECTLY THE RIBOSOMAL 16S rRNA POOL FROM BACTERIAL COMMUNITIES: A NOVEL PCR-INDEPENDENT APPROACH TO MICROBIAL DIVERSITY ANALYSES

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Abstract
The study of environmental microbial communities relies on a PCR-dependent amplification of genes entailing species identity as 16S rRNA, Such traditional approach is susceptible of biases depending on the level of primer matching in different species. Moreover, possible yet-to-discover species whose rRNA could differ enough from known ones would not even be revealed. To overcome these drawbacks we devised an approach consisting in direct sequencing of 16S ribosomal RNA without any primer- nor PCR-dependent step. The method was tested on a microbial community developing in an anammox bioreactor sampled at different time points. Resulting annotations were compared to those obtained by conventional PCR-based amplicon pyrosequencing. Major differences in proportions of dominant taxonomical divisions were observed, and an estimation of relative levels of bias resulting from the PCR-dependent method was obtained for each of the different phyla. The rRNAseq revealed also potential novel lineages and sequences not matching known ribosomal database records.

Introduction
The paper introduces a novel approach to study environmental bacterial diversity which overcomes the drawbacks bound to the current traditional methods. Those are based on PCR amplification of the 16S ribosomal RNA gene using 'universal' oligonucleotide primers. However, in such PCR-mediated analyses the result is indirect as it is dependent on the primer annealing efficiency. Since target sequence conservation is not universal, the DNA of some taxa are amplified better than others and the outcome is not proportional to the true abundances within the community (Kunin et al., 2010; Pinto and Raskin, 2012; Cai et al., 2013).

Materials and Methods

A pilot plant applying the anammox process available at the Eurotec Water Treatment Technologies, Padova, was the source of the biological material. Samples were collected from the anammox reactor at day 154 and day 189 from inoculation, corresponding, respectively, to a technical performance of 337 and 377 grams of total nitrogen abated per cubic meter per day. The MOBIO RNA PowerSoil® Total RNA isolation kit was used for RNA extraction. RNA was run in a 0.8 % low-melting point agarose gel and slices corresponding to the 16S rRNA band were cut out. Paired End libraries were prepared following the SOLiD Total RNA-seq kit guide and sequenced on a SOLiD 5500xl platform (Life Technologies inc.). SOLiD reads were aligned against reference datasets using the PASS software, version 1.64 (Campagna et al 2009). To correctly assign the SOLiD reads to their taxa, a two-steps procedure was designed. Firstly, only the uniquely-aligned reads, which correspond both to single or paired sequences that present a unique best hit against the reference dataset, were considered to obtain a preliminary group of putative subjects. Only subjects covered at least for the 10% of their total length (representing 150 bases of a 1500 full length 16S-rRNA) were selected to build a new 16S rRNA dataset for the subsequent step. In the second step all the SOLiD reads were re-aligned against the newly defined 16S rRNA dataset. Since this dataset represents just a small subset of all the known 16S sequences, many of the initially multi-mapped SOLiD reads accordingly presented a unique alignment against the subjects. The final dataset was created selecting those sequences covered at least for the 50% of their total length (corresponding to >750 bases in a 1500 bases-long molecule). As reference dataset, two 16S rRNA-genes databases were downloaded, RDP, release 9, and the 16S-rRNA gene sequences from the DDBJ, release 90.1. In order to obtain a database that could warrant the broadest span of representative biodiversity, the RDP and DDBJ databases were merged together and clustered at decreasing levels of similarity of 97,5%, 95%, 92%, 90%, 88%. The cluster analysis was carried out using the CD-HIT-EST tool of the CD-HIT package (Li and Godzik, 2006). The reference subjects identified by the SOLiD-reads were classified with the rRNA Taxonomy Binning workflow of CAMERA, using BLAST as aligner and the GreenGenes as online reference database. In parallel, in order to compare data with a standard PCR-dependent approach, the PowerSoil® DNA Total Isolation Kit, was used for genomic DNA isolation from the same samples. An universal primer mix for 16S rRNA genes amplification was used to suit the Roche 454-FLX Titanium sequencing system. Three forward primers, degenerated by one base, and three reverse primers, degenerated by three bases, were selected as the most adequate universal oligonucleotides. Their sequences are: F357: TACGGGAGGCHGCAG; R790: BWGGACTACCVGGGTATCT. For the 454 amplicon sequencing protocol, sequencing primer-A and multiplex identifier MID were added to the 5' of the F357 mix. Likewise, sequencing primer-B was added to the 5' of the R790 pool. Three replicate PCR-reactions were carried out for each sample. Each reaction was performed in 20 ul using 0.25 U of Phusion High-Fidelity DNA polymerase (Thermo Scientific), 454 amplicon reads were processed using Mothur version 1.22.2. Sequences where analyzed using the rRNA Taxonomy Binning workflow of CAMERA (Sun et al., 2011). Annotation was carried out using BLAST and the GreenGenes database as online reference dataset.

Results and Discussion

Regarding the PCR-based 454 reads, after trimming and filtering procedures, a total of 4.636 sequences for the first sampling point (day 154) and 3.397 sequences for the second (day 189) were obtained. From the direct RNA-seq protocol, after the cleaning procedure, a total of 43.259.527 (primer F3), 61.943.781 (primer F5) and 43.713.403 (primer F3), 53.468.401 (primer F5) SOLiD reads were obtained respectively for the first (day 154) and second (day 189) time points. The taxonomical classification at phylum level of both amplicons and rRNA-seq data is shown in Tab 1, which shows the marked differences in the results obtained by the two methods. Essentially, while the PCR-based pyrosequencing assigns to Proteobacteria the dominant abundance with 34.06 % and 43,4% for sampling time day 154 and day 189, respectively, their scores in the direct rRNA-seq are more than ten-fold lower. In these the overwhelmingly dominant phylum is instead Planctomycetes, amounting to 87.36 % and 86.44 respectively. Interestingly, Armatimonadetes were represented by more than 5% of the rRNA-seq reads while appearing scarcer in the PCR-dependent analysis (< 0.5%).The inverse trend is displayed by Chlorobi scoring 23.6% and 10.9% by PCR-based pyrosequencing and only 2.6% and 1.65 % by rRNA-seq.

	Day 154 sampling			Day 189 sampling		
Phylum	% (by PCR)	% (by RNA)	Ratio by PCR/ by RNA	% (by PCR)	% (by RNA)	Ratio by PCR/ by RNA
Acidobacteria	1,877	0,084	22,256	3,062	0,053	57,819
Actinobacteria	4,530	0,015	308,903	13,604	0,046	298,464
Bacteroidetes	1,359	0,151	9,020	1,590	0,399	3,989
Armatimonadetes	0,302	5,697	**0,053**	0,412	6,652	**0,062**
Chlorobi	23,598	2,595	9,093	10,984	1,648	6,662
Chloroflexi	2,071	0,867	2,387	1,914	0,978	1,953
Firmicutes	0,669	0,008	81,510	0,383	0,027	14,079
Gemmatimonadetes	4,659	0,206	22,612	6,302	0,177	35,642
Planctomycetes	24,892	87,362	**0,285**	14,694	86,459	**0,170**
Proteobacteria	34,060	2,705	12,593	43,463	3,301	13,166
Candidate Division BRC1	0,388	0,042	9,233	0,736	0,023	32,872
Candidate Division TM7	0,863	0,006	144,614	0,942	0,030	31,799

TABLE 1. Percent of the sequences obtained by PCR-based analysis and by rRNA-seq at the two sampling times and ratio of the two values pairs (% by PCR / % by rRNAseq). Only phyla for which a minimum of 10 sequences were available are reported in the table. Ratios resulting in values < 1 are highlighted in **boldface**.

The sampled microenvironment was deemed an ideal setting for a hypothesis-testing approach as the biochemical data reflected an overtly active anammox metabolism. Besides corroborating Planctomycetes as main players and confirming Chlorobi as associated group as proposed in the literature we detected a further relevant and supported occurrence of the phylum Armatimonadetes, not previously reported in anammox communities but apparently

important as it was the second most active and represented phylum after the Planctomycetes at both sampling times. This strengthens the suitability of the approach in estimating taxonomic groups that were rarely detected by techniques relying on PCR. The results also included a considerable number of rRNA reads that did not align with the databases. For the sampling time at day 159 those were 65.711.669 over a total of 105.203.308 (62.4%) and for the sampling time day 189 there were 73.694.386 over a total of 97.181.804 (75.8%). These high values can be regarded as another important clue conveyed by this novel approach whose potential, as enunciated, is that of enabling the possible discovery of novel taxa whose RNA features could not comply to known primer matching consensi. The direct RNAseq approach compared to a State-of-the-Art amplification-based sequencing has shown profound differences in the deduced community composition and has evidenced the extent of bias that currently used methods encounter when addressing unknown microbial diversity. The method, being based on the number of ribosomes is furthermore reporting which members of the community are actively engaged in functional metabolic activities within any given habitat community.

Keywords
rRNAseq, metagenomics, bacterial community, anammox bioreactor

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SPECIAL SESSION I

Young Researchers

SELECTED LECTURE

MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS-SPECTROMETRY (MALDI-TOF MS) BASED IDENTIFICATIONS OF MICROORGANISMS OF NON-CLINICAL ORIGIN

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Matrix-assisted laser desorption/ionization time-of-flight mass-spectrometry (MALDI-TOF MS) is an emerging tool for high-throughput and rapid microbial identification. Due to relatively higher accuracy, good database on clinically important microorganisms and low-cost of MALDI-TOF MS than other microbial identification methods, it has started replacing existing practices prevalent in clinical diagnosis. However, applicability of MALDI-TOF MS in the area of environmental and microbial diversity research is still limited mainly due to the lack of database on non-clinical microorganisms. Intense research activities in microbial diversity by conventional as well as by innovative high throughput methods has substantially increased the number of diverse microbial species known today. This important area of research is in urgent need of rapid and reliable method(s) for identification of microorganisms from various ecosystems. MALDI-TOF MS, in our opinion, appears to be most suitable method for such studies. Like other automated microbial identification systems (MIDI, Vitek, Biolog etc.) MALDI-TOF MS also rely on reference database for identification of microorganisms. Although instruments made by different manufacturers follow similar principle, the major differences remains in the procedure and algorithm used in creating their own reference database. Differences in approach to creating separate databases is reflected in the level of accuracy in identification of the same set of microorganisms (Carbonnelle et al., 2012). Failure in MALDI-TOF MS based identifications have been attributed mainly to lack of reference spectra in the databases associated with the instruments, or inability of the spectra to differentiate similar species (Seng et al., 2013). Therefore, there is a need to augment the existing MALDI-TOF MS databases with spectra of more microorganisms from different environments to increase wider application of this technology. This suggestion is based on the fact that positive correlations have been observed between number of reference spectra present in MALDI-TOF MS database and reliable identification (Calderaro et al., 2014).

Hundreds of millions of microorganisms populate the earth and each year the number of new validly published names of bacteria increases (Parte, 2014). A large number of already known (some unknown as well) microorganisms are isolated in microbial diversity and environmental microbiology studies. The MALDI-TOF MS platforms have very few spectra database of strains of non-clinical origin, resulting in a very low identification percentage (43-65%) for microbes isolated form soil, water and other environments (Table 1). Creating

database(s) incorporating reference MALDI-TOF MS spectra of such microorganisms is a challenging task which requires urgent attention. The creation of in-house database supplementing the limited commercial database has been proved to be highly advantageous for the identification of several bacteria, which are under-represented in the commercially available databases like *Borrelia*, *Brachyspira*, *Bradyrhizobium*, *Leptospira* and *Nocardia*.

Ecological sites	Total isolates	Identified (%)	Total genera
Insect guts, Western Ghats	975	508 (52.1)	22
Hot springs, Himachal Pradesh	1207	522 (43.2)	35
Wet land ecosystems, North West India	601	346 (57.6)	29
Various ecosystems, North East India	371	195 (52.6)	20
River sediments, North India	2026	1307 (64.5)	47
Extrem ecosystems, Orissa, Bihar and West Bengal	718	449 (62.5)	9
Mangroves, Eastern Ghats	838	393 (46.9)	23
Effluent treatment plants /contaminated sites	304	143 (47.0)	19
Marine environments, Arabian Sea	753	359 (47.7)	14

TABLE 1. MALDI-TOF MS based identification of microorganisms from different ecological sites of India.

Unfortunately, these in-house databases are not in public domain and remain inaccessible to other researchers. Development of an open access and universal database incorporating MALDI-TOF MS spectra of as many microorganisms as possible has been proposed as more appropriate than commercial and individual in-house databases. It is imperative that such databases be well curated and be continuously updated. We hope that more and more researchers will volunteer to make their in-house databases available in public domain. In addition to this, sample processing methods have also been reported to influence the MALDI-TOF MS spectra (Freiwald and Sauer, 2009). Different sample processing methods like, direct colony, formic acid extraction, trifluoroacetic acid extraction and physical disruption methods were used to get satisfactory results on identification of microbes using MALDI-TOF MS. It is desirable to have a standard well defined sample processing method which can be applied in majority of microorganisms in building high quality spectra database. It is also understood that a single sample processing method could not be applied for all types of microorganisms but deviation from an accepted standard sample processing method should be minimum.

In this article, we discussed different aspects of MALDI-TOF MS spectral database development and sample processing to improve microbial identification results from non-clinical origin. Since MALDI-TOF MS is rapid, easy to operate and less expensive, it may also play a bigger role in quality control and validation of microbial strains preserved in large culture collections, institutes and industries. Hence, it is anticipated that in the upcoming era of microbial culturomics MALDI TOF MS systems with extended databases (as advocated in earlier) will play a key role in bringing a revolution in microbial ecology and diversity studies as it did in the field of clinical diagnosis. It is expected that, with availability of

updated, error free and robust database along with optimized methods and protocols, this technique will be able to overcome all the challenges and will prove to be a valuable asset in the field of environmental microbiology, microbial ecology and taxonomy.

Keywords
MALDI-TOF spectral database, Culturomics, High-throughput identification.

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SELECTED LECTURE

BRINGING THE ITS BARCODE IN THE NGS FRAMEWORK

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The ITS (Internal Transcribed Spacer) region has been proposed as a universal "barcode" for Fungi (Schoch *et al*., 2012). It was selected after a screening of several DNA and rDNA potential marker regions, carried out by a multi-laboratory, multi-national consortium. It supports, and partially completes, the taxonomic information given by LSU (Large Sub Unity) rDNA region, previously proposed as species marker sequence (Kurtzman and Robnett, 1998).

ITS region has obvious advantages, due to the ease of anchoring the primers in conserved flanking regions of the rDNA operon. The operon has over 100 copies per genome which have been demonstrated to be somehow heterogeneous. This is due to the presence of different nucleotides in the same position of different copies (Henry, T. *et al*., 2000) and this is sometimes used as the basis for species discrimination.

This heterogeneity can be considered a problem or a source of additional information according to the experimental approach taken. This is particularly important when amplicon-based Next Generation Sequencing is undertaken. In fact, the sequence heterogeneity can produce an overestimation of the species variability or even of the global diversity in metagenomics samples. On the other hand, the study of the internal heterogeneity can be used for very high-resolution strain characterization (Chen, Y. C. *et al*., 2000), to track the strain spread out in medical and environmental conditions and to understand the mechanisms that have generated these variants. Unraveling this heterogeneity have become more and more crucial to establish if a set of isolates contains replicates of a few strains or strains all different from each other.

A set of ITS-LSU D1/D2 sequences were obtained using a new NGS technology, which permits very deep sequencing (over 500 X) (Figure 1 a) and compared with the ITS sequences obtained with the normal Sanger sequencing (Figure 1 b). All the reads obtained from this

new sequencing technique were assembled, using two different analysis routines, to obtain the consensus sequence:

1- *De Novo Assembly*: all the reads were assembled without any reference, in order to let the software find recognizable paired reads useful to generate one or more contigs; these assemblies were then aligned with ITS sequence, obtained by conventional sequencing, to highlight heterogeneous sites;
2- *Map to Reference*: all the reads were directly assembled using the ITS Sanger sequence as reference; the result is an alignment in which gaps and nucleotide substitutions are present and highlighted.

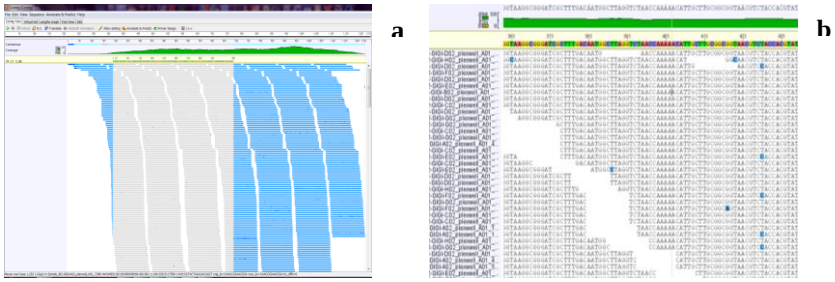

FIGURE 1. Contig Assembly (**a**) and alignment of the assembly with the ITS Sanger sequence (**b**) of the reads obtained from the Next Generation Sequencing of one of the tested strains.

The heterogeneity displayed by the reads was calculated for the whole sequence and in the four separated regions, ITS1, 5.8S rDNA, ITS2 and LSU within the D1/D2 domain, in order to evaluate whether the heterogeneity is spread out with the same degree throughout the two *loci* or if it is concentrated in specific areas.
These results will be useful to spread light in the mechanism of internal heterogeneity and to choose the regions for the evaluation of species diversity (low heterogeneity) and of species variability (high heterogeneity) to be used in metagenomics studies.

Keywords
ITS, barcode, heterogeneity, NGS, metagenomics

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SELECTED LECTURE

INITIAL STUDIES ON THE DIVERSITY OF SPALTING FUNGI IN THE SOUTHERN AMAZON FOREST OF PERU

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Introduction

Spalting is the coloration of wood by fungi, and is classified in three categories: bleaching, that is caused by white rot fungi, pigmentation, that is generated by secondary metabolites of Ascomycetes and the zone lines, which are mostly melanin molecules generated by Ascomycetes and Basidiomycetes (S.C. Robinson, Richter, & Laks, 2007). The most utilized spalting species are *Xylaria polymorpha*, which develops zone lines, *Scytalidium cuboideum*, which develops red pigmentation, *Scytalidium ganodermophthorum*, which has yellow pigmentation and *Chlorociboria aeruginosa* that has a blue-green pigment (S.C. Robinson, Richter, & Laks, 2008; Sara C. Robinson et al., 2014).

As many of these fungi, specially the ones that develop pigmentation, do not have a fruiting body, there is little research about them, specially in the Amazon rainforest. Peru, is a South American country known for its diversity, both fungal and otherwise. This research focused in the Madre de Dios area of the southern Peruvian Amazon. Potential spalting fungi were collectd, cultured, isolated, and sequenced. Most of the collected species were related to well-established North American fungi in the order of Helotiales, and within the genre *Scytalidium* and *Xylogone* (some *Xylogone* species have recently been reclassified as *Scytlidium* species). Wood species most commonly spalted included pashaco (*Macrolobium sp.*), in a secondary growth forest.

Materials and Methods

Samples were collected in the district of Las Piedras, in Madre de Dios, Peru; under the permit number 0328-2013-MINAGRI-DGFFS-DGEFFS, issued by the Peruvian Forest Service (SERFOR). The methodology of the collection consisted of locating dead logs and branches along the different trails that are located in the area. On each one of them, a longitudinal cut was made with a machete, until reaching the xylem. If zone lines or pigments were present, a sample of the stained wood was collected (varying sizes depending on amount of spalting). Tissue cultures ere made from the samples, later refined to single species pure cultures on 2% potato dextrose agar (PDA). Pure cultures were exported under the permit 002822 MINAGRI-DGFFS issued by SERFOR to the Forest Pathology laboratory at Oregon State University (OSU) to prepare the samples for sequencing. The DNA isolation was done with the QIAGEN® DNeasy Plant Mini Kit. PCR was performed using the hot start polymerase and cleaned with EXOsap-IT.. Samples were sequenced at the Center for

Genome Research and Biocomputing at OSU. Results were compared in the BLAST® webpage to identify the samples collected.

Results and Discussion

The sequenced fungi were divided on zone lines and pigmenting fungi, this classification was based by the kind of spalting that was observed at the moment of the collection,. The resulting sequences are shown in Table 1.

Spalting type	Identified fungus
Zone lines	*Xylaria guianensis* (Mont.) Fr. *Auricularia nigricans* (Fr.) Birkebak, Looney & Sánchez-García (syn *A. polytricha*) *Xylaria hypoxylon* (L.) Grev *Pestalotiopsis sp.* *Xylaria curta* Fr. *Peniophora sp.*
Pigments	*Scytalidium lignincola* Pesante *Xylogone sp.* *Scytalidium sp*. *Scytalidium ganodermophthorum* Kang, Singler, Y. W. Lee & S. H. Yun

TABLE 1. Results of the fungi identified classified by kind of spalting

Xylariales was the most common within the zone line producing fungi, and the genus *Xylaria* was the most frequent in this group. Two Basidiomycetes were also found is wood pieces with orange zone lines, identified as *A. polytricha* and *Peniophora* sp. Additional experiments are required to confirm that they produce the zone lines. For pigmenting fungi, the order Helotiales was the most frequent. The genus *Scytalidium* was heavily present in the area. This genus is related to the pigmenting species already studied in North America.

Conclusions

Most wood-pigmenting fungi found in the Amazon region of Madre de Dios, Peru, are Ascomycetes from the order Helotiales and the order Xylariales. The *Xylaria* genus was the most frequent species in zone line production, while the genus *Scytalidium* was the most common pigmenting fungi.

Keywords

Peruvian fungi, spalting, pigments, zone lines, sequencing

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SELECTED LECTURE

GUT MICROBIOTA AND METAGENOMIC DIVERSITY OF OMNIVORE, VEGETARIAN AND VEGAN HEALTHY SUBJECTS

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Introduction

The composition of intestinal microbiota is gaining importance in human health studies. The microbiota and their collective genomes, referred to as the microbiome, have a profound influence on human physiology and nutrition, and are crucial for human life (Bäckhed et al. 2005). Diet habits in particular appear to be an important factor that affects gut microbiota, in terms of abundance, composition and activity. However, until recently, not many studies have broadly and systematically considered the association between habitual diet and gut microbiota. Three main dietary habits have been recognized throughout the world: omnivore (O), ovo-lacto-vegetarian (VG) and vegan (V). Different kinds of food have been demonstrated to influence microbiota composition, as they provide substrates for bacterial proliferation and function as sources of bacterial contamination. Changing the intakes of the three main macronutrients (carbohydrates, proteins and fats) can significantly affect the composition of microbiota. To understand and exploit the impact of the gut microbes on human health and well-being it is necessary to decipher the content, diversity and functioning of the microbial gut community. In the present study, the fecal microbiota of 153 healthy volunteers, who followed O, VG and V diets, has been investigated by means of culture-independent approaches, namely RT-PCR-DGGE of 16S rRNA gene. In order to study the effect of dietary habits on the gut metagenome we applied shotgun sequencing to total fecal bacterial DNA.

Materials and methods

Healthy adult volunteers (51 per category) recruited from North to South Italy between 30-50 years of age and with a male:female ratio approximately 1:1, were recruited in 4 different locations in Italy: three locations in the north (Bologna, Parma and Turin) and one in the

south (Bari). Feces samples were home collect from each volunteer once per week for the 3 consecutive week and used for RNA and DNA extraction. cDNA were than used to amplify the variables V3 and V9 region of the 16S gene. The PCR products were then analyzed by DGGE. From a selection of 27 volunteers single-end DNA library construction and shotgun sequencing for the HiSeq 1500 platform were performed. Functional characterization of the shotgun sequence reads in the KEGG database was assessed in order to identify the relative abundance of specific metabolic pathways characteristic of each dietary group. The phylogenetic characterization of the shotgun sequences was also evaluated.

Results and discussion

The similarity matrixes obtained from dendrograms analysis of the RNA-DGGE fingerprints were used to build Projection on Latent Structures – Discriminant Analysis (PLS-DA). Concerning the dietary habits it was possible to observe a gradient of samples driving a certain degree of separation of omnivore from non-omnivore subjects. The rRNA DGGE profiles were very complex but only a few bands were specific in/of all three diets. The identification of dietary habit-specific bands showed the presence of members of the *B. fragilis* group in the O samples while *Faecalibacterium prausnitzii* was found to be a characteristic of VG. The pathway enrichment analysis of the metagenomes showed an increased abundance of genes involved in the riboflavin metabolism pathway (ko00740) in VG compared to O and one carbon pool by folate pathway (ko00670) compared to V. Moreover, V showed an increased abundance in genes involved in fatty acid biosynthesis (ko00071), amino sugar and nucleotide sugar metabolism (ko00520), butanoate (ko00650) and propanoate (ko00640) metabolism compared to O.

Correspondence Analysis (CA Fig.1) based on PCA of significant metabolic pathways (FDR < 0.01) showed a separation according to diet type.

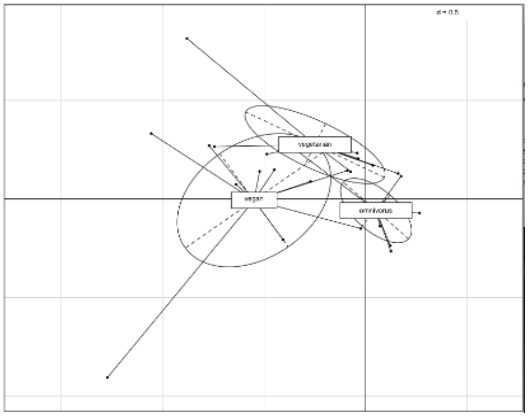

FIGURE 1. Correspondence analysis based on PCA of metabolic pathways. Subjects clustering was done according to the diet type

Sequences were also assembled and the resulting contigs were then searched for key microbial genes in butyrate, acetate and propanoate production, revealing significantly higher gene counts for short chain fatty acid (SCFAs) producing enzymes in V compared to VG and O. The protective role of SCFA against inflammatory diseases, immunomodulation, antimicrobial and anti-carcinogenic activities is well recognized. It is well reported that consumption of vegetable-based diets was significantly associated with increased levels of fecal SCFAs (De Filippis et al. 2015).
Taxonomic composition showed no clear separation of subjects based on diet. The abundance of *Roseburia*, *Ruminococcus*, *Prevotella* and *Lachnospira* were significantly different (more abundant) in V compared to O and VG. *Bifidobacterium* and *Bacteroides* were most abundant across the O samples (FDR < 0.01). Pair-wise Spearman correlations were calculated between microbial genera, and metabolic datasets. *Lachnospira* were positively correlated with pentose phosphate, galactose, aminosugar and nucleotide sugar metabolism pathway. However, *Ruminococcus* was found to be related with fatty acid biosynthesis and biosynthesis of unsaturated fatty acid pathways.

Conclusion
Changing the intakes of the three main macronutrients (carbohydrates, proteins and fats) can significantly affect the composition and the function of the gut metagenome and this study provides some evidence of the impact of healthy agrarian diets to establish effective pathways to prevent diseases.

Keywords
Fecal microbiota; Diet; rRNA DGGE; Gut metagenome

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SELECTED LECTURE

MUCOR SP. NOV. FROM TANGERINE FRUIT AND *MUCOR* SPP. FROM PLANT LEAVES IN KOREA

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Genus *Mucor* consists of approx. 10 species including *M. amphibiorum, M. circinelloides, M. hiemalis, M. hiemalis* f. *silvaticus, M. indicus* and *M. mucedo*. In the course of our survey of indigenous zygomycete fungi in Korea, four isolates of *Mucor*, EML-QT1, EML-CLB03, EML-CH83, and EML-BS5, have been isolated from tangerine fruit, turnip cabbage (known as kohlrabi), cowtail pine leaf and fir tree leaf as saprophytes, respectively. Sequence analysis by BLASTn search indicated that the isolates, EML-QT1, EML-CLB03, EML-CH83 and EML-BS5, were closest to *M. piriformis* (GenBank accession No. JN206031), *M. racemosus* (accession No. KJ589599), *M. mucedo* (accession No. JN206086) and *M. circinelloides* (accession No. KP132465) with identity values of 92.4% (439/475 bp), 100% (500/500 bp), 96.2% (858/891 bp) and 99.8% (535/536 bp), respectively. Especially, the EML-QT1 and EML-CH83 isolates were different from previously described *Mucor* species such as *M. piriformis* and *M. mucedo* in morphology as well as rDNA sequence. The sporangia of EML-QT1 isolate were smaller, reaching 129.3–158.7 μm wide × 136.9–165.4 μm long. The columellae were cylindrical-ellipsoidal, subglobose, and measured 66.8–81.7 × 70.9–86.4 μm in diameter. The columellae of EML-CH83 isolate were variable in shape and globose, subglobose to oval, or irregular, with small collarette. On the other hand, the columellae of the EML-BS5 isolate were subglobse, measured 19.3–23.4 × 19.7–24.1 μm. The sporangia were globose, yellow when young, yellowish brown at maturity, measured 33–67.4 x 35.3–70.8 μm. No zygospores were observed in all the isolates in this medium. Based on the morphological characteristics and sequence analysis of rDNA ITS and 28S rDNA regions, the EML-QT1, EML-CH83 isolates were identified as a new *Mucor* species, forming a separate clade in the phylogenetic tree. The EML-CLB03 and EML-BS5 isolates were identified as *M. racemosus* and *M. circinelloides*, respectively in Korea.

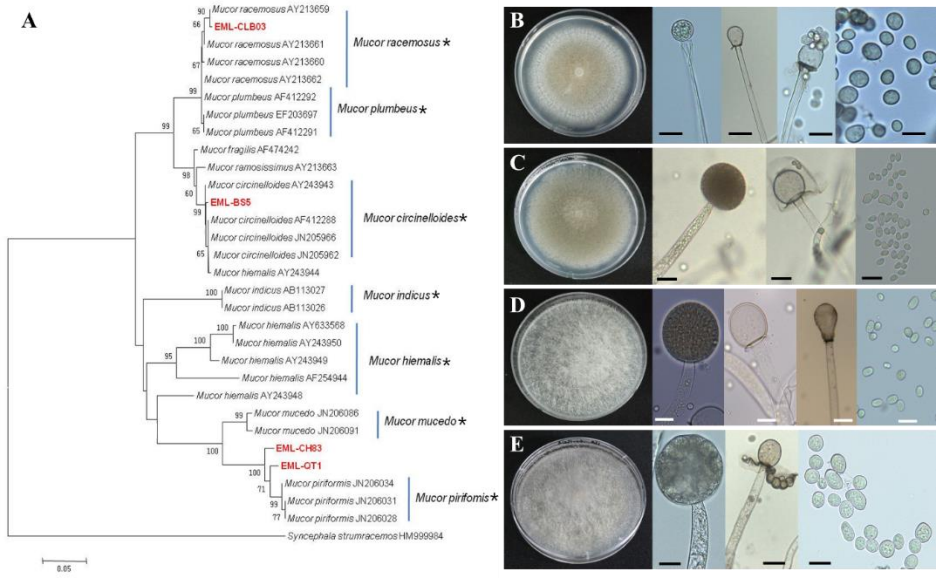

FIGURE1. Phylogenetic status of EML-QT1, EML-CH83, EML-BS5 and EML-CLB03 based on ITS-rDNA (A) sequence analyses, morphological characteristics of *Mucor racemosus* EML-CLB03 (B), *Mucor circinelloides* EML-BS5 (C), *Mucor* sp. EML-CH83 (D), *Mucor* sp. EML-QT1 (E). *Syncephalastrum racemosum was* used as an outgroup. Bootstrap values were shown above branches supported by more than 50% from 1,000 replications. *Classification by Jacobs K. & Botha A. (2008). Sacal bars=50 µm (BD), 20 µm (E).

Keywords

Mucor sp. nov., *Mucor racemosus*, *Mucor circinelloides*

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SELECTED LECTURE

TEMPERATE NATIVE GRASSLANDS WITH DIFFERENT FIRE HISTORIES DIFFER IN THEIR FUNGAL COMMUNITY COMPOSITION

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Introduction

Restoration of native grasses is a primary conservation goal in the highly endangered temperate grasslands of southern Victoria. Frequent, low-intensity fires have proven to promote long-term native grasses productivity, benefit inter-tussock native flora and reduce the potential invasion of exotic weeds (Morgan et al. 1999). However, the effect of repeated prescribed fires on structure and functioning of ecosystems, in particular the below-ground fungal communities, has not been addressed, despite the crucial role played by soil microbial communities and plant symbionts, such as mycorrhizal fungi (Pringle et al. 2009). The aim of our study is to fill this gap by investigating the mechanisms of response of soil microbial communities to different fire regimes.

Matherial and Methods

We investigated the role of Fires Interval (1-2 years, 2-3 years, >3 years) and Time-Since-Last-Fire (<1 month; 3-6 months; 6-18 months; >18 months) to assess direct and indirect effects of prescribed fires respectively, using the Automated rRNA Intergenic Spacer Analysis (ARISA) of PCR-amplified ITS fragments. The compositional state of fungal communities was compared among the sites and visualized using nonmetric multidimensional scaling (nMDS; Bray–Curtis) implemented in the PRIMER-6 software, while SIMPER function was used to more formally determine which variables contributed to the separation between groupings resolved by nMDS.

Results and Discussion

Variation in the soil fungal community structure was strongly linked to fire frequency, but not time-since-last-fire. Although differences in community composition were not significantly related to fire frequency (R = 0.15, p = *0.13*), there was significant minor overlap in fire frequencies of 2-3 yrs and >3 yrs (R = 0.67, p= 0.29). Similarity in community composition increased with decling fire frequency (unfrequently burnt: 59%; medium frequency: 53%; high frequency: 51%). This increasing compositional similarity suggests that when fire frequency exceeds 3 years, the fungal community undergoes biotic

homogenization (Olden & Rooney 2006). The observed compositional shifts are attributable to small changes in relative frequencies of common OTUs and – to a greater extent – changes in frequencies in the less frequent OTUs. Mechanistically, biotic homogenization is likely driven by the change in plant community composition resulting from altered fire regimes. Consistent with the homogenization observed at below-ground level, a shift in the overall vegetation compositional balance occurs following the fire frequency decline, resulting in an increasing plant community similarity (Zeeman et al. submitted). Given the strong specificity of plant-microbe associations among temperate grasslands grass species (Osanai et al. 2013), the loss of plant diversity in the infrequently burnt sites would explain the increased compositional similarity of the associated soil microbial communities.

Conclusions

As frequent fires contributes to the ecosystem resilience and resistance to perturbations, we speculate that fire intervals exceeding 3 years cause a loss of diversity which affects not only the native plants but their microbial counterparts as well. Since soil microbes represent the majority of biodiversity in terrestrial ecosystems and are intimately involved in moderating plant-plant competitive interactions, we suggest that changes in fungal community composition should be taken into account when assessing the impact of fire-mediated land management in temperate grasslands.

Keywords

Biotic Homogenization, fungal ARISA, Community Fingerprinting, Prescribed Burning

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SELECTED LECTURE

ANTICANCER METABOLITES OF ENDOBIOTIC FUNGUS TRICHOTHECIUM ROSEUM *AND ITS BIOCHEMICAL EFFECTS AS A STRONG CANDIDATE FOR MCF-7 BREAST CANCER CELL-LINE*

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Abstract

Seventy-five endophytic fungi were isolated during this study from eight medicinal plants from different altitudes in Saint Katherine Protectorate, South Sinai, Egypt. *Trichothecium roseum* and *Stachybotrys chartarum* recovered from *Achillea fragrantissima* and *Origanum syriacum* subsp. *sinaicum* respectively *were identified* on the basis of its morpho-molecular characteristics by comparing the ITS1 – 5.8S – ITS2 rDNA region sequence data with reference strains data deposited in GenBank. To explore the anticancer activity of *T. roseum* and *S. chartarum* metabolites, taxa have been cultivated on potato dextrose broth for 14 days on a rotary *shaker* at *180* rpm at 28°C, followed by extraction twice with ethyl acetate (EtOAc). The Median Lethal Dose (LD_{50}) of EtOAc and aqueous extracts of *T. roseum* and *S. chartarum* metabolites were 656, 178, 891.251 and 1158 mg/kg b.wt. of laboratory mice respectively. The therapeutic effects of EtOAc and aqueous extracts were evaluated against Ehrlich ascites carcinoma (EAC) cells of seventy female Swiss mice *in vivo*. Evaluated parameters included EAC-bearing mice body weight gain (BWG), tumor volume (TV), median survival time (MST) and percentage increased life span (%ILS). Moreover, their effects on some liver and kidney biochemical parameter and several tumor markers were also investigated. Both EtOAc and aqueous extracts of *T. roseum* significantly decreased BWG and TV, but significantly increased MST and % ILS, 23-27 and 48-71%, respectively. The results showed that *T. roseum* isolated from medicinal plants in arid Sinai is a strong candidate against MCF-7 breast cancer cell line.

Introduction

Endophytic fungi are symbiotically associated biota of living plant tissues without causing any immediate harm to their host (Petrini 1991) and are not host specific (Cohen 2006). Bioprospecting is generally described as the search for naturally occurring chemical compounds and biological material, especially in extreme or biodiversity-rich environments (Abdel-Azeem *et al.* 2012). The aim of this work is directed to survey anti-cancer active

metabolites produced by endobiotic fungi isolated from eight medicinal plants against *Ehrlich Ascites* Carcinoma (EAC) mice model.

Materials and Methods

A total number of 1500 plates were used for isolation of endobiotic fungi from eight medicinal plants by surface sterilization technique (Abdel-Azeem & Salem 2012) on six media after Atlas (2004). Taxonomic identification of fungal isolated using phenotypic characteristics down to the species level was mainly based on the relevant identification keys. Two endophytic fungi: *Trichothecium roseum* and *Stachybotrys chartarum* were chosen to survey their ability to produce potential anti-cancer metabolites. Fungi were grown on PDA medium at 28ºC for 5 days, and then were cultured PDB medium for 14 days at 25 ºC on a shaker at 180 rpm and filtrated. Liquid filtrate and grinded frozen mycelia were extracted with ethyl acetate, and both of aqueous and solvent layers were collected separately. Crude broth extract was obtained under reduced pressure and reconstituted in 5% dimethylsulfoxide in ethanol (v/v). The therapeutic effects of EtOAc and aqueous extracts were evaluated against Ehrlich ascites carcinoma (EAC) cells *in vivo* in which seventy female Swiss mice were divided into 7 groups (10 animals/group). Evaluated parameters included EAC-bearing mice body weight gain (BWG), tumor volume (TV), median survival time (MST) and percentage increased life span (%ILS). Moreover, their effects on some liver and kidney biochemical parameter and several tumor markers were also investigated (Nyland & Mattoon, 2002, Rudloff, 2005).

Results

A total number of 1274 CFU, which were assigned to 75 species, 32 genera and 8 new records to Egypt was isolated during the present study. Ascomycota was represented by 74 species (98.66% of the total isolated species) and only 1 for Zygomycota. Anamorphic Ascomycota came first by recording 45 species and teleomorphic Ascomycota came second by recording 29 species out of 75.

The sequences of the 18s rDNA region partial sequence of the *T. roseum* isolate and *S. chartarum* were 1012 and 1344 pb respectively. The 18S partial sequence data of the isolated *T. roseum* and *S. chartarum* isolates were more than 99% identical with the 18S partial sequence data of the reference strains no. U69892 and KC78690 deposited in GenBank respectively. Both EtOAc and aqueous extracts of *T. roseum* significantly decreased BWG and TV, but significantly increased MST and % ILS, 23-27 and 48-71%, respectively. The four fungal extracts showed anticancer activity more than 30%. Aqueous extract of *T. roseum* came first by recorded a maximum inhibition ratio of 63.89% followed by EtOAc extract (53.48%) without any changes in liver and kidney functions. The tumor markers for breast cancer (CA 15.3), ovarian cancer (CA 12.5), pancreas cancer (CA 19.9), carcinoembryonic antigen (CEA) and alpha-fetoprotein (AFP) were significantly reduced. Results showed that *T. roseum* isolated from medicinal plants is a strong candidate to control MCF-7 breast cancer cell-line and the present work contributed to the inventorying and conservation of fungal endobionts in Egypt.

Keywords

Bioprospecting, Breast Cancer, Conservation, Egypt, Sinai

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SELECTED LECTURE

LINK BETWEEN LACTOBACILLUS PENTOSUS *ADAPTATION TO OLIVE BRINE AND ITS ABILITY TO FORM BIOFILMS*

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Introduction

Table olive fermentation represents a stressful environment for microorganisms. The most adapted bacterial species to this niche are *Lactobacillus pentosus* and *Lactobacillus plantarum* even if the reasons of this ability are not completely understood. In a previous study, *L. pentosus* C11, a resistant strain to olive brine stresses, was mutagenized by random mutagenesis (Perpetuini et al., 2013). Five transposition mutants (*obaD*, *enoA1*, TT*obaAB*, TT*gpi*, TT*obaC*), unable to face olive brine conditions, were identified, allowing the detection of essential genes for *L. pentosus* C11 growth under table olives fermentation conditions. Two of these genes encode metabolic functions (*enoA1* and *gpi*), for the others, no function is predicted (*oba* genes). Since biofilm formation represents one of the main bacterial strategy to survive in stressful environments, in this study, the same strain and its derivative mutants were investigated for adhesion capacity and biofilm formation on olive skin during fermentation by plate counts, confocal microscopy, reverse transcription-quantitative PCR (RT-qPCR) and microbial adhesion to solvents (MATS) test.

Material and methods

Bacterial strains and culture media

Strains were routinely grown at 37°C in MRS medium (Oxoid), with erythromycin supplementation (5 mg/l) when necessary or YG medium (10 g/l yeast extract, 10 g/l glucose) at pH 6.0. The brine-derivative medium, used to grow bacteria in the stressful conditions, was supplemented with 20 g/l glucose and 20 g/l yeast extract (Oxoid), adjusted to pH 6.0, and pasteurized at 65 °C for 45 min.

Olive fermentations process and subsequent bacteria counts

Lactobacillus pentosus C11 (10^7 CFU/ml) was inoculated in sterile glass jars, containing 300 ml pasteurized brine (70 g/l NaCl, pH 4.0) and 300 g of olives. Jars were maintained at 25 °C for 2 months. The planktonic (non-adhering) cells were collected by centrifugation. To collect sessile (attached) bacteria, olive samples (300 g) were placed in one liter Erlenmeyer flask containing 300 ml of PBS and 10 g of glass beads (2 mm diameter) (Biosigma) which was agitated on a shaker at 200 rpm for 1 h at 4 °C to detach microorganisms from olive skin.

Then the liberated bacteria were harvested by centrifugation. Bacteria counts were made on the harvest sessile and planktonic cells by plating serial dilutions on MRS.

Biofilm assay on polystyrene

The ability to form biofilms was monitored by growing the cells (10^7 CFU/ml) in flat-bottom 6 wells cell culture plates (Costar, Corning, NY) containing 5 ml YG medium at pH 6.0 for 72 h as previously described (Kubota et al., 2009).

Microbial adhesion to solvents (MATS) test

The physicochemical characterization of bacterial surface properties was evaluated by the MATS method according to Bellon-Fontaine (1996).

Quantification of bacterial colonization of olive skin

Slices of olive skin (about 1 cm^2) were added in a tube containing 1 ml YG medium or brine-derivative medium and inoculated with *L. pentosus* WT and its mutants (10^7 CFU/ml). Cultures were incubated at 25 °C for 10 days. Then, the olive skin was removed from tubes, washed three times in PBS, placed in tubes containing 0.5 g glass beads (2 mm) and then vortexed for 5 min. Samples of detached cells were serially diluted in saline solution and plated for counting CFU/ml. Three independent cultures were made for each condition.

Confocal microscopy and image analysis

Examinations of biofilms were performed using a confocal laser scanning microscopy (CLSM) (Nikon, model Eclipse TE2000) with a Plan Apochromat VC 100x/1.4 numerical aperture (NA) oil-immersion objective (Nikon). Bacterial fluorescent labeling was carried out using 2 µg/ml of the fluorescent probe 4',6-diamidino-2-phenylindole (DAPI D-9542, Sigma) for 5 min. prior to visualization. Images were recorded using a 408 nm laser diode. Emission signal was collected from 455 to 505 nm. Digital image acquisition and analysis were performed with NIS Elements AR software (version 4.10.01).

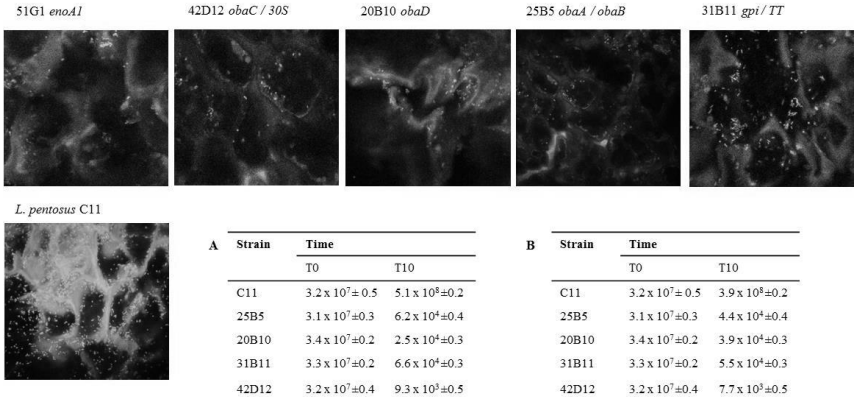
RNA extraction and RT-qPCR analysis

After two months of olive fermentation with *L. pentosus*, planktonic and sessile bacteria were recovered. Total RNA was extracted using the TriReagent (Sigma) as previously described (Licandro-Seraut et al., 2008). RT-qPCR and calculations of relative transcript levels (RTLs) were carried out as previously described (Perpetuini et al., 2013) and the genes *tpiA* and *rpoD* were used as internal calibrators.

Results and discussion

Confocal microscopy and plate counts revealed that sessile state is the main life-style of *L. pentosus* C11 during olive fermentation. The deduced percentage of *L. pentosus* cells adhered to olive skin is 98%, indicating that the sessile state represented the prevailing *L. pentosus* life-style during table olive fermentation (data not shown). The remaining planktonic cells could be considered as detached cells from the biofilm to establish new communities and then to extend the biofilm, as commonly observed (Davey and O'Toole, 2000). Since sessile state was shown to be the main life-style during table olive fermentation, it was of interest to compare the capacity of the WT *L. pentosus* and the *oba* mutants to form biofilms on abiotic surfaces. The mean cell number in biofilms was 2.5 x 10^8 CFU/well for the WT, while it was about 10-fold lower for the three mutants TT*obaAB*, *obaD* and TT*gpi*, and about 1000-fold

lower for mutants TT*obaC* and *enoA1* (data not shown). MATS test showed that this phenotype was probably due to an alteration of cell surface properties (data not shown). The five brine-sensitive mutants were assessed for their ability to form biofilm on olive skin after incubation in either a rich medium (YG) or a brine-derivative medium at pH 6.0. In brine-derivative medium, the WT formed a biofilm on the skin with a cell density of 5.1 x 10^8 CFU/cm^2 after 10 days (Fig. 1), while this aptitude was dramatically reduced for mutants with values ranging from 4.5 x 10^3 CFU/cm^2 to 6.6 x 10^4 CFU/cm^2. Confocal microscopy observation of olive skin in the presence of olive brine after 10 days of incubation revealed that the WT started to colonize olive skin forming a thin, multilayer biofilm with also few cells outside the matrix, while mutants only adhered sparsely to the olive skin (Fig. 1).



A

Strain	Time	
	T0	T10
C11	3.2 x 10^7 ± 0.5	5.1 x 10^8 ±0.2
25B5	3.1 x 10^7 ±0.3	6.2 x 10^4 ±0.4
20B10	3.4 x 10^7 ±0.2	2.5 x 10^4 ±0.3
31B11	3.3 x 10^7 ±0.2	6.6 x 10^4 ±0.3
42D12	3.2 x 10^7 ±0.4	9.3 x 10^3 ±0.5
51G1	3.1 x 10^7 ±0.2	4.5 x 10^3 ±0.5

B

Strain	Time	
	T0	T10
C11	3.2 x 10^7 ± 0.5	3.9 x 10^8 ±0.2
25B5	3.1 x 10^7 ±0.3	4.4 x 10^4 ±0.4
20B10	3.4 x 10^7 ±0.2	3.9 x 10^4 ±0.3
31B11	3.3 x 10^7 ±0.2	5.5 x 10^4 ±0.3
42D12	3.2 x 10^7 ±0.4	7.7 x 10^3 ±0.5
51G1	3.1 x 10^7 ±0.2	6.2 x 10^3 ±0.5

FIGURE 1. *L. pentosus* C11 and its mutants colonization of olive skin in presence of modified olive brine and colony forming units expressed as CFU/cm^2 in brine-derivative medium (A) and YG medium (B).

To investigate the role of *oba* genes in the biofilm formation, *L. pentosus* C11 was used as inoculum for olive fermentation and RT-qPCR was performed to compare the expression of *oba* genes in sessile and planktonic bacteria. *enoA1*, *obaC* and *gpi* genes were upregulated in sessile bacteria by 33, 13 and 9-fold respectively, while no change was observed for *obaA*, *obaB*, *obaD* and *obaE* (Fig. 2).

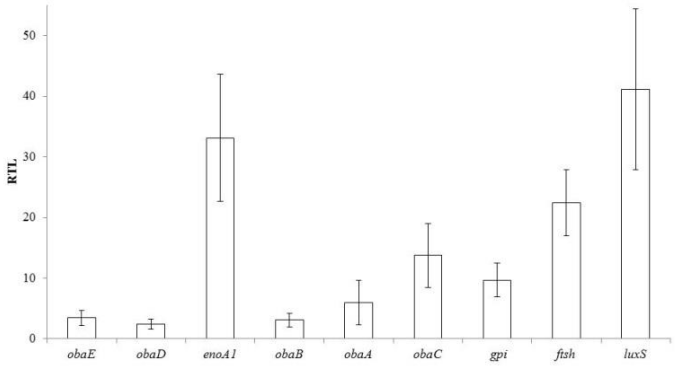


FIGURE 2. Relative transcript levels of *L. pentosus* C11 genes. Transcript levels of each gene are expressed as the relative fold change, with planktonic cells as the reference condition (fold change 1). Four biological repeats were performed, and bars indicate standard deviations.

Conclusions

This study proves that biofilm at the olive surface is the main lifestyle of *L. pensosus* C11 to achieve olive fermentation. The ability of this olive-adapted strain to form biofilm despite the antimicrobial compounds and the hydrophobic epicutilar wax of the olives is probably due to its surface properties and the help of adhesive surface proteins (ObaC, EnoA1, GpiA).

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SESSION II

THE COMPLEXITY OF FOOD ECOSYSTEMS: PHYSIOLOGY OF SINGLE STRAINS IN PURE COLTURE VS. COMPLEX CONSORTIA

PLENARY LECTURE

WOODEN TOOLS: COMPLEX RESERVOIRS OF MICROBIAL DIVERSITY FOR FOOD FERMENTATION

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Food fermentation exists for millenaries (Salque *et al*., 2012). An incredible number of raw material either vegetal or animal can be fermented to increase shelf life and to create diversified flavors. Up to date more than 5000 fermented foods (including fermented beverages) are listed in the world and the daily consumption is estimated between 50 to 400g per day and per capita (Tamang J.P. and Kailasapathy K., 2010). By an empirical approach, only based on their "five senses" and their creativity, our ancestors have created all the kind of fermented foods we know today, which still have a crucial and heartening place in our diet: wine, bread, cheese, fermented milks and butter, vinegar, olives, beer and so on….Most of them have deep cultural and territory roots, and local declensions. However, most of them are for few decades the object of strong industrialization and standardization. Indeed, since Pasteur (1865) and his demonstration of the involvement of "microbes" in the fermentation process, starters were developed to better control it, to avoid defects and to make it more repeatable in order to be able to increase the scale of production and shelf life. Indeed, between 1880 and 1930, in Europe, a rapid urbanization and industrialization occurred changing food demand and ways of production.

However, excellent fermented foods and beverages existed before the use of industrial starters. Unfortunately we tend to forget how elaborated and rich, almost artistic, are the artisanal practices of our ancestors, which were able to manage the microbial diversity, without even suspecting its existence! Most of these artisanal practices involved wooden tools: barrels, mess (kneading machine), vats, spoons, molds, cream separator, hoops, shelves,...Surprisingly the microbial ecology of wooden tools used in food fermentation has been rarely explored, as well as its involvement in the final quality. These last years several publications reported the presence of a rich biofilm on the surface of wooden vats used in dairy fermentations, in particular in French and Sicilian PDO cheeses (Licitra et al., 2007; Lortal et al; 2009; Didienne et al., 2012; Settanni *et al*., 2012; Scatassa *et al*., 2015) as well as the microbial ecology of shelves used in cheese ripening (Mariani et al., 2007). Thanks to recent molecular tools, a large microbial biodiversity was showed on the surface of the vats. Electron or confocal laser microscopy revealed extraordinary images of thick microbial biofilms covering the wood surface (figure 1), biofilm composed of many different species including lactic acid bacteria, (dominating), enterococci, high GC% bacteria like coryneforms, some gram negative; yeasts and moulds (Lortal et al., 2014). When raw milk is placed in the vat, a massive spontaneous inoculation by the vat biofilm occurs in few minutes

as it was demonstrated by putting microfiltrated milk into tina wooden vats (Lortal et al., 2009); the inoculated lactic acid bacteria composing the dominant part of the biofilm, contribute then significantly to the acidification step and the whole biofilm ecosystem to the subsequent ripening. The contact with the vat represents quantitatively an enrichment of the milk by a 'trained' microflora, supporting the affirmation of many cheese makers that wooden vats directly impacts final sensorial qualities and typicity. The microbial ecosystem of a given wooden vat is related to the cheese technology and is different for Salers, Ragusano, caciocavallo Palermito or Vastedda della valle des Belice. This complex ecosystem is stable in time in terms of dominant species, and for each species, several strains co-exist. Interestingly, molecular typing revealed that strains are farm/vat specific. All the authors above underlined the absence or extremely low levels of undesirable microorganisms like coliforms, and the complete absence of pathogens like Salmonella or Listeria. Qualitatively the composition of wooden vat biofilm was deeply explored. From four tinas (wooden vat used in the Sicilian PDO Ragusano)(Licitra *et al*., 2007; Lortal *et al*., 2009), 200 clones of the dominant species *S. thermophilus*, which is also the most metabolically active as shown by RT-PCR-TTGE, were isolated and characterize by pulsed-field gel electrophoresis (PFGE) and Multilocus sequence typing (MLST) in order to assess the number of strains as several are cohabiting inside the same vat. By comparing these isolated strains to 160 other *S. thermophilus* coming from all over the world, it was found that Sicilian Tina strains forms a completely separate cluster (Valence *et al*., unpublished data) and were thus unique with 17 completely new sequence types. Whole genome sequencing of some of these strains will help in understanding their specificity. Some technological properties were explored like the ability to produce antimicrobial compounds and phage resistance. Interestingly they were shown to be at least 4 times more resistant to phages when compared to commercial starters. Since that first attempt, the microbial ecology of several others wooden vats used in pasta filata Sicilian cheeses, from cow and ewe raw milks, were deeply explored (Settanni et al., 2012; Scatassa et al., 2015a; Scatassa et al., 2015b). By 16S DNA sequencing, a total of 16 different lactic acid bacteria (LAB) species were identified at dominating levels in these wooden vats, including *S. thermophilus*, various lactobacilli and Enterococci. 2 to 5 LAB or enterococci species coexist in a predominant within one wooden vat species (depending on the cheese technology concerned), *E.faecium* being systematically present. Clustering of strains was performed by RAPD, and strains belonging to the same species clustered closely. Some technological properties were assess for isolated strains (acidification, diacetyl formation, autolysis, proteolytic activity, and production of antimicrobial compounds). Interestingly, many strains of LAB and Enterococci were shown to produce bacteriocin-like inhibitory substances against pathogens, which thus contribute for sure to the safety of wooden vats. In the last case explored up to date, the Gerle, used in the French PDO Salers (Didienne *et al*., 2012), the predominant lactic acid bacteria were shown to be lactobacilli and leuconostoc, with the presence of yeasts and molds. Again a large biodiversity in the biofilm composition was observed and was correlated with management procedures.

All these molecular descriptions showed that this ancestral system is a reservoir of microbial diversity, obviously safe, and efficient in enriching raw milk. However, beyond these descriptions, many generic questions are still not solved. How the colonization of the wood takes place? How deep is the wood colonized and what is the long term "dialogue" *in situ* between the microbial biofilm and the wood? Is the wood itself, by its own compounds,

contributing to the final safety by inhibiting pathogens (as suggested by Miller *et al*., 1996)? What is the functional implications of the microbial community structure (Smid *et al*., 2014) of this complex spontaneous wood biofilm and its exact contribution to final cheese quality and typicity? As some of these cheese making are not continuous and even can be seasonal, how the biofilm survives without nutriment within the wood? Aand finally, how to optimize the management, cleaning of the wood to reassure safety agencies?

Indeed, despite the absence of pathogens, and the fact that wood has never been documented to be involved in any food borne disease outbreak, despite the invaluable technological qualities of this natural material (renewable, used from immemorial times, available everywhere, resistant, cheap, etc…), the *Codex Alimentarius* does not approve the use of wood in contact with food. The main argument is its irregular surface and porous structure, which make wood difficult to clean. Attempts by the FDA to forbid its contact with food and in particular milk and cheese is in the air. European harmonization is still not done and texts governing his use are mainly national; its use is for example under a provisory authorization in France and Italy at least, as the use of wood is mandatory in several PDO cheeses. Only recently appropriate technics to assess the surface contamination of wood were proposed in the literature (Ismael *et al*., 2014).

In conclusion, more science is urgently needed to reinforce safety and cleaning issues, to better understand the mechanisms underlying the establishment of this natural stable biofilm, the functional balance within the strains and their final contribution to the food typicity and nutritional value (Montel *et al*., 2014). Wooden tools is a source of unique strains for artisanal and PDO cheeses in many European countries. They are also at least absolutely crucial in many small scale fermentations in developing countries (Holzapfel et al., 2002; Motarjemi *et al*., 2012; Nout and Motarjemi, 1997).

For all these reasons, to ban wooden tools for hypothetical safety reasons would be a very detrimental decision. Science has now revolutionary tools to explore and manage microbial diversity (Cocolin and Ercolini, 2015). This is one of the role of scientists to contribute to informed decision. The case of wooden tools is now urgent.

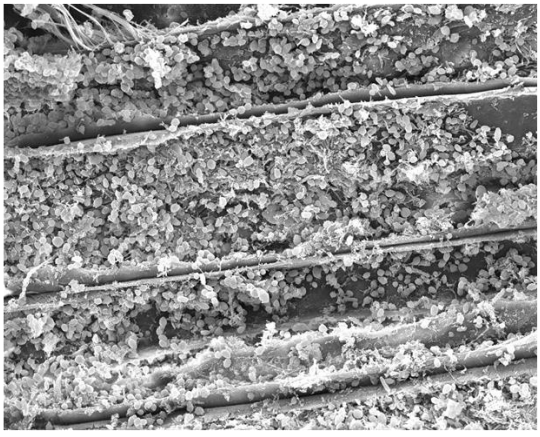

FIGURE 1. Biofilm of Tina wooden vat observed by scanning electron microscopy (from Lortal *et al.*, 2014)

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SELECTED LECTURE

MELATONIN AND TRYPTOPHAN-DERIVATIVES IN WINE: THE YEAST CONTRIBUTION DURING ALCOHOLIC FERMENTATION

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Introduction

Melatonin (N-acetyl-5-methoxytryptamine; MEL) is an indoleamine produced in animals, plants and microorganisms. In animals and plants, it is synthetized from L-tryptophan (TRP) metabolism via serotonin (Chattoraj et al., 2009), and it modulates the circadian and circannual rhythms, reproductive function, bone metabolism and turnover, via cell-receptor-mediated mechanisms. It shows a powerful antioxidant activity directly scavenging the free radical species (both reactive oxygen and nitrogen species) and stimulating the activity of antioxidant enzymes (Reiter et al., 2009). MEL has also been detected in bacteria (Manchester et al., 2000) and yeast (Sprenger et al., 1999) for which little is known on its biosynthesis.

MEL has been found in several foods, including and wine (Iriti et al., 2006; Vitalini et al., 2013), where MEL isomers (MIs) were also detected (Rodriguez-Naranjo et al., 2011; Gomez et al., 2012). Recently, in the attempt to determine the conformation of the most abundant melatonin isomer detected in red wine, we have identified it as tryptophan-ethylester (TEE) (Iriti and Vigentini, 2015). It was reported an increase of MEL and MIS during the alcoholic fermentation (AF) meaning the role of yeast is crucial (Rodriguez-Naranjo et al., 2011; Rodriguez-Naranjo et al., 2012). Investigations are necessary for a better comprehension of MEL synthesis yeast-mediated in enological conditions, including both Saccharomyces and non-*Saccharomyces* strains. For this purpose, the aims of this research were the screening of Saccharomyces and non-*Saccharomyces* strains in laboratory conditions and the monitoring of melatonin and its isomers in oenological conditions.

Material and methods

Nine yeasts were screened: *Saccharomyces cerevisiae* EC1118, IOC18-2007 and UMY255, *Torulaspora delbrueckii* CBS1146^T, UMY196 and UMY336, *Zygosaccharomyces bailii* ATCC36947^T, UMY991 and UMY598. To assess the production of MEL, its MIs and TEE by the yeast species, strains were cultivated in YNB medium containing 20 and 100 mg/L TRP, as precursor. Growth tests were performed in at 25°C in static condition inoculating the

cells in exponential phase at approximately 0.2 OD. Experiment lasted up to 6 days monitoring cell count, MEL, MIs and TEE production at 0, 24, 48, 72, 96 and 144 h. The experimental musts were prepared with two varieties of white (Chardonnay and White Muscat) and red (Merlot and Croatina) grapes grown in Piemonte (Italy). Before inoculation, musts were pasteurized at 100°C for 1 min by autoclave. Tryptophan content was assayed prior and after the thermal process to determine if pasteurization could affect its initial concentration. When necessary, ammonium sulfate and/or TRP were added to must. The must were inoculated at about 10^6 CFU/mL from yeast culture pre-inoculated in YPD. Melatonin, TRP and TEE were determined by UPLC-MS/MS analysis carried out on an Acquity UPLC separation module (Waters) coupled with a triple quadrupole mass spectrometer mod. Quattromicro (Waters). The mass spectrometer was operating in the electrospray ionization positive mode and the fragmentation transitions were $(m/z)^+$ 233→174, 159 and 131 for MEL, MIs and TEE, and $(m/z)^+$ 205→188, 146, 118 for TRP.

Results and discussion

Production of MEL, MIs and TEE was preliminary assessed by inoculating yeasts in a chemically defined medium. All the tested strains produced TRP derivatives, although with different kinetics and concentrations per biomass (Figure 1). Most strains showed increased levels in these metabolites at the highest TRP concentration (100 mg/l) in the medium. Only few strains, belonging to non-*Saccharomyces* species, accelerated the TRP derivative formation with the increasing of TRP concentration in the medium. The highest MEL production was detected in the exponential phase of growth, between 24-48 h from the inoculation. The only exception was *T. delbrueckii* UMY336 which showed an increased level in MEL accumulation after 72 h, at the early stationary phase. *Z. bailii* ATCC36947T released the highest amount of MEL (37.2 ng/10^9 cells), followed by *S. cerevisiae* UMY255 (14.1 ng/10^9 cells) and *T. delbrueckii* CBS1146T (9.5 ng/10^9 cells). The strain *Z. bailii* UMY991 e *Z. bailii* UMY598 did not synthetize melatonin in our experimental conditions. Two MIs, MI1 and MI2, were produced by cells at the lowest concentration of TRP (20 mg/l) in the medium (Figure 1). As previously observed for MEL, MI1 and MI2 concentrations increased with the increasing in TRP between 24-48 h from the inoculation. TEE was synthetized at any TRP concentration and increased with the increasing of the amino acid concentration in the medium and following a species- and strain-dependent behavior. *S. cerevisiae* strains showed a peak of TEE in the supernatant 24 h after inoculation. As reported for MEL and MIs, after reaching the maximum amount, TEE production decreased.

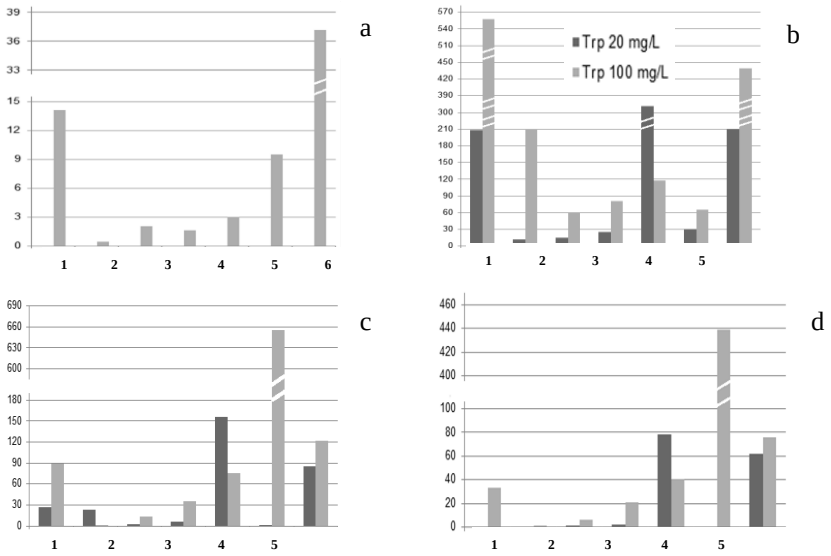

FIGURE 1. Concentrations of (a) MEL, (b) TEE, (c) MI1 and (d) MI2 in ng/10^9 cells. 1: *S. cerevisiae* UMY255; 2: *S. cerevisiae* EC1118; 3: *S. cerevisiae* IOC 18-2007; 4: *T. delbrueckii* UMY196; 5: *T. delbrueckii* UMY336; 6: *T. delbrueckii* CBS1146^T; 7: *Z. bailii* ATCC36947^T.

After a preliminary screening, three strains (*S. cerevisiae* EC1118, *T. delbruekii* CBS1146^T and *Z. bailii* ATCC36947^T) belonging to different species were selected for further determination of TRP derivative production during alcoholic fermentation. MEL, MI1 and MI2 were not detected in musts before the inoculations. On the contrary, TEE was found at very low concentrations ranging from 0.07 ng/mL to 0.33 ng/L except for Chardonnay must where it was not detected. During alcoholic fermentation, MEL, MI1 and MI2 were not produced by all the selected yeast strains in our experimental conditions. However, yeasts were all able to accumulate TEE up to 2.78 ± 0.39 ng/10^6 cells in Chardonnay inoculated with *T. delbruekii* CBS1146^T. Interestingly, a new compound was detected (RT 2.33 min) only in enological condition.

Conclusions

The results obtained in this work have confirmed that *S. cerevisiae* is able to release TRP derivatives (MEL, MIs and TEE) (Sprenger et al., 1999; Gomez et al., 2012; Rodriguez-Naranjo et al., 2012). Furthermore, we have shown, for the first time, that also non-*Saccharomyces* yeasts are able to produce MEL and other TRP derivatives. In particular, the levels and the types of these metabolites varied greatly, depending on the yeast strain and cell growth environment. In our oenological conditions, yeasts were unable to produce the same indoleamines (i.e. MEL and MIs). However, some yeasts synthesized high concentrations of a new MI and TEE in fermented musts. Further investigation will need to clarify the yeast pathways involved in MEL metabolism and the enological steps and conditions potentially effecting MEL, MIS and TEE synthesis in wine.

Keywords
Melatonin, Melatonin Isomers, Yeasts, Wine

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SELECTED LECTURE

ECOLOGICAL ENGINEERING OF MEAT MICROBIAL ECOSYSTEMS: FACTORIAL DESIGN OF COMPLEX MEAT PRESERVATIVE CULTURES FOR SPOILAGE REDUCTION

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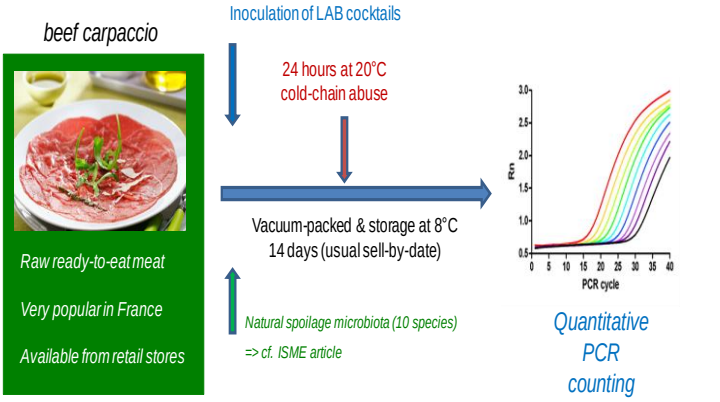
Introduction

The microbial spoilage of meat products with short shelf lives is responsible for a significant amount of food waste in Europe. Understanding this phenomenon could help food scientists to build ecological engineering strategies for improving the sustainability of this type of food. Meat spoilage is a complex ecological process which needs some theoretical input and a greater focus on ecological questions before new conceptual approaches to control the process could be done. Our recent work showed that meat spoilage involves poorly characterized bacterial communities (1). This study has significantly clarified the questions about the level of richness and evenness of these microbial ecosystems. Nevertheless, our ability to understand the mechanisms underlying *who does what* in the genesis of spoilage is weak.

Strategy

From this first analysis, we have moved forward to successfully perform spoilage-controlled ecological engineering. More precisely, to overcome the challenge of complexity, our strategy was based on using a specific species among those found on the natural meat bacterial ecosystems and to widely assess its intra-species diversity. Beef carpaccio was chosen as meat model. This meat product is traditionally eaten raw but due to a thin slicing, making a product with high contamination surface, beef carpaccio is a very edible product highly sensitive to spoilage. In a previous study we have shown that packaging may influence the microbiota of beef carpaccio (2). However, this analysis based on culture-dependent method revealed to be somewhat biased as few species among the most abundant ones were likely undetected (1). Furthermore, *lactobacillus sakei*, one of the most abundant species using culture-dependent method revealed to be a sub-dominant population using quantitative metagenomic analysis. This species which is known to harbor a wide intra-species diversity (3) and a potential for controlling the beef meat ecosystem toward lower spoilage (4) could then be used as a good candidate for testing ecological hypotheses of strains consortia versus single strains.

Or strategy is summarized in **Figure 1: Carpaccio Meat Model Used for the Experimental design of Testing Protective Cultures.** Based on quantitative metagenomic experiments, analysis were performed to evaluate bacterial richness and evenness describing the spoilage phenomena. Specific Bio-markers were designed to follow specifically the top10 species identified in beef carpaccio meat by quantitative PCR. We followed the spoilage microbiota naturally found in each carpaccio sample. Then, Factorial design was used to build various *L. sakei* strain consortia and to generate lineage-admixted synthetic microbial communities for ecological theory testing (second part of the figure). Plackett & Burman screening factorial design for evaluating the influence of genotypic-dependent *L. sakei* Strains Consortia. Strains X1 to X7 were originating from the three intra-species lineages lineages (3) (red= lineage 1; green=lineage2; blue=lineage 3).



	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Q
N_1	+1	+1	+1	-1	+1	-1	-1	...
N_2	-1	+1	+1	+1	-1	+1	-1	...
N_3	-1	-1	+1	+1	+1	-1	+1	...
N_4	+1	-1	-1	+1	+1	+1	-1	...
N_5	-1	+1	-1	-1	+1	+1	+1	...
N_6	+1	-1	+1	-1	-1	+1	+1	...
N_7	+1	+1	-1	+1	-1	-1	+1	...
N_8	-1	-1	-1	-1	-1	-1	-1	...

- 3 factorial designs = 21 strains studied in 21 cocktails of 4 strains
- Several repetitions = in total ~280 samples were analyzed and ~1400 measurements performed

Results

We hypothetized that a combination *Lactobacillus sakei* strains covering the three phylogenetic lineages would be more robust over storage time than one superstar strain. The screening has revealed unequal relative efficiency of strains towards the growth inhibition of spoilage microbiota with some strains having clear positive effects on growth inhibition, while others showed poor or even spoilage stimulating power. Four strains were selected and blended together to give a new combination. The new consortium of 4 strains was evaluated again. This analysis has revealed a strong synergic effect of the strains yielding a very efficient cocktail. The effect of reducing the growth capacity and final cell density of spoiling microbiota had clear visible effect on the beef carpaccio pieces of meat. Our result show that only specific combinations of strains are suitable for efficient spoilage control, meanwhile other combinations lead to unchanged spoilage status of the meat. This example will be useful for understanding what drives bacterial ecosystems toward the setting of spoilage or towards the preservation of meat. It will also be useful for extracting and comparing from these data functional differences between strains from synthetic consortia versus those occurring naturally. Our work is paving the way to a more rational, ecologically-based, design of complex preservative cultures.

Keywords

Biopreservative culture, meat spoilage, metagenomic, strains consortia, food spoilage ecosystems.

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SELECTED LECTURE

UNDERSTANDING THE BACTERIAL COMMUNITIES OF HARD CHEESE WITH BLOWING DEFECT

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Introduction

Hard cheese production require long ripening time creating the optimal conditions for spoiling microorganisms able to survive in the food matrix. Late blowing in cheese caused by butyric clostridia is a well-fitting example (Ingham et al., 1998; Klijn et al., 1995; Vissers, 2007). Different clostridial species alone or in association, have been related during time to the blowing problem, but few data are available about their dynamic changes in the cheese shape and their relationships all along the ripening period. In Italy, Grana Padano (GP) hard cheese is produced from raw cow's milk added with natural whey starter cultures and protected from clostridia spoilage by lysozyme addition (www.granapadano.com). The aim of the present work was to assess the microbial communities of spoiled hard cheese using new NGS technologies associated to quantitative and qualitative cultivation-independent techniques. These approaches have not yet been applied for the study of microbial communities involved in late blowing spoilage. Respectively, a PCR-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) approach to obtain a qualitative characterization of clostridia heterogeneity, a TaqMan qPCR on single *C. tyrobutyricum* species and an NGS approach based on Illumina MiSeq sequencing of total bacteria were applied to 83 Grana Padano cheese samples from nine production facilities, with or without the addition of lysozyme and with blowing defects appearance at different ripening times. Information gathered from this study could be useful to assess the effect of lysozyme as a preservative and to measure the effects of ripening time on clostridial population and their relationships with other bacterial species present in the cheese paste.

Materials and Methods

A total of 83 samples of hard cheese with different ripening times (1-23 months), positive or negative to lysozyme, and showing anomalous pastry defects and cavities were collected from the Grana Padano cheese production area in Northern Italy. Total bacterial DNA was extracted for each cheese sample using the bead-technology based FastDNA® SPIN kit and the Fast-Prep® Instrument. A 16S-based *Clostridium* cluster-I specific PCR and DGGE analysis was based on a 235 bp fragment amplification specific for the order of Clostridiales. Denaturing gradient gel electrophoresis (DGGE) was performed on the amplified fragments using an INGENY phorU-2 DGGE system. Only products migrating as a single band, were

PCR amplified, purified and sent to a commercial sequencing facility. The sequences were blasted in RDP database and in the GenBank using the NCBI BLAST program. The optimised TaqMan qPCR protocol for *C. tyrobutyricum* enumeration previously described (Bassi et al., 2013) was applied to all cheese samples. Then, a high-throughput sequencing approach was used on 40 selected samples (20 Lys- and 20 Lys+ samples) with different ripening time and clostridia composition. The TruSeq™rDNA sample preparation kit was applied for the amplicon library preparation, while the sequencing reaction was performed with a MiSeq Illumina instrumen with V3 chemistry, generating 300 bp paired-end reads. Sequences were then analysed with Mothur v.1.33.0 (Schloss et al., 2009). Downstream sequence analyses were then performed using both the operational taxonomic unit (OTU) and the taxonomy based approach.

Results and Discussion

Clostridium cluster I-specific amplicons were detected in 72 out of 83 (87%) defected cheese samples and in 1 out of 8 control unspoiled cheeses. Clostridia distribution in cheese samples with blowing defect was mainly affected by the presence or absence of lysozyme, while rare correlations have been found with the ripening times. In the 56 samples where lysozyme was added to milk, we found most frequently *C. butyricum* (50% of the samples) followed by *C. tyrobutyricum* (30%), *C. perfringens* (23%), *C. sporogenes* (21%) and *C. septicum* (5%). On the contrary, in the 35 cheeses made without lysozyme, *C. tyrobutyricum* was found to be the prevalent species in the majority of samples (77%) and, in most cases, it was the only detected species. Real-time quantitative PCR was applied for *C. tyrobutyricum* enumeration; in cheese samples manufactured with lysozyme, 45% were positive to *C. tyrobutyricum* with counts ranging from 2.0 to 9.2 log CFU/50 g. A higher prevalence of this species was observed in cheese without lysozyme (83% of samples) where only 6 samples were negative and counts varying from 2.0 to 8.9 log CFU/50 g. A total of 20 samples with lysozyme and 20 without lysozyme were randomly selected, DNA extracted and 16S rRNA genes amplified with universal primer for Bacteria. No significant differences according to ripening time, lysozyme or defect were found for all analyzed indexes. The eight most abundant bacterial genera found in the analyzed cheese samples with and without lysozyme, which account for 95% of the total bacterial populations, were *Lactobacillus* (65.3%), *Streptococcus* (14.4%), *Clostridium* (9.54%), *Brevibacterium* (1.5%), *Enterococcus* (0.97%), *Staphylococcus* (0.96%), *Acinetobacter* (0.77%) and *Chryseobacterium* (0.5%). Hierarchical clustering based on the abundance of bacterial genera, revealed three main clusters: cluster A, composed by 13 samples, was characterized by the highest proportion of sequences assigned to the *Clostridium* genus and a relevant presence of *Streptococcus* sequences, ascribed to *Streptococcus thermophilus*; cluster B where the genus *Lactobacillus* was predominant in 20 samples; cluster C, composed by three samples, where more than 50% of the sequences were assigned to *S. thermophilus*.

A crucial step for the reduction of cheese blowing defects caused by clostridia is to investigate the community of these anaerobic sporeformers and their ecological relationships with the other members of the cheese microbiota (Doyle et al., 2015). Results obtained with *Clostridium* cluster I-specific PCR-DGGE analysis provided a qualitative picture of the dominant clostridia biodiversity and suggested that clostridia, in case of blowing defect, are nearly ubiquitous members of the Grana Padano cheese ecosystem and are strictly related to the spoilage event. In general, outputs of NGS data regarding clostridial communities were

in accordance with those obtained by PCR-DGGE analysis even targeting different 16S hyper-variable regions and with a major resolution and quantitative power for the NGS approach. Analyses of NGS data defined three main clusters, the first (Cl-A) characterized by the dominant presence of *C. butyricum*, the second (Cl-B) by a co-presence of *C. butyricum* and *C. tyrobutyricum* and the third (Cl-C) by a higher prevalence of *C. tyrobutyricum*. Moreover, although the two analytical methods were not totally comparable, the quantitative data obtained from qPCR on *C. tyrobutyricum* were in accordance with the results of NGS analysis. In addition, *Clostridium* cluster I-specific PCR-DGGE data demonstrated that *C. tyrobutyricum*, previously described as the main responsible of hard cheese spoilage (Klijn et al., 1995; Le Bourhis et al., 2005), was the most frequent species, hosted in the 50% of total samples. Lysozyme, rather, seemed to influence bacterial distribution both in terms of *Clostridium* and *Lactobacillus*, the two most abundant genera, together with *Streptococcus*, found in the analyzed cheeses. Among clostridia, *C. tyrobutyricum* was negatively affected by lysozyme. Recent experiments in milk and RCM medium proved that lysozyme was particularly effective in limiting *C. tyrobutyricum* cells and spores growth respect to other clostridia (Avila et al., 2014). Otherwise, *C. butyricum* resulted the most prevalent species when lysozyme was added to milk. This observation confirmed the in vitro data (Avila et al., 2014) of high resistance of *C. butyricum* to this additive. Our data, based on both genus specific DGGE and NGS approaches revealed that *C. butyricum* is a common component of the clostridial population involved in late blowing of hard cheese. *C. sporogenes*, which was detected in approximately 20% of the analyzed samples, always associated to *C. butyricum* or *C. tyrobutyricum* and present in low amounts, seemed to be independent on lysozyme addition. Shifts in the *Lactobacillus c*ommunity were also observed in the

presence of lysozyme for both bacterial species of the primary lactose fermentation and NSLABs. An increase in the proportion of *L. delbrueckii* and obligate heterofermentative NSLABs was detected in the presence of added muramidase, while in its absence *L. helveticus* and other NSLABs, such as *L. rhamnosus*, *L. casei* and *L. buchneri*, were more abundant. Several ecological relationships were also found. Most of the samples from cluster Cl-C, characterized by a high abundance of *C. tyrobutyricum*, showed also the dominance of *S. thermophilus* and *L. rhamnosus*. A possible relationship between *S. thermophilus*, *L. rhamonosus* and *C. tyrobutyricum* could be explained by the effect on germination produced by L(+)lactate an end product of lactose metabolism by the first two bacteria that, alone or in association with amino acids, such as alanine, is the most effective germinant for *C. tyrobutyricum* (Bassi et al., 2009). Differently from other clostridia detected in cheese, *C. tyrobutyricum* is able to use lactate as energy source. This species metabolizes both D(-) and L(+) racemic forms of lactic acid, although D(-) lactate acid is more rapidly dissimilated to butyric acid when cells grow in RCM medium (Huchet et al., 1997). The analysis of the *C. tyrobutyricum* genomes (Bassi et al., 2013c; Jiang et al., 2013) revealed the presence of genes coding for both D and L lactate dehydrogenases and of an L-lactate permease. All the samples dominated by *C. butyricum* (cluster Cl-A) were also clustered in Lb-A, where *L. delbrueckii* was the most abundant *Lactobacillus*. Based on the energetic metabolism, it cannot be explained why the presence of *L. delbrueckii* relates to a higher prevalence of *C. butyricum*.

Conclusions

Our data indicated that in hard cheese presenting the typical signs of late blowing, cells of spoiling clostridial species coexisted with the LAB populations. DNA-based approaches indicated a correlation between the inhabitant LAB, both natural starters and NSLAB, and the prevalence of different species of clostridia. Moreover, our study showed that the use of lysozyme, added to affect spore germination and the vegetative cell outgrowth, shaped the species composition of the cheese bacterial communities of both LAB and butyric clostridia.

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SELECTED LECTURE

FOODMICROBIONET: A TOOL FOR THE VISUALISATION AND ANALYSIS OF THE STRUCTURE OF BACTERIAL FOOD MICROBIAL COMMUNITIES

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Introduction

High-throughput sequencing (HTS) targeting the 16S RNA gene or 16S rRNA has become the main approach for the culture independent analysis of microbial communities. It requires the extraction and partial purification of microbial DNA or RNA from foods, HTS of variable regions of the target using one of the several platforms available, analysis of the raw sequences by a variety of bioinformatic pipelines to identify Operational Taxonomic Units (OTUs) and for the presentation and analysis of abundance data using a variety of statistical tools. Although deposit of the sequence projects in public databases makes the raw data of such studies readily available, their analysis requires significant bioinformatic skills and computing resources, and no tool has been developed yet for the analysis and presentation of OTU abundance data obtained in different studies. Network analysis tools are increasingly being used for the presentation of OTU-sample networks and microbial interaction networks. In the first case, two types of nodes (sample and OTUs) are connected by edges representing abundance of OTUs in a given sample. Network analysis software (such as Cytoscape or Gephi) is then used to create information rich displays which efficiently allow to capture the similarity relationships among food samples and to identify core and sample or food group specific communities. Although a variety of node or network specific statistics may be used to characterize the topology of networks from different studies, these have been rarely calculated in food microbial ecology studies. Microbial abundance data can also be used to infer microbial co-occurrence and co-exclusion relationships, thus providing insight into the network of positive (commensalism, mutualism) and negative (competition, amensalism, parasitism) that shape microbial communities. This work describes the development of a data

repository and network analysis tool for the rapid exploration and analysis of bacterial food microbial communities data. The tool, which we named FoodMicrobionet, is made publicly available using an interactive interface at http://www2.unibas.it/parente/fmbn1_0web.

Materials and methods

Data included in the current version of FoodMicrobionet (1.0) include published studies on cheese and starter cultures (Ercolini et al., 2012; De Filippis et al., 2014; De Pasquale et al., 2014a, 2014b; Dolci et al., 2014), fermented milks (Marsh et al., 2013), raw and fermented meats (De Filippis et al., 2013; Greppi et al., 2015), sourdoughs (Ercolini et al., 2013; Rizzello et al., 2015), and olive fermentation (Cocolin et al., 2013). In addition, data from unpublished studies on undefined starters, fresh and ripened cheeses, sourdoughs and fresh meat products were also included. Most sequence data were obtained using a single platform (Roche Life Sciences 454 Junior) and processed using a common software (QIIME versions 1.6.0 or 1.8.0) and pipeline (de novo UCLUST pipeline for OTU picking, with RDP and Greengenes database for taxonomic assignment) in a single sequencing centre (Department of Agricultural Sciences, University of Naples Federico II) using the V1-V3 region of 16S DNA or cDNA as a target. OTU abundance tables were used to build edge (with edge weight representing the abundance of an OTU in a given sample) and node (with metadata for both OTUs and samples) tables, which were imported in Gephi 0.8.2 beta. Nodes statistics (degree, i.e. the number of samples in which a given OTU is found or the number of OTUs for a given sample; weighted degree, i.e. the abundance of a given OTU in a given sample; abundances summed to 100 for each sample node) were calculated and styles were applied to enhance the display: the colour of the node was attributed on the basis of a custom field containing families for OTUs and Food subgroup for samples; the size of the nodes was made proportional to the weighted degree of the node; edge thickness was made proportional to the weight of the connection. A Yfan Hu force based layout algorithm was applied to highlight similarities among food samples and to identify core and sample-specific communities. Simplified versions of the networks were obtained by filtering. The whole network was then exported for web visualisation using the Sigmajs exporter plugin of Gephi. Microbial interaction networks were inferred for selected groups of samples using the Conet (v1.0.7beta) app of Cytoscape 3.2.1 and topological properties of the networks were calculated using the Network Analyzer tool. Finally, Systat 13 was used for graphical analysis of selected properties of sub networks.

Results and discussion

The current version (1.0) of FoodMicrobionet includes 552 sample and 964 OTU nodes, with 18,115 OTU-sample interactions, and is by far the largest repository of data on bacterial communities in foods. The database can be used to rapidly extract information on food bacterial communities by exploiting the metadata in the nodes table. Examples are presented in Figure 1.

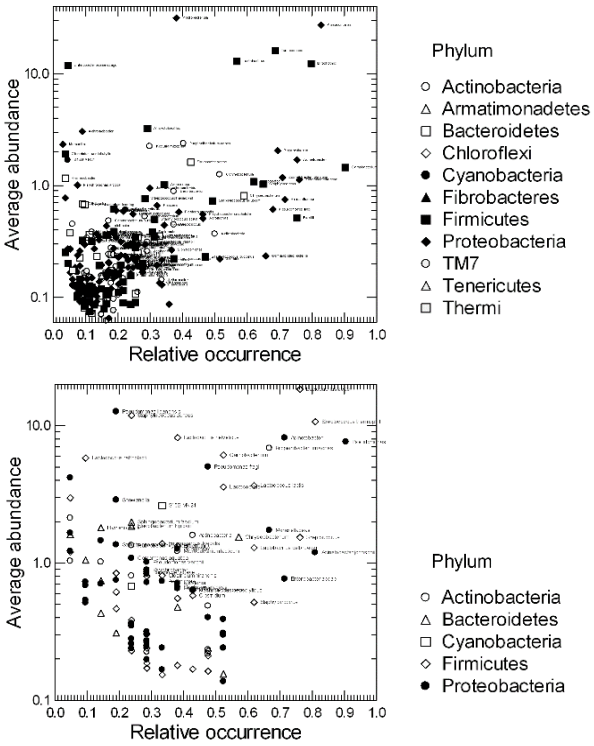

FIGURE 1. Average abundance (%) as a function of relative occurrence (an indication of how frequently an OTU occurs) in raw meat (left) and raw milk (right) samples extracted from FoodMicrobionet 1.0. Only OTUs occurring at a total abundance ≥1% are shown.

The network, built with Gephi or Cytoscape can be exploited by experienced user to create a variety of visualisation and to analyse the properties of sub networks extracted by filtering. Some examples are available at http://www2.unibas.it/parente/wordpress/?page_id=978. Even inexperienced users can exploit the web based visualisation to explore of the OTU-sample relationships and identify core, food type and sample specific microbial communities. Additional search tools and hyperlinks can be used for the rapid selection of food groups and operational taxonomic units and for the rapid access to external resources (NCBI taxonomy, digital versions of the original articles). Microbial interaction network analysis on selected datasets showed that the complexity (in terms of network size, average path length and modularity) of OTU-OTU networks increased with the complexity of the microbial community. It was lowest for kefir and for undefined starters and fresh cheeses, increased in surface ripened cheese, and was largest for raw meat samples. However, it was lower than that found in other microbial communities (human microbiome, soil and other environmental microbial communities).

Conclusions

While the combination of several datasets in a single repository is appealing, both the network visualisations and the meta-analyses should be interpreted with some caution. In fact, differences from sample handling through DNA extraction, variable regions of the 16S rRNA gene chosen as target, library preparation, sequencing technology, sequencing depth / sample coverage and bioinformatics path chosen for the analysis can have a strong impact on detection and abundance of OTUs. However, with these limitations in mind, FoodMicrobionet still provides significant advantages to the scientific community and to the food industry by making available a large set of curated data on the occurrence of different taxa in foods, facilitating the process of writing original articles and reviews and providing information for food process development.

Keywords

Food microbial communities; High-throughput sequencing; Network analysis; Microbial interaction networks.

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SELECTED LECTURE

MOLECULAR AND FUNCTIONAL DIVERSITY OF LACTIC ACID BACTERIA AND YEASTS CHARACTERIZING SOURDOUGH TUSCAN BREAD

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Introduction

Sourdough Tuscan bread is traditionally produced using type I sourdough, characterized by a spontaneous fermentative process based on backslopping. Sourdoughs are very complex biological ecosystems where lactic acid bacteria (LAB) and yeasts interact, often establishing stable associations. The typical sourdough LAB species are *Lactobacillus brevis*, *Lactobacillus pontis*, *Lactobacillus reuteri*, *Lactobacillus plantarum* and *Lactobacillus sanfranciscensis* which represents the species most frequently isolated. Several yeast species are also found beyond *Saccharomyces cerevisiae*, such as *Kazachstania exigua*, *Candida humilis*, two maltose-negative yeasts known to form a stable mutualistic association with *L. sanfranciscensis*, which is able to hydrolyze maltose (De Vuyst *et al*., 2014). The diversity of sourdough microbial communities depends on process technologies, types of flour and other ingredients traditionally associated with local culture and origin. Such diversity is at the basis of differential metabolic products, affecting nutritional, organoleptic and technological traits of baked goods. The aim of this study was to analyse the molecular and functional properties of the sourdough microbiota used to produce Sourdough Tuscan bread.

Material and methods

The sourdough analysed in this study was obtained by the Consortium "Sourdough Tuscan Bread" (Consorzio Pane Toscano a Lievitazione Naturale, CPT). 10 g of sourdough samples were homogenized in a stomacher bag containing 90 mL of saline peptone water for 2 min at 260 rpm. LAB were counted by plating on mMRS and on SDB supplemented with cycloheximide (100 mg/l). Yeasts counts were carried out on WL Nutrient and YEPD supplemented with chloramphenicol (100 mg/l). Colonies were randomly selected, purified by streaking four times onto the same medium used for isolation and maintained at –80 °C in 20% (v/v) glycerol. Isolates were analysed by molecular and functional methods. DNA from isolates and reference strains (*Lactobacillus panis* DSMZ 6035, *L. sanfranciscensis* DSMZ 20451, *Lactobacillus fermentum* DSMZ 20052, *L. brevis* DSMZ 20054, *L. plantarum* IMA B23, *Lactobacillus curvatus* IMA LB51, *S. cerevisiae* ATCC 32167, *Dekkera bruxellensis* IMA 1L, *C. humilis* DBVPG 6753, *K. exigua* DBVPG 6956) was extracted by "MasterPure™ Yeast DNA Purification Kit" (Epicentre®). LAB DNA was amplified using 27f and 1495r primers and digested with the restriction endonucleases *Alu*I, *Hinf*I and *Hae*III,

while yeast DNA was amplified using ITS1 and ITS4 primers and digested with the *Hinf*I and *Hae*III enzymes. The isolates and reference strains were grouped by comparing their restriction patterns and identified by sequences analysis.
For a functional characterization of isolates, phytase and protease activities were assessed using qualitatively methods on agar plates. To test phytase activity, yeasts were grown on PSMG medium containing 4 g/L Na-phytate, while the same medium added with 5% of fresh yeast extract at pH 5.6 was used for LAB (Jorquera *et al.*, 2008). To eliminate false positive results plates were counterstained using cobalt chloride (Bae *et al.*, 1999). Protease activity of yeasts and LAB was assessed on YED and mMRS containing 2% of skim milk, respectively. Yeast amylase activity was tested on YEP medium containing 1% soluble starch, pH 6.46 at 30°C using Lugol staining (Osimani *et al*., 2009). Sourdough microbial diversity was also investigated by PCR-DGGE, as described by Palla et al. (2015). This technique was also used to evaluate the effect of the manufacturing environment on sourdough microbiota, comparing the PCR-DGGE profiles obtained from the same sourdough maintained by different bakeries (Consortium "Sourdough Tuscan Bread") located in Quarrata, PT (A), Livorno (C), Casore del Monte, PT (D) along with that (B) maintained in our laboratories (DAFE, University of Pisa). These sourdoughs were refreshed using the same wheat flour type and the same protocol provided by the CPT procedural guideline.

Results and discussion
LAB and yeasts counts were 10^9 and 10^7 cfu/g, respectively, consistently with previous data reporting that the yeasts/LAB ratio in mature sourdoughs is generally 1:100. A total of 130 yeasts and 386 lactic acid bacteria were isolated in pure culture. Among them, 96 lactic acid bacteria and 68 yeasts were selected and characterized by Amplified Ribosomal DNA Restriction Analysis (ARDRA), RFLP analysis of the internal transcribed spacer regions (ITS) and sequence analysis. 16S rDNA sequence analysis identified all LAB isolates as *L. sanfranciscensis*, although ARDRA analysis detected a polymorphism within 16S rDNA gene. The combination of the three different ARDRA profiles allowed us to group the isolates into three ribotypes. One profile (49% of the isolates) corresponded to *L. sanfranciscensis* DSMZ 20451, a second profile (50% of the isolates) to *L. sanfranciscensis* DSMZ 20663, as reported by Foschino et al. (2001), while a third profile represented by only one isolate, was different when *Hinf* I was used. Among yeasts, 96% of the isolates were identified as *C. humilis* and only 4% as *S. cerevisiae.* Our data are consistent with previous findings on type I sourdoughs microbial communities. Interestingly, in our sourdough samples we identified only *L. sanfranciscensis* contrary to what found in other Tuscan bread sourdoughs, where this LAB species occurred in association with *Lactobacillus paralimentarius* (Bozza Pratese) and *Lactobacillus gallinarum* (Pane di Altopascio tradizionale) (Minervini *et al*., 2012). Among yeasts, *S. cerevisiae* is the most commonly species retrieved in sourdoughs from Central and South Italy. By contrast, *C. humilis* prevailed in our samples, in agreement with findings on Pagnotta del Dittaino PDO sourdough (Gullo *et al*., 2003), rye flour sourdoughs (Meroth *et al*., 2003) and traditional Italian sweet baked products sourdoughs (Lattanzi *et al*., 2013).
A preliminary screening of functional abilities of our isolates showed that the three strains identified as *S. cerevisiae* exhibited phytase, amylase and protease activities. Among *C. humilis* strains, 50% showed protease activity, while only 6% and 2% was able to solubilize

phytate and starch, respectively. Only one *C. humilis* strain showed all the activities tested. The ability to solubilize phytate was detected in 19% of LAB strains, while none was able to digest casein. Our data suggest that amylase activity is widespread among *S. cerevisiae* and occasionally found in *C. humilis*.

Microbial diversity of CPT sourdough, investigated by PCR-DGGE, confirmed the occurrence of *L. sanfranciscensis* associated with *C. humilis* and *S. cerevisiae* (Fig. 1). Moreover, the absence of microorganisms in the VBNC state was revealed. The same molecular method was used to compare microbial populations of CPT sourdough maintained in four different bakeries. PCR-DGGE profiles showed the presence of the same yeast and LAB communities in three sourdoughs (A, B and D). On the contrary, the fourth sourdough (C) showed different profiles. In particular, LAB communities were characterized by the absence of *L. sanfranciscensis* and by the presence of *Weissella cibaria*/*W. confusa*, *L. alimentarius*/*L. paralimentarius* and *L. pontis* (Fig. 1a). Yeast community was characterized only by *S. cerevisiae* (Fig. 1b).

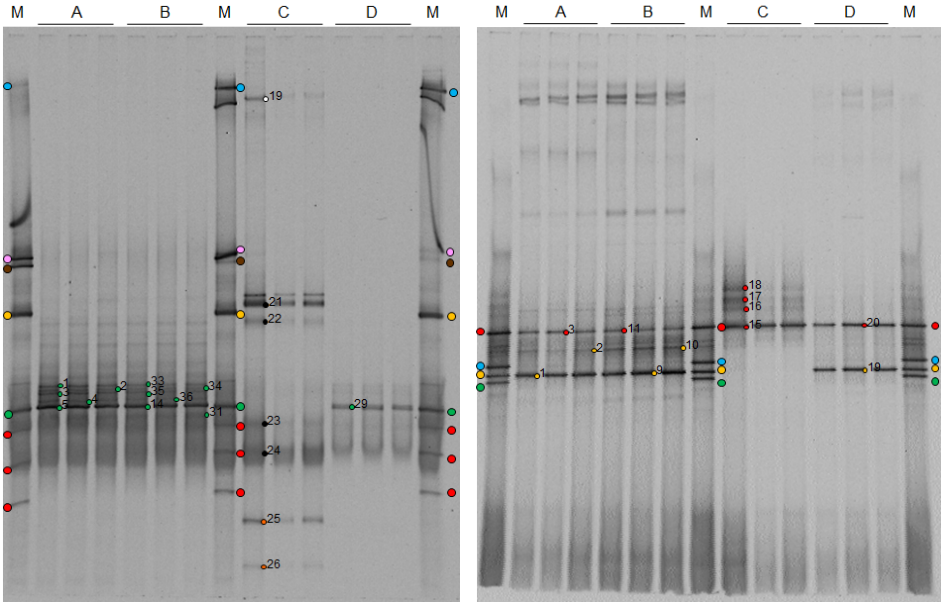

Figure 1. DGGE profiles of sourdough samples from different Tuscan bakeries (A, B, C, D). **(a)** LAB Marker (M): *Lb. plantarum* IMA B23, *Lb. brevis* DSMZ 20054, *Lb. curvatus* IMA LB51, *Lb. fermentum* DSMZ 20052, *Lb. sanfranciscensis* DSMZ 20451, *Lb. panis* DSMZ 6035. Numbered fragments were sequenced and colour indicates the sequence homology from GeneBank: *Lb. sanfranciscensis*, *Lb. pontis*, *W. cibaria/W. confusa*, *Lb. alimentarius/Lb. paralimentarius*. **(b)** Yeast Marker (M): *S. cerevisiae* ATCC 32167, *K. exigua* DBVPG 6956, *C. humilis* DBVPG 6753, *D. bruxellensis* IMA 1L. Sequenced fragments are marked with numbers and the colour indicates the sequence homology from GeneBank: *S. cerevisiae*, *C. humilis*.

Our results suggest that sourdough microbial communities structure remains stable when sourdoughs are refreshed using the same wheat flour type and the same protocol provided by the CPT procedural guideline. The different composition of yeast and LAB communities found in sourdough C suggests that changes in the procedure adopted by the bakery C may

have occurred. In conclusion, our findings demonstrate that PCR-DGGE represents a rapid and reliable tool to detect the correct maintenance of microbial communities characterizing Tuscan bread sourdough, resulting from an accurate application of the CPT protocol.

Keywords
Sourdough Tuscan bread, *Lactobacillus sanfranciscensis*, *Saccharomyces cerevisiae*, *Candida humilis*, PCR-DGGE.

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SPECIAL SESSION II

Young Researchers

SELECTED LECTURE

CHARACTERIZATION OF YEAST FLORA OF "HURMA" OLIVES USING MOLECULAR METHODS AND MID-IR SPECTROSCOPY

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Introduction

Among the olive varieties in Turkey, Erkence olives, grown in nearby area around Karaburun Peninsula of Izmir, go through a natural debittering phase on the tree during its ripening. As a result of this phase, the olives lose their bitter taste while still on the tree and have a dark brownish color in the inside and a wrinkled outer layer which are their differentiating appearance characteristics from olives that do not undergo this process. This naturally debittered olive type is known by the name of Hurma (Aktas et al., 2014). According to an old study performed in Greece with a similar type of olive, the debittering process was attributed to the action of a fungus, Phoma olea,which hydrolyses oleuropein, a bitter phenolic compound of olives (Kalogeras, 1932). There is no study in the literature related to the characterization of yeasts on this unique type of olive, Hurma. Until present, the characterization of yeasts associated with table olives has been made through biochemical and morphological methods, using the taxonomic keys (Kurztman and Fell, 1998). More recently, molecular methods and FTIR spectroscopy using chemometric techniques have been used for the identification of yeasts due to being rapid, easy and more precise methods for yeast identification. In order to understand the role of yeasts in maturation and debittering process of natural Hurma olives, characterization of olive yeasts from two olive types, Hurma and Gemlik, an olive variety which is commonly consumed as table olive, was aimed using molecular methods and mid-IR spectroscopy in comparison with cultural methods.

Materials and methods

Two different types of olives as well as their leaves were used in the analyses: Gemlik (GO) and Hurma (HO) olives, leaves of Gemlik (GL) and Hurma (HL) olive trees. Hurma olives were hand-picked from an olive orchard which is located in Karaburun Peninsula of Izmir, while an orchard in Izmir Institute of Technology campus area which is 30 km south of the first orchard was the place where Gemlik type was obtained. All olive and olive leaf samples were obtained during 8 weeks of maturation period from the end of October until the beginning of December for the two harvest years (2011 and 2012).

For characterization of yeast isolates, at first some macroscopic and microscopic morphological analyses and physiological tests were performed. Assimilation of nitrogen compounds by yeast isolates was also evaluated. Cultural identification was based on

established schemes of Kurtzman and Fell (1998). After DNAs of yeast cells were isolated, the amplification reactions were performed using ITS1 and ITS4 primers and for some unidentified species using NL1 and NL4 primers. After purification of PCR products, DNA fragments were sequenced using a BigDye Terminator Cycle Sequencing system (version 3.1, Applied Biosystems, Taiwan) according to the manufacturer's instructions.
The yeast colonies of olive samples were used in FTIR analysis after sub-culturing on agar media at 28°C for 48 h. All yeast species were scanned through an FTIR spectrometer (Perkin Elmer Spectrum 100, Wellesley, MA), having a horizontal attenuated total reflectance (HATR) accessory. The data from the FTIR spectrometer was analysed by using multivariate statistical techniques with SIMCA software (SIMCA P-10.5 Umetrics Inc. Sweden).

Results and discussion

In this work, totally 182 yeast strains were isolated, purified and evaluated using cultural, molecular methods and their spectra were obtained with FTIR spectrometer. Following the evaluation of the yeast strains by cultural methods and taxonomic key, amplification reaction was performed for all yeast isolates of both harvest years using ITS primers and for ITS-unidentified strains using NL primers. A total of 46 yeasts were identified in the first harvest year using molecular methods and the most identified yeast species in the first year were *Metschnikowia sp.* (18/46, 39%), followed by *R. mucilaginosa* (13/46, 28%) and *D. hansenii* (6/46, 13%). The distribution of HO yeasts (29/46) in this year were as mostly *Metschnikowia sp.* (17/29, 59%), followed by *D. hansenii* (6/29, 21%). In the second harvest year, a total of 136 yeasts were identified by molecular methods. *Aureobasidium sp.* was the most commonly found yeast in the second year both among all olive types (106/136, 78%) and on HO (19/25, 76%). Besides, yeasts different from the first year were also identified in the second year, including *Cryptococcus sp.* (6/136, 4.4%), *Sporidiobolus sp*. (6/136, 4.4%), *Wickerhamiella sp*. (4/136, 3%) and *Pseudozyma sp.* (3/136, 2.2%). When HO of two harvest years were compared in terms of yeast types it could be observed that only *Aureobasidium sp.* was the common yeast isolated during both years.
PCA of the FTIR data of olive yeasts were performed using the second derivative of the both full (4000-650 cm^{-1}) and partial (3030-2830 cm^{-1}, 1350-1200 cm^{-1} and 900-700 cm^{-1}) spectral regions. PCA results revealed that the best results for clustering were obtained for the species of *Metschnikowia* sp., *Aureobasidium* sp. and Candida sp. for the first year; whereas in the second year the best results for clustering were for the species of *Wickerhamiella sp.*, *Cryptococcus sp*. and *Aureobasidium* sp. It was concluded that characterization of yeasts using FTIR was a successful complementary method for molecular techniques.

Conclusion

This is the first study in the literature defining the yeast flora of HO that might be responsible for natural debittering on the tree by using molecular methods and FTIR spectroscopy in comparison with cultural methods. Comparison of yeast flora of HO for both harvest years revealed that there might be a link between natural debittering of HO on the tree and the yeast types. When the results of this study are compared with previous researches, it seems that the yeast flora of Hurma and Gemlik olive samples is more like Greek olive cultivars; however different yeast species from literature were also observed in HO.

Acknowledgement
This study was funded by Scientific and Technological Research Council of Turkey (TUBITAK-TOVAG Project No: 110O780).

Keywords
Molecular methods; mid-IR spectroscopy; yeast; characterization; hurma olive

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SELECTED LECTURE

THE USE OF FT-IR SPECTROSCOPY AND ITS SEQUENCING AS USEFUL TOOLS FOR STRAIN DEREPLICATION IN MEDICAL ENVIRONMENT

COLABELLA Claudia (1)*, ROSCINI Luca (1), TIECCO Matteo (1), CORTE Laura (1), TASCINI Carlo (3), VU Duong (4), MEYER Wieland (5), ROBERT Vincent (4), CARDINALI Gianluigi (1,2)

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Introduction

One of the basic questions in microbiology is how to tell that an isolate is an independent strain or just a replica of other known strains (Essendoubi et al., 2005). The introduction of modern molecular and spectroscopic techniques promises a huge increase in the "taxonomic resolution". At the same time a theoretical question raises: how different must two strains be to be considered different? "**Dereplication**" is the complex of analytical and interpretative steps deployed to assess the difference between isolates and to determine which group of identical isolates represents a strain (Roscini et al., 2010). On the other hand, an effective dereplication discriminates between strains considered identical. The efficacy of dereplication depends on the variability, on the independence of the employed markers and on their processing with bioinformatics tools (Carriconde et al., 2011). The assessment of the statistical probability of identity between two strains description and the development of high-throughput analytical pipelines are essential conditions to apply dereplication efficiently in the medical environment.

Strategy

The aim of this work was to improve the ability of the ITS barcode to discriminate between *Candida* isolates and strains, isolated in several wards of two different Italian Hospitals (Pisa and Udine). This approach was taken in order to investigate on the variability within cluster of isolates with identical ITS sequences. The ITS clusters were obtained using UPGMA tree based on the ITS distances of these isolates/strains. The internal heterogeneity of these clusters was investigated by FT-IR spectroscopy and analyzed via R statistical software. Example is presented in Figure 1.

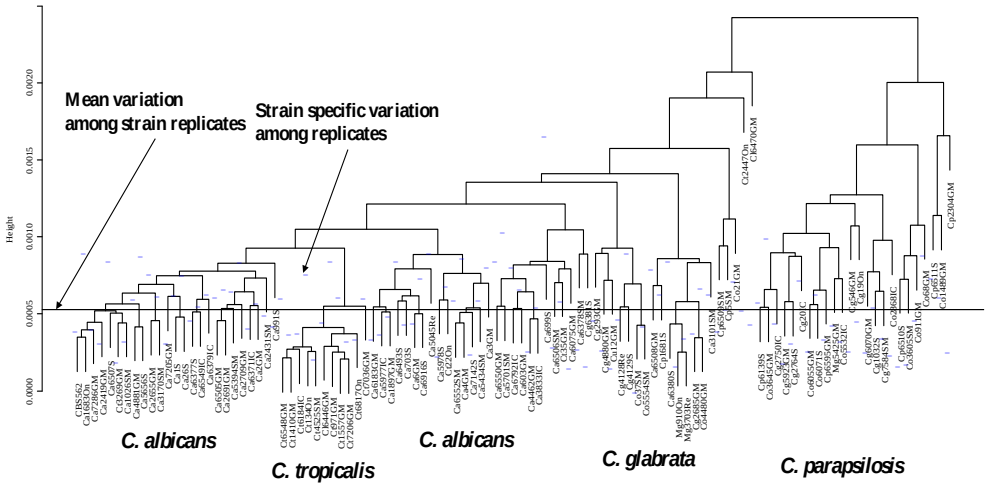

FIGURE 1. FT-IR cluster analysis of typing region A [700-800 cm^{-1}] of *Candida* strains isolated from Udine Hospital.

Results

The results showed that the isolates sharing the same ITS sequence could be separated with different degrees of statistical significance, giving the opportunity to medical microbiologists to track really identical isolates within the same ward and hospital, in order to assess their origin and spread-out. These findings point out the importance of the simultaneous use of molecular biology, spectroscopy and statistical analysis to define if a new isolate is just a replica of a known strain or a new strain.

Keywords

Strain dereplication, Medical environment, ITS cluster, FT-IR heterogeneity analysis.

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SELECTED LECTURE

BACTERIAL COMMUNITIES' DYNAMICS AND INTERACTIONS DURING POULTRY MEAT STORAGE TO IMPROVE FOOD QUALITY AND SAFETY

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Introduction

Meat products host a large diversity of microbial communities that can vary depending on seasonal changes and production processes. Bacterial contamination occurs mostly at the surface and on the skin during slaughtering processes (Luber, 2009). Bacteria contaminating meat originate from animal microbiota (feces, hide, skin, feathers), from plant environment (air, equipment, surfaces) and from human manipulators. Meats may encompass pathogenic or spoilage bacteria (Doulgeraki et al., 2012) which must be controlled to ensure safety and quality of the products. Then, processing steps and storage conditions shape the dynamics of this bacterial community. Effects of different treatments (temperature, chemicals decontaminations, marinating, or different preservation processes) have been studied in order to find strategies for fighting human pathogenic or spoilage species. For that purpose, challenge-tests are usually performed by inoculating food matrices from one batch with single strains or cocktails encompassing few strains that do not represent the natural and variable bacterial communities hosted by meat products. We developed a protocol to describe and study bacterial communities dynamics during meat products shelf life.

Material and methods

Chicken legs stored under different modified atmosphere packaging (MAP) commonly used in France, and from various origins were collected from supermarkets and stored at 4°C. Whole bacterial populations were collected at use-by-date (UBD) or 2/3 UBD and stored frozen at -80°C as aliquots. Bacterial diversity was determined by cultural methods on 23 samples and by 16S rDNA pyrosequencing on 10 out of the 23 samples. Frozen bacterial communities were inoculated on fresh meat and stored under two different MAP (with or without oxygen). Bacterial populations were monitored by cultural methods during 10 days of storage. DNA and RNA were extracted for metagenomic and metatranscriptomic analysis to evaluate genes and functions expressed in this complex food ecosystem.

Results and discussion

Total viable counts present on chicken legs varied among samples (10^3 - 10^8 CFU/g). Plating methods showed that lactic acid bacteria (LAB), *Brochothrix thermosphacta*, and *Pseudomonas* spp. were dominant. Data of 16S rDNA pyrosequencing (Figure 1) confirmed the presence of *B. thermosphacta,* and revealed that *Pseudomonas* was mainly represented by *P. extremaustralis* and *P. cedrina*. The dominant LAB were *Carnobacterium* and *Shewanella* species according to previous results (Nieminen et al., 2012). The predominance of *Pseudomonas* was correlated to meat packaging under high oxygen concentration, except when *B. thermosphacta* was dominant, suggesting a competition between these species (Figure 1). Interactions between different food spoilage bacteria such as *Pseudomonas* and *Shewanella* were previously reported (Gram et al., 2002).

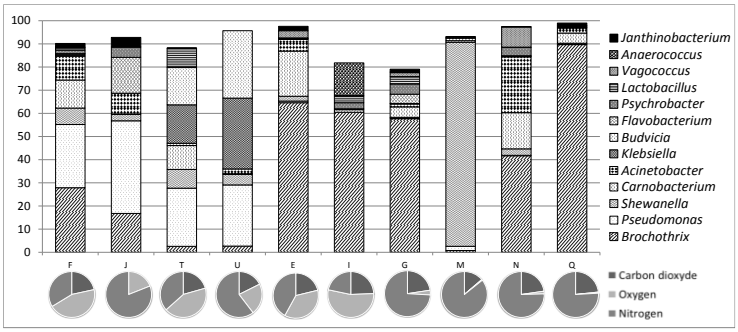

FIGURE 1. Relative abundance of observed genus (at least 0,1% of total reads) for 10 samples of chicken legs stored at 4°C until UBD. Modified atmosphere packaging compositions were reported for each sample.

Two bacterial communities with predominance of either *B. thermosphacta* or *Pseudomonas spp.* were used in reproducible challenge tests on meat. Bacterial populations stored frozen were able to colonize meat samples and overgrow the initial natural contamination. Metatranscriptomic and metagenomic analyses are in progress to evaluate the impact of storage atmosphere on these bacterial population dynamics and on the functions they express, and to propose optimized storage conditions of poultry meat.

Conclusion

An accurate method was developed enabling reproducible challenge-tests experiments on natural bacterial communities of poultry meat, for metatranscriptomic and metagenomic analysis of the influence of storage parameters on bacterial behavior.

Keywords

Food safety, Poultry meat, Microbial communities, Metagenomic, Metatranscriptomic.

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SELECTED LECTURE

ADMINISTRATION OF LACTOBACILLI AND BIFIDOBACTERIA ON APIS MELLIFERA L. *BEEHIVES TO INCREASE HEALTH OF THE BEE SUPER-ORGANISM*

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Introduction

In the last decades, honeybees have been afflicted by different kinds of biotic and abiotic stresses (pathogens, climate changes, pesticides), posing a serious threat to the agricultural field and natural ecosystems (Klein et al., 2007; Aizen et al., 2009). New sustainable strategies to help bees are envisaged, also considering the EU ban of antibiotics in beekeeping. The use of commensal bees gut microorganisms and their related secondary metabolites to re-establish the worn-out gut microbiota and control diseases spread are more and more taken into consideration. This work was aimed at the administration to bees of *Lactobacillus* and *Bifidobacterium* strains, previously isolated from healthy honeybee gut and possessing antimicrobial activity against bee pathogens (*Paenibacillus larvae*, *Melissococcus plutonius* and *Nosema ceranae*), in order to check their effect on health status and wellness of the beehive superorganism. Moreover, the microbial gut community of control and treated bees was evaluated.

Material and methods

Six strains deriving from bee gut (*Bifidobacterium asteroides* DSM 20431, *B. coryneforme* C155 *B. indicum* C449, *Lactobacillus kunkeei* Dan39, *L. plantarum* Dan91 and *L. johnsonii* Dan92) were mixed in a sucrose sugar solution 1:1 (w:v) and applied in field. The preparation was sprayed weekly over the frames during the four weeks preceding Linden honey flow on nine hives. Further nine hives were selected as control for a total of 18 beehives monitored. Both treated and control hives were standardized according to health status and genetic potential prior to the experiment beginning (Audisio and Benítez-Ahrendts, 2011). Gut samples, randomly collected among workers bees from both treated and control hives, were processed for microbial honeybee gut community analysis by qPCR, PCR-DGGE and NGS (IlluminaMiSeq sequencing).

Results and discussion

Improved honey, pollen and brood production were observed in treated hives, associated with interesting change in the microbial gut community. Linden stored honey was 59% higher than the control at the end of the blooming, 1 month after the end of the treatments. The brood

extension was registering an increase of 46% and the stored pollen an increase of 53%. These results are consistent with existing literature on probiotic application on honeybees, and can have a strong economic impact in the beekeeping sector, which is greatly suffering from bee collapse and decline. qPCR analyses, targeting *Bifidobacterium* and *Lactobacillus* spp., evidenced a slight but significant increase in treated samples. The cluster analysis on the PCR-DGGE profiles, targeting eubacteria, showed two distinct clusters, as a result of a shift in the microbial community between treated and control hives. Three major bacterial species were found, following band excision and sequencing: *Giliamella apicola*, *Snodgrassela alvi* and *Commensalibacter intestinii*. *G. apicola* and *S. alvi* represent important bee endosymbionts and are known to dominate honeybee gut microbiota together with lactobacilli. *C. intestinii*, which is particularly evident in the treated group, belongs to the family Acetobacteraceae, which have recently gained great attention for its functional role within insect gut. PCR-DGGE targeting lactobacilli showed few differences between the two groups and excision of relevant bands led to the identification of six species (*L. kullabergensis*, *L. kimbladii*, *L. apis*, *L. melliventris* and *L. kunkeei*), which are commonly isolated from the honeybee gut. PCR-DGGE targeting bifidobacteria is in progress. Interestingly, the results obtained by NGS showed some intriguing results. Beside the increase in *Bifidobacterium* levels, a significant increase (200%) in the Acetobacteraceae members was observed, while a slight decrease in the *Lactobacillus* genus was detected. The observed increase in *Bifidobacterium* levels is not surprising, as the administered probiotic cocktail contained three bifidobacteria. The slight decrease of members of the genus *Lactobacillus* can be explained considering that only *L. kunkeei* is a dominant species in the bee gut microbiota, whereas *L. johnsonii* and *L. plantarum* are less abundant. Unexpectedly, the observed increase of the Acetobacteraceae in the treated group could be considered a promising result since many members of the family have recently emerged as important endosymbionts for honeybees whose roles can range from metabolic to immunomodulation (Crotti et al., 2010).

Conclusions

The current work contributes to moving the probiotic concept within the insect world with a deep investigation of the bee gut microbiota. The designed bacterial treatment on honeybees led to an improvement of the hive products associated with relevant changes in the bee gut microbiota. These favorable results are promising for the beekeeping sector, which need new sustainable technologies to counteract pests and support honeybee health.

Keywords

Bifidobacterium spp., *Lactobacillus* spp., honeybees, PCR-DGGE, IlluminaMiSeq sequencing

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SELECTED LECTURE

A NEW ZYGOMYCETE SPECIES AND TWO NEW RECORDED FUNGI FROM DOKDO, KOREA

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Dokdo is located in the northeastern part of Ulleungdo, extremity of Korea and known as volcanic island. In total, 47 fungal isolates were isolated from a soil sample collected from the island, by dilution plating method. The isolates were identified on the basis of morphological characteristics on PDA and MEA media and rDNA ITS sequence analysis. The major genera includes *Absidia*, *Aspergillus*, *Bionectria*, *Cunninghanella*, *Fusarium*, *Metarhizium*, *Mortiellera*, *Mucor*, *Penicillium*, *Stemphylium* and *Trichoderma.* The % sequence identity (the number of matches/the complete alignment length) values via NCBI BLAST searching of EML-DDSF4, EML-MF30-1 and EML-IFS45-1 represented 100% (350/350) with *Mortierella oligospora* (GenBank accession no. JX976032), 98.3% (472/480) with *Metarhizium guizhouense* (GenBank accession no. HM055445) and 92.1% (456/495) with *Absidia* sp. (GenBank accession no. JQ683214), respectively. The EML-IFS45-1 isolate was identified as a new *Absidia* species belonging to Mucoraceae. In addition, three species of *M. oligospora* and *M. guizhouense* represented new records in Korea. The fungal diversity from Dokdo soil and their molecular phylogenetic status will be discussed in the presentation.

Keywords
Metarhizium guizhouense, *Mortierella oligospora*, *Absidia* sp. nov., Dokdo soil

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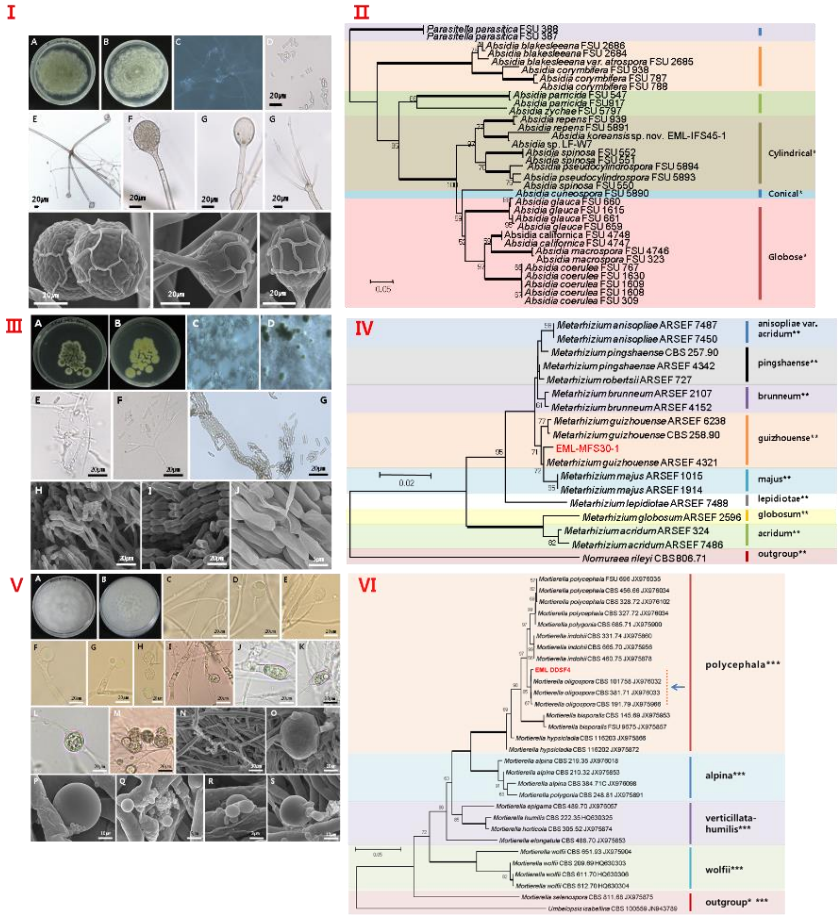

FIGURE 1. Morphology of *Absidia* sp. nov. EML-IFS45-1 (Ⅰ), *Metarhizium quizhouense* EML-MFS30-1 (Ⅲ) and *Mortierella oligospora* EML-DDSF4 (Ⅴ). Neighbor-Joining tree of alignment of the rDNA ITS sequence of *Absidia* sp. nov. EML-IFS45-1 (Ⅱ), combined data set of ITS rDNA and BT2 sequences of *Metarhizium guizhouense* EML-MFS30-1 (Ⅳ). *Mortierella oligospora* EML-DDSF4 (Ⅷ) isolate aligned SSU rDNA, ITS rDNA, and LSU rDNA sequences and related species from GenBank databases. *Parasitella parasitica* FSU 388 (Ⅱ), *Nomuraea rileyi* CBS 807.71 (Ⅳ) and *Umbelopsis isabellina* CBS 100559 (Ⅵ) were used as outgroup, respectively. Bootstrap values over 50% were shown at the above branches supported by 1,000 replications. * Classification by Hoffmann et al. (2007), ** Classification by Bischoff et al. (2009), *** Classification by Wagner et al. (2013) and Tamas *et al*. (2011).

SELECTED LECTURE

INFLUENCE OF INTRAPARTUM ANTIBIOTIC PROPHYLAXIS AGAINST GROUP B STREPTOCOCCUS *ON THE EARLY NEWBORN GASTROINTESTINAL COMPOSITION*

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Introduction

The first gut microbial population plays a critical role in the health of the host contributing to the development of the immunity, host response to pathogens, bioavailability of nutrients and host metabolic activity. Scientific evidences suggest that microbial dysbiosis of the intestine of the newborn can be directly correlated with several gut diseases (Di Gioia et al., 2014; Rodriguez et al., 2015). It is well known that colonization in the early days after birth is influenced by several factors (Fouhy et al., 2012), however, the administration of antibiotics to the mother during labor, referred to as intrapartum antibiotic prophylaxis (IAP), has not received much attention, although this practice is routinely used in group B *Streptococcus* positive women to prevent the infection of newborns. A recent study showed a significant reduction of bifidobacteria in newborns born to mothers subjected to IAP with respect to untreated mothers using qPCR on DNA extracted from feces (Aloisio et al., 2014). The advent of Next Generation Sequencing (NGS) has determined a revolutionary approach in the study of complex microbial community such as the human intestinal microbiota. In particular, it has allowed to reveal much about its structure and function giving a high throughput data, not allowed by other approaches. Recent studies analyzed the critical points that could affect the efficiency of NGS analysis, in particular, the choice of the primers used for amplifying the 16S rDNA target regions can lead to a selection of specific components of the intestinal microbiota at the expense of others (Milani et al., 2013). This work is aimed at evaluating, for the first time, the main effects of IAP on the whole microbiome composition of newborns at seven days after birth using a parallel sequencing targeted to seven different regions of the 16S rDNA gene.

Material and methods

The study was conducted on 20 newborns, 10 of them born by mothers positive to GBS and treated with 2 g of ampicillin at least 4 h before delivery (IAP group), whereas the other 10 were born to mothers negative to GBS (control group). Fecal samples of newborns were collected at 7 days after birth and used for DNA extraction using the QIAamp DNA Stool Mini Kit, with a slight procedure modification (Aloisio et al., 2014). Sequencing was performed with Ion PGM platform. Ion 16S Metagenomics kit was used to amplify the 7 different 16S hypervariable regions (V2,V3,V4,V6+7,V8,V9) to be sequenced. Obtained

data were analyzed using the Ion 16S Metagenomics Analyses module implemented in the Ion Reporter tool. Cluster analysis and biodiversity index were then calculated.

Results and discussions

The taxonomic identification carried out highlighted the presence of four different phyla in all samples (Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria), showing not homogeneous distributions between the two groups (IAP and control) and significantly different values of relative abundance. Particularly, Actinobacteria and Bacteroidetes were more represented in control group, while Proteobacteria were prevalent in IAP group. Moreover, a different distribution of Gram positive and Gram negative bacteria in the two groups reflect a selective trend correlated to the antibiotic treatment with a higher abundance of Gram negative microorganisms within the IAP group. This suggested that IAP impaired the colonization of Gram positive microbial groups, such as bifidobacteria and lactobacilli, which are known to exert a beneficial effect on the host. Even family composition displayed a different picture of relative abundance between the two groups, with a substantial larger amount of bacterial families in the control group compared to IAP one. These findings were supported by computation of two biodiversity indices (Chao1 and Shannon). In parallel, taking advantage of the sequencing of seven hypervariable regions of 16s DNA gene (V2,V3,V4,V6+7,V8,V9), it was possible to evaluate the efficiency of each primer pair and each region in describing the microbiota composition. The percentage of mapped reads was not homogenous among each hypervariable region. In particular the regions with the higher percentage of mapped reads were V2, V3 and V6+7 which represented >60% of the total amount of mapped reads. On the other hand, regions V4, V8 and V9 contributed to ~30%. In addition, the percentage of mapped reads was evaluated for each phylum detected by each amplified region, to compare their informative power. The elaboration of these data revealed that the V4 and V6+7 regions seemed the most accurate both for the number of assigned reads for sample and for classification resolution, while the other regions seemed to underestimate the diversity content.

Conclusions

In conclusion, the overall experimental outcomes showed that IAP has a notable impact on gut microbiota of newborn reducing microbial biodiversity and allowing a strong colonization of Proteobacteria and reducing Actinobacteria. Moreover, the innovative technical approach allowed us to conclude that the different hypervariable regions assayed differently explored the variability that characterize the intestinal microbiota.

Keywords

gut microbiota, intrapartum antibiotic prophylaxis**,** newborns, NGS, 16S rDNA gene

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SELECTED LECTURE

GENETIC DIVERSITY OF BROCHOTHRIX THERMOSPHACTA *AND FOOD SPOILAGE*

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Introduction

Understanding and controlling food spoilage represent today a scientific, economic, and ecological challenge: approximately 1/3 of food production is lost every year (Gustavsson *et al*., 2011) mainly due to microbial spoilage. Bacteria responsible for spoilage are diverse and their spoiling ability is strain-dependent and depending on the type of food, storage and processing conditions and on bacterial communities sharing food environment. *Brochothrix* the second genus belonging to the *Listeriaceae* family, with *B. thermosphacta* as the main species is a psychrotrohic and ubiquitous bacterium. It has been highlighted in many food matrices and is consistently associated with the spoilage process. *B. thermosphacta* is considered as one of the dominant organisms associated with spoilage of chilled meat and seafood products stored aerobically, vacuum packaged or under modified atmosphere. The result of its activity on these products is the production of metabolites responsible for the appearance of off-odors such as acetoin (Dainty and Mackey, 1996). Although this species was isolated from a wide variety of food matrices, few studies have focused on its genotypic diversity and on the genetic function involved in spoilage development.
The aim of this work was to evaluate the intraspecies diversity of *B. thermosphacta* isolates, issued from various ecological origins (meat, poultry, fish and environment).

Material and methods

A collection of 159 strains of *B. thermosphacta* isolated from bovine slaughterhouse environment and from different food matrices (milk, pork/beef/lamb/horse/poultry meats, and seafood products) was constituted from various laboratory collections (Table 1). Strains originating from food matrices were isolated from both fresh and spoiled products.
All the isolates and the reference strains *B. thermosphacta* DSMZ 20171^T, *B. thermosphacta* DSMZ 20599, and *B. campestris* DSMZ 4712T, and *L. innocua* CIP 8011 as an outgroup control strain, were genotyped by Rep-PCR (repetitive element palindromic PCR) using primer $(GTG)_5$ (Gevers *et al.*, 2001). *L. innocua* CIP 8011 was used as an outgroup control strain. The resulting DNA profiles were analyzed using the BioNumerics software Systems (Bio-Numerica 250 UPGMA Dice Correlation, Applied Maths, Sint-Martens-Latem,

Belgium). A dendrogram was generated from the Rep-PCR profiles. To assess the genotypic diversity of the *B. thermosphacta* collection, two coefficients of similarity were selected (60% and 80%) based on previous studies reported for this species (Xu *et al*., 2010; Papadopoulou *et al*, 2012).

Genotyping by other methods: Pulsed Field Gel Electrophoresis (PFGE) and Matrix Assisted Laser Desorption/Ionization – Time of Fly (MALDI-TOF) is in progress.

Food matrices	**Number of strains**	**Laboratory**
Horse meat	2	INRA, Jouy-en-Josas
Lamb meat	1	
Lamb + beef sausage	4	
Beef meat	25	UMR 1014 INRA-Oniris / INRA, Jouy-en-Josas
Bovine slaughterhouse environment	7	UMR 1014 INRA-Oniris Nantes
Poultry meat	48	
Pork meat	8	Laboratory of Microbiology and Biotechnology of Foods, University of Athens Greece
Sea bream	8	
Cod	4	IFREMER, Nantes
Shrimp	20	IFREMER / UMR 1014 INRA-Oniris Nantes
Salmon	30	
Milk	1	INRA, Aurillac
Unknown	1	INRA, Jouy-en-Josas

TABLE 1. Composition of the strain collection of *B. thermosphacta*

Results and discussion

The analysis of the dendrogram generated from Rep-PCR profiles (data not shown) revealed a significant genetic diversity within the strain collection. Indeed, 12 or 37 distinct genotypic clusters were generated with a coefficient of similarity of 60%, or 80%, respectively. This diversity is not related to the environment strains were isolated from: strains issued from the same ecological environment could belong to distinct genotypic groups. Conversely isolates from distinct ecological niches could belong to the same cluster. Therefore, no ecotype was observed. Preliminary results by other typing methods suggest no overlapping of Rep-PCR clusters and PFGE profiles: *B. thermosphacta* type strains DSMZ 20171^{T} and DSMZ 20599 belonging to the same Rep-PCR cluster presented different PFGE profiles. Nevertheless, PFGE confirmed the absence of ecotype.

Conclusion

The results of genotyping by Rep-PCR show that there is a significant diversity between the isolates. However, the concept of ecotype was not demonstrated. These results suggest that spoiling ability is correlated to strain properties rather than to food environment. After genotyping by others methods, Rep-PCR, MALDI-TOF, and PFGE clustering will be compared and confronted to spoilage properties of *B. thermosphacta* isolates to correlate spoiling functions and genotypes, before genome comparison.

Keywords
Brochothrix thermosphacta, spoilage, off-odors, genotypic diversity, food matrices.

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SELECTED LECTURE

PRELIMINARY ASSESSMENT TO THE FUNGAL COLONIZATION ON DOUGLAS-FIR, WESTERN RED CEDAR AND RED ALDER IN GROUND CONTACT EXPOSURE

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Introduction

Wood exposed in outdoor applications can remain wet for extended periods of time, although moisture level can vary depending on climatic conditions. Untreated wood in ground contact can quickly reach favorable moisture conditions that allow fungal colonization. The natural resistance of wood to biological degradation varies widely among wood species and even within the same species. Untreated western redcedar and Douglas-fir heartwood are less susceptible to decay, in contrast to the untreated sapwood of Douglas -fir or red alder. A diverse array of fungi can participate in the degradation process, but changes in fungal community composition over the course of decay are poorly understood of because the complexity of these communities and the absence of long-term studies. Such studies would help to better understand the patterns of fungal colonization in wood exposed under varying environmental regimes. These data could have important implications for improving wood performance under changing environmental conditions. In this study, patterns of fungal colonization of three wood species were assessed using a culture-dependent molecular approach in a ground-contact field test to provide preliminary insights into differences in fungal colonization patterns associated with climate and substrate.

Materials and Methods

Twenty-four stakes of Douglas-fir (*Pseudotsuga menziesii*) heartwood and sapwood, western redcedar (*Thuja plicata*), and alder (*Alnus sp.*) were prepared according to procedures described in American Wood Protection Association Standard E7-09 (AWPA, 2012). Stakes were installed in a field site in Corvallis, Oregon, US. Stakes were assessed for fungal colonization after 3, 6 and 9 months of exposure by cutting them into 50 mm-cubes at predetermined sampling points (above ground, ground line and below ground). The cubes were used for either moisture content determination or fungal isolation. Moisture content (MC) at time of harvest was determined gravimetrically. The cubes for fungal culturing were cut into six smaller cubes, flame-sterilized and placed into Petri-dishes with 1.5% malt extract agar (MEA) or 1.5% MEA with Benomyl added. Any fungi growing from the wood were

transferred to new MEA plates to obtain pure cultures that were grouped into morphological taxa. Fungal DNA was CTAB-extracted from representatives of each morphological taxon. The ITS region of the DNA was amplified by PCR with the universal ITS4 and ITS1-F primer set. All amplified DNA was checked in 1% agarose gels. The PCR products were cleaned and sequenced at the Center for Genome Research and Biocomputing of Oregon State University (CGRB). The nucleotide sequences were compared to sequences available on the National Center of Bioscience Informatics (NCBI) site using BLAST (Basic Local Alignment Search Tool) for identification to the nearest taxonomic group. Conditions for decay development were assessed using precipitation and temperature data obtained from the National Climatic Data Center (NCDC).

Results

A total of 327 pure cultures were obtained and grouped into 40 unique morphotaxa using the sequence matches from BLAST. The morphotaxa belonged to *Ascomycetes* (87%), *Basidiomycetes* (12%) and *Mucoromyetes* (0.6%) (Figure 1). *Phialophora mustea* (14%*)* was isolated 47 times and *Cadophora sp* (11.6%) was isolated 38 times. These species were dominant in isolations from wood with soft rot and wood in ground contact which may indicate that they were well established in the wood substrate. Only one *Basidiomycete* occurred in all three collections, *Trametes versicolor* (2.8%) that was isolated 9 times. Decay fungi such as *Postia placenta* and *Armillaria novoae* became more common after 6 months of exposure.

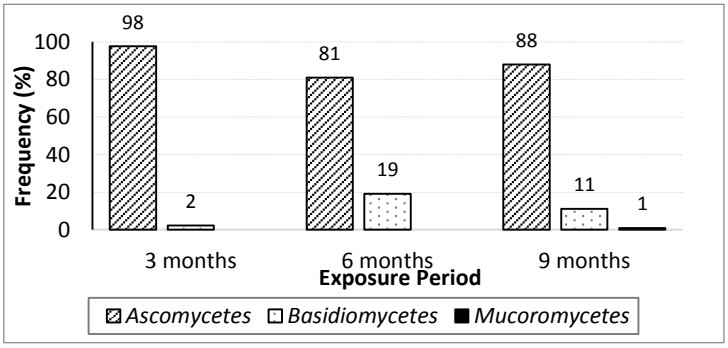

FIGURE 1. Fungal taxa frequency in stakes exposed in a field test near Corvallis, OR-USA for 3, 6 and 9 months.

Average MC of stakes at the three collection times increased from above ground (21%) to ground line (40%), and finally below ground (43%). Isolation frequency also increased as MC increased from above to below ground. These results show the importance of consistent moisture for fungal colonization. Isolation frequency also varied between wood types. Isolation frequencies were higher in non-decay resistant red alder (38%) and Douglas-fir sapwood (37%). Isolation frequencies in the moderately durable Douglas-fir heartwood and

highly durable western redcedar were 24 and 1%, respectively. Alpha diversity (Shannon Wienner Diversity Index) was higher after 6 months (2.80) of exposure than at 3 (1.81) or 9 months (2.42) of exposure. Alpha diversity was not related to the extent of exposure. However, diversity variations were higher at the ground line for all wood species. There was a significant (α=0.05) effect of the rainfall and temperature by exposure period on taxa frequency. These trials are on-going, but the preliminary results indicate that decay fungi are not initially a prominent component of the fungal flora.

Conclusions

Fungal colonization fluctuated seasonally in the wood substrate. Average rainfall and temperature at each exposure period significantly affected fungal community composition. Fungal frequencies were much higher below ground, with *Ascomycetes* dominating the fungal flora. This was coincident with the area where moisture conditions were most suitable for fungal attack.

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SELECTED LECTURE

THE LEAF MICROBIAL COMMUNITY DEGRADATION PROCESS AND ENDOPHYTIC BACTERIA

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Introduction

Bacteria and fungi play a key role in the leaves life cycle: from the early stage of development until the fall off during senescence. The leaf has more than one microbial community, one exposed to the surface (the phyllosphere) and a second within the tissue the endophytic community. The role of the endophytic bacteria is not well known. Some findings suggest that those bacteria may have a role in plant growth promotion, protection against biotic stress (i.e. parasite and pathogens), and against abiotic stress such as salt stress and drought (Hardolm et al. 2015). On contrast, more studies have been focused on last part of the leaves life cycle where the microorganism are involved in the organic matter decomposition. Even though there are several reports on this topic, there is a general lack of comprehensive knowledge including all the different aspect of the decomposition events. Aims of this study are: i) to investigate the season variability of the endophytic community associated with oak leaves and ii) to examine the different aspects of decomposition events in three plant species (rhododendron, beech and oak).

Material and methods

Endophytic community experiment: the aim of the experiment is to understand the influence of the season on the endophytic community, according to this aims the sampling events were done one for every season of 2014. The leaves surface were sterilized and then a fine powder was obtained using liquid nitrogen. DNA extraction was carried out with DNeasy Plant Kit (Qiagen).

Litter bag decomposition experiment. The plant species selected are oak, beech and rhododendron. There are three experimental sites located in South Tyrol (Italy),: i) the oak forest is near Monticolo lake, ii) the beech forest at San Genesio (BZ) and iii) the rhododendron forest area in Renon. The experiment uses litter bags of nylon with a mesh size of 40 µm, according to the experimental design of Aneja (Aneja et al. 2006). Every litter bag is processed in the same way: chemical analysis of the C and N, fixation for FISH, DNA extraction and shotgun metagenome on selected samples. Thermal Gravimetric Analysis (TGA) for characterization of the amount of biological polymers in the samples. Enzymatic essay are going to be performed on specific enzyme such as peroxidase, amylase and cellulose.

Results and discussion

Preliminary results (fig.1) show that season has an effect on the endophytic community in oak leaves and also the chemistry and the morphology of the leaves. The use of different tree in the sampling has no influence. This preliminary result suggests that the bacterial community is influenced by seasonality and we are going to gain more information with shotgun metagenome. With this technique we will be able to focus our attention on specific class of genes linked to nitrogen cycle, UV protection that are associated with the seasonality and are considered to provide a potential benefit to the plant.

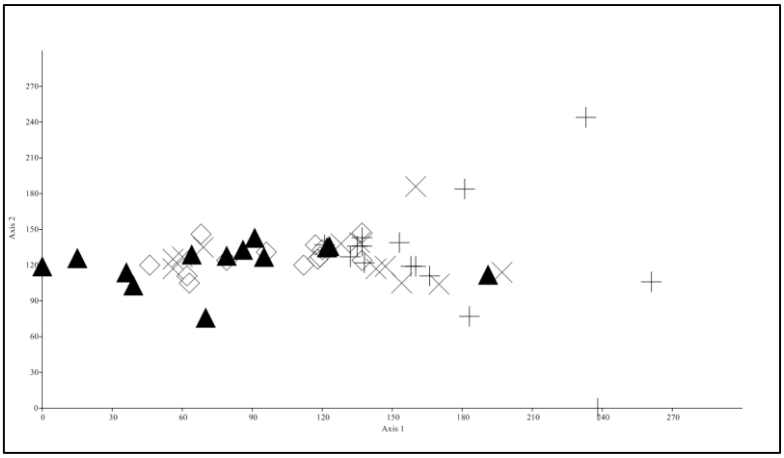

FIGURE 1. The figure shows the results of DCA (Detrented Correspondence Analysis) of all endophytic samples. Black triangle: spring; square: early summer; cross: late summer; plus: autumn.

Contrary to the endophytic community that is relatively simple, the decomposing community is complex and influenced by more than one factor, biotic and abiotic. The experiments will led to a more holistic view of the process due the long time observation and the differential approach that we are using. The study will focus on characterization of the litter, amount of lignin, cellulose and hemicellulose with Thermal Gravimetric Analysis (TGA). The transplantation experiment will give information of the Home Field Advantage (HFA). Transplantation will also simulated a new possible future scenario with different temperature and humidity. The enzymatic characterization focus on different type of enzyme involved in degradation of different polymers such as laccase, peroxidase, amylase and cellobiohydrolase.

Next to those classical microbiological techniques, the metagenome will be used to gain information on who is inside and which are their function. The application of this technique to both projects will led us to characterize the taxonomic composition and their gene contents in the two scenario. This will led also to focus on specific class of genes that are present on both dataset and that can play a role in the different stage of the life cycle of a leaf such as genes involved in the nitrogen cycle.

Conclusion

The microbial community is important from the early development of the leaf until the senescence. The study has showed as preliminary result that the community associated with the oak leaves is influenced by the season and by the chemical and morphological traits of the leaves. The microbial community associated with the senescence is more complex, composed by fungi and bacteria, with a succession of colonization. Here the study want to explore the decomposition of different plants with a transplantation experiment to study the HFA, the effect of different abiotic condition (temperature and humidity). The microbial community is going to be characterize on taxonomic levels, gene contents and the topology. The final goal is to have a broader view of the process not focusing on a specific aspect of the topic that will provide only partial information.

Keywords

Decomposition, endophytes, leaves, metagenome, microbial community,

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SESSION III

GENES AND FUNCTIONS IN COMPLEX MICROBIAL COMMUNITIES

PLENARY LECTURE

MINING METAGENOMES FOR NOVEL ENZYMES

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Emerging new DNA sequencing technologies and bioinformatics have immensely contributed to the discovery of microbial diversity in the environment. They, however, have not managed to go beyond the identification of homologs of "known", i.e. functionally characterised, enzymes in a rather limited number of environmental settings. Activity-centred enzyme discovery from metagenomes relying on naïve enzyme assays (in contrast to the metagenome sequence mining approach) reveals genuinely novel enzymes, but it is a low-throughput process, enzyme screens implemented therein are largely limited by general (easy-to-assay) tests and it relies on a very limited number of heterologous expression hosts. In the past two decades, microbial communities from almost two thousands different sites distributed all over the Planet have been examined for their genomic content, however, only in less than or 11% of metagenomic studies new enzymes have been isolated and, in most cases only partially, characterized, with marine environments as an origin of novel enzymes being largely undersampled [1]. A number of national and international projects are currently aiming at closing the increasing gap between the vast number of gene sequences in the databases and their functional analysis. The presentation will show a number of recent examples on activity-based enzyme discovery from marine microbial biodiversity hotspots, e.g. deep-sea hypersaline environments [2, 3] high-temperature habitats [4] and oil-polluted environments and petroleum-degrading microorganisms [5]. The analysis of existing drawbacks in metagenomic enzyme discovery will be given and the possible ways of their circumvention proposed.

Acknowledgements
The financial support by EU FP7 projects Kill-Spill (contract No. 312139), MAMBA (contract No. 226977), MicroB3 (contract No. 287589) and EU Horizon2020 Project INMARE (contract No. 634486) is greatly appreciated.

Keywords
Activity-based metagenomics, Enzyme screening

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SELECTED LECTURE

OCCURRENCE OF ANTIBIOTIC RESISTANCE GENES IN FECES AND SALIVA OF HEALTHY OMNIVORES, OVO-LACTO VEGETARIANS AND VEGANS

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Introduction

Antibiotic resistance (AR) is a concrete threat to human health, since bacteria, as highly adaptable organisms, are becoming increasingly resistant to antibiotics and this reduces the number of antimicrobial agents effective against human pathogens responsible for most bacterial infections. Dietary intake undoubtedly represents one of the main route for the entrance of antibiotic resistant bacteria and their genes into the human digestive tract. Dietary intakes are known to have an influence on the composition of the human gut microbiota, however very scarce are data currently available on the impact of dietary habits on human resistome.

Based on these premises, this study was aimed at investigating the effect of the omnivore, ovo-lacto vegetarian and vegan diet on the risk of introducing transferable AR genes into the human digestive tract. To this end, the bacterial DNA extracted from saliva and feces samples collected from 144 healthy volunteers (48 omnivores; 48 ovo–lacto vegetarians; 48 vegans) was screened with optimized PCR and nested-PCR assays for the occurrence of genes coding for resistance to antibiotics conventionally used in clinical practice, namely tetracyclines [*tet*(M), *tet*(K), *tet*(O), *tet*(S), *tet*(W)], macrolide-limcosamide-streptogramin [*erm*(A), *erm*(B), *erm*(C)], vancomycin (*vanA*, *vanB*) and ß-lactams (*mecA*, *blaZ*). The results of the molecular screening were statistically analyzed in order to define the incidence of diet on the frequency and distribution of AR genes.

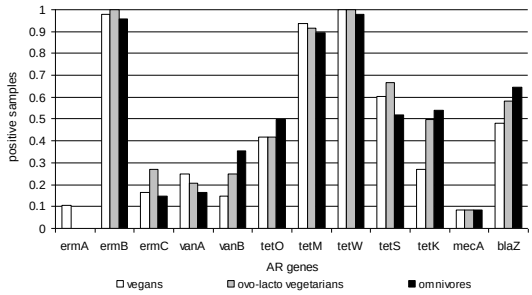
Material and methods

One hundred forty-four healthy non-smoker volunteers (84 females and 60 males) aged 18-59, who had followed a habitual omnivore, ovo-lacto-vegetarian or vegan diet for at least one year at the time of enrollment were recruited from 4 different locations situated in North (Turin, Bologna and Parma) and South (Bari) Italy, as part of a large research project titled "Microorganisms in foods and in humans: study of the microbiota and the related metabolome as affected by omnivore, vegetarian or vegan diets" funded by the Ministry of Education, Universities and Research (MIUR).

Saliva and feces were weekly supplied by the volunteers, for a time span of three weeks. Detailed procedures for sampling of saliva and feces have previously been detailed by De Filippis et al. (2014) and Ferrocino et al. (2015), respectively.
The collected samples were subjected to microbial DNA extraction using commercial kits, as previously described (De Filippis et al., 2014; Ferrocino et al., 2015).
Total DNA extracts from the 144 saliva and feces samples were amplified in PCR reactions targeting genes coding for the resistance to tetracyclines [*tet*(M), *tet*(W), *tet*(O), *tet*(S), *tet*(K)], macrolide-lincosamide-streptogramin B [*erm*(A), *erm*(B), *erm*(C)], vancomycin (*vanA*, *vanB*) and beta-lactams (*blaZ*, *mecA*). The sole samples giving negative PCR results were further subjected to nested PCR assays aimed at increasing the amplification sensitivity. Positive and negative controls were used in each PCR reaction. Five microliters of each PCR product were analyzed by electrophoresis in 1.5% (w/v) agarose gel. Gels were visualized under UV light and photographed.
The frequencies of the different AR genes were calculated as the ratio of positive samples to total number of samples. A contingency analysis based on the Likelihood Ratio $\chi 2$ test was used to test the influence of dietary habits on the occurrence of the screened AR genes. Statistical analysis was performed by JMP8 (SAS Institute Inc) and a $P \leq 0.05$ was regarded as statistically significant.

Results and discussion
In the last decades, the spread of antibiotic resistance genes in food related ecosystems has attracted the attention of the international scientific community, due to its implications for human health. However, to date, very scarce are data shedding light on the influence of diet on occurrence and spread of AR genes, especially those encoding for resistance to antibiotics routinely used in human therapy to contrast bacterial infections, as those considered in this study.
To the best of the author's knowledge, this is the first report on the impact of a long-term omnivore, ovo-lacto vegetarian and vegan diet on the human resistome, with a double focus on both the oral and gut metagenomes. Such a study was a part of a more ample research aimed at investigating the human microbiota and the related metabolome as affected by these three dietary habits.
The DNA extracted from saliva and feces of the recruited volunteers was first screened by PCR and, in case of negative result, by nested PCR with internal primers, for the occurrence of the AR genes of interest. As expected, the nested PCR assays were characterized by a highest sensitivity in respect to the corresponding PCR assays, allowing the number of positive samples to be increased.
In some cases, targeted genes (f.i. *mecA* in feces, *erm*(A) in saliva; *vanA* and *tet*(O) in both feces and saliva) could be amplified only by nested PCR, whereas for other genes, namely *erm*(B), *tet*(M), *tet*(W) and *blaZ*, most of the analyzed samples were positive in the first PCR assay. Only in a few cases (namely *vanB* and *mecA* in saliva), the targeted genes were not detected at all by neither PCR nor nested-PCR.
The frequencies of the screened AR genes in saliva and feces of omnivores, ovo-lacto-vegetarians and vegans are showed in Figure 1.

a)



b)

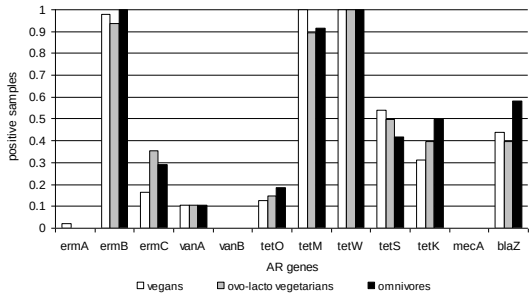


FIGURE 1. Comparison of the frequencies of AR genes in a) feces and b) saliva of vegans, ovo-lacto vegetarians and omnivores.

Overall, resistance genes to macrolide-lincosamide-streptogramin B and tetracyclines were prevalent in both types of biological samples, irrespective of the diet considered. The genes *erm*(B), *tet*(W) and *tet*(M) were detected with the highest frequency in both saliva and feces, followed by *tet*(S), *blaZ* and *tet*(K). These results are in agreement with those collected in previous studies, demonstrating the high spread of the same genes in food (Garofalo et al., 2007; Federici et al., 2014) and environmental ecosystems (Gueimonde et al., 2006; Seville et al., 2009; Card et al., 2014). As the three diets were concerned, a lack of interference by the omnivore and ovo-lacto vegetarian diet with an increased occurrence of the transferable resistances under study was seen. By contrast, a low interference of the vegan diet with the occurrence of some AR genes was found, with *tet*(M) and *erm*(A) notably prevalent in saliva and feces respectively, and *tet*(K) significantly less abundant in feces of this group of volunteers. These results match with those very recently published on the composition and metabolism of salivary (De Filippis et al. 2014) and fecal (Ferrocino et al., 2015) microbiota of the same volunteers, which clearly demonstrated a high similarity of the microbiota associated with the oral cavity and gut of subjects following the three diet regimes.

Conclusions

The impact of different diets on the human resistome was investigated. Feces and saliva of 144 healthy volunteers were screened for the occurrence of AR genes. The genes *erm*(B), *tet*(W) and *tet*(M) were detected with the highest frequency in both saliva and feces. In the latter samples, *erm*(A) and *mecA* occurred with the lowest frequency, whereas in saliva, *vanB* and *mecA* were not detected at all. The statistical analysis showed a correlation of the vegan diet with a prevalence of *erm*(A) in feces and *tet*(M) in saliva as well as a significantly lower occurrence of *tet*(K) in feces.

Research project funded under the PRIN program (2010-2011) of Ministero dell'Istruzione dell'Università e della Ricerca: "Microorganisms in foods and in humans: study of the microbiota and the related metabolome as affected by omnivore, vegetarian or vegan diets".

Keywords

Dietary habits; human feces; human saliva; antibiotic resistance genes; resistome;

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SELECTED LECTURE

METATRANSCRIPTOME RNA-SEQ ANALYSIS OF CHEESE SURFACE MICROBIOTA IDENTIFIES PHYSIOLOGICAL RESPONSES OCCURRING DURING RIPENING

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Introduction

The microbiota from the surface of smear-ripened cheeses includes a large variety of bacteria, yeasts and moulds, which activity contributes to the development of the typical organoleptic properties (flavour, texture and colour) and it also limits the growth of spoilage microorganisms such as *Pseudomonas*, or of pathogens such as *Listeria monocytogenes*. A better knowledge of the cheese surface microbiota would be useful for improving the control of its activity during cheese ripening and for improving the quality of the final products. For example, in many cases, microorganisms that are deliberately inoculated do not establish themselves at the surface of cheese. Furthermore, the growth and activity of these microorganisms is influenced by numerous factors, which are mainly unknown, and which are at the origin of variations in the organoleptic and sanitary quality of the final product. The cheese surface microbiota is also an interesting model system to address microbial ecology questions. Indeed, it is mainly composed of culturable microorganisms, its complexity is limited (typically 5-10 dominant species), it can be studied during a reasonable time-scale (a few weeks), and its activity is associated with changes in substrate composition that can be quantified.

For a long time, investigation of microbial physiology during cheese ripening was hampered by a lack of methods that could be applied to the solid and complex cheese matrix. However, during the last years, the development of efficient mRNA extraction methods from cheese, and the sequencing of the genomes of the most important cheese species, offers the possibility to investigate cheese microbiota by metatranscriptomic analysis. Such analyses can be performed after assembling sequencing reads, and the resulting contigs are then submitted to functional annotation (Lessard et al., 2014). Another possible approach is to perform short-read sequencing and to map the corresponding reads to reference genomes (Dugat-Bony et al., 2015). One advantage of the latter approach is that, due to the higher throughput of short-read sequencing, more reads are produced, which results in a higher sensitivity. In the present study, we aimed to test this approach on a reblochon-type cheese, and to use it to better characterize the activity of the cheese surface microbiota during ripening. For that purpose,

a database of reference genomes was constituted by including one reference genome for each of the five species that were used during cheesemaking, and RNA sequencing (RNA-seq) was performed after ribosomal RNA depletion.

Material and methods

Reblochon-type cheeses were manufactured at pilot-scale from pasteurized cow's milk, using five inoculated microorganisms: two lactic acid bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*), one surface bacterium (*Brevibacterium aurantiacum*), and two yeasts (*Debaryomyces hansenii* and *Geotrichum candidum*) (Castellote et al., 2015). After 5, 14, 19 and 35 days, cheese rinds were sampled, RNA was extracted and rRNA was depleted using the Ribo-zero™ Magnetic gold kit (yeasts and bacteria) (Epicentre). After reverse transcription, cDNA were sequenced using a short-read sequencing technology (Illumina HiSeq), generating approximately 75 million reads for each cheese sample. Reads were then mapped against the genome of the five species, and those that mapped on only one CDS sequence were kept for further analyses. For each CDS, the read numbers were normalized to the total number of CDS reads ("library" normalisation) or to the number of CDS reads from each species ("species" normalisation). Functional classification of the reference CDSs was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations. Differential gene expression analysis was done using the Bioconductor DESeq2 package in the statistical environment R.

Results

From day 5 to day 19, most of the sequencing reads (approx. 65%) mapped on the reference CDSs. However, at day 35, only about 35% of the reads mapped on the reference CDSs, which may due to the growth of adventitious microorganisms, to a decrease in rRNA removal efficiency or to the decrease of RNA integrity. When the detection cut-off for each CDS was fixed to a mean value of 10 reads per sample, a large proportion of the reference CDSs was detected. Indeed, depending on the strain, between 75 and 96% of the CDSs were detected, except for *B. aurantiacum*, for which the low CDS detection level (2%) was due to poor growth. Interestingly, an excellent repeatability of gene expression levels was obtained, as the coefficient of variation for cheese replicates (three separate cheeses sampled at the same ripening time) was typically about 15 to 30%. In addition, there was a very good correlation between the gene expression levels measured by RNA-seq and those measured by reverse transcription real-time PCR (for a set of 80 genes, R^2 of the linear regressions of the log of fold changes was 0.850). Only little changes were observed in the transcriptomes of the two lactic acid bacteria from day 5 to day 35, which may be explained by the fact that they were in the stationary growth phase during all that period. However, considerable changes were observed for the yeasts. For *G. candidum*, there was a higher expression level for genes involved in amino acid metabolism and lipid metabolism at day 35 than at the beginning of ripening, and the opposite was observed for genes involved nucleotide metabolism, in transcription and translation (FIGURE1).

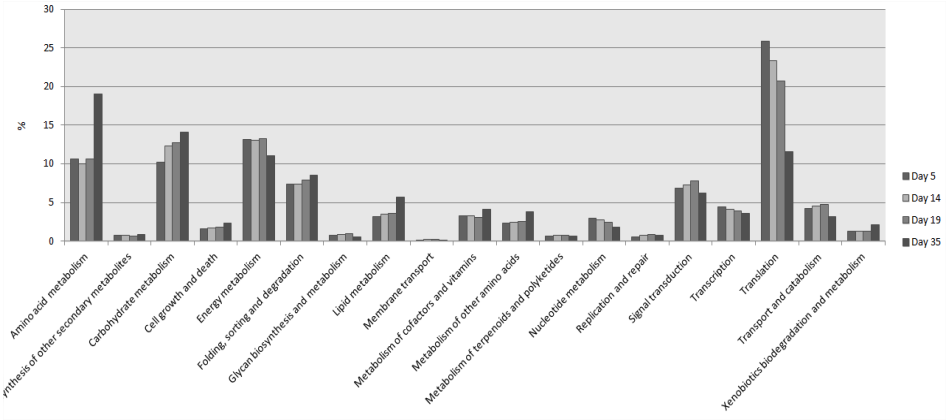

FIGURE 1. Functional classification of the *G. candidum* transcripts during cheese ripening. Functional classes were determined according to KEGG annotations of the CDSs.

Detailed examination of the induction/repression profiles of specific genes or groups of genes revealed interesting information regarding the metabolic adaptation of microorganisms to the evolution of the cheese environment during the ripening process. For example, expression of genes involved in detoxification (multidrug transporters) and iron transport increased at the end of ripening for *D. hansenii*. Expression of the mitochondrial genes and of genes involved in the electron transport chain decreased for both *G. candidum* and *D. hansenii*. Expression of the PMA1 gene (plasma membrane H+ ATPase) decreased for *G. candidum* and *D. hansenii*, probably as the result of the alkalinisation of the cheese medium. A large increase of expression of genes involved in NAD+ *de novo* biosynthesis was observed for *G. candidum*. Ammonia importers were more expressed at the beginning of ripening than at the end, whereas the opposite was observed for ammonia exporters, which is probably the consequence of the large ammonia accumulation in cheese. Gene expression profiles also revealed changes in amino acid catabolism during ripening, and different behaviours were observed for *D. hansenii* and *G candidum*. Some genes were selected to devise bioindicators, based on real-time PCR analyses, which are useful to characterize technological or biological activities during cheese ripening.

Conclusion

This work is an example showing how metatranscriptomic analyses provide insight into the activity of microbial communities for which reference genome sequences are available. Interestingly, reliable data were obtained even for cheese samples in which part of RNA was degraded, showing that a good RNA integrity is not mandatory for metatranscriptomic analysis. Such approach may be extended to more complex microbial communities. In the future, this will be facilitated by the increase of the throughput of DNA sequencing technologies, by the increase of the size of the corresponding reads, and also by a higher availability of the genome sequences of reference strains.

Acknowledgments
This work was supported by the ExEco program (a joint metatranscriptomic and biochemical approach of the cheese ecosystem: for an improved monitoring of the expression of a complex food ecosystem) (ANR-09-ALIA-012-01), funded by the French National Research Agency (ANR), and by the EcoStab grant from INRA métaprogramme MEM 2012. This work has benefited from the facilities and expertise of the high throughput sequencing platform of IMAGIF (Centre de Recherche de Gif - www.imagif.cnrs.fr). We thank Anne-Sophie Sarthou and Jessie Castellote for excellent technical assistance.

Keywords
Cheese, metatranscriptome, RNA-seq, reverse transcription-quantitative PCR, gene expression

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SELECTED LECTURE

IDENTIFICATION OF AMINE-DEGRADING NON-STARTER LACTIC ACID BACTERIA FROM SICILIAN AND APULIAN TYPICAL/TRADITIONAL CHEESES AND CHARACTERIZATION OF ENZYMATIC ACTIVITIES

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Introduction

Biogenic amines (BA) are low molecular weight organic compounds that can be present in fermented foods. A high content of biogenic amines in dairy products undermines food quality and safety and has toxicological consequences for consumers. Therefore, the potential role of microorganisms with amine degrading activity acquired a particular interest in the last few years to prevent or reduce biogenic amine accumulation in food products, especially fermented foods. BA include different classes on the basis of their structure: monoamines (including tyramine, 2 phenyl-ethyl amine and tryptamine), diamines, such as histamine, putrescine and cadaverine, and polyamines (spermine and spermidine) (Lee and Kim, 2013). These compounds are mainly produced by the decarboxylation of amino acids by microbial action. Microbial activity may often cause the formation of BA in food. It is reported that enterococci and hetero-fermentative lactobacilli are the main tyramine and histamine producers, respectively, but other LAB and some Gram-negative bacteria may also be involved in BA formation in cheese (Calzada et al., 2013). Therefore, the detection of bacteria possessing amino acid decarboxylase activity is important to estimate the risk of BA food content and to prevent biogenic amines accumulation in food products. Otherwise, some bacteria are able to degrade BA. Little is known about the enzymatic activities responsible for BA degradation, but the enzymes named Amine Oxidases (AOs) are involved in this process.

The amino oxidase activity of several bacteria dominating some ecosystems have been employed to reduce BA content in food (García-Ruiz et al. 2011). Two main classes of AOs have been described: flavin-containing monoamine oxidases and copper-containing amine oxidases (CuAO), generally found in a wide range of microbial, plant, and animal systems (Lee and Kim 2013). However, AOs have been characterized only from some bacterial genera (Zaman et al., 2010, Lee and Kim, 2013) and enzymes belonging to different classes may be involved in the degradation of BA. In order to select strains for application in the control of levels of biogenic amines during cheese ripening, the main purpose of this study

was the investigation of LAB strains isolated from Sicilian and Apulian typical/traditional cheese typologies for the expression of enzymatic activities involved in BA production and degradation.

Materials and Methods

The potential to produce BA was investigated and confirmed by a molecular approach, amplifying fragments of genes coding for tyrosine, ornithine, histidine, lysine and phenylalanine decarboxylases from LAB previously isolated from nine Apulian (Cacio, Caciocavallo Podolico Dauno, Caciocavallo Silano Protected Designation of Origin (PDO), Cacioricotta, Canestrato Pugliese PDO, Caprino di Biccari, Caprino di Castel Fiorentino, Pecorino Foggiano, and Vaccino), and eleven Sicilian (Caciocavallo Palermitano, Ragusano PDO, Caprino Girgentano, Fior di Capra, Fiore Sicano, Maiorchino, Pecorino Siciliano PDO, Piacentinu Ennese, Provola dei Nebrodi, Tuma Persa, and Vastedda della valle del Belice PDO) traditional cheeses and whose decarboxylase activity was previously tested by a phenotypical/microbiological approach (Guarcello et al., 2015).

Genomic DNA from *type strains* were used as positive controls for *tyrdcA* and *phedc*, *hdcA*, and *odc/cadA* genes, respectively. Amplified fragments were verified by sequencing by Eurofins Genomics s.r.l. (Milan) (that also synthesized all the oligonucleotides used in this study) and comparative searches by the Basic Local Alignment Search Tool program (BLAST) in GenBank/EMBL/DDBJ database (http://www.ncbi.nlm.nih.gov).

Only decarboxylase negative isolates were tested for their ability to degrade BA. To this purpose, all bacteria were tested for growth in chemically defined medium (CDM), prepared as described by Miladinov et al. (2001) with minor modifications. CDM was singly supplemented with the main biogenic amines, as the single nitrogen source. Growth in broth was confirmed recording optical density measurements at 600 nm (OD600), measured by a 6400 spectrophotometer (Jenway Ltd., Felsted Dunmow, UK) at 48 and 72h of incubation.

Presumptive amine-degrading/non-producer strains were differentiated by random amplification of polymorphic DNA-PCR (RAPD-PCR) analysis in a 25-µL reaction mix using primer M13. RAPDPCR profiles were analyzed with the pattern analysis software package Gelcompare II software version 6.5 (Applied-Maths, Sin Marten Latem, Belgium). Genotypic identification of LAB was carried out by 16S rRNA gene sequencing. PCR reactions were performed as described by Weisburg et al. (1991).

Degenerated primers, mapping in conserved regions of genes annotated as coding for amine oxidases from Firmicutes and other bacteria, were designed and tested in different combinations. Amplified fragments generated with the tested primer pairs were purified by QIA-quick purification kit (Qiagen) and cloned into the pGEM® -T Easy Vector (Promega, Milan, Italy) following manufacturer's instructions. Fragments of interest were purified by recombinant white colonies and sequenced.

Post-sequencing analysis included editing and translation of the obtained sequences and open reading frames prediction (ORFs) by the softwares ChromasPro v1.6 (Copyright 2003e2012 Technelysium Pty Ltd. Biotech Works Inc.) and pDRAW32 v1.1.114 (http://www.acaclone.com). Putative protein domains were located via use of the Pfam (http://pfam.sanger.ac.uk), Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de) and Conserved Domain Database (CDD) (http://www.ncbi.nlm.nih.gov) databases. Edited sequences were compared by a BLAST search in GenBank/EMBL/DDBJ database and aligned by ClustalW version 1.83.

Results and discussion

The occurrence of genetic determinants encoding amino acid decarboxylases was investigated by a molecular approach, also extending the analysis to activities that were not previously explored. About 36% of the PCR-screened isolates did not show the ability to produce BA. By an integrated microbiological and molecular approach, presumptive amine-degrading bacteria were found in 14 out of 20 cheeses, including all the Sicilian cheese typologies and just three Apulian typical dairy products (Caprino di Biccari, Cacio and Pecorino Foggiano). Furthermore, 34% of LAB showed the ability to grow in the CDM synthetic medium supplemented with at least one of the main BA, including 38% of Sicilian isolates and 25% of Apulian isolates. About 90% of our isolates appeared able to use at least two different BA as nitrogen source to grow. Eighty-one presumptive cheeseborne amine degrading/aminoacid decarboxylase-negative isolates were selected and genetically identified. As expected, bacteria belonging to various LAB genera (*Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Enterococcus*, *Pediococcus* and *Weissella*) and species were isolated from the different cheese samples. Some authors reported, infact, that strains belonging to the genera *Brevibacterium*, *Lactobacillus*, *Pediococcus* and *Micrococcus* are capable of reducing histamine, tyramine and putrescine concentrations in fermented food, such as wine and cheese (Callejon et al., 2014). Some authors have used BA-degrading bacteria in order to diminish BA in foods. In addition, a strain-specificity of degrading activities was noticed. The ability of microorganisms to decarboxylate amino acid is known to be highly variable (Landete et al., 2007) and this behavior was registered for two our *E. faecalis* strains isolated from the cheeses Vastedda della valle del Belíce and Pecorino Siciliano PDO The presence of producer microorganisms does not necessarily determine the formation of BAs in cheese, since proteolysis must also occur, the pH must be corrected, and the ripening period must be long enough (Fernández et al., 2007). To gain insight into the molecular determinants of the enzymatic activities responsible for amine degradation, we isolated and characterized genomic fragments including determinants for hydrolase and alpha-amylase activity, besides methyltransferase and exonuclease domains. We are investigating their potential role in amine degradation, in order to support the application of emerging methods based on cheese-borne LAB cultures as control measure to reduce BA accumulation in cheese.

Conclusions

Accumulation of BA in cheese and other foods is a matter of public health concern. In our work, we detected cheese-borne LAB with the potential to degrade BA. Further analysis are in progress to study their effective ability to reduce BA in cheese during ripening and to isolate the corresponding coding genes and to study their expression. Therefore, the study might support the application of emerging methods based on LAB cultures as control measure to reduce BA accumulation in cheese.

Keywords

Lactic acid bacteria, biogenic amines, degradation, cheese.

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SELECTED LECTURE

DEVELOPMENT OF A METHOD EMPLOYING A METABOLIC GENE TO MONITOR NON-STARTER LACTIC ACID BACTERIA STRAINS AND THEIR EVOLUTION DURING RIPENING OF CHEESE

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Introduction

Long ripened cheeses harbour a viable microflora at the end of ripening, mainly composed of non-starter lactic acid bacteria (NSLAB), deriving from the raw milk and dairy environment, contributing to the final characteristics of cheese (Settanni & Moschetti, 2010). Among NSLAB, the species *Lactobacillus rhamnosus*, *Lactobacillus casei* and *Lactobacillus paracasei* are regarded together as *L. casei* group, due to their phylogenetic and phenotypic relatedness (Felis & Dellaglio, 2007). These species were frequently found in Grana Padano (GP) and Parmigiano Reggiano (PR), becoming the dominant bacterial population during ripening (Gatti et al., 2014). The metabolic strategies adopted by *L. casei* group to grow and survive in the cheese environment, which is lactose free, probably rely on the activation of alternative metabolic pathways, to exploit different nutrient sources present in the cheese matrix. Recent studies performed on *L. rhamnosus* growing in a cheese model system (Lazzi et al. 2014) revealed an upregulation of the gene *spxB*, coding for pyruvate oxidase (POX), an enzyme that catalyses the oxidation of pyruvate to acetyl-phosphate, with subsequential ATP production. The role of respiratory metabolism in *L. casei* group is raising a growing interest (Zotta et al., 2014), and *spxB* gene is involved in this functionality. Given the potential role of *spxB* in bacterial growth during cheese ripening, its presence and diversity was explored over a *L. casei* group isolates collection through High Resolution Melt (HRM) analysis. Moreover, *spxB* sequence heterogeneity in *L. casei* group allowed to use this gene to target the metabolically active microflora with High-Throughput Sequencing (HTS). In order to get an overall depiction of microbial evolution during GP ripening, HTS was also performed on 16s rRNA sequence.

Materials and methods

The first part of the study was performed on 74 wild isolates belonging to *L. casei* group, isolated from cheese at different stages of ripening. A PCR screening on the genomic DNA

of the 74 strains confirmed the presence of *spxB* gene in all isolates. Then, 44 amplicons were sequenced, and the resulting sequences were aligned to generate a phylogenetic tree. HRM was performed, and dissociation curves were analyzed. Aligned melting curves and difference plot were obtained for the *L. casei*, *L. paracasei* and *L. rhamnosus* strains using the fluorescence of each strain, and a confidence level of 90%. The second part of the study was performed on GP cheese at different ripening ages (2, 6 and 12 months of ripening), coming from two different cheese-making (CM1 and CM2). RNA extraction was performed, and the RNA was reverse transcribed into cDNA, and used to prepare an amplicon library for 16s rRNA and *spxB* gene. The libraries were used for pyrosequencing on a GS Junior platform (454 Life Sciences, Roche, Italy) according to the manufacturer's instructions by using a Titanium chemistry.

Results and discussion

The presence of *spxB* gene in 74 isolates belonging to *L. casei* group was confirmed through PCR. The occurrence of this gene in a wide set of *Lactobacilli* has never been reported so far, therefore 44 amplicons were sequenced, and the sequences were compared showing an overall sequence homology of 84,96%. Recently, some studies regarding genetic typing of *L. casei* group, reported the application of HRM, a post-PCR method (Iacumin et al., 2015, Porcellato et al., 2012b). Since HRM is a fast and easy technique, we applied it to all the *L. casei* group strains but, differently from previous studies which focused on 16s rRNA gene, we targeted a metabolic gene. The resulting melting curves allowed to discriminate 4 species-specific variants, with distinctive melting temperatures (T_m), as shown in Figure 1.

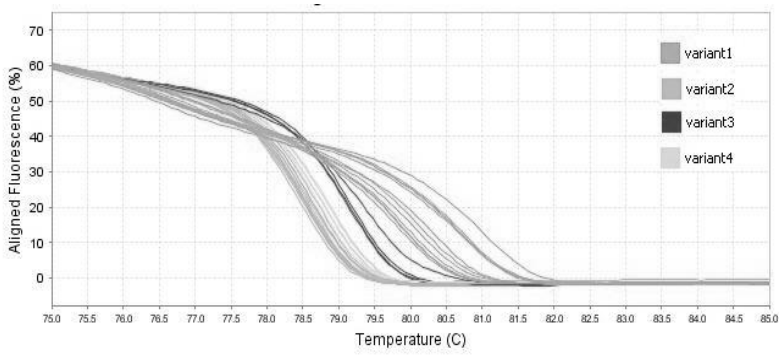

FIGURE 1. HRM analysis of spxB: aligned melt curves. Variant 1 indicates L. rhamnosus strains (Tm=80.5 ± 0.5 °C); variant 2 indicates L. casei (Tm=78.5 ± 0.1 °C); variants 3 (Tm=79,2 ± 0.1 °C) and 4 (Tm= 78,8 ± 0.1 °C) indicate L. paracasei strains.

To transfer the *in vitro* results to the *in situ* level, and prove the discrimination power of *spxB* even in a complex environment, such as cheese, HTS approach was followed. In particular, pyrosequencing was performed on the metabolically active microbiota of two different GP cheese-making, targeting 16S rRNA and *spxB* of *L. casei* group. The opportunity to perform HTS on cDNA extracted from long ripened cheese implement data from literature. The results for the 16s rRNA sequence revealed an evolution of the bacterial population during ripening, with the phylum *Firmicutes* representing, on average, 99.70% of the bacterial

species. Bioinformatic analysis led to the identification of 80 OTUs, whose distribution among the samples allows to clearly distinguish between samples belonging to CM1 and CM2. Among these, *L. casei* group represents, on average, 51% of the viable bacterial population. The other metabolically active species, with a relative abundance higher than 1% in both cheese-makings, were: *L. fermentum*, *L. helveticus*, *L. delbrueckii*, *S. thermophilus* and, to a lesser extent, *L. buchneri*. All the species showed fluctuation related to the ripening age of the cheese.

Regarding *spxB* gene, 76 sequence types were retrieved, with an overall similarity of 99,5%. The sequences were grouped into four macro-clusters, according to their sequence similarity to reference strains available in public databases: two clusters were identified as *L. rhamnosus*, consisting of 22 (LR1_1-22) and 25 (LR2_1-25) sequences, respectively, and two clusters gathered together *L. casei* and *L. paracasei*, consisting of 13 (PC1_1-13) and 16 (PC2_1-16) sequence types. Of these, 31 sequences were present with an abundance higher than 1%, and nine were present with an abundance more than 5%. Interestingly, a single sequence type is present in all the samples: it is PC2-5, belonging to the *L. casei*/*paracasei* group, and it increases in both cheese-making, representing one of the dominant genotypes (Fig.2). On the other hand, some sequences were exclusively present only in CM1 (LR1-1) or either in CM2 (LR1-9, LR1-20). Furthermore, 20 sequences were found in single samples, at an abundance >1%, with the highest diversity being present in the 12 mo. sample of CM1 (Fig. 2). These results underline the importance to explore the genetic variability of metabolic genes, to achieve strain typing in a microbiota characterized from a few dominant bacterial species, but with a high intraspecific biodiversity.

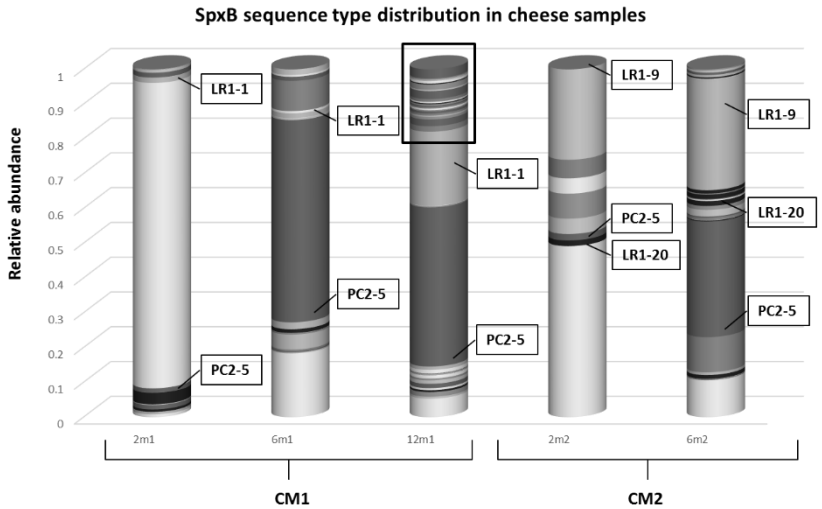

FIGURE 1. Relative abundance of sequence types of *spxB* among GP samples. The sequence types cited in the text are labelled. The black rectangle points to the elevate degree of diversity present among the *L. rhamnosus* sequence types in 12 mo. old sample.

Conclusions

SpxB proved to be a gene of ecological interest, which permits to monitor, at strain level, the metabolically active bacteria belonging to *L. casei* group, directly in cheese. Its expression was retrieved throughout the ripening, pointing to pyruvate oxidase activity encoded from *spxB* as a significant one for bacterial growth in long ripened cheese. So far, it is left to understand the metabolic significance of the differential evolution among the sequence types, as well as its effect on cheese ripening.

Keywords

Lactobacillus casei group, *spxB* gene, High Resolution Melting, cheese, High Throughput Sequencing

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SELECTED LECTURE

BACTERIAL DIVERSITY AND FUNCTIONALITY IN MINERAL SOILS OF EARLY SUCCESSIONAL STAGES IN A GLACIER ALPINE MORAINE

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Introduction

In high mountain environments, microbial communities are key-players of soil formation and pioneer plant colonization and growth. In the last ten years many researches has been carried out to highlight their contribution. Bacteria, fungi, archaea, and algae are normal inhabitants of the most common habitats of high altitude mountains, such as glacier surfaces, rock wall surfaces, boulders, glacier waters, streams, and mineral soils (Ciccazzo et al., 2015). Here, microbial communities are the first colonizers, acting as keystone players in elemental transformation, carbon and nitrogen fixation, and promoting the mineral soil fertility and the pioneer plant growth. Especially in the high mountain environments, these processes are fundamental to assess the pedogenetic processes in order to better understand the consequences of the rapid glacier melting and of the climate change.

Material and methods

The experimental site is located in Matsch/Mazia Valley (Italy), a lateral valley of Val Venosta, Central Alps. Two transects were defined at 2830 and 2824 m a.s.l. respectively. The first transect (T0) is 20 meters far from the glacier tongue and it is ice-free since 2010. The second transect (T1) is 70 m far from the glacier, in a mineral soil aging 50 years after glacier melting. In T0 we identified 6 sites, sampled in triplicates for molecular analysis. Each triplicate sample was constituted by one gram of mineral sand. The triplicate samples were taken 20 cm from each other, as triangle shape, while 200 grams of sands were taken inside the triangle for the chemical analysis. In T1 we identified 6 plots of one square meter each. We sampled each plot accordingly to a W-sampling strategy, having at the end a homogeneous mineral soil sample. This was used for molecular and chemical analysis.
Molecular analysis were done by ARISA fingerprintings and by MiSeq Illumina sequencing. ARISA was done as in Cardinale et al. (2004). DNA samples were sequenced with an Illumina MiSeq sequences: PCR amplification of environmental 16S rRNA genes was carried out using primer set for bacteria amplifying the V1-V3 variable regions (27Fmod 5'-AGRGTTTGATCMTGGCTCAG-3' and 519Rmodbio 5'-GTNTTACNGCGGCKGCTG-3') and then sequenced using the MiSeq Illumina, accordingly to the standard V3 protocols.

Template was quantified with suitable Real Time PCR reactions. Raw sequences were processed using the software QIIME. Reads shorter than 200 bp or longer than 800 bp were removed. Afterwards, reads were checked for quality. Sequences below the default quality score were removed. Filtered sequences were assigned to the different samples according to their barcode. Next, sequences were checked for chimeras with the Chimera Usearch. OTUs table were generated using demultiplexed sequences at 97% similarity and singletons were removed. Taxonomy were assigned using GreenGenes database. The matrix generated was exported for the downstream statistical analysis.
Bacteria functional diversity profile was inferred using PICRUSt software package (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States http://picrust.github.com). Briefly, picked closed-reference OTUs against the 13.5 Greengenes database (QIIME scripts: pick_closed_reference_otus.py) were analysed via PICRUSt according to the instructions furnished by the developers. The accuracy of metagenome predictions was tested trough the Nearest Sequenced Taxon Index (NSTI). The accuracy prediction is related to the presence of closely representatives bacterial genome. The lower values reveal a closer mean relationship.
PAST software was used to perform statistical analysis. Chao-1 index, Evenness and Richness were calculated on OTUs table deriving from pyrosequencing. Non metric Multidimensional Scaling (NMDS) on OTUs table normalized was performed by using Bray-Curtis dissimilarity distance. Venn diagram were generated using VENNY 2.0.

Results and discussion

The number of reads taken into the analysis was 7,372,393. The number of reads ranged from 118,296 (sample 105) to 1,591,839 (sample 90c). The average number of reads per sample was 307,183. The reads were assigned to 45,160 OTUs. We calculated Fisher's alpha, which is insensitive to divergent sample sizes, to estimate the number of species in the samples, and Shannon's evenness index. Fisher's species richness was on average higher in the T1 than in the T0 soils, and it scattered much more in the T0 soils than in T1 soils. Shannon's OTU distribution was more even in the T1 plots than in T0 (t-test and F-test, resp., alpha = 0.05). Interestingly the plots of T1 showed a less dispersive distribution than T0 samples, while the triplicate samples of T0 are closely related. Both the Detrended Correspondence Analysis of ARISA ribosomal ITS and of MiSeq 16S rRNA gene diversity data showed similar results (Fig. 1).

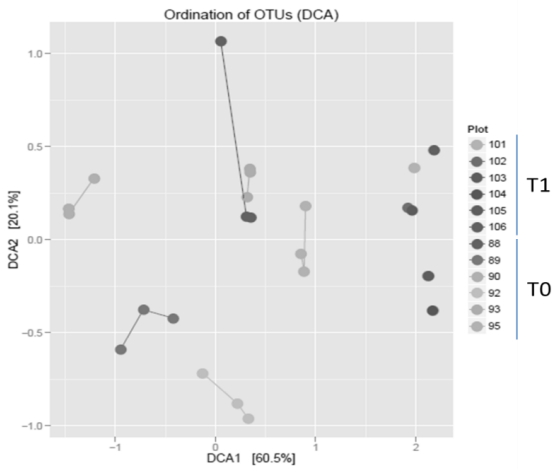

FIGURE 1. DCA (Detrended Correspondence Analysis) of bacterial community structure of transects T0 (5 years old soil) and T1 (50 years old soil) after Illumina MiSeq high throughput sequencing.

Samples from T0 were well separated by T1, indicating an important difference between the two microbial communities. There was also a gradient in bacterial diversity in T1, according to the geographical location. Finally, replicated samples of T0 were closely related, indicating that bacterial communities at microsite level are very similar, while a significant difference was visible among T0 sites.

By looking for taxonomic differences it was interesting to note that T0 and T1 shared only 24% of the overall bacterial OTU (996 OTU). At genus level, T1 and T0 shared 143 genera, while T0 had 85 unique genera, and T1 had only 17 unique genera: This result indicates that only well adapted bacterial genera can colonize the harsh and oligotrophic environment of T0. According to the taxon diversity analysis, in T0 there were the major representative belonging to Thermi, Cyanobacteria, and TM7 respect to T1, while T1 had more representative of *Planctomycetes* and *Verrucomicrobia*. Some of the OTU were significantly related to the geomorphology of the site, such as distance from the first T1 plot, as it was happened for some *Planctomycetes*. On the other hand representative involved in carbon recycling were scattered found between the two transects: In T0 there was the predominance of *Acidimicrobia*, *Acetobacteraceae* and *Methylophilales*, while in T1 Acidobacteria of Gp6 or of *Solibacteres*, or *Methylacidiphilales* were predominant. *Micrococcaceae*, known to produce pigments conferring resistance to irradiation, were predominant in T1. Interestingly the presence in T0 and T1 of N-involved bacterial taxa was significantly different between the two transects. Regarding diazotrophic bacteria, while T0 is mostly colonized by Cyanobacteria, T1 was colonized by a number of other N-fixers, such as *Burlholderia*, *Opitutaceae*, *Rhizobiaceae*, *Phyllobacteraceae*, *Bosea* sp., *Devosia* sp. or *Rhodoplanes* sp.. Even without any plant presence, *Bradirhyzobiaceae* were commonly present in all samples of T0 and T1.

Conclusions

Although its strong oligotrophic characteristics, glacier forefields appear to be complex systems where highly-specialized bacterial communities are actively involved in soil formation and fertilization, preparing microsites to the colonization of pioneer plants.

Keywords

Bacterial communities, Functionality, Alps, Glacier forefield, Illumina MiSeq.

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SESSION IV

NEW TOOLS AND STRATEGIES TO UNRAVEL THE COMPLEXITY OF MICROBIAL DIVERSITY

PLENARY LECTURE

BIODIVERSITY BIOINFORMATICS, CHALLENGES AND OPPORTUNITIES

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History

Until the end of the nineteen nineties scientists have been studying nature, biology, mathematics, physics, chemistry, etc with a diversity of tools that were producing data and volumes of information that were "consistent" or easily manageable by single researchers. Scientists were observing, describing and modeling using simple or advanced tools for their times but nothing that was really submerging them in terms of volumes at least. When Carl Linnaeus, the father of binomial nomenclature and of classical taxonomy, published the first edition of Systema Naturae in 1735, he was only 28 years old. He was considered as a master in botany, zoology and medicine. He was teaching botany and medicine, and in those days, it was possible to embrace such a broad scientific spectrum. Even if he certainly was an exceptional genius, we do not believe that this would be possible nowadays with the amount of information, the complexity and the diversity of knowledge that current scientific works are requiring.

In 1958, Frederik Sanger got a first Nobel Prize for his work on the structure of Insulin and got a second one in 1980 for the DNA sequencing technique bearing his name. A few years after, the sequencing of the yeast and later the human genomes received a lot of attention and were published in high impact papers but nothing like a Nobel Prize. Today, genome sequencing is becoming almost "trivial" and is not sufficient anymore to deserve publication in journals such as Nature, for example. Producing a human genome can almost be done overnight by a single sequencer at a very low cost.

Evolution

In 1989, when the first author started working as a young microbiologist isolating yeasts from the tropical forest of central Africa, the only existing tools to depict biodiversity were the microscope to observe morphological and sexual features as well as a number of physiological tests. Almost everyone could achieve these types of taxonomic, ecological and biodiversity studies without much advanced knowledge nor technical or financial means. All our scientific mentors and predecessors have been using the exact same techniques during their working life with minor and incremental evolutions only. The rise of DNA sequencing methods and their drop in prices made them affordable. However, they would never have had

the success that we know without the emergence of personal computers and associated software. Without the latter, aligning sequences would have been an almost impossible task if done by hand. This is therefore the combination of sequencing methods and software and computers that was the starting point of the current and ongoing revolution.
All this shows the fast evolution and paradigm changes that occurred in the last few decades. Things are not settling down at all and the production of raw data in humongous amounts by high throughput machines is just starting and will give scientists willing to use them, incredible opportunities and challenges at the same time. We believe that never before, in any scientific domain, the speed of evolution of techniques has been as high as today. The most conservative taxonomists maybe depressed, discouraged or scared by this incredible revolution because existing knowledge is constantly questioned and undergoes a fast process of obsolescence. This is not only the techniques that are changing but the generated results are questioning and changing the way we look at things. For example, species circumscriptions or concepts are heavily changed and often reduced in size due to the better discrimination power of DNA. Even more, some are strongly questioning the need to continue using species concepts, especially in the microbial world, since they are subjective agglomerates, not always fitting with many biological observations or even unnecessarily blurring the reality when trying to correlate features at that taxonomic level. There are suggestions or trends to use specimens, strains, isolates, or even DNA/RNA sequences based clustering instead of classical binomial taxonomy that is seen by some as instable and unable to answer their questions.

Researcher's behavior

Morphological, sexual, physiological, chemical, ecological and other basic metadata are more and more disregarded by some taxonomists or post-taxonomists in favor of the molecular ones. This understandable shift is potentially dangerous though because of the high risk of disconnection to the realities of the organisms by silico specialists that have never seen the original organism. They are therefore lacking basic descriptive, functional and contextual knowledge about them. This may lead to possible erroneous and disastrous scientific hypotheses and conclusions.
Another major issue of modern science and the behavior of a number of researchers is the race for high impact papers and the fact that produced data have a very short lifespan and are not maintained anywhere. One could call it "quick and run" science when the main goal of a paper is to publish in high ranking journals and when the data used to produce the paper are lost, abandoned or not archived properly. For some, the race for high impact means a lack of consistency in terms of research subjects sometimes guided by financial necessities, sometimes by personal ambition.

Databases

Until a few years ago, the only way to publish scientific results was to publish papers in scientific journals. This is still the major vehicle and essentially, the only seriously recognized one. People building databases are not acknowledge for their hard and systematic work since there are no impact factors associated with this activity. This is discouraging many to start doing it and others to continue the effort and maintain existing and valuable databases. This must certainly change because bioinformatics, and biodiversity informatics even more, are strongly depending on well-maintained, curated, dynamic and evolving reference

databases. Without data and, even better, without quality data, nothing would be possible. The work of database curators has to be recognized encouraged and valued. A few solutions are available to address this problem but let us cited two of them. First, using the number of hits to the websites using a database is a quite good indication of the usefulness of the project. A second possibility would be to promote the nano-publication (http://nanopub.org) concept where the usage of databases by machines to find information would automatically induce nano-citations rewarding researchers feeding databases with their data points (Mons and Velterop, 2009). Project funding should of course be based on the scientific merit of a project, the quality of the researcher based on Impact Factor, number of hits on their websites (independently measured by third parties, for example by Google Analytics) and/or the nano-publications associated with their databases and websites/web services.

Of course, not all databases are equally valuable but a number of important criteria have to be present to make a good one. Dynamic. A database is a "living organism" that needs to evolve over time. Not only in terms of number of records but also in terms of recorded characters or data points (fields). The emergence of new methods or technologies implies the addition of new fields and therefore the update, as far as possible, of existing records to avoid missing data or incomplete records. Format. Data formats are essential when analyzing the information. For example, one could store sizes in several ways, as discrete data like small, medium and large or as continuous data by recording the actual values. The latter options is always preferable to the first. Keeping the information as close as possible to their original or production format is always preferable over transformation that are not reversible. In some case though this is impossible like with the raw files produced by next generation sequencers that are too large for long term storage. Another major point regarding format is to try avoiding text fields that are mainly directed towards human reading while the future goes more in the direction of machine learning and automated data mining. Polyphasic. Store single data types can be interesting but the real value of great databases is to combine multiple characters (fields) associated with a single record (specimen, sample, etc) in order to allow correlation and statistical analysis on many criteria at the same time which one could call polyphasic data analysis. Volumes. All data produced by modern machine such as next generation sequencers are huge and are really challenging our storage capacities. Conventional relational databases are quickly showing their limits and new technologies have been implemented to support much larger data integration rates (MongoDB is an example of such data storage engine). Networking, Interoperability and Semantic Web. Even the most complete databases in terms of records and associated characteristics cannot contains all the needed information that a researcher may want. For example, sequence database are associated with strains or specimen data that have a number of characteristics present in other repositories and it may be interesting to link all these information points with climate, geographical or ecological data. This, based on the latitude, longitude where the organism was isolated. These data are stored in different databases that must be part of a network of interoperable repositories used, for example, by semantic web-like technologies.

In a recently published paper (Robert, Cardinali, Casadevall, 2015) we demonstrated the use of an historical culture collection database from the CBS-KNAW containing fungal strains information recorded over more than a century. Data such as geographic origin of the strains, minimum and maximum growth temperatures, fermentation and assimilation of a large

number of chemicals have been recorded for nearly 100.000 strains over that period. Recently the whole collection was screened and strains sequenced or DNA barcoded (internal transcribed spacer and ribosomal large subunit loci). All data were analyzed and combined to demonstrate a number of highly interesting facts and trends. Thanks to the large number of observations, we could show that the ability to grow at high temperatures was not monophyletic at all even if ascomycetous fungi were more likely to grow at higher temperatures than basidiomycetes, the latter being more frequent in higher latitudes. We also observed that there was a trend indicating that basidiomycetes collected in the last 2 or 3 decades are able to grow at higher temperatures than the ones isolated previously while ascomycetes are stable from that point of view. Increasing temperatures of the last 2 or 3 decades due to climate changes are even quite well correlated with the evolution of basidiomycetous fungi. We have suggested that there seems to be an adaptation of basidiomycetes to grow at increased temperatures. Of course, this remains a hypothesis that need to be confirmed. In June this year BMC indicated that this paper had already been downloaded 4330 times in a few months indicating its interest. All this would not have been possible without the hard work of many curators, researchers and technicians working at or with the CBS-KNAW culture collection since the end of the 19th century. It also shows that when recording data properly and systematically over a long period of time for a large number of samples, strains, specimens or items, the use of such data may not be directly used for its original purpose but could be used much later (several decades in our case for some data) for completely different objectives.

Intelligent speed

Having large, well formatted and curated databases is an important requirement but retrieving the information quickly and effectively is yet another challenge that mainly remains to be addressed. Let's take a simple example. A researcher interested to know the species name of an organism isolated from a soil sample based on its DNA sequence may go to the NCBI website and submit it to the blast engine. The alignment usually comes after a few seconds (around 5 seconds on average). For 10 sequences our last trial allowed us to get data in 28 seconds while for a batch of 100 sequences 175 seconds were needed. The problem started for batch requests containing 1000 sequences where 7004 seconds were needed (almost 2 hours). Extrapolations for 1 million sequences show that results may be obtained after 80 days! Knowing that a single sample coming from next generation sequencing (NGS) runs may produce millions of sequences, one can quickly see the problem that we are facing when dealing with urgent needs like in medical diagnostics or in monitoring scenarios where time and speed are directly correlated with live saving. New algorithms are being developed but to our knowledge there is still no magic tool and associated IT infrastructure that can sustain the submission of millions of samples coming daily from NGS machines and produce results in minutes. This an important gap for the future development of NGS as diagnostic or monitoring tool.

The so-called big data issue is already causing extremely serious challenges for (bio-)informaticians as well as for IT infrastructure specialists and this will only grow in the future. On the other hand the possibilities offered by the amount of data produced will likely lead to amazing discoveries. Very exciting times for people ready to jump into the silico world.

Training multi-disciplinary teams

Informatics and bioinformatics sensu lato offer incredible opportunities for the brave that are not afraid of spending days and nights finding solutions to difficult problems or bugs or developing new and innovative solutions, algorithms or software. One of the major problems and beauties at the same time of the field is its hybrid nature between mathematics, information technology, software developments, (molecular) biology and ecology, just to cite a few. It means that future bioinformaticians candidates will have to have serious training on the different aspects. This being said, even with advanced training, it is an illusion to think that one person will be able to manage and perfectly understand in all aspects (biology, molecular, algorithms, informatics, etc) and specialization is certainly unavoidable. It means that people will have to work in multi-disciplinary teams to achieve serious results. On the positive side, collaborative work will be encouraged leading to increased chances of discoveries with the addition of talents and heterosis effect that will be induced. On the other side, financing such large multi-disciplinary teams will be expensive and challenging. Finding common languages between different people training in other disciplines is yet another difficulty.

Conclusions

Financial aspects of building and maintaining the wanted infrastructure will require specific grants that are not really available currently. Funding organizations need to reserve specific grants for databasing, including long-term maintenance (with monitoring) for the ones of major interest. The same applies to people developing algorithms, new IT infrastructures, high-throughput machineries, etc.

Bioinformatics and biodiversity informatics have the potential to amaze the world with incredible discoveries and, to us, never in the history of science the rate of paradigms changes has been so high. New researchers will have tools that alchemists of the past would never have dreamed of using. Monitoring and diagnostic tools will allow to solve problems that were before considered as unachievable. Young people unafraid of intellectual challenges, and ready to work hard, should be strongly encouraged to embrace this new field knowing that it will not be an easy route but such an exciting one.

Keywords

Bioinformatics, software, database, challenges, biodiversity

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SELECTED LECTURE

POOLED LIBRARY PREPARATION FOR DEEP SEQUENCING OF DIVERSE MICROBIAL SAMPLES

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The sequencing of 16S and ITS regions from potentially diverse fungal samples represents an important set of biological and technical challenges. It is useful for many genotyping application, as well as for potential identification of pathogens, to be able to detect and quantify variants in complex microbial mixtures. Generally, for this task, the regions that are sequenced are hundreds of bp in length, which is within the achievable limits of Sanger/CE sequencing, but when the amplified regions come from non-clonal mixtures, the sensitivity and ability of Sanger to determine the presence and abundance of subtypes within the mixture is generally poor. Next-generation sequencing of such samples represents a logical alternative to Sanger in this regard, since the ability to observe variants in complex mixtures is a natural feature of deep sequencing. NGS approaches, however, have limitation of read length for assays derived from from direct sequencing of amplicons, and high barriers of cost-per-sample compared to Sanger for deep sequencing of longer constructs.

In this study, we explored the used of a novel pooled-based library preparation approach, plexWell™ that circumvents many of the read-length and cost-per-sample issues that are encountered in NGS sequencing of long amplicons. The key feature of this approach is the use of a "pooled library prep" step, in which a relatively large number of samples (e.g. a 96-well plate) are subjected to a DNA-barcoding step that randomly labels DNA in each well of a plate, and then the samples are pooled and a single library is created containing random fragments (and constituent barcodes) from all of the samples on the plate. We report here the results of a pilot study that involve sequencing of diverse samples of *Candida* using plexWell, comparison of the results with gold-standard data (Sanger), and initial conclusions on the utility of this approach for quantification and characterization of within-sample diversity.

The results of this pilot study demonstrate that pooled-based library preparation of long amplicons (~1.5 kb) can be effectively sequenced using the plexWell method. The library derived from *Candida* amplicons used in the study was sequenced with single-end 100bp reads on an Illumina MiSeq, to an average depth of 200x. Data were compared with Sanger traces obtained in parallel, and after mapping, concordance with Sanger calls was > 99%. In several cases it was further observed that nucleotide-level heterogeneity was present despite

an unambiguous A/C/G/T basecall from Sanger data. We discuss these results in the context of ongoing work to utilize this method for characterization of diverse microbial sequencing samples.

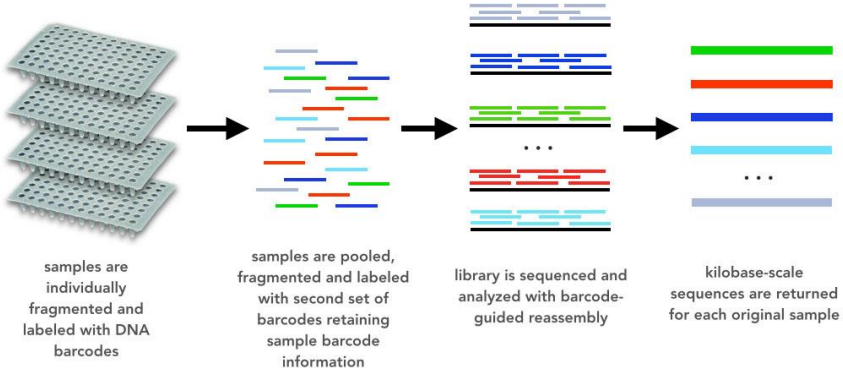

FIGURE 1. Schematic of the plexWell workflow. A 96-well plate of PCR products is labels with well-specific DNA barcodes, and then pooled, and a library is generated that preserves the well-specific DNA barcodes on each sequenced fragment.

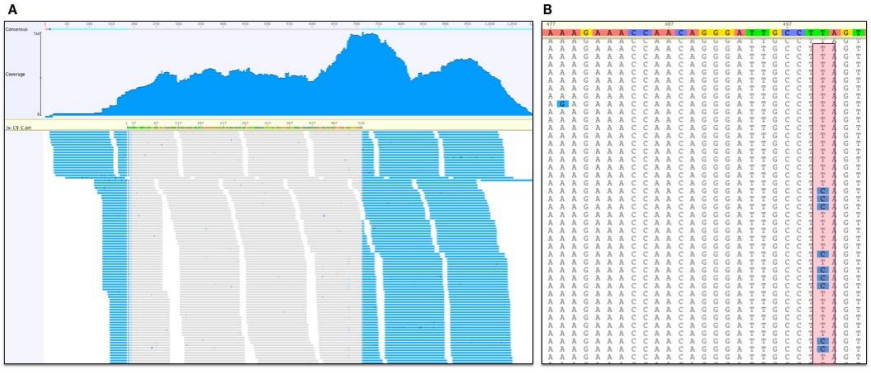

FIGURE 2. Examples of (A) alignment of *Candida* 16S ribosomal read (Sanger) to reads generated by plexWell, and (B) a heterogeneous position observed by NGS but uncalled by Sanger.

Keywords

Next-generation sequencing, sample preparation, deep sequencing

SELECTED LECTURE

16S rRNA-BASED HTS APPROACH TO MONITOR THE MICROBIOTA DEVELOPMENT DURING STORAGE OF BEEFBURGERS IN ACTIVE PACKAGING

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Introduction

Undesired microbial development in meat could appear during storage. Numerous storage methods had been applied to control the spoilage process. Among them, nisin-based active packaging have been developed as a powerful tool (La Storia et al., 2012). Species from Lactobacillales, Bacillales, Enterobacterales, Pseudomonadales and Vibrionales, appear to be the predominant spoilage microorganisms on meat/meat products in antimicrobial packaging (Sun et al., 2012). Indeed, it is poorly understood whether members of these microbial communities can really contribute to the spoilage process. High-throughput sequencing (HTS) is becoming an increasingly popular tool in food microbiology (Ercolini, 2013). In the present study, beef burgers were stored at 4°C under vacuum in nisin-activated antimicrobial packaging. From 0 to 21 days of storage, analysis were performed to determine the loads of the main microbial groups and to monitor the microbiota diversity by RNA-based DGGE and pyrosequencing.

Material and methods

Microbial analysis: a nisin-based antimicrobial solution (NS) at 2.5% (Nisin, Sigma) was prepared as described by Ercolini et al. (2010). The antimicrobial solution was coated manually on the inner part of 12 bags of Linear Low Density Polyethylene (LLDPE) (30×30cm^2) and on both sides of 72 LLDPE strips (30cm×10cm). Both bags and films were air dried (50°C) and used for the packaging of beef burger samples (100g each). Two independent batches were analyzed (A and B). A total of 324 burgers were prepared in 12 activated bags, 6 for each batch (treated, T). An equal non-activated series was used as control (C). The samples were VP before thermal sealing and stored at 4 °C. After 0, 1, 3, 5, 7, 14 and 21 days 6 samples from each bag were taken for microbial assessment as previously described (Greppi et al., 2015). *DGGE analysis:* RNA and DNA extraction, PCR and DGGE analysis were performed as described by Greppi et al. (2015). *Statistical analysis:* data from microbiological counts were analyzed by ANOVA with time or batch as the main factor while *t-test* was used to assess the differences between C and T samples at the same time and between C samples of the two batches. A combined data matrix including DNA and RNA fingerprints was obtained by Bionumerics 4.6 and dendrograms were retrieved by using Dice coefficient and UPGMA algorithm. The similarity distance matrix was used to build PLS-

DA by R package "mixOmics". *Pyrosequencing:* V1–V3 region of the 16S rRNA gene was amplified by using primers and PCR condition as described by Greppi et al. (2015). The amplicon pool was processed by using Titanium chemistry on a GS Junior platform (454 Life Sciences, Roche). *Bioinformatics:* raw reads were filtered according to 454 processing pipeline and analyzed through QIIME pipeline. 99% OTUs were picked against the Greengenes database of the 16S rRNA gene. Alpha and beta diversity and statistical analyses were carried out in R environment (www.r-project.org). Abundance of OTUs from two biological replicates of each sampling time was averaged. Filtered OTUs table (0.5% in at least 2 samples) was used to make a heatmap by R package "heatmap3". Filtered OTUs table (5%) was used to produce nodes and edge tables. The tables were imported in Gephi software and an OTU network was built. PICRUSt was used to predict abundances of gene families based on 16S data. OTUs were re-determined by using pick_closed_reference_otus.py script of QIIME with default parameters at 97% similarity against the Greengenes database. KEGG orthologs were then collapsed at level 3 of hierarchy, and the table was imported in "gage" Bioconductor package, to identify biological pathways over or under represented between T and C samples. Pair-wise Spearman correlations were calculated between OTUs and predict metagenomes.

Results and discussion

Comparing batches A and B, viable counts at time 0 in all the media appeared to be significantly higher in batch B ($P < 0.05$). Microbial load of meat depends on several factors (Nychas et al., 2008). For batch A, few differences between C and T samples were observed. Total viable counts and LAB were not affected by the use of the antimicrobial packaging and they increased in all the samples throughout the storage, reaching a final load of about 6 Log CFU/g. Few differences were observed in the count of yeasts, while no differences were detected for *Staphylococcaceae, Enterobacteriaceae* and moulds. On the other hand, an effect of the antimicrobial packaging on the main microbial population was observed for batch B. In particular, LAB increased from 4.4 to 6 Log CFU/g in C samples during storage, while in T the load was kept to about 4.4 CFU/g during the whole period; a significant reduction of the total viable counts of about 1 Log was observed at the end of storage. PLS-DA, as a function of nucleic acids, showed a certain gradient of separation between DNA and RNA samples, while those as a function of the batches presented a clear separation. To evaluate the viable population only the RNA data were further taken into account.

A total of 371,314 raw reads were obtained after 454 processing. 290,245 reads passed the filters applied through QIIME, with an average value of 5,023 reads/sample and an average length of 462 bp. The OTU network (FIG.1) showed that *Photobacterium phosphoreum*, *Lactococcus piscium, Lactobacillus sakei* and *Leuconostoc carnosum* were the major OTUs shared between C and T in both batches. From the size of the edges, it was possible to see how the relative abundance of the above OTUs increased, as affected by the VP time compared to the samples at day 0. *P. phosphoreum* increased from about 15 to 57% of the relative abundance in both batches (FIG.2), while *L. sakei* increased from 10 to 37%. *L. carnosum* were found in all the samples never lower than 5%. *P. phosphoreum* was previously reported as dominant of spoiled cod under modified atmosphere packaging (MAP) conditions and recently found as core OTUs of seafood community (Chaillou et al., 2014) while *L. piscium* and *Lb. sakei* have been recently found in a variety of meat products under MAP conditions (Rahkila et al., 2012). Through PCoA with a weighted UniFrac distance

matrix it was possible to show that samples from batch A grouped together and they were well separated from batch B on the basis of their microbiota. Comparing T samples from batch A to B, no differences in terms of composition were found, whilst C samples from batch A to B differed significantly ($P < 0.001$). ANOVA and g_test run through group_significance.py script of QIIME showed that *Kocuria rhizophila*, *Staphylococcus xylosus*, *L. carnosum* and *Carnobacterium divergens* were significantly more abundant in C of batch B compared to C of batch A. Those OTUs are sensitive to nisin treatment explaining the differences found between the two batches.

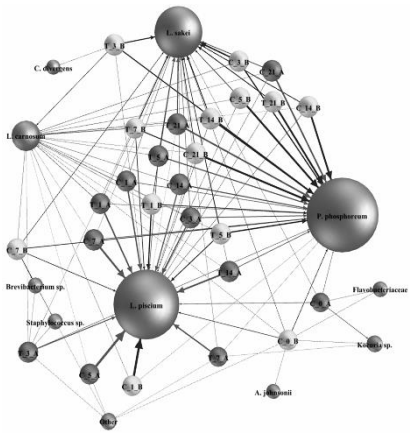

FIGURE 1. OTU network summarizing the relatioshinp between OTUs and samples.

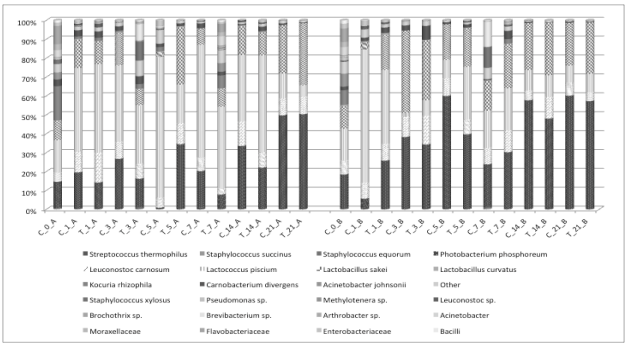

FIGURE 2. Incidence of the major taxonomic groups detected by pyrosequencing. OTUs with an incidence above 5% in at least 2 samples are shown.

Regarding the predicted metagenomes, the pathway enrichment analysis performed by "gage" showed an enrichment of propanoate metabolism (ko00640), butanoate metabolism (ko00650) biosynthesis of unsaturated fatty acids (ko01040) and sulfur metabolism (ko00920) in C samples compared to T from batch B, only. Plotting the correlation between

OTUs and predicted pathways of batch B it appeared that *L. carnsoum* and *Lb. sakei* were positively correlated with the metabolism of volatile fatty acids. *L. piscium* was mainly correlated with the biosynthesis of unsaturated fatty acids while *L. carnosum* was found to be linked to sulfur metabolism. On the other hand, despite the strong Spearman's correlation, the relationship between OTUs and predicted pathways was not statistically significant. The effect of *L. piscium* and *Lb. sakei* on the food matrix apperead to be related to the production of off-flavours (Hernandez-Maced et al., 2012).

Conclusions

The evidences presented showed that the nisin-based antimicrobial packaging was effective only as a function of the initial microbiota. The treatment impact was observed when microbiota sensitive to nisin were present in the samples at the beginning, independently of the initial load in the matrix. Only a few taxa can really play a role during the storage of beef burgers. Further, the use of nisin-based antimicrobial packaging can determined a reduction of the abundance of specific metabolic pathways related to the spoilage, with a potential impact on the prolongation of the shelf life.

Keywords

Antimicrobial vacuum packaging, meat, nisin, RT-PCR-DGGE, rRNA-based pyrosequencing.

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SELECTED LECTURE

FOURIER TRANSFORM INFRARED SPECTROSCOPY FOR EXPLORING MICROBIAL PHENOTYPIC DIVERSITY

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Introduction

Fourier transform infrared (FTIR) spectroscopy is a rapid, nondestructive technique that can be used to probe the total composition of intact microbial cells without the use of reagents. Complex yet distinct and reproducible spectral signatures or 'fingerprints' of microorganisms may be obtained for phenotyping purposes, even down to the strain level. Furthermore, the hyphenation of FTIR spectroscopy with mathematical and statistical methods has rendered the technique more versatile.

In this paper, we will present two studies demonstrating the high discriminatory power of FTIR spectroscopy in phenotyping: (a) FTIR shows the presence of characteristic and highly reproducible FTIR phenotypes for *Saccharomyces cerevisiae* gene knock-out strains, which lack genes known to be involved in lipid biosynthesis and which lack a detectable growth phenotype; (b) FTIR separates *Penicillium* isolates obtained from an apple juice production line according to phylogeny and source of isolation.

Material and methods

Yeast strains: We used *S. cerevisiae* homozygous diploid deletion strains in the BY4743 background with the genotype *MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0*, from the EUROSCARF stock center (http://www.uni-frankfurt.de/fb15/mikro/euroscarf/index.html). The analyzed 76 mutants corresponded to knock-outs of genes involved in lipid biosynthesis pathways. Moulds strains: We used *Penicillium* spp isolates obtained from the juice production line and reference strains.

Cultivation of gene knock-out strains for FTIR spectroscopy: 76 strains of the *S. cerevisiae* homozygote diploid gene knock-outs of the BY4743 series, stored deep-

frozen (-80°C) in 20% glycerol, were initially inoculated in 350 µl of SD medium (0.14% yeast nitrogen base without amino acids, 0.5% ammonium sulphate, succinic acid buffered at pH 5.8 and 2% glucose, 20 mg/l histidine, 20 mg/l methionine, 20 mg/l uracil, 20 mg/l lysine, and 100 mg/l leucine) in honeycomb microtiter plates and incubated for ~72 h at 30°C (termed pre-pre-culture). This procedure was repeated once (second incubation ~48 h, termed pre-culture). For experimental runs, pre-cultured strains were inoculated to an optical density OD of 0.03–0.1 in 350 µl of SD medium in honeycomb microtiter plates (as above) and cultivated for either 24 and 48 hours in a Bioscreen C analyzer (Labsystems Oy, Finland). The optical density (OD) was measured using a wide band filter (450–580 nm) and the incubation was set at 30.0°C (±0.1°C) with ten minutes pre-heating time. Plates were subjected to shaking at highest shaking intensity with 60 s of shaking every other minute. OD measurements were taken every 20 minutes. Except where otherwise stated, cell cultures were harvested in the stationary phase (after 24 and 48 h). The cell suspensions were transferred from the 100-well honeycomb plates to 96-well plates (with conical bottom) and the biomass was cleaned from the remaining growth medium by washing 4x with 0.1% NaCl solution in a WellWash AC microtiter plate washer (ThermoScientific, Waltham, MA). After the last washing cycle approximately 50 µl liquid remained in the wells.

Cultivation of moulds for FTIR spectroscopy: For the cultivation of moulds the high-throughput micro-cultivation system developed in EU R4SME Fp7 project FUST was used. The high-throughput micro-cultivation system comprises of 96-well microtiter plates (MTPs), a cover for MTPs (Sandwich covers (Enzyscreen, Netherlands), and a Clamp system (Enzyscreen, Netherlands), for mounting MTPs on the top of each other in the incubator-shaker. Cultivation was performed for 48 hours at 25°C under continuous shaking conditions. After cultivation mould samples were washed with deionized water and sonicated for homogenization.

FTIR spectroscopy analysis: After washing, 8 µl of the cell suspension was transferred onto IR-light-transparent Silicon 384-well microtiter plates, which were dried under moderate vacuum (0.9 bar) for 10 to 15 minutes to generate an even thin film suitable for IR measurements. A High Throughput Screening eXTension (HTS-XT) unit coupled to a Tensor 27 spectrometer (both Bruker Optik GmbH, Germany) was used for data acquisition. The spectra were recorded in transmission mode in the spectral region 4000 to 500 cm^{-1} with a resolution of 6 cm^{-1}, an aperture of 5.0 mm, taking 64 scans that were subsequently averaged. Prior to each sample measurement, background spectra of the Silicon substrate were collected in order to account for variation in water vapor and CO_2.

Data analysis: All FTIR spectra were pre-processed on the level of the second derivative using a nine point Savitzky-Golay algorithm, in order to enhance the spectral resolution. This was followed by Extended Multiplicative Signal Correction (EMSC) in order to separate physical light-scattering effects as baseline, multiplicative, linear and quadratic wavenumber dependent effects from chemical information in the spectra (Kohler et al 2005). Principal Component Analysis (PCA) was applied for studying phenotypic variation (Martens et al 2001). For calibrating FTIR spectral data for fatty acid measurement by GC analysis, power partial least squares regression (PPLSR) was used (Indahl, U.L.K et al 2009). All data analysis was done by in-house developed program codes in Matlab 8.0. (The MathWorks Inc., Natick, United States).

Results and discussion

In order to evaluate the ability of FTIR spectroscopy to distinguish between knock-out yeast strains, PCA was performed on each experimental run for 24 hours and 48 hours, separately. For growth on standard medium, the time point 24 hours is at the end of the exponential phase, while the time point 48 hours is in the stationary phase. The score plots for the first and second principal components are shown in Fig. 1a-d, for the two harvest times and two spectral regions, respectively. In Fig. 1a and b the score plots for the spectral region 2800 cm^{-1}–3100 cm^{-1} are shown for the harvest times 24 and 48 hours, respectively. In Fig. 1c and d the score plots for the spectral region 900 cm^{-1}–1800 cm^{-1} are shown for the harvest times 24 and 48 hours, respectively. Many of the strains show a dominant FTIR phenotype that is different from the wild type both for 24 hours and 48 hours cultivation time. Many of the strains exhibiting a distinct phenotype and being different to the wild type after 24 hours also show a distinct phenotype after 48 hours. The 24 hours phenotype and 48 hours phenotype are similar distinctive.

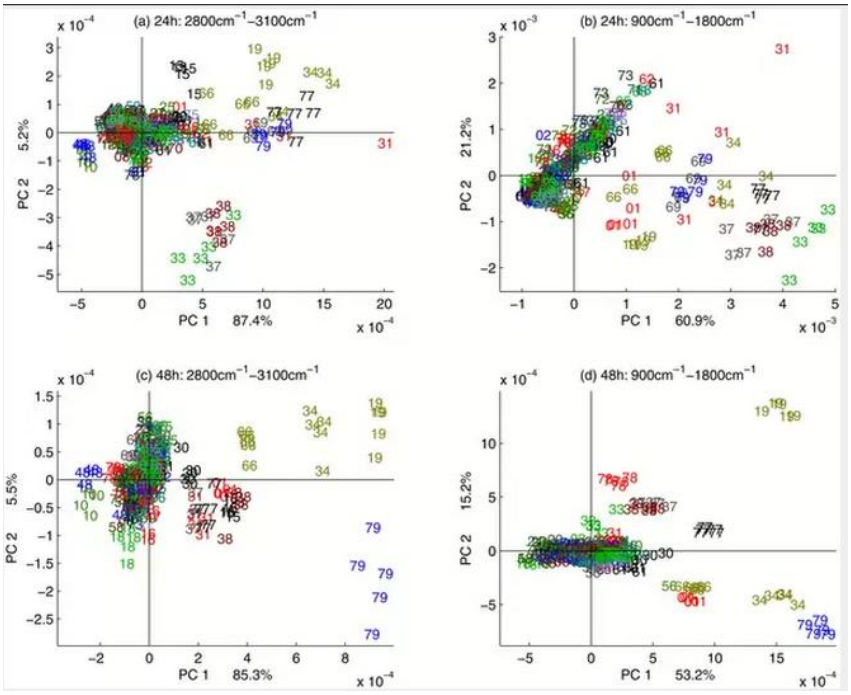

FIGURE 1. The first and the second scores of the PCA of one experimental run are shown for two harvest time points and two spectral regions

Conclusions

A recently developed high-throughput microcultivation approach for FTIR spectroscopic characterization of fungi allows the cultivation of several hundred strains under strictly controlled conditions (Shapaval et al 2010), (Shapaval et al 2013a), (Shapaval et al 2013b), The multivariate analysis showed that many of the examined knock-out strains showed characteristic and highly reproducible FTIR phenotypes despite having no detectable growth phenotype (Kohler et al. 2015) and the detected phenotypes were confirmed by more high-resolution GC-MS analysis. Hierarchical-cluster analysis of both FTIR fingerprints and ITS sequences of *Penicillium* isolates showed that highly reproducible FTIR fingerprints of the isolates mirror the source of isolation and thus provide an overview over their phenotypic diversity, while ITS sequencing shows only phylogenetic affiliation.

Keywords

FTIR spectroscopy, yeasts, moulds, phenotypic fingerprinting.

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SELECTED LECTURE

A COMPREHENSIVE SNAPSHOT OF PLANT NICHE ENVIRONMENTS SENSING AND ADAPTIVE REGULATION MODELS FOR LACTOBACILLUS PLANTARUM C2 THROUGH WHOLE TRANSCRIPTOME AND PHENOTYPIC MICROARRAY

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Introduction

Raw fruits and some vegetables possess intrinsic chemical and physical features that make them particularly hostile environments for bacteria. To cope with environmental conditions, microorganisms may adopt sophisticated adaptation mechanisms. The diversity of plant environments and of bacterial enzyme activities makes the microbial adaptation to plant niches markedly heterogeneous (Filannino et al., 2014). *Lactobacillus plantarum* is a highly heterogeneous and versatile lactic acid bacterium frequently found or used in vegetables and fruits fermentation.

In this study, we investigated the plant niche-specific traits of *L. plantarum* C2 during the late exponential growth phase and maintenance period. Carrot and pineapple juices were chosen as model systems representative of vegetables and fruits, respectively, and the rich medium MRS was used as the control.

Materials and methods

L. plantarum C2 was grown and maintained in carrot or pineapple juices to mimic the chemical composition of the respective raw matrices. De Man, Rogosa and Sharpe broth was used as the control medium for optimal growth. Whole-transcriptome analysis based on customized microarray profiles was used to determine altered transcription patterns in *L. plantarum* C2. Microarray profiles were compared with substrate utilization data gathered from high throughput phenotypic microarrays (OmniLog technology).

Results and discussion

Plant substrates exerted a transcriptional pressure and induced specific molecular and metabolic responses in *L. plantarum* C2. To provide an overview of the specific transcriptional reprogramming associated with growth and maintenance in each model system, we defined a set of putative KEGG pathways that were significantly enriched and that were associated with enriched genes. The DAVID annotation tool (a web-based tool developed for GO-ranking analysis) was used for pathways analysis. Regardless of the substrate, a common transcriptional response was associated with several biological processes required for growth and maintenance. Furthermore, *L. plantarum* C2 displayed distinct transcriptional adaptations in its core metabolic pathways for growth and

maintenance in carrot or pineapple juices. Carrot juice induced the expression of functional pathways in *L. plantarum*. These pathways allow bacteria to sense the environment, save energy and adopt alternate routes for the regeneration of the NAD cofactor. Therefore, the metabolism of carbon and of other essential elements may be coordinated. *L. plantarum* C2 cells under the acidic environment of fruit like pineapple developed a sophisticated regulatory network that combines several transcriptional regulators to coordinate optimal carbohydrate flow, amino acid and protein metabolism, pH homeostasis and membrane fluidity. Phenotypic dissimilarity in *L. plantarum* C2, across the different plant substrates and MRS medium, was the highest in carrot juice concerning the carbon metabolism, and in pineapple juice related to the nitrogen metabolism.

Conclusions

RNA and phenotypic microarray analyses revealed altered transcription patterns of genes encoding functions involved in primary metabolism, membrane transport, cofactors and vitamins metabolism, translation regulation, nucleotide metabolism, and fatty acid biosynthesis. Findings presented in this study support the conclusion that *L. plantarum* exhibits high levels of environmental niche specificity to sustain growth and survival in different plant-associated habitats. Findings contribute to the description of bacterial transcriptional adaptation to niches, and provide a more solid basis for selection the most suitable starters for fermentation of targeted matrices.

Keywords

Transcriptome, phenome, *Lactobacillus plantarum*, plant niches adaptation.

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SELECTED LECTURE

MALDI-TOF MS OF MICROBIAL MIXTURES: IMPRESSIONS OF ITS USABILITY FOR CULTURE-INDEPENDENT ANALYSES OF MICROBIAL DIVERSITY IN FOOD ECOSYSTEMS

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Introduction

In the course of the last two decades, the field of microbial ecology, with food microbiology in particular, has undergone profound changes due to a methodological shift from conventional culturing approaches towards the use of culture-independent techniques (Justé et al., 2008). This transition has shed new light on the diversity and population dynamics of the microbiota present by bypassing a number of limitations associated with culture-based approaches. Today a wide variety of, mostly genetic, profiling methods is available, already signifying an important step in a better understanding of the microbial composition of many foods. Yet, despite evident advantages, they also have their specific limitations: parts of the micro-organisms present still remain uncharted and often, these techniques do not excel in speed and cost-effectiveness preventing a more wide-spread and routine use like in the food industry.

A fast-paced evolving technique in the field of microbiology is matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Its high sensitivity and accuracy together with high throughput capacities have made it a state-of-the-art technique for systematic characterization and identification of large numbers of microbial isolates of various origin (Freiwald and Saurer, 2009). Besides, it has been suggested to apply MALDI-TOF MS as a new tool in polyphasic taxonomy for the delineation of novel micro-organisms (Vandamme and Peeters, 2014). However, still to date, its application for the culture-independent analysis of microbiota remains challenging and largely untouched. The fact that a given species can be identified through taxon-specific biomarker sets, i.e. mass peaks with a certain mass to charge (m/z) ratio (Fenselau, 2013), opens up perspectives to be able to discriminate and identify the different members within a community, however. With this, important advantages are the comparative simplicity of the mass spectra for a given species and the high reproducibility of the technique.

In this study, we investigated the effect of an increasing complexity of a microbial mixture on the quality of the resulting MALDI-TOF MS profiles and as a consequence the usability

of MALDI-TOF for the analysis of microbial communities such as those encountered in food ecosystems.

Material and Methods

To evaluate the usability of MALDI-TOF MS for the profiling of microbial communities we investigated the effect of an increasing complexity of a microbial mixture on the quality of the MS profiles. The complexity of the mixtures varied from two to twenty-one different species. Two sets of such mixtures were generated in parallel, if possible using a different strain between the two sets. Strains used in this study were obtained from the BCCM/LMG bacteria collection (Gent, Belgium, http://bccm.belspo.be). All strains were cultured using the same conditions: MRS agar; 24 hours; 37 °C; aerobic. Then, for each strain, standardized cell suspensions of 1.5 optical density at 590 nm in 0.85% NaCl were made. Mixtures were prepared by mixing 100 µL of these standardized solutions. Each set of mixtures was prepared in triplicate and analyzed three times with MALDI-TOF MS, resulting in a total of nine spectra per mixture. Similarly, for each isolate nine profiles were generated.

MALDI-TOF MS analysis was performed on cell extracts obtained according to the formic acid and acetonitrile cell extraction procedure as described by Freiwald and Sauer (2009). However, in the context of this study, the extraction was performed on the whole cell pellets (centrifugation for 2 minutes at 13.000 rpm) obtained from the cell solutions generated as describe before. Resulting bacterial cell extracts (1 µL) were spotted on a 384 Opti-TOF stainless steel MALDI-TOF MS target plate (AB Sciex) and dried at room temperature. Subsequently the sample spot was overlaid with 1 µL matrix consisting of a 0.5% (w/v) α-cyano-4-hydroxycinnamic acid (α-CHCA) in 50:48:2 acetonitrile:water:trifluoroacetic acid solution. The spotted plate was analyzed with the 4800 Plus MALDI TOF/TOF™ Analyzer (AB Sciex) in linear, positive-ion mode. Each generated spectrum resulted from 40 laser shots at 50 random positions within the measuring spot. MALDI-TOF mass spectra were generated in the mass range 2-20 kDa.

Analysis of the spectral data was performed visually and with Data Explorer 4.9 software (AB Sciex), BioNumerics 5.1 (Applied Maths, Belgium) for full spectra comparisons and MASCAP (Mass Spectrometry Comparative Analysis Package) in the MATLAB 7.0 environment for peak based analysis.

Results and Discussion

As expected, the complexity of MALDI-TOF MS profiles increased with growing numbers of species added to the mixture. However, spectral profiles remained of good quality (no smear or excessive background). This, together with a high reproducibility between the nine replicate spectra for each sample, permitted comparing mixtures of different species composition. Figure 1 shows a Pearson similarity curve-based UPGMA cluster of 3 averaged (of triplicate spots) entire MALDI-TOF MS profiles of the most complex mixtures composed of 18, 19, 20 and 21 different species.

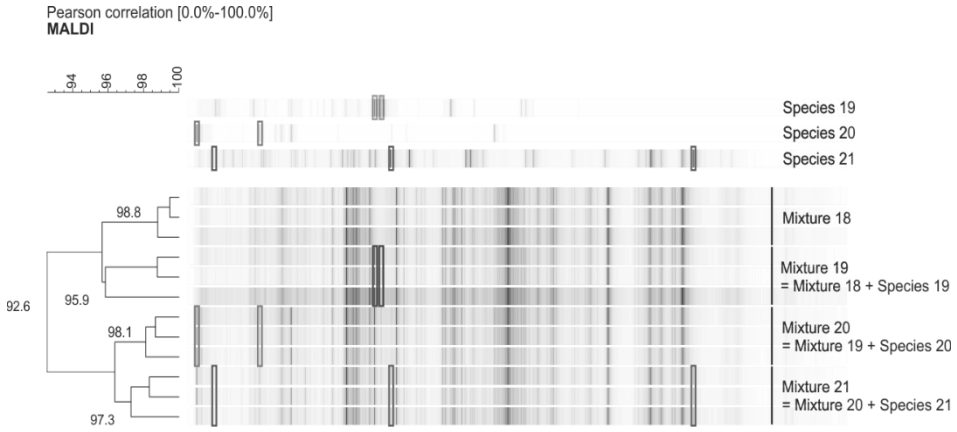

FIGURE 1. Quality, Reproducibility and Discriminatory power

High similarity values between the 3 averaged spectra of each mixture demonstrate a high reproducibility. Also, the different mixtures, even when only differing in one species, could easily be distinguished. In colored boxes, are specific peaks that could visually be attributed to the respective species added newest to the mixture. However, individual members could not always be retrieved visually from the community profile or by just comparing the full spectra. Sensitivity of MALDI-TOF was at least as high as for DGGE with a detection limit situated around 1%. Also here, actual sensitivity could be higher as this observation could also be biased by the software and visual restrictions (data not shown).

The aforementioned problems explain the need for more specialized data interpretation software allowing further in-depth analyses. A more detailed analysis was performed using MASCAP taking into account peak lists, instead of a full spectral analysis. This peak based analysis approach revealed more details with respect to the species present in the mixtures and regarding differences between the diverse mixtures. In Figure 2 the full MALDI-TOF MS profile of a mixture of 19 species is shown. On top, the 100% peak (i.e. peaks that occur in all 9 mass spectra of that mixture) profiles of that same mixture and the mixture missing the 19th species. Below are depicted the 100% peaks of species 19 with in red those peaks that were not present in any of the other 20 strains used in the dataset of this study. A similar approach was used to elucidate the composition of all mixtures by coupling peaks in the mixture profile to species specific peaks.

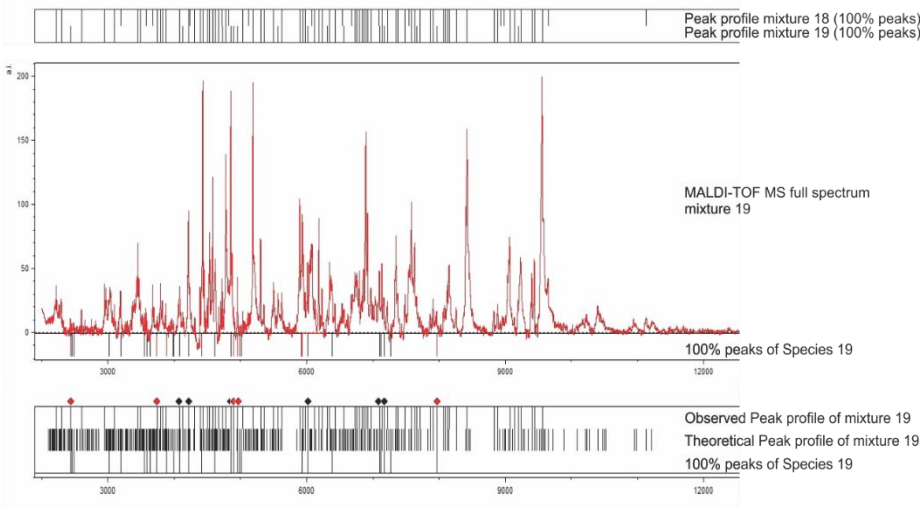

FIGURE 2. Peak based analysis of spectra using MASCAP

Conclusion

These preliminary results indicate potential for MALDI-TOF as a fast and high throughput application for the analysis of more complex microbial communities the culture-independent way with use, for instance, in the fast paced monitoring of microbial dynamics or for instant analysis of the effect of varying production process parameters on the microbial composition of food.

Keywords

MALDI-TOF MS; microbial diversity; culture-independent; high-throughput

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SESSION V

METAORGANISMS: FUNCTIONAL INTERDEPENDENCY OF MICROBIAL ASSOCIATIONS WITH PLANTS, ANIMALS AND HUMANS

PLENARY LECTURE

APPRAISAL OF MICROBIAL DIVERSITY FOR HEALTH: OF MICE, CATS AND MEN …

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Background

Gut microbiota plays a key role in the maintenance of homeostasis of host physiology, comprising development, metabolism and immunity. Profiling the composition and the gastrointestinal microbiome with a reliable methodology is of substantial interest to yield new insights into the pathogenesis of many diseases as well as to define new prophylactic and therapeutic interventions.

Recent developments in metagenomics have provided researchers with the tools needed to open the "black box" of microbiome science. These novel technologies have enabled the establishment of correlations between dysbiotic microbial communities and many diseases. Extended approaches and meticulous data interpretation will be important for resolution of these discrepancies. In this context, diagnostic tools and analytic solutions for research purposes are needed to support both clinical studies in humans and pre-clinical developments using laboratory rodents. The growing need to survey the tremendous microbial diversity in a culture independent manner, has led to the development of molecular methods through sequence profiling of part of conserved genes such as 16S rDNA, in various scientific fields including ecology (plants, animals), agronomy, biotechnology, and of course Human Health. Next-generation sequencing technologies providing unprecedented throughput of data, are now routinely used to assess bacterial community composition in complex samples. Depending if rough/basic bacterial signature or extensive resolution of taxonomic assignment of organisms is needed, the time and costs for 16S rRNA profiling *versus* full genome analysis or bacterial RNA sequencing may vary from 1 to 50. Here, we shortly report several applied examples dedicated to fecal samples from distinct origins (human, laboratory mouse and domesticated cat) together with considerations on inter-individual samples, illustrating the methodological strengths.

Methods

The individual murine fecal samples were freshly collected from ten BALB/c mice from Charles River (France) during defecation, immediately frozen in liquid nitrogen and stored at -80°C until further process. The feces samples (1-2 grams at two distinct sites in duplicate) from a single human healthy volunteer (43 years old, male) were collected at regularly time points, quickly frozen and store at -80°C. Finally, the single fecal sample of cat origin was taken from the freshly made kitty litter (Globule). All the samples were blindly processed for DNA extraction. Metabiote® kit has been used for library preparation according to

Genoscreen's recommendations (GenoScreen, Lille, France). Final libraries containing each 12 different samples identified by a SIM were amplified by emPCR as described in the GS Titanium Amplification Method Manuel Lib-L. Sequencing was performed on a GsFLX Instrument using version 2.9 software. Amplicon Libraries were each sequenced on one separate eighth of PicoTiterPlate (PTP) resulting in between 84 000 and 115 000 Passed Filter reads. Read length histogram (Figure 4) shows the typical achieved modal read lengths that is in agreement with the Metabiote® V3V4 amplicon length. MetabioteOneLine Pipeline has been used to assess microbial population definition, diversity and comparison. This pipeline comprises the following steps: preprocessing (SIM sorting, no mismatch in specific primer, read length selection, elimination of reads with ambiguous bases, signal quality filter, homopolymers exclusion), chimera detection, OTU clustering, comparison to the database Greengenes and taxonomic establishment base on the use of QIIME pipeline (Caporaso et al, 2010).

Results and Comments

We first report consistent analysis of samples from distinct origins: human, mouse & cat. A representative example of the corresponding human, cat and mouse microbial profiles respectively obtained at the phylum, family and genus level is shown on **Figure 1**. Obviously, the methodology allows identifying highly specific signatures for material from each origin. According to the phylum level, both Firmicutes (over 70%) and Bacteroidetes (20-25%) are detected in mice and men in ranges in agreement with expected results, while the substantial Proteobacteria (10%) found in human is restricted to a marginal group in mice. Mollicutes were only detected in mice samples. Surprisingly, Gram-negative species are negligibly detected in the cat fecal material where beside the major Firmicutes (85%), Actinobacteria are highly represented (15%). The latter is essentially assigned to *Bifidobacteria* species at the genus level, showing that extremely anaerobic strains are effectively identified. In line, near 50% of the feline bacterial community is made of *Clostridium* species while *Clostridiales* are part of 5% in human and 10% in mice. Data presented here show that methodology allows identifying highly specific signatures for material from each origin. Of note, mice fecal sample appear grossly more similar to humans than the cat, suggesting at least an appropriate use of these laboratory rodents for microbial-related studies and research purposes. However, attempts to reach a fully similar microbial profile in lab mice and men would ideally be necessary. This would require the generation of microbiota-humanized mice with steady and long-term maintenance of the symbiotic communities. Unfortunately, no evidence of such complete tolerance is achieved today and some specific human-derived species are probably unable to durably colonize the mouse digestive tract.

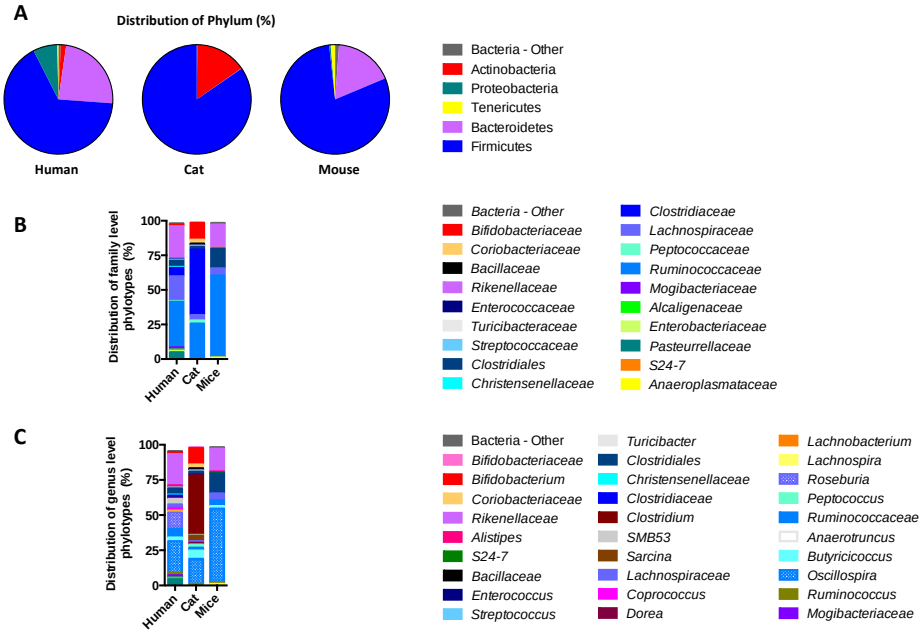

FIGURE 1.

We then report human intra-individual variations during short time course sampling. Structure of the intestinal microbiota varies substantially between individuals (Lozupone et al., 2012). Furthermore, the gut microbiota composition is dynamic and may endure slight variations following the day time activity, including work habits, sleeping period and obviously eating varied diets. We confirmed these events by addressing time course sampling of fecal replicates (two replicates at two distinct sites) on the same human specimen at 0, 24h, 30h and 48h. As shown in **Figure 2**, the composition of phylum, family and genus at the same time point demonstrates minor changes depending on the sampling site while, in contrast, replicates are similar "two by two". In contrast, more important variations are seen in respect with time. For example, although the core bacterial community is preserved all day long (*Rikenellaceae*, *Roseburia*, *Oscillospora*), the microbial profiling is clearly different after 24h, revealing an increase in *Ruminococcus.* Likewise, analysis at the 48h time point showed a higher proportion of the phylum Proteobacteria (corresponding to *Haemophilus* spp *from Pasteurrellaceae*), *Sutterella* and the clone SMB53 (candidate genus of *Clostridiaceae*) concomitantly with a drop in *Rikenellaceae.* Interpreting the sources and consequences of these changes is elusive and mostly speculative here. However, the subtle fluctuations could reasonably be attributed to the direct or indirect impact of ingested foodstuffs.

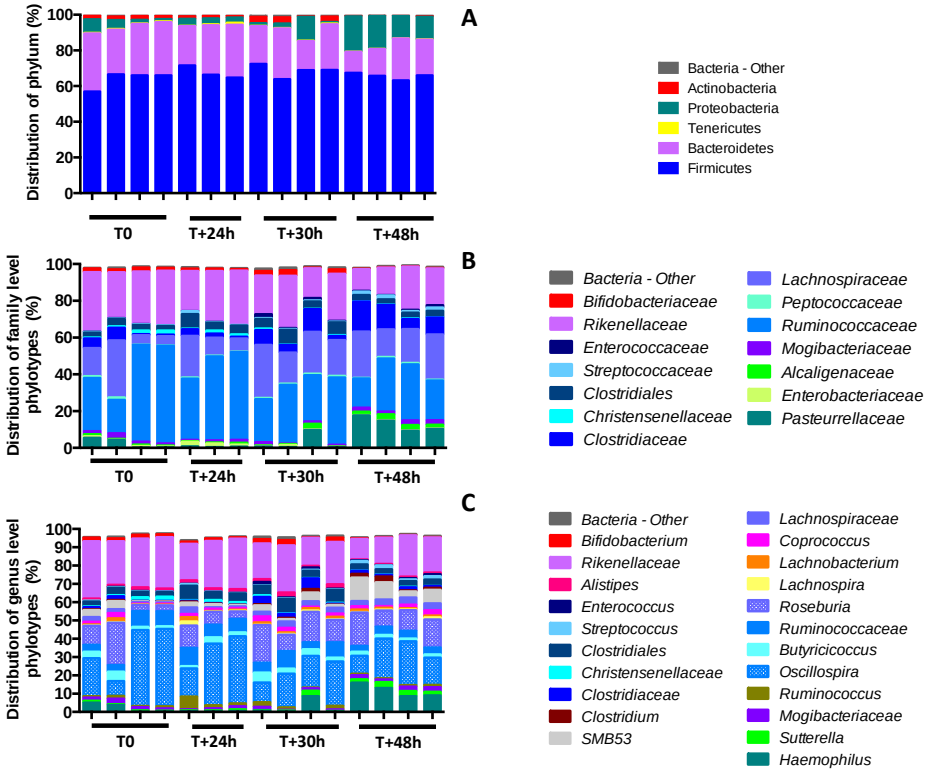

FIGURE 2.

Finally, we addressed the inter-individual variations in cohoused laboratory mice. A relative uniformity of biological responses is essential in murine experimental models worldwide. Individuality in gut microbiota composition is shaped by complex environmental and host genetic factors (Benson et al., 2010) and consequently, variable bacterial communities correspond to specificity in immune and metabolic pathways (Walker et al., 2014; Patterson and Turnbaugh, 2014). The composition (and activities) of intestinal symbiotic microbial consortia highly depends on the mice genetic backgrounds (Campbell et al., 2012) but huge variations between isogenic adult mice reared in different research institutions and providers are observed too (Friswell et al., 2010), as well as important seasonal changes. Moreover, individuality in mice microbiota profiles may also evoke concerns for research purpose. Here, we questioned the diversity among ten specimens from the same conventional laboratory cage following 12 days of acclimatization. **Figure 3** illustrates a detailed overvew of such individual profiles on the phylum, family and genus level. Abundances in *Firmicutes* can represent 60% to 90% while the *Bacteroidetes* range from 10 to 35%. Less frequent phyla such *Tenericutes* and *Proteobacteria* could or not be detected. For example, *Ruminococcus* spp are identified in only 6 mice from the group while four mice are *Alistipes* positive. Such diversity is constantly observed in cagemates from distinct providers upon the arrival and

following various diets or treatments (data not shown). Neither coprophagy nor long-term cohousing seems to be able to standardize this fact.

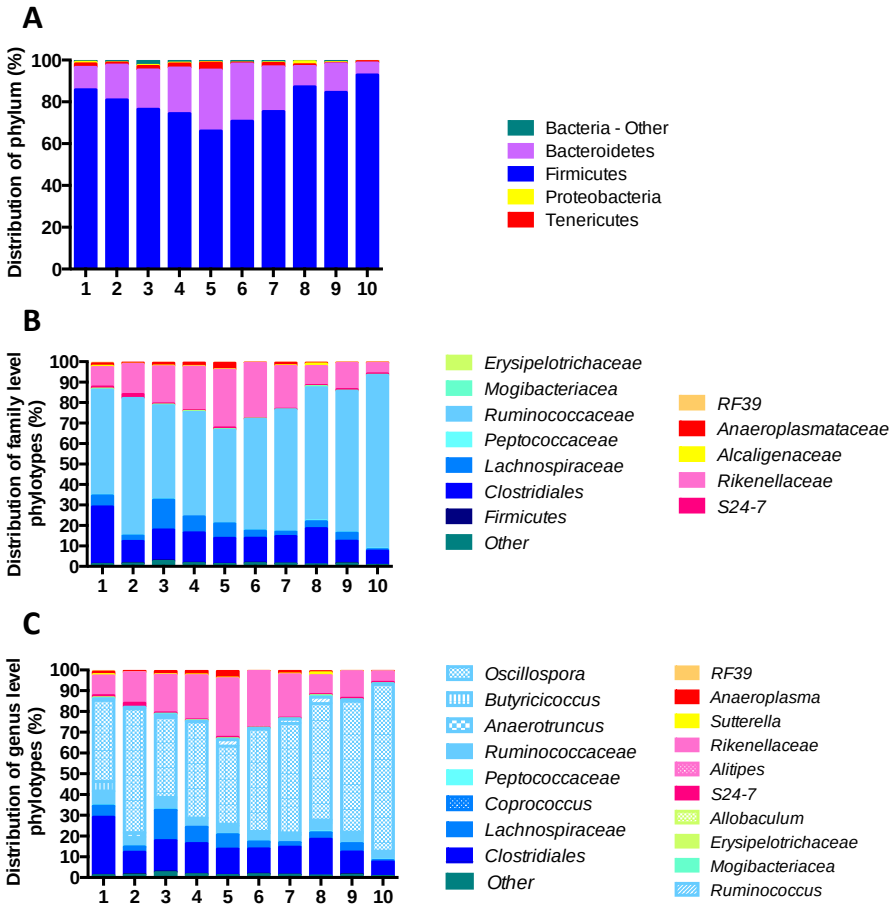

FIGURE 3

Conclusions

Knowing the composition of the microbial community alone does not necessarily lead to an understanding of its function and functional metagenomic and (meta)-metabolomic might be required. However, such analyses are helpful to explain discrepancies among individuals considering the microbial determinant of biochemical individuality. Without playing the role of Cassandra, pointing out the huge technical bias and both inter /intra-individual variations as well as time-related changes of gut microbial composition, the separate profiling of bacterial communities is of major interest for scientists and clinicians. Indeed, it allows further stratification of distinct responders both in modelling immune and infectious diseases and for personalized therapeutic interventions. Collectively, this process is useful for the

diagnostic of dysbiotic states and the follow-up of diet and treatments in clinical studies. In addition, it may clearly serve as corner stone for research purposes in microbiota-presumed diseases modeling in rodents, the latter being more realistic and thus fitting the 3Rs ethical rules (Richmond, 2000). Although the microbiome science needs a healthy dose of scepticism (Hanage, 2014), it also requires reliable and consistent tools for gold standard metagenomic analysis.

Keywords
Gut microbiota; health; phylotype profiling; individual variation.

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SELECTED LECTURE

BACTERIAL BIODIVERSITY ON SOLANUM TUBEROSUM *INOCULATED BY ENDOPHYTES*

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Introduction

The use of agrochemicals is not the only solution to increase crop productivity. In fact, the abuse of these products is decreasing the biodiversity of farmed land consequently lowering productivity. However, the plant houses inside, and the xylem vessels in particular, a vast microbial community that can be assimilated to our gut microbial communities, in which some organisms may have probiotic functions. We can use these microorganisms to stimulate plant growth and to increase the biodiversity of agricultural land.
Potatoes (*Solanum tuberosum*) are the main crop of the Fucino plain (L'Aquila, Italy) with a cultivation area of about 3000 hectares and an average annual production of 1,200,000 tons. We inoculated a mixer of selected endophytic nitrogen-fixing bacteria, plant growth promoters and protectors of some pathogens, on potato crops in the plain of Fucino.

FIGURE 1. Inoculated plant on the left, control plant on the wright.

Materials and Methods

Potato cv Agata was inoculated with a mixer of $1x10^6$ per species of *Azospirillum brasilense*, *Herbaspirillum seropedicae*, *Gluconacetobacter diazotrophicus* and *Burkholderia ambifaria*. The inoculum was absorbed on Agroperlite, an inert volcanic support, and was distributed at the sowing directly in the furrow. The presence of the four bacterial species

was checked in the soil before sowing, inside the plants at different growth stages and at the end of the harvest.
We sampled the soil before and after sowing of plants, treated and not with the inoculum and we analyzed its functional biodiversity (Eco-plates BIOLOG) and genetics biodiversity (DGGE). PCA (Principal Component Analysis) analysis, Simpson's Diversity Index (D), Simpson's Evenness Index (Ed), Range-weighted Richness (Rr) and Functional Organization (Fo) were analyzed by XLSTAT.

Results and Discussion

The bacterial species utilized were all present in the soil before the inoculum, except for *B. ambifaria*, actually, is a bacterium mostly associated with corn. This crop, in fact, was not cultivated in previous years in the field.
The results have shown that the soil biodiversity is increased by the presence of plants, either inoculated or not. The inoculum, instead, positively influenced the development of the aerial part of the plant (Figure 1), increased the size of the tubers, though not significantly increased the total productivity (biomass of the tubers). Inside inoculated plants (tubers, roots and shoot) we found concentrations of about $1x10^5$/ 10^7 bacteria of the inoculated species, while in the control plants these were nearly absent. This demonstrates the effectiveness of the selected strain in comparison with the autochthonous strains.
A PCA was performed to identify the relationship between soil samples inoculated with the microbial mix and the control soil and the importance of each variable in defining these relationships, at the level of substrates use. Figure 2 shows the biplot graph; it enables to represent simultaneously scores and loadings in the space of two main components, to be able to understand the relationships that exist between them.

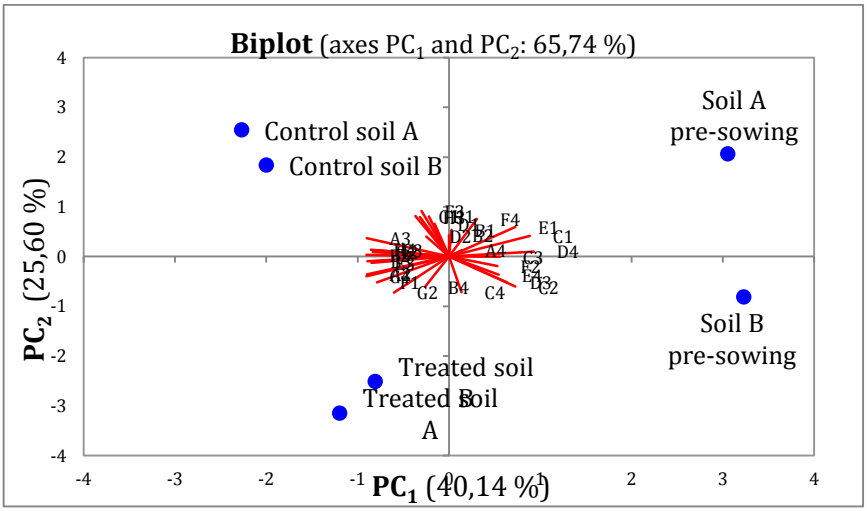

FIGURE 2. PCA Biplot graph. On x-axis there is the PC_1 (the first principal component), on y-axis there is the PC_2 (the second principal component).

Figure 2 shows that the soil before the sowing is metabolically different from the sown one, also the soil treated with the inoculum is different from the control soil, because they are

correlated with different types of substrates. Again, the presence of the plant increases the metabolic activity of the soil microbial community.
The result of DGGE gel is shown in Figure 3. There are a high number of bands, higher in the control and treated soils. The values of position and intensity of the bands were statistically processed to calculate the biodiversity indices of the microbial community, reported in Table 1. The value of richness (Rr) of soil is much lower than the pre-sowing soil samples at harvest. This demonstrates that the activity of the soil at the time of sowing still suffers from low metabolic activity due to the low winter temperatures and the absence of the plant. From table 1 we can also see how the reachness is greater in the control soil compared to treated soil. Observing the value of the Simpson diversity index (1-D) and Uniformity index (Ed) we see how the microbial community of Control and Treated soil samples show a high degree of diversity and uniformity (index values !-D and Ed> 0.9). Also from this value we can say that the effect of the inoculum has not brought imbalances at the community level of the soil; on the contrary, its increased uniformity (Ed), demonstrates that the inoculated bacteria are crucial for increasing biodiversity within the soil.

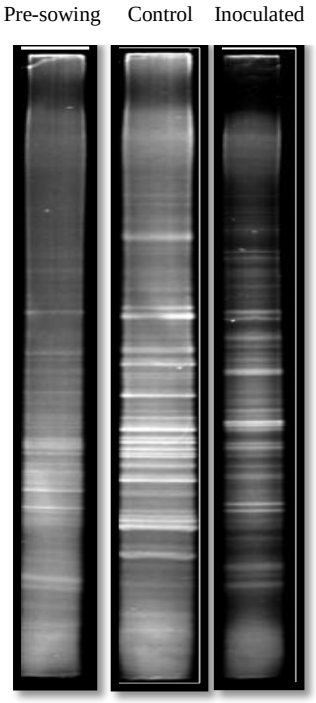

FIGURE 3. DGGE gel

Samples	Rr	1-D	Ed
Pre-sowing	31,9345	0,926039	0,983916
Control	155,475	0,953489	0,986368
Treated	121,912	0,950206	0,985399

TABLE 1. Biodiversity indexes

Keywords
Soil microbial biodiversity, plant growth promoting rhizobacteria, Principal Component Analysis, Denaturing Gradient Gel Electrophoresis

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SELECTED LECTURE

LACTIC ACID BACTERIA ARE ENDOPHYTIC COMPONENTS OF DURUM WHEAT PLANT FOLLOWING THE WHOLE LIFE CYCLE FROM SOIL TO FLOUR

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Introduction

Wheat is a well-characterized crop with a worldwide economic relevance, whose associated bacteria could influence the plant growth (Bulgarelli et al., 2013). Several bacteria contaminate grains, and hence flour, affecting the quality of leavened baked goods (De Vuyst et al., 2009). Some spore-forming bacteria (e.g. *Bacillus* sp.) cause rope spoilage in bread, but they can be inhibited by the use of sourdough (Valerio et al., 2012). Lactic acid bacteria (LAB), the dominant sourdough microorganisms, originate from flour and bakery environment. The current abundance of literature has not yet elucidated the origin of LAB (Minervini et al., 2014), but it suggests that LAB contaminating flour are a part of the endophytic microbiota of cereals. This study aims to assess with culture-dependent and -independent approaches the composition of the wheat plant microbiota during the different phases of growth, and to establish the extent of flour contamination by endophytic lactic acid bacteria and other *Firmicutes*, which from the field become relevant for sourdough fermentation.

Materials and methods

Epigeous (leaves/spikes) and hypogeous organs of durum wheat (cultivars Odisseo and Saragolla) were sampled at tillering, stem elongation, booting, flowering, milk development, and physiological maturity. Epiphytic and endophytic bacteria were separated from each other and subjected to extraction of DNA. DNA was also extracted from grain and flour (processed wheat) of both cultivars and used as template, together with Firmicutes-specific primers, in next generation sequencing analyses performed through Illumina MiSeq by Research and Testing Laboratory (Lubbock, TX). Weighted and unweighted UniFrac distance matrices and OTU tables were used to perform ADONIS and ANOSIM statistical tests through the compare_category.py script of QIIME to verify the microbial populations in the different plant organs. Endophytic LAB extracted from plant organs and

Results and discussion

Beta-diversity indices showed that the composition of *Firmicutes* in durum wheat spikes was similar to that of processed grains. *Bacillus*, *Exiguobacterium*, *Paenibacillus*, *Staphylococcus*, *Lactobacillus*, *Streptococcus*, *Enterococcus*, and *Lactococcus* were the core

genera of wheat plant and processed samples (data not shown). All the above-mentioned *Firmicutes* were previously identified in wheat flour (Ercolini et al., 2013; Minervini et al., 2015; Rizzello et al., 2015). Based on the results of this study, the spike or grain microbiota strongly contaminated the related flour. However, when flour is used for producing sourdough, only the most adapted microorganisms (mainly consisting of lactobacilli) are selected, leading to mature sourdough, whose microbiota greatly differs from that of flour (Ercolini et al., 2013). Within the core microbiota of wheat plant and processed samples, the relative abundance of each genus was affected by the plant organs, the cultivars, and the phenological stages. Overall, *Bacillus* was the most abundant genus in roots, especially at the epiphytic level. In this study, *Bacillales* were found as dominant OTUs in the Saragolla flour. Saragolla showed a lower relative abundance of OTUs belonging to *Lactobacillus* in roots, leaves, and spikes than did Odisseo durum wheat (data not shown). Saragolla had the fastest increase of OTUs belonging to LAB in roots and, especially, at epiphytic levels (leaves and spikes) during booting. At the endophytic level (spikes), Saragolla showed the highest number of OTUs belonging to *Lactobacillus* during milk development. However, the relative abundance of *Lactobacillus* markedly decreased at physiological maturity, probably because of the decrease of a_w found at that stage. The LAB composition of spikes directly affected the bacterial community of processed wheat samples (grain and flour). As hypothesized, the OTUs found as endophytic bacteria of grains (*Lactobacillus*, *Lactococcus*, *Enterococcus*, and *Streptococcus*) were also found in the flour. In most of spontaneous sourdoughs, lactococci, eneterococci and streptococci are present only as intermediate organisms, whereas lactobacilli frequently dominate this ecosystem (Ercolini et al., 2013). In this study *L. plantarum*, one of the key-bacteria of sourdough, was the only species identified in both cultivars at all the phenological stages. Culture-dependent analyses confirmed *L. plantarum* as the endophytic LAB species found in both cultivars, at all phonological stages, as well as in the processed wheat samples. Several isolates of this species at different phonological stages of Odisseo (Fig. 1) and Saragolla (Fig. 2) wheat as well as in the processed wheat samples showed the same RAPD fingerprint. Besides *L. plantarum*, mesophilic (e.g., *L. coryniformis*) and thermophilic (*L. helveticus* and *L. delbrueckii*) lactobacilli were mainly isolated in the first phenological stages and in the milk development and physiological maturity stages, respectively (data not shown).

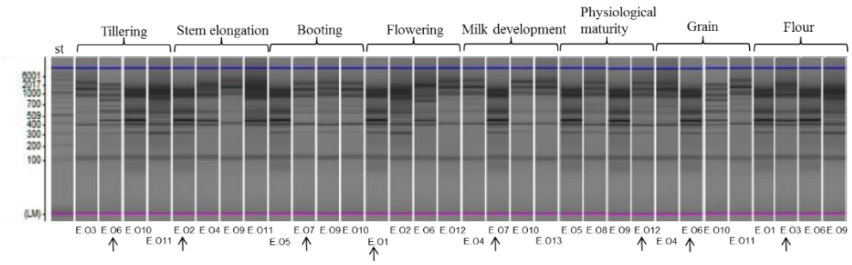

FIGURE 1. Representative RAPD-PCR profiles of *Lactobacillus plantarum* isolated at the endophytic level from leaves and spikes, grain, or flour of Odisseo durum wheat. Primer M13 was used for RAPD-PCR analysis. A 2-logDNAladder (0.1 to 10.0 kb) was used as a molecular size standard (st). Capillary electrophoretic profiles were singly acquired by MultiNA. Strains isolated from different phenological stages and processed wheat and showing similar RAPD-PCR profiles are indicated by arrows.

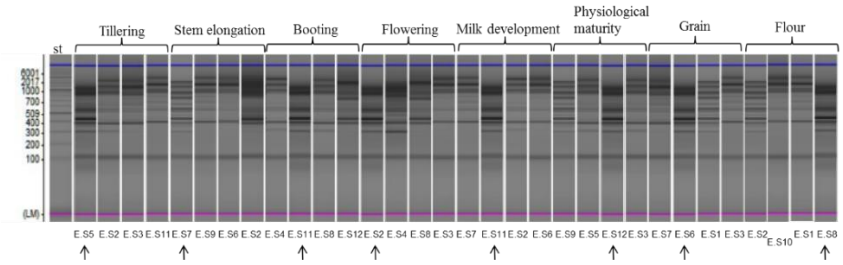

FIGURE 2. Representative RAPD-PCR profiles of *Lactobacillus plantarum* isolated at the endophytic level from leaves and spikes, grain, or flour of Saragolla durum wheat. Primer M13 was used for RAPD-PCR analysis. A 2-logDNAladder (0.1 to 10.0 kb) was used as a molecular size standard (st). Capillary electrophoretic profiles were singly acquired by MultiNA. Strains isolated from different phenological stages and processed wheat and showing similar RAPD-PCR profiles are indicated by arrows.

Conclusions

The results of this study highlight that the wheat microbiota differed from that of the grains and flour. Only few microorganisms (e.g. *L. plantarum*) identified in wheat plant play a role in sourdough.

Keywords

Wheat; endophytic bacteria; lactobacilli; flour; sourdough

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SELECTED LECTURE

GILL-BACTERIA ASSOCIATION IN DUAL-BREATHING ANIMALS LIVING IN MANGROVE ECOSYSTEM

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Introduction

Tropical mangroves are coastal habitats with harsh environmental conditions predominantly due to tidal cycles that determine sharp gradients of salinity, oxygen and nutrient availability [1]. Mangrove sediment is characterized by high concentrations of biogenic sulfide and natural toxic compounds such as tannins and polyphenols produced by plants to protect themselves from fouling. Among the animals that can cope with such challenging stresses, crabs are extremely well adapted representing the most abundant resident taxa in the mangrove all over the world. Beyond these stresses, crabs has also have to face a poor nutrient diet due to the very high C:N and C:P ratio of the food sources available. Despite this, they were able to colonize all ecological niches evolving different life-styles that range from strictly aquatic to arboreal, undergoing different levels of terrestrialisation. This adaptation is driven by major functional evolution of the gills toward a lung-based structure supporting bimodal breathing and catabolite excretion [2]. As common in extreme environments, animals resort to specific symbiosis essential for their homeostasis and survival. In mangrove forests sessile animals like the shipworm *Neoteredo reynei*, a wood-boring mollusk, harbors nitrogen-fixing bacteria within bacteriocytes inside gills, while the giant marine ciliate *Zoothamnium niveum* hosts chemoautotrophs sulphur-oxidizing bacteria [3]. Due to these premises, we investigate the reason for the success of mangrove crabs, hypothesizing that bacteria-gill symbiosis played a major force in shaping the unique adaptation of crabs to cope with the challenges of the mangrove ecosystem.

Materials and Methods

To test our hypothesis, we focused on two key stone mangrove crab species along the latitudinal distribution of the West Indian Ocean mangroves: the ocypodid *Uca urvillei* and the sesarmid *Perisesarma guttatum* [4]. These mangrove crabs were sampled across a large latitudinal range at their southernmost, northernmost and equatorial distribution sites on the South African (ZA) and Kenyan (KY) Indian Ocean coasts and the Red Sea Saudi Arabian (KSA) coast respectively. To explore the bacteria-gill association we used scanning and

transmission electron microscopy techniques followed by molecular analysis on the 16S rRNA gene, specifically PCR-denaturing gradient gel electrophoresis and Illumina sequencing. We used fluorescence *in-situ* hybridization (FISH) as a diagnostic tool to visualize the bacterial classes retrieved by molecular analysis on the gill surface of the two crab species investigated.

Results and Discussion

In both species, electron microscope images reveal thickenings in all lamellae to avoid collapse, and the consequent interruption of oxygen exchange in air (Fig 1). This confirms the bimodal nature of this organ. However, the most important result is the complete coverage of gills by a layer of bacteria (Fig 1). They showed a specific location on the gill surfaces with a uniform colonization pattern between the regularly spaced lamellae (Fig. 1). TEM images reveal the tight connection between bacteria and the gill lamellae with electrodense filaments that seem to firmly anchor the cells to the surface (Fig 1). Molecular analysis detected the constant presence of a complex core microbiome specific to each of the two species along the latitudinal transect (Fig 2A-C). At all three latitudes, the two communities were dominated by uncultured *Actinobacteria* (ranging between 39 to 50%) distantly related to the genus *Ilumatobacter*. Another relevant group belonging to the *Alphaproteobacteria* class, the *Rhodobacteraceae* was represented by different OTUs along the overall transect. The main abundance of these two bacterial classes (*Actinobacteria* and *Alphaproteobacteria*) has been confirmed by FISH (*Actinobacteria* in Fig. 2D and E).

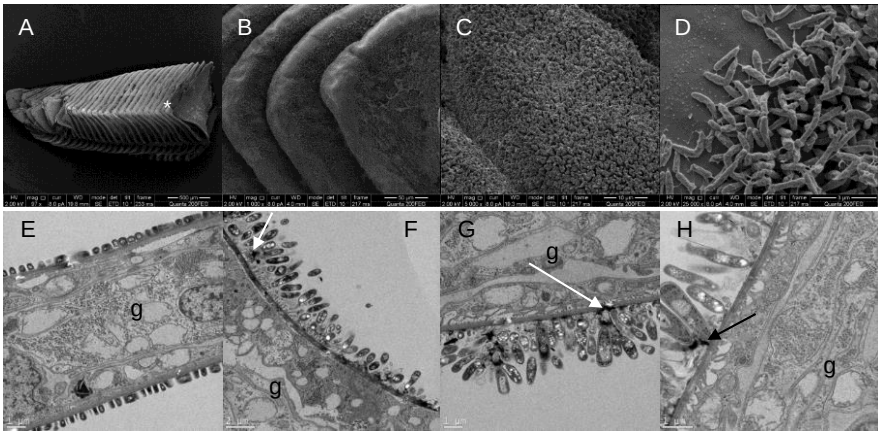

FIGURE 1. (**A**) Whole *Uca* crab gill showing the thickened portion (*) of all the lamellae in order to prevent their collapse in air. (**B**) Detail of the *Uca* gill lamellae. (**C** and **D**) Details of the extremely dense bacterial coverage on the *Uca* gill surface. (**E, F, G, H**) Detailed cross sections of the gill (g) showing the complex structure of the bacterial layer covering the external part. Arrows highlight the electrodense filaments that bacteria use to tightly adhere to the gill surface. The scale bars correspond to 500 μm in (**A** and **B**), 10 μm in (**C**) and 3 μm in (**D**), 1 μm in (**E, G** and **H**) and 2 μm in (**F**).

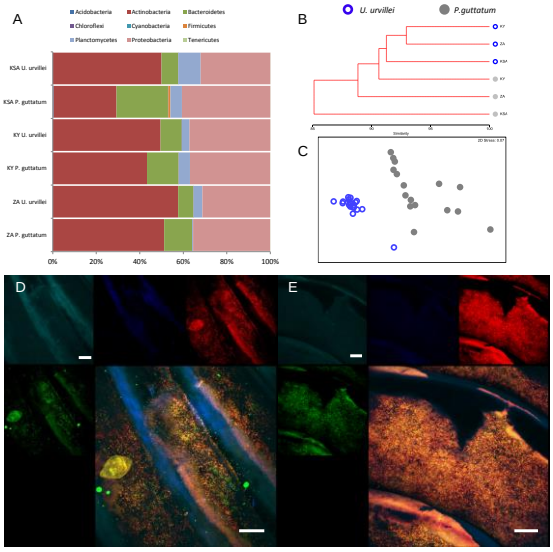

FIGURE 2. (A) The 16S rRNA gene sequencing revealed the dominance of two main bacterial classes, *Actinobacteria* and *Proteobacteria*. The bacterial communities assemblage is constant all along the latitudinal transect and it is discriminated only by crab species (**B** and **C**). Metagenomic analyses were confirmed by FISH analyses (**D** *U. urvillei* and **E** *P. guttatum*) where a massive presence of *Actinobacteria* (green probes) on the overall bacterial layer (red probes) was detected. The scale bars correspond to 50 μm in (**D** and **E**).

Conclusion

We provide here the first evidence of a novel bacterial-arthropod symbiosis contributing new insights into the understanding of the biology of mangrove crabs [4]. Our results show that in both species a constant and thick layer of bacteria entirely cover the gill lamellae designated for oxygen exchange and excretion. This can be apparently counterintuitive but we hypothesize that the function of these bacteria could play a major role in sulfide detoxification, nitrogen recycling and metabolic homeostasis, in the light of their taxonomic identity, localization on the gill surfaces and the biogeographical pattern observed along the latitudinal gradient.

Acknowledgments

This research was supported by the baseline research funds to Daniele Daffonchio - King Abdullah University of Science and Technology.

Keywords

Symbiosis, Bimodal-breathing, Mangrove, Gill-Bacteria association, Adaptation

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SELECTED LECTURE

MICROBIOTA AND METABOLOME SIGNATURES IN PLANT-BASED COMPARED TO OMNIVORE DIETS

DE FILIPPIS Francesca (1), PELLEGRINI Nicoletta (2), VANNINI Lucia (3, 4), JEFFERY Ian B. (5, 6), LA STORIA Antonietta (1), LAGHI Luca (3, 4), SERRAZANETTI Diana I. (4), DI CAGNO Raffaella (7), FERROCINO Ilario (8), LAZZI Camilla (2), TURRONI Silvia (9), COCOLIN Luca (8), BRIGIDI Patrizia (9), GOBBETTI Marco (7), O'TOOLE Paul W. (3, 4), ERCOLINI Danilo (1)*

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Introduction

The role of diet in shaping the gut microbiota is widely recognized (Aldenberg and Wu, 2014). Although it is well established that a sudden diet modification can promptly cause a change in the gut microbiome (Wu et al., 2011; David et al., 2014), and there is a general agreement on the association of some *taxa* with vegetable-rich (*Prevotella*) or protein/fat-rich diets (*Bacteroides/*Clostridia) (Wu et al., 2011; David et al., 2014), little is known about the structure of the gut microbiota in individuals with defined dietary habits such as strict vegans or vegetarians compared to omnivores. Nevertheless, vegan (V) and vegetarian (VG) dietary patterns are increasing their popularity. Mediterranean diet (MD), common in the Western Mediterranean culture, can be considered an omnivore diet characterized by a high consumption of fruit, vegetables, legumes, nuts and minimally processed cereals with well-known positive effect on the health. Since the intestinal microbiome can be considered a useful biomarker of long-term consumption of healthy or unhealthy diets (Aldenberg and Wu, 2014), it is important to determine if and to what extent long-term dietary choices can impact on the composition of the microbiota and how this can influence the production of beneficial microbial metabolites.

Materials and methods

A cohort of 153 apparently healthy volunteers was assembled comprising 51 VG, 51 V, and 51 O. Daily food and beverage consumption was recorded and the level of adherence to the Mediterranean dietary pattern was assessed using a 11-Unit dietary score based on tertiles (Agnoli et al., 2011). Fecal and urinary metabolome were analysed by gas-chromatography mass spectrometry-solid-phase microextraction (GC-MS/SPME) and NMR analyses. The

microbial diversity was assessed by pyrosequencing of the V1-V3 region of the 16S rRNA gene and the sequences were analysed by using QIIME 1.8.0 software (Caporaso et al., 2010).

Results and discussion

Analyzing the adherence level to the MD, the subjects were divided in 3 groups with low, medium and high adherence to MD. Only 11% of the subjects had low adherence level, while also 30% of the omnivores showed high MD adherence rates. This remarkable adherence to the Mediterranean diet makes the cohort a good model for a population following a healthy Western diet. The overall structure of the microbiota was similar in subjects across the different diet types. Using PAM clustering, we identified 3 enterotypes (ET), accordingly to a previous report (Arumungam et al., 2011). The three clusters were associated with abundance of *Bacteroides* (ET1), *Blautia*/*Coprococcus* (ET2) and *Prevotella* (ET3) (Figure 1). The enterotype clustering was not affected by the diet type as all three ETs included omnivores, vegetarian as well as vegan subjects showing that the diet is not associated with a global change in the microbiota. Similarly, the *Prevotella*/*Bacteroides* ratio was not diet-dependent in this dataset.

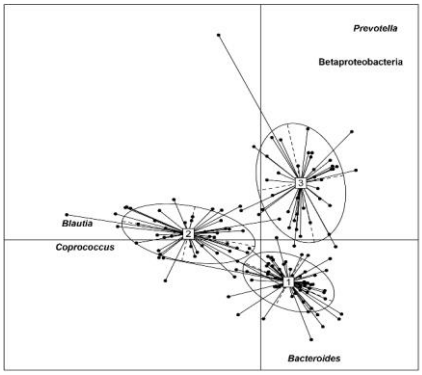

FIGURE 1. Between-class analysis, which visualizes results from PCA and clustering based on Jensen-Shannon distance of the fecal samples analyzed in this study showing a stratification of samples in three enterotypes (ET).

sPLS-DA regression based on significantly different dietary patterns showed a diet-based separation of Firmicutes (ET2) away from the Bacteroidetes (ET1 and ET3) enterotypes (Figure 2A). Consistently, significant differences were observed between ETs based on energy from carbohydrates and intake of vegetable proteins (higher in ET1 and ET3, $P<0.01$) and of total, saturated and monounsaturated fat and animal proteins (higher in ET2, $P<0.01$). The data indicate that the Bacteroidetes ETs are more linked to an agrarian diet and the Firmicutes ET to an omnivore diet; accordingly, higher Firmicutes/Bacteroidetes abundance ratios were found in most of the omnivores. Morover, associations were found between specific *taxa* and nutrient/food intake. *Veillonella*, *Roseburia*, *Haemophilus*, *Lachnospira* and *Prevotella* were correlated with vegetable-based diets, in particular with the consumption of pulses, fruit and vegetables and the intake of nutrients typical of vegetable foods, like fibre, starch, folate, beta-carotene and vitamin C. *Lachnospira* and *Prevotella* also displayed strong negative correlations with the omnivore diet. *Blautia*, *Adlercreutzia*, *Enterococcus*, *Alistipes*, *Lactobacillus*, *Streptococcus*, *Ruminococcus* (from the *Lachnospiraceae* family, L-*Ruminococcus*) correlated positively with the typical omnivore foods, such as meat, fish,

preserved meat, milk, cheese and nutrients like vitamin D, cholesterol, animal fats and proteins.

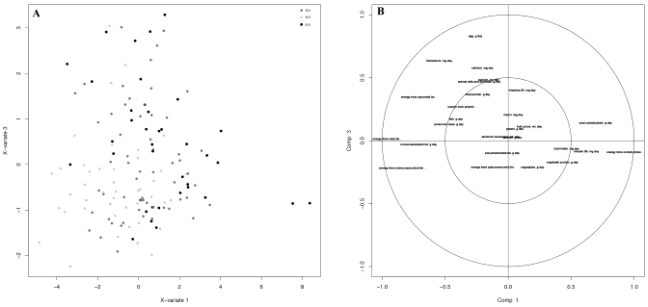

FIGURE 2. Score plots of sPLS-DA model based on dietary information showed a gradient based on *Firmicutes* (ET2) and *Bacteroidetes* (ET1 and ET3) relative abundance.

Moreover, there was a very clear relationship between fecal metabolome and diet. In particular, the levels of SCFA were increased in vegans and vegetarians, as well as in subjects with higher adherence to the MD. SCFA are produced by the intestinal microbiota during fermentation of undigested polysaccharides and have a well-documented protective role (Louis et al., 2014). These associations were corroborated by the strong correlations between SCFA and the consumption of fruit, vegetables, legumes ($P<0.05$) and fibre ($P<0.001$). Conversely, urinary trimethylamine oxide (TMAO) levels, linked to risk of atherosclerosis and cardiovascular disorders and produced by the gut microbiota from choline and carnitine (Wang et al., 2011), were significantly lower in VG and V ($P<0.0001$) compared to O subjects. Interestingly, the levels of TMAO were positively correlated with *taxa* associated to animal-based diet.

Conclusions

In this study, we provided a concrete evidence for the inter-connection between dietary patterns, gut microbiota and microbial metabolites. The consumption of fruit and vegetables through a MD offers encouraging possibilities to achieve beneficial microbiome-related metabolomic profiles in subjects consuming an omnivore diet.

Keywords

Gut microbiota; gut metabolome; short-chain fatty acids; vegan diet; vegetarian diet.

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SELECTED LECTURE

BACTERIAL DIVERSITY AND FUNCTIONAL SERVICES WITHIN THE RHIZOSHEATH OF A DESERT PLANT

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Introduction

A specific group of plants called xerophytes has evolved to adapt to extreme environments such as hot-deserts where limited water and nutrient availability, high temperature and salt accumulation represent key environmental challenges. Xerophytes developed special physiological and morphological features making them able to cope with strong water and nutrient limitations. In particular, drought tolerant species present complementary traits aiding (i) the limitation of water loss, (ii) the storage of water inside the plant tissues and (iii) the increment of water uptake trough the root system [1,2]. During periods of extended drought, which often correspond to rather high soil surface temperatures, the ability of plants to maintain a positive water balance can be severely challenged. The main player in this balance is the root system. Many of the perennial plants growing in desert sands possess a well-developed roots system characterized by the presence of a rhizosheath [3]. This structure is made and maintained by the root 'metaorganism' (the assemblage of persistent root hairs, root tissues and the associated microorganisms), its secretions and the root air-entrapped sand particles, resulting in a cylindrical sheath around the root core. All these components are suggested to be involved in increasing moisture retention, limiting desiccation and protecting the root central core [4]. It has also been determined that the rhizosheath provide a favorable "nanohabitat" for soil bacteria proliferation, including some that promote nutrient availability [5]. Despite the recognized important role of root-bacteria association in the root system homeostasis, only few data are available about the identification and the functional role of the microorganisms associated to the rhizosheath structure.

In this work we study the sand grain rhizosheath of the xeric plant *Panicum* sp. growing in the sand dunes of the Saharan Grand Erg Oriental in the South of Tunisia. To evaluate the microbiome contribution to the development of such unique root structure and in supporting plant growth under drought, we have analyzed the bacterial diversity associated with the root

system compartments together with the metabolisms and physiological profiles of these communities.

Materials and Methods

Intact roots of three *Panicum* sp. plants were collected from the sand dunes near by the Ksar Ghilane oasis in the South Tunisian Sahara desert. Under aseptic conditions the roots were dissected into three different fractions: the rhizosphere (R) composed of the sand grains loosely attached to the external surface of the rhizosheath, the rhizosheath (RS) composed by root hairs enwrapping sand grains, and the internal root core (E) that was surface sterilized to avoid contaminations from the rhizosheat. The un-vegetated sand 4 m far from the plants (B fraction) was sampled as control. All samples were collected under sterile condition and were stored at 4°C for microbiological isolation or at -20°C for molecular analysis.

In order to characterize the rhizosheath structure, stereo- and low temperature scanning electron (Cryo-SEM) microscopy was performed on the samples. To describe the structure and composition of the bacterial community associated to the root system fractions E, RS and R a metaphylogenomic analysis (Pyrotag) of the 16S rRNA genes has been done on the total extracted DNA. Furthermore, the root tissues and the rhizosheath have been used to obtain a collection of bacterial strains using different media. The isolated strains were identified and tested in vitro for their potential activity in plant protection and promotion as described in Marasco et al [6].

Results and Discussion

The *Panicum* sp. root system (Fig 1A) is characterized by a central root core covered by a compact sand-rhizosheath (Fig. 1B and C) composed by a complex of alive/dead root hairs able to entangle in a complex network individual sand grains (Fig. 1C and D). The roots 'metaorganism' is supposed to exude a mucous material that enwrap the sand grains (Fig. 1E) contributing to cement the different components and form a sleeve-like structure upholstering the root core. Microscopy observations demonstrated the presence of several prokaryotic cell morphologies on the root core surface (Fig. 1F), indicating that *Panicum* root is a suitable niche for bacterial proliferation.

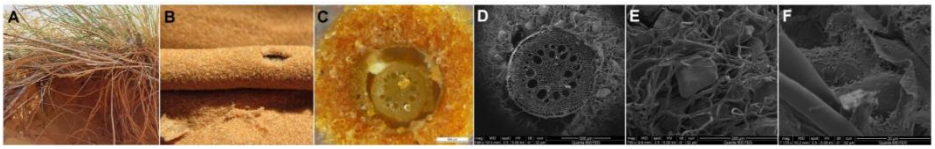

FIGURE 1. (A) Root system of Panicum sp. plant grown in Sahara desert dune. (B) Detail of a single root covered by the sand rhizosheath. (C) Stereomicroscope image of a root cross-section. (D, E and F) Cryo-SEM image performed on root cross section, rhizosheath surface and root core surface, respectively. The scale bars correspond to 500 μm in (C) and (D), 200 μm in (E) and 20 μm in (F).

A 16S rRNA gene metaphylogenomic analysis was initiated to explore the structure of the bacterial communities associated to the *Panicum* root system compartments. While the root-free sand (B) resulted inhabited by a complex microbiome represented by a relatively complex OTU97 pattern, the root system compartments were represented by a more specific community enriched with certain bacterial classes that presented a different spatial distribution in the different root compartments (E, RS and R) (Fig. 2A). According to a

bipartite network analysis (Fig. 2B), the plant compartments determine selective pressures that shape the observed community structure 'manipulating' the bacterial diversity naturally present in the bulk sand (B). In particular, the phylogenetic affiliation revealed that while the endosphere (E) of the root core was dominated by *Gammaproteobacteria* mainly represented by the *Pseudomonas* genus, the R and RS fractions were enriched mainly by *Actinobacteria* of the families *Micrococcaceae* and *Streptomycetaceae*.

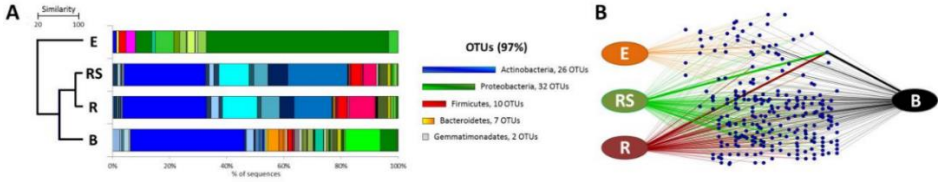

FIGURE 2. (A) Cluster analysis of metaphylogenomic data obtained on the partial 16S rRNA gene sequences considering the OTU97. (B) Network analysis of OTU97 showing connections between the bacterial communities associated to the three root compartments (E, R and RS) and the sand un-vegetated soil (B).

The microbial collection generated from the *Panicum* root system (E and RS) included a total of 300 bacterial strains representing 3 classes, 13 families, 19 genera and 48 species. The isolates, including oligotrophic and halotolerant strains, were affiliated to low GC gram-positive bacteria (38% in E and 21% in RS), *Proteobacteria* (12% in E and 6% in RS) and *Actinobacteria* (50% in E and 73% in RS). A differential distribution pattern of the main bacterial taxa among the two compartments was observed in the cultured collection as well. To estimate the functional influence of bacteria on the root, the plant protection and growth promotion potential of the isolates was characterized in vitro by evaluating their ability to produce auxin and siderophore, to solubilize phosphate, to release EPS and tolerate abiotic stresses such as salt and drought. Twenty percent of the isolates exhibited multiple PGP activities, which may affect plant growth directly, indirectly or synergistically. In particular, the tested isolates showed the potential of (i) bio-stimulation of plant growth through auxin production, (ii) bio-fertilization through production and solubilization of nutrients (P, Fe and N) and (iii) resist to salinity and low water availability. The most versatile PGP strains capable of exopolysaccharide production (EPS) were tested in microcosm experiments demonstrating their capability to enhance water retention of sand.

Conclusions

The complex nature of the microbiome associated to *Panicum* sp. root system suggests that bacteria are adapted to the environmental conditions of the plant rhizosheath that appears to attract and select a unique root–bacterial communities, adapted to drought conditions and contributing to water stress resistance. Overall we advance here the hypothesis that bacteria can be an important factors involved in rhizosheath formation and homeostasis.

Acknowledgments
This research was supported by the EU project BIODESERT and King Abdullah University of Science and Technology.

Keywords
Rhizosheath, Bacterial association, Extreme environment, Drought resistance, Plant-bacterial adaptation

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SESSION VI

BRINGING THE MICROBIAL COMPLEXITY IN THE INDUSTRIAL ENVIRONMENT

PLENARY LECTURE

INTEGRATING MICROBIOLOGY AND ENGINEERING FOR BIOREMEDIATION

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Bioremediation refers to the decontamination of soil, water bodies and sediments through biological means. Intrinsic bioremediation is used to describe the natural attenuation of pollutants without any human intervention. Quite often the natural attenuation rates of contaminants in the environment are very low and hence, human intervention is required to enhance biodegradation rates. This human intervention to enhance biodegradation rates is what turns bioremediation from a natural attenuation process to a decontamination technology. In this paper the challenges that need to be overcome for a successful application of bioremediation are presented through several practical examples where the integration between the fields of microbiology and engineering is demonstrated.

Biostimulation or bioaugmentation?

Bioaugmentation is defined as the inoculation of contaminated soil, sediment or sludge with isolated strains or consortia with specific organic compound degrading capabilities to enhance in-situ or ex-situ bioremediation applications. Bioaugmentation is primarily used to overcome the problem of lack of microbes expressing the appropriate catabolic potential to mineralize target contaminants.

Bioaugmentation is equivalent to increasing the metabolic capabilities of the microbiota present in the soil. Such an increase is actually the result of an enlargement of the genetic capacity present at the site. In that respect, bioaugmentation corresponds to an increase in the gene pool and, thus, the genetic diversity of the site. In principle, this genetic diversity could be increased by augmenting the microbial diversity (Dejonghe et al. 2001).

A bioaugmentation attempt is considered successful if the rate and percent of pollutant removal is increased and if the augmented species are not outgrown by the indigenous populations.

Excluding certain xenobiotics, one can forcefully claim that the indigenous microbial populations are most of the time capable to degrade organic pollutants if suitably stimulated. In such cases, bioaugmentation can only be justified if the requested time period for bioremediation is very short. Bioaugmentation at very high numbers ($>10^9$ cells/mL) is expected to yield satisfactory results in the short term, although the presence of a large indigenous degrading microbiota and nutrient availability in the contaminated soil may render bioaugmentation totally unnecessary (Cavalca et al. 2002). If on the other hand, time is not of essence, the indigenous populations will most likely yield the desired results in the longer run.

When dealing with soils contaminated with xenobiotics, the potential benefits of bioaugmentation become more and more apparent. It should be pointed out here that in situations where there is a nutrient imbalance, bioaugmentation should always be combined with biostimulation (Figure 1).

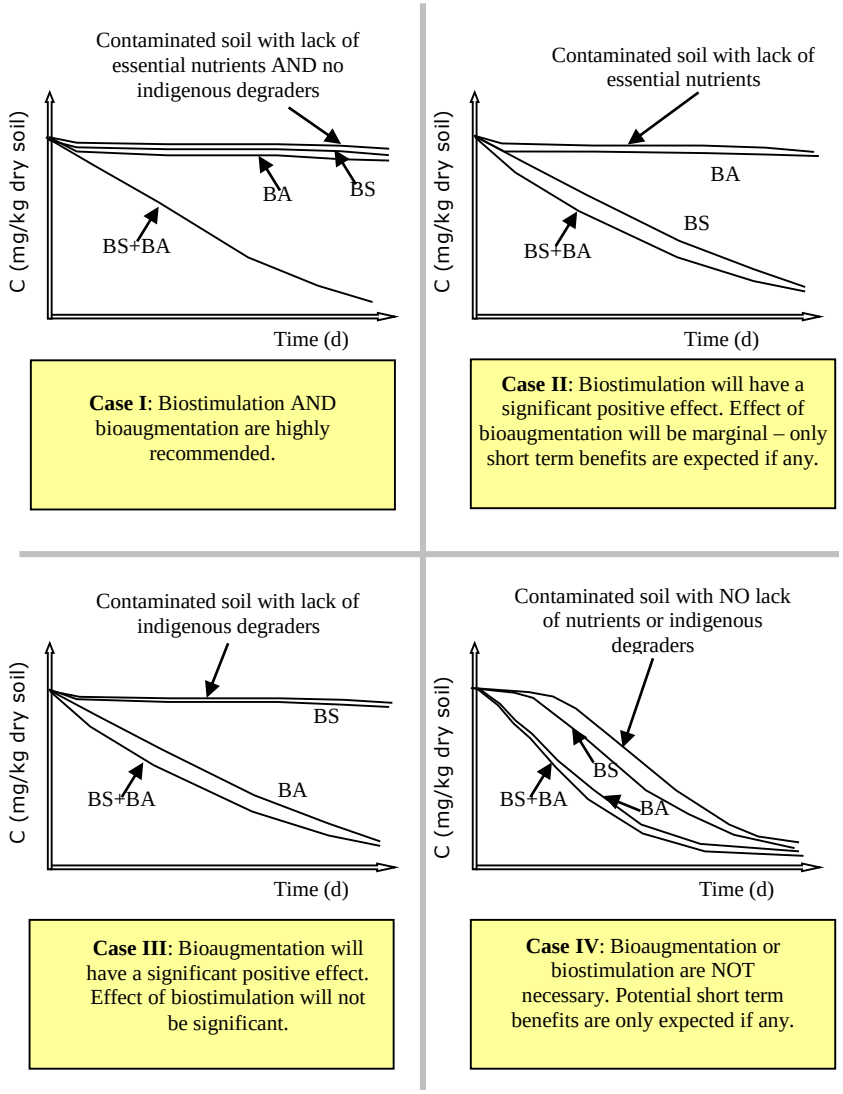

FIGURE 1. Effect of bioaugmentation and biostimulation on biodegradation of organic contaminants (not to scale) for four different cases. Abbreviations of treatments: BA=Bioaugmentation, BS=Biostimulation, BS+BA=Bioaugmentation and biostimulation (source: Kalogerakis, 2005)

Oxygen – the usual suspect…

There is a strong interest in aerobic degradation as the rates are much higher compared to the corresponding anaerobic processes. The inherent assumption is that dissolved oxygen levels are maintained above their critical levels by a sufficient supply of oxygen. At typical ambient temperatures, the concentration of DO is about 8 to 15 mg/L whereas typical organic substrates can be found in concentrations of 50 g/L or more. Namely the amount of dissolved oxygen which is readily available to the microorganisms is >1000 less! As a result, oxygen is depleted very rapidly and hence, developed technologies aim at increasing the transfer of the oxygen from the gas phase (gas bubble) to the liquid phase (dissolved oxygen). Several designs for air distributors are commercially available that match specific needs.

Innovative solutions include the introduction of H2O2 in the liquid phase; fenton like reagents; low power electrochemically generated O2.

Biosurfactants and other stimulants

One of the rate limiting steps that results in overall low biodegradation rates is due to the limited bioavailability of the absorbed contaminants in the soil. In order to increase the bioavailability either of the following options may be adopted (Kalogerakis, 2011):

(i) Surfactants can be added together with nutrients at the beginning of the remediation cycle. Concentrations below the critical micelle concentration (CMC) do not have any noticeable effect on bioavailability. On the other hand, much higher concentrations than CMC may have a negative effect on the biodegradation rate due to toxicity.

(ii) Alternatively solvents can also be used for increasing the availability and bioavailability of low solubility-, hydrophobic pollutants in soil remediation. In particular, immiscible (in water) and non degradable solvents with affinity for hydrophobic contaminants can help in attracting the molecules of contaminants adsorbed onto soil, transfer the contaminant into the solvent phase, and afterwards to facilitate the exchange of contaminant between the solvent to the aqueous phase where microorganisms can finally degrade the pollutant

(iii) Use of biosurfactants is considered a better choice. They act as emulsifiers that facilitate desorption of hydrophobic contaminants. High biosurfactant levels can be accomplished either by maintaining a high microbial concentration resulting in high production of biosurfactants or by adding commercially available biosurfactants directly into the bioreactor. In general, biosurfactants are more expensive compared to usual chemical agents.

Another reason for slow overall biodegradation rates is the recalcitrance of specific organic contaminants or complex mixtures of them. The strategy to improve the overall process is based primarily on selecting a combination of different processes (Kalogerakis, 2011). Typical examples are:

(i) A sequence of biological processes, for example, anaerobic followed by aerobic treatment. The optimum sequence and the switching point is determined by performing a series of experiments during the biodegradation test phase.

(ii) Oxidizing agents can be added to the soil to partially break down recalcitrant contaminants. Fenton's reagent has been successfully used to enhance high molecular weight PAHs degradation in slurry phase bioreactors.

(iii) A combination of chemical / biological processes is the most promising alternative. In particular, the use of advanced oxidation processes (AOPs) as a pre-treatment step where the powerful hydroxyl radicals ($OH^{\bullet}$) break down the contaminants into more easily biodegradable species is highly recommended.

(iv) Finally, bioaugmentation may prove to be a very useful strategy for xenobiotics particularly at the start up of the operation (Wang et al. 2004).

Nano et al. (2003) have proposed the sequential use of slurry-phase bioreactors followed by solid-phase bioremediation to improve overall economics and effectiveness of treatment of diesel-decontaminated soils. They showed that a low hydraulic retention time and a low slurry recycle ratio allows for a better utilization of the bioreactor volume. A 7-day slurry-phase bioreactor treatment was shown to provide enough contaminant removal allowing the soil drawn from the slurry-phase bioreactors to be fed effectively to the solid-phase bioreactors for completing the soil cleanup. Similarly, Kuyukina et al. (2003) reported on the use of slurry phase bioremediation followed by landfarming for the remediation of crude oil-contaminated soil. Slurry-phase biotreatment resulted in an 88% reduction in oil concentration after 2 months. The resulting reactor product, containing approximately 25g/kg of total recoverable petroleum hydrocarbons (TRPH), was then loaded into landfarming cells where further decontamination to the level of 1.0 to1.5 g/kg of TRPH was achieved after 5 to 7 weeks.

In situ versus ex situ approaches

Despite the apparent economic attractiveness of in-situ bioremediation technologies for the treatment of contaminated soils, ex-situ approaches are generally preferable for reasonably accessible contaminated soils as they allow much tighter control of the bioremediation process and better estimates of the residual contamination at the end of the treatment period. Ex-situ bioremediation (i.e., landfarming, windrow composting, biopiles, in-vessel composting and slurry-phase bioremediation) is particularly suited for hot spot treatment if they are reasonably accessible (typical depths less than 5 m).

Concluding remarks

Ex-situ bioremediation methods are and will continue to be more commonly used than in-situ ones if the contaminated soil is reasonably easily accessible. Windrows and biopiles are the most frequently used methods for soil remediation, particularly for hydrocarbon-contaminated soils. Both are reasonably well-understood processes (especially for petroleum hydrocarbons), but it is still difficult to accurately predict end-points. Often it is not clear whether contaminant reduction is due to biodegradation, volatilization (e.g., BTEX), leaching or dilution.

Proprietary additives are somewhat unnecessary for a vast majority of contaminants! Standard fertilizers and bulking agents can do the job of stimulating the indigenous populations. Bioaugmentation is quite often not necessary. However, the initial biodegradation of recently contaminated soils with xenobiotic compounds may benefit from bioaugmentation by accelerating the initial biodegradation rates. In situations where the time

period for bioremediation is of essence, a suitable bioaugmentation strategy may prove to be quite beneficial for several contaminants.
If we extrapolate current industrial practice, one should expect the following trends to continue in the future: (i) the use of windrow composting and biopiles will continue to grow; and (ii) slurry phase bioreactors will find only niche applications (e.g., hot spot treatment) because of higher costs.

Keywords
Bioremediation, biostimulation, oxygenation, biosurfactants

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SELECTED LECTURE

EXPLORING AND FTIR-ING YEAST DIVERSITY TOWARDS THE DEVELOPMENT OF SUPERIOR STRAINS FOR LIGNOCELLULOSIC ETHANOL

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Introduction

Robust yeast strains with high inhibitors tolerance remain a critical requirement for the production of lignocellulosic bioethanol. These stress factors are known to severely hinder yeast growth and fermentation performance (Jönsson et al., 2013). This study aims at the design of industrial yeast suitable for the lignocellulose-to-bioethanol route. Grape marc was selected as extreme environment to search for innately tolerant yeast because of its limited nutrients, exposure to solar radiation, weak acid and ethanol content (Favaro et al., 2013 and 2014). One hundred and twenty *Saccharomyces cerevisiae* strains were recently described to have inhibitors, temperature and osmotic tolerance greater than those exhibited by industrial strains and showed remarkable potential for bioethanol production.

With the aim of further investigating their industrial fitness, a Fourier Transform InfraRed Spectroscopy (FTIR)-based bioassay was employed to explore the yeast metabolomic and viability responses to inhibitors. Three different strains of *S. cerevisiae* (Fm17, Fm84 and DSM70049) were chosen as representative for the top, medium and low inhibitors tolerance. The yeast were exposed to acetic acid, formic acid, furfural and 5-hydroxymethyl-2-furaldehyde (HMF) and analysed by a FTIR-based assay to detect the extent of the metabolomic stress, the type of response and the induced mortality. To deeper investigate the effect of the co-presence of inhibitors on yeast metabolism, FTIR analysis was also conducted on yeast cells challenged with mixtures composed by increasing dosages of each single inhibitory compound.

Materials and methods

FTIR experiments were carried out as described in Corte et al. (2010). Four inhibitors were used at increasing concentrations (mM): 30, 60, 120 and 240 for acetic acid; 13, 27, 53 and 106 for formic acid; 7, 14, 29 and 56 for furfural; 7, 15, 30 and 59 for HMF. Each dose of inhibitor was reported in the manuscript as relative concentration (RC) of the third level considered as the highest concentration of these inhibitors found in lignocellulosic hydrolysates. Inhibitors were formulated also into four mixtures (RC_{25}, RC_{50}, RC_{100}, RC_{200}) obtained adding every toxic compound at the concentrations reported above. Inhibitors were added to the cells of *S. cerevisiae* Fm17, Fm84 and DSM70049 and the control was obtained by exposing the cells to distilled sterile water. Cells were incubated 1 h at 25 °C in a shaking incubator at 50 rpm, then yeast cells were prepared for FTIR measurements. All tests were carried out in triplicate. Synthetic Stress Indexes (SIs) were calculated according to Corte et al. (2010) for the Global Stress Index (GSI) and four different spectral regions: fatty acids (W1), amides (W2), mixed region (W3) and carbohydrates (W4). The biocidal activity tests were carried out to compare the metabolomic damages with the loss of viability. 100 µL of each cells suspension prepared for FTIR analysis were serial diluted to determine the viable cell counting, in triplicate, on YPD (Yeast Peptone Dextrose) plates. The biocidal effect was measured as % of cell mortality induced at different concentrations.

Results and discussion

In order to search for superior yeast, a collection of 120 thermo-tolerant *S. cerevisiae* strains was assessed for inhibitor tolerance when grown in complex and minimal broths with increasing concentrations of inhibitory compounds (Favaro et al., 2013 and 2014). Through the distribution analysis of their ability to withstand inhibitors, three different strains (Fm17, Fm84 and DSM70449) were selected as representative for the uppermost, medium and low inhibitors tolerance and analysed with a FTIR-based bioassay.

Firstly, the study of the metabolomic alteration induced by single inhibitors on yeast was carried out by calculating Stress Indexes (SIs), and coupled with cell mortality (%) of the three strains, when exposed to increasing concentration of single inhibitory compounds (data not shown). In general, at low inhibitors concentrations (RC_{25} and RC_{50}), the comparison between metabolomic and mortality data allowed to define three different categories of response. Fm17 showed low mortality values and relatively low metabolomic responses, the typical behavior of a resistant strain. On the contrary, DSM70449 displayed the highest mortality and the lowest responses, the pattern of a sensitive strain. Fp84 had low mortality values and high metabolomic changes. This third behavior is potentially interesting in the case of secondary metabolites of industrial importance, but is likely to trigger a waste of metabolic energy deployed for resistance rather than for ethanol production.

Inhibitors mixtures severely reduced cell viability with the exception of RC_{25} (Figure 1), a partially biocidal concentration causing 26, 44 and 64% mortality in Fm17, Fp84 and DSM70449, respectively. The evolution of SIs showed that the metabolomic response was proportional to the different strain resistance level, further supporting the original classification of these three strains as resistant, intermediate and sensitive, respectively. Indeed, RC_{25} pointed out to three different types of response where the sensitive yeast (DSM70449) gave low metabolomic response with a Global Stress Index (GSI) of 0.6 arbitrary units (a.u.) and high mortality (64%), the tolerant strain Fm17 low metabolomic

response (GSI: 0.9 a.u.) and low mortality (26%), and the intermediately tolerant strain Fp84 exhibited high metabolomic response (GSI: 2.4 a.u.) and mortality level of nearly 44%.
To evaluate whether positive (synergistic or additive) or negative (antagonistic) interactions occurred between inhibitors affecting yeast cell viability, the observed mortality (OM) induced by the mixtures were compared with the sum of those caused by each inhibitor at the same concentration (expected mortality, EM). The exposure to inhibitors mixtures (RC_{25} and RC_{50}) produced OM<EM, indicating that some sort of mechanism induces antagonism among inhibitors. However, only the RC_{25} with Fm17 and Fp84 strains could be used to make an accurate evaluation, as the EM values of the other combinations exceeded 100% mortality. In the strain Fm17, the OM (25.8%) was 37.6% less than the EM (63.4%), with $p = 0.000238$. Similarly, strain Fp84 showed an OM value (43.7%) 16.8% lower than EM (60.5%) ($p = 0.002342$). These findings confirmed that an antagonistic effect can be observed and measured at low cocktail concentrations.

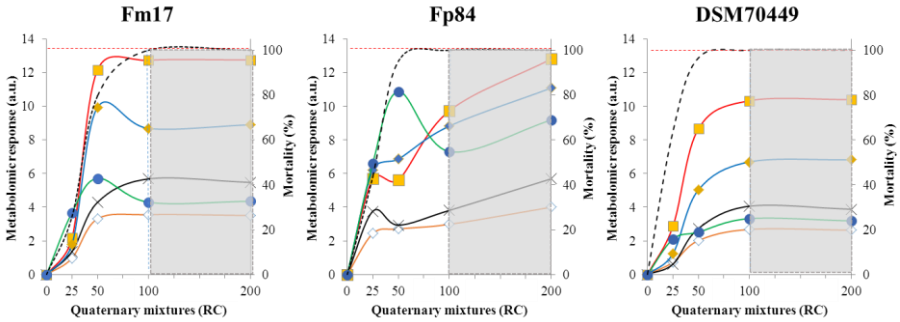

FIGURE 1.SIs of *S. cerevisiae* Fm17, Fp84 and DSM70449 subjected to increasing RCs of quaternary mixtures. ◊ represents GSI, × W1 region, ■ W2 region, ♦W3 region, ●W4 region, dashed line represents mortality. *Post mortem* chemical reaction of cellular components is indicated by a grey box. a.u. stands for "arbitrary units".

Metabolomic metrics confirmed this hypothesis and allowed to quantitatively evaluate the type of interactions among inhibitors also at metabolomic level, for all strains and RCs. In order to assess a hypothetical antagonism at the metabolic level, an Absolute Reduction Indicator (ARI) was proposed as the difference between the sum of the metabolomic responses, induced separately by each inhibitor, and that of the mixture. Positive ARI values indicate antagonism, negative synergism and figures close to 0 additivity. With few exceptions, ARI values were positive indicating variable intensity of antagonism among inhibitors for the three strains at RC_{25} and RC_{50} in all the considered spectral regions (W1-W4 region) and for the whole spectrum (GSI). These data strengthened that the three strains have different metabolomic responses to inhibitors. Moreover, both cell mortalities and metabolomic data confirmed the presence of antagonistic effect among the inhibitors acting, as mixtures, on yeast cells. Although these results are not in accordance with the related literature on the additive and synergistic effects of inhibitors on microbial metabolism (Jönsson et al., 2013), this study represents, to our knowledge, one of the first reports on the antagonistic effect exerted by inhibitors in mixtures.

Conclusions

This paper demonstrated that FTIR is a powerful tool for assessing industrial fitness in yeast. Moreover, this is the first account on qualitative and quantitative evaluations of the antagonistic effects of quaternary inhibitors mixtures on metabolomic responses and viability reduction in *S. cerevisiae*. Further studies using binary and ternary mixtures are in progress to deeply understand the mechanism sustaining this novel phenomenon and to assist strain selection towards the development of superior yeast suitable for the lignocellulosic ethanol.

Keywords

Lignocellulosic bioethanol, industrial yeast, inhibitors, FTIR, antagonism

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SELECTED LECTURE

DIVERSITY IN THE RESPONSE OF AEROBIC METABOLISM TO ENVIRONMENTAL FACTORS AMONG DIFFERENT WINE YEAST SPECIES

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Introduction

The steady increase in alcohol levels in wine is one of the main challenges faced by the oenological industry. High alcoholic strength might impair wine sensory quality, or discourage consumers, concerned about health or road safety. The problem is complex and is being addressed by researchers involved in all the stages of wine production (Teissedre, 2013). In this context, development of low alcohol yield *Saccharomyces cerevisiae* yeast strains has been a hot topic for several years (Cadiere et al. 2011; Varela et al. 2012; Tilloy et al. 2014), but modifying ethanol yields in this species has proven to be tough.

The current interest of the wine industry on alternative yeasts, and the metabolic diversity they offer, open interesting alternatives to genetic modification. Our research group proposed using the respiratory metabolism of these alternative yeast species for reducing the alcohol content of wine (Gonzalez, et al. 2013). We have now addressed the impact of three easily manageable environmental factors (temperature, nitrogen source, and oxygen supply level) on the aerobic metabolism in synthetic must of some of these yeasts.

Materials and methods

A commercial *S. cerevisiae* wine yeast strain, EC1118, *Metschnikowia pulcherrima* CECT 12898, *Candida sake* CBS 5093, and *Kluyveromyces lactis* CECT 10669. Batch cultures were performed in a defined medium containing 200 g/L glucose, 6 g/L citric acid, 1.7 g/L YNB without amino acids and ammonium sulphate, 0.018 g/L myo-inositol and different concentrations of ammonium chloride. The pH of medium was adjusted to 3.5 with NaOH. Experiments were performed in a DASGIP parallel fermentation platform, with 200 mL medium, 250 rpm, and a gas flow of 1.0 L/h. The cultures were sparged with either pure air or mixtures of air, O_2 and N_2, to get 10 %, 21 % and 50 % oxygen content in the inlet gas. Exhaust gas was cooled in a condenser and the instant concentrations of O_2 and CO_2 in the exhaust gas recorded with a GA4 gas analyser.

The concentrations of glucose, glycerol, ethanol, and acetic acid were determined by HPLC. Two calculations were performed with analytical data from the 72 h samples. Alcohol level reduction was calculated as the difference between the expected increase in ethanol content, according to sugar consumed up to this time point and the actually measured ethanol content. Even though this alcohol level reduction value is useful for comparison between conditions and strains, it is probably overestimated, since part of the ethanol produced will have been lost by stripping. In addition, an integrative parameter, Efficacy (efficacy for alcohol level

reduction) was designed to simplify comparisons between strains or growth conditions. It was calculated as follows: Efficacy= AR x2 x(0.5-AA). Were AR is the alcohol level reduction (expressed as % ABV), and AA is acetic acid content (g/L). Oxygen consumption and CO_2 production were determined by taking into account the inlet and exhaust gas flows, and their respective concentrations in air and in the off gas. Instant values were integrated over time. Respiration quotient (RQ) was calculated as que molar ratio between CO_2 production and oxygen consumption. An orthogonal design was used to get the best combination of factors with the minimal number of experiments. Orthogonal design and conjoint analysis were performed with the IBM SPSS Statistics v19 software.

Results and discussion

Clear differences were found for ethanol yield values among the four yeast strains (Figure 1). The distribution for *S. cerevisiae* is narrower, and values, clearly higher than for any other yeast. This trend towards high ethanol production under most culture conditions is in agreement with the well described metabolic features and the evolutionary history of this species (Piškur, et al. 2006). For acetic acid yield, the highest median value and overall distribution were also observed for *S. cerevisiae* (Figure 1). This species was the highest acetic acid producer in eight out of the nine growth conditions tested (data not shown). This is in agreement with our previous results (Morales, et al. 2015); and confirm acetic acid production as the major drawback of using *S. cerevisiae* under aerated winemaking conditions. In contrast, the low median and distribution of values observed for acetic acid yield in *C. sake* (Figure 1) suggest this strain might be an interesting option for alcohol level reduction by respiration.

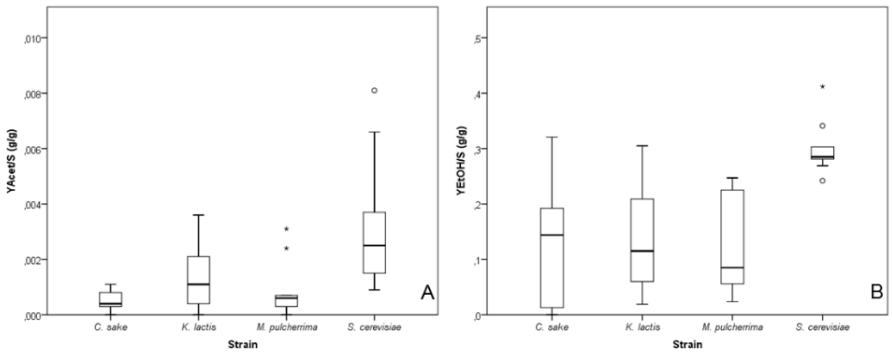

FIGURE 1. Boxplots for acetic acid yield on glucose (A), and ethanol yield on glucose.

Statistical analysis revealed that glucose consumption was favored both by increasing nitrogen availability (YAN) and increasing fermentation temperature, with quite similar relative impact for all the strains in the study (Table 1). However, a clear effect of increasing oxygen supply on glucose consumption was only shown for *C. sake*. Surprisingly, YAN shows the highest relative impact on ethanol production for all the strains apart *K. lactis*. On the other side, increasing oxygen supply shows a negative impact on ethanol yield for all strains but *C. sake*. This suggests that the stimulation of sugar uptake induced by oxygen in this species might go beyond its respiratory capacity, resulting in glycolytic overflow.

Finally, *K. lactis* is the only yeast strain for which oxygen supply appears as the most relevant factor affecting ethanol yield (Table 1).
"Efficacy" was defined in this work as a specific parameter related to alcohol level reduction, taking into account sugar consumption, as well as ethanol and acetate production. *C. sake* showed the best distribution of Efficacy values; while *S. cerevisiae* showed the lowest values in almost all the conditions analyzed. The analysis showed a positive correlation of Efficacy with oxygen supply, for all the strains tested (Table 1). However, the relative impact was different for each strain, with *C. sake* sowing the strongest dependence on oxygen supply, while *K. lactis* showed very limited impact. YAN appeared as a relevant variable to take into account for Efficacy in all yeast strains tested (Table 1). Nevertheless, the direction of the correlation depends on the strain; it is positive for *M. pulcherrima* and *K lactis*, and negative for the other two strains. Similarly, Efficacy is affected by fermentation temperature in opposite directions depending on the strain, negatively for *S. cerevisiae* and positively for the other yeasts (Table 1). The lowest impact of temperature on Efficacy was observed for *C. sake*.

		C. sake	*K. lactis*	*M. pulcherrima*	*S. cerevisiae*
Ethanol Yield	Nitrogen source	62	34	52	44
	Temperature	19	15	17	30
	Oxygen	19	51	31	26
Acetic Acid Yield	Nitrogen source	24	36	38	15
	Temperature	6	26	29	48
	Oxygen	70	38	33	37
Alcohol reduction	Nitrogen source	35	47	30	27
	Temperature	18	46	49	61
	Oxygen	47	7	21	12
Efficacy	Nitrogen source	41	45	32	46
	Temperature	15	42	50	35
	Oxygen	44	13	18	19

TABLE 1. Importance of three environmental factors on different fermentation parameters for different yeast species.

Conclusions

We have analyzed the impact of three easily manageable environmental factors on the production of the main fermentation metabolites by four yeast strains during the fermentation of synthetic grape must. The different levels for each environmental factor were chosen considering a potential use for alcohol level reduction by sugar respiration. Surprisingly, oxygen supply was not the main driver of the differences observed for most parameters and strains. However, we must be aware that conclusions of this analysis cannot be extrapolated beyond the range of values assayed. Especially considering that anaerobic conditions were not assayed, since respiration would not take place under total oxygen deprivation. This extreme condition would have had a strong impact on the final result. In addition, both the relevance of each factor, and the direction they affect Efficacy, are different for each strain.

As a trend, increasing oxygen supply would positively contribute to alcohol level reduction and Efficacy of the process. Finally, *C. sake* CBS 5093 appears as the most promising strain for alcohol level reduction by respiration. Using this strain the drawbacks related to volatile acidity under aerated conditions will be completely avoided.

Keywords
Volatile acidity; Alcohol level reduction; sugar respiration; aerated fermentation

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SELECTED LECTURE

CHARACTERIZATION OF SURFACE-ACTIVE MOLECULES PRODUCED BY MARINE BACTERIAL ISOLATES

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Introduction

Biosurfactants (BS) are amphipathic compounds produced by a variety of microorganisms. These compounds could be of low-molecular weight type, which are generally glycolipids and lipopeptides, and high-molecular weight type which are mainly lipopolysaccharides, lipoproteins or a combination of both. The high-molecular weight BSs, frequently called bioemulsifiers (BE), are associated with production of stable emulsions, but do not always exhibit lowering of the surface or interfacial tension. Owing to the advantages the microbial surfactants have over their conventional counterparts, like biodegradability, low toxicity and production from renewable substrates (Lima *et al.*, 2011), these compounds have broad range of potential applications. However, their commercialization is still limited due to their low yields and high costs of production. Hence, selection of microorganisms producing BSs highly active and/or at high yields remains the key factor which could help overcoming these bottlenecks. Furthermore, given the need for surface-active compounds with potential application for bioremediation under marine environmental conditions, interest in the selection of BSs-producing marine microbes has been increasing recently. Microbes from marine habitats represent indeed an excellent opportunity to not only discover novel BSs, but also molecules that are likely to function in marine ecosystems since they have developed adaptative strategies to cope with temperature extremes, low water activity and nutrient availability as well as high pressure. Hence, these microbes could be a source of BSs able to act in saline, high-pressure environments; criteria required in the event of oil spills occurring at high depth (Jackson *et al.*, 2015). In this work, we report on the characterization of BS/BE produced by bacteria isolated from marine sediments. The BE showed interesting characteristics including high activity and stability both in time and under different stress conditions, which highlights the suitability of the isolates as candidates for the development of BE formulations to be used in combatting oil spills in marine environment.

Materials and methods

The marine bacterial isolates used in this study were obtained from sediment samples collected from three sites (harbors) located in the south of Italy. Screening for BS/BE production was performed on cell-free culture supernatants after bacterial growth in modified mineral salt medium (mMSM) broth containing (g/l): glucose, 1; Na_2HPO_4, 0.7; KH_2PO_4, 0.9; $NaNO_3$, 2; $MgSO_4 \cdot 7H_2O$, 0.4; $CaCl_2 \cdot 2H_2O$, 0.1; NaCl, 30 and 2 ml of trace element solution [per liter, 2 g $FeSO_4 \cdot 7H_2O$, 1.5 g $MnSO_4 \cdot H_2O$, 0.6 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$]. The

surface activity of was evaluated using three methods: i) *Emulsification index* (EI24%) of different organic solvents (estimated as the height of the emulsion layer, divided by the total height, multiplied by 100); ii) *drop collapse assay* (on Parafilm® surface) and iii) measurement of *interfacial surface tension (IFT)* with a drop shape analysis system DSA30 using the pendant drop technique. Tween 80, SDS (sodium dodecyl sulfate) and CTAB (hexadecyl-trimethyl-ammonium bromide), each at final concentration of 0.5%$_{w/v}$, were used as a positive controls while deionized water and non-inoculated growth medium as negative controls, respectively. All screening assays were performed in duplicate as independent experiments.

In order to assess the stability of the BE activity, culture supernatants were supplemented with 300 g/l NaCl or subjected to autoclaving (121°C for 20 min) and the emulsifying activity was evaluated. Furthermore, the stability of the emulsions produced was monitored for eight months after incubation at room temperature. All the experiments were carried out in triplicate. The results were reported as residual emulsification activity (REA) (%) expressed as follows: REA (%)=EI_t/EI_{24} $x100$; where EI_t (%) is the EI (%) value at time t; and compared with those obtained with positive controls.

Partial purification of BS/BE from supernatants was performed by acid precipitation. Preliminary chemical characterization of BS/BE was carried out by recording the infra-red spectrum (FT-IR) of the dried partially purified BE over the wavenumber range 450–4000 cm^{-1} using a Perking Elmer Spectrum One FT-IR spectrometer (transmission mode) after milling with KBr pellet.

Results and discussion

A total of 79 bacterial isolates having different colony morphology were obtained from marine sediments collected in three harbors in the Med sea after several successive streaking on mMSM agar medium. After bacterial growth on mMSM broth in the presence of glucose as main carbon source (at 20 or 30°C), 34 isolates were shown to grow quickly in liquid medium and were then considered for the BS/BE screening resulting in the selection of 15 isolates as best surface active molecules producers. The maximum emulsifying activity was observed between 48 and 72 h of incubation of the different isolates. In particular, the highest emulsification index was equal to 75±1.7% after growth in mMSM with 1%$_{w/v}$ glucose as the only carbon source.

Stability of the produced crude BE was evaluated from cell-free culture supernatants after exposure to low water activity as well as to high temperature and pressure. Most of the isolates were shown to produce stable surface-active molecules showing residual activities of up to 100% after autoclaving or in the presence of 5M NaCl. The common chemical surfactants SDS, CTAB or Tween 80 showed a reduced emulsifying activity at this NaCl concentration while no effect of autoclaving was observed. BE that are not affected by NaCl concentrations up to 300 g/l have been described in few bacteria including *Paenibacillus* (Gudiña *et al.*,2015) and *Geobacillus* (Zheng *et al.*, 2011); but the strains were not from marine origin. Moreover, the emulsions formed showed a high stability at room temperature, maintaining up to 100% of the original emulsification activity over a period of 8 months under standard conditions or in the presence of 300 g/l of NaCl. Comparable extended stability in time has been previously observed for the BE produced by marine *Pedobacter* sp. strain MCC-Z where the emulsions remained stable for four months (Beltrani *et al.*, 2015),

while no reports on extended stability even under such low water activity conditions has benne reported yet, to the best of our knowledge. Partially purified BE, obtained by acid precipitation from cell-free culture supernatants after growth in mMSM-glucose medium, were subjected to FT-IR analysis for identification of the main functional groups present. The FT-IR spectra of most of the isolates, suggested that the produced BS/BE belong to the glycopeptide/glycoprotein and glycolipid classes of BS.
The selected isolates were also tested for their growth and surface-active molecules production on a relatively cheap and highly available carbon source. All the isolates were able to grow on mMSM with 2%$_{v/v}$ soybean oil as main carbon source and produce surface-active molecules. In specific, the highest emulsification index was equal to 69.17±1.2% and some of the produced molecules were able to reduce the surface tension of the medium from 72.66±0.3 to 29.60±1.15 mN/m. Also in this case, the produced emulsions were stable up to eight months of incubation at room temperature retaining up to 98% of their activity and for some of the isolates the emulsification activity was stable in the presence of 300 g/l NaCl.
The results obtained highlight that the microbes from marine habitats could be a source of BS able to act under extreme conditions; criteria required in the event of oil spills occurring at high depth (Jackson *et al.*, 2015). Moreover, the development of BS formulations to be applied in the case of oil spill would benefit from the results obtained in this study. Indeed, sprydrying or lyophilization could be applied for the recovery/conservation of BS and in both procedures, the molecules are subjected to low water activity conditions.
With regard to the isolates identity, partial 16S rRNA gene sequencing revealed the bacteria to be mainly from the *Marinobacter* genus. Although there are many reports on the production of surface-active molecules from marine bacteria, to the best of our knowledge, there have been no reports on the characterization of surface-active molecules production from the *Marinobacter* species. In this genus there are more than 33 validated species, but literature regarding these bacteria deals mainly with the description of the species while no reports on the putative biotechnological potential of these non-pathogenic bacteria are available.

Conclusions
The results obtained here highlight the potential of the produced surface-active molecules obtained from marine bacteria for application in various industrial sectors. In specific, owing to their activity and stability under harsh non-conventional conditions including high salt concentrations and temperature extremes, the BS/BE produced here would be very interesting for the development of formulations to be applied in for the bioremediation of oil spills under marine environment.

Acknowledgements
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Keywords
Biosurfactants, bioemulsifiers, marine bacteria, oil spill

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SELECTED LECTURE

METAGENOMIC AND ARISA PROFILING OF THE WINE MICROBIAL CONSORTIUM AND ITS FUNCTIONAL POTENTIAL

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Abstract

The current study employed traditional culture-dependent methods in conjunction with culture-independent methods for an in-depth analysis of the wine microbiome. Automated Ribosomal Intergenic Spacer Analysis (ARISA) and metagenomic analysis revealed higher diversity than culture-dependent studies. A high diversity of filamentous fungi including members of the genera *Alternaria*, *Aspergillus, Neofusicoccum*, *Botryotinia*, *Penicillium* and *Mucor*, and common wine yeasts of the genera *Candida*, *Metschnikowia*, *Aureobasidium* and *Pichia* were identified in the microbiome. However, taxonomic assignment of ARISA peaks was a challenge and could result in underestimation of the diversity due to overlapping peaks. Functional analysis of the metagenomic data showed that genes related to metabolism accounted for the majority of the sequences (35.6%), followed by poorly characterized categories (28.3%), cellular processes and signalling (18.4%), and information storage (17.8%). The Streptophyta (mainly *Vitis vinifera*) accounted for 60% of the functional gene pool, while the Ascomycota and Basidiomycota, accounted for 22% and 4%, respectively. A high frequency of glycosidases, endoglucanases and chitinases mainly of filamentous fungal origin in the metagenome was evident. However, due to insufficient sequence depth, contig assembly was not possible and therefore only short fragments of these genes were obtained. The presence of these enzymes in wine yeast isolates was also evaluated using plate screen assays. Chitinases and glucanases were observed in several yeast species of the genera *Candida*, *Pichia*, *Metschnikowia* and *Rhodotorula*, while β-glucosidases were not prevalent. This work is a major step toward the understanding of genetic potential of the wine microbiome and forms the basis for a rational approach to improve yeast isolation strategies and identification of genes encoding hydrolases relevant in winemaking. Future work will address issues of sequence depth and retrieval of full gene sequences from the metagenome.
Keywords: microbiome, metagenomics, microbial diversity, wine/grape must

Introduction

Vitis vinifera is one of the most widely cultivated fruit crops which has been extensively studied from various foci including plant pathology, viticultural practices, as well as winemaking. The grapevine plant hosts a plethora of epiphytic and endophytic microorganisms that play various roles in the productivity and disease resistance the plant. In addition, the grape associated microorganisms are pivotal in winemaking as they are involved in the conversion of grape sugars as well as other macromolecules to ethanol, CO_2 and aroma active compounds.

The grapevine microbial ecosystem comprises filamentous fungi, yeasts and bacteria. The possible contribution of these microorganisms to the "terroir" has sparked a new quest for in-depth evaluation of the vineyard, grapevine and wine microbiome. Consequently, recent research endeavours have employed high-throughput second generation sequencing technologies to study the microbial diversity (Bokulich et al., 2014, David et al., 2014; Pinto et al., 2014; Taylor et al., 2014). These studies have indeed revealed a higher diversity compared to culture-based methods and even early culture-independent techniques such as DGGE. In fact, some authors have suggested that culture-based methods might miss approximately 95% of the community (Taylor et al., 2014). This diversity represents a treasure trove of biocatalysts that could be exploited to enhance plant health and wine quality. The current study aimed to tap into this diversity by using shot-gun whole metagenomic analysis to explore the functional and structural diversity microbial diversity associated with Cabernet sauvignon grapes from a biodynamic vineyard.

Materials and Methods

Vitis vinifera L. Cabernet sauvignon grapes were collected from a biodynamic vineyard. The samples were crushed and destemmed under sterile conditions. Yeast isolation and enumeration were performed by plating out serial dilutions of the grape must sample onto Wallerstein nutrient agar supplemented with 200 mg/L biphenyl and 34 mg/L chloramphenicol. Yeast isolates were identified by PCR-RFLP followed by sequencing of representatives of unique RFLP profile groups. For metagenomic analysis, freshly crushed must was centrifuged and the pellet washed with a solution containing 0.15 M NaCl, 0.1 M EDTA, and 2% (w/v) polyvinylpyrrolidone. Genomic DNA was extracted using a CTAB-based method and Amplified Ribosomal Intergenic Spacer analysis (ARISA) was performed. In addition, 500 ng DNA was used for generation of a sequencing library according to the GS-FLX Rapid Library preparation kit, followed by pyrosequencing on the Roche GS-FLX system using Titanium chemistry. Post-sequencing quality filtering, length filtering, dereplication, OTU picking, taxonomic assignments and functional analysis was performed on the MetaGenome Rapid Annotation Subsystems Technology pipeline (Meyer et al., 2008).

Results and discussion

A suite of culture-dependent methods, ARISA and metagenomic analysis was used to evaluate the structural functional diversity of the microbiome associated with Cabernet sauvignon grapes from a South African biodynamic vineyard. A comparison of the fungal community composition shows that the metagenomic approach revealed the highest diversity (Figure 1A). A total of 74 fungal species could be detected in the metagenome compared to 11 yeast isolates and 14 ARISA peaks detected. The data identified filamentous fungi to be the dominant taxa in the grape must fungal community (Figure 1B. A functional analysis of the grape must metagenome revealed that *Vitis vinifera* accounted for approximately 60% of the gene pool, followed by the ascomycetous and basidiomycetous fungi. This indeed is in agreement with what the grape must matrix consists of, since ripe berries are known to harbour a higher proportion of ascomycetes (Bokulich et al., 2014; David et al., 2014). In addition, the filamentous fungi that colonize the grape berry endosphere are mostly ascomycetes (Pinto et al., 2014; Taylor et al., 2014). Glycosidases and other enzymes of

oenological interest such as endoglucanases, pectinases and chitinases were well represented in the metagenome (Figure 1C). Plate screening of several yeasts isolates confirmed the presence of these activities in members of the genera *Pichia*, *Candida* and *Metschnikowia*. These enzymes are relevant for grape juice processing and for the release of bound aroma precursors.

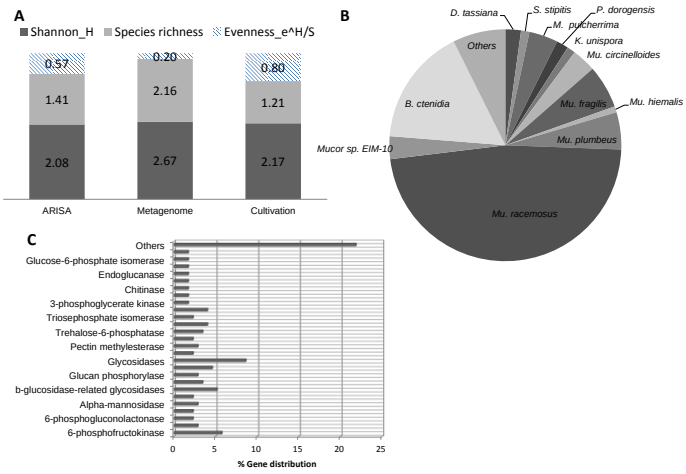

FIGURE 1. A diversity analysis of the fungal community in the Cabernet sauvignon grape must using different methods (**A**) and the major fungi detected in the metagenome (**B**). The relative abundance and distribution of genes involved in carbohydrate transport and metabolism in the Cabernet sauvignon metagenome (**C**).

Conclusion

The current study explored the use of shotgun sequencing in conjunction with culture-based methods and community fingerprinting to tap into the grape berry metagenome and evaluate its functional potential. The approach proved valuable for identification of genes encoding novel enzymes of oenological relevance, which can possibly be retrieved through various strategies and further exploited.

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SELECTED LECTURE

BIO-HYDROGEN PRODUCTION OF CLOSTRIDIUM ACETOBUTYLICUM *DSM 792 AND THE pSOL1 DEFICIENT MUTANT MU56 IN DEPROTEINIZED CHEESE WHEY.*

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Introduction:
Dark fermentation of whey derived from cheese manufacturing could represent an opportunity to increase the value of this dairy byproduct (Davila-Vazquez et al. 2009). Today over the 25% of the whey produced in the European Union is used for human consumption but it becomes a byproduct after extraction and concentration of the protein fraction. The bio-hydrogen production can actually represent a future prospect of renewable energy (Das and Veziroglu, 2008). In this work the ability of growing and producing hydrogen was demonstrated for the *Clostridium acetobutylicum* DSM 792 type strain and for its pSOL1 deficient mutant Mu56, using deproteinized whey derived from Grana Padano cheese production as substrate. The mutant Mu56 lacks the 210-Kb plasmid pSOL1 which carries the genes responsible for acetone, butanol and ethanol production, solventogenesis pathway (Jones and Woods, 1986). This causes the interruption of the solventogenic pathway and the redirection of the electrons flow toward the production of hydrogen, with the consequent increasing in the yield (Oh et al. 2009). The loss of the pSOL1 megaplasmid in Mu56 was demonstrated by PCR and southern blot and it was confirmed by genome analysis. The higher yield achieved in deproteinized whey from *C. acetobutylicum* Mu56, compared to the parent strain DSM 792, was demonstrated using two independent analysis: the BHP and the water displacement methods.

Materials and methods
Clostridium acetobutylicum DSM 792 and the pSOL1 deficient mutant Mu56 were cultured anaerobically in RCM medium and in deproteinized whey. The whey came from the Grana Padano cheese making process and it was deproteinized by industrial process using reverse osmosis and ultrafiltration. The pSOL1 deficient mutant Mu56 was obtained by serial subculture at 37°C in RCM medium in anaerobic conditions. The loss of the megaplasmid pSOL1 was checked by amplification of the aldehyde/alcohol dehydrogenase genes. Southern blot analysis was carried out to confirm the loss of pSOL1 and a fragment of the *adhE* gene was used to prepare the specific probe. The genomic DNA of *C. acetobutylicum* mutant Mu56 was fragmented at 500 bp and sequenced at Fasteris facilities (Geneve, Switzerland); MiSeq instrument operated in V3 chemistry in paired-reads 300x2. Contigs

were ordered and aligned against the reference genome of *C. acetobutylicum* DSM 792. MAUVE (Darling et al., 2004) and RAST (Aziz et al., 2008) software was utilized to inspect the loss of pSOL1 megaplasmid comparing the assembled contigs of the deficient mutant strain against the genome of the type strain DSM 792 *C. acetobutylicum*. SNPs analysis was performed with kSNP3.0 (Gardner et al., 2015). Volumetric analysis was used to compare *C. acetobutylicum* type strain and the mutant Mu56 and hydrogen amount was measured by gas chromatography. Biochemical hydrogen potential (BHP) was assessed using the AMPTS (Automatic Methane Potential Test System, Bioprocess control, SE).

Results and Discussion

C. acetobutylicum pSOL1 deficient mutant Mu56 was obtained by repetitive cells subculture. The loss of the megaplasmid was inspected by PCR technique. Specific primers were used to amplify a 452 bp and a 549 bp fragments of the *adhE2* and the *adhE* genes, respectively, which are part of the coding region responsible of the solventogenic pathway and harbored by the pSOL1 megaplasmid. The attended PCR products were obtained using the genomic DNA of *C. acetobutylicum* DSM792 as template while no PCR products resulted from the amplification of the Mu56 DNA. The southern blot was carried out to confirm the result of PCR amplification reactions. The *adhE* gene was used as probe and a high signal resulted in the pattern of digested genomic DNA of *C. acetobutylicum* DSM 792. No signal was detected in digested DNA of the mutant Mu56, confirming the loss of the megaplasmid pSOL1. Complete genome analysis was performed for the mutant strain Mu56 and the available genome in GenBank of *C. acetobutylicum* DSM792 was used as comparison. The analysis confirmed the loss of pSOL1 megaplasmid in Mu56 strain. Whole genome of *C. acetobutylicum* Mu56 was screened for the possible presence of SNPS. In total 134 SNPs were detected: 21 were unannotated genomic regions and 113 were coding-protein annotated regions. Among these 87 genes in *C. acetobutylicum* Mu56 were found to contain SNPs, a probable consequence of the 56 subculture steps carried out to obtain the cured strain. Some of this SNPs could contribute in the metabolic changes occurring in Mu56 strain. It is well known that the loss of pSOL1 cause the interruption of the solventogenic pathway. The results of Gas Chromatography Volatile Fatty Acids analysis were a further confirmation because butanol was not detected in Mu56 metabolites. Hydrogen production was evaluated by BHP analysis using deproteinized whey as substrate Fig.1. The production of H_2 in Mu56 strain started before the parent strain DSM 792. The Mu56 H_2 yielded 4,41±0,01 mol H_2/mol lactose while DSM 792 produced 3,81 ±0,089 mol H_2/mol lactose.

The number of cells at the end of the fermentation process was 7,51±0,04 log cells ml^{-1} for Mu56 and 8,06±0,03 log cell ml^{-1} for the DSM 792 type strain, therefore the hydrogen yield of Mu56 resulted 18,96% higher than that of DSM792, if correlated with the number of the cells. . SEM analysis were carried out on both strains and it was evident that the loss of pSOL1 caused morphological changes in cells, the interruption of sporogenesis and an increase in the degeneration process, with highlighted autolysis in strain Mu56.

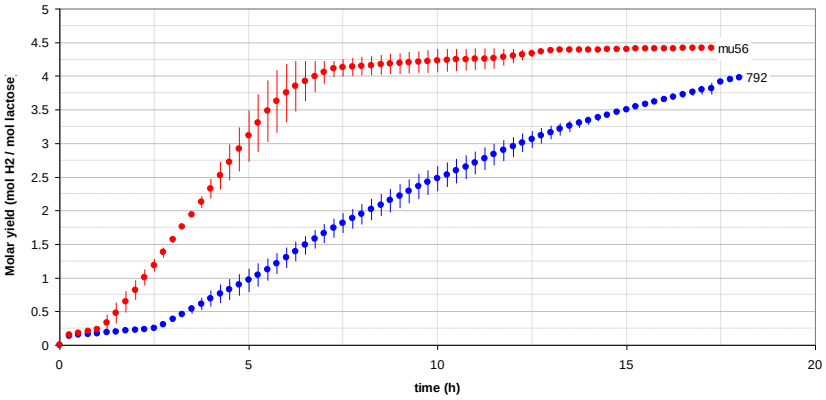

FIGURE 1.

Conclusions

Bio-hydrogen was produced using deproteinized cheese whey. The loss of pSOL1 in *C. acetobutylicum* Mu56 caused the interruption of the solventogenic pathway and an increase in hydrogen production in buffered condition, if correlated to the number of the number of cells. Genome analysis confirmed the loss of the megaplasmid and the investigation of SNPs revealed some mutations which could contribute to changes occurred to *C. acetobutylicum* mutant strain Mu56.

Keywords

Hydrogen, renewable source, dark fermentation, *Clostridium acetobutylicum*, cheese whey

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POSTERS

SESSION I

PS1-01

THE ROLE OF AMINO ACIDS IN THE MODULATION OF BIOFILM FORMATION IN SACCHAROMYCES CEREVISIAE *FLOR YEASTS*

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Microbial biofilms are generally undesired in clinical realms, food processing, drinking water distribution systems, but they may be also desired in food processes that include cheeses and sausages maturation and biological aging of wine. In this contest, small molecules with inhibiting and dispersing properties towards bacteria and yeast biofilms are becoming always more studied. Here, we studied the interaction of *Saccharomyces cerevisiae* flor yeasts used as biofilm-forming model yeasts with several amino acids chosen for their different physiochemical characteristics. Particularly through dose response and adhesion to plastic we analyzed the ability of cellular growth in flor yeast strains, wild type and mutants. Dose response analyses showed variability in the effect of amino acids toward strains. Cationic and sulphuric amino acids showed the highest inhibition activity of flor yeasts to adhesions to polystyrene (biofilm formation). On the contrary, the presence of hydroxylic amino acids positively modulated the biofilm formation.

ACKNOWLEDGMENTS
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PS1-02

TINA BIOFILMS AS SOURCE OF MICROORGANISMS INVOLVED IN VOLATILE ORGANIC COMPOUNDS PRODUCTION OF RAGUSANO CHEESE

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This study aimed at investigating the composition of biofilm microbiota of *Tina* used for Ragusano cheese-making. In addition, the ability of biofilm microbiota of *Tina* to generate volatile organic compounds (VOCs) in milk samples was evaluated by incubating milk in the *Tina* under Ragusano cheese making conditions. *Tina* biofilms from eleven facilities producing Ragusano cheese under traditional conditions were analyzed. A polyphasic approach, based on culture–dependent and independent methods was used. VOCs were identified and quantified by GC/MS-SPME, coupled to Smart Nose and GC/O. All biofilm samples showed a significantly higher (P<0.05) counts of streptococci, compared to other microbial groups. *Streptococcus thermophilus* was the dominant species in both biofilm and

in milk samples incubated with *Tina* biofilms. Other lactic acid bacteria genera (*Lactobacillus*, *Lactococcus*, *Enterococcus* and *Leuconostoc*) were variously identified within the eleven facilities. Low levels of *Pseudomonas* spp. and yeasts counts were detected, whereas coliforms and presumptive pathogens (*Listeria monocytogenes* and *Salmonella* spp.), were not detected. Several strong correlations ($r>0.6$; $P<0.05$) were found between some lactic acid bacteria of *Tina* biofilms and VOCs produced during incubation of milk samples under Ragusano cheese-making conditions. In details, positive correlations were found between *Enterococcus hirae* and alcohols, *Lactococcus lactis*, *Lactobacillus plantarum*, *Lactobacillus casei* and *Lactobacillus delbrueckii* and aldeydes, and *Lactobacillus fermentum*, *Lactobacillus helveticus* and *Lactobacillus hilgardii* and ketones. The findings of this study demonstrate that *Tina* biofilm represents a source of lactic acid bacteria, which significantly contributes to the synthesis of VOCs in Ragusano cheese.

PS1-03

BACTERIAL STRAINS FROM THE "Terra dei Fuochi": ISOLATION, SELECTION AND EFFECT ON THE DYNAMIC OF MICROBIAL POPULATIONS AFTER THEIR APPLICATION TO THE CONTAMINATED SOILS

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The environmental contamination due to accumulation in the soil of persistent organic xenobiotics substances chemicals such as hydrocarbons is a problem that is becoming increasingly important in the world. Technologies based on the use of microorganisms able to degrade the organic pollutants, allow to accelerate the natural detoxification processes in the soil environment and represent an alternative method of environmental remediation compared to the harmful traditional methods. The aim of this study was to isolate and select microorganisms potentially able to remove hydrocarbons from contaminated soils in the "*terra dei fuochi*" (Campania region, Italy) and also the dynamic of microbial populations after inocula application. Selective and differential substrates to isolate new microorganisms were developed on the basis of contaminants identified in the pilot sites. A total of ninety-six microbial strains were isolated and identified by polyphasic approach on the basis of their phenotypic, biochemical and molecular characterization. Isolated strains were also tested for their ability to grow on selective minimal medium with a mixture of different PAHs as sole carbon source and characterized for their bioemulsifier production and the ability to grow in biofilm on hydrophobic surfaces. Obtained results showed a wide range of bacterial species able to metabolize contaminants with potential use in soil remediation as well as to produce bioemulsion. On the basis of all results, the two strains *Bacillus megaterium* EL5, able to degrade up to 30.4% of PAHs (phenanthrene) in 21 days, and *Bordetella petrii* EL12B, showing high emulsification properties (Emulsification Index=0,95) and able to degrade up to 70.7% of phenanthrene in 21 days, were selected to produce the bacterial formulate to use in *in situ* experiments. PCR-DGGE as well as high-throughput sequencing were performed to highlighted the microbial dynamic and diversity in the contaminated soils before and after

microbial inoculation.

ACKNOWLEDGEMENTS
This work was supported by Research Project LIFE11/ENV/IT/275-ECOREMED.

PS1-04

STUDY OF FUNCTIONAL MICROBIAL GROUPS AND MICROBIAL COMMUNITY IN A SMALL-SCALE BIOMETHANATION PROCESS OF ORGANIC MUNICIPAL SOLID WASTE

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The biomethanation process involves the conversion of organic waste to methane by anaerobic digestion carried out by a cooperation of numerous microorganisms with different functional capacities. The quality and quantity of produced methane depends on the metabolic transformations conducted by microorganisms during the process. In this work, a small-scale anaerobic digestion of organic municipal solid waste was carried out in ten pilot digesters (100L) to supervise the microbial populations occurred during the process and the relationship between microbial communities and biogas production. Eight anaerobic digesters were inoculated with animal manure and other two were used as non-inoculated controls. Samples were analysed before anaerobic digestion and after 26 days of experimentation. Bacterial diversity was evaluated by PCR-DGGE and the generic and functional microbial groups were also enumerated by using differential selective media. Biogas production, pH and temperature were also determined. Preliminary results obtained from PCR-DGGE and cluster analysis, performed to estimate the percentage of similarity of microbial populations, showed that the bacterial populations were mostly influenced by municipal organic waste different treatments. Moreover, microbial populations involved in initial step of biogas process such as cellulolytic, amylolytic, pectinolytic, proteolytic bacteria were influenced by waste composition and decreased of 1-2 Log CFU/g at the end of the process. *Methanosarcina* spp. and *Methanobacterium* spp. were the most abundant methanogenic species detected showing a concentration of about 8 and 9 Log CFU/g, respectively. Interesting was the inverse correlation observed between acid percentages and methane production. In fact, methane was detected only in the digesters with pH>4.30. Results obtained showed that is important to take under control microorganisms which exhibited very different metabolic capacities to understand the desired reactions and to obtain an initial community evenness, important factors for stable operation of mixed microbial cultures.

This work was supported by Provincia di Avellino, NHP-ESCo and SIB.

PS1-05

FUNGICIDES AND WINE FERMENTATIONS: EVIDENCES OF COMPLEX EFFECT ON THE WINE MICROBIOTA

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In this work we investigated the repetitive stuck fermentations in a winery located in the Apulian region, South of Italy. Apart from microbial starters and the presence of spoilage microrganisms, a panel of pesticides formulations used in the pest management of the winery vineyards were considered. One of the fungicides tested against *Saccharomyces cerevisiae* was identified as a potential inhibitor of yeast growth. The selected commercial preparation contained metalaxil-M and folpet as the principal active ingredient. Monitoring the weight loss during alcoholic fermentation, we demonstrated that the fungicide preparation, at a dosage corresponding to the half of the EU limit in grapevine, compromised the beginning of alcoholic fermentation in wine (e.g. 10 fold reduction of CO_2 released in the first two days). Furthermore, our findings were confirmed on eight strains of *S. cerevisiae* of which six strains are commonly used in oenology and two autochthonous strains from Apulian wines, suggesting that sensivity to the studied fungicide is not a strain-specific character. In order to improve our understanding on the effect on the wine microflora and, consequently, on wine quality and safety, we tested the effect of the selected fungicide on a panel of strains belonging to species of oenological interest such as non-*Saccharomyces* yeasts, and malolactic bacteria. No inhibition was observed on malolactic bacteria. In general, the pesticide inhibited also non-*Saccharomyces*. However, non-*Saccharomyces* strains able to grow when exposed to the fungicide were observed. The addition of the selected fungicide formulation increased the risks related to i) so called 'wine diseases' and ii) biogenic amines formation in presence of producer strains. Our results, for the first time, demonstrated that fungicide preparations might cause a clear inhibition of the beginning of alcoholic fermentation. Overall, the use of fungicide in viticulture/enology should be properly managed, even considering the risks of economic losses due to stuck fermentations.

PS1-06

MOLECULAR CHARACTERIZATION OF FUNGAL BIODIVERSITY AND EARLY IDENTIFICATION OF FUNGI ASSOCIATED WITH OIL PALM DECAY, PARTICULARLY GANODERMA BONINENSE

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The oil palm, *E. guineensis Jacq.*, is the main oleaginous plant cultivated in the world. The fungi *Ganoderma boninense* has been identified as the main harmful phytopathogen of these

cultures in South-East Asia. It is responsible of major economic losses due to weakening, necrosis and death of the host plant. This fast-growing agro-industry in the tropical belt is now confronted to the emergence of *Ganoderma* in Cameroon. However, the incidence and epidemiology of the disease, as well as the species involved, differ from what has been observed in Asia. This research project aims to characterize the diversity and the genetic structuration of aggressive *Ganoderma* species found in plantation sites in Cameroon, using the ITS, nuc-SSU, nuc-LSU and mt-SSU molecular markers for phylogenetic analysis, complemented by a morpho-anatomic description of the samples. During this project, 43 samples of *Ganoderma spp*. have been collected on oil palm trees in 5 plantation sites of the SOCAPALM group in Cameroon, from which 40 pure culture isolates have been produced. A phylogeny based on the molecular marker nuc-SSU data of our samples has been reconstructed.

PS1-07

EVALUATION OF ANTIBIOTIC-RESISTANT ENTEROCOCCI IN CONSTRUCTED WETLANDS SYSTEM FOR WASTEWATER REUSE IN AGRICULTURE

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Constructed wetlands (CWs) are engineered systems which reproduce the natural water remediation processes occurring in nature in order to reuse the final effluent for agricultural purposes. The aims of this study were to monitor the sanitation efficiency of a full-scale horizontal sub-surface flow CW combined with UV treatment and to evaluate the Antibiotic-Resistance (AR) in enterococci, isolated, both in influent and in three different effluents, in 12 consecutive months. For this purpose, the microbiological indicators *Escherichia coli*, total coliforms and enterococci were monitored by plating counts. Moreover, the levels of enterococci sensitivity to different antibiotics molecules were measured using microdilution assay. The results showed that the levels of microbiological indicators were significantly lowered in water samples collected downstream the CW system, compared to the water samples collected upstream the CW system. While the CW system allowed an efficient reduction in *E. coli* population, reaching a final value below the Italian limits required for wastewater reuse in agriculture, a moderate reduction was achieved for enterococci count. The taxonomic identification of isolates reveled the dominance of the species *Enterococcus faecalis* both in influent ad effluents, followed by *Enterococcus faecium* and *Enterococcus hirae*. Most of enterococci isolates showed high AR to most of the tested antibiotics and variable sensibility to glycopeptides and beta-lactam both in influent and effluent samples. Strong differences were also observed among sampling times with a higher AR levels monitored in the period from April to June. In conclusion, while the CW system considered in the present study is suitable for the water reuse in agriculture, according to the Italian legislation, the presence of enterococci at high concentrations underlined the persistence of this bacterial group in water environment with a potential risk to serve as a genetic reservoir of transferable AR, a matter of concern for public health.

PS1-08

YEAST CULTURABLE DIVERSITY OF SOUTH TYROLEAN ALPINE SOILS

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Cold environments have been extensively studied since 1960s for their microbial biodiversity. Early studies reported the occurrence of bacterial communities in such habitats; only in the last fifteen years the presence of yeast populations has been investigated. Cold-adapted yeasts isolated from cold environments were regarded either as psychrophilic or psychrotolerant organisms, which are both metabolically active at low temperatures. Therefore the study of cold-adapted yeasts may result interesting for their taxonomy, ecology, physiology, as well as for their potential biotechnological application. In this regard soils from four forest sites located in the Italian Alps in South Tyrol were sampled in spring and autumn 2014 at different altitudes (from about 600 to 2,000 m a.s.l.). The influence of different abiotic parameters (e.g. geo-morphological, micro-climatic, physical and chemical conditions) on yeast culturable diversity was taken into consideration. Seven-hundred and sixty-four yeast strains were isolated at four different incubation temperatures (4, 10, 20 and 30°C). The isolates were identified by sequencing their D1/D2 domains of 26S rRNA gene and ITS. Sequences were compared with those published in GenBank database (BLASTN freeware from www.ncbi.nlm.nih.gov/BLAST). The results of identification reported that Basidiomycota predominated the yeast diversity in these ecosystems: in particular the species *Trichosporon porosum*, *Cryptococcus terricola* and *Cryptococcus podzolicus* accounted for over 50% of the total isolates. A few abiotic parameters apparently affected the frequency of isolation of some culturable species. All yeast strains were checked for their optimal growth temperature: these tests confirmed the psychrophilic or psychrotolerant status of the organisms. The expression of some extracellular cold-active enzymatic activities of biotechnological relevance was also observed.

PS1-9

THE EFFECT OF EXTRACELLULAR SUGARS EXTRACTION ON THE 3D-STRUCTURE OF BIOLOGICAL SOIL CRUSTS FROM DIFFERENT ECOSYSTEMS

ROSSI Federico (1), FELDE Vincent (2), UTEAU-PUSCHMANN Daniel (3), COLESIE Claudia (4), FELIX-HENNINGSEN Peter (3), PETH Stephan (5), DE PHILIPPIS Roberto (1)

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Biological Soil Crusts (BSC) are complex microbial communities that play key ecological roles in many ecosystems of the world. In drylands, they affect water redistribution and affect hydrological cycles. This very important ecological feature is mostly owed to the extracellular polymeric substances (EPS) which are a very important microbial metabolical product. In a previous study, the extraction of EPS from BSC, using a novel non- destructive method, led to a significant change in the water sorptivity (Rossi et al. 2012). This was concluded to be possibly due to changes in the crust pore structure, due to to the lack of the stabilizing effect of the EPSs.To ascertain this, we examined possible effects of EPS-extraction on soil structure using 3D-computed micro-tomography. In this study, we considered four different BSC types collected in Svalbard, Germany, Israel and South Africa, having varying grain sizes and species compositions. For each one, we physically cut out a small piece (1cm) and CT-scanned it at a high resolution. After putting it back in the dish, the whole crust sample was treated for EPS-extraction, the small piece removed again and re-scanned in order to detect possible effects of the treatment. Our results showed that the EPS-extraction method had varying extraction efficiencies: while in some cases the amount removed was barely significant, in other cases higher quantities were recovered. Notwithstanding no difference in micro-structure could be detected, and no distinct patterns of change in the structure of the crusts was detected. At the same time, CT-scan analysis allowed to detect differences between the four different crust types. This leads us to conclude that the change in sorptivity after EPS removal is related more to a change of the hydrophobicity of the very surface of the crust, rather than a change of the inner structure.

References Rossi, F. et al. (2012) *Soil Biol Biochem* 46, 33–40.

SESSION II

PS2-01

TEMPERATURE VARIATION IN CHEESE RIPENING IS ASSOCIATED WITH SIGNIFICANT CHANGES IN THE MICROBIOME STRUCTURE AND ACTIVITIES

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Caciocavallo Silano is a traditional Italian pasta-filata cheese produced according to a back-slopping procedure. In this study, we used 16S rRNA and total RNA shotgun sequencing to study the microbiota and the metatranscriptome of this cheese during the ripening. A first experiment suggests that cheese ripening follows a gradient from the core towards the crust, driven by non-starter lactic acid bacteria (NSLAB) growth: from 10 to 60 days of ripening, lactobacilli progressively increased in the cheese core. Moreover, metabolic pathways related to protein and amino acid catabolism were over-expressed in the cheese core, while carbohydrates metabolism was enriched on the crust. Based on these results, we tried to accelerate the ripening process through increasing the temperature (from 16 to 20 °C, condition B) or decreasing the relative humidity (RH) (from 75 to 65%, condition C). 16S rRNA sequencing showed that the higher temperature promoted NSLAB growth in the cheese core. Therefore, we further studied the metatranscriptome of samples of cheese core and crust ripened in standard conditions (A) and at higher temperature (B) for 10, 20 and 30 days. Moreover, proteolysis was studied through Liquid Chromathography - Mass Spectrometry (LC-MS) and the volatile profile was determined by Solid Phase Microextraction Gas Chromathography coupled to Mass Spectrometry (SPME-GC/MS). Metatranscriptome of cheese core and crusts clearly clustered separately. Interestingly, genes encoding for proteases, peptidases, amino acid and peptide transporters were enriched in the core of samples ripened at 20 °C. Accordingly, a decrease in high molecular weight peptides and an increase in many volatile compounds concentration were detected in samples B compared to the control. This study provided an in-depth description of the distribution of microbial activities driving cheese ripening, suggesting that the increase in the temperature may accelerate the ripening process through promoting NSLAB growth.

PS2-02

COEXISTENCE OF LACTIC ACID BACTERIA AND POTENTIAL SPOILAGE MICROBIOTA IN A DAIRY-PROCESSING ENVIRONMENT

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Food processing plants are populated by microbial consortia that play a fundamental role in food quality and safety. In this study, a culture-independent high-throughput amplicon sequencing of both 16S and 26S rRNA was used to investigate the microbial ecosystem of a dairy plant and cheeses produced therein. Cheese samples and surface swab-samples from equipment used for their production were collected. Investigating the interactions between cheese and environmental microbiota can be fundamental in understanding how these interactions may have a role in the process of fermentation, influencing cheese quality and safety. The microbiota of environmental swabs showed a high diversity, including more than 200 operational taxonomic units (OTUs) with variable relative abundances (0.01-99%) depending on the species and samples. A core microbiota shared by 70% of the samples was found and a co-existence of lactic acid bacteria (LAB) and possible spoilage-associated bacteria was highlighted. The most representative core members were *Streptococcus thermophilus*, *Pseudomonas*, *Acinetobacter* and *Psichrobacter*; while the most abundant OTUs in the yeasts group were *Kluyveromyces marxanius*, *Yamadazyma triangularis*, *Trichosporon faecale*, and *Debaryomyces hansenii*. Beta diversity analysis showed that, based on the composition of the microbiota, the samples grouped according to their nature that showing a high level of similarity. Moreover, differential distribution of metabolic pathways between the two categories of samples were shown by predicted metagenomes.

PS2-03

OCCURRENCE AND DISTRIBUTION OF BRETTANOMYCES BRUXELLENSIS *IN WINE ENVIRONMENT*

ORO Lucia (1), CANONICO Laura (2), COMITINI Francesca (1), CIANI Maurizio (1)

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Brettanomyces bruxellensis yeasts represent a one of the most important microbial causes of wine spoilage in worldwide. Nowadays, the origin, presence and diffusion to of these spoilage yeasts is still uncertain and debated. The aim of the work was to evaluated the presence of *B. bruxellensis* on the surface of the grape berries through enrichment and selective media and compare with those isolated in the winery environment using molecular fingerprinting and physiological characteristics. The strains were submitted to molecular analysis with RAPD-PCR (M13, M14) and minisatellites (PIR1, PIR3). Results of typing procedures showed that the 15 strains of *B. bruxellensis*, coming from the vineyard, were grouped into six clusters, while the 28 strains, isolated from the cellar, were grouped in ten biotypes. After the comparison, three biotypes exhibited a full correspondence between strains isolated in grape surfaces and winery. Interestingly, one of these, includes 5 strains coming from grapes and 14 from winery strains indicating that this genotype is dominant in this specific ecological niche (44% of total strains). Afterward further physiological characterizations were carried out on the population of *B. bruxellensis* in synthetic must and the results showed that there is a correspondence of the ethylphenol concentration between strains within the various groups.

PS2-04

OCCURRENCE OF BIOGENIC AMINES PRODUCING AND DEGRADING BACTERIA IN PECORINO CHEESES

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Under normal condition the consumption of foods or beverages containing biogenic amines (BAs) have not toxic effects because they are rapidly detoxified by the activity of the amine oxidizing enzymes (MAO and DAO). However in presence of high BAs content, in allergic individuals or if MAO inhibitors are applied the detoxification system is not capable of metabolizing BAs, inducing toxicological risks. The presence of BAs in foods is due to the enzymatic decarboxylation of free amino acids by microorganisms that possess this activity. Recently the use of food microorganisms able to degrade BAs once they have been synthesized has been proposed. In order to isolate and characterize BA-degrading bacteria, Pecorino cheese samples were screened. Among the isolates only 24 strains were found able to degrade tyramine/histamine, 14 of which belonging to different *Enterococcus* species (*E. faecium*, *E. italicus*, *E. casseliflavus* and *E. gallinarum*), 6 to *Leuconostoc mesenteroides* and 2 to *Corynebacterium variabile*. None was positive for the *hdc* gene, while 8 had *tdc* gene, indicating their potential to produce tyramine. In order to evaluate their capacity of producing and degrading tyramine and histamine the strains were suspended in phosphate buffer containing precursors or the two BAs. The analyses of supernatants by HPLC showed that all the strains did not produce histamine, while 8 strains produced tyramine. As regards BA-degrading ability the strains showed a great variability, ranging from 0 to 44% degradation for tyramine and from 4 to 32% for histamine. However only 2 strains reduced the histamine content of about 30% and four strains tyramine of about 40%. The two strains with high histamine oxidase activity shown no or low ability to oxidize tyramine.

PS2-05

BACTERIAL AND FUNGAL COMMUNITIES ASSOCIATED WITH FRESH FRUITS AND CLADODES OF MEDITERRANEAN **OPUNTIA FICUS-INDICA** *(L.) CULTIVARS*

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Opuntia ficus-indica (L.) Mill. native to Mexico, is widely distributed in arid and semi-arid regions of South and Central America, Africa and the Mediterranean area. Due to the high ecological adaptability, *O. ficus-indica* is able to growth in all arid and semi-arid environments. Given the trend of the Mediterranean area towards the global desertification

and the decline of water resources, the cultivation of *O. ficus-indica* is constantly increasing since may have a role in effective food production systems as fruit and vegetative crop. Despite the wide use of fresh cactus fruits and young cladodes investigations into the ecology of fungi and bacteria associated with fruiting plants is largely lacking. Fruits and cladodes were collected in September 2014 from 12 distinct *O. ficus-indica* organically certified cultivars among four major distinct Mediterranean regions (n = 3 for each region) across South of Italy (Puglia, Sicily and Sardinia) and North of Tunisia (Jendouba, Kasserine and Le kef) where *O. ficus-indica* is largely represented. The epiphytic and endophytic microbial diversity was investigated through a culture-dependent approach with molecular identification. Deep community pyrosequencing approach, targeted at the 16S rRNA and ITS R1 loci, to examine the richness and composition of bacterial and fungal communities associated with *O. ficus-indica* was also adopted. Complementary community-level physiological profiles determined by the use of Biolog EcoPlates confirmed the diversity and richness of *O. ficus-indica* microbial community. This study highlighted, through a comprehensive and comparative approach, the dominant microbiotas of 12 Mediterranean *O. ficus-indica* cultivars, which determined some of the peculiarities of the geographical community structure.

PS2-06

ANTIMICROBIAL RESISTANCE PROFILES OF ENTEROCOCCUS *POPULATIONS ISOLATED FROM PECORINO DI FARINDOLA CHEESE*

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Pecorino di Farindola is a traditional cheese produced in artisan plants in the Abruzzi region, in the east side of the Gran Sasso mountain, Central Italy. Ninety *Enterococcus* isolates were collected from raw milk, curd and cheeses at different ripening stages up to a maximum of 150 days. All isolates were identified by 16S rRNA gene sequencing. Additionally, antibiotic resistance profile of strains to 18 antimicrobial agents was determined using minimum inhibitory concentration (MIC). The MIC results were interpreted according to CLSI breakpoints. Moreover the genetic determinants (*vanA*, *vanB*, *vanC1* and *vanC2/C3*) for vancomycin resistance were investigated. The dominant species were *Enterococcus faecalis* (64 strains) and *Enterococcus faecium* (14 strains), followed by *Enterococcus gallinarum*, *Enterococcus hirae* and *Enterococcus casseliflavus*. All strains, except for *E. faecium*, showed high resistance to streptogramin. *Enterococcus gallinarum* and *E. faecalis* strains showed a moderate resistance to tetracycline (30-36%), rifampin (20%), erythromycin (18%). Furthermore *E. faecalis* strains had a moderate resistance also to gentamycin and streptomycin. The strains of *E. faecium* showed a low resistance (7%) to some of the mentioned antibiotics. No strain had the *vanA* and *vanB* genes. *Enterococcus gallinarum* and *E. casseliflavus* strains were resistant to vancomycin (100%), as confirmed by the presence of *vanC1* and *vanC2/C3*, respectively. Even if enterococci do not represent a serious risk to

consumers, they are becoming resistant to an increasing number of clinically important antibiotics, such as those reported in this study. As *Enterococcus* populations are a considerable proportion of the microbiota present in cheeses produced with ewes raw milk in Mediterranean countries, their antimicrobial resistance profile should be thoroughly investigated for a careful safety evaluation.

PS2-07

SELECTION OF VAL-PRO-PRO (VPP) AND ILE-PRO-PRO (IPP)–PRODUCER LACTOBACILLUS *STRAINS FOR DEVELOPING FUNCTIONAL YOGURT ENRICHED OF BIOACTIVE PEPTIDES*

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Aim of this study was to evaluate non-starter lactobacilli (NSLAB) isolated from cheeses for their ability to release ACE-inhibitory and antioxidant peptides from milk caseins. We considered 34 NSLAB isolated from Parmigiano Reggiano (PR) and 5 from Pecorino Siciliano. After a preliminary screening, two NSLAB strains from PR, namely *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331, were selected based on their proteolytic phenotype, and they were used to inoculate sterile cow milk. The fermentation process was monitored by measuring viable cell population, kinetic of acidification, consumption of lactose, and synthesis of lactic acid. Milk fermented with *Lb. casei* PRA205 exhibited higher radical scavenging (1184.83 ± 40.28 mmol/L trolox equivalents) and stronger ACE-inhibitory (IC50 = 54.57 μg/mL) activities than milk fermented with *Lb. rhamnosus* PRA331 (939.22 ± 82.68 mmol/L trolox equivalents; IC50= 212.38 μg/mL). Similarly, *Lb. casei* PRA205 showed the highest production of ACE-inhibitory peptides Val-Pro-Pro and Ile-Pro-Pro, which reached concentrations of 32.88 and 7.52 mg/L after 87 and 96 h of milk fermentation, respectively. Previously, *Lb. casei* PRA205 has been demonstrated to have high resistance to GI tract, antibiotic susceptibility, and adhesive phenotype (Solieri et al., 2014). These evidences overall suggested that *Lb. casei* PRA205 may be a promising culture to produce functional foods with high amounts of IPP and VPP, antioxidant activities, and a high content in health-promoting viable cells. Future perspective is the application of probiotics in yogurt as carrier food in order to obtain probiotic yogurt enriched of VPP and IPP, as well as of healthy viable cells of strain PRA205.

Reference

Solieri, L.,Bianchi, A., Mottolese, G., Lemmetti, F., Giudici, P., 2014. Tailoring the probiotic potential of non-starter Lactobacillus strains from ripened Parmigiano Reggiano cheese by in vitro screening and principal component analysis. Food Microbiol. 38, 240–249.

PS2-08

TOWARDS THE DEFINITION OF MINIMAL INHIBITORY CONCENTRATIONS FOR HALOBACTERIUM SALINARUM

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Archaea are prokaryotic organisms thriving mostly at extreme environments and sharing characteristics with both bacteria and eukaryotes. Within the Archaea domain, the *Halobacteriaceae* family comprises species that are mainly found in solar salterns, marine environments and salted food products. A major representative of this group is *Halobacterium salinarum*. Recently, these microorganisms have raised an increasing scientific interest, since they have been found in both food and human intestinal mucosa. Very little is known about their antibiotic resistances, and it is thus important to establish microbiological breakpoints for *Halobacteriaceae*, as done in the past for bacteria. At present only few works have studied antibiotic resistance in halophilic Archaea, but the Minimal Inhibitory Concentrations (MICs) proposed by these studies can't be considered acceptable since they didn't use at least 50 different strains for each species, as the European Committee on Antimicrobial Susceptibility (EUCAST) requires. Fifty-two different strains of *Halobacterium salinarum* were isolated and characterised by RAPD (Random Amplification of Polymorphic DNA) and 16S rRNA analyses within a study on the microbial ecology of animal casings. We assessed the microbiological breakpoints of these isolates and other three collection strains against anisomycin, ciprofloxacin, clindamycin, erythromycin, novobiocin, rifampicin and trimethoprim. While for rifampicin, ciprofloxacin and trimethoprim the MICs previously found have been confirmed, for the other antibiotics we obtained for clindamycin, novobiocin and anisomycin MICs lower than the reported ones, while for erythromycin they were higher. In particular, we observed that *H. salinarum* is mainly susceptible to antibiotics that inhibit DNA replication rather than those which interact with protein synthesis. High variability among strains was mainly found with novobiocin. This work provides new insights about MICs for *H. salinarum*, a strain that is often found along the food chain, and which can be a first model for the assessment of antibiotic resistance profiles in Archaea.

PS2-09

MOLECULAR AND TECHNOLOGICAL CHARACTERIZATION OF COAGULASE NEGATIVE STAPHYLOCOCCI INVOLVED IN THE RIPENING OF NATURALLY FERMENTED LLAMA MEAT SAUSAGES FROM NORTHWESTERN ARGENTINA

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Llama meat fermented sausages are traditional products consumed in the Andean region of

South America. During sausage fermentation, lactic acid bacteria (LAB) and coagulase negative staphylococci (CNS) play an important role in meat preservation and fermentation processes and for this reason are the most microorganisms used as starter cultures in meat fermentation. The aim of the present study was to identify at species level and characterize for technological and safety characteristics, CNS isolated from fermented llama sausage (without addition of starter cultures), produced in a pilot-plant (P) and on artisanal (A) scale, to select potential starter cultures. A total of 140 colonies were isolated from Baird Parker medium, but only 55 isolates were genetically ascribed to *Staphylococcus* genera belonging to 6 *Staphylococcus* species: *Staphylococcus equorum* and *Staphylococcus saprophyticus* were the predominant followed by *Staphylococcus capitis*, *Staphylococcus pasteuri* and *Staphylococcus xylosus* and *Staphylococcus warneri.* Both productions exhibited a high variability in RAPD fingerprints: 14 and 15 strains were clustered in P and A production respectively. The CNS strains were studied to determine technological properties (nitrate reductase, proteolytic and lipolytic activities; and the ability to grow at the temperature and pH values of fermenting sausage, and at high NaCl concentrations) and safety characteristics (amino acid decarboxylase, enterotoxigenic activities and antibiotic susceptibility) by traditional methods and molecular approach. We evaluated the minimum inhibition concentrations (MIC) for eight antibiotics: ampicillin, ofloxacin, oxacyllin, kanamycin, erythromycin, tetracycline, cephaloridine and vancomycin antimicrobial. The MIC results showed high resistance for the first three antibiotics and susceptibility for the last two. Nitrate reductase activity was observed in 87% of *S. equorum*, whereas was absent in all *S. saprophyticus* and *S. warneri* strains. The data will be presented and discussed.

PS2-10

WHAT MICROBIAL DIVERSITY FOR CONTROLLING PATHOGEN BACTERIA IN CHEESES?

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The objective is to point out that microbial diversity can be an asset to ensure safety of raw milk cheeses. It will be developed through two examples dealing with the control of *Listeria monocytogenes* and of Shigatoxin producing *Escherichia. coli* (STEC). In first, natural microbial consortia from raw milk and rind surface of St- Nectaire cheese can act as a barrier against *L. monocytogenes* which developed mainly during ripening. One of these consortia was composed of 32 species. By methodology of successive simplifications, it has been shown that the association of at least 4 species (*Lactobacillus plantarum , Leuconostoc citreum, Ln. pseudomesenteroides* and *Macrococcus caseolyticus*) was necessary to achieve a similar inhibition, even if individual strains were not inhibitory *in vitro* . Natural inhibitory consortia against the STEC have not yet been identified. Then another strategy relying on screening of dairy bacterial strain's collection (147 strains) has been adopted. Screening in model cheese was more efficient than in vitro test to select several individual strains of lactic acid bacteria and one strain of *Hafnia alvei* that can inhibit the STEC O26:H11 and O157:H7 strains in uncooked pressed cheese. Synergy in the inhibition by association of strains of *H.*

alvei , *Lb. plantarum* and *Lactococcus lactis* at high level of milk inoculation was observed since 6h of cheese making in Saint -Nectaire type cheese . This association was not effective in type Cantal and blue cheese technologies but other associations including *Leuconostocs* were inhibitory in theses cheeses. Anti - STEC activities were not associated with pH decrease. In conclusion, it is primordial to maintain the biodiversity from milk production to cheeses because these ecosystems fit into natural bio-preservation and constitute a reservoir of anti-pathogenic strains.

PS2-11

MICROBIOLOGICAL, PHYSICO-CHEMICAL AND SENSORIAL CHARACTERIZATION OF ARTISANAL NICASTRESE GOAT CHEESE

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Nicastrese cheese is a traditional cheese produced in a small scale in the Nicastro area of Calabria (Italy), using raw goat's milk. No selected starter cultures are added and the cheese manufacture is carried out using artisanal procedures. The present study provides, for the first time, a complete view of the microbial diversity and dynamics involved during cheese manufacturing and ripening. Samples of raw goat's milk, curd and cheese (30 and 60 days old), derived from different farms, were investigated through a polyphasic approach. Physico-chemical and sensorial parameters and volatile organic compounds (VOCs) were studied. To identify the bacteria population two hundred and fifteen strains were isolated from selective culture media, clustered using Restriction Fragment Length Polymorphism and identified by 16S rRNA gene sequencing. According to 16S rRNA gene sequencing, *Lactococcus lactis* represented the dominant species both in milk and in curd samples, while *Lactobacillus plantarum* and *Lactobacillus casei* dominated thought the cheese ripening. Bacterial community determined by a culture-independent approach revealed differences among samples and an increase in bacterial diversity during cheese ripening, highlighting the presence of several lactobacilli. Similarly, significant differences were detected among samples for physico-chemical and sensorial parameters, indicating that raw milk has a pivotal role in the cheese gross composition. Moreover, different VOCs (free fatty acid, esters, aldehydes, ketones and alcohols) were identified in the volatile fraction of Nicastrese goat cheeses by SPME extraction and GC-MS analyses. In particular, free fatty acids and esters exhibited a significant increase through ripening.

PS2-12

YEASTS DIVERSITY IN SPECK: ASSESSMENT OF THEIR CONTRIBUTION TO PROTEOLYSIS AND AROMA GENERATION

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The occurrence of non starter microorganisms and particularly yeasts on cured meat has been widely described. However, their contribution to the biochemical changes that lead to the typical properties and sensory characteristics of dry cured meats remains poorly defined. Among dry meat products, Speck Alto Adige is characterized by several selective conditions including dehydration, curing and smoking which can affect the product microbiota. In order to assess the possible role of yeasts in Speck Alto Adige, yeast consortia were evaluated throughout the main processing phases in 2 companies. Also the proteolytic profiles (by SDS-PAGE electrophoresis) and the accumulation of volatile organic compounds (VOC – by SPME-GC/MS analysis) were determined. A total of 164 isolates were identified, being *Debaryomyces hansenii* (86.6%) the dominant species followed by *Yarrowia lipolytica* (11%). RAPD-PCR fingerprinting revealed the prevalence of a specific biotype of *D. hansenii* in one of the 2 companies which seems to be related to the selective pressure generated by the specific processing conditions adopted. In particular the curing and smoking conditions used resulted in a greater persistence over ripening of nitro/nitroso compounds, phenols and furanes which accounted also for a lower accumulation of microbial metabolites. The comparison between in vitro proteolytic and SPME-GC/MS profiles, generated by selected strains of *D. hansenii* and *Y. lipolytica*, and those of the samples collected during processing and ripening showed that several bands and VOC were common to Speck and yeasts profiles, while some signals specifically characterized *Y. lipolytica* or *D. hansenii* profiles.

PS2-13

LACTIC ACID BACTERIA FROM PIACENTINU ENNESE *CHEESE: CHARACTERIZATION AND IDENTIFICATION THROUGH A POLYPHASIC APPROACH*

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It is generally accepted that lactic acid bacteria (LAB) play a significant role during the cheese ripening, especially in those produced under traditional procedures. Among them, *Piacentinu Ennese* is an artisanal cheese produced in the Enna area of Sicily (Italy) using raw cow milk without addition of any starter cultures. The typical properties of this artisanal

cheese are mainly due to their richer indigenous NSLAB population. In the present study, almost one-hundred lactic acid bacteria (LAB) strains were isolated from fresh, 6 and 8 months ripened *Piacentinu Ennese* cheeses, provided from 3 different farms, in order to select strains to be use as starter cultures . Firstly, the LAB strain were screened for technological and safety aspects i.e. acidifying activity, sensitivity to different class of antibiotics, biogenic amine production and antimicrobial activity versus different pathogens (*Listeria monocytogenes, Escherichia coli, Staphylococcus aureus, Pseudomonas* spp). Then the strains were characterized and identified using a polyphasic approach, which combine a molecular fingerprinting technique (GTG5-PCR), a proteomics approach (MALDI-TOF MS) and sequencing analysis of the 16S rRNA gene. Results revealed that 90% of the strains exhibited good acidifying activity in skim milk; most of the strains (78%) showed variable resistance to the antibiotics tested and only few strains exhibited high resistance. All strains were not able to produce biogenic amines and 65% of the strains showed antimicrobial activity against the pathogens tested. Molecular characterization revealed that most of the strains isolated from fresh cheese were ascribed to *Lactococcus lactis* species, while several NSLAB were detected in the 6 and 8 ripened cheese. In detail, most strains consisted of *Pediococcus pentosaceus*, *Lactobacillus plantarum*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus*. In addition to these dominant groups, some isolates identified as *Lactobacillus pentosus* and *Streptococcus macedonicus* were retrieved from the old cheese samples.

PS2-14

ANIMAL CASINGS AS A SOURCE OF MICROORGANISMS INVOLVED IN MEAT FERMENTATIONS: EVIDENCE FROM CULTURE-BASED AND HIGH-THROUGHPUT MOLECULAR METHODS

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Natural casings are portions of animal intestines that have been used for centuries in the manufacturing of many typical dry-fermented sausages. Because of their intestinal origin, casings have a high microbial load, but their possible role as inoculants of microorganisms that can play a role in the ripening of dry-fermented sausages has not been investigated yet in detail. Here, we analyzed by means of culture-dependent methods and Illumina high-throughput sequencing of 16S rRNA amplicons the bacterial ecology of hog, cow and ovine casings at different stages of their preparation for sausages production. We also compared the bacterial ecology of casings, meat mixtures and sausages at different ripening stages in the production chains of *Salame Mantovano*, a typical Italian dry-fermented sausage. Culture-based methods relied on the isolation and characterization of strains on different typical media, while for the molecular methods multi-million reads were originated and analyzed after amplification and Illumina MiSeq sequencing of 16S rRNA amplicons. From the animal casings, several strains of *Staphylococcus*, *Lactobacillus*, *Bifidobacterium*, *Vagococcus* and *Clostridium* were counted in significant amounts, isolated and characterized

at phylogenetic level. High-throughput sequencing analyses revealed a high bacterial diversity, which differed strongly between casings of different animal species. It was also found that the technological processes had a strong impact on the casings bacterial ecology, with a significant reduction of undesired microorganisms, and an increase in the proportion of lactobacilli and staphylococci. Finally, the analyses on the production chain of *Salame Mantovano* revealed that several strains found in the casings before stuffing were also detected in the final ripened products, thus confirming our main hypothesis. This work shows the importance of the use of natural casings in the manufacturing of typical dry-fermented products, and highlights the role of high-throughput sequencing technologies as powerful tools to gain a better comprehension of food fermentations.

PS2-15

PROTECTIVE EFFECT OF LACTIC ACID BACTERIA CULTURES ON SPOILAGE MICROBIOTA OF FRESH CHEESE

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Lactic acid bacteria (LAB) have been used to inhibit undesirable bacteria in ferment foods. Different studies showed how adjunct cultures are active against spoilage bacteria and pathogens in different foods. In cheese the spoilage can affect the shelf life of the product with smear on the cheese surface and packaging blowing. Recently cultured-independent methods, and in particular next generation sequencing (NGS), have been used to evaluate the complete microbiota in complex ecosystems and we applied this technique to better understand the microbial community in fresh cheese at the end of its shelf life. The cultivation based methods and NGS analysis over the shelf life period, showed that the spoilage microbiota at 8°C and 14°C is composed of primarily by species of *Enterobacteriaceae* and *Moraxellaceae*. To limit the outgrowth of these bacteria we tested in *in vitro* cheese model 13 different lactic acid bacteria (LAB). Three out of 13 bacteria, namely *Lactobacillus rhamnosus* RH05, *L. sakei* LK04 and *Carnobacterium maltoaromaticum* CNB04, were the most effective in inhibiting Gram negative bacteria and so they were assessed in industrial trials either alone or in combination. Soft cheese with and without adjunct cultures were prepared and stored at 8°C and 14°C until the end of the shelf life. Traditional counting colonies and next generation sequencing demonstrated that the use of adjunct cultures reduces the growth of spoilage microbiota at both temperatures of storage. In particular during industrial experiments *Carnobacterium maltoaromaticum* CNB04 showed the most evident activity against psychotropic spoilage microbes.

PS2-16

PREVALENCE OF ANTIMICROBIAL RESISTANT BACTERIA IN THE FOOD CHAIN

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It is known that the use of antibiotics in animal farming acts as selective pressure on bacterial populations, inducing the outgrowth of antimicrobial resistant (AMR) strains. Less information is available on the spread of these bacteria and of the AMR genes in the food chains and their fate and during food fermentation. Moreover, few data are available on the consumer exposure to AMR through foods. To investigate on it, we used two approaches: first the analysis AMR populations from milk and dairy fermented foods, then the use of genomics for the detection of AMR determinants in food associated bacteria. We focused on three antibiotics of human or veterinary importance: ampicillin, tetracycline and erythromycin. A total of 208 AMR strains were isolated from dairy products belonging the species *Streptococcus lutatiensis*, *Lactobacillus salivarius, Lactococcus lactis, Enterococcus faecalis, Staphylococcus xylosus*, *Staphylococcus saprophyticus*, *Bacillus licheniformis*, *B. cereus Pseudomonas aeruginosa*, *Obesobacterium proteus*, *Hafnia alveae, Shigella spp*, *Escherichia coli*, *Klebsiella pneumonia* and *Proteus mirabilis*. The genetic determinants responsible for resistance were identified and the mostr represented were *ermB*, *ermC*, *tetM*, and *blaZ* and genes for extended-spectrum β-lactamases. Multidrug resistant strains were isolated. The genomic analyses of species of *Enterococcus, Lactobacillus*, *Streptococcus* and *Staphylococcus*, targeted to the detection AMR genes and mobile elements, plasmids, transposons and ICE (integrative conjugative elements), revealed the presence of a scattered presence of AMR determinants, with a higher frequency in *Enterococcus*. These results highlight a risk associated to AMR bacteria in food and underline the emergency of deeper understanding the role of food AMR populations as risk factor for human health.

SESSION III

PS3-01

A SCREENING APPROACH TO ASSESS ANTIBIOTIC RESISTANCE AND PRESENCE OF RELATED GENES IN COMPLEX MICROBIAL COMMUNITIES

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Four *scotta-innesto* natural starter cultures (SR30, SR56, SR63, SR74) for Pecorino Romano cheese, collected on different Sardinian dairy plants and lyophilized in the 1960s, were tested for antibiotic susceptibility. To screen the complex microbiota for antibiotic-resistant bacteria and resistance genes, reactivated and concentrated cultures (about 5x108 cfu/ml) were inoculated (105 cfu/ml) in IST-lactose and LSM broth (ISO 10932:2010) containing tetracycline, erythromycin, clindamycin, ampicillin, gentamycin, chroramphenicol, or tylosine at different concentrations (EFSA, 2012). By this screening, only one culture (SR56) out the four tested was suspected to contain LAB resistant to tetracycline and erythromycin. To estimate the load of presumptive antibiotic-resistant LAB, decimal dilutions of SR56 were inoculated in broth with tetracycline (4, 8, 16 mg/l), erythromycin (2, 4, 8 mg/l), and without antibiotics as control. Only cocci-shaped bacteria grew in broths with antibiotics. The estimated load of presumptive tetracycline- and erythromycin-resistant cocci was 104 cfu/ml and 103 cfu/ml, respectively. A pool of colonies, picked up from plates streaked with an aliquot of growth-positive broths, was tested by PCR for *Enterococcus faecium*, *Enterococcus faecalis*, *Enterococcus durans*, *Streptococcus thermophiles*, tetM, ermA and ermB genes. Only *E. faecium* and *tet*M were detected. Furthermore, 30 *E. faecium*, out of the 80 isolated from plates containing antibiotics, harboured *tet*M: these isolates turned out to belong to 3 different *Sma*I pulsotypes, 27 out of 30 shared the same profile. Pecorino Romano cheese samples at 5 and 8 months of ripening (minimum time for consumption), experimentally made with a starter composed by SR56 and SR30, were seeded in IST agar medium with 8 mg/l tetracycline, erythromycin or clindamycin, and without antibiotic. Bacterial loads in antibiotic-containing media were in the order of 102 cfu/g in spite of a load of 107 cfu/g in control medium. No *E. faecium* were isolated from plates with antibiotics.

PS3-02

SELECTION OF OXYGEN-TOLERANT MUTANTS THROUGH ADAPTIVE EVOLUTION OF LACTOBACILLUS CASEI *N87*

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Lactobacillus casei is a lactic acid bacterium used in the production of fermented and

functional foods. Several strains of *L. casei* were able to grow under aerobic and respiratory conditions, expressing phenotypes with more competitive (higher stress survival and biomass production) features. In this study, an evolution experiment was performed to evaluate the genotypic and phenotypic adaptation of respirative strain *L. casei* N87. Sixteen propagations (~ 45 generations) were carried out in aerobic and respiratory conditions. A total of 660 colonies were randomly isolated, cultivated in anaerobiosis (AN), aerobiosis (AE) and respiration (RS), and screened for their tolerance or sensitivity to one or more conditions. The progress of propagations promoted biomass production and rate of oxygen uptake, indicating the increase of oxygen-tolerant phenotypes during the adaptive cultivations. Ten mutants were selected and again propagated in anaerobiosis, aerobiosis and respiration to evaluate kinetics of growth, biomass production, oxygen consumption, catalase activity and tolerance of oxidative stress. The mutant AE-51 (sensitive to aerobic and respiratory growth, with the lowest μmax and oxidative stress tolerance) and mutants AE-284 and RS-337 (with the best growth and oxidative stress performances) were used for whole-genome sequencing (Illumina HiSeq2000). Several single nucleotide polymorphisms (SNPs) were detected in mutants. SNPs principally affected upstream sequences of mobile elements and tRNA genes (tRNASer and tRNAVal). In AE-284 and RS-337, point mutations were also found in upstream regions of aryl-alcohol dehydrogenase (involved in metabolism of aromatic compounds), and only in respirative RS-337 on the upstream sequence of NAD(P)HX dehydratase (involved in the removal of toxic hydrated forms of NADH and NADPH). Evolution towards aerobic and respiratory lifestyle did not cause nonsense modifications (stop codons and truncated proteins) in mutants. Given the short duration of selection it is likely that respiration-competent *L. casei* strains may naturally evolve under conditions favouring respiration.

PS3-03

GENES AS BIOINDICATOR IN COMPLEX HUMAN-IMPACTED ECOSYSTEMS

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Bioindicators are organisms that rapidly reveal the quality of environment, by highlighting the effect of environmental changes on a population, community, habitat or even on an ecosystem. On the basis of this characterization, bioindicators are useful in assessing additive, synergistic and antagonistic effects upon living organisms within a broad pollutant-based spectrum of complex environments. Even several bioindicators are used worldwide, the utilization of bacteria is quite limited, mainly due to different theoretical and conceptual constrains representing a very novel topic. The aims of the work were i) to determine the presence of multi-contaminants from agricultural, municipal and industrial activities in complex freshwater environments through the differential shifts of the microbial community structures ii) to examine the antibiotic resistance spread in freshwater channels using Class I

integrons as bioindicators. In Zhangye (Gansu Province, China) we analyzed rhizobacterial sediment samples of *Phragmites australis* (common reed)-associated rhizosphere in sites affected by different types and levels of pollution. Bacterial community structures were grouped according to different land usages, resulting in an effective diagnostic tool, capable of detecting a broad spectrum of pollutant. Furthermore, the potential utilization of Class I integrons was tested to investigate the link between the dissemination of mobile genetic elements and antibiotic resistance in the same study area. Class I integrons were found in all the polluted sampling sites, with a higher occurrence of Class I integrons carrying various gene cassettes in the areas affected by agricultural pollution. In contrast, Class I integrons were absent in the unpolluted areas.

PS3-04

*EXOGLUCANASE GENES (*WaEXG1 *and* WaEXG2*) ARE INDUCED IN* WICKERHAMOMYCES ANOMALUS *WHEN THE YEAST IS GROWN ON GRAPE BERRIES AND GRAPE BERRIES INOCULATED WITH* BOTRYTIS CINEREA

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The use of yeasts, including *Wickerhamomyces anomalus*, as biocontrol agents against fungi responsible for postharvest diseases of fruits and vegetables has been investigated for the past two decades. Among a variety of mechanisms, the production and secretion of glucanases have been reported to play a role in the ability of yeast to inhibit other fungi. Some yeasts also produce different types of "killer toxins" that presumably provide a competitive advantage against other microbes. Two killer toxins produced by *W. anomalus* have been previously demonstrated to be exoglucanases that are coded by the genes, *WaEXG1* and *WaEXG2*. The objective of the present study was to determine the expression of these genes when *W. anomalus* was grown on halved grape berries that were either non-inoculated or inoculated with spores of *Botrytis cinerea*, or in minimal media supplemented with cell walls of the pathogen. Expression profiles over a 48 h period of incubation were analyzed by RT-qPCR utilizing gene-specific primers and compared to glucanase gene expression in the NYDB (control). Results indicated that expression of *WaEXG1* at 48 h was 3.5, 3 and 4 fold greater than the control at T0, in the control, non-inoculated grape berries, and inoculated grape berries, respectively. Expression of *WaEXG1*in a minimal medium supplemented with cell walls of *Botrytis*, was also induced after 48 h, but at a much lower level than in the other treatments. An increase in the expression of *WaEXG2* over a 48 h period was also observed, with the highest levels observed in the controls and the lowest in the minimal medium containing *Botrytis* cell walls. These results suggest that while both glucanase genes respond to the nutritional environment, *WaEXG1* may be more responsive to the presence of a specific pathogen.

PS3-05

PRELIMINARY EVIDENCES FOR QUORUM SENSING INVOLVEMENT IN IN VITRO *CALCIUM CARBONATE PRECIPITATION BY* BURKOLDERIA AMBIFARIA

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Quorum sensing is a process of cell-to-cell communication widely employed by a variety of gram-positive and gram-negative bacterial species to coordinate communal behavior. Quorum sensing requires production, secretion, and detection of extracellular signal molecules called autoinducers, some of which are used for intraspecies communication, while others promote interspecies communication. Bacteria use quorum sensing to regulate a variety of phenotypes, such as biofilm formations, toxin production, exopolysaccharide production, virulence factor production and motility. The aim of this preliminary research was to study the involvement of this cell-to-cell communication in laboratory calcium carbonate precipitation by Gram negative bacteria. We tested the production of AHL autoinducers by seven Gram negative bacterial calcifying strains named C3, C5 and C14 isolated from a soil sample (L'Aquila, Central Italy), A7, A11 and A14 from moonmilk (Grotta Nera, Central Italy) and *PHP7* strain of *Burkholderia ambifaria* from corn rhizosphere (Nancy, France), by cross streaking them with an AHL- strain of *Chromobacterium violaceum* (CV026). Only the strain PHP7 induced the production of violacein in *Chromobacterium violaceum* (CV026). After, we produced an AHL- mutant of the strain PHP7 of *Burkholderia ambifaria*. The AHL- mutant grown on B-4 agar plates lost the calcifying capability of the wild type strain. Calcium carbonate crystals produced by the wild type strain of *Burkholderia ambifaria* showed at SEM observation the presence of geometrically streamed bacterial imprints. All these preliminary evidences suggest that the activation of quorum sensing-regulated genes may be a prerequisite for *in vitro* bacterial calcite precipitation, in some instances.

PS3-06

GENETIC DIVERSITY AND nifH *GENE AMPLIFICATION OF ENDOPHYTES ISOLATED FROM* ORYZA ALTA *AND* ORYZA OFFICINALIS

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Rice (*Oryza sativa* L.) being the primary staple food feeds more than two fifth population of the world and its demand is increasing around 12% annually. Endophyte play key roles in physiological processes of host plant influencing enhancement of biotic and abiotic stress resistance. They improve plant growth by helping host in fixation of atmospheric nitrogen. Two hundred and forty endophytic strains were isolated from leaves, stem and roots of wild

rice *Oryza alta* and *Oryza officinalis* under aerobic condition. One hundred and thirteen strains were screened as diazotroph by acetylene reduction assay (ARA) and their cluster representatives were further corroborated by *nifH* gene amplification. The isolated diazotrophs were grouped into XII clusters by SDS-PAGE of whole cell protein pattern which was further confirmed by insertion sequence-based polymerase chain reaction (IS-PCR) finger printing pattern. The sequencing and phylogenetic analysis of 16S rDNA of the cluster representative strains showed the great genetic diversity and belongs to genera *Enterobacter*, *Klebsiella*, *Raoultella*, *Burkholderia*, *Pseudomonas* and *Acinetobacter*. In addition physiological, biochemical and phenotypic characterization using API 20EN Kit and Biolog microplate also supported the strains diversity. Their phylogenetic analysis based on 16S rRNA gene sequences was done to locate their closest relatives. Results further confirmed the endophytic nature and nitrogen fixing ability of the isolated strains. Representative strain B48 isolated from *O. alta* belongs to genus *Raoultella* of family *Enterobacteriacae* and it is reported for the first time in wild rice. Moreover the strains B69 and B80 have the potential of novelty which need further exploration.

PS3-07

ASSESSMENT OF MANGANESE- AND HEME-DEPENDENT CATALASE IN LACTOBACILLUS CASEI

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Catalase reduces oxidative stress by degrading hydrogen peroxide to molecular oxygen and water. Genes encoding for heme-dependent catalase are present in 23 species of lactic acid bacteria, while those for manganese-catalase only in 13 species. Available genomes of *Lactobacillus casei* do not include sequences annotated as heme- or Mn-catalase and, recently, several recombinant approaches have been used to express these proteins in *L. casei* and improve oxidative stress tolerance. In this study, catalase activity was detected in 4 *L. casei* strains. Whole-genome sequencing (Illumina HiSeq2000) confirmed the presence of both heme-catalase (1461-bp gene) and Mn-catalase (936-bp gene) and two different PCR-products were clearly found in the strains. The effect of cultivation (anaerobiosis or aerobiosis) and supplementation with hemin, manganese and/or iron was investigated (24 factorial experiment in chemically defined medium) on the catalase production of respirative strain *L. casei* N87. Kinetics of growth, enzymatic activity, tolerance of oxidative stress and expression of heme- and Mn-catalase genes were measured in all conditions. The presence of cofactors and respirative growth improved biomass production in *L. casei* N87. Catalase genes were differently expressed on the basis of manganese and hemin supplementation, confirming the different nature of two enzymes. Total catalase activity and tolerance of oxidative stress were higher in respiratory cells when both manganese and hemin were added. However, relevant gene expression and enzymatic activities were found in Mn-supplemented anaerobiosis. Lack of iron in the substrate significantly reduced catalase activity and expression levels in all conditions. Acquisition of catalase genes is probably due to genetic

element transfer from other lactobacilli or pediococci sharing the same ecological niches. Exploitation of *L. casei* strains with intrinsic catalase activities may be a relevant feature for health (prevention of human diseases) and food (reduction of rancidity and discolouration) related applications.

SESSION IV

PS4-01

MACRO- AND MICRODIVERSITY IN MODEL NATURAL MILK STARTERS

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Natural milk cultures (NMC) are undefined starters produced by milk pasteurization, high temperature incubation and backslopping, and are used for several cheese varieties.
Our objective was to monitor macro- and micro-diversity of NMCs over repeated reproduction using amplicon targeted next-generation sequencing (NGS). Raw milk was obtained from 8 cheese plants and used to produce NMCs. DNA extraction was carried out at cycles 4 and 7 for all cultures and 13 for selected cultures. Macrodiversity was evaluated by NGS of the 16S rRNA gene. The NMCs clustered in three groups, one dominated by *S. thermophilus*, the other by *L. delbrueckii* and a third by both species. Two subdominant OTUs (*Lact. lactis*, *Lactococcus* sp.) were present in most samples at 0.5-3%. Others (*Streptococcus* sp., *Enterobacteriaceae*, *Raoultella* sp., *S. parauberis*, *Lact. raffinolactis*) were found at 0.05-3% in ≤10% of the samples. With one exception, no clear clustering between cultures belonging to different cycles nor any clear evolution of communities structure was evident. A significant co-exclusion relationship was found between *L. delbrueckii* and *Pseudomonas* while co-occurrence relationships were found among a group of minor OTUs. No relationship with NMCs' pH was found. The micro-diversity of *S. thermophilus* populations was estimated by NGS of a partial sequence from the phosphoserine phosphatase gene. With two exceptions a single sequence type (ST) dominated (>99%) the NMC. For some cultures a single ST remained dominant over reproduction cycles. In others, the same ST dominated in early cycles but was replaced by another at later stages. While the overall structure of microbial communities in NMC is quite stable, with only two thermophilic species dominating, a number of sub-dominating species (mesophilic LAB and *Proteobacteria*) are able to persist even under the rather selective conditions used. NGS of the serB gene offers potential application for monitoring the dynamics of *S.thermophilus* populations.

PS4-02

DIVERSITY OF MARASMIUS *(BASIDIOMYCOTA) SECTION* SICCI *FROM PHU HIN RONG KLA NATIONAL PARK, THAILAND*

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Marasmius is a genus of saprobic macrofungi found on leaf litter in worldwide. Since Thailand is locate on tropical region that have the mega-diversity of organism including fungi. All previous work on the diversity of Thai *Marasmius* were conducted in upper-north region of Thailand. Phu Hin Rong Kla National Park, located in lower northern Thailand, has different types of forest and also diverse fungal habitats. Owing to the research regarding *Marasmius* from Phu Hin Rong Kla National Park is limited; therefore, species diversity of *Marasmius* from this area were studied. Based on morphology and the internal transcribed spacer (ITS) of rRNA gene, 17 taxa of *Marasmius* section *Sicci* were found. Six of them, *Marasmius thailandicus*, *M. umbelliformis*, *M. haematocephalus f. sinapis*, *M. mandeangensis*, *M. mungkood* and *M. rongklensis* are new taxa while the others, *M. persicinus* and *M. ferrugeneus* are reported for the first time in Thailand.

PS4-03

POTENTIAL OF SCALAR FERMENTATION WITH STARMERELLA BACILLARIS *(SYNONYM* CANDIDA ZEMPLININA*) AND* SACCHAROMYCES CEREVISIAE *FOR THE PRODUCTION OF SANGIOVESE WINE*

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The role of non-*Saccharomyces* yeasts during the must alcoholic fermentation must was re-valued since they can positively contribute to wine quality. Unfortunately, fermentation conducted with natural flora diminished predictability of the process and of the wine features. For this, the combined use of non-Saccharomyces and *Saccharomyces cerevisiae* cultures gained interest. Among non-*Saccharomyces*, *Starmerella bacillaris* (*Candida zemplinina*) seems to have a positive role in the winemaking. Aim of this work was to investigate *Starmerella bacillaris* strain in scalar fermentation with *Saccharomyces cerevisiae* for producing Italian Sangiovese wines. These were compared to those obtained with a commercial *Saccharomyces cerevisiae*. The wines were analyzed for pH, alcohol strength, total and volatile acidity, residual sugars, mannoproteins, biogenic amines, ethyl carbamate and volatile compounds. *Starm. bacillaris*, inoculated in must at 6 log CFU/ml, developed at 25°C up to five days from inoculation, after that *S. cerevisiae*, inoculated in scalar fermentation, was able to carry on the fermentation. No significant difference was recorded for the wine chemical-physical features except for the alcohol strength (11% for the wine obtained with *S. cerevisiae*, 9.8% for wines from scalar fermentation). The principal component analysis of the volatile data showed that wines from scalar fermentations were characterized by an increased content of short and medium chain fatty acid-esters and significant higher mannoprotein content. The highest histamine content was detected in wines from scalar fermentation (2.7 ppm), althought this value slightly exceeded the restricted German limit (2 ppm). The results outlined the synergic effects of the scalar fermentation for the production of wines characterized by highest contents in mannoproteins and specific volatile compounds. Although, the scalar fermentation resulted in wines with

2.7 ppm of histamine, the combined use of the two species permitted to have the lowest alcoholic strength, meeting the consumer tendency.

PS4-04

THE PHYLOGENETIC ANALYSIS OF ANONOXYBACILLUS *AND* BREVIBACILLUS *STRAINS;* BREVIBACILLUS GELATINI *SP.NOV., ISOLATED FROM HOT SPRING IN TURKEY*

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Some hot springs located in west of Turkey were investigated with respect to presence of thermophilic microorganisms. On the basis of the 16S rRNA gene sequence analysis, the 8 isolates are members of the genus *Anoxybacillus*, the other 8 isolates are members of the genus *Brevibacillus*. Based on *rpoB* sequence similarity analysis, isolates TF15, PDF18, TH4 and D1041 are strains of A. gonensis; isolates PDF1, PDF2, PDF15 andTH5 are strains of *A. kamchatkensis*. On the basis of hypervariable regions (HV) similarity analysis of the 16S rDNA, isolate PDF17, PDF27 and PDF29 are strains of *B. thermoruber*, *B. parabrevis*, *B. borstelensis*, respectively. PDF22 and PDF28 are strains of *B. agri*. PDF4 and PDF11 are identical and the two strains may be strains of a new species of *Brevibacillus*. DNA–DNA relatedness was tested to determine the relationship between strains PDF4T and PDF10. Strain PDF4T exhibited a high level of DNA–DNA relatedness with strain PDF10 (92 %), which supported them belonging to the same species. DNA–DNA hybridization was performed for strain PDF4T with the type strains of the most closely related species and strain PDF4T exhibited relatively low levels of DNA–DNA relatedness with the closest type strains, namely *B. formosus* DSM 9885T (32 %), *B. choshinensis* DSM 8552T (30 %), *B. reuszeri* DSM 9887T (18 %), *B. brevis* DSM 30T (40 %), *B. parabrevis* DSM 8376T (46 %), *B. limnophilus* DSM 6472T (34 %), *B. nitrificans* DSM 26674 (36 %)T, and *B. agri* DSM 6348 (59 %)T. These values are clearly below the 70 % cut off point recommended for the delineation of genomic species. Thus, our results support the placement of strain PDF4T within a separate and previously unrecognized species, for which the name *Brevibacillus gelatini* sp.nov is proposed. The type strain is PDF4T.

PS4-05

THE SIMPLICITY OF WELL-KNOWN TECHNIQUES TO UNDERSTAND THE COMPLEXITY OF UNKNOWN MICROBIAL COMMUNITIES

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Nowadays, molecular biology has become an indispensable tool to study bacterial species constituting complex microbial communities, not detectable with classic microbiology

techniques because not cultivable or present in too low concentration. Three traditional scotta-innesto natural starter cultures (SR30, SR56, SR63), collected on different Sardinian dairy plants and lyophilized in the 1960s, were revitalised in 2013-2014 to be characterized both from a microbiological and a technological point of view. Two starter mix were constituted by mixing SR30+SR56 (Mix A), and SR30+SR63 (Mix B), to produce experimental Pecorino Romano PDO cheese A (CA) and B (CB), respectively. Moreover, a commercial selected starter (CSS) was employed to produce a control cheese (CC). All of the above starters were used to perform three manufactures in three consecutive weeks, in different periods of the year. Genomic DNA was extracted by a commercial kit, FTA®/CloneSaver cards, and phenol/chloroform/isoamyl alcohol, from SR30, SR56, SR63, CSS, thermized milk, and cheeses from a representative batch, at different sampling points (24h, 1, 3, 5 and 8 months). The aim of this work was to evaluate the complexity of microbial communities, while assessing the effectiveness of different DNA extraction methods with respect to different molecular techniques (genus/species-specific PCR, Rep-PCR(GTG)5, RAPD-PCR(M13) and PCR-DGGE(V3 region)), using culture-dependent methods as support. DNA extraction methods affected the performances of the different molecular biology techniques, but DGGE. Compared with cheeses, in milk a higher number of species was detected by DGGE. Rep- and RAPD-PCR fingerprints of starters and cheeses were grouped by BioNumerics software with respect to samples origin. Particularly, a high similarity was observed between CA and CB profiles, which were very different from CC. The combination of the different classic and molecular techniques applied turned out to be very effective to understand the complexity of microbial communities colonizing milk, starters and cheeses.

PS4-06

PRESENCE, ANTIMICROBIAL RESISTANCE AND GENETIC DIVERSITY OF CAMPYLOBACTER *SPECIES ISOLATED FROM BROILER CHICKENS*

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Campylobacter bacteria are the most commonly reported cause of acute bacterial enteritis in EU and broiler chickens are considered a major source of this pathogen. The aim of this study was investigated on the presence of Campylobacter spp both in broiler and slaughter to evaluate a possible correlation between these two environments. Six broiler farms located in the central Italy were undergone to experimentation using microbial and molecular approaches. Twenty-five cloacal swabs and fifteen neck skins samples from each farms was detected using ISO 10272-1:2006. There was a discrepancy between the presence of *Campylobacter* in farm and in slaughter with a great abundance in farm and scarcity in slaughter. Indeed, *Campylobacter* war detected in all farms (with exception of farm C) of about 42% of positive cloacal swabs. For each positive cloacal swabs and neck skins samples three purified colonies were isolated (121 strains) and identified by multiplex PCR. Resulted showed that *C. coli* (67,7%) was most common, followed by *C.jejuni* (19,8%), *C. lari* (1%)

and mixed *C. coli* and *C. upsaliensis* (9,1%). The molecular characterization obtained by AFLP showed the presence of three major clonal groups between *C. jejuni* species and four clonal groups were found between *C. coli* isolates. Therefore, AFLP analysis was considered a suitable method for population studies of *Campylobacter*. Also antimicrobial susceptibility for ciprofloxacin, nalidixic acid, erythromycin, streptomycin, gentamicin, tetracycline and chloramphenicol was investigated: all strains of *C. coli* had a similar profile while only the isolates from a farm were resistant to erythromycin. Differences were also seen in the *C.jejuni* isolates from two different farms since in one they were resistant to streptomycin.

PS4-07

COLLETOTRICHUM *SPECIES ASSOCIATED WITH CULTIVATED CITRUS ANTHRACNOSE IN TUNISIA*

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The genus *Colletotrichum* includes many plant pathogenic species causing diseases, commonly known as anthracnose, on a wide range of crops of major importance. In citrus, *Colletotrichum* species may be saprobic, endophytic or pathogenic. Three citrus diseases caused by different *Colletotrichum* species have been reported worldwide: anthracnose, post-bloom fruit drop and key lime anthracnose. None of these diseases have previously been reported in Tunisia. In this study, twenty-one isolates of *Colletotrichum* sp. obtained from stems of different cultivated Citrus varieties showing anthracnose-like symptoms in Tunisia were characterized. Multilocus phylogenetic analyses based on internal transcribed spacer (ITS), glyceraldehydes 3-phosphate dehydrogenase (GAPDH), glutamine synthetase (GS) and ApMat sequences, revealed that all Tunisian isolates belonged to the *C. gloeosporioides* species complex. Two main clades of isolates were identified within the complex, with 19 isolates corresponding to *C. gloeosporioides* and two a putative new species. Among the isolates belonging to the first clade, one isolate showed high closeness with an Australian *C. gloeosporioides* isolate from *Citrus sinensis*. The two isolates corresponding to the new species were both obtained from *Citrus reticulata*. Pathogenicity tests confirmed the three groups of isolates to be pathogenic and revealed a significant (pathogen x host) interaction and an eventual specificity in causing infection on fruits and detached leaves after wounding or without wounding according to the inoculated varieties. These results are in agreement with previous studies in Italy that found that *Colletotrichum gloeosporioides* was the predominant pathogen of cultivated citrus species. In addition, one new potential *Colletotrichum* species that causes anthracnose of citrus was identified.

PS4-08

FOCUSING ON DRAWBACKS IN MANAGING THE COMPLEXITY OF MICRO-ALGAL COMMUNITY

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This contribution underlies the complexity in studying and managing mixed micro-algal populations naturally evolved from seawater with the aim to improve managing of this type of cultures. Nowadays the attention of researchers is paid on the extreme specialization of microbial pure cultures. The simplification of the microbiological actors playing in a process has been considered the way to gain the best results in producing food, beverages, and industrial products. Microalgae are involved, among others, in the production of alternative sources of energy. The majority of literature concerns study and selection of pure micro-algal strains using classical methodologies of observation, counting, isolation, and storage. A few studies shift the attention from pure cultures to micro-algal communities and its possible exploitation. The microbial complexity, in fact, has a key role in maintaining a "climax" stable state with resilience to environmental changes; moreover, the interaction among the different species composing a stable population can lead to an improvement of the culture performances. In the establishment of natural mixed micro-algal populations, various hurdles have been experienced. Throughout the evolution of three seawater samples - collected along the coast of Reggio Calabria (South Italy) - a natural changing consisting in appearing of some species and disappearing of others until the establishment of stable mixed populations was observed. The different species clumped together forming macroscopic and persistent aggregates that impaired the achievement of growth curves for each population present in the culture - due to the drawbacks encountered in using cell counting chambers - the recognition of the different type of cells and so the identification by classical key and the achievement of pure cultures by micromanipulation - due to the persistence of the aggregates - and the appropriate cryostorage - due to various responses of different micro-algae species to different cryoprotectants.

PS4-09

ACETIC ACID BACTERIA FROM UNIMORE MICROBIAL CULTURE COLLECTION: BIOLOMICS PLATFORM AS A TOOL FOR THEIR INDUSTRIAL EXPLOITATION

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Unimore Microbial Culture Collection includes a pool of acetic acid bacteria (AAB) strains collected since 2000. Main isolation sources of the UMCC AAB strains are wine vinegars, balsamic vinegars, cereal vinegars, fermented juices and kombucha tea. During the years, methods to recover representative isolates and preserve phenotypic stability were improved

within UMCC, in order to investigate the key traits useful for their industrial exploitation. Acetic acid bacteria have attractive features useful in performing conventional and innovative processes/products. They incompletely oxidize sugars, alcohols and polyols to the corresponding oxidation products and they are exploited by pharmaceutical, medical, chemical and food-grade industries for the production of acetic acid, dihydroxyacetone, gluconic acid, 2-keto-L-gulonic acid and cellulose. Phenotypic changes, mainly regarding ethanol oxidation, acetic acid resistance and cellulose production, have been detected, consequently, suitable preservation methods and check procedures are performed in UMCC. The AAB strains are identified and characterized by a polyphasic approach and molecular typing are performed by fingerprinting techniques (ERIC/PCR and GTG5). Target strain performance and fermentation assays are developed at pilot scale to provide industrial strains. The BioloMICS (BioAware) platform of UMCC allows a comprehensive data management that combines phenotypic and molecular traits with industrial strain performance.

PS4-10

IDENTIFICATION OF L. RHAMNOSUS *LIVE MICROORGANISMS IN FOOD PRODUCTS BY ABSOLUTE QUANTIFICATION OF* SPXB *CDNA USING REAL-TIME PCR*

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Lactic acid bacteria (LAB) constitute an heterogeneous group of genera that share many physiological features. They are characterized by the capacity of fermenting sugars primarily into lactic acid via homo-fermentative or hetero-fermentative metabolism. Lactobacilli occur in habitats such as wine, milk, meat, vegetables, cereal grains and they have been used in food fermentation processes for centuries. LAB are also present in mucosal surfaces, in the intestinal tract, the vagina and the oral cavity of humans and animals. Thanks to some of their metabolic properties LAB are used in the production of fermented foods. In these processes, they significantly contribute to the flavor, texture, nutritional value and microbial safety of the final fermented product. Because of these reasons, LAB find many applications in food industry. Many strains of LAB have been commercially exploited because they can provide competitiveness against pathogenic bacteria colonizing gastrointestinal tracts therefore there are many food applications that depend on their probiotic effects. Nowadays there is a large variety of products claiming health benefits due to the presence of probiotic bacterial strains. Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (FAO/WHO Food and Agriculture Organization and World Health Organization, 2001). *L. rhamosus* has an important role both as a probiotic and in the production of aromas in dairy products. In this work we have developed a rapid method for the identification of *L. rhamnosus* “live microorganisms” in food commercial product by absolute quantification of cDNA of SpxB gene using real-time PCR.

PS4-11

THE COMPLEX MICROBIAL COMMUNITY OF WITHERED BERRIES OF CV. CORVINA AS REVEALED BY METAGENOMIC ANALYSIS

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Corvina is the most important of Verona's red grape varieties and forms the basis for the production of unique wines, such as Amarone and Recioto of the Valpolicella area (Italy). The distinctive features of such wines are linked to the peculiar winemaking techniques and especially to the post-harvest grape withering process, when the grapes are partially dried in attics for three/four months to concentrate sugars up to about 27% (w/v). Dehydratation increases the concentration of constituent substances in berries, determining also specific expression of grape genes. During this process, grapes are colonized with a complex microbiota to an extent which depends on environmental temperature and humidity. In this study, a whole metagenome sequencing (WMS) approach was used to analyse the microbial consortia present on Corvina berries at the end of the withering process performed in two different conditions ("traditional" or "fast"). Representative berry samples were collected and washed to avoid grapevine DNA contamination. Bioinformatic analyses, performed on reads and scaffolds, revealed that traditionally withered berries were dominated by *Clostridiales* and *Pseudomonadales*, while the fast procedure determined the presence of *Enterobacteriales* and *Lactobacillales*. On the contrary, few consistent differences characterized the eukaryotic fraction, dominated by Aspergillus and *Penicillium* for both samples. Interestingly, the "binning" procedure revealed 15 most abundant genomes characterizing the two conditions. These results provide insights into the microbial community of Corvina withered berries and reveal relevant variations attributable to environmental withering conditions. Further studies will be performed to determine whether the different microbial compositions could lead to significant chemical variations of the musts, with an impact on the organoleptic properties of wine. This study underlines how novel technologies, like WMS, could open novel perspectives in the knowledge and management of traditional processes as the withering process of Corvina, with an impact on the winemaking of important Italian wines.

PS4-12

YEASTS ISOLATED FROM ORGANIC OR BIODYNAMIC GRAPES: GENOMIC AND PROTEOMIC IDENTIFICATION

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Wine is the product of complex interactions between yeasts and bacteria that commence in the vineyard and continue throughout the fermentation process until packaging. Although grape cultivar and cultivation provide the foundations of wine flavour, microorganisms, especially yeasts, have impact on the subtlety and individuality of the flavour response. Consequently, it is important to identify and understand the ecological interactions that occur between the different microbial groups, species and strains. Moreover, recently a wide literature highlights the positive role of non-*Saccharomyces* yeasts in the definition of a more complex aroma profile which contributing to sensory characterization of wines. The main goal of this study was to characterize the yeast populations of grape musts produced from biodynamic or organic agriculture by two approaches: genomics and proteomics. Moreover, the suitability of MALDI-TOF-MS Biotyper for the identification of non-clinical samples was verified. The genomics was performed by using PCR reaction (Sanger sequencing) with the amplification of ITS region and the proteomics was carried out through MALDI-TOF-MS Biotyper, which employed as analytical and typing expression profiling of yeast, yeast-like species and strain variants. About 130 isolated strains from fermenting must were identified and both MALDI-TOF MS Biotyper and sequencing showed a more complex yeast population belonging to *Saccharomyces* and non-*Saccharomyces* genera of spontaneously fermented products compared to driven fermentation. The sequencing identified all the tested strains while the MALDI TOF MS Biotyper identified up to the 70-80% of the isolated yeasts (dependently on the considered sample), probably due to the incomplete libraries of the instruments. Indeed, this study could support the widening of the libraries with peptide profiles of food interest microorganisms in order to increase the potential applications of the proteomic approach currently limited to clinical areas.

PS4-13

MICROBIOLOGICAL AND CHEMICAL HEALTH RISK ASSOCIATED TO BIVALVES COLLECTED IN THE CENTRAL ADRIATIC SEA

SCHIRONE Maria (1), SUZZI Giovanna (1), TOFALO Rosanna (1), PERPETUINI Giorgia (1), VISCIANO Pierina (1), BERTI Miriam (2), SCORTICHINI Giampiero (2), GROTTA Lisa (3), MARTINO Giuseppe (1)

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Bivalves consumption can be associated to some health risks due to the natural accumulation of microbiological or chemical hazards by their filter-feeding system. These hazards are regularly monitored as they must be lower than the legal limits established by European Commission, and food producers decide the necessary sampling and testing frequencies as a part of their procedures based on hygiene control principles. The aim of this study was to analyze samples of *Chamelea gallina* harvested in the Central Adriatic Sea, in terms of pollutants and microbiological contents. In particular, the food safety criteria set for live bivalve mollusks (i.e. *Salmonella* and *Escherichia coli*) and the possible presence of *Norovirus* in clams collected at different sampling sites along the Abruzzi region coast

(Central Italy) were investigated. Among pollutants, the determination of different compounds belonging to polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), commonly referred to as dioxins, was assessed due to their high toxicity. All samples complied with the microbiological criteria, as well as *Norovirus* was never detected in the examined clams. The maximum levels fixed for sum of PCDD/Fs (3.5 pg/g wet weight) were also respected. The values found in clam samples from the different sites ranged from 0.024 to a maximum of 0.051 pg/g wet weight. The results of this study confirmed that the health risks for consumers are low even if a risk based monitoring program and the management of bivalves growing areas should always be assessed and suitable in a future scenario in which microbiological criteria are adopted in food legislation.

PS4-14

CACIOTTA AND CACIOCAVALLO CHEESE RIND MICROBIOTA: DIVERSITY, COMPOSITION AND ORIGIN

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Caciotta and Caciocavallo were traditional cheeses produced in the Southern of Italy from cows' milk with the application of starter cultures. The composition of their rind microbiota and their changes during ripening have not previously investigated. In the present study we investigated the bacterial community structure of the two cheeses during manufacture and ripening using a colture dependent and independent approaches, based on next generation sequencing of 16S rRNA gene amplicons. In particular, the aims of this study were to determine the relationships between the microbiota of the milk, surfaces of the dairy equipments and ripening rooms and the rind microbiota of the cheeses. An high diversity were found in the analysed samples. Several OTUs were present in all samples and include mostly lactic acid bacteria (LAB), especially *Streptococcus* and *Lactobacillus* species. Abundance and diversity of these genera differed between the two cheeses and in response to the ripening environment. Also a large number of non LAB genera could be identified and including *Staphylococcus* spp., *Marinomonas* spp., *Cobetia* spp., *Chryseobacterium, Propionibacterium,* and *Brochotrix* spp. The results of the environmental sampling showed that *Streptococcus thermophilus* was the species found at the highest level in all samples analysed and he also dominate the microbial community of the brine and may survive over time. Overall, the microbial composition of the milk used, the equipment's of the production area, the brine, the ripening rooms, determined the bacterial community composition, especially on the rind.

PS4-15

MICROBIAL DIVERSITY OF A SALINE SOILS IN A NATURAL ENVIRONMENT ON SOUTH ITALY

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Spatial and time-related heterogeneity of complex soil microbial communities represents a thorny and challenging issue to investigate. The argument becomes even more complex when the attention goes to the microbial ecology of "extreme" environments. The present study was focused on metagenomic DNA analysis of a soilwhere some ecological contrasting variables acted as strong drivers in fungal and bacterial spatial distribution. A salt-affected soil was studied to evaluate the relationships between microbial community structure, soil features and DNA extraction effectiveness. 16 soils samples from A horizons were collected according to a random simple sampling scheme. Bacterial, archaeal, and fungal communities were characterized by their 16S rDNA genes with T-RFLP method. Pyrosequencing-based analysis of the V2-V3 16S rRNA gene region, to identify changes in bacterial diversity and community structure was performed by means of 454 massive sequencing approach. Chemical and physical soil analyses were also performed. The choice of DNA extraction and purification method had a significant effect upon bacterial, archaeal, and fungal molecular characterization from soil mainly because salts affected DNA extraction procedures. The different concentration of salt, and calcium sulfate in soil influenced the structure and distribution of the microbial community which resulted highly variable also when comparing very close areas. Soil salinity thus represented both a natural gradient that defined the structure and distribution of microbial species in the environment, but also a critical element that introduced variability in the yield and quality of DNA extraction and purification. Robust techniques that allow the evaluation of the effects of environmental matrix on the representativeness of the extracted DNA will have to be developed in order to better understand the diversity of so heterogeneous systems.

PS4-16

DIVERSITY OF ARBUSCULAR MYCORRHIZAL FUNGI IN APPLE ORCHARDS UNDER DIFFERENT AGRICULTURAL MANAGEMENT SYSTEMS

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Arbuscular mycorrhizal fungi (AMF) are beneficial soil symbionts accomplishing key functions in the complex networks of belowground/aboveground biotic interactions, as they live in association with the roots of most plant families, including fruit trees. AMF deliver

many essential agroecosystem services, representing fundamental elements of soil fertility and productivity in low-input agricultural systems. However, little is known on the biodiversity of AMF communities in relation to agricultural practices in apple orchards. In South Tyrol, where the great majority of Italian apple production is located, many growers adopt integrated fruit production management techniques, while organic and biodynamic management systems are increasing. In this work different molecular tools, such as molecular cloning and sequencing techniques of partial SSU rDNA gene, as well as denaturing gradient gel electrophoresis (PCR-DGGE) were used to gain knowledge on the structure and dynamics of AMF communities in integrated, organic and biodynamic apple orchards in South Tyrol. The sequence types detected corresponded to both known and unknown AMF species, including *Glomus*, *Rhizophagus*, *Funneliformis*, *Diversispora*, *Claroideoglomus* and *Paraglomus* species. The data obtained utilising both molecular techniques showed that the different farming systems were able to shape AMF communities in apple orchards, suggesting that low-input agricultural systems, where AMF diversity is higher, may benefit from mycorrhizal symbioses in terms of soil fertility and ecosystem services.

PS4-17

PTR-TOF-MS AS A NEW STRATEGY TO MONITOR MICROBIAL VOLATOME: CULTURABLE MEDIA, FOOD NICHES, AND MICROBIAL INTERACTIONS

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Analytical tools for the identification and quantification of volatile organic compounds (VOCs) produced by microbial cultures have countless applications in an industrial and research context which are still not fully exploited. The various techniques for VOC analysis generally arise from the application of different scientific and technological philosophies, favoring either sample throughput or chemical information. Proton Transfer Reaction-Mass Spectrometry (PTR-MS) represents a valid compromise between the two aforementioned approaches, providing rapid and direct measurements along with highly informative analytical output. The present work report the main applications of PTR-MS in the microbiological field of fermented food.

Keywords: mass spectrometry; PTR-MS; microorganism; volatile organic compound; VOC; microbial starters.

PS4-18

VIROME-ASSOCIATED ANTIBIOTIC RESISTANCE GENES IN FRESHWATER AQUACULTURE

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Due to their structural characteristics in extracellular phase, bacteriophages persist quite successfully in the environment and are resistant to natural or man generated stressors. Aquatic compartments may therefore play a significant role in driving antibiotic resistance genes (ARGs) transfer. The project is aimed to highlight the impact of viral populations in the spreading of ARGs in aquaculture. Upstream and downstream water samples from an experimental salmonid aquaculture were treated with a tangential flow filtration system to separate and concentrate viral-like particles and microbial cells. Then viromes were investigated through shotgun NGS while DNA from microbial communities underwent NGS(1)6S rRNA gene profiling. Bioinformatic analysis was performed using QIIME and Meta-Vir tools, CARD, NCBI, and COG database. Upstream and downstream samples were characterized by limited changes in viral community composition at family level, while a large variation in bacterial population was observed. The relative abundance of AR drug classes did not significantly change whereas the taxonomic distribution of ARGs and bacterial metabolic genes in the viromes were remarkably different between upstream and downstream samples thereby reflecting the taxonomic composition and diversity of the bacterial communities. Interestingly, an inverse relationship between the relative abundance of genes belonging to a bacterial family in the virome and the same bacterial family abundance in the microbioma was observed. Furthermore, the frequency of AR resistant colonies showed an inverse relationship with respect to the abundance of specific virome-associated ARGs.

PS4-19

ASPERGILLUS FOETIDUS *MEDIATED SYNTHESIS OF SILVER NANOPARTICLES AND ITS EFFECT ON THE GROWTH AND SOME BIOCHEMICAL PARAMETERS OF* ASPERGILLUS FOETIDUS

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The synthesis of silver nanoparticles has been carried out by using the extracellular filtrate of the fungal strain, *Aspergillus foetidus* MTCC8876. The live cell filtrate of fungi has been used as reducing agent in the process of nanoparticles synthesis. In 50 ml of cell filtrate, AgNO3 stock solution was added to make it final concentration as (1) mM of $AgNO_3$ and allowed to shake in an incubator for several hrs. in dark. The synthesis of silver nanoparticles (SNP) was identified primarily as change of the color of the extracellular filtrate and confirmed with the help of the study of UV-Vis spectroscopy. The synthesized nano particles have further been characterized byfollowing thedifferent biophysical techniques. The results obtained from the study of antifungal activities of the silver nanoparticles are found to be very significant. Growth of *Aspergillus foetidus* both in liquid and solid Czapek Dox media have been studied in presence of silver nanoparticles. Medium free dry biomass of the fungi was collected and the same have been used for the assay of catalase, lipid per oxidation, thiol

& protein content. The growth of the fungus was found to be remarkably decreased with the increase of concentration of SNP and it was found that the activities of enzymes and contents of all of the biochemical parameters were found to be changed which may be considered as a possible mechanistic of mode of action of antifungal activity of biosynthesized silver nanoparticles.

PS4-20

PYROSEQUENCING ANALYSIS OF THE FUNGAL BIODIVERSITY IN TABLE OLIVES

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The biochemistry and microbial ecology of *PDO* Aloreña de Málaga table olive fermentations were investigated in two different industries (1 and 2) of province of Málaga (Spain). Monitoring of the physicochemical parameters such as pH, acidity, salt, color and texture of fruits was carried out during the entire process. No differences were detected in physicochemical parameters, which were characteristics for this type of elaboration. The microbial ecology was investigated using both culture-dependent and culture-independent methods in fruit and brine. The initial yeast population was different between both industries. While industry (1) showed counts around 4-5 $\log_{10}$ cfu/mL during the first month of fermentation, industry 2 presented lower concentrations (1-3 log10 cfu/mL), although, both locations reached the same yeast populations at the end of storage period (>5 log10 cfu/mL). Bar-coded pyrosequencing analysis of the ITS rDNA region identified 109 and 118 operational taxonomic units (OTUs) in fruits and brines, respectively, and revealed the presence of yeast genera previously identified in table olives such as *Pichia*, *Candida*, *Debaryomyces* and *Sacharomyces* sp., but also, this is the first time that other predominant genera such as *Zygostorulaspora*, *Penicillium* and *Aspergillus* have been identified in this type of table olives. Small differences were found between most abundant yeasts in fruits and brines, during the fermentation process and between both industries.

PS4-21

BACTERIAL DIVERSITY IN CANTAL-TYPE CHEESE REVEALED BY 16S rRNA-BASED METABARCODING SEQUENCING

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The sensory quality of cheese is the result of the composition and activities of microbial communities in interaction with the milk biochemical components. Dairy cow's feeding

influences the composition of milk fat, which in return affects the sensory quality of cheeses. The role of microorganisms in this interaction is still poorly understood. The objective of this study is to evaluate the impact of cows feeding on the composition and dynamics of microbial communities in connection with the fat composition of milk and cheese. Two balanced cows groups were conducted in two contrasting farming systems: Bota with high botanical diversity grasslands, and Pepi with less diversified meadows and a low intake of concentrates. The raw or pasteurized milk produced in each system was processed into uncooked pressed Cantal type cheese (3 replicates) at two periods (beginning and end of summer). A culture-dependent approach revealed no difference in level between the main microbial groups between milks and cheeses of the two farming systems. In parallel, a metabarcoding approach based on massive sequencing the (1)6S rRNA gene has shown that the bacterial diversity of cheese rind is higher than that of the inner part. Bacterial balances between July and September cheeses were different. These changes especially affected the lactobacilli group. The α diversity of raw milk cheeses in July is higher in Pepi than in Bota cheeses, and vice versa for the September cheeses. Ongoing analyses will determine whether the observed differences in cheese are associated with differences in microbial and biochemical composition (especially fat) of milk from the two farming systems.

PS4-22

TWO NOVEL CLADES OF GENUS GANODERMA *FROM MAHARASHTRA, INDIA*

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Ganoderma is the large cosmopolitan genus of polypore bracket mushroom belonging to the family Ganodermataceae with more than 300 species. In this study mature stage *Ganoderma* samples were collected from the Konkan region of Maharashtra (Western Ghats) a biodiversity hotspot of India. The DNA was isolated and Internal Transcribe Spacer (ITS) region and mitochondrial small sub unit (mtSSU) regions of rDNA were amplified and sequences. Phylogenetic analysis of ITS, mtSSU and combined dataset of ITS and mtSSU was carried out by Maximum Parsimony (MP) method using PAUP* V10.4 beta software. The robustness of the clade member generated in MP tree (PAUP) was further confirmed by Maximum Likelihood (ML) method in MrBayes 3.2.2 and RAxMLGUI V1.3. Macro and micro morphological characters were note down and microscopic observations were made by taking free hand sections of fruiting body passing through hymenium. Measurements of hyphae (binding and skeletal), basidiospores, were recorded using standard calibrated ocular eyepiece. Overall topology of the maximum parsimony (MP) tree is similar with previously published trees. Phylogenetic study isolates fall in to distinct 4 groups in which, 3 groups belongs to laccate isolates and 1 group belongs to non-laccate isolates. Out of these 4 groups two groups are known i.e. group 1 (laccate) is already knows as Asian *G.lucidum* complex / *G. multipileum* and group 4 (non-laccate) was identified as *Ganoderma applanatum* / *australe* complex. The remaining two laccate groups were reported as novel groups from India with high bootstrap support. The topology of the ITS, mtSSU and combined ITS and

mtSSU dataset were very much similar to each other. Out of four distinct clades two were already known and two were novel from India. Both novel groups were from laccate group. The ITS phylogeny resolved only terminal relationship while mtSSU phylogeny resolved terminal as well as basal relationship.

Keywords: ITS, mtSSU, *Ganoderma*, medicinal mushroom, phylogeny, rDNA, Parsimony

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PS4-23

MOLECULAR SYSTEMATICS OF THE MEDICINAL MUSHROOM GANODERMA *FROM KERALA, INDIA USING INTERNAL TRANSCRIBE SPACER REGION OF rDNA*

VARGHESE Ruby (1), VARGHESE Nibu (1), NAIR C.K.K. (1), **DALVI Yogesh** (1)

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Ganoderma Karstern is an important group of medicinal and plant pathogenic fungi. It is a large cosmopolitan genus of polyporus bracket mushroom belonging to the family Ganodermataceae and has been used in traditional Chinese herbal medicine for over 4000 years. Wide varieties of morphological characters are exhibited by species belonging to these subgroups and more than 330 species were reported in literature. However, due to the complex morphology of the genus, only 148 species have been validated to date. To characterize medicinal mushroom *Phellinus* and *Ganoderma* collected from Western Ghats of Kerala using Internal Transcribed spacers of ribosomal DNA. Mature fruiting bodies *Ganoderma* were collected from various localities of Central Travancore regions of Kerala (Western Ghats) a biodiversity hotspot of India. Genomic DNA samples were extracted from fruiting body. The Internal Transcribe Spacer (ITS) regions of rDNA were amplified with specific primers to get amplicon of 500 to 780 bp. Parsimony analysis was performed using PAUP*with published reference sequences. Macro and micro morphological characters were note down and microscopic observations were made by taking free hand sections of fruiting body passing through hymenium. Measurements of hyphae (binding and skeletal), basidiospores, were recorded using standard calibrated ocular eyepiece. The images were processed using Digimizer V4.2.6 (2014) image analysis software (MedCalc Software). ITS phylogeny separats *Ganoderma* with the distinct clades of laccate and non-laccate species. South Indian specific novel groups are present. Phylogenetic results were compared with morphological data. Both the data support subgenus *Ganoderma* (laccate) and subgenus *Elfvingia* (non-laccate). Phylogenetically related isolates have similar morphological characters. This is the first attempt to understand phylogeny of *Ganoderma* from Kerala, South India using ITS rDNA region. Present study could improve our understanding of complex morphology and genetic diversity of diverse *Ganoderma* species occurring in Kerala.

Keywords: ITS, *Ganoderma*, medicinal mushroom, phylogeny, rDNA, Kerala.

PS4-24

MOLECULAR SYSTEMATICS OF THE MEDICINAL MUSHROOM PHELLINUS *FROM KERALA, INDIA USING INTERNAL TRANSCRIBE SPACER REGION OF rDNA*

VARGHESE Nibu (1), DALVI Yogesh (1), VARGHESE Ruby (1), GANLEY Austen R. D. (2), LAMROOD Prasad (3), JITE Paramjit K. (3), MALPATHAK Nutan P. (3)

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Phellinus and related genera are belongs to the family Hymenochaetaceae, well known for its medicinal & ecological importance. They are plant pathogens which causes white pocket and heart-rot. Many species have been used as folk medicine in India, Korea and China because of their biochemical and pharmaceutical actions. These mushrooms have different geographic origin and ecological niche and hence correct taxonomical identification of species and genera is difficult with morphological criteria alone. Present study aims molecular approach to characterized *Phellinus* and related genera on the basis of internal transcribed spacer regions (ITS) of ribosomal gene repeats in their genome. Samples were collected from different parts of Kerala which comes under Western Ghats of India (a biodiversity hotspots). ITS regions were amplified and sequenced by using specific primers for all collected samples. The amplification of ITS regions produced a DNA fragment of 500 to 780 bp in all strains. To determine the affinities of these collections with existing species, parsimony analysis was performed using PAUP* with published reference sequences. This was the first attempt to review the concept of Indian species of *Phellinus s.l* on the basis of rDNA analysis and numerical taxonomy of morphological data, especially from the Western Ghats of Kerala. A total 4(1)8 ITS reference sequences were used in phylogenetic analysis which were represented from 3(1) different countries like Korea, China, Japan, Germany, Finland, USA, Estonia, Canada, Italy, Greece, UK, Turkmenia, Philippines, India, Slovenia, Russia, Sweden, Costa Rica, Australia, Cuba, France, Argentina, Thailand, Czech Republic, Mexico, Taiwan, Israel, Lituvania, Turkey, Ukraine and Belarus. These reference sequences represent a total 16 generic groups in the family Hymenochetacea viz. *Phellinus s. l.*, *Phellinus s.s.*, *Inonnotus s.l*, *Inonotus s.s.*, *Inocutis*, *Pseudochaetae*, *Fomitiporia*, *Onnia*, *Pseudoinonnotus*, *Porodaedalea*, *Hymenochaetae*, *Fuscoporia*, *Phellinidium*, *Xanthoporia*, *Phylloporia* and *Phellopilus* were used as reference backbone to understand the relationship of the study isolates with other genera. The result of above analysis suggested that the ITS phylogenetic analysis of the study isolates using two methods i.e. MP and ML in three softwares (PAUP*, MrBayes and RaXML) distinctly separated out study isolates into a novel clade with high confidence value (bootstrap) indicating that this novel clade might be specific to India.

Keywords: ITS, *Phellinus*, medicinal mushroom, phylogeny, rDNA, Parsimony

PS4-25

NEW APPROACH TO ASSESS LACTIC ACID BACTERIA COMPATIBILITY DURING CHEESE-MAKING

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Compatibility tests constitute an effective way to assess interactions among lactic acid bacteria: *(a)* it is necessary to avoid strain combinations displaying mutually inhibitory properties; *(b)* it may be beneficial to combine strains based on their ability to coexist. To study the compatibility of starter cultures and adjunct cultures mixed to control the production of mozzarella cheese, a tube containing 10 ml of UHT milk was inoculated in triplicate with three different strains in pure and mixed culture: the size of the inoculum in the mixed culture was at the 5% level for the starter strain and at the 10% level for the adjunct strain. The cultures were incubated at 37°C and after 90 and 180 minutes were analysed. The pH was determined; effectively, any interaction occurring in mixed cultures is reflected by acid production. The acidification of growth medium in batch culture is a good reflection of bacterial growth, which is why pH measurement is sometimes used to track growth [1]. In addition, the antioxidant activity was also measured by analysing the radical scavenging activity using a spectrophotometric decolourization assay (ABTS) [2]. The preliminary results and the relevant related literature are discussed considering, above all, the advantages for dairy industry and the consequences for starter and adjunct culture selection deriving from the possibility to simply predict interaction among lactic acid bacteria during cheese-making.

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PS4-26

CULTURE-DEPENDENT AND CULTURE-INDEPENDENT CHARACTERIZATION OF POTENTIALLY FUNCTIONAL BIPHENYL DEGRADING BACTERIAL COMMUNITY IN RESPONSE TO EXTRACELLULAR ORGANIC MATTER FROM KOCURIA RHIZOPHILA

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In this study,the extracellular organic matter (EOM) from Kocuria rhizophila has been exploited to biostimulate the growth of the autochthonous microbial community for

eventually enhancing the depletion of polychlorinated biphenyls (PCBs) in historically contaminated sediments (6.260±9.3 10−3µg PCB/ g dry weight). Biostimulation consisted of the amendment to the sediments of the resuscitation promoting factor of the Kocuria rhizophila for increasing the possibility to cultivate those bacterial candidates that in natural environment, survive under a wide variety of stress conditions by entering a 'viable but non-culturable' (VBNC) state, in which cells are intact and alive but fail to grow either in their natural environment and on bacteriological media; however, consisting in bacterial candidates possibly exploitable for bioaugmentation of contaminated matrices with a bacterial population with metabolic traits of interest. The effects of the amendment of the extracellular organic matter (EOM) from Kocuria rhizophila to the contaminated sediments have been analyzed in terms of (1) the relative abundance of selected bacterial groups with reference to untreated sediments by quantitative real-time PCR, and of (2) the characterisation of pure cultures unique to the enrichment culture derivingfrom sediments with EOM amendment. The amendment of the extracellular organic matter (EOM) from Kocuria rhizophila determined mainly the enrichment of the Actinobacteria. Some isolates belonging to the genus of interest has been isolated, cultivated on bacteriological media and analysed for their capacity to deplete biphenyl in vitro.

PS4-27

INCIDENCE OF PLASMODIOPHORA BRASSICAE *AND THE COMPOSITION OF ITS RACES IN SOILS OF POLAND*

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Oilseed rape is susceptible to a number of diseases that cause significant economic losses to farmers. Clubroot caused by protozoa species *Plasmodiophora brassicae* Woronin is now one of the biggest threats to healthiness of oilseed rape plants. In Poland the presence of clubroot was reported on 250 thousand hectares of agricultural soils. The pathogen was found in all main growing areas of oilseed rape. The aim of this work was to determine the incidence and recognize races of *P. brassicae* in the soils of Poland. The pathogen was gathered from root tumors of oilseed rape plants in different regions of Poland. The collection of samples included new variants breaking the currently used resistance gene(s). The samples were collected from 67 fields of winter oilseed rape located in 13 provinces of Poland, mainly Pomerania, Varmia & Mazuria, Lower Silesia and Opole region. The tumors were frozen or air-dried and propagated on the susceptible genotype of *B. rapa* var. *pekinensis* variety 'Granaat'. To examine current population of clubroot in Poland we have used reference forms proposed by Somè et al. (2003) as well as cv. Mendel - a cultivars with resistance Crr genes. The assessment was done using 0 to 4 scale, where 0 was a healthy plant with fully developed roots and 4 was a small plant with roots changed to a club. We have determined 7 races of *P. brassicae* in Poland. The most common races were P1 (52%) and P3 (36%). Molecular detection of *P. brassicae* using Real-time PCR showed very high incidence of this microorganism in numerous soils. The incidence of clubroot depended on soil pH, intensity of oilseed rape cultivation as well as soil moisture.

Aknowledgement
Experimental work was funded by the Ministry of Agriculture and Rural Development of Poland, project number 50.

PS4-28

MICROBIAL POPULATIONS OF LEPTOSPHAERIA *SPP. ON F1* BRASSICA *HYBRIDS*

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Stem canker of brassicas (blackleg), caused by the fungal complex *Leptosphaeria maculans-L. biglobosa* is one of the most destructive diseases of oilseed rape (*Brassica napus*) worldwide. In numerous countries, including Poland both pathogen populations co-exist and they can jointly lead to severe disease symptoms as well as yield losses. The incorporation of *L. maculans* resistance into *Brassica* lines with desirable agronomic and quality traits is a major objectives in breeding programs. At present (1)4 R-genes effective against *L. maculans* have been reported (*Rlm1-Rlm10* and *LepR1-LepR4*) from various *Brassica* species.The aim of this study was to look for the genetic resistance to stem canker in *Brassica* F(1) hybrids in field conditions. The experiment was done in Dłoń (N51o41'22.0", E 17o04'23.0"), Wielkopolska (Great Poland) region. F1 generations of interspecific hybrids between three *B. napus* cultivars and *B. carinata*, *B. fruticulosa*, *B. rapa* ssp. *chinensis*, *B. rapa* ssp. *pekinensis* as well as *B. napus* cultivar with *Rlm7* gene were evaluated. The determination of *Leptosphaeria* species was studied using Loop-mediated DNA Amplification (LAMP) method. For this purpose leaf samples were collected from plants of hybrid lines. Disease incidence was assessed in two seasons (autumn 2014 and spring 2015), on 50 plants per replicate, according to the scale 0-5. The genotypes differed with their reaction to the pathogen. In both seasons, the cultivar with *Rlm7* resistance gene showed significantly less phoma leaf spotting symptoms, as compared to genotypes with no *Rlm7*. The pathogen population of fungi causing blackleg of oilseed rape in Poland was composed of *L. maculans* and *L. biglobosa*. The isolates on leaves of genotypes without *Rlm7* resistance gene were mainly identified as *L. maculans* (83%), whereas the isolates obtained from cultivars harbouring *Rlm7* resistance gene were scarce (8%) and belonged mostly to *L. biglobosa*.

Aknowledgement
Experimental work was funded by the Ministry of Agriculture and Rural Development of Poland, project number 54.

PS4-29

YEAST DIVERSITY IN NOSOCOMIAL ENVIRONMENTS: WHEN DISENTANGLING THE COMPLEXITY CAN HELP SAVING LIVES

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Many fungal species, widely present in the natural environment, are opportunistic pathogens with a significantly increasing incidence over the past decades. These infections are often associated with the ability to form biofilm on implanted biomaterials and/or host surfaces, with important medical implications due to the increased drug resistance of sessile cells. A 270 nosocomial isolates screening of opportunistic yeast was carried out with a 96 well plate-based method, for their ability to form biofilm. Each isolate was re-identified with ITS sequencing, confirming the species identification. Contingency analysis was used to investigate the relationships between the frequency of isolation of a determined species, its biofilm–forming ability and the environment of isolation. *Candida albicans* displayed the highest biofilm forming frequency (90.63%), followed by *Candida tropicalis* (75%) and *Candida parapsilosis* (41%). Interestingly, the least frequently isolated species did not form biofilm. In Pisa Hospital, isolates biofilm-forming outweigh the non-forming in all wards, with a ratio approximately around 3.55, with the exception of Rehabilitation and Oncology ones. *C. albicans* is the species most often isolated in Specialistic Medicine, Surgery and ICU, followed by *C. parapsilosis*. *C. albicans* results the species most often isolated also in all wards of Udine Hospital, followed by *C. parapsilosis* and *C. glabrata* in General and Specialist Medicine. Most of the less common species are found only in General Medicine ward. These data suggest that the species frequency in the nosocomial environment is strongly linked to the biofilm-forming ability. Furthermore, the regression analysis showed that the same species behave differently in the two environments analyzed. Understanding the environmental distribution of opportunistic fungal species and their resistance to antifungal agents has important clinical implications in saving lives.

SESSION V

PS5-01

INTESTINAL MICROBIOTA AND THE DEVELOPMENT OF SOME HUMAN DISEASES: CURRENT ADVANCES AND FUTURE OPPORTUNITIES

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Compositional changes of the intestinal microbiota are correlated with several chronic conditions, including autism (AS), nephropathy (e.g., IgAN), celiac disease (CD) and the group of obesity-associated pathologies called metabolic syndrome (e.g., type II diabetes). Bacteria interact with each other and the host through substrate fermentation and metabolites production. Compared to healthy individuals (HC), the fecal microbiota and metabolome are different in AS, CD, IgAN and type II diabetes patients. Dietary interventions with fibre and/or probiotics could represent a future opportunity to restore a balanced microbiota in AS, IgAN, CD and type II diabetes patients. Fibre consumption appears to have particularly important effects on both host health and microbiota composition and activity. Indeed, many of the postulated benefits of high-fibre diets appear to be mediated via the intestinal microbiota. Elevated fibre consumption promotes gut microbial production of beneficial compounds like short chain fatty acids (SCFA) (important for regulating appetite, adiposity and inflammation), while simultaneously reducing putrefactive fermentation of proteins and formation of toxic products such as secondary bile acids. Beta-glucans, mainly found in barley and oat bran, positively influenced fecal microbiota and metabolome. Two months of dietary intervention of HC volunteers with β-glucans (3 g/day) modulated the composition and the metabolic pathways of the intestinal microbiota, leading to an increased amount of SCFA (e.g. 2-methyl-propanoic acid, acetic, butyric, hexanoic and propionic acids). The administration of probiotics for two months in CD patients was positively correlated with the markers of host health such as BMI, blood pressure, blood glucose/lipid, insulin resistance, QoL self-assessments and colonic function.

PS5-02

COMPLEX NETWORKS OF NUTRITIONAL INTERACTIONS BETWEEN PLANT BENEFICIAL SYMBIONTS AND ASSOCIATED BACTERIA

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Arbuscular mycorrhizal fungi (AMF) form mutualistic symbiosis with the roots of most food crops and play a key role in plant nutrient uptake, while protecting host plants from biotic and abiotic stresses. The establishment and efficiency of mycorrhizal symbioses are affected

by bacteria living in association with AMF spores (Agnolucci *et al.* 2015), hyphae and mycorrhizal roots (mycorrhizosphere), which show different functional abilities, including indole acetic acid (IAA) production, nitrogen fixation, solubilization of phosphate and phytates. By using a culture-independent approach, PCR-DGGE and sequence analysis, we previously identified bacterial species belonging to *Arthrobacter, Bacillus, Herbaspirillum, Massilia, Pseudomonas, Rhizobium, Streptomyces* associated with *Rhizoglomus intraradices* IMA6 spores. Here, a culture-dependent approach was utilized to isolate such bacteria and detect their functional traits. From a pool of 374 strains, 122 were selected and characterized for P-solubilizing, chitinase, nitrogen fixing activity and siderophores and IAA production. Phosphatase and phytase activities were detected in 73% and 100% of Actinobacteria, in 74% and 83% of chitinolytic bacteria and in 44% and 52% of nitrogen-fixers. The emerging picture of mycorrizospheric interactions is one of a previously unimagined complexity, where different partners of a tripartite association - host plants, AMF and bacteria - may positively interact and provide new multifunctional benefits. Indeed, AMF associated bacteria may be transported along hyphae to the relevant soil volume explored, where they may enhance nutrient availability, control plant pathogens and promote plant growth. Further studies should investigate whether different compositions of AMF-associated bacterial communities may determine differential performances of AMF isolates, in order to select the best AMF/bacteria combinations to be utilised as biofertilisers and bioenhancers.

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PS5-03

PROFILING OF GUT METHANOGENIC ARCHAEA IN LACTATING PIGLETS USING ILLUMINA-BASED 16S rRNA GENE SEQUENCING: EFFECTS OF DIETARY PECTIN

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Methane-producing archaea play a key ecological role within the digestive system of humans and animals: they feed off of hydrogen and other byproducts of bacterial metabolism thus regulating carbohydrate fermentation in the gut. Although it is well established that diet is an important determinant of gut microbiota composition, to date there are only limited data describing how gut methanogens are affected by diet in humans. In this study, we investigated the archaeal community in the feces of neonatal piglets fed either a control or a pectin-containing milk diet (10.0 g/L) using 16S rRNA gene deep sequencing and real-time PCR. We sought to determine if pectin supplementation could affect the archaeal population in the gut of such a model animal. Analysis of the archaeal community revealed that in control piglets the dominant methanogens were members of the genus *Methanobrevibacter*, followed by*Methanosphaera cuniculi*. The diet composition had no clear impact on the total number of either methanogens or total archaea, as assessed by real-time PCR. The main effect of pectin supplementation was on the relative composition of the fecal methanogenic species,

with a reduction of the *Methanobrevibacter spp.* proportion and an increase of *Methanosphaera cuniculi* relative abundance. The response on the population structure level appeared to be individual, as different piglets responded differently to pectin supplementation. The putative impact of this microbial shifts on the health of the host is unknown, anyway this study provides new information on the physiological significance of some fiber used in human nutrition.

PS5-04

ADDITIVE APARTIR OF COMPOSITE MULTIENZIMATICOS EXOGENOUS RUMINAL MICROBIOTA MULTIPLIER

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This is a technology applied to the technical field of the feeding of ruminant animals. This allows to increase the production of meat and milk, substantially from cellulosic descartes. The additive allows you to use higher amounts of fiber in confinement or field with supplementation of balanced systems or conventional grains. It generates with this additive exo -enzymes to supplement the rumen with power -ups of the natural bacterial microbiota of the rumen. Thus, form multi-enzyme complexes, called *cellulosomes* or domains of cohesion between natural microbial populations in the rumen and these enzymes contained in the additive. The proportion of union is part of additive with 10 parts of bacterial microbiota in the rumen, improving the performance of 45% efficiency above mentioned almost 78%. Proof of this on analysis of manure was found far less fermentable organic matter, indicating a greater digestion, greater use of cellulose and hence more production. These cultivated species of fungi, which recombine in different proportion are grown separately in halls of culture; from there, the product of fruiting on cellulosic substrates gets the active principles of this additive for additional cultivation of yeast, vitamins, minerals, essential amino acids and excipient to obtain the final product. The difference inventive it is production of exo -enzymes on cellulosic substrates, vitro, these species then both exo -enzymes and fungal remains of these species cultured in the laboratory, produce, synthesize them, and percentage combined with other compounds for use as an additive exogenous and increase efficiency. This is the border limit microbial performance increase, without affecting the exogenous fungal population natural microbiota of the rumen.

PS5-05

EVALUATING THE CONTRIBUTION OF THE GUT BACTERIAL COMMUNITY OF THE WOOD-BORING BEETLE **PSACOTHEA HILARIS HILARIS** *TO THE HOST PHYSIOLOGY*

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The xylophagous larvae of the yellow-spotted longicorn beetle *Psacothea hilaris hilaris* attack plants belonging to the *Moraceae* family, in particular *Ficus* (including fig trees) and *Morus* spp. (such as mulberries). In native countries the insect is mainly associated to mulberry trees and is already known as a serious problem for sericulture, while its preference for fig trees in Italy could threaten fig cultivation in the Mediterranean area. In the present work, with the aim to characterize the bacterial community associated to the gut of wild and laboratory-reared *P. h. hilaris* larvae and to assess its contribution to the host physiology, we applied cultivation-independent and -dependent methods. Specifically, PCR-Denaturant Gradient Gel Electrophoresis (DGGE) has been used to verify if the diet or the gut tract could have an effect on the composition of the gut community. Results showed that wild larvae and larvae reared on artificial diet in presence of antibiotics and preservatives owned richer communities than larvae reared on diet, not exposed to antibiotics and/or preservatives. A significant difference in the bacterial community composition has been showed between the midgut and the hindgut, likely due to the different physiological conditions. By the establishment of a collection of bacterial isolates from the guts of wild larvae, the possible contribution of the isolates to the host physiology has been investigated by *in vitro* assays. It has been shown that they can contribute to the host physiology through carbon or nitrogen uptake i.e. contributing to the cellulose digestion, exploiting the by-products of the plant cell wall degradation, converting waste molecules (such as uric acid and urea) or proteins to ammonia and smaller peptides, or even fixing atmospheric nitrogen. In conclusion, *P. h. hilaris* harbors many gut commensals that could contribute to the host physiology, utilizing the food source.

PS5-06

THE GUT BACTERIAL COMMUNITY ASSOCIATED TO THE BLACK SOLDIER FLY, HERMETIA ILLUCENS

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World population is growing rapidly and concurrently the protein demand is increasing, constituting a serious concern for the future. Although, insects are commonly used as food source in many Eastern countries, only recently the awareness that insects can be a high potential source of animal proteins for feed and food production has been prompting research activities and interest from the scientific community. In this perspective, the prepupae of the black soldier fly (BSF), *Hermetia illucens* (Diptera: *Stratiomyidae*), could be used as feedstuff for the aquaculture: the prepupal stage contains indeed a very high percentage of protein and fat. Moreover, BSF larvae, being able to grow on different organic matrices and consuming twice their weight a day, can contribute to the bioconversion of food waste disposal in a sustainable manner. The insect microbiome plays many essential roles for the

host metabolism and biology, being involved in different aspects of the host life such as nutrition, immunity and reproduction. Here, we investigated the bacterial diversity associated to the gut of BSFs sampled at different life stages (larval, prepupal and adult ones) and reared on different diets (standard, fruit-waste derived and vegetable-waste derived ones) by the use of cultivation-independent and -dependent methods. By the use of PCR-Automated Ribosomal Intergenic Spacer Analysis (ARISA), the diversity of the bacterial communities associated to BSF guts has been assessed to evaluate the possible influence of the diet and life stage on it. By the use of cultivation-dependent techniques, we established a bacterial collection and the contribution of the host commensals to the host metabolism was evaluated.

PS5-07

IN VITRO *EVALUATION OF THE EFFECT OF DIETARY FIBERS ON THE METABOLOMIC PROFILE OF HUMAN MICROBIOTA*

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Foods contain thousands of compounds which, upon digestion and metabolism, give rise to complex physiological reactions and interactions with the intestinal microbiota resulting in the metabolites present in body fluids such as plasma, urine and feces. Several studies have demonstrated that different foods influence the gut ecosystem. In fact, dietary components are susceptible for metabolism by the intestinal microbial community, particularly affecting the growth and the metabolic activity of the dynamic bacterial populations. Fiber-rich diets or dietary interventions with fiber and/or functional foods, i.e. pre- and probiotics, contribute to the maintenance of health or prevention of diseases through the direct action of the intestinal microbiota. In general, pre- and probiotic administration aims at increasing the end products of carbohydrate fermentation, i.e. short chain fatty acids (SCFA), while reducing proteolytic fermentation resulting in the formation of potentially toxic metabolites. The aim of this study was to evaluate *in vitro* the potential effects of supplementation of different dietary fibers (DF) and prebiotics on fecal metabolomic profiles of healthy humans. Batch culture fermentations of fecal slurries of a healthy individual with added DF or prebiotics were performed and the metabolome detected through gaschromatographic coupled with mass-spectrometry and solid phase microextraction (SPME/GC-MS) analysis. Among the 90 molecules belonging to different chemical groups that were detected, some alchools and ketones in addition to SCFA (acetic, butyric and propionic acids) were associated to DF. Moreover, changes in the metabolic profiles induced by the DF supplementation were compared to those obtained by using wheat bran previously fermented with lactic acid bacteria in order to evaluate whether any of the dietary biomarkers was accumulated thus suggesting that bran fermentation can promote a prebiotic effect.

SESSION VI

PS6-01

CHARACTERIZATION AND TECHNOLOGICAL POTENTIAL OF LACTIC ACID BACTERIA ISOLATED FROM ALGERIAN GOAT'S MILK

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Many wild lactic acid bacteria were isolated from goat's milk collected from different areas in Western Algeria. The strains were identified using phenotypical, biochemical and physiological properties. API system and SDS - PAGE technique was also used in identification of the strains. The strains were screened for production and technological properties such as acid production, aminopeptidase activity, autolytic properties, antimicrobial activity and exopolysaccharide production. In general most tested isolates showed a good biomass separation; as for the production of the lactic acid, results revealed that our strains are weakly acidifying; nevertheless, lactococci showed a best acidifying activity compared to lactobacilli. Aminopeptidase activity was also weak in most strains; but, it was generally higher for lactobacilli compared to lactococci. Autolytic activity was generally higher for most strains, more particularly lactobacilli where we recorded values of 71.13% and 70% of autolysis rate respectively in Lactobacillus rhamnosus strains 9S10 and 9S7. Antimicrobial activity was detected in 50% of the isolates, particularly in lactobacilli where 80% of strains tested were able to inhibit the growth of other strains. The survey of the profile of the texture, the proteolysis as well as the development of the flaveur in the Domiati cheese made on the basis of our isolated strains have been led during the ripening.
The sensory assessment shows that the cheese salted in milk received the best scores in relation to cheese salted after drainage. Textural characteristics, such as hardness, cohesiveness, gumminess and chewiness decreased in the two treatments during the 60 days of ripening. Otherwise, it has been noted that adhesiveness and adhesive force increased in the cheese salte in milk.

Keywords: Lactic acid bacteria; Technological properties; Acidification; Bacteriocin; Exopolysaccharides (EPS); textural properties.

PS6-02

ANTIMICROBIAL ACTIVITY OF CINNAMOMUM ZEYLANICUM *ESSENTIAL OIL AND GRAPE SEED EXTRACT AGAINST* CLOSTRIDIUM PERFRINGENS *TYPE A INOCULATED IN LYONER-TYPE SAUSAGES DURING REFRIGERATED STORAGE*

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Plant-based products are outstanding alternatives to antimicrobial preservatives, and their use in foods meets consumer demands for minimally processed natural products. The objective of this work was to study the effect of cinnamon (*Cinnamomum zeylanicum*) essential oil (CEO) and grape seed extract (GSE) at two concentrations (0.02% and 0.04% v/w for CEO; 0.08% and 0.16% v/w for GSE) against *Clostridium perfringens* type A (RITCC 2752) inoculated in Lyoner-type sausages stored at 4°C for 40 days. The CEO extracted by hydrodistillation and analyzed by gas chromatography–mass spectrometry (CG–MS) method. The stock culture of the bacteria was prepared with Fluid Thioglycollate Medium (FTG Medium) and Brain Heart Infusion medium (BHI) enriched by blood serum of horse under anaerobic conditions at 37 °C for 24 h. The lyoner samples were inoculated with a microorganism culture to obtain an initial level of 106 CFU/g viable cells. Silicone was attached on different points on the surface of the product package. After drying, the grown culture of the target microorganism was injected with a sterile needle and syringe in a laminar flow biosafety cabinet. For the enumeration of *C. perfringens*, 50 g of samples were weighed and transferred into sterile stomaching bags. Then 450 ml of sterile peptone water was added (0.1% w/v) and homogenized in a Stomacher (Seward Stomacher 400 Circulator, UK) with 400 strokes/min for 2.5 min at room temperature. Stomached slurries were decimal serially diluted in peptone water (0.1% w/v), and aliquots (100 µl) of the sample dilutions were spread on sulfite polymyxin sulfadiazine agar (SPS agar, Merck). The plates were incubated at 37 °C for 24 to 48 h under anaerobic conditions (anaerobic jars with GasPak system type A; anaerobic atmosphere generator, Merck) and the colonies were counted. According to compositional analysis of the CEO, 17 chemical compounds were identified, representing 93.15% of the total EO. The major compound groups were Cinnamaldehyde (80.42%), *α*-Copaene (2.731%) and *trans*-Calamenene (2.166%). CEO at all concentrations, and the combination of CEO and GSE, had significantly effect on population of *C. perfringens* ($P< 0.05$) compared to control samples at the end day of storage period. But the samples with GSE alone had no statistically significant effect on target microorganism ($P> 0.05$). The most dramatic effect was observed in samples elaborated with 0.04% CEO with 0.16% GSE where the bacterial population was reduced 4.06 log10 CFU/g after the entire storage time. The results showed that when the combination of CEO and GSE be used as natural additive to control *C. perfringens* in Lyoner-type sausage, subsequently synthetic additive could be used at minimal amounts, which goes according to current market trends.

PS6-03

THE FIRST WORLD SOURDOUGH LIBRARY: A BANK OF MICROBIAL DIVERSITY

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Traditional sourdoughs represent an immense source of microbial diversity, resulting from the combination of ingredients, protocols of propagation, and house microbiota. Such microbial diversity strongly affects the peculiar qualities of the derived baked goods. The microbial community of traditional sourdough is often subjected to unpredictable

fluctuations that may be not always beneficial to the quality of the product (2). Based on these premises, the first World Sourdough Library was conceived and created in Saint Vith (Belgium). It aims at preserving all the components of sourdough: protocols of propagation, pure microbial cultures, and the sourdoughs themselves, in a frozen state. Currently the Sourdough Library is composed of more than 70 sourdough samples collected in different Countries and microbiologically and biochemically characterized. *Lactobacillus sanfranciscensis* and *Saccharomyces cerevisiae* were the red tread for most of the sourdoughs. Overall, bacterial diversity was higher than yeasts. Besides *L. sanfranciscensis*, *Lactobacillus plantarum* and *Leuconostoc* sp. were identified, with a frequency varying from country to country and from sample to sample. Unusual bacterial species (*Lactobacillus xiangfangensis* and *Lactobacillus diolivorans*) were found in sourdoughs collected in France. In some sourdoughs *S. cerevisiae* was replaced by *Candida humilis* or by species of the *Kazachstania* genus. Three sourdough collected in Hungary showed the presence of *Saccharomyces uvarum*, *Candida zemplinina* and *Metschnikowia* sp., which are rarely encountered in the sourdough ecosystem.

PS6-04

STABILITY AND REUSABILITY OF MALTASE PRODUCED BY BACILLUS LICHENIFORMIS *KIBGE-IB4 USING IMMOBILIZED ENZYME TECHNOLOGY*

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Maltase (α- glucosidase) catalyzes the degradation of maltose into glucose and plays a central role in food industries. Partially purified maltase from *Bacillus licheniformis* KIBGE-IB4 was used for strong interaction within anionic polysaccharide (alginate) in the form of beads. The calcium alginate beads having 2.0 mm bead size showed higher activity. The stability of maltase against different temperatures was increased after entrapment and entrapped maltase showed higher resistance against different temperatures as compared to free maltase. The entrapped maltase showed admirable recycling efficiency and retained more than 60 % of its initial activity even after third cycle. The results suggest that the approach of matrix entrapment within calcium alginate beads of maltase is a promising bioprocess technology to construct bioreactor for practical food industrial application.

PS6-05

APPLICATION OF HIGH-THROUGHPUT SEQUENCING TO EXPLORE THE EVOLUTION OF THE BACTERIAL COMMUNITY IN AN INDUSTRIAL TANNERY WASTEWATER TREATMENT PLANT

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In the frame of the BIOSUR project (LIFE11 ENV/IT/075), a one-year monitoring of an industrial tannery wastewater treatment plant was performed, both from chemical-physical and biological points of view. This study was conducted to investigate the evolution of the bacterial community of activated sludge in the same treatment plant during 2013. Bacterial diversity was analyzed by the use of the Illumina MiSeq platform for sequencing hypervariable regions in the 16S rRNA genes. Bioinformatic analysis of the obtained 12,429,502 paired-ends reads was performed using the QIIME pipeline. The assembled 3,874,044 contigs were binned into 5,029 Operational Taxonomic Units (OTUs) that were assigned to taxonomic groups by the use of the Silva 111 database. Results indicate that the bacterial diversity of active sludge was maintained at very high levels throughout the whole year. The dominant bacterial phylum was Proteobacteria, which accounted for 45%. Multivariate analysis showed distinct clustering of samples. The first group, corresponding to samples collected from January to July 2013, was detached from the second group, corresponding to samples collected from September, after a period of drastic decrease of work in tanneries. The Shannon diversity index (H) indicated a slight increase in the diversity level in the second period. Therefore, although a slight effect of seasonality is also visible, we observed a stronger effect of the re-colonization after the summer stop of the plant on the microbial community structure. Also, the parameter that appeared to have the greatest influence on nitrifying and sulfur oxidizing bacteria was the temperature.

PS6-06

EXPLORING THE SULFUR-OXIDIZING POTENTIAL OF MICROBIAL BIOMASS IN A TANNERY WASTEWATER TREATMENT PLANT.

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Tannery wastewater contains high concentrations of pollutants, mostly sulfides, which are usually chemically removed by expensive and inefficient methods. Biological methods, based on sulfur-oxidizing bacteria activity, have been proven to be more convenient, but are still poorly used. In the frame of the BIOSUR project (LIFE11 ENV/IT/075), a reactor was set up and developed in order to explore the sulfur-oxidizing potential of primary and biological sludge biomasses in a tannery wastewater treatment plant. Selective conditions were set in a reactor by incremental addition of sulfides (2-4 mg/l) and low pH (2-4). Primary and biological sludge of the plant were used as inoculum. The developing microbial community was characterized and monitored by means of T-RFLP fingerprinting, clone-library construction of 16S rRNA coding genes, Fluorescent *In Situ* Hybridization and isolation in pure culture. Obtained results show the evolution of a selected, specialized sulfur-oxidizing biomass from the onset of the reactor to the stationary phase. In this scenario, about 76% of screened clones are represented by bacteria whose 16S rRNA gene sequence shows affiliation with that of members of the genus *Halothiobacillus*, well-known as Sulfur Oxidizing Bacteria (SOB). The same results has been also confirmed by isolation in SOB selective medium and FISH experiments. Additionally, also members of other sulfur-oxidizing genera have been detected (i.e. *Thioclava*, *Ochrobactrum*). The parameters mostly

shaping the structure of the microbial community are pH, sulfides and clorides. Moreover, results indicate primary sludge as main reservoir of sulfur-oxidizing bacteria. Our data clearly show a wide potential for microbiological sulfur-oxidation, mainly residing in the primary sludge of the tannery wastewater plant, which could be conveniently used for sulfide removal.

PS6-07

CELL-RECYCLE BATCH PROCESS OF SCHEFFERSOMYCES STIPITIS *AND* SACCHAROMYCES CEREVISIAE *CO-CULTURE FOR SECOND GENERATION BIOETHANOL PRODUCTION*

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Lignocellulosic materials such as agro-residues are attractive feedstock for bioethanol production. In order to establish a low-cost bioethanol production process from lignocellulosic residues, efficient conversion of the two dominating monomer sugars, glucose and xylose, to ethanol should be achieved. However, the most commonly used yeast in bioethanol production, *Saccharomyces cerevisiae*, is able to convert only hexose sugars such as glucose and is not able to co-ferment glucose and xylose. In this context, the use co-culture of *Scheffersomyces stipitis* (*Pichia stipitis*) and *S. cerevisiae* would be advantageous for optimal xylose fermentation, where glucose is effectively fermented by *S. cerevisiae* and remained xylose can be efficiently fermented by *S. stipitis.* In the present work we investigated on glucose and xylose syrup fermentation, using *S. stipitis* and *S. cerevisiae* co-culture evaluating yeast interaction, inoculation ratio and aeration condition to increase bioethanol production. In this regard, three *S. cerevisiae* strains were evaluated in co-culture with *S. stipitis* CBS 5773 at different ratio. Optimal condition was *S. cerevisiae* EC1118 and *S. stipitis* co-culture, while 1% of oxygen concentration was found the best condition for ethanol production (bioreactor trials). To increase ethanol production with *S. cerevisiae/S. stipitis* co-culture cell-recycle batch process was evaluated. Using this process was achieved the maximum ethanol production (9.73 g/l) and ethanol yield (0.42 g/g) showing a ten-fold increase of ethanol productivity in comparison with batch process (2.1 g/l/h). In these conditions a stabilization at steady state condition of the cells ratio of *S. cerevisiae/S. stipitis* (1:5) was obtained. Dissolved oxygen concentration is crucial to achieve a proper co-culture ratio able to optimize second generation bioethanol production.

PS6-08

EFFECTS OF SIZE AND STARTER CULTURES ON THE RIPENING AND FINAL CHARACTERISTICS OF FERMENTED SAUSAGES

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Fermented sausages are the result of complex microbiological activities and biochemical transformations, mainly due to microorganisms used as starter cultures and natural microflora. Also the formulation, the mincing degree, the diameter and the size of sausage, the RH/temperature applied during maturation and the length of ripening play a role in affecting the product final characteristics. Many of the transformations taking place during ripening are necessary to obtain the final peculiar characteristics while others, such as aminoacid decarboxylation, can affect fermented sausage safety.
This work was aimed to the evaluation of the effects of sausage diameter and starter cultures on the characteristics of products during fermentation and ripening. In particular, the same meat butter was stuffed into two different synthetic casings (108X600 mm and 50X250 mm) after inoculation with two different LAB starter (*Lactobacillus sakei* and *Pediococcus pentosaceus*). Samples were periodically analysed during fermentation up to the end of ripening for microbiological counts, chemico-physical parameters, aroma profile, biogenic amines content and proteolysis and lipolysis patterns. The results obtained evidenced the importance on the final characteristics of the products of the sausage size and starter cultures added. The comprehension of the role of the main productive variables on the formation of flavour profile and the other product characteristics is a crucial step for understanding the peculiarity of typical productions and for optimizing the overall sausage quality.

PS6-09

SPONTANEOUS FERMENTATION OF A CHEESE-TYPE VEGAN PRODUCT: EVALUATION OF THE MICROBIAL COMMUNITY

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In the recent years vegetarian and vegan diets have been more widespread because of a higher attention and perception of consumers to nutritional and ethical aspects. In particular, cheese substitutes obtained by soy (such as tofu) or nuts are actually present on the market. In this context, the aim of this study was to characterize a cheese-type vegan product obtained by nuts. In particular, nuts previously soaked for 8 hours were grounded while adding water. The bulk obtained was let to ferment for 48 hours and then added with salt and lemon juice. The fresh product was split into 200g-molds and dried overnight at 42°C. After this period,

the products were stored for 7 days at 5°C before packaging under modified atmosphere. Samples were periodically collected throughout the whole process in order to monitoring microbial counts of selected groups (LAB, yeasts and moulds, enterobacteria, enterococci and staphylococci), pH, aw, organic acids, biogenic amines and volatile molecules profiles. Results regarding microbial counts showed that the predominant microflora was represented by LAB, with counts of 8 log cfu/ml already after soaking. After 24 hours they reached levels of about 9 log cfu/ml and remained stable in the following steps. LAB isolates were purified and identified by sequencing 16S rRNA region. The results showed that the spontaneous fermentation of this product is mainly due to heterofermentative LAB such as leuconostocs and *Weissella spp*. After 24 and 48 hours of fermentation, also some strains of *Pediococcus pentosaceus* were detected. Regarding pH, the initial value of the bulk was about 6.0 and decreased until 4.5 after fermentation. The volatile molecules profiles of the vegan cheesed were mainly characterized by the presence of acetic acid, ethyl alcohol, ethyl acetate and benzaldehyde.

PS6-10

CHARACTERIZATION OF SOURDOUGH FOR THE PRODUCTION OF COLOMBA, AN ITALIAN TRADITIONAL SWEET-LEAVENED BAKED GOOD

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Colomba is a traditional Italian sweet-leavened baked product, manufactured according to specific procedures starting from a sourdough continuously refreshed. This sourdough is the result of a complex ecosystem of lactic acid bacteria (LAB) and yeasts and its use can improve sensory quality and shelf-life of resulting products. The aim of this work was to assess the community profile of the sourdough through metagenomics analysis and compare these results with parameters used for the characterization of such habitats.
Culture-dependent and culture-independent microbiological analysis together with metabolite analyses of sourdoughs were performed. In particular, different samples, taken throughout a 19 h process of sourdough maturation, were analysed for the determination of yeast and LAB counts, pH, aw, carbohydrates, organic acids content and volatile profile. Moreover, metagenomics analysis were carried out for bacteria (16s) and yeast (ITS) species. The quality of some leavened sourdough baked goods is not always consistent, unless a well propagated sourdough starter culture is used for the dough fermentation. Many factors may influence the composition of the sourdough microbiota and the persistence of species and strains associations. The strict cooperation evidenced between LAB and yeasts has important technological and organoleptic consequences on the product. The data obtained in this study allowed a deeper comprehension of microbial community evolution during sourdough maturation, fermentation and the production of this traditional fermented product.

PS6-11

MEDITERRANEAN WHEAT CULTIVARS: SOURCE OF MICROBIAL DIVERSITY FOR SOURDOUGH STARTER DESIGN

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Iranian, Tunisian, and Albanian flours obtained from local wheat cultivars were collected from the countries of origin. The main chemical and technological properties of the flours were investigated. Gliadin and glutenins were characterized by 2-dimensional electrophoresis. The flours were used to make spontaneous sourdoughs according to the backslopping procedure, aiming at investigating the microbial communities. Lactic acid bacteria (LAB) were typed and identified, showing that strains of *Pediococcus pentosaceus*, *Weissella cibaria*, *Lactobacillus plantarum*, and *Leuconostoc mesenteroides* were the most abundant. LAB strains were characterized and selected based on the assessment of metabolic traits related to the optimal technological properties (kinetics of growth and acidification, quotient of fermentation, and proteolytic activity). Pools of selected LAB were used to design mixed starters. Compared to spontaneous fermentation, the use of selected starters favored the increase of the concentrations of organic acids and free amino acids, phytase and antioxidant activities, and textural properties of the breads. The results of these studies showed the peculiarity of LAB microbiota in cultivars of different Mediterranean areas, and the potential of autochthonous LAB strains to be used as selected starters to extend and exploit the use of sourdough biotechnology.

PS6-12

RUMEN AS NATURAL ECOSYSTEM OF NOVEL BACTERIAL STRAIN FOR BIOLOGICAL PRODUCTION OF SUCCINIC ACID FROM RENEWABLE SOURCES

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Succinic acid is widely used as precursor of many important chemicals for a broad range of industrial applications such as in the biodegradable plastics, pharmaceuticals, polymers. Today, succinic acid is mainly produced by chemical process, but due to high production cost and environmental concerns, there is growing interest in the synthesis of succinic acid based on biotechnological alternatives such as microbial fermentation from renewable sources such as lignocellulosic biomass. In this context, the present work aims to isolate and characterize new wild-type bacterial strains able to synthetize high yields of succinic acid and to assess their ability to production, through the tuning of different environmental conditions. Fourteen rumen samples collected from animals different for breed, age and origin were inoculated in selective enrichment broths. After incubation, bacterial diversity was evaluated by PCR-DGGE and the isolation of wild-type bacterial strains was performed using selective media.

The DGGE profiles obtained were used to evaluate the level of similarity between the different samples by cluster analysis that showed a high biodiversity among samples. A total of 364 putative succinic acid-producing bacteria were isolated and identified. The molecular identification showed a predominance of genera belonging to the family *Enterobacteriaceae* (41.8%), followed by Lactic acid bacteria (27.8%), *Bacillus* (22%), to a lesser extent *Acinetobacter*, *Staphylococcus*, and *Comanomonas* (5%). Isolates were characterized for their ability to produce succinic acid. Very interesting is the ability of the new isolated strain *Cosenzaea myxofaciens* BPM1 to produce succinic acid. Moreover, to optimize the production of succinic acid, different fermentation conditions were tested. The new strain *Cosenzaea myxofaciens* BPM1 was able to produce succinic acid also when pretreated hydrolysed lignocellulosic biomass was used as substrate for fermentation.

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PS6-13

STUDY OF METABOLIC PROCESS AND COMPOSITION OF THE MICROBIAL COMMUNITY FROM A TMAH CONSUMING LAB-SCALE REACTOR

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Biological treatment of industrial wastes is a reliable process to water recycling and to remove polluting organic compounds before safely release into the environment. Understanding the ecology and the metabolic chain of the reactor community is necessary to find the best operating conditions of the process. An aerobic tetra-methyl-ammonium (TMAH) degrading reactor was run during a week, and the consumption of TMAH was daily monitored by liquid chromatography. The reactor was filled with a mineral medium enriched with 50 mg TMAH as C and N source, and an aerobically digested sludge as inoculum, for a final C/N ratio of 20/1. TMAH totally disappeared after a week, while ammonia increased in the medium and the pH was routinely lowered with H2SO4. When all TMAH disappeared in the medium air intake was stopped and denitrification occurred. This suggest that ammonia is a source of energy for the reaction, and a product of the TMAH degradation. Denaturing Gradient Gel Electrophoresis (DGGE) give a representation of the bacterial fraction selected by the culture media and shows that species related to the TMAH removal efficiency were enriched, in comparison to the original sample. Using selective culture media, those species were isolated and identified by 16s-rDNA amplification and sequencing. The difference between the original community and the selected TMAH degrading one was analyzed, and compared with unrelated TMAH degrading bacteria isolated from native soil, in order to relate the TMAH abatement with highly efficient species, which can be used to improve the process and for gene-mining applications.

PS6-14

FUNCTIONAL SCREENING OF NON-CONVENTIONAL YEASTS (NCYS) FOR THEIR ENE-REDUCTASE (ER) ACTIVITY

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Due to the growing trend aimed at replacing conventional chemical approach with more sustainable ("green") processes, the development of new biocatalysts for reducing electron-poor alkenes is becoming important to enlarge the portfolio of microorganisms (and related enzymes) to be used for producing fine chemicals, pharmaceuticals, agrochemicals and fragrances. Considering their high metabolic diversity in the present study a functional screening of a number of non-conventional yeasts (NCYs), belonging to the genera *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Kazachstania*, *Kluyveromyces*, *Lindnera*, *Meyerozyma*, *Nakaseomyces*, *Pichia*, *Trichosporon*, *Vanderwaltozyma* and *Wickerhamomyces*, all isolated from natural habitats and conserved in the Industrial Yeasts Collection of the University of Perugia, Italy (www.dbvpg.unipg.it), was performed to analyze their capability to reduce C=C double bonds occurring in five compounds (characterized by different electron-withdrawing groups, i.e., ketone, ester and nitrile): 1) 4-phenylbut-3-en-2-one; 2) methyl-3-phenylprop-2-enoate; 3) 3-phenylprop-2-enenitrile; 4) 4-(4-chlorophenyl)but-3-en-2-one; and 5) 1,3-diphenylprop-2-en-1-one. A structure-activity-relationship was apparently observed. Almost all the NCYs herein studied exhibited the ability to reduce ketones, whereas no reducing activity toward both esters and nitriles has been observed. The substitution of the methyl group (compound 1) with a phenyl group (substrate 5) led to a considerable increase of the number of active yeasts. The same effect, although in a minor extent, was observed with the chlorinated ketone (compound 4). Noteworthy, all the NCYs showed only ER activity, which gave exclusively the reduction of C=C double bond. The higher conversions are generally obtained for the compound 5. The results showed that the biocatalytic ability of NCYs was strain-dependent.

PS6-15

CYTOTOXIC ACTIVITY AND SURVIVAL OF SPORE-FORMING BACTERIA ASSOCIATED TO BREAD-MAKING PROCESS

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Raw materials used in bread - making process may be a rich source of spore - forming bacteria whose presence after cooking may represent a spoilage concern for bakery industries and a risk to consumer health.The aim of this study was to investigate the toxigenic potential of 54 spore- forming bacterial strains isolated from bread ingredients and bread, mainly of the *Bacillus* genus, and their resistance to a thermal treatment reproducing thebread cooking

process to ascertain if they could represent a health concern for consumers. The potential toxigenicity of the strains was evaluated by screening the cytotoxic activity on HT - 29 cells using bacterial culture filtrates after growing bacterial cells in BHI and in the bread - based medium BEB. The results showed a high cytotoxic activity of *B. cereus* strains, although it was lower in BEB medium. PCR analyses detected the presence of genes involved in the production of NHE, HBL or CytK toxins in *B. cereus* strains, while none of the tested strains contained the gene for cereulide production. Production of NHE and HBL toxins was also confirmed by specific immunoassays only for *B. cereus* strains. Cytotoxic activity of 13 strains belonging to *B. amyloliquefaciens* (7 strains), *Paenibacillus* spp. (3) *B. mojavensis* (1), *B. simplex* (1) and *B. pumilus* (1) was also detected. Interestingly, *B. cereus* strains assigned to phylogenetic group IV exhibited a thermal resistance markedly lower than *B. cereus* group III; furthermore, *B. amyloliquefaciens* strains almost completely survived the heat treatment, but showed a low cytotoxic activity. It is also relevant that single strains belonging to *B. mojavensis* and *B simplex* showed a cytotoxic activity higher after growth in BEB than in BHI and a spore resistance enough to survive the bread cooking process. In conclusion, our study indicates that spore forming bacteria could represent a risk to consumer health related to strains able to produce toxic substances and to survive bread cookin conditions

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PS6-16

EXPLORING MICROBIAL DIVERSITY OF A BREWERY FULL SCALE ANAEROBIC DIGESTER TO LOOK FOR ROBUST AND EFFICIENT H2-PRODUCING MICROBES

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Bio - hydrogen, obtained by fermentation of organic substrates, is considered an interesting lternative renewable energy. However, the industrial scale H2 production from organic waste by fermentative process is far to be realized as technical and economical limitations have still to be solved. Low H2 yields, lack of industrially robust organisms and high cost of sub stratesare the major limiting factors [1,2]. This research aims at the development of microorganisms and/or microbial consortia suitable for the industrial co version of low cost organic materials into H2. To look for microbes with both interesting hydrogen fermentative raits and proper robustness, granular sludge from a brewery full scale Upflow Anaerobic Sludge Blanket (UASB) digester was selected as promising environment because of being at industrial scale and processing complex substrates. One hundred and twenty bacterial strains, previously isolated from heat treated granular sludge having, high hydrogen yields [3], were genetically identified by 16S rDNA sequencing and screened for extracellular hydrolytic profile on cellulose, hemicellulose, starch, pectin, lipids, protein. The isolates exhibited a broad range of hydrolytic activities and the most interesting strains were assessed for their H2 - production performances from glucose. The top twenty H2 - performing microbes were then evaluated in H2 - production trials using complex polysaccharides (such

as starch and cellulose) as main carbon source. Few strains produced high H2 levels and yields and were evaluated also on organic fraction of municipal solid waste, which is mainly composed by starch, cellulose and protein. The strains produced promising H2 yields and could be considered as good candidates towards the development of industrially relevant microbes for the conversion of organic waste into H2.

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PS6-17

DEVELOPMENT OF INDUSTRIAL CELLOBIOSE FERMENTING SACCHAROMYCES CEREVISIAE *STRAINS FOR THE BIOETHANOL PRODUCTION FROM LIGNOCELLULOSIC BIOMASS*

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Increasing attention has been recently devoted to the production of bioethanol from lignocellulosic biomass. However, lignocellulose is expensive to process because of the need for costly pre - treatments and large dosages of commercial enzymes. Moreover, lignocellulose trereatment results in the formation of inhibitors affecting the following fermentation hase. So far only limited efforts have been spent on selecting yeast able to both olerate inhibitors and efficiently ferment sugars [1]. Further, to reduce production costs, a fermenting yeast capable of producing one or more of the enzymes required for the pre - treatments, is needed. The aim of his study as to develop industrial yeast capable of processing cellobiose into ethanol, by expressing heterologous β - glucosidase genes in *S. cerevisiae* strains. β - glucosidases, splitting cellobiose into glucose, play a major role in the cellulose hydrolysis by eliminating the inhibitory activity of cellobiose on other enzymes involved in the saccharification of cellulose. Three fungal β - glucosidases, BGL1 from *Saccharomycopsis fibuligera* , BGL2 and BGL3 from *Phanerochaete chrysosporium* , were previously indicated as highly hydrolytic on cellobiose [2]. Therefore, their genes were singly δ - integrated into the chromosome of three *Saccharomyces cerevisiae* strains, namely Fm17, M2n and MEL2, selected for their industrial robustness. Enzymatic activity of the recombinant β - glucosidases secreted by the obtained recombinants showed interesting results when evaluated *in vitro* on cellobiose. The engineered strains could grow using cellobiose as the sole carbon source and efficiently converted it into ethanol. Their fermenting activity is being evaluated on native cellulosic substrates, supplemented with customized cellulase enzyme cocktails necessary for hydrolising cellulose together with the secreted β - glucosidase. This will allow to select the best fermenting strains and optimize the process conditions to achieve the greatest ethanol yield from cellulosic substrates.

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PS6-18

INVESTIGATING BIODEGRADATION OF PETROLEUM-DERIVED PLASTICS BY ANAEROBIC MICROBIAL CONSORTIA

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The increasing production and use of petroleum-derived plastics has led to environmental pollution problems due to plastic wastes accumulation. In the marine environment, it has been estimated that, about 60-80% of the total amount of marine litter derive from petroleum-based plastic. In EU, plastic wastes are currently disposed off mainly in landfill (38 %); the remaining part is recycled (26 %) or incinerated for energy recovery (Plastics Europe 2012). Plastics biodegradation could be among the eco-friendly techniques for safe handling and management of plastic wastes. However, there are very scarce studies on biodegradation of synthetic plastics. In this work, anaerobic microbial communities were enriched from surfaces of waste plastics deriving from compost and landfill plants located in different EU countries, and tested for their ability to degrade four petroleum-derived plastics namely polyethylene (PE), polypropylene (PP), polystyrene (PS) and polyvinyl chloride (PVC). Biodegradation assays were carried out in the presence of the virgin (non-pretreated) plastic films as main carbon source in four different media targeting nitrate-reducing bacteria, sulphate-reducing bacteria and archaea. Biodegradation was evaluated by different chemical analyses followed by gravimetric measurements while microbial growth was monitored by analysing gas production and evaluating biofilm formation on film surface. Chemical and microscopic analyses applied to all different plastic films showed that partial biodegradation was observed only in the case of PVC films at 7 months of incubation. The biodegradation activity was detected at the following sampling points where a weight loss percentage of up to 18% was observed compared to abiotic controls that exhibited a maximum of 3.9%; after 18 months of incubation. Analysis of active microbial communities is in progress.

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PS6-19

MICROBIAL COMMUNITY STRUCTURE AND DYNAMICS OF TWO-STAGE ANAEROBIC DIGESTER CHANGING THE FEEDSTOCK FROM ENERGY CROP TO ANIMAL WASTE

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Two-stage anaerobic digestion (AD) is a promising technology for the co-production of biohydrogen and biomethane from different feedstocks by complex microbial communities. Energy crops, crop residues and animal manure are now commonly co-digested to enhance the biogas production and quality. However, even the partial replacement of the energy crops with agro-industrial waste could drastically decrease the cost of the process. To do this without impairing the efficiency, the dynamics and assembly of the microorganisms underpinning the process must be understood and related to the operational changes of the AD. Here, the performance and the microbial community structure of a full-scale AD, consisting of a hydrogenogenic/acidogenic (HA) and a methanogenic (M) reactor, were monitored for 128 days. During this time, the percentage of chicken manure in the feedstock mixture was slowly increased from 1.6 to 7.5 to replace part of the energy crops. This strategy allowed lowering the cost of the feedstock whilst maintaining the energy production constant, though the C/N ratio decreased from 14 to 11. Denaturing Gradient Gel Electrophoresis, followed by band sequencing, revealed that the *Eubacterial* community shifted from species belonging to *Clostridiaceae* to members of the *Ruminococcaceae* in HA reactor, while M reactor harbored mainly species related to the *Erysipelotrichale*. Within the *Archaea*, *Methanobacteria*, *Methanofollis* and *Methanosarcina* spp. were always detected, but in M reactor *Methanofollis* became dominant at the end of the survey. Moving window analyses suggested that during the changes in the feedstock mixture selective pressures were exerted on the *Eubacterial* and *Archaeal* communities into HA and M reactors, respectively. However, natural fluctuations of the communities started again once the operating conditions stabilized. In conclusion, the energy crops could be safely replaced by low quality feedstock, but the process should be done slowly so that the microbes have time to acclimatize to the new conditions.

PS6-20

ISOTOPIC FINGERPRINTING AND GENOMICS AS INTEGRATED TOOLS FOR CHARACTERIZATION AND MONITORING OF CONTAMINANT BIODEGRADATION AT INDUSTRIAL SITES

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The work to be presented is focused on effective integration of chemical, isotopic and genetic analysis of microbial consortia in soil and aquifers to better assess in situ contaminant degradation, to evaluate the occurrence of specific biodegradation processes, to identify relevant microorganisms and to monitor the key processes during bioremediation. The communication will refer mainly to activity carried out at two sites contaminated by organo-chlorinated compounds. In the first case Compound Specific 13C Isotope Analysis (CSIA) data on chloroalkanes is providing a relevant background of the history of biodegradation and natural attenuation at the site when integrated with laboratory data on the active microbial consortia active on the contaminants. Work on this second aspect has been carried out for almost a decade in collaboration with two academic laboratories. An integration of the

diverse set of data is being used to guide site remedial strategies. The second case concerns an aquifer contaminated by low levels of monochlorobenzene (MCB). Microcosms with groundwater and sediments from the above-mentioned site were set up and incubated under aerobic and anaerobic conditions for more than 60 days. Biostimulated microcosms were also prepared adding nutrients, or electron donors. Heat-killed or chemically killed microcosms were also set up and referred to as abiotic controls. Concerning the biotransformation process, MCB ($\approx$ 110 μg mL^{-1}) was generally completely removed in microcosms enriched with N and P within only 7 days, while a slower degradation kinetic was observed in the anamended microcosms (80% removed within 60 days). These results confirmed that a natural attenuation could occur at the contaminated site under specific oxidative conditions. However, the degradation process could be limited by the depletion of nutrients. CSIA analysis confirmed negligible isotope fractionation under oxidative conditions as already reported in previous studies. Thus, isotopic fingerprinting based on chlorine isotopes fractionation was planned (analysis still in progress). High-throughput sequencing (Illumina) analysis and quantitative PCR were performed to gain insights into the structure of the microbial community and to quantify the copy number of possible taxonomic and functional biomarkers, which can be coupled with isotopic fingerprints for a complete assessment of biodegradation processes *in situ*. Based on these two cases as examples conclusions will be drawn on opportunities and difficulties encountered so far on the application of laboratory studies in actual processes of environmental remedy.

SESSION VII

PS7-01

DEGRADATION OF THE MYCOTOXIN OCHRATOXIN A BY BACTERIAL STRAINS ORIGINATING FROM CONTAMINATED VINEYARD SOILS

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Ochratoxin A (OTA) is a mycotoxin denoted by a nephrotoxic activity contaminating several foodstuffs. Nowadays, the biological systems for OTA degradation to the less toxic OTa aroused great interest by the scientific community. In the present study, bacteria able to biodegrade OTA were isolated from soil samples collected in OTA - contaminated vineyards. Soil samples were collected from five vineyards of Negroamaro and Primitivo grape cultivars in Salento (Southern Italy). They were cultured in appropriate media added with OTA, mycotoxin degradation was determined by HPLC/FLC analysis and bacterial colonies were isolated by plating. clonal relationships between isolates was assessed by using an automated rep - PCR System and then each strain was identified by 16S rRNA gene sequencing. A Total of 225 bacterial isolates were able to convert OTA in OTα. The Molecular analysis of the above isolates showed the presence of 27 different strains (rep-PCR profiles). The Sequence analysis of the 16S - rRNA gene indicated that they belonged to five bacterial genera: *Pseudomonas*, *Leclercia*, *Pantoea*, *Enterobacter* and *Acinetobacter* . Additional assessment of OTA - degrading capacity of the 27 strains indicated that only the *Acinetobacter calcoaceticus* strain 396.1 and the *A. sp.* Strain neg1 conserved the above property: both strains were further studied thus showing that they were able to convert 82% and 91% OTA Into OTα In 6 days at 24°C, respectively. The Occurrence of OTα as the sole OTA - product was established by LC – MS/MS. This Is the first description on OTA Biodegradation under aerobic conditions and moderate temperature by bacterial strains from agricultural soils. These Microorganisms might be used to detoxify OTA Contaminated feed and could be a resource for the development of a new enzymatic detoxification system

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PS7-02

A SURVEY ON YEAST SPECIES OCCURRING IN THE OLIVE OIL EXTRACTION PROCESS

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During the olive oil extraction process, the yeasts contaminating the olives' carposphere pass on into the oil along with the solid particles of the fruit and the micro-drops of the vegetation water. The yeast species, able to persist in freshly produced oil, can remain active during the conservation period and, according to their metabolic capabilities, can either improve or worsen the oil quality. Studies on the yeast ecology during the olive oil extraction process are lacking. Therefore, the aim of this study was to quantify and identify the yeast populations occurring in different samples (olives, crushed and kneaded pastes, centrifuged oil and pomaces) collected during 14 olive oil extraction processes were carried out in the same oil mill at the beginning, in the middle, and at the end of the same harvest year. The results showed that the yeast concentrations associated with the olives exhibited quite similar values independently of the sampling day, while the yeast concentrations occurring in the pastes, unfiltered oil, and pomaces in the first day-sampling were significantly lower than those found in the last day (ANOVA, $p<0.05$). Similarly, the yeast species identified (by RFLP-rITS and sequencing rRNA genes) in pastes, unfiltered oil, and pomaces samples were more numerous than in olive samples (10 and 3 respectively) and their isolation frequencies changed over the sampling time. Therefore, the occurrence of various yeast species in olive oil extraction process was dependent not only on yeasts contaminating the olives but also on yeasts colonizing the plant for oil extraction. These findings suggest a progressive contamination of the oil mill plant that selects some yeast species. In particular, the two-phase decanter during pastes centrifugation determined the oil contamination with *Yamadazima terventina* and *Candida adriatica*, two yeasts able to influence the oil quality.

PS7-03

RESPONSE MECHANISMS OF ESCHERICHIA COLI *TO SUB-LETHAL CONCENTRATIONS OF THYME ESSENTIAL OIL, CARVACROL, 2-(E)-HEXANAL AND CITRAL*

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In *vitro* studies showed that thyme essential oil and two of its main components, such as carvacrol and thymol, as well as volatile aldehydes such as 2-(*E*)-hexenal and citral, are characterized by a strong antimicrobial activity both in model and food systems. Their application as food preservatives is very promising but it requires a deeper knowledge about their mechanisms of antimicrobial action. In order to more consciously use these molecules and their components in the food industry it is necessary to better comprehend the stress response induced by the addition of these molecules on pathogenic and spoilage microorganisms. In this context, the main aim of this work was to study the stress response, due to 1 hour exposure to sublethal concentrations of thyme essential oil, carvacrol, citral and 2-(E)-hexenal, in the middle of exponential growth phase, in the model bacteria, *Escherichia coli* K12 using DNA microarray technology. The data obtained proved that 1h exposition to sublethal concentrations of the natural antimicrobials strongly affect the global gene expression in *E. coli* for all antimicrobials used. In the latter, the modification of the

expression in genes involved in metabolic pathways as well as fatty acid biosynthesis suggesting that the cytoplasmic membrane of *E. coli* is the major cellular target of essential oils and their components.

PS7-04

*IDENTIFICATION OF NON-*LISTERIA *SPP. FROM SELECTIVE AGAR MEDIA USED FOR THE DETECTION OF* LISTERIA MONOCYTOGENES *IN READY-TO-EAT VEGETABLES*

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Listeria monocytogenes species, frequently isolated from soil, vegetation and water, is consistently associated with human illness. With growth temperature from 0°C to 45°C, it is a key foodborne pathogen in chilled, refrigerated and ready-to-eat foods. Food safety criteria applicable to ready-to-eat foods able to support the growth of *L. monocytogenes* are established in Regulation (EC) No. 2073/2005, amended in 2007. There are current ISO horizontal methods for the detection and enumeration of *L. monocytogenes* in food. ISO 11290-1:1996 is a method for the detection, while ISO 11290-2:1998 is a method for enumeration. Both methods were amended in 2004 to include modified media. However, conventional methods of detection for *Listeria* spp. can take up to five days to obtain a result, not fully compatible with the very short shelf-life of ready to eat vegetables. Aim of the study was the detection of *L. monocytogenes* in ready-to-eat sliced artichokes by conventional plating method and the identification of a representative number of presumptive pathogen isolates by genus- and species-specific PCR reactions and by 16S rRNA gene sequencing. *Listeria* spp. detection was performed, in parallel, on DNA extracted directly from artichoke samples by real-time PCR with the *mericon* Listeria spp Kit (QIAGEN S.r.l., Italy), which is commercialized for the specific and sensitive detection of several *Listeria* species in enrichment cultures of food samples. The results evidenced that none of the presumptive *Listeria* isolates on selective media belonged to *Listeria* spp. and, according to sequencing results, the isolates represented 4 different bacterial species belonging to *Cellulosimicrobium*, *Microbacterium* and *Curtobacterium* genera.

PS7-05

DIVERSITY OF ARBUSCULAR MYCORRHIZAL *FUNGAL COMMUNITIES IN MULTI-SPECIES ORGANIC COVER CROPS*

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Scanty information is available on the impact of diversified cover crops on native arbuscular mycorrhizal fungi (AMF). To study the diversity and phylogenetic structure of AMF communities in multi-species and monospecific organic cover crops in Central Italy, we analysed 54 root systems of host plants from six experimental plots. In the multi-species cover crop treatment host plants included *Vicia villosa* Roth, *Trifolium alexandrinum* L., *Trifolium incarnatum* L., *Avena* sp. and *Phacelia tanacetifolia* Benth., while only *V. villosa* was present in the monospecific cover crop. We detected 376 AMF sequences, spanning the regions V3 and V4 of the nuclear SSU rRNA gene, using AML1 and AML2 primers. Based on 97% sequence similarity, 15 Glomeromycetes operational taxonomic units (OTUs) were found, encompassing seven different AMF genera. Four OTUs, representing 88% of sequences, were shared among all plant species. They referred to the genera *Funneliformis*, *Claroideoglomus* and *Acaulospora.* Three singletons found in *V. villosa*, *Avena* sp. and *T. alexandrinum* in the multi-species cover crop treatment, referred to *Glomus*, *Diversispora* and *Archaeospora*, respectively. Although each plant species hosted a similar number of sequence types either if grown alone, as *V. villosa* in the monospecific cover (7), or grown together (6-10), the total number of OTUs from the monospecific (7) was lower than from the multi-species cover crop (15). AMF community structure in the roots of *V. villosa* grown either as single cover crop species or together with the other four species, did not show any differences, as revealed by PERMANOVA analysis, which, by contrast, detected a significant difference (P=0.035) among AMF communities hosted by plants of multi-species crop treatment. Our data highlight the important role played by cover crop diversity in promoting AMF diversity, which is fundamental for biological soil fertility and the delivery of ecosystem services.

PS7-06

SEQUENCE HETEROGENEITY OF LARGE SUBUNIT AND INTERNAL TRANSCRIBE SPACER DOMAINS IN THE DNA OPERON ENCODING FOR THE RIBOSOMAL RNA OF OGATAEA UVARUM SP. NOV.

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A yeast strain isolated during a large-scale study on vineyard-associated yeast strains from Apulia (Southern Italy) was subjected to sequence analysis of the large subunit (LSU) and internal transcribed spacer (ITS) domains of its DNA operon encoding for the ribosomal RNA (rDNA). The two molecular marker sequences indicated that this strain could not be attributed to any known species and it was described as the type strain of *Ogatea uvarum* sp.nov. Moreover, the molecular assays showed several secondary peaks in the ITS2 sequence, but not in the LSU D1/D2. In the aim to test whether these peaks were due to the internal heterogeneity of the DNA operon encoding for the rDNA, the two domains themselves and the clones from them derived after PCR amplification were sequenced. The analyses on the internal variants of ITS and LSU showed a significant variability, although within that predictable among different strains of the same yeast species. In this *Ogataea*

uvarum Sp. Nov., ITS was more variable than LSU especially in the ITS2 region. The heterogeneity revealed by this strain was then judged in the frame of its potential consequence in NGS-based environmental metagenomic studies, in which the variability among operons can lead to biodiversity overestimation and to incorrect identification at the species level. The above findings are discussed in the light of the diverse analytical approaches for fungi identification based on sequence similarity. The results of this study show that the internal variability of the rDNA operon requires careful consideration before being used in future NGS metagenomic investigations and emphasizes the need of specific models to interpret the concept of fungal species, when the reproductive barriers represented by exclusively sexual reproduction are not present.

PS7-07

BIOLOG AND PCR-DGGE ANALYSIS TOWARDS THE IDENTIFICATION OF FUNCTIONAL AND GENETIC DIVERSITY OF "MONTEPULCIANO D'ABRUZZO" VINEYARD SOIL MICROBIAL COMMUNITIES ALONG SEASONAL CHANGES

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The association of soil micobial communities with chemical soil parameters and weather conditions was studied in vineyards under two different management conditions: grassy soils and soils without grass cover. The vineyard is a complex agro-ecosystem consisting of a close connection between the microorganisms, the soil and the plant. The study was conducted on six different types of "Montepulciano D'Abruzzo" soils and on two sampling, the first in autumn and the second in spring. The results obtained from Biolog Ecoplate system, showed that the Average Well Color Development (AWCD) index and the Shannon index were higher in samples collected in autumn, so the functional diversity had a seasonal influence. The carbon sources mostly used by soil microbes were carbohydrates and carboxylic acids. DNA was extracted by soil samples and analyzed by using Denaturing Gradient Gel Electrophoresis (DGGE) of PCR-amplified partial 16S rRNA genes. The results were analyzed using the Simpson's diversity index (1-D), Simpson's Evenness index (Ed) and Range-weighted richness (Rr). The geneticic biodiversity, thus obtained, resulted correlated with the grass cover of soils and with the seasonality. The DGGE technique allowed also the identification of the microbial community through sequencing of excised bands. The results allowed monitoring the spatial/temporal changes in microbial community structure and provided a view of the dominant microbial species of the sample. The detection of the microbial composition was useful also to evaluate the possibility of inoculating vineyard soils with Plant Growth Promoting Rhizobacteria (PGPR), replacing the chemical fertilization. PGPR is a group of bacteria that, interacting with the soil, root system and the tissues of the host plant, is capable of applying beneficial effects on it.

PS7-08

EACH VINE CULTIVAR, ITS WINE YEAST: EXPLORING THE BIODIVERSITY OF GEORGIAN STRAINS FOR A PRECISION OENOLOGY

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In Georgia the wine production is still carried out in every rural village by traditional practices that foresee spontaneous fermentation with a long maceration time. A survey on wine yeast biodiversity in six different areas was investigated by sampling grapes, musts and wines deriving from nine different native vine cultivars. One hundred and seventy six isolates were identified by ITS RFLP and partial sequencing of D1/D2 domain 26S rDNA. 14 different species were identified and the isolates ascribed to *S. cerevisiae* were subtyped by the analysis of interdelta region polymorphism. After a screening based on technological characters (fermentation rate, alcohol tolerance, development in liquid medium and resistance to sulfur dioxide) and quality traits (acetic acid, glycerol and H2S production), three strains belonging to *S. cerevisiae*, *T. delbrueckii*, *K. marxianus* species were selected and separately inoculated (10^6 CFU/mL) in musts made from Rkatsiteli (white cultivar) and Saperavi (red cultivar) healthy grapes in order to carry out experimental micro-vinifications in a Georgian cellar. Chemical analysis and microbial counts were performed to monitor the fermentative process. The identification of the isolates showed the co-presence of other yeasts naturally present in the musts but the inoculated strains have dominated the transformation. While the precision viticulture is currently applied to optimize the vineyards performance, in maximizing grape yield and quality, the precision enology that would exploit the potential technological of wild strains hidden in yeast biodiversity, still remains a matter of research activities. Actually, the best expression of the varietal character of a wine may depend on the enzymatic activities and metabolites production of microorganisms taking part in the transformation of the must and in the aging of the wine.

YesVite project: Yeasts for the Sustainability in Viticulture and Oenology FP7-PEOPLE-2013-IRSES

PS7-09

MYCORRHIZATION, MYCELIAR GROWTH AND BASIDIOSPORES GERMINATION OF SOME SPECIES OF **BOLETACEAE**

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Family Boletaceae represent a wide group of fungi characterized by fruit-bodies which produce an high number of spores for the reproduction of the species. Only few studies focus their attention on the spore germination processes and many aspects are already yet to understand. Moreover, their ability to form mycorrhizas with plants is important for better

understand the factors that most influence the production of fruit-bodies. In this study we focus the attention on six different species: *Boletus edulis, Leccinum duriusculum, Suillus granulatus, Suillus luteus, Suillus grevillei* and *Xerocomus badius* collected in Val Grande, Piemonte, Italy. Spores collected from fresh fruit-bodies showed a low germination rate (under 1%), but cells remained alive and turgid till 6 month of conservation at -18°C and 4°C. Many ectomycorrhizas were observed on the lateral roots of seedlings after 2 month of incubation at 20°C and 16h of light. These results showed the ability of some of these species to produce completely formed mycorrhizas *in vitro*. Growing mycelia of *Suillus* spp. on different agar media without a specific element each showed that the growing rate is affected by the presence of NaCl and by the concentration of nitrogen, phosphorus and magnesium source. Achieve the best medium for each mushroom was important to guarantee fast growth and viable mycelium. Future applications could be the production of mycorrhizal plants and the creation of inocula with spore and mycelium for mushrooms cultivation, in order to improve the fungal productivity of the forests and carry out a bioremediation process for the restoration of forest areas.

PS7-10

DIFFERENT RESPONSE OF BACTERIAL AND ARCHAEAL SOIL MICROBIAL COMMUNITIES ON THE APPLICATION OF BIOCHAR ON A SHORT TERM CROPPING SYSTEM

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We evaluated the effect of the application of different quantities of biochar on soil (0, 2, 4, 8 % w/w) on Bacterial, Archaeal and Ammonia Oxidizing Archaeal (AOA), microbial communities during a greenhouse experiment on zucchini cropping system. Bacterial communities were assessed by ARISA, while archaeal and AOA communities were analyzed by t-RFLP Quantitative analysis was performed by Q-PCR on bacterial and archaeal communities. Free soil, soil surrounding roots and rhizosphere soil were separately sampled and analyzed at the beginning and end of 4 months culture cycle. Qualitative and quantitative analysis of ARISA peaks revealed that eubacterial communities were mainly shaped by rhizosphere effect and no significant effect of different char addiction was found. Q-PCR confirmed rhizosphere effect on bacterial communities. Archaeal communities were found to be influenced both by soil fraction (particularly free soil from other root-associated fractions - $P<0.001$), and added biochar ($P<0.001$). Q-PCR results were consistent with quantitative analysis of t-RFLP profiles. Principal difference were evidenced between untreated and 4-8% amended soil, while by comparing intermediate treatments no significant difference in archaeal communities was evidenced. Since archaeal communities showed a major influence of both soil fraction and % char added, we focused the attention on AOA, stated that they may dominate niches sustained by ammonification of organic material and control the rate limiting step of ammonia oxidation in soil. Differently from what observed

considering the overall archaeal community structure, focusing on this specific functional group of Archaea (AOA) we could detect significant differences also between the rhizosphere and soil surrounding roots fractions. About biochar effect, even if a general influence on AOA communities was not evidenced, when more detailed evaluation of separate soil fraction was performed, soil surrounding roots and rhizosphere showed AOA communities to be influenced by char treatment, while free soil AOA were not ($P<0,05$).

PS7-11

HIGH-THROUGHPUT ASSESSMENT OF BACTERIAL COMMUNITIES IN RIPARIAN SOILS DESIGNED FOR DECONTAMINATION OF ANTHROPOGENIC NITROGEN

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Riparian buffer strips are used to protect aquatic ecosystems from the input of anthropogenic nitrogen. This effect is linked to several factors, including plant uptake, soil properties and the presence and activity of denitrifying microorganisms residing both in soil and in the rhizosphere of the plants [1, 2]. Aim of this work was to assess the total bacterial community of riparian strips soil, and to identify any significant correlation between bacterial populations, treatments and soil chemical properties. A total of twenty samples from buffer and external areas were sampled at three different depths (surface 0-15 cm, medium 45-60 cm and deep 80-100 cm) in two different seasons (spring and autumn). DNA was amplified with tagged primers targeting the V3-V4 region of 16S rRNA and sequenced with Illumina MiSeq operating in 300 bp X2 paired-reads module. Sequences processing resulted in 2.5 millions high quality sequences, which were reduced to 74632 in each of the 20 samples in order to avoid biases related with the analyses of samples with differing sizes. Good's coverage values were $\geq$ 99.9% for all samples, thus showing that the sequencing effort was enough to capture the vast majority of bacterial diversity. Dominating phyla were *Acidobacteria*, *Chloroflexi* and *Proteobacteria*, followed by *Firmicutes*, *Bacteroidetes*, *Nitrospirae* and *Verrucomicrobia*. Multivariate analyses on OTUs abundances identified a number of significant differences related to the soil type (control or buffer strip) and to the depth. A number of OTUs showed significant correlations with organic carbon and potential nitrification data, while Spearman correlation analyses identified inverse significant correlations between organic carbon, *in situ* denitrification and bacterial richness. Additional analyses are undergoing to better assess further interactions between Illumina high-throughput sequences and chemical data.

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PS7-12

BIOTECHNOLOGICAL POTENTIAL OF FUNGAL VOLATILE ORGANIC COMPOUNDS PRODUCED BY ENDOPHYTIC FUNGI FOR THE BIOLOGICAL CONTROL OF BOTRYTIS CINEREA *AND* ASPERGILLUS OCHRACEOUS

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With the increasing consumer demand of fruits and vegetables free of fungicide residues, it's necessary to look for control/management methods of diseases in fruits and vegetables without risks to consumers and the environment. A promising alternative to reduce the use of synthetic fungicides is the mycofumigation, which consists in the use of antimicrobial volatile organic compounds (VOCs) produced by fungi. Some species of filamentous fungi have been reported together with the potential for producing antimicrobial VOCs able to kill a wide range of microorganisms. The objective of this study was to isolate, in different habitats, fungal isolates that emit antimicrobials VOCs able to kill important phytopathogenic fungi associated with post-harvest diseases in fruits and vegetables. In a Brazilian's tropical forest fragment (Parque Estadual da Serra do Brigadeiro and Reserva Florestal Mata do Paraíso - MG) decaying plant material (litter) was collected together with healthy tissue from coffee plants, to isolate fungi associated with litter and endophytic fungi in coffee plant branches. The potential of antimicrobial volatile emission of the cultures obtained were tested through Petri plate trials, subdivided into two compartments. One compartment was utilized to cultivate the obtained isolate, and the other compartment with the plant pathogenic fungus. *Botrytis cinerea*, *Aspergillus ochraceus* and *Rhizopus* sp. were used as phytopathogenic fungi. None of the 283 isolates obtained from litter influenced the growth of the colony of phytopathogenic fungi. However, eight endophytic isolates obtained from coffee plants inhibited the growth of *B. cicerea* and *A. ochraceus*. The isolates will be tested *in vivo* for the control of gray mold in fruits and vegetables as also for the control of *A. ochraceous* on coffee beans. Financial support: FAPEMIG, CNPq and CAPES.

PS7-13

FTIR SPECTROSCOPY FOR HIGH-THROUGHPUT SCREENING IN MICROBIAL BIOTECHNOLOGY

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Globally there is a dramatic increase in the demand for food, energy, materials and clean water since natural resources are limited. As a result, industries are looking for ways to find new sources of biomass, reduce rest materials and to improve resource efficiency. Microorganisms have a high potential to be used as bio-factories for the production of primary and secondary metabolites that represent high-value bio-products (enzymes,

polyunsaturated fatty acids, bio-plastics, glucans, chitosan, chitin etc.) (1). In order to find good microbial producers, to design suitable substrates and to perform process optimization, rapid analytical techniques for quantifying target bio-products in microbial cells are needed. We have developed a high-throughput approach based on microcultivation and FTIR spectroscopy that facilitates the screening of microorganisms, substrates and process conditions for the optimization of the production of different high-value metabolites. Currently, the developed approach is in use for screening of filamentous fungi in order to find oleaginous strains with the ability to produce polyunsaturated fatty acids (2) and monitoring of substrate utilization.

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PS7-14

THE ITALIAN GROUP OF WINE MICROBIOLOGY (GMV): VALIDATION OF A SYNTHETIC MEDIUM FOR THE CHARACTERIZATION OF WINE STRAINS OF SACCHAROMYCES CEREVISIAE

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The Italian group of wine microbiology (GMV) was reconstituted in 2014 with a mission to collect all the skills concerning wine making. The partners of the group GMV are 19 universities and two research centers. The main purpose is to build a benchmark for science and wine industry, able to offer applicative solutions and disseminate the numerous activities carried out by different Italian research centers on issues of "Wine Microbiology". At present, the group is working both for research dissemination, with the publication of joint articles in the journal Frontiers in Microbiology, and for a common experiment involving 17 partners. The purpose of this experiment is to validate a synthetic medium, similar to grape must, for the characterization of wine strains belonging to *Saccharomyces cerevisiae* species. For this reason, a common protocol has been developed in order to assess its effectiveness to obtain reproducible and statistically valid results. Furthermore, this multicenter experiment will assess the extent of experimental differences due to the fermentation in different laboratories with the same strain. The development of a validated medium and of the

confidential limits for fermentation data are expected to improve the comparison of experimental data obtained in different centers and to obtain a shared protocol for wine strainevaluation. The first preliminary results will be elucidated and discussed.

PS7-15

MICROBIAL TECHNOLOGY IN YOUR HAND. LIGHTER ORGANIC HYDROGEN GAS

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In the present work we describe an innovative lighter organic hydrogen gas working by microbial activities and the fermentation of organic substrates (1). The lighter comprising a container having a refill valve, a release valve of the gas to be turned on and a button that controls the release valve, and ignition means suitable to trigger the combustion of hydrogen with oxygen outside air; it is characterized in that the container has a partition wall permeable to only gas which divides said container into two sections, a first section containing a support on which adheres a biomass consisting of fermenting liquids mixed in microorganisms (heterotrophic bacteria or anaerobic non-photosynthetic bacteria/photosynthetic algae), which is positioned in the first section of the refill valve and a second section that contains the hydrogen gas produced by said microorganisms and the drain valve of the gas that is opened by the manual device. More, the lighter organic hydrogen gas is characterized in an inert and non-biodegradable support with cross-linked structure on which adheres the microbial biomass. When lighter organic hydrogen gas works by phototropic microorganisms the container is made of transparent material; otherwise, if lighter organic hydrogen gas works in dark fermentation, the container is made of non-transparent material and heterotrophic bacteria are used.

References

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PS7-16

DIVERSITY OF SPOILAGE FUNGI IN DAIRY PRODUCTS AND THEIR ENVIRONMENT

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In the dairy industry, significant economic losses result from the spoilage of dairy-products by yeasts and filamentous fungi. In order to develop efficient means to prevent and control

fungal spoilage, it is important to characterize the diversity of fungal contaminants present in the dairy environment and in spoiled products. In the present study, the diversity of fungi present in 95 spoiled dairy products including milk, fresh cheese, hard cheese, yoghurt, fermented milk drink, butter, cream and cream cheese (116 isolates) as well as 59 isolates obtained after air-sampling of the dairy environment was investigated. Depending on the fungal genera, filamentous fungi were identified after sequencing of the ITS region and partial β-tubulin, EFα and calmodulin genes while the D1-D2 domain of the 26S rRNA gene was used for yeasts. A high diversity was found among fungal isolates. Indeed, 25 genera representing 67 species were identified out of 175 isolates, with *Penicillium* being the most prevalent (51.4% of total isolates), followed by *Cladosporium*, *Candida*, *Mucor* and *Microascus*. At the species level, *Penicillium commune* and *Penicillium bialowiezense* were the most prevalent filamentous fungi, representing 10.2% and 7.4% of total fungal isolates, respectively. Among yeast species, *Meyerozyma guilliermondii*, *Trichosporon asahii* and *Yarrowia lipolytica* were the most frequent spoilage yeasts in dairy products, each representing 2.8% of total fungal isolates. Moreover, fungal species such as *Penicillium antarticum* and *Penicillium salamii* were identified for the first time in dairy products. Numerous species were both identified in spoiled products and after air-sampling suggesting that dairy environment acts as a primary source of contamination. In conclusion, this study provided useful information on the occurrence of fungal contaminants in dairy products and should help to develop adequate strategies for combating fungal spoilage.

PS7-17

EXPLORATION OF BACTERIAL DIVERSITY OF WESTERN AND OUTER HIMALAYA REGIONS FOR IDENTIFICATION AND CHARACTERIZATIONS OF GOLD NANOPARTICLES SYNTHESIZING BACTERIA

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Microbial diversity of the western and outer Himalaya region is mostly unexplored towards the gold nanoparticles synthesis by bacteria so far. The microorganisms play an integral and often unique role in the functioning of the ecosystems in maintaining a sustainable biosphere and productivity. Exploration of Himalaya region for assessment of beneficial bacterial species can potentially provide us novel microbes with newer apllications, which may be highly efficient and significant to microbial and biotechnology industry. In the present study gold mines located at outer Himalaya and four hot water springs namely Manikaran, Kasol, Kalath, Vashisht and Madi near Rhontang Pass located at western Hinalaya were explored towards for isolation of gold nanoparticles bacteria. A total of eighty seven samples were collected in form of soil, pebbles, stalagmite and rock matting were collected from different sites of Himalaya regions. Eighty five bacterial isolates were obtained, which were studied for various morphological characters. Each bacterial isolate was screened individually for its ability to synthesize gold nanoparticles and only 38 bacterial isolates, from goldmine and Vashisht hot water spring water samples were shown to possess gold nanoparticles synthesizing activity. Formation of gold nanoparticles was confirmed by colour change of the solution from yellow to purple/red wine colour. Out of thirty eight gold nanoparticles

synthesizing bacterial isolates only eleven bacterial isolates were selected for further studies in the basis of maximum gold nanoparticles synthesis activity *viz.*, GBI-1, GBI-2, GBI-3, GYI-4, GYI-5, GYI-10, GYI-11, GPI-2, GPI-3, GPI-5 and GPI-6. These eleven bacterial isolates were further investigated for biochemical characters. Genomic DNA was isolated only from three selected bacterial isolates *viz.*, GBI-1, GPI-2 and GBI-3 and amplification was carried out successfully using PCR-16S rRNA gene technology. Sequencing of the PCR products was done using similar primers, and the nucleotide sequences obtained were blasted using online NCBI BLASTn program and bacterial isolates were identified as *Bacillus thuringensis*, *Bacillus licheniformi* and *Bacillus flexus*. Invitro gold nanoparticles were synthesized which were characterized using various techniques such as, Fourier transform infrared spectroscopy (FTIR), Transmission electron microscope. FTIR spectrum showed the presence of the functional groups. Transmission electron microscope results confirmed that gold nanoparticles were spherical in nature, monodispersed as well as dispersed in small groups. In this study, we reported Utilization of bacterial diversity for the development of an easy bioprocess for synthesis of GNPs of desired size and shape. The study demonstrates that there is a possible protein present in the isolated strains which reduces Au3+ to Au0 through an electron shuttling mechanism leading to the synthesis of nearly monodispersed GNPs. This green route of biosynthesis of GNPs is a simple, economically viable and an eco-friendly process. Gold nanoparticles are used for delivering molecules into cells to slow down cancer cells. Nanoparticles also have apllication in improved targeted drug delivery, gene therapy, magnetic resonance imaging contrast agents and biochemical war fare detection.

Keywords: Gold nanoparticles, Transmission electron microscope, Fourier Transform Infrared

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PS7-18

THE MICROBIOME OF ASTRAGALUS GOMBIFORMIS *(POM.): DIVERSITY AND PLANT GROWTH PROMOTION FUNCTIONALITIES*

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The structure of the microbiome associated to the medicinal plant *Astragalus gombiformis (Pom.)*, exposed to arid conditions in the hot desert of Kssar ghilan (south Tunisia), was assessed. Three fractions were analyzed with a combination of a culture and a molecular based analysis; rhizosphere and root surrounding soil fractions were compared to an uncultivated soil. According to 16S rRNA gene Denaturing Gradient Gel Electrophoresis, the structure of rhizosphere bacterial community was highly diverse followed by the root surrounding soil, indicating a selective pressure determined by the plant metabolisms. Similarly, culturable bacteria genera showed different distribution into the three fractions. 16S rRNA gene sequences recorded using unculturable and cuturable dependent approaches, clustered the bacterial community to *Firmicutes* phyla followed by *Actinobacteria*, whereas, *Proteobacteria* was the major encountered phyla within the uncultivated soil. Therewith,

isolates colonizing the microbiome of *Astragalus gombiformis (Pom.)* presented stress resistance capabilities. Moreover, bacterial isolates associated to rhizosphere and root surrounding soil fractions, were more potent for different Plant Growth Promotion (PGP) traits, as revealed by *in vitro* evaluation. The medicinal plant *Astragalus gombiformis (Pom.)* microbiome revealed a strong functional homeostasis of the associated beneficial bacteria. Among the most active isolates, four strains *Brevibacterium sp*., *Micrococcus sp*., *Bacillus sp*. and *Terribacillus sp*. were selected and tested *in vivo* to promote the viability and growth of tomato. Strains were inoculated, separately and in consortium, on tomato seeds. The strain *Micrococcus sp*. and the consortium displayed the most efficient effects on the harvested plant growth. This study provides an intriguing first view of PGP bacteria associated with *Astragalus gombiformis (Pom.)* micobiome for arid lands sustainability purpose.

PS7-19

DIVERSITY AND PLANT GROWTH PROMOTING ACTIVITY OF BACTERIAL ENDOPHYTES ASSOCIATED TO CITRUS TREE ROOTS

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Plants have a diverse internal microbial biota colonizing their internal tissues that has been shown to have an important influence on plant growth and health. The present study examined the diversity and the plant growth promoting (PGP) abilities of endophytic bacteria associated to citrus roots growing in different locations of Tunisia. A total of 105 endophytic bacteria were isolated from roots tissues. The diversity of the bacterial community, analyzed by the amplification of the internal transcribed spacers 16S-23S rRNA allowed the distinction of 32 different fingerprinting ITS profiles. Partial 16S rRNA gene sequences clustered the identified strains into three major phyla: *Proteobacteria, Actinobacteria* and *Firmicutes*. Culture-independent bacterial community assessment, using Denaturing Gradient Gel Electrophoresis (DGGE) method, revealed the prevalence of *Proteobacteria,* and *Firmicutes*. With a lower extent, members of *Actinobacteria* and C*yanobacteria* were also detected. Furthermore, isolates were assessed for PGP cabilities, in order to select promising PGP candidates. These strains could be exploited in biofertilizer formulates to sustain crop production. The overall data highlighted a diverse range of bacteria inhabiting citrus roots tissues which could contribute to tissue homeostasis.

PS7-20

THERMUS ANATOLIENSIS SP.NOV., *A THERMOPHILIC BACTERIUM FROM GEOTHERMAL WATERS OF BUHARKENT, TURKEY*

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Gram stain-negative, lack of motility, catalase- and oxidase- positive strain, designated MT1T was isolated from Buharkent hot spring in Aydin, Turkey. Its taxonomy was investigated using a polyphasic approach. The strain grew at 45-80 °C (optimum, 65 °C), at pH 5.5 - 10.5 (optimum, pH 7.5) and in the presence of 2.0 % NaCl (w/v). 16S rRNA gene sequence analysis revealed that the strain belonged to the genus *Thermus;* strain MT1T detected low-level similarities of 16S rRNA gene sequences (below 97 %) compared with all other species in this genus. The predominant fatty acids of strain MT1T were iso-C15 : 0 (43.0 %) and iso-C17 : 0 (27.4 %). Polar lipid analysis revealed a major phospholipid, one major glycolipid, one prominent aminophospholipid, two minor aminolipids, one minor phospholipid and several minor glycolipids. The major isoprenoid quinone was MK-8. The DNA G+C content of MT1T was 69.6 mol %. On the basis of a taxonomic study using a polyphasic approach, strain MT1T is considered to represent a novel species of the genus *Thermus*, for which the name *Thermus anatoliensis* sp. nov. is proposed. The type strain is MT1T (=NCCB 100425T =LMG 26880T).

PS7-21

ISOLATION AND DETERMINATION OF LIGNIN DEGRADING BACTERIA FROM SOIL SAMPLES COLLECTED FROM DIFFERENT AREAS OF TRABZON

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Lignin is the most abundant natural raw material available on Earth. It represents 30% of all the non-fossil organic carbon on Earth. Lignin is found as a major component of lignocellulose in plant cell walls and is extremely resistant to chemical and biochemical breakdown. The recalcitrance of the lignin component of lignocellulose is a limitation in the utilization of plant biomass for biotechnological processes. Consequently, there is considerable interest in the identification of novel organisms and catalysts for lignin breakdown. The microbial pretreatment of lignin is a natural process which no adverse environmental consequences foreseen. Biological pretreatment employs microorganisms and their enzymatic machineries to break down lignin. Several species of bacteria has been determined that degrading lignocellulosic material but industrial applications of these bacteria and their enzymes are still limited. In this work, soil samples collected from Trabzon and the surrounding area were screened for the bacteria with lignin degrading enzymatic activities. Soil samples were obtained from forestry wastes, chicken pen and a graveyard. The samples were inoculated into LB medium containing 5% of lignin. Lignin degrading samples were determined and subcultured into LB agar plates for pure colonies. Based on differencies of colony morphologies, 12 isolates were selected for identification. 16S rDNA analyses revealed that the isolates represents 4 *Acinetobacter calcoaceticus*, 2 *Commomonas testosteroni*, 1 *Pseudomonas fluorescens*, 1 *Aeromonas media*, 1 *Paracoccus yeei*, 1 *Enterobacter ludwigii*, 1 *Enterobacter cloacae* and 1 *Enterobacter* sp. strains. PCR reactions

were carried out with appropriate primers for determining the genes encoding lignin peroxidase. In the first step lignin peroxidase gene had been found in *Acinetobacter calcoaceticus*. This gene had succesfully clonned, expressed and purified. Characterization and optimization of the enzyme will be done for industrial applications. New PCR primers will be designed for amplifying the lignin degrading enzymes of the other strains.

PS7-22

BREVIBACILLUS GELATINI SP.NOV., *ISOLATED FROM HOT SPRING IN TURKEY*

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Two Gram-positive, moderately thermophilic, endospore-forming, rod-shaped, motile bacteria were isolated from Camkoy hot spring in the provinces of Aydın, Turkey and were characterized in order to determine their phylogenetic positions. These strains were designated as PDF4T and PDF10. 16S rRNA gene sequence analysis revealed that the two strains belonged to the genus *Brevibacillus*; strain PDF4T showed highest sequence similarity to strain PDF10 (99.5 %), *Brevibacillus* brevis DSM 30T (98.9 %), *Brevibacillus parabrevis* DSM 8376T (98.6 %), ***Brevibacillus formosus*** DSM 9885T (98.5 %), *Brevibacillus agri* DSM 6348T (98.4 %), *Brevibacillus nitrificans* DSM 26674T (98.2 %), *Brevibacillus limnophilus* DSM 6472T(98.1 %), *Brevibacillus choshinensis* DSM 8552T (98.1 %), *Brevibacillus reuszeri* DSM 9887T (97.9 %). The predominant fatty acids of strains PDF4T and PDF10 were anteiso-C15:0 (60.0 %) and iso-C15:0 (22.3 %). The polar lipids of strain PDF4T consisted of dihosphatidylglycerol (DPG), phosphatidylglycerol (PG), phospatigylethanolamine (PE), phosphatidylmonomethylethanolamine (PME); an unknown phospholipid, two unknown lipid, an unknown aminophospholipid, and two unknown aminolipid. The major isoprenoid quinone was MK-7. The DNA G+C content of PDF4T was 51.7 mol %. DNA-DNA hybridization showed less than 60 % relatedness between strain PDF4T and type strains of the most closely related species given above. Thus, our results support the placement of strain PDF4T within a separate and previously unrecognized species. Based on these data, the two strains are considered to represent a novel species of the genus *Brevibacillus*, for which the name *Brevibacillus gelatini* sp.nov is proposed. The type strain is PDF4T.

PS7-23

DEVELOPMENT OF A MULTI-SPECIES SEMI-LIQUID SOURDOUGH STARTER

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Sourdough is a mixture of flour and water fermented by lactic acid bacteria (LAB) and yeasts. Sourdough LAB fermentation increases the water-binding and gas-retention of pentosans, improves texture changes, contributes directly to flavor (proteolytic activity), increases the loaf volume, delays starch retrogradation and bread firming and inhibits ropiness by spore-forming bacteria and molds. Sourdough LAB fermentation may also produce nutritionally active compounds from flour proteins, such as peptides and amino acid derivatives with various functionalities in humans (antioxidant, anti-hypertensive, anti-inflammatory activities). Despite of these benefits, sourdough processes are rarely used in the industrial manufacture where fast leavening are required. Nevertheless, to avoid losing the sensorial and nutritional properties of sourdoughs, several industrial bakeries have been recently interested in semi-liquid preparations containing LAB. Therefore, the aim of this work was to obtain a multi-species semi-liquid sourdough starter consisting of LAB with high peptidase activity and then potentially able to increase free amino acid and small peptides with antioxidant activity in doughs. Three LAB strains (*Lactobacillus plantarum* DZB108, *Lactobacillus farciminis* DZB19, *Lactobacillus rossiae* R206) were chosen because of their high peptidase activities according to *in vitro* assays. Fermentations with a commercial *Saccharomyces cerevisiae* were carried out inoculating the LAB strains as axenic or mixed cultures in semi-liquid doughs obtained with four different flours (wheat, durum wheat, rice, buckwheat), using neutral and acidified sterile semi-liquid doughs as control. Peptidase, antioxidant, acidification and microbial growth capabilities were assayed in each semi-liquid dough and the results were statistically treated. No negative interaction between microorganisms compromising growth and acidification capability was observed. Peptidase activities of the strains in axenic cultures and mixed cultures were the same regardless of the flour used, while the antioxidant activities were dependent on the type of flour. The strains can be used to develop a multi-species semi-liquid sourdough starter.
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PS7-24

POTENTIAL OF LACTOCOCCUS LACTIS*, NISIN PRODUCING STRAIN, AND THYME ESSENTIAL OIL USED AS INNOVATIVE WASHING SOLUTION AT INDUSTRIAL LEVEL TO IMPROVE SAFETY AND QUALITY OF MINIMALLY PROCESSED LAMB'S LETTUCE*

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Chlorine is often applied at industrial level for washing of fresh vegetables. However, chlorine is unable to guarantee safety of minimally processed vegetables due to its low efficacy against pathogenic and spoilage microorganisms. Moreover, chlorine implies the formation of carcinogenic chlorinated compounds and vapors having adverse health effects such as the increase of microbial chlorine resistance. For this, some European countries have prohibited the use of chlorine for the disinfection of the raw materials used for the production

of minimally processed vegetables. Plant essential oils, their components and biocontrol cultures were proved as alternative tools to chlorine to control foodborne pathogens without detrimental effects on shelf life and sensorial properties of minimally processed lamb's lettuce at lab scale level. The best performances were demonstrated by a selected culture of *Lactococcus lactis*, nisin producer, and thyme essential oil added alone or in combination, respectively at 6 log CFU/ml and 250 ppm, in the washing solution of lamb's lettuce. The innovative solutions were scaled up at industrial level in comparison with chlorine disinfection processes (120 ppm). The products were packed following the standard company procedures and stored at 6°C. During the storage the quality parameters and the shelf life were determined in relation to the washing solution applied. Also the effects of the biocontrol culture and/or thyme essential oil addition on lamb's lettuce microbiota were studied by culture dependent and independent methods (pyrosequencing). The results showed no significant differences of the cell loads of mesophilic aerobic bacteria, yeasts, total and fecal coliforms in relation to the washing solution adopted. No significant differences were also recorded for color and texture parameters considered. By contrast the use of biocontrol agent and/or the thyme essential oil determined significant spoilage population shifts and significantly affected the volatile molecule profiles and the sensory features of the products.

PS7-25

DETERMINATION OF KRAFT LIGNIN DEGRADATION RATE OF VARIOUS BACTERIA

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The aromatic polymer lignin is well known for its resistance to microbial degradation because of its high molecular weight and the presence of various linkages. White rot fungi have attracted widespread attention because of their powerful lignin-degrading enzymatic systems. However, the use of fungi in industrial applications isn't feasible due to the structural hindrance caused by fungal filaments; the requirement of particular culture conditions, such as humidity, aeration, temperature, and pH, that aren't compatible with industrial processing environments; the requirement of a long lag period, which thus results in very slow lignin degradation and the need for additional food sources, such as glucose and nitrogen, to support the fungal growth. In contrast, bacteria are worth studying for their ligninolytic potential due to their immense environmental adaptability and biochemical vesatility. This study is about the isolation of the bacteria degrading lignin out of soil samples contaminated with petrol, and the identification of species. Eight different isolates were obtained from the soil samples, and according to the analysis, it was determined that L1, L2, and PT21 isolates were the different strains belonging to species of *Enterobacter carcerogenus*, L3 and L4 were different strains of *Enterobacter ludwigii*, PT22 isolate belonged to *Citrobacter sedlakii*, PT41 belonged to *Citrobacter farmeri*, and G1 isolate was the strain belonging to the species of *Klebsiella pneumonia*. The Kraft lignin degradation ratios of L1, L2, L3, L4, PT21, PT22, PT41 and G1 were identified to be 37 %,14 %, 20 %, 43 %, 48 %, 51%, 28%, and 60% respectively. This study found that the strain G1 have a strong potential for KL degradation.

Additional studies have to be made to determine the optimum conditions for maximum lignin degradation of the strains. In addition, ligninolytic enzymes from these strains could be extracted and evaluated.

PS7-26

DETERMINING THE DECOLORIZATION CAPACITY OF SEVERAL BACTERIA THAT ISOLATED FROM FERTILIZER CONTAMINATED SOIL

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Dyes and dyestuff are used in textile, cosmetic, pharmaceutical and leather industries but are of primary importance to textile manufacturing whose wastewaters may contain a variety of these pollutants. The release of dyes has caused concern because of their environment and health effects. Several of these dyes are very stable to exposure to light, water and many chemicals. They are also difficult to degrade by microorganisms, but bioremediation is still seen as an attractive solution due to its reputation as a low-cost and environmentally friendly technology, compared to chemical and physical treatment processes. When considering the enzyme sources especially fungi draw attention. On the other hand, the stability of fungi are not good in practical treatment under extreme environmental and substrate conditions, such as higher pH, oxygen limitation, and high extraction and lignin concentrations. Bacteria are worthy of being studied for their ligninolytic potential due to their immense environmental adaptability and biochemical versatility. For this reason, the ligninolytic enzyme studies are focus on the bacteria in the recent years. This study is about the isolation of the bacteria degrading lignin out of soil samples contaminated with fertilizer. Seven different isolates were obtained from the soil samples, and identification of species were carried out according to the 16S rRNA analysis. As the efficiency of isolates in waste dye removal was examined, they were observed to degrade RBBR at 7-80 %. Lignin peroxidase enzyme obtained from C1 isolate decolorized the dyes to the proportions between 15-60 % by using them as substrate. The best result is obtained from methylene blue as 60%. Based on these results the isolates could be used for removal of waste dyes.

PS7-27

FONTIBACILLUS PULLULANOLYTICUS SP.NOV., *ISOLATED FROM SOIL*

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Gram stain-negative, motility, catalase- and oxidase- positive strain, designated DSHK107T was isolated from soil of Cukurova University campus in Adana, Turkey. Its taxonomy was investigated using a polyphasic approach. The strain grew at 20 – 42 °C (optimum, 30 °C),

at pH 6.0 – 9.0 (optimum, pH 7.0) and in the presence of 2.0 % NaCl (w/v). 16S rRNA gene sequence analysis revealed that the strain belonged to the genus *Fontibacillus;* strain DSHK107T showed highest sequence similarity to type strains *Fontibacillus aquaticus* GPTSA 19T (97.8 %) and *Fontibacillus panacisegetis* P11-6T (97.0 %). The predominant fatty acid of strain DSHK107T was anteiso-C15 : 0 (46.7 %). The polar lipids of strain DSHK107T consisted of dihosphatidylglycerol (DPG), phosphatidylglycerol (PG), phospatigylethanolamine (PE), four unknown phospholipid (PLs), four unknown lipid (ULs), three unknown glycolipids (GLs), two unknown aminophospholipid (PNs), an unknown aminolipid (AL) and an unknown aminophosphoglycolipid (APGL). The major isoprenoid quinone was MK-7. The DNA G+C content of DSHK107T was 42.1 mol %. DNA–DNA hybridization showed that the strain DSHK107T shared low DNA-DNA relatedness with *F. aquaticus* GPTSA 19T and *F. panacisegetis* P11-6T (47 and 59.3 %, respectively). Thus, our results support the placement of strain DSHK107T within a separate and previously unrecognized species. On the basis of a taxonomic study using a polyphasic approach, strain DSHK107T is considered to represent a novel species of the genus *Fontibacillus*, for which the name *Fontibacillus pullulanolyticus* sp. nov. is proposed. The type strain is DSHK107T (=NCCB 100560T =DSM 100116T).

PS7-28

STUDY OF THE BACTERIAL DIVERSITY OF FOODS: LH-PCR VERSUS PCR-DGGE

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In the food microbiology field, there is a continuous and increasing interest in profiling microbial communities in food ecosystems, in order to better understand and lead food fermentation, to preserve foods from spoilage and also to investigate the ecology of food-borne pathogens. Until 30 years ago, the growth of microorganisms on synthetic media was the only way to perform a microbiological investigation of foods. Following the invention of PCR it became possible to develop several molecular techniques aimed at identifying food-borne microorganisms without using cultivation. These methods, called "culture-independent techniques", analyze nucleic acids (DNA and/or RNA) extracted directly from food microbial cells in order to study the microbial ecology and dynamics occurring in food ecosystems. Among the various culture-independent methods based on PCR the Polymerase Chain Reaction - Denaturing Gradient Gel Electrophoresis (PCR-DGGE) and Length Heterogeneity - Polymerase Chain Reaction (LH-PCR) techniques have been extensively applied and show great potential for outlining the microbial diversity of fermented and unfermented foods. However, to the authors' knowledge, these two techniques have never been compared by analyzing both the total microbial DNA and the RNA extracted directly from foods. Based on the above premises, the present study was aimed at comparing the bacterial diversity of several fermented and unfermented foods disclosed by the two culture-independent methods, LH-PCR and PCR-DGGE. To that end, the total microbial DNA and RNA was extracted directly from fourteen selected foods and domain A of the variable regions V1 and V2 of the

16S rRNA gene was analyzed through LH-PCR and PCR-DGGE. Finally, the results of these analyses were also compared with bacterial viable counts obtained after bacterial growth on suitable selective media.

PS7-29

DIVERSITY ANALYSIS AND ANTIMICROBIAL POTENTIAL OF MICROORGANISMS FROM CHANDRA TAL LAKE AND SURAJ TAL LAKE IN THE INDIAN TRANS-HIMALAYAS

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The high altitudes fresh water lakes located in the Indian trans-Himalayas with extreme climatic conditions, strong solar radiations and very little anthropogenic activities offer perfect platform to explore the untapped microbial diversity for new antimicrobials. Sediment and water samples were collected from Chandra Tal Lake and Suraj Tal Lake of Lahaul and Spiti region in the Indian trans-Himalayas. A total of, 690 bacteria and 138 fungi were isolated on different media, after incubation at 28 °C for 7 days and 10 °C for 30 days. Results of ERIC-PCR based fingerprinting of the isolates indicated higher genetic diversity among bacteria and fungi from Chandra Tal Lake samples in comparison to Suraj Tal Lake samples. Sequencing of 16S rRNA gene of representative strains of bacterial ERIC types revealed the presence of 43 bacterial genera including 148 species, while ITS region sequencing of representative strains of fungal ERIC types indicated 22 genera with 36 species. Members of actinobacteria were most dominating among bacteria in all samples of both Lakes, except sediment sample of Suraj Tal Lake for which members of firmicutes were dominating. Among fungi, the members of order helotiales were dominating in Chandra Tal Lake samples and hypocreales in Suraj Tal Lake samples. Five isolates exhibited less than 98% 16S rRNA gene sequence identity to their closest neighbor in EzCloud database indicating them as putative new species. Screening of all isolates for antimicrobial activity by agar overlay method against a set of 10 test strains resulted in to the selection of 113 bacterial and 25 fungal isolates with antagonistic properties against two or more test strains. The isolates with antimicrobial activity were more from sediment samples in comparison to water samples. The study revealed huge cultivable microbial diversity in the two high altitude lakes with potential antimicrobial activities.

PS7-30

UNRAVELING THE EFFECTS OF FOOD-RELATED ENGINEERED NANOPARTICLES ON THE GUT INTERACTIVE ECOSYSTEM (NANOGUT)

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The ever-growing exploitation of nanotechnologies and innovative nanomaterials in food and agricultural applications is rising a number of safety, environmental, ethical, policy and regulatory issues, becoming a priority field for scientific research. At present, packaging is the major food-related area of nanoparticles' application (NPs, particles between 1 and 100 nanometers in size), increasing human oral exposure to estimated consumes of over a trillion man-made food-related NPs every day per person in developed countries. In addition, the growing awareness of the importance of the gut microbiome in health and disease, and the recognition that intestinal microflora exists as a biofilm with characteristics different from its planktonic counterpart, highlights the need to consider how deeply microbial and in particular bioflm-structures ecology can affect human health. However, a little number of published studies considered both the complexity of anaerobic intestinal biofilms and their interactions with food-related NPs and literature on the safety of oral exposure to food-related NPs and their effects on human-relevant biological systems provides insufficient reliable data. The NanoGut project funded by Fondazione Cariplo aims to elucidate the effects of sub-lethal concentrations of food-related NPs on the gut interactive ecosystem, potential toxicity mechanisms, and create the scientific know-how to develop leading edge methodologies vital for the nanosafety assessment. NanoGut involves the development of an interactive *in vit*ro gut ecosystem model composed by: 1) Caco-2 intestinal epithelial cell; 2) anaerobic mono- and multi-species intestinal biofilm; 3) silver nanoparticles (AgNPs), chosen as the more representative engineered nanoparticles used in food packages and conservation, will be used as related-food NPs models; 4) a probiotic bacterium (*Bacillus subtilis natto*).

PS7-31

THE ROLE OF STAPHYLOCOCCUS EQUORUM *IN INFLUENCING THE CHARACTERISTICS OF A MOULD RIPENED BLUE-VEINED RAW MILK CHEESE*

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Lactococcus lactis and *Penicillium roqueforti* are used as starter cultures in blue cheese making. However, the presence of *Staphylococcus equorum*, a common starter culture in fermented meat products, has been reported in mature cheeses. In the current study, the interaction between the starters *Lc. lactis* and *P. roqueforti* and non-starter *S. equorum* was examined in model cheeses made by the procedure for Stilton cheese, but using UHT milk to control the microbiota composition precisely. Different combinations of *Lc. lactis*, *P. roqueforti* and *S. equorum* were used to determine the role of *S. equorum* in influencing cheese characteristics. In one model *S. equorum* was added into the milk 1.5 h after *P. roqueforti* addition. In the other it was smeared on the surface of the cheese immediately after un-moulding. One month ripened cheeses were analysed for viable counts, texture and flavour volatiles using SPME GC-MS. The results showed that in cheeses without *P. roqueforti* addition, the presence of *S. equorum* introduced in both ways could inhibit the

surface growth of contaminant *P. roqueforti* and also slowed its growth in cheeses with added *P. roqueforti*. In the surface-smear cheeses this resulted in a paler coloured crust and firmer textured cheese. The addition of *S. equorum* in the initial process made the cheese core softer. Flavour volatile analysis showed *S. equorum* did not affect the production of typical blue cheese flavour compounds, but different parts of the cheese produced different compounds and acid was produced in the crust only. Therefore, adding *S. equorum* during fermentation should be avoided to allow *P. roqueforti* growth and sporulation, but its presence as a smear at the beginning of ripening could have a positive effect on cheese texture. Its ability to prevent *Penicillium* surface growth could be beneficial for white cheeses where this is an undesirable flaw.

PS7-32

TOXIN-ANTITOXIN SYSTEM IN PLASMID FROM DAIRY L. RHAMNOSUS

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Lactobacillus rhamnosus is a non-starter lactic acid bacterium that plays a significant role during cheese ripening, leading to the formation of flavor. In long-ripened cheeses it persists throughout the whole time of ripening due to its capacity to adapt to environmental conditions. The metabolic response of *L. rhamnosus* to different ecosystems, particularly in cheese dairy environment, is still poorly understood. In a recent work it was reported an upregulation of genes related to a *L. casei* plasmid during the growth of *L. rhamnosus* strains in a cheese-like medium. It is known that plasmids often carry genes that might be essential for survival under harsh conditions, encoding important traits, such as enzymes involved in secondary metabolic pathways, conferring adaptive advantages to host strains. The aim of this work was to better explore the plasmid sequences identified in two *L. rhamnosus* strains isolated from cheese at different month of ripening by employing *in silico* analysis and Northern blot experiments. Interestingly, both the plasmids extracted from different *L. rhamnosus* strains contain a specific sequence region recognized as toxin-antitoxin (TA) system. This system is generally involved in plasmid maintenance, but recently some authors suggested that it can also function as stress response element. By different complex mechanisms this system prevents the proliferation of plasmid-free cells. This cellular stress-response has not yet been studied in food lactic acid bacteria, which have high nutritional demanding and often grow in hostile environments, where resources are limiting. By this study, for the first time, we demonstrate the presence of this interesting evolutionary system in *L. rhamnosus.*

PS7-33

CHANGES IN MICROBIAL POPULATION IN CORN SILAGE DURING ANAEROBIC FERMENTATION AND AEROBIC CHALLENGE

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Silage has in the past been studied intensely focusing on chemical analyses, dry matter loss and aerobic stability, while whole microbial population has only been analyzed to a minor extent. Today we have an insight into the main fermentation process, which has supplied tools for significant improvements, but to fully understand the process, we need to obtain a more complete picture of the microbiological flora during the ensiling process. For this purpose metagenomics is a powerful tool as it gives the possibility to analyze microbial communities regardless of the cultivation ability of the single members. The FoodGenomics collaboration between Chr. Hansen and some of the leading academic groups within Next Generation Sequencing work, the Center for GeoGenetics, University of Copenhagen, and the Center for Biological Sequence Analysis, Technical University of Denmark, aims at increasing the quality of fermented food and feed. It utilizes the metagenomics approach to link microbial flora composition to measures of quality with emphasis on the role of the addition of specific microbes. Data will be presented on laboratory scale corn silage trials with corn retrieved from Danish farms.

PS7-34

PELAGIC AND SYMPAGIC BACTERIAL COMMUNITIES ASSOCIATED WITH PLEURAGRAMMA ANTARCTICA *NURSERY AREAS (ROSS SEA, ANTARCTICA)*

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RAISE Project, funded by the Italian Program for Research in Antarctica (PNRA), aims at increasing the knowledge about the ecology of Antarctic silverfish *Pleuragramma antarctica*. Antarctic silverfish is the most abundant species in the Ross Sea, the most productive area of Antarctic region and, at the same time, the sea considered most pristine in the world. This key species is potentially threatened by both increasing human disturbance and climatic changes, and many aspects of its ecology are still unknown. Up to now, the only documented nursery area of such species is Silverfish Bay, in the Ross Sea, where *P. antarcticum* eggs are found every year in spring, at high concentrations, under the ice cover that shelters sea surface. That environment is characterized by the presence of the so-called platelet ice, made of blades that accumulates under the ice pack. It is not yet clear if such environment offers a reproductive advantage to silverfish, e.g. protecting eggs and larvae

from predators. The activity illustrated in this work, part of RAISE Project, aims at better characterizing this particular nursery area, studying the bacterial communities present in the water (pelagic) and on ice (sympagic) of Silverfish Bay and adjacent areas. In particular, this study aims at identifying bacterial species associated with this area, evaluating bacterial community structure (i.e. richness, biodiversity) and pointing out their possible role in the characterization of *P. antarctica* nursery environment. State-of-the-art sequencing techniques, based on pyrosequencing of ribosomal DNA, have been optimized and applied to environmental samples collected in Antarctica. Preliminary results presented in this study illustrates the differences between pelagic and sympagic bacterial communities associated with silverfish nursery areas in comparison with neighbouring areas where eggs of that species are not usually found.

PS7-35

INTERACTIONS BETWEEN LISTERIA MONOCYTOGENES *AND* PENICILLIUM ROQUEFORTI *IN MILK: A PICTURE TAKEN BY REAL-TIME PCR IN MONOCULTURE, CELLS VS CELLS AND SPENT CULTURE CONDITIONS*

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Listeria monocytogenes represents one of the main food safety concerns and, among foods that can result in contamination, Gorgonzola cheese focuses many attentions. In this product, the growth of *Penicillium roqueforti*, added as a secondary starter to pasteurized raw milk, leads to an increase of pH due to the mould proteolitic activity during the ripening. This could favor the contamination by spoilage and pathogen microorganisms, among which *L. monocytogenes*. The behavior of *L. monocytogenes* is widely studied and modelled in different conditions but the factors that regulate what happens when it coexists with other microflora are not clear. The competition with lactic acid bacteria usually reflects the "Jameson Effect", with the simultaneous growth stop of both microflora when the dominant one reaches the stationary growth phase. Instead, in Gorgonzola cheese, where the contribution of the mold is remarkable, it has been demonstrated that *L. monocytogenes*, if present, besides not being inhibited can even grow. In order to contribute to the ambitious goal of understanding microbial interactions in foods, the aim of this work was to monitor, in different conditions, the behavior of microorganisms in milk by quantitative Real-Time PCR. In particular, in a first experimental set up, *L. monocytogenes* kinetics were followed by contacting the cells with *P. roqueforti* at different stages of mold growth. In a following trial, the effect due to mold metabolites, released in milk during different growth stages, on *L. monocytogenes* behaviour was evaluated. Data obtained revealed how *L. monocytogenes* and *P. roqueforti* show a different behavior when co-coltured compared to growth in monoculture.

PS7-36

BILE RESISTANCE AND ANTIGENOTOXIC ACTIVITY OF FOOD-ASSOCIATED MICROBES

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Recently, several studies are focusing on food-associated microbes that, being ingested in a viable form, as a normal component of the diet, can interact with the human host, eventually by transiently colonizing its gut.In this work, lactic acid bacteria (LAB) and yeasts, representing the prevailing microorganisms associated with 28 foods, including fruit and vegetables, consumed without any cooking in different diets, were identified till strain level and characterized for some properties dealing with "probiotic" functions. The transit tolerance of 130 strains, mainly belonging to *Lactobacillus plantarum*, *Lactobacillus sakei*, *Candida zeylanoides*, and *Torulaspora delbrueckii*, was determined by exposing washed cell suspensions at 37°C to a simulated gastric juice (pH 2.0), containing pepsin (0.3% w/v) and to a simulated small intestinal juice (pH 8.0), containing pancreatin (1 g/1) and bile extract (0.5%), thus monitoring changes in total viable count. In particular, 18 strains of *L. plantarum* mainly isolated from table olives, cheeses and sourdough, were considered for tolerance towards conjugated bile acids and bile salts at different concentrations (0.15-3.6 w/v) and with relation to the presence and distribution of *bsh* genes, ranging from two to four, among the strains preliminarily investigated. The acid-bile tolerant strains were also studied by SOS-Chromotest to assess the antigenotoxic activity against the model genotoxin 4-nitroquinoline-1-oxide (4-NQO). While different studies investigated such a property in LAB, it is worth noting that only few researches dealt with the acid-bile tolerance and the antigenotoxic activity of yeasts. Interestingly, the supernatants from bacteria- or yeast-genotoxin co-incubations exhibited a variable, strain dependent, suppression on SOS-induction produced by 4-NQO on the tester strain *Escherichia coli* PQ37 (*sfi*A::*lac*Z) exceeding, in many cases, the value of 75%. Our experimental results can contribute to shed light on some interesting functional properties of GRAS, food-associated microbes, ingested with foods as a part of the human diet.

PS7-37

CHARACTERIZATION OF WINE YEASTS DURING WINE PROCESS USING DIFFERENT TECHNIQUES

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Study of the microorganisms that colonise the skin of grapes has been an important topic in microbial taxonomy of especially yeasts associated with vines and vineyards. It is known that yeast microbiota on grapes and in musts is influenced by factors such as climatic conditions, geographical location of the vineyard and grape variety. Molecular methods have been used

for the identification of yeasts from wines and the most relevant molecular methods used in the identification of yeast species are based on the variability of the ribosomal genes 5.8S, 18S and 26S. Previous results have demonstrated that the complex ITS regions (non-coding and variable) and 5.8S rRNA gene (coding and conserved) are useful in measuring close fungus phylogenetic relationships. Mid-infrared spectroscopy is a rapid technique which provides highly specific biochemical fingerprints of microorganisms and coupled with different chemometrics analyses offer a wide range of applications including detection, taxonomic level classification and characterization. The objective of this study was identification of yeast flora of 7 wine samples (red, rose and white) through entire wine process from must until the end of fermentation using molecular methods in comparison with cultural methods followed by mid-IR spectroscopic techniques to monitor the diversity of yeasts during a wine process. As a result, identified yeast species included *M.pulcherrima* (2/19, 11%), *P.membranifaciens* (3/19, 16%), *H.uvarum* (1/19, 5%) and *S.cerevisiae* (13/19, 68%) during the whole process. Multivariate analysis of the data showed that *S.cerevisiae* isolates formed a cluster which were probably starter cultures and this cluster was generally separated from the other three yeasts which were isolated at the beginning of wine process. Therefore, it was concluded that FTIR could be succesfully used as a complementary method of molecular techniques for differentiation of wine yeast species isolated at different steps of wine process and monitoring the food process microbiologically.

PS7-38

LACTOBACILLUS PENTOSUS *AND* PICHIA MEMBRANIFACIENS, *THE DOMINANT SPECIES IN BIOFILMS FROM NATURAL GREEN OLIVES GORDAL VARIETY*

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This work examines the microbial communities adhered to the epidermis of natural green olives, Gordal variety, in order to study the microbial biodiversity present in olive biofilms. The fermentation process was microbiologically monitored for 90 d. At this time, formation of true biofilms in the surface of fermented fruits was confirmed by scanning electron microscopy. Then, samples of olive fruits were withdrawn and treated by means of stomacher and sonication for biofilm disaggregation and subsequent recovery of the lactic acid bacteria and yeast populations. The use of the stomacher for 1 min was the most effective treatment to release the lactic acid bacteria from biofilms (6.6 log10 cfu·g-1), whereas sonication for 5 min was the most efficient method for recovery of yeasts (up to 3.5 log10 cfu·g-1). Molecular identification and characterization of isolates revealed that among lactic acid bacteria, *Lactobacillus pentosus* was the only species found, showing two different genotypes. In the other hand, *Pichia membranifaciens* was the predominant yeast species. A greater predisposition of the bacteria (due to their higher counts obtained) than the yeast for integration into these biofilms was noticed.

PS7-39

STRESS RESPONSE MECHANISMS OF LISTERIA MONOCYTOGENES *EXPOSED TO CITRAL, CARVACROL, (E)-2-HEXENAL AND THYME ESSENTIAL OIL*

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Essential oils and theirs constituent are volatile compounds produced by different species of plants as secondary metabolites. Their antimicrobial properties are well documented but their mechanisms of action are not completely understood, and also their use in food industry cannot be performed without a clear knowledge of their effects on the metabolism and cellular constituents of pathogenic microorganisms. In this context, the principal aim of this research was to study the stress response, triggered by one hour of exposure to sublethal concentrations of thyme essential oil, carvacrol, citral and 2-(E)-hexenal, in the middle of exponential growth phase, in *Listeria monocytogenes* SCOTT A considered as model bacteria,. To achieve this purpose, a gene-targeted and proteomic approaches were performed by using, respectively, RT-PCR assays and LC-MS/MS shotgun experiments. The results obtained showed that the addition of the natural antimicrobials, at different concentrations, induced an evident perturbation of the involved genes expression in different metabolic pathways. Moreover, proteomic analysis showed that the target pathogen respond to sublethal doses of essential oils using specific adaptation mechanisms to the environmental stress conditions. These results will deeply highlight the mechanisms used by *L. monocytogenes* in food matrix, after the exposure or during growth, in presence of essential oils or bio-active compounds. The knowledge of these mechanisms will allow a better management of natural antimicrobials use in food systems.

PS7-40

STUDY OF THE BACTERIAL BIODIVERSITY IN SPANISH-STYLE GREEN TABLE OLIVE FERMENTATION BY RT-PCR–DGGE ANALYSIS

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In this work, we have study the bacterial biodiversity through the fermentation of Manzanilla and Gordal variety olives processed as Spanish-style (lye treated olives) at the industrial level. For this purpose, samples obtained from brines and biofilms at different times of fermentation (0, 15 and 90 days) were analysed by RT-PCR-DGGE to determine viable cells by an independent culture approach. Three species (*Vibrio vulnificus*, *Lactobacillus plantarum* and *Lactobacillus parafarraginis*) were present in samples obtained from both

olive varieties, while *Lactobacillus sanfranciscensis* and *Halolactibacillus halophilus* were only detected in Gordal olives, and *Staphylococcus epidermidis* was only found in Manzanilla olives. *Vibrio vulnificus* and *Lactobacillus plantarum* were the most important species because of their presence in all samples from biofilms at the end of fermentation process. This is the first time that *Vibrio vulnificus* has been identified in table olive processing. Further studies are necessary to determine the influence of unusual species during table olive processing.

PS7-41

BACTERIAL DIVERSITY DURING THE WINE PRODUCTION WITH TWO CENTRAL EUROPEAN GRAPE VARIETIES: BLAUFRÄNKISCH (FRANKOVKA MODRÁ) AND GRÜNER VELTLINER (VELTLINSKÉ)

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The bacterial diversity during the wine production steps involving two Slovak grape varieties (Frankovka modrá and Veltlínske zelené) was assessed using culture-dependent and culture–independent strategies. The bacterial strains were isolated from grapes (G), initial-phase must (M1), 7-10 days-fermented must [middle fermenting must (M2)] and must in the end-fermentation phase, almost wine (M3) using four different agar plate media: R2A, GYC (for the cultivation of acetic acid bacteria), de Man – Rogosa – Sharpe agar (MRS; for the cultivation of lactic acid bacteria, anaerobic conditions) and MRS-Tomato (MRS-T; for the cultivation of *Oenococcus* strains; anaerobic conditions). The isolated microflora was clustered by fluorescence-ITS PCR (f-ITS) and then members of individual clusters were identified by amplification of 16S rRNA gene and by its sequencing. The total DNA was extracted from all wine-related samples (G, M1, M2 and M3) and analyzed by a combination of semi-nested PCR with DGGE and the construction of a clone library for each sample. The results evidenced a complex microflora in both wine varieties and the two investigation strategies (culture-dependent and culture–independent) demonstrated their complementarity. The culture-dependent analysis evidenced mainly the members belonging to the class Alphaproteobacteria (*Gluconobacter* spp., *Acetobacter* spp.) and Bacilli (*Lactobacillus* spp., *Leuconostoc* spp., *Bacillus* spp.). Sporadically, members of the class Gammaproteobacteria and Actinobacteria were identified. The culture-independent approach displayed the predominant presence of Gammaproteobacteria followed by Alphaproteobacteria, Actinobacteria and Betaproteobacteria. This method detected only few Bacilli members on Frankovka modrá grape, which belonged to the genus *Bacillus*. To our knowledge, this is the first report describing the bacterial diversity of these two wine varieties and evidencing the great complexity of bacterial flora during wine fermentation.

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PS7-42

BARRELLED EWES' CHEESE: INVESTIGATION OF MICROBIAL DIVERSITY AND VOLATILE AROMA-ACTIVE COMPOUNDS

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Long-ripened barrelled ewes' cheese is mixed with lump cheese produced from pasteurized cows' milk in order to produce winter bryndza cheese, which is a sort of PGI Slovakian bryndza cheese. The microbial diversity of five different barreled ewes' cheese batches was estimated by cultivation using different agar media (de Man – Rogosa – Sharpe, M17, glucose-tryptone-yeast extract, Chromocult C medium, Baird-Parker, and yeast extract-glucose-chloramphenicol). The total DNA was also extracted from all barrelled ewes' cheese samples and analysed by a combination of semi-nested PCR with DGGE and the construction of a clone library for each sample. Aroma-active compounds were analysed in barrelled ewes' cheese and in cows' lump cheese by gas chromatography – olfactometry (GC-O) supported by gas chromatography – mass spectrometry (GC-MS). Microbiological cultivation analysis evidenced the predominance of lactococci, lactobacilli and *Geotrichum* spp., while levels of coliforms and coagulase-positive staphylococci were found to be acceptably low. The DGGE approach combined with the construction of bacterial and fungal clone libraries allowed the identification of 29 prokaryotic taxa, with the majority of clones being *Lactococcus lactis* subsp. *lactis* and *Streptococcus thermophilus*, and 30 eukaryotic taxa, with the majority of clones being *Galactomyces geotrichum* and *Galactomyces candidum*. The chemical investigation of barrelled ewes' cheese by GC-O revealed 39 aroma-active compounds, or mixtures in case of co-elution. Butanoic acid, ethylbutanoate, isovaleric acid, ethylhexanoate, hexanoic acid, methyloctanoate and δ-decalactone were detected at high intensities in all samples. Marked differences were observed comparing the aroma-active compounds profile of barrelled ewes' cheese with that from the shorter-ripened May bryndza cheese, containing 30 compounds specific only for barrelled ewes' cheese. The data obtained demonstrate that barrelled ewes' cheese is a product with specific microflora and a very specific rich profile of aroma-active volatile compounds.

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PS7-43

GENOME MINING AND PROTEOMICS APPROACH CONFIRMS THE PRODUCTION OF ANTIFUNGAL PEPTIDES IN LACTOBACILLUS PLANTARUM *UC8491*

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Lactic acid bacteria (LAB) able to inhibit food-spoilage fungi are of greatest interest for the food industry. Since, the antifungal activity of LAB remains poorly understood, need to be continuously study to develop new commercial formulations applicable to the control of food-borne yeasts and molds. The whole genome of a bacteriocin producing strain *Lactobacillus plantarum* UC8491 able to inhibit foodborne pathogenic bacteria and spoilage fungi in fermented food (cheese, yogurt and vegetable products) was sequenced. The draft genome has a size of 3.30 Mb and contain 334 subsystems, according to the RAST analysis. The presence of the plantaricin biosynthesis cluster containing the *pln* genes organized in five operons: *plnABCD; plnEFI*; *plnJKLR*, *plnMNOP*, and *plnGHSTUV* in the genome of *L. plantarum* UC8491 was confirmed. A bottom-up proteomic approach was carried out through QTOF mass spectrometry from cell free supernatant and from a band excised on Tricine-SDS-PAGE gel corresponding to the halo of *Penicillium* inhibition. Peptides were filtered through a 10 kDa membrane, reduced and alkylated, then separated via nanoscale CHIP liquid chromatography in reverse mode, and analyzed in data-dependent tandem mass spectrometry. Spectra were processed in SpectrumMill versus Bactibase and Bagel database. The analysis of the fraction $\leq$10 kDa resulted in the identification of several peptides belonging to bacteriocins (in particular class IIc) or related proteins, such as Bioactive peptide 1; acidocin, pyiocin/carocin, lantibiotic, ABC transporter, ATP-binding protein, Enterocin AS-48 and divergicin 750. Peptides with homology to colicins were also identified. This results are in accordance with the Bagel database analysis, showing the presence in the genoma of *L. plantarum* UC 8491 of more than 30 small ORF from putative bacteriocin from Class IIb and IIc diffretent than plantaricin. Results obtained in this study reveled that this strain produces additional antimicrobial peptides, probably responsible of its antifungal activity.

PS7-44

BIODIVERSITY OF MICROFUNGI IN THE HYPERSALINE URMIA LAKE NATIONAL PARK (NW, IRAN)

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The National Park of Urmia Lake represents a unique ecosystem, owing to the special ecological conditions prevailing on the region which ranges from a normal to extreme environmental conditions. In present study, the biodiversity of soil microfungi in this region was explored using a combination of morphological and molecular data. Soil samples were

collected from Urmia lake basin and islands insides the lake. Filamentous fungi and yeast isolates were recovered using routine mycological methods and were subsequently subjected to morphological examination. Sequences data for ITS-rDNA region and D1/D2 domain of the 26S rDNA gene were generated for the representative isolates. Our results revealed a rich diversity of filamentous fungi and yeast species in the sampled areas. A total number of 36 filamentous fungi species belonging to 22 genera and 27 yeast species belonging to 10 genera were identified. The filamentous fungi included: *Acrostalagmus*, *Alternaria*, *Arthrinium*, *Aspergillus*, *Bipolaris*, *Botrytis*, *Beauveria*, *Chaetomidium*, *Chaetomium*, *Cladosporium*, *Embellisia*, *Exophiala*, *Fusarium*, *Gymnoascus*, *Penicillium*, *Stachybotrys*, *Sarocladium*, *Talaromyces*, *Trichoderma*, *Trichothecium*, and *Ulocladium*. Additionally, two *Aspergillus* species and one *Talaromyces* which represent yet undescribed new species are reported. Among the yeasts species, five genera namely *Candida*, *Debaryomyces*, *Metschnikowia*, *Meyerozyma* and *Torulaspora* resided in ascomycota and five genera *viz.*, *Cryptococcus*, *Holtermanniella*, *Rhodotorula*, *Rhodosporidium* and *Trichosporon* resided in basidiomycota. Basidiomycetous yeast species were more prevalent and 22 species out 27 belonged to this group and only five ascomycetous species were identified. Members of the genus *Cryptococcus* represented the most dominant species (14 out of 27). In addition several isolates which apparently represent new species were identified. The majority of yeast isolates could grow on culture medium supplemented with 10% NaCl; while, 18 isolates could grow even in higher concentration of NaCl (15%). All together our results showed a rich biodiversity of microfungi in Urmia lake basin.

PS7-45

FUNCTIONAL ANALYSIS OF CLOSTRIDIUM SPOROGENES *SPORE CORTEX LYTIC ENZYME CWLJ*

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Members of the bacterial orders *Bacillales* and *Clostridiales* survive starvation by forming metabolically dormant spores, which represent the most environmentally robust cell-type found in nature. However, spores constantly monitor their environment, via receptor proteins that are localised deep within the spore, and which under suitable conditions will irreversibly trigger the process of spore germination and the resumption of vegetative growth. During this process, the depolymerisation of the thick layer of peptidoglycan (cortex) is essential in permitting hydration and the re-establishment of metabolism in the spore core. Two classes of peptidoglycan lysins are involved in cortex hydrolysis during germination: cortex-lytic enzymes (CLEs), including SleB and CwlJ, typically only recognise intact spore peptidoglycan, and are essential for initiating cortex hydrolysis; cortical fragment lytic enzymes (CFLEs), which are catalytically active against peptidoglycan fragments generated by CLE activity. In contrast to detailed studies conducted on cortex hydrolysis *Bacillus*,

knowledge of the germination mechanisms that underpin spore germination in the *Clostridia* is lagging behind. In *C. sporogenes* milk-isolated strain UC9000, used as the non-toxigenic counterpart of *C. botulinum*, CLEs were identified from analyses of the genome and the functional analysis of CwlJ cortex lytic enzyme was performed. CwlJ gene was cloned into an expression vector for *E. coli* and protein expression examined. Expression levels were examined initially by fluorescence microscopy and SDS-PAGE analyses. CwlJ activity against decoated spores was tested and causes an OD drop of 65% when added to decoating spores. HPLC analysis of the fragments generated from CwlJ could help to identify the enzyme hydrolytic bond specificities. Achieved data could be usefull to characterize the function of Cwlj in in *Clostridium sporogenes* germination.

PS7-46

BIOTECHNOLOGICAL TREATMENT FOR THE BAKERY TO INCREASE DOUGH STRUCTURE, SHELF-LIFE AND SENSORY PROPERTIES

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Gluten is the major factor involved in the structural properties of bakery products. Thanks to its ability to create aggregates, it gives dough viscosity, elasticity and cohesion, all features responsible for the baking performance. Gluten is composed of extensible, viscous gliadins and rigid, elastic glutenins. Moreover, the gliadin protein fraction is the main factor responsible for the development of celiac disease (CD) and other non-celiac gluten sensitivities. Nowadays, the development of new technologies in the food industry aims to both improve products already marketed, and to develop new gluten-free products. The purpose of our work was to set up a biotechnological process based on the combined use of cross-linking enzymes and selected microbial consortia to produce new gluten free bakery products with improved shelf-life, and sensory properties. Cross-linking enzymes, able to organize and create protein networks, are suitable for the food industry as protein modifiers. Among those enzymes, Transglutaminase (TGase) is surely a potentially interesting tool for its capacity to enhance the cohesiveness and elasticity of the dough, solving the problem of lack of consistency of gluten-free flours. Our research showed that the microbial TGase (from *Streptoverticillium mobaraense*) has the capacity to modify wheat flour proteins and determine protein network formation responsible for a major molecular structural stability of the product. The TGase effect on flour mainly involved gluten and globulin fraction modification. The results showed that the enzyme activity in the presence of a selected microbial consortium of lactic acid bacteria and yeasts (*Lactobacillus sanfrancisciencis* and *Candida milleri*) caused synergic effects on the sensory, rheological and shelf-life features of the products. These features resulted significantly affected also by formulation and process variables. Based on our results, we are evaluating the gluten-free flours of corn, rice, amaranth and lentil, to develop a new bakery product for people with gluten sensitivities.

PS7-47

STUDY OF THE BACTERIAL BIODIVERSITY IN TABLE OLIVES

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The objective of this research was to characterize the biochemistry and microbiology of spontaneous *PDO* Aloreña de Málaga table olive fermentations and define the bacterial biodiversity during the process. The study was carried out in two different locations in Malaga province (Spain) following the fermentation during 4 months. Biochemical analysis of olives showed a progressive increase in free acidity as consequent of a spontaneous fermentation, with pH and salt values characteristic of this type of product. Both locations showed a similar trend in microbiology enumeration by plating in selective media. Yeasts were the most abundant microorganisms during the time, while lactic acid bacteria (LAB) cell count revealed low concentration or even were not detected. The novel of this research is the study of the microbial ecology of olives fermentation using bar-coded pyrosequencing of 16S rDNA to characterize bacterial diversity. The bacterial α- and β-diversity differed between fruits and brines being higher for fruits especially at the beginning of the fermentation process and the opposite for the brines. Bacterial diversity changed during fermentation time, and a total of 323 and 150 operational taxonomic units (OTUs) were identified in fruits and brines, respectively. The predominant genera were *Celerinantantimonas*, *Pseudomonas*, *Propionibacterium*, and diverse *Acetobacteraceae*. Some differences in the bacterial diversity were found between industries, special at the beginning of the process, whereas no differences were found for the rest of physicochemical parameters such as color and texture in the final product.

PS7-48

MICROBIAL COMMUNITIES FLUCTUATIONS IN MANURED SOIL

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The aim of this study was to evaluate the effect of manure fertilization on soil microbial communities structure after three manure amendments on corn field. Foregoing manure spreading was periodically performed two times per year on field. Since continuous manure spreading could results in nitrate pollution and accumulation on soil and water, the nitrogen input given to soil with manure was limited to 170 kg N ha-1 per year, according to the Nitrates Directive. In the present work, we first investigated if manure spreading on soil field at two different time points (May 2014 and October 2014) could affect the microbial structure. Then, at the third time point (May 2015), we observed if microbial communities restored and if their structure was comparable to that observed at the first time point. Soil

was sampled 20 days after each amendment and promptly analysed. Amended and never-amended soils were compared. Phospholipids fatty acids (PLFA) analysis was performed. Terminal-branched saturated PLFA (a15:0, i15:0, i16:0, i17:0, and a17:0) were used as markers for Gram-positive bacteria whereas monounsaturated PLFA (16:1ω7c, 18:1ω7c, 18:1ω9c) and cyclopropyl saturated PLFA (cy17:0, cy19:0) for Gram-negative bacteria. The sum of signature PLFA for Gram-positive and Gram-negative bacteria is referred to total bacterial PLFA. The quantity of the PLFA 18:2ω6,9 was used as an indicator of fungal biomass. Separation and quantification of the PLFA were carried out using a gas chromatograph. Temporal temperature gel electrophoresis (TTGE) fingerprints of 16S rRNA, protozoa, archaea and fungi were performed. Gels were digitalized and pairwise analysis was performed by calculation of Pearson correlation indices through unweighted pair group method using arithmetic averages (UPGMA). Statistical analysis was carried out using Pearson correlation indices for significance calculation through Permutation test in order to compare the effect of treatments and time. This investigation could be useful to better understand about microbial fluctuations in soil in order to prevent loss on soil fertility and microbial quality, however, future investigation are still necessary.

PS7-49

CRISPR/CAS9 SYSTEM AS A VALUABLE GENOME EDITING TOOL APPLICABLE TO WINE YEASTS

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Saccharomyces cerevisiae has an extensive repertoire of molecular methods for the genetic analysis of laboratory strains. Although this has widely contributed to the interpretation of gene functionality within haploid genomes, the genetic basis of desirable metabolic pathways in industrial diploid and polyploid yeast strain is still poorly understood. The genetic engineering of industrial yeasts is currently undergoing major changes due to the development of a marker-free, high-throughput, and multiplexed genome editing approach inspired to the bacterial immune systems: the "Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) immune system". CRISPR/Cas9 allows the knockout or the tuning of expression of specific targeted genes and pathways. The system requires an endonuclease (Cas9) that is guided to a specific DNA sequence by a guide RNA (gRNA). The assembling of a ribonucleoprotein complex (Cas9/gRNA) can produce cuts in specific sites of eukaryotic genomes and generates different combinations of gene deletions through a single transformation event. CRISPR/Cas9 is gaining significant attention because of its biotechnological applications and its potential to boost innovation. The wine industry could particularly gain an advantage from this engineering system; indeed, molecular studies should help understanding the contribution of *Saccharomyces* and non-*Saccharomyces* species to several wine features such as those linked to wine quality and safety (aroma and off-flavours compounds, ethanol and glycerol production, sulphur dioxide resistance, toxic compound formation, etc.). In this study we outline a strategy to modify wine yeasts by CRISPR/Cas9 approach. To prove the

robustness of this system and to offers an engineering pipeline for further gene editing in specific metabolic pathways relevant for the wine production, two commercial strains of *S. cerevisiae* (EC1118 and AWRI 796) have been genetically engineered in the arginine degradation pathway to generate strains with a reduced urea and ethyl-carbamate production.

PS7-50

EFFECTS OF HIGH PRESSURE HOMOGENIZATION ON THE TECHNOLOGICAL FEATURES OF NISIN-PRODUCER LACTOCOCCUS LACTIS *STRAINS IN RAW MILK*

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Numerous lactic acid bacteria strains have been identified as protective cultures to enhance the safety of dairy products, also due to their ability to produce bacteriocins. In addition, high pressure homogenization (HPH) has previously been indicated as a key tool to enhance some metabolic and technological features of lactobacilli and probiotic strains. In this context, the aim of this research was to evaluate whether HPH may be effective to improve the technological performance and bacteriocinogenic ability of 11 different nisin-producer *Lactococcus lactis* strains in raw milk. In particular, it was evaluated the effects of HPH treatment at 100 MPa on the fermentation kinetics, volatile molecule profiles and antilisterial activity of selected lactococci inoculated in raw milk. Among the tested strains, *L. lactis* subsp. *lactis* CBM21 and 9FS16 exhibited fermentation kinetics compatible with industrial processes, high production of bacteriocins and antilisterial activity in milk. Moreover, these strains showed complex volatile molecules profiles, including alcohols, aldehydes and acids, that have an important influence on the organoleptic characteristics of dairy products. The application of the hyperbaric treatment to milk inoculated with the selected biocontrol agents allowed to further increase their antagonistic activity by both improving their growth kinetics and raising the sensitiveness of *Listeria monocytogenes* Scott A to nisin. Notably, the HPH treatment increased the amount of volatile molecules produced by the lactococcal cultures. The results of this study indicate the effectiveness of HPH treatment to induce positive technological modifications on the strains CBM21 and 9FS16, making them of particular interest to be used as co-starters for improving the quality and safety characteristics of short and medium-ripened cheeses, as well as promoting their differentiation.

PS7-51

DIVERSITY OF METABOLOMIC RESPONSES OF BACTERIA AND YEASTS TO CATIONIC SURFACTANTS

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Cationic surfactants are relevant antimicrobial and sanitizing agents the interest of which is increasing due to their possible application as biocide or growth inhibitors in various conditions. Mechanisms of action proposed for these compounds against microbial cells are partly dependent on the surfactant structure but mainly on the target cell type. Hence, there is a great need for the development of tools and experiments that will enable study of these important antimicrobial agents interacting with the variety of microbial cell envelope components at the molecular level of the intact organisms. In this work, we investigate the metabolomic responses of the yeasts *Saccharomyces cerevisiae* and *Candida albicans* and the bacteria *Escherichia coli* and *Listeria innocua*, challenged by synthetic tropinium-head surfactants. These surfactants were chosen for their high biocidal activity between a large set of synthetic molecules. In fact, tropinium-head molecules have a bulky and rigid hydrophilic headgroup that increases their biocidal efficacy probably acting on the cell membranes. The cell species were chosen as important yeasts in the food and clinical environment and the other two represent the Gram negative and positive bacteria. Metabolomic, conductometric and microbiological analyses, confirmed that the biocidal effects largely depend on cell structure and metabolism. Statistical analysis allowed us to correlate the induced mortality with the metabolomic cell response, highlighting different modes of action. In general, gaining insights in the interactions between fine structural properties of surfactants and the microbial diversity can allow tailoring these compounds for the various operative conditions.

PS7-52

HIGH-THROUGHPUT SCREENING FOR ANTIFUNGAL ACTIVITIES OF BACTERIAL AND FUNGAL ISOLATES IN A CHEESE-LIKE MEDIUM

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Fungal spoilage is one of the causes of consequential losses in the dairy industry. In this context, the use of bioprotective cultures can be an alternative or a complementary approach to be considered. Lactic acid bacteria (LAB) and propionibacteria, as well as some fungal species, can exhibit antifungal activities with large differences in activity between strains. Therefore, it is necessary to develop high-throughput screening methods to test a large number of strains and find the most efficient ones. In the present study, we developed a miniaturized high-throughput screening technique to rapidly detect antifungal activities in a cheese-like model. This model, distributed in a 24-well plate, consisted of 5-fold concentrated whole milk ultrafiltration retentate (final fat concentration of 45%), rennet (0.03%) and inoculated with a mesophilic lactic commercial starter and a pH indicator. Each well of the plate could be considered as a miniature cheese of ~2 g. Potent antifungal isolates were cultured in two dairy media; (i) a 10%-reconstituted low heat skim milk supplemented with 45% anhydrous milk fat (LH) and (ii) a 6-fold concentrated milk ultrafiltration permeate sterilized by 0.22 µm filtration and complemented with 10 g/l yeast extract and a pH indicator (UF). After cultivation, cultures (100 µl) were deposited on the miniature cheese surfaces followed by inoculation in duplicate with 50 spores or cells of 4 different fungal targets, *e.g.*,

Mucor racemosus, *Galactomyces geotrichum*, *Penicillium commune* and *Yarrowia lipolytica*, and incubation at 12°C for up to 15 days. We screened 505 bacterial isolates belonging to *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Leuconostoc* and *Propiobacterium* genera and 198 fungal isolates belonging to 28 genera. This high-throughput screening for antifungal activity revealed that 52 and 216 bacteria, and, 53 and 89 fungi, inhibited at least one fungal target after cultivation in UF and LH, respectively. Among the 4 tested fungal targets, *P. commune* was the most frequently inhibited fungus while only few isolates were able to inhibit *M. racemosus* or *Y. lipolytica*. This method opens new possibilities to screen microorganisms for antifungal activities. These results also underline the importance of the culture and screening media used on the expression of antifungal activities by bacteria or fungi.

PS7-53

DIVERSITY OF YEASTS ISOLATED FROM VITIS VINIFERA SSP. SYLVESTRIS *(GMELIN) HEGI GRAPE-BERRIES IN THE MEDITERRANEAN BASIN*

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Vitis vinifera L. ssp. *Sylvestris* (Gmelin) Hegi is the only ancestral grapevine species in Europe and it is recognized as the dioecious parental generation of today's cultivars. It constitutes an important phytogenetic resource, but urbanization and industrialization of territories, climatic change and the arrival in Europe of pathogens and pests have led it to be included on the IUCN Red List of Threatened Species in 1997. Although numerous studies about the current health status of this species have been carried out, at best of our knowledge, no studies on microbial populations of grape-berry surfaces have been done. The present work has been focused on the study of yeast occurrence and diversity on grape-berries collected from wild vines (*V. vinifera* ssp. *Sylvestris*). Final outputs have allowed: *i)* to obtain precise information about yeast communities; *ii)* to provide an objective framework for the classification of the broadest range of species according to their extinction risk; *iii)* to select,actractive yeast strains for their biotechnological potential, offering new opportunities to winemakers. Grape-berries from wild vines were carefully harvested throughout three Mediterranean Basin countries, Spain, Italy and Georgia, in different sites for each area. In all, more than 2880 epiphytic and endophytic yeast colonies were isolated and identified as belonging to more than 20 species, including *Saccharomyces cerevisiae*, by 26 rDNA D1/D2 domains and ITS region sequencing. Moreover, despite of some species appeared in the three countries, yeast ecology differed in the sampled regions. This study highlights the biodiversity potential of pristine environments that still represent a fascinating avenue for the investigation of gene evolution and that offer a platform to face common problems in winemaking.

PS7-54

INFLUENCE OF SALT AND STORAGE PACKAGING CONDITIONS ON THE SPOILAGE MICROBIAL DIVERSITY OF RAW PORK SAUSAGES

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Both salt concentration and packaging type can modify the nature and the dynamic of microbial ecosystems in meat products. The purpose of this work was to investigate the influence of these two parameters on the bacterial communities of raw pork sausages using a metagenomic approach and to correlate these data with sensory analysis. Two concentrations of sodium chloride were tested: normal content (2 % w/w) *vs*. a 25 % reduction (1.5 % w/w) and stored either under vacuum or modified atmosphere. Sausages were stored at 8 °C during 21 days until their use-by date. Spoilage assessment was performed by sensory and physicochemical analyses. To characterize the bacterial diversity, the 16S rDNA of five replicates per condition was amplified for pyrosequencing at a scale of 15,000 reads per library. Reads were then clustered into Operational Taxonomic Units (OTU). Spoilage was characterized by an important greying of the products and a production of gas and off-odours defined as rancid, sulphurous and sour. It was more pronounced for samples under modified atmosphere and easier to be ascertained with the lowest salt content. The 20 samples provided 387,111 bacterial 16S rRNA sequences which were analyzed and binned into 78 OTUs. Packaging atmosphere influenced the average bacterial richness with 22 OTUs ± 6 identified in vacuum-packed meat and dropped to 16 OTUs ± 4 in modified atmosphere-packed meat. The richness was influenced mainly in the sub-dominant population whereas the core dominant species, similar in all samples were composed of *Lactobacillus sakei*, *Lactococcus piscium*, *Carnobacterium divergens*, *Serratia proteamaculans* and *Brochothrix thermosphacta*. However, salt reduction turned out to induce a significant drop in species diversity evenness among the core dominant species and thus led to a faster spoilage event. This knowledge will help in defining strategies to offset salt reduction meanwhile keeping the safety of meat products.

PS7-55

SPECTRUM OF COELOMYCETOUS *FUNGI IDENTIFIED IN CLINICAL SETTINGS FROM UNITED STATES OF AMERICA (USA)*

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Recent reports indicate the increasing number of infections, ranging from superficial to systemic, due to coelomycetous fungi. These fungi produce the asexual spores (conidia) within different sort of fruiting bodies (conidiomata). As their conidia are infrequently dispersed by phenomena producing aerosols, the way of the infection acquisition is mostly

by traumatic implantation of plant contaminated materials. In this work we have attempted to provide the molecular and phenotypic identification and the current taxonomy placement for the coelomycetous fungi isolated from clinical specimens. We had studied 230 isolates received by the Fungal Testing Laboratoty (UTHSC, San Antonio, Texas) from different clinical centers distributed along USA. For morphological identification, the isolates were grown and characterized on oatmeal agar (OA) and malt extract agar (MEA) 4% up to 14 days at 25ºC. The microscopic features were obtained from direct wet mounts (using water and lactic acid, alternatively) and slide cultures. For molecular identification the D1 and D2 domains of 28S nrRNA gene were amplified and sequenced, followed by a Megablast search for all nucleotide sequences. The alignment of the nucleotide sequences was performed by MEGA6, and the phylogenetic reconstruction was made using the maximum-likelihood and Bayesian inference with MEGA6 and MrBayes computer programs, respectively. The results allowed us to identify 59 genera of coelomycetous fungi from clinical samples, all belonging to the subphylum Pezizomycotina but pertaining to different orders, pertaining most of the isolates to the genera *Epicoccum*, *Neoscytalidium*, *Paraconiothyrium*, *Peyronellaea* and *Phoma*. Due to the high biodiversity of this set of coelomycetous fungi isolated from clinical specimens, only the combination of phenotypic and molecular tools was usefulness to identify these taxa.

PS7-56

CHARACTERIZATION OF BIOCONTROL ACTIVITY OF INDIGENOUS YEAST STRAINS FROM PATAGONIA, ARGENTINA, AGAINST POSTHARVEST DISEASES OF SWEET CHERRY AND BERRIES

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Indigenous mould and yeasts diversity of stored sweet cherries, raspberry and blackberry from Patagonia (Argentina) were studied for identify main rot fungi and potential antagonistic biocontrol yeasts. Ten antagonistic yeastswere the most promising isolates to be used as biocontol agents against and the most virulent postharvest pathogens identified during this study (*Penicillium crustosum* and *Mucor piriformis*) at 0°C. *Cryptococcus adeliensis, Aureobasidium pullulans, Cryptococcus victoriae* and *Cystofilobasidium capitatum* reduced the decay incidence by *P. crustosum* more to 36.66% and severity in more 50%, while for *M. piriformis, Cryptococcus albidosimilis, C. victoriae, Meyerozyma guillermondii, Guehomyces pullulans, A. pullulans* and *C. capitatum* reduced their incidence in more to 30% and severity in more 50% in cherries. Yeast synergism was also evaluated in co-inoculation assays on cherries stored at 0°C for 30 days. *C. albidosimilis and A. pullulans* combination was the most effective against both pathogenshowever, their combined application did not improve the level of control.Foreign commercial yeast used as a reference in assays, only reduced 10% to incidence to Penicillium and 30% to Mucor in the same conditions. Yeasts were not able to reduce the incidence of moulds artificially inoculated in berries, but in natural infection assays *M. guillermondii, C. albidosimilis* and *C. capitatum* were effective

causing more to 30% of disease reduction.The mechanisms by which yeast isolated enhanced the biocontrol efficacy and are being evaluated. These regional yeasts isolates that shows higher biocontrol activity are promising tools for formulation protocols as an industrial perspective.

PS7-57

AUTOCHTHONOUS YEASTS FOR UNIQUE WINES – AN EXPERIENCE IN UMBRIA

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It is shown that the microorganisms involved in the production of many foods and beverages (cheeses, meats, wines) contribute to determining the quality and contribute to giving those products their typical characteristics and uniqueness of local products.
The project found its basis in studies conducted by the University of Perugia, which in the past have led to the identification and isolation of microbial indigenous biotypes that may represent important factors of innovation in wine production of Umbria. These accessions are still in storage in the germplasm bank at the microbiological - 3A Agrifood Technology Park of Umbria (3A-PTA) and at the former Department of Applied Biology of the University of Perugia.
The overall objective of the project is the introduction of product innovation in the wine sector through:

- Verification of the validity of the starter microbiological (native wine yeasts) under conditions of actual production of wines with the involvement of some regional wineries;
- The production of batches of wine yeasts tested and ready to use;
- The production of batches of wine yeasts for organic wine production using a BIO protocol;
- The use of indigenous wine yeasts for the production of local wines.

The project was conducted at the following Umbrian wineries: Cardinali Giorgio Farm, Cantine Giorgio Lungarotti, Cantina Novelli, Cantina Todini, Cantina Cardeto, Cantina Perusia, Cantina Moretti Omero. The experiments carried out in this project have shown the validity of autochthonous strains in being able to withstand and adapt to the conditions of fermentation, in producing correct wines and, above all, impressing distinctive aromatic notes.

AUTHOR INDEX

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Biological Complexity according to the Stanford Encyclopedia of Philosophy is much more than being complicated: "Ecological systems are supposed to be not merely complicated, they are "**complex**". While much has been written about an alleged new science of complexity in recent years, no compelling operational distinction has yet been presented to distinguish complexity from complicatedness. In general, complex systems are supposed to exhibit "emergent" properties, that is those that, in some sense or other, resist reductionist explanation.
These few sentences outline the difficulty of facing the problem of complexity in Biological Diversity, that involves all the branches of experimental Sciences, but also epistemology and other philosophical disciplines. The study of Microbial Diversity is further complicated by the enormous amount of diversity and variability and by the problem that microbes are invisible to the naked eye.
It is therefore clear that the study of Microbial Diversity, with all its complexity, is a serious challenge at the methodological and substance level.

- The Methodological Challenge
Microbiologists have used the pure culture approach since C.E. Hansen first introduced the technique more than 130 years ago. Now meta-genomics, meta-metabolomics and meta-proteomics allow for direct studies of alpha and beta diversity displayed by microbial communities. These high-throughput techniques produce data at such a rate that current analytical pipelines are often unable to keep the pace, calling for new interpretation approaches.

- The Substance Challenge
Microbial diversity is present and has an effective action in almost all types of environment, including human body, plant and animals, food, natural and agricultural environments, poses the challenge of a detailed description of the microbiomes and of their evolution over the time. The awareness of rapid microbial circulation poses a further challenge to elucidate this aspect, by comparing microbial diversity among different environments.

This volume collects all abstracts of key note, plenary, selected oral and poster presented from October 27th to 29th at the Microbial Diversity Conference in Perugia, Italy. This is the third edition of the event organized by the Italian Society of Agricultural, Food and Environmental Microbiology (SIMTREA). Scientists from 36 countries have contributed to this event and are the authors of this book that represents an up-to-date state of the art of the problems, the potential solutions, the current understanding and the huge vastness of yet to understand phenomena related to the Microbial Diversity.

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