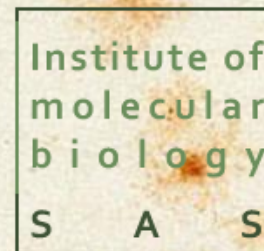
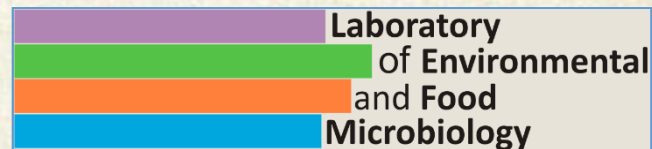


- Visegrad Fund
-
-

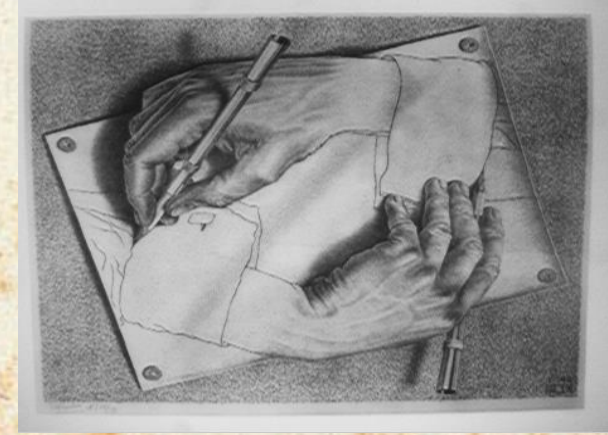
Protection of books by microbial Next Generation Sequencing diagnosis and application of Essential Oils disinfection

Domenico Pangallo





Laboratory of Environmental and Food Microbiology



- Detection of food-borne pathogens by molecular methods
- Detection of non-declared components in food by DNA analysis
- Characterization of the autochthon microflora in traditional food

- Investigation of the biodegradative microflora in art objects
- Study of the biodegradative activities
- Archive documents analysis
- Conservation strategies

Typical Microflora present in Indoor Art Objects and Environment

FUNGI

Acremonium sp., *Alternaria tenuis*, *Alternaria solani*, *Alternaria alternata*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus tamari*, *Aspergillus versicolor*, *Cladosporium elatum*, *Cladosporium cladosporoides*, *Cladosporium herbarum*, *Cephalosporium sp.*, *Curvularia lunata*, *Chaetomium globosum*, *Chaetomium succineum*, *Fusarium roseum*, *Fusarium solani*, *Fusarium oxysporum*, *Geothrichum sp.*, *Gliocadium sp.*, *Mixotrichum sp.*, *Monilia macrospora*, *Mucor racemosus*, *Mycoderma sp.*, *Myrothecium verrucaria*, *Ophistoma sp.*, *Paecilomyces variabilis*, *Penicillium bevicompactum*, *Penicillium frequentans*, *Penicillium chrysogenum*, *Pestalotia oxyanthi*, *Phoma glomerata*, *Rhizopus nigricans*, *Trichothecium roseum*, *Trichothecium sp.*, *Trichoderma viride*, *Trichoderma longibrachiatum*, *Trichoderma lignorum*, *Ulocladium botrytis*, *Verticillium chlamydosporium*, *Verticillium albo-atrum*, *Scopulariopsis brevicaulis*, *Scopulariopsis acremonium*, *Stachybotrys atra*, *Spicaria sp.*

BACTERIA

Aeromonas caviae, *Aeromonas sp.*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus circulans*, *Cellulomonas sp.*, *Cellulomonas cellasea*, *Cellulomonas cellulans*, *Cellvibrio mixtus*, *Chromobacterium sp.*, *Cytophaga aurantiaca*, *Flavobacterium breve*, *Micrococcus luteus*, *Micrococcus roseus*, *Micrococcus varians*, *Pseudomonas fluorescens*, *Pseudomonas elongata*, *Streptococcus sp.*, *Streptomyces rimosus*, *Staphylococcus sp.*, *Clostridium sp.*, *Vibrio sp.*, *Xanthomonas sp.*

It seems that the fungal community has the biggest responsibility and rule for air contamination and biodegradation of art.

It is possible to study the microbial communities by two different strategies:

- Culture-dependent

Establish suitable conditions to isolate and cultivate a microbe species.



- Culture-independent

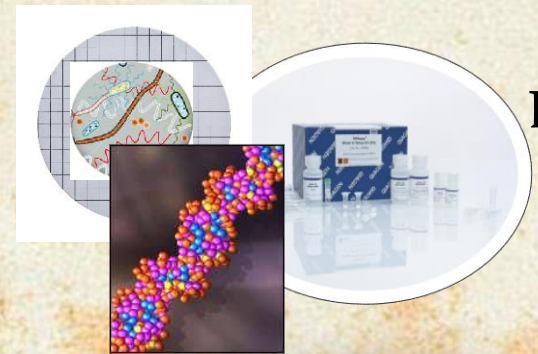
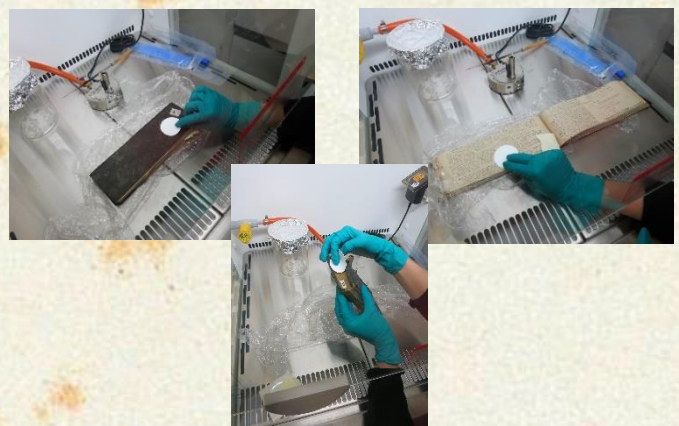
Relies on **molecular methods** to study microbes within their environments.



1st AIM

Microflora detection from book samples

Sampling
with nitrocellulose
membrane
4 Samples



DNA extraction



PCR amplification
~ 700 bp



Bacterial 16S rRNA
Fungal 28S rRNA



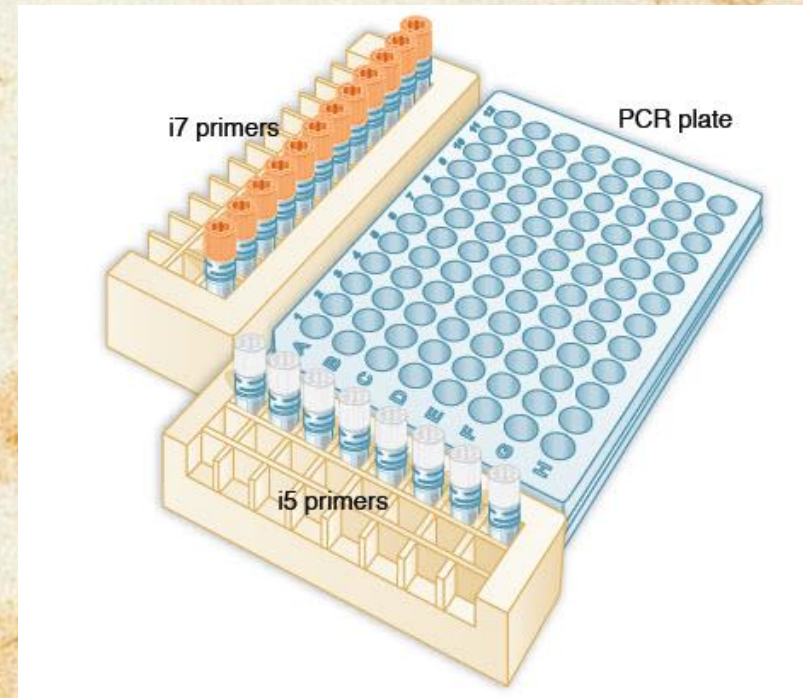
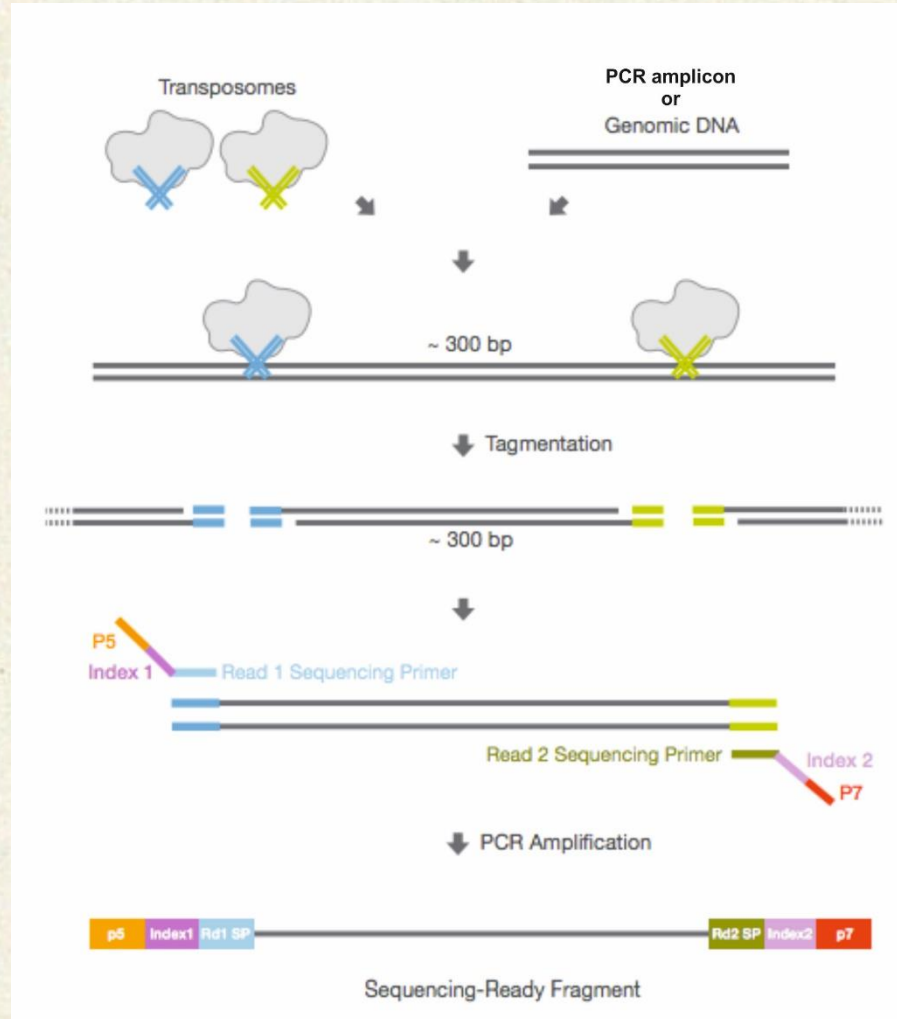
PCR purification



**Ready for NGS
analysis**

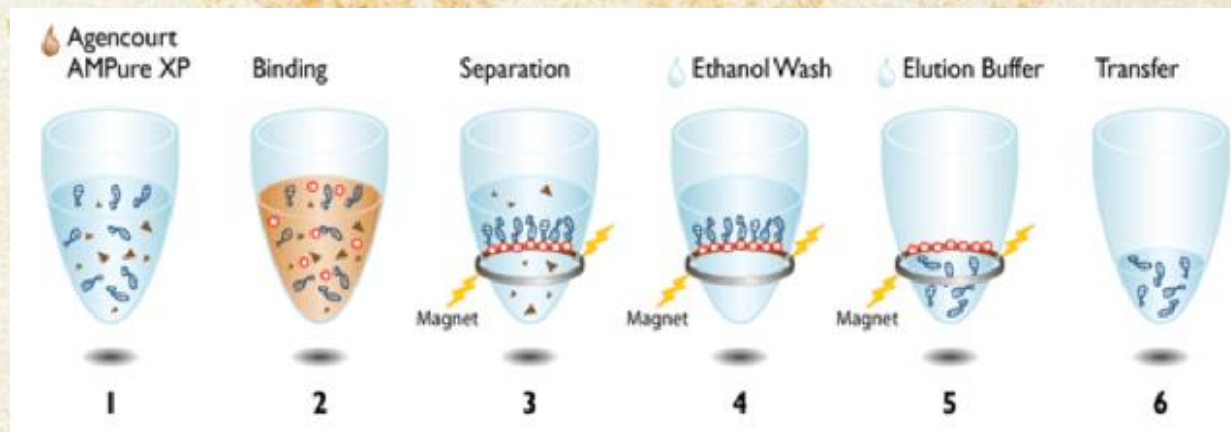
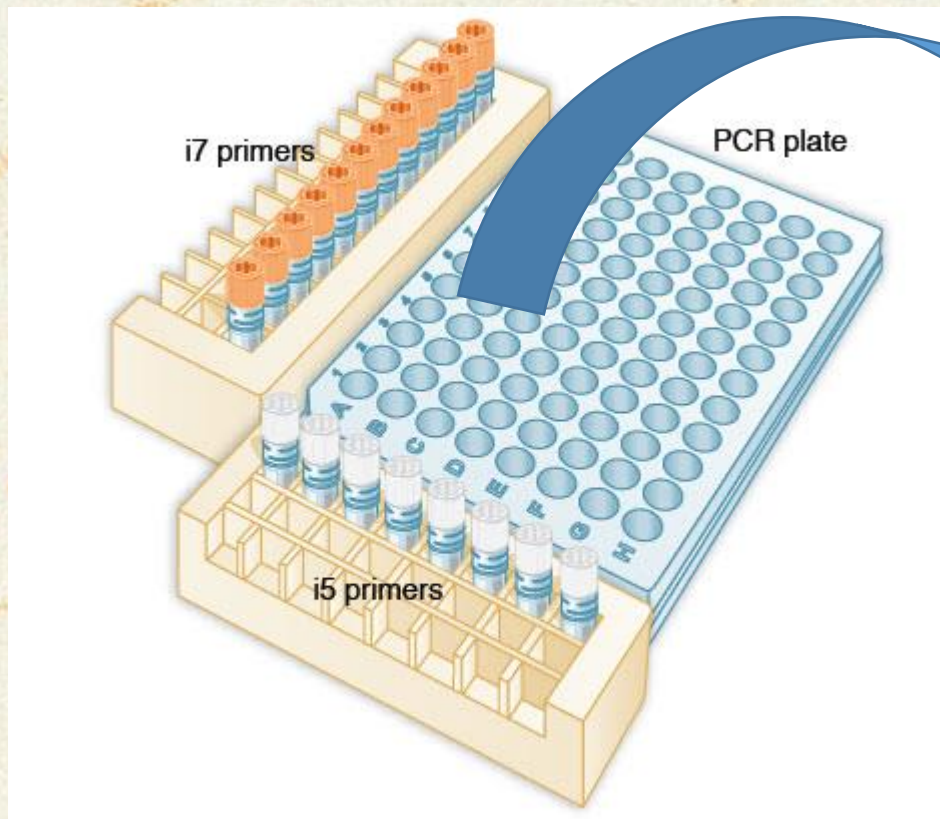
Library preparation

by Nextera XT Index kit

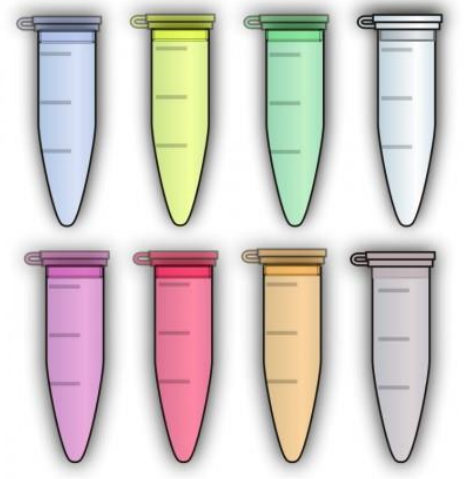
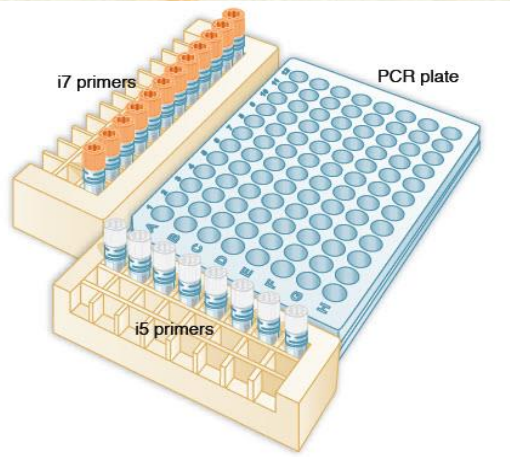


INDEX 1 (i7) SEQUENCE	INDEX 2 (i5) SEQUENCE
N701 TAAGGCGA	S501 TAGATCGC
N702 CGTACTAG	S502 CTCTCTAT
N703 AGGCAGAA	S503 TATCCTCT
N704 TCCTGAGC	S504 AGAGTAGA
N705 GGACTCCT	S505 GTAAGGAG
N706 TAGGCATG	S506 ACTGCATA
N707 CTCTCTAC	S507 AAGGAGTA
N708 CAGAGAGG	S508 CTAAGCCT
N709 GCTACGCT	
N710 CGAGGCTG	
N711 AAGAGGCA	
N712 GTAGAGGA	

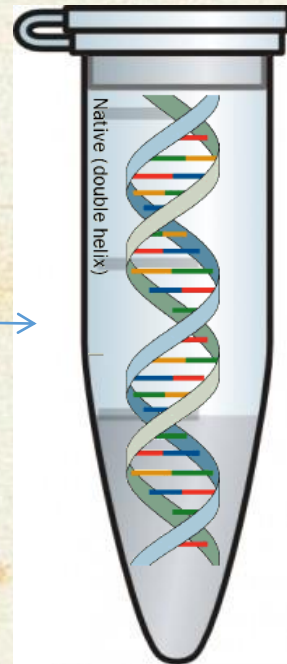
PCR purification



DNA sample pooling, denaturation and dilution



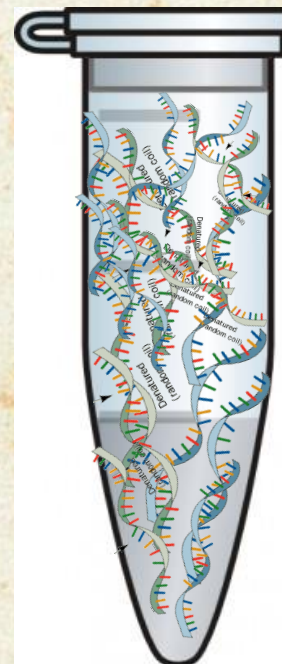
dsDNA
libraries
(2nM)



dsDNA
libraries
(2nM)



denaturation
(0,2N NaOH)
1st dilution



ssDNA
(1nM)

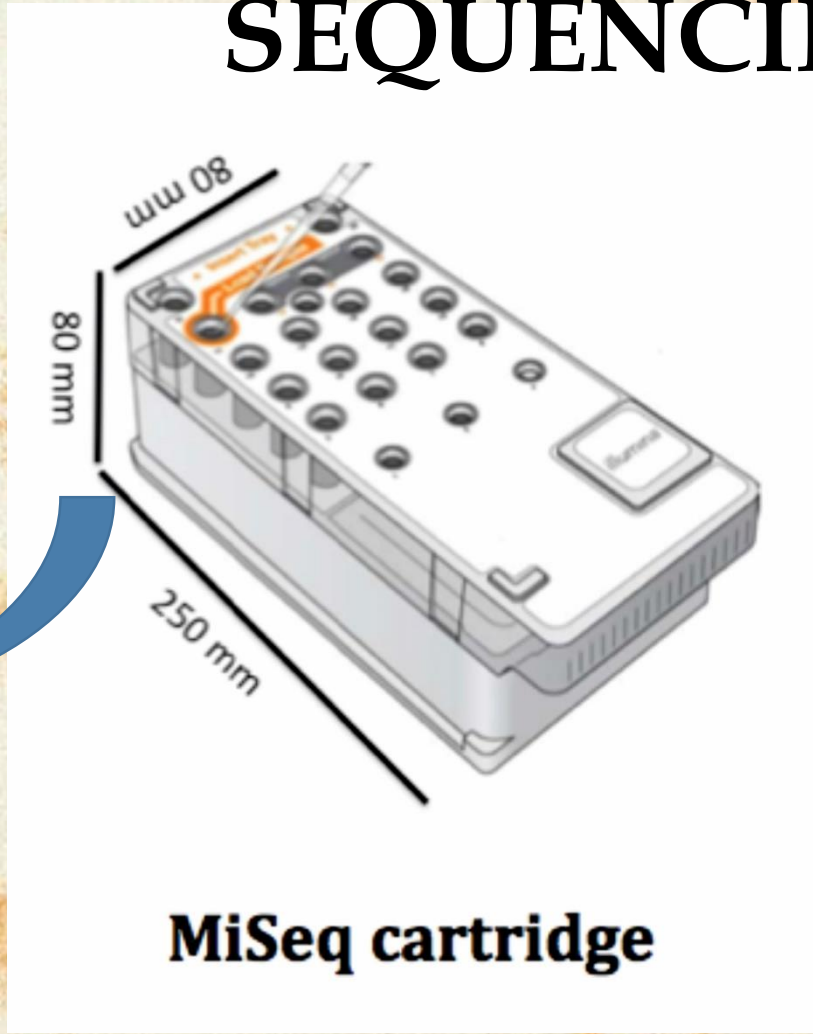


2nd dilution
(hybridization
buffer)

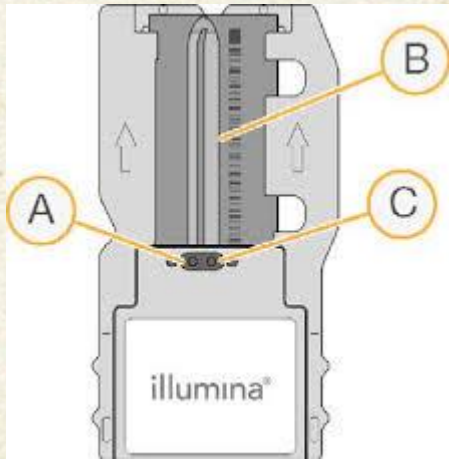


ssDNA
(10-12pM)

SEQUENCING

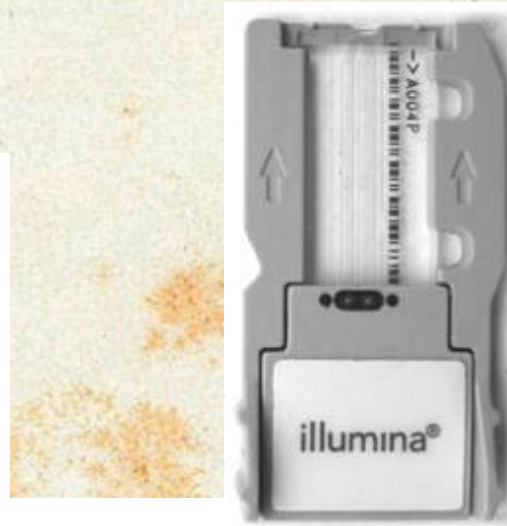
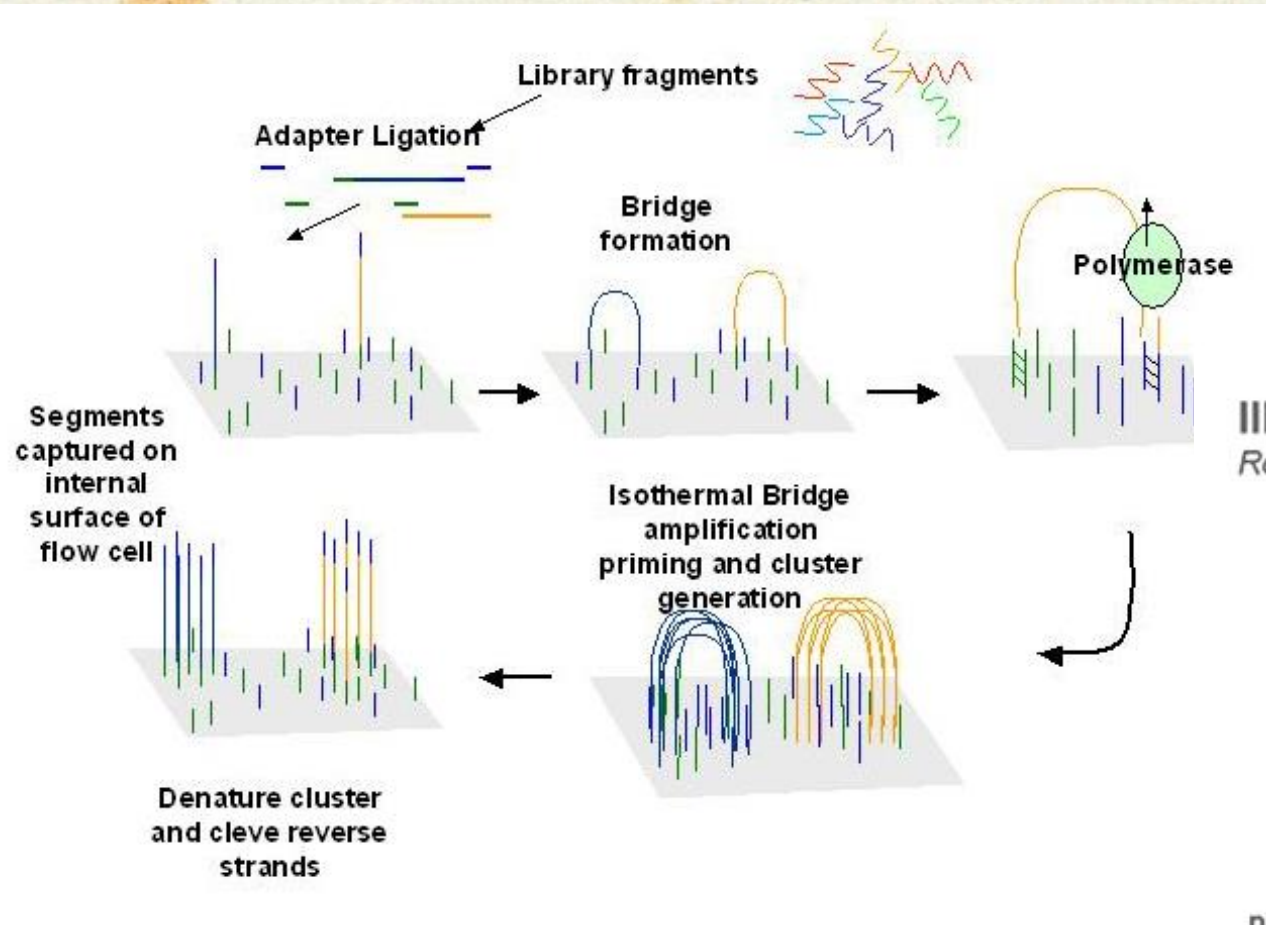


MiSeq cartridge



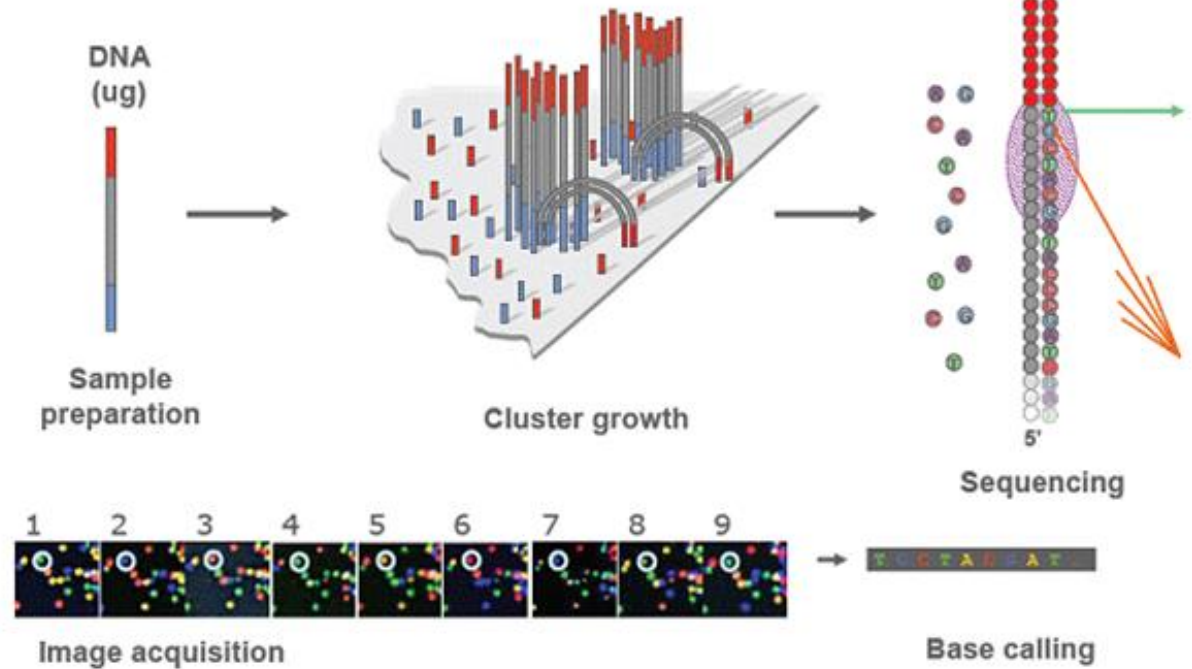
MiSeq chip

On MiSeq chip



Illumina Sequencing Technology

Robust Reversible Terminator Chemistry Foundation



Amazing Bioinformatics Work

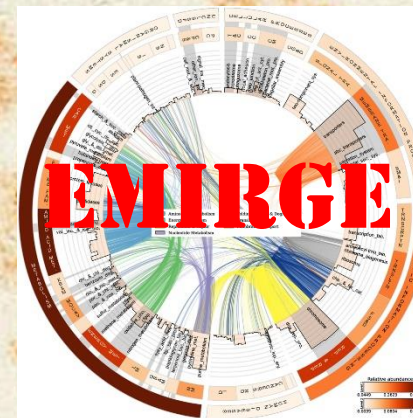
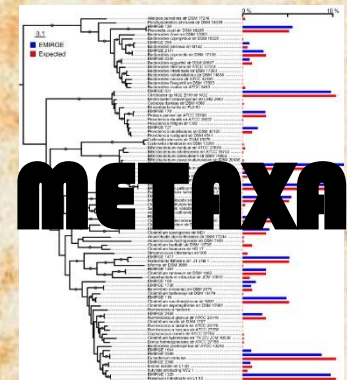
BaseSpace[®]
Genomics Cloud Computing

powered by illumina[®]

Software

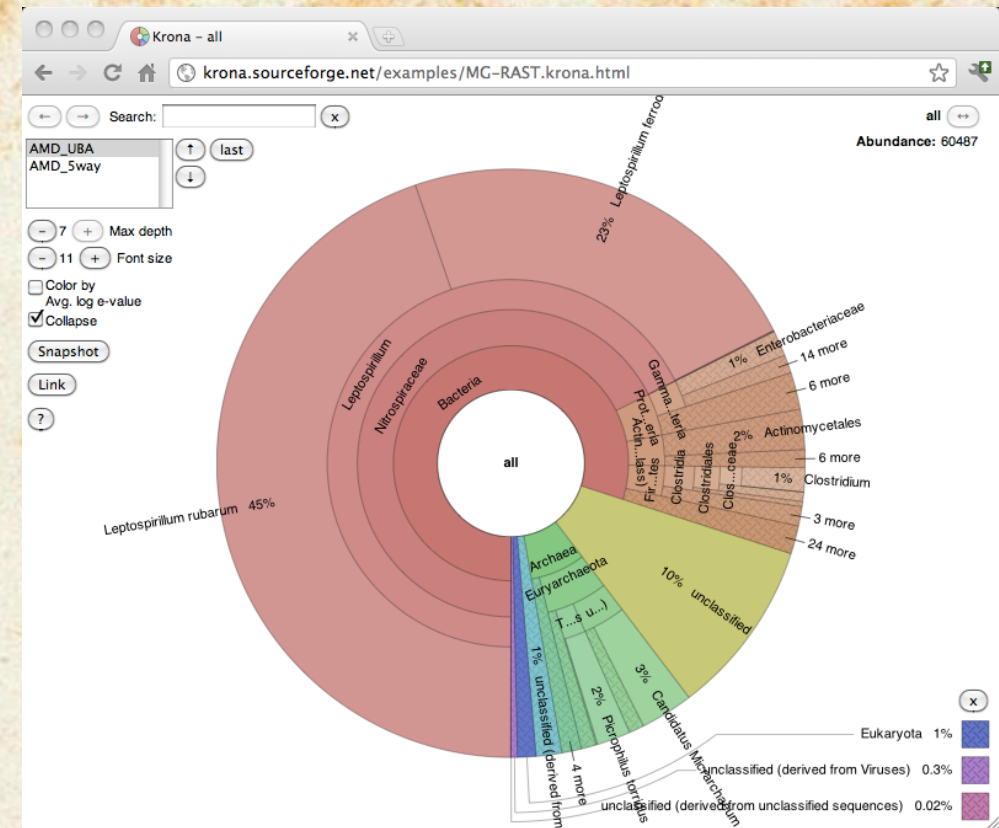
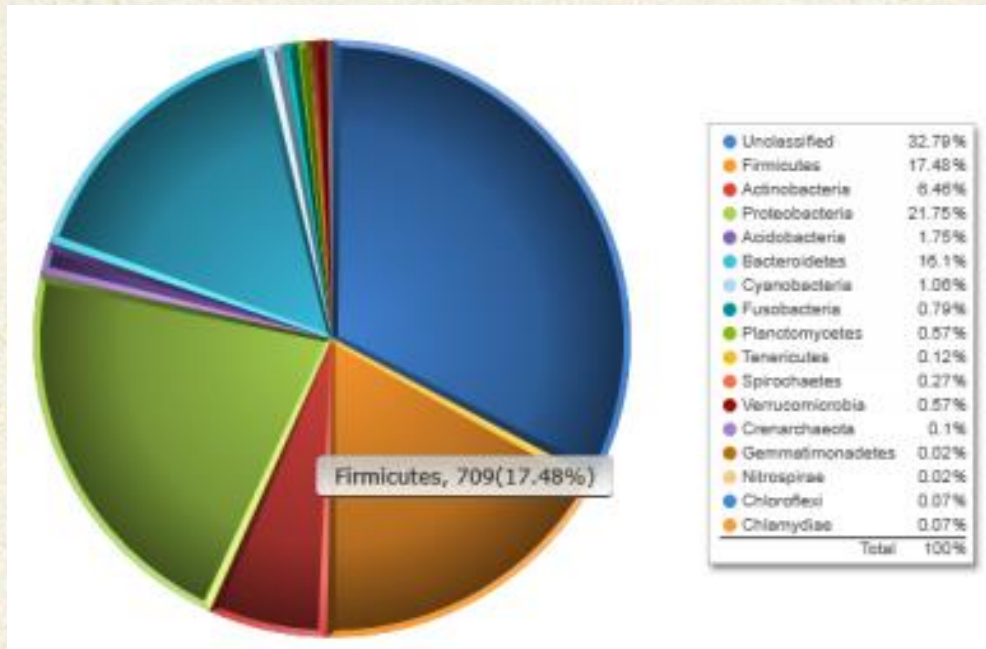


Databases



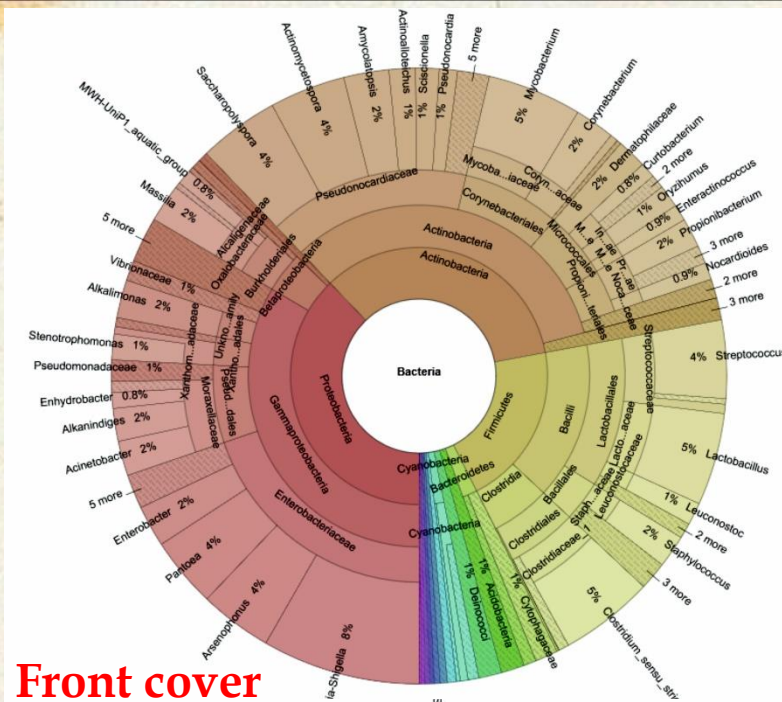
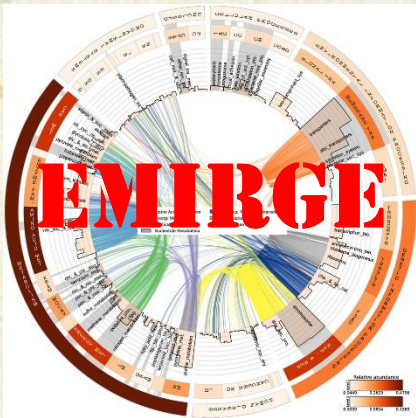
Taxonomic classification

- Assigns taxonomic classification to each read
- 6 taxonomic levels (Domain, Phylum, Class, Order, Family, Genus)

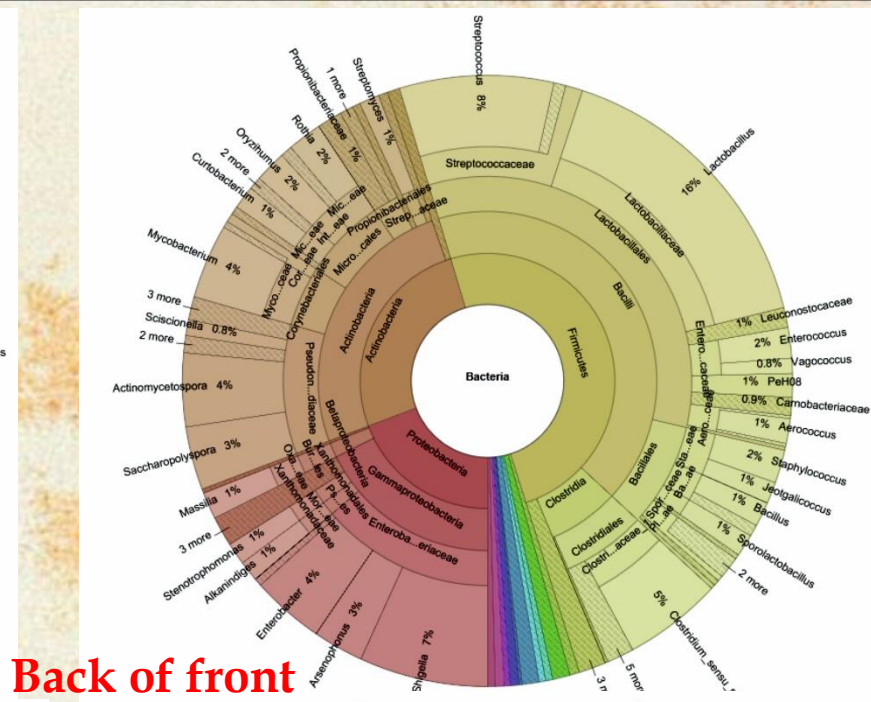


Our results

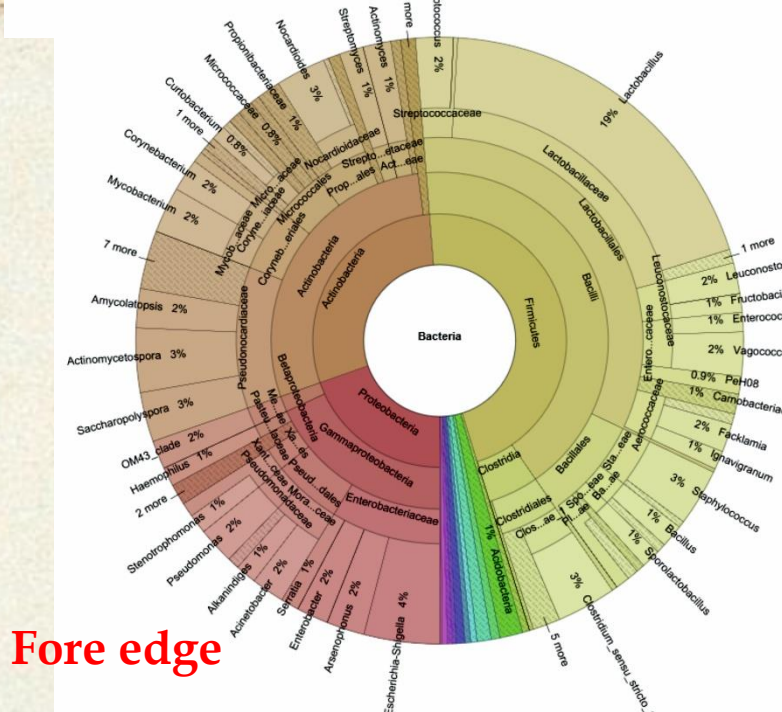
Bacterial 16S rRNA



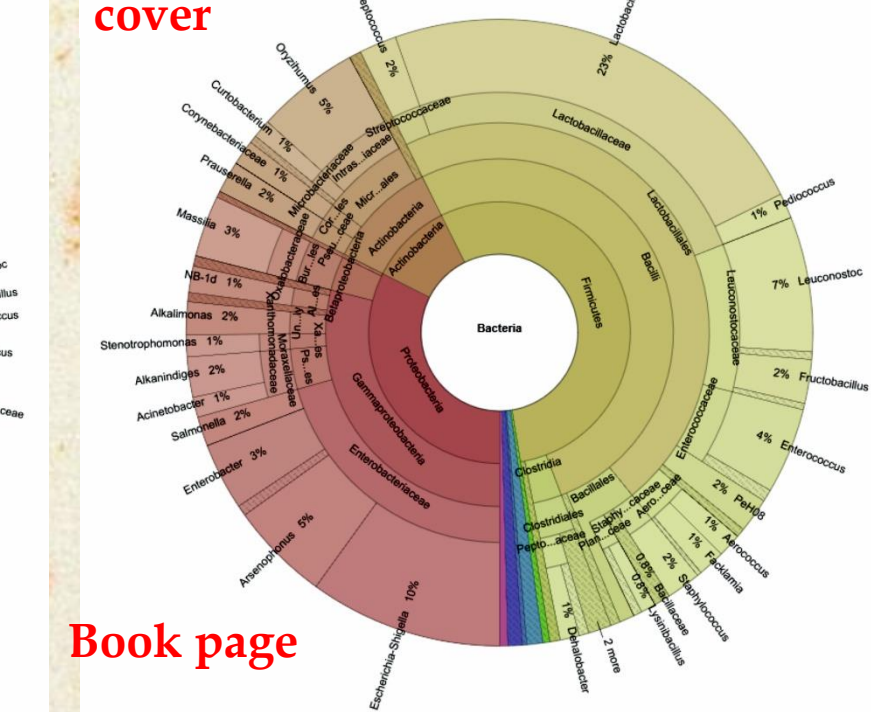
Front cover



Back of front cover



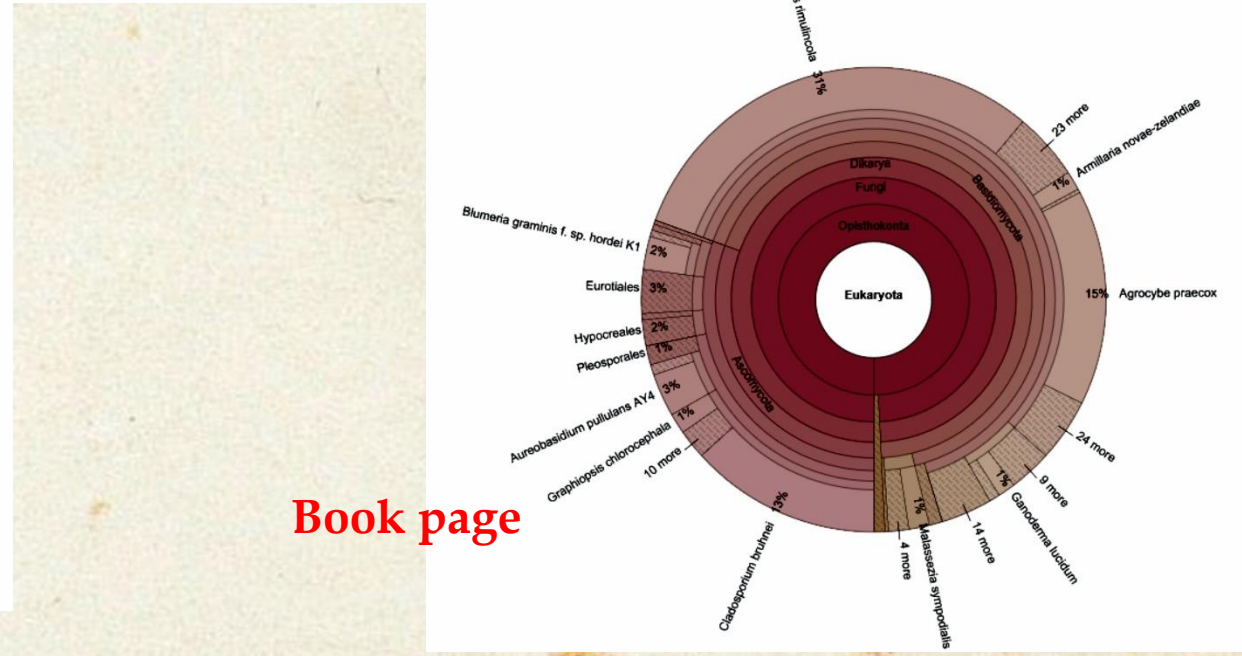
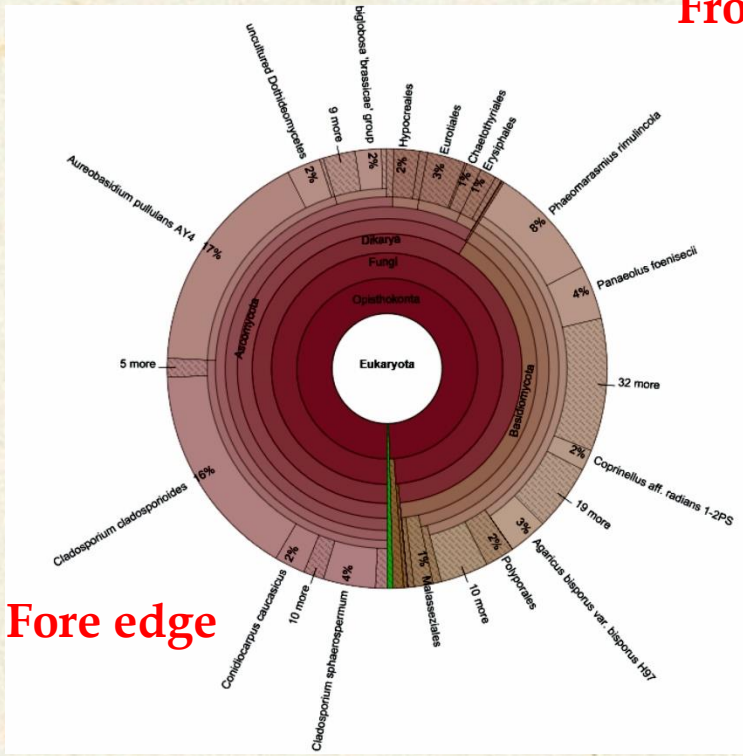
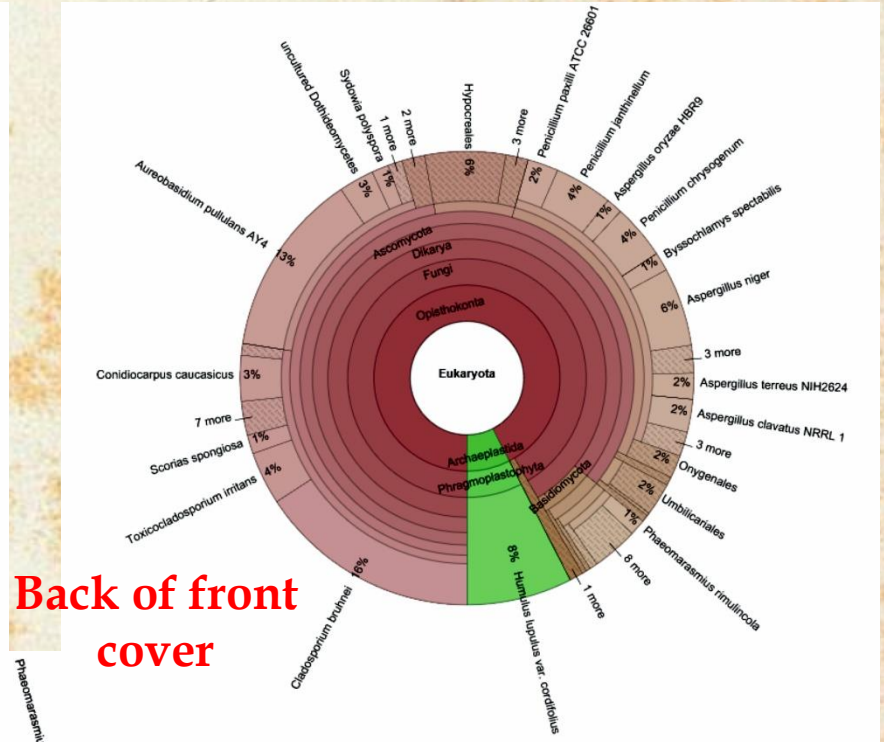
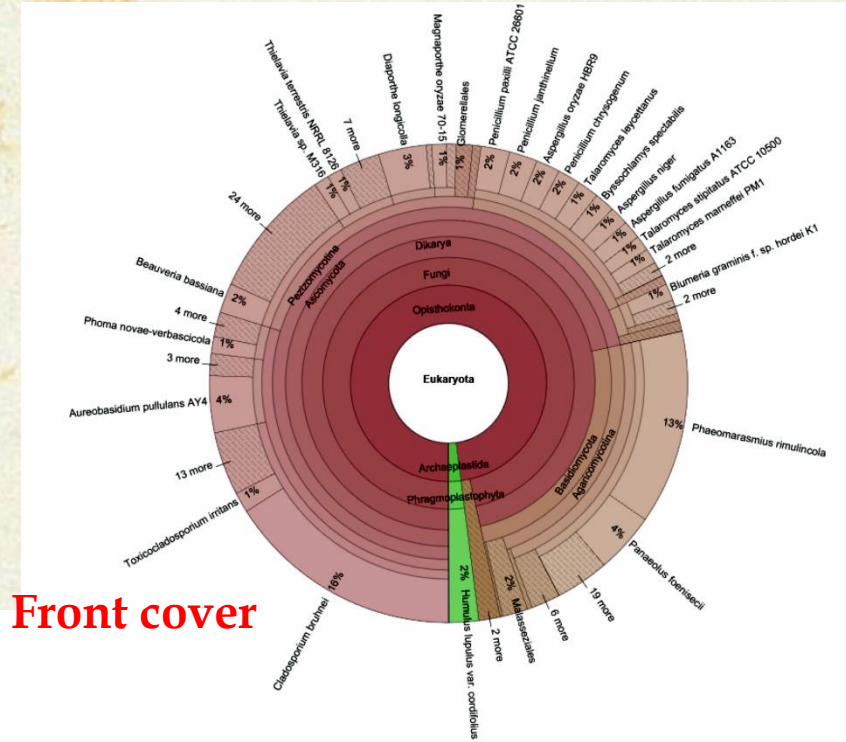
Fore edge



Book page

Our results

Fungal 28S rRNA



BACTERIA

Gammaproteobacteria

Escherichia-Shigella, Arsenophonus, Enterobacter, Alkanindiges, Stenotrophomonas, Vibrio.....

Firmicutes

Lactobacillus, Leuconostoc, Enterococcus Staphylococcus, Pediococcus, Carnobacterium, Facklamia, Aerococcus.....

Actinobacteria

Pseudonocardiaaceae, Corynebacteriaceae, Mycobacteriaceae, Microbacteriaceae, Intrasporangiaceae, Micrococcaceae, Dermatophilaceae, Propionibacteriaceae, Nocardioideaceae, Glycomycetaceae, Streptomycetaceae, Bogoriellaceae, Actinomycetaceae and Micromonosporaceae

Betaproteobacteria

Massilia, Rugamonas, Hydrogenophaga, Sideroxydans

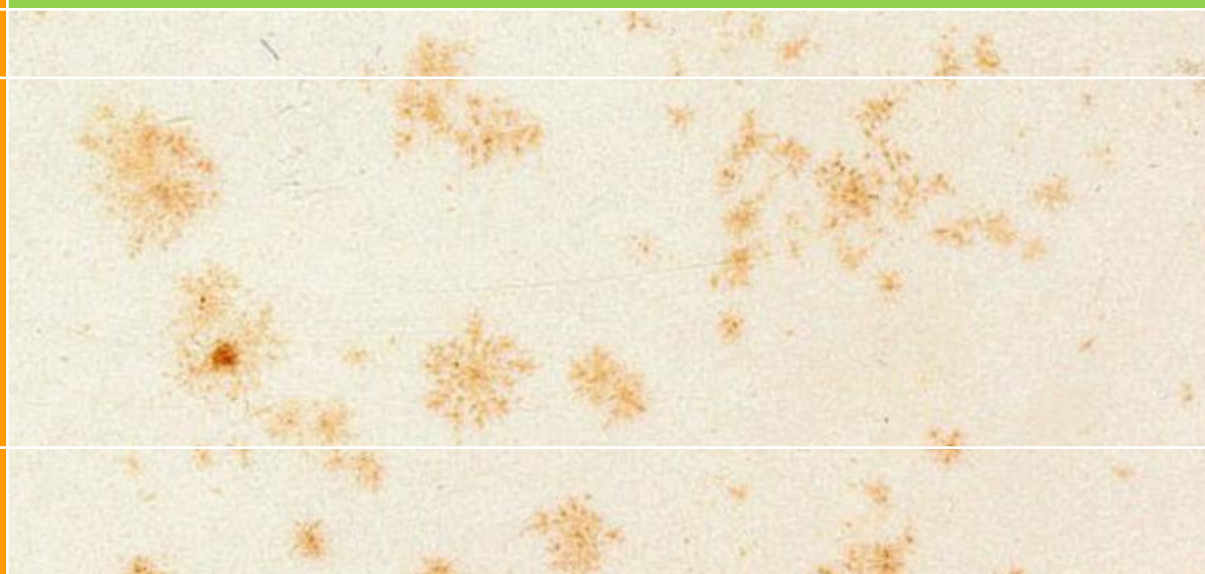
FUNGI

Ascomycota

Cladosporium bruhnei, Toxicocladosporium irritans, Aureobasidium pullulans, Phoma novae-verbascicola, Scorias spongiosa, Conidiocarpus caucasicus, Penicilli, Aspergilli.....

Basidiomycota

Phaeomarasmium rimulincola, Agrocybe praecox, Armillaria novae-zelandiae, Ganoderma lucidum, Stropharia coronilla, Schizophyllum commune, Armillaria luteobubalina, Bovista nigrescens, Arrhenia auriscalpium.....



Others AIMS

- The aim of this study was evaluation of the antibacterial, antifungal activity of six essential oils against different genera of bacteria and fungi; cyto /genotoxic activity in human cell line
- Disinfection of books

Microorganisms and Essential oils tested by *in vitro* experiment

Bacteria

Escherichia coli
Salmonella typhimurium
Yersinia enterocolitica
Staphylococcus aureus
Listeria monocytogenes
Enterococcus faecalis

Fungi

Alternaria alternata
Aspergillus fumigatus
Chaetomium globosum
Cladosporium cladosporioides
Penicillium chrysogenum

Essential Oils



Oregano



Thyme



Clove



Arborvitae

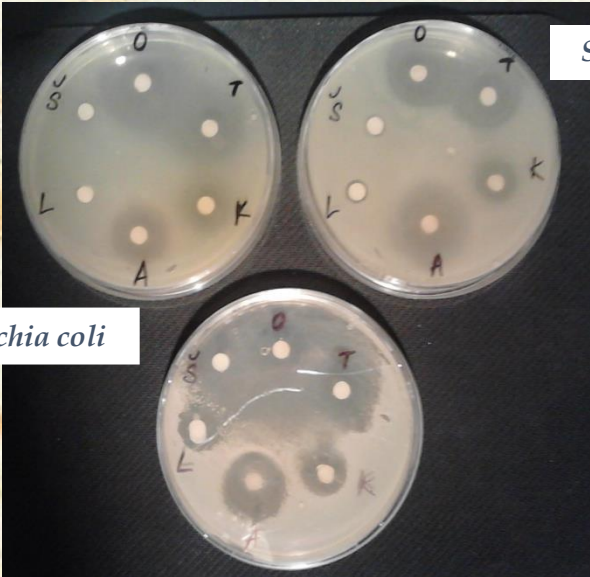


Lavender



Clary sage

Antibacterial effect



Salmonella typhimurium

Escherichia coli

Staphylococcus aureus

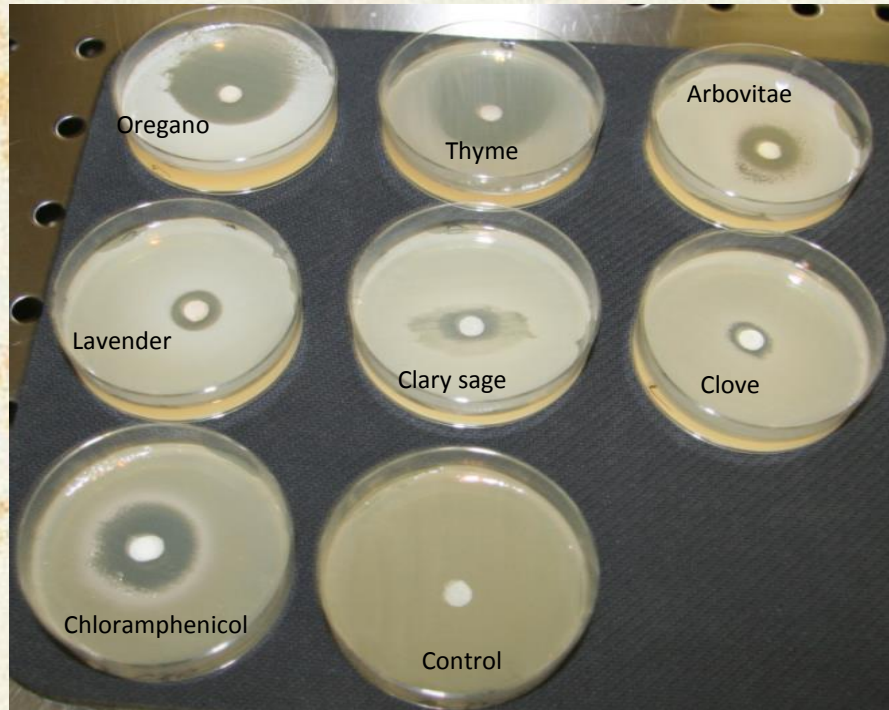
Antibacterial activity of Eos tested by disc-diffusion method

EOs	Zone of inhibition (mm)					
	<i>E. coli</i>	<i>Salmonella</i>	<i>Y. enterocolitica</i>	<i>St. aureus</i>	<i>L. monocytogenes</i>	<i>Ent. fecalis</i>
Oregano	5	10	8	12	7	5
Thyme	5	10	8	12	7	5
Clove	2	3	5	3	3	2
Arborvitae	5	7	10	5	3	2
Levander	1	1	1	2	1	1
Clary sage	1	1	1	2	1	1
Chloramphenicol	3	1	3	5	3	3

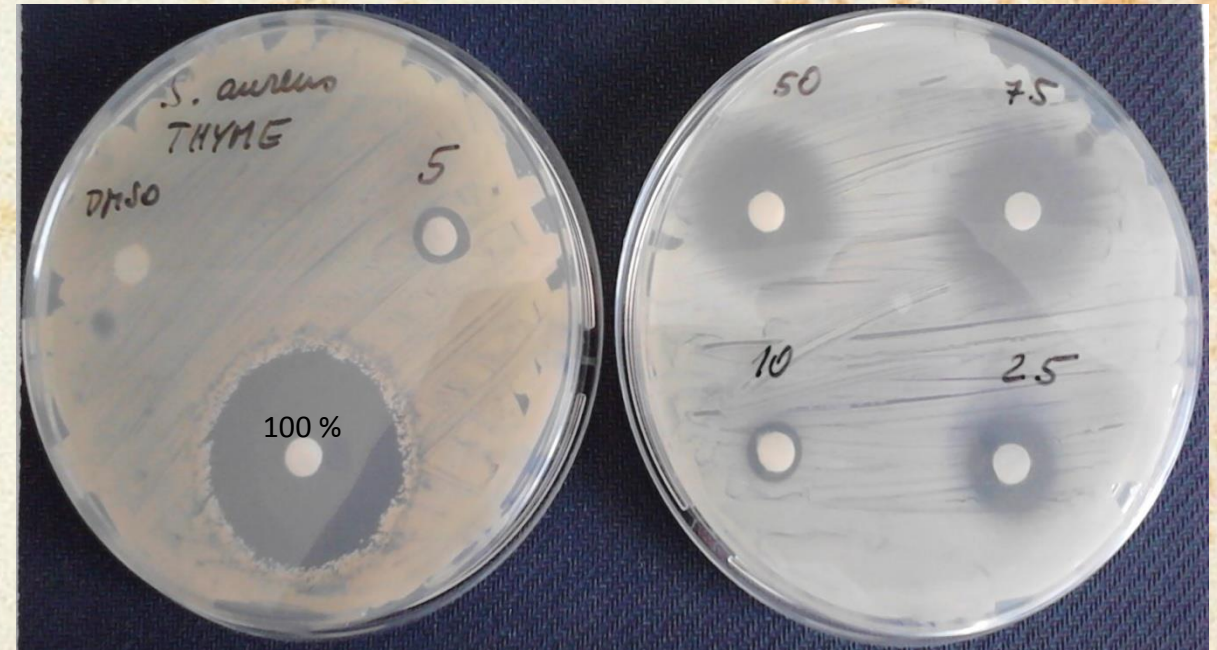
Disc diffusion assay identifies essential oils with antimicrobial activity. O - Oregano, T - Thyme, K - Clove, A - Arbovitae, L - Lavander, S - Clary sage

The diameters of inhibition zones against six bacterial strains. EOs concentration [5 µl / disc (6 mm)] Chloramphenicol concentration (30 µg / disc)

Screening for Antibacterial Activity



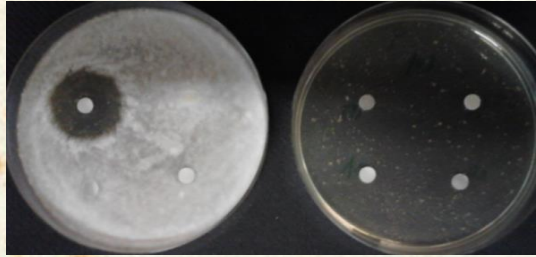
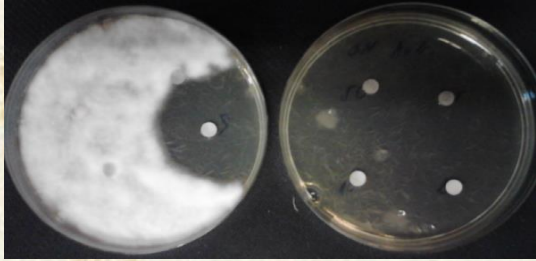
Disc diffusion assay identifies essential oils with antimicrobial activity. Antibiotic at a concentration of **30 μg / disc** (Chloramphenicol) and **pure essential oils (5 μl)** were tested against *S. aureus*



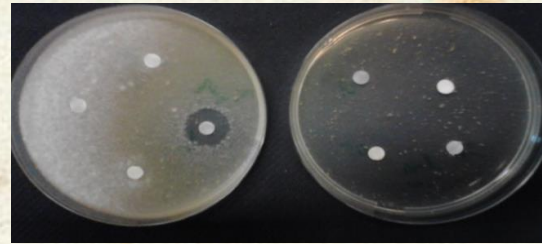
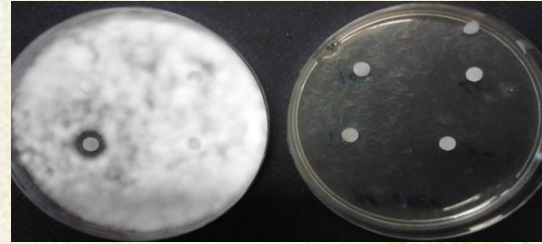
Effect of different concentrations (**100%, 75% diluted, 50%, 25%, 10% and 5%**) of thyme essential oil against *S. aureus*

Antifungal effect

Chaetomium globosum



Chaetomium globosum

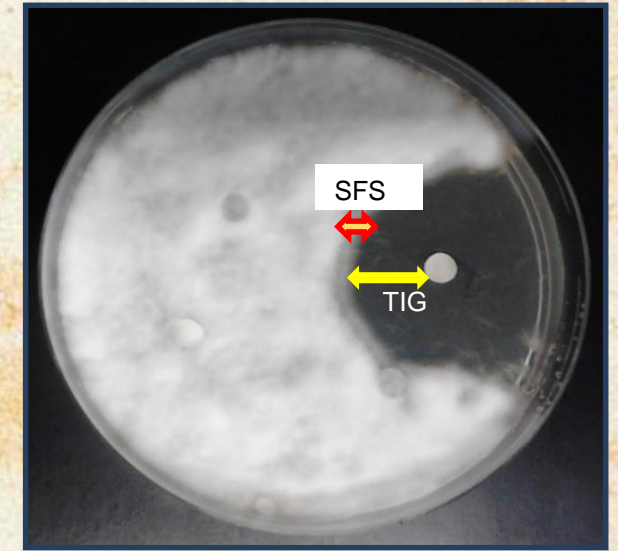


Penicillium chrysogenum

Antifungal activity of arborvitae essential oil (75%, 50%, 25%, 10% -right side and 5% -left side) dissolved in DMSO against *Chaetomium* and *Penicillium* strain.

Penicillium chrysogenum

Antifungal activity of the oregano essential oil (75%, 50%, 25%, 10% -right side and 5%-left side) dissolved in DMSO against *Chaetomium* and *Penicillium* strain.



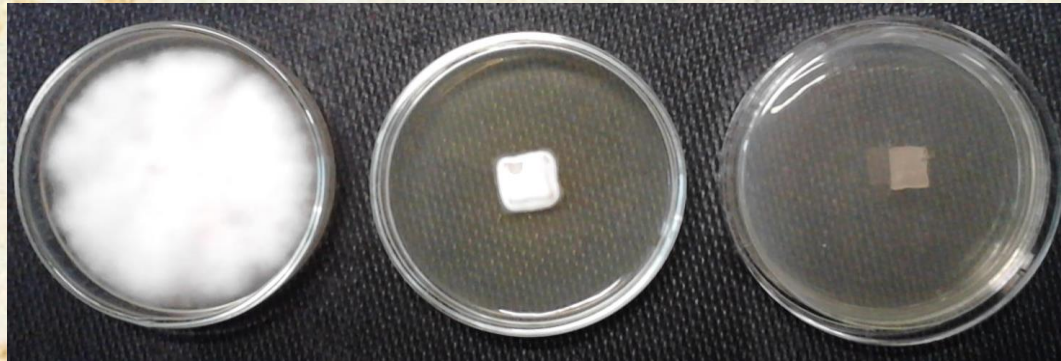
Antifungal activity of the arborvitae essential oil (5%) dissolved in DMSO against *Chaetomium globosum*. TIG: indicate Total Inhibition of Growth, SFS: indicate Stop of Fungal Sporulation.

Inhibitory effect of EOs on mycelial growth of fungi "in vitro"

Control

MIC

MFC



Chaetomium globosum

MIC - minimal inhibitory concentration (the lowest concentration of EO that completely prevent the visible fungal growth) - **Agar dilution methods / mycelium squares**

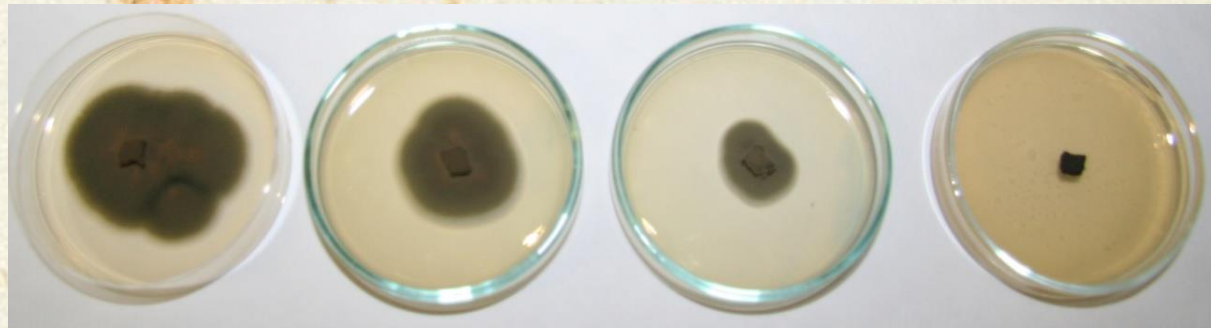
MFC - minimal fungicidal concentration (the lowest concentration at which no colony growth was observed after subculturing into fresh medium) - **Agar dilution methods / mycelium squares**

Control

1% EO

10% EO

50% EO



Cladosporium cladosporioides

Control

1% EO

10% EO

50% EO



Alternaria alternata

antifungal activity of thyme EO

Evaluation of antifungal activity: microatmosphere method

Chaetomium globosum suspension



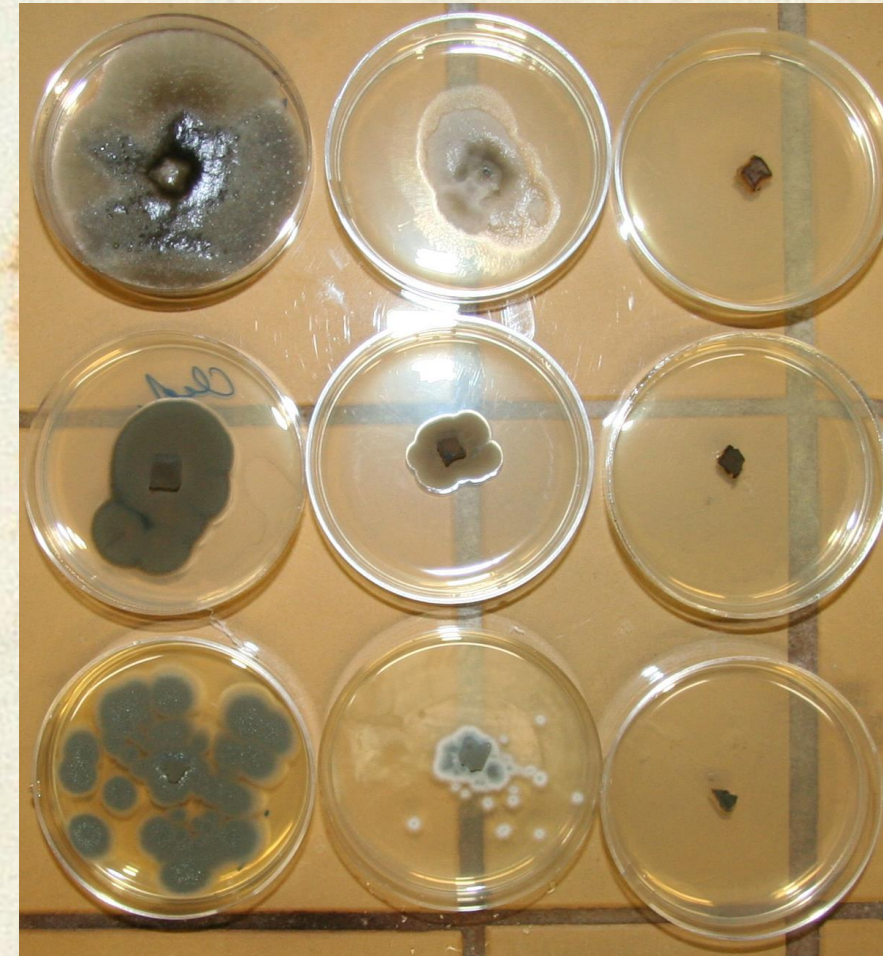
A B C



Penicillium chrysogenum suspension

The antimicrobial activity of volatile vapor of **oregano** at different concentration (A- control, B- 5%, C- 75% at dose level of 1 μ l / ml air space - 20 μ l / 20 ml total volume of plate). Fungi suspensions of conidia of each strain were inoculated with a final concentration of 10^5 conidia / ml

A B C



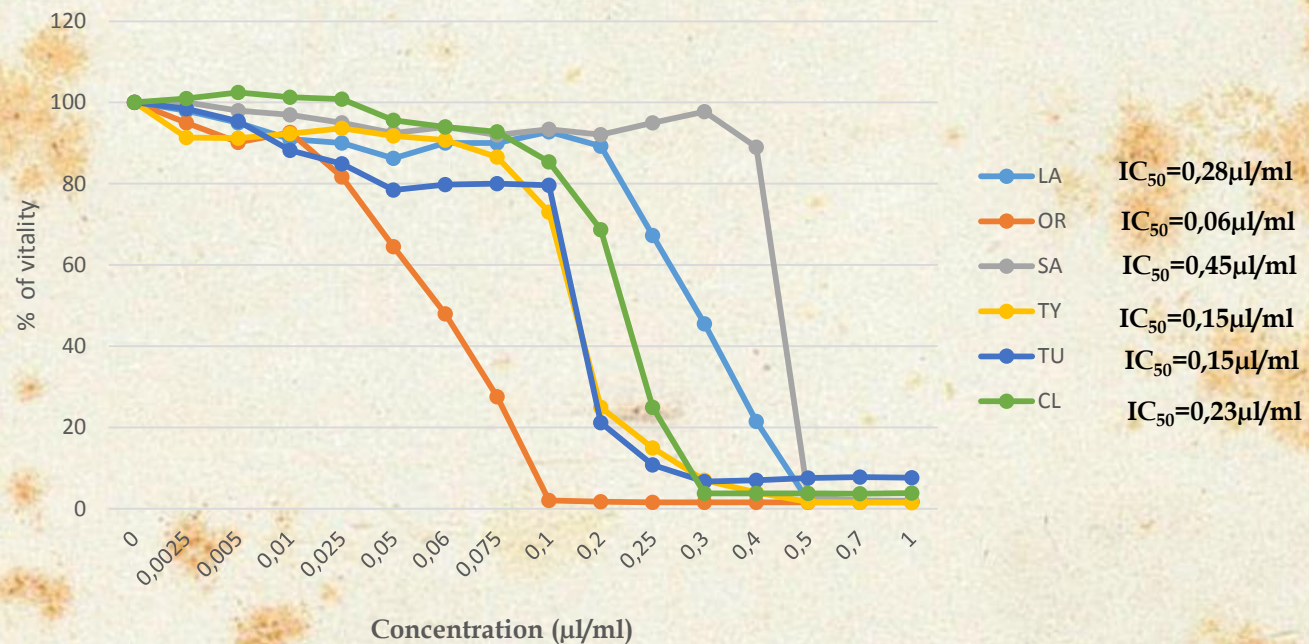
Alternaria alternata

Cladosporium cladosporioides

Penicillium chrysogenum

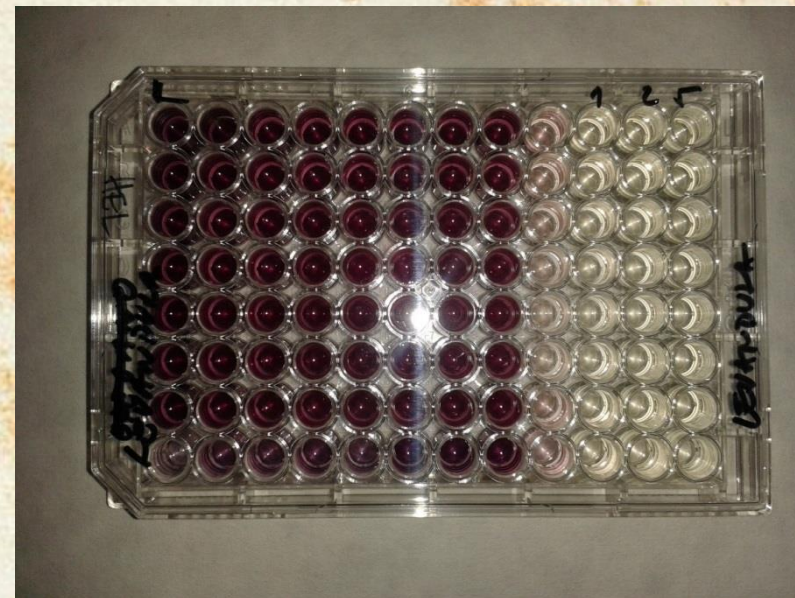
Mycelial (**squares**) growth inhibition of volatile vapor of **thyme** essential oil at different concentration against fungal strains on Malt Extract agar (MEA) plate (A- control, B- 5%, C- 75% at dose level of 1 μ l / ml air space).

Cytotoxic activity



Effect of the test substances on cell viability of human embryo lung cells (HEL) evaluated by MTT assay

Lavender



Oregano

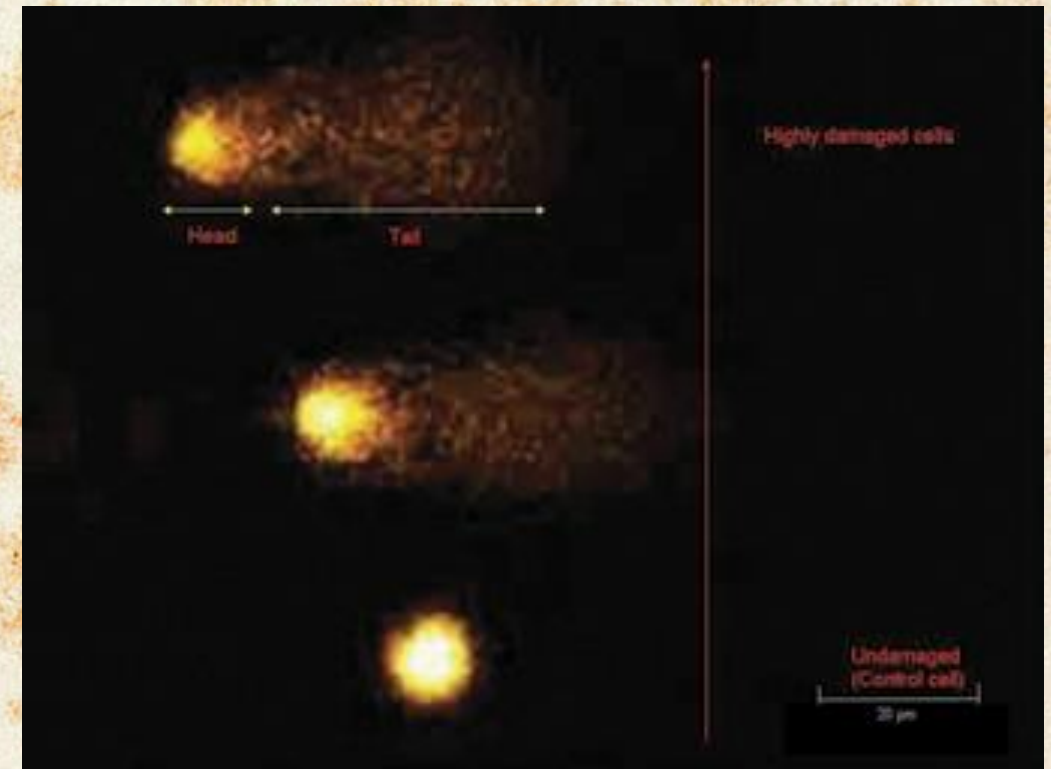


Genotoxic analysis

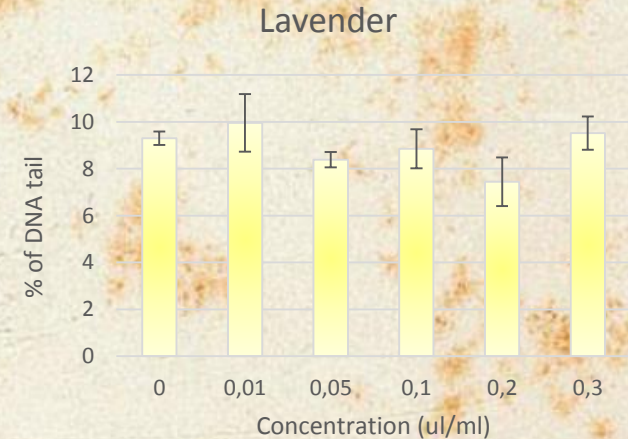
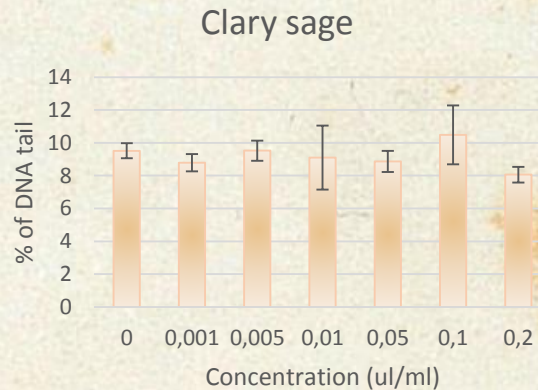
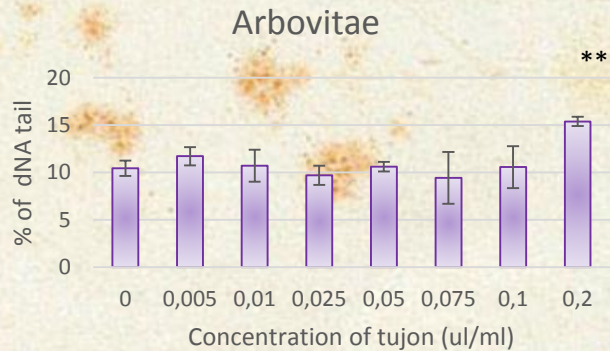
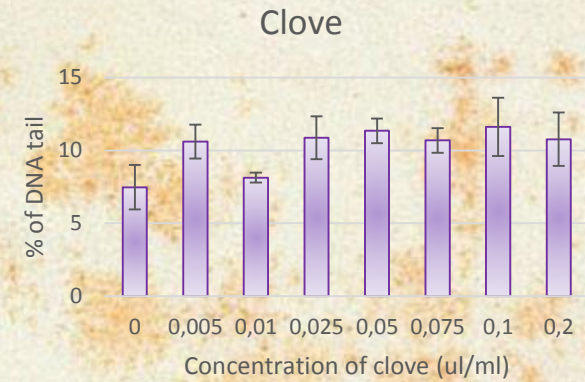
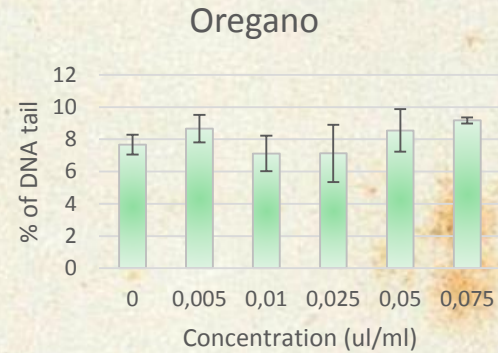
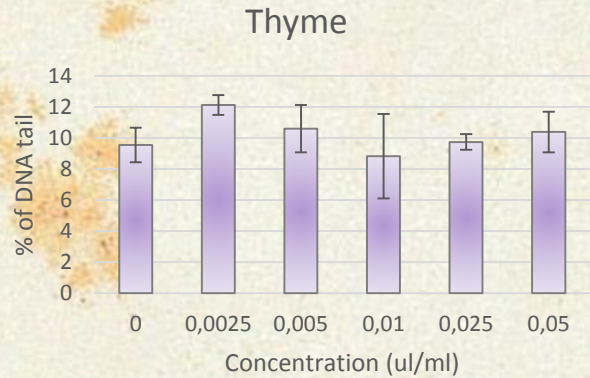


METAFER system

Comet assay

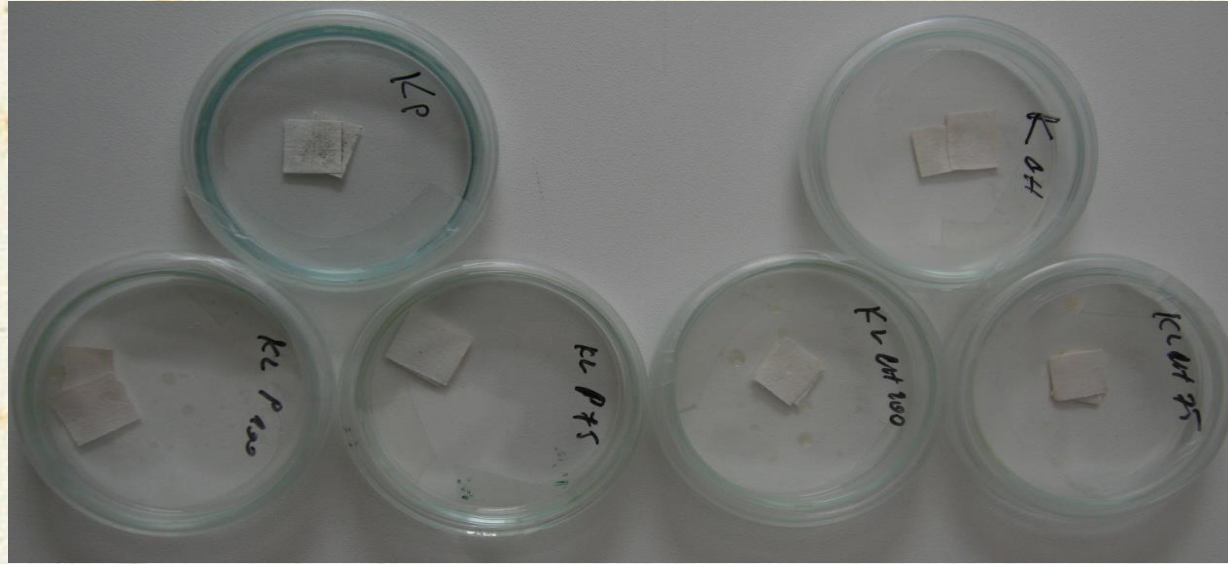


Genotoxic results



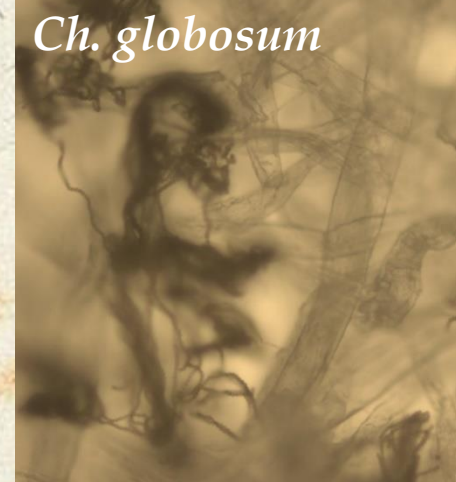
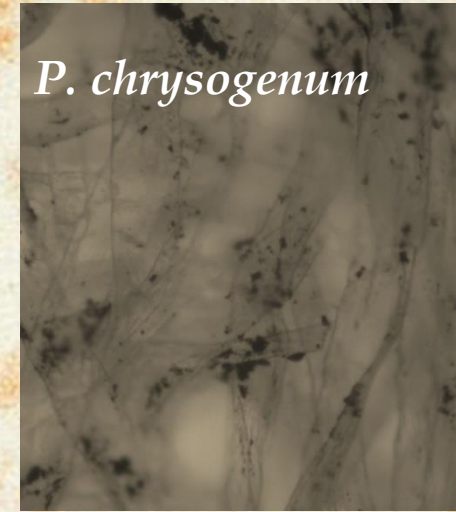
Comet data (% tail DNA) of human embryo lung cells (HEL) treated with different concentrations of the test substances

Influence of EOs to microfilamentous fungi grown on paper



Micro-atmosphere experiment (Whatman 1 paper with *Ch. globosum* or *P. chrysogenum*)

Before



After



Influence of EO vapour for optical characteristics of albumen photographs and paper

Whatman paper samples	ΔE_{ab}^*
Paper_Oregano_5	0
P_O_25	1
P_O_75	0
P_Thyme_5	1
P_T_25	0
P_T_75	1
P_Arbovitae_5	1
P_A_25	2
P_A_75	4
P_Clove_5	1
P_CL_25	1
P_CL_75	1

Microatmosphere method testing

Spectro-Densitometer SpectroDens (Diatech)

$\Delta E_{ab}^* = 0.5 - 2$ critical noticeable colour equality

$\Delta E_{ab}^* = 2 - 4$ colourful difference, which is accepted in direct comparing

$\Delta E_{ab}^* = 4 - 8$ colourful difference, which is accepted in undirect comparing

$\Delta E_{ab}^* > 8$ massive colourful difference

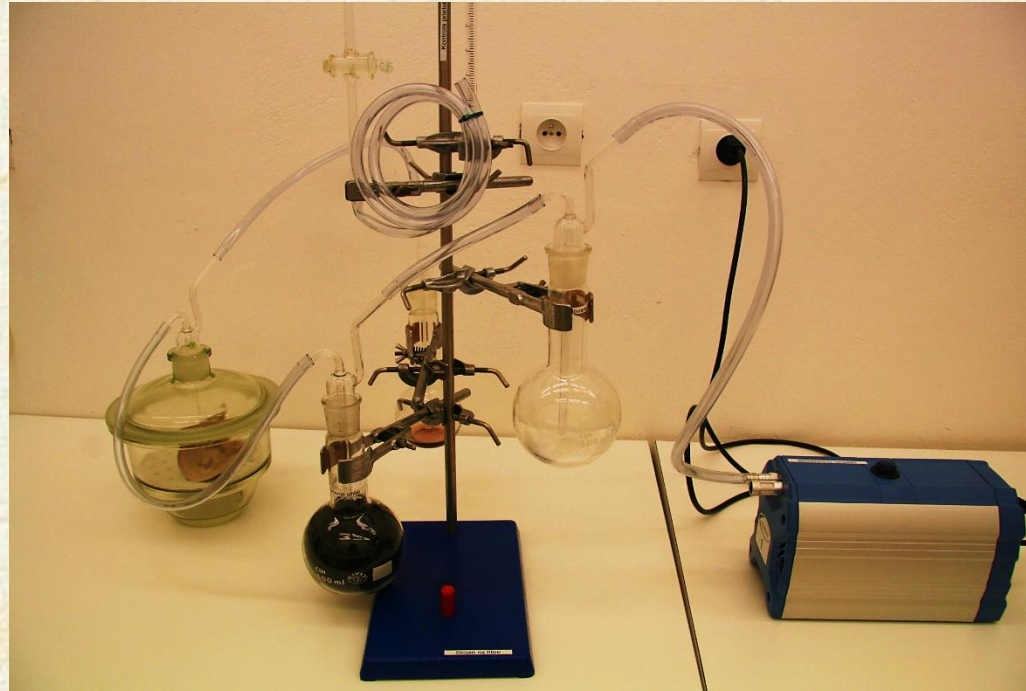
Albumen photo samples	ΔE_{ab}^*
Photograph_Oregano_5	4
Ph_O_25	3
Ph_O_75	1
Ph_Thyme_5	3
Ph_T_25	2
Ph_T_75	1
Ph_Arbovitae_5	7
Ph_A_25	10
Ph_A_75	13
Ph_Clove_5	10
Ph_CL_25	7
Ph_CL_75	17

ΔE_{ab} total colour difference $\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2}$

Conclusion of *in vitro* testing

- The obtained results show that the oregano, thyme, clove, arborvitae, lavender, clary sage oils demonstrate inhibitory activity against clinical and environmental strains
- Oregano, thyme, clove and arborvitae were the most active against all tested bacteria and fungi
- Volatile vapour of essential oils showed the strong antifungal potential
- Based on the results of the cytotoxicity and genotoxicity assays the six essential oils can be considered safe at low concentration for human health
- Thyme doesn't cause color changing in paper and albumen photo models

Application of EO vapor and their use for books disinfection



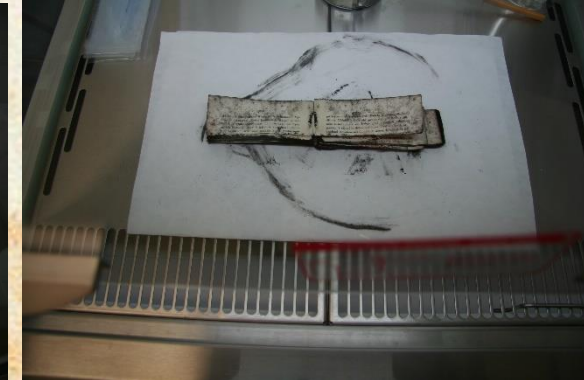
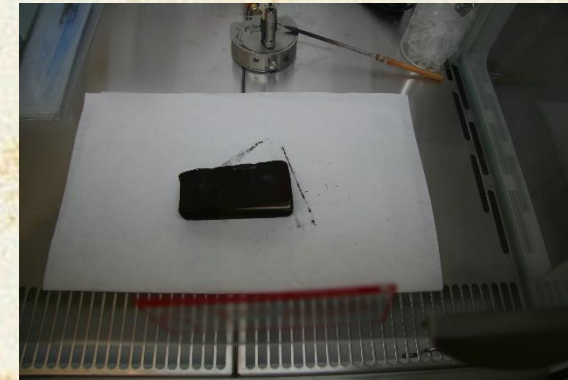
Equipment for EO vapor testing - Laboratory disinfection line

For the first attempt 10% diluted Thyme essential oil was used

Books Before and After EO treatment

Czech Book Before

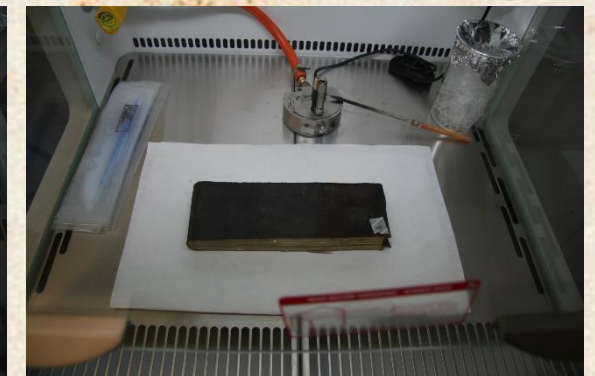
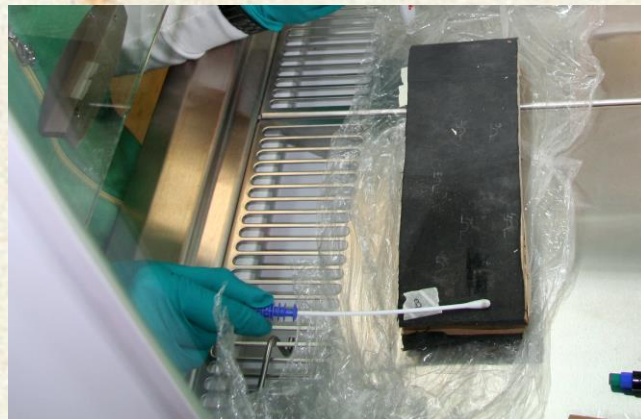
Czech Book After



Artificially contaminated with *Aspergillus niger*, *Penicillium aurantiogriseum* and *Cladosporium herbarum*

Polish Book Before

Polish Book After



Methods to detect the presence of Microorganisms



Bioluminescence
ATP Determination



RNA Detection

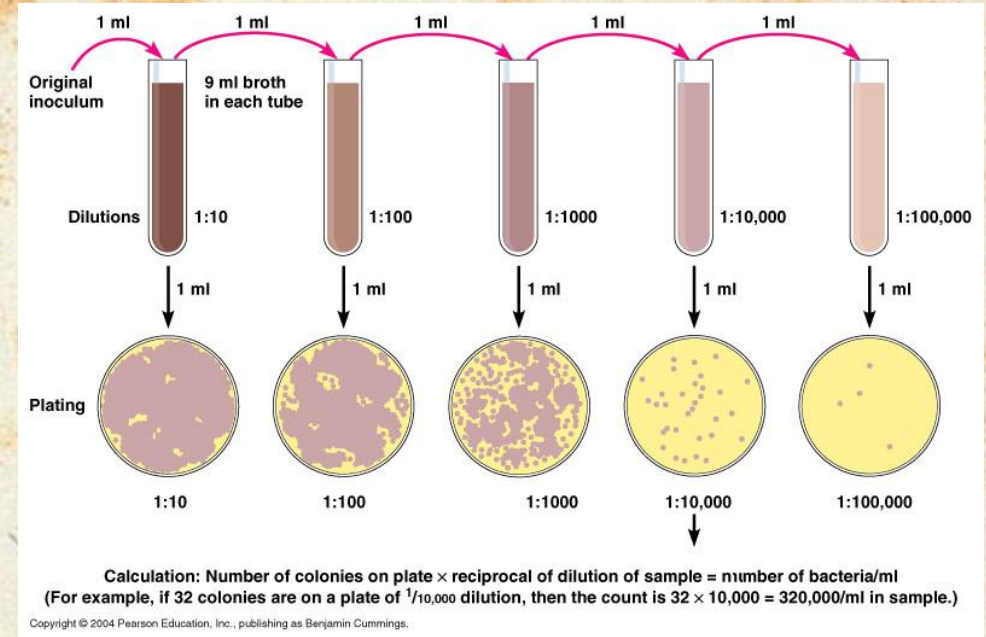


Plate counting
CFU / cm²

TSA and MEA

Results

Bioluminescence ATP

Book Samples	RLU _{sample}	
	Before cleaning	After cleaning
Polish Book (PB) front cover	190	176
PB - paper on the back of front cover	182	158
PB - fore edge	297	194
PB - page	177	159
Czech Book (outside)	2189	2026
Czech Book (inside)	3930	250

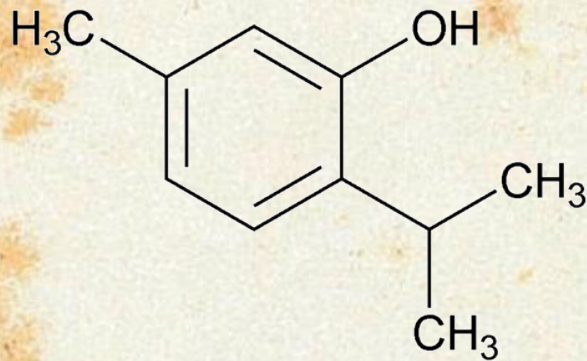
RNA detection

Book Samples	RNA (ng/μl)	
	Before cleaning	After cleaning
Polish Book (PB) front cover	1064	16.9
PB - paper on the back of front cover	121	5.9
PB - fore edge	92	2.5
PB - page	452	2.5

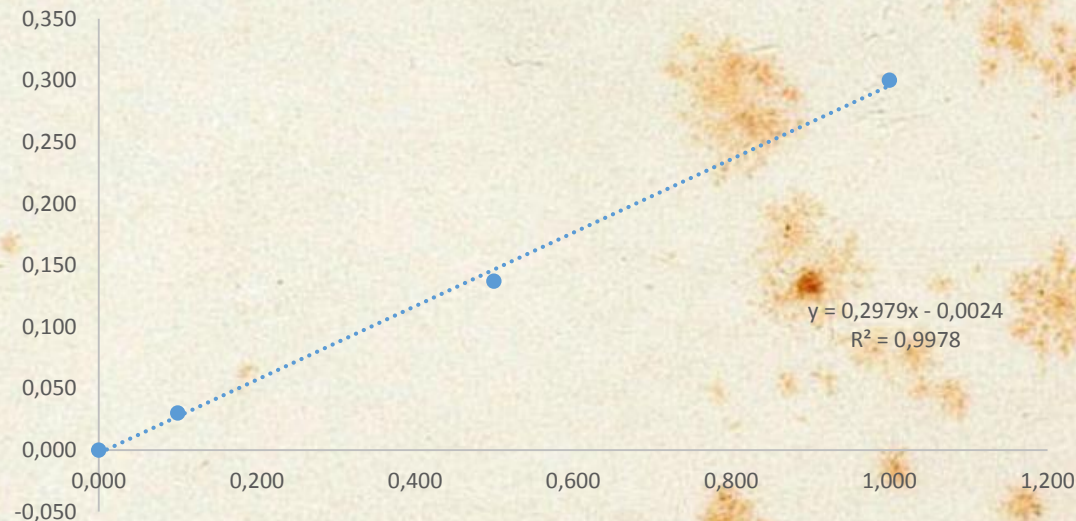
Colonies Forming Units

Book Samples	CFU / 25 cm ²	
	Before cleaning	After cleaning
Polish Book (PB) front cover	1530	0
PB - paper on the back of front cover	950	0
PB - fore edge	485	0
PB - page	40	0

Spectrophotometric determination of Thymol



Oxidation of **2,4-dinitrophenylhydrazine** with potassium periodate and coupling with **Thymol** in alkaline medium to form an intense violet water soluble dye that is stable and has a maximum absorption at 570 nm [*Spectrophotometric Determination of Thymol in Pharmaceutical Preparations Via Oxidative Coupling Reaction with 2,4-dinitrophenylhydrazine in the Presence of Potassium Periodate*, Ghadah Fadhil, Department of Chemistry, College of Science, University of Baghdad, Baghdad, Iraq, *Iraqi Journal of Science*, 2014, Vol 55, No.1, pp: 27-34].





Impinger

Results

A – 0,0048

B – 0,0045

mg/dm³

Oleum thymii in the air

C – 0,0016 (0,02% - **critical** is **1%**)

mg/cm²

Oleum thymii in the paper

Thank you for your Attention





And nice evening.....

Der Kuss - G. Klimt

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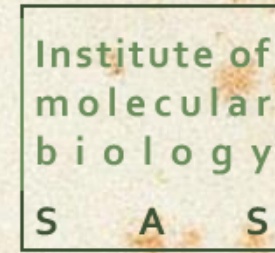
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