VYTAUTAS MAGNUS UNIVERSITY
NATURE RESEARCH CENTRE

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THE STRUCTURE OF FRUIT MYCOBIOTA AND FUNCTIONING MECHANISMS OF ITS COMPONENTS – KILLER SYSTEMS OF *SACCHAROMYCES* GENUS YEASTS

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ABBREVIATIONS

ATP – adenosine triphosphate

BCIP – 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt

CWI – cell wall integrity pathway

dNTP – deoxynucleoside triphosphate

dsRNA – double stranded RNA

EDTA – ethylenediaminetetraacetic acid

gDNA – genomic DNA

GTP – guanosine triphosphate

GO – gene ontology

HOG – high osmolarity glycerol pathway

IgG – immunoglobulin G

ITS – internal transcribed spacer

MAPK – mitogen-activated protein kinase

NA – nucleic acids

NBT – nitro blue tetrazolium chloride

NGS – next generation sequencing

OD – optical density

PCoA – principal coordinate analysis

PCR – polymerase chain reaction

PIP_2 – phosphatidylinositol 4,5-bisphosphate

PVDF – polyvinylidene fluoride

SAGA – Spt-Ada-Gcn5-acetyltransferase complex

SDS – sodium dodecyl sulfate

Tris – tris(hydroxymethyl) aminomethane

VLP – virus-like particles

YEPD – yeast extract peptone dextrose

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following co-authored original publications in 6 journals referred in the *Clarivate Analytics Web of Science* (CA WoS) database. Throughout the text, the publications are referred to using the Roman numerals.

I. Lukša, J., **Vepštaitė-Monstavičė, I.**, Yurchenko, V., Serva, S., Servienė, E. (2018) High content analysis of sea buckthorn, black chokeberry, red and white currants microbiota – A pilot study. *Food Research International*, 111: 597-606.

II. Lukša, J., **Vepštaitė-Monstavičė, I.**, Apšegaitė, V., Blažytė-Čereškienė, L., Stanevičienė, R., Strazdaitė-Žielienė, Ž., Ravoitytė, B., Aleknavičius, D., Būda, V., Mozūraitis, R., Servienė, E. (2020) Fungal microbiota of sea buckthorn berries at two ripening stages and volatile profiling of potential biocontrol yeast. *Microorganisms*, 8(3): 456.

III. **Vepštaitė-Monstavičė, I.**, Lukša, J., Stanevičienė, R., Strazdaitė-Žielienė, Ž., Yurchenko, V., Serva, S., Servienė, E. (2018) Distribution of apple and blackcurrant microbiota in Lithuania and the Czech Republic. *Microbiological Research*, 206, 1-8.

IV. **Vepštaitė-Monstavičė, I.**, Lukša, J., Konovalovas, A., Ežerskytė, D., Stanevičienė, R., Strazdaitė-Žielienė, Ž., Serva, S., Servienė, E. (2018) *Saccharomyces paradoxus* K66 killer system evidences expanded assortment of helper and satellite viruses. *Viruses*, 10: pii: E564.

V. Servienė, E., Lukša, J., **Vepštaitė-Monstavičė, I.**, Urbonavičius, J. (2016) A quick and reliable method for genome-wide host factor screening of *Saccharomyces cerevisiae* killer toxins. *Biologija*, 4, 268-275.

VI. **Vepštaitė-Monstavičė, I.,** Lukša, J., Servienė, E. (2021) Interaction of host factors in response to yeast K2 toxin stress – attractiveness for plant protection. *Zemdirbyste-Agriculture*, 4. DOI 10.13080/z-a.2021.108.040.

AUTHOR CONTRIBUTIONS IN THE CORRESPONDING PAPERS

IVM collected fruits and berries, performed cultivable yeast isolation and identification, prepared microbial gDNA for NGS and participated in metagenomic analysis (**I-III**). **IVM** was involved into isolation of yeast killer strain AML-15-66, performed characterization of K66 toxin and identification of genetic factors affecting susceptibility to the toxin (**IV**). **IVM** was involved into validation of genome-wide host factor screening (**V**). **IVM** constructed and characterized double mutants as well as analyzed interactions of host factors in response to the

action of K2 toxin (**VI**). **IVM** together with colleagues analyzed the data, designed figures, and wrote the papers (**I-VI**).

LIST OF CONFERENCE PRESENTATION ON THE DISSERTATION TOPIC

1C. Ravoitytė, B., Stanevičienė, R., **Vepštaitė-Monstavičė, I.**, Sederevičiūtė, A., Lukša, J., Strazdaitė-Žielienė, Ž., Servienė, E. (2019) Effects of temperature and pH on *Saccharomyces paradoxus* killer yeasts. *Proceedings of XXIX international conference on yeast genetics and molecular biology*, 378.

2C. Stanevičienė, R., Lukša, J., **Vepštaitė-Monstavičė, I.**, Strazdaitė-Žielienė, Ž., Servienė, E. (2019) Yeasts-commensals in the sea buckthorn ecosystem. *Proceedings of 8t^h congress of European microbiologists FEMS*, 1446.

3C. Lukša, J., **Vepštaitė-Monstavičė, I.**, Stanevičienė, R., Strazdaitė-Žielienė, Ž., Serva, S., Servienė, E. (2019) High content analysis of microbiota on medicinal properties possessing berries. *Proceedings of 8th congress of European microbiologists FEMS*, 1445.

4C. Servienė, E., Stanevičienė, R., **Vepštaitė-Monstavičė, I.**, Lukša, J., Strazdaitė-Žielienė, Ž., Apšegaitė, V., Butkienė, R., Aleknavičius, D., Blažytė-Čereškienė, L., Būda, V., Mozūraitis, R. (2019) Sea buckthorn berry-related yeasts and their volatiles. *Proceedings of FEBS3+ conference of Latvian, Lithuanian and Estonian Biochemical societies*, 107.

5C. **Vepštaitė-Monstavičė, I.**, Lukša, J., Stanevičienė, R., Strazdaitė-Žielienė, Ž., Yurchenko, V., Serva, S., Servienė, E. (2018) Metataxonomic analysis of berries-associated microorganisms. *Proceedings of EMBO conference "Experimental approaches to evolution and ecology using yeast and other model systems"*, 111.

6C. **Vepštaitė-Monstavičė, I.**, Lukša, J., Stanevičienė, R., Strazdaitė-Žielienė, Ž., Yurchenko, V., Serva, S., Servienė, E. (2018) Metataxonomic analysis of berries-associated microorganisms. *Proceedings of the XVth International Conference of Lithuanian Biochemical Society,* 70.

7C. **Vepštaitė-Monstavičė, I.**, Lukša, J., Urbonavičius, J., Servienė, E. (2018) Interaction of yeast genetic factors in response to *Saccharomyces cerevisiae* K2 toxin stress. *Proceedings of international conference „Microbial stress: to molecules and back“*, 78.

8C. **Vepštaitė-Monstavičė, I.**, Lukša, J., Stanevičienė, R., Strazdaitė-Žielienė, Ž., Žilakauskis, A., Naumov, D., Yurchenko, V., Serva, S., Servienė, E. (2017) Biogeografijos įtaka obuolių ir juodųjų serbentų mikrobiotai. *Proceedings of 10th young scientists conference „Bioateitis: gamtos ir gyvybės mokslų perspektyvos”*, 4.

9C. **Vepštaitė-Monstavičė, I.**, Stanevičienė, R., Lukša, J., Strazdaitė-Žielienė, Ž., Servienė, E. (2017) Yeast communities on fruits and berries of Lithuania. *Proceedings of 28th international conference on yeast genetics and molecular biology (ICYGMB),* 307-308.

10C. Konovalovas, A., Aitmanaitė, L., **Vepštaitė-Monstavičė, I.**, Stanevičienė, R., Servienė, E., Serva, S. (2017) New double-stranded RNA viruses from *Saccharomyces sensu stricto*. *Proceedings of 28th international conference on yeast genetics and molecular biology (ICYGMB),* 309-310.

11C. Konovalovas, A., Žilakauskis, A., **Vepštaitė-Monstavičė, I.**, Servienė, E., Serva, S. (2016) Evolutionary relationship of ubiquitous *Saccharomyces cerevisiae* dsRNA viruses. *Proceedings of EMBO conference "From functional genomics to systems biology",* 199.

12C. **Vepštaitė-Monstavičė, I.**, Stanevičienė, R., Lukša, J., Strazdaitė-Žielienė, Ž., Naumovas, D., Žilakauskis, A., Cimalova, S., Servienė, E. (2016) The impact of biogeography on diversity of yeast and killer viruses. *Proceedings of EMBO conference "Experimental approaches to evolution and ecology using yeast and other model systems"*, 124.

13C. Servienė, E., Lukša, J., **Vepštaitė-Monstavičė, I.**, Stanevičienė, R., Urbonavičius, J., Serva, S. (2016) Importance of external and cellular environment for the functioning of yeast killer toxins. *Proceedings of EMBO conference "Experimental approaches to evolution and ecology using yeast and other model systems"*, 113.

14C. Lukša, J., **Vepštaitė-Monstavičė, I.**, Stanevičienė, R., Strazdaitė-Žielienė, Ž., Žilakauskis, A., Konovalovas, A., Serva, S., Servienė, E. (2016) Persistence of killer viruses in the natural environment. *Proceedings of 7th EMBO conference*, 182.

SCIENTIFIC PROBLEM

Yeasts are widespread unicellular eukaryotic fungal microorganisms, abundant in the air, water, soil and on various parts of plants, especially on fruits and berries that are rich in sugars (Abdelfattah *et al.*, 2016a; Kurtzman *et al.*, 2011). The abundance and distribution of yeasts on fruits and berries depends on plant species, the ripening stage, climatic conditions and geographical location, type and acidity of soil, good farming practices, use of herbicides and other agricultural chemicals, etc. (Pinto *et al.*, 2015, 2014; Pretorius, 2000; Zhang *et al.*, 2021). Thus, the discovery of microorganisms residing on our favorite fruits and berries, and evaluation of their role in our health is becoming more relevant. For this purpose, research of strawberries, mandarins, sweet and sour cherries, pears, plums, apples, mulberries has been carried out (Abdelfattah *et al.*, 2016a, 2016b; Clooney *et al.*, 2016; Droby *et al.*, 2016; Janisiewicz *et al.*, 2014; Lukša and Servienė, 2020; Stanevičienė *et al.*, 2021; Volschenk *et al.*, 2016), however, it is only a small part in the context of the great diversity of fruits and berries consumed worldwide. The structure of microbiota may differ between the plants of the same species, thus it is relevant to increase efforts put on the investigation of these communities and factors shaping the structure of fungal microorganisms‘ communities.

Certain yeasts possess killer trait and produce toxins that are killing sensitive yeasts (Bewan and Makower, 1963; Makower and Bewan, 1963) and in this manner, killer yeasts can regulate the structure of microbiota. Killer toxins are encoded in several genetic elements: in linear DNA, chromosome, and double-stranded RNA (Magliani *et al.*, 1997). Yeasts of *S. cerevisiae* and *S. paradoxus* species harbor the most identified dsRNA-encoded killer toxins (Rowley, 2017), that are investigated in detail for possible application in medicine, pharmacy, food industry, environment protection, etc. (Boynton, 2019; Hatoum *et al.*, 2012; Meinhardt and Klassen, 2009; Polonelli *et al.*, 2000; Van Vuuren and Jacobs, 1992). *S. cerevisiae* dsRNA-encoded toxins (K1, K2, and K28) have been more extensively investigated (primary sequence, biochemical properties, mechanism of action) than *S. paradoxus* toxins (Dignard *et al.*, 1991; Magliani *et al.*, 1997; Rodríguez-Cousiño *et al.*, 2017, 2011; Schmitt and Tipper, 1990; Servienė *et al.*, 2012). The gap of knowledge of *S. paradoxus* toxins exist, since their mechanisms of action and cellular response have not been described.

Upon the action of killer toxins, stress response pathways, linked to the cell wall biogenesis, functions of vacuoles and mitochondria, and ion homeostasis are activated in the yeast target cell. Among activated signalling pathways, two major pathways emerge, namely high-osmolarity glycerol (HOG), and cell wall integrity (CWI) (Servenė *et al.*, 2012). Usually,

these pathways are coactivated, thus it is important to reveal the interplay between genetic factors involved in response to the killer toxin. Genetic factors, involved in sensitivity/resistance to dsRNA-encoded K1, K2, and K28 toxins have been identified in genome-wide screening studies (Carroll *et al.*, 2009; Pagé *et al.*, 2003; Servienė *et al.*, 2012). A lot of these factors are also involved in HOG and CWI pathways. However, analogous analysis involving killer toxins of *S. paradoxus* yeasts has not been performed previously.

This work has extended the knowledge about mycobiota of widely consumed fruits and berries: composition of fungal microorganisms‘ communities of white, black, and red currants, chokeberries, sea buckthorn berries, and apples, depending on the plant, fruit ripening stage, and external environment as well as prevalence of yeast viral killer systems (**I-III**). Investigation of abundance of yeasts on the surface of fruits and berries led to the identification of *S. paradoxus* AML-15-66 strain, producing dsRNA-encoded K66 toxin (**IV**). A novel viral dsRNA killer system was described, genetic factors involved in the resistance to K66 toxin were identified (**IV, V**). The interplay between HOG and CWI pathways and response to dsRNA-encoded *S. cerevisiae* K2 toxin was revealed (**VI**). *S. paradoxus* K66 and *S. cerevisiae* K2 killer systems were compared based on the action and cellular response mechanisms (**IV**, **VI**).

THE AIM AND MAIN TASKS OF THE STUDY

The aim of doctoral thesis is to evaluate the structure of mycobiota present on fruits and berries and to perform the screening for killer systems of *Saccharomyces* spp. yeasts and investigate their mechanisms of functioning.

The basic tasks:

1. To examine the composition and factors shaping mycobiota of fruits and berries.
2. To evaluate the prevalence of yeast viral killer systems on sea buckthorn, black chokeberry, currant, and apple.
3. To characterize isolated from natural environment *Saccharomyces paradoxus* K66 killer system and to investigate functionality of the toxin.
4. To determine the receptors of *S. paradoxus* K66 toxin in the yeast cell.
5. To identify the genetic factors modulating resistance of yeast cells to *S. paradoxus* K66 toxin.
6. To investigate the interaction of yeast genetic factors and mechanisms of cellular response to *Saccharomyces* spp. viral killer systems.

NOVELTY AND SIGNIFICANCE OF THE STUDY

1. Total mycobiota analysis of sea buckthorn berries, chokeberries, white, red and black currants, and apples grown in Lithuania was performed utilizing next generation sequencing for the first time. The structure and diversity of fungal microorganisms' communities, the abundance of yeast viral killer systems were investigated in depth. Mycobiota of apples and black currants grown in Lithuania and Czech Republic were compared in the geographical context.
2. *S. paradoxus* AML-15-66 strain, harboring newly identified and described dsRNA-encoded viral killer system, was isolated from the natural environment.
3. For the first time a comprehensive study of the functioning of *S. paradoxus* K66 toxin was performed: optimal toxin action conditions and antagonistic activity were evaluated, primary K66 toxin receptors in the cell wall were identified.
4. A newly developed "survival" method was used to identify genetic factors, determining resistance to *S. paradoxus* K66 toxin.
5. The interplay between genetic factors participating in CWI and HOG pathways and involved in response to *Saccharomyces cerevisiae* K2 toxin was revealed and the strenght of these interactions was described for the first time.
6. Comparative analysis of the action of *Saccharomyces* spp. toxins and cellular response to viral killer systems was performed by revealing substantial similarities and differences between mechanisms.

Investigation of the structure of mycobiota present on fruits and determination of potentially pathogenic and beneficial fungal microorganisms is important in farming to predict and control plant diseases, for a longer shelf life of the harvest; to ensure the safety of consumption of fruits and berries in the food industry. Research of killer systems and their mechanisms of action enables the possibility to apply killer toxins for biocontrol of pathogens. Well-described killer yeasts can be used in fermentation processes, cheese, and wine production, etc. Investigation of the functioning of virus-based yeast killer systems is significant for understanding of viruses' spreading and the search of antiviral compounds.

STATEMENTS TO BE DEFENDED

1. The structure of mycobiota of red, white, and black currants, chokeberries, sea buckthorn berries, and apples is influenced by the internal and external environment of the host-plant.
2. Viral killer systems prevail in *Saccharomyces* genus yeasts, isolated from fruits and berries.
3. Changes of beta-1,6-glucan levels in yeast cell wall alter binding efficiency of *S. paradoxus* K66 toxin.
4. The genetic background of the target cell determines the response to the action of *S. paradoxus* K66 toxin.
5. Interaction between factors involved in the cell wall integrity and high-osmolarity glycerol pathways is crucial in the response formation to toxins produced by *Saccharomyces* genus yeasts.
6. Genetic factors involved in the functioning of killer systems of *Saccharomyces* genus yeasts and sensitivity to toxins are not identical.

1 BRIEF LITERATURE OVERVIEW

1.1 The prevalence of fungal microorganisms on fruits and berries

Yeasts are widespread unicellular eukaryotic fungal microorganisms, proliferating by cellular division or sexually via generation of spores (Walker, 2009). Yeasts are abundant in the air, water, soil, on various parts of plants, e.g. on leaves, flowers, and especially on berries and fruits. (Abdelfattah *et al.*, 2016a; Kurtzman *et al.*, 2011). The abundance and distribution of yeasts on fruits and berries depends on climatic conditions, geographical location, plant species and the ripening stage, herbicides, and other agricultural chemicals, etc. (Pinto *et al.*, 2015, 2014; Pretorius, 2000; Zhang *et al.*, 2021). Aberrations from the usual seasonality result in changes of habitat and ecological niche of microorganisms, leading to a decrease in diversity (Saleem and Moe, 2014). The quality of berries and fruits, including the number of bioactive compounds, also depends on plant growth conditions (Borowska and Brzóska, 2016; St George and Cenkowski, 2007). Yeast diversity on fruits is influenced by contact with the soil, insects, and animals capable to transfer microorganisms from one surface to another (Stefanini *et al.*, 2012; Valero *et al.*, 2007).

Fungal microorganisms are grouped into beneficial, e.g. *Cryptococcus*, increasing resistance to other microorganisms attacking the plant, and harmful, e.g. *Phoma*, phytopathogenic, causing great economic loss (Abdelfattah *et al.*, 2018, 2016a; Droby *et al.*, 2016; Liu *et al.*, 2013). Microorganisms residing on plants can induce diseases spreading in the food chain or can influence human health by affecting changes in the diversity of the gut microbiome or stimulating immunity response (Berg *et al.*, 2014; Higgins *et al.*, 2018; Hirt, 2020). Interaction between different microorganisms influences microbiological diversity of the plant and ecological and evolutionary processes (Alvarez-Pérez and Herrera, 2013; Friesen *et al.*, 2011). Microorganisms, distributed on the surface of fruits, stems, leaves, or inner plant tissues, affect plant development and adaptation. In this way, the potential of plant adaptation for food production is increased (Abdelfattah *et al.*, 2016a; Barrow *et al.*, 2008; Kumar *et al.*, 2021; Zhang *et al.*, 2021).

Fungal composition depends on the plant species (Pinto *et al.*, 2014). The geographical location and farming practices are also among the factors strongly affecting the diversity of microorganisms (Leff and Fierer, 2013). So far, the biogeographical distribution of microorganisms was mostly analyzed on grapes, since these berries are applied to winemaking (Pinto *et al.*, 2015; Setati *et al.*, 2012; Wang *et al.*, 2015; Griggs *et al.*, 2021). Barata and

colleagues analyzed composition of microbiota based on the grape ripening stage and determined that *Aureobasidium*, *Cryptococcus,* and *Rhodotorula* fungal microorganisms dominate on unripe berries, while *Hanseniaspora, Metschnikowia,* and *Pichia* yeasts – on ripe grapes (Barata *et al.*, 2012). Composition of mycobiota of grapes was also analyzed according to the location, i.e. examining vineyards located in different places (Bokulich *et al.*, 2013; Setati *et al.*, 2012; Taylor *et al.*, 2014). Hall and Wilcox investigated microbiota of grapes from Washington and New York (USA) and Tasmania (Australia). In all samples *Candida*, *Pichia,* and *Hanseniaspora* were among the most prevalent genera of fungal microorganisms (Hall and Wilcox, 2018). Bokulich *et al.* determined the structure of microbiota of grape must from two different vineyards in California (USA). Dominating fungal microorganisms *Cladosporium* spp., *Botryotinia fuckelania*, *Penicillium* spp., *Davidiella tassiana*, *Aureobasidium pullulans*, and populations of *H. uvarum*, *S. cerevisiae*, and *C. zemplinina* yeasts were identified in both vineyards, however their abundance differs (Bokulich *et al.*, 2013).

In recent years, plants beneficial to human health (chokeberries, currants, sea buckthorn berries, etc.) and their products are gaining more attention, since they are rich in vitamins, minerals, fatty acids, antioxidants, etc. Thus it is important to identify, what microorganisms are found on these plants (Agbarya *et al.*, 2014; Boeing *et al.*, 2012; Borowska and Brzóska, 2016; Chauhan *et al.*, 2007). Diversity of microorganisms on plums, pears, apples, sweet and sour cherries, strawberries, mulberries, oranges has been investigated (Abdelfattah *et al.*, 2016a, 2016b; Clooney *et al.*, 2016; Droby *et al.*, 2016; Janisiewicz *et al.*, 2014; Lukša and Servienė, 2020; Stanevičienė *et al.*, 2021; Volschenk *et al.*, 2016). On Czech mulberries *Hanseniaspora*, *Cryptococcus*, *Phoma*, and *Cladosporium* genera were the most abundant, whereas dominating fungal microorganisms‘ species were *Hanseniaspora uvarum*, *Cryptococcus laurentii,* and *Cryptococcus magnus* (Lukša and Servienė, 2020). Stanevičienė and colleagues compared fungal mycobiota structure of sour and sweet cherries grown in private plantations and bought in the supermarket. More *H. uvarum* yeasts were found on sour than on sweet cherries, while more *Cladosporium cladosporioides* were on sweet cherries. *Microcyclosporella mali*, *Ramularia citricola,* and *Neosetophoma rosae* fungal microorganisms were detected only on sweet cherries. *Aureobasidium*, *Metschnikowia*, *Taphrina*, *Dothiora,* and *Cladosporium* dominated on sweet and sour cherries grown in plantations, whereas *Aureobasidium*, *Vishniacozyma*, *Rhodotorula*, *Hanseniaspora, Cladosporium,* and *Filobasidium* were detected on sweet cherries purchased in the food store (Stanevičienė *et al.*, 2021). Research group of Abdelfattah and colleagues investigated the abundance of fungal microorganisms on different parts of mature apple: peel, wounded flesh, ends of calyx and

stem. Fungi of *Penicillium* genus dominated on the apple peel, while *Alternaria* – on ends of stem and calyx (Abdelfattah *et al.*, 2016).

Based on metagenomics analysis, the microbial diversity of fruits and berries is high; however, dominate several groups (Leff and Fierer, 2013; Montesinos *et al.*, 2015). Early research of plant microbiota involved isolation of microorganisms and there was a lack of knowledge on the total composition of fungal microorganisms and bacteria (Valero *et al.*, 2007; Volschenk *et al.*, 2016). Next generation sequencing (NGS), metagenomics analysis accomplished with bioinformatics enabled investigation of microorganisms' communities, including non-cultivable cultures. NGS recently became one of the main tool in the food industry uncovering the dynamics of microorganisms' communities in stages of growth, production, and distribution (Abdelfattah *et al.*, 2016b; Higgins *et al.*, 2018; Leff and Fierer, 2013). High-throughput sequencing technology enabled the effective use of microorganisms for the enrichment of food safety and quality (De Filippis *et al.*, 2018). Nevertheless, knowledge of microorganisms' diversity and distribution in natural habitat remains limited.

1.2 Yeast killer systems

Certain yeasts possess killer trait and produce killer toxins – proteins or glycoproteins, interacting with receptors in the surface of the sensitive cells – that are killing sensitive cells of the same or/and other yeast species. Toxin-producing yeasts are immune to their own toxin (Klassen *et al.*, 2017; Magliani *et al.*, 1997). Killer strains possess their toxins encoded in one of the three elements: chromosome, linear double-stranded DNA plasmid, or double-stranded RNA (Magliani *et al.*, 1997). Toxins encoded in dsRNA are produced by diverse yeast species: *Saccharomyces cerevisiae*, *S. paradoxus*, *S. uvarum*, *S. mikatae*, *S. bayanus*, *S. kudriavzevii*, *Hanseniaspora uvarum*, *Torulaspora delbrueckii*, *Ustilago maydis*, *Zygosaccharomyces bailii*, *Pichia membranifaciens, Metschnikowia pulcherrima,* etc. (Bedir and Kuleasan, 2021; Drinnenberg *et al.*, 2011; Golubev, 2005; Ramírez *et al.*, 2017; Rodríguez-Cousiño *et al.*, 2017; Rowley *et al.*, 2016; Santos *et al.*, 2011; Villalba *et al.*, 2016; Weiler and Schmitt, 2003). The vast majority of dsRNA-encoded killer toxins are identified in *S. cerevisiae* and *S. paradoxus* yeasts (Rowley, 2017). Killer yeasts are common in nature; they are found in all continents, including Antarctica, and on various surfaces (from plants to insects, soil, water, etc.). Laboratory toxin-producing strains are defined in more detail than strains found in natural habitats (Boynton, 2019).

Four *S. cerevisiae* dsRNA-encoded toxins – K1, K2, K28, and Klus – are best described so far, and are different in primary protein sequence, biochemical properties, and mechanisms of action (Dignard *et al.*, 1991; Mannazuu *et al.*, 2019; Rodríguez-Cousiño *et al.*, 2011; Schmitt

and Tipper, 1990; Servienė *et al.*, 2012). K1 and K2 toxins kill sensitive yeast cells in two steps: they bind to the primary receptors β-1,6 glucans in the cell wall (Hutchins and Bussey, 1983; Lukša *et al.*, 2015), afterward, toxin aims to secondary plasma membrane receptor Kre1, resulting in the formation of ion channels and subsequent loss of potassium ions and ATP molecules (Breinig *et al.*, 2002; Martinac *et al.*, 1990; Orentaite *et al.*, 2016; Schaffrath *et al.*, 2018; Schmitt and Breinig, 2002). K28 toxin binds to α-1,3-mannoproteins, located in the cell wall, interacts with plasma membrane-located receptor Erd2, and enters the cell via endocytosis. Intracellular K28 toxin is transferred to the cell nucleus and inhibits DNA synthesis, leading to cell cycle arrest in G1/S stage (Reiter *et al.*, 2005; Schmitt and Tipper, 1995). The mechanism of action of Klus toxin is unrevealed (Rodríguez-Cousiño *et al.*, 2013, 2011).

The majority of yeast killer toxins are encoded in dsRNA viruses, named M satellites. They are maintained by the helper dsRNA virus L-A (Wickner, 1996; Wickner *et al.*, 2013). M virus encodes for toxin, responsible for the killer phenotype. For the effective functioning of a killer system, well-organized cooperation of L-A and M viruses is essential. L-A virus possesses 4,6 kbp segment, coding for major structural capsid protein Gag, which is acting by encapsulation of L-A and M dsRNAs into icosahedral structure VLPs, and for Gag-Pol phusion protein, which is essential for replication and encapsulation of both viruses (Dinman *et al.*, 1991; Icho and Wickner, 1989; Wickner *et al.*, 2013). Several variants of *S. cerevisiae* L-A virus exist – LA-1, LA-2, LA-28, and LA-lus. The identity of their nucleotide sequences is 74 % (Konovalovas *et al.*, 2016; Rodríguez-Cousiño *et al.*, 2013). Certain relationships between L-A and M viruses were revealed (LA-1 and M1, LA-2 and M2, LA-28 and M28, LA-lus and Mlus) (Aitmanaitė *et al.*, 2021; Konovalovas *et al.*, 2016; Rodríguez-Cousiño *et al.*, 2017, 2013).

Four *S. cerevisiae* M dsRNA viruses have been described to date (M1, M2, M28, and Mlus) (Schaffrath *et al.*, 2018). The genome of M virus is 1,6-2,4 kbp size and codes for preprotoxin, which undergoes maturation to become a toxin (K1, K2, K28, and Klus). M dsRNA viruses do not show sequence homology, even though the structural organization of genomes is similar. In the 5‘-terminal region of positive strand is an open reading frame, encoding toxin precursor. Unique AU-rich site followed by variable length non-coding 3‘-terminal site, possessing *cis*-acting elements, required for viral RNA polymerase to perform encapsulation and replication (Rodríguez-Cousiño *et al.*, 2017).

Saccharomyces paradoxus is the closest relative of *S. cerevisiae* yeasts and is prevalent in natural habitats (Rodríguez-Cousiño *et al.*, 2017). It was formerly believed that *S. paradoxus* killer toxins are encoded in chromosomes and only recently dsRNA viruses (L-A and M) were

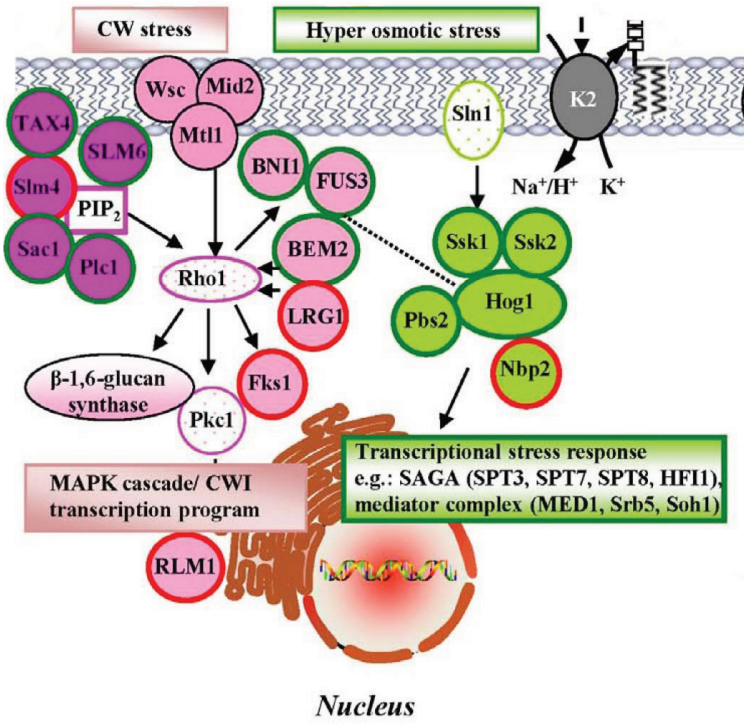
found in this species (Naumov *et al.*, 2005). Genomes of L-A and M dsRNA viruses are similar to those found in *S. cerevisiae* strains: L-A size is 4,6 kbp, and M size varies from 1,6 to 2,8 kbp (Chang *et al.*, 2015; Rodríguez-Cousiño *et al.*, 2017). Six different *S. paradoxus* killer viruses are known – M1L, M21, M28, M45, M62, and M74. They encode novel toxins and their structure and functions are not fully described yet (Fredericks *et al.*, 2021; Rodríguez-Cousiño *et al.*, 2017).

1.3 Cell stress response pathways

In response to killer toxins, yeast cell can activate several stress response pathways, linked to cell wall biogenesis, ion/pH homeostasis, functioning of vacuoles and mitochondria (Carroll *et al.*, 2009; Pagé *et al.*, 2003; Servienė *et al.*, 2012). *S. cerevisiae* cells possess the cell wall integrity, hyperosmotic pressure, phosphoinositol, and pheromone signaling pathways to quickly react to stress-inducing factors: changes in osmotic pressure, heat shock, oxidative stress, pH alterations, nutrient starvation, toxins, etc. (Drogen *et al.*, 2020; Gasch *et al.*, 2000; Levin, 2011; Schuh and Audhya, 2012). Response to stimulus is coordinated via metabolism, gene expression, cellular division, morphological changes, and apoptosis (Elion, 2000).

Protection from stress induced by *S. cerevisiae* K2 toxin involves activation of cell wall integrity (CWI) and high osmolarity glycerol (HOG) pathways (1 Fig., Servienė *et al.*, 2012). HOG signaling pathway is activated by Hog1 kinase, which induces initiation of cytoplasmic (ion flow control, glycerol transport, metabolism of enzymes, protein translation) and nuclear adaptation (changes in cell cycle, gene transcription control) responses (Dihazi *et al.*, 2004; Rodríguez-Peña *et al.*, 2010; Saito and Posas, 2012). The core of HOG pathway is mitogen-activated protein kinase cascade, namely Ssk2, Ssk22, Ste11, Pbs2, and Hog1 (Saito and Posas, 2012).

During stress, a signal is transduced via sensor proteins from the cell surface to Rho1 GTP-ase, which is the major regulator of CWI pathway, responsible for the biogenesis of the cell wall (1 Fig.). Rho1 GTP-ase recruits physiological response via Pkc1-MAPK cascade, 1,3-glucan synthases Fks1 and Gcs2, formin Bni1, and Sec3 effector, resulting in total remodeling of the cell wall (Feldmann, 2012; Levin, 2005). Rho1 GTP-ase is regulated by factors Lrg1, Bem2, Sac7, and Bag7. Phosphoinositol pathway proteins Tax4, Slm4, Slm6, Sac1, and Plc1 in concert with PIP_2 and Rho1 stimulate CWI pathway (Levin, 2011).



1 Fig. *K2 toxin entrance into the target cell and main cell response patterns (modified from Servienė et al., 2012)*

K2 toxin binds to the primary receptors β-1,6-glucans located in the cell wall. K2 is recognized by secondary receptor Kre1 and is integrated into the cell membrane, resulting in disruption of electrochemical ion gradient and loss of K^+ ions. Perturbation of the integrity of the plasma membrane activates several stress response pathways, namely, CWI (pink), HOG (green), and phosphoinositol pathway (purple). Rho1 and Pkc1 transduce stress signal from the plasma membrane to nucleus, leading to activation of specific transcriptional programs. PM – plasma membrane, CWI – cell wall integrity pathway, HOG – high osmolarity glycerol pathway

Many cellular factors affect the formation of response to toxin-induced stress. Genome-wide screening studies revealed hundreds of genetic factors, both shared and unique, involved in response to the majority of toxins synthesized by *S. cerevisiae* yeasts (Carroll *et al.*, 2009; Pagé *et al.*, 2003; Servienė *et al.*, 2012). It was found that sensitivity to K1 toxin is determined by 268 gene products, involved in the biosynthesis of the cell wall components, lipids and sterols, secretion pathway (Pagé *et al.*, 2003). 332 gene products are related to sensitivity to K2 toxin. Genes conferring resistance are linked to the biogenesis of the cell wall, mitochondrial functions, while genes associated with the increased sensitivity are involved in osmosensing and cell wall stress signaling, ion transport, and homeostasis (Servienė *et al.*, 2012). Deletions of certain genes linked to activation of stress signals and protein degradation resulted in increased sensitivity to K28 toxin, while the elimination of particular genes important to endocytosis, lipid structure, and cell wall biogenesis pathways led to resistance to K28 toxin (Carroll *et al.*, 2009).

Genome-wide *S. cerevisiae* analysis revealed that many factors, participating in HOG and CWI pathways are linked to response to K1, K2, and K28 toxins. Inactivation of Hog1 or its main partners Pbs2, Ssk1, and Ssk2 results in higher sensitivity to all of these toxins (Carroll *et al.*, 2009; Pagé *et al.*, 2003; Servienė *et al.*, 2012). Hog1 is tightly interconnected with transcription of many mediating transcriptional activators and repressors, thus the loss of this kinase leads to distinct responses to killer toxins (Saito and Posas, 2012). Deletion of RNA polymerase II transcription factor Δ*iwr1* or polymerase mediator complex subunit Δ*srb5* confers increased resistance to *S. cerevisiae* K1, K2, and K28 killer toxins (Carroll *et al.*, 2009; Pagé *et al.*, 2003; Servienė *et al.*, 2012). Elimination of transcription factors Δ*soh1* or Δ*sfp1* leads to higher sensitivity to K2 and K28 toxins (Carroll *et al.*, 2009; Servienė *et al.*, 2012). Removal of Nbp2, negative regulator of Hog1, responsible for connection of Ptc1 phosphatase into the Pbs2/Hog1 complex, results in resistance to K1 and K2 toxins (Pagé *et al.*, 2003; Servienė *et al.*, 2012). Genome-wide analysis of yeast single mutants revealed a lot of transcriptional regulators (Med1, Snf2, Spt3, Spt7, Spt8, etc.), involved in the cellular response to K2 toxin only (Servienė *et al.*, 2012). Cell wall stress activates CWI response pathway, resulting in cell wall remodeling (Levin, 2011). Transcriptional reprogramming changes the transcription of genes taking part in biogenesis of cell wall, energy generation, metabolism, signal transduction, and stress response (Sanz *et al.*, 2017). Disruption of GTPase-activating Lrg1 and Bem2 proteins leads to sensitivity to K2 toxin. Phenotype change of Δ*bem2* cells might be related to structural defects of the cytoskeleton. Upon elimination of Bni1 and its activator Fus3 kinase, susceptibility to K2 toxin also increases. Inactivation of Rho1 effector Lrg1 leads to increased resistance to K2 toxin, probably because of diminished activity of 1,3-beta-D-glucan synthase Fks1 and subsequent alterations in the cell wall structure. Removal of Rlm1 transcription factor also results in higher resistance to the K2 toxin (Servienė *et al.*, 2012).

HOG response pathway is related to yeast cell adaptation to hyperosmotic stress, while CWI signaling cascade is activated upon cell wall perturbations. In some cases, hyperosmotic stress, heat shock, low pH, and oxidative stress provoke activation of both pathways. It confirms that HOG and CWI pathways can be positively compatible with one another (Rodríguez-Peña *et al.*, 2010). HOG collaborates with CWI pathway by promoting cell wall reorganization via regulation of specific genes required for biosynthesis (Udom *et al.*, 2019). To control various stress conditions and adaptive responses MAPK pathways and their components interact to enhance response flexibility (Fuchs and Mylonakis, 2009; Saxena and Sitaraman, 2016). Interaction of genes, where one gene is repressed or enhanced by one or more other genes is called epistasis (He *et al.*, 2010). Genes are epistatic, when the phenotype of mutation in one gene is changed because of the mutation in another gene (Batenchuk *et al.*, 2010; Steidle *et al.*,

2020). Epistasis is dependent on both genetic and environmental contexts. Environmental stress may have a long-lasting effect on epistasis (Batenchuk *et al.*, 2010; de Visser *et al.*, 2011). *In silico* research has proved that negative interaction usually occurs between genes with overlapping functions, while the positive interaction is seen between genes responsible for functionally different metabolic pathways (He *et al.*, 2010). Epistasis is often applied to describe functional interactions between genes in model organisms (Phillips, 2008; Xu *et al.*, 2011). A limited number of components enforce the same genetic factors to take part in both pathways, e.g. Ste11 belongs to both HOG and CWI pathways (Saxena and Sitaraman, 2016). There is still a gap of knowledge about the interaction mechanisms of MAPK pathways.

2 MATERIALS AND METHODS

The materials and methods used in this work are described in corresponding publications (**I-VI**).

2.1 Collection of samples

During 2015-2018 in Lithuania and Czech Republic were collected ripen fruits and berries (apples, black, red and white currants, chokeberries, sea buckthorn berries), identification of fungal microorganisms inhabiting the surface of collected plant material was performed. Currants were collected in July, chokeberries and apples – in August, sea buckthorn berries – in July and September (**I-III**).

2.2 Sample preparation for metagenomic analysis

Fruits and berries were washed with 0,05 M phosphate buffer. Genomic DNA (gDNA) from the sediments of the collected outwashes was extracted using „Genomic DNA purification kit“ („Thermo Fisher Scientific Baltics“, Vilnius, Lithuania) according to manufacturer‘s recommendations. The quality and quantity of gDNA were evaluated using the „Nanodrop 2000“ spectrophotometer („Thermo Fisher Scientific Baltics“, Vilnius, Lithuania) (**I-III**).

2.3 DNA amplification, library preparation, and bioinformatic analysis

To identify fungal microorganisms, gDNA ITS2 (ribosomal DNA) region was amplified using specific primers: ITS3-KYO2 (5′-GATGAAGAACGYAGYRAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Toju *et al.*, 2012). The library was constructed in Macrogen Inc. (Seoul, South Korea) or BaseClear (Leiden, Netherlands), using „Illumina“ adapters („Illumina“, San Diego, CA, USA). Amplicon library was validated with DNA bioanalyzer „Agilent Technologies 1000“, and quantitative validation was performed by quantitative PCR method, according to „Illumina“ recommendations. Amplicons were sequenced using the „Illumina MiSeq V3“ (2 × 300 bp) platform (Macrogen, Seoul, South Korea; BaseClear, Leiden, Netherlands) (**I-III**).

Bioinformatics analysis of microorganisms' communities was performed using FLASH 1.2.11 (Magoč and Salzberg, 2011), CD-HIT-OTU 4.5.5 (Li *et al.*, 2012), and QIIME v. 1.8 (Caporaso *et al.*, 2010) software (**I-III**).

2.4 Enrichment and identification of cultivable yeasts

Approximately 30 g of aseptically collected berries and fruits were incubated in 5 % dextrose solution at 22 ºC for 15 days. Serial dilutions of selectively enriched yeasts in „Ringer" solution („Merck", Kenilworth, UK) were transferred on YEPD plates with chloramphenicol and incubated at 25 ºC for 2-3 days (**II, III**). Based on the morphology of the colonies (e.g. color and shape), yeast-like colonies were selected, morphologically similar (by color, colony shape) yeast-like colonies were analyzed by light microscope „Leica DM750" (Wetzlar, Germany) and examination proceeded to molecular identification.

2.5 Molecular identification of yeast species

Genomic DNA was extracted from freshly grown yeast cells using „Genomic DNA purification kit" („Thermo Fisher Scientific Baltics", Vilnius, Lithuania) according to the manufacturer's instructions. Specific ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers were employed to amplify the region between 18S and 28S rRNA genes. PCR reaction volume 50 µL: 5 µL 10x buffer solution, 1 µL 2 mM dNTP mixture, 1 µL direct ITS1 and 1 µL reverse ITS4 primers (10 µmol/L), 2,5 U/µL „Dream Taq" DNA polymerase (all materials from „Thermo Fisher Scientific Baltics", Vilnius, Lithuania), 1 µL (5 ng) gDNA and sterile distilled water to reach a final volume of 50 µL. PCR amplification was conducted in „Esco" thermocycler using following conditions: initial denaturation for 5 min. at 94 ºC, followed by 25 cycles of denaturation for 1 min. – 94 ºC, elongation for 1 min. 30 s – 53 ºC and synthesis for 2 min. – 72 ºC. The final synthesis stage occurred for 10 min. at 72 ºC. PCR products were analyzed by 1 % agarose gel electrophoresis (**II-IV**).

PCR products were subjected to reaction with *HinfI* and *CfoI* restriction endonucleases, obtained fragment profiles were analyzed by 2 % agarose gel electrophoresis. PCR products showing different profiles were purified using the „GeneJet PCR purification kit" („Thermo Fisher Scientific Baltics", Vilnius, Lithuania) according to the manufacturer's recommendations and sequenced using ITS1 and ITS4 primers in „BaseClear" (Leiden, Netherlands). Generated

sequences were compared with sequences available in the EMBL-EBI database (http://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html) (**II-IV**).

2.6 Determination of yeast killing trait and susceptibility

To detect killer phenotype, a stroke of tested yeast strain was transferred on plates with the lawn of MB medium with sensitive *S. cerevisiae* α‘1 strain (2×10^6 cells/plate) or strains of other yeast species. To determine susceptibility, tested strains are transferred into the lawn, while killer strains are stroke onto the lawn. Formation or absence of lysis zones – indicators of killer phenotype or susceptibility phenotype – was registered after incubation at 25 ºC for 2 days (**IV-VI**).

2.7 Double stranded RNA extraction

Total RNA extraction was performed using a previously published method (Fried and Fink, 1978) with several modifications. Freshly grown yeast cells were collected and washed with 50 mM EDTA, adding 1/10 of the initial yeast culture volume. Collected cells were suspended in 50 mM TrisCl pH 9.3 and 1 % β-mercaptoethanol solution, using 1/10 of the initial yeast culture volume, and incubated at room temperature for 15 minutes. TES (0.01 M TrisCl pH 7.5, 0.01 M EDTA, 0.2% SDS, and 0.1 M NaCl) buffer (2/10 of the initial volume) and equal part of phenol were added to the pelleted cells, vortexed thoroughly, and incubated for 45 min. with shaking at room temperature. Phases were separated by centrifugation. Nucleic acids (NA) were precipitated by adding an equal volume of isopropanol and 1/10 volume of 3M sodium acetate pH 5.2 to the separated aqueous phase. Sediments were washed with 75 % ethanol and dissolved in nuclease-free water. DNA was eliminated by adding DNAse I and 1/10 volume of reaction buffer, followed by incubation at 37 ºC for 30 minutes, and subsequent DNAse I inactivation. NA were analyzed by 1 % agarose gel electrophoresis.

Total RNA was incubated with 2.8 M LiCl at 4 °C for 16-24 hours. Single-stranded RNA was eliminated by centrifugation for 45 min. 18 000 × *g*. Double-stranded RNA was precipitated by adding an equal volume of isopropanol and 3 M NaCl (1/10 of volume). Sediments were washed with 75 % ethanol and dissolved in nuclease-free water. Extracted dsRNA L-A and M fractions were analyzed by 1 % agarose gel electrophoresis (**IV**).

2.8 Extraction of killer toxins produced by *Saccharomyces* spp. yeasts

S. paradoxus AML-15-66 strain was cultivated in synthetic toxin extraction medium, pH 4,8 for 4-6 days at 18 ºC, until 0.6-0.8 optical density (OD600). The supernatant was filtered through sterile polyvinylidene fluoride (PVDF) membrane with 0.22 µm pore diameter („Millipore“, Bedford, MA, USA) and 100-fold concentrated using pressure-based „Amicon PM-10“ filtration system, with 10 kDa membrane molecular size permeability(„Sigma-Aldrich“, St. Louis, MO, USA) (**IV**). In the same manner, *S. cerevisiae* K2 toxin was produced and extracted, besides that *S. cerevisiae* M437 strain was grown in synthetic toxin extraction medium, pH 4.0 (Servienė *et al.*, 2012; **V**).

2.9 Functional K66 toxin tests

The activity of K66 toxin was investigated in different acidity MB media (pH from 3.2 up to 6.0). Activity in temperatures of 4 ºC, 15 ºC, 20 ºC, 25 ºC, 30 ºC, and 37 ºC was analyzed (**IV**).

2.10 Screening of yeast genetic factors and bioinformatic analysis

S. cerevisiae strains with single gene deletions (YKO library) and parental BY4741 strain were seeded to form an overlay in plates with MB medium, pH 4.8 (2×10^6 cells/plate). Equal size wells were formed in plates and filled with 100-fold concentrated toxin solution. After 2 days of incubation at 25 ºC, lysis zones formed around wells with *S. paradoxus* K66 or *S. cerevisiae* K2 toxin in plates with YKO library cells were compared with the zone in a plate with control BY4741 strain (Servienė *et al.*, 2012; **IV, VI**). This test was adapted to K66 toxin investigation from the screening method used for K2 toxin (**V**). Drops of tested toxin, or toxin-producing cells can also be placed onto the overlay of sensitive yeast cells (**V**).

Gene ontology (GO) analysis was performed using „BiNGO 3.0.3“ plugin in „Cytoscape 3.6.1“ software (Maere *et al.*, 2005). P value significance score was calculated using hypergeometric test, using the Benjamini and Hochberg false discovery rate correction (Haynes, 2013) (**IV**).

Networks of interacting genetic factors were visualized using the „STRING 10“ database (http://string-db.org) (Szklarczyk *et al.*, 2011). Networks were created using the “confidence view” option and the strength of the associations is reflected in line thickness. Medium confidence score – 0,4 was chosen (**IV, VI**).

2.11 K66 toxin binding to *S. cerevisiae* single gene deletion mutants and polysaccharides

Freshly grown BY4741 (control) and cells of tested *S. cerevisiae* strains were incubated at 4 ºC with 100-fold concentrated K66 toxin. To evaluate K66 binding to polysaccharides, namely chitin, laminarin, pullulan, and pustulan, concentrated K66 toxin was mixed with each polysaccharide. Samples were incubated for 1 h at 25 ºC. The supernatant was checked using the well assay. Lysis zones were evaluated after 2 days of plates incubation at 25 ºC (**IV**).

2.12 Construction and validation of double mutants

PCR product, possessing *HIS3* gene with 3ʿ- and 5ʿ- flanking sequences, was amplified using forward F-HphN-delHog1 (5′-GGAACAAAGGGAAAACAGGGAAAACTACAACTATCGTATATAATAATGCGTACGC TGCAGGTCGAC-3′) and reverse R-HphN-delHog1 (5′-CAAAAAGAAGTAAGAATGAGTGGTTAGGGACATTAAAAAAACACGTTTAATCGAT GAATTCGAGCTCG-3′) primers. pYM15 plasmid served as DNA matrix („Euroscarf"). PCR reaction mixture volume 100 µL: 50 µL 2x „DreamTaq Green PCR Master Mix" („Thermo Fisher Scientific Baltics", Vilnius, Lithuania), 3.2 µL of each primer (10 µmol/L), 100 ng of plasmid DNA and nuclease-free water to the final volume of 100 µL. PCR cassette amplification was conducted in „Esco " thermocycler according to this program: initial denaturation for 3 min. at 95 ºC, followed by 30 cycles of denaturation for 30 s – 95 ºC, elongation for 30 s – 54 ºC, and synthesis for 2 min. 40 s – 72 ºC. The final synthesis step lasted for 4 min. at 72 ºC. PCR product was purified using „GeneJet PCR purification kit" („Thermo Fisher Scientific Baltics", Vilnius, Lithuania) according to manufacturers' instructions and analyzed by 1 % agarose gel electrophoresis (**VI**).

To integrate the PCR cassette into the yeast genome by homologous recombination, yeast transformation was performed by LiCl method (Guthrie and Fink, 1991) with several modifications. Positive transformants with *HOG1* gene replaced into *HIS3* were analyzed after incubation at 30 ºC for 5 days (**VI**).

To confirm *HOG1* deletion, protein electrophoresis in 10 % SDS-polyacrylamide gel and Western blot hybridization, using specific primary anti-Hog1 antibodies (1:3000) and specific anti-rabbit IgG secondary antibodies (1:5000) were performed. The membrane was visualized by means of a colorimetric NBT/BCIP system („Thermo Fisher Scientific Baltics", Vilnius, Lithuania), according to manufacturers' recommendations (**VI**).

2.13 Yeast growth rate determination

Growth of tested cultures was observed by measuring absorption at 600 nm wave length, starting from 0.1 OD. Experiment was done in triplicate. Growth rates were calculated using „GraphPad Prism“ software (Olivares-Marin *et al.*, 2018) (**VI**).

3 RESULTS AND DISCUSSION

3.1 Prevalence of fungal microorganisms and killer systems in fruit-berry ecosystems

For the first time the diversity of fungal microorganisms on the surface of Lithuanian chokeberries, white and red currants, sea buckthorn berries (**I**), Lithuanian and Czech apples, and black currants (**III**) was determined using a metagenomics approach. Sea buckthorn mycobiota was analyzed at early and late harvesting periods (**II**). The dependence of mycobiota on fruits and berries, their ripening stage, and the habitat was analyzed (**I-III**).

3.1.1 Fungal microbiota dependence on berries

Fungal microorganisms identified on the surface of chokeberries belong to dominating Ascomycota (66,2 %) and Basidiomycota (32,2 %) phyla (2A Fig. and S2 Table, **I**). Ascomycota phylum was represented by Dothideomycetes and Taphrinomycetes, while Basidiomycota – by Tremellomycetes, Microbotryomycetes, and Exobasidiomycetes (2B Fig., **I**). From 34 detected families Davidiellaceae (20,8 %), Dothioraceae (7,9 %), and Taphrinaceae (3,5 %) were dominating. These families are represented by *Cladosporium* (20,8 %) and *Taphrina* (3,5 %) genera. Basidiomycota phylum at genus level was represented by *Cryptococcus* (10,9 %) and *Rhodotorula* (9,1 %) (Fig. 2D, **I**). Low amounts of *Phoma*, *Mrakiella*, *Lewia,* and *Hanseniaspora* genera were detected (**I**). 45,6 % of fungal microorganisms were not identified at the genus level (Fig. 2D and Table S2, **I**).

On red and white currants were detected 2 prevailing eukaryotic microorganisms‘ phyla, 59 families, and 106 genera. Alike on sea buckthorn berries and chokeberries, dominant phylum on both currants – Ascomycota (68,3 % on red and 57,3 % on white currants), and less – Basidiomycota (28,2 % and 39,7 %, accordingly) (Fig. 2A, **I**). The most abundant class – Dothideomycetes (53,1 % on red and 51,5 % on white currants) (Fig. 2B, **I**), possessing Dothioraceae and Davidiellaceae families (Fig. 2C, **I**). Glomerellaceae (9,1 %) family, represented by *Colletotrichum* genus, was found only on red currants. On both currants dominant generas were *Cladosporium* (17,3 % on red and 20,7 % on white currants), *Cryptococcus* (11,5 % and 21 %, respectively), *Phoma* (4,8 % and 8,3 %) and *Rhodotorula* (1,6 % and 3 %) (Fig. 2D, **I**). Unidentified fungal microorganisms on red currants composed 40,2 %, on white – 31,8 % (Fig. 2D and S2 Table, **I**).

On the surface of sea buckthorn berries 4 dominant fungal microorganisms‘ phyla, 58 families, and 108 genera were found. Alike on chokeberries, white and red currants Ascomycota (89,4 %) was dominant, following Basidiomycota (8,2 %) (Fig. 2A, **I**). Dothideomycetes prevail at class level, Tremellomycetes and others were also reported (Fig. 2B, **I**). At the family rank, Dothioraceae (78 %) was dominant, significantly less Davidiellaceae (2,4 %), and even less Taphrinaceae and Saccharomycodaceae were found (Fig. 2C, **I**). A large part, even 87,9 % of fungal microorganisms were unidentified at the genus level (Fig. 2D and Table S2, **I**).

For the first time differences of fungal microorganisms‘ communities on sea buckthorn berries were evaluated at different ripening stages (**II**). It was determined that the composition of fungal microorganisms on ripe and unripe sea buckthorn berries is distinct. On unripe and ripen sea buckthorn berries 4 phyla of fungal microorganisms prevail, 69 orders, and 144 families (Table S1, **II**). Ascomycota dominates on both sea buckthorn samples (85 % on unripe and 97,6 % on ripen berries), significantly less Basidiomycota was found (10,6 % and 1,7 %, respectively) and other fungi (Fig. 2A, **II**). The impact of the ripening stage for the diversity of fungal microorganisms was firstly observed at the class rank. A large proportion of Dothideomycetes was found in both samples (54,0 % on unripe and 44,6 % on ripe berries) (Fig. 2B, **II**). On unripen berries Taphrinomycetes (18,4 %) and Tremellomycetes (7,6 %) was commonly detected, whereas on ripe – Saccharomycetes (47,6 %). At the family level on sea buckthorn berries of both ripening stages prevails Aureobasidiaceae, while on unripe berries Taphrinaceae was also found, and on ripe – Metschnikowiaceae (Fig. 2C, **II**). In total 196 genera were identified, on unripen berries *Aureobasidium* (31,4 %), *Taphrina* (18,4 %), and *Cladosporium* (8,1 %) prevailed, while on ripe – *Metschnikowia* (44,5 %) and *Aureobasidium* (40,6 %) (Fig. 2D, **II**). *Aureobasidium* genus members were more common on ripen than on unripe sea buckthorn berries. *Aureobasidium* species show biocontrol activity towards *Botrytis*, *Bacillus*, *Colletotrichum*, and *Penicillium* phytopathogens (Grube *et al.*, 2011). *Taphrina* are known as biotrophic plant pathogens, inducing plant diseases, activating different plant hormones (Tsai *et al.*, 2014). Members of *Cladosporium* can produce antifungal compounds or cause plant and human diseases (Sandoval-Denis *et al.*, 2016; Wang *et al.*, 2013).

A part of yeasts unidentified by the metagenomic analysis was cultivated under selective conditions and species were determined using molecular biology tools (gDNA extraction, ITS region amplification, restriction fragment length polymorphism analysis, and sequencing). From the surface of white and red currants, chokeberries, and ripe sea buckthorn berries, *Hanseniaspora uvarum* and *Pichia kudriavzevii* yeasts were amplified. *Pichia anomala*, *Metschnikowia pulcherrima* yeasts were detected on red currants and chokeberries. *Candida zemplinina* was identified on white currants and sea buckthorn berries (**12C, 14C**). On the latter,

Candida californica was also found. *Pichia membranifaciens* was identified on sea buckthorn berries and chokeberries (**6C**). *Pichia nakasei* was only found on sea buckthorn berries. The vast majority of cultivable yeasts, not detected on other berries were observed on chokeberries: *Hanseniaspora* spp., *Kazachstania* spp., *Torulaspora delbrueckii*, *Zygosaccharomyces microellipsoides*, *Zygotorulaspora florentina*, *Lachancea* spp., *Saccharomyces cerevisiae*, and *Saccharomyces paradoxus*. *S. cerevisiae* was also found on sea buckthorn berries (**6C**).

From the surface of unripe and ripe sea buckthorn berries more than 20 yeast strains were amplified using enrichment strategy. *Aureobasidium pullulans* was common on both sea buckthorn samples. *Metschnikowia pulcherrima* was most prevalent on ripe berries, whereas *Cryptococcus wieringae* – on the contrary: was more abundant on unripe sea buckthorn berries. The least abundant species on both sea buckthorn samples was *Hanseniaspora uvarum* (**II**). Yeasts assigned to *Hanseniaspora* and *Metschnikowia* genera possess antagonistic properties against molds that promote fruit rotting (Janisiewicz *et al.*, 2001; Liu *et al.*, 2010; Tilocca *et al.*, 2020). *M. pulcherrima* can act as a biocontrol agent by active competition for nutrients and synthesis of volatile organic compounds (Contarino *et al.*, 2019; Parafati *et al.*, 2015).

PCoA analysis revealed the differences between the composition of fungal microorganisms‘ communities of sea buckthorn berries, chokeberries, and white and red currants. Mycobiotas of red and white currants were grouped together, while those of sea buckthorn berries and chokeberries were clearly separated from one another and from both currants (Fig. 3B, **I**). It means that the fungal composition of both currants is similar but distinct from sea buckthorn berries and chokeberries, which also possess different microbial populations. Mycobiotas of mature and unripe sea buckthorn berries were clearly separated in PCoA analysis (Fig. 1, **II**). Thus, sea buckthorn berries have different fungal communities at distinct ripening stages.

The diversity of fungal microorganisms, differences in composition, and abundance are usually dependent on plant and the ripening stage. Occurence of fungal microorganisms on sea buckthorn berries, chokeberries, red and white currants is important to maintain the balance between potentially beneficial and pathogenic microorganisms in nature. It is relevant for the enhancement of plant growth conditions and protection from bacterial and fungal diseases. In addition, increased demand for natural, minimally processed plant-based foods, encourages elaboration of detailed plant mycobiota analysis (**I, II**).

3.1.2 Distribution of fungal microorganisms based on plant habitat

Analysis of metagenomics data revealed that the composition of fungal microorganisms on apples grown in Lithuania and Czech Republic is similar: Ascomycota phylum (86,3 % on Lithuanian and 86,8 % on Czech apples) is dominant, and Basidiomycota (12,5 % and 11,2 %, respectively) (Fig. 3A, **III**) is present at small level. At the class rank, Saccharomycetes (70,2 % on Lithuanian and 63 % on Czech apples) prevailed (Fig. 3B, **III**) and was not described at genus and species levels (Table S2, **III**).

Slight differences between the distribution of fungal microorganisms on Lithuanian and Czech apples were observed at genus level: *Cryptococcus* (5,7 %), *Cladosporium* (4,2 %), and *Hanseniaspora* (3,3 %) prevail on fruits cultivated in Lithuania, while in Czech Republic *Hanseniaspora* (17,6 %) was largely detected. Certain genera were found only in one country: *Stachybotrys* was detected on Lithuanian apples, while *Wickerhamomyces* – on Czech apples (Fig. 4B, **III**). These differences may be determined by distinct climatic conditions. A small proportion of *Pichia terricola*, *P. fermentans*, *Torulaspora delbrueckii,* and *Saccharomyces cerevisiae* yeasts were detected by next-generation sequencing but they were identified in enrichment cultivation experiments (Table S2, **III; 14C**). The abundance of fermenting yeasts depends on plant‘s habitat and the ripening stage of fruit. A lower number of fermenting yeasts were determined by slightly earlier harvesting time when the fruit surface was intact and the amount of sugar available for yeasts was low. *Hanseniaspora* sp., *Pichia* sp., *Metschnikowia pulcherrima, Wickerhamomyces* and *Saccharomyces cerevisiae* yeasts were also isolated from apples, plums and pears after incubation in fermentation conditions for 15 days (Vadkertiová *et al.*, 2012). *Wickerhamomyces* sp. and *Metschnikowia* sp. yeasts can act as biocontrol agents and protect plants from pathogens, and regulate the composition of microbiota (Abdelfattah *et al.*, 2016b; Muccilli and Restuccia, 2015; Parafati *et al.*, 2015; Vadkertiová *et al.*, 2012).

Distribution of fungi at phylum level on Lithuanian and Czech black currants is similar and not differ from apples of both countries: Ascomycota (71,5 % on Lithuanian and 92,2 % on Czech black currants) dominates, and Basidiomycota (24,2 % and 7 %, accordingly) is detected at lower amount (Fig. 3A, **III**). At the class rank, Dothideomycetes are found on Lithuanian berries, on Czech – Saccharomycetes, and a slightly less of Dothideomycetes (Fig. 3B, **III**). The broad diversity of fungal microorganisms was observed at family and genus ranks on black currants harvested in Lithuania and Czech Republic. On Lithuanian black currants dominate *Cladosporium* (45,4 %) and *Cryptococcus* (15,2 %) yeast genera, while on Czech – *Hanseniaspora* (48,5 %), *Cladosporium* (5,2 %) and *Rhodotorula* (1,5 %) yeasts (Table S2, **III**). Thus, the mycobiota of black currants is different in a geographical context. *Hanseniaspora* sp.

is a less fermenting yeast species, also found on the surface of grapes, strawberries, and apples (Graça *et al.*, 2015; Santo *et al.*, 2012). *Cladosporium* spp. is a representative of Dothideomycetes, which was identified on all tested samples of fruits and berries, and mostly abundant on Lithuanian black currants. *Cladosporium* spp. is widely distributed on various plant surfaces (Abdelfattah *et al.*, 2016a, 2016b). *Cryptococcus* and pigmented *Rhodotorula* yeasts were not only found on apple and black currants tested in this work but also on strawberries (Abdelfattah *et al.*, 2016a), grapes (Barata *et al.*, 2012; Setati *et al.*, 2012), pears, plums, and apples (Vadkertiová *et al.*, 2012). *Rhodotorula* sp. forms biofilm on the surface of grapes throughout the process of ripening (Lederer *et al.*, 2013). *Cryptococcus* is established as a typical yeast community member at the early fruit ripening stage (Janisiewicz *et al.*, 2014; Vadkertiová *et al.*, 2012). These fungal microorganisms are often isolated from fruit outwashes and are employed as biocontrol measures to protect the harvest from diseases during transportation and storage (Liu *et al.*, 2013). *Hanseniaspora*, *Rhodotorula*, and *Dioszegia* are beneficial for plants fungal microorganisms, while members of *Phoma*, *Lewia*, *Colletotrichum*, *Septoria*, and *Taphrina* belong to potential pathogens (Cissé *et al.*, 2013; Eyal, 1999; Phalip *et al.*, 2006; Renker *et al.*, 2004).

Using selective enrichment conditions fermentative *Hanseniaspora* spp., *H. uvarum*, *Metschnikowia* spp., *S. cerevisiae*, *P. fermentans*, and *P. terricola* yeasts were found on Lithuanian and Czech black currants and apples (Table S3, **III; 12C, 14C**). *M. pulcherrima*, *P. membranifaciens,* and *T. delbruecki* yeasts were absent only on Lithuanian black currants, *P. kudriavzevii* – only on Czech, and *Wickerhamomyces anomalus* – on Lithuanian apples. *Candida boidinii* yeasts were detected only on Lithuanian apples. *S. paradoxus* was only found on black currants and apples grown in Lithuania but not in Czech Republic. More *S. cerevisiae* than *S. paradoxus* yeasts were identified on the surface of black currants and apples (**III; 12C, 14C**).

According to PCoA analysis data, fungal microorganisms communities from apples collected in different countries were grouped together, indicating their similarity. Lithuanian and Czech black currants‘ mycobiotas are clearly separated from one another and those of apples‘ from both countries. It points out to different mycobiota compositions between black currants from both countries and Lithuanian and Czech apples mycobiota (Fig. 2B, **III**).

Conducted metagenomic research of fruits and berries revealed that the diversity of fungal microorganisms is more dependent on the plant than on the growth location of fruits or berries. Besides, plant growth conditions play a major role in mycobiota‘s diversity. Quantitative differences between fungal microorganisms‘ populations were likely not only affected by climatic conditions but also by the ripening stage (**III**).

3.1.3 The occurence of viral killer systems on fruits and berries

Cultivation enrichment of yeasts sometimes enables the discovery of toxin-producing species: *Pichia membranifaciens*, *P. kluyveri*, *P. inositovora*, *P. anomala*, *P. farinosa*, *Hansenula mrakii*, *H. saturnus*, *Tilletiopsis albenscens*, *Kluyveromyces lactis*, *Cryptococcus humicola*, *S. cerevisiae*, *S. paradoxus,* etc. (Marquina *et al.*, 2002). Killer toxins despatch sensitive yeast strains and in concert with volatile compounds and antifungals affect the structure of yeast community on fruits and berries (Boynton, 2019; Buser *et al.*, 2014; Contarino *et al.*, 2019; Droby *et al.*, 2009; Freimoser *et al.*, 2019; Parafati *et al.*, 2017).

Investigation of yeasts isolated from the surface of black, red and white currants, apples, chokeberries, sea buckthorn berries led to the discovery of 104 killer trait possessing strains (**12C, 14C**). The majority of killer yeasts were identifien in *P. kluyveri*, *P. anomala,* and *P. membranifaciens* species. Killing phenotype was observed in certain members of *Metschnikowia* spp., *Saccharomyces* spp., *H. uvarum*, *T. delbrueckii*, *Kwoniella shandongensis*, *Kazachstania bulderri*, *Kazachstania africana*, *Zygotorulaspora florentina*, and *Aureobasidium pullulans* species.

Out of 104 killer strains, 15 have dsRNA-encoded systems. Viral killer systems were identified in *S. cerevisiae*, *S. paradoxus*, *P. membranifaciens*, *H. uvarum*, and *Metschnikowia* sp. yeasts (**12C, 14C**). The majority of virus-based dsRNA systems belong to members *S. paradoxus* (5) and *S. cerevisiae* (5) species. Three dsRNA-encoded killer systems were reported in *Metschnikowia* genus, and one in both, *P. membranifaciens* and *H. uvarum* yeasts.

S. paradoxus killer strains were amplified from the surface of white (1) and black currants (2), apples (1), and chokeberries (1) (**IV**). Enrichment of yeasts from red currants yielded only one *S. cerevisiae* killer strain, and two strains from each, apple and sea buckthorn (**12C, 14C**). *Metschnikowia* genus yeasts possessing dsRNA-encoded killer toxins were amplified from white (1) and black (1) currants, as well as chokeberry (1) (**12C, 14C**). *H. uvarum* (1) and *P. membranifaciens* (1) killer strains were found exclusively on apples (**12C**).

Rodriguez-Cousino and colleagues reported that the size of L-A virus genome in *S. cerevisiae* and *S. paradoxus* species is 4,6 kbp (Rodríguez-Cousiño *et al.*, 2017). The size of both L-A viruses of investigated *Saccharomyces* genera strains was similar – about 4,5 kbp (**12C, 14C**). Investigated *S. cerevisiae* yeasts had M dsRNA from 1,6 kbp to 1,8 kbp in size (**12C, 14C**), consistent with sizes described in the literature (1,6-2,4 kbp) (Rodríguez-Cousiño *et al.*, 2017). Certain isolated *S. paradoxus* strains have larger M virus genomes than *S. cerevisiae*, since 2,8 kbp M dsRNA was detected. Size of M virus dsRNA in tested *S. paradoxus* strains

varies from 1,7 kbp to 2,8 kbp (**12C, 14C**) and corresponds to published data (1,6-2,8 kbp) (Rodríguez-Cousiño *et al.*, 2017).

3.2 Characterisation of *Saccharomyces paradoxus* K66 killer system

Up to 20% of yeasts, isolated from the surface of fruits and berries, exhibit the killing phenotype (Boynton, 2019). From the surface of serviceberries (lot. *Amelanchier ovalis*) *Saccharomyces paradoxus* AML-15-66 killer strain, producing newly discovered dsRNA-encoded K66 toxin, was identified and described (**IV**). This type of viral killer system was also found on apples and black currants (**12C, 13C**).

Yeasts strain AML-15-66 was isolated from the enriched spontaneous fermentation of serviceberries. Tested strain was confirmed belonging to *S. paradoxus* species by sequencing of ITS region (Fig. S1A, **IV**) and restriction fragment length polymorphism analysis (Fig. S1B, **IV**). Since *S. paradoxus* yeasts can potentially produce killer toxins, the killing activity of AML-15-66 strain was tested on a lawn of sensitive *S. cerevisiae* α‘1 strain (pH 4,0). Yeasts killer toxins can be encoded in a chromosome, linear double-stranded DNA (dsDNA) plasmid, and viral double-stranded RNA (dsRNA) (Magliani *et al.*, 1997). Toxins encoded in dsRNA and produced by *Saccharomyces* spp. yeasts have been studied the most extensively (Rowley, 2017). Total RNA extraction from *S. paradoxus* AML-15-66 strain and analysis confirmed that K66 toxin is also encoded in M dsRNA, 1,7 kbp in size. Preprotoxin sequence analysis revealed three potential Kex2 protease hydrolysis sites: Arg59, Arg181, and Arg239 (Fig. 3, **IV**). Sites of disulfide bonds remain ambiguous. In the K66 protein sequence five N-glycosylation sites were found, four of which correspond to those found in K21 toxin (Rodríguez-Cousiño *et al.*, 2017). 141 amino acid residue has the highest modification probability (**IV**).

It was determined that *S. paradoxus* AML-15-66 yeasts exhibit killing activity against *S. cerevisiae* strains when growth medium pH value varies from 3.6 to 5.6 (Table 1, **IV**). Partially purified *S. paradoxus* K66 viral protein activity was detected from pH 3.6 to pH 5.2, with the highest activity between pH 4.4 and pH 4.8. K66 toxin is inactive at pH values 3.2, 5.6, and 6.0 (**IV**). In an acidic environment (pH 4.0 and pH 3.6) toxin activity diminishes to 38 % and 25 %, accordingly, while at pH 5.2 50 % of protein activity remains. Analysis of survival of yeast cells upon exposure to K66 toxin at temperatures from 4 ºC to 37 ºC, revealed that purified protein is active at 15-30 ºC, with optimal activity at 20 ºC, whereas is inactive at limiting 4 ºC and 37 ºC temperature (**IV**). Determined values of optimal toxin action correlate with the toxin stability and activity values of other described killer toxins. Mycoviral killer toxins are usually active from pH 4.0 to pH 5.4 at a temperature below 30 ºC (Meinhardt and Klassen, 2009; Muccilli and

Restuccia, 2015). Conducive conditions for settlement of *Saccharomyces* killer species were found on fruits with lower pH at optimal 20-30 ºC temperature (Lukša *et al.*, 2016).

S. cerevisiae killer toxins have a narrow scope of targets since they only inhibit a few strains of the same genus or species (Mannazzu *et al.*, 2002). Klus toxin is an exception because it acts against a broader specter of yeasts (Rodríguez-Cousiño *et al.*, 2011). *S. paradoxus* K66 toxin has a similar killing activity, alike *S. cerevisiae* killer yeasts: acts against *Saccharomyces* but not other yeast genera. The strongest K66 toxin killer activity was observed against sensitive *S. cerevisiae* α‘1 and BY4741 strains. From all tested killer strains, K66 toxin the most effectively killed *S. cerevisiae* M300 strain, producing K28 viral protein. The weakest killing activity was observed against *S. cerevisiae* M437 yeast cells, producing K2 toxin. The tested toxin does not kill *Pichia* spp., *Hanseniaspora* spp., *Candida* spp., and *Torulaspora* spp. yeasts. *S. paradoxus* AML-15-66 strain, producing K66 toxin, is sensitive to the action of *S. cerevisiae* K2 toxin and resistant to *S. cerevisiae* K1, K28, Klus, and *S. paradoxus* K21 mycotoxins (**IV**).

3.3 Screening for genetic factors modulating the action of *S. paradoxus* K66 toxin

To determine genetic factors, significant to the action of *S. paradoxus* K66 toxin and involved in the formation of resistance, phenotypic changes of 526 *S. cerevisiae* strains, bearing single gene deletion, upon exposure to K66 toxin were recorded (**IV**). The tested gene deletions were selected based on the previously published data of *S. cerevisiae* K1, K2, and K28 toxins (Carroll *et al.*, 2009; Pagé *et al.*, 2003; Servienė *et al.*, 2012). Screening for genetic factors affecting the action of K66 toxin was conducted employing a novel method, which was previously used for detection of genetic factors, involved in response to *S. cerevisiae* K2 killer toxin formation (**V**). Placing yeast from the YKO library onto the lawn with toxin-producing cells or purified toxin (survival test) enhances the method sensitivity, experiment speed, and costs, in comparison with the traditional killing assay. This novel screening method is applied for the identification of genetic factors, involved in the action of environmental killer yeast toxins. What is more, survival test enables detection of previously unidentified factors, with the unknown roles in toxin response pathways (**V**).

During the screening, mutants of 125 *S. cerevisiae* genetic factors demonstrated distinct levels of phenotypic response to K66 toxin: 73 were more resistant than the control BY4741 strain, while the remaining 52 were more sensitive (**IV**). All genetic factors, determining sensitivity and resistance to K66 toxin, were grouped. The largest groups involved genes, linked to the cell wall biogenesis (15), membrane formation, secretion, and transport (16), chromatin structure and gene expression (18), and translation (13) (Fig. 5, **IV**). Removal of 19 genes

resulted in the same phenotype in response to *S. cerevisiae* K1, K2, K28, and *S. paradoxus* K66 toxins. Different response to K66 and some *S. cerevisiae* toxins was formed in 13 tested single gene mutants. 85 % of genetic factors, identified in the K66 toxin screen were also shared with K1 and K2 toxins, likely because of the similar mechanism of the action. 19 genetic factors were shared in both *S. paradoxus* K66 and *S. cerevisiae* K28 toxin screens. Several products of these genes are involved in endocytosis, while the role of remaining, regarding K66 toxin, is unclear (**IV; 13C**).

Gene ontology (GO) analysis revealed statistically significant enrichment of genes, associated with the cell wall biogenesis, osmotic stress response, and signaling pathways. The importance of cell wall components to the functioning of K66 toxin suggests that K66 acts by disruption of ion homeostasis. It is supported by the importance of a high number of osmotic-stress-related genes (**IV**).

Integrated functional interaction network (medium confidence score) of tested proteins was acquired from the STRING 10 database (Fig. 6, **IV**). This work demonstrated that the majority of identified genetic factors are responsible for stress response and signaling pathways, cell wall biogenesis, belong to ribosomal components, and are linked to translation, the membrane and protein transmembrane transport (**IV**).

3.4 Identification of *S. paradoxus* K66 toxin primary receptors

To determine *S. paradoxus* K66 toxin primary receptors in the cell wall, viral protein binding to yeast cells with different levels of β-1,3- and β-1,6-glucans (*in vivo* assay), and polysaccharides with distinct glucan links (*in vitro* assay) were investigated (Fig. 7, **IV**). It was found that Δ*aim26*, Δ*smi1,* and Δ*kre1* strains, possessing lower amount of β-1,6-glucans bind from 35 % to 42 % fewer K66 toxin molecules, in comparison with control BY4741 cells (Fig. 7A, **IV**). K66 toxin killing activity does not depend on the β-1,3-glucan level in the cell wall (amount of β-1,3 glucan in Δ*aim26* strain is the same as in the wild type cells, in Δ*smi1* – 50 % decreased, and in Δ*kre1* – 10 % increased) (Fig. 7B, **IV**). When the level of β-1,6 links rises, like in Δ*bud27*, Δ*map1,* and Δ*end3* cells*,* K66 toxin binding efficiency increases by about 30 %, regarding control cells.

Defects of the cell wall structure affect K66 toxin binding efficiency, like in the case of *S. cerevisiae* K1 and K2 toxins (Lukša *et al.*, 2015; Novotná, 2004; Pagé *et al.*, 2003; Servienė *et al.*, 2012). Correlation between genes, deletions of which resulted in diminished K66 toxin binding activity and enlarged resistance to the toxin, was observed. This was expected, since toxin firstly interacts with the components of cell wall and plasma membrane. Genes, deletions

of which results in higher toxin binding efficiency, correlate with increased sensitivity to K66 toxin (**IV**).

To establish the importance of β-1,6 glucans for K66 toxin binding *in vivo*, toxin ability to bind polysaccharides with different glucan links was evaluated (Fig. 7C, **IV**). After K66 toxin incubation with laminarin (β-1,3 and β-1,6 links), pustulan (β-1,6 link), pullulan (α-1,4 and α-1,6 links), and chitin (β-1,4 link) the remaining toxin activity was investigated using lysis zone assay by employing sensitive *S. cerevisiae* α‘1 cells. Free toxin forms lysis zones. Competitive inhibition of viral protein activity shows that only β-1,6-glucan in pustulan binds to the K66 toxin. Its activity was completely abolished with pustulan. None of the remaining tested polysaccharides was binding K66 protein, it was confirmed by lysis zones equal in size to the control sample without polysaccharides.

In vitro and *in vivo* specific binding investigations confirmed that the primary receptor of *S. paradoxus* K66 toxin is β-1,6-glucan (**IV**), as of *S. cerevisiae* K1 and K2 toxins (Hutchins and Bussey, 1983; Lukša *et al.*, 2015).

3.5 Interaction of yeast genetic factors in response to *Saccharomyces* spp. viral killer systems

S. cerevisiae K2 toxin binds to the cell wall and interacts with the plasma membrane, resulting in the leakage of K^+ ions and other molecules (Marquina *et al.*, 2002; Pagé *et al.*, 2003). Disorders of HOG and CWI signaling pathways disrupt cell wall integrity mechanisms and osmoregulation, leading to changes of sensitivity to K2 toxin (**VI**). Based on previous data of K2 toxin action modulating genetic factors (Servienė *et al.*, 2012), 12 single gene mutations of genes (Δ*ssk1*, Δ*ssk2*, Δ*soh1*, Δ*slm4*, Δ*rlm1*, Δ*lrg1*, Δ*med1*, Δ*spt3*, Δ*spt8*, Δ*bem2*, Δ*fus3*, Δ*tax4*) involved in CWI and HOG pathways, and changing sensitivity to K2 toxin were selected (Table 1, **VI**). *SSK1* and *SSK2* genes are strongly associated with *HOG1* and elimination of these genes leads to sensitivity to *S. cerevisiae* K1, K2, K28, and *S. paradoxus* K66 toxins (Carroll *et al.*, 2009; Pagé *et al.*, 2003; Servienė *et al.*, 2012; **IV, VI**). Single Δ*soh1* mutant is sensitive, whereas Δ*slm4* mutant is resistant to K2 and K28 toxins but remains as wild type upon treatment with K1 and K66 toxins (Carroll *et al.*, 2009; Pagé *et al.*, 2003; Servienė *et al.*, 2012; **IV, VI).** Mutations Δ*rlm1* and Δ*lrg1* are beneficial for the target cell since they determine greater resistance to *S. cerevisiae* K2 and *S. paradoxus* K66 toxins (Servienė *et al.*, 2012; **IV, VI**). Elimination of *MED1*, *SPT3*, *SPT8*, *BEM2*, *FUS3,* and *TAX4* genes results in sensitivity to K2 toxin, but not to K1, K28, or K66 toxins (Carroll *et al.*, 2009; Pagé *et al.*, 2003; Servienė *et al.*, 2012; **IV, VI**). Selected genes belong to the HOG pathway based on GO (**VI**).

To investigate the relationship between genetic factors involved in HOG and CWI signaling pathways, *HOG1* gene was eliminated from single gene mutants by homology recombination (Janke *et al.*, 2004) and replaced into *HIS3* gene (Fig. 1C, **VI**). Twelve strains posessing double mutations were generated: Δ*ssk1*-Δ*hog1*, Δ*ssk2*-Δ*hog1*, Δ*soh1*-Δ*hog1*, Δ*slm4*-Δ*hog1*, Δ*rlm1*-Δ*hog1*, Δ*lrg1*-Δ*hog1*, Δ*med1*-Δ*hog1*, Δ*spt3*-Δ*hog1*, Δ*spt8*-Δ*hog1*, Δ*bem2*-Δ*hog1*, Δ*fus3*-Δ*hog1*, and Δ*tax4*-Δ*hog1* (**VI**).

The growth of yeast cells depends on pH, temperature, growth medium, and changes at the genetic level (Salari and Salari, 2017). To determine, whether elimination of *HOG1* gene from single gene mutants results in yeast growth rate change, the growth rate of double mutants in rich YEPD medium was evaluated (Fig. 3, **VI**). The elimination of *HOG1* gene from the cell genome does not significantly change growth rate parameters, in comparison with the parental BY4741 strain (Δ*hog1* growth rate is 0,4 ± 0,06 h^{-1}, and BY4741 – 0,49 ± 0,05 h^{-1}), similarly to the elimination of other tested single genes. However, growth rates of certain double mutants were significantly different, in comparison with single mutants: growth rates increased (about 40 %) in Δ*spt8*-Δ*hog1* and Δ*spt3*-Δ*hog1* double mutants, regarding single mutants and the wild type strain. The growth rate of double Δ*bem2*-Δ*hog1* mutant increased by 30 %, compared to the strain with a single *BEM2* deletion (**VI**). Elimination of *HOG1* gene from Δ*soh1* and Δ*rlm1* single mutants resulted in 25 % decreased growth rate. No other statistically significant growth rate differences between single and double mutants were detected. Data on growth rates can be used to determine interactions between genetic factors and evaluate epistatic effect. Strong associations, resulting in great changes of growth parameters of double mutants compared to respective single mutants, are rare. Weak associations are more common, as was confirmed in our investigation, showing weak differences between single and double mutants or respective genes (Jakubowska and Korona, 2012). Nevertheless, even small growth parameter differences play a role in yeast viability and reaction to various stress factors, especially exposure to the killer toxin (**VI**).

Genetic interaction between genetic factors of HOG and CWI pathways was analyzed using the sensitivity-based lysis zone method upon cell exposure to K2 toxin (**VI**). Stress provokes HOG pathway activation, which regulates glycerol synthesis and cell wall integrity (Saito and Posas, 2012). Genetic interactions in defective cells were evaluated by comparing sensitivity to K2 toxin of mutant strains with single and double gene deletions (Fig. 4, **VI**). Sensitivity to K2 toxin was increased in the majority of tested single mutants (Δ*ssk1*, Δ*ssk2*, Δ*soh1*, Δ*spt3*, Δ*spt8*, Δ*bem2*, Δ*fus3*, Δ*tax4,* and Δ*hog1*), in comparison to parental BY4741 strain. Increased sensitivity of the majority of double mutants to the toxin was observed in comparison to single mutants but remained at the same sensitivity level as Δ*hog1* mutant (**VI**).

Elimination of *HOG1* gene from Δ*ssk1* and Δ*ssk2* cells, where gene products of deleted genes interact in the same signaling pathway, did not cause significant changes upon K2 toxin treatment (Fig. 4, **VI**). Soh1 and Med1 are RNA polymerase II mediator complex subunits (Boube *et al.*, 2002). Sensitivity of the double Δ*soh1*-Δ*hog1* mutant increased in comparison with the respective single mutant strain, while the response to K2 toxin in Δ*med1*-Δ*hog1* cells remained the same as in Δ*med1* strain. Removal of *HOG1* gene from cells with Δ*spt3* or Δ*spt8* that have disrupted SAGA transcriptional regulation (Wu *et al.*, 2004), increased the sensitivity to K2 by 50 %, regarding the respective single mutants. Elimination of Hog1 kinase from strains defective in CWI pathway, namely Δ*bem2* and Δ*tax4* strains, resulted in increased sensitivity to K2 toxin – in Δ*bem2*-Δ*hog1* cells – by 35 %, and even more in Δ*tax4*-Δ*hog1* cells. Thus, phenotypic response to K2 toxin of the mentioned double mutants did not change and was independent of gene products, involved in the same or distinct signaling pathways. Only an increase in the sensitivity level was observed (**VI**).

Only three single mutants, where products of eliminated genes are involved in the CWI signaling pathway, changed the response to K2 toxin stress, upon removal of *HOG1* gene (**VI**). Single Δ*rlm1*, Δ*lrg1,* and Δ*slm4* mutants are more resistant to K2 toxin than parental BY4741 strain, while Δ*hog1* is sensitive to K2 toxin. Double Δ*rlm1*-Δ*hog1*, Δ*lrg1*-Δ*hog1,* and Δ*slm4*-Δ*hog1* mutants are sensitive to K2 toxin alike single Δ*hog1* strain. Genes are epistatic to each other, when the phenotypic effect associated with one certain mutation is replaced by another gene mutation (Batenchuk *et al.*, 2010; Steidle *et al.*, 2020). Thus, Δ*hog1* mutation is epistatic to Δ*rlm1*, Δ*lrg1,* and Δ*slm4* single mutants (**VI**).

Protein-protein interaction network was generated using STRING data analysis program (Szklarczyk *et al.*, 2011) and high throughput *S. cerevisiae* genome database (https://www.yeastgenome.org). Interaction network with a medium confidence level includes 24 links (Fig. 5, **VI**). Line thickness corresponds to the strength of the link and includes functional and physical interactions. Hog1 directly strongly interacts with HOG signaling pathway regulator Ssk1 and CWI pathway mediator Rlm1. Direct Hog1 interactions with MAP kinase Ssk2, transcriptional regulators Spt3 and Spt8, alike with Rho1 GTPase activating Bem2 protein are slightly weaker. Hog1 links with other CWI and HOG proteins analyzed in this work are indirect: interact via protein kinases Fus3 and Lrg1, RNA polymerase II complex, and transcription regulator Soh1, phosphoinositol signaling pathway regulators Tax4 and Slm4. Based on STRING data it was determined that other factors, potentially interacting in HOG and CWI pathways can be identified (**VI**).

3.6 Comparison of functioning of *S. cerevisiae* and *S. paradoxus* killer systems

Mechanism of action of *S. cerevisiae* dsRNA-encoded K1 and K2 toxins occurs in two stages. Initially, the killer protein binds to the primary receptor β-1,6-glucan, located in the cell wall (Hutchins and Bussey, 1983; Lukša *et al.*, 2015). Afterward, toxins are attracted to secondary plasma membrane receptor Kre1 and form selective cation channels, which leads to the loss of K^+ ions and ATP molecules, intracellular pH becomes more acidic and the cell dies (Breinig *et al.*, 2002; Martinac *et al.*, 1990; Orentaite *et al.*, 2016; Schaffrath *et al.*, 2018; Schmitt and Breinig, 2002). This work proved that β-1,6-glucan is also the primary receptor of *S. paradoxus* K66 toxin (**IV**). Based on the same primary receptor, one can presume that action of *S. paradoxus* K66 toxin at the cell wall level is similar to those of *S. cerevisiae* K1 and K2 toxins.

Inactivation of *KRE1* gene in target yeast cells leads to resistance to *S. cerevisiae* K1 and K2 toxins (Pagė *et al.*, 2003; Servienė *et al.*, 2012). Investigation of Δ*kre1* strain phenotype upon exposure to K66 toxin revealed that cell also becomes resistant, like it is to K1 and K2 toxins (**IV; 13C**). Thus, at the plasma membrane level, the action of *S. paradoxus* K66 toxin is also similar to those of *S. cerevisiae* K1 and K2 toxins. Based on the obtained data, the action of K1, K2, and K66 toxins towards yeast cells is uniform, however, certain functionality indicators are different.

Optimal action of *S. cerevisiae* K1 toxin is observed at temperature lower than 23 ºC, at pH 4.3-4.7 (Dignard *et al.*, 1991; Kurzweilová and Sigler, 1993), while optimal conditions for *S. cerevisiae* K2 toxin is 20-25 ºC and pH 4,0-4,4 (Lebionka *et al.*, 2002; Lukša *et al.*, 2016; Pfeiffer and Radler, 1984). *S. paradoxus* K66 toxin performs best at pH 4.4-4.8 and 20 ºC temperature (**IV**). Thus, optimal K66 toxin killing conditions are more similar to K1, than to K2 toxin.

Killer toxins induce stress by attacking the target cell and cell can respond in several ways: by regulating ion/pH homeostasis, the activity of mitochondria and vacuoles, cell wall biogenesis, by activating specific cell wall integrity, and hyperosmotic pressure pathways, etc. (Brewster *et al.*, 1993; Levin, 2011; Servienė *et al.*, 2012). To protect itself from the lethal effect of *S. cerevisiae* K2 toxin, the cell activates CWI and HOG pathways via main MAPK cascade genetic factors (Servienė *et al.*, 2012). According to the similarity of action mechanism, one can presume that in response to *S. paradoxus* K66 toxin cells also activate CWI and HOG signaling pathways (**7C**).

Analysis of single mutants of genetic factors, involved in HOG and CWI pathways, revealed similarities and differences between responses to *S. cerevisiae* K2 and *S. paradoxus*

K66 toxins. About one-fourth of strains, lacking one of the genetic factors (Lrg1, Rlm1, Nbp2, Ssk2) show the same phenotype, and a similar number of single mutants (lacking Slm6, Hog1, Plc1, Ssk1, or Pbs2) exhibit different sensitivity levels. Elimination of about half of the genetic factors (Fks1, Bem2, Fus3, Bni1, Tax4, Slm4, or Sac1), involved in both pathways, resulted in phenotypic changes after exposure to K2 toxin, while phenotype remained alike the wild type after K66 toxin treatment (Servienė *et al.*, 2012; Table S1, **IV**). Thus, the cell is more sensitive to the stress, induced by *S. cerevisiae* K2 than *S. paradoxus* K66 toxin.

To identify factors contributing to the cell wall biogenesis and required for the action of *S. cerevisiae* and *S. paradoxus* killer systems, sensitivity analysis of yeast mutants with single gene deletions was performed (Table S1, **IV**; **13C**). Cell phenotypes were different upon exposure to distinct toxins produced by *Saccharomyces* spp.; differences between sensitivity and resistance formation to known *S. cerevisiae* K1, K2, K28, and environmental *S. paradoxus* toxins were determined (Table S1, **IV**; **13C)**. It was proved that genetic factors, involved in the synthesis of the cell wall components, modification and transfer, interact structurally or functionally and determine the efficiency of the action of killer toxins (**13C**).

A part of genetic factors involved in response to *S. cerevisiae* K2 and *S. paradoxus* K66 killer systems, according to STRING and GO analysis, are assigned to the functional group of the cell wall biogenesis, response to stress and signaling pathways (Servienė *et al.*, 2012; **IV**), however a significant portion of genetic factors does not match this classification. In response to K66 toxin, genetic factors are assigned to the translation process, linked to membrane and protein transmembrane transport, cell cycle, glycosylation, and signal transduction (**IV**). Response to K2 toxin requires genetic factors responsible for the structure of mitochondria, mitochondrial translation, sugar metabolism, chromatin structure and gene expression, proton, phospholipid transport and ATP synthesis, catabolism of proteins linked to the endoplasmic reticulum (Servienė *et al.*, 2012).

Comparison of the activity of *S. cerevisiae* K1, K2 and *S. paradoxus* K66 toxins revealed similarities of the action at the cell wall and membrane levels (overlapping receptors, activation of similar stress response pathways). An acidic environment and a temperature of 20-23 ºC are necessary for the optimal functioning of both *S. cerevisiae* and *S. paradoxus* toxins. However, the reaction of the target-cell to the *Saccharomyces spp.* synthesized toxins differ (varies the number of genetic factors involved in the formation of resistance and the level of susceptibility).

CONCLUSIONS

1. The mycobiota of currants, chokeberries, sea buckthorn berries and apples as well as the diversity of cultivated yeasts depends on the host-plant species, habitat, and ripening stage.
2. Fifteen percent of isolated killer yeasts maintain viral dsRNA-based killer systems prevailing in *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* yeasts.
3. K66 toxin produced by *S. paradoxus* AML-15-66 strain is encoded in dsRNA. Toxin killing activity against *S. cerevisiae* yeasts is optimal at 20 ºC temperature, pH 4.8.
4. Primary K66 toxin binding receptors in the yeast cell wall are β-1,6-glucans.
5. Yeast genetic factors, associated with the cell wall and membrane biogenesis, chromatin structure and gene expression, secretion and transport, osmotic stress response, and signaling pathways, are involved in the formation of resistance to *S. paradoxus* K66 toxin.
6. Genetic factors involved in the cell wall integrity and high osmolarity glycerol pathways as well as responding to the action of *S. cerevisiae* K2 toxin exhibit physical and functional interactions of different intensity.
7. Mechanisms of the action of *S. cerevisiae* K2 and *S. paradoxus* K66 toxins and response formation are similar, while the involvement of genetic factors and the level of response to toxin-induced stress differ.

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SANTRAUKA

MOKSLINĖ PROBLEMA

Mielės – gausiai gamtoje paplitę vienaląsčiai eukariotiniai grybiniai mikroorganizmai, randami ore, vandenyje, dirvožemyje ir ant įvairių augalo dalių, ypač ant vaisių ir uogų dėl juose esančių cukrų (Abdelfattah *et al.*, 2016a; Kurtzman *et al.*, 2011). Mielių paplitimas bei pasiskirstymas priklauso nuo augalo rūšies, sunokimo stadijos, klimato sąlygų ir geografinės padėties, dirvožemio tipo ir rūgštingumo, geros auginimo praktikos, herbicidų bei kitų žemės ūkyje naudojamų cheminių medžiagų ir kt. (Pinto *et al.*, 2015, 2014; Pretorius, 2000; Zhang *et al.*, 2021). Siekiant išsiaiškinti, kokie grybiniai mikroorganizmai vyrauja ant vaisių bei uogų tirtos braškės, mandarinai, vyšnios, trešnės, kriaušės, slyvos, obuoliai, šilkmedžio uogos ir kt. (Abdelfattah *et al.*, 2016b, 2016a; Clooney *et al.*, 2016; Droby *et al.*, 2016; Janisiewicz *et al.*, 2014; Lukša and Servienė, 2020; Stanevičienė *et al.*, 2021; Volschenk *et al.*, 2016), tačiau palyginti su pasaulyje egzistuojančia vaisių gausa ištirta visai nedaug. Be to, mikrobiotos sudėtis gali skirtis net ant tos pačios rūšies augalo, todėl tikslinga intensyvinti mikroorganizmų bendrijų bei jas lemiančių veiksnių tyrimus.

Kai kurios mielės pasižymi biocidinėmis savybėmis ir sintetina toksinus, žudančius jautrias mielių ląsteles (Bewan and Makower, 1963; Makower and Bewan, 1963), ir tokiu būdu gali reguliuoti mikrobiotos sudėtį. Biocidinių toksinų genetinė medžiaga koduojama keliais būdais: linijinėje DNR plazmidėje, chromosomoje ir dvigrandininėje RNR (Magliani *et al.*, 1997). *Saccharomyces cerevisiae* ir *S. paradoxus* rūšyse identifikuota daugiausia dgRNR koduojamų biocidinių toksinų (Rowley, 2017), kurie detaliau nagrinėjami dėl pritaikomumo medicinoje, farmacijoje, maisto pramonėje, aplinkos apsaugoje ir kt. (Boynton, 2019; Hatoum *et al.*, 2012; Meinhardt and Klassen, 2009; Polonelli *et al.*, 2000; Van Vuuren and Jacobs, 1992). *S. cerevisiae* sintetinami dgRNR koduojami toksinai (K1, K2 ir K28) išanalizuoti išsamiau (pirminė seka, biocheminės savybės, veikimo mechanizmas) nei *S. paradoxus* toksinai (Dignard *et al.*, 1991; Magliani *et al.*, 1997; Rodríguez-Cousiño *et al.*, 2017, 2011; Schmitt and Tipper, 1990; Servienė *et al.*, 2012). Apie pastaruosius žinoma nedaug, o veikimo ir ląstelinio atsako mechanizmai visai netirti.

Biocidiniams toksinams veikiant mielių ląsteles, įjungiami atsako į stresą keliai, susiję su ląstelės sienelės biogeneze, vakuolių ir mitochondrijų veikla, jonų homeostaze. Aktyvuojami ir signaliniai atsako keliai, iš kurių pagrindiniai yra hiperosmotinio slėgio (HOG) ir ląstelės sienelės vientisumo (CWI) (Servenė *et al.*, 2012). Šie keliai gali būti aktyvuojami pavieniui arba

kartu, tačiau nėra aišku, kaip juos koordinuojantys genetiniai veiksniai sąveikauja tarpusavyje. Atlikus viso genomo analizę, nustatyti jautrumą/atsparumą dgRNR koduojamiems *S. cerevisiae* K1, K2 ir K28 toksinams formuojantys genetiniai veiksniai, kurie dalyvauja HOG ir CWI keliuose (Carroll *et al.*, 2009; Pagé *et al.*, 2003; Servienė *et al.*, 2012). Analogiška viso genomo analizė *S. paradoxus* sintetinamiems toksinams iki šiol nebuvo atlikta.

Šiame darbe pateikta nauja informacija apie dažnai vartojamų vaisių ir uogų mikobiotą: išanalizuota baltųjų, juodųjų ir raudonųjų serbentų, aronijų, šaltalankių bei obuolių grybinių mikroorganizmų sudėtis, kuri priklauso nuo augalo bei vaisiaus sunokimo stadijos ir radimvietės. Išnagrinėtas mielių virusinių biocidinių sistemų paplitimas ant tiriamų vaisių (**I-III**). Tiriant mielių paplitimą ant vaisių ir uogų paviršių identifikuotas *S. paradoxus* AML-15-66 kamienas, sintetinantis dgRNR koduojamą K66 toksiną (**IV**). Detaliai apibūdinta naujai rasta dgRNR virusinė biocidinė sistema, nustatyti atsparumą jai lemiantys genetiniai veiksniai (**IV, V**). Įvertinta HOG ir CWI atsako kelių sąsaja veikiant *S. cerevisiae* dgRNR koduojamu K2 toksinu (**VI**). Palygintos *S. paradoxus* K66 ir *S. cerevisiae* K2 biocidinės sistemos pagal veikimo ir ląstelinio atsako mechanizmus (**IV**, **VI**).

TIKSLAS IR UŽDAVINIAI

Darbo tikslas – įvertinti vaisių-uogų mikobiotų struktūrą ir atlikti *Saccharomyces* spp. biocidinių sistemų paiešką bei ištirti jų funkcionavimo mechanizmus.

Darbo uždaviniai:

1. Ištirti vaisių-uogų mikobiotų sudėtį bei ją lemiančius veiksnius.
2. Įvertinti mielių virusinių biocidinių sistemų paplitimą ant šaltalankių, aronijų, serbentų bei obuolių.
3. Apibūdinti iš gamtinės aplinkos išskirtą *Saccharomyces paradoxus* K66 biocidinę sistemą bei ištirti toksino funkcionalumą.
4. Nustatyti *S. paradoxus* K66 toksino receptorius mielių ląstelėje.
5. Identifikuoti genetinius veiksnius, lemiančius ląstelių atsparumą *S. paradoxus* K66 toksinui.
6. Ištirti mielių genetinių veiksnių sąveiką ir palyginti ląstelinio atsako mechanizmus reaguojant į *Saccharomyces* spp. virusines biocidines sistemas.

DARBO NAUJUMAS IR REIKŠMĖ

1. Pirmą kartą atlikta Lietuvoje augančių šaltalankių, aronijų, baltųjų, raudonųjų ir juodųjų serbentų bei obuolių visuminė mikobiotų analizė taikant naujos kartos sekoskaitą. Detaliai ištirta nuo augalo priklausoma mikroorganizmų bendrijų sudėtis ir įvairovė bei mielių virusinių biocidinių sistemų paplitimas. Geografiniu atžvilgiu palygintos Lietuvoje ir Čekijoje sunokusių obuolių ir juodųjų serbentų mikobiotos.
2. Iš gamtinės aplinkos išskirtas *S. paradoxus* AML-15-66 kamienas, kuriame aptikta ir apibūdinta nauja dgRNR koduojama virusinė biocidinė sistema.
3. Pirmą kartą atliktas išsamus *S. paradoxus* K66 toksino funkcionavimo tyrimas: įvertintos optimalios veikimo sąlygos ir antagonistinis aktyvumas, nustatyti pirminiai receptoriai, kuriais K66 toksinas jungiasi prie ląstelės sienelės.
4. Naujai sukurtu „išgyvenamumo“ metodu identifikuoti genetiniai veiksniai, lemiantys ląstelių atsparumą *S. paradoxus* K66 toksinui.
5. Nustatytos CWI ir HOG atsako keliuose dalyvaujančių genetinių veiksnių sąsajos reaguojant į *Saccharomyces cerevisiae* K2 toksiną bei pirmą kartą aprašytas šių sąveikų stiprumas.
6. Atlikta palyginamoji *Saccharomyces* spp. toksinų veikimo bei ląstelių atsako į virusines biocidines sistemas analizė, atskleidžiant esminius mechanizmų panašumus bei skirtumus.

Vaisių visuminių mikobiotų struktūros tyrimai bei potencialiai patogeninių ir naudingų grybinių mikroorganizmų nustatymas svarbus žemės ūkyje – prognozuojant ir suvaldant augalų ligas, ilgesnį laiką išlaikant derlių, maisto pramonėje – siekiant užtikrinti saugų vaisių ir uogų vartojimą. Biocidinių sistemų funkcionavimo mechanizmų tyrimai suteikia galimybę pritaikyti toksinus patogenų biokontrolei. Ištirtos biocidinės mielės gali būti pritaikomos fermentacijos procesuose, vyno bei sūrio gamyboje ir kt. Mielių virusinių sistemų veikimo mechanizmų tyrimai reikšmingi virusų plitimo supratimui bei antivirusinių medžiagų paieškoje.

GINAMIEJI TEIGINIAI

1. Raudonųjų, baltųjų ir juodųjų serbentų, aronijų, šaltalankių bei obuolių mikobiotos sudėtį veikia augalo-šeimininko vidinė bei išorinė aplinka.
2. Ant vaisių ir uogų paplitusiose *Saccharomyces* genties mielėse yra aptinkamos virusinės biocidinės sistemos.

3. Beta-1,6-gliukanų pokyčiai mielių ląstelės sienelėje keičia *Saccharomyces paradoxus* K66 toksino prisijungimo efektyvumą.
4. Ląstelės-taikinio genetinė aplinka lemia atsaką į *S. paradoxus* K66 toksino veikimą.
5. Ląstelės sienelės vientisumo ir hiperosmotinio slėgio keliuose dalyvaujančių veiksnių sąveika yra būtina formuojant atsaką į *Saccharomyces* genties mielių sintetinamus toksinus.
6. *Saccharomyces* genties mielių biocidinių sistemų funkcionavimui įtakos turintys genetiniai veiksniai bei jautrumas toksinams nėra tapatūs.

MEDŽIAGOS IR METODAI

Naudotos medžiagos ir metodai aprašyti publikacijose (**I-VI**).

Vaisių ir uogų mėginių surinkimas. 2015-2018 metais Lietuvoje ir Čekijoje surinkti sunokę vaisiai ir uogos (obuoliai, juodieji, raudonieji ir baltieji serbentai, aronijos, šaltalankiai) bei atliktas jų paviršiuje paplitusių grybinių mikroorganizmų identifikavimas. Serbentai buvo rinkti liepą, aronijos ir obuoliai – rugpjūtį, o šaltalankiai – liepą ir rugsėjį (**I-III**).

Mėginio paruošimas metagenominei analizei. Vaisiai ir uogos plaunami 0,05 M fosfatiniu buferiniu tirpalu. Iš gautų nuosėdų išskiriama genominė DNR (gDNR), naudojant genominės DNR išskyrimo rinkinį „Genomic DNA purification kit“ („Thermo Fisher Scientific Baltics“, Vilnius, Lietuva) ir vadovaujantis gamintojo rekomendacijomis. Išskirtos gDNR kiekis ir kokybė įvertinami spektrofotometru „Nanodrop 2000“ („Thermo Fisher Scientific Baltics“, Vilnius, Lietuva) (**I-III**).

Grybinių mikroorganizmų DNR padauginimas, bibliotekos paruošimas ir bioinformatinė analizė. Siekiant nustatyti grybinius mikroorganizmus, padaugintas gDNR ITS2 (ribosominės DNR) regionas naudojant specifinius pradmenis: ITS3-KYO2 (5′-GATGAAGAACGYAGYRAA-3′) ir ITS4 (5′- TCCTCCGCTTATTGATATGC-3′) (Toju *et al.*, 2012). Amplikono biblioteka paruošta Macrogen (Seulas, Pietų Korėja) arba BaseClear (Leiden, Olandija) kompanijose, naudojant „Illumina“ adapterius („Illumina“, San Diegas, JAV). Parengtos bibliotekos patikrinimas atliktas DNR bioanalizatoriumi „Agilent Technologies 1000“, o kiekybiškai DNR biblioteka įvertinta kiekybinio PGR metodu pagal „Illumina“ rekomendacijas. Amplikonų sekoskaita vykdyta naudojant „Illumina MiSeq V3“ (2 × 300 bp) (Macrogen, Seulas, Pietų Korėja; BaseClear, Leiden, Olandija) (**I-III**).

Mikobiotų bioinformatinė analizė atlikta naudojant FLASH 1.2.11 (Magoč and Salzberg, 2011), CD-HIT-OTU 4.5.5 (Li *et al.*, 2012) ir QIIME v. 1.8 (Caporaso *et al.*, 2010) programas (**I-III**).

Mielių kultivavimas ir identifikavimas. Apie 30 g steriliai surinktų vaisių ir uogų 15 dienų buvo laikomi 5 % dekstrozės tirpale 22 ºC temperatūroje. Atrankiai padaugintų mielių praskiedimai atlikti „Ringer“ tirpalu („Merck“, Kenilworth, JK) ir paskleisti ant agarizuotos YEPD terpės su chloramfenikoliu. Inkubuojama 2-3 dienas 25 ºC temperatūroje (**II, III**). Įvertinus užaugusių kolonijų morfologinius požymius, pvz., spalvą ir formą, buvo atrinktos į mieles panašios kolonijos, kurios analizuotos šviesiniu mikroskopu „Leica DM750” (Wetzlar, Vokietija) ir toliau identifikuotos molekulinės biologijos metodais.

Mielių rūšių nustatymas molekulinės biologijos metodais. Iš šviežios mielių kultūros išskiriama genominė DNR, naudojant gDNR gryninimo rinkinį „Genomic DNA purification kit“ („Thermo Fisher Scientific Baltics“, Vilnius, Lietuva) pagal gamintojo rekomendacijas. Specifiniais ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) ir ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) pradmenimis padauginamas regionas tarp 18S ir 28S rRNR genų. PGR reakcijos mišinio tūris 50 µL: 5 µL 10x buferinio tirpalo, 1 µL 2 mM dNTP mišinio, po 1 µL tiesioginio ITS1 ir atvirkštinio ITS4 pradmenų (10 µmol/L), 2,5 U/µL „Dream Taq“ DNR polimerazės (visi reagentai iš „Thermo Fisher Scientific Baltics“, Vilnius, Lietuva), 1 µL (5 ng) gDNR ir sterilaus distiliuoto vandens iki 50 µL. PGR atliekama „Esco“ termocikleryje: pradinė denatūracija – 5 min. 94 ºC, 25 ciklai kartojant denatūraciją 1 min. – 94 ºC, elongaciją 1 min. 30 s – 53 ºC ir sintezę 2 min. – 72 ºC temperatūroje. Galutinis sintezės etapas vykdomas 10 min. 72 ºC temperatūroje. Gauti PGR produktai analizuojami elektroforezės būdu 1 % agarozės gelyje (**II-IV**).

PGR produktai veikiami *HinfI* ir *CfoI* restriktazėmis, gauti profiliai tikrinami elektroforezės būdu 2 % agarozės gelyje. Skirtingus restrikcinio kartografavimo profilius turintys PGR produktai išgryninami naudojant „GeneJet PCR purification kit“ rinkinį („Thermo Fisher Scientific Baltics“, Vilnius, Lietuva) pagal gamintojo rekomendacijas ir naudojant ITS1 ir ITS4 pradmenis „BaseClear“ įmonėje (Leiden, Netherlands) nustatomos jų DNR sekos. Gautos sekos palyginamos su pateiktomis EMBL-EBI duomenų bazėje (http://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html) (**II-IV**).

Mielių biocidiškumo ir jautrumo nustatymas. Siekiant nustatyti biocidinį fenotipą, tiriamas mielių kamienas tašku užsėjamas ant agarizuotos MB terpės giluminio sluoksnio su jautriu *S. cerevisiae* α‘1 kamienu (2×10^6 ląstelių/lėkštelėje) arba kitų mielių rūšių kamienais. Nustatant jautrumą, tiriamos mielės sėjamos giluminiu būdu, o testeriniai kamienai taškuojami. Lizės zonų susidarymas ir biocidinis bei jautrumo fenotipas analizuojamas po 2 dienų inkubavimo 25 ºC temperatūroje (**IV-VI**).

Dvigrandininės RNR išskyrimas. Suminės RNR išskyrimas vykdomas pagal anksčiau publikuotą Fried and Fink (1978) metodą su keliais pakeitimais. Šviežiai užaugintos mielių

ląstelės surenkamos centrifuguojant ir praplaunamos pridedant 1/10 pradinio ląstelių tūrio 50 mM EDTA. Ląstelės surenkamos ir suspenduojamos 1/10 pradinio tūrio 50 mM TrisCl pH 9,3 su 1 % β-merkaptoetanolio, inkubuojamos 15 min. kambario temperatūroje. Ląstelės surenkamos, pridedama 2/10 pradinio tūrio TES (0,01 M TrisCl pH 7,5, 0,01 M EDTA, 0,2 % SDS ir 0,1 M NaCl) buferinio tirpalo, lygia dalimi pridedama fenolio ir inkubuojama 45 min. vartant. Fazės atskiriamos centrifuguojant. Nukleorūgštys (NR) išsodinamos iš vandeninės fazės pridedant vieną tūrį izopropanolio ir 1/10 tūrio 3M natrio acetato pH 5,2. Nuosėdos praplaunamos 75 % etanoliu ir ištirpinamos vandenyje be nukleazių. DNR pašalinama pridėjus DNAzės I ir 1/10 tūrio jos reakcijos buferinio tirpalo. Inkubuojama 30 min. 37 ºC temperatūroje ir DNAzė I inaktyvuojama. Nukleorūgštys išfrakcionuojamos 1 % agarozės gelyje.

Suminės RNR frakcija per naktį inkubuojama su 2,8 M LiCl 4 °C temperatūroje. Viengrandininės RNR pašalinamos centrifuguojant 45 min. 18 000 × *g*. DgRNR išsodinama iš vandeninės fazės naudojant 1/10 tūrio 3 M NaCl ir vieną tūrį izopropanolio. Nuosėdos praplaunamos 75 % etanoliu ir ištirpinamos vandenyje be nukleazių. Išskirtos dgRNR frakcijos analizuojamos elektroforezės būdu 1 % agarozės gelyje (**IV**).

***Saccharomyces* spp. sintetinamų toksinų išskyrimas.** *S. paradoxus* AML-15-66 kamienas auginamas pH 4,8 sintetinėje toksino išskyrimo terpėje 4-6 dienas 18 ºC temperatūroje, kol pasiekia 0,6-0,8 optinius vienetus (OD600). Supernatantas filtruojamas per sterilią polivinildenfluorido (PVDF) membraną, kurios porų skersmuo yra 0,22 µm („Millipore“, Bedford, MA, JAV) ir 100 kartų koncentruojamas per slėgio pagrindu veikiančią „Amicon PM-10“ sistemą, kurios membranos molekulinio svorio pralaidumas 10 kDa („Sigma-Aldrich“, St. Louis, MO, JAV) (**IV**). Analogiškai išskiriamas *S. cerevisiae* K2 toksinas, tik jį sintetinantis M437 kamienas auginamas sintetinėje toksino išskyrimo terpėje, pH 4,0 (Servienė *et al.*, 2012; **V**).

K66 toksino funkcionalumo tyrimas. K66 toksino aktyvumas tirtas skirtingo rūgštingumo MB terpėje (pH nuo 3,2 iki 6,0). Temperatūrinis aktyvumas analizuotas 4 ºC, 15 ºC, 20 ºC, 25 ºC, 30 ºC ir 37 ºC temperatūrose (**IV**).

Mielių genetinių veiksnių atranka ir bioinformatinė analizė. *S. cerevisiae* pavienių genų deleciniai kamienai (YKO biblioteka) bei tėvinis BY4741 kamienas giluminiu būdu užsėjami į MB, pH 4,8, mitybinę terpę (2×10^6 ląstelių/lėkštelėje), kurioje į suformuotus šulinėlius įpilama 100 kartų sukoncentruoto tiriamojo toksino. Po 2 dienų inkubavimo 25 ºC temperatūroje įvertinamos *S. paradoxus* K66 ir *S. cerevisiae* K2 toksino suformuotos skaidrios zonos ir palyginamos su lizės zona ant BY4741 kontrolinio kamieno (Servienė *et al.*, 2012; **IV, VI**). Šis tyrimas pritaikytas K66 toksino svarbių genetinių veiksnių atrankai panaudojant K2 toksinui

sukurtą atrankos metodą (**V**). Tiriamasis toksinas taip pat gali būti užlašinamas arba toksiną sintetinantis kamienas taškuojamas ant giluminio jautraus mielių kamieno sluoksnio (**V**).

Genų ontologijos (GO) analizė atlikta naudojant „BiNGO 3.0.3“ papildinį „Cytoscape 3.6.1“ platformoje (Maere *et al.*, 2005). P reikšmės patikimumas apskaičiuotas hipergeometriniu testu naudojant Benjamini ir Hochberg klaidingų atradimų dažnio pataisą (Haynes, 2013) (**IV**).

Sąveikaujančių genetinių veiksnių tinklai nubraižyti naudojant „STRING 10“ duomenų bazę (https://string-db.org/) (Szklarczyk *et al.*, 2011). Tinklai sukurti pagal „patikimumo vaizdo“ parinktį programoje, kai sąsajų stiprumas žymimas skirtingo storio linijomis. Pasirinktas vidutinio patikimumo rodiklis – 0,4 (**IV, VI**).

K66 toksino surišimas su *S. cerevisiae* pavienes iškritas turinčiais kamienais ir polisacharidais. Šviežiai užaugintos kontrolinio BY4741 ir tiriamųjų *S. cerevisiae* kamienų kultūros inkubuojamos su 100 kartų sukoncentruotu K66 toksinu 4 ºC temperatūroje. Siekiant nustatyti K66 toksino surišimą su polisacharidais, chitinas, laminarinas, pululanas ir pustulanas sumaišyti su sukoncentruotu K66 toksinu. Mėginiai inkubuojami 1 val. 25 ºC temperatūroje. Supernatantas tikrinamas duobučių metodu. Po 2 dienų inkubavimo 25 ºC temperatūroje analizuojamos skaidrios lizės zonos (**IV**).

Dvigubų mutantų konstravimas ir patikra. PGR produktas, turintis *HIS3* geną su 3ʿ- ir 5ʿ- galų sekomis, padaugintas naudojant tiesioginį F-HphN-delHog1 (5′-GGAACAAAGGGAAAACAGGGAAAACTACAACTATCGTATATAATAATGCGTACGC TGCAGGTCGAC-3′) ir atvirkštinį R-HphN-delHog1 (5′-CAAAAAGAAGTAAGAATGAGTGGTTAGGGACATTAAAAAAACACGTTTAATCGAT GAATTCGAGCTCG-3′) pradmenis. pYM15 plazmidė naudota kaip matricinė DNR („Euroscarf“). PGR reakcijos mišinio tūris 100 µL: 50 µL 2x „DreamTaq Green PCR Master Mix” („Thermo Fisher Scientific Baltics”, Vilnius, Lietuva), 3,2 µL kiekvieno pradmens (10 µmol/L), 100 ng plazmidinės DNR ir vandens be nukleazių įpilama iki 100 µL. PGR kasetės padauginimas vykdomas „Esco“ termocikleryje nurodytomis sąlygomis: pradinė denatūracija vykdoma 3 min. 95 ºC temperatūroje, po to seka 30 ciklų kartojant denatūraciją 30 s – 95 ºC, elongaciją 30 s – 54 ºC bei DNR sintezę 2 min. 40 s – 72 ºC temperatūroje. Galutinis sintezės etapas vykdomas 4 min. 72 ºC temperatūroje. PGR produktas išgrynintas, naudojant PGR gryninimo rinkinį „GeneJet PCR purification kit” („Thermo Fisher Scientific Baltics”, Vilnius, Lietuva) pagal gamintojo rekomendacijas ir analizuojamas elektroforezės būdu 1 % agarozės gelyje (**VI**).

Siekiant į mielių genomą įterpti PGR kasetę homologinės rekombinacijos būdu, mielių transformacija atlikta LiCl metodu (Guthrie and Fink, 1991) su keliomis modifikacijomis.

Teigiami transformantai su *HOG1* geno pakaita *HIS3* genu analizuojami po 5 dienų inkubavimo 30 ºC temperatūroje (**VI**).

Norint patvirtinti *HOG1* geno iškritą, atlikta baltymų elektroforezė 10 % NDS-poliakrilamidiniame gelyje ir „Western“ hibridizacija, naudojant specifinius pirminius anti-Hog1 antikūnus (1:3000) ir specifinius antrinius prieš triušio IgG veikiančius antikūnus (1:5000). Membrana ryškinama naudojant kolorimetrinę NBT/BCIP sistemą („Thermo Fisher Scientific Baltics”, Vilnius, Lietuva) pagal gamintojo rekomendacijas (**VI**).

Mielių augimo greičio nustatymas. Tiriamųjų kultūrų augimas stebimas matuojant absorbciją ties 600 nm bangos ilgiu, startuojant nuo 0,1 optinio vieneto. Eksperimentas atliktas 3 kartus. Augimo greičiai apskaičiuoti naudojant „GraphPad Prism“ programinę įrangą (Olivares-Marin *et al.*, 2018) (**VI**).

REZULTATAI IR JŲ APTARIMAS

Grybinių mikroorganizmų ir biocidinių sistemų paplitimas vaisių-uogų ekosistemose. Pagal metagenominės analizės duomenis pirmą kartą nustatyta grybinių mikroorganizmų įvairovė ant lietuviškų aronijų, baltųjų ir raudonųjų serbentų, šaltalankių (**I**) bei lietuviškų ir čekiškų obuolių ir juodųjų serbentų paviršių (**III**). Išanalizuota mikobiotos priklausomybė nuo vaisiaus ir uogos, jų prinokimo stadijos bei augimvietės (**I-III**).

Mikobiotos priklausomybė nuo uogos. Ant aronijų paviršiaus identifikuoti grybiniai mikroorganizmai priklauso vyraujantiems Ascomycota (66,2 %) ir Basidiomycota (32,2 %) skyriams (2A pav. ir S2 lentelė, **I**). Ascomycota skyrių atstovauja Dothideomycetes ir Taphrinomycetes, o Basidiomycota – Tremellomycetes, Microbotryomycetes ir Exobasidiomycetes (2B pav., **I**). Iš 34 nustatytų šeimų dominuoja Davidiellaceae (20,8 %), taip pat yra Dothioraceae (7,9 %) ir Taphrinaceae (3,5 %). Šias šeimas atstovauja *Cladosporium* (20,8 %) ir *Taphrina* (3,5 %) gentys. Basidiomycota skyrių genties lygmenyje reprezentuoja *Cryptoccocus* (10,9 %) ir *Rhodotorula* (9,1 %) (2D pav., **I**). Mažais kiekiais aptiktos *Phoma*, *Mrakiella*, *Lewia* ir *Hanseniaspora* gentys (**I**). Genties lygmenyje neidentifikuoti 45,6 % grybinių mikroorganizmų (2D pav. ir S2 lentelė, **I**).

Ant raudonųjų ir baltųjų serbentų išskirti 2 vyraujantys grybinių mikroorganizmų skyriai, 59 šeimos ir 106 gentys. Kaip ir aronijų atveju, dominuojantis skyrius ant abiejų serbentų – Ascomycota (68,3 % ant raudonųjų serbentų ir 57,3 % ant baltųjų), mažiau randama Basidiomycota (atitinkamai 28,2 % ir 39,7 %) (2A pav., **I**). Gausiausia klasė – Dothideomycetes (53,1 % ant raudonųjų serbentų ir 51,5 % ant baltųjų) (2B pav., **I**), kuriai priklauso Dothioraceae

ir Davidiellaceae šeimos (2C pav., **I**). Glomerellaceae (9,1 %) šeima, kurią atstovauja *Colletotrichum* gentis, aptikta tik ant raudonųjų serbentų. Ant abiejų serbentų dominuojančios gentys yra *Cladosporium* (17,3 % ant raudonųjų ir 20,7 % ant baltųjų serbentų) ir *Cryptococcus* (atitinkamai 11,5 % ir 21 %), taip pat randama *Phoma* (4,8 % ir 8,3 %) ir *Rhodotorula* (1,6 % ir 3 %) (2D pav., **I**). Neidentifikuoti grybiniai mikroorganizmai ant raudonųjų serbentų sudarė 40,2 %, o ant baltųjų – 31,8 % (2D pav. ir S2 lentelė, **I**).

Ant šaltalankių paviršiaus identifikuoti 4 dominuojantys grybinių mikroorganizmų skyriai, 58 šeimos ir 108 gentys. Kaip ir aronijų bei baltųjų ir raudonųjų serbentų atveju dominuoja Ascomycota (89,4 %), taip pat identifikuota Basidiomycota (8,2 %) (2A pav., **I**). Klasės lygmenyje vyrauja Dothideomycetes, mažiau randama Tremellomycetes ir kitų (2B pav., **I**). Šeimos taksonominiame range vyrauja Dothioraceae (78 %), ženkliai mažiau randama Davidiellaceae (2,4 %), šiek tiek identifikuota Taphrinaceae ir Saccharomycodaceae (2C pav., **I**). Net 87,9 % grybinių mikroorganizmų genties lygmenyje neidentifikuoti (2D pav. ir S2 lentelė, **I**).

Pirmą kartą ištirti mikobiotų skirtumai ant šaltalankio uogų paviršiaus skirtingų nokimo stadijų metu (**II**). Nustatyta, kad prinokusių ir žalių šaltalankio uogų grybinių mikroorganizmų sudėtis skiriasi. Ant žalių ir prinokusių šaltalankių vyrauja 4 eukariotinių mikroorganizmų skyriai, 69 eilės ir 144 šeimos (S1 lentelė, **II**). Ascomycota dominuoja ant abiejų šaltalankių mėginių (85 % ant neprinokusių uogų ir 97,6 % ant prinokusių), ženkliai mažiau randama Basidiomycota (atitinkamai 10,6 % ir 1,7 %) ir kitų grybų (2A pav., **II**). Pirmiausiai uogų sunokimo įtaka grybinių mikroorganizmų sudėčiai pastebima klasės lygmenyje. Didelis kiekis Dothideomycetes aptiktas abiejuose mėginiuose (54,0 % ant žalių ir 44,6 % ant sunokusių uogų) (2B pav., **II**). Ant nesunokusių uogų dažniausiai randama Taphrinomycetes (18,4 %) ir Tremellomycetes (7,6 %), o ant prisirpusių – Saccharomycetes (47,6 %). Šeimos lygmenyje ant abiejų nokimo stadijų šaltalankių vyrauja Aureobasidiaceae, tik ant neprinokusių dar randama ir Taphrinaceae, o ant prinokusių – Metschnikowiaceae (2C pav., **II**). Iš viso identifikuotos 196 gentys, iš kurių ant neprinokusių uogų gausiai rasta *Aureobasidium* (31,4 %), *Taphrina* (18,4 %), *Cladosporium* (8,1 %), o ant prinokusių – *Metschnikowia* (44,5 %) ir taip pat *Aureobasidium* (40,6 %) (2D pav., **II**). Pastarosios genties atstovai dažniau randami ant prinokusių nei ant žalių šaltalankių uogų. *Aureobasidium* rūšys biocidiškai veikia *Botrytis*, *Bacillus*, *Colletotrichum* ir *Penicillium* fitopatogenus (Grube *et al.*, 2011). *Taphrina* genties grybai yra biotrofiniai augalų patogenai, lemiantys augalų ir vaisių ligas, dėl kurių aktyvuojami skirtingi augalo hormonai (Tsai *et al.*, 2014). *Cladosporium* atstovai gali sintetinti priešgrybines medžiagas arba sukelti ligas (Sandoval-Denis *et al.*, 2016; Wang *et al.*, 2013).

Dalis metagenominės analizės būdu neidentifikuotų mielių nustatytos kultivuojant atrankiomis sąlygomis ir analizuojant molekulinės biologijos metodais (gDNR išskyrimas, ITS regiono padauginimas, restrikcinis kartografavimas ir sekoskaita). Nuo tirtų baltųjų ir raudonųjų serbentų, aronijų bei sunokusių šaltalankių paviršiaus padaugintos *Hanseniaspora uvarum* ir *Pichia kudriavzevii* mielės. *Pichia anomala*, *Metschnikowia pulcherrima* mielės rastos ant raudonųjų serbentų bei aronijų. *Candida zemplinina* identifikuota ant baltųjų serbentų ir šaltalankių (**12C, 14C**). Ant pastarųjų identifikuota ir *Candida californica*. *Pichia membranifaciens* nustatyta ant šaltalankių ir aronijų (**6C**). *Pichia nakasei* specifiškai rasta tik ant šaltalankio uogų. Daugiausia kultivuojamų mielių nustatyta aronijų paviršiuje: *Hanseniaspora* spp., *Kazachstania* spp., *Torulaspora delbrueckii*, *Zygosaccharomyces microellipsoides*, *Zygotorulaspora florentina*, *Lachancea* spp. ir *Saccharomyces cerevisiae* bei *Saccharomyces paradoxus*. *S. cerevisiae* taip pat rasta ir ant šaltalankių (**6C**).

Nuo žalių ir prinokusių šaltalankio uogų paviršiaus praturtintomis sąlygomis padauginta virš 20 mielių kamienų. *A. pullulans* gausiai randama abiejuose šaltalankio uogų mėginiuose. *M. pulcherrima* daugiausia randama ant sunokusių uogų, o *C. wieringae* – priešingai: dažniau randama ant neprinokusių šaltalankių. Mažiausiai paplitusi ant abiejų šaltalankio mėginių yra *Hanseniaspora uvarum* (**II**). *Hanseniaspora* ir *Metschnikowia* gentims priklausančios mielės antagonistiškai veikia vaisių puvinį sukeliančius pelėsius (Janisiewicz *et al.*, 2001; Liu *et al.*, 2010; Tilocca *et al.*, 2020). *M. pulcherrima* veikia kaip biokontrolės veiksnys, nes aktyviai konkuruoja dėl maistinių medžiagų ir sintetina lakiuosius organinius junginius (Contarino *et al.*, 2019; Parafati *et al.*, 2015).

Pagal PCoA analizės duomenis buvo nustatyti surinktų šaltalankių, aronijų ir baltųjų bei raudonųjų serbentų grybinių mikroorganizmų sąstato skirtumai. Raudonųjų ir baltųjų serbentų mikobiota sugrupuota kartu, o šaltalankių ir aronijų aiškiai atskirta viena nuo kitos ir nuo abiejų serbentų (3B pav., **I**). Tai reiškia, kad abiejų serbentų grybinė sudėtis panaši, bet skiriasi nuo šaltalankių ir aronijų, kurių mikobiotos taip pat skiriasi tarpusavyje. Iš skirtingo nokimo stadijose tirtų šaltalankių PCoA analizės nustatyta, kad sunokusių uogų mėginiai yra aiškiai atskirti nuo neprinokusių (1 pav., **II**). Apibendrinus gautus rezultatus, galima teigti, kad nevienodo sunokimo šaltalankio uogų mikobiotos skiriasi.

Grybinių mikroorganizmų įvairovės, sudėties ir paplitimo skirtumai dažnai priklauso nuo paties augalo bei vaisiaus sunokimo lygmens. Mikobiotos pasiskirstymas ant šaltalankių, aronijų, raudonųjų ir baltųjų serbentų svarbus potencialiai patogeninių ir naudingų mikroorganizmų balanso išlaikymui gamtoje. Tai aktualu gerinant augalų auginimo sąlygas ir apsaugant juos nuo bakterinės ir grybinės kilmės ligų. Be to, didėjant natūralaus, minimaliai

apdoroto ir be cheminių medžiagų poveikio užauginto augalinio maisto paklausai, reikalinga išsami augalų mikobiotos analizė (**I, II**).

Grybinių mikroorganizmų priklausomybė nuo radimvietės. Pritaikius metagenominius tyrimų metodus nustatyta, kad grybinių mikroorganizmų sudėtis ant Lietuvoje ir Čekijoje užaugintų obuolių yra panaši: dominuoja Ascomycota (86,3 % ant lietuviškų ir 86,8 % ant čekiškų obuolių), aptinkama ir Basidiomycota (atitinkamai 12,5 % ir 11,2 %) (3A pav., **III**). Klasės range vyrauja Saccharomycetes (70,2 % ant lietuviškų ir 63 % ant čekiškų obuolių) (3B pav., **III**), kuri pagal metagenominius duomenis neapibūdinta genties ir rūšies lygmenyse (S2 lentelė, **III**).

Nedideli skirtumai tarp grybinių mikroorganizmų pasiskirstymo ant lietuviškų ir čekiškų obuolių stebimi genties lygmenyje: *Cryptococcus* (5,7 %), *Cladosporium* (4,2 %) ir *Hanseniaspora* (3,3 %) vyrauja ant Lietuvoje užaugintų vaisių, o Čekijoje daugiausia aptinkama *Hanseniaspora* (17,6 %). Kai kurios gentys rastos tik vienoje šalyje: ant lietuviškų obuolių identifikuota *Stachybotrys*, o ant čekiškų – *Wickerhamomyces* (4B pav., **III**). Šie skirtumai gali būti dėl nevienodų klimato sąlygų. *Pichia terricola*, *P. fermentans*, *Torulaspora delbrueckii* ir *Saccharomyces cerevisiae* mielių mažai aptikta naujos kartos sekoskaitos metu, tačiau identifikuotos tiriant fermentuojančias mieles praturtintomis sąlygomis (S2 lentelė, **III; 14C**). Fermentuojančių mielių gausa priklauso nuo aplinkos, kurioje vaisius auga, ir prinokimo stadijos. Mažus fermentuojančių mielių kiekius ant obuolių lėmė šiek tiek ankstesnis derliaus nuėmimo laikas, kai vaisiaus paviršius buvo nepažeistas ir mielėms pasiekiamo cukraus kiekis ribotas. *Hanseniaspora* sp., *Pichia* sp., *Metschnikowia pulcherrima*, *Wickerhamomyces* sp. ir *Saccharomyces cerevisiae* mielės taip pat rastos ant obuolių, slyvų ir kriaušių po 15 dienų inkubavimo praturtinoje terpėje fermentuojančiomis sąlygomis (Vadkertiova *et al.*, 2012). *Wickerhamomyces* sp. bei *Metschnikowia* sp. mielės gali veikti kaip biokontrolės veiksniai ir apsaugoti augalus nuo patogenų, taip pat reguliuoti augalų mikrobiotos sudėtį (Abdelfattah *et al.*, 2016b; Muccilli and Restuccia, 2015; Parafati *et al.*, 2015; Vadkertiová *et al.*, 2012).

Grybinių mikroorganizmų pasiskirstymas skyriaus range ant lietuviškų ir čekiškų juodųjų serbentų yra panašus, taip pat daug nesiskiria nuo abiejose šalyse surinktų obuolių: dominuoja Ascomycota (71,5 % ant lietuviškų juodųjų serbentų ir 92,2 % ant čekiškų), randama Basidiomycota (atitinkamai 24,2 % ir 7 %) (3A pav., **III**). Klasės lygmenyje ant lietuviškų uogų randama Dothideomycetes, ant čekiškų – Saccharomycetes, šiek tiek mažiau – Dothideomycetes (3B pav., **III**). Didelė grybinių mikroorganizmų įvairovė tarp Lietuvoje ir Čekijoje užaugintų juodųjų serbentų stebima šeimos ir genties ranguose. Ant lietuviškų juodųjų serbentų vyrauja *Cladosporium* (45,4 %) ir *Cryptococcus* (15,2 %) mielių gentys, o ant čekiškų – *Hanseniaspora* (48,5 %), mažiau aptinkama *Cladosporium* (5,2 %) ir *Rhodotorula* (1,5 %) (S2 lentelė, **III**).

Taigi, juodųjų serbentų mikobiota skiriasi geografiniu atžvilgiu. *Hanseniaspora* sp. yra mažai fermentuojanti mielių rūšis, kuri taip pat randama vynuogių, braškių ir obuolių paviršiuje (Graça *et al.*, 2015; Santo *et al.*, 2012). *Cladosporium* sp. – Dothideomycetes atstovas, identifikuotas ant visų tirtų vaisių ir uogų mėginių, o daugiausiai jo rasta ant lietuviškų juodųjų serbentų. *Cladosporium* sp. aptinkamas ant įvairių augalo dalių, todėl šių mielių gausa nestebina (Abdelfattah *et al.*, 2016a; 2016b). *Cryptococcus* ir pigmentuotos *Rhodotorula* mielės rastos ne tik ant šiame eksperimente tirtų obuolių ir juodųjų serbentų, bet ir ant braškių (Abdelfattah *et al.*, 2016a), vynuogių (Barata *et al.*, 2012; Setati *et al.*, 2012), kriaušių, slyvų, obuolių (Vadkertiova *et al.*, 2012). *Rhodotorula* sp. formuoja bioplėvelę ant vynuogių paviršiaus visų nokimo stadijų metu, taip apsaugodama uogas nuo išdžiūvimo ir išorinio mikroorganizmų poveikio (Lederer *et al.*, 2013). *Cryptococcus* pripažinta kaip tipinė mielių bendruomenės sudedamoji dalis ankstyvojoje vaisių nokimo stadijoje, kai vaisiai dar nesunokę (Janisiewicz *et al.*, 2014; Vadkertiová *et al.*, 2012). Šie grybiniai mikroorganizmai dažnai išskiriami iš vaisių nuoplovų ir gali būti biokontrolės veiksniais, apsaugančiais nuo ligų derliaus transportavimo ir saugojimo metu (Liu *et al.*, 2013). *Hanseniaspora*, *Rhodotorula*, *Dioszegia* – augalams palankūs grybiniai mikroorganizmai, o *Phoma*, *Lewia*, *Colletotrichum*, *Septoria*, *Taphrina* atstovai priklauso potencialiems patogenams (Cissé *et al.*, 2013; Eyal, 1999; Phalip *et al.*, 2006; Renker *et al.*, 2004).

Mielių kultivavimo praturtintomis sąlygomis metu ant lietuviškų ir čekiškų juodųjų serbentų bei obuolių rastos *Hanseniaspora* spp., *H. uvarum*, *Metschnikowia* spp., *S. cerevisiae*, *P. fermentans* ir *I. terricola* fermentuojančios mielės (S3 lentelė, **III; 12C, 14C**). *M. pulcherrima*, *P. membranifaciens* ir *T. delbruecki* mielės neidentifikuotos tik ant lietuviškų juodųjų serbentų, *P. kudriavzevii* nerasta tik ant čekiškų obuolių, o *Wickerhamomyces anomalus* – ant lietuviškų. Tik ant lietuviškų obuolių nustatytos *Candida boidinii* mielės. *S. paradoxus* būdingos tik Lietuvoje auginamiems juodiesiems serbentams ir obuoliams, nes ant čekiškų vaisių neidentifikuotos. Ant juodųjų serbentų ir obuolių paviršiaus daugiau aptinkama *S. cerevisiae* nei *S. paradoxus* (**III; 12C, 14C**).

Išanalizavus PCoA duomenis, nustatyta, kad obuolių iš skirtingų šalių mikobiota sugrupuota kartu, vadinasi, yra panaši. Lietuviškų ir čekiškų juodųjų serbentų mikobiota aiškiai atskirta viena nuo kitos ir nuo abiejų obuolių. Tai reiškia, kad abiejų šalių juodųjų serbentų grybinių mikroorganizmų įvairovė skiriasi tarpusavyje ir nesutampa su Lietuvoje ir Čekijoje užaugintų obuolių mikobiotos sąstatu (2B pav., **III**).

Atlikus vaisių ir uogų metagenominius tyrimus parodyta, kad grybinių mikroorganizmų įvairovė labiau priklauso nuo vaisiaus ar uogos tipo nei nuo jų augimo vietovės. Be to, didelę įtaką grybinių mikroorganizmų įvairovei daro augalo aplinkos sąlygos. Tikėtina, kad ne vien

klimatinės sąlygos lėmė mikobiotų kiekybinius skirtumus, ne mažiau svarbi ir analizuojamų uogų bei vaisių sunokimo stadija (**III**).

Virusinių biocidinių sistemų paplitimas ant vaisių ir uogų. Praturtintomis sąlygomis kultivuojant mieles, kartais randamos toksinus sintetinančios rūšys: *Pichia membranifaciens*, *P. kluyveri*, *P. inositovora*, *P. anomala*, *P. farinosa*, *Hansenula mrakii*, *H. saturnus*, *Tilletiopsis albenscens*, *Kluyveromyces lactis*, *Cryptococcus humicola*, *S. cerevisiae*, *S. paradoxus* ir kitos (Marquina *et al.*, 2002). Kileriniai toksinai žudo jautrius mielių kamienus ir kartu su lakiaisiais junginiais, priešgrybinėmis medžiagomis veikia mielių sudėtį ant vaisių ir uogų (Boynton, 2019; Buser *et al.*, 2014; Contarino *et al.*, 2019; Droby *et al.*, 2009; Freimoser *et al.*, 2019; Parafati *et al.*, 2017).

Tiriant nuo juodųjų, raudonųjų ir baltųjų serbentų, obuolių, aronijų, šaltalankių paviršiaus išskirtas mieles, aptikti 104 biocidines savybes turintys kamienai (**12C, 14C**). Daugiausia jų identifikuota *P. kluyveri*, *P. anomala* ir *P. membranifaciens* mielėse. Biocidinių savybių turi ir kai kurie *Metschnikowia* spp. ir *Saccharomyces* spp. atstovai, *H. uvarum*, *T. delbrueckii*, *Kwoniella shandongensis*, *Kazachstania bulderri*, *K. africana*, *Zygotorulaspora florentina*, *A. pullulans* kamienai.

Nustatyta, kad iš 104 biocidinių kamienų 15 turi dgRNR koduojamas sistemas. Virusinės biocidinės sistemos identifikuotos *S. cerevisiae*, *S. paradoxus*, *P. membranifaciens*, *H. uvarum* ir *Metschnikowia* spp. mielėse (**12C, 14C**). Daugiausia virusinių sistemų aptikta *S. paradoxus* (5) ir *S. cerevisiae* (5) mielių kamienuose. Trys dgRNR koduojamos biocidinės sistemos rastos *Metschnikowia* gentyje, po vieną identifikuota *P. membranifaciens* ir *H. uvarum* mielėse.

S. paradoxus biocidiniai kamienai padauginti nuo baltųjų (1) ir juodųjų serbentų (2), obuolio (1) ir aronijos (1) paviršių (**IV**). Ant raudonųjų serbentų praturtintomis sąlygomis rastas vienas *S. cerevisiae* biocidinis kamienas, po 2 aptikti ant obuolių ir šaltalankių (**12C, 14C**). *Metschnikowia* genties dgRNR koduojamos biocidinės mielės padaugintos nuo baltųjų (1) ir juodųjų (1) serbentų bei aronijų (1) (**12C, 14C**). *H. uvarum* (1) ir *P. membranifaciens* (1) biocidiniai kamienai rasti tik ant obuolio (**12C**).

Rodríguez-Cousiño su kolegomis teigia, kad L-A genomo dydis *S. cerevisiae* ir *S. paradoxus* rūšyse yra 4,6 kbp (Rodríguez-Cousiño *et al.*, 2017). Šiame darbe tirtų abiejų *Saccharomyces* genčių kamienų L-A dydis yra panašus – apie 4,5 kbp (**12C, 14C**). *S. cerevisiae* tirtų mielių M dgRNR dydis yra nuo 1,6 kbp iki 1,8 kbp (**12C, 14C**) ir atitinka minimus literatūroje (1,6-2,4 kbp) (Rodríguez-Cousiño *et al.*, 2017). Kai kurie aptikti *S. paradoxus* kamienai turi didesnius M genomus nei *S. cerevisiae*, nes identifikuota 2,8 kbp M dgRNR. Tirtų *S. paradoxus* kamienų M dydis varijuoja nuo 1,7 kbp iki 2,8 kbp (**12C, 14C**) ir atitinka literatūroje skelbiamus duomenis (1,6-2,8 kbp) (Rodríguez-Cousiño *et al.*, 2017).

***Saccharomyces paradoxus* K66 biocidinės sistemos tyrimai.** Iki 20 % nuo vaisių ir uogų paviršiaus padaugintų mielių turi biocidinį fenotipą (Boynton, 2019). Nuo medlievos (lot. *Amelanchier ovalis*) paviršiaus padaugintas, identifikuotas bei aprašytas *Saccharomyces paradoxus* AML-15-66 biocidinis kamienas, sintetinantis naujai rastą dgRNR koduojamą K66 toksiną (**IV**). Šio tipo virusinės biocidinės sistemos aptiktos ir ant obuolių bei juodųjų serbentų (**12C, 13C**).

Mielių kamienas AML-15-66 išgrynintas iš praturtinto spontaninio medlievos raugo, kuriame atrankiai padaugintos fermentuojančios mielės. ITS regiono sekoskaitos (S1A pav., **IV**) ir restrikcinio kartografavimo būdu kamienas identifikuotas kaip *S. paradoxus* (S1B pav., **IV**). Kadangi *S. paradoxus* potencialiai gali sintetinti biocidinius toksinus, ant jautraus *S. cerevisiae* α‘1 giluminio sluoksnio (pH 4,0) patikrintas ir nustatytas AML-15-66 kamieno biocidinis aktyvumas. Mielių biocidiniai toksinai gali būti koduojami chromosomoje, linijinėje DNR plazmidėje ir virusinės kilmės dvigrandininėje RNR (Magliani *et al.*, 1997). Daugiausia ištirti *Saccharomyces* spp. sintetinami biocidiniai toksinai, kurie koduojami dgRNR (Rowley, 2017). Iš *S. paradoxus* AML-15-66 kamieno išskyrus RNR, nustatyta, kad K66 toksinas taip pat koduojamas dgRNR, kurios M dydis yra apie 1,7 kbp. Pagal preprotoksino sekos analizės duomenis nustatytos trys potencialios Kex2 proteazės hidrolizės vietos: Arg59, Arg181 ir Arg239 (3 pav., **IV**). Disulfidinių tiltelių formavimo vietos išlieka dviprasmiškos. K66 baltymo sekoje nustatytos penkios N-glikozilinimo vietos, iš kurių keturios sutapo su K21 toksino (Rodríguez-Cousiño *et al.*, 2017). Didžiausia tikimybė yra modifikuoti 141-oje padėtyje esančią aminorūgštį (**IV**).

Nustatyta, kad *S. paradoxus* AML-15-66 mielės biocidiškai veikia *S. cerevisiae* kamienus, kai auginimo terpės pH reikšmė kinta nuo 3,6 iki 5,6 (1 lentelė, **IV**). Iš dalies išgryninto *S. paradoxus* sintetinamo K66 virusinio baltymo aktyvumas nustatytas pH ribose nuo 3,6 iki 5,2, o veikimo pikas yra tarp pH 4,4 ir 4,8. Kai pH reikšmės 3,2, 5,6 ir 6,0, K66 toksinas neaktyvus (**IV**). Rūgštesnėje terpėje (pH 4,0 ir pH 3,6) toksino aktyvumas sumažėja iki 38 % ir 25 % atitinkamai, o pasiekus pH 5,2, išlieka 50 % baltymo aktyvumo. Analizuojant mielių ląstelių išgyvenamumą veikiant K66 toksinu temperatūrose nuo 4 ºC iki 37 ºC, nustatyta, kad išskirtas baltymas aktyvus 15-30 ºC temperatūrų intervale, efektyviai veikia esant 20 ºC, bet neaktyvus esant ribinėms 4 ºC ir 37 ºC temperatūroms (**IV**). Nustatyti optimalūs toksino veikimo rodikliai koreliuoja su kitų biocidinių toksinų aktyvumo ir stabilumo rodikliais. Mikovirusų kilmės kileriniai baltymai dažniausiai aktyvūs nuo pH 4,0 iki pH 5,4 žemesnėje nei 30 ºC temperatūroje (Meinhardt and Klassen, 2009; Muccilli and Restuccia, 2015). Palankios sąlygos įsitvirtinti *Saccharomyces* biocidinėms rūšims nustatytos žemesnio pH vaisiuose, esant optimaliai 20-30 ºC temperatūrai (Lukša *et al.*, 2016).

S. cerevisiae kileriniai toksinai turi siaurą tikslinį taikinių diapazoną, nes slopina tik kelis tos pačios genties kamienus ar rūšis (Mannazzu *et al.*, 2002). Išimtis yra Klus toksinas, kuris veikia platesnį mielių spektrą (Rodríguez-Cousiño *et al.*, 2011). *S. paradoxus* K66 tipo toksinas pasižymi panašiu žudymo spektru kaip ir *S. cerevisiae* biocidinės mielės: veikia *S. cerevisiae* ląsteles, tačiau nežudo kitų mielių genčių. K66 toksinas stipriausiai biocidiškai veikia jautrias *S. cerevisiae* α‘1 ir BY4741 mieles. Iš visų tirtų biocidinių kamienų K66 toksinas aktyviausiai žudo *S. cerevisiae* M300 padermę, sintetinančią K28 virusinį baltymą. Silpniausiai biocidiškai veikia *S. cerevisiae* M437 mielių ląsteles, sintetinančias K2 toksiną. Tiriamasis toksinas nežudo *Pichia* spp., *Hanseniaspora* spp., *Candida* spp. ir *Torulaspora* spp. mielių. *S. paradoxus* AML-15-66 kamienas, sintetinantis K66 tipo toksiną, yra jautrus *S. cerevisiae* K2 toksino poveikiui bei atsparus *S. cerevisiae* K1, K28, Klus ir *S. paradoxus* K21 mikotoksinams (**IV**).

***S. paradoxus* K66 toksino veikimą lemiančių genetinių veiksnių atranka.** Siekiant nustatyti genetinius veiksnius, svarbius *S. paradoxus* K66 toksino veikimui ir dalyvaujančius ląstelių atsparumo virusiniam baltymui formavime, atlikta 526 *S. cerevisiae* vienetines genų iškritas turinčių kamienų patikra pagal fenotipo pokytį veikiant K66 toksinu (**IV**). Tiriamieji vienetiniai mutantai atrinkti išanalizavus anksčiau paskelbtus duomenis su *S. cerevisiae* K1, K2 ir K28 toksinais (Carroll *et al.*, 2009; Servienė *et al.*, 2012; Pagé *et al.*, 2003). K66 toksino veikimą lemiančių veiksnių atranka vykdyta pagal naujai sukurtą *S. cerevisiae* biocidiniams toksinams svarbių genetinių veiksnių atrankos metodą, kuris pirmiausia buvo pritaikytas K2 toksino modelinės sistemos analizei (**V**). Įrodyta, kad mielių vienetines iškritas turinčios bibliotekos užsėjimas ant agarizuotos terpės su giluminiu tiriamąjį biocidinį toksiną sintetinančio kamieno arba su išskirto toksino sluoksniu (išgyvenamumo tyrimas) padidina vykdomos atrankos jautrumą ir greitį bei sumažina tyrimo kaštus palyginti su tradiciniu biocidiškumo nustatymu. Sukurtas naujas atrankos metodas pritaikomas siekiant identifikuoti gamtinėje aplinkoje rastų biocidinių kamienų veiklą lemiančius veiksnius. Be to, išgyvenamumo tyrimu galima identifikuoti anksčiau nenustatytus genetinius veiksnius, kurie dar gali būti nežinomi, kaip dalyvaujantys atsako į toksino sukeltą stresą keliuose (**V**).

Atrankos metu nustatyti 125 *S. cerevisiae* genetiniai veiksniai, kurių iškritas turintys kamienai rodo skirtingo lygio fenotipinį atsaką į K66 toksino poveikį: 73 iš jų atsparesni negu kontrolinis BY4741 kamienas, o 52 – jautresni (**IV**). Visi jautrumą ir atsparumą K66 toksinui lemiantys genetiniai veiksniai suskirstyti į grupes. Didžiausioms grupėms priklauso genai, susiję su ląstelės sienelės biogeneze (15), membranos formavimu, sekrecija ir transportu (16), chromatino struktūra bei genų raiška (18) ir transliacija (13) (5 pav., **IV**). 19 genų pašalinimas lemia vienodą mielių fenotipą atsakant į *S. cerevisiae* K1, K2, K28 ir *S. paradoxus* K66 toksinų poveikį. Skirtingą atsaką į K66 ir kai kurių *S. cerevisiae* sintetinamų toksinų poveikį formuoja

13 vienetinių mutantų. K66 toksino patikros metu 85 % identifikuotų genetinių veiksnių buvo bendri ir K1 bei K2 toksinams galimai dėl panašaus jų veikimo mechanizmo. Nustatyta tik 19 bendrų genetinių veiksnių, svarbių *S. paradoxus* K66 ir *S. cerevisiae* K28 toksinų poveikiui. Kelių iš šių genų produktų funkcijos susijusios su endocitoze, o likusios K66 toksino atžvilgiu yra neaiškios (**IV; 13C**).

Genų ontologijos (GO) analizė parodė statistiškai reikšmingą genų, susijusių su ląstelės sienelės biogeneze, atsaku į osmotinį stresą ir signalinius kelius, praturtinimą. Išreikšta ląstelės sienelės komponentų svarba K66 toksino funkcionalumui leidžia daryti prielaidą, kad K66 veikia sutrikdydamas jonų homeostazę. Tai patvirtina ir nustatytas didelis su atsaku į osmotinį stresą susijusių genų kiekis (**IV**).

STRING 10 duomenų bazėje sukurtas tirtų baltymų tarpusavio sąsajų tinklas, esant vidutinio lygio patikimumo įverčiui (6 pav., **IV**). Parodyta, kad identifikuoti genetiniai veiksniai priklauso ribosominiams komponentams, yra susiję su atsaku į stresą ir signaliniais keliais, ląstelės sienelės biogeneze, transliacijos procesais ir membrana bei baltymų pernaša per ją (**IV**).

***S. paradoxus* K66 toksino pirminių receptorių paieška.** Siekiant nustatyti *S. paradoxus* K66 toksino pirminius receptorius ląstelės sienelėje, ištirtas virusinio baltymo jungimasis su skirtingą β-1,3- ir β-1,6-gliukanų kiekį turinčiomis mielių ląstelėmis (*in vivo* tyrimas) bei su nevienodas gliukanų jungtis turinčiais polisacharidais (*in vitro* tyrimas) (7 pav., **IV**). Tyrimo metu nustatyta, kad Δ*aim26*, Δ*smi1* ir Δ*kre1* vienetines iškritas turintys kamienai su sumažėjusiu β-1,6-gliukanų kiekiu suriša nuo 35 % iki 42 % mažiau K66 toksino molekulių palyginti su kontrolinėmis BY4741 ląstelėmis (7A pav., **IV**). Biocidinis K66 toksino aktyvumas nepriklauso nuo β-1,3-gliukanų koncentracijos ląstelės sienelėje (β-1,3 gliukanų kiekis Δ*aim26* kamiene yra kaip laukinio tipo ląstelėse, Δ*smi1* – 50 % sumažėjęs, o Δ*kre1* – 10 % padidėjęs) (7B pav., **IV**). Kai mielių ląstelės sienelėje β-1,6 jungčių kiekis padidėja kaip Δ*bud27*, Δ*map1* ir Δ*end3* atvejais, K66 toksino prisijungimas padidėja apie 30 % palyginti su kontrolinėmis ląstelėmis. Ląstelės sienelės struktūros defektai daro didelę įtaką K66 toksino prisijungimo prie ląstelių efektyvumui kaip ir *S. cerevisiae* K1 bei K2 toksinų atvejais (Lukša *et al.*, 2015; Novotná, 2004; Pagé *et al.*, 2003; Servienė *et al.*, 2012). Nustatyta koreliacija tarp genų, kurių iškrita lėmė sumažėjusį K66 toksino surišimą ir padidėjusį atsparumą toksinui. To buvo tikėtasi, nes toksinas pirmiausia sąveikauja su ląstelės sienele ir plazminės membranos komponentais. Genai, kurių iškritos lemia padidėjusį toksino surišimą, koreliuoja su padidėjusiu jautrumu K66 toksinui (**IV**).

Siekiant patvirtinti duomenis apie β-1,6 gliukanų svarbą surišant K66 toksiną *in vivo*, įvertintas toksino gebėjimas tiesiogiai surišti polisacharidus, turinčius skirtingas gliukanų jungtis (7C pav., **IV**). Po K66 toksino inkubavimo su laminarinu (β-1,3 ir β-1,6 jungtys), pustulanu (β-

1,6 jungtis), pululanu (α-1,4 ir α-1,6 jungtys) ir chitinu (β-1,4 jungtis) likutinis toksino aktyvumas patikrintas lizės zonų metodu ant jautrių *S. cerevisiae* α‘1 ląstelių. Nesurištas toksinas formuoja skaidrias lizės zonas. Konkurencinis virusinio baltymo veikimo slopinimas parodo, kad tik pustulane esantys β-1,6-gliukanai suteikia prisijungimo vietas K66 toksinui. Jo aktyvumas visiškai panaikintas naudojant pustulaną. Nė vienas iš kitų tirtų polisacharidų nesuriša K66 baltymo, lizės zonos susidarė tokio paties dydžio kaip kontrolinio mėginio be polisacharido.

In vitro ir *in vivo* specifinio surišimo tyrimai patvirtino, kad pirminiai *S. paradoxus* K66 toksino receptoriai yra β-1,6-gliukanai (**IV**), kaip ir *S. cerevisiae* K1 bei K2 toksinų (Hutchins and Bussey, 1983; Lukša *et al.*, 2015).

Mielių genetinių veiksnių sąveika formuojant atsaką į *Saccharomyces* spp. virusines biocidines sistemas. *S. cerevisiae* K2 toksinas jungiasi su ląstelės sienele ir sąveikauja su plazmine membrana, taip lemdamas K^+ ir kitų molekulių pašalinimą iš ląstelės (Marquina *et al.*, 2002; Pagé *et al.*, 2003). HOG ir CWI signalinių kelių sutrikimai pažeidžia ląstelės sienelės vientisumą palaikančius mechanizmus ir osmoreguliaciją, todėl pakinta ląstelės jautrumas K2 toksinui (**VI**). Atsižvelgiant į anksčiau nustatytus K2 toksino veikimą lemiančius genetinius veiksnius (Servienė *et al.*, 2012), atrinkti 12 su ląstelės sienelės vientisumo ir hiperosmotinio slėgio keliais susijusių vienetines genų iškritas turinčių kamienų Δ*ssk1*, Δ*ssk2*, Δ*soh1*, Δ*slm4*, Δ*rlm1*, Δ*lrg1*, Δ*med1*, Δ*spt3*, Δ*spt8*, Δ*bem2*, Δ*fus3*, Δ*tax4*), kurių ląstelių jautrumas keičiasi paveikus K2 toksinu (1 lentelė, **VI**). *SSK1* ir *SSK2* genai stipriai susiję su *HOG1* ir jų pašalinimas lemia ląstelių jautrumą *S. cerevisiae* K1, K2 ir K28 bei *S. paradoxus* K66 toksinui (Carroll *et al.*, 2009; Pagé *et al.*, 2003; Servienė *et al.*, 2012; **IV, VI**). Vienetinis Δ*soh1* mutantas jautrus, o Δ*slm4* atsparus K2 ir K28 toksinams, bet išlieka laukinio tipo veikiant K1 ir K66 (Carroll *et al.*, 2009; Pagé *et al.*, 2003; Servienė *et al.*, 2012; **IV, VI).** Mutacijos Δ*rlm1* ir Δ*lrg1* yra naudingos, lemiančios didesnį atsparumą *S. cerevisiae* K2 ir *S. paradoxus* K66 toksinams (Servienė *et al.*, 2012; **IV, VI**). *MED1*, *SPT3*, *SPT8*, *BEM2*, *FUS3* ir *TAX4* genų pašalinimas lemia ląstelių jautrumą veikiant K2 toksinu, tačiau kiti trys toksinai įtakos nedaro (Carroll *et al.*, 2009; Pagé *et al.*, 2003; Servienė *et al.*, 2012; **IV, VI**). Atrinkti genai pagal GO priklauso osmosinio slėgio signaliniam keliui (**VI**).

Siekiant ištirti HOG ir CWI signaliniuose keliuose dalyvaujančių genetinių veiksnių sąsajas, homologinės rekombinacijos būdu (Janke *et al.*, 2004) iš vienetines genų iškritas turinčių mielių pašalintas *HOG1* pakeičiant jį *HIS3* genu (1C pav., **VI**). Sukurta 12 dvigubas mutacijas turinčių kamienų: Δ*ssk1*-Δ*hog1*, Δ*ssk2*-Δ*hog1*, Δ*soh1*-Δ*hog1*, Δ*slm4*-Δ*hog1*, Δ*rlm1*-Δ*hog1*, Δ*lrg1*-Δ*hog1*, Δ*med1*-Δ*hog1*, Δ*spt3*-Δ*hog1*, Δ*spt8*-Δ*hog1*, Δ*bem2*-Δ*hog1*, Δ*fus3*-Δ*hog1* ir Δ*tax4*-Δ*hog1* (**VI**).

Kadangi mielių ląstelių augimas priklauso nuo pH, temperatūros, mitybinės terpės, taip pat nuo pokyčių genetiniame lygmenyje (Salari and Salari, 2017), siekta nustatyti, ar *HOG1* geno pašalinimas iš mielių pavienių genų mutantų lemia augimo parametrų pakitimus. Nustatytas defektuotų ląstelių augimo greitis praturtintoje YEPD mitybinėje terpėje (3 pav., **VI**). *HOG1* geno pašalinimas iš ląstelės genomo reikšmingai nedaro įtakos augimo parametrams palyginti su BY4741 kamienu (Δ*hog1* augimo greitis yra 0,4 ± 0,06 val.$^{-1}$, o BY4741 – 0,49 ± 0,05 val.$^{-1}$). Palyginus BY4741 kamieno augimo greitį su vienetines genų iškritas turinčių kamienų augimo greičiais, ženklūs pokyčiai taip pat nenustatyti. Kai kurių dvigubas mutacijas turinčių mielių kamienų augimo greičiai reikšmingai pasikeitė palyginti su vienetines genų iškritas turinčiais ir laukinio tipo kamienu: statistiškai patikimai (apie 40 %) padidėjo Δ*spt8*-Δ*hog1* ir Δ*spt3*-Δ*hog1* augimo greičiai. Δ*bem2*-Δ*hog1* kamieno augimo greitis padidėjo 30 % palyginti su vienetinę *BEM2* iškritą turinčiu (**VI**). Pašalinus *HOG1* geną iš Δ*soh1* ir Δ*rlm1* vienetinių mutantų, jų augimo greičiai sumažėjo 25 %. Kitų statistiškai reikšmingų augimo greičio pokyčių tarp vienetines ir dvigubas mutacijas turinčių mielių kamienų nenustatyta. Duomenys apie augimo greičius gali būti naudojami siekiant nustatyti genetinių veiksnių sąveikas bei įvertinti epistatinį poveikį. Stiprios sąveikos, lemiančios ženklius dvigubas mutacijas turinčių mielių kamienų augimo parametrų pokyčius, palyginti su atitinkamais pavienes genų iškritas turinčiais, yra retos (Jakubowska and Korona, 2012). Silpnos sąsajos yra žymiai dažnesnės. Tai patvirtino ir mūsų tyrimas, parodantis silpnus dvigubų mutantų augimo greičio pokyčius palyginti su atskiras genų iškritas turinčiomis mielėmis. Nepaisant to, ir nedideli augimo parametrų skirtumai daro įtaką mielių gyvybingumui ir reakcijai į įvairius stresinius veiksnius, ypač į biocidinį toksino poveikį (**VI**).

Genetinė sąveika tarp HOG ir CWI kelyje dalyvaujančių genetinių veiksnių analizuota jautrumu pagrįstu lizės zonų metodu ląsteles paveikus K2 toksinu (**VI**). Sukeltas stresas aktyvina HOG kelią, kuris reguliuoja glicerolio sintezę ir veikia ląstelių sienelių vientisumą (Saito and Posas, 2012). Genų sąveikos defektuotose ląstelėse įvertinamos lyginant vienetinių ir dvigubų mutantų jautrumą K2 toksinui (4 pav., **VI**). Daugumos tirtų vienetinių mutantų (Δ*ssk1*, Δ*ssk2*, Δ*soh1*, Δ*spt3*, Δ*spt8*, Δ*bem2*, Δ*fus3*, Δ*tax4* ir Δ*hog1*) jautrumas K2 toksinui padidėjo palyginti su tėviniu BY4741 kamienu. Didžioji dalis dvigubų mutantų buvo jautresni toksinui palyginti su vienetiniais mutantais, tačiau išliko tame pačiame jautrumo lygyje kaip ir Δ*hog1* (**VI**).

Išveiklinus *HOG1* geną iš Δ*ssk1* ir Δ*ssk2* ląstelių, kurių pašalintų genų produktai sąveikauja tame pačiame signaliniame kelyje, patikimai reikšmingi pokyčiai, paveikus K2 toksinu, nenustatyti (4 pav., **VI**). Soh1 ir Med1 yra RNR polimerazės II tarpininko komplekso subvienetai (Boube *et al.*, 2002). Dvigubo Δ*soh1*-Δ*hog1* mutanto jautrumas padidėjo palyginti

su atitinkamu vienetinę geno iškritą turinčiu kamienu, o Δ*med1*-Δ*hog1* atsakas į K2 toksiną išliko kaip Δ*med1*. *HOG1* geno pašalinimas iš pažeistų SAGA transkripcijos reguliacijos ląstelių Δ*spt3* ar Δ*spt8* (Wu *et al.*, 2004) padidino jautrumą K2 toksinui 50 % palyginti su vienetiniais mutantais. Pašalinus kinazę Hog1 iš defektuotų Δ*bem2* ir Δ*tax4* kamienų, Δ*bem2*-Δ*hog1* jautrumas K2 toksinui padidėjo 35 %, o Δ*tax4*-Δ*hog1* – dar daugiau. Taigi, minėtų dvigubų mutantų atsako į K2 toksiną fenotipas nepriklausomai nuo genų produktų įsitraukimo į tuos pačius ar skirtingus signalinius kelius nepakito. Nustatytas tik jautrumo lygio padidėjimas (**VI**).

Tyrimo metu tik trys vienetiniai mutantai, kurių pašalintų genų produktai dalyvauja CWI signaliniame kelyje, pakeitė atsaką į K2 toksino sukeltą stresą, kai buvo pašalintas *HOG1* genas (**VI**). Vienetiniai mutantai Δ*rlm1*, Δ*lrg1* ir Δ*slm4* atsparesni K2 toksinui nei tėvinis BY4741 kamienas, o Δ*hog1* jautrus K2 toksinui. Dvigubi Δ*rlm1*-Δ*hog1*, Δ*lrg1*-Δ*hog1* ir Δ*slm4*-Δ*hog1* mutantai yra jautrūs K2 toksinui kaip ir vienetinis Δ*hog1* kamienas. Genai apibūdinami kaip epistatiniai vienas kitam, kai fenotipinį poveikį, susijusį su tam tikra mutacija, pakeičia antroji geno mutacija (Batenchuk *et al.*, 2010; Steidle *et al.*, 2020). Taigi, nustatyta, kad Δ*hog1* mutacija yra epistatinė Δ*rlm1*, Δ*lrg1* ir Δ*slm4* vienetines iškritas turintiems kamienams (**VI**).

Baltymo-baltymo sąveikų tinklas nubraižytas naudojant STRING duomenų analizės programą (Szklarczyk *et al.*, 2011) ir didelio našumo *S. cerevisiae* genomo duomenų bazę (https://www.yeastgenome.org). Sąveikų tinklas su vidutiniu patikimumo įverčiu apima 24 ryšius (5 pav., **VI**). Linijos storis parodo sąsajos stiprumą bei apima funkcinę ir fizinę sąveiką. Hog1 stipriu tiesioginiu ryšiu sąveikauja su HOG signalinio kelio reguliatoriumi Ssk1 ir CWI kelio tarpininku Rlm1. Tiesioginė Hog1 sąsaja su MAP kinase Ssk2, transkripcijos reguliatoriais Spt3 ir Spt8, kaip ir su Rho1 GTPazę aktyvuojančiu Bem2 baltymu šiek tiek silpnesni. Hog1 jungtys su kitais šiame darbe analizuojamais CWI ir HOG kelių dalyviais yra netiesioginės: sąveikauja per baltymų kinazes Fus3 ir Lrg1, per RNR polimerazės II komplekso ir transkripcijos reguliatorių Soh1, per fosfoinozitolinio signalinio kelio reguliatorius Tax4 ir Slm4. Pagal STRING analizės duomenis galima atrasti ir kitų potencialiai HOG ir CWI keliuose sąveikaujančių veiksnių (**VI**).

***S. cerevisiae* ir *S. paradoxus* biocidinių sistemų funkcionavimo palyginimas.** *S. cerevisiae* dgRNR koduojami K1 ir K2 toksinai veikia jautrias ląsteles dviem etapais. Pirmiausia biocidinis baltymas prisijungia prie pirminių ląstelės sienelės receptorių β-1,6-gliukanų (Hutchins and Bussey, 1983; Lukša *et al.*, 2015). Po to toksinai priartėja prie plazminės membranos antrinių Kre1 receptorių ir formuoja katijonams atrankius jonų kanalus, per kuriuos netenkama K^+ jonų ir ATP molekulių, viduląstelinis pH tampa rūgštesnis ir ląstelė žūva (Breinig *et al.*, 2002; Martinac *et al.*, 1990; Orentaite *et al.*, 2016; Schaffrath *et al.*, 2018;

Schmitt and Breinig, 2002). Šiame darbe įrodyta, kad *S. paradoxus* sintetinamo K66 toksino pirminiai receptoriai taip pat yra β-1,6-gliukanai (**IV**). Pagal pirminių receptorių sutapimą galima daryti prielaidą, kad *S. paradoxus* K66 veikimas į ląstelės sienelę yra panašus kaip *S. cerevisiae* K1 ir K2 toksinų.

Kai mielėse išveiklintas *KRE1* genas, ląstelė tampa atspari *S. cerevisiae* K1 ir K2 toksinų poveikiui (Pagé *et al.*, 2003; Servienė *et al.*, 2012). Tiriant Δ*kre1* kamieno fenotipą paveikus K66 toksinu, nustatyta, kad ląstelė tampa atspari kaip ir K1 bei K2 atvejais (**IV; 13C**). Taigi plazminės membranos lygmenyje *S. paradoxus* K66 toksino veikimas taip pat panašus į *S. cerevisiae* K1 ir K2. Pagal turimus duomenis K1, K2 ir K66 toksinų veikimas į mielių ląstelę nesiskiria, tačiau kai kurie jų funkcionalumo rodikliai nesutampa.

S. cerevisiae K1 toksinas optimaliai veikia esant žemesnei nei 23 ºC temperatūrai, kai pH 4,3-4,7 (Dignard *et al.*, 1991; Kurzweilová and Sigler, 1993), o *S. cerevisiae* K2 toksino optimalios veikimo sąlygos yra pH 4,0-4,4 ir 20-25 ºC temperatūra (Lebionka *et al.*, 2002; Lukša *et al.*, 2016; Pfeiffer and Radler, 1984). *S. paradoxus* K66 toksinas geriausiai žudo esant pH 4,4-4,8 ir 20 ºC temperatūrai (**IV**). Taigi, K66 toksino optimalaus veikimo sąlygos panašesnės į K1 nei į K2 toksino.

Biocidiniai toksinai veikdami ląstelę-taikinį sukelia jai stresą, į kurį ląstelė reaguoja keliais būdais: reguliuodama jonų/pH homeostazę, mitochondrijų bei vakuolių veiklą, ląstelės sienelės biogenezę, aktyvuodama specifinius ląstelės sienelės vientisumo, hiperosmotinio slėgio kelius ir kt. (Brewster *et al.*, 1993; Levin, 2011; Servienė *et al.*, 2012). Ląstelė apsisaugodama nuo *S. cerevisiae* K2 toksino poveikio įjungia minėtus CWI ir HOG kelius per pagrindinius MAPK kaskadose dalyvaujančius genetinius veiksnius (Servienė *et al.*, 2012). Pagal veikimo mechanizmų panašumą galima daryti prielaidą, kad reaguodamos į *S. paradoxus* K66 toksiną ląstelės taip pat aktyvina CWI ir HOG signalinius kelius (**7C**).

Išanalizavus HOG ir CWI keliuose dalyvaujančių genų produktų vienetinių mutantų fenotipo skirtumus veikiant *S. cerevisiae* K2 arba *S. paradoxus* K66 toksinu, nustatyti atsako į šiuos toksinus panašumai ir skirtumai. Maždaug ketvirtadalio genetinių veiksnių (Lrg1, Rlm1, Nbp2, Ssk2) vienetines iškritas turinčių kamienų fenotipai sutampa, panašus kiekis (Slm6, Hog1, Plc1, Ssk1, Pbs2) skiriasi jautrumo lygiu. Maždaug pusės abiejuose keliuose svarbių veiksnių (Fks1, Bem2, Fus3, Bni1, Tax4, Slm4, Sac1) vienetines iškritas turinčių kamienų fenotipai paveikus K2 toksinu pasikeičia, o K66 atžvilgiu lieka laukinio tipo (S1 lentelė, **IV;** Servienė *et al.*, 2012). Taigi, ląstelė jautriau reaguoja į *S. cerevisiae* K2 nei į *S. paradoxus* K66 toksino sukeliamą stresą.

Siekiant nustatyti tikslinius su ląstelės sienelės biogeneze susijusius veiksnius, reikalingus *S. cerevisiae* ir *S. paradoxus* biocidinių sistemų veikimui, atlikta mielių vieno geno iškritos

mutantų jautrumo analizė (S1 lentelė, **IV**; **13C**). Ląstelių fenotipas skiriasi paveikus skirtingų *Saccharomyces* genties rūšių sintetinamais toksinais: nustatyti jautrumo ir atsparumo formavimosi skirtumai tarp žinomų *S. cerevisiae* K1, K2, K28 ir išskirtų iš natūralios aplinkos bei gamtinių *S. paradoxus* toksinų (S1 lentelė, **IV**; **13C)**. Įrodyta, kad genetiniai veiksniai, susiję su ląstelės sienelės komponentų sinteze, modifikavimu ir perkėlimu, sąveikauja struktūriškai arba funkciškai ir lemia biocidinių toksinų veiksmingumą (**13C**).

Formuojant atsaką į *S. cerevisiae* K2 ir *S. paradoxus* K66 biocidinių sistemų poveikį, dalis genetinių veiksnių pagal STRING ir GO analizę bendrai priskiriami ląstelės sienelės biogenezės, atsako į stresą ir signalinius kelius funkcinei grupei (**IV**; Servienė *et al.*, 2012), tačiau nemažai jų ir nesutampa. K66 toksino atveju atsakui svarbūs genetiniai veiksniai priskirti transliacijos procesui, susieti su membrana ir baltymų pernaša per ją, ląstelės ciklu, glikozilinimu ir signalo perdavimu (**IV**). Paveikus K2 toksinu, nustatyta, kad atsake dalyvauja genetiniai veiksniai atsakingi už mitochondrijų struktūrą ir transliaciją, cukrų metabolizmą, chromatino struktūrą ir genų raišką, protonų, fosfolipidų pernašą ir ATP sintezę, su endoplazminiu tinklu susijusių baltymų katabolizmą (Servienė *et al.*, 2012).

Palyginus *S. cerevisiae* K1, K2 ir *S. paradoxus* K66 biocidinių sistemų funkcionalumą nustatyti toksinų veikimo panašumai ląstelės sienelės ir membranos lygmenyse (sutampa receptoriai, aktyvuojami streso atsako keliai). Tiek *S. cerevisiae*, tiek *S. paradoxus* toksinų optimaliam veikimui būtina rūgštinė aplinka ir 20-23 ºC temperatūra. Tačiau išryškėjo taikinio-ląstelės reakcijos į *Saccharomyces* spp. sintetinamų toksinų sukeltą stresą skirtumai (skiriasi genetinių veiksnių, dalyvaujančių atsparumo formavime, skaičius bei jautrumo lygis).

IŠVADOS

1. Serbentų, aronijų, šaltalankių ir obuolių mikobiota ir kultivuojamų mielių įvairovė priklauso nuo augalo-šeimininko, vaisių prinokimo stadijos bei radimvietės.
2. Tarp išskirtų biocidinių mielių penkiolikoje procentų yra aptinkamos virusinės dgRNR koduojamos biocidinės sistemos, vyraujančios *Saccharomyces cerevisiae* ir *Saccharomyces paradoxus* mielėse.
3. Vaisių-uogų paviršiuje identifikuoto *S. paradoxus* AML-15-66 kamieno sintetinamas K66 toksinas yra koduojamas virusinėje dgRNR. Toksino optimalios veikimo sąlygos yra 20 ºC temperatūra ir pH 4,8.
4. Pirminiai mielių ląstelės sienelėje esantys receptoriai, prie kurių jungiasi *S. paradoxus* K66 toksinas, yra β-1,6-gliukanai.

5. Genetiniai veiksniai, susiję su mielių ląstelės sienelės ir membranos biogeneze, chromatino struktūra ir genų raiška, sekrecija ir transportu, signaliniais keliais, atsaku į osmotinį stresą, dalyvauja atsparumo *S. paradoxus* K66 toksinui formavime.
6. Ląstelės sienelės vientisumo ir hiperosmotinio slėgio kelių genetiniai veiksniai, reaguojantys į *S. cerevisiae* K2 toksino veikimą, sudaro skirtingo stiprumo fizines ir funkcines sąveikas.
7. *S. cerevisiae* K2 ir *S. paradoxus* K66 toksinų veikimo bei atsako formavimo mechanizmai yra panašūs, tačiau skiriasi genetinių veiksnių įsitraukimas bei mielių reakcijos lygis į toksinų sukeltą stresą.

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4. Novickij, V., Zinkevičienė, A., Stanevičienė, R., Gruškienė, R., Servienė, E., **Vepštaitė-Monstavičė, I.,** Krivorotova, T., Lastauskienė, E., Sereikaitė, J., Girkontaitė, I., Novickij, J. (2018) Inactivation of *Escherichia coli* using nanosecond electric fields and nisin nanoparticles: a kinetics study. *Frontiers in microbiology*, 9: 3006.
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6. Gylienė O., Servienė E., **Vepštaitė I**., Binkienė R., Baranauskas M., Lukša J. (2015) Correlation between the sorption of dissolved oxygen onto chitosan and its antimicrobial activity against *Escherichia coli*. *Carbohydrate Polymers*, 131: 218-223.
7. Lukša, J., **Vepštaitė, I.,** Servienė, E. (2015) Association between cell wall-related processes and functionally non-annotated factors important for K2 susceptibility. *Biologija*, 61(2), 43-49.

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PAPER I

High content analysis of sea buckthorn, black chokeberry, red and white currants microbiota – A pilot study

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Research article

High content analysis of sea buckthorn, black chokeberry, red and white currants microbiota – A pilot study

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ABSTRACT

The high potential of sea buckthorn, black chokeberry, red and white currants in healthy food industry boosted interest in the plant cultivation. The present study is the first work providing comprehensive information on microbial populations of these berries. Next Generation Sequencing allowed identification of eukaryotic and prokaryotic microorganisms prevalent on specific berries, including uncultivable microorganisms. Our study revealed the broad diversity of berries-associated bacterial and fungal microorganisms. Analysis of representative microbial OTUs showed a clear separation among inhabitants of sea buckthorn, black chokeberry and both currants, indicating plant-defined differences in the composition of the bacterial and fungal microbiota. Among the microorganisms distributed on tested berries, we documented potentially beneficial fungi and bacteria along with potential phytopathogens or those harmful for humans. Thus, plant microbiota appears to be highly relevant for the evaluation of the microbiota impact on food quality and human health.

1. Introduction

In recent years, the exploration of various plants and their products for improving human health grew steadily (Agbarya, Ruimi, Epelbaum, Ben-Arye, & Mahajna, 2014; Basu, Rhone, & Lyons, 2010; Boeing et al., 2012). Among the beneficial berries black chokeberry, sea buckthorn, and currants are particularly popular (Basu et al., 2010; Borowska & Brzóska, 2016; Chauhan, Negi, & Ramteke, 2007).

Hippophae rhamnoides L., the common sea buckthorn, is the most widespread species of the genus *Hippophae* common in Europe, Asia, and North America. Sea buckthorn is a popular garden and landscaping shrub preventing soil erosion and reducing pollution (Li, Du, & Guo, 2015). The berries of sea buckthorn have high contents of vitamins (C, E and K), carotenoids, flavanols, and sugars. They are used in food industry, medicine and cosmetics (Li & Schroeder, 1996; Patel, Divakar, Santani, Solanki, & Thakkar, 2012).

Black chokeberry, *Aronia melanocarpa* (Michx.) Ell., is widely planted in natural and domesticated environments of North America, Northern and Eastern Europe as ornamental plants. They are also used as a source for food or beverage production. Berries are loaded with essential phytonutrients, vitamins, antioxidants and bioactive agents, exhibiting antimicrobial, anticancer and antiviral activity (Baum, Howard, Prior, & Lee, 2016; Borowska & Brzóska, 2016; Liepiņa, Nikolajeva, & Jākobsone, 2013).

Red currant (*Ribes rubrum* L.) and white currant (the cultivar of *Ribes rubrum*) are native across Europe, Asia, and North America. These berries are usually cultivated for food and beverages production. Both fruits are rich in vitamin C and K, minerals, as well as organic acids and polyphenols, capable to boost the immune system, help fight infections, reduce a risk of heart disease and cancer (Mikulic-Petkovsek, Schmitzer, Slatnar, Stampar, & Veberic, 2012; Wojdyło, Oszmiański, Milczarek, & Wietrzyk, 2013).

The quality of fruits and berries along with the content of active components depend on the cultivation and climatic conditions during vegetation, application of agrochemicals, hydration and harvest time (Borowska & Brzóska, 2016; George & Cenkowski, 2007). Microorganisms colonizing the surface of fruits, leaves, stems or living within tissues have a major influence on plant development, adaptation and evolution, in turn affecting plant potential in food production (Abdelfattah, Wisniewski, Droby, & Schena, 2016; Barrow, Lucero, Reyes-Vera, & Havstad, 2008). The phytopathogenic microorganisms are responsible for significant economic losses, while others are considered beneficial by inducing resistance in the host (Abdelfattah, Wisniewski, Droby, & Schena, 2016; Droby, Wisniewski, Teixidó,

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0963-9969/

Spadaro, & Jijakli, 2016). Moreover, plant-associated microorganisms may cause foodborne diseases or may have relevant effect for human health by contributing to the diversity within gut microbiome or by stimulating immune response (Berg, Erlacher, & Grube, 2015; Higgins et al., 2018). The distribution of microorganisms on plants is defined by many factors, including plant species, ripening stage, climatic conditions and application of agrochemicals (Pinto et al., 2014, 2015, Pretorius, 2000). Changes in the planting regime alter microbial habitat and ecological niche, leading to the loss of microbial diversity and functional traits (Saleem & Moe, 2014).

According to metagenomic studies, the diversity of the microbiota on fruits and vegetables is high (100–1000 operational taxonomic units, OTUs) with only a few dominant groups (20–50 OTUs) (Leff & Fierer, 2013; Montesinos, Frances, Badosa, & Bonaterra, 2015). In the past, studies of the plant microbiota were based on isolation/culture techniques, thus missing on overall fungal microorganism and bacteria composition (Valero, Cambon, Schuller, Casal, & Dequin, 2007; Volschenk, du Plessis, Duvenage, & Korsten, 2016). The Next Generation Sequencing (NGS) and metagenomic approaches, complemented by the bioinformatics analyses, have made it possible to assay microbial communities, including the organism's refractory to cultivation. The NGS methods have gained increasing attention in recent years in studying of the microbial community dynamics at different points of food production chain, starting from cultivation of food sources, manufacturing and distribution (Abdelfattah, Wisniewski, Li Destri Nicosia, Cacciola, & Schena, 2016; Higgins et al., 2018; Leff & Fierer, 2013). High-throughput sequencing technologies have potential to advance effective application of microbial resources for improving food quality and safety (De Filippis, Parente, & Ercolini, 2018). Recent comprehensive NGS-based microbiome analyses were performed on several fruits and berries, such as grapes, apples, blackcurrants, strawberries, and oranges (Abdelfattah, Wisniewski, Droby, & Schena, 2016; Abdelfattah, Wisniewski, Li Destri Nicosia, et al., 2016; Clooney et al., 2016; Droby et al., 2016; Vepstaite-Monstavice et al., 2018). Nevertheless, our understanding of the diversity of the producer-associated microbial communities, the factors that influence the composition of these communities and the distributions of individual taxa across producer types, in particular among representatives of difficult-to-cultivate taxa, is still limited.

The broad interest in sea buckthorn, black chokeberry, red and white currants requires an investigation of the microbial communities, colonizing the surface of these berries. The goal of the current study was to identify the composition of bacterial and fungal microorganisms associated with the black chokeberry, sea buckthorn, red and white currants harvested in Lithuania. The high-throughput identification and quantification of microflora composition provided information relevant for plant disease management, increasing the yield of the desired crop, and uncovered the potential role of microbiota in berries-based food production.

2. Materials and methods

2.1. Ethics statement

The collection of berries was carried out on private lands with land owners' permission to conduct the study on sites. It did not involve endangered or protected species.

2.2. Sampling of the berries and DNA extraction

Sea buckthorn *Hippophae rhamnoides* L. berries were aseptically collected from the private farm located in the Vilnius region of Lithuania (GPS coordinates: 54°75′20.0”N, 25°27′99.6″E) in the mid-September 2016. Black chokeberry *Aronia melanocarpa* (Michx.) Ell. were collected from the Klaipeda region of Lithuania (GPS coordinates: 55°59′70.0”N, 21°59′60.7″E) in the mid-August 2016. Red currant (*Ribes rubrum*) and white currant (the cultivar of *Ribes rubrum*) were sampled from the Ignalina region of Lithuania (GPS coordinates: 55°34′23.0”N, 26°16′46.8″E) in the mid-July 2016. All plants did not receive any chemical treatment during growing and harvesting period. The samples were collected into sterile plastic bags and processed within 2–4 h after harvesting. The berries of interest (about 300 g) were placed in 500 mL of sterile 0.05 M phosphate buffer pH 6.8 for 30 min at room temperature with shaking at 120 rpm. Outwashes were filtered through 420 μm filters, centrifuged at 12,000 × *g* for 20 min, and pellets were stored at −20 °C until subsequent analysis.

For metagenomic analysis, 40 mg of pellet per sample were used. DNA isolation from collected sediments was performed using a Genomic DNA purification kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) in accordance with the manufacturer's protocol. The quantity and quality of extracted DNA were determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).

2.3. Bacterial and fungal DNA amplification and amplicon library preparation

DNA samples from sea buckthorn, black chokeberry, red and white currant microbiota were amplified using the specific primers: for fungal microorganisms ITS3-KYO2 (5′-GATGAAGAACGYAGYRAA-3′) and ITS4 (5′- TCCTCCGCTTATTGATATGC-3′) (Toju, Tanabe, Yamamoto, & Sato, 2012); for bacteria S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′) (Klindworth et al., 2013). Amplicon library preparation was performed at Macrogen Inc. (Seoul, Korea) using modified Illumina adapters (Illumina, San Diego, USA). The validation of prepared library was performed on Agilent Technologies Bioanalyzer DNA 1000, the quantification of DNA library templates performed using qPCR according to the Illumina Protocols. Amplicons were sequenced at Macrogen Inc. (Seoul, Korea) using Illumina MiSeq V3 (2 × 300 bp).

2.4. Data processing and analysis

The bioinformatics pipelines, FLASH v1.2.11 (Magoc & Salzberg, 2011), CD-HIT-OTU v4.5.5 (Li, Fu, Niu, Wu, & Wooley, 2012), QIIME v1.8 (Caporaso et al., 2010), were used to process and analyze the sequence data. Preliminary processing of the data was performed using default parameters of FLASH v1.2.11: sequences with a minimum quality score of 25 were filtered and paired-end reads were merged. Sequences were denoised, chimeric sequences were identified and removed, and the remaining reads were clustered into the Operational Taxonomical Units (OTUs) with a minimum 97% similarity threshold, using the CD-HIT-OTU v4.5.5 (Li et al., 2012). The most abundant sequences in each OTU were used for the taxonomy assignments using the RDP for 16S rDNA (Cole et al., 2014; Wang, Garrity, Tiedje, & Cole, 2007) and the UNITE for ITS (Kõljalg et al., 2013) databases as references. The QIIME v1.8 (Caporaso et al., 2010) was used to generate rarefaction curves demonstrating richness of population. For a downstream analysis, the OTU table was rarefied at an even depth (unassigned sequences and chloroplasts were discarded, 62,652 number of reads used for 16S rRNA rarefaction and 134,622 number of reads - for ITS2). Alpha diversity was calculated using observed species, Shannon, Good's coverage and Chao1 estimates (Caporaso et al., 2010). Weighted UniFrac metrics were used to evaluate β-diversity and Principal coordinates analysis (PCoA) as implemented in QIIME v1.8 was used to relate the bacterial and fungal microorganism community composition to sample types (Lozupone & Knight, 2005). The heatmap was generated using ascendant hierarchical clustering based on Euclidian distances (in XLSTAT 2018.04.20).

2.5. Nucleotide sequence accession number

The sequencing data are available at the Sequence Read Archive

(SRA) of the National Center for Biotechnology Information (NCBI), under accession number SRP108325.

3. Results

3.1. Diversity and richness of microbial communities

Illumina MiSeq sequencing generated 455,374 high quality 16S rRNA gene amplicons and 862,565 ITS reads for the black chokeberry, sea buckthorn, red and white currants (Table 1). The clustering of the sequences generated a total of 1888 OTUs (498 for bacterial V3-V4 and 1390 for fungal ITS2) (Table 1). The total number of detected bacterial OTUs varied from 68 to 214, while that for fungal OTUs ranged from 217 to 491 in the tested berries samples. In both prokaryotic and eukaryotic sequences, the highest number of OTUs was observed in red currants and the lowest in white currants. In agreement with OTU data, the Shannon's Diversity and the Chao1 estimates also revealed that red currant berries had a higher bacterial and fungal microorganism diversity than other berries. The ratio between the number of the obtained and the expected OTUs (predicted by Chao1) was used to determine the coverage for the microbial communities: it was above 94% in all cases, indicating that a good coverage was achieved. Rarefaction curve showed that the numbers of OTUs were saturated in all samples and enough for further community analysis (Fig. S1).

3.2. Characterization of sea buckthorn's bacterial and fungal microorganism communities

We proceeded to explore the taxonomic profiles of the sea buckthorn-associated bacterial and fungal microorganisms. In total, six bacterial phyla (35 families and 56 genera) and four fungal phyla (58 families and 108 genera) were identified. The dominant phylum across an entire prokaryotic microorganism population was Proteobacteria (71.1%) (Fig. 1A), mainly represented by Alphaproteobacteria and Gammaproteobacteria at the class level (Fig. 1B). Across the eukaryotic microorganism population, Ascomycota was the dominant phylum accounting for 89.4% of the total number of detected sequences, followed by Basidiomycota (8.2%) (Fig. 2A). The major group of OTUs within the Ascomycota belonged to the class Dothideomycetes, while the Basidiomycota was represented by members of the class Tremellomycetes (Fig. 2B).

At higher taxonomic resolution, bacterial community was mostly dominated by the families Enterobacteriaceae (31.4%), Microbacteriaceae (16.3%) and Pseudomonadaceae (14.1%) (Fig. 1C), exemplified by the most abundant genera *Pantoea* (16.8%), *Okibacterium* (14.4%) and *Pseudomonas* (14.1%), respectively (Fig. 1D, Table S1). Fungal microorganism community on sea buckthorn was dominated by Dothioraceae (78%), followed by Davidiellaceae (2.4%) and traces of Taphrinaceae and Saccharomycodaceae (Fig. 2C). At the genus level, the vast majority of sea buckthorn-associated fungal microorganisms (87.9%) were described as unidentified (Fig. 2D, Table S2).

3.3. Black chokeberry microbiota composition

Of the six bacterial phyla detected in the present study on black chokeberry, the dominant phyla were Proteobacteria (59.9%) and Bacteroidetes (27.7%). The rest of the community consisted of Actinobacteria (7.3%), Firmicutes (2.4%), and others (Fig. 1A). At the class level, prokaryotic microorganisms mainly belonged to Alphaproteobacteria, Cytophagia and Gammaproteobacteria (Fig. 1B). Among 33 bacterial families identified (Table S1), Sphingomonadaceae (27.7%) and Cytophagaceae (23.6%) were the most abundant (Fig. 1C), represented by the genera *Sphingomonas* (27.1%) and *Hymenobacter* (23.2%) (Fig. 1D). The rest of the community consisted of *Acinetobacter*, *Pseudomonas*, *Variovorax*, *Pantoea*, *Frondihabitans* and *Mucilaginibacter* (Fig. 1D; Table S1).

The fungal microorganism associated with black chokeberry belonged to three phyla, where Ascomycota (66.2%) and Basidiomycota (32.2%) were the most abundant (Fig. 2A, Table S2). The first of them was represented by Dothideomycetes and Taphrinomycetes, the second one - by Tremellomycetes, Microbotryomycetes and Exobasidiomycetes (Fig. 2B). Of the 34 fungal families identified on black chokeberry, Davidiellaceae (20.8%) dominated, along with Dothioraceae (7.9%) and Taphrinaceae (3.5%) (Fig. 2C). These families were represented by *Cladosporium* (20.8%) and *Taphrina* (3.5%). Representatives of Basidiomycota at the genus level were *Cryptococcus* (10.9%) and *Rhodotorula* (9.1%) (Fig. 2D). Other fungal microorganisms, such as *Phoma*, *Mrakiella*, *Lewia* and *Hanseniaspora*, were detected at low frequencies. About half of the black chokeberry-associated fungal microorganisms (45.6%) were unidentified at the genus level (Table S2).

3.4. Composition of red and white currant bacterial and fungal microorganism communities

The bacterial community profile analysis showed a total of eight phyla, 51 families and 82 genera distributed on red and white currants. Bacterial phyla, dominating on currant berries, were ascribed to Proteobacteria (56.2% on red and 57.5% on white currant, respectively), Bacteroidetes (28.4% and 26.6%, respectively) and Actinobacteria (11.5% and 13.8%, respectively) (Fig. 1A). At the class level, they were represented by Alpha-, Beta- and Gammaproteobacteria, along with Cytophagia and Actinobacteria (Fig. 1B). The broad diversity of bacteria was evident at the family and genus level (Fig. 1C, D). Most of the family-level OTUs belonged to Cytophagaceae (17.5% on red and 18.1% on white currant, respectively), Sphingomonadaceae (15.1% and 11.3%), Oxalobacteraceae (10% and 8.2%), Methylobacteriaceae (10.3% on both currants), Pseudomonadaceae (5.7% and 7.6%), Microbacteriaceae (7.8% and 10.4%) and Comamonadaceae (4.4% and 9.5%) (Fig. 1C). Dominating genera on both

Table 1
Summary of metagenomic surveys conducted on black chokeberry, sea buckthorn, red and white currant berries.

Samples	Target region	High quality reads	OTUs	Chao1	Coverage	Shannon diversity
Black chokeberry	V3–4	125,380	79	80.5	0.9814	4.15
	ITS2	230,205	236	238	0.9916	4.08
See buckthorn	V3–4	142,450	137	137.5	0.9964	3.20
	ITS2	262,515	446	471.6	0.9457	2.39
Red currant	V3–4	117,755	214	215.5	0.9930	5.64
	ITS2	224,754	491	494.2	0.9935	4.34
White currant	V3–4	69,789	68	69	0.9855	5.03
	ITS2	145,091	217	220	0.9864	4.11
	Prokaryotic	455,374	498			
	Eukaryotic	862,565	1390			
	Total	1,317,939	1888			

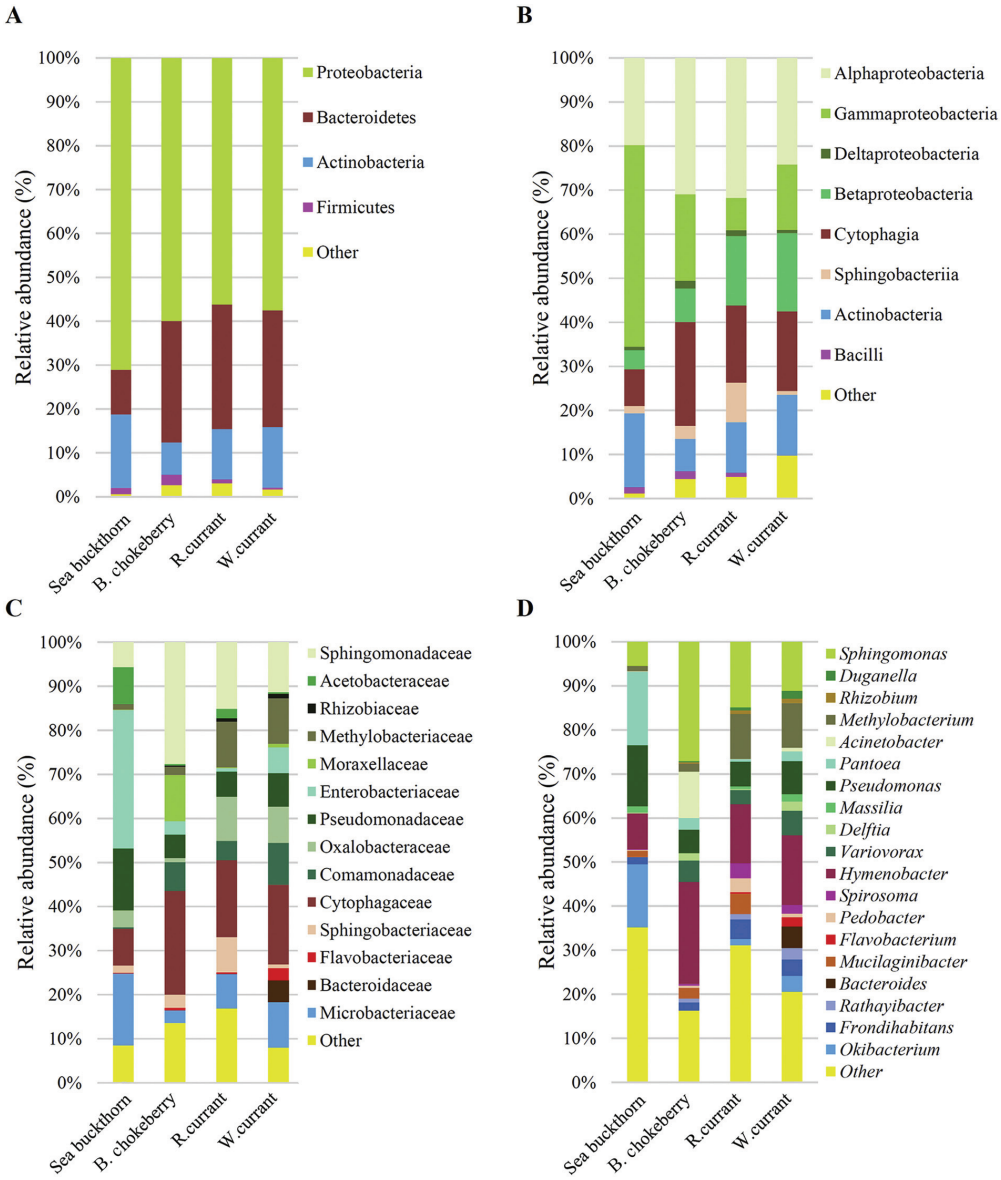


Fig. 1. Prokaryotic microbial community distribution on sea buckthorn, black chokeberry, red and white currant. A – phylum level, B – class, C – family, D – genus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

currants analyzed were *Hymenobacter*, *Sphingomonas* and *Methylobacterium*, followed by *Pseudomonas*, *Frondihabitans* and *Variovorax* (Fig. 1D). Some genera were observed on red currant berries only, such as *Mucilaginibacter* (4.6%), or exceptionally on white currants, such as *Bacteroides* (4.9%) (Table S1).

The fungal microorganisms distributed on red and white currants belonged to two phyla, 59 families and 106 genera. Similarly to the sea buckthorn and black chokeberry, the dominant phylum was Ascomycota (68.3% on red and 57.3% on white currant, respectively), followed by Basidiomycota (28.2% and 39.7%) (Fig. 2A). The most abundant class was Dothideomycetes (53.1% and 51.5% on red and white currants, respectively) (Fig. 2B), represented by Dothioraceae and Davidiellaceae families (Fig. 2C). Some families, as Glomerellaceae (9.1%), were detected on red currant only (Fig. 2C), represented by *Colletotrichum* (Table S2). The most dominant genera on both currants were *Cladosporium* (17.3% on red and 20.7% on white currant) and *Cryptococcus* (11.5% and 21%, respectively), followed by *Phoma* (4.8% and 8.3%) and *Rhodotorula* (1.6% and 3%) (Fig. 2D). Unidentified at the genus level fungal microorganisms were present on both plants (40.2% and 31.8% for red and white currant, respectively) (Table S2).

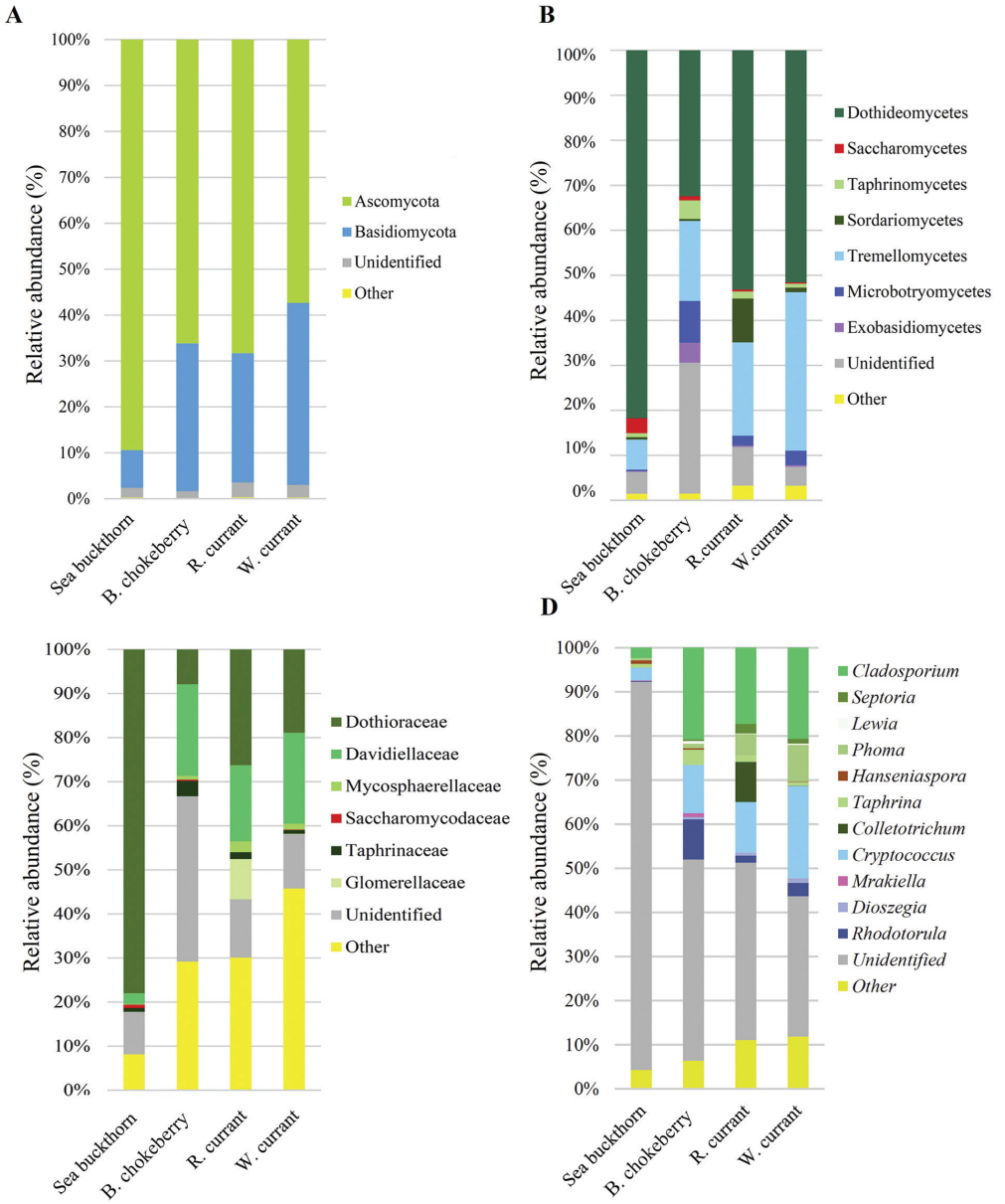


Fig. 2. Fungal microorganism community distribution on sea buckthorn, black chokeberry, red and white currant. A – phylum level, B – class, C – family, D – genus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Comparison of bacterial and fungal microorganism communities on sea buckthorn, black chokeberry, red and white currants

The Principal Coordinate Analysis (PCoA), performed with the representative OTUs, displayed a clear separation of sea buckthorn, black chokeberry and both currants, indicating a difference in the composition of the bacterial and fungal microorganism communities (Fig. 3). A certain distinction was observed between both currants based on the structure of the bacterial population (Fig. 3A). This was likely influenced by several bacterial genera present exclusively on one type of the berries (*e.g. Mucilaginibacter* - on red currant berries, *Bacteroides* - on white currant). The clustering of red and white currant bacterial community into the separate sections of PCoA plot from sea buckthorn and black chokeberry can be explained by the increased number of several less abundant genera. The fungal microbiota of red and white currants clustered together, while the communities of the fungal microorganisms on sea buckthorn and black chokeberry were clearly separated from each other and both currants (Fig. 3B).

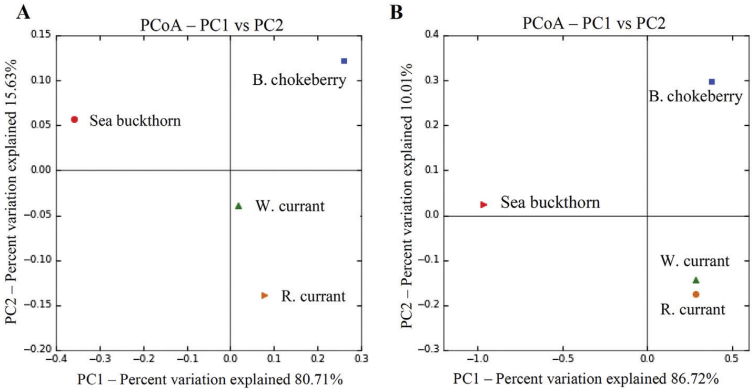


Fig. 3. Principal coordinates analysis (PCoA) of the relative abundance of bacterial (A) and fungal microorganism (B) OTUs.

The heatmap diagram illustrated distribution of the most abundant fungal and bacterial OTUs on various fruit surfaces (Fig. 4). Among bacteria community, OTUs of *Methylobacterium* (OUT12, OTU23 and OTU30), *Pedobacter* (OTU36) and *Frondihabitans* (OTU14, OTU32) inhabited mainly red currant, while those belonging to *Flavobacterium* (OTU33), *Duganella* (OTU24) and *Bacteroides* (OTU61) were more abundant on white currant. Higher abundance of OTUs matching in GenBank to *Okibacterium* (OTU11) and *Pantoea* (OTU4) was documented on sea buckthorn as compared to both currants and black chokeberry. OTUs from the *Acinetobacter* (OTU22), *Bacillus* (OTU89), *Kineasporia* (OTU42) and *Variovorax* (OTU20) bacteria were more abundant on black chokeberry as compared to other berries tested (Fig. 4A). Among *Pseudomonas* genera, different distribution of closely-related microorganisms is observed, e. g. *Pseudomonas* OTU5 is present mainly on sea buckthorn surface, while OTU8 distributed similarly on sea buckthorn and red currant. Differently developed OTUs from *Hymenobacter* genera also possess various distribution: OTU25 and OUT123 is inhabiting mainly black chokeberry, OTU105 is related to white currant and OTU148 inhabiting red currants.

Among fungal microorganism community, OTUs assigned to *Hanseniaspora* (OTU0) and *Cystofilobasidiales* (OTU55) were more prevalent on sea buckthorn samples comparing to other fruits tested. Conversely, we found a significantly higher proportion of OTUs belonging to *Taphrina* (OTU14), *Lewia* (OTU66), *Rhodotorula* (OTU16, OTU18) and *Mrakiella* (OTU61) on black chokeberry compared to sea buckthorn and both currants. OTUs from *Phoma* (OTU13), Tremellales (OTU10, OTU28) and *Articulospora* (OTU60) were more distributed on both currants as compared to other berries tested, while *Knufia* (OTU36), *Septoria* (OTU19) and *Colletotrichum* (OTU12) were more prevalent on red currant (Fig. 4B). Some OTUs of the same genera developed differently and vary on inhabitation pattern, e. g. *Cryptococcus* OTU17 is present mainly on black chokeberry, OTU4 inhabits both currants, OTU11 is distributed on sea buckthorn and OTU23 is more prevalent on black chokeberry and white currants.

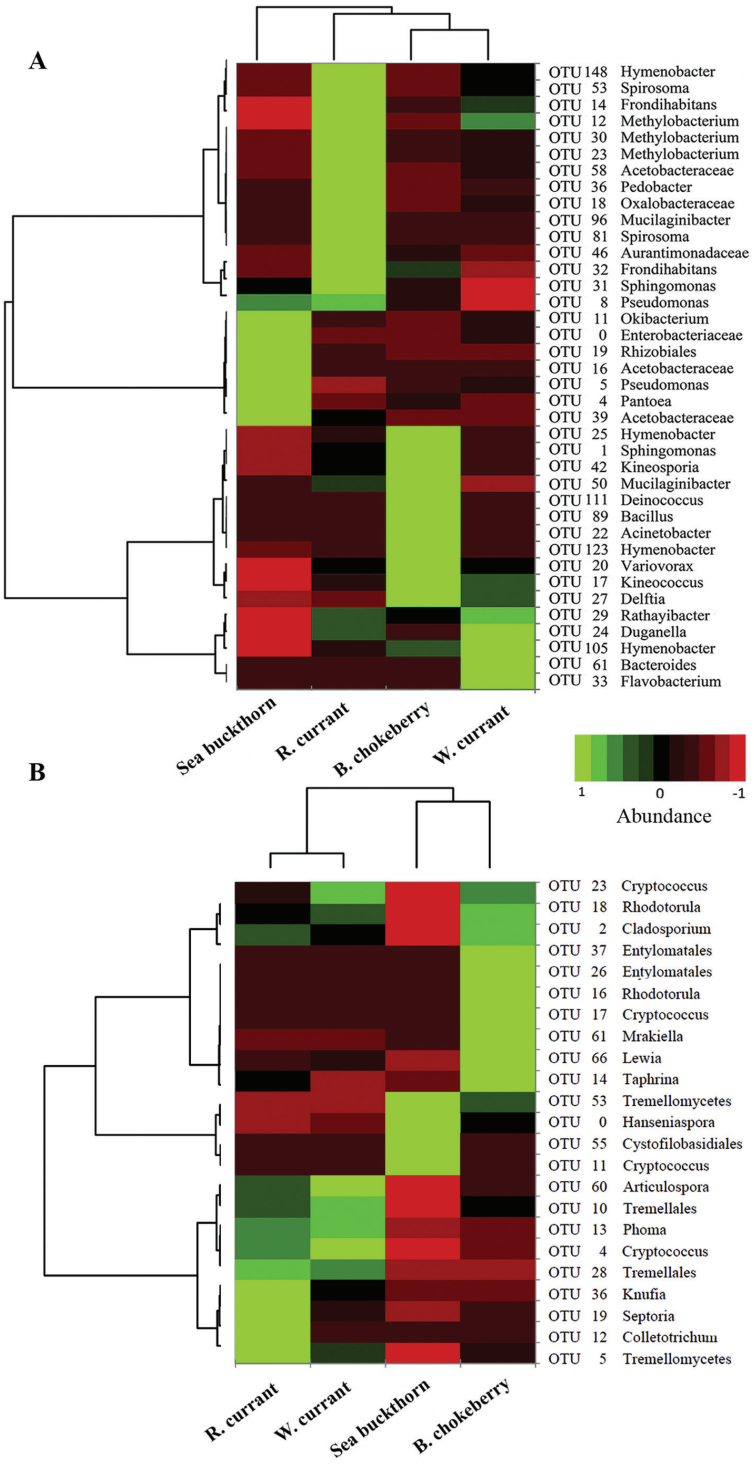
4. Discussion

The current study for the first time uncovered the structure of microbial populations associated with sea buckthorn, black chokeberry, red and white currant berries. For a comprehensive and culture-free analysis, we have applied Next Generation Sequencing and observed the wide diversity of prokaryotic and eukaryotic microorganisms on all berries tested.

According to our data, sea buckthorn, black chokeberry and both currants were dominated by fungal microorganisms prevalent on intact fruits due to the early harvesting time. In grapes, it was previously demonstrated that the intact berries were dominated by basidiomycetous yeasts, such as *Cryptococcus*, *Rhodotorula*, *Sporobolomyces* and the yeast-like fungus *Aureobasidium pullulans* (Barata, Malfeito-Ferreira, & Loureiro, 2012). During the ripening, the oxidative or weakly fermentative ascomycetous populations, such as of *Candida*, *Hanseniaspora*, *Metschnikowia*, and *Pichia* increased in frequency (Barata et al., 2012). *Cladosporium* and *Cryptococcus* were the most abundant genera observed in our study on black chokeberry, red and white currant berries. This is not surprising since *Cladosporium* are considered as ubiquitous fungi, with some species causing plant or human diseases (Sandoval-Denis et al., 2016; Wit et al., 2012), while others providing numerous antifungal agents (Wang et al., 2013). The abundant presence of *Cryptococcus* may be relevant, considering that species of this genus have been used as biocontrol agents against many pathogens (Chand-Goyal & Spotts, 1996; Hashem, Alamri, Hesham, Al-Qahtani, & Kilany, 2014). The genus *Cryptococcus* also encloses species capable of biofilm formation, thus reducing fungal cell susceptibility to heat, cold and UV irradiation (Martinez & Casadevall, 2007). Of note, certain species of *Cryptococcus* can cause infections in humans and animals, e. g., nervous system mycoses or pulmonary diseases (Bernal-Martinez et al., 2010).

It is generally accepted that more than 98% of microorganisms present in nature cannot be isolated (Lucero, Unc, Cooke, Dowd, & Sun, 2011). Therefore, the most informative estimates of the fungal diversity in plants are obtained from the metagenomic sequence data (Handelsman & Alvarez-Cohen, 2007). When fresh-cut fruits such as apple, plum, pear, or orange were analyzed by culture-dependent techniques, only microbial populations of limited diversity, mainly consisting of yeasts and molds, were isolated (Graca et al., 2015; Janisiewicz, Jurick, Peter, Kurtzman, & Buyer, 2014; Vadkertiova, Molnarova, Vranova, & Slavikova, 2012; Volschenk et al., 2016). Employment of the high-throughput sequencing techniques allowed detection of not only prevalent microorganisms, but also species present at low abundance or unculturable, thus overcoming the limitations of the culture-dependent approaches (Clooney et al., 2016; Pinto et al., 2015; Vepstaite-Monstavice et al., 2018). According to our data, the majority of fungal microorganisms present on sea buckthorn were represented by uncultured *Aureobasidium*. On the other berries, uncultured Ascomycota (such as uncultured Metschnikowiaceae and uncultured *Taphrina*) were detected. Recently, it was demonstrated that some *Aureobasidium* species demonstrated biological control activity against leaf pathogens, especially against mold such as *Botrytis* and *Bacillus* bacteria (Grube, Schmid, & Berg, 2011; Parsa et al., 2016). *Metschnikowia* also includes species commonly found on the fruit surface and acting as biocontrol agents against different pathogens (Parafati, Vitale, Restuccia, & Cirvilleri, 2015).

We have identified beneficial fungal microorganisms, such as



(caption on next page)

Fig. 4. Heatmap of bacterial and fungal microorganisms OTU abundance on sea buckthorn, black chokeberry, red and white currant. The colour intensity is proportional to the relative abundance of bacterial and fungal microorganism OTUs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Hanseniaspora, *Rhodotorula* and *Dioszegia*, distributed at a low frequency. *Rhodotorula* spp. were previously found on grapes during all ripening stages and shown to be capable of producing biofilms on surfaces of berries, thus protecting them from pathogens (Lederer, Nielsen, Toldam-Andersen, Herrmann, & Arneborg, 2013). *Hanseniaspora* spp. displays antagonistic properties against the development of molds responsible for fruit spoilage (Liu et al., 2010). *Dioszegia* spp. is referred as a beneficial microorganism associated with arbuscular mycorrhizal fungi and possessing antagonistic activities (Renker, Blanke, Borstler, Heinrichs, & Buscot, 2004). From the potential pathogens for plants and/or humans, *Phoma*, *Lewia*, *Colletotrichum*, *Septoria*, *Taphrina* were identified (Aveskamp, de Gruyter, & Crous, 2008; Cisse et al., 2013; Eyal, 1999; Phalip, Hatsch, Laugel, & Jeltsch, 2006; Wang et al., 2013).

The presence of fungal microorganisms can potentially alter the composition of bacterial communities and *vice versa* (Grube et al., 2011). Among the prokaryotic consortia on all berries tested, we have identified microorganisms either potentially beneficial or pathogenic. *Pantoea* and *Pseudomonas* were abundant on sea buckthorn. It was reported previously that both genera contain species possessing antibacterial and antifungal activity (Ligon et al., 2000; Trotel-Aziz, Couderchet, Biagianti, & Aziz, 2008). However, certain *Pantoea* or *Pseudomonas* species, distributed in diverse ecological niches, including water, soil, fruits, vegetables and foodstuffs, are recognized plant, animal or human pathogens (Coutinho & Venter, 2009; Cruz, Cazacu, & Allen, 2007; Higgins et al., 2018; Pukatzki, Kessin, & Mekalanos, 2002). Bacteria from the genera *Sphingomonas* and *Hymenobacter* residing on black chokeberry, red and white currants could be advantageous for plants due to their ability to induce plant resistance and promote growth, as well as by exhibiting antagonism against food-spoilage bacteria (Kim et al., 1998; Mageswari, Subramanian, Srinivasan, Karthikeyan, & Gothandam, 2015; Sukweenadhi et al., 2015). Similar beneficial traits may be possessed by other bacteria documented in our study, such as *Massilia* spp. (Ofek, Hadar, & Minz, 2012), *Acinetobacter* spp. (Suzuki, Sugawara, Miwa, & Morikawa, 2014), *Flavobacterium* spp. (Sang & Kim, 2012), *Methylobacterium* spp. (Ryu et al., 2006). Some species of abovementioned genera may induce metabolic processes that cause food to be unsuitable for human consumption (Ercolini, Russo, Nasi, Ferranti, & Villani, 2009; Rawat, 2015). Since microorganisms cohabiting sea buckthorn, black chokeberry and both currants at the species level were undescribed or assigned to the uncultured bacterium, it is plausible to suggest that some species undesirable for plants and humans are also present at a low frequency.

Co-existence of the different microbial population on the plants is driven by the multiple forces. It is dependent on produced enzymatic or bioactive compounds and generates competition for the nutrients (Pinto et al., 2014). The distribution of fungal and bacterial microorganisms on sea buckthorn, black chokeberry and both currants was showed to be essential to understand the existing balance of pathogenic and beneficial microorganisms. This is highly relevant for the development of strategies for plant cultivation and disease management. Furthermore, an increasing demand of natural food, minimally processed and prepared without chemical preservatives, requires comprehensive analysis of microbiota residents of the plants. The technology of production of natural food therefore should be bounded with the understanding of the balance among beneficial and pathogenic microorganisms, ubiquitously present even under good farming practices. The limited processing underscores the significance of quality of raw material for preparation of food truly valuable for human health.

5. Conclusion

In this study, we analyzed bacterial and fungal microorganism populations associated with the black chokeberry, see buckthorn, red and white currant berries harvested in Lithuania by applying NGS techniques. Differences in diversity, composition and overall prevalence of eukaryotic and prokaryotic microorganism were dependent on the host plant. Among prokaryotic and eukaryotic consortia, we have identified potentially beneficial and pathogenic microorganisms. Obtained data substantiate understanding of the interactions between resident microflora and plant. The study uncovers the importance of microbiota in berries-based food production and deepens knowledge of their ecological and medical potential.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2018.05.060.

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Author contributions

Investigation: IVM, JL.
Bioinformatic analysis: JL, ES.
Data curation and analysis: ES, SS, VY.
Funding acquisition: ES.
Writing - original draft: SS, ES.
Writing - review & editing: SS, VY, ES.

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PAPER II

Fungal microbiota of sea buckthorn berries at two ripening stages and volatile profiling of potential biocontrol yeast

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Fungal Microbiota of Sea Buckthorn Berries at Two Ripening Stages and Volatile Profiling of Potential Biocontrol Yeasts

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Abstract: Sea buckthorn, *Hippophae rhamnoides L.*, has considerable potential for landscape reclamation, food, medicinal, and cosmetics industries. In this study, we analyzed fungal microorganism populations associated with carposphere of sea buckthorn harvested in Lithuania. An amplicon metagenomic approach based on the ITS2 region of fungal rDNA was used to reveal the ripening-affected fungal community alterations on sea buckthorn berries. According to alpha and beta diversity analyses, depending on the ripening stage, sea buckthorn displayed significantly different fungal communities. Unripe berries were shown to be prevalent by *Aureobasidium*, *Taphrina*, and *Cladosporium*, while ripe berries were dominated by *Aureobasidium* and *Metschnikowia*. The selected yeast strains from unripe and mature berries were applied for volatile organic compounds identification by gas chromatography and mass spectrometry techniques. It was demonstrated that the patterns of volatiles of four yeast species tested were distinct from each other. The current study for the first time revealed the alterations of fungal microorganism communities colonizing the surface of sea buckthorn berries at different ripening stages. The novel information on specific volatile profiles of cultivable sea buckthorn-associated yeasts with a potential role in biocontrol is important for the development of the strategies for plant cultivation and disease management, as well as for the improvement of the quality and preservation of the postharvest berries. Management of the fungal microorganisms present on the surface of berries might be a powerful instrument for control of phytopathogenic and potentially antagonistic microorganisms affecting development and quality of the berries.

Keywords: *Hippophae rhamnoides*; fungal communities; volatile organic compounds

1. Introduction

Sea buckthorn (*Hippophae rhamnoides L.*) is garden and landscaping shrub widespread in Europe, Asia, and North America, distinguished for good adaptation to barren soils and harsh climates [1]. The ecological impact of sea buckthorn in preventing soil erosion, improving its properties, and reducing pollution has been demonstrated [2]. Economically, the plant species has been considered as a source of healthy nutritious food, giving rise to an increased demand for medicine and cosmetics [3,4].

Cultivation of sea buckthorn is developing due to the request for its berries with a high content of minerals, different vitamins, specific valuable oils, phenolics, and essential fatty acids, as well as low level of both fructose and glucose [5]. Phytochemicals and essential oil generated from sea buckthorn berries and seeds reduce inflammation; have antibacterial, anticancer, and radioprotective activity; relieve pain; and promote regeneration of tissues [3,6,7].

The microenvironments coherent with plants and fruits are highly colonized by different communities of bacterial and fungal microorganisms [8]. Plants provide nutrient-rich and stable surrounding conditions vital for the development of microbiota. In turn, epiphytic microorganisms produce secondary metabolites that may enhance plant immunity to unfavorable conditions and diseases [8–10]. Numerous microorganisms due to the production of volatile organic compounds (VOCs) have been found to promote plant growth and improve crop productivity [11]. Volatile production, secretion of antifungal compounds, and killer toxins allow for fruit-associated microorganisms to demonstrate increased antagonistic activity, an ability to modulate carposphere microbiota structure, and might mediate interactions with insects [12–16]. The role of bacterial and fungal microorganisms as producers of chemical indicators for the attraction of insects and their communication has also been revealed [17,18]. Microorganisms occupy a trophic level between plants and insects. Insects from several orders are feeding on fruits, while yeasts use insects either as hosts or vectors [17]. The chemical composition of each blend of volatiles may vary depending on the producing yeast and the ecological niche where the cross-talking species are growing [14,16].

The spreading of microorganisms on plants is determined by many factors, such as climatic conditions, application of agrochemicals, plant species, and ripening stage [19–21]. Changes in the planting regime alter microbial habitat and diversity, affect functional traits [22]. Comprehensive next-generation sequencing-based (NGS) microbiome analysis accomplished on several fruits and berries—such as apples, blackcurrants, grapes, olives, strawberries, oranges, etc.—is mainly focused on plant-conditioned prevalence [23–28]. The biogeographic distribution of microbiota communities has been revealed on apple, blackcurrant, and grape [21,28]. The effect of the ripening stage on the development of the microbial population was investigated in grapes, mango, nectarines and plums [8,29–31]. Analysis of grape bacterial microbiota defined changes in the structure and size of the population occurring during the ripening process. The levels of bacteria raised gradually and reached their highest value when the berries became overripe [8]. Yeasts are sparsely observed in the early stage of fruit development, but they increase in number as fruits ripen [32]. The yeast genera occurring on most mature nectarine fruit (e.g. *Hanseniaspora*, *Pichia*, and *Zygosacharomyces*) were significantly different from those on fruit at earlier stages of development [29].

It must be noticed that few works dealing with the characterization of sea buckthorn-associated microbial communities were published [33,34]. By analyzing representative microbial operational taxonomic units (OTUs) prevalent on sea buckthorn berries, the plant-defined composition of bacterial and fungal microbiota was recorded [33]. The impact of plant age and season was investigated in sea buckthorn plantation on the rhizosphere microbial community only [34,35].

To the best of our knowledge, there is no information on the ripening affected sea buckthorn carposphere-associated fungal community's alterations and release of volatile organic compounds by the yeasts isolated from this plant. Therefore, the aims of this study were (i) to investigate the changes of the sea buckthorn-associated fungal microbiota depending on the ripening stage of the berries, (ii) to isolate and cultivate potential biocontrol yeasts distributed on unripe and ripe sea buckthorn berries, and (iii) to identify pattern of volatiles produced by the selected cultivable yeast species. The obtained information on the ripening-affected changes of sea buckthorn-associated fungal microbiota composition, and distribution of potentially beneficial and phytopathogenic microorganisms, may be important to develop effective plant disease control strategies. Due to high demand of sea buckthorn berries in food industry and increased request of unprocessed food, the metagenomics data appear to be highly relevant for the evaluation of the impact of microbiota and specific microorganisms on food quality and human health. Isolated cultivable yeasts might encourage new opportunities for

developing natural tools for disease management, postharvest protection, and the quality of the sea buckthorn berries with potential in food production. The data on volatile profiling of selected yeasts could be relevant for improving plant productivity and generating new environmentally friendly formulations for pest control and plant disease management.

2. Materials and Methods

2.1. Ethics Statement

The collection of sea buckthorn berries was authorized by the private owner, fully acknowledged in a paper. Endangered or protected species were not involved; therefore, no specific permissions were required.

2.2. Sample Collection

Hippophae rhamnoides L. berries were aseptically sampled from the organic private plantation located in the Molėtai region of Lithuania (GPS coordinates: 55°15′12.2″N, 25°26′23.1″E). The sea buckthorn berries were collected from three different places of sea buckthorn plantation separated by more than 50 meters. The berries were randomly selected from three bushes and combined into one biological replicate at each location. The experiment was carried out at two different growth stages: at the beginning of berry ripening (UB) in July and ripe berries (RB) in September 2018. The samples were collected into sterile plastic bags, transported to the laboratory and processed within 2–3 h after harvesting.

For metagenomic analysis, 300 g of the berries were placed into flasks with 500 mL of sterile 0.05M phosphate buffer (pH 6.8) for 30 min and incubated at room temperature with shaking at 120 rpm. Outwashes were filtered through 420 µm filters and followed centrifugation at 12,000× g for 20 min. The pellets were stored at −20 °C for subsequent DNA extraction.

For cultivable fungal microorganisms isolation, 15 g of the berries were placed in a 30 mL of liquid MD medium (2% dextrose, 1% (NH4)2SO4, 0.09% KH2PO4, 0.05% MgSO4, 0.023% K2HPO4, 0.01% NaCl, 0.01% CaCl2) for 24 h at room temperature with shaking at 100 rpm. Outwashes were serially diluted in MD medium and plated on extract-peptone-dextrose (YPD)-agar plates (1% yeast extract, 1% peptone, 2% dextrose, 2% agar) containing 50µg mL^{-1} chloramphenicol and incubated for 3–5 days at 25 °C. Morphologically distinct yeast-like colonies were applied for molecular identification.

2.3. Metagenomic Analysis of Sea Buckthorn Fungal Microbiota

For metagenomic analysis, DNA was isolated from collected sediments (40 mg of pellet per sample) using a Genomic DNA purification kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) and following the manufacturer's protocol. The quantity and quality of extracted DNA were measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). DNA samples of fungal microorganism populations were amplified using the ITS2 region-specific primers: ITS3-KYO2 (5′-GATGAAGAACGYAGYRAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [36]. Amplicon libraries were prepared using modified Illumina adapters (www.illumina.com), validated on an Agilent Technologies Bioanalyzer DNA 1000 and paired-end sequenced (2 × 300 bp) using Illumina MiSeq platform (BaseClear B.V. Leiden Netherlands). Complete data sets were submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (Accession number PRJNA590349).

2.4. Bioinformatics Analysis

Sequences were pre-processed, quality filtered, and analyzed using QIIME2 version 2018.4 [37]. Forward and reverse reads were trimmed with Cutadapt 2.8 [38]. Primer-free fastq files were processed using Divisive Amplicon Denoising Algorithm 2 (DADA2) plugin implemented in QIIME2. Reads with higher than 2.6 expected error rates were discarded. Chimeric sequences were eliminated using

'consensus' method. Alpha and beta-diversity analyses were performed with the q2-diversity plugin in QIIME2 at a sampling depth of 43458. Principal coordinates analysis (PCoA) was performed based on non-phylogenetic Bray–Curtis distances and visualized with the make_2d_plots.py script. To assign taxonomy of obtained Amplicon sequence variants (ASV) we used the QIIME2 q2-feature-classifier plugin and the v7.2 UNITE database as the reference at 97% identity [39].

2.5. Cultivable Yeast Identification

Yeast strains were streak-plated from single colonies onto YPD-agar medium supplemented with 50 μg mL^{-1} chloramphenicol and incubated for 3 days at 25 °C. The colors and the structure of the colonies were recorded. For the microscopy study, slides were prepared from 1-week-old cultures and observed under the light microscope (Leica DM750, Wetzlar, Germany), by recording micrographs with the digital camera (Leica ICC50 HD, Wetzlar, Germany).

DNA was isolated from fresh yeast culture (24 h) by using the Genomic DNA purification kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) following the manufacturer's instructions. For identification of yeast, PCR amplification of the region between the 18S rRNA and 28S rRNA genes was performed using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers. The reaction mixtures (50 μL) contained 5 μL of DreamTaq green buffer, 1 μL of 2 mM dNTP mix, 1 μL of each primer (10 μmol L^{-1}), 2.5 unit of DreamTaq DNA polymerase (all from Thermo Fisher Scientific Baltics, Vilnius, Lithuania) and 1 μL of DNA template (5 ng). The PCR conditions were as follows: initial denaturation at 94 °C for 5 min; 25 cycles of 94 °C for 1 min, 53 °C for 1 min 30 s, 72 °C for 2 min; a final elongation at 72 °C for 10 min. For preliminary yeast species identification, PCR products were digested with *Cfo*I and *Hinf*I enzymes and restriction profiles were checked by 1% agarose gel electrophoresis. PCR products were purified using a GeneJet PCR purification kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania), following the manufacturer's instructions and sequenced using ITS1 and/or ITS4 primers at BaseClear (Leiden, Netherlands). The generated sequences were compared with those found in the FASTA network service of the EMBL-EBI database (http://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html) and deposited in the National Center for Biotechnology Information (NCBI) under accession numbers MN700626, MN700627, MN700628, and MN700629.

2.6. Sampling and Identification of Volatile Organic Compounds (VOCs) Produced by Yeasts

The solid-phase micro-extraction (SPME) technique was applied to collect the headspace volatiles of yeasts. Yeasts were cultivated in polystyrene Petri dishes (Ø 55 mm × 14 mm) on YPD-agar (14 mL) for 2 days at 25 °C. For sampling background volatiles, YPD-agar plates without yeast were used as control samples. SPME fiber coated with a polydimethylsiloxane-divinylbenzene polymer (DVB/PDMS, 65 mm coating layer thickness, Supelco, Pennsylvania, USA) was used. Before each collection, purification of SPME fibers was conducted at 240 °C for about 10 min in a GC injector. The needle of SPME syringe was placed above the yeast layer through a small hole made in a Petri dish; the fiber was exposed to the headspace for 60 min at room temperature. After sampling, the fiber was inserted for 2 min into the injection port of gas chromatograph for desorption of the volatiles.

The volatiles collected were analyzed using Shimadzu gas chromatograph GC-2010 coupled with Shimadzu mass selective detector MS-QP 2010 Plus (Kyoto, Japan). In the GC, Restek Stabil-Wax column (30 m × 0.25 mm × 0.25 μm, Bellefonte, PA, USA) was used. The oven temperature was programmed as follows: the initial temperature was maintained isothermally at 40 °C for 1 min; afterward, it was raised to 200 °C at a rate of 5 °C min-1, then increased to 240 °C at a rate of 10 °C min-1, and maintained isothermally for 11 min. The GC injector temperature was set at 240 °C. Helium was used as carrier gas (1.5 mL min^{-1}). The relative amount of each of the compounds was counted based on the area of the chromatographic peak. The volatile compounds were identified according to their mass spectra and their retention indexes in a NIST version 2.0 mass spectral library (National Institute

of Standards and Technology, USA) as well as co-chromatography of synthetic standards. Software GCMS solution version 2.71 (Shimadzu, Kyoto, Japan) was applied for data analysis.

2.7. Statistical Data Analysis

Nonparametric Mann–Whitney U test was applied to evaluate differences of volatile amounts between yeast and control samples, i.e., YPD-agar plates without yeast, using the Statistica 6.0 program package (StatSoft, Inc., Tulsa, OK, USA). To assess and visualize the associations between odor blends of four yeast species and volatile compounds, the principal component analysis (PCA) was performed using Canoco 4.5 software (Biometris Plant Research International, Wageningen, The Netherlands). Amounts of styrene, 2,5-dimethyl pyrazine, 2-ethylhexanol and methoxy-phenyl-oxime derived from yeast samples did not differ from those collected from the control; hence, they were not included in PCA analysis. Significantly smaller amounts of the compounds collected from the control samples were subtracted from the amounts released by yeasts. By Canoco 4.5 software, absolute amounts expressed as area under chromatographic peak were log-transformed, scaled dividing each value by its standard deviation and centered.

3. Results

3.1. Diversity and Richness of Fungal Microorganism Communities

To characterize the diversity of fungal microorganism community distributed on sea buckthorn at different ripening stages, we harvested berries from the three distinct shrubs at private organic plantation and generated a combined sample as one biological replicate. For metagenomics analysis, three biological replicate samples of unripe berries (UB) obtained from different locations of the sea buckthorn plantation and three corresponding to ripe berries (RB) were used. Sequencing of the ITS2 rRNA partial gene region of DNA samples generated a total of 726,634 reads with an average of 121,105 sequences per sample (range 897,20-164,922) (Table 1). Following quality filtering, 454,538 sequences were recovered. The clustering of the sequences generated a total of 1615 unique amplicon sequence variants (ASVs) (1014 [338±70, hereafter median for 3 samples ± standard deviation] for fungal ITS2 from unripe berries and 601 [200±35.6] ASV for ripe berries). The observed number of ASV reveals a significant difference in fungal community richness between UB and RB sample groups (ANOVA p=0.038).

Table 1. Total sequences obtained for eukaryotic microbial community for unripe (UB) and ripe (RB) sea buckthorn samples.

	Reads Obtained	High Quality Reads	ASV	Shanon Diversity	Pielou Eveness	Simpson Index
UB1	103,024	49,891	300	5.26	0.64	0.94
UB2	164,922	110,782	419	5.00	0.58	0.91
UB3	89,720	43,458	295	5.37	0.66	0.94
RB1	114,814	76,641	190	3.71	0.49	0.86
RB2	135,098	92,822	240	3.795	0.48	0.85
RB3	119,056	80,944	171	3.21	0.44	0.79
Total	726,634	454,538	1615			

The microbial community analysis according to the nonparametric Kruskal–Wallis test showed a statistically significant decrease (*p*-value 0.046) in fungal microorganism diversity amongst RB samples in comparison to UB samples. In agreement with ASV data, Shannon's diversity and Simpson indexes estimate that unripe sea buckthorn berries have a higher fungal microorganism diversity than ripe berries. Rarefaction curve approached plateaus indicating that the sequencing depth was sufficient to capture the microbial diversity of each sample (Figure S1). Principal coordinate analysis (PCoA) accomplished with the representative ASVs demonstrated a clear separation of UB and RB

samples, indicating a difference in the composition of the fungal microbiota (Figure 1). Clustering of triplicate sample sequence libraries using the Bray–Curtis dissimilarity index revealed consistency of microbial community structure among replicates.

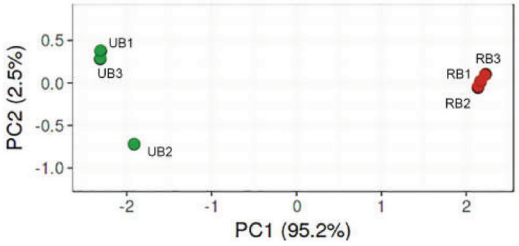


Figure 1. Principal coordinate analysis (PCoA) of the relative abundance of fungal microorganism amplicon sequence variants (ASVs) associated with unripe (UB) and ripe (RB) sea buckthorn berries. PCoA plot is based on unweighted UniFrac distance metrics.

3.2. Composition of Fungal Microbiota on Unripe and Ripe Sea Buckthorn Berries

A total of four phyla, 69 orders, and 144 families were identified in the present study (Table S1). Based on the average abundance analysis, the dominating phyla on both sea buckthorn samples were Ascomycota (85.0% for UB and 97.6% for RB, respectively) followed by the Basidiomycota (10.6% and 1.7% in UB and RB, respectively) and other fungi (Figure 2A; Table S1). The impact of ripening on the fungal microorganism community was notable starting at the class level. Dothideomycetes were present in large numbers in both samples (54.0% for UB and 44.6% for RB, respectively) (Figure 2B). However, on unripe berries (UB) the most abundant classes were Taphrinomycetes (18.4%) and Tremellomycetes (7.6%) when in RB samples Saccharomycetes (47.6%) prevailed. At the family level, in UB samples we found dominating Aureobasidiaceae and Taphrinaceae, while RB samples were prevalent by Aureobasidiaceae and Metschnikowiaceae (Figure 2C). In total, 196 genera were identified in this study; the most abundant were *Aureobasidium* (31.4%), *Taphrina* (18.4%), and *Cladosporium* (8.1%) for UB samples, and *Metschnikowia* (44.5%) with *Aureobasidium* (40.6%) for RB samples (Figure 2D).

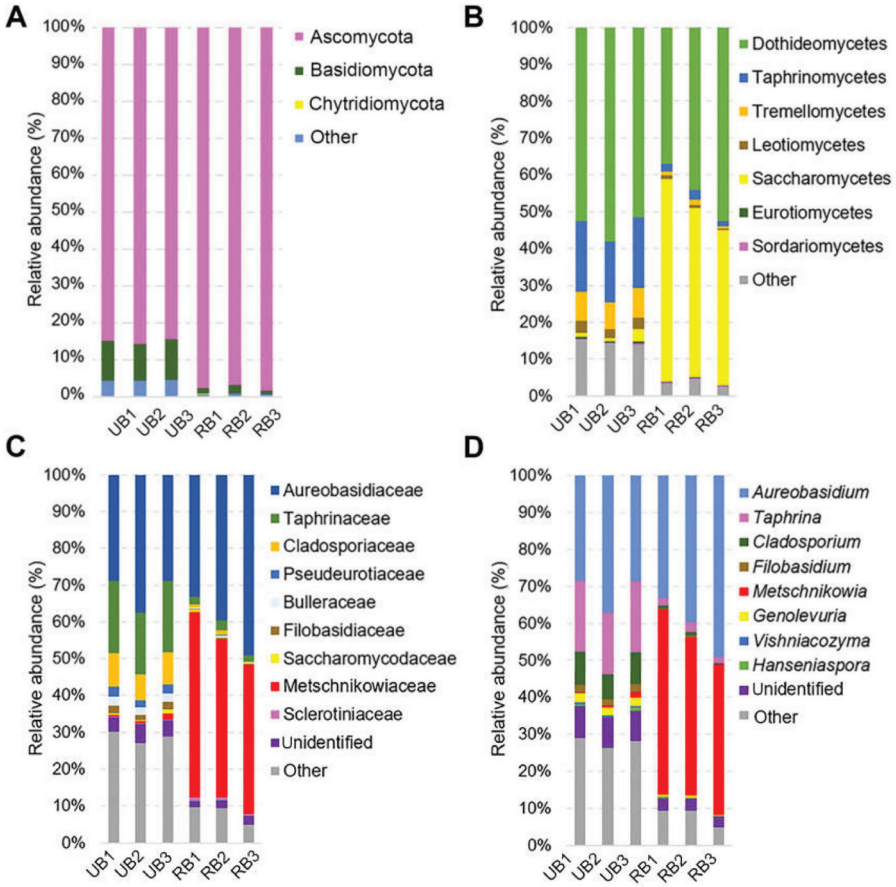


Figure 2. Fungal microorganism community distribution on sea buckthorn unripe (UB) and ripe (RB) berries. Relative abundance of sequences classified at the phylum (**A**), class (**B**), family (**C**), and genus (**D**) level. The taxonomic groups comprising less than 1% of the total composition were assigned to "Other".

The Venn diagram presents the distribution of unique fungal ASVs in UB and RB samples (Figure 3). A total of 887 unique ASVs found in this study, 198 were shared by both UB and RB samples, while 493 ASVs were exclusive to UB and 196 to RB samples, respectively.

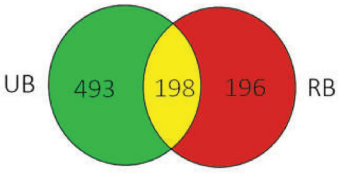


Figure 3. Venn diagram illustrating the number of unique and shared amplicon sequence variants (ASVs) among sea buckthorn samples at different ripening stages. UB – unripe sea buckthorn berries, RB – ripe berries.

A heatmap diagram illustrates the distribution of the most abundant fungal ASVs (Figure 4). Hierarchical cluster analysis showed that the microbial community structure displays different patterns

between UB and RB groups, while samples collected within the same ripening stage feature similar community structure. The core microbiomes detected in both samples comprised of ASV1 and ASV3 assigned to *Aureobasidium pullulans* (Figure 4; Table S2). At the beginning of ripening, besides the *Aureobasidium pullulans* the most abundant ASVs were ASV4 (7.6%) and ASV14 (2.4%) (both *Taphrina carpini*), ASV10 (4.7%) (Taphrina), ASV6 (6.8%) (*Cladosporium*), and ASV20 (1.6%) (*Filobasidium wieringae*). During the ripening of the berries, their prevalence diminished to less than 1%. A dramatic change was observed between the beginning and the end of the ripening period, mainly due to the increase of fungal microorganisms belonging to *Metschnikowia spp*. (ASVs 2, 5, 7, 9, 11, 22, and 30). The overall abundance of ASVs assigned to *Metschnikowia* in RB berries reached 44%, while on the unripe berries they consisted only 0.7%.

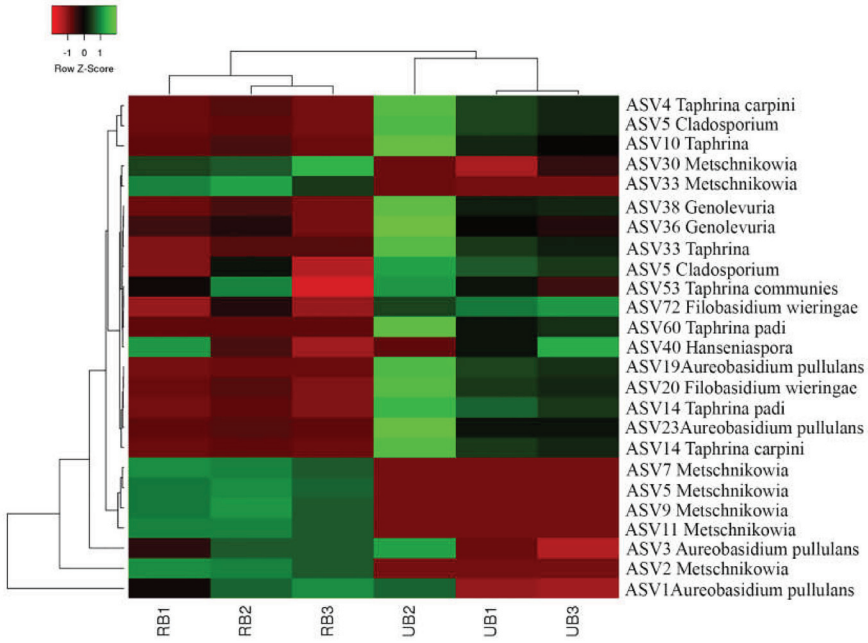


Figure 4. Heatmap of fungal microorganism's unique amplicon sequence variants (ASVs) abundance on sea buckthorn. The color intensity is proportional to the relative abundance of fungal microorganism ASVs.

3.3. Sea-Buckthorn-Associated Cultivable Potential Biocontrol Yeasts and Their Produced Volatiles

By applying cultivation techniques, more than 20 yeast strains were isolated from the surface of sea buckthorn berries. Yeasts were subjected for primary species identification by morphological colony analysis and bright field microscopy with following fingerprinting of PCR-amplified sequences of internal transcribed spacers 1 and 2 including 5.8S ribosomal RNA gene. For further analysis of VOC production, selected strains proceeded sequencing-based species level confirmation and deposition of sequences in NCBI.

Four yeast strains, belonging to different genera identified by metagenomics analysis of UB and RB microbiota with referred potential biocontrol properties [15,16,40] (Table S1, Figure 5), were selected for VOCs analysis. Morphologically different SB-18-2 and SB-18-25 yeast strains were recovered from UB berries while SB-18-34 and SB-18-31 yeast strains were isolated from RB berries. Basing on the electrophoretic analysis, similar size (about 600 bp) ITS regions were PCR-amplified from SB-18-2 and SB-18-25 strains, and differed in *HinfI* and *CfoI* restriction patterns (Figure 5). The sequence identity

match of SB-18-2 yeast was 99.64%, comparing to that of *Aureobasidium pullulans* culture YY11 deposited in GenBank. SB-18-25 yeast strain was assigned to *Cryptococcus wieringae* (syn. *Filobasidium wieringae*) basing on RFLP profile and 99.48% similarity to *C. wieringae* CBS:11709 culture. Following the genomic DNA purification, 380 bp and 700 bp PCR products were amplified from SB-18-34 and SB-18-31 yeast strains, respectively. Their PCR-RFLP profiles corresponded to *Metschnikowia* and *Hanseniaspora* yeast species [41]. According to the high similarity to sequences depositedin GenBank, SB-18-34 strain was identified as *Metschnikowia pulcherrima* and SB-18-31 as *Hanseniaspora uvarum* yeast species (Figure 5).

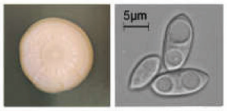
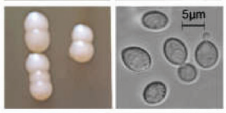
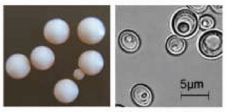
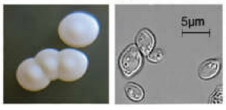
Species / Acc. no.	Morphological analysis	Profiles of 5.8S-ITS-RFLP analysis (bp)			Reference / Acc. no.	Identity (%)
		PCR product	*Hinf*I	*Cfo*I		
Aureobasidium pullulans SB-18-2 / MN700626		600	300, 180, 120	180, 180, 100, 100	*A. pullulans* / KR912244.1	99.64
Cryptococcus wieringae SB-18-25 / MN700627		600	250, 200, 150	320, 280	*C. wieringae* / FN824493.1	99.48
Metschnikowia pulcherrima SB-18-34 / MN700628		380	200, 180	200, 100, 80	*M. pulcherrima* / KY104204.1	100
Hanseniaspora uvarum SB-18-31 / MN700629		700	350, 200, 150	300, 300, 100	*H. uvarum* / MN371888.1	99.85

Figure 5. Identification of yeasts isolated from sea buckthorn surface.

Aureobasidium pullulans SB-18-2 belongs to genus, which is the most abundant in UB samples (31.4%), and of significant representation (40.6%) in RB samples. *Metschnikowia pulcherrima* SB-18-34 represents genus, which is the most abundant (44.5%) in RB samples. *Cryptococcus wieringae* SB-18-25 belongs to genus, more prevalent (1.9%) on the surface of UB berries than on the RB berries (0.2%). *Hanseniaspora uvarum* SB-18-31 is a member of genus, which is the least dominant among the selected genera with only 0.5% and 0.2% occurrence in UB and RB samples, respectively.

The gas chromatographic-mass spectrometric analysis revealed 29 compounds that were exclusively present in the headspace of four yeast species and occurred significantly more abundant compared to those of control samples. The yeast released volatiles were composed of 11 esters, 6 alcohols, 5 volatile fatty acids, 3 ketones, 2 aromatics, and 2 compounds bearing both ester and aromatic moieties (Table 2).

Table 2. Odor blends of four yeast species and control sampled by SPME technique.

No.	Compound	CAS No.	RI	GR	*H. uvarum*	*M. pulcherrima*	*A. pullulans*	*C. wieringae*	Control
1	Ethyl acetate	141-78-6	898	ES	26.26 ± 4.63	8.98 ± 0.28	0	0	0.06 ± 0.02
2	Ethanol	64-17-5	902	OH	4.58 ± 0.36	4.89 ± 0.42	6.26 ± 0.82	0.39 ± 0.03	0.10 ± 0.02
3	Ethyl propionate	1105-37-3	915	ES	1.00 ± 0.05	0.24 ± 0.01	0	0	0.01 ± 0.001
4	2-Methylprop-1-yl acetate	110-19-0	985	ES	0.31 ± 0.09	0.07 ± 0.01	0	0	0
5	Ethyl butanoate	105-54-4	1013	ES	0.05 ± 0.03	0	0	0	0
6	Ethyl 2-methylbutanoate	7452-79-1	1033	ES	0.02 ± 0.01	0	0	0	0
7	Ethyl 3-methylbutanoate	108-64-5	1050	ES	0	0	0.04 ± 0.01	0	0
8	Butyl acetate	123-86-4	1063	ES	0.28 ± 0.03	0	0	0	0.10 ± 0.02
9	2-Methylpropanol	78-83-1	1095	OH	0.42 ± 0.07	0.98 ± 0.08	1.04 ± 0.13	1.70 ± 0.11	0
10	3-Methylbutyl acetate	123-92-2	1105	ES	13.28 ± 0.5	0.40 ± 0.02	0.15 ± 0.05ns	0.07 ± 0.01ns	0.10 ± 0.07
11	Butanol	71-36-3	1149	OH	0	0	0.13 ± 0.03	0.09 ± 0.01	0
12	2-Heptanone	110-43-0	1170	KT	0	0	0.07 ± 0.01	0.06 ± 0.01	0
13	3-Methylbutyl propionate	105-68-0	1176	ES	0.62 ± 0.09	0	0	0	0
14	2-Methylbutanol	137-32-6	1207	OH	1.63 ± 0.28	1.77 ± 0.24	0.84 ± 0.18	6.47 ± 1.21	0
15	3-Methylbutanol	123-51-3	1213	OH	3.28 ± 0.37	5.95 ± 0.56	4.07 ± 0.51	10.39 ± 0.36	0.21 ± 0.11
16	Ethyl hexanoate	123-66-0	1224	ES	0.02 ± 0.01	0	0	0	0
17	Styrene*	100-42-5	1238	AR	0.20 ± 0.04ns	0.13 ± 0.03ns	0.07 ± 0.01ns	0.16 ± 0.01ns	0.14 ± 0.06ns
18	2-hydroxy-3-butanone	513-86-0	1273	KT	0.15±0.04	0.14 ± 0.01	0	0.06 ± 0.01	0
19	2,5- Dimethyl pyrazine*	123-32-0	1316	PY	0.02±0.01ns	0.01 ± 0.001ns	0.03 ± 0.01ns	0.04 ± 0.01ns	0.03 ± 0.02ns
20	6-Methyl 5-hepten-2-one	110-93-0	1327	KT	0	0	0	0.93 ± 0.10	0
21	Ethyl octanoate	106-32-1	1430	ES	0.12 ± 0.01	0	0	0	0
22	Acetic acid	64-19-7	1449	FA	1.15 ±0.25	0	0.01 ± 0.008	0.07 ± 0.02	0
23	6-Methyl-5-hepten-2-ol	1569-60-4	1461	OH	0	0	0	0.08 ± 0.02	0
24	2-Ethylhexanol*	104-76-7	1488	OH	0.02 ± 0.001ns	0.02±0.01ns	0.02±0.001ns	0.02±0.004ns	0.03±0.01ns
25	Propionic acid	1979-09-04	1528	FA	0	0	0	0.03 ± 0.004	0
26	2-Methylpropionic acid	79-31-2	1562	FA	0	0	0	0.18 ± 0.04	0
27	Butanoic acid	107-92-6	1634	FA	0.03 ± 0.01	0	0	0.03 ± 0.01	0
28	3-Methylbutanoic acid	503-74-2	1703	FA	0.07 ± 0.02	0.01 ± 0.001	0.06 ± 0.04	0.71 ± 0.25	0
29	Methoxy-phenyl-oxime*		1767	IM	0.03 ± 0.01ns	0.01 ± 0.003	0.01 ± 0.004	0.03 ± 0.01ns	0.03 ± 0.01ns
30	2-Phenylethyl acetate	103-45-7	1795	ES/AR	3.52 ± 0.64	0.04 ± 0.01	0	0	0
31	2-Phenylethyl propionate	122-70-3	1858	ES/AR	0.11 ± 0.02	0	0	0	0
32	Phenylmethanol	1960-12-08	1856	AR	0	0.03 ± 0.01	0	0	0
33	2-Phenylethanol	1960-12-08	1894	AR	3.60 ± 0.26	3.45 ± 0.65	2.28 ± 0.45	0.21 ± 0.03	0.03 ± 0.01

Compounds indicated by * mark were excluded from PCA; CAS No—chemical abstract service number; RI—retention index (DB-Wax fused silica capillary column 30 m x 0.25 mm i.d., 0.25 μm film thickness); GR—group of chemical compound; ES—ester; OH—alcohol; KT—ketone; AR—aromatic; PY—pyrazine; FA—fatty acid; IM—imine; all values in the columns headed by yeast names are the absolute amounts expressed as areas under the chromatographic peaks and have to be read as numbers times 100000 ± standard error of the mean followed by the "ns" are not significantly different compare to control (nonparametric Mann–Whitney U test, $P < 0.05$); control samples were obtained by collecting background volatiles from YPD-agar plates without yeast.

The principal component analysis revealed that the volatile blend of any of the four yeast species was clearly separated from each other. The first principal component axis accounted for 56% of the total variation and to a large extent explained separation between volatile blends of *C. wieringae* versus *H. uvarum* yeasts and *A. pullulans* versus *M. pulcherrima* yeasts (Figure 6A). Volatile profiles of *C. wieringae* yeasts were characterized by five alcohols (9, 11, 14, 15, and 23), three volatile fatty acids (25, 26, and 28), and two ketones (12 and 20) which were exclusively present or occurred in the large amounts compared to those released by *H. uvarum* yeasts (Figure 6B, Table 2). Ten esters were unique for the volatile blends of *H. uvarum* yeasts in addition to acetic acid (22), 2-hydroxy-3-butanone (18), and 2-phenylethanol (33) with amounts larger compared to those of *C. wieringae* yeasts (Figure 6B, Table 2). *M. pulcherrima* yeasts released the larger amounts of esters (1, 3, 4, and 10), while *A. pullulans* yeasts released the higher amounts of two alcohols (ethanol (2), butanol (11)) (Table 2).

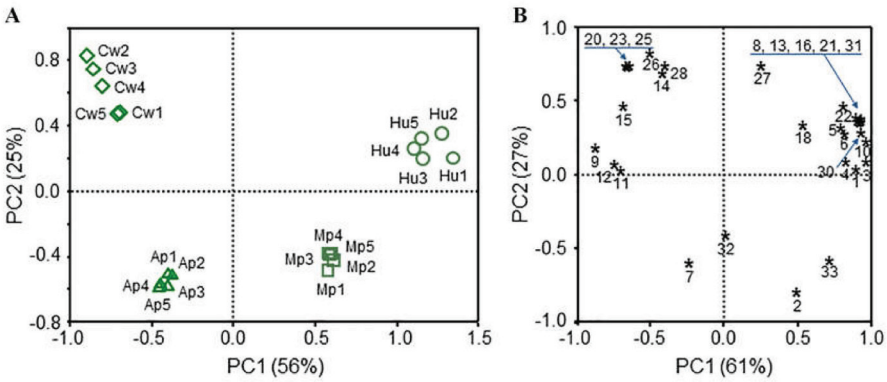


Figure 6. Associations between blends and their components of four species of yeasts populating sea buckthorn berries. Associations are visualized by principal component analysis (PCA). (**A**) Distribution of odor blends released by *C. wieringae* (Cw, diamond shape), *A. pullulans* (Ap, triangle shape), *M. pulcherrima* (Mp, square shape), and *H. uvarum* (Hu, circle shape). n=5 for each species. (**B**) Distribution of volatile compounds composing the blends. Stars represent the volatile compounds indicated by numbers. Names of the compounds are listed in Table 2.

The second PC axis accounted for 25% of the total variation and explained the separation between volatile blends of *A. pullulans* and *C. wieringae* species and in part between volatile blends of *M. pulcherrima* and *H. uvarum* yeasts (Figure 6A). 6-Methyl 5-hepten-2-one (20), 6-methyl-5-hepten-2-ol (23), propionic acid (25) and 2-methylpropionic acid (26) were unique volatiles released by *C. wieringae*, while only ethyl 3-methylbutanoate (7) was specific for *A. pullulans* (Figure 6B, Table 2). Ethyl butanoate (5), ethyl 2-methylbutanoate (6), 3-methylbutyl propionate (13), ethyl hexanoate (16), ethyl octanoate (21), and 2-phenylethyl propionate (31) were exclusively present in volatile blends of *H. uvarum* yeasts, and phenylmethanol (32) was unique for *M. pulcherrima* (Figure 6B, Table 2).

4. Discussion

The current study, for the first time, uncovered the structure of fungal microorganism communities colonizing the surface of sea buckthorn berries at different ripening stages in natural habitat. For a comprehensive analysis, we have applied next generation sequencing and observed the wide diversity of eukaryotic microorganisms on tested berries. The total abundance of fungal microorganisms changed significantly during the period of fruit ripening. The microbial alpha-diversity—estimated by the Simpson's index, Shannon's diversity index, and Pielou's evenness index—was highest among the fungal community on unripe berries. The analysis of the fungal microbiota structure revealed clear differentiation between unripe and mature berries, as demonstrated by the Bray–Curtis beta-diversity

analysis. According to our data, unripe sea buckthorn berries were dominated by *Aureobasidium*, *Taphrina*, *Cladosporium*, and *Filobasidium* (syn. *Cryptococcus*) genera microorganisms, while on ripe berries prevailed *Aureobasidium* and *Metschnikowia* (Table S1, Figure 2). It was previously demonstrated that *Cryptococcus*, *Rhodotorula*, *Sporobolomyces*, and *Aureobasidium pullulans* yeasts were associated with the early stage of maturation of fruits and berries, such as apples, grapes, nectarines, etc. [29,32,42]. In our previous work, it was demonstrated that Dothioraceae family fungal microorganisms prevailed on sea buckthorn berries, which at the lower taxonomic level were attributed to uncultured *Aureobasidium* [33]. Our results were partially consistent with previous findings, since most fungal microorganisms present on sea buckthorn were represented by *Aureobasidium*. However, the overall structure of fungal microbiota varies most likely depending on the differences in climatic conditions, berry harvesting period (previously—September 2016; in this study—July 2018), localization of sea buckthorn plantation, etc.

Various microorganisms act as biological control agents of phytopathogens and have a role in postharvest decay and toxin contamination by emitting volatile organic compounds [40,43]. Several biocontrol yeasts (e.g., *Candida oleophila, Aureobasidium pullulans, Metschnikowia fructicola, Cryptococcus albidus, Saccharomyces cerevisiae*) have already been used for the production of commercial plant protection products, encouraging the deeper exploration of the fascinating traits and application possibilities of other yeasts as well [16,44,45]. Even the different strains of the same yeast species are able to produce highly different amounts of the same VOCs (e.g., *A. pullulans* [43]), thus it is important to investigate volatiles of yeasts strains within the species that are already proven to possess biocontrol traits (like *Aureobasidium pullulans* [43,46], *Metschnikowia pulcherrima* [46], and *Hanseniaspora uvarum* [47]), or are less investigated, but closely related to bioactive yeasts (e.g., potential biocontrol yeast *Cryptococcus wieringae* which belongs to the same genus as *C. albidus*, *C. laurentii*, and C. flavus [16,48–50]).

Aureobasidium pullulans is well-known yeast-like fungus hosting on plants, mostly present in a cooler climate like that of central Europe and less related to grapes grown in warm areas [51]. It was revealed that some *Aureobasidium* species demonstrated biological control activity against phytopathogens, such as *Botrytis*, *Bacillus, Colletotrichum, Penicillium* [43,52,53]. The antagonistic activity of *A. pullulans* mainly includes nutrient competition and production of volatile organic compounds or glucanases, chitinases, and extracellular proteases [40,46]. *Taphrina* and *Cladosporium* were the next numerous fungal microorganisms observed on unripe sea buckthorn berries in our study (Table S1, Figure 2). *Taphrina* genus fungi are known as biotrophic plant pathogens that cause plant and fruit disease symptoms, which may be ascribed to the ability to synthesize and modulate different plant hormones [54]. *Cladosporium* is considered as ubiquitous fungi, with some species providing numerous antifungal agents [55], while others causing plant or human diseases [56,57]. *Cryptococcus* encloses species producing biocontrol agents against many pathogens [16,50], while certain species can cause infections in humans and animals [58].

During the ripening of different fruits and berries, the oxidative or weakly fermentative yeast populations—such as *Candida*, *Hanseniaspora*, *Metschnikowia*, and *Pichia*—increased in frequency [42]. *Hanseniaspora* spp. have been frequently found on the surface of different fruits, e.g. apples, grapes, or strawberries [59,60]. Numerous yeasts belonging to the *Hanseniaspora* genus display antagonistic properties against the development of fruit spoilage causing molds [40,61]. *Metschnikowia* spp. isolated from fruit are sugar-loving yeasts and their populations are most likely stimulated by juices diffusing from the damaged surface of mature fruit [42]. This yeast genus includes several biocontrol agents, which are very effective against fruit spoilage fungi [62]. The biocontrol abilities of *M. pulcherrima* have been mainly assigned either to competition for nutrients or to the production of volatile organic compounds [15,46]. The competition of *M. pulcherrima* for iron was reported to play a substantial role in biocontrol interactions [63]. *Metschnikowia* spp. are linked to several groups of nectarivorous insects, suggesting that this specificity could be correlated to the services provided by the yeast to the insect or vice versa [64,65].

Yeasts produce chemo-diverse blends of volatiles comprised of aldehydes, ketones, alcohols, volatile fatty acids, esters, aromatics, heterocyclic compounds, hydrocarbons, terpenoids, and some others [66,67]. Ethanol is probably the most known volatile metabolite of yeasts derived from carbohydrate catabolism and fermentation [66]; it is one of the major volatiles collected from the headspace of *H. uvarum*, *M. pulcherrima*, and *C. wieringae* yeasts. Amounts of ethyl acetate, another metabolite of carbohydrate catabolism, prevailed in the VOCs produced exclusively by *H. uvarum and M. pulcherrima* yeasts. It is a valuable solvent for extracting compounds with antimicrobial properties [68] and possess minor inhibitory effect for *Botrytis cinerea* [69]. In addition to ethyl acetate, 10 esters were unique for the volatile blends of *H. uvarum* yeasts; these compounds originated from amino acid synthesis and degradation pathways [66]. Prevalence of esters in volatile blends of *H. uvarum* has been reported in previous studies [70,71]; however, ethanol being the third largest component of the blend in our experiments, has not been determined by Babcock et al. [71] or Piper et al. [70]. High production of ethanol, 3-methylbutanol, 2-phenylethanol, ethyl acetate, and 3-methylbutyl acetate were reported for *M. pulcherrima* and *A. pullulans* yeasts [15]. The data published are comparable with the results of our study in terms of large production of alcohols by both yeast species; however, we were not able to detect ethyl acetate in the headspace of *A. pullulans*. To date, there is no report on VOCs of *C. wieringae* yeast. Volatile organic compounds produced by *A. pullulans* that were found in our study (namely 2-phenylethanol, 2-methylpropanol, 2-methylbutanol, and 3-methylbutanol) had been previously shown to control postharvest fruit pathogens (*B. cinerea, Colletotrichum acutatum, Penicillium expansum, Penicillium digitatum*, and *Penicillium italicum*) [43]. All these alcohols were produced by *A. pullulans* SB-18-2, *M. pulcherrima* SB-18-34, *C. wieringae* SB-18-25, and *H. uvarum* SB-18-31 but in different amounts, suggesting the potential biocontrol capabilities of these strains. 2-Phenylethanol is one of the main VOCs produced by various yeast species (e.g. *Cyberlindnera jadinii, Lachancea thermotolerans, Candida intermedia, Candida friedrichii, Saccharomyces cerevisiae, Wickerhamomyces anomalus)* affecting numerous pathogens (e.g. Aspergillus carbonarius, Aspergillus flavus, Aspergillus ochraceus, Penicillium digitatum, Phyllosticta citricarpa) (see [40] and references therein). Among tested strains, *H. uvarum* SB-18-31 and *M. pulcherrima* SB-18-34 produced the highest amount of 2-phenylethanol, whereas *A. pullulans* SB-18-2 excelled at ethanol generation, while volatilome of *C. wieringae* SB-18-25 was dominated by 3-methylbutanol. Ethyl acetate was the dominating volatile of *H. uvarum* SB-18-31 and *M. pulcherrima* SB-18-34. 2-Phenylethyl acetate was produced in significant amounts (comparing to other tested yeast strains) by *H. uvarum* SB-18-31 and had been previously reported to contribute to growth inhibition of *A. ochraceus* growth and ochratoxin A production during processing of coffee [47].

Due to the extremely sensitive olfaction system, insects are capable to use microbial volatiles to search for suitable feeding and oviposition sites [18,72]. Emissions from microorganisms associated with different development stages of fruits and berries contribute to the information about habitat suitability [73] and it was demonstrated that insects recognized yeast communities based on their specific volatile profiles [74–76]. Most of the volatile compounds (2-phenyl ethanol, 2-methylbutanol, 3-methylbutanol, ethyl acetate, 2-phenylethyl acetate, ethyl propionate, and others) produced by yeast in our study had been suggested to play a role in modulating behavior of *Rhagoletis batava* flies [67]. Examples of microbial VOC application for pest control already demonstrated their high potential for use in integrated pest management programs [77]. Identification of attractive and repellent odors from the yeasts associated with berries at preferred and unsuitable stages for pest insect feeding and oviposition would provide a base for the development of environment-friendly pull-push pest control for *Rhagoletis batava* flies, a major pest of the sea buckthorn.

5. Conclusions

The metagenomic approach based on the sequencing of ITS2 region of fungal rDNA revealed changes in the fungal community associated with the ripening of sea buckthorn berries. Investigation of alpha and beta diversity of the fungal community, analysis of representative microbial ASVs

showed a clear separation among inhabitants of unripe (UB) and ripe berries (RB). Based on results of metagenomics analysis, unripe berry samples prevalently had *Aureobasidium*, *Taphrina*, and *Cladosporium* genera yeasts; while ripe berries were dominated by *Aureobasidium* and *Metschnikowia*. Potential biocontrol yeasts *Aureobasidium pullulans* and *Cryptococcus wieringae* were isolated from sea buckthorn surface at the early harvesting stage, while *Metschnikowia pulcherrima* and *Hanseniaspora uvarum* were amplified from ripe berries and applied for the analysis of produced volatile organic compounds. Eleven esters, six alcohols, five volatile fatty acids, three ketones, two aromatics, and two compounds bearing both ester and aromatic moieties were isolated from the headspaces of four yeast species and identified by gas chromatography and mass spectrometry techniques. Principal component analysis revealed that volatile blends of all four yeast species were clearly separated from each other.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/8/3/456/s1, Figure S1: Rarefaction curves at a genetic distance of 3% for each sample, Table S1: Fungal taxonomy abundance count for sea buckthorn samples at different ripening stages from phylum to species level, Table S2: Relative abundance of the most common ASVs.

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PAPER III

Distribution of apple and blackcurrant microbiota in Lithuania and the Czech Republic

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Distribution of apple and blackcurrant microbiota in Lithuania and the Czech Republic

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ABSTRACT

The microbial assemblies on the surface of plants correlate with specific climatic features, suggesting a direct link between environmental conditions and microbial inhabitation patterns. At the same time however, microbial communities demonstrate distinct profiles depending on the plant species and region of origin. In this study, we report Next Generation Sequencing-based metagenomic analysis of microbial communities associated with apple and blackcurrant fruits harvested from Lithuania and the Czech Republic. Differences in the taxonomic composition of eukaryotic and prokaryotic microorganisms were observed between plant types. Our results revealed limited geographic differentiation between the bacterial and fungal communities associated with apples. In contrast, blackcurrant berries harvested from different regions demonstrated high diversity in both bacterial and fungal microbiota structures. Among fungal and bacterial microorganisms, we identified both potentially beneficial (*Cryptococcus, Hanseniaspora*, *Massilia*, *Rhodotorula, Sphingomonas*) and phytopathogenic microorganisms (*Cladosporium*, *Pantoea*, *Phoma*, *Pseudomonas, Septoria, Taphrina*) indicating their important roles in ecological and evolutionary processes.

1. Introduction

Plants host many microorganisms that colonize the surface of fruits, leaves, flowers and stems, as well as within their tissues (Abdelfattah et al., 2016a). The distribution of microorganisms on fruits is defined by a continuum of factors, including plant species, geographic location, climatic conditions, ripening stage and the application of agrochemicals (Pretorius, 2000; Pinto et al., 2014, 2015). The microorganism community year-to-year is characterized by the appearance of many new patterns, indicating that the behavior of most of the strains is not perennial. Fungi and bacteria inhabiting the fruit surface may be transported from the soil to the plants by insects and other animal species (Valero et al., 2007; Stefanini et al., 2015). On the other hand, some microorganisms, particularly yeast, could be permanent residents on fruits employing the latter as depositorium for survival and propagation. Microorganisms naturally associated with fruits may be beneficial and induce resistance in the hosting plant (e.g. *Cryptococcus*, *Sphingomonas*) or phytopathogenic and responsible for significant economic losses (e.g. *Phoma*, *Pantoea*) (Coutinho and Venter, 2009; Liu et al., 2013; Abdelfattah et al., 2016a). The interactions between different microorganism species may influence the structure of microbial communities inhabiting the fruit surface and through either direct or indirect impact on the plant can mediate many ecological and evolutionary processes (Friesen et al., 2011; Alvarez-Perez and Herrera, 2013).

The fungal and bacterial communities can be very diverse and will be defined by the associated plant species (Pinto et al., 2014). However, geographic location and farming practice also significantly influence microbial diversity (Leff and Fierer, 2013). Until now, the biogeographic distribution of microbiota communities has been studied mainly on grapes, the essential resource for wine production (Setati et al., 2012; Pinto et al., 2015; Wang et al., 2015). Only a limited number of studies on microorganisms residing on plums, apples, pears, cherries, and strawberries have been reported (Janisiewicz et al., 2014; Abdelfattah et al., 2016a, 2016b; Clooney et al., 2016; Volschenk et al., 2016). Few of them were dedicated to comparison of fruit-associated fungal communities differing in location (Setati et al., 2012; Bokulich et al., 2014; Taylor et al., 2014).

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The domesticated apple (*Malus pumila* Mill.) is a worldwide-grown major temperate fruit crop. Like many other fruits, apple is colonized by a number of different microorganisms and could be affected by several different phytopathogens (Teixidó et al., 1999; Graca et al., 2015). Current knowledge about the apple microbiota is limited and largely focused on species that cause disease and thus pose economic threats (Teixidó et al., 1999; Abadias et al., 2006). Another research focus is related to natural antagonists that could be used as biological control agents against phytopathogens (Piano et al., 1997). To date, only one comprehensive report on the fungal community associated with organic and conventionally grown apples in the state of Washington, USA, has been published (Abdelfattah et al., 2016b). It was demonstrated that the phylum Ascomycota was dominant on apples, followed by Basidiomycota and Chytridiomycota. Communities of fungal microorganisms differ depending on the parts of the apple fruit (e.g. *Cryptococcus* and *Alternaria* were most abundant on the stem and calyx; *Penicillium* – in peel and wounded flesh; while *Mycosphaerella* was found exclusively in the calyx). Bacterial communities associated with apples (in Colorado, USA) consisted of two most abundant groups – Microbacteriaceae and Sphingomonadaceae (Leff and Fierer, 2013). The apples used for that studies were purchased from a local supermarket or grocery store, thus analysis was conducted not immediately after collection. It is possible therefore that external conditions such as fruit storage and transportation as well as period of time before performing molecular analysis may have impacted the structure of microbiota due to decreasing survival of fruit-associated microorganisms or involving contaminating ones.

Blackcurrant (*Ribes nigrum* L.) is a native temperate crop widely cultivated both commercially and domestically in the major part of Europe and northern Asia. Even in the USA, there is a growing interest in expanding *Ribes* production (Hummer and Dale, 2010). The berries are rich in polyphenols and vitamin C, thus are attractive for regulation of the gut and intestinal microbiota, protecting against anti-inflammatory degenerative disorders or even cancer in humans (Paredes-Lopez et al., 2010; Tabart et al., 2012). The blackcurrants or their extracts are also widely used in food and beverage manufacturing. The broad interest in growing and application of blackcurrants demand investigation of the microbial communities colonizing the surface of these berries. To the best of our knowledge, no reports on the blackcurrant fungal and bacterial microbiota have been presented thus far.

The objective of the present study was to identify the composition of the bacterial and fungal microbiota closely associated with apples and blackcurrants collected in Lithuania and the Czech Republic. The identification and quantification of fruit and berry microflora expanded current knowledge about the structure of plant-associated bacterial and fungal communities, and revealed the biogeographic distribution of microbiota on apples and blackcurrants, as well as provided valuable information on the impact of environmental factors on the distribution of these microbial populations.

2. Materials and methods

2.1. Ethics statement

The collection of samples was carried out on private land and the owner of the land gave permission to conduct the study on site. It did not involve endangered or protected species.

2.2. Sampling of the fruits and DNA extraction

The domesticated apples (*Malus pumila* Mill.) were aseptically collected in the late-August 2016 on the private farms located in the Vilnius region of Lithuania (GPS coordinates: 54°75′20.0″N, 25°27′99.6″E) and Ostrava region of the Czech Republic (GPS coordinates: 49°83′03.9″N, 18°17′47.3″E). Blackcurrants (*Ribes nigrum* L.) were sampled from the Ignalina region of Lithuania (GPS coordinates: 55°34′23.0″N, 26°16′46.8″E) and Ostrava region of the Czech Republic (GPS coordinates: 49°83′03.9″N, 18°17′47.3″E) in the mid-July 2016. The fruits were collected into sterile plastic bags and processed within 2–4 h after harvesting. Fruits of interest (300 g) were placed in 500 mL of sterile 0.05 M phosphate buffer pH 6.8 for 30 min (in the case of blackcurrants) and 2 h (for apples) with shaking at120 rpm. Outwashes were filtered through 420 µm filters, centrifuged at 12,000 g for 20 min, and precipitates were stored at −20 °C until subsequent analysis.

For metagenomic analysis, 40 mg of pellet per sample was used. DNA isolation from collected sediments was performed using a Genomic DNA purification kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) in accordance with the manufacturer's instructions. The quantity and quality of extracted DNA were determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).

2.3. Bacterial and fungal DNA amplification and amplicon library preparation

DNA samples from apples and blackcurrant microbiota were amplified using the primers specific for fungi and bacteria. For identification of fungal microorganisms, the ITS2 region of ribosomal DNA was amplified using ITS3-KYO2 (5′-GATGAAGAACGYAGYRAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers (Toju et al., 2012). For bacteria identification, the V3-V4 region of the 16S rRNA gene was amplified with primers S-D-Bact-0341-b-*S*-17 (5′-CCTACGGGNGGCWGCAG-3′) and S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′) (Klindworth et al., 2013). Amplicon libraries were prepared using modified Illumina adapters (www.illumina.com), validated on an Agilent Technologies Bioanalyzer DNA 1000 and sequenced using Illumina MiSeq V3 (2 × 300 bp) (Macrogen Inc., Seoul, Korea). All sequences obtained during this work are available at the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI), under accession number SRP108314.

2.4. Data processing and analysis

The bioinformatics pipelines, FLASH 1.2.11 (Magoc and Salzberg, 2011), CD-HIT-OTU 4.5.5 (Li et al., 2012), and QIIME v. 1.8 (Caporaso et al., 2010), were used to process and analyze the obtained sequence data. Preliminary processing of the data was performed using the default parameters of FLASH 1.2.11: sequences with a minimum quality score of 25 were filtered and paired-end reads were merged. Sequences were denoised, chimeric sequences were identified and filtered, and the remaining reads were clustered into the Operational Taxonomical Units (OTUs) with a minimum 97% similarity threshold, using the CD-HIT-OTU 4.5.5 (Li et al., 2012). The most abundant sequences in each OTU were used for the taxonomy assignments using the RDP (Ribosomal Database Project) (Wang et al., 2007; Cole et al., 2014) and the UNITE (Koljalg et al., 2013) databases as references. For downstream analysis, the OTU table was rarefied at an even depth to reduce biases in sequencing depth. Alpha diversity was calculated using observed species, Shannon, Good's coverage and Chao1 estimates (Caporaso et al., 2010). Weighted Unifrac algorithm was used to evaluate β-diversity (Lozupone and Knight, 2005). Principal coordinates analysis (PCoA), as implemented in QIIME v. 1.8, related the bacterial and fungal microbiota composition to sample types and examined the distance between different ecosystems.

2.5. Cultivable yeast enrichment and identification

The aseptically collected apple and blackcurrant fruits (30 g each) were kept in 5% dextrose solution for 15 days at a temperature of 22 °C. Serial dilutions were made in a Ringer solution (Merck, Kenilworth, United States), plated on YEPD-agar plates (1% yeast extract, 1% peptone, 2% dextrose, 2% agar) containing 50 µg/mL chloramphenicol

and incubated for 2–3 days at 25 °C. Randomly selected colonies with yeast-like morphology were used for molecular identification. DNA was isolated from fresh yeast culture (24 h) by using Genomic DNA purification kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) in accordance with the manufacturer's instructions. For identification of yeast, the region between the 18S rRNA and 28S rRNA genes was amplified using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers. The PCR was performed in a total reaction of 50 µL, consisting of 5 µL DreamTaq green buffer, 1 µL of 2 mM dNTP mix, 1 µL of each primer (10 µmol/L), 2.5 unit of Dream Taq DNA polymerase (all from Thermo Fisher Scientific Baltics, Vilnius, Lithuania), 1 µL of DNA template (5 ng) and sterile distilled water up to 50 µL. PCR amplification was carried out by Esco thermocycler, according to the following PCR conditions: an initial denaturation at 94 °C for 5 min, followed by 25 cycles of 94 °C for 1 min, 53 °C for 1 min 30 s and 72 °C for 2 min. The final extension was carried out at 72 °C for 10 min. The PCR products were digested with CfoI and HinfI enzymes and tested by 1% agarose gel electrophoresis. PCR products differing in restriction profiles were purified using a GeneJet PCR purification kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania), according to the manufacturer's instructions and sequenced using ITS1 and/or ITS4 primers at BaseClear (Leiden, Netherlands). The obtained sequences were compared with those found in the FASTA network service of the EMBL-EBI database (http://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html).

3. Results

3.1. Diversity and richness of microbial communities

In this study, we assessed and compared the microbial communities of apples and blackcurrants sampled from Lithuania and the Czech Republic by DNA massive parallel sequencing of 16S rDNA for bacteria and ITS2 for fungal analysis. After quality evaluation, a total 1,466,580 high quality sequences were recovered (967,110 eukaryotic and 499,470 prokaryotic sequences). The clustering of the sequences generated a total of 1378 OTUs (940 [264 ± 95, hereafter median for 4 samples ± standard deviation] for fungal ITS2 and 438 [99 ± 24] for bacterial V3-V4) (Table 1). The total number of OTUs detected in individual samples varied from 85 to 328. Based on analysis of prokaryotic sequences, the highest number of OTUs was detected in blackcurrants sampled in Lithuania (Table 1). In agreement with OTU data, the Shannon's Diversity and the Chao1 estimates also revealed that blackcurrant berries had a higher bacterial diversity than apples. The analysis of eukaryotic sequences indicated that the Apple_CZ sample had the highest number of fungal OTUs, followed by Blackcurrant_CZ, Apple_LT and Blackcurrant_LT (Table 1).

The ratio between the number of the obtained and the expected OTUs (predicted by Chao1) was used to determine the coverage for the microbial communities. It was over 90% in all cases, indicating a good coverage. Rarefaction curve showed that the numbers of OTUs were saturated in all samples, making them suitable for further community analysis (Fig. 1).

Principal Coordinate Analysis (PCoA) performed with the representative OTUs showed a clear separation of apple and blackcurrant, indicating a difference in the composition of the bacterial and fungal microbiota (Fig. 2). The differences were mostly observed at the lower taxonomic levels (see Supplementary Tables S1 and S2 in the online version at DOI: http://dx.doi.org/10.1016/j.micres.2017.09.004). Bacterial microbiota of apples was clustered into a similar plot (Fig. 2A), while fungal microbiota of regionally distinct apples was slightly separated (Fig. 2B). The communities of prokaryotic and eukaryotic microorganisms on blackcurrants sampled in Lithuania and the Czech Republic were clearly separated in PCoA plots from each other and from apples (Fig. 2).

3.2. Composition of apple bacterial and fungal microbiota

The populations of fungal and bacterial microbiota at the phylum level were similar on both apples (Apple_LT and Apple_CZ) collected from the geographically distinct regions of Lithuania and the Czech Republic. The dominant phylum across the entire eukaryotic microorganism population was Ascomycota (86.3% and 86.8% in Apple_LT and Apple_CZ respectively), supplemented by Basidiomycota (12.5% and 11.2%) (Fig. 3A I). Regarding the bacterial population, Proteobacteria (95.8% on Apple_LT and 96.9% on Apple_CZ) dominated in both localities (Fig. 3A II, Table S2 in the online version at DOI: http://dx.doi.org/10.1016/j.micres.2017.09.004). The bacterial diversity was evident when we analyzed sub-phyla distribution (Figs. 3 B II, 4 A). Gammaproteobacteria dominated on both apple samples (81.6% in Apple_LT and 75.8% in Apple_CZ) (Fig. 3B II), represented by Enterobacteriaceae (48.9% in Apple_LT and 34.2% in Apple_CZ) and Pseudomonadaceae (32.6% and 41.3% respectively) (Fig. 4A). Fungal microorganisms mainly consisted of Saccharomycetes (70.2% in Apple_LT and 63.0% in Apple_CZ) (Fig. 3B I), which were not characterized at the genus (Fig. 4B) or at species level and were assigned to uncultured *Metschnikowiaceae* spp. (66.7% and 44.3% respectively) (see Supplementary Table S2 in the online version at DOI: http://dx.doi.org/10.1016/j.micres.2017.09.004). Slight differences of distribution of apple-associated fungal microorganisms were observed only at the genus level. From identified fungal microorganisms, *Cryptococcus* (5.7%), *Cladosporium* (4.2%) and *Hanseniaspora* (3.3%) dominated in Lithuania, while on apples sampled in the Czech Republic, *Hanseniaspora* represented the most abundant fraction (17.6%). Some genera were detected only in one location: for example, on apples grown in Lithuania, we observed *Stachybotrys*, while in the Apple_CZ sample – *Wickerhamomyces* (Fig. 4B). Same cultivable yeast species, such as *Issatchenkia terricola*, *Pichia fermentans*, *Torulaspora delbrueckii*, *Saccharomyces cerevisiae*, were weakly distinguished by NGS analysis (see Supplementary Table S2 in the online version at DOI: http://dx.

Table 1
Total sequences obtained for eukaryotic (ITS2) and prokaryotic (V3-V4) microbial community for apple and blackcurrant samples.

Samples	Target region	High quality reads	OTUs	Chao1	Coverage	Shannon diversity
Apple_LT	ITS2	251,647	225	225.13	0.9994	2.73
	V3-V4	117,934	90	91	0.989	2.31
Apple_CZ	ITS2	305,477	328	339.33	0.9666	2.91
	V3-V4	141,529	104	115	0.9043	2.59
Blackcurrant_LT	ITS2	128,244	85	92.5	0.9297	2.93
	V3-V4	117,227	150	160	0.9375	5.4
Blackcurrant_CZ	ITS2	281,742	302	306.23	0.9862	2.98
	V3-V4	122,780	94	96	0.9792	4.95
	Eukaryotic	967,110	940			
	Prokaryotic	499,470	438			
	Total	1,466,580	1,378			

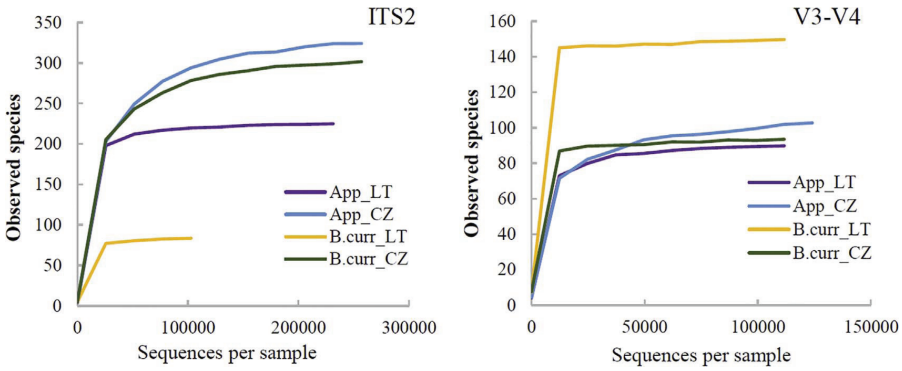

Fig. 1. Rarefaction curves at a genetic distance of 3% for each sample. ITS2 sequences from the analysis of the population of eukaryotic microorganisms and V3-V4 region sequences from the analysis of the community of prokaryotic microorganisms.

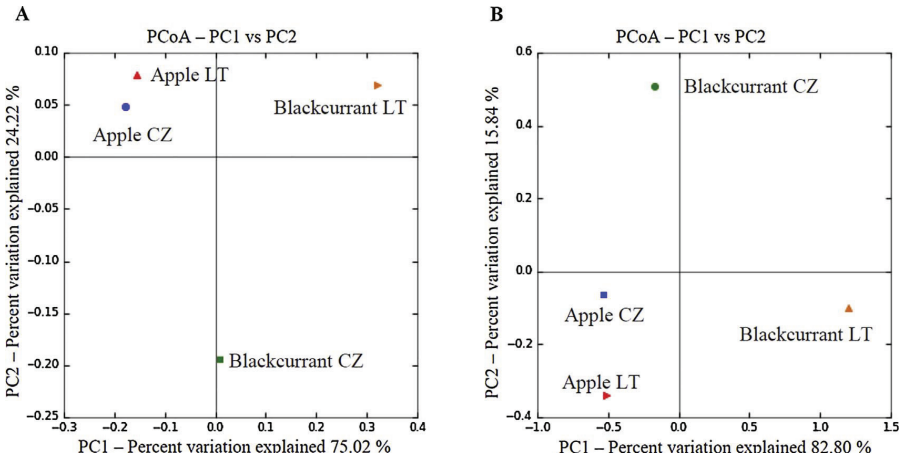

Fig. 2. Principal component analysis (PCoA) profiles based on the structure of bacterial (A) and fungal microorganisms (B) community.

doi.org/10.1016/j.micres.2017.09.004), but were detected after isolation from both apple samples by applying enrichment and cultivation techniques (see Supplementary Table S3 in the online version at DOI: http://dx.doi.org/10.1016/j.micres.2017.09.004).

3.3. Composition of blackcurrant bacterial and fungal microbiota

The distribution of fungal microorganisms at the phylum level was similar on blackcurrant berries located in both Lithuania and the Czech Republic. The dominant phylum across the entire eukaryotic microorganism population was Ascomycota (71.5% and 92.2% in Blackcurrant_LT and Blackcurrant_CZ respectively), though it also contained Basidiomycota (24.2% and 7.0%, respectively) and other unidentified fungi (Fig. 3A I). The distribution and abundance of bacteria varied depending on sampling geography (Fig. 3A II). Blackcurrants sampled in Lithuania were dominated by Firmicutes (35.4%), Proteobacteria (26.9%) and Actinobacteria (20.0%). However, on the blackcurrant berries harvested in the Czech Republic, the most abundant bacterial phylum was Proteobacteria (71.8%).

The broad diversity and variation of bacterial and fungal microorganisms among the blackcurrant samples harvested in Lithuania and the Czech Republic were evident at the family and genus level (Fig. 4). Among the dominant bacterial OTUs, Staphylococcaceae (27.1%), Flavobacteriaceae (7.2%) and Moraxellaceae (5.7%) were the most abundant families in Blackcurrant_LT, while the Blackcurrant_CZ sample was dominated by Enterobacteriaceae (20.4%), followed by Oxalobacteraceae (8.4%), Pseudomonadaceae (7.8%), Sphingomonadaceae (7.4%), Comamonadaceae (7.0%), Cytophagaceae (7.0%), Acetobacteraceae (6.1%), and Sphingobacteriaceae (5.4%) (Fig. 4A). The analysis of distribution of fungal microorganisms on blackcurrants harvested in Lithuania revealed that the most dominant genera were *Cladosporium* (45.4%) and *Cryptococcus* (15.2%), while the berries collected from the Czech Republic were dominated by *Hanseniaspora* (48.5%), followed by *Cladosporium* (5.2%) and *Rhodotorula* (1.5%) (Fig. 4B; see Supplementary Table S2 in the online version at DOI: http://dx.doi.org/10.1016/j.micres.2017.09.004).

4. Discussion

The distribution of microorganisms depends on plant species and may be affected by growing, ripening and storage conditions (Pinto et al., 2015). However, it is difficult to establish major and specific factors responsible for driving the divergence of bacterial and fungal communities (Leff and Fierer, 2013) since different factors have a cumulative effect. The current study evaluated the distribution of bacterial and fungal microorganisms found on apple fruits and blackcurrant berries, grown in distinct regions in Lithuania and the Czech Republic. None of the sampled plants received any chemical treatment

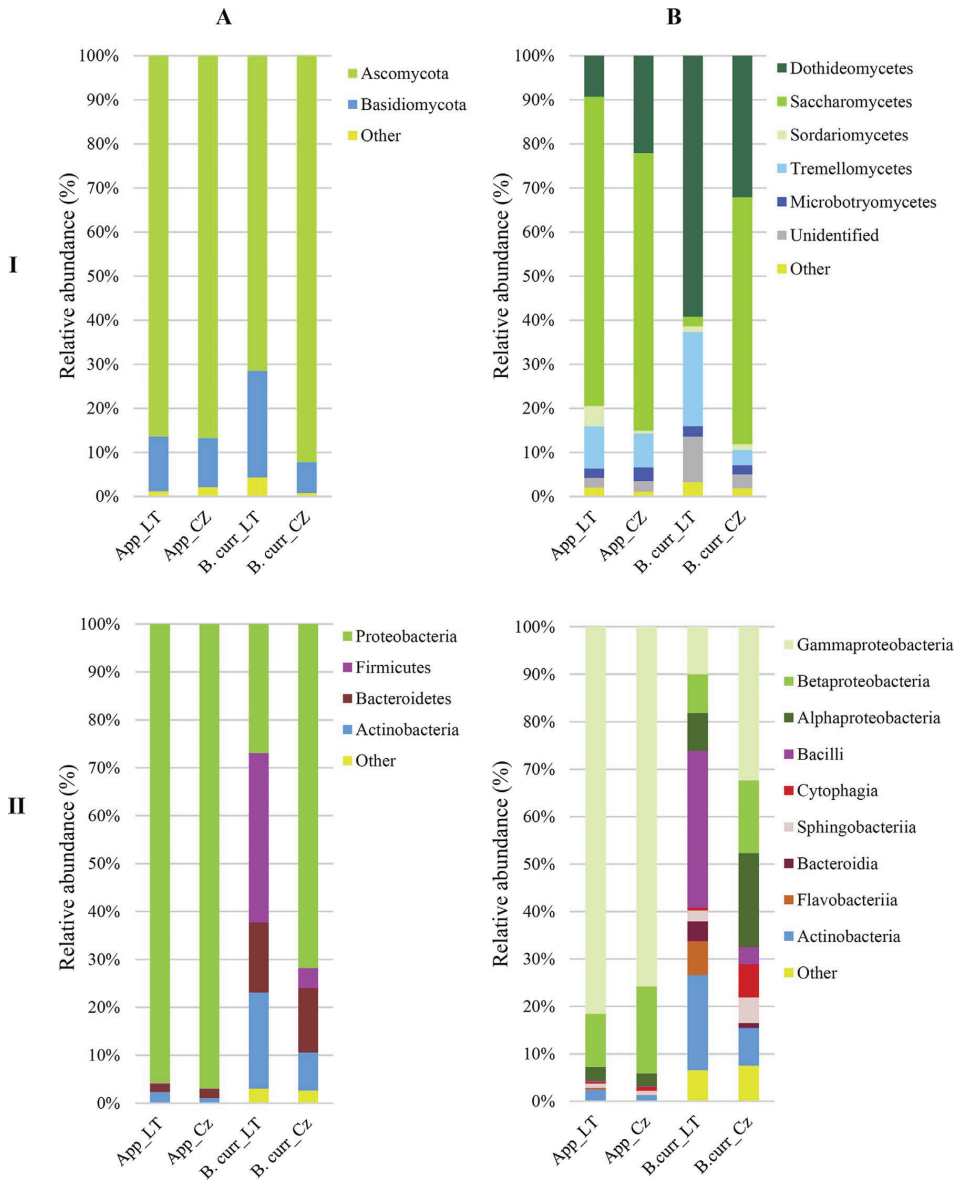

Fig. 3. Eukaryotic (I) and prokaryotic (II) microbial community distribution at the phylum (A) and class (B) level.
App_LT – apples sampled in Lithuania, App_CZ – apples collected in the Czech Republic; B. curr_LT – blackcurrant berries sampled in Lithuania, B. curr_CZ – blackcurrant berries collected in the Czech Republic.

and were analyzed immediately after sampling, thus minimizing external impact on the composition of microbiota.

We found that the bacterial communities from the apple surface were dominated by Gammaproteobacteria, mostly represented by the family Enterobacteriaceae. This is barely surprising, given that Gammaproteobacteria (as many other bacteria) is recognized initial degraders of organic matter contributing to the release of nutrients such as phosphorus and nitrogen (Sarr et al., 2017). However, our data differ from previous observations (Leff and Fierer, 2013), where the most abundant bacterial class on apples (purchased from a grocery store in Boulder, CO, USA) was Alphaproteobacteria, mostly the Sphingomonadaceae family with Enterobacteriaceae detected in lower frequency. By applying culture-dependent techniques, no Enterobacteriaceae were detected on apples that had been fresh-cut and purchased from different supermarkets of Spain (Abadias et al., 2008). In our case, irrespective of geographical location (samples from both Lithuania and the Czech Republic), more than two thirds of species were representatives of Gammaproteobacteria, such as *Pantoea* spp. and uncultured *Pseudomonas* (see Supplementary Table S1 in the online version at DOI: http://dx.doi.org/10.1016/j.micres.2017.09.004). Certain *Pantoea* or

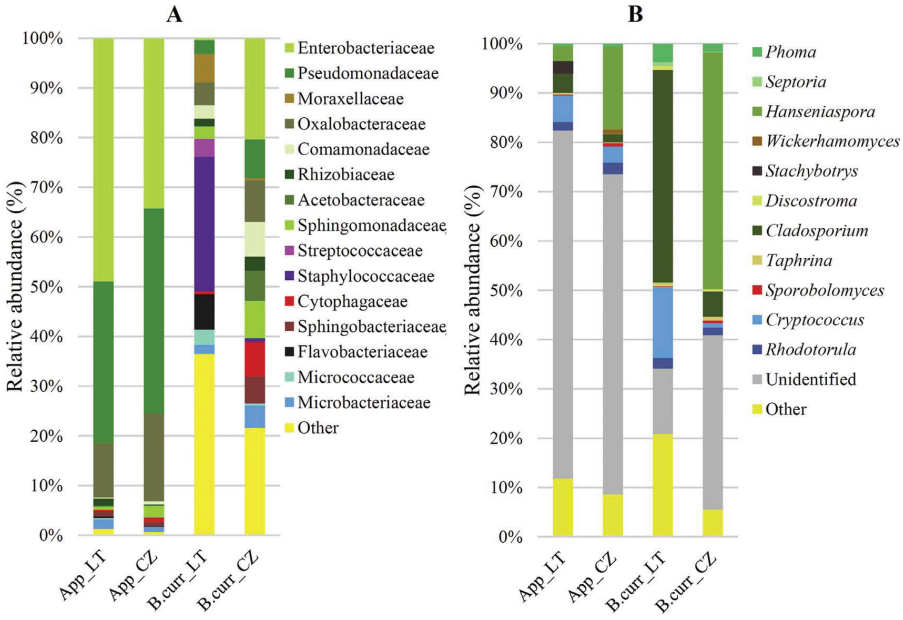

Fig. 4. Relative abundance of major bacterial families (A) and fungal microorganism genera (B) present on apples and blackcurrants sampled in Lithuania and the Czech Republic.

Pseudomonas species are well-known plant pathogens responsible for economic losses (Coutinho and Venter, 2009). On the other hand, they could produce antibacterial and antifungal agents protecting hosts from disease (Ligon et al., 2000; Enya et al., 2007). The rest of the bacterial population consisted of representatives of different genera, such as *Duganella*, *Massilia*, *Sphingomonas*, etc., which could be beneficial to plants due to their ability to induce plant resistance and promote growth (Kim et al., 1998; Ofek et al., 2012).

The bacterial community from the blackcurrant surface was more divergent in comparison to the apples. The microorganisms on blackcurrants located in Lithuania differed at the lower taxonomic level (family, genus or species) from berries harvested in the Czech Republic. Uncultured bacteria of *Staphylococcus* became more abundant on Lithuanian berries, followed by *Acinetobacter*, *Streptococcus*, *Flavobacterium*, etc. Uncultured *Tatumella* and *Pantoea* representing Enterobacteriaceae dominated on blackcurrants from the Czech Republic, followed by *Gluconobacter*, *Massilia*, *Lactobacillus*, *Methylobacterium,* etc (see Supplementary Table S1 in the online version at DOI: http://dx.doi.org/10.1016/j.micres.2017.09.004). These differences could be stipulated by distinct climatic conditions and the ripening stage of berries. Both potential plant pathogens and beneficial bacteria could be inferred among observed microorganisms. Taking into account that human pathogenic bacteria can adapt to plant hosts (Abdelfattah et al., 2016a), there is a chance that such kind of bacteria were also present on the berries tested in our study.

By focusing on the communities of fungal microorganisms, we identified Saccharomycetes to be the major class associated with apples in both locations and blackcurrants collected in the Czech Republic. Blackcurrant berries sampled in Lithuania were dominated by Dothideomycetes. Saccharomycetes were mainly represented by *Hanseniaspora uvarum* and uncultured *Metschnikowiaceae. Hanseniaspora* sp. (see Supplementary Table S2 in the online version at DOI: http://dx.doi.org/10.1016/j.micres.2017.09.004), characterized by low fermentative activity, have been frequently found on the surface of different fruits, e.g. grapes, strawberries or apples (Santo et al., 2012; Graca et al., 2015). *Metschnikowia* also includes species commonly found on the fruit surface and acting as biocontrol agents against different plant pathogens (Parafati et al., 2015). They can strongly antagonize the growth of various filamentous fungi and bacteria by depleting iron in the growth medium (Liu et al., 2013). Using metagenomic analysis, other members of Saccharomycetes, such as *Wickerhamomyces anomalus*, *Saccharomyces cerevisiae*, *Issatchenkia terricola* and *Pichia fermentans* were detected at low level (see Supplementary Table S2 in the online version at DOI: http://dx.doi.org/10.1016/j.micres.2017.09.004) or were notable only after culture enrichment (see Supplementary Table S3 in the online version at DOI: http://dx.doi.org/10.1016/j.micres.2017.09.004). Abundance of yeast is dependent on the fruit development stage and changes along with fruit ripening. Most likely, the low quantities of fermenting yeast found on the surface of our apples could be due to the early harvesting time, when the fruits were undamaged and accessibility to sugar sources was limited. On the other hand, *Hanseniaspora* sp., *Pichia* sp., *Metschnikowia pulcherrima*, *Saccharomyces cerevisiae* were documented on apples after enrichment of samples during only 15 days at fermenting conditions (Vadkertiova et al., 2012). *Wickerhamomyces* and *Pichia* spp. can live both outside and within fruit tissues and even low quantities, acting as biocontrol agents, could regulate the structure of plant microbiota (Vadkertiova et al., 2012; Muccilli et al., 2013; Abdelfattah et al., 2016b).

Cladosporium spp., as representatives of Dothideomycetes, were identified in all samples and in exceptionally high quantity on the blackcurrant berries sampled in Lithuania (see Supplementary Table S2 in the online version at DOI: http://dx.doi.org/10.1016/j.micres.2017.09.004). The abundant presence of this genus was not surprising, considering that it is ubiquitous fungi detected on the surface of different plants (Abdelfattah et al., 2016a, 2016b). In agreement with microbiota studies performed on strawberries (Abdelfattah et al., 2016a), grapes (Barata et al., 2012; Setati et al., 2012), apple, pear, and plum (Vadkertiova et al., 2012), *Cryptococcus* and pigmented yeast *Rhodotorula* were also found on the apples and currants in our experiments. *Cryptococcus* was recognized as a typical constituent of the yeast community and association with the early state of maturation

of fruits has been reported (Janisiewicz et al., 2010; Vadkertiova et al., 2012). These ubiquitous fungi have often been isolated from fruit washings and have been identified as biocontrol agents for management of the postharvest diseases (Liu et al., 2013). *Rhodotorula* sp. can be found on grapes during all ripening stages and produce biofilms on berry surfaces (Lederer et al., 2013).

Our results demonstrated differences in bacterial and fungal microbiota diversity across fruits and berries. The bacterial communities on apples were relatively uniform and similar to one another regardless of geographic location. The blackcurrant bacterial populations were divergent in the regional context and, in comparison with apples, demonstrated that both sampling location and plant species influenced bacterial community composition. A similar tendency was observed in the communities of fungal microorganisms, which were more similar on apples located in different regions than on blackcurrant berries. Our data on apples agree with several previous studies, where it has been demonstrated that the distribution of phyllosphere bacterial communities has minimal geographic differentiation (Redford et al., 2010). Likewise, our observed regional effect on blackcurrant microbiota is consistent with biogeographical correlation of grape wine microbial communities (Pinto et al., 2015). Microorganisms strongly differ across plant species, likely due to variations in metabolites, physical characteristics and symbiotic interactions with the host plant and other microbial inhabitants (Lindow and Brandl, 2003; Hunter et al., 2010). Shifts in the community composition could occur during the time of transport from the field to the grocery store and into hands of the final consumer (Leff and Fierer, 2013; Abdelfattah et al., 2016b). This could explain why our data on apple microbiota structure differ from previous studies conducted on apples purchased from the local supermarket. Moreover, whether conventional or organic farming, an exposure of plants to chemical treatments have been documented to alter the fungal and bacterial microbiota composition (Leff and Fierer, 2013; Abdelfattah et al., 2016b).

Author contributions

Investigation: IVM, JL, RS, ZSZ, VY
Bioinformatic analysis: JL, ES
Data curation and analysis: ES, SS, VY
Funding acquisition: ES
Writing – original draft: SS, ES
Writing – review & editing: SS, VY, ES

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PAPER IV

***Saccharomyces paradoxus* K66 killer system evidences expanded assortment of helper and satellite viruses**

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Article

Saccharomyces paradoxus K66 Killer System Evidences Expanded Assortment of Helper and Satellite Viruses

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Abstract: The *Saccharomycetaceae* yeast family recently became recognized for expanding of the repertoire of different dsRNA-based viruses, highlighting the need for understanding of their cross-dependence. We isolated the *Saccharomyces paradoxus* AML-15-66 killer strain from spontaneous fermentation of serviceberries and identified helper and satellite viruses of the family *Totiviridae*, which are responsible for the killing phenotype. The corresponding full dsRNA genomes of viruses have been cloned and sequenced. Sequence analysis of SpV-LA-66 identified it to be most similar to *S. paradoxus* LA-28 type viruses, while SpV-M66 was mostly similar to the SpV-M21 virus. Sequence and functional analysis revealed significant differences between the K66 and the K28 toxins. The structural organization of the K66 protein resembled those of the K1/K2 type toxins. The AML-15-66 strain possesses the most expressed killing property towards the K28 toxin-producing strain. A genetic screen performed on *S. cerevisiae* YKO library strains revealed 125 gene products important for the functioning of the *S. paradoxus* K66 toxin, with 85% of the discovered modulators shared with *S. cerevisiae* K2 or K1 toxins. Investigation of the K66 protein binding to cells and different polysaccharides implies the β-1,6 glucans to be the primary receptors of *S. paradoxus* K66 toxin. For the first time, we demonstrated the coherent habitation of different types of helper and satellite viruses in a wild-type *S. paradoxus* strain.

Keywords: *Saccharomyces paradoxus*; *Totiviridae*; dsRNA virus; killer system

1. Introduction

Yeasts constitute a large group of microorganisms characterized by the ability to grow and survive in stressful conditions and to colonize a wide range of environmental ecosystems [1]. The secretion of yeast killer toxins confers a competitive edge to the producer strain by excluding other yeasts from shared habitat without direct cell-to-cell contact [2]. Rather than providing immediate advantages for the respective host, killer toxin-immunity systems also have to be considered as important players in the autoselection system [3]. Recently, the mutual incompatibility of double-stranded RNA virus-based killer systems and the RNA interference mechanism in yeast has been demonstrated [4].

Double-stranded RNA-based killer systems have been described in different yeast species, such as *Saccharomyces cerevisiae*, *S. paradoxus*, *S. uvarum*, *Ustilago maydis*, *Zygosaccharomyces bailii*, *Hanseniaspora uvarum*, and *Torulaspora delbrueckii* [5–7]. The mycoviruses of the *Totiviridae* family are incapsulated into virus-like particles (VLPs) and stably persist in the host cell without causing cell lysis; they are transmitted by vegetative cell division or through sexual fusion [5,8–10]. Most killer toxins are encoded by dsRNA viruses called M satellites, which depend for their propagation and maintenance on an L-A helper virus [8,11]. Among the representatives of the genus *Saccharomyces*, the killer phenomenon has been studied most extensively in *S. cerevisiae*, where four different viral-originated killer toxins (K1, K2, K28, and Klus) have been described [12–15]. For effective functioning of the killer system, well organized communication between the functionally distinct ScV-LA and ScV-M viruses is required. The L-A virus has a 4.6 kb segment that encodes the major structural capsid protein Gag, encapsulating either L or M virus dsRNA in icosahedral structures, and the Gag-Pol fusion protein responsible for replication and encapsidation [11,16,17]. There are several variants of L-A (ScV-LA-1, ScV-LA-2, ScV-LA-28 and ScV-LA-lus), with an average of 74% identity in nucleotide sequences, associated with different M viruses and displaying distinct phenotypic properties [14,18]. Four different *S. cerevisiae* M dsRNA viruses have been described (ScV-M1, ScV-M2, ScV-M28 and ScV-Mlus) so far [15]. Certain relationships between L-A and M viruses have been observed (LA-1 and M1, LA-2 and M2, LA-lus and Mlus, LA-28 and M28) [14,18,19] and the possible role of the toxin-producing M viruses in selecting the L-A variants to support them has been proposed [19]. A certain heterogeneity in L-A itself has also been reported with functional phenotypic variants that exhibited differences in maintaining the K1 and K2 phenotypes with the involvement of MKT genes [20]. The observed exceptions of specificity were essentially limited to laboratory strains or hybrids, as well as strains featuring significantly elevated amounts of L-A dsRNA or proteins encoded by this virus [19,20]. The association of distinct L-As with different M viruses suggests their co-evolution, leading to the propensity of a particular L-A virus to maintain a certain type of M virus [14,19].

The M viral genome is 1.6–2.4 kb in size and encodes a specific preprotoxin, which is subsequently processed into a mature protein (K1, K2, K28, and Klus). The M dsRNA viruses show no sequence homology to each other, though the organization of their genomes is strikingly similar. The positive strand contains an open reading frame (ORF) in the 5′-terminal region that encodes for the toxin precursor, followed by a unique internal AU-rich region, and a 3′-terminal non-coding region of variable length possessing *cis* signals for encapsidation and replication by the viral RNA polymerase [6]. Different killer toxins are secreted glycoproteins lacking amino acid sequence conservation and adopting diverse cell killing mechanisms. The proposed mechanism of the action for the K1 and K2 toxins is a two-step process, whereby the killer protein first binds to the primary cell wall receptor-β-1,6-glucan [21,22], then, at the second step, the toxins approach a plasma membrane receptor and form lethal cation-selective ion channels [15,23–25]. In contrast, the K28 toxin binds to α-1,3-mannoproteins positioned in the cell wall, then interacts with a plasma membrane receptor Erd2 and enters the cell by endocytosis. In the cell, the K28 toxin travels to the nucleus by retrograde passage and blocks DNA synthesis causing G1/S cell cycle arrest [26,27]. The lethal mechanism of the Klus toxin is yet to be uncovered [14,28].

Many host genes affect the maintenance of *S. cerevisiae* L-A and M viruses [11], as well as the performance of viral killer toxins [29–31]. The SKI family genes block expression of the non-polyadenylated viral mRNA [32]. Species-specific virus restriction factor Xrn1p (encoded by SKI1 gene) appears to co-evolve with totiviruses to control viral propagation in *Saccharomyces* yeasts [33]. MAK family gene products are necessary for L-A and M propagation [12,34,35]. The exact function and interplay of these genes in virus replication and maintenance is not fully understood [36,37]. On the other hand, the presence of dsRNA viruses impacts the expression of numerous host genes [38,39], many of them tightly integrated into cellular metabolism. The phylogenetic analysis uncovers that at least some mycoviruses co-evolve with their hosts, suggesting a close interaction between participants [40,41]. Viruses and their hosts exist in a constant state of genetic conflict, where an

advantage for one party is often a disadvantage for the other [33]. The stable persistence of L-A virus in some 20% of wild *S. cerevisiae* [42] cells suggests a generally detrimental impact to the host, probably because of consumption of energy and material resources [11]. In addition, M dsRNAs are rather rare in wild strains [42,43].

Saccharomyces paradoxus is the closest relative of the domesticated yeast *S. cerevisiae*, mainly found in the wild [6]. Killer toxins of *S. paradoxus* have been considered as chromosome-coded for a long time and it is only relatively recently that dsRNA viruses (L and M) in such yeast have been discovered [44]. The sizes of L and M dsRNAs genomes are similar to those of *S. cerevisiae* strains. The degree of nucleotide variation in different types of L-A viruses ranged from 73% to 90%, depending on the geographical location of *S. paradoxus* strains [6,9]. The essential features of *S. cerevisiae* L-A viruses-frameshift region and encapsidation signal-remain conserved in all *S. paradoxus* L-A variants investigated thus far. The encoded Gag-Pol proteins demonstrate 85% to 98% amino acid identity. At least five different *S. paradoxus* killer toxin-producing viruses (SpV-M21, SpV-M28, SpV-M45, SpV-M62, and SpV-M74) have been identified. They encode toxins differing in sequences from any of those previously known, while structural-functional characterization has not been accomplished yet [6].

In this study, we performed cloning and structure-functional analysis of the *S. paradoxus* SpV-LA-66 and SpV-M66 viruses, recently isolated from the natural environment. Comparison of the SpV-LA-66 sequence and phylogenetic analysis demonstrated its close relationship to SpV-LA-28 and SpV-LA-21 viruses. The SpV-M66 virus-encoded preprotoxin sequence analysis allowed prediction of the structural elements of the active K66 killer protein. The *S. paradoxus* K66 toxin was isolated and the essential parameters of protein activity were investigated. For the first time, we have identified genetic factors, involved in the functioning of the *S. paradoxus* virus-originated K66 toxin, and those important for the susceptibility of the target cell. The gene products, connected to cell wall organization and biogenesis, as well as involved in the regulation of response to osmotic stress, were demonstrated as significantly enriched. By performing in vivo and in vitro toxin binding assays, we demonstrated that β-1,6 glucans could play the role of primary receptors for the *S. paradoxus* K66 toxin. We concluded that SpV-LA-66 and SpV-M66 represent a previously undescribed combination of helper and satellite viruses in the wild-type *S. paradoxus* AML-15-66 strain.

2. Materials and Methods

2.1. Strains and Media

The killer strain employed in this study (*Saccharomyces paradoxus* AML-15-66) was originally isolated from spontaneous fermentation of serviceberries (*Amelanchier ovalis* Medik.). The following yeast strains were used for the killer assay: *S. cerevisiae* α'1 (*MATα leu2-2* (*KIL-0*)) [45], M437 (*wt, HM/HM* (*KIL-K2*)) [46], K7 (*MATα arg9* (*KIL-K1*)) [47], MS300 (*MATα leu2 ura 3-52* (*KIL-K28*)) [13], SRB-15-4 (wt, HM/HM (KIL-Klus) (laboratory collection), BY4741 (*MATa; his3 D1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0* (*KIL-0*)) (Thermo Scientific Molecular Biology, Lafayette, CO, USA), and *S. paradoxus* T21.4 strain (kindly provided by Dr. G. Liti, Université Côte d'Azur, CNRS, INSERM, IRCAN, Nice, France). The curing of yeast strains from dsRNA viruses was accomplished as described in [39].

For identification of yeast, the regions between the 18S rRNA and 28S rRNA genes containing two non-coding spacers (ITS-A and ITS-B) separated by the 5.8S rRNA gene were PCR-amplified using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers [48], and sequenced at Base Clear (Leiden, ZH, The Netherlands). The obtained sequences were compared with those found in the FASTA network service of the EMBL-EBI database (http://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html). Screening for genetic factors modulating the K66 toxin activity was performed with a *S. cerevisiae* single ORFs deletion strains (BY4741 background, *MATa*; *his3 D1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) (Thermo Scientific Molecular Biology, Lafayette, CO, USA).

Yeast strains were grown in standard YEPD medium (1% yeast extract, 2% peptone, 2% dextrose, 2% agar). For the killing assay, MBA medium (0.5% yeast extract, 0.5% peptone, 2% dextrose) was

used, adjusted to appropriate pH 3.2–6.0 with the 75 mM phosphate-citrate buffer and supplemented with 0.002% methylene blue dye. For toxin preparation, liquid synthetic medium SC (2% dextrose, 0.2% K_2HPO_4, 0.1% $MgSO_4 \times 7H_2O$, 0.1% $(NH_4)_2SO_4$, 1.29% citric acid, 2.76% $Na_2HPO_4 \times 12H_2O$) was used, containing 5% glycerol, adjusted to appropriate pH 3.2–6.0.

2.2. Assay for Killing/Resistance Phenotypes

For detection of killing phenotype, the tested *S. paradoxus* strain was spotted on the MBA agar plates seeded with a lawn of the sensitive *S. cerevisiae* strain BY4741 (2×10^6 cells/plate) or strains of different yeast species. After incubation of the plates at 25 °C for 3 days, clear zones of growth inhibition surrounding the killer cells were evaluated and interpreted as a killer activity.

The sensitivity/resistance tests were performed by spotting *S. cerevisiae* killer strains onto the MBA plates with an overlay of the *S. paradoxus* strain AML-15-66. The absence of lysis zones indicates a resistant phenotype, while non-growth zones around the different types of killer toxins producing colonies were attributed to the sensitive phenotype [49].

2.3. Viral dsRNA Isolation From Yeast

Total extraction of nucleic acids from yeast was based on the previously described method [50] with modifications. *S. paradoxus* culture was grown in YEPD media overnight at 30 °C. Cells were collected by centrifugation for 5 min at 5000× *g* at 20 °C and washed with 1/10 part of the starting volume of the culture media supplemented by 50 mM EDTA. Cells were collected and re-suspended in 1/10 part of the starting volume of TB buffer (50 mM Tris-HCl pH 9.3; 1% β-mercaptoethanol) and incubated for 15 min at the room temperature. Cells were pelleted and re-suspended in 2/10 part of the starting volume of TES buffer (10 mM Tris-HCl pH 8.0; 100 mM NaCl; 10 mM EDTA; 0.2% (*w/v*) SDS). Subsequently, an equal volume of phenol (pH 5.2) preheated to 80 °C was added, and the suspension was vigorously shaken for 45 min at room temperature. Afterwards, an equal volume of chloroform was added and mixed thoroughly. The mix was subjected to centrifugation at 18,000× *g* for 45 min at 4 °C and separated by pipetting. Nucleic acids were pelleted from an aqueous fraction by adding 1 volume of isopropanol supplemented with 1/10 volume of 3 M sodium acetate (pH 5.2) and subjected to centrifugation at 18,000× *g* for 10 min at 4 °C. The resulting pellets were washed with 75% ethanol and dissolved in DEPC-treated water. Re-suspended nucleic acids were separated in 1% agarose gels and visualized by staining with ethidium bromide.

For double-stranded RNA (dsRNA) preparation, an isolated 700 μg of the total nucleic acid fraction was incubated in 2.8 M LiCl overnight at 4 °C [51]. The single-stranded nucleic acids were removed by centrifugation at 18,000× *g* for 45 min at 4 °C. The aqueous phase was substituted with 1/10 volume of 3 M NaCl and 1 volume of isopropanol, dsRNA pelleted by centrifugation at 18,000× *g* for 10 min at room temperature, washed with 75% ethanol and dissolved in DEPC treated water. The resulting L and M dsRNAs were visualized by gel electrophoresis and gel-purified using GeneJet Gel Extraction Kit (Thermo Fisher Scientific, Vilnius, Lithuania).

2.4. cDNA Synthesis, Amplification, and Cloning

Viral dsRNA cDNA synthesis and amplification were performed as described [52], with modifications. PC3-T7 loop primer (5′-GGATCCCGGGAATTCGGTAATACGACTCACTAT ATTTTTATAGTGAGTCGTATTA-3′) was ligated to gel-extracted dsRNA following the primer:dsRNA molar ratio of 250:1. The ligation reaction was performed by T4 RNA ligase (Thermo Fisher Scientific, Vilnius, Lithuania) in the supplier's buffer with additional adding of up to 20% PEG-6000, 10% DMSO, 0.01% BSA and 20U RiboLock RNase Inhibitor (Thermo Fisher Scientific, Vilnius, Lithuania) overnight at 37 °C. DsRNA with ligated primers was purified using a GeneJet PCR Purification Kit (Thermo Fisher Scientific, Vilnius, Lithuania), before cDNA synthesis step was denatured by adding of DMSO to a final concentration of 15% (*v/v*), heating at 95 °C for 2 min, and immediately transferred onto the ice for 5 min. Prepared dsRNA was reverse-transcribed using Maxima Reverse Transcriptase

(Thermo Fisher Scientific, Vilnius, Lithuania). Alkaline hydrolysis of residual RNA was performed and cDNA strands re-annealed at 65 °C for at least 90 min followed by gradual cooling to 4 °C for 2 h The cDNA was amplified using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Vilnius, Lithuania) with PC2 primer (5′-CCGAATTCCCGGGATCC-3′) by using the manufacturer's recommended cycling conditions, with an initial step carried at 72 °C for 2 min added. PCR products were cloned into the pUC19 vector (Thermo Fisher Scientific, Vilnius, Lithuania), sequenced at Base Clear (Leiden, ZH, The Netherlands) and the obtained sequences blasted against known sequences in the NCBI database.

2.5. Sequence Analysis

A maximum likelihood phylogenetic tree was constructed using the IQ-Tree v1.6.3 [53] with automatic selection of best-fit amino acid substitution and site heterogeneity models. LG + R3 proved to be the best-fit model. Edge support was estimated with bootstrap test (1000 replicates). Phylogenetic tree visualization was performed using Fig-Tree v1.4.3 program (http://tree.bio.ed.ac.uk/software/figtree/). Phobius server http://phobius.sbc.su.se/ [54] was employed for transmembrane topology identification. Sites of N-glycosylation in the protein sequences were identified using NetNGlyc web server (http://www.cbs.dtu.dk/services/NetNGlyc/). Conservative domains of the protein were determined using Pfam database [55]. DIANNA server (http://clavius.bc.edu/~clotelab/DiANNA) [56] was employed for disulfide bond connectivity prediction.

2.6. Analysis of the Partially Purified K66 Toxin for Thermal and pH Activity

S. paradoxus strain AML-15-66 was grown in synthetic SC-medium at various pH in the range of 3.2–6.0 for 4 to 6 days at 18 °C until reaching comparable cell density (OD600: 0.6–0.8). Yeast cells were separated by centrifugation at 3000× *g* for 10 min and filtration of supernatant through a 0.22 µm sterile PVDF membrane (Millipore, Bedford, MA, USA). The supernatant was then filtered using pressure-based Amicon system (membrane MWCO 10 kDa, Sigma-Aldrich, St. Louis, MO, USA) and serial centrifugations followed through Amicon ultra centrifugal filters with different cut-offs (10 and 30 kDa). The preparation of partially purified K66 toxin was used for assessment of optimal toxin activity and screening for modulators.

To determine K66 toxin activity at different pH values, MBA plates adjusted to pH values between 3.2 and 6 were seeded with the sensitive *S. cerevisiae* strain α'1 (2×10^6 cells/plate) and incubated at 25 °C in the presence of aliquots of the toxin (100 µL) extracted from killer protein-producing strain grown at appropriate pH and concentrated 100-fold. The inhibition zones were determined on triplicate plates after 2–5 days of incubation and the mean of remaining toxin activity was expressed in percent. Toxin preparation obtained at pH 4.8 was used for temperature activity measurement following the method described in [57].

2.7. Screening for Modulators of K66 Activity and Bioinformatic Analysis

The sensitivity was tested by either depositing 100 mL of concentrated 100-fold K66 toxin into 10 mm diameter "punched-wells" in the agar plate or spotting K66-producing cells onto the MBA medium overlaid with the yeast strain of interest (2×10^6 cells/plate). Plates were incubated for 2 days at 25 °C, and the diameter of the lysis zones measured and compared with those formed on BY4741 overlay. The screening was repeated 3 times.

The GO-term analysis was performed using the BiNGO 3.0.3 plug-in embedded into the Cytoscape 3.6.1. platform [58]. Significance *p* values were calculated with the hypergeometric test, using the Benjamini and Hochberg false discovery rate (FDR) correction for the enrichment of each GO term. Fold enrichment (F.E.) was determined by dividing the frequency of specific gene cluster to the total frequency for each GO term.

Network diagrams were generated using STRING web resource (version 10.5, http://string-db.org) [59]. Our created network uses the "confidence view" option of the program,

where stronger associations are represented by thicker lines. The experiments-based active prediction method was used, and the medium confidence score (0.400) was utilized.

2.8. Evaluation of K66 Toxin Binding to the Yeast Mutants and Different Polysaccharides

S. cerevisiae BY4741 as control and mutant strains from *S. cerevisiae* deletion library were cultivated at 30 °C in YEPD medium overnight. 2×10^6 cells were sedimented by centrifugation at 3000× *g* for 10 min and washed with 1 mL SC medium, pH 4.8 and incubated with 500 µL of 100-fold concentrated K66 toxin at 4 °C for 1 h. The supernatant was collected by centrifugation 1 min 10,000× *g* and tested by the well-test. After 2 days incubation at 25 °C the diameter of lysis zones was measured.

For analysis of K66 binding to different polysaccharides, 9 mg of chitin, laminarin, pullulan, or pustulan were mixed with 100 µL of 100-fold concentrated K66 toxin. Samples were incubated for 1 h at 25 °C. After centrifugation (1 min 10,000× *g*), 100 µL of supernatant was transferred into the wells on MBA medium (pH 4.8) containing α'1 cells. Lysis zones were analyzed after 2 days of incubation at 25 °C.

2.9. GenBank Accession Numbers

The SpV-L-A66 and SpV-M66 cDNA nucleotide sequences appear in NCBI/GenBank under GenBank accession no. MH784501 and MH784500, respectively.

3. Results

3.1. Characterization of the S. paradoxus Killer Strain

The yeast strain AML-15-66 was isolated from spontaneous fermentation of serviceberries. Based on the ITS region sequencing data and RFLP-PCR profiles, the strain was identified as *S. paradoxus* (Figure S1). We observed that AML-15-66 exhibits killing activity against *S. cerevisiae* non-killer and different types of killer virus-possessing strains at pH ranging from 3.6 to 5.6 (Table 1).

Table 1. Killing phenotype of *S. paradoxus* AML-15-66 strain. Diameter of zone of inhibition in mm: +++ (3–2.5), +++/- (2.5–2), ++ (2–1.5), ++/- (1.5–1), + (1–0.5), +/- (0.5–0).

Target Strain (Killer Type)	Killing Phenotype of *S. Paradoxus* AML-15-66							
	pH							
	3.2	3.6	4.0	4.4	4.8	5.2	5.6	6.0
S. cerevisiae ά 1 (K0)	-	+/-	+	++	+++	++/-	-	-
S. cerevisiae BY4741 (K0)	-	+/-	+	++	+++/-	+	-	-
S. cerevisiae K7 (K1)	-	-	+/-	+	+	+/-	-	-
S. cerevisiae K7 [L-M-] (K0)	-	-	+/-	+	++/-	+	-	-
S. cerevisiae M437 (K2)	-	-	+/-	+/-	+/-	+/-	-	-
S. cerevisiae M437 [L-M-] (K0)	-	-	-	+/-	+	+/-	-	-
S. cerevisiae CRB-15-4 (Klus)	-	-	-	+/-	+	+	+/-	-
S. cerevisiae CRB-15-4 [L-M-] (K0)	-	-	-	+/-	+	+	+/-	-
S. cerevisiae MS300 (K28)	-	-	+	+	++	+	-	-
S. cerevisiae MS300 [L-M-] (K0)	-	-	+	++/-	++	++/-	+/-	-
S. paradoxus AML-15-66 (K66)	-	-	-	-	-	-	-	-
S. paradoxus AML-15-66 [L-M-] (K0)	-	-	+/-	+	++/-	+/-	-	-
S. paradoxus T.21.4 (K21)	-	-	-	-	-	-	-	-

The strongest killing activity was determined at pH 4.4–4.8 against non-killer *S. cerevisiae* strains α'1 and BY4741. The killing phenotype against various types of killer toxin-producing strains was weaker, compared to that of the killer-free strains. *S. paradoxus* AML-15-66 strain demonstrated the lowest activity against *S. cerevisiae* M437 cells maintaining ScV-M2 virus (Table 1) and was not active against tested *Pichia*, *Hanseniaspora*, *Candida*, and *Torulaspora* spp. *S. paradoxus* AML-15-66 strain was

found to be resistant to the action of *S. cerevisiae* K1, K28, and Klus mycotoxins as well as to the K21 toxin, produced by *S. paradoxus* T21.4 strain, while susceptible to the action of *S. cerevisiae* K2 toxin produced by strain M437 only (Figure S2A).

3.2. Double-Stranded RNA Viruses from the S. Paradoxus Strain

To delve into the nature of the killing phenotype, we extracted dsRNAs from AML-15-66 and performed electrophoretic analysis (Figure S3). The size of observed L and M dsRNAs was compared to that of the dsRNAs isolated from reference strains of *S. cerevisiae* K7 (LA-1, M1), M437 (LA-lus, M2), MS300 (LA-28, M28), and SRB-15-4 (LA-lus, Mlus). The size of the L fraction was about 4.6 kb and thus highly similar to all L dsRNAs, while M dsRNA was about 1.6 kb and thus close to M2 dsRNA. We named these viruses SpV-LA-66 and SpV-M66, respectively. Purified dsRNA of the SpV-LA-66 virus was used as a substrate for primer ligation, subsequent reverse transcription, and cDNA amplification. In total, the genome of the SpV-LA-66 virus was found to possess 4580 nucleotides. Like other known L-A viruses, SpV-LA-66 genome features two overlapping open reading frames, which encode capsid protein Gag and RNA dependent RNA polymerase Gag-pol, formed by ribosomal frameshift. All features inherent for L-A viruses, such as conservative frameshift region, packing and replication signals, and catalytic histidine residue required for cap-snatching were present in the SpV-LA-66 genome sequence. Tentative ORFs coding for the Gag-pol and Gag proteins were compared with corresponding fragments of *S. cerevisiae* and *S. paradoxus* dsRNA sequences. At the nucleotide level, all entries display 74 to 92% identity (Figure 1).

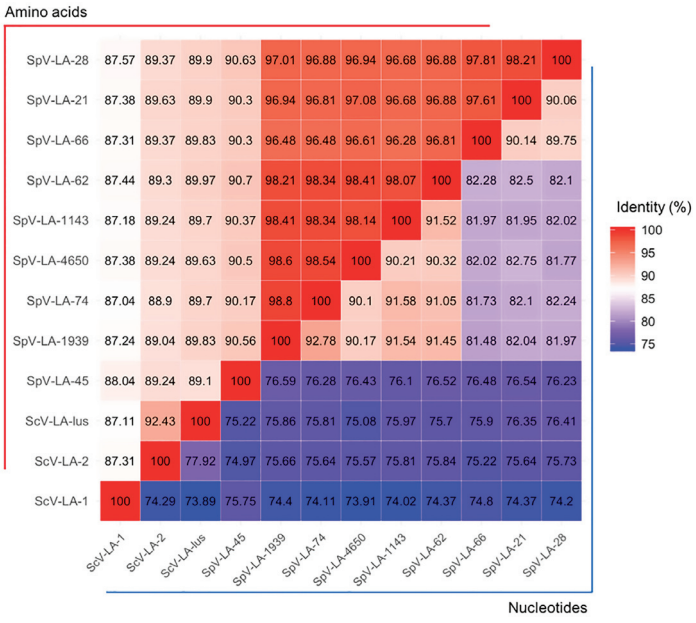

Figure 1. The similarities of *S. cerevisiae* and *S. paradoxus* dsRNA L-A virus-encoded Gag-pol proteins. ORFs coding for the Gag-pol proteins were compared with corresponding fragments of *S. cerevisiae* and *S. paradoxus* dsRNA sequences, namely: GenBank entry J04692 for the ScV-LA-1 virus, KC677754 for ScV-LA-2, and JN819511 for ScV-LA-lus, KU845301 for SpV-LA-28 (formerly attributed to *S. cerevisiae*), KY489962 for SpV-LA-21, KY489963 for SpV-LA-45, KY489964 for SpV-LA-74, KY489965 for SpV-LA-4650, KY489966 for SpV-LA-1939, KY489967 for SpV-LA-1143, KY489968 for SpV-LA-62. Identity at nucleotide level is represented in the lower right triangle, amino acid level–in the upper left triangle, framed by corresponding blue and red lines.

Similarity at the amino acid level is higher: coat proteins (Gag) are 88 to 99% homologous (Figure S4), while RNA polymerases (Gag-pol) are 87 to 98% homologous (Figure 1). Proteins originating from SpV-LA-66, SpV-LA-28 (initially reported as ScV-LA-28, origin recently updated by [6]) and SpV-LA-21 are the most closely related and comprise a separate cluster in relation to other L-A viruses (Figure 2). The remaining L-A viruses from *S. paradoxus* comprise another cluster. Altogether, *S. paradoxus* L-A viruses are significantly more homogenous than those from *S. cerevisiae*.

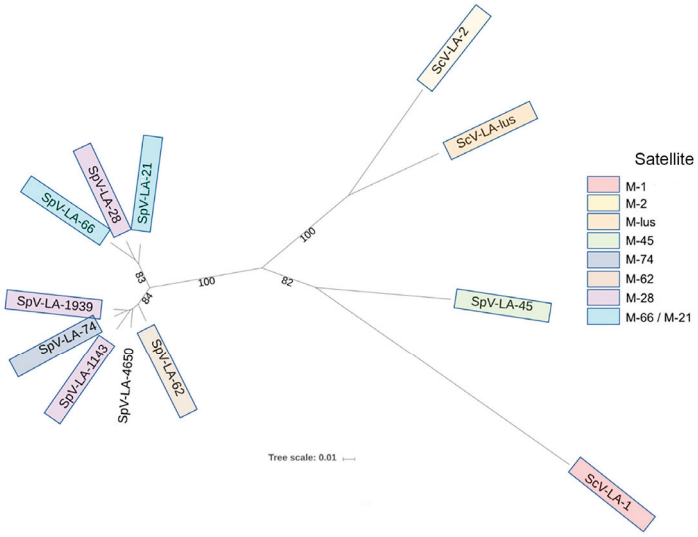

Figure 2. The phylogenetic tree of dsRNA-encoded Gag-pol proteins from *S. cerevisiae* and *S. paradoxus* yeasts.

In the same AML-15-66 strain, we discovered and cloned a 1553 bp long M dsRNA, named as SpV-M66. Sequence analysis of M66 satellite shows 86% identity in nucleotides to *S. paradoxus* SpV-M21 virus (GenBank MF358732) (Figure S5). The SpV-M66 genome consists of 5′-end 4 bp non-translating region, single ORF of 1,038 bp, which has 92% aa identity to K21 killer preprotoxin (GenBank ATN38270), about 60 bp polyA region and 330 bp non-translating region located at 3′-end (Figure S6). We expressed the complete ORF sequence from SpV-M66 in *S. cerevisiae* BY4741 strain and confirmed that the encoded protein confers the host with the killer activity (Figure S2B). Sequence analysis of preprotoxin revealed three potential recognition sites for Kex2 protease ending at amino acids Arg59, Arg181, and Arg239 (Figure 3, Figure S5); prediction of disulfide bridge formation sites remains ambiguous. Up to five putative protein N-glycosylation sites were found in the sequence of the K66 protein, four of them overlapping with those for K21. The amino acid at 141 position has the highest probability to be modified. A hydrophobicity profile reveals three transmembrane domains (from 21 to 42 aa, 62 to 85 aa, and 97 to 119 aa) in the K66 protein (Figure 3). In the C-proximal part of K66 precursor, a conservative Pfam family domain DUF5341 of presumably unknown function has been identified.

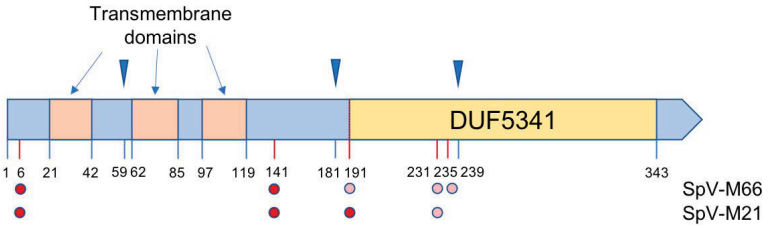

Figure 3. Features of the protein coded by SpV-M66. Blue triangles above the picture mark Kex2 sites. Predicted transmembrane domains and DUF5341 conservative Pfam family domain are marked within the picture. Dots below the picture mark putative glycosylation sites for K66 and K21 proteins, color intensity corresponds to the value of reliability index of the given position. Feature-linked positions of amino acids are indicated.

3.3. Effect of pH and Temperature on the Action of the S. Paradoxus K66 Toxin

The activity of partially purified *S. paradoxus* K66 viral protein was assayed on a lawn of *S. cerevisiae* strain α'1 with adjusted pH value from 3.2 to 6. K66 toxin exhibits killing activity in a narrow pH range between 3.6 and 5.2, with an activity peak at pH 4.4–4.8 (Figure 4A).

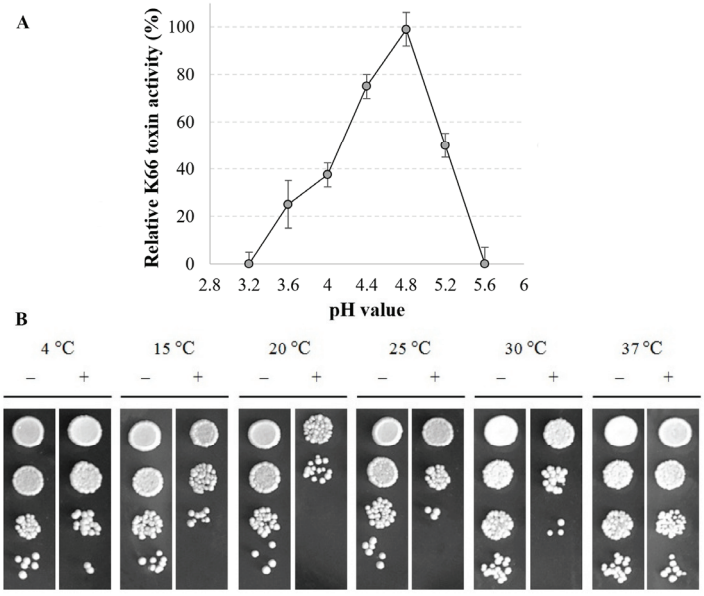

Figure 4. Impact of pH and temperature on K66 toxin functionality. (**A**) Sensitive *S. cerevisiae* strain a'1 was seeded into MBA medium (2×10^6 cells/plate, adjusted to pH values between 3.2 and 6) and 100 µL of the concentrated K66 toxin poured into 10 mm wide wells, cut in the agar layer. Plates were incubated for 2 days at 25 °C, non-growth zones around the wells measured and expressed as mean of three independent experiments in percent ± SD. (**B**) Sensitive yeast a'1 cells (5×10^5 cells) were mixed with 500 µL of 100-fold concentrated K66 toxin or the same volume of heat-inactivated K66 toxin and incubated for 24 h at different temperatures. Yeast cells were then serially diluted and spotted onto YPD-agar plates following for 2 days incubation at 25 °C.

More acidic pH values of 4.0–3.6 result in a reduction of toxin activity up to 38% and 25% respectively, and more basic than optimal pH 5.2 results in about 50% of toxin activity remained (Figure 4A). By analyzing yeast cells that survived the treatment by K66 protein at different

temperatures (from 4 °C to 37 °C), we found that extracted viral protein is active at temperatures between 15 °C and 30 °C, with optimal temperature of 20 °C (Figure 4B).

3.4. Genetic Factors Modulating the Functionality of the Viral K66 Toxin

To determine the genetic factors important for the action of *S. paradoxus* K66 toxin and involved in the formation of cellular resistance to the viral agent, we screened 526 *S. cerevisiae* single-gene deletion mutants, previously demonstrated to alter the functioning of *S. cerevisiae* K1, K2, or K28 toxins [29–31].

We identified 125 *S. cerevisiae* YKO library mutants demonstrating different degrees of phenotypic response to the K66 toxin (Table S1), of which 73 were more resistant than the control strain BY4741 and 52 more sensitive to the toxin treatment. We manually annotated groups of all identified mutants resistant or susceptible to K66 toxin. The largest groups contain genes associated with cell wall organization and biogenesis (15), membrane formation/secretion/transport (16), chromatin organization/gene expression (18), and translation (13) (Table S1, Figure 5). Deletions of 19 genes were common in all four screens performed and cause resistance/sensitivity alterations in the cells, not depending on the toxin type. In 13 mutants, different responses to K66 and some *S. cerevisiae* toxins were recorded (Table S1, Figure S2C). 85% of modulators identified in *S. paradoxus* K66 screen (106 gene products) were also identified in screens of *S. cerevisiae* K2 or K1 toxins.

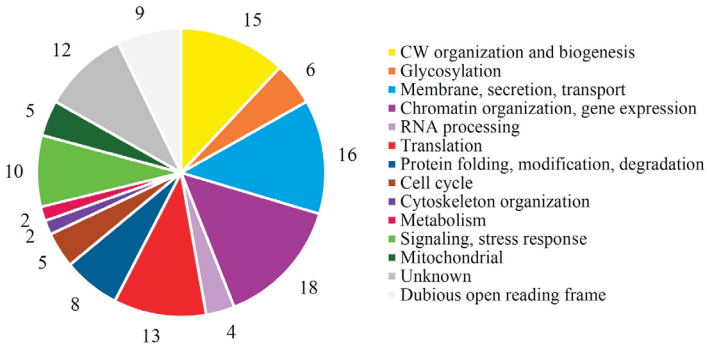

Figure 5. Distribution of cellular processes and cellular components involved in the action of K66 toxin. The number of genes identified in each class is indicated.

The GO-term analysis ("biological process") reveals a statistically significant enrichment in genes involved in cell wall organization and biogenesis (F.E. (fold enrichment) of 4.1, $p < 5.4 \times 10^{-6}$), response to osmotic stress (F.E. of 5.6, $p < 2.7 \times 10^{-3}$), and signaling pathway (F.E. of 3.3, $p < 1.3 \times 10^{-3}$) (Table S2).

Based on published high-throughput datasets, we built a protein-interconnection network and documented that the majority of all identified genetic factors were a part of one main functional cluster, at medium confidence level (0.4) (Figure 6). Most of the observed proteins are involved in stress response and signaling processes, cell wall organization and biogenesis, belong to ribosomal components or translation machinery, and are connected to membranes or protein transport.

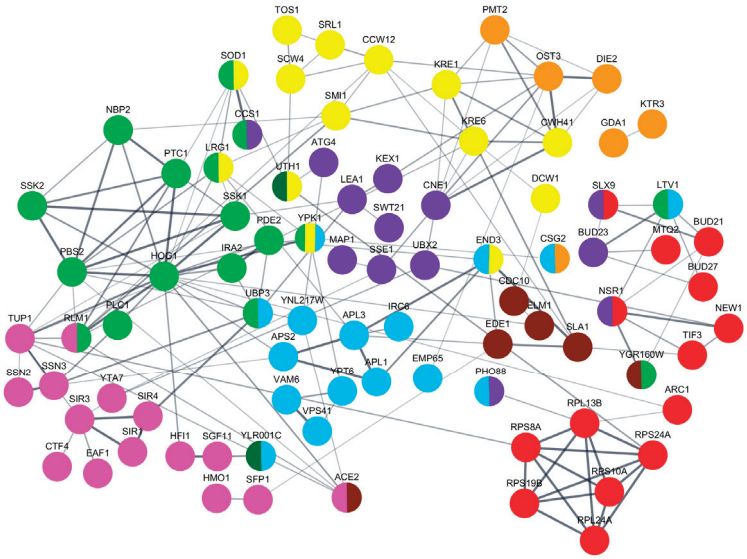

Figure 6. Interconnections of gene products involved in the modulation of susceptibility to K66 toxin. An integrated functional interaction network is obtained from STRING database. Subnetworks of proteins associated with ribosomes/translation (red), signaling and stress response (green), chromatin organization and gene expression (purple), glycosylation (orange), CW organization/biogenesis (yellow), cell cycle (brown), membrane and transport (light blue), RNA and protein modification (dark blue), and mitochondrial (dark green) are represented.

3.5. Targeting of the Viral K66 Killer Protein to the Cell Wall

To correlate the K66 toxin binding with the cell wall composition, we investigated the binding properties of the viral protein to mutant cells with altered levels of β-1,3 and β-1,6 glucans [21,29]. The level of glucan content was based on that reported in [21,29]. During this study, we determined that mutants with decreased levels of β-1,6 glucans Δ*aim26*, Δ*smi1*, and Δ*kre1* bind from 35% to 42% less of the K66 toxin molecules than control cells (BY4741) (Figure 7A). The killing activity of K66 is independent of β-1,3 glucan concentration in the cell wall (β-1,3 glucan amount in Δ*aim26* is as in *wt*, Δ*smi1*—50% decreased and in Δ*kre1*—10% increased). When mutant cells have increased amount of β-1,6-linkages at the cell wall, as in Δ*bud27*, Δ*map1*, and Δ*end3* mutants, K66 binding is boosted at about 30% over the control cells level. The defects in cell wall structure resulted in a major impact on the efficiency of K66 toxin binding to the cells, as in the case of K2 and K1 killer toxins [21,29,31,60]. We observed a good correlation between the genes whose deletion led to decreased K66 toxin binding and increased resistance to the toxin. Similarly, genes whose deletion led to increased toxin binding correlated with increased sensitivity towards the K66 toxin (Figure 7B).

To confirm the in vivo data on the importance of β-1,6 glucans as a binding target of K66, the ability of toxin to directly complex the polysaccharides bearing different glucan linkages was evaluated. After incubation with either laminarin (consisting of β-1,3 and β-1,6 linkages), pustulan (β-1,6 linkage), pullulan (α-1,4 and α-1,6 linkages), or chitin (β-1,4 linkage), residual toxin activity was tested by well test on the sensitive *S. cerevisiae* strain α'1 (Figure 7C). Unbound toxin forms clear lysis zones around the well. The competitive inhibition of the action of viral protein demonstrated that the β-1,6-glucan exclusively present in pustulan provides binding sites for the K66 toxin. K66 toxin activity was completely abolished by pustulan only.

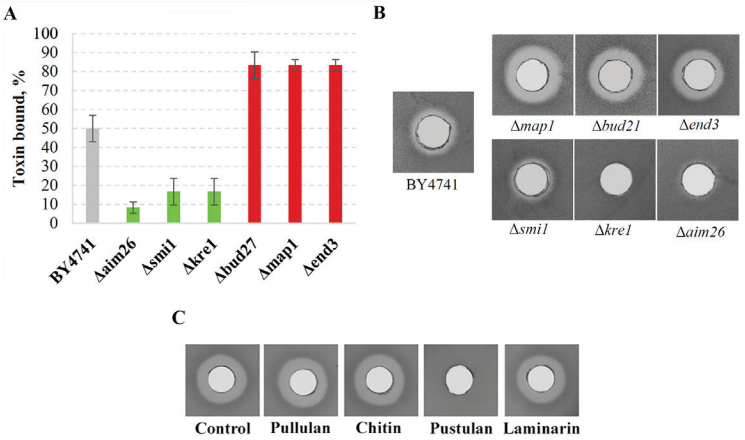

Figure 7. K66 toxin binding to yeast mutants with altered levels of β-glucans and different polysaccharides. (**A**) Cells of different yeast mutants (2×10^6 each) were incubated with 500 µL of concentrated K66 toxin, the remaining toxin activity was measured by the well assay. After incubation with the indicated yeast strain, the unbound K66 toxin is able to kill sensitive tester strain α'1, seeded in the MBA plates. The size of the formed lysis zones was converted to the relative toxin activity and subtracted from the total activity to calculate the binding efficiency. The data are averages ± standard deviations (SD) (n = 3). (**B**) Responses of deletion strains to the action of K66 toxin were measured in the well assay using BY4741 strain in a lawn. (**C**) Nine milligrams of each polysaccharide (chitin, laminarin, pullulan, or pustulan) in 100 µL of concentrated K66 toxin preparation was incubated for 1 h at 25 °C and residual toxin activity was analyzed in the well assay using sensitive α'1 strain in the lawn.

None of the other polysaccharides tested exhibited K66 protein binding effects, forming lysis zones equal to that of control sample without polysaccharide added. The in vitro and in vivo approaches used here to access the binding specificity suggest that type β-1,6-glucans can play the role of primary cell surface receptor for the K66 toxin.

4. Discussion

Our work for the first time provides deep insight into the composition and functioning of the *S. paradoxus* viral killer system. The study stemmed from the comprehensive analysis of the viral sequences, analyzed the genetic factors of the host and targets of the virus-encoded killer toxin.

The high similarity between *S. paradoxus* L-A viruses has been reported recently [6]. This observation is in line with the high relatedness observed previously between *S. paradoxus* dsRNA virus-possessing strains based on the distance matrix of PCR profiles with the microsatellite primer $(GTG)_5$ [61]. Here, we demonstrate that L-A viruses from *S. paradoxus* discovered so far are significantly more homogenous than those from *S. cerevisiae.* Within *S. paradoxus* L-A viruses, two clades can be confidently separated: LA-28 type, including SpV-LA-66, SpV-LA-21 and SpV-LA-28, and the rest of *S. paradoxus* L-A viruses, except for the SpV-LA-45.

No sequence homology between different *Saccharomyces sensu stricto* yeast dsRNA M viruses is detected, except for the high similarity of *S. paradoxus* SpV-M66 and SpV-M21 viruses. Even though SpV-LA-66 and SpV-LA-28 are highly related, no homology between SpV-M66 and SpV-M28 in nucleotide or in amino acid level can be documented. Genome organization of SpV-M66 resembles all so far described *S. cerevisiae* and *S. paradoxus* dsRNA M viruses [19,28,62]. The coding region is located at the 5′ terminus, followed by an A-rich sequence and non-coding region with secondary stem-loop structure important for encapsidation at the 3′ terminus. The ORF encoding for a K66 preprotoxin features three potential recognition sites of Kex2 protease, acting in the late Golgi compartment.

Thus, the maturation process of K66 toxin may proceed by two alternative scenarios: first, by removing pre-pro-sequence and potential γ-peptide (from 182 till 239 aa) and forming a disulfide-bonded α/β heterodimer. A similar structural organization is typical for almost all known *S. cerevisiae* killer toxins, except for the K2 killer protein lacking γ-subunit [15,62]. In the second scenario, the Kex2 protease may not cleave at the position 239 and, similar to the K2 killer protein, γ-peptide is not released. In this case, the β subunit will start from the amino acid position 182, retaining the integral DUF5341 domain. Future studies are needed to fully understand the role of DUF5341 in the killing phenotype of K66, as this domain is found in numerous proteins of *Ascomycota*, including the N-terminal part of KHS killer toxin. Of special interest is the presence of the DUF5341 domain identified within C-terminal part of the K2 toxin [63]. However, the patterns of DUF5341 sequence similarity to K66 and K2 do not match, making direct sequence comparison not possible. Therefore, K66 and K2 toxins appear to share the same DUF5341 domain core. Three transmembrane helixes detected in the K66 predict this protein to form an ion channel after reaching the plasma membrane, similar to *S. cerevisiae* K2 and K1 toxins [15,25]. The possibility of the K66 toxin acting in monomeric form could not be excluded, as the probability level of disulfide bond prediction is rather low. ER retention motif, typical for K28 protein and essential for its activity [37] was not found in the structure of K66, separating the organization and therefore the modes of action of these toxins further on.

BLAST search revealed K66 homologues in different yeast strains. We found that all ORFs coding homologues of K66 in *S. cerevisiae* are at the telomeric region of chromosome 5 and code for YER187W-like proteins. In some strains, YER187W ORF contains an in-frame stop codon (for example, in *S. cerevisiae* BY4741). YER187W ORF neighboring YER188W is homologous to Kbarr-1 killer toxin (GenBank KT429819), encoded by *Torulaspora delbrueckii* dsRNA Mbarr-1 killer virus. An identity of some regions of *T. delbrueckii* Mbarr-1 genome with the putative replication and packaging signals of most of the M-virus RNAs was observed, suggesting the evolutionary relationship [7]. Several chromosomal ORFs with homology to *S. cerevisiae* Klus, K1, or K2 preprotoxins have been observed in yeasts before, suggesting that the M virus might have originated from the host messenger RNAs, been encapsidated and replicated by the L-A virus-encoded RNA polymerase after acquiring sequences needed for both events, probably from the genome of the L-A virus itself [14]. In addition, it has been demonstrated that genes of dsRNA viruses from *Totiviridae* and *Partitiviridae* have widespread homologues in the nuclear genomes of eukaryotic organisms, such as plants, arthropods, fungi, nematodes, and protozoa, suggesting that viral genes might have been transferred horizontally from viral to eukaryotic genomes [8,64].

Despite the striking similarity in virus genome organization and toxin maturation, the modes of action of the viral toxins are clearly distinct [15]. There are differences between toxins with respect to killing, interaction with the host cells, and immunity formation mechanisms. Mycovirus-originated killer proteins are usually active between pH 4.0 and 5.4 and at a temperature below 30 °C [1,65]. The favorable conditions for the establishment of *Saccharomyces* killer species have been found on fruits where the pH is moderately low [49,66,67], growing best in a natural environment with optimal temperature range of 20 °C to 30 °C [57]. These conditions strongly correlate with activity and stability profile of most secreted killer toxins and are in line with our data pointing to the optimal activity of *S. paradoxus* K66 toxin at a temperature of 20 °C at pH about 4.8. The *S. cerevisiae* killer toxins have narrow target range, inhibiting only strains or species within the same genus [68], except for the Klus toxin, which executes the activity against broader spectrum of yeast [28]. *S. paradoxus* killer strain AML-15-66 exhibits similar features towards the majority of *S. cerevisiae* yeast, demonstrating killing activity against *S. cerevisiae* cells but being not active against other yeast genera tested. The AML-15-66 strain possessed the lowest activity against *S. cerevisiae* K2 toxin-producing strain M437, suggesting relation of both toxins, while K28-bearing *S. cerevisiae* strain M300 was killed the most efficiently among all killer strains tested. At the same time, AML-15-66 was sensitive to the action of *S. cerevisiae* K2 toxin, probably due to the high activity of this toxin [69,70], and completely resistant to other known killer types of *S. cerevisiae* K1, K28, and Klus.

The genetic screen performed on *S. cerevisiae* single-gene deletion strains revealed 125 gene products important for the functioning of the *S. paradoxus* K66 toxin and involved in the target cell susceptibility. Previous yeast genome-wide screens performed with all known dsRNA virus-originated *S. cerevisiae* killer toxins revealed a 753 gene set, contributing to the functioning of viral agents: 268 for the K1, 332 for the K2, and 365 for the K28 [29–31]. Page and colleagues [29] determined that the resistance to the *S. cerevisiae* K1 toxin is mostly conferred by gene products linked to the synthesis of cell wall components, secretion pathway, and cell surface signal transduction. Mutant cells with an increased amount of β-1,6-glucans in the cell wall, those unable to grow at high osmolarity, and bearing compromised stress response pathways, exhibited increased sensitivity to the K1 toxin [29]. The K2 toxin executed a similar mechanism of action as K1; therefore, changes in the cell wall structure and proper functioning of mitochondria remain crucial for the killing phenotype. The cells defective in HOG and CWI signaling pathways, with affected maintenance of pH and ion homeostasis, demonstrated hypersensitivity to the K2 toxin [31]. Even for the action of the different-by-mechanism K28 toxin, interrupted cell wall biogenesis process and lipid organization led to increased resistance, while most genes related to hypersensitivity to K28 toxin are those involved in stress-activated signaling and protein degradation [30]. In this study, based on manual annotation, the largest groups of K66 modulators have been identified as those connected to cell wall organization and biogenesis, membrane formation and transport, chromatin organization, and gene expression. GO analysis and protein interconnection networks highlighted the importance of cell wall structure for the functioning of *S. paradoxus* K66 toxin and allowed us to speculate that K66 acts via the disruption of ion homeostasis, since genes involved in regulation of osmotic stress response were highly represented in the screen. Importantly, 85% of modulators identified in the *S. paradoxus* K66 screen (106 gene products) were involved in the functioning of *S. cerevisiae* K1 or K2 toxins as well, pointing to a high similarity of their action. At the same time, only 19 unique modulators common for both *S. paradoxus* K66 and *S. cerevisiae* K28 were found. Functions of several of those gene products are connected to endocytosis and, therefore, remain unclear in the context of *S. paradoxus* K66 toxin predicted function. Until the relevant experiments are carried out, the possibility that the K66 toxin possesses yet another unique mode of the action cannot be excluded.

Yeast cell wall is a primary target for cytotoxic activity of most mycotoxins, and different components of the cell wall could play the role of receptors [71]. Mutants with altered cell wall biogenesis process demonstrated the most resistant phenotype to the action of *S. paradoxus* viral protein K66. This is expected, because the toxin primarily interacts with the cell wall and components of the plasma membrane. By investigating the efficiency of the K66 toxin binding to *S. cerevisiae* mutant cells with altered levels of β-1,3 and β-1,6 glucans in vivo and performing competition experiments with the polysaccharides, bearing different glucan linkages, we observed a good correlation between the level of β-1,6 glucans and binding ability. This highlights the potential of β-1,6 glucans to act as primary targets on the cell wall. The similar genetic factors related to cell wall organization and biogenesis processes modulate the functioning of *S. cerevisiae* K1 and K2 toxins [29,31]. Even more, β-1,6-glucan was originally proposed to be a cell wall receptor for *S. cerevisiae* K1 and K2 toxins [21,22]. Therefore, we propose that β-1,6 glucans could play the role of primary receptors of *S. paradoxus* K66 toxin, further relating its action to that of *S. cerevisiae* K1 and K2 toxins.

Our finding that the majority of L-A viruses from *S. paradoxus* are significantly more homogenous than those from *S. cerevisiae*, might substantiate an important evolutionary crossroad between wild *S. paradoxus* and domesticated *S. cerevisiae* yeast. It remains to be discovered the reason or driving force for the extreme level of homology of L-A viruses from *S. paradoxus*, if any: genomes of *S. paradoxus* were shown to be at least several times more diverse than *S. cerevisiae* genomes [72]. One can envision the evolutionary pressure from the satellite M dsRNA virus; however, many of *S. paradoxus* killer systems are rather diverse in terms of toxin specificity [9,19]. At the same time, *S. paradoxus* SpV-LA-45 is more similar to *S. cerevisiae* counterparts, than to L-As from *S. paradoxus*, extending further limits of L-A virus variability in host species. *S. cerevisiae* features at least four killer systems, maintained by

corresponding L-A variants [15,37], therefore highlighting a route for possible diversification of L-A viruses within species. Here, fitness pressure determined by the domestication of *S. cerevisiae* should not be overlooked.

Ample specific combinations of *S. paradoxus* killer systems cast doubts on homogeneity within killer-helper duos of *S. paradoxus*. The specificity paradigm in maintenance of M satellite virus by corresponding L-A virus only has been challenged by the *S. cerevisiae* K2 killer system, where two distinct L-A type viruses were found to support the ScV-M2 virus in wild strains [14,28]. This paradigm is further refused by the *S. paradoxus* K66 killer system, consisting of the previously unreported combination of LA-28 type SpV-LA-66 virus and M1/M2 type SpV-M66 virus. To the best of our knowledge, this is the first report of LA-28 type helper virus maintaining other than the M28 type satellite virus in a wild-type strain. The presented uniqueness of the K66 killer system raises questions of the true limits and the factors behind helper-satellite compatibility and distribution. Genetic background is essential for the consolidating of any virus in a cell; the diversity of yeast killer systems should obey the cellular context, unique for each and every host species. Results from this study extend our knowledge on the *Totiviridae* viruses in *Saccharomyces sensu stricto* yeasts and functioning of killer systems, urging exploration of new horizons of their diversity.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4915/10/10/564/s1. Figure S1: Identification of *S. paradoxus* AML-15-66 strain, Figure S2: Characterization of killing and immunity features of K66 toxin-producing *S. paradoxus* strain, Figure S3: Electrophoretic analysis of dsRNAs extracted from different killer yeast, Figure S4: The similarities of *S. cerevisiae* and *S. paradoxus* dsRNA L-A virus-encoded Gag proteins, Figure S5: Sequence alignment of SpV-M66 and SpV-M21 genomes, Figure S6: Nucleotide sequence of the SpV-M66 genome and amino acid sequence of the putative ORF of K66 toxin, Table S1: Mutant strains with an altered *S. paradoxus* K66 killer toxin phenotype, Table S2: GO terms in biological process for genes involved in the functioning of *S. paradoxus* K66 toxin.

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PAPER V

A quick and reliable method for genome-wide host factor screening of *Saccharomyces cerevisiae* killer toxins

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A quick and reliable method for genome-wide host factor screening of *Saccharomyces cerevisiae* killer toxins

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Numerous yeasts produce toxic compounds to fight the competitors. Such compounds include small molecules (like antibiotics), antibiotic peptides, and also larger proteins, including killer toxins. Their ability to affect the cell depends on the host factors modulating the killing activity. Here we describe a robotics-based method to advance the genome-wide screening for the host factors affecting sensitivity of budding yeast to the killer toxins using the K2 system as the model. We demonstrate that arraying the mutant library on the agar plates containing the K2 killer toxin-producing strain and/or purified toxin ("survival" assay) increases the sensitivity and speed of the screen and decreases the costs compared to the traditional "killer" assay. We show the applicability of a new screening method of searching for the host factors using a killer strain isolated from agricultural plant environment. In addition, the "survival" assay allows identification of previously undetected factors that could be the "missing links" in the pathways of toxin-induced cellular responses.

Keywords: *Saccharomyces cerevisiae*, K2 toxin, genome-wide screen, pinning robot

INTRODUCTION

A large variety of yeast secrete different classes of compounds aimed at preventing the growth of the competitors (Meinhardt, Klassen, 2009). These compounds include small molecules, antibiotic peptides, and even proteins. Killer toxins are the fungicidal proteins produced by different genera like *Williopsis*, *Pichia*, *Kluyveromyces*, and *Saccharomyces*. Nowadays, a particular interest in yeast killer strains and corresponding secreted toxins has emerged. Both of those have found application in industry (food protection from spoiling, wine production), agriculture (phytopathogen control), or medicine (creation of new generation vaccines and antibiotics, development of antifungal immunotherapies) (Goretti et al., 2009; Rodriguez-Cousino et al., 2011; Hatoum et al., 2012; Magliani et al., 2012; Maqueda et al., 2012). It is only rather recently that significant progress in the identification of killing principles and strategies, mechanisms of immunity, and approaches for the practical application of such proteins has been made.

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Several genome-wide screens searching for the factors altering sensitivity to killer proteins have been performed. Irrespective of the origin of the toxin, *Saccharomyces cerevisiae* libraries of non-essential gene mutants are usually used. They are sometimes complemented by a set of conditional alleles of essential genes. In this way genome-wide screens involving the K1 and K28 killer toxins from *S. cerevisiae*, HM-1 toxin from *Williopsis saturnus*, γ-toxin from *Kluyveromyces lactis*, and also the killer peptide derived from *Pichia anomala* were carried out (Pagé et al., 2003; Conti et al., 2008; Huang et al., 2008; Carroll et al., 2009; Miyamoto et al., 2011).

Performing the experiments with the *W. saturnus, K. lactis*, and *P. anomala* toxins, colonies from the *S. cerevisiae* libraries were manually arrayed on the agar plates, and the growth of mutants was further tested either in solid or liquid media (Conti et al., 2008; Huang et al., 2008; Miyamoto et al., 2011). In the case of K1 and K28 toxins, a different approach was used. Here each mutant strain was incorporated into a separate agar plate and the resistance to the toxins was scored by spotting on top the purified toxin or the toxin-producing strain with subsequent measuring of the inhibition zones ("killer" assay) (Pagé et al., 2003; Carroll et al., 2009).

In this work we combined two above-mentioned methods and developed both fast and reliable assay to screen for the *S. cerevisiae* mutants with altered sensitivity to K2 killer toxin. Furthermore, we demonstrated the sensitivity of the robotic system, which allowed us to identify additional mutants missed in the traditional "killer" assay. In addition, the applicability of the "survival" assay for searching for the host factors was demonstrated using *S. cerevisiae* killer toxin Kz isolated from the agricultural plant environment.

MATERIALS AND METHODS

Robot-aided screening of the mutants ("survival" assay)

About 4750 single nonessential gene deletion mutants consisting of BY4741 (*MAT*a *his3Δ1 leuΔ2 metΔ0 uraΔ3*) derivatives were arrayed on YPD+G418 agar plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar, 200 μg mL^{-1} G418) in the 96 colony format. The strains to be tested were then transferred either onto YPD agar plates (Plus Plates, Singer Instruments, Roadwater, UK), or into the liquid YPD medium in 96-well plates (Cellstar, cat. no. 655180, Greiner Bio-one, Germany), 150 μL/well, and grown for one day (18–24 h) at 30 °C. The agar YPD-grown cells were then deposed onto plates containing 40 mL of MB-agar medium (0.5% yeast extract, 0.5% peptone, 2% dextrose, adjusted to pH 4 with 75 mM phosphate-citrate buffer supplemented with 0.002% methylene blue dye) mixed with either 2×10^5 or 2×10^6 cells of overnight pre-grown K2 toxin-producing strain M437 (wt, HM/HM [kil-K2]) (Gulbiniene et al., 2004). A similar strategy was used for screening of *S. cerevisiae* Kz toxin-producing strain isolated from the agricultural environment.

In parallel, liquid YPD-grown cells were transferred onto MB-agar plates containing either 300 U or 600 U (as determined in Gulbiniene et al., 2004) of partially purified K2 toxin (Lebionka et al., 2002). All plates were incubated for two days (42–48 h) at 25 °C. The size and the colour of the colonies grown on the agar supplemented with M437 cells were evaluated visually, and pictures were taken using a PowerShot SX220 HS digital camera (Canon, Tokyo, Japan). The respective blue (corresponding to increased sensitivity to K2 toxin) and white (increased resistance) colonies, differing in colour from their neighbours and those in the control plate (no toxin), were selected as candidates for altered sensitivity to K2 toxin. The pictures of colonies grown on the agar plates containing purified K2 toxin were taken using either a Genosmart transilluminator (VWR International) or a Pathway™ 435 automated microscope (BD Biosciences, USA). Reduced in size or non-growing on the MB-agar with 300 U of K2 toxin colonies were selected as hypersensitive mutants, while the colonies growing at 600 U of toxin (no growth for wild-type control) were selected as the resistant strains.

Verification of the phenotypes of the selected mutants ("killer" assay)

About 2×10^6 cells of respective strain, selected using the "survival" assay, were grown overnight in liquid YPD, mixed with 10 mL of melted and pre-cooled to 45 °C MB-agar, and then poured into a standard 50-mm-diameter plastic Petri dish. Afterwards the inhibition zones were established either by (1) patching on the top of the agar the K2 toxin-producing strain M437, (2) deposing 5 µL (0.5 U) of purified K2 toxin, or (3) filling the 10-mm-wide cut into the agar wells with 100 µL (10 U) of K2 ("well" assay). The plates were incubated for two days at 25 °C, and non-growth zones of mutants were established. Identical approaches of the "killer" assay were used for the verification of deletion mutants detected in the "survival" assay and involved in resistance formation to Kz toxin. The summary of the screening procedure in both "killer" and "survival" assays is represented in Fig. 1.

RESULTS AND DISCUSSION

Screening procedure

Dominating in the vineyard-winery ecosystems, S. cerevisiae K2 killer yeasts produce toxin that is lethal to sensitive or other type of the killer toxins producing yeast strains (Gulbiniene et al., 2004). The ability to kill or to protect from the toxic action depends on the general features of the toxin and is influenced by the cellular self-defence mechanisms (Meinhardt, Klassen, 2009).

In order to identify encoded factors of budding yeast that mediate sensitivity to K2 toxin, we first established the "survival" assay using

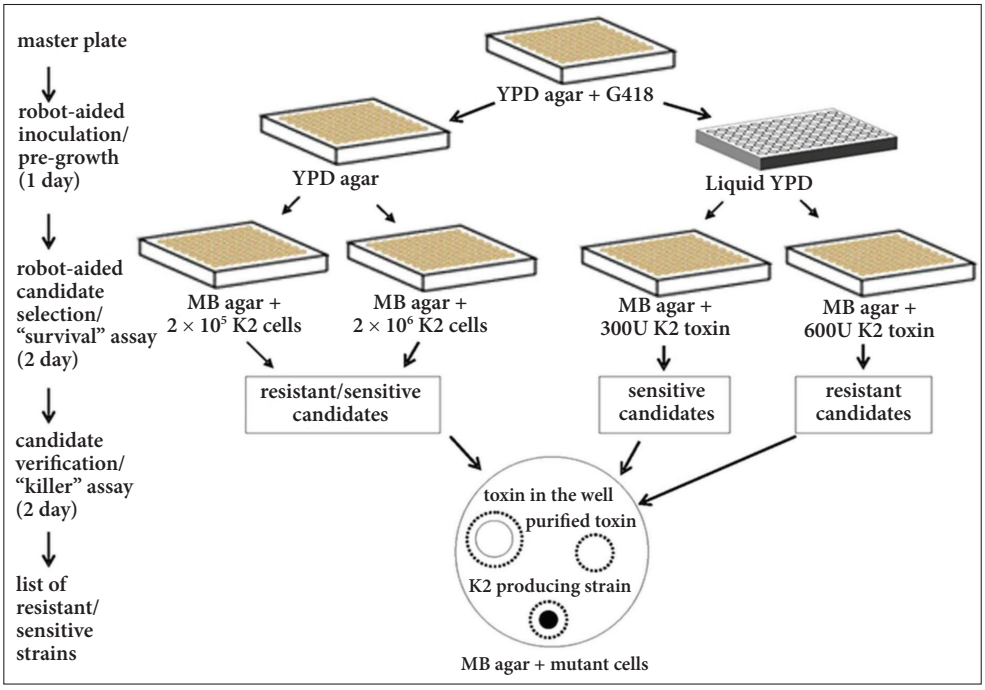

Fig. 1. YPD+G418. The cells were transferred either onto YPD-agar or into YPD liquid and grown overnight. Then the cells from YPD-agar were replicated onto the MB-agar, seeded with an overlay of the K2 toxin producing strain M437; liquid YPD pre-grown cells were transferred onto the MB-agar containing the K2 toxin. Respective resistant and sensitive mutants were selected. Verification of candidates was performed using the "killer" assay by deposing either purified K2 toxin into the agar-cut wells, or spotting either the toxin or K2 toxin-producing strain on top of the agar

the RoToR pinning robot (Singer Instruments, Roadwater, UK), and then performed the "killer" assay to verify the candidate strains. Figure 2 depicts the principal scheme of the selection procedure, from the robotic screen of the yeast libraries to the verification of candidates and, finally, obtaining the hits.

Selection of the factors affecting K2 toxin resistance

The respective blue (corresponding to increased sensitivity to K2 toxin) and white (increased resistance) colonies, differing in colour from their neighbours and those in the control plate (no toxin) (Fig. 3A) were selected as candidates for altered sensitivity to K2 toxin. Reduced in size or non-growing on MB-agar with 300 U of K2 toxin colonies (compared with the control plate) were selected as hypersensitive mutants (Fig. 3B), while colonies, growing at 600 U of toxin (no growth for wild-type control) were selected as the resistant strains (Fig. 3C). Using such a "survival" assay with the K2 killer producing strain, we found 311 strains demonstrating marked (big white colonies) resistance and 52 strains demonstrating weak (small whitish colonies) resistance.

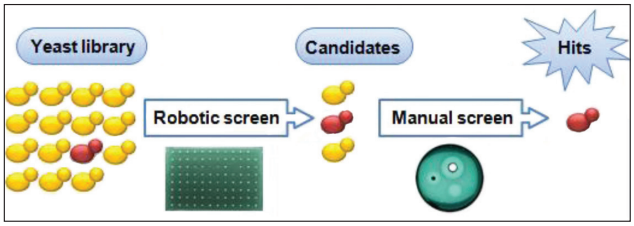

Fig. 2. The principal scheme for the selection of the candidate genes from the yeast mutant libraries

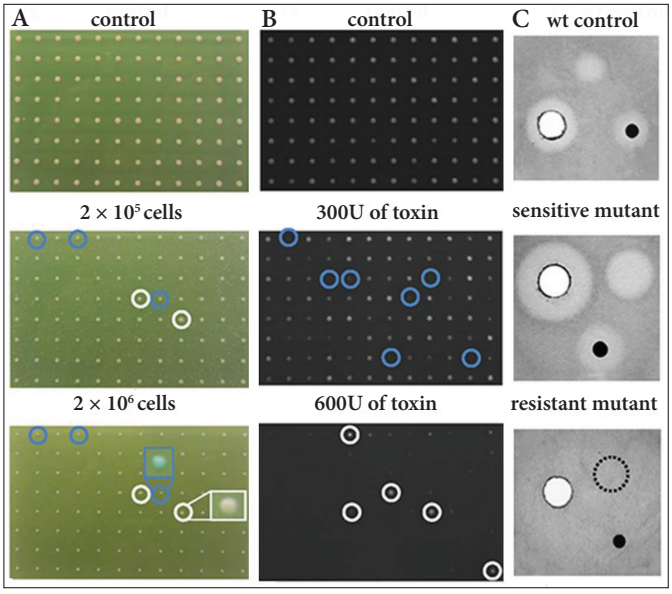

Fig. 3. Selection of *S. cerevisiae* mutants with altered sensitivity to K2 toxin. (A) Agar plates containing the K2 toxin-producing cells and the arrayed colonies of mutants. Resistant colonies are encircled in white, sensitive colonies – in blue. Images of typical sensitive and resistant colonies are magnified. (B) Agar plates containing purified K2 toxin inside and the arrayed colonies of mutants on top. The colonies are encircled as above. (C) Examples of the "killer" assay results for the wild type and extremely sensitive and resistant mutants

At the same time, 209 mutants exhibited marked (deep blue colonies), and 74 ones weak (blue colonies) hypersensitivity. When K2 toxin was used in a similar assay, 232 strains with marked (big colonies), and 61 strains with weak (small colonies) resistant phenotype were selected. Also, we discovered 287 strains with marked (traces of/no colonies) and 38 ones with weak (smaller than the wild-type colonies) hypersensitive phenotypes. These data are summarized in the "survival" assay part of Fig. 4. Some of the mutant strains show phenotypic differences from wt cells when K2 producing strain but not K2 toxin was used, and *vice versa*. For some other mutants such phenotypes were observed in both cases. In total, about 850 strains demonstrating reliable growth rate/colour differences from the parental strain in at least one of two approaches of the initial screening procedure were further tested using the "killer" assay.

Verification of K2 killer phenotypes using the "killer" assay

Verification and quantitative evaluation of K2 killer resistance phenotypes of the candidates selected from the "survival" assay was performed using 3 approaches ("killer" assays). Figure 3C shows the examples of the extremely sensitive and resistant mutants and corresponding wild-type control. Using this method, a list of 332 mutants, phenotypically different from the wild type strain BY4741, was established. About two-thirds of the selected mutants demonstrated either marked K2 resistance (<2 mm radius of non-growth zones in the "well" assay) or sensitivity (>3.5 mm radius of non-growth zones). The remaining one-third of the mutants showed weak resistance (2–2.5 mm of non-growth zones) or sensitivity (3–3.5 mm of non-growth zones), whereas the wild type strain demonstrated non-growth zones of about 2.5–3 mm. These data are summarized in the "killer" assay part of Fig. 4. The list and analysis of the aforesaid 332 mutants has been published (Servienė et al., 2012).

Surprisingly, some 520 of 850 candidates discovered in the "survival" assay were missing in the "killer" assay. This phenomenon can be attributed to the use of robotics resulting in higher sensitivity, and the variation in the growth rates of different mutants.

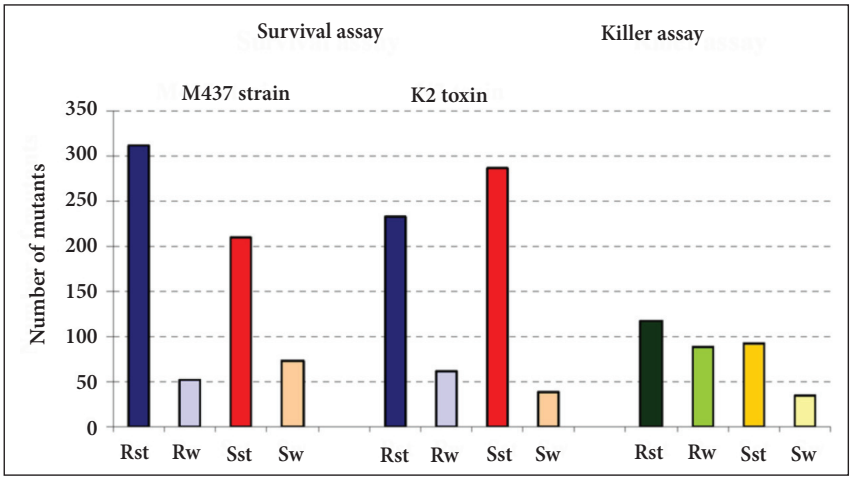

Fig. 4. Distribution of the resistant and hypersensitive mutants selected by the "survival" and "killer" assays. Deep blue denotes strong resistant (Rst), whereas light blue – weak resistant (Rw) mutants obtained using the "survival" assay. Red denotes strong (Sst), and pink – weak (Sw) hypersensitive mutants selected by the same approach. Strong resistant hits (Rst) from the "killer" assay are marked in deep green, while the weak ones (Rw) are marked in light green. The sensitive hits (Sst and Sw) from the same assay are marked in different shades of yellow, respectively

Advantages of the use of the pinning robot

Here we demonstrate the advantages of the use of the pinning robot for the selection of mutants with altered sensitivity to the K2 killer toxin. In comparison to the K1 and K28 killer toxin genome-wide screens where only the "killer" assays were used, fast and sensitive "survival" assay-based screen using either K2 toxin producing strain or the purified K2 toxin was first performed. We see several advantages of this method compared to traditional inhibition zone approach. First, it reduces the initial number of the candidates to be verified by the traditional "lysis zone" method about 5.5x (approx. 850 strains instead of 4750), thereby replacing tedious handwork and reducing the cost of the growth medium used as well as increasing reproducibility of the colony arraying. Secondly, the use of both the K2-toxin producing strain and the purified K2 toxin in parallel increases the sensitivity of screening and allows selection of the candidates possessing even a weak phenotype. We found that 85% of mutants demonstrating a strong phenotype, confirmed by the "killer" assay, could be picked using only a K2 toxin-based "survival" assay (Servienė et al., 2012). This demonstrates that in the majority of cases, such strategy of screening could be used for the selection of marked candidates. In contrary, for the detection of weak phenotype possessing candidates, both "survival" assay approaches should be used. Seventy-five percent of weak mutants confirmed by the "killer" assay demonstrated more pronounced phenotype in the K2-producing strain-based "survival" assay than the one using the K2 toxin (Servienė et al., 2012). The former assay is especially useful for testing of slowly growing mutants with altered physiology because of mild conditions achieved by slow but continuous action of the toxin produced by the killer cells. In addition, we demonstrated potential of survival assay-based screen for searching of host factors modulating sensitivity to *S. cerevisiae* killer toxin Kz isolated from agricultural plant environment. When such killer toxin producing strain was used as a selection agent, colonies differing from their neighbours in colour and those in the control plate (no toxin) were distinguished (Fig. 5).

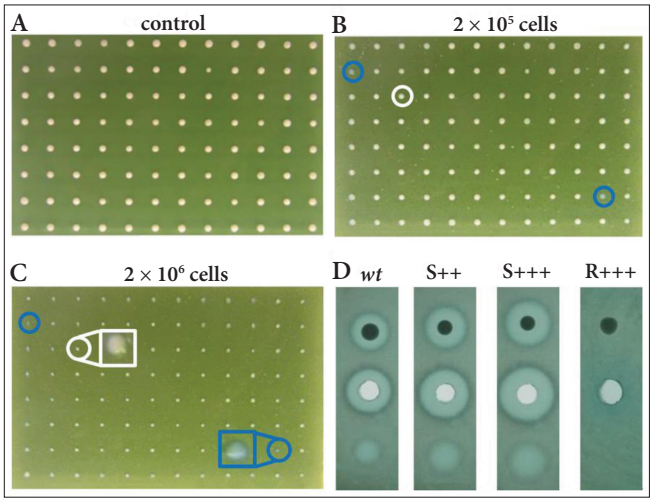

Fig. 5. Applicability of the "survival" assay for screening of the *S. cerevisiae* mutants with altered sensitivity to Kz toxin. (A) Nonessential gene deletion mutants deposited on MB-agar plates. (B, C) Agar plates containing the Kz toxin-producing cells (2×10^5 and 2×10^6 cells/plate) and the arrayed colonies of mutants. Resistant colonies are encircled in white, sensitive colonies – in blue (images of typical sensitive and resistant colonies are magnified). (D) Detected in the "survival" assay, sensitive (S++, S+++) and resistant (R+++) mutants were verified by the "killer" assay

The respective blue (corresponding to increased sensitivity to Kz toxin) and white (increased resistance) colonies were verified by the "killer" assay (Fig. 5D). We believe that the possibility to alternate harsh and mild test conditions could also be used for screening of the yeast essential gene libraries (e. g., yTHC, DAmP, Ts/Cs). Thirdly, the introduction of several independent testing methods (identification of resistant and hypersensitive strains by the use of the pinning robot and verification of the phenotype by applying the "killer" assay) instead of repeating the same experiment significantly increases the reliability of genome-wide screening. Even if the pinning robot is not available, the screening scheme could be used with affordable manual pinning tools.

CONCLUSIONS

In this work we developed a new method to screen the yeast gene deletion library for factors affecting toxin susceptibility and demonstrated its advantages. We showed that the majority of mutants could be selected using only the pinning robot (the "survival" assay). In addition, this method allows identification of mutants not detected by the traditional "killer" assay. Those factors could be "missing" links in the pathways of toxin-induced cellular responses that are currently under investigation.

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GREITAS IR PATIKIMAS ŠEIMININKO VEIKSNIŲ, MODULIUOJANČIŲ MIELIŲ ŽUDANČIŲ TOKSINŲ POVEIKĮ, ATRANKOS METODAS

Santrauka

Daugelis mielių rūšių gamina toksinus, skirtus kovai su konkurentais. Tokios medžiagos gali būti mažos molekulės (antibiotikai), peptidai ir didesni baltymai – žudantys toksinai. Jų poveikis ląstelei priklauso nuo šeimininko veiksnių, moduliuojančių žudymo lygį. Mes aprašėme roboto panaudojimu pagrįstą metodą, pagerinantį šeimininko veiksnių atranką iš *S. cerevisiae* mielių genomo, kai modeliu buvo pasirinktas K2 toksinas. Nustatyta, kad kamienų iš mielių mutantų bibliotekos auginimas ant agaro lėkštelių su K2 toksiną išskiriančiu kamienu ir / arba išgrynintu toksinu („išgyvenimo“ metodas) padidina atrankos greitį ir jautrumą bei sumažina savikainą, palyginti su tradiciniu „žudymo“ metodu. Pademonstruotas naujo patikros metodo pritaikymas šeimininko koduojamų veiksnių paieškai naudojant iš žemės ūkio augalų aplinkos išskirtą žudantį mielių kamieną. „Išgyvenimo“ metodas taip pat leidžia nustatyti anksčiau neišskirtus veiksnius, kurie gali būti toksino indukuoto ląstelės atsako „trūkstamos grandys“.

Raktažodžiai: *Saccharomyces cerevisiae*, K2 toksinas, atranka iš genomo, kolonijas pernešantis robotas

PAPER VI

Interaction of host factors in response to yeast K2 toxin stress – attractiveness for plant protection

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Interaction of host factors in response to yeast K2 toxin stress – attractiveness for plant protection

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Abstract

Killer toxin-producing yeasts are important in agriculture, as they may be used for the biological control of field and post-harvest bacterial and fungal diseases of plants. The foundation for the development and application of killer yeast as plant protection agents is understanding the mechanisms underlying killer toxin-conditioned biocontrol activity and the tolerance to toxin-driven stress. This study aimed to determine the interactions between genetic effectors of cell wall integrity (CWI) and high osmolarity glycerol (HOG) pathways under the stress conditions caused by *Saccharomyces cerevisiae* dsRNA-originated K2 toxin. Genetic interaction studies have used different measures of fitness including the relative growth rate and toxin susceptibility. During the research, 12 double deletion mutants were created by applying homologous recombination approach, and their growth parameters and response to toxin action were analysed. The majority of double gene deletion strains demonstrated insignificant (less than 30%) changes in growth rate compared to single gene mutants. Only elimination of *HOG1* gene in strains Δ*spt3* and Δ*spt8* resulted in significant increase of the growth rate reaching about 0.6 h^{-1}. K2 toxin sensitivity increased in most of the double mutants, independent of the involvement of gene products into the same or different signalling pathways thus pointing to weak interactions of gene products. *HOG1* mutation alters the phenotype (from resistant to sensitive) of mutants Δ*rlm1*, Δ*lrg1* and Δ*slm4* only and are epistatic to these CWI pathway effectors. In addition to the functional analysis, network of proteins involved in K2 toxin response was generated uncovering HOG and CWI players interconnected or acting through mediators.
This study deepens insight into the K2 toxin response-modulated genetic interactions and provides data important for practical application of killer yeasts.

Key words: yeast, toxin-driven stress, connections of signalling pathways, biocontrol mechanisms.

Introduction

Direct yield losses caused by plant pathogens significantly reduce global agricultural productivity. Therefore, the protection of crops against plant diseases caused by microbial pathogens is of primary importance (Pawlikowska et al., 2019). Chemicals are typically applied to prevent crop infections; however, nowadays such consensus has changed. It is widely recognized that the use of chemicals increases resistance of pathogens, negatively affects human health and causes environmental pollution (Abbey et al., 2019). Thus, biocontrol yeasts, producing volatile organic compounds, enzymes or antagonistic features possessing killer toxins, have been explored as a promising alternative to chemical fungicides (Contarino et al., 2019).

The widespread killer phenomenon in yeast is based on the secretion of killer toxins, lethal to the wide spectrum of fungi and bacteria (Belda et al., 2017). Killer toxins have been mainly studied with respect to the spoilage control in the food industry and treatment of clinical infections (Freimoser et al., 2019). Several toxins were shown to inhibit phytopathogens and proposed for plant protection (Perez et al., 2016). Nevertheless, further investigations are needed to evaluate toxin specificity and efficiency, to assess effects on beneficial microorganisms and mechanisms underlying the tolerance to toxin-driven stress.

Most extensively killer phenomenon has been studied in budding yeasts *Saccharomyces cerevisiae*, where dsRNR virus-originated killer toxins K1, K2, K28 and Klus have been described (Schmitt, Breinig, 2006; Rodríguez-Cousiño et al., 2011). Despite some similarities in their production, killer toxins have different primary sequences, biochemical properties and modes of action. *S. cerevisiae* K1 and K2 toxins act by disrupting the structural and/or functional integrity of the plasma membrane leading to the death of sensitive yeast strains (Breinig et al., 2002; Schmitt, Breinig, 2002; Lukša et al., 2015; Orentaite et al., 2016). K28 toxin kills the host cells by irreversibly blocking DNA synthesis and by triggering G1/S arrest and apoptosis (Eisfeld et al., 2000). The mode of the action of Klus mycotoxin has not been established yet (Rodríguez-Cousiño et al., 2011).

Many cellular factors play important roles in modulation of the response to toxins. By performing genome-wide screen, hundreds of such effectors, both unique and common for the most of *S. cerevisiae* produced toxins, were identified (Pagé et al., 2003; Carroll et al., 2009; Servienė et al., 2012). The susceptibility of *S. cerevisiae* yeast to K1 toxin was shown to be conferred by 268 gene products mainly related to the synthesis of cell wall components, secretion pathway, lipid and sterol biosynthesis and cell surface signal transduction (Pagé et al., 2003). To be implicated in K2 toxin susceptibility, 332 gene products were demonstrated. Genes involved in resistance were connected to cell wall and membrane structure and/or biogenesis, mitochondrial function, while genes involved in hypersensitivity encoded products active in osmosensory and cell wall stress signalling, ion transport and maintenance of homeostasis (Servienė et al., 2012). Genes, whose deletion caused hypersensitivity to K28 toxin, were related to stress-activated signalling and protein degradation, whereas resistant mutants were clustered to endocytic, lipid organization and cell wall biogenesis pathways (Carroll et al., 2009).

Multiple *S. cerevisiae* genome-wide screens revealed that numerous genetic factors related to high osmolarity glycerol (HOG) and cell wall integrity (CWI) stress response pathways are involved in susceptibility to all three killer toxins. HOG signalling pathway is modulated by Hog1 mitogen-activated protein kinase (MAPK) acting through cascades of MAPK and regulator proteins and inducing cytoplasmic and nuclear responses (Rodríguez-Peña et al., 2010; Saito, Posas, 2012). It was observed that inactivation either of Hog1 or its main partners Pbs2, Ssk1 and Ssk2 increased the sensitivity to K1, K2 and K28 toxins (Pagé et al., 2003; Carroll et al., 2009; Servienė et al., 2012). Hog1 kinase is tightly associated with the transcription machinery via numerous stress-mediating transcriptional activators or repressors; therefore, interruption of their functionality affects cellular response to the action of killer toxins.

Single mutants of RNA polymerase II transcription factor Δ*iwr1* or a subunit of polymerase mediator complex Δ*srb5* conditioned hypersensitivity to all three yeast killer toxins. Meanwhile, deletion of either transcription factors Δ*soh1* or Δ*sfp1* leads to the increased sensitivity to K2 and K28 toxins. Absence of Hog1 negative regulator Nbp2, recruiting the phosphatase Ptc1 to the Pbs2/Hog1 complex, caused resistance of the yeast cells to K1 and K2 toxins. The genome-wide screen of yeast knock-out mutants revealed numerous transcription regulators such as Med1, Snf2, Spt3, Spt7, Spt8, etc. to be important for the cellular response to K2 toxin action only (Pagé et al., 2003; Carroll et al., 2009; Servienė et al., 2012).

The CWI pathway, responding to cell wall stress conditions, transmits signal from cell surface sensors to the Rho1 GTPase, which mobilizes a physiological response through MAPK cascade and a variety of effectors leading to substantial remodelling of the cell wall (Levin, 2011). Through the transcriptional reprogramming, yeasts modulate the expression of genes important for the cell wall biogenesis, metabolism, energy generation, signal transduction and stress (Sanz et al., 2017). It was demonstrated that inactivation of GTPase activating proteins Lrg1 and Bem2 resulted in the altered susceptibility to K2 toxin. Deletion of *BEM2* gene caused increased sensitivity to K2 toxin possibly because of the defects in the cytoskeleton organization involving the formin Bni1. Elimination of Bni1 and its activating protein kinase Fus3 also increased the sensitivity to K2 toxin. The inactivation of Rho1 effector Lrg1 led to the increased K2 toxin resistance, probably due to the decreased 1,3-beta-D-glucan synthase Fks1 activity and subsequent alterations in the cell wall structure. The elimination of transcription factor Rlm1, responding to Rho1 signals, resulted in the increased K2 toxin resistance (Servienė et al., 2012).

The HOG pathway is mainly involved in the adaptation of yeast cells to hyperosmotic stress, then the cell wall integrity pathway is activated under cell wall instability. However, several stressful conditions such as hyperosmotic stress, heat shock, low pH and oxidative stress activate both pathways suggesting that they can be positively coordinated (Rodríguez-Peña et al., 2010). HOG pathway plays a collaborative role with the CWI pathway in inducing cell wall remodelling via the upregulation of specific cell wall biosynthesis genes (Udom et al., 2019). To manage diverse stress conditions and coordinate adaptive responses, the MAPK pathways and their components crosstalk and enhance the signalling capabilities (Fuchs, Mylonakis, 2009; Saxena, Sitaraman, 2016). Given the limited number of components, crosstalk among signalling pathways could arise from the sharing particular members, e.g., Ste11 role in HOG and CWI pathways (Saxena, Sitaraman, 2016). However, for most of these situations, there is a lack of mechanistic insight and little information about the connections between these MAPK pathways could be found.

In this study, the data on interactions of genetic factors from CWI and HOG pathways under stressful conditions induced by *S. cerevisiae* K2 toxin were presented. To deepen insight into the role of genetic factors in the biocontrol activity of K2 toxin and cell resistance formation mechanisms, double mutants of HOG and CWI effectors were generated, their growth and toxin susceptibility features were analysed, and the profiles of interactions were uncovered. Understanding the mechanisms conferring toxin-driven biocontrol activity may be attractive for the application of killer yeast in plant disease control.

Materials and methods

The experiment was carried out in 2015–2020 at the Nature Research Centre, Lithuania.

Strains and culture media. Parental *Saccharomyces cerevisiae* strain BY4741 (*MAT*a, *his3*Δ*1*, *leu2*Δ*0*, *met15*Δ*0*, *ura3*Δ*0*) and the non-essential haploid deletion strains (Δ*spt3*, Δ*spt8*, Δ*med1*, Δ*soh1*, Δ*ssk1*, Δ*ssk2*, Δ*fus3*, Δ*bem2*, Δ*lrg1*, Δ*slm4*, Δ*tax4*, Δ*rlm1*) derived from BY4741 by replacing single open reading frames by KanMX4

module were purchased from Thermo Fisher Scientific (USA). To isolate K2 toxin, *S. cerevisiae* M437 (*HM/HM [Kil-K2]*) was used (Naumov, Naumova, 1973).

Yeast strains were grown in standard yeast extract-peptone-dextrose (YPD) medium (1% yeast extract, 2% peptone and 2% dextrose) and complete minimal (CM) medium (0.67% YNB (yeast nitrogen base without amino acids) and 2% dextrose). To test K2 toxin susceptibility, methylene blue agar (MBA) medium (0.5% yeast extract, 0.5% peptone, 2% dextrose and 2% agar), adjusted to pH 4 with 75 mM phosphate-citrate buffer and supplemented with 0.002% methylene blue dye, was used. For the isolation of K2 toxin, synthetic complete (SC) medium (2% glucose, 6 mM K_2HPO_4, 8 mM $MgSO_4$ and 8 mM $(NH_4)_2SO_4$), adjusted to pH 4 with 75 mM phosphate-citrate buffer and containing 5% glycerol, was used.

Polymerase chain reaction (PCR)-cassette preparation. PCR product containing *HIS3* gene with flanking *HOG1* 3' and 5' sequences was amplified by forward F-HphN-delHog1 (5′-GGAACAAAGGGAAAACAGGGAAAACTACAACTATCGTATATAATAATGCGTACGCTGCAGGTCGAC-3′) and reverse R-HphN-delHog1 (5′-CAAAAAGAAGTAAGAATGAGTGGTTAGGGACATTAAAAAAACACGTTTAATCGATGAATTCGAGCTCG-3′) primers and using pYM15 plasmid DNA as a template (www.euroscarf.de). The PCR was performed in a total reaction of 100 µL consisting of 50 µL 2x DreamTaq Green PCR Master Mix (Thermo Fisher Scientific), 3.2 µL of each primer (10 µmol L^{-1}), 100 ng of plasmid DNA and nuclease-free water. Amplification of PCR-cassette was carried out by PCR Thermal Cycler (ESCO) according to the following conditions: an initial denaturation at 95°C for 3 min followed by 30 cycles of 95°C for 30 s, 54°C for 30 s and 72°C for 2 min 40 s. The final extension was carried out at 72°C for 4 min. The PCR products were purified using GeneJet PCR purification kit (Thermo Fisher Scientific) according to the manufacturer's instructions and analysed by 1% agarose gel electrophoresis.

Yeast transformation. For homologous recombination-based PCR-cassette insertion, yeast transformation was performed according to the LiCl method (Guthrie, Fink, 1991) with several modifications. Yeast strain of interest was grown in liquid YPD medium until reaching 0.5 OD (optical density). Cells were collected by centrifugation at 4000 rpm for 5 min and washed by Tris-EDTA (TE) buffer. For competent cell preparation yeasts were incubated in 0.1 M LiCl/TE buffer at 30°C for 1 h, then followed by centrifugation at 800 rpm for 5 min and resuspension of the pellet in 0.1 M LiCl/TE buffer.

For transformation of the yeast cells, *HIS3* encoding PCR-cassette (5 µl) was mixed with competent cells (25 µl) and incubated at 30°C for 30 min. Then two volumes of 50% PEG-4000 in TE buffer were added into transformation mixture and followed incubation at 30°C for 1 h and at 42°C for 20 min. After transformation, cells were suspended in 1 mL of liquid YPD medium and incubated overnight at 30°C with 100 rpm agitation. For *HIS3* selection, cells were sedimented by centrifugation at 4000 rpm for 1 min, resuspended in 100 µl of remaining YPD medium and plated onto selective minimal dextrose (MD) agar plates supplemented with vitamins, ammonium sulphate, methionine, leucine and uracil and 200 µg mL^{-1} G418 (geneticin) but lacking histidine. Positive transformants with replaced *HOG1* gene with *HIS3* were analysed after incubation of plates at 30°C for 5 days.

Detection of Hog1 deletion in yeast cells. For detection of Hog1 deletion, overnight yeast cells of interest were collected by centrifugation at 10 000 rpm for 1 min and washed by SC medium (pH 4.0). Sedimented by centrifugation, yeast cells (5×10^7 CFU $sample^{-1}$) were resuspended in 1 ml of K2 toxin extract and incubated at 20°C for 1 h with 40 rpm agitation. Then, cells proceeded washing by Tris-HCl buffer (pH 7.5) and centrifugation at 10 000 rpm for 1 min. The collected yeast cells were mixed with 200 µL of 0.1 M NaOH (sodium hydroxide) solution and incubated at 20°C for 10 min. Cells were sedimented by centrifugation at 14 000 rpm for 2 min, suspended in 50 µL of 2x SDS-PAGE sample buffer and followed by heat denaturation at 85°C for 5 min. The supernatant was applied for SDS-PAGE by resolving proteins on 10% SDS-polyacrylamide gel and subsequent Western blotting. The gel was transferred onto polyvinylidene fluoride (PVDF) membrane, blocked for 1 h at room temperature with 5% of milk powder in TTBS containing 0.05% Tween 20 and washed three times with TTBS. Western blot analysis was carried out using primary anti-Hog1 antibody (1:3000) and horse radish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (1:5000). Membrane was visualised by colorimetric signal detection using NBT/BCIP system (Thermo Fisher Scientific) according to the manufacturer's recommendations.

Detection of yeast growth parameters. For growth rate measurements, overnight single and double yeast mutant cultures were inoculated into YPD medium starting from 0.1 OD_{600} and grown at 30°C with 100 rpm agitation. Culture growth was monitored by measuring absorbance at 600 nm every hour during a 24 h period. All the experiments were carried out in triplicate. Growth rates of mutants were calculated using software *GraphPad Prism* (Olivares-Marin et al., 2018).

K2 toxin preparation. The K2 toxin producing *S. cerevisiae* strain M437 was grown in SC medium for 4 days at 18°C with 40 rpm shaking. Cells were collected by centrifugation at 5000× *g* for 10 min. The supernatant was filtered through a 0.22 µm diameter sterile PVDF membrane and concentrated 100-fold by ultrafiltration through an Amicon PM-10 membrane. Such toxin isolate was used for yeast susceptibility assay.

Susceptibility to K2 toxin. The sensitivity of single and double mutants to K2 toxin was tested by depositing 100 µL of concentrated 100-fold toxin preparate into 10 mm diameter "punched wells" in the MBA medium plates overlaid with the yeast strain of interest (2×10^6 cells $plate^{-1}$). Plates were incubated for 2 days at 25°C. The diameter of the lysis zones was measured and compared with that formed on strain BY4741 overlay.

Protein network construction. Network diagrams were generated using web resource STRING v11 (https://string-db.org/) (Szklarczyk et al., 2011). The experiments-based active prediction method was used, and the medium confidence score (0.4) was utilized. Our created network uses the "confidence view" option of the program, where stronger associations are represented by thicker lines.

Statistical analysis. Detection of growth rate and susceptibility assay were carried out in triplicate, and the data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for the estimation of the statistical significance; the differences were considered significant at $p < 0.05$.

Results and discussion

Saccharomyces cerevisiae K2 toxin binds to the cell wall and interacts with the plasma membrane causing outflow of potassium ions and other cellular molecules (Lukša et al., 2015; Orentaite et al., 2016). Defects in high osmolarity glycerol (HOG) and cell wall integrity (CWI) signalling pathways results in altered susceptibility of yeast to the action of K2 toxin (Servienė et al., 2012). It was demonstrated that general cellular factors of both pathways are physically and functionally interconnected and coordinate their responses by managing stressful conditions (Rodríguez-Peña et al., 2010). Many genetic factors may be involved in conditions-dependent negative (when mutants are synergistically deleterious) or positive (when the combination is less severe than is expected from independent effects) interactions. These factors may act directly or through mediators (Díaz-Mejía et al., 2018). The information on the nature of the connections between most of the gene products from HOG and CWI pathways, which are involved in formation of cellular susceptibility to K2 toxin, is scattered. Therefore, to deepen insight into the role of cellular factors in the biology of K2 toxin, double mutants of HOG and CWI genes were generated, and their growth and toxin susceptibility features were compared with single mutants as well as the profile of conditions-dependent interactions were uncovered.

Construction of yeast double mutants. For further analysis, 13 yeast single gene deletion strains related to the response of cell wall integrity and hyperosmotic stress pathways and having altered susceptibility to K2 toxin were selected (Table).

Table. Description of gene names, functions and susceptibility to *Saccharomyces cerevisiae* K1, K2 and K28 toxins as well as *S. paradoxus* K66 killer protein (according to Pagé et al., 2003; Carroll et al., 2009; Servienė et al., 2012; Vepštaitė-Monstavičė et al., 2018)

Gene ID	Description	*S. cerevisiae*			*S. paradoxus*
		K1	K2	K28	K66
		single mutant phenotype			
HOG1	Mitogen-activated protein (MAP) kinase involved in osmoregulation	S	S	S	S
SSK1	Cytoplasmic response regulator, part of a two-component signal transducer that mediates osmosensing	S	S	S	S
SSK2	MAP kinase of the HOG1 mitogen-activated signalling pathway	S	S	wt	S
SOH1	Subunit of the RNA polymerase II mediator complex	wt	S	S	wt
MED1	Subunit of the RNA polymerase II mediator complex	wt	S	wt	wt
SPT3	Subunit of the SAGA and SAGA-like transcriptional regulatory complexes	wt	S	wt	wt
SPT8	Subunit of the SAGA transcriptional regulatory complex; mutants sensitive to osmotic stress	wt	S	wt	wt
BEM2	Rho GTPase activating protein involved in signal transduction and cellular morphogenesis	wt	S	wt	wt
FUS3	Mitogen-activated serine/threonine protein kinase involved in pheromone-dependent signal transduction during mating	wt	S	wt	wt
TAX4	EH domain containing protein involved in cell wall organization, inositol lipid-mediated signalling	wt	S	wt	wt
RLM1	Component of the protein kinase C-mediated MAP kinase pathway involved in the maintenance of cell integrity	wt	R	wt	R
LRG1	GTPase-activating protein (GAP) involved in the Pkc1p-mediated signalling pathway that controls cell wall integrity	wt	R	wt	R
SLM4	Subunit of EGO/GSE complex, phosphatidylinositol-3,4-bisphosphate binding	wt	R	R	wt

Note. Gene description was adapted from *Saccharomyces* Genome Database (SGD) (www.yeastgenome.org); S – increased sensitivity to toxins is marked, R – resistant phenotype, wt – similar level as in parental strain BY4741.

Well-known Hog1 protein kinase-related regulators Ssk1 and Ssk2 were chosen. The abolishment of those genes determines hypersensitivity to *S. cerevisiae* K1, K2 and K28 toxins as well as to *S. paradoxus* K66 protein (Table). Among chosen strains, there are Δ*soh1* (gene encoding subunit of the RNA polymerase II mediator complex), which is sensitive to K2 and K28 toxins and strain Δ*slm4* (gene encoding subunit of EGO/GSE complex), which is

resistant to K2 and K28 toxins. Mutations Δ*rlm1* and Δ*lrg1* are beneficial and confer higher resistance to *S. cerevisiae* K2 and *S. paradoxus* K66 toxins. Both gene products are involved in the maintenance of the cell wall integrity. Other selected strains, deficient in transcription regulators or signal transducers (Med1, Spt3, Spt8, Bem2, Fus3 or Tax4), modulating the expression of genes important for cell wall biogenesis, demonstrate hypersensitivity to K2 toxin only.

To obtain cells carrying two gene deletions, a homologous recombination approach was used (Janke et al., 2004). For *HOG1* gene replacement, the *HIS3* gene was PCR amplified from pYM15 plasmid by adding sequences flanking *HOG1* gene (Figure 1A). According to electrophoretic analysis, the purified PCR fragment size was about 1700 bp (Figure 1B). Amplified PCR cassette was transformed into single gene deletion yeast cells, and homologous recombination based *HOG1* gene deletion was generated (Figure 1C).

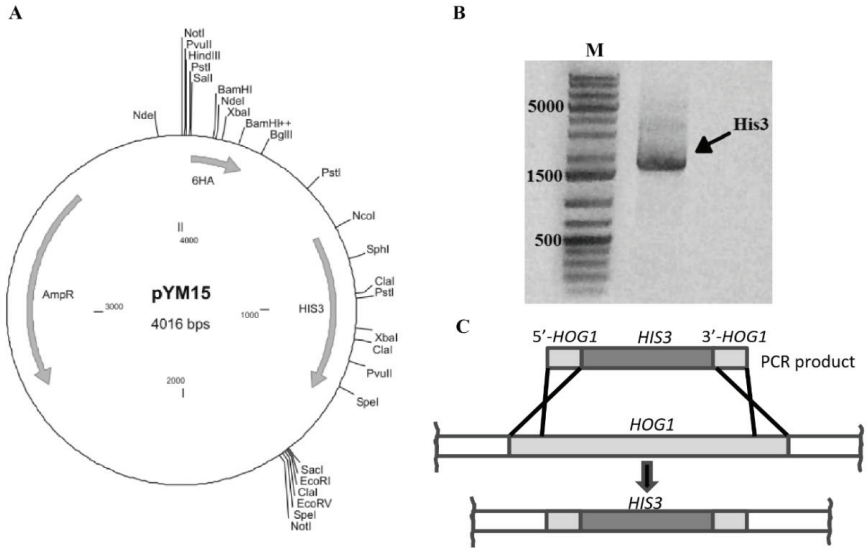


Figure 1. Principles of double mutants' construction: restriction map of pYM15 plasmid-encoding marker gene *HIS3* (www.euroscarf.de) (A), electrophoretic validation of PCR-amplified *HIS3* (B), scheme of *HOG1* replacement by *HIS3* using homologous recombination (C)

Transformed cells were selected by the growth on SC medium without histidine (Figure 2A). Afterwards, the viable colonies were tested for the absence of Hog1 protein (48.9 kDa) by Western blot hybridization using specific primary anti-HOG1 and horse radish peroxidase (HRP)-conjugated anti-rabbit IgG as secondary antibodies (Figure 2B).

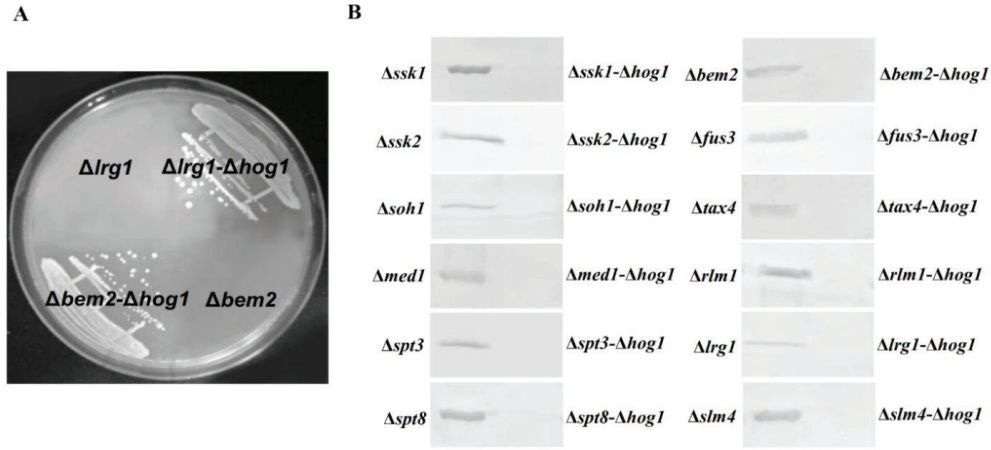
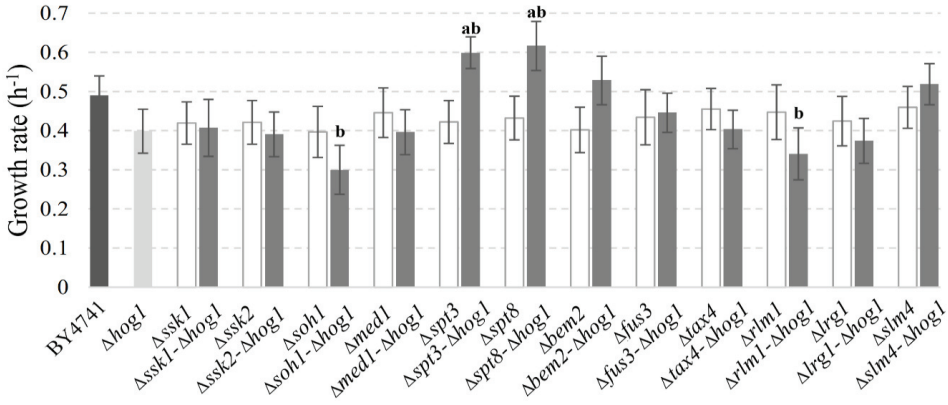


Figure 2. Validation of constructed *Saccharomyces cerevisiae* double mutants by growth on complete minimal medium without histidine (A) and by absence of Hog1 protein using Western blot hybridization (B)

Determination of yeast growth rates. Yeast growth profile depends on growing conditions such as pH, temperature and medium type, but growth variability even more depends on yeast species and genetic background (Salari, Salari, 2017). To determine whether the removal of *HOG1* gene from yeast single mutants does affect yeast growth parameters and viability, the growth rates of single and double mutants incubated in rich YPD medium were evaluated and compared to the parental strain BY4741 (Figure 3).



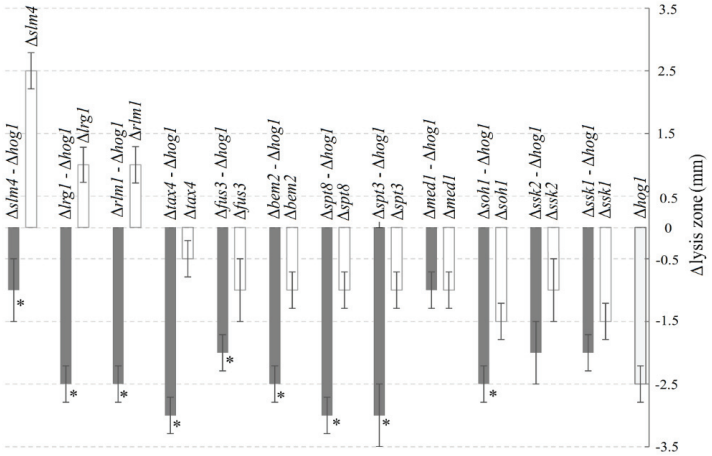
Note. Growth rates of mutants were calculated from three experiments, and the data were expressed as mean ± SD; significant differences ($p < 0.05$) between the growth rate of the double *vs* single mutants and parental strain BY4741 are marked a and b, respectively.

Figure 3. Growth rates of *Saccharomyces cerevisiae* single and double mutants

It was observed that deletion of *HOG1* gene by itself does not significantly change the growth rate compared to the parental strain BY4741: growth rate of mutant Δ*hog1* was 0.4 ± 0.06 h^{-1} and strain BY4741 – 0.49 ± 0.05 h^{-1}. The comparison of parental strain growth rate *vs* tested single gene mutants also did not show remarkable differences. However, the growth rates of some double mutants were affected significantly in comparison with single deletions. The significant increase (about 40%) of the growth rate was observed in the case of strains Δ*spt8*-Δ*hog1* and Δ*spt3*-Δ*hog1* comparing to a single mutant and to *wt*. The growth rate of strains Δ*bem2*-Δ*hog1* increased up to 30% comparing to single *BEM*2 gene deletion.

After elimination of *HOG1* gene, the doubling speed of yeast cells Δ*soh1* and Δ*rlm1* was reduced by 25%. No other significant differences between single and double gene mutants' growth rates were observed. The data on the rate of growth can be used, when the goal is to find individual interactions or estimate the mean epistatic effect. It was reported that interactions leading to strong alterations of fitness and growth parameters of double mutants in relation to those of the respective single mutants are generally rare, and weak interactions are more abundant (Jakubowska, Korona, 2012). During our experiment, also most double gene deletion strains demonstrated weak changes in growth rates *vs* individual gene deletion yeasts. Nevertheless, such changes in growth parameters may have an impact on yeast viability and response to various stressful factors, especially killer toxin action.

Response of double mutants to K2 toxin. For analysis of genetic interactions between the players of HOG and CWI pathways in response to K2 toxin action, a sensitivity-based approach was applied. The created K2 toxin-driven stress accomplishes activation of the HOG pathway, which regulates glycerol synthesis and affects cell wall integrity. Gene interactions in mutant cells were evaluated by comparing single and double mutant susceptibility to K2 toxin (Figure 4). Most tested single gene mutants (Δ*ssk1*, Δ*ssk2*, Δ*soh1*, Δ*spt3*, Δ*spt8*, Δ*bem2*, Δ*fus3*, Δ*tax4*, Δ*hog1*) demonstrated increased sensitivity to K2 toxin in relation to parental strain BY4741. The toxin susceptibility of most double mutants increased in comparison to single mutants but remained at a similar level to single mutant Δ*hog1*.



Note. Toxin formed lysis zones were measured and compared to the ones obtained on parental strain BY4741; negative values mean that lysis zones were smaller than displayed on parental strain, positive – larger than wt; all data were expressed as mean ± SD; * – corresponds to significant difference ($p < 0.05$) in K2 toxin sensitivity of double and single mutants.

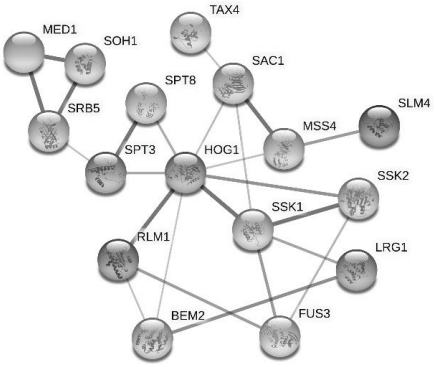
Figure 4. Sensitivity of yeast single and double mutants to *Saccharomyces cerevisiae* K2 toxin

HOG1 gene deletion from cells Δ*ssk1* and Δ*ssk2*, which interact in the same signalling pathway, had no significant changes upon K2 toxin treatment (Figure 4). Soh1 and Med1 both are subunits of the RNA polymerase II mediator complex (Boube et al., 2002). The sensitivity of a double mutant Δ*soh1*-Δ*hog1* increased comparing to single gene deletion, while Δ*med1*-Δ*hog1* response to K2 toxin remained as Δ*med1*. Elimination of *HOG1* gene from defective in SAGA transcriptional regulation cells Δ*spt3* or Δ*spt8* K2 toxin sensitivity increased about 50% comparing to single mutants (Wu et al., 2004). Deletion of Hog1 protein kinase from defective in CWI pathway strains Δ*bem2* and Δ*tax4* augmented the killer toxin sensitivity phenotype for Δ*bem2*-Δ*hog1* about 35% and for Δ*tax4*-Δ*hog1* even higher. Thus, the K2 toxin response phenotype in the mentioned above double mutants, not depending on the involvement of gene products into the same or different signalling pathways, did not change, only the increase in sensitivity level was observed.

Our observations agree with those of other researchers indicating the abundance of weak genetic interactions with an average effect close to zero or moderately positive (Jakubowska, Korona, 2012). In our research, only three single gene mutants, whose gene products are involved in CWI signalling, changed their response to K2 toxin, when additional deletion of *HOG1* gene was generated. Single mutants Δ*rlm1*, Δ*lrg1* and Δ*slm4* are more resistant to K2 killer protein than the parental strain BY4741, while the mutant Δ*hog1* is sensitive to K2 toxin. Importantly, that double mutants Δ*rlm1*-Δ*hog1*, Δ*lrg1*-Δ*hog1* and Δ*slm4*-Δ*hog1* are as sensitive to K2 toxin as the single gene deletion strain Δ*hog1*. The genes may be defined as epistatic to one another, when the phenotypic impact associated with a given mutation is altered by the presence of a second gene mutation (Batenchuk et al., 2010; Steidle et al., 2020). Thus, our results indicated that the mutation Δ*hog1* is epistatic to Δ*rlm1*, Δ*lrg1* and Δ*slm4*.

The sign and strength of interactions may change reflecting on the activation or inactivation of different pathways across environments. Thus, epistasis depends on both genetic and environmental context (Batenchuk et al., 2010). In silico study, He et al. (2010) found that negative interactions occur more frequently between genes with overlapping functions, whereas positive interactions are observed between functionally distinct metabolic pathways. Some studies have looked at the effect of environmental stress on the form of epistasis and pointed out that environmental conditions can have long-term effects on epistasis (de Visser et al., 2011).

Interconnections of gene products involved in K2 toxin response. To generate protein-protein interaction network, a STRING analysis was applied (Szklarczyk et al., 2011), which used high throughput datasets available in the *S. cerevisiae* Genome Database (https://www.yeastgenome.org). The network was drawn with medium confidence score and included overall 24 linkages. The line thickness represents the strength of data support and includes both functional and physical interactions (Figure 5). Hog1 has strong direct associations with HOG pathway regulator Ssk1 and CWI pathway mediator Rlm1.



Note. Stronger associations are represented by thicker lines; confidence score = 0.4.

***Figure 5*.** Interaction network of proteins from cell wall integrity (CWI) and high osmolarity glycerol (HOG) pathways involved in K2 toxin susceptibility

The direct connections of Hog1 protein kinase with MAPK Ssk2 and transcription regulators Spt3 and Spt8 as well as Rho1 GTPase activating protein Bem2 were slightly weaker. In this experiment, the linkages of Hog1 with other players of CWI and HOG pathways analysed were indirect. They were mediated through protein kinases (for Fus3 and Lrg1), RNA polymerase II complex and transcription regulators (for Soh1) and phosphatidylinositol signalling regulators (for Tax4 and Slm4). STRING analysis performed in this experiment suggests that there are still many interacting factors to be discovered between HOG and CWI pathways. More investigations are needed to detect small individual effects of epistasis.

Conclusions

1. The majority of created double gene deletion strains had minor changes in growth rate compared to individual gene mutants, and only strains Δ*spt3* and Δ*spt8* in combination with Δ*hog1* had a significant increase of growth rate.

2. The sensitivity towards K2 toxin increased in most generated double mutants thus suggesting weak interactions of all investigated gene products. Phenotype changes from resistant to sensitive in mutants Δ*rlm1*, Δ*lrg1* and Δ*slm4* caused by *HOG1* gene deletion showed epistatic effect on this cell wall integrity (CWI) pathway effectors.

3. The network of proteins involved in K2 toxin response uncovered interconnected or acting through mediators high osmolarity glycerol (HOG) and CWI players.

4. The obtained results deepen insight into mechanisms underlying the tolerance to *Saccharomyces cerevisiae* K2 toxin-caused cellular stress and toxin-driven biocontrol activity thus highlighting the potential of killer yeast in plant protection.

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Šeimininko veiksnių sąveika formuojant atsaką į mielių K2 toksino sukeltą stresą – pritaikomumas augalų apsaugai

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Santrauka

Toksinus sintetinančios mielės yra svarbios žemės ūkyje, nes gali būti panaudotos biologinei augalų apsaugai nuo bakterinių ir grybinių ligų. Biocidinio toksino veikimo ir ląstelių atsako toksino sukeltam stresui mechanizmų išaiškinimas yra itin svarbus, siekiant plėsti biocidinių mielių kaip augalų apsaugos priemonės pritaikomumą.
Tyrimo metu siekta nustatyti sąveiką tarp genetinių veiksnių iš ląstelės sienelės vientisumo (CWI, angl. *cell wall integrity*) ir atsako į hiperosmotinį stresą (HOG, angl. *high osmolarity glycerol*) signalinių kelių, paveikus *Saccharomyces cerevisiae* K2 toksinu. Tiriant genetinę sąveiką buvo naudoti du įverčiai – santykinis augimo greitis ir ląstelių jautrumas toksinui. Homologinės rekombinacijos būdu sukurta 12 mutantų su dviejų genų iškritomis ir išanalizuoti jų augimo parametrai bei atsakas į toksino sukeltą stresą. Dauguma dvigubų mutantų parodė nereikšmingus (mažiau nei 30 %) augimo greičio pokyčius, palyginti su vienetiniais mutantais. Tik Δ*spt3* ir Δ*spt8* kamienuose *HOG1* geno pašalinimas augimo greitį esmingai padidino iki 0,6 val^{-1}. Daugelio dvigubų mutantų jautrumas K2 toksinui padidėjo, nepriklausomai nuo signalinio kelio priklausomybės; tai rodo silpną genų produktų sąveiką. *HOG1* geno pašalinimas pakeitė tik mutantų Δ*rlm1*, Δ*lrg1* ir Δ*slm4* fenotipą iš atsparaus į jautrų, tuo patvirtindamas epistatinį poveikį šiems genetiniams efektoriams. Siekiant papildyti funkcinės analizės duomenis, sukurtas K2 toksino sukelto streso atsake dalyvaujančių HOG ir CWI baltymų tinklas ir atskleistos jų sąveikos. Nustatyta, kurie HOG ir CWI keliuose dalyvaujantys genų produktai sąveikauja tiesiogiai, o kurie per baltymus tarpininkus.
Tyrimo rezultatai pagilina žinias apie sąveikas genetinių veiksnių, moduliuojančių atsaką į K2 toksino sukeltą stresą, ir suteikia svarbios informacijos praktiniam biocidinių mielių pritaikymui.

Reikšminiai žodžiai: mielės, toksino sukeltas stresas, signalinių kelių ryšys, biokontrolės mechanizmas.

Iglė Vepštaitė-Monstavičė

The structure of fruit mycobiota and functioning mechanisms of its components – killer systems of *Saccharomyces* genus yeasts

Vaisių mikobiotos struktūra ir jos komponentų – *Saccharomyces* genties mielių biocidinių sistemų funkcionavimo mechanizmai

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