VILNIUS UNIVERSITY

RAMINTA PRANCKUTĖ

EVALUATION OF LACTIC ACID AND THERMOPHILIC BACTERIA ANTIBACTERIAL ACTIVITY AND COMPATIBILITY WITH PREBIOTIC OLIGOSACCHARIDES FOR DEVELOPMENT OF NEW SYNBIOTICS

Summary of doctoral dissertation

Biomedical sciences, biology (01B)

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VILNIAUS UNIVERSITETAS

RAMINTA PRANCKUTĖ

PIENARŪGŠČIŲ IR TERMOFILINIŲ BAKTERIJŲ ANTIBAKTERINIO AKTYVUMO IR SUDERINAMUMO SU PREBIOTINIAIS OLIGOSACHARIDAIS NAUJŲ SINBIOTIKŲ KŪRIMUI ĮVERTINIMAS

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INTRODUCTION

Nowadays consumers are more and more concerned about food quality and its health benefits (Gautam and Sharma, 2009; Abriouel *et al*., 2011; Mills *et al*., 2011; Balciunas *et al*., 2013; Yang *et al*., 2014; Dixit *et al*., 2016). Accordingly, functional food industry is rapidly expanding (Al-Sheraji *et al*., 2013; Dixit *et al*., 2016). Functional food products provide health benefits due to their probiotic, prebiotic, postbiotic or synbiotic components (Siró *et al*., 2008; Walsh *et al*., 2014; Pandey *et al*., 2015; Tomar *et al*., 2015). However, availability of such products is restricted due to the relatively high prices (Alvarez-Sieiro *et al*., 2016).

Probiotics are defined as "live microorganisms, which when consumed in adequate amounts, confer a health benefit on the host" (Dobson *et al*., 2011). Due to their antibacterial properties probiotics are mainly used for modulation of intestinal microflora. Most bacteria with probiotic properties belong to a group of Lactic Acid Bacteria (LAB) (Saad *et al*., 2013; Dixit *et al*., 2016; Dahiya *et al*., 2017). LAB produces many antibacterial compounds, including bacteriocins (Dobson *et al*., 2011; Ghosh, 2012; O'Shea *et al*., 2012; Saad *et al*., 2013; Walsh *et al*., 2014; Tomar *et al*., 2015; Alvarez-Sieiro *et al*., 2016; Ahmad *et al*., 2017). For this reason, LAB and/or their produced antibacterial substances are considered as a great alternative to conventional antibiotics and are widely used in food industry, medicine, veterinary and agriculture (Mills *et al*., 2011; O'Shea *et al*., 2012; Saad *et al*., 2013; Pandey *et al*., 2015; Dixit *et al*., 2016; Woraprayote *et al*., 2016; Ahmad *et al*., 2017).

One of the biggest concerns in today's health care is a widespread resistance of food spoilage and pathogenic microorganisms to conventional antibiotics. For this reason, research for new antimicrobial compounds has been one of the main objectives during the last decades. Bacteriocins are one of the best alternatives (Balciunas *et al*., 2013; Yang *et al*., 2014; Perez *et al*., 2014; Arqués *et al*., 2015; Cavera *et al*., 2015; Sumi *et al*., 2015; Egan *et al*., 2016; Ahmad *et al*., 2017; Kaškonienė *et al*; 2017; Maina *et al*., 2017). These usually small, cationic or amphiphilic molecules kills sensitive microorganisms by interacting with negatively charged cell envelope structures and consequently creating pores and/or inhibiting cell wall biosynthesis (Snyder and Worobo, 2014, Cavera *et al*., 2015; Ahmad *et al*., 2017). It is more difficult to evolve resistance to these mechanisms

comparing with conventional antibiotics, which act mainly on enzymes (Perez *et al*., 2014; Sumi *et al*., 2015; Maina *et al*., 2017).

The biggest potential of bacteriocin application is in food industry due to their specific activity spectra, nontoxicity and degradability by digestive proteases (Yang *et al*., 2014; Perez *et al*., 2014, Arques *et al*., 2015; Cavera *et al*., 2015; O'Connor *et al*., 2015; Egan *et al*., 2016; Woraprayote *et al*., 2016). Moreover, most LAB and/or their produced bacteriocins are recognized as safe food additives (GRAS) (Mills *et al*., 2011; Yang *et al*., 2014; Alvarez-Sieiro *et al*., 2016; Egan *et al*., 2016; López-Cuellar *et al*., 2016). But the main advantage of bacteriocins is their stability in wide temperature and pH ranges and activity in nano- or pico- concentrations. Also, they are tasteless and odorless which makes them even more favorable for food applications. These properties allow the application of bacteriocins together with or instead of other food processing factors (Mills *et al*., 2011; Perez *et al*., 2014; Arqués *et al*., 2015; O'Connor *et al*., 2015; Egan *et al*., 2016; Woraprayote *et al*., 2016). In this way food is not only protected from undesirable microorganisms, but also retains its quality and organoleptic properties. It also allows to prolong food shelf life with decreased amounts of chemical preservatives which are the main requirements of today's consumers (Arques *et al*., 2015; Egan *et al*., 2016; Woraprayote *et al*., 2016; Maina *et al*., 2017).

Among bacteriocins produced by gram-positive bacteria ones produced by LAB are the most extensively researched due to the long history of LAB use in food industry as probiotic or starter cultures (Mills *et al*., 2011; Gemechu, 2015; Alvarez-Sieiro *et al*., 2016; Egan *et al*., 2016; López-Cuellar *et al*., 2016; Maina *et al*., 2017). However, the main drawback of LAB bacteriocins is their inactivity against gram-negative bacteria and limited stability in high pH values which forces the search of new bacteriocins, characterized with broader antibacterial spectra and higher stability (Gillor *et al*., 2008; Mills *et al*., 2011; O'Connor *et al*., 2015; Ahmad *et al*., 2017; Kaškonienė *et al*., 2017; Maina *et al*., 2017).

Thermophilic *Geobacillus* sp. bacteria are especially attractive sources of antibacterial compounds as their produced bacteriocins are often active not only against gram-positive, but also against gram-negative bacteria and even spores (Cavera *et al*., 2015; Egan *et al*., 2016). Moreover, due to their thermophilic nature their bacteriocins usually are characterized with even better stability in high temperatures and wide pH

ranges comparing to LAB bacteriocins (Pokusaeva *et al*., 2009; Pranckutė *et al*., 2015). These properties are especially important when aiming to reduce the use of chemical preservatives in food industry in order to meet consumers' demands and lower the cost of functional foods (Mills *et al*., 2011; Arques *et al*., 2015; Alvarez-Sieiro *et al*., 2016; Egan *et al*., 2016; Maina *et al*., 2017). Furthermore, due to the ribosomal synthesis and genetically organized structure bacteriocins are amenable to genetic engineering allowing the creation of effective bacteriocin producers (Mills *et al*., 2011; Perez *et al*., 2014; Cavera *et al*., 2015; O'Connor *et al*., 2015; Egan *et al*., 2016; Maina *et al*., 2017) and to lower their application costs.

Prebiotic non-digestible oligosaccharides (OS) selectively stimulate the growth and/or functions of probiotic bacteria. Therefore, they are mainly used to modulate the composition of gut microflora, to increase survivability of probiotic bacteria during their passage through the digestive tract and to stimulate their proliferation in the gut (Roberfroid *et al*., 2010; Al-Sheraji *et al*., 2013; Saad *et al*., 2013; Slavin, 2013; Walsh *et al*., 2014; Valcheva and Dielaman, 2016; Dahiya *et al*., 2017). It is shown that prebiotics can also stimulate antibacterial activity of probiotic bacteria, including increased bacteriocin production (Chen *et al*., 2007; Vamanu and Vamanu, 2010; Patel and Goyal, 2012).

The aim of the research

The main purpose of this work was to evaluate thermophilic and lactic acid bacteria strains' probiotic potential, compatibility with prebiotic oligosaccharides and their influence on strains' antibacterial activity and effectiveness of new symbiotic combinations and possibilities of their application in food industry.

The following tasks have been formulated to achieve this aim:

1. To evaluate the growth and antibacterial activity of lactic acid bacteria type strains and yogurt isolates and thermophilic 118 strain.
2. To evaluate the ability of investigated strains to assimilate prebiotic oligosaccharides (palatinose, inulin and α-cyclodextrin (CD)) and their influence on antibacterial activity.
3. To evaluate the influence of sporulation on antibacterial activity of 118 strain.

4. To evaluate physicochemical properties and nature of antibacterial substance produced by 118 strain.
5. To evaluate the influence of oligosaccharides on antibacterial activity spectra of investigated strains against pathogenic bacteria.
6. To evaluate the antagonistic activity between investigated lactic acid and thermophilic bacteria and their effect on eukaryotic cells.

Scientific novelty and practical significance

Although probiotics and prebiotics are extensively studied and applied in food and other industries (Ghosh, 2012; Al-Sheraji *et al*., 2013; Saad *et al*., 2013; Slavin, 2013; Dixit *et al*., 2016), relatively little is known about their compatibility and effectiveness as potential synbiotics (Saminathan *et al*., 2011; Tomar *et al*., 2015; Pandey *et al*., 2015). Even less attention is focused on the influence of prebiotics on bacteriocin production (Chen *et al*., 2007; Vamanu and Vamanu, 2010; Patel and Goyal, 2012) and there is virtually no data showing the possibility to modulate the antibacterial activity of probiotic bacteria against pathogens using prebiotics.

Commercial oligosaccharides (OS) investigated in this work (palatinose, inulin and α-cyclodextrin) are used as prebiotics (Mussatto and Mancilha, 2007; Roberfroid *et al*., 2010; Ghosh, 2012; Al-Sheraji *et al*., 2013; Saad *et al*., 2013; Li *et al*., 2014), but very little is known about the influence of palatinose and α-cyclodextrin on probiotic bacteria growth (Mussatto and Mancilha, 2007; van Zanten *et al*., 2012) and even less – about their influence on the antibacterial activity of probiotic bacteria. Although inulin is one of the most comprehensively studied prebiotic OS (Roberfroid *et al*., 2010; Patel and Goyal, 2012; Saad *et al*., 2013; Valcheva and Dielaman, 2016), but majority of its research is orientated to the stimulation of *Bifidobacterium* sp. bacteria (Roberfroid *et al*., 2010; Slavin, 2013) while its influence on the growth and antibacterial activity of bacteria from other genera, such as *Lactobacillus* and *Lactococcus*, is explored only in few researches (Chen *et al*., 2007; Goderska *et al*., 2008; Vamanu and Vamanu, 2010; Kunova *et al*., 2011; Al-Sheraji *et al*., 2013).

Thermophilic 118 bacterial strain investigated in this work is closest to *Aeribacillus pallidus* (previously assigned to *Geobacillus* sp. (Miñana-Galbis *et al*., 2010)). Recently, antibacterial activity of these bacteria has been described (Muhammad

and Ahmed, 2015), but according to the data provided and the results obtained in this work it can be concluded that the described strain does not match the one used in this work. Therefore, bacteriocin produced by *Aeribacillus* sp. bacteria is described for the first time in this work.

In this work, the ability of thermophilic bacteria to assimilate OS and the influence of OS on the antibacterial activity of thermophilic bacteria was investigated for the first time. Moreover, it was shown that 118 strain was sensitive to, but not active against investigated LAB bacteria. These results showed the probiotic potential of the thermophilic 118 strain as it and/or its produced bacteriocin could be applied in food industry together with probiotic or starter LAB cultures harmlessly. Among thermophilic spore-forming bacteria, probiotic status has been proposed only to *Geobacillus thermoleovorans* strain S1 (Mahdhi *et al*., 2011). Activity against food spoilage and pathogenic bacteria, nontoxicity to eukaryotic cells, stability in high temperature and wide pH ranges shows a huge potential of 118 strain and its bacteriocin for the application in food industry, which would allow the reduction of the usage of physical and chemical food preservation treatments and lower the food protection costs without harming food quality and safety.

Some bacteria species belonging to genus *Geobacillus*, especially *Geobacillus stearothermophilus*, are recognized as common and important canned and dairy food contaminants (Gopal *et al*., 2015; André *et al*., 2017). All strains investigated in this work were highly active against *G. stearothermophilus*, which makes them very attractive for combating these food spoilage bacteria.

Thesis statements:

1. Lactic acid and thermophilic bacteria strains are capable of assimilating prebiotic oligosaccharides which positively affects the antibacterial activity.
2. Thermophilic 118 and lactic acid bacteria strains are active against pathogenic and/or food spoilage bacteria.
3. Prebiotic oligosaccharides can modulate antibacterial activity spectra and/or intensity against pathogenic bacteria.
4. Antibacterial substance produced by thermophilic 118 strain is a bacteriocin characterized with properties beneficial for food applications.
5. Investigated strains, characterized with antibacterial activity, can be used in combination with prebiotic oligosaccharides for the creation of new effective synbiotics.

MATERIALS AND METHODS

Microorganisms used in this study

Lactic Acid Bacteria (LAB) isolates investigated in this work were isolated from probiotic yogurts: "Actimel" ("Danone") (A isolates) and "Bifi" ("Rokiškio pienas") (B isolates). Yogurts were diluted using routine tenfold dilution method. Suspensions of 10^6, 10^7 and 10^8 dilutions were streaked on the solid LAB selective deMan-Rogosa-Sharpe (MRS) medium (Merck) and incubated at 30 °C for 24-48 h anaerobically in anaerobic jar with "Anaerocult A" gas pack (Merck).

Type LAB strains were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany) microorganism collection (Table 1).

Table 1. Type LAB strains used in this work.

Strain name	**DSM number**	**Abbreviation used in this work**
Lactobacillus acidophilus	20079	LA
Lactobacillus delbrueckii subsp. *bulgaricus*	20081	LD
Lactobacillus curvatus	20010	LC
Lactobacillus sakei subsp. *sakei*	20017	LS
Lactococcus lactis subsp. *lactis*	20481	LL
Lactococcus lactis subsp. *lactis*	20729	LL2

Table 2. Bacterial strains and yeast species used in this work for determination of investigated antibacterial substances antibacterial activity spectra and effect on eukaryotic cells.

Pathogenic bacterial strains		
	Gram+	*Bacillus cereus* ATCC 11778
		Bacillus cereus DSM 12001
		Bacillus subtilis ATCC 6633
		Clostridium perfringens
		Enterococcus faecalis ATCC 29212
		Enterococcus faecium 402-3/03
		Enterococcus faecium ATCC 19434
		Listeria monocytogenes ATCC 19117
		Staphylococcus aureus ATCC 25923
		Staphylococcus aureus MRSA
		Streptococcus pyogenes ATCC 19615
		Streptococcus pyogenes MPK 2331
	Gram-	*Klebsiella pneumoniae* ATCC 13883
		Pseudomonas aeruginosa ATCC 27853
		Salmonella enteritidis ATCC 13076
		Salmonella typhimurium ATCC 14028
Yeast		*Saccharomyces cerevisiae*
		Candida albicans
		Candida lusitania
		Candida guiliermondii

Thermophilic 118 bacterial strain was picked from bacterial strains collection of Department of Microbiology and Biotechnology, Vilnius University. *Geobacillus stearothermophilus* NUB36187, acquired from *Bacillus* Genetic Stock Center (BGSC, Nr.: 9A11) (Zeigler, 2001) was used as indicator strain in antibacterial activity experiments.

Cultivation media and conditions

All strains were inoculated into liquid media adding 5% (v/v) of cell suspension (1.2 OD_{590}), prepared in appropriate sterile medium. Isolated and type strains of *Lactobacillus* sp. were cultured in de Man, Rogosa, Sharp (MRS) broth (Merck) or basal MRS (bMRS) (Saminathan *et al.*, 2011). Type strains of *Lactococcus* sp. were grown in broth No. 92 (TY) (DSMZ culture medium list), which consisted of Tryptone Soya Broth (TSB, Merck), supplemented with 0.3% Yeast Extract (YE, Merck). Commercial granulated media (Merck) were used for routine strains refreshment, growth of biomass for inoculation and determination of antibacterial activity. All LAB strains (except LA and LD strains, Table 1) were cultivated aerobically. LA and LD strains were grown under anaerobic conditions, using "Anaerocult A" oxygen absorbent (Merck) in plastic bags and jars (Merck) at 37 °C temperature. Type LS and LC strains were grown at 30 °C, with 150 rpm agitation. A and B yogurt isolates were cultivated without additional aeration (facultative anaerobes) at 30 °C temperature. *Lactoccocus* sp. strains were grown in TSB or TY media at 37 °C, with 150 rpm agitation.

Thermophilic strains were grown aerobically at 55 °C temperature with 180 rpm agitation. For routine strains refreshment, growth of biomass for inoculation and determination of antibacterial activity of thermophilic strains Nutritive Bullion (NB) media was used (g/L): peptone from casein, 10; meat extract, 5; NaCl, 5. In growth, different carbon source assimilation and antibacterial activity evaluation experiments mineral mM9 or minimal minYE media were used. mM9 consisted of (ml/L): 5x salt solution, 200; 1 M $MgSO_4$, 2.0; 1 M $CaCl_2$, 0.1; 1000x vitamin stock solution, 1; 10x amino acid stock solution, 100; and D-glucose 2.5 g/L. Salt stock solution consisted of (g/L): KH_2PO_4, 15; NaCl, 2.5; NH_4Cl, 5.0. Composition of vitamin stock solution (mg/L): D-biotin, 10; myo-inositol, 100; niacin, 100; pyridoxine, 100; riboflavin, 100; thiamine, 100. Composition of amino acid stock solution is described in Leejeerajumnean *et al.*

(2000). minYE medium consisted of (g/L): yeast extract, 2; Na_2HPO_4, 5.24; KH_2PO_4, 2.77; $MgSO_4 \cdot 7H_2O$, 0.25; NH_4Cl, 2.0; D+Glucose, 2.5. Depending on the performed experiment, 0.22 μm filter sterilized (BioRad) $MnSO_4 \cdot 4H_2O$ stock (1000x) was added to the cultivation medium. Manganese stock solution was prepared as described by Kaunietis (2012). All salts, peptone, yeast and meat extracts and D+Glucose were purchased from Merck company; amino acids and vitamins – from Sigma Aldrich and Merck companies.

In cultivation experiments with different carbon sources, cultivation media (TY, bMRS, mM9 or minYE) were prepared from separate components without adding glucose. Three different commercially available OS were used in this study: inulin (Alfa Aesar), palatinose hydrate (TCI America) and α-cyclodextrin (CD) (Merck). 10 % (w/v) stock solutions of these OS and D+Glucose were prepared in sterile distilled water and filter sterilized with 0.22 μm filters (BioRad). The sterile OS stock solutions were added to sterilized appropriate medium to obtain final concentration of each OS of 1% (w/v) (for LAB strains) (Saminathan *et al*., 2010) or 0.25 % (for 118 strain). D+Glucose (Merck), which was a favorable carbon source for all investigated strains, was used as control. Experiments were carried out repeatedly and standart deviation values were estimated.

Yeasts (Table 2) were cultivated in YPD medium, consisting of (g/L): yeast extract, 10; peptone, 20; dextrose/glucose, 20, at 30 °C (Digaitiene *et al*., 2012). Antibacterial activity tests against pathogenic bacteria (Table 2) were carried out at Institute of Infectious Disease and Pathogenic Microbiology and National Public Health Surveillance Laboratory (NPHSL).

PCR conditions and primers used in this study

PCR conditions were applied as follows: 1) pre-denaturation at 95 °C, 1–5 min; 2) denaturation at 95 °C, 1 min; 3) annealing at 48–65 °C (depending on the primers used), 2 min; 4) extension at 72 °C, 3–4 min; 5) final extension at 72 °C, 7 min; and preservation at 4 °C. Stages 2–4 were repeated 29 or more times, depending on the experiment. The amplified products were detected by agarose gel electrophoresis and visualized under UV light (Sambrook and Russel, 2001). MassRuler DNA Ladder Mix™ (1 kb) and GeneRuler DNA Ladder Mix™ (100 bp) (Thermo Fisher Scientific) were used as DNA size markers. For PCR products extraction and purification GeneJET Gel Extraction Kit™ and GeneJET

PCR Purification Kit™ (Thermo Fisher Scientific) were used. PCR products were sequenced at the Sequencing Center of VU Life Science Center (Lithuania).

Table 3. Primers used in this study. NA – not applicable, Tm – primer melting temperature.

Primer	Sequence	Tm, °C	Expected product (size, bp)	Source
1BACTP	F: 5'- TGA AGA TGT ATT TGG GTG CGT -3' R: 5'- CAG GAG TTG CTG GTG TTG TT -3'	57 58	Lacticin 3147 (257)	Digaitiene *et al.* (2012)
2BACTP	F: 5'- GCG CCT GCA GGG CTT TCT TTC GAT CAC GAT-3' R: 5'- GCG CGT CGA CGG TTC GAT AGT TCG TGC TT -3'	75 75	Pediocin PA (541)	
3BACTP	F: 5'- CTA TGT ACA CCC GGT TGT AA -3' R: 5'- TTT ATG AAC TAG GCG AAT CA -3'	56 52	Nisin (590)	
4BACTP	F: 5'- ACA GGT GGA AAA TAT TAT GGT A -3' R: 5'- TTT TGC TTA TTA TTT ATT CCA G -3'	55 51	Sakacin P (150)	
m2405	F: 5'- CAA CAT ATG GCC AAG CGA CGC -3' R: 5'- CGA GAA TTC CGC TAC TAT TTC CC -3'	63 63	Hypotethical protein from *L. paracasei* ATCC 334 (≤ 300)	Kuo *et al.* (2013)
m2406	F: 5'-TCA CAT ATG AAA AAG AAA TTT GAT TGT GCT-3' R: 5'- TAA GAA TTC GCC CAC TTC TTT AC -3'	63 59		
m2163	F: 5'- AAA CAT ATG AAA CGA AAG TGC CCC AAA AC-3' R: 5'- TTT GAA TTC GCG ACG ATC TCT TGA ACA TTC-3'	66 68		
m2386	F: 5'- ATT CAT ATG GAC AGC ATC CGT GAT GTT TC -3' R: 5'- TTT GAA TTC GCT GCC AGA ACA AGT TGG TT -3'	68 68		
m2393	F: 5'-GCT CAT ATG GAA AAC GGT GGT TTA TGG TCA-3' R: 5'- AAA GAA TTC GGA ATC CCA GAA TGG CAG C -3'	69 69		
BACTA164-NIZLL	F: 5'- ATG AGT ACA AAG ATT TTA ACT TGG ATT GGT-3' R: 5'- ATA AAC GAA TGC ACT TAT GAT GTT ACT GT -3'	64 63	Nisin	Swetwiwathana *et al.* (2009)
BACTWNC-NIZLL	F: 5'- CCG GAA TTC ATA AGG AGG CAC TCA AAA TG -3' R: 5'- CGG GGT ACC TAC TAT CCT TTG ATT TGG TT -3'	69 69	Nisin A	Millette *et al.* (2008)
LacS	F: 5'- ATG GAA TTR TTR CCR ACK GCY GCY GTY YTR TA-3' R: 5'- ATG RTG TTT RGC NSW RTA YTT -3'	72 54	Lacticin S	Rodrígues *et al.* (1995)
AcdT	F: 5'- ATG ATT TCA TCT CAT CAA AAA ACG -3' R: 5'- CTA AAAACC GTC AGT ATA ACG AAG GC -3'	57 65	Acidocin 8120 (141)	This work
CurA	F: 5'- GCG CAG GAA TGA TTT CTG TAG GC -3' R: 5'- GCT CTG CCT TCA AAT TAG ACC CTC -3'	65 65	Curvacin A (179)	
CurA2	F: 5'- GCG CAG GAA TGA TTT CTG TAG GC -3' R: 5'- TGG GAT CAT TTG GCG TCT GC -3'	65 60	Curvacin A and imun. gene (837)	
SakA	F: 5'- GCG CAG AGG AGA TTC TTA GTT ATG -3' R: 5'- CAT TCC AGC TAA ACC ACT AGC CC -3'	64 65	Sakacin A (197)	
SakP	F: 5'- GCA GAA GTA ACA GCA ATT ACA GGT GG -3' R: 5'- CGC TAG CGT ATT CTT AGA ATA GTG TGC -3'	66 67	Sakacin P (622)	
BOXA1R	R: 5'-CTA CGG CAA GGC GAC GCT GAC G-3'	52	NT	Versalovic *et al.* (1994)
27F 1495R	F: 5'-GAG AGT TTG ATC CTG GCT CAG-3' R: 5'-CTA CGG CTA CCT TGT TAC GA-3'	50.5 47.5	16S rDNR	Link*

* https://www.ncbi.nlm.nih.gov/pubmed/10079532

Primers used in this work (Table 3) were synthesized in Metabion laboratory.

Partial identification of bacteria and their produced antibacterial substances

Gram staining and KOH tests of thermophilic isolates were performed as described by Chandra and Mani (2011), except Safranin (Merck) was used instead of Carbol Fuschin in Gram staining.

For partial identification of LAB yogurt isolates and thermophilic 118 strain, genomic DNA of LAB strains was extracted using GeneJET DNA Extraction Kit^{TM} (Thermo Fisher Scientific). Genomic DNA of thermophilic 118 strain was extracted by standard technique as described by Sambrook and Rusell (2001). Universal bacterial 16S rRNA gene primers (Table 3) were used for PCR reactions. PCR products were purified and sequenced at the Sequencing Center of VU Life Sciences Center (Lithuania). *In silico* data analysis was performed with NCBI BLAST algorithm.

In order to determine the number of different strains among yogurt isolates, BOX-PCR was performed, using universal BOXA1R primer (Table 3). PCR conditions were applied as described by Versalovic *et al*. (1994). Results were analyzed in agarose gel electrophoresis (Sambrook and Rusell, 2001).

Partial identification of antibacterial substances produced by LAB strains was carried out using 17 pairs of primers (Table 3), described in scientific publications or constructed *in silico* according to well-known bacteriocin-encoding gene sequences (NCBI, GenBank) using Lasergene v7.1 (DNASTAR, JAV) software package.

Antibacterial activity evaluation.

Agar overlay method. The antibacterial activity of strains was determined using the agar overlay method with modifications. Isolated and type strains were tested against each other. Strain tested for antibacterial activity was grown overnight in the middle of Petri dish with an appropriate agar medium. Then the layer of agar medium with inoculated presumably sensitive strain was poured on top of the grown culture (Aween *et al*., 2012; Maria and Janakiraman, 2012). The agar medium with inoculated strain was prepared by adding 20% (v/v) of 1.2 OD_{590} culture suspension into an appropriate agar media.

Agar well diffusion assay. 0.5 cm diameter wells were made with sterile plastic pipette tips in the agar media with inoculated sensitive strain. The agar media with indicator strain was prepared by adding 20% (v/v) of culture inoculum into an appropriate agar media. Inoculum was prepared of 1.2 OD_{590} for LAB and 1.5 OD_{590} for thermophilic

9A11 indicator strain. LAB culture samples were taken every 2 h (in case of LA strain – every 4 h) from the beginning of cultivation in liquid media. Samples of thermophilic 118 strain were taken every hour. Samples were centrifuged (13 400 × g, 5 min) and the serial twofold dilutions of the cell free supernatants were made. The amount of 100 µl of every dilution was poured into the prepared wells and plates were incubated overnight at the temperature appropriate for the used indicator strain. Clear zones round the wells after incubation indicate the inhibitory activity, which was expressed quantitatively as arbitrary units per milliliter (AU/ml) and was calculated as the reciprocal of the highest dilution showing definite inhibition (Kormin *et al*., 2001), using formula $AU/ml=(1ml\times V^{-1})/D^{-1}$, where V is the volume of the sample poured into the well (ml), and D is the highest dilution of the sample giving the last clearly visible inhibition zone (Hyronimus *et al*., 1998).

Preparation of crude protein extracts (CPEs)

118 strain was cultivated in liquid mM9 or minYE medium at 55 °C with 180 rpm agitation till the late exponential phase. Cells were discarded by centrifugation (10 000 × g, 4 °C, 20 min). Culture fluid was saturated with ammonium sulfate to final 80 % (w/v) concentration and incubated on magnetic stirrer at 5-8 °C temperature for 12 h or overnight. Protein precipitate was collected by centrifugation (20 000 × g, 50 min). Pellets were suspended in small amount (under 10 ml) Tris-HCl buffer (pH 7.5; 50 mM) (Moreno *et al*., 2000; Rosenberg, 2005). Ammonium sulfate was removed from the protein solution by dialysis (Rosenberg, 2005), using Snake Skin Dialysis Tubing, 3.5 MWCO (Thermo Fisher Scientific). Dialysis was performed at 5-8 °C temperature. Obtained protein suspension was concentrated with PEG 35 000 (Sigma-Aldrich). Protein concentration of CPEs was determined spectrophotometrically using Coomassie Plus (Bradford) Protein Assay Kit™ (Thermo Fisher Scientific).

Determination of CAPEs physicochemical properties

Enzymatic treatment. CPEs were treated with the following enzymes at final concentration of 1 mg/ml: proteinase K, trypsin, α-chymotrypsin and β-chymotrypsin (Merck); pronase E, ficin, papain α-amylase and lipase (Sigma-Aldrich). All enzymes were dissolved in appropriate buffers according to the suppliers and literature (Moreno *et al*., 2000). Sample and enzyme mixtures were incubated for 2 h at 37°C. Enzymatic activity was stopped by incubation for 5 min at 100 °C (this treatment did not affect CPEs'

antibacterial activity). The remaining antibacterial activity was determined by agar well diffusion assay (Moreno *et al*., 2000). In control mixture, 50 mM Tris-HCl, pH 7.5 buffer was used instead of enzyme solution. Effect of enzyme solutions on the growth of indicator strain was also tested.

Sensitivity to various temperatures. In order to determine the impact of temperature changes on antibacterial activity, CPEs were incubated at different temperatures: 60, 70, 80, 90, 100 °C (for 30 min) and 121°C (for 15 and 30 min). The remaining antibacterial activity was determined by agar well diffusion assay. Samples were also kept at +4°C and -20 °C temperatures for 2 weeks in order to evaluate the stability of antibacterial substance. Untreated samples were taken as respective controls (Lee *et al*., 2001; Kumar *et al*., 2010).

pH treatment. Aiming to determine antibacterial substance's stability within wide pH range, CPEs were mixed with series of 100 mM buffers (ratio 1:1): pH 3-6 – citrate (Merck), pH 7 – Na phosphate (Merck), pH 8-10 – Tris-HCl (Sigma-Aldrich). Buffers were chosen and prepared according to the literature (Lee *et al*., 2001). Sample and pH buffer mixtures were incubated for 4 h at 4°C. After incubation, pH of sample mixtures was restored with 0.5 M Tris-HCl buffer, pH 7.5. The remaining antibacterial activity in pH treated samples was determined by agar well diffusion assay. In control mixture, 50 mM Tris-HCl, pH 7.5 buffer was used instead of pH buffer.

Treatment with detergents. CPEs were mixed with 1 % (w/v) detergent solutions (Tween-20, Tween-80, Triton X-100, SDS, Urea) at ratio 1:1 and incubated for 5 h at 37 °C (Kumar *et al*., 2010). Detergent solutions were prepared in 50 mM Tris-HCl, pH 7.5 buffer. The remaining antibacterial activity in detergent-treated samples was determined by agar well diffusion assay. In control mixture 50 mM Tris-HCl, pH 7.5 buffer was used instead of detergent solution.

Tricine Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (T-SDS-PAGE) and zymogram preparation

CPEs were analyzed using T-SDS-PAGE method, using 4 % T, 3 % C concentrating and 49.5 % T, 6 % C separating gels (Schägger, 2006). For reducing conditions 4x T-SDS-PAGE dye with 6% mercaptoethanol was used (Schägger, 2006). PageRuler Unstained Low Range Protein Ladder™ (Thermo Fisher Scientific) was used

as molecular mass marker. Samples were loaded in two recurring wells. After electrophoresis one part of the gel was stained with PageBlue Protein Staining Solution™ (Thermo Fisher Scientific), and the second one was used for zymogram preparation. This part of PAGE gel was fixed in fixing solution (20 % isopropanol (Roth); 10 % acetic acid (Merck); 70 % water) for 2 h. Afterwards the gel was washed with distilled water for at least two hours with gentle mixing, changing water every 15-20 min. Then the gel was placed in a sterile Petri dish and overlaid with agar NB media inoculated with indicator strain (preparation of the medium with indicator strain is described above). Prepared zymogram was incubated at 60 °C temperature for 6-12 h. After incubation zymogram was compared to the stained part of the gel. Clear zone in the zymogram indicates the position of antibacterial protein in the gel.

RESULTS AND DISCUSSION

1. Lactic Acid Bacteria (LAB) research

1.1. Isolation and partial identification of LAB yogurt isolates

Two probiotic yogurts: "Actimel" (Danone) and "Bifi" (Rokiškio pienas) were used for the isolation of probiotic bacteria. Yogurts were chosen according to variety and amounts of bacteria based on producers' information. 20 isolates were isolated from each yogurt and marked with capital letter (A or B) depending on the yogurt title and number (1 to 20). As morphology of all colonies did not differ, 9 *Lactobacillus* sp. isolates were randomly chosen for further studies: 4 A and 5 B isolates. Approximate amount of 10^8 colony forming units (CFU) in 1 ml of yogurts was determined, coinciding with the information given by the producers and meeting probiotic product requirements (Pan *et al.*, 2009).

Yogurt isolates were partially identified by 16S rDNA sequence comparison using NCBI BLAST algorithm. Although performing alignment in "Nucleotide collection (nt)" database the 16S rDNA sequences of two *Lactobacillus* sp. species with 100 % similarity were presented in the NCBI BLAST alignment results (*Lactobacillus casei* and *Lactobacillus paracasei*), it should be noted that both these species are phenotypically and phylogenetically very similar (they both belong to *L. casei* group) and phylogenetic status of these species was a subject of discussion for a long time (Dellaglio *et al*., 2002; Judicial Commission, 2008). Therefore, strains and, accordingly, their 16S rDNA sequences in NCBI database, are very often wrongly assigned to these species (Kuo *et al*., 2013; Smokvina *et al*., 2013), and 16S rDNA sequences of BL23, Zhang, LC2W bei BD-II strains, which were among the first 5 alignment results, in NCBI database assigned to *L. casei* specie, actually belong to *L. paracasei* specie (Toh *et al*., 2013). However, when sequences alignment was performed in "16S ribosomal RNA (Bacteria and Archaea)" database, in all cases 16S rDNA sequences showed the highest similarity belonged to *L. paracasei* species. These results coincide with the information given by the producers of "Bifi" yogurt. However, there should be *L. casei* Danone bacteria in "Actimel" yogurt, but not *L. paracasei*.

In order to determine the number of different strains among yogurt isolates, BOX-PCR analysis was performed. Two different profiles coinciding with the isolation source were observed analyzing obtained PCR products in agarose gel (Fig. 1).

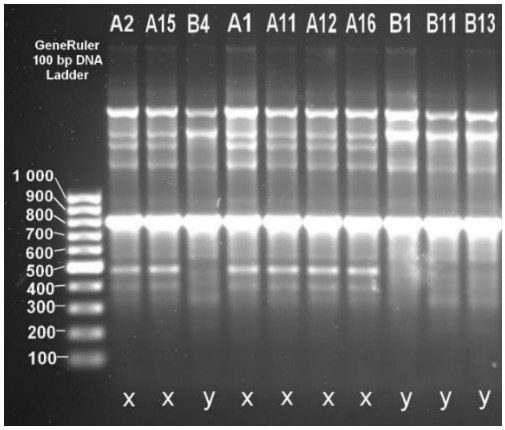


Fig. 1. BOX-PCR analysis results of LAB yogurt isolates in agarose gel. Isolate numbers are indicated at the top; on the left – GeneRuler™ DNA Ladder 100 bp (Thermo Fisher Scientific) molecular mass marker with indicated fragment masses; at the top – types of profiles (x and y indicates different profiles).

According to these results it can be concluded that yogurt isolates belonged to *L. paracasei* specie and represented two separate strains (A and B).

1.2. Evaluation of antibacterial activity of LAB yogurt isolates and type strains

Three isolates from every group (A and B) were used for antibacterial activity evaluation between yogurt isolates. Using agar overlay method it was determined that all tested strains formed clear zones inhibiting the growth of other overlaid isolate (isolates were tested against each other) (Table 4).

Table 4. Results of antibacterial activity evaluation of yogurt isolates. "+" – clear zone around producing isolate is less than 5 mm; "++" – clear zone is ≤ 10 mm; "+++" – clear zone is >10 mm.

		Sensitive isolate					
		A11	**A15**	**A20**	**B1**	**B4**	**B13**
Producing isolate	**A11**		+	++	+	+	+
	A15	+		++	+	+	+
	A20	+	+		+	+	+
	B1	++	++	+++		++	++
	B4	++	++	+++	+		+
	B13	+++	+++	+++	++	++	

According to the results it can be concluded that both A and B group isolates produced antibacterial substances. Sizes of inhibition zones showed that antibacterial substances produced by B group isolates were more active and/or secreted in higher

amounts or A isolates were more sensitive to antibacterial substances produced by B isolates. Although isolates of both A and B groups belonged to the same species, the observed differences in interspecies antagonism and antibacterial activity values could be explained by the fact that these groups represented two separate strains. According to literature, antibacterial activity spectrum and/or the level of activity caused by bacteriocin secretion can vary not only at the specie but also at the strain level (Moreno *et al*., 2000; Nuryshev *et al*., 2016).

Antibacterial activity between yogurt isolates and LAB type strains was also phenotypically evaluated (Table 5).

Table 5. Results of antibacterial activity evaluation between yogurt isolates and LAB type strains. "+/--" – clear zone around producing isolate is fairly visible, "+/-" – clear zone is less than 5 mm; "+" – clear zone is ≤ 10 mm; „++" – clear zone is ≤ 15 mm "+++" – clear zone is >15 mm.

		Sensitive strain								
		A11	**B13**	**A20**	**LA**	**LD**	**LL**	**LL2**	**LC**	**LS**
Producing strain	**A11**		+	++	+	+++	+++	+++	++	++
	B13	+++		+++	+	+++	+++	+++	++	++
	A20	+	+		++	+++	+++	+++	++	++
	LA	+/-	+/-	+/-		++	+	+	+/-	+
	LD	-	-	-	-		-	-	-	-
	LL	+/--	+/--	+/--	+/--	+		+	+	+
	LL2	++	+	++	+	+++	++		+++	+++
	LC	-	+/-	+/-	+/-	++	+	+		+
	LS	+	+/-	+	+/-	+	+	+	+	

All investigated LAB type strains (except LD) were characterized with antibacterial activity against other LAB (Table 5). Activity against closely related bacterial strains and/or species is one of the main traits of bacteriocins (Gillor *et al*., 2008; Chatterjee and Raichaudhuri, 2017; Kaškonienė *et al*., 2017). Therefore, it could be presumed that antibacterial activity of investigated LAB strains was determined by the secretion of bacteriocins.

1.3. Identification of antibacterial substances secreted by LAB

One of the possibilities to identify antibacterial substances is to search for bacteriocin-coding genes in the genomes of analyzed bacteria using PCR method. Therefore, 17 pairs of primers (Table 3) were used in PCR reactions with 15 LAB strains (5 type and 9 isolates) genomic DNA. Obtained PCR products are listed in Table 6.

Table 6. PCR products, obtained with all investigated LAB strains genomic DNA and bacteriocin specific primers (Table 3). ND – no data.

Bacterial strain (table 2.1)	Primers used (table 2.2)	Expected size of PCR product (bp)	Actual size of PCR product (bp)
Lactobacillus delbrueckii subsp. *bulgaricus* (LD)	1BACTP	257	~ 250
All yogurt isolates (A and B groups)	1BACTP	257	~ 1 700
Lactobacillus acidophilus (LA)	3BACTP	590	~ 250
A group yogurt isolates	3BACTP	590	~ 2 100
Lactococcus lactis subsp. *lactis* (LL)	3BACTP	590	~ 1 100
Lactococcus lactis subsp. *lactis* (LL2)	3BACTP	590	~ 600
Lactococcus lactis subsp. *lactis* (LL2)	BACTWNC-NIZLL	ND	~ 210
All yogurt isolates (A and B groups)	m2163 m2386 m2393 m2405 m2406	≤ 300	≤ 300
L. delbrueckii subsp. *bulgaricus* (LD)	CurA2	622	~1 400

Results of obtained PCR products size and sequence analysis can be clustered in four groups:

1) 2 PCR products coincided with nisin genes (3BACTP and BACTWNC-NIZLL) by their size and BLAST analysis results confirmed that LL2 strain most likely secreted nisin.
2) PCR products obtained using 5 primer pairs specific to *L. paracasei* ATCC 334 bacteriocins (m2163, m2386, m2393, m2405, m2406) (Kuo *et al*., 2013). Sequences of these products were homological to *L. paracasei* bacteriocin genes, allowing the assumption that observed antibacterial activity of A and B yogurt isolates was determined by the production of these bacteriocins and also confirming the identity of yogurt isolates.
3) PCR products obtained with primers constructed according to known bacteriocin genes (nisin (3BACTP), except the case of strain LL2 genomic DNA; lacticin 3147 (1BACTP); curvacin A and its immunity gene (CurA2)). These products did not match bacteriocin genes by their size and sequence analysis.
4) No PCR products were obtained in PCR reactions with genomic DNA of all investigated strains and primers constructed for known bacteriocin genes: pediocin PA, sacacin A, sacacin P (two pairs of primers), lacticin S, acidocin 8120 and curvacin A (Table 3).

1.4. LAB ability to assimilate OS

A11 and B13 yogurt isolates, representing different strains of *L. paracasei* species, were selected for further studies, according to the results of LAB antibacterial antagonism

and BOX-PCR analysis (Chapters 1.1 and 1.2). Three commercial prebiotic OS (inulin, palatinose and α-cyclodextrin (CD)) were used in this work. Glucose was used as control carbon source (Pranckutė *et al*., 2014).

Results presented in Fig. 2 indicate that both A11 and B13 yogurt isolates least efficiently assimilated palatinose. It was shown that *L. paracasei* 1195 strain was also unable to assimilate palatinose (Kaplan and Hutkins, 2003). While A11 isolate also ineffectively assimilated inulin, B13 isolate grew with this OS almost equally efficiently as with glucose, only in case of inulin exponential growth phase began approximately 4 h later than with glucose (Fig. 2). Ability of *L. paracasei* subsp. *paracasei* 8700:2 strain to assimilate inulin and FOS with various degree of polymerization (DP) was also described in literature (Makras *et al*., 2005), but also noted that OS assimilation is specific at species and even strain level (Makras *et al*., 2005; Kunova *et al*., 2011; Saminathan *et al*., 2011; Gänzle and Follador, 2012; Chung *et al*., 2016). This explaned observed differences between OS assimilation abilities of yogurt isolates, in spite of the fact that all isolates belonged to the same species.

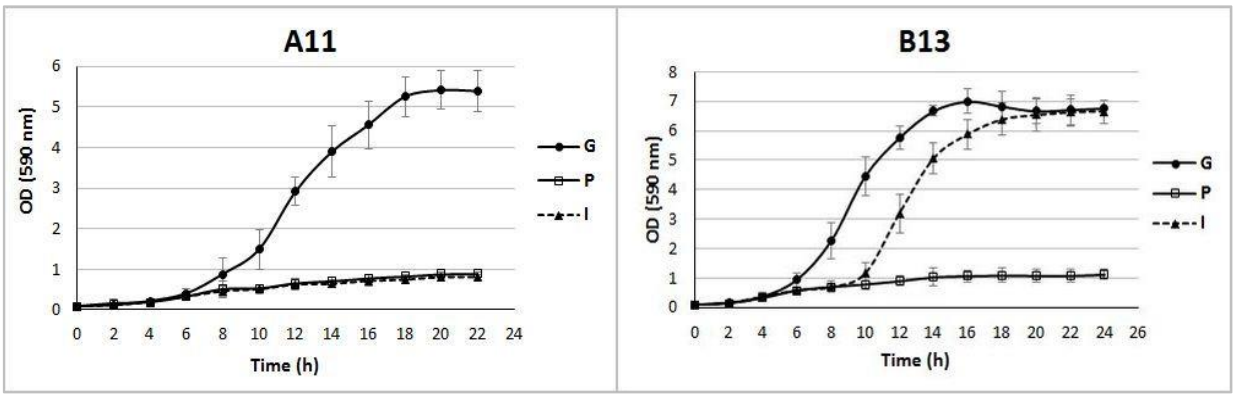


Fig. 2. OS influence on the growth of yogurt isolates A11 and B13. G – glucose, P – palatinose, I – inulin. Concentration of carbon source is 1% (w/v).

After addition of CD bMRS medium becomes opaque and culture OD changes can not be objectively evaluated. Therefore, ability of yogurt isolates to assimilate this OS was evaluated by counting CFU from culture samples grown with CD and glucose (for comparison), plated on solid MRS medium. Results have shown that both A and B group isolates did not assimilate CD: in 4 h samples of cultures grown with CD and glucose, CFU counts were of the same order (10^7 CFU/ml), but in 12 h samples with glucose, CFU counts increased up to 10^8 CFU/ml, while in samples with CD, concentration remained at 10^7 CFU/ml.

Analyzing the ability of type strains to assimilate OS revealed that palatinose and inulin were best assimilated by LL strain, but less effectively than glucose (Fig. 3). Also, exponential growth phase in the medium with palatinose lasted much longer (14 h) compared to exponential phase with glucose (8 h) (Fig. 3), showing that for assimilation of palatinose, LL strain most likely required metabolic rearrangement which slowed down the growth of the culture. Moreover, while cultivating this strain with CD as a sole carbon source, maximal reached OD_{590} was even higher compared with glucose, although in this case maximal OD_{590} was reached faster (Fig. 3). Therefore, it was showed that LL strain grew more efficiently on CD, but while glucose is easier assimilated, LL strain grew on it faster than on CD.

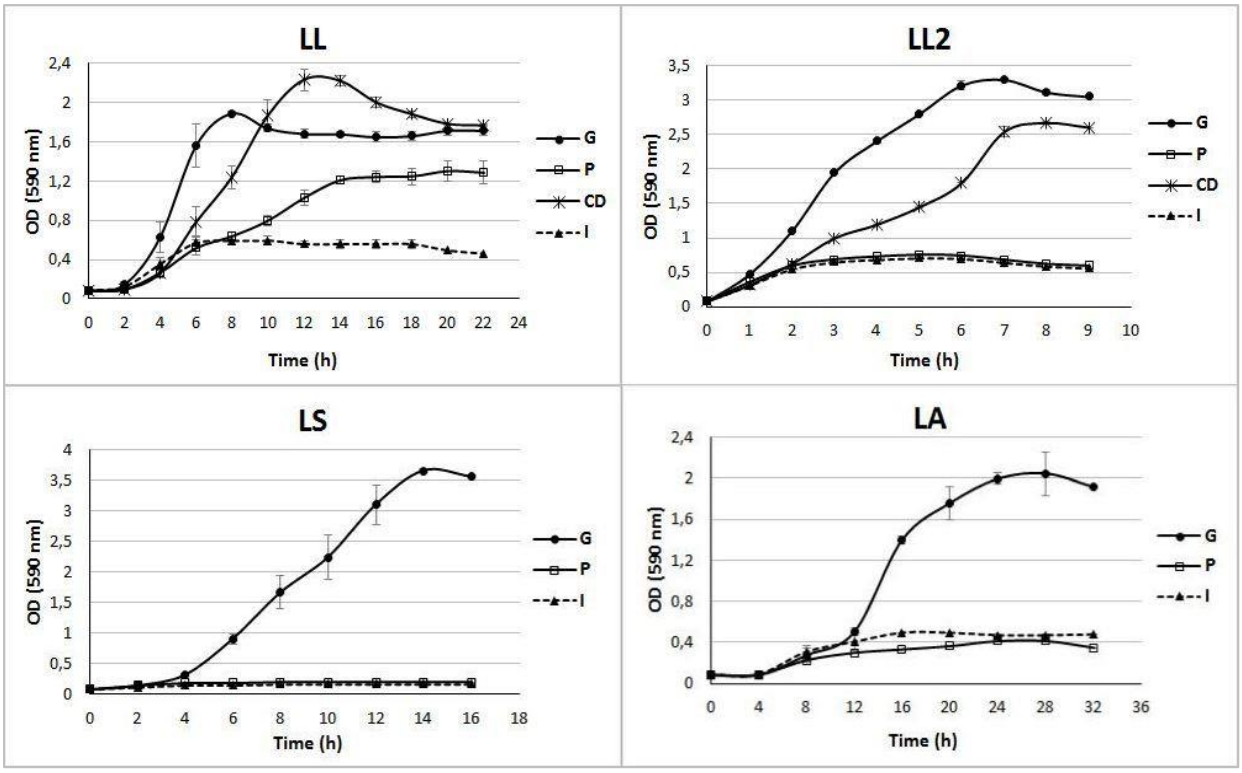


Fig. 3. OS influence on the growth of LAB type strains (LL, LL2, LS and LA, Table 1). G – glucose, P – palatinose, I – inulin, CD – α-cyclodextrin. Concentration of carbon source is 1% (w/v).

LL2 strain equally inefficiently assimilated inulin and palatinose – at the beginning of stationary phase only 0.5 OD_{590} was reached. As in the case of LL strain, LL2 strain was also capable of assimilating CD, but less efficiently than glucose (Fig. 3). Although, higher OD values using CD as a carbon source were observed during cultivation of LL2 strain, but compared to maximal OD values reached during growth on glucose, it can be concluded that CD was better assimilated by LL strain than LL2 strain.

Analysing the ability of *Lactobacillus* type strains to assimilate OS revealed that LS strain was completely unable to assimilate both palatinose and inulin. However, higly efficient growth on glucose has confirmed that cultivation conditions were suitable for this strain (Fig. 3).

LA strain also ineffectivelly assimilated these OS, although according to the literature *L. acidophilus* should be able to assimilate palatinose (van Zanten *et al*., 2012; Gänzle and Follador, 2012) and inulin (Goderska *et al*., 2008; Gänzle and Follador, 2012). LA is an obligatelly anaerobic bacterial strain and successful establishment of anaerobic conditions was proven by the effective culture growth on glucose (Fig. 3), showing that LA culture growth on OS was not observed only due to the strains inability to assimilate them but not due to inadequate cultivation conditions.

These results show that investigated LAB strains reacted to the used OS as carbon sources very differently. This opens an opportunity to modulate the growth of separate *Lactobacillus* sp. and *Lactococcus* sp. strains in mixed populations.

1.5. OS influence on antibacterial activity of LAB type strains and yogurt isolates

In this part of work the influence of prebiotic OS (inulin, palatinose and CD) on the antibacterial activity of investigated LAB strains was evaluated. Athough all tested strains characterized as sensitive to antibacterial substances produced by yogurt isolates (Table 5) were used for antibacterial activity evaluation of A11 and B13 isolates, no inhibition zones were observed. There are cases described in literature when LAB bacteriocin production was observed only during cultivation on solid medium, but not in the liquid one. However, in some cases (not all) bacteriocin production in liquid media can be stimulated by optimizing cultivation conditions (Heng *et al*., 2007; Maldonado-Barragán *et al*., 2009; O'Shea *et al*., 2012). Therefore, A11 and B13 isolates were cultivated in liquid medium applying different aeration conditions, pH values (5.2 – 7.2), Tween-80 concentrations (1 %; 0.5 %; 0.1 %), using mineral salt additives (Kaunietis, 2012) or different cultivation media (mM9), but antibacterial activity was not observed in any cases.

Inhibition zones were observed using *G. stearothermophilus* 9A11 strain as indicator, but only growing on glucose as a carbon source: in case of A11 isolate, small

10 AU/ml activity was observed from the 20th growth hour and in case of B13 isolate – from the 18th hour and was equal to 20 AU/ml.

During experiments with type LAB strains, antibacterial activity was determined only in cases of LL2 and LA strains, using LC and LS strains respectively (Table 1) as indicators as they were characterized being the most sensitive to antibacterial substances secreted by LL2 and LA strains (Table 5). Results of the OS influence on antibacterial activity are presented in tables 7 and 8 (Pranckutė *et al*., 2014).

Table 7. OS influence on the growth and antibacterial activity of *L. lactis* subsp. *lactis* (LL2) strain. AU/ml – arbitrary units of antibacterial activity. OD_{590} – culture optical density measured at 590 nm wawe length. Concentration of carbon source – 1 % (w/v).

Time (h)	**Glucose (C)**		**Palatinose**		**Inulin**		**CD**	
	AU/ml	**OD_{590}**	**AU/ml**	**$OD5_{90}$**	**AU/ml**	**$OD5_{90}$**	**AU/ml**	**OD_{590}**
2	0	1,11	0	0,59	0	0,55	0	0,63
4	0	2,41	0	0,73	0	0,68	0	1,20
6	40	3,22	0	0,74	0	0,70	80	1,80
8	320	3,12	20	0,74	20	0,59	320	2,67
10	160	3,06	10	0,59	10	0,56	320	2,61

Results of antibacterial activity evaluation showed that even in the cases of inefficient OS assimilation, antibacterial activity was still observed (Tables 7 and 8). While LL2 strain assimilated CD less efficiently than glucose (lower OD_{590} values were reached), antibacterial activity values were very similar (Table 7), indicating that relative antibacterial activity in medium with CD were higher compared to activity when cultivated with glucose. However, during LL2 growth on palatinose and inulin as sole carbon sources, only 20 AU/ml antibacterial activity was determined coinciding with LL2 strain's inability to assimilate these OS (Chapter 1.4).

Although LA strain was unable to efficiently assimilate both palatinose and inulin, antibacterial activity in culture grown on palatinose was at least 4 times higher, while maximal OD_{590} values were even lower compared to inulin (Table 8). Moreover, when comparing antibacterial activity and maximal reached OD_{590} values in cultures grown with palatinose and glucose, relative antibacterial activity with palatinose was estimated even 2.5-fold higher than with glucose (Table 8). These results showed that although LA strain's growth on palatinose as a carbon source was inefficient, but the production of antibacterial substances was much more intense compared to glucose. However, overall

maximal antibacterial activity reached during the culture growth with palatinose was two-fold lower (160 AU/ml) than with glucose (320 AU/ml) (Table 8).

Table 8. OS influence on the growth and antibacterial activity of *L. acidophilus* (LA) strain. AU/ml – arbitrary units of antibacterial activity. OD_{590} – culture optical density measured at 590 nm wave length. Concentration of carbon source – 1 % (w/v).

Time (h)	Glucose (C)		Palatinose		Inulin	
	AU/ml	OD_{590}	AU/ml	OD_{590}	AU/ml	OD_{590}
4	0	0,08	0	0,08	0	0,08
8	160	0,27	80	0,22	20	0,31
12	160	0,50	80	0,30	20	0,40
16	320	1,40	160	0,33	10	0,50
20	320	1,76	160	0,36	0	0,50
24	320	2,00	160	0,41	0	0,47
28	160	2,05	160	0,41	0	0,47
32	0	1,91	0	0,35	0	0,47

These results showed that although the investigated OS in most cases can not maintain as efficient culture growth as glucose, palatinose and CD have a considerable positive effect on LA and LL2 strains' (respectively) antibacterial activity.

1.6. Catabolic repression experiments

Strains, which expressed antibacterial activity during cultivation in liquid media (A11, B13, LL2 and LA; Chapter 1.5) were used in catabolic repression experiments (Pranckutė *et al*., 2016). These strains were cultivated using two carbon sources simultaneously – glucose and one of OS. OS were chosen according to the highest growth and/or antibacterial activity values of culture samples determined in previous studies (Chapters 1.4 and 1.5).

Comparing the growth of B13 isolate with glucose and both carbon sources (glucose and inulin) slight diauxic growth was observed (Fig. 4): untill 18^{th} hour, which was the beginning of the stationary phase when grown with glucose, in both cases (with glucose and both carbon sources) the growth was similar. Afterwards, in culture with both carbon sources the second exponential growth begins, showing that when all glucose is exhausted, assimilation of inulin begins. *L. paracasei* 1195 diauxic growth with glucose and fructooligosaccharides (FOS) was also described in the literature (Goh, 2007) and inulin is considered as long chain FOS (Gänzle and Fallador, 2012). On the other hand, during cultivation of A11 isolate, diauxic growth was not observed (Fig. 4): in both cases OD changes were similar showing that while glucose was still present in the medium inulin

was not assimilated. However, A11 isolate assimilated inulin very inefficiently and this assimilation rate could be insufficient for culture to transit into secondary exponential growth.

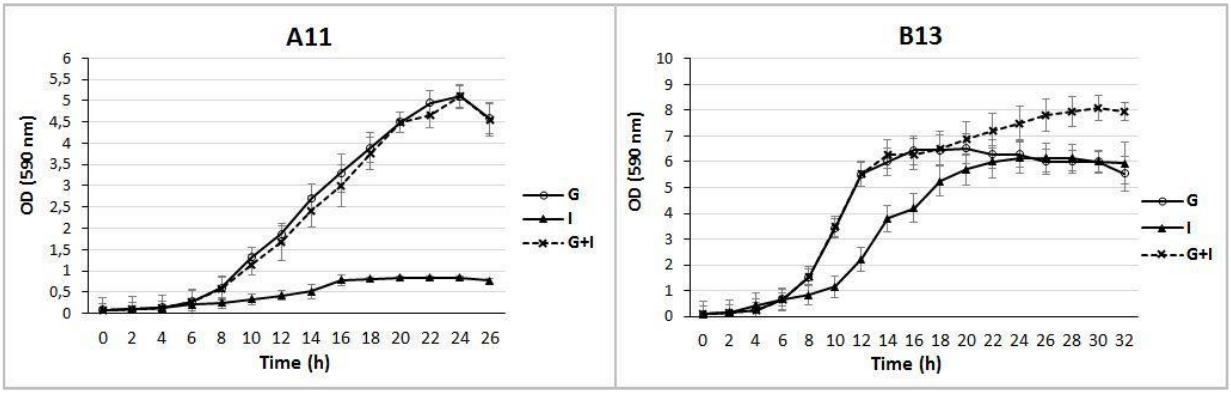


Fig. 4. Influence of two carbon sources (glucose (G) and inulin (I)) on the growth of A11 and B13 yogurt isolates. Concentration of every carbon source is 1 % (w/v).

Values of antibacterial activity coincided with the cell density changes. In case of A11 isolate in both variants (with glucose and with both carbon sources) antibacterial activity of 10 AU/ml was observed starting from 20th hour of growth. In case of B13 isolate, in medium with glucose 10 AU/ml activity was observed from 16th hour and remained unchanged till the end of growth. While in the medium with both carbon sources 10 AU/ml activity was also detected starting from 16th hour, antibacterial activity has doubled (20 AU/ml) and remained such till the end of growth after culture transition into the second exponential growth starting from 24th hour (Fig. 4).

During LA strain cultivation with two carbon sources (glucose and palatinose) diauxic growth was not observed but higher cell density values were reached (up to 1.8 OD_{590}) compared to ones measured in the medium with only glucose (up to 1.4 OD_{590}) (Fig. 5), showing that when growing with both carbon sources, assimilation of palatinose most likely began when glucose was still present in the medium. Therefore, the second logarithmic phase was not observed (Fig. 5). According to the literature, assimilation of different carbon sources in LAB is regulated hierarchically, but in some cases of *Lactobacillus* sp. bacteria catabolic repression manifests not always and particular carbon sources can be assimilated simultaneously with glucose (Sánchez *et al*., 2010; Gänzle and Follador, 2012). However, in both cases (with glucose and both carbon sources) no differences in the expression of antibacterial activity were observed: the highest activity (1280 AU/ml) was reached at 24th hour and remained stable till the end of growth.

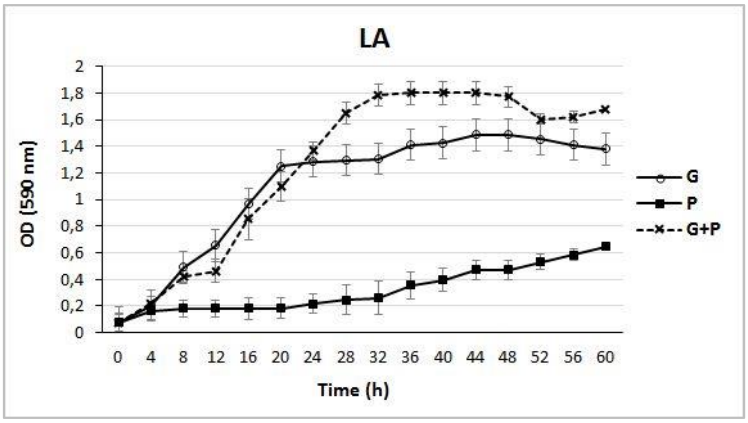


Fig. 5. Influence of two carbon sources (glucose (G) and palatinose (P)) on the growth of LA strain. Concentration of every carbon source is 1 % (w/v).

The most prominent diauxic growth was observed during cultivation of LL2 strain simultaneously with glucose and CD (Fig. 6). When cultivating LL2 strain with glucose and CD separately, in both cases cell density changes remained similar during entire growth (40 hours). While LL2 culture remained in stationary growth phase starting from 16th hour of cultivation in media with either glucose or CD, in culture with both carbon sources the second clearly distinguishable exponential growth phase began and lasted till 38th hour. During this second exponential growth maximal reached cell density was approximately 4.5-fold higher compared to the growth with separate carbon sources (Fig. 6). It shows that LL2 strain firstly consumed all glucose present in the medium and only after that – CD, which was assimilated more efficiently. These results coincided with literature where better assimilation of oligodextrans (DP from 3 to 9) compared to glucose by *L. lactis* bacteria was described (Grimoud *et al.*, 2010), but no data regarding the assimilation of cyclodextrins is available to date.

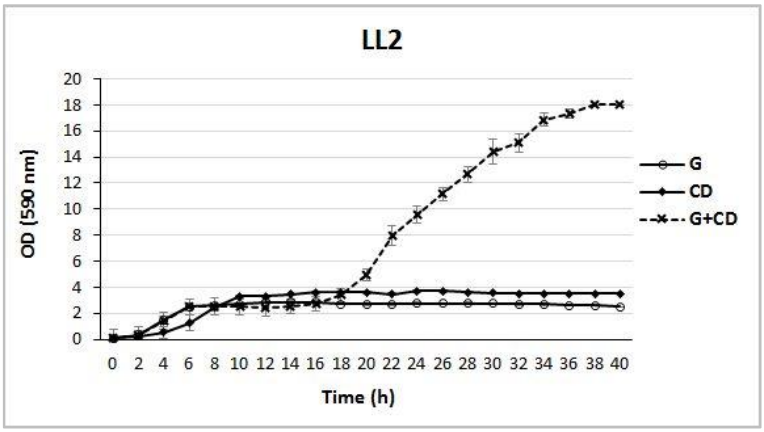


Fig. 6. Influence of two carbon sources (glucose (G) and CD) on the growth of LL2 strain. Concentration of every carbon source is 1 % (w/v).

Results of antibacterial activity evaluation showed that CD as a sole carbon source increased the antibacterial activity of LL2 strain significantly: in medium with CD maximal recorded activity (10240 AU/ml) was two-fold higher than in media with glucose or with both carbon sources (5120 AU/ml), although in this case even 4.5-fold higher cell density was reached (Fig. 6).

These results showed that combination of two carbon sources can greatly increase cell density reached by LL2 strain, which is very important aiming when aiming to apply this strain as probiotics since at least 10^7 CFU/ml viable bacteria should be present in the food product for its probiotic status confirmation (Pan *et al*., 2009).

2. Investigation of thermophilic 118 strain

2.1. Selection and partial identification of thermophilic strain with antibacterial activity

Thermophilic bacteria strain investigated in this work was selected from culture collection of Department of Microbiology and Biotechnology, Vilnius University. Strains of this collection were isolated from surface soil above the oil pools in Lithuania (Pranckutė *et al*., 2015).

Antibacterial activity of isolates was tested by agar overlay method using *G. stearothermophilus* 9A11 strain as indicator. Antibacterial activity between isolates was also evaluated (Pranckutė *et al*., 2015). One of 7 isolates with the highest antibacterial activity was chosen for further studies. This isolate (named 118 strain) was facultative thermophile (growth range: 45-60 °C) (Pranckutė *et al*., 2015).

Partial identification of 118 strain was performed by 16S rDNA sequence alignment in NCBI database. The top ten results of NCBI BLAST analysis with the highest similarity (99 %) were ribosomal gene sequences of *Aeribacillus/Geobacillus pallidus* or uncultured *Geobacillus* sp. bacteria. Therefore, it can be concluded that 118 strain was closest to *Aeribacillus pallidus* species.

Bacillus pallidus sp. nov. name was granted for the first time to thermophilic bacteria isolated from thermally treated wastewaters (Scholz *et al*., 1987). According to phylogenetic analysis and other characteristics of genus *Geobacillus* (Nazina *et al.,* 2001), it was proposed to rename *Bacillus pallidus* sp. nov. as *Geobacillus pallidus* sp. nov. with

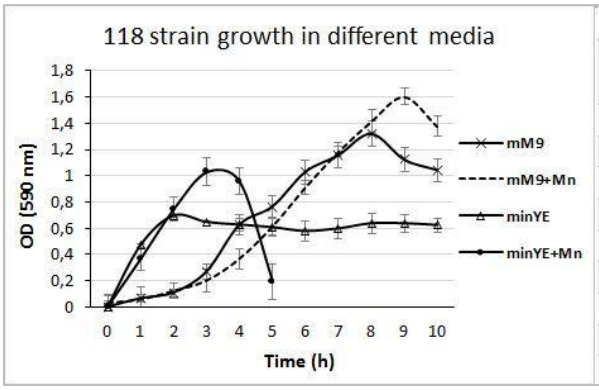
type strain H12^T (=ATCC 51176^T=DSM 3670^T=LMG 19006^T) (Banat *et al*., 2004). However, due to the discrepancies in phylogenetic analyses, DNA G+C content, fatty acid and polar lipid profiles, in 2010 it was proposed to rename *Geobacillus pallidus* sp. nov. as *Aeribacillus pallidus* gen. nov., comb. nov., with type strain H12^T (=ATCC 51176^T=DSM 3670^T=LMG 19006^T) (Miñana-Galbis *et al*., 2010).

In cooperation with University of Groningen, the genome of 118 strain was sequenced and deposited at GenBank database (Accession No.: LVHY00000000). Two putative bacteriocin encoding genes were identified using RAST server and BAGEL3 (Kaunietis *et al*., 2016).

2.2. Optimization of cultivation conditions for 118 strain

In order to extract antibacterial substances, suitable culture medium and growth conditions for optimal antibacterial substance production should be firstly assessed as it is well known that environmental conditions can have a huge influence on production of antibacterial substances and bacteriocins among them (Gautam and Sharma, 2009; O'Shea *et al*., 2012; Garsa *et al*., 2014). Media with the lowest amount of additional proteins were chosen for further preparation of crude protein extracts (CPEs). Minimal medium is also useful in order to create starvation conditions as bacteriocin production is often induced by nutrient depletion as a mean of competition (Heng *et al*., 2007; O'Shea *et al*., 2012; Snyder and Worobo, 2014; Egan *et al*., 2016). Therefore, mineral mM9 (with no additional proteins) and minimal minYE media were used in cultivation and antibacterial activity evaluation experiments of 118 strain.

Nutrients depletion also induces spore formation. It is known that in endospore-forming bacteria bacteriocin production and spore formation are often associated (Abriouel *et al*., 2011). Sporulation of *Bacillus* sp. bacteria can be induced by Mn^{2+} required for the activity of enzymes participating in the catabolism of certain carbohydrates. In the absence of Mn^{2+} intermediates of carbon compound metabolism start to accumulate in the cell inhibiting sporulation process (Vasantha and Freese, 1979). Accordingly, $MnSO_4$ additive was used in 118 strain's cultivation experiments in order to determine if Mn^{2+} induces sporulation of this strain and its influence on antibacterial activity. Cultivation experiments were performed in both media with and without $MnSO_4$ supplement. Results are presented in Fig. 7.



Antibacterial activity (AU/ml)				
Time (h)	mM9	mM9 +$MnSO_4$	minYE	minYE +$MnSO_4$
2	0	0	40	20
3	10	20	80	80
4	20	80	80	640
5	20	80	320	160
6	40	160	160	-
7	80	320	160	-
8	160	160	80	-
9	160	80	80	-
10	80	80	80	-

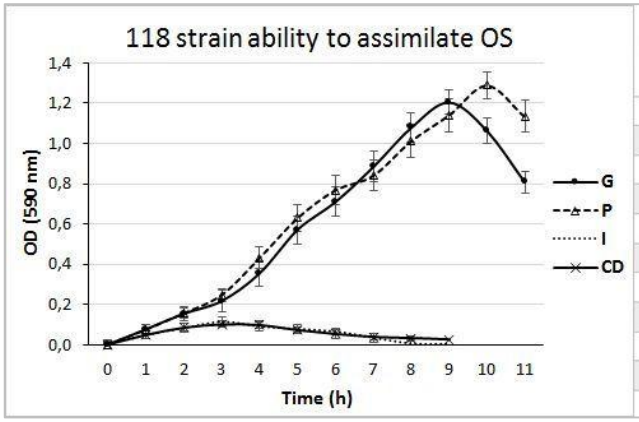
Fig. 7. Evaluation of growth and antibacterial activity of 118 strain using different cultivation media. Mn – $MnSO_4$ supplement.

Results showed that 118 strain is capable of growing and producing antibacterial substances in both media (Fig. 7). According to the estimated culture density values (Fig. 7), higher antibacterial activity was observed in minYE medium. Furthermore, exponential growth in minYE medium with $MnSO_4$ lasted only 3 h (Fig. 7). Typically, sporulation is induced when all nutrients are consumed and culture transits into stationary phase (Sonenshein, 2000; Phillips and Strauch, 2002; Gonźalez-Pastor, 2010). Precisely at this stage of culture growth the highest antibacterial activity values were recorded (Fig. 7). Such significant effect of manganese exposure on culture growth time was not observed in mM9 medium variants, most likely due to the extremely minimal composition of the medium, although increased antibacterial activity was determined earlier in mM9 medium with $MnSO_4$. These results showed that manganese ions induced 118 strain's sporulation which positively affected the expression of antibacterial activity. More efficient bacteriocin production during sporulation was also shown in cases of some other endospore-forming bacteria (Touraki *et al*., 2012; Berić *et al*., 2014; Huang *et al*., 2016).

2.3. OS influence on growth and antibacterial activity of 118 strain

Antibacterial substance produced by 118 strain was investigated aiming to evaluate its potential for application in food industry. Therefore, cultivation experiments using prebiotic OS as carbon sources were performed in order to estimate their influence on the growth and antibacterial activity of this strain. Minimal mM9 with $MnSO_4$ supplement was used. This medium was chosen based on its minimal composition and more extended exponential growth, compared to minYE medium (Fig. 7), allowing to evaluate the influence of carbon source on the culture growth more accurately.

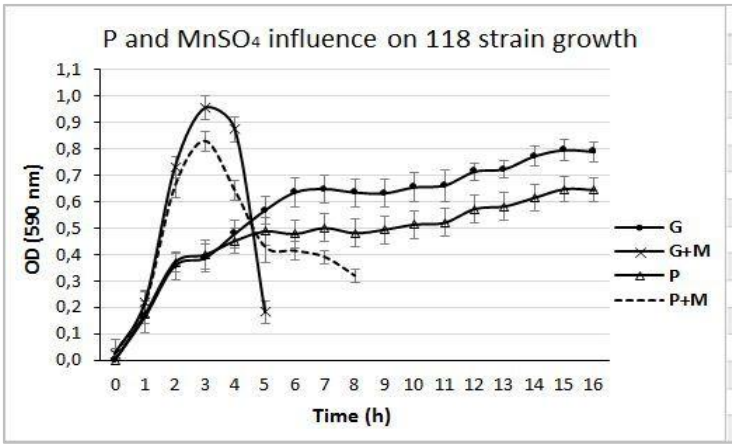
It was determined that from all used OS the assimilation of palatinose by 118 strain was the most efficient (Fig. 8), but this strain was completely unable to assimilate CD and inulin (0.1 OD_{590} was reached), even though during such inefficient growth antibacterial activity was still observed (Fig. 8).



Time (h)	Antibacterial activity (AU/ml) G	P	I	CD
0	0	0	0	0
1	0	0	0	0
2	40	160	160	20
3	80	160	320	10
4	640	640	80	10
5	640	640	0	10
6	640	640	0	0
7	1280	1280	0	0
8	640	1280	0	0
9	640	1280	0	0
10	640	1280	0	0

Fig. 8. Results of 118 strain's growth and antibacterial activity during cultivation in mM9 medium with different carbon sources. G – glucose, P – palatinose, I – inulin, CD – α-cyclodextrin. Concentration of carbon source – 0.25 % (w/v).

According to these results only glucose and palatinose were used as carbon sources for cultivation experiments in other medium (minYE), which was chosen for extraction of antibacterial substances. Obtained results (Fig. 9) showed that in both cases growth tendencies were similar and strain was capable of assimilating palatinose almost as efficiently as glucose.



Time (h)	Antibacterial activity (AU/ml) G	G+M	P	P+M
1	0	0	0	0
2	80	20	80	40
3	160	80	160	320
4	160	640	320	640
5	320	160	160	1280
6	160	-	160	640
7	160	-	160	640
8	80	-	160	320
9	80	-	80	-
10	160	-	80	-
11	80	-	160	-
12	160	-	160	-
13	320	-	320	-
14	320	-	320	-

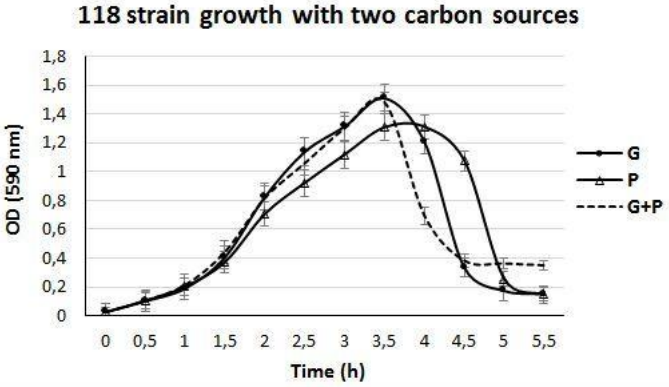
Fig. 9. Results of 118 strain's growth and antibacterial activity during cultivation in minYE medium with different carbon sources (glucose (G) or palatinose (P)) and with or without $MnSO_4$ supplement. Concentration of carbon source – 0.25 % (w/v).

Antibacterial activity values replicated culture growth tendencies (Fig. 9). According to cell density values during cultivation with palatinose as a carbon source,

antibacterial activity was estimated higher compared to glucose (Fig. 9), showing that palatinose positively affected 118 strains antibacterial activity.

2.4. Catabolic repression experiments

Cultivation of 118 strain in minYE medium with $MnSO_4$ supplement using two carbon sources (glucose and palatinose) simultaneously and separately was performed in order to determine the regulation of carbon sources assimilation by this strain (Fig. 10).



	Antibacterial activity (AU/ml)		
Time (h)	G	P	G+P
0	0	0	0
0,5	40	10	40
1	40	160	320
1,5	160	160	320
2	640	640	640
2,5	640	640	640
3	640	640	1280
3,5	1280	1280	2560
4	1280	2560	5120
4,5	1280	5120	2560
5	640	2560	1280

Fig. 10. Results of 118 strain's growth and antibacterial activity during cultivation in minYE medium with $MnSO_4$ supplement using two carbon sources (glucose (G) and palatinose (P)). Concentration of each carbon source – 0.25 % (w/v).

Although during cultivation with both carbon sources diauxic growth was not observed and OD values were similar to ones using only glucose, higher antibacterial activity values were estimated when growing the strain with palatinose and both carbon sources (Fig. 10). These results indicated a high compatibility of 118 strain with palatinose and possibility to apply this strain in protection and shelf life extenssion of food products in which palatinose is used as prebiotic or sugar replacement.

2.5. Extraction of antibacterial substances produced by 118 strain

Both pure and only partially purified bacteriocins can be applied in food industry (Mills *et al*., 2011; Balciunas *et al*., 2013; López-Cuellar *et al*., 2016). Therefore, crude protein extracts were prepared in order to evaluate the influence of different carbon sources and sporulation induction on properties of antibacterial substance in protein mixture and to determine whether these factors can induce production of additional antibacterial substances. minYE medium was used for isolation of crude protein extracts (CPEs) as

there are very little additional proteins in its composition and efficient growth and antibacterial activity was observed (Fig. 7 and 9).

4 CPE variants were prepared: using glucose or palatinose as sole carbon sources and in both cases – with or without $MnSO_4$ supplement. Protein concentrations and antibacterial activity values of CPEs are presented in Table 9.

Table 9. Protein concentrations and antibacterial activity evaluation of CPEs obtained from cultures of 118 strain. G (glucose) and P (palatinose) indicate used carbon source; M – $MnSO_4$ supplement.

Sample	**G**	**GM**	**P**	**PM**
Protein concentration (mg/ml)	21,52	15,39	18,02	20,65
Antibacterial activity (AU/ml)	40960	40960	20480	40960

Similar protein concentrations were estimated in all 4 CPEs (Table 9). Lower antibacterial activity of P sample was probably caused by experimental variation owning to both biological and technical effects.

2.6. Determination of molecular mass, quantity and sensitivity to reducing agents of antibacterial substances produced by 118 strain

In order to determine whether sporulation and different carbon source has any influence on the quantity of produced antibacterial substances, all 4 CPEs were analyzed using T-SDS-PAGE and zymography methods (Fig. 11, A).

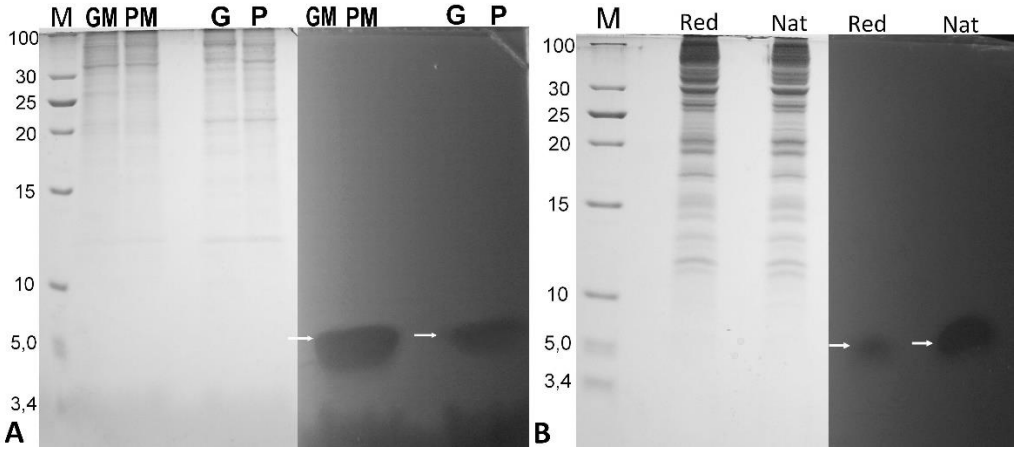


Fig. 11. Analysis of 118 strain's CPEs by T-SDS-PAGE and zymography methods. **A** – analysis of CPEs. CPE variants are indicated at the top: G (glucose) and P (palatinose) denote used carbon source; M – $MnSO_4$ supplement. **B** –evaluation of 118 strains antibacterial substance's sensitivity to reducing conditions. Applied conditions are as indicated at the top: Nat – native conditions; Red – reducing conditions. In both pictures PAG parts stained with PageBlue are on the left side, zymogram – on the right side. Molecular mass marker (M) – "PageRuler^{TM} Unstained Low Range Protein Ladder" (molecular masses are indicated at the right, kDa). Arrows indicates inhibition zones in zymogram.

Obtained different protein profiles confirmed that manganese ions induce 118 strain's sporulation during which cell metabolism, gene expression and accordingly – protein secretion, changes considerably (Phillips and Strauch, 2002; Egan *et al*., 2016). It was determined that in all cases 118 strain produced one antibacterial substance with approximate molecular mass of 5 kDa which coincided with the most common molecular masses of bacteriocins described in literature (Heng *et al*., 2007; Perez *et al*., 2014; Egan *et al*., 2016).

Considerably smaller inhibition zone was observed when sample was fractionated in reducing conditions (Fig. 11, B), meaning that disulfide bonds important for antibacterial activity manifestation were most likely present in the structure of investigated antibacterial substance.

2.7. Evaluation of physicochemical properties of antibacterial substance produced by 118 strain

According to previous results (Chapter 2.6) only one CPE (GM) was used for the evaluation of physicochemical properties of target antibacterial substance since the highest relative antibacterial activity was estimated in this CPE sample (Table 9).

Enzymatic treatment

The effect of proteolytic, amilolytic and lipolytic enzymes on antibacterial activity of CAP was evaluated. Proteinase K and pronase E almost completely diminished antibacterial activity of CPE, while ficin and papain reduced it two-fold. As all these enzymes are proteolytic, the obtained results confirmed the proteinaceous nature of investigated antibacterial substance. However, other proteolytic enzymes (trypsin and both chymotrypsins) did not affect antibacterial activity of CPE. It could be explained by the fact that bacteriocins are usually small peptides and the length of their amino acid sequence could be insufficient for specific recognition sites of these more specific enzymes.

As neither lipase or α-amilase had any effect on antibacterial activity of CPE it can be concluded that no carbohydrate or lipid moieties important for the activity are present in the structure of investigated antibacterial substance.

Effect of different temperatures

Thermostability of bioactive substance is extremely important for its application in food industry. Therefore, effect of different temperatures on CPE antibacterial activity was evaluated. It was determined that investigated antibacterial substance remained 100 % active after 30 min treatments in temperatures ranging from 70 to 100 °C while the most commonly applied food pasteurization temperatures are 70-80 °C (Gautam and Sharma, 2009). Also, 100 % activity was retained after incubation for two weeks at storage conditions (-20 and 4 °C) usually applied for most food products.

Even up to 12.5 % of antibacterial activity remained after application of sterilization conditions (121 °C for 15 min) which makes this substance extremely favorable for application in ultra-high temperature treated food (for example, ultra-high temperature treated milk or canned food) (Egan *et al*., 2016) protection and shelf life extension.

Sensitivity to various pH values and detergents

pH values in food industry are often very diverse so it is important that bioactive substance is resistant to pH changes. Therefore, stability of investigated antibacterial substance at pH values ranging from 3 to 10 was evaluated. In all cases 100% remaining activity was determined.

As some of detergents are used in food industry and agriculture (Food Safety Comission, 2007; EFSA, 2012), their influence on CPE's antibacterial activity was also evaluated. As some of used detergents (NDS, Tween-20 and Triton X-100) inhibited the growth of 9A11 indicator strain, only Tween-80 and Urea were used in this experiment.

It was determined that Tween-80 (polysorbate-80) and Urea did not affect antibacterial activity of CPE. These results are useful when aiming to apply the investigated antibacterial substance in food industry or agriculture as Tween-80 is used in various food products as emulsifier (Food Safety Comission, 2007). Moreover, polysorbates are often applied in combination with bacteriocins (Liang *et al*., 2005) as they can enhance their antibacterial activity (Gänzle *et al*., 2000; Cleveland *et al*., 2001). Meanwhile, Urea is used as rumen feed additive (EFSA, 2012).

Judging by the estimated molecular mass, physicochemical properties, sensitivity to reducing conditions and secretion during exponential growth phase it can be presumed

that antibacterial substance produced by 118 strain is most likely a bacteriocin belonging to I or II class (Heng *et al*., 2007; Perez *et al.*, 2014; Chatterjee and Raichaudhuri, 2017). Similar results were obtained during investigations of other bacteriocins produced by thermophilic or thermotolerant bacteria, such as thermophilin 1277 (Kabuki *et al*., 2007), thermophilin 347 (Vilani *et al*., 1995) and bacteriocins produced by *G. stearothermophilus* strains (Pokusaeva *et al*., 2009).

3. Determination of antibacterial activity spectra

3.1. Evaluation of antibacterial activity spectra of LAB strains

Samples from LAB cultures grown with OS and glucose were analyzed against food born pathogenic bacteria using agar well diffusion method. Only strains (A11 and B13 yogurt isolates and LA and LL2 type strains) characterized with antibacterial activity during cultivation in liquid media with OS were analyzed (Chapter 1.5). Pathogenic bacteria strains which were inhibited by at least one of the samples and estimated antibacterial activity values, are listed in the Table 10.

Table 10. Activity of culture samples of investigated LAB type strains and yogurt isolates grown with glucose and OS against pathogenic bacteria. "-"– inhibitory activity was not observed. G – glucose, P – palatinose, I – inulin, CD – α-cyclodextrin.

	Antibacterial activity (AU/ml)							
Tested strain	**A11**		**B13**		**LA**		**LL2**	
Sample / **Pathogen**	**G**	**I**	**G**	**I**	**G**	**P**	**G**	**CD**
Bacillus cereus DSM 12001	10	-	10	-	10	-	20	40
Enterococcus faecalis ATCC 29212	10	-	-	-	-	-	20	80
Enterococcus faecium 402-3/03	-	-	-	-	-	-	20	40
Enterococcus faecium ATCC 19434	10	-	-	-	-	-	20	80
Listeria monocytogenes ATCC 19117	-	-	10	-	-	-	40	40
Pseudomonas aeruginosa ATCC 27853	-	-	-	-	20	-	-	-
Staphylococcus aureus ATCC 25923	-	-	-	-	-	-	10	80
Streptococcus pyogenes ATCC 19615	-	-	-	-	-	-	80	80
Streptococcus pyogenes MPK 2331	10	10	40	40	160	20	640	640

None of the investigated LAB strains were active against used gram-negative pathogenic bacteria (Table 2) as is commonly stated in the literature (Mills *et al*., 2011; Maina *et al*., 2017). The only exception was LA strain, characterized with weak activity (20 AU/ml) against *P. aeruginosa* ATCC 27853 (Table 10). Activity of *L. acidophilus* strains against *P. aeruginosa* bacteria was also described in several other studies (Bernet-

Camard *et al*., 1997; Lonkar *et al*., 2005; Jebur, 2010; Jamalifar *et al*., 2011; Bharal and Sophal, 2013; Abdulla, 2014; Mahmood *et al*., 2014).

All samples of investigated LAB strains grown with glucose were active against *Streptococcus pyogenes*, a pathogenic bacteria able to cause notorious infections (Gillor *et al*., 2008; Stevens *et al*., 2016) and *Bacillus cereus* – a common meat and dairy product spailage bacteria (Abriouel *et al*., 2011; Sumi *et al*., 2015). However, only activity of *L. acidophilus* against *S. pyogenes* was shown in few researches (Bharal and Sophal, 2013; Abdulla, 2014). Although two strains of *S. pyogenes* were used, only samples of LL2 strain inhibited both of their growth (Table 10). Judging from the estimated values of antibacterial activity (Table 10) it can be presumed that *S. pyogenes* MPK 2331 strain is more sensitive to antibacterial substances produced by investigated LAB strains. These results coincide with literature where it is shown that sensitivity of pathogenic strains to antibacterial substances is specific and can vary between different strains (Meghrous *et al*., 1999; Swetwiwathana *et al*., 2009; Saraiva *et al*., 2014).

LL2 strain was characterized with the broadest antibacterial spectrum – it was active against half of the tested pathogenic strains. Samples of LL2 strain grown with glucose and CD were active against all other tested gram-positive bacteria (at least against one of the used strains) (Table 10). While, antibacterial spectra of both samples did not differ, 2- to 4-fold higher antibacterial activity values were determined for CD sample (Table 10). These results indicated that while CD can considerably increase the growth of LL2 strain, it can also enhance its antibacterial activity against pathogenic bacteria, which makes this combination even more favorable as potential synbiotic.

Although both investigated yogurt isolates (A11 and B13) belong to the same species (Chapter 1.1), their antibacterial activity spectra were not identical (Table 10), confirming that A and B isolates represented different strains. Many examples were described when antibacterial activity spectra vary even among different strains of the same species (Moreno *et al*., 2000; Abdulla, 2014; Nuryshev *et al*., 2016).

In most cases antibacterial activity of yogurt isolates and LA strain was determined only in samples of culture grown with glucose (Table 10). This can be explained by the inability of these strains to effectively assimilate OS leading to insufficient culture growth and, accordingly, poor production of antibacterial substances

(Chapters 1.4 and 1.5). Therefore, overall antibacterial activity of samples obtained with OS could be too small for inhibition of the growth of pathogenic bacteria.

Antibacterial spectra determined in this work in most cases did not coincide and/or were narrower comparing to the ones described in the literature. Despite specificities of antibacterial activity spectra and pathogenic strain sensitivity to antibacterial substances, mentioned before, inconsistencies of pathogenic strains used in this work and in other studies may also have an influence on the observed differences. Furthermore, estimated antibacterial activity of tested samples was relatively small (Table 10) and could be insufficient for complete evaluation of antibacterial spectra. These facts could also explain why in cases where two strains of the same pathogenic bacteria species were tested, antibacterial activity was detected only against one of them. On the other hand, even narrower antibacterial spectrum was determined in the research where primers specific for *L. paracasei* ATCC 344 bacteriocins were described (Kuo *et al*., 2013) compared to antibacterial spectra of yogurt isolates most likely producing these bacteriocins (Chapter 1.3).

3.2. Determination of antibacterial activity spectrum of 118 strain

Effect of bacteriocin produced by 118 strain on food-borne pathogens was also evaluated using agar well diffusion method. Sensitive pathogenic strains and estimated antibacterial activity values are presented in Table 11.

Table 11. Activity of 118 strain's CPE against pathogenic bacteria.

Sensitive pathogenic bacteria strain	Antibacterial activity of CPE (AU/ml)
Bacillus cereus ATCC 11778	20
Enterococcus faecalis ATCC 29212	10
Staphylococcus aureus ATCC 25923	10
Streptococcus pyogenes MPK 2331	40

It was determined that antibacterial substance produced by 118 strain was active against 4 (Table 11) of 16 tested pathogenic bacterial strains (Table 2). Narrow antibacterial specter is characteristic to bacteriocins (Heng *et al*., 2007; Gillor *et al*., 2008; Chatterjee and Raichaudhuri, 2017). However, in all cases detected antibacterial activity of CPE was relatively low (up to 40 AU/ml) comparing to the value estimated in previous experiments (Table 9) most likely due to the lower sensitivity of tested pathogens comparing to the highly sensitive 9A11 indicator strain. This could explain why

antibacterial activity was observed only against one (Table 11) of the two tested pathogenic bacterial strains of the same species (Table 2).

While no data exists describing bacteriocins or bacteriocin-like substances (BLIS) produced by *Aeribacillus pallidus* (or previously used names of this species), it is difficult to objectively evaluate the antibacterial spectrum of 118 strain determined in this work. Only one group of researches had investigated antibacterial substance produced by *A. pallidus* strain, and although it was proteinaceous, it was a secondary 37 kDa metabolite belonging to peptide antibiotics and not bacteriocin (Muhammad and Ahmed, 2015). Therefore, it can be concluded that antibacterial activity of 118 strain was determined by the different substance and it was not appropriate to compare their antibacterial spectra.

3.3. Evaluation of antibacterial activity between LAB and thermophilic strains and toxicity to eukaryotic cells

Antibacterial activity between investigated LAB and thermophilic strains was evaluated using agar overlay method. Since both thermophilic strains did not inhibit the growth of any of the tested LAB strains, only results of their sensitivity to antibacterial substances produced by LAB is presented in Table 12.

Table 12. Results of antibacterial activity between investigated LAB and thermophilic strains. "-" – no inhibition zone was detected; "+" – clear zone around producing strain is ≤5 mm; "++" – clear zone is ≤ 10 mm; "+++" – clear zone is ≤15 mm; "++++" – clear zone is >15 mm. NA – not applicable.

	Strains producing antibacterial substances							
Sensitive strains	**LL2**	**LL**	**LA**	**LC**	**LS**	**A11**	**B13**	**118**
118	++	+	++	++	++	+++	+++	NA
9A11	+++	-	+++	++++	++++	++++	++++	+++

It was shown that 118 did not inhibit the growth of any of the tested LAB strains but was sensitive to all the LAB strains used. While most of investigated LAB strains can be used as probiotics (Saad *et al*., 2013; Dixit *et al*., 2016) these results showed a great potential of 118 strain and/or its produced bacteriocin for protection of probiotic food products.

Nearly all of the tested strains (except LL strain) were characterized with high antibacterial activity against *G. stearothermophilus* 9A11 bacteria (Table 12). While *G. stearothermophilus* is a common caned and dairy products spoiling bacteria (Gopal *et al*.,

2015; André *et al*., 2017), obtained results showed potential application of investigated strains for combating these food spoilage bacteria.

Effect of antibacterial substances produced by all investigated (LAB and thermophilic 118) strains on eukaryotic (yeast) cells was determined by agar overlay method. Four different yeast species (*Saccharomyces cerevisiae* and 3 *Candida* species, Table 2) were used and in all cases no inhibitory effect was determined.

Limited data in the literature describing effect of LAB on eukaryotic cells is available (Gerez *et al*., 2009; Dalié *et al*., 2010; Digaitiene *et al*., 2012; Nuryshev *et al*., 2016), especially due to the production of proteinaceous substances. Most of these researches were focused on LAB effect on fungi causing mold, while effect on yeast cells was investigated only in few studies, showing *L. lactis* subsp. *lactis* activity against *Candida guilliermondii* (Nuryshev *et al*., 2016), *L. acidophillus* against *Candida albicans* (Grimoud *et al*., 2010) and ability of few *L. paracasei* strains to inhibit the growth of *Candida lusitaniae* (Schnürer and Magnusson, 2005). Although the same yeast species were used in this study, inhibitory activity was not determined, most likely due to the inconsistency of used strains or relatively low activity of tested samples.

Also no inhibitory effect of 118 strain on yeast cells was detected. However, as mentioned before, there is virtually no data about *A. pallidus* inhibitory spectrum in the literature. Therefore, it cannot be concluded if inhibitory effect was not observed due to the low antibacterial activity of tested sample or this bacteria species is not active against yeast cells.

Nontoxicity to eukaryotic (yeast) cells, inactivity against probiotic LAB, ability to assimilate prebiotic OS and inhibit the growth of food spoilage and pathogenic bacteria due to bacteriocin production, which is considered as one of the main advantage of probiotic bacteria (Gillor *et al*., 2008; Dobson *et al*., 2011; O'Shea *et al*., 2012; Gemechu, 2015; Tomar *et al*., 2015), show a great probiotic potential of investigated thermophilic 118 strain.

CONCLUSSIONS

1. Lactic acid bacteria yogurt isolates (A11 and B13) and type strains (*Lactobacillus acidophilus* DSM 20079 and *Lactococcus lactis* subsp. *lactis* DSM 20719) and thermophilic 118 strain were able to assimilate prebiotic oligosaccharides which positively affected strains' antibacterial activity.
2. α-cyclodextrin enhanced antibacterial activity of *L. lactis* subsp. *lactis* DSM 20179 strain against pathogenic bacteria.
3. All investigated strains were active against pathogenic (*Streptococcus pyogenes*) and food spoilage (*Bacillus cereus* and *Geobacillus stearothermophilus*) bacteria.
4. Antibacterial substance produced by 118 strain was a bacteriocin belonging to I or II class and showing stability in high temperature and wide pH range, resistance to detergent treatment and activity against pathogenic and food spoilage, but not against probiotic lactic acid bacteria.
5. Certain combinations of investigated bacteria with prebiotic oligosaccharides – B13 yogurt isolate with inulin, *L. acidophillus* DSM 20079 with palatinose, *L. lactis* subsp. *lactis* DSM 20719 with α-cyclodextrin and potentially probiotic thermophilic 118 strain (*Aeribacillus* sp.) with palatinose – could be applied in food industry as effective synbiotics.

LIST OF PUBLICATIONS

The thesis is based on the following original publications:

1. **Pranckute R**, Kaunietis A, Kuisiene N, Čitavičius D. Development of synbiotics with inulin, palatinose, α-cyclodextrin and probiotic bacteria. *Polish J Microbiol*. 2014;63(1):33-41
2. **Pranckute R**, Kaunietis A, Kananavičiute R, Lebedeva J, Kuisienė N, Šaleikienė J, Čitavičius D. Differences of antibacterial activity spectra and properties of bacteriocins, produced by *Geobacillus* sp. bacteria isolated from different environments. *J Microbiol Biotechnol Food Sci*. 2015;5(2):155-161
3. **Pranckute R**, Kaunietis A, Kuisiene N, Čitavičius DJ. Combining prebiotics with probiotic bacteria can enhance bacterial growth and secretion of bacteriocins. *Int J Biol Macromol*. 2016;89:669-676

Other publications:

1. Kaunietis A, de Jong A, **Pranckutė R**, Buivydas A, Kuipers OP. Draft genome sequences of two *Geobacillus* species strains, isolated from oil wells and surface soil above oil pools. *Genome Announc*. 2016;4(5):e01129-16.

CONFERENCE PRESENTATIONS

Dissertation theme was presented in 3 international conferences (in one of them – with oral presentation):

1. **Pranckutė R**, Kaunietis A, Kananavičiūtė R, Lebedeva J, Čitavičius D. Antibacterial spectrum and properties of bacteriocins of *Geobacillus* spp. Bacteria. BAMP 2013: International scientific conference on bacteriocins and antimicrobial peptides, 21-23 May, 2013, Košice, Slovakia. (Oral presentation)
2. **Pranckutė R**, Kuisienė N, Čitavičius DJ. Physicochemical properties of bacteriocin produced by thermophilic *Geobacillus* sp. strain, European Biotechnology Congress 2016, 5-7 May, 2016, Riga, Latvia.

Other conferences (as co-author):

1. Kaunietis A, Čitavičius D, **Pranckutė R.** Optimization of the secretion of bacteriocins and the bacterial cell growth of *Geobacillus* sp. 15 Strain. FEMS 2015: 6th Congress of European Microbiologists, 7-11 June, 2015, Maastricht, The Netherlands.
2. **Pranckute R**, Klusaite A, Bukelskis D, Kuisiene N. Antibacterial activity of microorganisms isolated from Krubera-Voronja cave. 3rd Congress of Baltic Microbiologists 2016 (CBM2016), 18-21 October, 2016, Vilnius, Lithuania.

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2016.04.01 - 2016.12.31	"Microbiome of the World's deepest cave: analysis of variety and antimicrobial activity". MIP-005/2014. Duration – 3 years (2014.03.01. - 2016.12.31). Financing institution – Research Council of Lithuania (RCL). Supervisor – Prof. N. Kuisienė.

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SANTRAUKA

Išaugęs šių dienų vartotojų funkcionalaus maisto poreikis bei išplitęs patogeninių ir maistą gadinančių bakterijų atsparumas įprastiems antibiotikams skatina ne tik naujų antibakterinių medžiagų paiešką, bet ir jas produkuojančių bakterijų bei funkcionalaus maisto komponentų (prebiotikų) suderinamumo ir jų įtakos antibakterinio aktyvumo raiškai tyrimus, siekiant sukurti efektyvius sinbiotikus.

Šiame darbe buvo tirti tipiniai pienarūgščių bakterijų (LAB) kamienai ir jogurtų izoliatai bei termofilinis sporas formuojantis 118 kamienas. Darbo metu buvo parodyta, kad dalis tirtų pienarūgščių ir termofilinis bakterijų kamienas geba įsisavinti prebiotinius OS ir/arba jie teigiamai veikia kamienų antibakterinį aktyvumą, o α-ciklodekstrinas padidina *Lactococcus lactis* subsp. *lactis* DSM 20179 kamieno antibakterinį aktyvumą ir prieš patogenines bakterijas.

Visi tirti bakterijų kamienai gebėjo inhibuoti bent dviejų (*Bacillus cereus* ir *Streptococcus pyogenes*) iš 16 tirtų patogeninių bei maistą gadinančių bakterinių kamienų augimą. Taip pat, pasižymėjo stipriu aktyvumu prieš termofilines endosporas formuojančias *Geobacillus stearothermophilus* bakterijas, kurios yra dažnos konservuotus ir pieno produktus gadinančios bakterijos. Šie rezultatai praplečia tirtų kamienų ir/arba jų sekretuojamų antibakterinių medžiagų pritaikymo galimybes.

Nustatyta, kad nei vienas iš tirtų bakterinių kamienų bei jų sekretuojamų antibakterinių medžiagų nepasižymi toksiškumu eukariotinėms (mielių) ląstelėms. Taip pat parodyta, kad termofilinis 118 bakterijų kamienas yra neaktyvus prieš, bet jautrus tirtų LAB antibakteriniam poveikiui, todėl yra perspektyvus taikymui probiotinių maisto produktų apsaugai ir galiojimo laiko prailginimui. Šie rezultatai rodo probiotinį iki šiol neaprašytą termofilinio, *Aeribacillus* sp. priklausančio, 118 kamieno potencialą.

Termofilinio 118 kamieno sekretuojama antibakterinė medžiaga yra maždaug 5 kDa molekulinės masės, baltyminės prigimties, yra sekretuojama eksponentinio augimo metu ir jos raiška susijusi su sporuliacija, struktūroje galimai turi disulfidinių jungčių ir pasižymi stipriu aktyvumu prieš producentui artimas bakterijas. Remiantis šiais duomenimis galima teigti, kad 118 kamieno sekretuojama antibakterinė medžiaga yra I arba II klasės bakteriocinas, pasižymintis pritaikymui maisto pramonėje naudingomis savybėmis: stabilumu aukštose temperatūrose ir plačiose pH reikšmėse, atsparumu

detergentų poveikiui ir aktyvumu prieš patogenines bei maistą gadinančias bakterijas. *Aeribacillus* sp. bakterijų sekretuojamas bakteriocinas šiame darbe aprašytas pirmą kartą.

Darbo metu nustatyti perspektyviausi tirtų antibakteriniu aktyvumu pasižyminčių bakterijų ir prebiotinių oligosacharidų deriniai – jogurto izoliato B13 ir inulino, *Lactobacillus acidophilus* DSM 20079 ir palatinozės, *L. lactis* subsp. *lactis* DSM 20719 ir α-ciklodekstrino bei termofilinio 118 kamieno (*Aeribacillus* sp.) ir palatinozės – gali būti pritaikomi maisto pramonėje kaip efektyvūs sinbiotikai.