LACTICASEIBACILLI AND LACTOCOCCI FROM SLOVAK RAW GOAT MILK AND THEIR POTENTIAL*

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Strains potential such as bacteriocin activity, biofilm formation ability, growth in skim milk, susceptibility to antibiotics, tolerance to bile and low pH as well as enzyme production was tested in the species *Lacticaseibacillus paracasei* and *Lactococcus lactis* detected in Slovak raw goat milk. The strains showed mostly low-grade biofilm formation ability, susceptibility to antibiotics and sufficient tolerance to oxgall/bile. *Lacticaseibacillus* (*Lcb.) paracasei* ZM-1, ZM-2 and *Lactococcus* (*Lc.*) *lactis* PD MO 1/8 showed high tolerance to pH 3 (67 %, 83 % and 63 %, respectively). The strains showed bacteriocin activity against the principal indicator *Enterococcus avium* EA5 (inhibition zone ranging 5–24 mm). A concentrated substance of *Lcb. paracasei* LPa ML 12/1 (pH 6.3) inhibited EA5 strain (inhibition activity 100 AU ml^{-1}). *Lcb. paracasei* ZM-1 and ZM-2 also produced a high amount of β-galactosidase (40 nmol). Although the strains indicated their beneficial potential, additional testing is needed; some tests are in processing for further possible application of selected strains in dairy.

goat milk, lactocaseibacilli, lactococci, biofilm, inhibition activity

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INTRODUCTION

Goat milk contains 'naturally occurring' microbiota significant from both beneficial and spoilage aspects depending on bacterial genera (Schirru et al., 2012). Although some studies including characterization of lactococci and lactobacilli in Slovak goat milk have been completed (Klapacova et al., 2015; Tomaska et al., 2015), more information on their benefits are needed. Thanks to progressive identification methods, more new genera and species, including also lactobacilli, have recently been taxonomically allotted or re-classified. Till March 2020, the genus *Lactobacillus* had been comprised of 261 species. Lactobacilli belong to phylum Firmicutes, class Bacilli, order Lactobacillales, family Lactobacillacae with the genera *Lactobacillus*, *Paralactobacillus* and *Pediococcus*. However, based on a polyphasic approach, the genus *Lactobacillus* was re-classified into 25 genera (Zheng et al., 2020) no meaning *Lactobacillus delbruckei* group, *Paralactobacillus* and 23 novel genera. The species *Lacticaseibacillus paracasei* is a common inhabitant of dairy environment (Zheng et al., 2020); some representatives of this species are known to have probiotic character or they can produce bacteriocins or other bioactive peptides and often are involved in probiotic products (Nes et al., 2014; Zommiti et al., 2020). Lactococci belong to the same phylum, class, and order as lactobacilli, however they are involved in the family Streptococcacae and genus *Lactococcus;* they are predominantly isolated from the dairy environment (Zheng et al., 2020). Lactic acid bacteria (LAB), covering also the genera *Lactococcus* and

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Lactobacillus (*Lacticaseibacillus*), have been in use for centuries in the fermentation of food, but also as a natural competitor to other microbiota that share the same niche (R e i s et al., 2012). The antimicrobial/competitive activity of LAB is due to the production of metabolites such as lactic and acetic acids, ethanol, diacetyl, hydrogen peroxide and carbon dioxide (R e i s et al., 2012; P u r e v d o r j et al., 2017).

The total number of goats in Slovakia was estimated at 18 491 heads at the end of 2019, showing an increasing tendency if compared with e.g. year 2016 (17 493 heads) (L a u k o v a et al., 2020a). Goat breeding is often associated with 'sale from the yard' because goats are undemanding animals. Goat milk and products from goat milk are very popular. Goat milk is higher in calcium content in comparison with e.g. cow milk. It contains also magnesium, sodium, phosphorus, copper, zinc and even trace elements such as manganese and chromium. There are also vitamins (A, B1, B2, B12, C, D, E, K, and folic acid), short chain fatty acids and medium chain fatty acids, e.g. butyric acid, but mostly capronic, caprylic, caprinic acids and also palmic acid, linolenic and arachidonic acids (U h r í n et al., 2002). In comparison to the proteinaceous complex of cow milk, that of goat milk involves a higher percentage of the amino acids threonine, isoleucine, lysine, tyrosine, cysteine and valine. Goat milk is an appropriate source of animal protein to produce food derived ACE inhibitory peptides (M a r u y a m a et al., 2006). In goat dairy products, Slovak dairy industry uses mostly commercial cultures. Mainly protective bacterial cultures, including bacteriocin-producing beneficial LAB, appear to be a promising tool (F i e l d et al., 2018; V a t a s c i n o v a et al., 2020). Goat milk itself or its products enriched with beneficial substances can be indicated as 'functional food'. Therefore, to choose an autochthonous strain with beneficial properties for proper use is preferred.

The aim of this study was to evaluate some parameters of selected lactocaseibacilli and lactococci detected in Slovak raw goat milk with the view of their further possible application in dairy. In the study, the following properties were tested: biofilm formation ability, antibiotic profile, enzyme production, haemolysis, tolerance to bile and low pH, growth in skim milk and inhibition activity (bacteriocin-like activity).

MATERIAL AND METHODS

Sampling and strains identification

First, a total of 53 samples of raw goat milk were collected from healthy animals bred in the central and eastern regions of Slovakia. Sampling was performed as previously described by L a u k o v a et al. (2021). Fifty-one samples were collected from individual animals. Two pooled raw milk samples were collected from 132 goats; althogether 283 goats were sampled. Sampling was performed by our colleagues from the Dairy Research Institute (DRI) in Žilina and the isolates were supplied to the Laboratory of Animal Microbiology at the Institute of Animal Physiology, Centre of Biosciences of the Slovak Academy of Sciences, for identification. In DRI, milks were treated using the standard microbiological method specified by the International Organization for Standardization (ISO/TS11133-1:2009). They were diluted in Ringer solution (1 : 9, Merck, Germany) and spread onto de Man-Rogosa-Sharpe agar (MRS) (pH 6.2; Merck) to detect lactocaseibacilli and on M17 agar (Difco, USA) for lactococci isolation. They were cultivated at 37 °C for 48 h. Different grown colonies were picked up from both media, checked for purity using Gram staining and a BA400 microscope (Motic, Germany). Pure colonies ($n = 20$) were submitted for identification using the matrix-assisted laser desorption ionisation time-of-flight spectrometry identification system (MALDI-TOF MS; Bruker Daltonics, USA) based on protein 'fingerprints' (A l a t o o m et al., 2011). Lysates of bacterial cells were prepared according to the producer's (Bruker Daltonics) instruction. The results were evaluated using the MALDI Biotyper 3.0 (Bruker Daltonics) identification database. Taxonomic allocation was evaluated on the basis of highly probable species identification (value score 2.300–3.000), secure genus identification/probable species identification (score 2.000–2.299) and probable genus identification (score 1.700–1.999). Positive controls were those included in the Bruker Daltonics identification database. Identical colonies evaluated with the same MALDI-TOF score value were excluded. For subsequent testing, the identified strains were maintained on MRS agar (Merck) and on M17 agar (Difco) and stored using the Microbank system (Pro-Lab Diagnostic, Canada). Based on their previously tested technological parameters, eight strains (*Lactocaseibacillus paracasei* ML12/1, *Lcb. paracasei* ZM-1, ZM-2, *Lactococcus lactis* subsp. *lactis* MK1/1, MK1/3, MK1/7, PDMO1/8, PDMO1/9) were selected for testing in the laboratory to obtain their complete characteristics.

Biofilm formation

A quantitative biofilm plate assay was used to test the biofilm formation ability of the identified strains as follows: one colony of the strain grown on MRS (Merck) agar and/or M17 (Difco) agar at 37 °C overnight was transferred into 5 ml of Ringer solution (Merck; pH 7.0, 0.75 % w/v) to reach McFarland Standard one corresponding to 1.0×10^8 cfu ml^{-1}. A volume of 100 µl from that culture was transferred into 10 ml of MRS and/or M17 broth. A volume of 200 µl of standardized culture was inoculated into a polystyrene microtiter plate wells (Greiner ELISA 12

Table 1a. Enzyme production by *Lactococcus lactis* strains (expressed in nmol)

Lactococcus lactis MK1/1			*Lactococcus lactis* MK1/3			*Lactococcus lactis* MK1/7		
1	control	0	1	control	0	1	control	0
2	alkaline phosphatase	5	2	alkaline phosphatase	10	2	alkaline phosphatase	5
3	esterase	5	3	esterase	5	3	esterase	5
4	esterase lipase	10	4	estease lipase	5	4	esterase lipase	5
5	lipase	10	5	lipase	5	5	lipase	5
6	leucine arylamidase	10	6	leucine arylamidase	0	6	leucine arylamidase	10
7	valine arylamidase	0	7	valine arylamidase	0	7	valine arylamidase	0
8	cystine arylamidase	0	8	cystine arylamidase	0	8	cystine arylamidase	0
9	trypsin	0	9	trypsin	0	9	trypsin	0
10	α-chymotrypsin	0	10	α-chymotrypsin	0	10	α-chymotrypsin	5
11	acid phosphatase	10	11	acid phosphatase	30	11	acid phosphatase	20
12	naphthol-AS-BI-phosphohydrolase	10	12	naphthol-AS-BI-phosphatase	10	12	naphthol-AS-BI-phosphatase	0
13	α-galactosidase	5	13	α-galactosidase	5	13	α-galactosidase	5
14	β-galactosidase	5	14	β-galactosidase	5	14	β-galactosidase	5
15	β-glucuronidase	0	15	β-glucuronidase	0	15	β-glucuronidase	0
16	α-glucosidase	5	16	α-glucosidase	0	16	α-glucosidase	5
17	β-glucosidase	20	17	β- glucosidase	5	17	β- glucosidase	10
18	N-acetyl-β-glucosaminidase	0	18	N-acetyl-β-glucosaminidase	0	18	N-acetyl-β-glucosaminidase	0
19	α-mannosidase	5	19	α-mannosidase	5	19	α-mannosidase	5
20	α-fucosidase	5	20	α-fucosidase	5	20	α-fucosidase	5

Table 1b. Enzyme production by *Lactococcus lactis* and *Lacticaseibacillus paracasei* strains (expressed in nmol)

Lactococcus lactis PD MO 1/8			*Lactococcus lactis* PD MO 1/9			*Lacticaseibacillus paracasei* LPa ML 12/1		
1	control	0	1	control	0	1	control	0
2	alkaline phosphatase	5	2	alkaline phosphatase	5	2	alkaline phosphatase	10
3	esterase	5	3	esterase	5	3	esterase	20
4	esterase lipase	5	4	esterase lipase	5	4	esterase lipase	20
5	lipase	5	5	lipase	5	5	lipase	10
6	leucine arylamidase	10	6	leucine arylamidase	0	6	leucine arylamidase	10
7	valine arylamidase	0	7	valine arylamidase	0	7	valine arylamidase	10
8	cystine arylamidase	0	8	cystine arylamidase	0	8	cystine arylamidase	10
9	trypsin	0	9	trypsin	0	9	trypsin	10
10	α-chymotrypsin	5	10	α-chymotrypsin	0	10	α-chymotrypsin	5
11	acid phosphatase	20	11	acid phosphatase	10	11	acid phosphatase	20
12	naphthol-S-BI-phosphohydrolase	0	12	naphthol-S-BI-phosphohydrolase	0	12	naphthol-S-BI-phosphohydrolase	30
13	α-galactosidase	5	13	α-galactosidase	5	13	α-galactosidase	10
14	β-galactosidase	5	14	β-galactosidase	5	14	β-galactosidase	10
15	β-glucuronidase	0	15	β-glucuronidase	0	15	β-glucuronidase	10
16	α-glucosidase	5	16	α-glucosidase	10	16	α-glucosidase	10
17	β glucosidase	5	17	β-glucosidase	5	17	β-glucosidase	10
18	N-acetyl-β-glucosaminidase	0	18	N-acetyl-β-glucosaminidase	0	18	N-acetyl-β-glucosaminidase	10
19	α-mannosidase	5	19	α-mannosidase	5	19	α-mannosidase	10
20	α-fucosidase	5	20	α-fucosidase	5	20	α-fucosidase	10

Table 1c. Enzyme production by *Lacticaseibacillus paracasei* strains (expressed in nmol)

Lacticaseibacillus paracasei ZM-1			*Lacticaseibacillus paracasei* ZM-2		
1	control	0	1	control	0
2	alkaline phosphatase	10	2	alkaline phosphatase	10
3	esterase	20	3	esterase	20
4	esterase lipase	20	4	esterase lipase	20
5	lipase	10	5	lipase	10
6	leucine arylamidase	40	6	leucine arylamidase	40
7	valine arylamidase	40	7	valine arylamidase	40
8	cystine arylamidase	10	8	cystine arylamidase	10
9	trypsin	20	9	trypsin	20
10	α-chymotrypsin	10	10	α-chymotrypsin	10
11	acid phosphatase	10	11	acid phosphatase	10
12	naphthol-AS-BI-phosphohydrolase	30	12	naphthol-AS-BI-phosphohydrolase	30
13	α-galactosidase	10	13	α-galactosidase	10
14	β-galactosidase	40	14	β-galactosidase	40
15	β-glucuronidase	10	15	β-glucuronidase	10
16	α-glucosidase	30	16	α-glucosidase	20
17	β-glucosidase	10	17	β-glucosidase	10
18	N-acetyl-β-glucosaminidase	10	18	N-acetyl-β-glucosaminidase	10
19	α-mannosidase	10	19	α-mannosidase	10
20	α-fucosidase	10	20	α-fucosidase	10

Well Strips, 350 µl, flat bottom; Frickenhausen GmbH, Germany) and incubated at 37 °C for 24 h. The biofilm formed in the microtiter plate wells was washed twice with 200 µl of deionized water and dried for 40 min at room temperature. The remaining attached bacteria were stained for 30 min at room temperature with 200 µl of 0.1 % (m/v) crystal violet in deionized water. The dye solution was aspirated away and the wells were washed twice with 200 µl of deionized water and dried for 30 min at room temperature. The dye bound to the adherent biofilm was extracted with 200 µl of 95 % ethanol and stirred. A 150 µl aliquot was transferred from each well and placed in a new microplate well for absorbance (A) measurement at 570 nm using an Apollo 11 Absorbance Microplate reader LB 913 (Apollo, Berthold Technologies, USA). Each strain and condition was tested in two independent tests with 12 replicates. A sterile culture medium (MRS/M17) was included in each analysis as a negative control. *Streptococcus equi* subsp. *zooepidemicus* CCM 7316 was used as a positive control (kindly provided by Dr. Eva Stykova, University of Veterinary Medicine and Pharmacy in Košice, Slovakia). The biofilm formation was classified as highly- positive ($A_{570} \geq 1$), low-grade positive ($0.1 \leq A_{570} < 1$) or negative ($A_{570} < 0.1$) according to Chaieb et al. (2007) and Slizova et al. (2015).

Enzyme production testing, haemolysis test

Enzyme production was tested by using a commercial API-ZYM system (BioMérioux, France). The evaluated enzymes (Table 1) followed the manufacturer's recommendation (1: alkaline phosphatase, 2: esterase (C4), 3: esterase lipase (C8), 4: lipase (C14), 5: leucine arylamidase, 6: valine arylamidase, 7: cystine arylamidase, 8: trypsin, 9: α-chymotrypsin, 10: acid phosphatase, 11: naphthol-AS-BI-phosphohydrolase; 12: α-galactosidase, 13: β-galactosidase, 14: β-glucuronidase, 15: α-glucosidase, 16: β-glucosidase, 17: N-acetyl-β-glucosaminidase, 18: α-mannosidase, 19: α-fucosidase). Inocula (65 µl) of McFarland Standard one suspensions were pipetted into each well of the kit. Enzyme activities were evaluated after 4 h of incubation at 37 °C and after the addition of Zym A and Zym B reagents. Colour intensity values from 0 to 5 and their relevant value in nanomoles (nmol) were assigned for each reaction according to the colour chart supplied with the kit.

Haemolysis was controlled by streaking the cultures on Brain heart agar supplemented with 5 % of defibrinated sheep blood. Plates were incubated at 37 °C for 24–48 h. The presence/absence of clearing zones around the colonies was interpreted as α-, β-/(negative) γ-haemolysis (Semedo et al., 2003).

Antibiotic phenotype testing

Two methods were used to evaluate the antibiotic susceptibility/resistance. Using the disk diffusion method, the evaluation was done according to the manufacturers' instruction involved in the Clinical and Laboratory Standard Institute guidelines (CLSI, 2020). The inhibition zone was expressed in mm. Moreover, the E-test/strip method was used following the EFSA (2012) recommendation. The minimum inhibition concentration (MIC) was assessed in µg. Antibiotic disks and strips used were as follows: novobiocin (Nov) (5 µg, Becton and Dickinson), ampicillin (Amp) (10 µg, Fluka, Oxoid), erythromycin (Ery) (15 µg, Becton and Dickinson), chloramphenicol (C), tetracycline (T), vancomycin (Van), rifampicin (R) (30 µg, Oxoid) and gentamicin (Gn) (120 µg, Becton and Dickinson). Comparison with the reference strains included in the manufacturer's instruction (Becton and Dickinson) was used as positive control. Briefly, the strains were cultivated in MRS (Merck or BH broth (Difco)) at 37 °C overnight. A 100 µl volume was plated onto MRS and/or BHA agar with 5 % of defibrinated sheep blood and antibiotic disks or E-test strips were applied. Antimicrobial free agar plates were included as control to check the growth of strains.

Tolerance to bile and low pH

The tolerance of strains in a bile environment was checked in MRS broth (Merck) and/or BH broth (Difco) enriched with 5 % oxgall/bile (Difco) according to Gilliland, Walker (1990). Overnight cultures of tested strains were inoculated (0.1 %) into MRS broth without and with oxgall/bile and incubated at 37 °C for 24 h. Viable cells of tested strains were counted at time 0 and after a 24-hour incubation of an appropriate dilution (in Ringer solution, Merck) plated on MRS and BHA agars (Merck and Oxoid). The resulting values were counts (surviving cells) of tested strains calculated at time 0 and after 24 h expressed in cfu ml^{-1}.

Tolerance to low pH (3.0) was tested according to Arboleya et al. (2011). Tubes with MRS broth and/or BH infusion (pH was adjusted to value 3) were inoculated with 0.1 % overnight culture of tested strains, cultivated at 37 °C and growth/surviving of strains (absorbance, A_{600}) was measured at time 0 (before cultivation) and after 180 min. After A_{600} measuring and plating (MRS agar, BH agar), surviving cell counts were calculated and expressed in cfu ml^{-1}.

Inhibition activity testing

The bacteriocin-like activity was first tested by the qualitative method according to Skalka et al. (1983). Briefly, BH agar (1.5 %, v/w) was used and BHA and MRS agar (0.7 %, v/w) were used for overlay. Testing was first provided against the principal (susceptible) indicator strain from our laboratory, *Enterococcus avium* EA5. After that, concentrated samples of each one strain were prepared in 60 ml of MRS. They were grown at 37 °C overnight (A_{600}, 0.812–0.983), centrifuged at 10 000 *g* for 30 min. Supernatants were divided into 2 × 20 ml and pH was adjusted at 4.5 and 6.3. Then the supernatant (treated with EDTA III to neutralize the substance) was concentrated using Concetrator Plus (Eppendorf AG, Germany) at 45 °C for 4 h. The inhibition ability of the concentrate was tested again using an agar spot test (De Vuyst et al., 1996) against the indicator EA5. The inhibition activity was expressed in AU ml^{-1} meaning a twofold dilution of the substance which inhibits the indicator strain.

Table 2. Identification score evaluation, biofilm formation ability and haemolysis of selected strains

Strains	MALDI-TOF[1]	Biofilm test[2]	Haemolysis[3]
Lacticaseibacillus paracasei			
ZM-1	2 193	nt	γ
ZM-2	2 041	0.104 ± 0.32	γ
ML12/1	2 004	0.125 ± 0.35	γ
Lactococcus lactis* subsp. *lactis			
MK1/1	1 700	0.132 ± 0.36	γ
MK1/3	1 827	0.126 ± 0.35	γ
MK1/7	1 712	0.100 ± 0.31	γ
PDMO1/8	1 900	0.089 ± 0.03	γ
PDMO1/9	1 904	0.113 ± 0.34	γ

MALDI-TOF = matrix-assisted laser desorption ionisation time-of-flight spectrometry, nt = not tested

[1]taxonomic allocation was evaluated on the basis of secure genus identification/probable species identification (score 2.000–2.299) and probable genus identification (score 1.700–1.999)

[2]biofilm formation was classified as highly positive ($A_{570} \geq 1$), low-grade positive ($0.1 \leq A_{570} < 1$) or negative ($A_{570} < 0.1$) according to Chaieb et al. (2007) and Slizova et al. (2015); values are means ± SD

[3]strain display α (alfa), β (beta) and negative γ (gamma) haemolysis (Semedo et al., 2003)

Growth in skim milk

Tubes containing skim milk (Difco) were inoculated with 0.1 % overnight culture of tested strains, cultivated at 37 °C. Growth/surviving of strains (absorbance, A_{600}) was measured at time 0 (before cultivation) and at 24 h (end of cultivation). After A_{600} measuring and plating (MRS and/or BHA agar), the surviving cell count was culculated and compared, expressed in cfu ml^{-1}.

Table 3. Surviving of lacticaseibacilli and lactococci in low pH and oxgall/bile. Counts of surviving bacterial cells are expressed in colony forming units (CFU) per ml (values are means ± SD)

Strains	Bile		pH 3.0	
	0 h	24 h	0 min	180 min
ZM-1	4.54 ± 0.24	5.39 ± 0.09	6.32 ± 0.39	4.48 ± 0.48
ZM-2	4.54 ± 0.24	5.39 ± 0.09	6.21 ± 0.33	3.96 ± 0.96
ML 12/1	2.00 ± 0.00	5.00 ± 0.00	5.05 ± 0.50	0.00 ± 0.00
MK1/1	4.46 ± 0.03	7.06 ± 0.02	5.25 ± 0.05	1.15 ± 0.15
MK1/3	1.76 ± 0.02	5.00 ± 0.00	5.54 ± 0.06	1.15 ± 0.15
MK1/7	4.15 ± 0.15	7.74 ± 0.04	4.98 ± 0.03	1.00 ± 0.00
PDMO1/8	1.76 ± 0.06	5.00 ± 0.00	4.74 ± 0.04	3.00 ± 0.00
PDMO1/9	1.54 ± 0.06	5.00 ± 0.00	5.23 ± 0.08	1.80 ± 0.50

RESULTS

The evaluation score assessment based on the MALDI-TOF spectrometry allotted the tested strains in the species *Lacticaseibacillus* (*Lactobacillus*) *paracasei* and *Lactococcus lactis* subsp. *lactis*. While the strains of *Lcb. paracasei* were evaluated with the score corresponding with secure genus identification/probable species identification (2.000–2.299) (Table 2), lactococci were taxonomicaly allotted in the species *Lactococcus lactis* subsp. *lactis* with the score ranging from 1.700 up to 1.999 corresponding with the probable genus identification. Strains were haemolysis-negative (γ-haemolysis) (Table 2).

Strains were low-grade positive ($0.1 \leq A_{570} < 1$) or negative ($A_{570} < 0.1$) (Table 2). *Lcb. paracasei* strains showed a well balanced low-grade biofilm formation ability with values 0.104 ± 0.32 and 0.125 ± 0.35 for ZM-2 and LPa ML 12/1 strains, respectively. Among lactococci, PD MO 1/8 strain was non biofilm-forming, and the others were evaluated as low-grade producers (Table 2). Among all strains tested, *Lc. lactis* subsp. *lactis* MK1/1 showed the highest value (0.132 ± 0.36). The activity of *Streptococcus equi* subsp. *zooepidemicus* CCM 7316, the positive control strain, was (in this run) 0.128 ± 0.38.

Lacticaseibacilli and lactococci tested were found to produce slight and high amount of β-galactosidase; especially strains *Lcb. paracasei* ZM-1, ZM-2 and LPa ML 12/1 (10–40 nmol) (Table 1a–c). Only a slight amount of alkaline phosphatase was noted (5–10 nmol). For many other enzymes (Table 1a–c) no production or only a slight production was evaluated. Chymotrypsin, trypsin, β-glucosidase or β-glucuronidase represent enzymes which are often indicated as markers for some disease. Therefore, their production in evaluated strains is not requested. Lactococci and *Lcb. paracasei* LPa ML 12/1 did not produce trypsin or α-chymotrypsin or they showed production in a limited amount (5 nmol); however, *Lcb. paracasei* ZM-1 and ZM-2, sufficient producers (40 nmol) of the beneficial enzyme β-galactosidase, produced 20 nmol and 10 nmol of trypsin and α-chymotrypsin, respectively. However, damaging enzyme β-glucuronidase was not produced by lactococci. In *Lcb. paracasei* strains its 10 nmol volume was evaluated (Table 1a–c).

Lacticaseibacilli (ZM-1, ZM-2, LPa ML 12/1) were susceptible to antibiotics tested using both disk and E-test/strip methods. In the case of disk method, the inhibition zone size ranged from 10 up to 29 mm. MIC for Gn reached 8 µg; for R 0.064 µg; for Ery 0.03 µg; 0.5 µg was MIC for T, 14 µg for Van except strains *Lcb. paracasei* ZM-1 and ZM-2, which were Van resistant and 4 µg for C, except LPa ML 12/1 strain which was C resistant. MIC 0.25 µg was reached at Amp testing, meaning susceptibility. In the case of lactococci, using disk method, inhibition zones ranged from 10 up to 33 mm; lactococci were mostly susceptible to antibiotics used, except strains *Lc. lactis* PD MO 1/8 resistant to Ery and PD MO 1/9 and MK1/7 resistant to R. In lactococci, MIC for Van was 0.50 µg; for C it was 2 µg; 0.12 µg was MIC for Gn, 0.25 µg for Amp, 0.12 µg for T and 0.06 µg for Ery. Lactococci MK1/3 and MO1/8 reached MIC 0.06 µg resp. 0.08 µg in the case of R and strains MK1/1, MK1/7 and PD MO 1/9 were R resistant also using E-test/strips.

Lactococci and lacticaseibacilli showed sufficient surviving in 5% oxgall/bile medium. Their growth increased from time 0 to 24 h after counting of surviving cells (Table 3). Lactococci MK1/1 and MK1/7 reached the highest colony forming unit count (7.04 resp. 7.74 log10 cfu ml^{-1}).

Following the low pH test, pH 3 was tolerated by most strains, their growth was sufficient after 180 min; in the case of lacticaseibacilli, the best tolerance to pH 3 showed strains ZM-1 and ZM-2 (67% and 83%, respectively) (Table 3). The least survival in low pH 3 showed strain LPa ML 12/1 after 180 min (0), but after 5 h still it was 2.0 log 10 cfu ml^{-1} (not shown in Table 3). Regarding lactococci, the best surviving

Table 4. Inhibition activity of tested strains. Values are in mm (inhibition zone size) and in AU ml^{-1} (inhibition activity)

	Producing strain							
	PDMO1/9	MK1/1	MK1/7	PDMO1/8	MK1/3	ML12/1	ZM-1	ZM-2
EA5[a]	5	5	5	0	9	10	24	23
EA5[b]	neg	neg	neg	neg	neg	100	neg	neg

neg = negative

[a]concentrates with pH 6.3 were not active, they did not inhibit indicator strain *Enterococcus avium* EA 5 growth using the method according to De Vuyst et al. (1996); however, using the method according to Skalka et al. (1983) inhibition zones ranged from 5 up to 24 mm;

[b]concentrates with pH 4.5 were not active, they did not inhibit indicator strain *Enterococcus avium* EA 5 growth, except the concentrate produced by *Lacticaseibacillus paracasei* LPa ML 12/1. Its inhibition activity was 100 AU ml^{-1} against EA5 strain using the method according to De Vuyst et al. (1996)

demonstrated *Lc. lactis* PD MO 1/8 (63%; Table 3); surviving of the other lactococci in low pH medium was 20% resp. 29% (Table 3).

The inhibition activity (Skalka et al., 1983) in all producing/tested lacticaseibacilli and lactococci was found against the principal indicator strain *E. avium* EA5 with inhibition zone ranging from 5 up to 24 mm. Only *Lc. lactis* subsp. *lactis* PD MO 1/8 did not form any inhibition zone. Among lactococci, PD MO 1/9 strain reached inhibition zone sizing 9 mm, the other lactococci showed inhibition with zones sizing 5 mm. Among lacticaseibacilli, inhibition zones ranged from 10 up to 24 mm (Table 4). When substances were concentrated, surprisingly they did not show inhibition activity against EA5 strain, except *Lcb. paracasei* LPa ML12/1 strain (pH 6.3), in which activity of 100 AU ml^{-1} was measured using agar spot test (Table 4). The concentrates of the other strains with pH 6.3 were not active, the same as those with pH 4.5 (Table 4).

Lacticaseibacilli and lactococci grew well in skim milk; their counts increased from 4.68 ± 0.02; 5.15 ± 0.15 cfu ml^{-1} at time 0 up to counts 5.00, 5.15 cfu ml^{-1} after 24 h.

DISCUSSION

In March 2020, the genus *Lactobacillus* was comprised of 261 species. Lactobacilli belong to the family Lactobacillacae and to different genera (Zheng et al., 2020), in our case to the genus *Lacticaseibacillus.* Lactococci are involved in the family Streptococcacae and genus *Lactococcus.* However, they both belong to the same phylum Firmicutes, class Bacilli, and order Lactobacillales (Zheng et al., 2020). Besides *Lacticaseibacillus* (previously *Lactobacillus*) *paracasei*, goat milk is a source of the other species of lactobacilli (*Lactobacillus johnsonii*, *Lactiplantibacillus* (*Lactobacillus*) *plantarum*, *Lacticaseibacillus* (*Lactobacillus*) *casei* (Tomaska et al., 2015). They also belong to the group of LAB producing lactic acid (Zheng et al., 2020). *Lactococcus lactis* strains can be commonly found in raw milk but also in fermented dairy products. E.g. Zamfir et al. (2016) demonstrated that *Lc. lactis* represented over 70 % of strains found in Romanian raw milk and fermented dairy products.

Biofilm formation in strains is studied from two aspects. It is a beneficial property in probiotic strains; in the case of spoilage strains it is understood to be a virulent factor, but both are associated with strains protection. It means, in beneficial strain protection itself gives the strain possibility to act beneficially; in pathogens, biofilm protects strain and it is then more difficult to inhibit it. In this study, lacticaseibacilli and lactococci showed mostly a low-grade biofilm formation ability. Salas-Jara et al. (2016) reported biofilm testing in beneficial/probiotic lactobacilli species as a new challenge for development of probiotic use. Moreover, in *Lactococous lactis* biofilm forming strain, detailed studies were carried out to find a cholate-stimulating effect in its biofilm formation (Zaidi et al., 2011). There are facts that contribute to beneficial character of strains tested when further used in dairy.

Having in mind, probable incorporation of strains in any product from safety aspect or as functional food, to test their properties is necessary; among them also production of beneficial enzymes, e.g. β-galactosidase and oppositely not beneficial enzymes as well (e.g. β-glucuronidase or α-chymotrypsin), etc. Lacticaseibacilli and lactococci tested were found to produce slight but also high amount of β-galactosidase, especially *Lcb. paracasei* ZM-1, ZM-2 and LPa ML12/1 (10–40 nmol). Enzyme β-galactosidase (lactase) is glycoside hydrolase enzyme that catalyzes the hydrolysis of β-galactosides into monosacharides. This enzyme is important for organic as it is a key provider in production of energy and a source of carbons through the break down of lactose to galactose and glucose. It is very important for lactose-intolerant community as it is responsible for making lactose-free milk or products (Abou-Soliman et al., 2020). Nowadays, more than 70 % of the world`s population suffer from the inability to digest lactose. Therefore, those β-galactosidase-

producing strains can be used in products because of this purpose. On the other hand, alkaline phosphatase is the enzyme playing role in metabolism within liver and skeleton. In human blood it works as a marker for hepatitis or osteomalacia diagnosis. Therefore, a slight amount of that one enzyme found in tested strains is a promising result. Esterase, esterase lipase, acidic phosphatase or naphthol-AS-BI-phosphohydrolase represent hydrolases. In general, strains tested were not associated with high amount of undesirable enzymes; oppositely, they produced beneficial β-galactosidase. Those *Lcb. paracasei* strains ZM-1, ZM-2 strains, biofilm-producing with β-galactosidase production and technological properties were used in lactic acid drink preparation (D r o n c o v s k y et al., 2019); acidity and senzoric properties were not changed after a 21-day storage condition at 4 °C with their count 7.0 log10 cfu ml^{-1} (D r o n c o v s k y et al., 2019).

Antibiotic susceptibility, tolerance in bile environment and low pH are criteria previously established for selection of further beneficial/probiotic bacteria (N e m c o v a , 1997; E F S A , 2012). In spite of phenotype testing of these properties, strains in this study sufficiently tolerate low pH as well as bile and they are susceptible to antibiotics. So, they have a platform to be developed in their further application, although more other tests are required.

Regarding the strains growth in milk, D r o n c o v s k y et al. (2019) reported that lactococci MK1/3, PD MO 1/9 and especially lacticaseibacilli ZM-1 and ZM-2 reached overnight in goat milk 9.0 log10 cfu ml^{-1} and after storing for 22 days at 5 °C it was up to 7.0 log10 cfu ml^{-1}. Also Z a m f i r et al. (2016) showed cells count of *Lc. lactis* 19.3 in cow or soya milk attaining up to 10.0 log10 cfu ml^{-1}. They also found fast acidification of *Lc. lactis* 19.3 in milk; this property can be advantageous for the development of functional starter culture with a reduced fermentation time and a reduced contamination by spoilage microbiota.

Bacteriocin substances from lactobacilli or lactococci are known to inhibit dominantly Gram-positive bacteria (N e s et al., 2014). In dairy industry, listeriae, staphylococci are those which are spoilage, but the same holds also for enterococci and streptococci or Gram-negative coliforms associated with mastitis. Z a m f i r et al. (2016) showed that *Lc. lactis* 19.3 even produces nisin (lantibiotic bacteriocin) with a bactericidal effect against *Listeria monocytogenes*. In general, there is not so much literature associated with bacteriocins produced by *Lcb. paracasei* from milk or dairy products. T o l i n a c k i et al. (2010) presented a bacteriocin produced from *Lcb.* (previously *Lactobacillus*) *paracasei* subsp. *paracasei* isolated from home-made hard cheese traditionally manufactured in the village Ubli in Montenegro. It was found to inhibit growth of the other *Lcb. paracasei*, but also *L. monocytogenes, Streptococcus pyogenes* and shigellae. It is a small peptide with approximate molecular mass of 4 kDa. M i a o et al. (2014) also described a novel bacteriocin FL produced by *Lcb. paracasei,* however subsp. *tolerans* from kefir, that has been heat stable with a broad antimicrobial spectrum. C u k r o w s k a et al. (2009) selected *Lcb. paracasei* strain LOCK 0919 which presented antagonistic activity against pathogenic bacteria and it is tolerant to low pH and bile. In functional animal studies, one strain induced cytokine production towards Th1 cell-mediated antiallergic response. Our substances from lacticaseibacilli and lactococci were only preliminarily/partially studied; however, our aim was to show their benefit as lactococci and *Lcb. paracasei* isolated from Slovak raw goat milk. It is continued in their detail studies to learn more about their real inhibition spectrum. However, it is the first study reporting on bacteriocin activity by *Lcb. paracasei* or lactococci isolated from Slovak raw goat milk.

CONCLUSION

The strains detected in Slovak raw goat milk, *Lacticaseibacillus paracasei* and *Lactococcus lactis,* mostly showed a low-grade biofilm forming ability and antibiotic susceptibility, sufficient survival in 5% oxgall/bile, sufficient growth in skim milk, high tolerance to low pH, and beneficial inhibition/bacteriocin activity. Concentrated bacteriocin substance of *Lcb. paracasei* LPa ML 12/1 (pH 6.3) manifested inhibition activity against the principal indicator strain *Enterococcus avium* EA5 (100 AU ml^{-1}). Moreover, *Lcb. paracasei* ZM-1 and ZM-2 produced 40 nmol of beneficial hydrolase enzyme β-galactosidase.

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