Fortification of Goat Milk Yogurts with Encapsulated Postbiotic Active Lactococci

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Article

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Abstract: The species *Lactococcus lactis* is the bacterium extensively used in dairy industry. This bacterium is generally recognized as safe which was added to the European Food Safety Authority Qualified Presumption of Safety list. The major functions of this species in dairy fermentation are production of lactic acid from lactose, citric acid fermentation and hydrolysis of casein. But the representatives of this species producing bacteriocin substances can exert an inhibitory effect against spoilage bacteria. The aim of this study was to test three lactococcal strains isolated from raw goat milk for their postbiotic activity and to test their stability in goat milk yogurts in encapsulated form for further application. To reach the aims, validated methods were used. Three postbiotic active *Lactococcus lactis* subsp. *lactis* strains (identified by Blastn 16S rRNA analysis) from raw goat milk produced bacteriocin substances which in their concentrated form inhibited the growth of indicator enterococci and staphylococci up to 97.8 % with inhibitory activity up to 800 AU/mL. The encapsulated (freeze dried) lactococci fortified goat milk yogurts showed sufficient stability and survival in yogurts. The highest amount was reached using the strain MK2/8 (8.1 ± 0.0 cfu/g log 10) not influencing yogurt pH.

Keywords: goat milk; bacteriocin; dairy products; supplementation; lactococci

1. Introduction

Lactococci represent one of the most important group of lactic acid bacteria (LAB) used in dairy industry, and especially, the species *Lactococcus lactis* [1, 2]. The species *L. lactis* belongs to the phylum Firmicutes, to the class Bacilli, to the order Lactobacillales, to the family Streptococcacae and to the genus *Lactococcus* [3]. Representatives of this species are used in processing of fermented dairy products such as yogurts, cheeses, and/or cream, etc. Fermented food has been understood as healthy food which plays an important role in the maintainance of human health status [4]. And some representatives of the species *L. lactis* have been demonstrated to be a promising candidates for the delivery of functional proteins because of its non-invasive and non-pathogenic character [5]. Lactococci have been documented to have a long history of safe use, supported by recognition of Generally Recognized as Safe by the Food and Drug Administration or Qualified Presumption of Safe (QPS) by European Food Safety Authority (EFSA) [6]. Moreover, the species *L. lactis* is one of the best characterized low GC Gram-positive bacteria with detailed knowledge on genetic, metabolism and biodiversity [7-8]. Besides suitable technological functions of some representatives of the species *L. lactis*, there are also strains with probiotic and/or postbiotic character. As it is well known, probiotics are defined as non-pathogenic bacterial strains which induce health benefits on the host when ingested in adequate amount [9]. These beneficial strains can possess postbiotic activity. It means, they can produce bacteriocins (substances of proteinaceous character) with antimicrobial (inhibitory)

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activity [10]. The bacteriocin-producing, beneficial/probiotic strains can have an advantage in competitive interactions with the spoilage bacteria in the food matrix [11].

Recently, bacteriocins have been belonged to the group of postbiotics. Postbiotics are defined as preparations of inanimate microorganisms and/or their components that confers a health benefit on host [12]. Because goat husbandry as well as goat milk production and its processing have a long tradition in Slovakia and products made from goat milk are popular among population and they are friendly consumed, the aim of this study was to test three strains of lactococci isolated from raw goat milk in their encapsulated form as well as to test their stability and/or effect in goat milk yogurts. Freeze drying is the simplest form of encapsulation [13, 14]. In addition, suitable application form of beneficial strains is one of the most important condition for application purposes. Fortified yogurts can be then supposed as functional food to bring health-promoting and supporting benefit for consumer.

2. Materials and Methods

2.1. Isolation and Identification of Lactococci

Twenty-seven raw goat milk samples were collected from healthy goats in central region of Slovakia as previously described by Lauková et al. [15]. To treat milk samples, the standard microbiological dilution method was applied specified by the International Organization for Standardization (ISO). Raw goat milk samples were diluted in Ringer solution (pH-7.0, Merck, Darmstadt, Germany). Dilutions were plated onto MRS agar (De Man-Rogosa-Sharp agar, Merck, pH 6.3), BHI agar (pH 7.0, Difco-BD company, Sparks, MD, USA) and/or M17 agar (Difco, pH 6.9) and incubated at 37 °for 24/48 h to detect lactococcal colonies. Then picked up colonies were checked for purity using BHI agar (Difco) enriched with sheep blood and based on technological properties [16], three strains were selected for more detail study: MK2/2, MK2/7 and MK2/8 (in co-operation with our colleagues from Dairy Research Institute in Žilina, Slovakia). At our laboratory, these strains were controlled for hemolysis using the method described by Lauková et al. [15] and Semedo-Lemsaddek et al. [17]. As next step the strains were submitted for sequence analysis.

2.2. DNA Extraction, PCR Amplification and Sequencing

The detail description of the method was used in our previous study [18]. Briefly, the genomic DNA was extracted from pure colonies by using DNAzol direct (Molecular Research Centre Inc., Cincinnati, USA) following the instructions of manufacturer. The 16S ribosomal RNA genes from isolates were amplified by PCR using the universal primers (Merck-Sigma Aldrich, Darmstadt, Germany) such as Bac27F (5-AGAGTTTGATCMTGGCTCAG-3) and 1492R (5-CGGYTACCTTGTTACGACTT-3). The PCR mixture (50 µL) contained 2 µL of DNA shield and 46 µL of a reaction mixture comprising One Taq 2x Master Mix with Standard Buffer (New England Biolabs, The United Kingdom), diluted in water for molecular biology (PanReac AppliChem, Darmstadt, Germany) to 1x concentration and 1 µL of each primer (concentration 33 µM). The PCR conditions (thermocycler-TProfesional Basic, Biometra GmbH, Göttingen, Germany) used were as follows: 94 ºC for 5 min followed by 30 cycles of denaturation at 94 ºC for 1 min, annealing at 55 ºC for 1 min, and primer extension at 72 ºC for 3 min, then at 72 ºC for 10 min. Aliquot PCR product was separated by horizontal 3% (w/v) agarose gel electrophoresis in Tris-acetate-EDTA buffer (pH 7.8) and visualized with GelRed (Biotium, Inc., Hayward, CA, USA). Amplified product was sent in low bind tube at minimal volume 15 µL for purification and sequencing in both directions using 1492R and Bac27F primers (Microsynth, Wien, Austria). The 16S rRNA sequence was validated and assembled by Geneious 8.0.5 (Biomatters, Auckland, New Zealand) and subjected to BLASTn analysis (https://BLAST.ncbi.nlm.nih.gov/BLAST.cgi).

2.3. Enzymatic Profile of Selected Lactococci

The following enzymes involved in the API-ZYM panel system (BioMerieux, Marcy lEtoile, France) were tested: alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine

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arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphtol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. The method was previously described by Lauková et al. [16]. Briefly, the volume 65 µL of tested strain inoculum (Mac Farland stage 1) was transferred into each well of the test panel plate. The panel plate was incubated at 37 °C for 4 h. Then a drop of reagents Zym A and Zym B was added in each well. Enzyme activity was assessed according to color intensity evaluation (0-5). Then a relevant value in nanomol (nmol) was assigned for each reaction according to the color chart supplied with the kit.

2.4. Susceptibility to Antimicrobials

Susceptibility to antimicrobials (antibiotics) was assayed using two methods; agar diffusion test with antibiotic disks; as a second method E test/strip diffusion recommended by the EUCAST (European Committee on Antimicrobial Susceptibility testing system) [19] was applied.

Using agar diffusion method, BHI agar enriched with blood (Difco) was used. Broth cultures of testing strains (100 µL) were spread on agar surface and the following antibiotic disks (12) were tested (as recommended by disks supplier): clindamycin (2 µg), novobiocin (5 µg), penicillin (10 IU), ampicillin (10 µg), erythromycin (15 µg), streptomycin (25 µg), rifampicin (30 µg), vancomycin (30 µg), kanamycin (30 µg), chloramphenicol (30 µg), ticarcillin (75 µg) and gentamicin (120 µg). Disks were supplied by Oxoid (Basingstoke, The United Kingdom), except kanamycin supplied by Lachema (Czech Republic). The agar plates with disks were cultivated overnight at 37 °C and then susceptibility (inhibitory zones diameter) or resistance to antibiotics was evaluated according to the EUCAST.

In case of E-strip method, the minimum inhibitory concentration (MIC) was established providing the following antibiotic strips: penicillin (0.016-256 µg/mL), chloramphenicol (0.016-256 µg/mL), gentamicin (0.064-1024 µg/mL), rifampicin (0.032-32 µg/mL), streptomycin (0.064-1024 µg/mL), erythromycin (0.015-256 µg/mL), kanamycin (0.016-256 µg/mL), vancomycin (0.016-256 µg/mL), tetracycline (0.016-256 µg/mL), and ampicillin (0.016-256 µg/mL). M17 agar (Difco) plates were seeded with overnight broth culture (BHI, Difco) of tested strain (100 µL). Antibiotic strips were placed on the surface of plates. *E. faecalis* ATCC2921 and/or *Lactococcus lactis* CCM 1881 were included as the positive control strains.

2.5. Biofilm-Forming Ability of Lactococci

This parameter was tested using a quantitative plate assay according to Chaieb et al. [20] and Slížová et al. [21] as previously described by Lauková et al. [19]. Pure colony of the strain grown on M17 agar (Difco) overnight at 37 °C was inoculated into 5 mL Ringer solution (pH 7.0, 0.75% w/v). The suspension corresponded to $1x10^8$ cfu/mL. The volume 100 µL from this dilution was transferred into microtiter plate wells (Greiner ELISA 12 well strips, Frickenhausen GmbH, Germany). After incubation of plate at 24 h at 37 °C, the forming biofilm in the microtiter wells plate was washed twice with 200 µL of deionized water and dried at room temperature for 40 min. Staining of attached bacteria was followed with 200 µL of 0.1= crystal violet in deionized water at 25 C° for half hour. Then, the dye solution was aspirated away and the wells were washed twice with 200 µL of deionized water. After water removal, the plate was dried for half hour at room temperature. The dye bound to adhered biofilm was extracted using 200 µL of 95% ethanol. The volume 150 µL was then transferred from each well in to a new microtiter plate for absorbance (A_{570}) measuring by the use of Apollo 11 absorbance reader LB 913 (Apollo, Berthold, technologies, Oak Ridge, TN, USA). Two independent runs with 12 replicates were measured including also negative control (BHI broth). *Streptococcus equi* subsp. *zooepidemicus* CCM 7316 was positive control (provided by Dr. Styková from University of Veterinary Medicine and Pharmacy in Košice, Slovakia). Biofilm-forming ability of lactococci was evaluated according to the classification [19-21]; highly positive ($A570 \geq 1.0$), low-grade positive ($0.1 \leq A570 \leq 1.0$) and negative ($A570 \leq 0.1$).

2.6. Concentrated Bacteriocin Substance Preparation and Postbiotic Activity Testing

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Lactococci (0.1%) inoculum were inoculated in the volume 40 mL of MRS broth (Merck, Darmstadt, Germany, pH 6.5-7). They were incubated at 37 °C for 24 h to reach A_{600} up to 1.0 MK2/2-0.834, MK2/7-0.977, MK2/8-0.984. The grown cultures were centrifuged (*10 000 x g*) for half hour. The pH of supernatants was adjusted to 5.5-6.0. The cell-free supernatants were treated by addition of EDTA/Chelaton III (Sigma, Germany) and heated at 80 °C for 10 min to eliminate effect of other organic substances. Then they were concentrated using Concentrator Plus (Eppendorf, Hamburg, Germany) to achieve concentrated substances (4.0 mL, CBs). The inhibitory activity was tested using agar spot test [22]. Briefly, as bottom agar layer, BHI agar (Difco) was used and as semi-solid agar for surface overlaying was used 0.7 % M17 agar (Difco). The inhibitory activity was expressed in arbitrary unit per mL (AU/mL) and corresponds with the highest dilution of concentrated substance which caused growth inhibition of indicator strain. The following strains (162) were used as indicators: the principal indicator *Enterococcus avium* EA5 (from piglets feces), 8 vancomycin-resistant enterococci originated from food (kindly provided by Dr. Bírošová , Slovak Technical University in Bratislava, Faculty of Chemical and Food Technology), 4 staphylococci from raw goat milk (our strains), 11 staphylococci from different sources, *S. felis*- 16 fecal strains from cats, *S. chromogenes* -13 fecal strains from cows, one strain *S. haemolyticus* from cow, 2 strains of *S. sciuri* from feces, 30 canine strains of *S. pseudintermedius*, 34 strain of methicillin-resistant *S. aureus* from pigs, 5 human origin *S. aureus* Met^R and one from cow, then 11 strains of *E. faecalis* from poultry feces, 9 canine fecal *E. faecium* strains highly resistant to aminogylcosides, 17 human *E. faecium* HLAR (those strains were provided by Dr. Troscianczyk from University in Lublin, Poland, Table 2).

2.7. Freeze Drying Process (Encapsulation) of Lactococci for Their Application in Yogurts

To encapsulate lactococci, freeze drying method was used as the simplest form of encapsulation [13] previously described by Lauková et al. [14]. Lactococci (rifampicin-labelled variants) were grown in 300 mL of M17 broth (pH 6.9) overnight at 37 °C to reach an absorbance A_{600} up to 1.0. Then the appropriate volume of grown lactococcal cultures were mixed with skim milk in small flasks in a ratio 1:1 (Simandl company, Czech Republic). Flasks were frozen (at -80°C) and freeze-drying was processed using a Micro Modulyo 230 freeze dryer (Thermo-electron corporation, Asheville NC28804, USA). The cell count in the powder was checked using standard microbiological method, meaning dilutions in Ringer solution (Merck, Germany). Dilutions were spread on a M17 agar with rifampicin (Difco) to count lactococci after incubation at 37 °C for 24-48 h.

2.8. Surviving and Stability of Postbiotic Active, Encapsulated Lactococci in Goat Milk Yogurts

Fresh goat milk white yogurts (150 g) used in experiment were bought from the commercial market network. As indicated on the product label, they contain commercial yogurt culture with 3.5% of fat with the following parameters: energy 254 kJ/61kcal, fat 3.7 g of which saturated fatty acids participated with 2.2 g, carbohydrates 3.9 g, sugar value of which was 2.0 g. Proteins content in yogurts formed 3.7 g, salt 0.26 g altogether for 100 g of the product. The encapsulated lactococci were checked for cell count before application. Then 0.5 g of each one encapsulated *L. lactis* strain was applied in the experimental yogurts. The control yogurts were absent of lactococci MK 2/2, MK2/7 and MK2/8. Before strains application, yogurt samples were diluted in peptone water and controlled for absence of non-requested bacteria by spreading on Mac Conkey agar (Difco) to control coliforms. Yogurts were coliforms absent. Yogurts were also checked for streptococci and lactic acid bacteria count which was determined in colony forming units per gram in log 10-up to 5.1, respectively 8.1 CFU/g log 10 in both. The cells of applied lactococci were checked on M17 agar enriched with rifampicin (100 μg/mL) to differ them from other lactococci. The LAB were counted on MRS agar (Merck, Germany). Sampling was performed after 24 h of application, then at day 7, 10 and 14 when experimental control was stopped because of declared expiration time for goat milk yogurts. For plating, yogurts were sampled (one g), mixed in peptone water using Stomacher-Masticator (IUL, Barcelona, Spain) in ratio 1:9. After dilutions, the appropriate dilutions were spread on their formerly indicated media according to ISO. In addition, pH values were measured using a Checker-pH tester

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(Hanna Instruments Incorporation, Woonsocket, USA). The initial pH was in range from 3.0 to 3.90 (Table 3). Yogurts were placed in the fridge during tested (14 days) period.

3. Results

3.1. Taxonomy and Enzyme Profile of Lactococci

Taxonomy of the strains based on BLASTn analysis allotted all three strains to the species *Lactococcus lactis* reaching percentage identity (BLASTn 16S rRNA) sequence for the strain MK2/2 to 99.82% with the sequence of the strain *Lactococcus lactis* in GenBank (MT545096). In the strain MK2/7 was reached 99. 47% identity with KX880980.1. Finally, the strain MK2/8 was assessed with the identity 99.82 % to KX880977.1.

Regarding the enzyme evaluation, lactococci did not produce nor damaging nor beneficial enzymes; meaning that only *L. lactis* MK2/7 produces 5 nmoL of beneficial enzyme β-galactosidase. The tested lactococci did not produce the enzyme β-glucuronidase and /or N-acetyl- β-glucosaminidase. No production of trypsin and α-chymotrypsin was detected and/or up to 5 nmoL in the strains MK2/7 and MK2/8. The other enzymes were not produced.

3.2. Susceptibility to Antibiotics (Antimicrobials) and Biofilm-Forming of Lactococci

Detecting antibiotic phenotype in lactococci by the use of disk diffusion method, the strains were susceptible to clindamycin (2 μg) with the zone size from 17 to 23 mm. Regarding the novobiocin (5 μg), the zone size ranged from 15 to 17 mm. In case of penicillin (10IU), susceptibility range was high, from 10 to 32 mm. Susceptibility to ampicillin (10 μg) was indicated in strains with zone sizes from 15 to 32 mm. Lactococci were susceptible to erythromycin (15 μg) with zone sizes in diameter 20-26 mm, to streptomycin (25 μg) inhibitory zones ranged from 11 to 15 mm, to rifampicin (30 μg) were zones in size 12-17 mm, and to vancomycin (30 μg) reached zones 13-18 mm. Regarding the kanamycin (30 μg) zones measured from 16-20 mm, for chloramphenicol (30 μg) were zones in range 23-27 mm, for ticarcillin (75 μg) zones measured 12-30, and for gentamicin (120 μg) 17-23 mm. In general, zones size range were balanced in lactococci; however, the highest inhibitory zone (the highest susceptibility) was measured in the strain MK2/2 (12-32 mml). In case of MK2/7 (10-25 mm) and for MK2/8, zone sizes measured from 11 to 27 mm.

Using E-strip method, lactococci were susceptible to all antibiotics tested with different MIC (Table 1). Only for the strain MK2/7 (in case of Kan) and for the strain MK2/2 regarding Str were the strains no susceptible no resistant as well (Table 1).

Assessing biofilm-forming ability, the strains MK2/2 and MK2/7 showed low-grade ability to form biofilm (0.122 ± 0.005 for the strain MK2/2; 0.120 ± 0.003 for the strain MK2/7) and the strain MK2/8 did not show biofilm-forming ability (0.099 ± 0.0). When comparing to the positive indicator strain CCM 7316, biofilm was measured in value 0.336 ± 0.219.

Table 1. Summary of susceptibility to antibiotics of lactococci using E-test. (MIC,minimal inhibitory concentration is expressed in μg).

Strain	Kan	Str	Pnc	Chc	Ery	Rif	Tc	Va	Gn	Amp
MK2/2	4/**S**	96/S/R	0.094/**S**	5/**S**	0.047/**S**	6/**S**	0.25/**S**	0.38/**S**	2/**S**	0.016/**S**
MK2/7	96/**S**/R	24/**S**	0.38/**S**	6/**S**	0.064/**S**	0.08/**S**	0.25/**S**	1.5/**S**	8/**S**	0.19**S**
MK2/8	10/**S**	3/**S**	0.064/**S**	12/**S**	0.50/**S**	0.08/**S**	0.38/S	3/**S**	0.094/**S**	0.094/**S**

Kan, kanamycin; Str: streptomycin (0.064-1024 μg/mL); Pnc: penicillin, (0.016-256 μg/mL), Chc: chlorampheni. col, (0.016-256 μg/mL); Ery: erythromycin (0.015-256 μg/mL**);** Rif: rifampicin, (0.032-32 μg/mL**);** Tc: tetracycline (0.016-256 μg/mL, Va: vancomycin (0.016-256 μg/mL), Gn: gentamicin, (0.064-1024 μg/mL), amp, ampicillin (0.016-256 μg/mL), S, susceptible, R/S dubious, Explanation: 4/S means MIC=4 μg and it is susceptible.

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3.3. Postbiotic Activity Testing of Concentrated Substances Produced by Lactococci

Postbiotic potential of concentrated bacteriocin substances produced by tested lactococci is summarized in Table 2. As indicated formerly, altogether 162 indicator bacteria were included in testing, 46 enterococci and 116 staphylococci from various sources. The growth of 45 enterococcal strains out of 46 (97.8 %) was inhibited by treatment with postbiotic substances from lactococci. The principal indicator strain *E. avium* EA5 was inhibited using each one CBs (800 AU/mL). Enterococci were inhibited not depending on their source of isolation, food-derived but also fecal strains and also not depending on their species. Inhibition of the strains species *E. faecalis, E. faecium, E. gallinatrum, E. casseliflavus* was detected and inhibitory activity reached up to 400 AU/mL. Antimicrobial activity of postbiotic CBs from all three lactococci against enterococci was well-balanced except the strain *E. faecalis* 220 which was not inhibited by CBs MK2/2.

Among staphylococcal indicators used (116), the growth of 94. 8% was inhibited. In case of CBs MK2/2 110 out of 116 staphylococci were inhibited.The same number of strains was inhibited using CBs MK2/7 (110 out of 116, Table 2), and 111 staphylococcal indicators was inhibited by the substance CBs MK2/8, 95.7%); again postbiotic activity of all three CBs was well balanced including one more strain inhibited in case of CBs MK2/8. The highest inhibitory activity reached 800 AU/mL. Regarding the species inhibition in staphylococci, the representatives of the *S. pseudintermedius* were inhibited by all three CBs with inhibitory activity up to 400 AU/mL in case of CBs MK2/7. Almost all 16 species strains of *S. felis* were inhibited; in case of CBs MK2/2 only the growth of two strains was not inhibited and in case of CBs MK2/7 and MK2/8 only one strain in each *S. felis* 62-1, and *S. felis* 16 was not inhibited. The species strains *S. chromogenes* were inhibited by use of all three CBs; however, only low inhibitory activity (100 AU/mL) was measured. Surprisingly, also *S. aureus* strains isolated from human samples (11) were inhibited with inhibitory activity up to 400 AU/mL. In case of food-derived *S. aureus* strains (milk, cheese) also inhibition was noted with activity up to 200 AU/mL. Only one strain was not inhibited using CBs MK2/7. When methicillin-resistant staphylococci from pig feces were treated with lactococcal postbiotic active CBs, at least 31 out of 34 strains were inhibited (Table 2) (100-400 AU/mL). *S. aureus* from cow feces was inhibited by three CBs (100 AU/mL) as well as *S. sciuri* of human origin and *S. haemolyticus* from cow (155SHLK39). And, finally also the species strains *S. arlettae*, *S. delphini* and *S. schleiferi* isolated from raw goat milk as postbiotic active lactococci were inhibited. The most susceptible among these three species strains were the strains *S. arletatte* (up to 800 AU/mL).

Table 2. Inhibitory activity of postbiotic substances from lactococci (expressed in arbitrary unit per milliliter, AU/mL).

Indicators	MK2/2	MK2/7	MK2/8
E. avium EA5	1/1, 800	800	800
E. gall.	3/3, 100,400,100	100,400,100	100,200,100
E. cassel.	4/4, 100,100,100,100	100,100, 100,100	100,0,100,100
E. faecium VRE13	1/1, 100	100	100
E. faecalis	11/10, 100-200	11/11, 100-400	11/11, 100-200
E. faecium	9/9,100-400	9/9, 100-400	9/9,200-400
E. faecium	17/17, 100-200	17/17, 200-400	17/17, 200-400
S. felis	16/14, 100-400	16/15, 100-800	16/15, 100-800
S. chromogenes	13/13, 100	13/13, 100	13/13, 100
S. aureus, human	6/5, 100	6/4, 100-200	6/6, 100
S. aureus, milk, cheese	4/4,100	4/3,100-200	4/4,100-200
S. aureus, human	5/5,100-200	5/5,100-400	5/5, 100-200
S. aureus, cow	1/1, 100	1/1, 100	1/1, 100
S. aureus, pig	34/32, 100	34/33,100-400	34/31,100-200
S. arlettae	2/2, 400,800	2/2, 400,800	2/2, 400

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S. schleiferi	1/1,400	1/1,100	1/1,200
S. delphini	1/1, 400	1/1, 800	1/1,800
S. sciuri human	2/1,100	2/1,100	2/1,100
S. haemolyticus cow	1/1,100	1/1,100	1/1,100
S. pseudintermedius	30/30,100-200	30/30,100-400	30/30,100-200

E. gall.:*E. gallinarum* VRE10, *E. gallinarum* VRE18, *E. gallinarum* VRE16, *E. faecium* VRE13 food-derived strain, *E. faecalis* isolated from canine feces, *E. faecium* HLAR 9/9 from poultry feces, *E. faecium*: human multiresistant strains, *S. felis* isolated from feces of cats, *S. chromogenes:* feces of cows, *S. aureus*: source is human, milk and cheese, *S. arlettae, S. schleiferi*, *S. delphini* from raw goat milk, *S. sciuri* from mastitis, MRSA *S. aureus* from pig feces 34, *S. aureus* , 5 human strains, *S.aureus* from cow: 1, x/x means number of tested strains/number of inhibited strains; e.g. 800 means inhibitory activity in AU/mL; Some of strains were kindly privided from our colleagues as indicated in Material and Method part.

3.4. Surviving and Stability of Postbiotic Active, Encapsulated Lactococci in Goat Milk Yogurts

Before starting of yogurts supplementation, microbial backround in yogurts was analyzed with the impact on amylolytic cocci and lactic acid bacteria (LAB). The pH of yogurts was the same 3.30 ± 0.1 -3.90 ± 0.1. The initial value of lactococci in encapsulated form reached 7.95 cfu/g (log 10) for the strain MK2/2, and 7.84 cfu/g for the strains MK2/7 and MK2/8. The strain MK2/7 (4.94 ± 0.2 cfu/g) was the most established strain in yogurts after 24 h, followed with the strain MK2/2 (2.65± 0.1 cfu/g, log 10; Table 3). The strain MK2/8 was detected only in amount 1.30 ± 0.1 log 10 cfu/g after 24 h. However, at day 7, the strain MK2/8 increased in experimental yogurt with difference 2.69 log cycles), in MK2/2 was found increase with difference 1.95 log cycle and for yogurt MK2/7 was increased with difference 1.62 log cycle. At day 10, the strain MK2/2 increased in yogurts to amount 5.85 ± 0.3 cfu/g log 10 with difference 1.25 log cycle comparing to day 7. In the strain MK2/7 was increase with difference 0.78 log cycle and in the strain MK2/8 it was 2.1 log cycle increase. Finally, at day 14, the count of the strain MK2/2 in yogurts was almost the same as at day 10 and it reached 5.78 ± 0.6 cfu/g (log 10). The count of MK2/7 was not higher than at day 10 and reached up to 7.0 cfu/g log 10. However, the highest establishment of applied strain was detected in the strain MK2/8; it reached 8.1 cfu/g (log 10). The counts of amylolytic cocci were in correlation with counts of applied strains after 24 h; therefore, they were the lowest in yogurts enriched with the strain MK2/8, then MK2/2 and the highest in yogurts with the strain MK2/7 (Table 3). Their count increased also in control yogurts. At day 7, the highest increase in amylolytic cocci was noted in yogurts with the strain MK2/8 (difference 3.83 log cycle), while in yogurts with the strain MK2/2 amylolytic cocci were almost in the same count and in case of yogurts with the strain MK2/7 their increase with difference 1.4 log cycle was noted. At day 10, amylolytic cocci in control yogurts were almost in the same amount, slightly increased. Their count was almost the same in case of the strains MK2/2, MK2/7 and MK2/8. Finally, at day 14, amylolytic cocci reached 9.51 ± 0.6 cfu/g in control yogurts; increase was shown also in case of yogurts with the strain MK2/2 (9.1 cfu/g), and in yogurts with the strains MK2/7 (10.1 ± 1.2 cfu/g). The count of the strain MK2/8 was almost at the same level as at day 10 (Table 3). The counts of lactic acid bacteria were high and continually increased during experimental time (Table 3). The pH in yog urts was corresponded with microbiota account and it was not negatively influenced.

4. Discussion

The species *Lactococcus lactis* is the bacterium extensively used in dairy industry [23]. This bacterium is recognized as safe (GRAS-generally recognized as safe) and also it was added to the European Food Safety Authority Qualified Presumption of Safety (QPS) list [24]. The major functions of this species in dairy fermentation are production of lactic acid from lactose, citric acid fermentation and hydrolysis of casein [1]. Moreover, the representatives of this species which produces bacteriocin substances can exert an inhibitory effect against several spoilage bacteria [25]. E.g. Sanca et al. [26] presented the probiotic strain *L. lactis* subsp. *lactis* L2 with inhibiting activity against pig pathogens in vitro. Lacticin 3147, a broad-host range, two-component bacteriocin produced by *L. lactis* subsp. *lactis* DPC3147 isolated from Irish kefir-like grain was found to act on the cytoplasmic membrane of

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sensitive cells, forming pores. It inhibits listeriae, staphylococci, streptococci and clostridia [27] but not Gram-negative bacteria. Anti-staphylococcal effect of lactococcal CBs was also demonstrated in vitro in this study involving various staphylococcal species strains. In addition, also enterococci were inhibited. It means, lactococcal CBs showed inhibitory effect against Gram-positive bacteria; however, up to now inhibitory effect against Gram-negative indicator strains has not been tested. Even et al. [28] reported that the application of *L. lactis* in the dairy industry can prevent *S. aureus* food poisoning to control enterotoxin production. Wu et al. [29] reported the strains *L. lactis* RWP-3 and RWP-7 to prevent bacterial infections and optimize the intestinal microbiota of humans and animals as well through its ability to inhibit growth of pathogens. E.g. milk fermented by *L. lactis* NRRL B-50571 and B-50572 are able to reduce blood pressure and blood lipids [29]. Lactococci presented in this study showed sufficient surviving and stability in goat milk yogurts applied in encapsulated (freeze-dried) form. Akbar and Anal [30] isolated the strain *L. lactis* subsp. *lactis* from fermented milk which was used as bio-control agent against *S. aureus*.

Table 3. Fortification of goat milk yogurts with postbiotic active lactococci and their stability and surviving (in colony forming unit per gram, cfu/g log 10 ± SD.

Sampling	pH	Lactococci tested	Amylolytic cocci	LAB
Control/24	3.35 ± 0.1	nt	7.06 ± 0.1	7.1 ± 0.0
MK2/2	3.30 ± 0.0	2.65 ± 0.1	7.1 ± 0.0	8.96 ± 0.5
MK2/7	2.99 ± 0.1	4.94 ± 0.2	8.17 ± 0.0	8.1 ± 0.0
MK2/8	3.34 ± 0.1	1.30 ± 0.1	5.1 71 ± 0.0	6.95 ± 0.7
Control/7 day	3.33 ± 0.1	nt	8.38 ± 0.5	8.46 ± 0.5
MK2/2	3.21 ± 0.1	4.60 ± 1.1	7.83 ± 1.0	8.61± 065
MK2/7	2.93 ± 0.1	6.56 ± 0.5	10.1 ± 1.5	8.30± 0.3
MK2/8	3.35 ± 0.1	4.0 ± 0.0	8.93 ± 085	7.48± 0.1
Control/10 day	3.33 ± 0.1	nt	8.98 ± 0.7	9.18 ± 0.5
MK2/2	3.21 ± 0.1	5.85 ± 0.3	7.1 ± 0.0	9.52 ± 0.1
MK2/7	2.92 ± 0.1	7.34 ± 0.5	10.5 ± 1.5	8.91 ± 0.2
MK2/8	3.35 ± 0.1	6.1 ± 0.0	8.1 ± 0.0	9.48 045
Control/14 day	3.33 ± 0.1	nt	9.51 ± 0.6	8.70 ± 0.5
MK2/2	3.21 ± 0.1	5.78 ± 0.6	9.1 ± 0.0	9.20 ± 1.0
MK2/7	3.29 ± 0.1	6.93 ± 0.8	10.1 ± 1.2	9.69 ± 0.9
MK2/8	3.35 ± 0.1	8.1 ± 0.0	8.88 ± 0.9	9.41 ± 0.8

The strains for fortification should fulfill safety aspects. Enzyme profile analysis in the beneficial strains is one among important parameters and also one among markers of the strain characteristic and safety. Based on the enzyme type, its production and/or no production can indicate if it is beneficial. Lactococci tested did not produce damaging enzymes which can indicate their safe habitat. Some damaging enzymes e.g. N-acetyl-β-glucosaminidase, α-chymotrypsin serve as disease markers. Bacterial β -glucuronidase can play even role in colon cancer. Therefore, zero value of that enzyme measured in tested lactococci also contributes to their safety habitat. Similarly, beneficial and postbiotic (bacteriocin) active strain *E. durans* ED26E/7 isolated from ewe milk lump cheese did not produce that enzyme [31].

To distinguish strains susceptible to antibiotics and/or intrinsically resistant or those having acquired resistance, antibiotic profile testing is required, MIC evaluation including. Postbiotic active lactococci were found susceptible to antibiotics. Similarly, Floréz et al. [32] tested the strains of *L. lactis* isolated from dairy and they found them susceptible to erythromycin, chloramphenicol and vancomycin and other tested antibiotics. Lactococci are easily adaptable bacteria to specific environment determinants which can indicate them for several uses [29]. The important in case of each one application strain is form used as well as its surviving and stability in the product.

L. lactis can be used e.g. as a natural preservative and anti-botulism agent in cheeses [33]. Yogurt is a popular fermented dairy product and especially that produced from goat milk has possessed

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nutritional and nutraceutical benefits. E.g. protein present in yogurt can be easily digested. It is proven to reduce cholesterol levels. It can lessen the risk of type 2 diabetes by improving insulin sensitivity and glucose tolerance. It also can improve bone density. There are all points which lead to use yogurts as functional food especially those enriched with beneficial microbiota. In our previous studies, selected beneficial strains such as *Lactiplantibacillus plantarum* LP17L/1 and/or *Lacticaseibacillus paracasei* LPa12/1 [14] were checked for their additional benefits in model mice Balb/c and/or food-derived animals such as broiler rabbits to influence also health status and/or e.g. via immunological parameters. No mortality was detected in mice. In case of broiler rabbits and LP17L/1 strain, the total lactic acid bacteria and amylolytic streptococci were significantly increased ($p<0.001$). Lower GPx values were measured in the experimental rabbits in comparison with control animals, which means that this strain did not induce oxidative stress. Phagocytic activity in blood of rabbits was not negatively influenced [34]. Moreover, in case of LP17L/1 strain the protective effect against *Trichinella spiralis* infection was detected associated with the increased oxidative metabolism of peritoneal macrophages which activated the metabolic activity of macrophages during migration of newborn larvae [35]. In each of this aim application form and stability of strains is in paramount of interest. Encapsulation of beneficial strains is useful form which as also indicated here can fulfill request for stability of strains in environment. Sufficient growth of LPa12/1 strain in goat milk yogurts was reported in our previous study [14]. And in future also lactococci studied here will be checked to spread their functional character.

In spite of the fact that some strains of *L. lactis* are well established as commercial probiotics, it is still needed to refine their benefits. Among them is involved also their postbiotic activity. Thakur et al. [36] reported that milk is considered a significant source of bioactive peptides (postbiotics) and has the potential to be utilized in the production of nutritional supplements owing to its beneficial health impacts on humans. There also anticarcinogenic properties were mentioned. Therefore, our study is promising contribution in the benefit of goat milk and its products fortified with beneficial postbiotic lactococci.

5. Conclusions

Three postbiotic active strains of the species *Lactococcus lactis* subsp. *lactis* were isolated from Slovak raw goat milks. Concentrated bacteriocin substances produced by these strains (CBs) inhibited the growth of 97.8 % enterococcal indicator strains with inhibitory activity up to 800 AU/mL. Among 116 staphylococcal indicators, the growth of 94. 8% was inhibited. Inhibitory activity was not influenced by the strains species. The taxonomy of lactococci was confirmed using BLASTn 16S rRNA analysis reaching percentage identity sequence from 99. 47% up to 99.82% with the sequences of the strains *Lactococcus lactis* in GenBank. Lactococci did not produce damaging enzymes. *L. lactis* MK2/7 even produces 5 nmoL of beneficial enzyme β-galactosidase. The strains MK2/2 and MK2/7 showed low-grade ability to form biofilm and lactococci were mostly susceptible to tested antibiotics. Fortification of yogurts made from goat milk with encapsulated lactococci showed their sufficient stability and surviving with most dominant inhabitant MK2/8 strain. It indicates their perspective as functional additives.

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