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original scientific paper

Lactococci of Local Origin as Potential Starter Cultures for Traditional Montenegrin Cheese Production

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Summary

The aim of this study is to characterise and examine the biochemical properties of 40 *Lactococcus lactis* strains isolated from indigenous Montenegrin dairy products in order to explore their potential to be used as starter cultures for producing typical Montenegrin cheese, such as 'bijeli sir', 'masni sir' and 'njeguški sir'. Their safety regarding the production of biogenic amines, the presence of antimicrobial resistance and the antibacterial activity against relevant pathogens and spoilage microorganisms has also been tested. Based on the characterisation, all strains belong to *L. lactis* ssp. *lactis*. Out of these 40 strains, 23 displayed rapid acidification ability and proteolysis. However, none of the strains exhibited the ability of lipid degradation. Most of the strains were not associated with any health risk investigated. Summing up, a large percentage (27.5 %) of the tested strains showed good properties. These strains should be further examined for their possible application as specific starter cultures in the production of indigenous cheese in Montenegro.

Key words: *Lactococcus lactis* ssp. *lactis*, traditional Montenegrin cheese, starter cultures, technological properties, safety assessment

Introduction

Traditional manufacture of cheese from raw milk is an important feature of the Montenegrin culture. These types of cheese have a versatile taste, aroma and consistency when compared to industrially produced ones. The manufacture is generally carried out from unpasteurised milk, without the addition of defined starter cultures and by non-standardised technologies. Primarily, the activity of lactic acid bacteria, normally present in the milk and the production environment during fermentation and ripening, determines texture, aroma and flavour of these dairy products (*1*). Thus, this unique microflora adds to the specificity of cheese produced in the particular locality (*2–4*). Defined starter cultures, however, work faster and more reliably than the natural fermentation microbiota. They contribute to more predictable products with a positive impact on product quality and consumer health (*5*). Since there is no commercial production of starter cultures for Montenegrin soft and hard cheese types, only universal cultures from international manufacturers can be used. But such cultures are tailored for the needs of other markets and would not lead to products with typical properties of traditional Montenegrin cheese such as 'bijeli sir', 'masni sir' and 'njeguški sir' (*6*).

Of all lactic acid bacteria, particularly *Lactococcus lactis* plays a decisive role in the cheese making process (*7*).

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Thus, carefully selected strains of this species are major components of starter cultures for dairy fermentation (*8*). Testing lactococci isolated from indigenous fermented milk products could lead to the development of starter cultures with the necessary properties for the production of traditional Montenegrin cheese that is well accepted by the local population. After proper investigation, these microorganisms may find their application as starter cultures in the Montenegrin dairy industry for the production of safe and consistent cheese with designated geographical origin that could be placed on the international market.

The first stage in carefully selecting starters is to establish the precise identity of an isolate at genus and species level (*9*). Possible duplicates can be excluded by an analysis at strain level (*10*). Next to the correct identification, technological performance and stress resistance have to be characterised when screening for effective starters (*9*). The most important role of *L. lactis* strains in cheese production is their ability to rapidly produce lactic acid, which promotes the coagulation and formation of curd. Another essential characteristic is the possession of a proteolytic enzyme system enhancing the development of cheese flavour. Additionally, some lactococci can produce other aromatic compounds such as diacetyl and acetoin through the metabolism of citrate or lipids (*11*). Next to these technical aspects, a number of metabolites such as lactic acid and bacteriocins exhibit antimicrobial properties, which contribute to the safety of cheese or suppress spoilage (*12*). Furthermore, strains selected as starters must be safe. In this respect, possible hazards to human health such as the release of toxic compounds (*9*) or their ability to disseminate resistance determinants (*13*) should be verified.

Bearing in mind the importance of local starter cultures for the production of traditional cheese, we examined the potential use of *L. lactis* strains isolated from indigenous Montenegrin dairy products as starter cultures considering the properties mentioned above.

Materials and Methods

Bacterial strains and growth conditions

Lactococci were isolated previously (as described below) during the analysis of the diversity of lactic acid bacteria in Montenegrin soft (*e.g.* 'bijeli sir', 'masni sir') and hard (*e.g.* 'njeguški sir') cheese as well as in spontaneously fermented milk.

The strains were maintained in glycerol at –80 °C and resuscitated in M17 broth (Merck, Darmstadt, Germany) according to Terzaghi and Sandine (*14*) at 30 °C overnight. This procedure was repeated twice. The resulting culture broth was used as precursor for the following tests, unless otherwise stated. In total 40 *Lactococcus* strains (Fig. 1) were included in the present study.

Isolation and identification at species and strain level

In brief, dilutions of the dairy products were inoculated on de Man, Rogosa and Sharpe (MRS; Merck) agar (*15*), and M17 agar (Merck) according to Terzaghi and Sandine (*14*). Both media were anaerobically incubated at 30 °C for 2–5 days. Representative colonies were identified at species level (primers gadB21 and Gad7) (*16*) and characterised at strain level (random amplification of polymorphic DNA (RAPD) primers M13 and 1283) (*17*,*18*) using polymerase chain reaction (PCR)-based methods. Species-specific PCR was performed as described by Nomura *et al.* (*16*). RAPD-PCR for typing the isolates at strain level was conducted using a PCR mix (25 μL) containing 1 μL of DNA, 2.5 μL of 10× PCR buffer (Finnzymes, Vantaa, Finland), 0.5 μL of deoxyribonucleotide triphosphates (dNTPs; 10 mM), 0.5 μL of DNA polymerase (2 U/μL; Dynazyme, Finnzymes), 18.5 μL of sterile distilled water and 2 μL of one of the two RAPD primers (10 pmol/μL). The following PCR program was used: 95 °C for 5 min, then 45 cycles at 95 °C for 60 s, 36 °C for 60 s, 72 °C for 60 s, ending with 72 °C for 8 min. PCR products were analysed by electrophoresis on a 2 % agarose gel. The obtained RAPD patterns were additionally processed and analysed using the BioNumerics software v. 6.6.4 (Applied Maths, Sint-Martens-Latem, Belgium). Subsequently, a phylogenetic tree was created using the unweighted pair group method with arithmetic mean (UPGMA) by combining and clustering the fingerprint types of both RAPD primers (Fig. 1).

Phenotypic characterisation of strains and determination of their stress resistance

Production of CO_2

The production of CO_2 from glucose was investigated using a modified method of Winn *et al.* (*19*). Briefly, broth cultures of lactococci (50 μL) were inoculated into a test tube containing 10 mL of M17 broth containing 1 % glucose (Torlak, Belgrade, Serbia) and a Durham's tube. After 24–48 h of incubation at 30 °C, the gas production was observed. If the gas accumulated in the Durham's tube to more than one third of its capacity, the test was considered positive.

Bacterial growth on M17 agar at different temperatures

The M17 agar was inoculated with the *L. lactis* strains and incubated for 72 h at 4, 10 and 45 °C. Afterwards, the agar was examined for bacterial growth by looking for the presence of colonies.

Bacterial growth in M17 broth with 6.5 % NaCl or at pH=9.6

Bacterial growth was monitored over 5 days by measuring the absorbance using the Bioscreen C system (Labsystems, Helsinki, Finland), which includes an incubator, shaking functions and a photometer. Firstly, 300 μL of M17 broth supplemented with 6.5 % NaCl (Roth, Karlsruhe, Germany) or with pH value adjusted to 9.6 were transferred to each well of two honeycomb plates (respectively 10×10 wells; Labsystems). Afterwards, a volume of 15 μL of the broth culture of lactococci was pipetted into each well and the plates were incubated at 30 °C in the Bioscreen C system. The absorbance of the cell suspensions was measured automatically at 600 nm in regular intervals of 30 min. Before each measurement, the culture wells were automatically shaken for 10 s at normal speed. The experiments were carried out in duplicate. Average values of the duplicate absorbance measurements were calculated and used to generate growth curves for each studied strain.

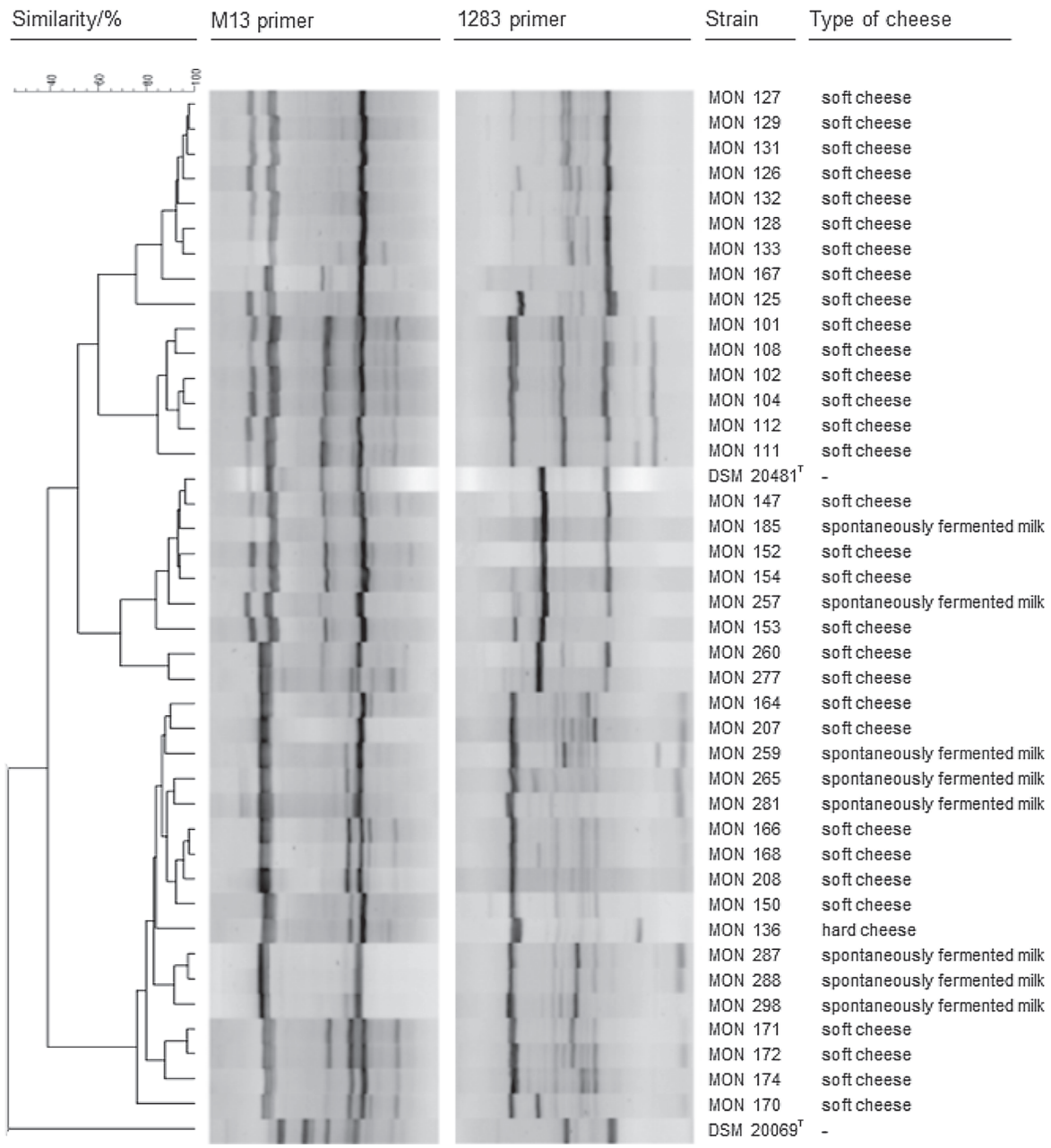

Fig. 1. Composite dataset of RAPD patterns obtained for 40 *Lactococcus lactis* test strains and two type strains, demonstrating genetic variability. MON=tested strains; DSM=Deutsche Sammlung von Mikroorganismen (German Collection of Microorganisms): DSM 20481^T=*Lactococcus lactis* ssp. *lactis*, DSM 20069^T=*Lactococcus lactis* ssp. *cremoris*. Pearson's correlation: optimisation: 1 %, curve smoothing: 0 %, M13 active zone: 20–90 %; 1283 active zone: 30–100 %

Production of exopolysaccharides

The production of exopolysaccharides (EPS) was checked by looking for mucous *L. lactis* colonies grown on M17 agar. Additionally, such colonies were extended with an inoculation loop to detect the production of long filaments. If there were no slimy colonies or the formation of long filaments was missing, it was concluded that the strain did not produce EPS.

Citrate utilisation

Lactococci were streaked on Simmons citrate agar (Oxoid, Hampshire, UK), containing citrate and bromothymol blue as pH indicator. An increase in pH due to the reduction of citrate and the production of alkaline by-products resulted in a colour change from green to blue.

Acetoin production

Methyl red-Voges Proskauer (MR-VP) broth (Oxoid) was inoculated with the microorganisms and incubated at 30 °C for 72 h. After incubation, 2.5 mL of the incubated broth were taken and 0.6 mL of VP reagent A (5 % α-naphthol in absolute ethanol; Roth) and 0.2 mL of VP reagent B (40 % KOH in distilled H_2O; Merck) were added, mixed thoroughly and left at room temperature for 10–30 min. The production of acetoin was indicated by a colour change of the medium to red.

Technological performance of strains

Acidification and post-acidification ability in milk

The acidification and post-acidification ability of each strain was tested by inoculating 50 μL of broth culture in 50 mL of ultra-high temperature (UHT) processed skimmed cow's milk with 2.8 % fat (Imlek, Belgrade, Serbia). Incubation was done at 30 °C for 48 h and the resulting pH values were measured after 2, 4, 6, 8, 12 and 24 h (*6*). The ability of post-acidification was investigated by measuring the pH of the inoculated skimmed milk after 48 h of incubation.

Activity of β-galactosidase

The activity of β-galactosidase was measured on *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) disks according to the manufacturer's instructions (*20*) with minor modifications. In brief, *L. lactis* colonies grown on M17 agar were emulsified in 0.1 mL of 0.85 % NaCl solution containing one ONPG disc. The appearance of yellow colour after incubation at 30 °C for 24 h indicated β-galactosidase activity.

Proteolytic activity

Lines of *L. lactis* strains were streaked on the surface of 3 % nutritive agar (Torlak) supplemented with 3 % sterile skimmed milk (with 1.5 % fat; Imlek). The inoculated medium was incubated at 30 °C for 24 h. The presence of clear transparent zones around the colonies indicated proteolytic activity.

Lipolytic activity

Tributyrin agar (Torlak) was inoculated with 100 μL of decimal dilutions of tested strains by pour plate method. Transparent zones around the colonies after an incubation for 72 h at 30 °C implied lipolytic activity.

Properties of strains contributing to the safety of cheese

Antimicrobial activity of lactococci against selected microorganisms

The antimicrobial activity of lactococci against relevant pathogens and spoilage microorganisms was tested by an agar streak and spot method according to Domig *et al.* (*10*) with minor modifications. Strains or isolates of *Staphylococcus aureus*, *Listeria* spp., *Escherichia* spp., *Enterobacter* spp., *Serratia* spp. and *Pseudomonas* spp., originating from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) or isolated from the same dairy products as the *Lactococcus* strains, were used as indicator microorganisms. *L. lactis* colonies incubated for 24 h in brain heart infusion (BHI) agar (Oxoid) were transferred to 5 mL of 0.85 % NaCl solution. Subsequently, the inoculum was adjusted to McFarland standard no. 1 and streaked in two parallel lines about 20 mm apart on the centre of a BHI agar plate. After incubating the plates for 24 h at 32 °C, inocula of indicator microorganisms were prepared in the same way as those of the lactococci. Of these, 5 μL were dropped between the two pre-incubated *Lactococcus* streaks and separately on the margin of the BHI agar plates as growth control. The plates were again incubated at 32 °C for 24 h and evaluated afterwards. The absence of growth of the indicator microorganisms between the two *Lactococcus* streaks and growth at the margin of the agar plates indicates the presence of antimicrobial substances.

Subsequently, the agar well diffusion assay was used to screen for bacteriocin-producing isolates among lactococci which inhibited the growth of *Staphylococcus aureus* in the previous test. *S. aureus* DSM 1104 and *S. aureus* DSM 20231 from the DSMZ collection as well as one isolate from cheese (SA 17) were applied as indicator bacteria. These indicator microorganisms were inoculated in 15 mL of BHI broth, whereas MRS broth was utilised for the test strains. All were incubated overnight at 32 °C. The analysis was conducted according to Yang *et al.* (*21*) with minor modifications. Hence, 1 mL of each indicator bacterium was transferred to 15 mL of liquid BHI agar maintained at 50 °C and immediately poured into a Petri dish. After solidification, four wells (5 mm in diameter) were cut and 35 μL of cell-free supernatant (CFS) from a *Lactococcus* strain were added to each well.

CFSs were prepared as follows: 13 mL of the incubated broth were centrifuged at 14 000×*g* for 5 min (Eppendorf, Hamburg, Germany). The supernatant was filtered through a sterile 0.22-μm syringe filter and 35 μL of this sterile CFS were added to the first well. The remaining CFS was adjusted to pH=6.5 to rule out pH effects due to the presence of organic acids. This neutralised CFS was filtered and 35 μL were added to the second well. Subsequently, the neutralised CFS was treated with catalase (1 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) at 25 °C for 30 min to eliminate the possible inhibitory activity of H_2O_2, filtered and transferred to the third well. The fourth well was filled with 35 μL of 1 mg/mL of nisin (Sigma-Aldrich), which was used as positive control. After incubation at 37 °C for 24 h the growth inhibition was determined.

Screening for decarboxylase genes

Bacterial DNA was obtained using the Peqgold DNA isolation kit (Peqlab, Erlangen, Germany) (*22*) according to the manufacturer's protocol. The primer pairs JV17HC/JV16HC (*23*), P1/P0303 (*24,25,* respectively) and ODCf/ODCr (*26*) were used to allow the detection of genes involved in the production of histamine (histidine decarboxylase, HDC), tyramine (tyrosine decarboxylase, TDC) and putrescine (ornithine decarboxylase, ODC). PCR reactions were performed as described above with minor modifications: 0.25 μL of DNA polymerase (2 U/μL), 1 μL of each primer (10 pmol/μL) and 18.75 μL of sterile distilled water were used instead of the previously mentioned amounts. The following PCR program was used: 95 °C for 5 min, then 30 (HDC), 35 (TDC) or 40 (ODC) cycles at 95 °C for 60 s, 48 °C (HDC) or 55 °C (TDC and ODC) for 60 s, 72 °C for 60 s ending with 72 °C for 8 min. PCR products were analysed by electrophoresis on a 2 % agarose gel.

PCR products of interest were purified with the PCRExtract Mini Kit (5 Prime GmbH, Hilden, Germany) and subjected to commercial sequencing (Eurofins MWG Operon, Ebersberg, Germany). Sequence compilation and comparison were performed using the nucleotide basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (NCBI) (*27*).

Antimicrobial susceptibility testing

The antimicrobial susceptibility of all strains against ampicillin (AMP), vancomycin (VAN), gentamicin (GEN), kanamycin (KAN), streptomycin (STR), erythromycin (ERY), clindamycin (CLI), tetracycline (TET) and chloramphenicol (CHL) were determined by broth microdilution according to the ISO 10932/IDF 233:2010 method (*28*) with minor modifications. All antibiotics originated from Sigma-Aldrich and were dissolved in water for preparing stock solutions, except for erythromycin and chloramphenicol. To dissolve these two antibiotics, 95 % ethanol was used in volumes as low as possible. Subsequently, stock solutions were diluted in water to obtain solutions with preliminary concentrations in the ranges of (in μg/mL): 0.03–32 (ERY), 0.06–64 (CLI), 0.25–256 (AMP, VAN and CHL), 0.5–512 (GEN and TET), 1–1024 (STR) and 2–2048 (KAN). A volume of 50 μL of each solution was dispensed in the wells of the microtiter plates.

Bacterial inocula were prepared by suspending colonies from 24-hour incubated M17 agar in 5 mL of 0.85 % NaCl solution. Subsequently, inocula were adjusted to McFarland standard no. 1 and diluted in a ratio of 1:500 in double strength Iso-Sensitest broth (Oxoid) for inoculation of the microtiter plates by adding 50 μL of diluted inoculum to each well. This resulted in the final antibiotic concentration ranges of (in μg/mL): 0.01–16 (ERY), 0.03–32 (CLI), 0.12–128 (AMP, VAN and CHL), 0.25–256 (GEN and TET), 0.5–512 (STR) and 1–1024 (KAN). After incubating the plates under anaerobic conditions at 32 °C for 48 h, the minimum inhibitory concentration (MIC) was read as the lowest concentration of an antimicrobial agent at which visible growth was inhibited. The accuracy of susceptibility testing was monitored by parallel use of the quality control strain *Lactococcus lactis* ATCC 19435.

Results and Discussion

Characterisation of strains

L. lactis ssp. *cremoris* and *L. lactis* ssp. *lactis* as well as its diacetyl-forming biovariety *L. lactis* ssp. *lactis* biovar. diacetylactis are the most common *L. lactis* subspecies found in dairy environments (*8*,*29*). *L. lactis* ssp. *lactis* is usually differentiated from *L. lactis* ssp. *cremoris* by its tolerance to higher temperatures and salt concentrations (*8*). Only the latter species can produce EPS, which is useful for the modification of texture and rheological properties in dairy products (*29*). All tested *L. lactis* strains did not create EPS and two thirds of the strains (67.5 %) were able to grow in the presence of 6.5 % NaCl. Moreover, *L. lactis* ssp. *lactis* can be distinguished from its diacetyl-forming biovariety on the basis of citrate fermentation, which leads to the production of aromatic compounds such as diacetyl and acetoin. Because none of the strains could degrade citrate or produce acetoin, all strains were assigned to *L. lactis* ssp. *lactis* (Table 1).

Furthermore, the inability to produce CO_2 from glucose and to grow in the presence of 6.5 % NaCl, at 45 °C or at pH=9.6 was considered to be specific for lactococci (*30*). These criteria are often used to differentiate lactococci from enterococci. Except for the lack of CO_2 production, which is typical for homofermentative lactococci and a

Table 1. Phenotypical and biochemical properties of 40 tested *Lactococcus lactis* strains

Tested strain	CO_2 production	EPS production	Acetoin production	Citrate utilisation	Growth conditions				
					t/°C			*w*(NaCl)/%	
					4	10	45	6.5	pH=9.6
MON101	–	–	–	–	–	+	+	+	+
MON102	–	–	–	–	–	+	–	+	+
MON104	–	–	–	–	–	+	+	+	+
MON108	–	–	–	–	–	+	–	+	+
MON111	–	–	–	–	–	+	+	+	+
MON112	–	–	–	–	–	+	+	+	+
MON125	–	–	–	–	–	+	–	–	–
MON126	–	–	–	–	–	+	–	+	–
MON127	–	–	–	–	–	+	–	–	–
MON128	–	–	–	–	–	+	–	–	–
MON129	–	–	–	–	–	+	–	–	+
MON131	–	–	–	–	–	+	–	–	–
MON132	–	–	–	–	–	+	–	+	–
MON133	–	–	–	–	–	+	–	–	–
MON136	–	–	–	–	–	+	–	–	–
MON147	–	–	–	–	–	+	+	+	–
MON150	–	–	–	–	–	+	–	+	–
MON152	–	–	–	–	–	+	–	+	–
MON153	–	–	–	–	–	+	–	+	–
MON154	–	–	–	–	–	+	–	–	–
MON164	–	–	–	–	–	+	–	+	+
MON166	–	–	–	–	–	+	–	+	–
MON167	–	–	–	–	–	+	–	+	+
MON168	–	–	–	–	–	+	–	+	–
MON170	–	–	–	–	–	+	–	+	–
MON171	–	–	–	–	–	+	–	+	–
MON172	–	–	–	–	–	+	–	–	–
MON174	–	–	–	–	–	+	–	–	–
MON185	–	–	–	–	–	+	–	+	–
MON207	–	–	–	–	–	+	–	–	–
MON208	–	–	–	–	–	+	–	+	–
MON257	–	–	–	–	–	+	–	+	–
MON259	–	–	–	–	–	+	–	–	–
MON260	–	–	–	–	–	+	–	+	–
MON265	–	–	–	–	–	+	–	+	–
MON277	–	–	–	–	–	+	–	+	–
MON281	–	–	–	–	–	+	–	+	–
MON287	–	–	–	–	–	+	–	+	–
MON288	–	–	–	–	–	+	–	+	–
MON298	–	–	–	–	–	+	–	–	–

EPS=exopolysaccharides

desired trait for the production of full-fat cheese without holes (*31*), the other criteria could not be totally verified within this study (Table 1). Thus, 67.5 % of the strains grew in the presence of 6.5 % NaCl, 12.5 % grew at 45 °C and 22.5 % at pH=9.6. Similar results were obtained by Facklam and Elliott (*32*), who recommend a careful interpretation of weak test results, and Corroler *et al.* (*33*), who assume that not enough strains have been characterised to consider an inability to grow at pH=9.6 or in the presence of 6.5 % NaCl to be specific for lactococci. Additionally, many industrially important traits may be unstable as they are plasmid mediated in *L. lactis* (*34*). However, growth of lactococci in the presence of 6.5 % NaCl and at higher or lower temperatures would not be surprising, because these microorganisms survive in hostile conditions and are commonly exposed to many stresses (*33*). Being able to grow at low temperatures, lactococci can continue to keep the product from spoiling and inhibit the growth of pathogenic microorganisms (*35,36*). None of the strains was able to grow at 4 °C, but all grew at 10 °C (Table 1). Furthermore, growth at 45 °C, shown by 12.5 % of the tested strains, could indicate their adaptation to local climatic conditions (*37*). For the manufacturing of traditional products, which are usually made with high NaCl concentrations (*38–40*), growth in the presence of high NaCl concentrations would also be a good criterion.

Technological suitability of strains as starter cultures

Acidification and rapid production of lactic acid are the most important criteria in the selection of starter cultures for cheese production (*3,41–43*). Strains with good acidification ability decrease the pH of milk from its normal value of 6.6 to a pH=5.3 within 6 h or to a pH<4.8 or 4.65 within 24 h of incubation at the optimal temperature (*44*). Taking this essential characteristic into account, the tested strains were classified into three groups based on their acidification ability in UHT milk with 2.8 % fat. The first group consisted of 23 (57.5 %) fast milk-coagulating strains, which lowered the pH of milk below 4.65 after 24 h of incubation. This group represents a collection of potential starter microorganisms. The second group with slower acidification ability (pH=4.65) comprised one milk-coagulating strain (2.5 %). Sixteen strains (40 %) that were characterised by a slow acidification ability (pH>4.65) comprised the third group. With these strains no coagulation of milk was observed within 24 h; moreover, 37.5 % of them were not able to coagulate the milk even within 48 h of incubation (Table 2).

The storage life of fermented products is largely influenced by the post-acidification ability of the used starters (*30*). Hence, attention is also paid to post-acidification properties when selecting starter cultures. Within this study the post-acidification was calculated by the difference of the pH value after 24 and 48 h of incubation. Strains with a difference between 0 to 0.49 pH units were considered as strains with a low or absent post-acidification ability (*44*). This is a good feature from the perspective of their possible use as starter cultures because the consistency and sensory properties of the product will be maintained (*45*). All strains in the first and second group had weak post-acidification ability (Table 2).

Lactose is the main carbohydrate metabolised by two different pathways in lactococci to provide energy. The common pathway employs a lactose-specific phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS). The resulting lactose phosphate is hydrolysed by phospho-β-D-galactosidase to glucose and galactose-6--phosphate, which are simultaneously metabolised to pyruvate. Pyruvate is mainly converted into lactic acid (*46*). The amount of lactic acid is sufficient to overcome the buffering system of milk during an extended incubation (48–72 h) resulting in an acidic environment (*41*).

The second pathway of lactose utilisation employs a carbohydrate-specific permease and β-galactosidase, importing and cleaving lactose to glucose and galactose, which are further metabolised (*46*). ONPG is a compound structurally similar to lactose except that glucose has been replaced by *ortho*-nitrophenyl. Strains possessing both enzymes are able to hydrolyse ONPG into galactose and *ortho*-nitrophenol. *Ortho*-nitrophenol is a chromophore that is colourless when bound to D-galactopyranoside, but yellow in its free form (*20*). Eighteen strains (78.3 %) of the 23 potential starter cultures showed the ability to decompose ONPG (Table 2). These lactococci are capable of carrying out a rapid acidification in milk (*47–49*).

All lactococci of the first and second group and 25 % of the third group could degrade casein (Table 2). Proteolysis is crucial in the process of cheese ripening and undoubtedly the most important biochemical process for the formation of taste and smell of cheese. Thus, the ability to break down casein plays a key role in the selection of cheese starters. The degradation of casein by proteinases and peptidases from starter cultures leads to small peptides and free amino acids. These are the main precursors of volatile compounds which are required for the development of the specific cheese flavour. Next to this, proteolysis is important for the desired texture (*50*, *51*). Lactococci also require proteins, peptides and amino acids for their own growth. Since the content of free amino acids in milk is not sufficient, they depend on their own proteolytic system utilising casein. However, the occurrence of non-proteolytic lactococci, which do not grow after depletion of all free amino acids, was also reported (*52*).

Next to proteolysis, lipolysis of milk fat by lactic acid bacteria influences the cheese flavour development (*53*). Generally, lipolysis refers to the hydrolysis of triglycerides by the enzyme lipase and results in the formation of free fatty acids, which may be precursors of aromatic components such as methyl ketones, secondary alcohols, esters and lactones (*54,55*). Compared to other bacterial groups, lactic acid bacteria are considered as weakly lipolytic (*56*). This corresponds to our findings, as all tested strains did not display transparent zones around the colonies on tributyrin agar after incubation (Table 2). Also a *L. lactis* strain investigated by Katz *et al.* (*53*) did not show any lipase activity, whereas Meyers *et al.* (*57*) observed a weak one in some lactococci.

Cheese safety issues

The antimicrobial activity of LAB is due to their production of organic acids, carbon dioxide, hydrogen peroxide, diacetyl, fatty acids, bacteriocins, bacteriocin-like

Table 2. Technological properties of 40 tested *Lactococcus lactis* strains

Tested strain	Group	*t*/h						β-galactosidase activity	Proteolysis	Lipolysis
		2	4	6	8	24	48			
		pH (milk at 30 °C)								
MON101	1	6.19	5.77	5.20	4.56*	4.08	4.03	+	+	–
MON102	1	6.34	6.20	6.03	5.69	4.46*	4.21	+	+	–
MON104	1	6.28	5.94	5.31	4.58*	4.10	4.06	+	+	–
MON108	1	6.24	5.78	5.18	4.51*	4.09	4.07	+	+	–
MON111	1	6.32	6.05	5.58	4.88	4.10*	4.04	+	+	–
MON112	1	6.25	6.10	5.69	5.07	4.11*	4.03	+	+	–
MON125	3	6.39	6.31	6.26	6.18	5.28	4.17*	–	–	–
MON126	1	6.30	5.80	5.16	4.62*	4.17	4.11	+	+	–
MON127	3	6.32	6.04	5.69	5.43	4.69*	4.31	–	+	–
MON128	3	6.33	6.31	6.21	6.23	6.05	5.87	–	–	–
MON129	1	6.38	6.24	6.11	6.04	4.29*	4.12	+	+	–
MON131	3	6.34	6.26	6.18	6.13	6.04	5.85	–	–	–
MON132	1	6.25	5.97	5.61	5.37	4.62*	4.27	–	+	–
MON133	3	6.34	6.35	6.33	6.35	5.05	4.24*	–	–	–
MON136	1	6.28	5.87	5.40	5.12	4.49*	4.31	+	+	–
MON147	1	6.29	6.05	5.49	5.08	4.40*	4.16	–	+	–
MON150	3	6.30	6.09	5.96	6.67	5.12	4.76*	–	+	–
MON152	1	6.28	5.98	5.44	5.08	4.40*	4.18	–	+	–
MON153	1	6.33	6.09	5.57	5.26	4.56*	4.26	–	+	–
MON154	1	6.29	5.75	5.40	5.20	4.55*	4.29	–	+	–
MON164	1	6.18	5.45	4.60*	4.31	4.05	4.04	+	+	–
MON166	1	6.32	5.96	5.50	5.22	4.42*	4.19	+	+	–
MON167	1	6.33	5.90	5.43	5.15	4.55*	4.28	+	+	–
MON168	3	6.31	6.21	6.15	6.10	5.70	5.29	+	+	–
MON170	1	5.96	5.39	5.11	4.90*	4.30	4.13	+	+	–
MON171	3	6.27	6.16	6.09	6.03	5.61	4.62*	+	–	–
MON172	3	6.25	6.12	6.09	6.00	5.60	4.42*	+	–	–
MON174	3	6.31	6.21	6.16	6.04	5.71	5.42	+	–	–
MON185	1	6.28	5.88	5.15	4.47*	4.10	4.07	+	+	–
MON207	3	6.29	6.20	6.11	6.06	5.72	5.44	–	–	–
MON208	1	6.26	5.85	5.40	5.18	4.47*	4.22	+	+	–
MON257	3	6.38	6.29	6.29	6.23	6.24	6.13	–	–	–
MON259	3	6.40	6.30	6.28	6.20	5.24	4.32*	–	–	–
MON260	3	6.29	6.10	5.97	5.75	5.08	4.68*	+	+	–
MON265	1	6.23	5.80	5.48	5.28	4.56	4.37*	+	+	–
MON277	3	6.37	6.20	6.05	5.78	5.18	4.76*	+	–	–
MON281	1	6.24	5.88	5.41	5.19	4.50*	4.35	+	+	–
MON287	2	6.33	6.11	5.65	5.34	4.65*	4.42	+	+	–
MON288	1	6.26	5.89	5.37	5.21	4.59*	4.44	+	+	–
MON298	3	6.39	6.26	6.21	6.12	5.33	4.29*	+	–	–

After 24 h of incubation, the pH of milk decreased to: pH<4.65 (group 1), pH=4.65 (group 2) and pH>4.65 (group 3)
*coagulation

substances and others (*21*,*58*). Within our study none of the lactococci showed any activity against the Gram-negative indicator bacteria (*Enterobacter* spp., *Pseudomonas* spp. and *Serratia* spp.). According to Rodríguez *et al.* (*59*), LAB bacteriocins are generally inactive against Gram-negative bacteria due to their resistance conferred by the outer membrane. The test strains were also less effective in reducing the growth of two *Listeria* spp. strains (DSM 15675 and DSM 20649), which may be related to the production of organic acids or hydrogen peroxide. However,

seven of the 40 *Lactococcus* strains were able to inhibit the growth of *Staphylococcus aureus* strains and isolates (DSM 1104, DSM 20231 and SA17) (Table 3). Nevertheless, characterising these strains for antibacterial compounds using the agar well diffusion assay, no inhibition zone could be observed. Similar results were reported by Ammor *et al.*

Table 3. Antimicrobial activity of 40 tested *Lactococcus lactis* strains

Tested strain	Group	*Staphylococcus aureus*			*Listeria* spp.		*Escherichia* spp.		*Enterobacter* spp.		*Serratia* spp.		*Pseudomonas* spp.	
		DSM 1104	DSM 20231	SA17	DSM 15675	DSM 20649	EC7	EC9	DSM 30053	EN3	DSM 4487	EN1	DSM 1117	DSM 50090
MON101	1	–	–	–	(+)	(+)	–	–	–	–	–	–	–	–
MON102	1	–	–	–	(+)	(+)	–	–	–	–	–	–	–	–
MON104	1	–	–	–	(+)	(+)	–	–	–	–	–	–	–	–
MON108	1	–	–	–	(+)	(+)	–	–	–	–	–	–	–	–
MON111	1	–	–	–	(+)	(+)	–	–	–	–	–	–	–	–
MON112	1	–	–	–	(+)	(+)	–	–	–	–	–	–	–	–
MON125	3	–	–	–	(+)	(+)	–	–	–	–	–	–	–	–
MON126	1	–	–	–	(+)	(+)	–	–	–	–	–	–	–	–
MON127	3	–	–	–	(+)	(+)	–	–	–	–	–	–	–	–
MON128	3	–	+	–	(+)	(+)	–	–	–	–	–	–	–	–
MON129	1	–	–	–	(+)	(+)	–	–	–	–	–	–	–	–
MON131	3	–	–	–	(+)	(+)	–	–	–	–	–	–	–	–
MON132	1	–	–	–	(+)	(+)	–	–	–	–	–	–	–	–
MON133	3	–	–	–	(+)	(+)	–	–	–	–	–	–	–	–
MON136	1	–	–	–	(+)	(+)	–	–	–	–	–	–	–	–
MON147	1	–	–	–	(+)	(+)	–	–	–	–	–	–	–	–
MON150	3	–	–	–	(+)	(+)	–	–	–	–	–	–	–	–
MON152	1	–	–	(+)	(+)	(+)	–	–	–	–	–	–	–	–
MON153	1	–	–	–	(+)	(+)	–	–	–	–	–	–	–	–
MON154	1	–	–	–	(+)	(+)	–	–	–	–	–	–	–	–
MON164	1	–	–	+	(+)	(+)	–	–	–	–	–	–	–	–
MON166	1	(+)	(+)	+	(+)	(+)	–	–	–	–	–	–	–	–
MON167	1	(+)	(+)	+	(+)	(+)	–	–	–	–	–	–	–	–
MON168	3	(+)	(+)	+	(+)	(+)	–	–	–	–	–	–	–	–
MON170	1	–	(+)	+	(+)	(+)	–	–	–	–	–	–	–	–
MON171	3	+	(+)	+	(+)	(+)	–	–	–	–	–	–	–	–
MON172	3	+	(+)	+	(+)	(+)	–	–	–	–	–	–	–	–
MON174	3	(+)	+	+	(+)	(+)	–	–	–	–	–	–	–	–
MON185	1	–	–	+	(+)	(+)	–	–	–	–	–	–	–	–
MON207	3	(+)	+	+	(+)	(+)	–	–	–	–	–	–	–	–
MON208	1	–	(+)	+	(+)	(+)	–	–	–	–	–	–	–	–
MON257	3	–	–	–	(+)	(+)	–	–	–	–	–	–	–	–
MON259	3	–	–	(+)	(+)	(+)	–	–	–	–	–	–	–	–
MON260	3	(+)	–	+	(+)	(+)	–	–	–	–	–	–	–	–
MON265	1	–	–	–	(+)	(+)	–	–	–	–	–	–	–	–
MON277	3	(+)	–	+	(+)	(+)	–	–	–	–	–	–	–	–
MON281	1	–	–	–	(+)	(+)	–	–	–	–	–	–	–	–
MON287	2	–	–	+	(+)	(+)	–	–	–	–	–	–	–	–
MON288	1	–	–	+	(+)	(+)	–	–	–	–	–	–	–	–
MON298	3	–	–	+	(+)	(+)	–	–	–	–	–	–	–	–

After 24 h of incubation, the pH of milk decreased to: pH<4.65 (group 1), pH=4.65 (group 2) and pH>4.65 (group 3); DSM=Deutsche Sammlung von Mikroorganismen (German Collection of Microorganisms); SA17, EC7, EC9, EN3 and EN1=isolates from Montenegrin dairy products; –=no inhibition, *i.e.* growth like the control, (+)=weak inhibition, *i.e.* growth between that of the control and no growth, +=total inhibition, *i.e.* no growth

(*60*), as they could only detect antimicrobial activity on agar but not in the cell-free supernatant fluids. These authors had several hypotheses for this phenomenon such as the adsorption of bacteriocin-like substances by filters, their attachment to the cell wall or the inability of the strains to produce inhibitors in liquid cultures (*60*).

Table 4. Safety evaluation of 40 tested *Lactococcus lactis* strains

Tested strain	Group	Biogenic amine gene			Antimicrobial susceptibility								
					MIC/(μg/mL)								
		HDC	TDC	ODC	AMP	VAN	GEN	KAN	STR	ERY	CLI	TET	CHL
MON101	1	–	–	–	0.5	0.5	4	16	128*	0.25	0.5	0.5	4
MON102	1	–	–	–	0.5	0.5	8	16	128*	0.25	0.5	0.5	4
MON104	1	–	–	–	0.5	0.5	4	16	64*	0.25	0.25	0.5	4
MON108	1	–	–	–	0.5	0.5	4	16	64*	0.25	0.5	0.5	4
MON111	1	–	–	–	0.5	0.5	8	16	128*	0.25	0.25	0.5	4
MON112	1	–	–	–	0.5	0.5	4	16	64*	0.25	0.25	0.5	4
MON125	3	–	–	–	0.5	0.5	2	8	32	0.25	0.12	0.25	4
MON126	1	–	–	–	0.5	0.5	4	8	32	0.25	0.25	0.25	4
MON127	3	–	–	–	0.5	0.5	4	8	32	0.25	0.25	0.25	4
MON128	3	–	–	–	0.5	0.5	4	8	32	0.25	0.25	0.25	4
MON129	1	–	–	–	0.5	0.5	2	8	256*	0.25	0.12	0.25	4
MON131	3	–	–	–	0.5	0.5	4	8	32	0.25	0.25	0.25	4
MON132	1	–	–	–	0.5	0.5	2	8	32	0.25	0.12	0.25	4
MON133	3	–	–	–	0.5	0.5	4	16	32	0.25	0.12	0.25	4
MON136	1	–	–	–	0.25	0.5	2	8	16	0.25	0.06	0.5	8
MON147	1	–	–	–	0.25	0.5	2	4	16	0.25	0.12	0.25	4
MON150	3	–	–	–	0.25	0.5	1	4	32	0.25	0.06	0.5	2
MON152	1	–	–	–	0.5	0.5	2	4	32	0.25	0.12	0.5	4
MON153	1	–	–	–	0.25	0.5	2	16	16	0.25	0.12	0.5	4
MON154	1	–	–	–	0.25	0.5	2	8	32	0.25	0.12	0.5	4
MON164	1	–	–	+	0.5	0.5	2	8	32	0.25	0.06	0.5	4
MON166	1	–	–	–	0.5	0.5	4	16	16	0.25	0.06	0.5	8
MON167	1	–	–	–	0.25	0.5	4	16	32	0.25	0.25	0.5	8
MON168	3	–	–	–	0.5	0.5	2	8	32	0.25	0.12	0.5	4
MON170	1	–	–	–	0.5	0.5	2	8	32	0.25	0.06	0.5	8
MON171	3	–	–	–	0.5	0.5	4	16	64*	2*	0.25	0.5	8
MON172	3	–	–	–	0.5	0.5	2	8	32	0.25	0.06	0.5	8
MON174	3	–	–	–	0.5	1.0	1	4	128*	2*	0.12	0.5	4
MON185	1	–	–	–	0.25	0.5	2	4	16	0.25	0.06	0.25	8
MON207	3	–	–	–	0.5	0.5	2	8	32	0.25	0.06	0.5	8
MON208	1	–	–	–	0.5	0.5	2	4	16	0.25	0.06	0.5	8
MON257	3	–	–	–	0.5	0.5	2	4	32	0.25	0.06	0.25	8
MON259	3	–	–	–	0.25	0.5	2	8	32	0.25	0.06	0.5	4
MON260	3	–	–	–	0.5	0.5	4	16	64*	0.25	0.12	0.5	4
MON265	1	–	–	–	0.25	0.5	4	16	16	0.25	0.06	0.5	4
MON277	3	–	–	–	0.5	0.5	4	16	64*	0.25	0.12	0.5	4
MON281	1	–	–	–	0.25	0.5	4	8	32	0.25	0.12	0.5	4
MON287	2	–	–	–	0.5	0.5	4	16	64*	0.25	0.06	1	8
MON288	1	–	–	–	0.25	0.5	4	16	64*	0.25	0.06	0.5	4
MON298	3	–	–	+	0.25	0.5	2	8	64*	0.25	0.12	0.5	4

After 24 h of incubation, the pH of milk decreased to: pH<4.65 (group 1), pH=4.65 (group 2) and pH>4.65 (group 3); MIC=minimum inhibitory concentration; HDC=histidine decarboxylase, TDC=tyrosine decarboxylase, ODC=ornithine decarboxylase; AMP=ampicillin, VAN=vancomycin, GEN=gentamicin, KAN=kanamycin, STR=streptomycin, ERY=erythromycin, CLI=clindamycin, TET=tetracycline, CHL=chloramphenicol; *resistant according to EFSA (*61*)

The biogenic amines most commonly found in fermented dairy products are histamine and tyramine, but putrescine is also frequently detected (*62*). Thus, PCR was used to amplify fragments of genes coding for HDC, TDC and ODC. While no amplicons for HDC and TDC could be observed, two strains were positive for ODC (Table 4). The PCR products of these strains were subjected to sequencing for confirmation. The obtained sequences verified the result as they corresponded to fragments of the ODC gene, detected in *Oenoccocus oeni* strains (GenBank accession no. FR751075.1-FR751079.1) (*27*). According to Ladero *et al.* (*63*), *L. lactis* is one of the main putrescine producers in cheese. However, only *L. lactis* strains producing putrescine by the agmatine deiminase (AgDI) pathway and not by the ornithine decarboxylase (ODC) pathway have been described (*63*). Although some *L. lactis* strains have been reported as potential histamine or tyramine formers (*64*), none of the relevant genes was found within our study. Similarly, Priyadarshani and Rakshit (*64*) could not observe any histamine and tyramine formation in their tested *L. lactis* strains.

Using the cut-off values of EFSA (*61*) to distinguish resistant from susceptible strains, all lactococci were susceptible to the tested antibiotics except for streptomycin and erythromycin (Table 4). While only two strains slightly exceeded the cut-off value of erythromycin, approximately one third of the investigated strains can be classified as resistant to streptomycin according to EFSA (*61*). However, the typical MIC distribution for wild type organisms covers three to five adjacent 2-fold dilution steps surrounding the modal MIC (*65*). This applies to the streptomycin MIC distribution obtained within our study (Table 4). As a wild type organism is defined as a strain which does not harbour acquired resistance to the examined antibiotic (*66*), the higher streptomycin MICs might be natural in *L. lactis*. High streptomycin MIC values for lactococci were also observed by Fernández *et al.* (*8*), Toomey *et al.* (*67*) and Rodríguez-Alonso *et al.* (*68*). Nevertheless, the possibility of the presence of antibiotic resistance genes should not be ignored and needs further investigations. For instance, a decreased susceptibility to erythromycin due to a multidrug transporter encoded by the gene *mdt*-(A) was shown in *L. lactis* (*69*). Otherwise the erythromycin-resistance gene *erm*(B) could likewise be responsible for the higher MIC values of the two *Lactococcus* strains, as this gene was also found in intermediate-level resistant (2–16 μg/mL) *L. garviae* isolates (*70*).

Conclusions

Our study suggests that 11 (27.5 %) of the 40 tested *Lactococcus lactis* ssp. *lactis* strains have a potential to be used as starter cultures for the production of traditional Montenegrin cheese. Of course, further studies such as an investigation of antimicrobial resistance determinants have to be done to assure the innocuousness of these strains. For a successful application of these well characterised lactococci in dairy industry, it is also necessary to determine the biochemical reactions that take place during fermentation and ripening of the product due to the activity of these bacteria. Thus, after inoculating milk with these strains, changes of microbial, physical, chemical and sensory characteristics during fermentation and ripening of cheese as well as the final product should be monitored.

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