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Review paper

Biopreservation of traditional raw milk cheeses with an emphasis on Serbian artisanal cheeses and their historical production

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A b s t r a c t: Cheese is one of the oldest food products with preservation based on fermentation, the most common and perhaps the oldest biotechnology. It relies on the biochemical action of lactic acid bacteria (LAB) and is regarded as health-friendly by consumers. Some autochthonous LAB, worldwide and in Serbia, have been characterized as effective producers of antimicrobial compounds such as low-molecular-weight metabolites, hydrogen peroxide, bacteriocins, and bacteriocin-like molecules, and so have demonstrated great potential as food preservatives . The raw milk cheese microbiota, as a good source of novel bacteriocinogenic LAB with high diversity of microbial activity, is key in controlling the microbial load in cheese and achieving diverse sensory characteristics.

Keywords: *biopreservation, lactic acid bacteria, raw milk cheese.*

1. Antimicrobial potential of lactic acid bacteria – an attractive model of biopreservation

The global food industry is continually changing and evolving in order to meet consumers' needs. Consumers want food that is convenient: high-quality, fresh (minimally processed), natural (preservative free), undoubtedly safe and with extended shelf life. Both consumer and food legislative needs call for innovative approaches to preserving food. Therefore, food processors and the scientific community have to explore and implement novel food preservation systems.

Biopreservation is defined as the extension of shelf life and enhanced safety of foods by the use of natural or controlled microbiota and/or antimicrobial compounds (*Schillinger et al.*, 1996; *Stiles*, 1996). Fermentation, the most common and historically-rooted form of biopreservation, relies on the biochemical action of lactic acid bacteria (LAB). Moreover, fermentation, perhaps the oldest biotechnology, has been utilized for millennia (*Ross et al.*, 2002) and is regarded as health-friendly by consumers.

The LAB are a heterogeneous group of organisms functionally related by their ability to convert hexoses into lactic acid during homo- or heterofermentative metabolism. Although LAB do not compromise a distinct taxonomic group, they are phylogenetically closely related, with their small genomes and simplified metabolic pathway for carbohydrate fermentation (*Pfeiler and Klaenhammer*, 2007). The ecological distribution of LAB is extensive: they are indigenous to food-related habitats but also associated with the mucosal surfaces of animals (*Makarova et al.*, 2006). Molecular studies revealed that considerable genetic adaptation has occurred during the coevolution of LAB with their diverse habitats (*Mayo et al.*, 2008). The transition toward a nutrient-rich lifestyle (e.g. milk) to gene loss and metabolic simplification (reduction). Also, LAB adaptation to milk has resulted in acquisition and duplication of genes involved in the metabolism of carbohydrates and amino acid transport, thus ensuring the genetic traits dedicated to efficient exploitation of milk's nutrients (*Makarova et al.*, 2006).

The major antimicrobial compounds produced by LAB are organic acids (lactic acid, acetic acid). Rapid acidification is one of the major criteria for selection of LAB starter strains that are utilized in

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the dairy industry. However, it is an important parameter in achieving the microbiological stability of fermented food if we bear in mind that, generally, food safety is guaranteed as soon as the pH value reaches 4.2 or below (*Holzapfel*, 2002). The antimicrobial effect of acids is exerted by interfering with maintenance of cell membrane potential, inhibiting active transport and reducing intracellular pH, thus hindering a variety of metabolic pathways (*Kashket,* 1987; *Lorca and De Valdez*, 2009). Moreover, specific strains of LAB are characterized as effective producers of other antimicrobial compounds such as low-molecular-weight metabolites (reuterin, diacetyl, and fatty acids), hydrogen peroxide, bacteriocins, and bacteriocins-like molecules (*Suskovic et al.*, 2010). Among these, the bacteriocins, proteinaceous compounds with antimicrobial activity against pathogenic and spoilage bacteria, have demonstrated great potential as food preservatives.

2. Bacteriocins

Bacteriocins are defined as small, heat-stable, ribosomally synthesized antimicrobial peptides with a narrow or broader spectrum of activity (*Cotter et al.*, 2005). It is reasonable to assume that numerous bacteriocins exist in nature, but industrially important LAB have been mainly exploited as a huge reservoir for bacteriocins, as they have earned the "generally recognized as safe – GRAS" (*FDA*, 1988) status due to their long tradition of safe use in fermented food. Over the years, various schemes have been introduced in order to classify the bacteriocins of Gram-positive bacteria. *Cotter et al.* (2005) suggested a more radical modification of the previous classification schemes where bacteriocins can generally be classified into one of two groups on the basis of whether they undergo post-translational modifications: Class I (modified – lantibiotics) and Class II (unmodified – non-lantibiotics), as opposed to Klaenhammer's (*Klaenhammer*, 1993) four class scheme. The mode of antimicrobial action of bacteriocins differs among classes including membrane permeability, interference with cell wall synthesis, or dependence on a receptor molecule required for binding, inhibition of sugar-uptake system and efflux of intracellular solutes (*Perez et al.,* 2015). It was generally assumed that most bacteriocins were not active against Gram-negative bacteria due the integrity of their lipopolysaccharide outer membrane (*Stevens et al.*, 1991; *Perez et al.,* 2015). In the same studies, the antimicrobial activity was attributed to bacteriocin production as shown by susceptibility of inhibitor substances to degradation by proteolytic enzymes .

Bacteriocins are qualified to be promising tools in the biopreservation due to several characteristics (*Perez et al.,* 2015):

i) Inherent tolerance to thermal stress
ii) Activity over a wide pH range
iii) No adverse effect on quality and flavor
iv) Easy degradation by proteolytic enzymes, which minimizes the development of resistance mechanisms. Occasional resistance is observed, probably due to intrinsic ability of cells to change lipid composition of membrane, but it is unclear if this phenomenon is generated by spontaneous mutation (*Crandall and Montville*, 1998; *Vadyvaloo et al.*, 2002; *Nes and Johnsborg*, 2004).
v) Suitability to bioengineering due to their primary metabolic nature (*Perez et al.,* 2014).

It is noteworthy that very few commercial bacteriocinogenic protective cultures are marketed today owing to the difficulty of developing cultures that are efficient and effective in food systems. *In situ* bacteriocin production is favored as it does not require specific legislative approval, but it does require that the producer strain is well adapted to the food matrix, and capable of active growth and bacteriocin expression. The effectiveness of bacteriocin activity in food is affected by numerous factors: interference with food matrix, enzymatic degradation, and retention of bacteriocin molecule by components of the food system, the antagonistic effect of background microbiota, slow diffusion and insolubility due to inadequate physicochemical parameters and uneven distribution of bacteriocin in heterogeneous food matrix (*Cleveland et al.,* 2001; *Gálvez et al.,* 2007).

Before the introduction of genetic studies, screening for bacteriocins relied on functional assays, in which potential producer organisms were tested for antimicrobial activity against selected indicator organisms. It is not an ideal solution because not only is bacteriocin production plasmid-encoded (which implies instability due to plasmid loss), but it has also been recognized that regulatory mechanisms of bacteriocin synthesis are subject to temperature control (*Diep et al.,* 2000). Therefore, a major obstacle in screening and choosing novel bacteriocin-producing LAB is that their optimal growth temperature can differ from their optimal temperature for bacteriocin production (*Nes and Johnsborg*, 2004).

Bearing in mind the ubiquity of bacteriocin production, the ecological consideration of this trait has been established, although it is not fully understand what, precisely, that ecological role is (*Riley et al.*, 2003). Subinhibitory concentrations of structurally diverse inhibitor molecules affected transcription of many bacterial genes not necessarily linked to stress response, suggesting that antimicrobial compounds may function as signal molecules in natural habitats when produced at low concentration – a phenomenon previously known as hormesis (*Calabrese and Baldwin*, 2002). So far, it has been suggested that at least some bacteriocins have a dual role, acting as inhibitors at high concentrations but also as signaling compounds at lower concentrations, thus playing a role in mediating microbial population and community interactions (*Fajardo and Martinez*, 2008). Based on comparative genomic analysis of LAB, it has been recognized that molecular systems responsible for bacteriocin production are evolving in response to adaptation, reflecting the long-term existence of LAB in complex, highly competitive microbial communities (*Makarova et al*., 2006). Ecologically speaking, bacteriocin production provides LAB with greater competitiveness.

In recent years, extensive activities of the scientific community have been focused on the antimicrobial properties of LAB (*Leroy and De Vuyst*, 2004; *De Vuyst and Leroy*, 2007; *Suskovic et al*., 2010). Considering consumer aversion to traditional chemical preservatives, which is forcing food processors to reconsider their preservation technology and seek alternative tools, the use of bacteriocins is likely to expand in the future. However, many bacteriocins have not been fully characterized and therefore cannot be extensively exploited in the food industry. To be wholly characterized, bacteriocins have to be tested for a variety of parameters including spectrum of antimicrobial activity, mode of action, sensitivities to heat, pH, proteolytic enzymes, salt and detergents, determination of molecular mass, amino-acid composition and sequence, determination of the genetic basis of the bacteriocin production and secretion (*Todorov*, 2009). Additionally, great effort has to be devoted to fully address the question of efficient *in situ* bacteriocin expression and consequently to optimize the efficiency of bacteriocin production. The heterologous expression of bacteriocins from LAB could be an affordable solution (*Jiménez et al*., 2015), as advances in genome sequencing of LAB favor the genetic manipulation of these bacteria .

3. Raw milk cheeses: a bastion against undesirable microorganisms

Recently, there has been a considerable interest in locally-sourced, fresh, organic, natural and sustainable products. One product that ideally embodies these consumer expectations is raw milk cheese. *Paxson* (2008) stated that the raw milk cheese processing could be considered as biotechnology for localism.

Cheese is a living and dynamic ecosystem that involves the growth of complex microbial consortia. The complexity of cheese microbiota is due to abundant bacterial associations and biotic interactions favored by the structure and physicochemical heterogeneity of the cheese matrix. The unique chemosensory profile of cheese, as well as shelf-life, quality and safety are largely determined by composition and evolution of cheese microbiota (*Irlinger and Mounier*, 2009). A recent study (*Bokulich and Mills*, 2013) showed that environmental microbiota originating from the cheese processing facility are capable of establishing in the surface microbiota of wash-rind cheeses and most likely impact the sensory properties of cheese. This established site-specific in-house microbiota with potential to be continuously transferred to successive batches of cheese confirmed the basic ecological principles: "everything is connected to everything else" and "environmental selection." According to *Marcellino* (2003), processing parameters introduced through the traditional manufacturing of raw milk cheeses are the major driving forces in selection of specific cheese microbiota, far more so than distinct geographical areas with their inherent characteristics (pedioclimatic conditions, pasture). Therefore, *Marcellino* (2003) considered raw milk cheese as a nature-culture hybrid.

There is a general consensus that cheeses are safer than other unfermented dairy products due to the interplay of various abiotic and biotic factors. After comprehensive summary of the epidemiological literature related to raw milk cheese outbreaks, *Donnelly* (2001) stated there is no compelling data indicating that mandatory pasteurization would result in a safer product. Moreover, some microbiologists argued that pasteurization diminishes the principle of competitive exclusion, and provides an environment which supports pathogen growth. According to EFSA-ECDC (*EFSA-ECDC*, 2016), non-compliance with microbiological criteria established for *Listeria monocytogenes* was primarily related to soft and semi-soft cheeses made from pasteurized milk, which implied post-processing contamination. However, there are on-going debates about the safety of raw milk cheeses, that raise what *Mintz* (2002) recognized as a conundrum of democratic capitalist societies: "how to provide protection of public health on one hand, yet maintain freedom of choice on the other". Moreover, anthropologists and sociologists, especially in the USA, introduced the notion of microbiopolitics as a theoretical frame for understanding debates over the food localism, nutrition, health and safety of raw milk cheeses (*Paxson*, 2008). In Paxson's theory of post-Pasteurian culture, the hyperhygienic Pasteurian claim is that only milk pasteurization makes cheese safe. In contrast, post-Pasteurians, including defenders of traditional cheeses, rely on the complex microbial ecology of cheese as an effective safety strategy. They claim that high diversity of microbial activity coupled with local know-how of the manufacturing process are keys for controlling the microbial load in cheese and achieving diversification of sensory characteristics (*Montel et al*., 2014). *Panari and Pecorari* (1999) stated, with particular reference to traditional processing of Parmigiano-Reggiano: "These processing systems are continually aiming at improvements in quality and only the best technologies persist through the centuries; furthermore, they have accepted corrective practices (as in the modern Hazard Analysis and Critical Control Points [HACCP] protocol) suitable for customers' requirements, including hygienic traits".

Available data from scientific literature and reports from the Center for Disease Control indicate *L. monocytogenes*, verocytotoxin-producing *Escherichia coli* (VTEC), *Staphylococcus aureus*, *Salmonella* and *Campylobacter* as the main microbiological hazards associated with raw milk cheese outbreaks, whereas *Salmonella*, followed by VTEC were the most common etiological agents (*Verraes et al*., 2015). According to EFSA-ECDC (*EFSA-ECDC*, 2016), out of 592 foodborne outbreaks with strong evidence, cheese was implicated in 1.5% of cases, which was the same prevalence as for the sweets and chocolate category. In the industrialized world, the low prevalence of food-borne outbreaks due to consumption of dairy products, including raw milk cheeses, is achieved by successful implementation of overall management of infectious disease in dairy herds, HACCP implementation, and active surveillance throughout the food chain with adherence to GHP and GMP.

Raw milk cheese processing is an illustrative example of empirical application of hurdle technology. Fermentation as a key biochemical process results in decrease of pH due to acid production, redox potential reduction, and nutrition depletion. The proper development of acidity is the most important process control tool that determines cheese safety. The phases of curd processing and pressing favor syneresis and consequently lead to decrease in water activity (a_w). The salting, besides redirecting the biochemical processes, additionally reduces a_w. The interplay of these physicochemical parameters establishes a hostile environment for any pathogens possibly introduced to the cheese matrix due to contamination. During the ripening process, the well adapted autochthonous LAB become established in high numbers, owing to their resistance to reduced pH, decreased a_w and high salt concentration. The main advantage of autochthonous LAB compared to other microbial groups is their potential to effectively compete for limited sources of nitrogen (*Sieuwerts et al*., 2008) due to efficient proteolytic and transport systems. The hurdle effect of natural LAB microbiota on undesirable contaminants seems to be highly variable and is accomplished by competing for nutrients (competitive interactions) and producing inhibitory compounds (organic acids, volatile compounds, H_2O_2) and/or antimicrobial compounds (bacteriocins) (*Irlinger and Mounier*, 2009).

4. Antimicrobial potential of autochthonous LAB microbiota isolated from raw milk cheeses worldwide

Numerous studies considered raw cheese microbiota as a good source of novel bacteriocinogenic LAB strains (*Franciosi et al*., 2009; *Dal Bello et al*., 2010; *Ortolani et al*., 2010). *Moraes et al.* (2012) confirmed that most (93%) of the enterococcal isolates originating from raw milk and cheese in Minas Gerais state, Brazil, harbored at least one lantibiotic or enterocin gene but as the bacteriocinogenic isolates also carried virulence genes, the authors emphasized the need for careful evaluation of their application in food systems.

Mojsova et al. (2015) reported that enterococci isolated from Macedonian traditional cheeses showed antimicrobial activity predominantly against *L. monocytogenes*, and among bacteriocinogenic enterococcal isolates, enterocin P, cytolysin and enterocin A were the most frequently detected structural genes. The authors concluded that although screened enterococcal isolates could be of great technological potential as protective cultures in the cheese industry, it is necessary to ensure that potentially applicable isolates are free of virulence factors.

Enterococcus faecalis isolates from Italian traditional cheeses were evaluated for their antimicrobial potential against foodborne spoilage and pathogenic bacteria and against LAB commonly used as starter cultures in dairy fermentation (*Silvetti et al.*, 2014). The analyzed enterococcal isolates inhibited *Bacillus cereus*, *E. coli*, *L. monocytogenes*, *S. aureus*, and *Clostridium sporogenes*, whereas LAB were moderately antagonized. In that study, by applying a molecular approach, only one enterococcal isolate was found to be enterocinogenic, as the structural gene for enterocin AS-48 was the only one amplified. The authors assumed that the antimicrobial properties of other, phenotypically positive isolates could be due to another non-enterocin inhibitory compound.

By applying the spot-on-the-lawn test, *Tulini et al*. (2013) evaluated the antimicrobial potential of *Lactobacillus paraplantarum* FT259, isolated from Brazilian semi-hard artisanal cheese, on a variety of food-related bacteria and LAB. *L. paraplantarum* FT259 inhibited *L. monocytogenes*, *Listeria innocua* and *Lactobacillus sakei* but was not active against Gram-negative bacteria or staphylococci.

Achemchem et al. (2005) examined Jben, a soft, farmhouse goat's cheese made in Morocco, as a potential source of LAB bacteriocin producers. Among the isolates, *Enterococcus faecium* F58 was selected for further study according to its broad inhibitory spectrum against *Listeria*, *Staphylococcus*, *Bacillus*, *Clostridium*, *Brochothrix* and *Lactococcus*, although none of the Gram-negative bacteria examined were inhibited. The results obtained concerning the characterization of inhibitory substance(s) produced by *E. faecium* F58 strongly suggested the proteinaceous nature of compound(s), which was named enterocin F58. The high thermostability of enterocin F58 and activity over a wide pH range justified its potential use for biopreservation in food systems.

Out of 2,227 LAB isolated from 27 Peruvian artisanal cheeses, 0.9% showed an inhibitory effect against *L. monocytogenes* CWBI-B2232 (*Gálvez et al*., 2009). As no change in inhibitory activity was observed after acid neutralization and treatment with catalase of the cell-free supernatants (CFS), the authors assumed that the antimicrobial effect of autochthonous LAB was due to bacteriocin-like substances, which was further confirmed by proteolytic digestion of the CFS.

Milioni et al. (2005) successfully recovered the anti-staphylococcal *Lactococcus plantarum* LpU4 from an Italian traditional Pecorino cheese. The confirmed the anti-staphylococcal effect of autochthonous LAB strains is a matter of utmost concern considering that 9.9% of all food-related outbreaks reported in the EU in 2015 were caused by staphylococcal toxins and cheese was the most commonly implicated food vehicle in the strong-evidence outbreaks caused by staphylococcal toxins (*EFSA-ECDC*, 2016).

Cosentino et al. (2012) reported that bacteriocin-producing strains *of Lactococcus lactis* subsp. *lactis* isolated from traditional Sardinian goat and sheep dairy products, growing in co-culture *with L. monocytogenes*, were able to reduce the *Listeria* counts by approximately 4 log units compared to the control. Comparable results were obtained by *Coelho et al.* (2014), who noticed that bacteriocinogenic *E. faecalis* isolates from a traditional Azorean artisanal cheese (Pico cheese), were able to control growth of *L. monocytogenes* in fresh cheese by decreasing the *Listeria* count by approximately 4 log during the storage period of 7 days.

The anti-listerial effect of bacterial communities in cheese has been frequently observed (*Eppert et al.*, 1997; *Maoz et al.*, 2003; *Mayr et al.*, 2004). Individually, however, the bacteriocinogenic strains isolated from complex anti-listerial microbiota did not show any inhibition of the *Listeria* growth (*Eppert et al.*, 1997), which emphasized the importance of bacterial interaction *in situ* (*Roth et al.*, 2010; *Demarigny and Gerber*, 2014).

Mezaini et al. (2009) concluded that *Streptococcus thermophilus* T2, isolated from Algerian traditional cheese, Raib, had antimicrobial potential toward the closely-related Gram-positive bacteria, *L. innocua* and *E. faecalis*, and is a promising candidate to help improve microbiological microbiological safety and increase the shelf life of traditional dairy fermented food.

5. Antimicrobial potential of autochthonous LAB microbiota isolated from raw milk cheeses in Serbia

Cheese is one of the oldest food products, and cheese production, dating from 8000 years ago, is a classic method of food preservation (*Savic et al.*, 2009). Serbia, as well as other Balkan countries, has a long history in the production of traditional dairy foods, including cheese **(***Golic et al*., 2013). In the Middle Ages, Serbian cheese was manufactured by the Vlach people during summer. Back then, cheese was a rare and expensive food, and so was considered to be food for the nobility. Cheese was not only an important food in Serbian cuisine, but it was also used as a valuable payment mechanism, being exchanged for salt and craft products. Moreover, during the reign of Emperor Dusan in the 1300s, a cheese impost was implemented, which was given as a donation to monasteries (*Marjanovic-Dusanovic*, 2004). Documents from 1417 suggest that Serbia exported cheese to Dubrovnik, then a well-known trade center. Two main cheese types produced in Serbia were white brined cheeses and much later, hard cheese, the so-called kachkaval (*Pejic*, 1956). According to historical data, the tradition of kachkaval (Tzintzar language the word ″kač″ means cheese) cheese making was introduced by nomads from Greece to locals on Stara Planina Mountain about 100 years ago (*Mijacević and Bulajic*, 2004). Nomadic sheep breeders known as Crnovunci (Blackwool people) started to produce kachkaval by enclosing white cheese in a sheep's stomach, bellows in hot water, then later mixing and salting the cheese. This manufacturing process was passed on to the people of Dojkinci village, who improved it and maintain it today (*Mijacevic and Bulajic*, 2004; *Veljovic et al.*, 2007). Kachkaval was exported to Vienna and Budapest. Zlatar cheese, white brined hard cheese, has been produced traditionally in Serbia for centuries (*Veljovic et al*., 2007). Other brined cheeses, including Sjenica cheese, Svrljig cheese, Golija cheese, Pirot cheese, Sara cheese and Homolj cheese, are traditionally manufactured in households in mountain regions, while Sombor cheese originates in the northern, flat plains of Serbia (*Dozet et al*., 1996; *Mijacevic and Bulajic*, 2008; *Vucic et al*., 2008; *Stevanovic et al.*, 2016).

It is not only a long-lasting manufacturing tradition that makes cheeses from Serbia special. The originality and specificity of the cheeses reflect the presence of natural LAB microbiota originating from raw milk and the specific geographic region (*Radulovic et al*., 2016). Specificities of microclimate, characteristics of the raw milk and sublethal stresses introduced through the manufacturing process (milk acidification, renneting, whey drainage, salting, and ripening) selectively favor development of LAB with unique phenotypic characteristics (*Mijacevic et al*., 2005). Their metabolic activity results in end products with specific sensory attributes. Accordingly, autochthonous cheeses might be considered as unique ecological entities (*Licitra*, 1997). Autochthonous, artisanal cheeses in Serbia are produced in households from raw milk (cow's, sheep's and goat's) or milk mixtures, without the use of commercial starter cultures.

Reduction of pH due to production of organic acids (primarily lactic acid) is the main preservation mechanism of LAB. *Veljovic et al.* (2007) and *Nikolic et al.* (2008) showed reasonable acidification autochthonous LAB isolates from Zlatar cheese showed reasonable acidification, able to decrease cheese pH to 4.8 after 5 to 7 h. LAB strains tolerant to high salt concentrations and able to grow at low pH, thus forming the predominant microbiota in cheese, have an important role in the ripening process (*Begovic et al*., 2011). Some LAB isolated from Zlatar cheese produced from sheep's or cow's milk were extremely tolerant to high salt concentrations as well as low pH, meaning they competed well with a variety of bacterial species, including spoilage microorganisms (*Terzic-Vidojevic* et al., 2007; *Veljovic et al*., 2007; *Terzic-Vidojevic et al*., 2013). *Mijacevic et al*. (2003) showed that LAB isolated from kachkaval were resistant to high temperature, low pH and high salt as result of their long-term adaptation to the technological conditions of cheese processing.

In most cases, autochthonous LAB in raw milk cheeses from the Balkan region and Serbia, as part of it, are mesophiles such as *Lactococcus* spp., *Lactobacillus* spp., and *Enterococcus* spp. or thermophiles such as *S. thermophilus* (*Terzic-Vidojevic et al*., 2007; *Veljovic et al.,* 2007; *Terzic-Vidojevic et al.,* 2009). LAB diversity in raw milk cheeses from Serbia is also reflected in the production of different antimicrobial substances like bacteriocins, hydrogen peroxide, and diacetyl which are antagonistic to a wide bacterial spectrum (*Veljovic et al.,* 2007).

The ability of LAB to produce bacteriocins enables these bacteria to grow competitively in mixed bacterial populations (*Topisirovic et al*., 2007). A study conducted on 253 samples of Zlatar cheese, production of which is not standardized, and which can be purchased only in local markets or from artisanal producers, reported that 70 *L. lactis* subsp. *lactis*, *E. faecalis* or *Lactobacillus paracasei* subsp. *paracasei* isolates produced proteinaceous, antimicrobial, possibly bacteriocin-like substances (*Topisirovic et al*., 2007). Comparable results were obtained another study, where 87 of 253 LAB isolates produced antimicrobial proteinaceous compound, possibly bacteriocin-like substances (*Veljovic et al.,* 2007). Enterococci isolated from Zlatar cheese as well as from Pirot kachkaval (an artisanal cheese from Stara Planina Mountain, made from raw cow's milk without starter culture) were reported to be producers of enterocins (*Topisirovic et al.,* 2007; *Terzic-Vidojevic et al.,* 2009). The importance of enterocins lies in their antimicrobial activity against Gram-positive bacteria such as *L. monocytogenes* and *L. innocua*, which was confirmed by *Nikolic et al.* (2008), who isolated enterococcal strains with antilisterial activity from Bukuljac, a homemade goat's cheese. *L. paracasei* subsp. *paracasei* isolated from homemade Sjenica cheese from the Pester Plateau, Serbia, produced bacteriocin BacSJ, a heat-stable proteinaceous bacteriocin, with retained activity after treatment at 100°C for 1 hour at pHs from 2 to 11. All lactococcal and enterococcal isolates from Vlasina cheese, traditionally made from raw sheep's and goat's milk, were bacteriocin producers, and 53.4% of LAB isolates from white pickled cheeses from rural South Morava and mountainous regions of Eastern Serbia and Golija Mountain were bacteriocin producers active against *S. aureus* (*Golic et al*., 2013; *Terzic-Vidojevic et al*., 2013). Bacteriocins produced by LAB isolate from Vlasina cheese survived for 30 minutes at 63.5°C (*Terzic-Vidojevic et al*., 2013). Antimicrobial compounds produced by lactococcal and enterococcal isolates from artisanal raw milk cheese produced on Stara Planina Mountain showed activity against *L. lactis* subsp. *lactis* and *L. paracasei* subsp. *paracasei* (*Begovic et al*., 2011).

LAB isolated from Zlatar cheese produced nisin-like substances that inhibited Gram-positive *S. aureus* and *L. monocytogenes*, as well as spore-forming bacteria including *Clostridium* spp. and *Bacillus* spp. Due to the fact that nisin is natural preservative, approved and commercially used in over 50 countries around the world, the safety of Zlatar cheese can be attributed to production of these substances (*Veljovic et al*., 2007).

Nikolic et al. (2008) studied the antimicrobial activity of *Lactobacillus* isolates (largely *L. paracasei* subsp. *paracasei*), a producer of the bacteriocin SJ, from Bukuljac cheese,with effect on *Lactobacillus paracasei* subsp. *paracasei*, as well as *Salmonella* Enteritidis.

LAB production of diacetyl is an interesting technological property due to the contribution of this compound to the buttery aroma of fermented cheeses. Moreover, diacetyl is recognized as an antimicrobial substance, although at concentrations which exceed its acceptable sensory level. However, synergistic effects, with diacetyl in combination with other antimicrobial factors, may be an acceptable solution (*Lee and Jin,* 208). Diacetyl inhibits the growth of Gram-negative bacteria by reacting with arginine binding protein, thus affecting arginine utilization (*Jay,* 1982). *Terzic-Vidojevic et al.* (2015) showed that the most of the lactococcal isolates from white pickled and fresh soft cow's milk artisanal cheeses in Serbia and Croatia were producers of diacetyl.

6. Conclusion

In conclusion, bacteriocinogenic isolates are common among the autochthonous LAB microbiota of Serbian raw milk cheeses. Further studies are encouraged to evaluate the application of these strains/bacteriocins to enhance the safety and quality of raw milk cheese.

Conflict of interest. The authors declare that they have no conflicts of interest.

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