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Antimicrobial activity of bacteriocins of Lactic Acid Bacteria on Listeria monocytogenes, Staphylococcus aureus and Clostridium tyrobutyricum in cheese production

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Abstract

The generally accepted concept of the necessity of producing safe foods has indirectly influenced the decision to replace chemical preservatives with natural ones. Bacteriocins, and in particular those synthesized by lactic acid bacteria (LAB) in the food industry, are considered to be their effective replacement. In controlling the growth of microbial pathogens and/or the occurrence of pathogenic bacteria in food, with the permitted nisin and pediocin, a significant antibacterial effect has been shown for most LAB bacteriocins. However, the use of purified bacteriocins as bio preservatives in cheese production is limited. To inhibit the growth of bacteria *L. monocytogenes, S. aureus* and *C. tyrobutyricum* in cheese, bacteriocinogenic LAB strains contained in primary, adjunct or protective culture are much more acceptable in cheese production.

Key words: bacteriocins and bacteriocinogenic LAB strains, inhibition, *Listeria monocytogenes, Staphylococcus aureus, Clostridium tyrobutyricum,* cheese

Introduction

Primarily due to the high resistance of pathogenic bacteria to antibiotics, bacteriocins have become equally important for the food industry (De Vuyst and Leroy, 2007; Mokoena, 2017; Teixeira Barbosa et al., 2017), human (Mathur et al., 2015; Sivaraj et al., 2018; Dreye et al., 2019; Lopetuso et al., 2019) and veterinary medicine (Lagha et al., 2017; Abdelfatah et al., 2018; Vieco-Saiz et al., 2019).

Consequently, compared to previous periods, the number of studies on the common and specific properties of bacteriocins, especially those synthesized by lactic acid bacteria (LAB), has increased significantly (Perez et al., 2014; Samaržija, 2015; Alvarez-Sieiro et al., 2016; Teixeira Barbosa et al., 2017; Tumbarski et al., 2018; Venegas-Ortega, 2019; Rahmeh et al., 2019).

In the food industry, LAB bacteriocins are considered as a real alternative to traditional food additives (Samaržija et al., 2009; Cotter et al., 2013; Perez et al., 2014). However, due to technological constraints, and especially due to legal restrictions, only nisin and pediocin PA-1/AcH are allowed in the production of food (Favaro et al., 2015). The criteria for authorizing the use of bacteriocins in food production are numerous and restrictive: (i) the microbial strain that forms it must have GRAS (Generally Regarded as Safe) status or QPS (Qualified Presumption of Safety) status, (ii) have a broad spectrum of inhibitory activity, (iii) exhibit highly specific activity, (iv) show no adverse effect on health, (v) not have the ability to transmit antibiotic resistance, (vi) demonstrate positive effects on improving the safety, quality and taste of food, (vii) have good stability in a wide range of temperatures and pH values, and (viii) have optimal solubility and stability in a specific type of food (Cotter et al., 2005; De Vuyst and Leroy, 2007; Leroy and De Vuyst, 2010; Silva et al., 2018). In addition, once isolated bacteriocins become susceptible to inactivation due to environmental and physio-chemical conditions, which limits their use. However, encapsulation of bacteriocins using nanotechnology is considered as a good strategy for creating a protective barrier from environmental conditions and/or temporarily increasing its activity against target microbial species (Tumbarski et al., 2018; Venegas-Ortega et al., 2019). The use of LAB bacteriocin in the food industry is determined by the type of food and the technology of its processing. In this regard, bacteriocins can be used to improve food quality and safety: (i) by inoculation of bacteriocin containing active LAB strains in the form of primary, adjunct or protective culture, (ii) by adding a pre-fermented product containing bacteriocinogenic LAB strains, (iii) by direct addition of purified or partially purified bacteriocin, and (iv) indirectly by incorporating bacteriocin into the packaging protective film (De Vuyst and Leroy, 2007; Borges and Teixeira, 2016).

In general, cheese is considered a safe food because of its physicochemical characteristics and the antimicrobial activity of its microbiome against pathogenic bacteria (Fox et al., 2000; Farkye and Vedamuthu, 2002; Irlinger et al., 2015). Despite that, according to data available for 2015 published by the European Food Safety Authority (EFSA) and the European Centre for Disease Control (ECDC), epidemic poisoning caused by consumption of cheese contaminated with pathogenic bacteria and/or their toxins has been reported in many countries (EFSA and ECDC, 2016). This is especially true for the consumption of soft cheeses (≥50 % moisture) contaminated with staphylococcal enterotoxins or *Listeria monocytogenes* (Choi et al., 2016; Babić et al., 2018). The potential use of bacteriocin as a bio preservative in these types of cheeses seems justified. On the contrary, for semi-hard (39 % - 50 % moisture) and hard cheeses (<39 % moisture), which generally do not support the growth of pathogenic bacteria after two months of ripening (Cogan and Beresford, 2002) the effect of bacteriocin may be significant for growth inhibition of *Clostridium* spp., which cause late blowing defect.

Compared to other types of fermented foods, the use of bacteriocins in cheese production is limited, and the study of their actual effect in the cheese matrix is extremely complex. This claim is equally true regardless of whether they are used to prevent the growth of pathogenic bacteria or microbial spoilage agents, or to improve the ripening and sensory properties of cheese.

This review is a contribution to the analysis of scientific results on the effect of purified or semi-purified bacteriocins and bacteriocinogenic LAB strains on the growth inhibition of pathogenic bacteria *S. aureus*,

L. monocytogenes and *C. tyrobutyricum* in cheese. Also, the paper highlights research topics in the field that currently capture the interest of the professional and scientific public.

Bacteriocins of lactic acid bacteria

In general, bacteriocins are a heterogeneous group of ribosomally synthesized bioactive antimicrobial peptides or proteins of many types of gram-positive and gram-negative bacteria, including certain types of archaea (O'Connor and Shand, 2002; Nandane et al., 2007; Samaržija, 2015). Most bacterial species (~99 %) synthesize at least one bacteriocin. These can be post-translationally modified by cellular enzymes or excreted from the bacterial cell into the environment unmodified (Yang et al., 2014). For the bacterial cell which forms them, bacteriocins have a primarily protective function against other microbial species competing for the same source of nutrients. The formation of bacteriocin is an evolutionarily inherited bacterial ability of an effective mechanism of self-defence. It is thought that a bacterial cell uses bacteriocin for survival in competition with closely related species within a specific ecological habitat. Therefore, in most cases the inhibitory activity of bacteriocins is directed only at closely related species. However, certain bacterial species, or more specifically their strains, also have the ability to produce bacteriocins with broad inhibitory spectrum which act against different microbial species (Yang et al., 2014; Karpiński and Szkaradkiewicz, 2016; Tulini et al., 2016).

Bacteriocins synthesized by LAB are usually thermostable small peptides that have a narrower or broader spectrum of inhibitory activity against other bacteria, including antibiotic-resistant species. According to the available data, more than 230 LAB bacteriocins are currently described, half of which have been identified at the protein DNA level. In addition, 785 putative sets of genes responsible for bacteriocin synthesis, including ribosomal and post-translationally modified antimicrobial peptides, were identified on the basis of the fully described genomes for the 12 genera of LAB, which was not previously the case (Alvarez-Sieiro et al., 2016). Classification and mechanism of antibacterial action of bacteriocins of LAB for their potentially wider application in the food industry, animal husbandry, aquaculture, medicine, veterinary medicine, the pharmaceutical industry or cosmetics industry are described in detail in several excellent review articles (Cotter et al., 2013; Yang et al., 2014; Egan et al., 2016; López et al., 2016; Alvarez-Sieiro et al., 2016; Teixeira Barbosa et al., 2017; Silva et al., 2018; Tumbarski et al., 2018; Lepetuso et al., 2019; Choyam et al., 2019). In this context it is also important to highlight the fact that certain strains of *Enterococcus* genus synthesize, different by chemical structure, bacteriocins of the common name enterocins. More than 30 have been isolated so far, the most studied of which is enterocin AS-48 synthesized by *Enterococcus faecalis* (Hanchi et al., 2018). Notwithstanding the fact that enterococci are pathogenic in some cases and most of them (~88 %) are antibiotic resistant, modern genotyping techniques make it possible to isolate their safe bacteriocinogenic strains (Arbulu et al., 2016; Xi et al., 2018). Depending on the chemical structure, some of them are classified into established groups and some still cannot be classified. Many enterocins have been found to have a strong inhibitory effect on foodborne pathogenic bacteria (Favaro et al., 2014). Their antibacterial activity is preserved over a wide range of pH values (2-12), at higher heating temperatures (100 °C/1 hour) and with the use of chemical agents (Ribeiro et al., 2017).

The use of bacteriocins of LAB in cheese production

Compared to the number of studies on the possible use of LAB bacteriocin in preventing the growth of microbial spoilage agents and pathogenic bacteria in food, the number of such studies in cheese production is relatively small. The reasons for this are multiple, but in this area they are primarily determined by: (i) number of types (>2200) and groups of cheeses (soft, semi-hard, hard, extra hard, smear-ripened cheeses, cheeses with moulds ...) and (ii) by the use of microbial cultures that limit the use of purified

and semi-purified bacteriocins and inoculation with active LAB strains bacteriocin. Additionally, unambiguous descriptions of the effect of bacteriocin on the quality and health of cheese are limited by its specific technology (Favaro et al., 2015; Blaya et al., 2018; Yeluri Jonnala et al., 2018). In other words, in the production of cheese is used both raw and pasteurized milk, microbial population of each individual cheese in the same species is unique, and there are considerable differences in their proportions of protein, fat and salt (up to 6 %), moisture content, pH value (~4.2-7) and ripening temperature (~6-8 and 20-24 °C). Therefore, assessment of the effect of bacteriocins and/or bacteriocinogenic strains of LAB is extremely complex.

Within the relatively small number of studies on the benefits of bacteriocin as a bio-preservative in cheese production, the largest number is concerned with determining their ability to inhibit the growth of the pathogenic bacteria *L. monocytogenes* and *S. aureus*, and *Clostridium* spp. In cases of epidemic cheese poisoning, *L. monocytogenes* and *S. aureus* are its most commonly isolated pathogens (de Oliveira et al., 2018). Otherwise, ubiquitous *L. monocytogenes* is the only species of the foodborne *Listeria* genus that belongs to the group of intercellular pathogenic bacteria. Mortality caused by infection with this pathogenic bacterium for pregnant women, the elderly and immunocompromised persons can be up to 30 % (Buchanan et al., 2017; Heir et al., 2018). In addition to pathogenicity, of particular importance for cheese production are their physiological growth abilities: (i) in the environment with a salt concentration higher than 10 % (a_w 0.92), (ii) in the pH range from 4.3 to 10.0, and (iii) of survival in brine (25.5 % NaCl) up to 4 months at 4 °C temperature (Ryser, 2011).

Among the groups of cheeses, the most favourable environments for the growth of *L. monocytogenes* are: (i) fresh (casein and albumin) cheeses, (ii) soft cheeses with the mould rind (for example, Brie, Camembert) and (iii) smear-ripened cheeses (for example, Münster, Reblochon). The intrinsic and extrinsic properties of these groups of cheeses support its growth throughout maturation and/or viability (Farkye and Vedamuthu, 2002; Amato et al., 2017; Jackson et al., 2018). On the contrary, in semi-hard and hard cheeses the presence of *L. monocytogenes* is extremely rare (Cogan and Beresford, 2002). Of the coagulase-positive staphylococci, only enterotoxic *S. aureus* strains that secrete thermostable enterotoxin into the food are considered pathogens (Johler et al., 2015; Cousin et al., 2018; Fisher et al., 2018). The bacterium *S. aureus*, like *L. monocytogenes*, belongs to the group of ubiquitous organisms. Therefore, in addition to *E. coli, Shigella, Bacillus* and *Clostridium*, it is the leading cause of food intoxication (EFSA, 2016). The risk of cheese contamination with staphylococcal enterotoxins is also determined by its physiological growth ability over a wide range of temperatures (7-48 °C) and pH values (4-10). Also, compared to most other species, *S. aureus* in the growth medium tolerates the lowest amount of available water (a_w 0.83-0.86) and very high salt concentrations (15-20 %). The *S. aureus* also has a high capacity to develop antibiotic resistance (Samaržija et al., 2007; Asperg and Zangerl, 2011; Medveďová and Valík, 2015). However, compared to other pathogens that mostly contaminate cheese, the reproduction number of *S. aureus* for human enterotoxin poisoning is relatively large (~10^5 cfu/mL/g).

The bacteria *Clostridium* spp., and in particular the *C. tyrobutyricum* species, are the most commonly isolated causes of late blowing of semi-hard and hard cheeses (Panelli et al., 2013). Late blowing is a characteristic microbial mistake of cheese textures, which happens at the end of ripening, sofor the dairy industry, primary it has an economic significance. Among the microbial species that can cause late blowing defects in semi-hard (e.g. Gouda, Edam) and hard cheeses (e.g. Emmental, Gruyère), *C. tyrobutyricum* is its most commonly isolated agent (Aureli et al., 2011; Ivy and Weidmann, 2014). Namely, the pH value of most types of semi-hard and hard cheeses is between 5.2 and 5.3, and the optimum pH for growth and propagation of this bacterium is 5.8. In contrast, bacteria *C. butyricum, C. beijerinckii* or *C. sporogenes* have an optimal pH value of growth and multiplication between 6.5 and 7.0. Therefore, as an individual species, these bacteria are less often isolated causative agents of late blowing. *C. tyrobutyricum* is also a cause of late blowing of extra hard cheeses such as Grana Padano, Parmigiano Reggiano, Pecorino Sardo or Pag cheese, which are ripening for at least six months (Gomez-Torres et al., 2015; D'Incecco et al., 2018). The initial number of this bacterium in raw milk required for the late blowing defect is

extremely low. For example, 5-10 spores *of C. tyrobutyricum* in a litre of milk are enough to cause late blowing of Gouda. However, depending on the type, the number of spores isolated from the spoiled cheese is between 10^3 and 10^6 g^{-1} (Samaržija et al., 2007). In addition to milk, the main source for the occurrence of *Clostridium* spp. in cheese is reusable brine.

In cheese production, the main advantage of using LAB bacteriocins is the real possibility that these naturally occurring non-toxic preservatives (Cotter et al., 2013; Choyan et al., 2019) replace chemical preservatives. The wider selection of bacteriocin is another advantage over conventional preservatives. This applies in particular to the use of bacteriocinogenic strains in the composition of primary, adjunct or protective cultures (Beshkova and Frengova, 2012; Ben Said et al., 2019). Unlike purified and/or semi-purified bacteriocins, the use of bacteriocinogenic cultures is not limited by law (Arqués et al., 2015). Also, unlike purified ones, bacteriocinogenic strains in the culture composition do not bind to protein and/or milk fat in cheese, and there is no negative effect on its sensory quality (Favaro et al., 2014). However, there are cultures available on the market that contribute to the quality and safety of cheese, and have a relatively low sensitivity to digestive protease, without changing sensory characteristics (Barreto Penna and Todorov 2016; Ben Said et al., 2019). Consequently, in recent years, research has been intensified on the isolation and description of new strains of LAB, bacteriocinogens, for their potential use in cheese production based on the results of previous studies. Therefore, the results of previous studies on the effectiveness of bacteriocin LAB on the growth inhibition of *L. monocytogenes*, *S. aureus* and *Clostridium* spp. in the production of fresh, semi-hard and hard cheese are presented below.

Fresh and soft cheeses

Fresh and soft cheeses are more susceptible to contamination by *L. monocytogenes* and *S. aureus* than other cheese types. This is due to their high water content (~67-80 %) and the high pH value for soft cheeses, especially those that ripen through the activity of moulds or bacterial smears on the surface. Nisin, PA-1/AcH pediocin, lacticin 3147 and enterocins alone or in combination with other antimicrobial methods such as the use of high pressure or lysozyme supplementation, significantly reduce the initial number of these pathogens in these cheeses (Sobrino-López and Martín-Belloso, 2008; Ben Said et al., 2019). The use of commercial nisin, with or without other protective procedures, has proven to be effective in preventing the growth of *L. monocytogenes* for most types of these cheese groups (10^2-10^6 cfu g^{-1}). Purified nisin (50 IU/g^{-1}) used as an additive in an edible protective coating, has been found effective in inhibiting the growth of *L.monocytogenes* present in the number of ~10^6 cfu g^{-1} in albumin cheeses. Respectively, this protective combination can eliminate the appearance of *L. monocytogenes* in cheese stored at 4 °C for the first seven days (Martins et al., 2010). The occurrence of *L. monocytogenes* in white brine cheeses can be completely eliminated by the addition of nisin (1000-1500 IU mL^{-1}) into pasteurized milk and a subsequent heat treatment (63 °C/5 min) of finished cheese packed into vacuum plastic bags (Al-Holy et al., 2012). The combination of nisin and heat successfully inhibits the growth of this bacterium in cheese within 8-10 weeks, regardless of whether its storage temperature is 4 or 10 °C. This protective measure is also effective in cases of high initial brine contamination with *L. monocytogenes* (10^6 cfu mL^{-1}). In the production of fresh, non-cultured cheese, the addition of purified nisin (500 IU mL^{-1}) to pasteurized milk effectively inhibits the growth of *S. aureus* (Felicio et al., 2015). Primarily, this study confirmed that for fresh cheeses, and especially those with extended shelf life, nisin effectively reduces their numbers to levels insufficient for enterotoxin formation.

To inhibit the growth of *L. monocytogenes* and *S. aureus* in fresh cow cheese within 96 hours of storage at 4 °C, Kondrotiene et al. (2018) researched the effect of three different strains of *Lactococcus lactis* isolated from raw goat's milk which synthesize nisin Z. Individual bacteriocinogenic strains (~10^8 cfu mL^{-1}) in form of culture (2 %) were added to raw and pasteurized cow milk.

Regardless of whether fresh cheese (up to four days of shelf life) is produced from raw or pasteurized milk, all tested strains have proven to be effective biological preservatives in controlling the growth of these pathogenic bacteria. The addition of purified lacticin 3147 (10 % w/v) to fresh cheese

artificially inoculated with *L. monocytogenes* (10^4 cfu mL^{-1}) within 5 min at a temperature of 30 °C can eliminate up to 40 % of its initial population and after 120 min for ca. 85 % (Morgan et al., 2001). The authors speculate that lacticin 3147 may completely inhibit the growth of *L. monocytogenes* by prolonged incubation of fresh cheese at 30 °C. In controlling the growth of *L. monocytogenes* in fresh cheese there are also bacteriocinogenic strains that synthesize enterocins when used as adjunct culture (1-2 %). High antilisterial activity was confirmed for bacteriocinogenic strains of enterococci and *L. lactis* with added genes for enterocin formation (Achemchem et al., 2006; Liu et al., 2008; Khan et al., 2010).

Smear ripened cheeses

In controlling the growth of *L. monocytogenes* (10^2-10^4 cfu g^{-1}) on the surface of smear ripened cheeses, the dispersion of bacteriocinogenic protective culture on the cheese surface was proven effective. A culture composed of a transconjugated *L. lactis* strain that forms lacticin 3147 and lacticin 481 has a faster and more effective inhibitory effect on the growth of that pathogenic bacterium compared to cultures in which the bacteriocinogenic strain has the ability to form only one of these two bacteriocins. In addition, regardless of the strain used, growth inhibition of *L. monocytogenes* has no effect on the smear microbial developement (O'Sullivan et al., 2003a; O'Sullivan, et al., 2006). Loessner et al. (2003) found that the use of bacteriocinogenic *L. plantarum* strains on smear ripened cheeses was not effective in inhibiting the growth of *L. monocytogenes* when continuously used over a longer period. Namely, most strains of this pathogenic bacterium have a high potential for developing pediocin resistance. Therefore, the authors suggest that in protective culture, these strains are occasionally replaced by those that have the ability to form bacteriocins with different chemical structures. For example, strains that synthesize nisin or lacticin. In controlling the growth of *L. monocytogenes* on the surface of smear ripened cheeses, the influence of bacteriocinogenic strains of enterococci was investigated (Martín-Platero et al., 2009). The advantage of enterocin compared to other bacteriocins is the extremely rapid antibacterial effect (within 30 min), the narrow spectrum of activity (mainly against enterococci and *Listeria* spp.) and the same activity in the pH range 4.0-8.0. For example, in the production of Münster cheese, Izquierdo et al. (2009) found a strong antilisterial activity for *E. faecium* WHE 81, which forms several types of bacteriocins. In these studies, a strain of *E. faecium* WHE 81 (~10^5 cfu mL^{-1}) was added to the surface of the cheese on the seventh day, simultaneously with the smear culture (*Debaryomyces hansenii* and *Brevibacterium linens*). The initial *L. monocytogenes* number on the cheese surface (10^2 cfu g^{-1}) was reduced to a population of <50 cfu g^{-1} which was no longer able to initiate its growth. On the other hand, *E. faecium* WHE 81 which naturally exists on the surface of Münster cheese, had no negative effect on the course of its ripening.

Semi-hard and hard cheeses

Compared to the growth and survival of *S. aureus* in fresh and soft cheeses, in almost all semi-hard and hard cheeses its number decreases during ripening. Most commonly, after 30 days of ripening, these cheeses are no longer positive for *S. aureus*, regardless of the initial contamination level of milk and/or coagulum (Samaržija et al., 2007). However, this does not mean that they do not contain staphylococcal enterotoxins (SE) at a concentration sufficient to intoxicate the human body. Pinto et al. (2011) on semi-hard traditional Brazilian Minas Serro cheese have confirmed that a low nisin concentration of 500 IU mL^{-1} added to raw milk before coagulation can reduce the initial bacterial count of *S. aureus* from ~10^4 cfu mL^{-1} for 2 log units in the first seven days of ripening. That is, to a level insufficient to produce a toxic enterotoxin concentration. The results of these studies are particularly relevant for the production of semi-hard and/or hard cheeses made from raw milk, where it can be expected to occur in numbers ≥10^5 cfu mL^{-1}. Rodríguez et al. (2005) investigated the influence of a protective culture with the transconjugant *L. lactis* strain forming pediocin and nisin on growth control of *S. aureus* in semi-hard cheese. Pasteurized milk was artificially inoculated with *S. aureus* (~10^6 cfu mL^{-1}), and a protective culture (1 %) was added in addition to commercial mesophilic culture (1 %). The cheese matured in plastic bags under vacuum for 30 days. The protective culture reduced the initial *S. aureus* number by 0.98 log units after 30 days of ripening. Scannell et al. (2000) determined the efficacy of nisin incorporated in protective packaging

on the growth control of *S. aureus* in Cheddar cheese packed in slices under vacuum. The significance of these studies is in finding that nisin incorporated in protective packaging retains its activity for a period of 3 months, regardless of the storage temperature.

Despite numerous studies, there is still no universal method for the elimination of *C. tyrobutyricum*, a cause of late blowing of semi-hard and hard cheeses (D'Incecco et al., 2015; Talukdar et al., 2017). In practice, preventive methods commonly used for this purpose are bactofugation, high hydrostatic pressure, microfiltration or the addition of nitrates and nitrites or lysozyme.

Studies on the effectiveness of LAB bacteriocin, as a different antimicrobial component, in growth inhibition of vegetative cells and germination of *C. tyrobutyricum* spores are limited by: (i) abundance of types of semi-hard and hard cheeses, (ii) differences in the intrinsic and extrinsic properties of cheeses (e.g. a_w, pH, salt concentration, ripening temperature) and (iii) significant physiological differences between *C. tyrobutyricum* strains (Ruusunen et al., 2012). In other words, high physiological variability and different growth abilities were found between the strains of this species, under stressful conditions. To eliminate spores and vegetative cells of *C. tyrobutyricum* in this cheese group, Ávila et al. (2014) compared the effectiveness of lysozyme, reuterin and sodium nitrate alongside nisin. The results of these studies confirmed that reuterin (0.51-32.5 mM) and nisin (0.05-12.5 μg mL^{-1}) in cheese production may probably be good preventive options for its growth control. The use of mesophilic culture with the bacteriocinogenic strain *L. lactis* subsp. *lactis* (1 %), which forms nisin Z, has proven to be effective in preventing the growth of *C. tyrobutyricum* (~10^6 spores g^{-1}) in semi-hard cheese (Rilla et al., 2003). During the 30 days of ripening (12 °C, 90 % relative humidity), the initial *C. tyrobutyricum* number in the cheese was reduced to ~10^3 cfu g^{-1}. On the contrary, in the cheese produced by commercial culture (control group), its number increased to >10^7 cfu g^{-1} in the same period. Mathot et al. (2003) researched the influence of the bacteriocinogenic strain *Streptococcus thermophilus* (~10^7 cfu mL^{-1}) in the production of hard cheese in combination with *Lactobacillus delbrueckii* subsp. *lactis* in primary thermophilic culture on the growth of *C. tyrobutyricum* (10^2-10^4 spores mL^{-1}). In the experiment, gas formation was not determined within 20 days of cheese ripening. On the contrary, in the control samples gas formation was visible after 8 days (10^4 spores mL^{-1}), or after 14 days (10^2 spores mL^{-1}). For inhibition of *C. tyrobutyricum* spore growth in semi-hard cheese (maturing for 8 weeks) Bogovič Matijašić et al. (2007) tested the effectiveness of the probiotic strain *Lactobacillus gasseri* K7 (Rif +). At the same time, commercial thermophilic culture (*Streptococcus thermophilus*), *L. gasseri* K7 (Rif +) (~10^7 mL^{-1}), and *C. tyrobutyricum* spores (~10^3 mL^{-1}) were added to pasteurized milk. After 6 weeks of ripening (15-17 °C), the average concentration of butyric acid in the control cheese compared to the experimental one was significantly higher (1.43 vs 0.70 g kg^{-1}). The probiotic strain *L. gasseri* K7 (Rif +) maintained its initial number until the end of cheese ripening and had no inhibitory effect on *S. thermophilus*.

Although no specific bacteriocin was confirmed by these studies, the results confirmed that *L. gasseri* K7 (Rif +) can effectively inhibit the growth of vegetative cells and prevent the development of *C. tyrobutyricum* spores in semi-hard cheese. In general, the selection of probiotic LAB strains to determine their inhibitory activity against undesirable microbial species is considered desirable since most or all of them belong to bacteriocinogenic strains (Zamberlin et al., 2012; Samaržija, 2015; Choyam et al., 2019). Combination of high-pressure homogenization (HPH) and nisin has also been shown to be effective in controlling the growth of *Bacillus* spp. and *Clostridium* spp. The assumption is that the inactivation of these spores is due to (i) synergetic effect of nisin and HPH on spore inactivation or (ii) induction of spore germination by HPH after which the nisin has a lethal effect on them (Egan et al., 2016).

Isolation and identification of bacteriocinogenic strains of LAB

Research on the isolation and identification of bacteriocinogenic strains of LAB for their potential use in cheese production began about twenty years ago, and has intensified in recent years (Pogačić et al., 2010). Namely, on the basis of present knowledge, it is considered that bacteriocinogenic strains of BMK contained in primary, adjunct or protective culture in cheese production can have a much wider

application than their purified or semi-purified bacteriocins. Kabuki et al. (2006) found that bacteriocin thermophilin 1277 synthesized by the strain of *Streptococcus thermophilus* SBT1277, when isolated from raw milk, has antimicrobial activity against some types of LAB and bacteria causing spoilage, *C. butyricum, C. sporogenes* and *B. cereus*. The importance of these studies lies in the fact that the optimum temperature for the formation of thermophilin 1277 for this bacterial strain is 35 °C, and that it is significantly lower at higher (45 °C) and lower (30 °C) temperatures. In this respect, the bacteriocinogenic strain of *S. thermophilus* in the primary or adjunct culture may replace its non-bacteriocinogenic strain in the growth control of, for example, *Clostridium* spp. in semi-hard and hard cheeses. From raw sheep milk used in production of PDO Zamorano cheese, Bravo et al. (2009) isolated 10 strains of *L. lactis* subsp. *lactis* having structural genes for the simultaneous synthesis of nisin and lacticin 481. The authors believe that bacteriocinogenic LAB strains that simultaneously synthesize two different bacteriocins are better candidates for the composition of protective cultures than those that synthesize only one. With the same aim of isolating bacteriocinogenic LAB strains as potential candidates in the culture composition, Dal Bello et al. (2010) isolated 1000 isolates from indigenous products (cheeses and meat) of north-western Italy (Piedmont). For 98 of them the bacteriocin synthesis abilities were confirmed, and for 56 isolate inhibitory activity against more than one indicating bacterial species (*L. monocytogenes*, *S. aureus*, *C. tyrobutyricum*, *E. coli*, *S. enteritidis*). Of the 55 lactococcal isolates, for 40 of them the ability to synthesize nisin (A or Z) or lacticin (481) and lactococacin B was confirmed. Simultaneous synthesis of two bacteriocins was confirmed for 8 isolates of *L. lactis*, (nisin A and Z), 1 strain *L. lactis* (lacticin A and nisin A), 1 strain of *L. lactis* subsp. *cremoris* (nisin Z and lactococcin B). Of the 22 bacteriocinogens of *Enterococcus faecium* isolates, four have been confirmed for the synthesis of two enterocins (A and P). Soliman et al. (2011) explained at the nanomolar level the mode of antimicrobial activity of the two-peptide plantaricin S formed by the strain *Lactobacillus plantarum* LPCO10 against pathogenic bacteria [*L. monocytogenes* (2 strains), *E. faecalis* (2 strains), *S. aureus* (2 strains)]. For potential protective application in the production of raw milk cheeses, Milioni et al. (2015) tested 35 strains of *L. plantarum* isolated from traditionally produced sheep Pecorino cheeses. Among them, the *L. plantarum* LpU4 strain, which synthesizes plantaricin LpU4 and exhibits strong inhibitory activity against pathogenic *S. aureus* strains of different phenotypic resistance was separated. Plantaricin LpU4 also shows a complete antibacterial efficacy under conditions similar to those used in the production of sheep milk cheese (pH 4.8-5.6, NaCl ~3 %, maturing at room temperature). Macaluso et al. (2016) used the biofilm of 20 wooden boilers to produce traditional raw sheep (Pecorino Siciliano and Vastedda della Valle del Belice) and cow milk cheeses (Caciocavallo Palermitano), without the addition of culture. Out of 669 isolates, 37 showed strong inhibitory activity against *L. monocytogenes*.

According to several authors, the antilisterial effect of these isolates can be used to incorporate them into the production system to increase the safety of the formed microbial biofilm during cheese production. Rumjuankiat et al. (2015) successfully isolated and described three new bacteriocins (plantaricin KL-1X, KL-1Y, KL-1Z) from the bacterial strain *L. plantarum* KL-1. These bacteriocins confirmed their stability in the medium with pH values of 2 to 12 and at temperatures of 25 °C, 80 and 100 °C/30 min and 121 °C/15 min. At the same concentrations, plantaricin KL-1Y exhibits higher inhibitory activity against indicator gram positive and gram negative bacteria (*B. coagulans, B. cereus, L. innocua, S. aureus, E. coli* O157:H7) than nisin. At the same time, nisin has the same antibacterial activity as KL-1Y only against *B. coagulans* and *B. cereus*. Azizi et al. (2017) suggest *Lactobacillus plantarum* strains as potential candidates in primary or adjunct cultures, which simultaneously produce plantaricin A and EF, and which have shown antagonistic activity against indicator bacteria *S. aureus*, *L. innocua* and *E. coli*. Bacteriocinogenic *L. plantarum* strains were isolated from indigenous Iranian cheese made from raw milk. Other than that, *L. plantarum* that forms at least six different plantaricins can also inhibit many LAB types, such as clostridia and propionic bacteria naturally occurring in the environment (Tumbarski et al., 2018). In particular, this knowledge is important for controlling the growth in the incidence of the secondary microbial LAB population on cheese.

Specifically, the predominant microbiotas of ripening cheeses are LAB in the composition of primary and or adjunct cultures and those originating in the production environment (nonstarter or NSLAB). During the 3 to 9 month ripening period NSLAB can be present in numbers up to 8 log cfu g^{-1}. Although important for ripening, their metabolism may cause errors or inconsistencies in the quality of cheese, depending on the dominant species or strain (Blaya et al., 2018). Rahmeh et al. (2019) suggest the use of bacteriocinogenic strains of *Pediococcus pentosaceus* CM16 and *Lactobacillus brevis* CM22 isolated from raw camel milk as potential candidates in protective cultures. Semi-purified bacteriocins of both these strains show good technological characteristics such as thermostability and activity in the pH range of 2-10; in addition to strong antilisterial activity. Peirotén et al. (2019) propose new strains of bifidobacteria, *B. breves* INIA P734 and *B. bifidum* INIA P671 as potential candidates for composition of adjunct cultures. These strains are common to mothers and infants during breastfeeding. In addition to technological stability and the absence of a negative effect on cheese quality, these strains also show resistance to gastrointestinal conditions.

These strains of bifidobacteria can also increase the functional value of cheese, since almost all probiotic bacteria form bacteriocins with protective function (Samaržija, 2015). Scatassa et al. (2017) found that LAB derived from the environment of Sicilian dairy industry could be a good source of new bacteriocinogenic strains controlling the growth of *L. monocytogenes* in cheese. An *in vitro* study showed a strong antilisterial activity in tested strains of *L. lactis, L. rhamnosus* and *E. faecium* which did not adversely affect the quality of Pecorino Siciliano cheeses. Tulini et al. (2016) tested the antimicrobial and proteolytic activity of LAB isolates from raw cow, goat and buffalo milk and cheeses in the south eastern region of Brazil to determine potential candidates for culture composition. *Streptococcus uberis* (3 strains) isolates were characterized as bacteriocinogens, and *Weissella confusa, W. hellenica, Leuconostoc citreum*, and *L. plantarum* showed a strong antifungal activity. Additionally, all these isolates also showed a strong proteolytic activity. Particular relevance of these studies lies in the idea that, during the selection of new potential strains for culture composition, those are chosen that simultaneously show the protective but also a strong proteolytic, lipolytic or glycolytic activity, necessary for the proper course of cheese ripening. Arbulu et al. (2016) hypothesized that birds belonging to the Griffon Vulture family, feeding solely on carcasses without displaying any health problems, could be a good source of bacteriocinogenic LAB strains with potential biotechnological applications. Molecular identification confirmed that the most LAB with antimicrobial activity isolated from the faeces of that bird belongs to enterococci (91 %), of which *E. faecium* accounts for 40 %. Most (75 %) have genes for multiple bacteriocin production. Among these isolates, an enterocin HF-forming strain of *E. faecium* M3K31 was identified as an effective bio-preservative candidate in the control of bacterial growth of *Listeria* spp. according to a safety assessment prescribed by The European Food Safety Authority (EFSA, 2012), *E. faecium* M3K31 does not show antibiotic resistance, is free of potential virulence factors, and is sensitive to peptidases. Compared to enterocin A, enterocin HF has significantly higher potential for *Listeria* spp.

Recently, the Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) method has been successfully used to rapidly isolate and identify potentially new bacteriocinogenic LAB strains or pathogenic bacteria associated with food contamination. For example, using the MALDI-TOF MS method, it is possible to identify all pathogenic species associated with food contamination (Pavlović et al., 2013) or identify LAB from non-conventional yogurt (Karaduman et al., 2017) or traditional French Maroilles cheese at species or subspecies level, using the same protocol, all within 16 hours (Nacef et al., 2017). To identify potential bacteriocinogenic candidates for protective cultures, Kanak et al. (2018) used the MALDI-TOF MS method to rapidly classify 150 LAB isolates at the species level, isolated from 21 traditionally produced cheeses in Turkey. According to the results of the MALDI-TOF MS method, the dominant LAB types of these cheeses are *E. faecium* (34 %) and *E. faecalis* (25 %). However, only *E. faecalis* (6 strains) and *E. faecium* (3 strains) showed antimicrobial activity against pathogenic bacteria (*L. monocytogenes, S. aureus, E. Coli* O157: H7, *C. sakazakii, B. cereus* and *S. Typhimurium*). That is, the authors identified three strains of *E. faecium* as one candidate, one of which has a strong

inhibitory effect on *L. monocytogenes*, one against *E. coli* O157: H7 and one against *C. sakazakii*. The importance of these results and the results of Nacef et al. (2017), Karaduman et al. (2017) is in the application of the relatively new, fast and reliable MALDI-TOF MS method in identifying the microbial diversity of LAB from different ecological niches. The MALDI-TOF MS method enables comparison of the obtained profiles with reference species and rapid classification of isolates. In other words, this method enables easier detection of new bacteriocinogenic LAB strains as potential candidates for the composition of cheese cultures.

Conclusion and future perspectives

Based on previously conducted research on the growth control of pathogenic bacteria in *L. monocytogenes, S. aureus*, and the cause of late blowing *C. tyrobutyricum*, the use of bacteriocin LAB alone or in combination with other preventive methods has proven to be effective. However, for the wider use of LAB bacteriocin in cheese production, future research must be based on the so-called *multi-omic* (genomic, transcriptomics, proteotomics, metabolomics) approach. In other words, to understand the complex cheese ecosystem, these studies must include a combination of genomic and post-genomic studies of LAB and other microbial species involved in cheese ripening as a single integral system: genes, environmental conditions, biochemical pathways, proteins and metabolites, microbial functions and interactions that take place during cheese production and ripening (Blaya et al., 2018). Also, with innovative methods such as MALDI-TOF MS, isolation of new effective bacteriocinogenic or antifungicidal strains from different ecological niches and food samples is significantly faster compared to the previous period. In this regard, regardless of abundance of types (>2200) and groups of cheeses, it is only through an interdisciplinary approach that the true potential of LAB bacteriocin on the sensory, nutritional and health quality of the cheese can be determined. With this approach, it is realistic to expect that, in growth control of undesirable microbial species in cheese, it will soon be possible to select bacteriocins: (i) of narrower and broader spectrum of antimicrobial activity (ii) most effective for a particular type or group of cheese or (iii) most effective when combined with other preventive procedures. It will also be possible to select new bacteriocinogenic LAB strains which have a beneficial effect on the ripening and sensory quality of the cheese or can inhibit the growth of microbial pathogens.

Antimikrobna aktivnost bakteriocina bakterija mliječne kiseline prema Listeria monocytogenes, Staphylococcus aureus i Clostridium tyrobutyricum u proizvodnji sira

Sažetak

Opće prihvaćen koncept o nužnosti proizvodnje zdrave i sigurne hrane neizravno je utjecao na odluku da se i kemijski konzervansi zamijene prirodnim. Bakteriocini, a osobito oni koje sintetiziraju bakterije mliječne kiseline (BMK) u prehrambenoj se industriji smatraju njihovom učinkovitim zamjenom. U kontroli rasta mikrobnih uzročnika kvarenja i/ili pojavnosti patogenih bakterija u hrani, uz dopušteni nizin i pediocin i za većinu je do sada opisanih i pročišćenih bakteriocina BMK utvrđen značajan antibakterijski učinak. Međutim, primjena pročišćenih bakteriocina kao biokonzervansa u proizvodnji sira je limitirana. Za inhibiciju rasta bakterija *L. monocytogenes, S. aureus i C. tyrobutyricum* u siru, znatno su prihvatljiviji bakteriocinogeni sojevi BMK sadržani u primarnoj, dopunskoj ili protektivnoj kulturi od pročišćenih bakteriocina.

Ključne riječi: bakteriocini i bakteriocinogeni sojevi BMK, inhibicija, *Listeria monocytogenes, Staphylococcus aureus, Clostridium tyrobutyricum,* sir

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