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Safety evaluation and identification of key genes from nisin operon in bacteriocinogenic strains isolated from goat milk

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ABSTRACT

Lactococcus lactis bacteriocin producers' strains were differentiated by repPCR. Applied chemicals, pH and temperature showed specific effect on the bacteriocin stability. Addition of CFS to the actively growing cultures of *Listeria monocytogenes* ScottA, *Lactobacillus sakei* ATCC 15521 or *Enterococcus faecium* ATCC 19434 resulted in the complete inhibition of bacterial growth. Different levels of adsorption of bacteriocin produced by studied strains to the test organisms were observed on bacteriocin and test organisms' specific manner, affected by temperature, pH and selected chemicals, important results for the prediction of potential optimal application of studied bacteriocins. Only between 200 AU/mL and 400 AU/mL were levels of adsorption of the bacteriocins to the cells surface on producers' cells. The five tested strains resulted negative for the presence of different bacteriocins genes, except for nisin. Moreover, the targeted genes, parts of nisin operon, were detected partially in the DNA of DF2Mi and DF60Mi, suggesting that the bacteriocin(s) produced by these two strains differ from nisin. Studied strains were evaluated for presence of different virulence, antibiotics resistance and biogenic amines production associated genes, an important feature for their safety evaluation. Interaction between studied strains and commercial medicaments were evaluated in order to better model their potential application as probiotics.

1. Introduction

Lactic acid bacteria (LAB) belong to one of the most explored microbial groups by the food industry, having essential role in the preparation of different fermented products, particularly in the dairy sector. Production of yoghurt by *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus termophilus* is a school-book example of transformation of milk in fermented products by microorganisms (Tamime & Robinson, 2004). With the development of applied food microbiology, different species from the group of LAB were proposed and successfully used as starter cultures for the production of different fermented milk products. Among these, *Lactobacillus acidophilus*, *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, *Lactobacillus rhamnosus, Leuconostoc mesenteroides* and *Lactobacillus plantarum* stand out, as they harbor all required technological and safety characteristics (Paula, Jeronymo-Ceneviva, Todorov, & Penna, 2015; Powell, Witthuhn, Todorov, & Dicks, 2007). As starter cultures, LAB can play complex roles in the dairy fermentation processes. Besides essential for the texture of the final product, they are responsible for the organoleptic characteristics and degradation of lactose, in addition to production of antimicrobial compounds that confer safety to the product and bioactive peptides that promote human health (Atanasova et al., 2021). However, LAB can cause the degradation of citrate to aspartate, diacetyl and acetoin, influencing the quality and safety of the final product.

The role of LAB in fermented food products is well related to the safety of these products, due to production of several antimicrobial compounds, including bacteriocins (Chikindas, Weeks, Drider, Chistyakov, & Dicks, 2018). According to the definition of bacteriocins, they are modified antibacterial peptides synthesized in the ribosome, without post translation modifications, with activity against genetically related species (Chikindas et al., 2018; Todorov, Franco, & Tagg, 2019). Bacteriocins-producing LAB have been isolated from different ecological environments and their bacteriocins were characterized and evaluated in different research projects, not only as food biopreservatives, but as

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promising therapeutically tools for control of human and other animals infection diseases (Osuntoki, Gbenle, & Olukoya, 2003; Todorov, Cavicchioli, et al., 2019; Çetinkaya, Osmanafaoflu, & Çökmüo, 2003). Moreover, Chikindas et al. (2018) proposed that bacteriocins can play more complex roles in the microbial universe, such as interspecies signaling molecules and can have even inhibitory effects on specific eukaryotic cells.

However, some LAB strains cannot be considered safe for application in fermented products, since they have well documented records indicating pathogenicity. Even some species traditionally accepted as GRAS contain strains that have been shown to be potential health risk as they harbor virulence genes in the DNA (Muñoz-Atienza et al., 2013). Several putative virulence factors have been described in LAB, especially in enterococci (Duprè, Zanetti, Schito, Fadda, & Sechi, 2003; Martín-Platero, Valdivia, Maqueda, & Martínez-Bueno, 2009; Vankerckhoven et al., 2004). Moreover, a number of antibiotic-resistant LAB strains were described in the literature (Martín-Platero et al., 2009; Leavis, Bonten, & Willems, 2006). Other concerns are the possibility of transfer of these genes to non-pathogenic microorganisms by conjugation (Eaton & Gasson, 2001) and production of biogenic amines by decarboxylation of amino-acids as part of the natural metabolic process (Bover-Cid & Holzapfel, 1999). In addition to the negative effect to the health of the consumers, biogenic amines in fermented food products can be considered indication for presence of spoilage organisms (ten Brink, Damink, Joosten, & Huis in 't Veld, 1990). The evaluation of safety of LAB for application in food fermentation processes needs to be considered as milestone in the portfolio of the newly isolated microorganisms and evaluated as beneficial strains.

Different research papers have focused on the potential role of bacteriocinogenic LAB for biopreservation of dairy products, including control of spoilage and growth of pathogenic bacteria (Messens, Verluyten, Leroy, & de Vuyst, 2003; Pingitore, Todorov, Sesma, & Franco, 2012). The ability of LAB to produce bacteriocins can be regarded as an important criterion in the selection of starter cultures (Paula et al., 2015) and try to fuel the increasing consumer demand for naturally preserved food products (Pingitore, Todorov, Sesma, & Franco, 2012).

In a previous study we have reported on isolation and identification of *Lc. lactis* strains from goat milk samples, with bacteriocinogenic properties (Furtado et al., 2009). Strains of interest were identified based on 16s rRNA partial gene sequencing and proven to be producers of antimicrobial metabolites with proteinaceous nature. The aim of the present study was to evaluate the safety of the bacteriocinogenic *Lc. lactis* strains and to further characterize the expressed bacteriocins.

2. Material and methods

2.1. Bacteriocin-like inhibitory substances (BLIS) producing strains

The study was conducted with five BLIS-producing *Lc. lactis* strains isolated from goat milk collected in an organic farm (Furtado et al., 2009). *Listeria monocytogenes* Scott A, *Lactobacillus sakei* ATCC 15521 and *Enterococcus faecium* ATCC 19434 were used as target sensitive strains. The strains were grown in MRS (*Lc. lactis*, *Lb. sakei and E. faecium*) or BHI (*L. monocytogenes*) for 24 h at 37 °C and stored at 80 °C in presence of 30% glycerol as crioprotector. Before use, the cultures were propagated at least 2 times in MRS or BHI for 24 h at 37 °C.

The selected *Lc. lactis* strains were submitted to fingerprinting by random amplification of polymorphic DNA (RAPD) and repPCR analysis for differentiation of the strains. Total DNA was extracted using ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA) following the manufacturer recommendations. The extracted DNA was quantified by a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA). RAPD PCR was performed with primers OPL-02 and OPL-08 (Kit L of the RAPD® lomer kits, Operon Biotechnologies, Cologne, Germany) and repPCR with primer (5'- $(GTG)_5$–3′). PCR amplifications were performed as described by Todorov, Ho, Vaz-Velho, and Dicks (2010) and Valledor, Bucheli, Holzapfel, and Todorov (2020). The resulting amplicons were separated by electrophoresis on 1.5% (w/v) agarose gels in 0.5 × TAE buffer at 100V for 2 h. Gels were stained in 0.5 × TAE buffer supplemented with 0.5 μg/mL ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA). Banding patterns were analysed using Gel Compare, Version 4.1 (Applied Maths, Kortrijk, Belgium).

2.2. Confirmation of BLIS production

The capability of the selected strains to produce BLIS/s was rechecked according to dos Santos et al. (2020). Cell free supernatant (CFS) from 24 h old cultures, grown in MRS at 37 °C were obtained after centrifugation (10 000×*g*, 10 min, 4 °C), correction of pH to 5.5–6.5 and heat-treatment at 80 °C for 10 min. For evaluation of bacteriocinogenic activity, 10 μL of treated CFSs were spotted on the surface of plates containing BHI supplemented with 1% agar and cultures of *L. monocytogenes* ScottA, *Lb. sakei* ATCC 15521 or *E. faecium* ATCC 19434 (app 10^6 CFU/mL). Plates were incubated at 37 °C for 24 h and formation of inhibitions zones larger than 3 mm of target cultures was considered evidence for bacteriocin/BLIS production. Levels of bacteriocin/BLIS activity were expressed according to dos Santos et al. (2020) as AU/mL based on critical dilution assay, taking in consideration type of performed dilutions, last dilution presenting inhibition zone of at least 3 mm in diameter and volume of deposited bacteriocin/BLIS containing CFS in the test.

2.3. Characterization of the BLIS

The obtained CSFs were tested for inhibitory activity against a list of LAB strains and *L. monocytogenes* from different serological groups. Additionally, the CSFs were evaluated for resistance to treatment with chemicals commonly used in the food industry and/or in laboratory practice in bacteriocins/BLIS studies. The CFSs were added of Triton X-100 (Sigma Aldrich), Triton X-114 (Sigma Aldrich), Tween 20 (Merck, Darmstadt, Germany), Tween 80 (Merck), NaCl (Sigma Aldrich), SDS (Sigma Aldrich), urea (Merck) or EDTA (Merck) (all 10 mg/mL) and incubated for 30 min at 37 °C. The pH of the CFSs was corrected to 6.0 when needed, and then tested for activity against *L. monocytogenes* ScottA, *Lb. sakei* ATCC 15521 and *E. faecium* ATCC 19434.

In addition effect of pH and temperatures on stability of bacteriocins/BLIS produced by studied strains was evaluated according to dos Santos et al. (2020). The CFSs obtained as described before were exposed to 30, 37, 45, 80 and 100 °C for 20, 60 and 120 min and 121 °C for 20 min. For the evaluation of the effect of pH on stability of the evaluated bacteriocins/BLIS, pH of the CFS was corrected to 2.0, 4.0, 6.0, 8.0 and 10.0, incubated for 60 min, pH was corrected to 6.0 when needed and followed by testing for activity against *L. monocytogenes* ScottA, *Lb. sakei* ATCC 15521 and *E. faecium* ATCC 19434.

2.4. Dynamics of growth inhibition of the target cultures

In these tests, 200 mL of BHI or MRS were inoculated with 2% (v/v) *L. monocytogenes* ScottA or *Lb. sakei* ATCC 15521 or *E. faecium* ATCC 19434 and incubated at 37 °C. After 3 h (early exponential growth phase), 20 mL of filter-sterilized CSFs obtained from 24 h cultures of the five *Lc. lactis* strains (pH adjusted to 6.0) were added to the target cultures and incubated again at 37 °C. The optical density at 600 nm was measured at selected intervals for the following 9 h. Cultures without addition of CFS served as controls. After 12 h from the beginning of the experiments, 1 mL of each culture was withdrawn, submitted to decimal dilutions and plated on BHI or MRS for determination of presence of viable cells of *L. monocytogenes* ScottA, *Lb. sakei* ATCC 15521 or *E. faecium* ATCC 19434.

2.5. Reduction of viable cells of target microorganisms by the BLIS

L. monocytogenes ScottA, *Lb. sakei* ATCC 15521 and *E. faecium* ATCC 19434 were grown in 10 mL of MRS at 37 °C until reaching the stationary phase (OD_{600nm} 0.8–1.0). Cultures were centrifuged (5000×*g*, 5 min, 4 °C), washed with sterile saline (0.85% NaCl, *m/v*) and re-suspended in same volume of sterile saline. Equal volumes of these cell suspensions were mixed with 0.22 μm filter sterilized (Millipore, Burlington, MA, USA) CFSs (pH 6.0), and incubated for 1 h at 37 °C. Viable cell numbers (CFU/mL) were determined before and after incubation, by plating on BHI or MRS supplemented with 2% agar. Suspensions of the target microorganisms without addition of bacteriocin/BLIS served as positive controls.

2.6. Adsorption of BLIS to target microorganisms and to producer cells

The adsorption of the bacteriocins/BLIS produced by the selected *Lc. lactis* strains to *L. monocytogenes* ScottA, *L. monocytogenes* 620 4b, *L. monocytogenes* 637 1/2c, *Lb. sakei* ATCC 15521 and *E. faecium* ATCC 19443 was evaluated according to Todorov (2008). Strains L. *monocytogenes* 620 4b and 637 1/2c are food isolates of the culture collection of University of Sao Paulo, Sao Paulo, SP, Brazil. The target organisms were cultured for 18 h in BHI or MRS broth at 37 °C and cells obtained by centrifugation (8000×*g*, 15 min, 4 °C) were washed twice with sterile 5 mM sodium phosphate buffer (pH 6.5) and re-suspended in the same buffer to reach OD at 600 nm equal to 1.0. The obtained cell suspensions were mixed with equal volume of the CFSs (pH 6.0), prepared as described above, and incubated for 1 h at 37 °C. After removal of the cells by centrifugation (8000×*g*, 15 min, 25 °C), the residual bacteriocin/BLIS activity in the supernatant (unbound bacteriocin/BLIS) was determined by the spot-on-the-lawn approach, as described before. The % of adsorbed bacteriocins/BLIS were determined as:

$$\%\ \text{adsorption} = 100\left(\frac{\text{bacteriocin activity after treatment}}{\text{original bacteriocin activity}}\ \text{x}\ 100\right)$$

Moreover, the influence of temperature (4 °C, 25 °C, 30 °C and 37 °C), pH (4.0, 6.0, 8.0 and 10.0) and presence of 1% (w/v) of the selected chemicals (NaCl, tween 20, tween 80, glycerol and SDS) related to the adsorption of the studies bacteriocins/BLIS to *L. monocytogenes* ScottA was evaluated according to Todorov (2008). The experiments set-up was similar to the one described before, except that in the investigation for the effect of pH on adsorption of bacteriocins/BLIS to target microorganisms, the test organisms cells suspensions were prepared with sterile ultrapure water (MilliQ, Waters, Millipore) and pH corrected with 1M NaOH or 1M HCl.

2.7. Adsorption of BLIS to producer cells

Adsorption of bacteriocins/BLIS to producer cells was evaluated according to Yang, Johnson, and Ray (1992): 18 h-old cultures of studied strains grown in 10 mL MRS at 37 °C were adjusted to pH 5.0 with 1M NaOH, the cells harvested by centrifugation (8000×*g*, 15 min, 4 °C) and washed with an equal volume of sterile 0.1 M sodium phosphate buffer (pH 6.5). The obtained cells were re-suspended in equal volume (10 mL) 100 mM NaCl, pH 2.0, and stirred for 1 h at 4 °C. CFS was obtained after centrifugation (3000×*g*, 30 min, 4 °C) and pH adjustment to 7.0 with sterile 1 M NaOH. Antimicrobial activity in the original CFS (before treatment) and after experimental set-up was tested as described above. The ratio between the amount of bacteriocin/BLIS liberated in the growing environment and that adsorbed to the surface of the producer cells was calculated.

2.8. Bio-molecular screening for presence of bacteriocin genes

Total DNA was isolated from the five *Lc. lactis* strains and quantified as described before. PCR reaction were performed targeting genes encoding for known bacteriocins including nisin, pediocin PA-1, enterocin A, enterocin P, enterocin B and enterocin L50B (Valledor et al., 2020), curvacin A and sakacin P, lactocin A, lactocin 481, lactocin B, lactocin M, lactocin GQ, lactocin 3147 (Todorov et al., 2017), sakacin G1 and sakacin G2 (Todorov, Furtado, Saad, Tome, & Franco, 2011). The applied primers and conditions for the PCR reactions were performed as described previously (Todorov et al., 2011, 2017; Valledor et al., 2020) with some modifications in the annealing temperature, according to the used primers. The amplified products were separated by agarose gels electrophoresis in 0.5 × TAE buffer, and stained with 0.5 μg/mL ethidium bromide (Sigma Aldrich).

2.9. Screening for presence of nisin operon

Functionality of nisin expression was evaluated by screening for presence of different genes in the nisin operon (Table 1) in the total DNA of the *Lc. lactis* strains, extracted and quantified as described before. PCR analyses were also conducted targeting different regions of *Tn5276*. PCR reactions were performed according to Perin, Todorov, and Nero (2016), following protocols proposed by Al Khatib, Lagedroste, Fey, Kleinschrodt, Abts, & Smits (2014), Olasupo, Schillinger, Narbad, Dodd, and Holzapfel (1999), Pisano et al. (2015), Veljovic et al. (2007) and Li and O'Sullivan (2002). The amplified products were separated by agarose gels electrophoresis in 0.5 × TAE buffer, and stained with 0.5 μg/mL ethidium bromide (Sigma Aldrich).

2.10. Assessment of safety of isolates

The five *Lc. lactis* strains were submitted to PCR reactions targeting the virulence genes *gel*E (gelatinase production), *hyl* (hyaluronidase), *asa*1 (aggregation substance), *esp* (enterococcal surface protein), *cyl*A (cytolisin), *efa*A (endocarditis antigen), *ace* (adhesion of collagen), *van*A and *van*B (both related to vancomycin resistance) and the amino-acid decarboxylase genes *hdc*1 and *hdc*2 (both related to histidine decarboxylase), *tdc* (tyrosine decarboxylase), and *odc* (ornithine decarboxylase). Tests were performed according to Martín-Platero et al. (2009), Rivas et al. (2005) and Vankerckhoven et al. (2004). The obtained amplified products were separated by 0.8–2.0% (w/v) agarose gels electrophoresis in 0.5 × TAE buffer, and stained with 0.5 μg/mL ethidium bromide (Sigma Aldrich).

2.11. Effect of commercial drugs and antibiotics on growth of the strains

The commercial drugs listed in Table 2, obtained from local drugstores (Bulgaria and Brazil) and solubilized in sterile distilled water were tested for their effect on growth of the strains. The strains were cultured in 10 mL MRS broth at 37 °C for 18 h and added to MRS supplemented with 1.0% (*w/v*) agar to achieve 10^6 CFU/mL. After solidification of the medium, each drug (10 μL) was spotted onto the surface, and incubated at 37 °C for 24 h. Presence of inhibition zones, at least 3 mm in diameter, were considered as evidence for growth inhibition. The minimal inhibition concentration (MIC) was determined using serial two-fold dilutions of the drug presenting inhibitory properties, according to Carvalho, Kruger, Furtado, Todorov, and Franco (2009), considering inhibition zones of at least 3 mm in diameter.

3. Results and discussion

3.1. Differentiation of the producers and characterization of BLIS

In the initial report (Furtado et al., 2009) 5 bacteriocinogenic strains of *Lc. lactis* were isolated from goat milk samples from farm located in Sitio Recantinho, Ibiúna, SP, Brazil. All 5 bacteriocin/BLIS producing strains were Gram-positive and catalase-negative. All fermented carbohydrates by homofermentative metabolitic way, and no production of CO_2 was recorded from the fermentation of glucose or gluconate. Good

Table 1
Primers used in this study for detection of genes in DNA from *Lc. lactis* DF2Mi, DF3Mi, DF5Mi, DF6Mi and DF60Mi.

Primer sequence	Target gene	Function	Reference	Results				
				DF2Mi	DF3Mi	DF5Mi	DF6Mi	DF60Mi
For detection of nisin operon genes								
F: ATG AGT ACA AAA GAT TTC AAC TT R: TTA TTT GCT TAC GTG AAC GC	*nisQ*	structure	Veljovic et al. (2007)	+	+	+	+	+
F: CTA GTT CCT GAA TAA TAT AGA G R: TAT TAA GGC CAC AAT AAG	*nisABTCIPRK*	structure and maturation	Olasupo et al. (1999)	-	+	+	+	-
F: AGA GAA GTT ATT TAC GAT CAA C R: ATC TGA CAA CAA ATC TTT TTG T	*nisB*	maturation	Olasupo et al. (1999)	-	+	+	+	-
F: CGC TTT GCT ATG GAG ACG AAT R: GAG CTC CTA TGC CAA ATG TA	*nisB*	maturation	Li and O'Sullivan (2002)	-	+	+	+	-
F: GAA GAA TAC ATG AAA TGA GG R: TAA CTT TCC AGC TGT CCC	*nisT*	maturation	Li and O'Sullivan (2002)	-	+	+	+	-
F: TTC AGA GCA ATA TGA GG R: TAT TAA GGC CAC AAT AAG	*nisC*	maturation	Olasupo et al. (1999)	-	+	+	+	-
F: GAC AAT CTT GAT TTC TAG TGC TCA GTT GCA CGA AAT AAG TAA AG R: CTT TAC TTA TTT CGT GCA ACT GAG CAC TAG AAA TCA AGA TTG TC	*nisI*	immunity	Al Khatib et al. (2014)	-	-	-	-	-
F: ATT GTG GCC TTA ATA GGG R: TAG CGA CTT GTC AGA AGC	*nisI*	immunity	Li and O'Sullivan (2002)	-	-	-	-	-
F: CAG TGC CAT GGG TAA AAA ATA TTC AAT GCG R: CTT AGA GAA TTC TCT AAT GAG	*nisRK*	regulation	Veljovic et al. (2007)	-	+	+	+	–
F: CAG GTG CTA CAA GAT ATC AG R: ACA ACT CCG CAA TAC CAT CAG	*nisF*	immunity	Li and O'Sullivan (2002)	+	+	+	+	+
F: CAA ATA GGC GGC CGC ATG CAG GTA AAA ATT CAA AAT CTT TCT AAA ACA TAT AAA G R: GAA TTC GAG CTC CAC AAG AAA AAA TAC TTT ATC TAA TCT TTT TTT TAG	*nisFEG*	immunity	Al Khatib et al. (2014)	-	-	-	-	-
For detection of genes for virulence and biogenic amines production								
F: TAT GAC AAT GCT TTT TGG GAT R: AGA TGC ACC CGA AAT AAT ATA	*gelE*	gelatinase	Vankerckhoven et al. (2004)	-	-	-	-	-
F: ACT CGG GGA TTG ATA GGC R: GCT GCT AAA GCT GCG CTT	*cylA*	cytolisin	Vankerckhoven et al. (2004)	-	-	-	-	-
F: ACA GAA GAG CTG CAG GAA ATG R: GAC TGA CGT CCA AGT TTC CAA	*hyl*	hyaluronidase	Vankerckhoven et al. (2004)	-	-	-	-	-
F: GCA CGC TAT TAC GAA CTA TGA R: TAA GAA AGA ACA TCA CCA CGA	*asa*1	aggregation substance	Vankerckhoven et al., 20	+	+	-	-	+
F: AGA TTT CAT CTT TGA TTC TTG R: AAT TGA TTC TTT AGC ATC TGG	*esp*	enterococcal surface protein	Vankerckhoven et al. (2004)	+	-	-	-	-
F: GCC AAT TGG GAC AGA CCC TC R: CGC CTT CTG TTC CTT CTT TGG C	*efa*A	endocarditis antigen	Martín-Platero et al. (2009)	-	-	-	-	+
F: GAA TTG AGC AAA AGT TCA ATC G R: GTC TGT CTT TTC ACT TGT TTC	*ace*	adhesion of collagen	Martín-Platero et al. (2009)	-	-	-	-	-
F: TCT GCA ATA GAG ATA GCC GC R: GGA GTA GCT ATC CCA GCA TT	*van*A	vancomycin resistance	Martín-Platero et al. (2009)	-	-	-	-	-
F: GCT CCG CAG CCT GCA TGG ACA R: ACG ATG CCG CCA TCC TCC TGC	*van*B	vancomycin resistance	Martín-Platero et al. (2009)	-	-	-	-	-
F: AGA TGG TAT TGT TTC TTA TG R: AGA CCA TAC ACC ATA ACC TT	*hdc*1	histidine decarboxylase	Rivas et al. (2005)	-	-	-	-	-
F: AAY TCN TTY GAY TTY GAR AAR GAR G R: ATN GGN GAN CCD ATC ATY TTR TGN CC	*tdc*2	histidine decarboxylase	Rivas et al. (2005)	-	-	-	-	-
F: GAY ATN ATN GGN ATN GGN YTN GAY CAR G R: CCR TAR TCN GGN ATA GCR AAR TCN GTR TG	*tdc*	tyrosine decarboxylase	Rivas et al. (2005)	-	-	-	-	+
F: GTN TTY AAY GCN GAY AAR CAN TAY TTY GT R: ATN GAR TTN AGT TCR CAY TTY TCN GG	*odc*	ornithine decarboxylase	Rivas et al. (2005)	-	-	-	-	-

+ detection of targeted gene; - gene was not detected.

growth was recorded at 25, 30 and 37 °C. Slow growth was recorded for all strains at 15 °C and no growth at 45 °C. In present study, the investigations with these strains progressed, and RAPD-PCR and repPCR indicated that the five strains are different and not replicas of one or more strains (Fig. 1).

Isolation of different bacteriocinogenic strains from same environmental samples is not unusual fact. Boza and cheeses can be serving as example as source of various bacteriocinogenic strains (Favaro et al., 2014; Todorov, 2010; Von Mollendorff, Todorov, & Dicks, 2006). Despite isolated from goat milk in the same farm, the strains used in this study originated from different milk samples, from different animals. All five strains were active against *L. monocytogenes* from different serological groups, but when tested for the antagonistic activity against other test organisms, a difference was recorded (Furtado et al., 2009), suggesting production of different bacteriocins, or modifications of the same bacteriocin. It is important to underline the highly specific activity against *L. monocytogenes* and low antimicrobial action against different tested LAB. One of the advantages of bacteriocins in comparison with antibiotics is their selectivity and narrow spectrum of activity (Simons, Alhanout, & Duval, 2020).

When the effect of different chemicals, pH and temperature on the stability of the bacteriocins/BLIS produced by five studied *Lactococcus* strains is considered, slight differences were observed, reinforcing the possibility that different, or naturally modified variants, are produced (Table 3a). Bacteriocins/BLIS produced by DF2Mi and DF60Mi, were affected by presence of EDTA. However, other 3 bacteriocins/BLIS

Table 2
Effect of commercial drugs on the growth of *Lc. lactis* Df2Mi, DF3Mi, DF5Mi, DF6Mi and DF60Mi, presented as diameter of inhibition zones in millimeters and Minimal Inhibition Concentration (MIC).

Brazilian commercial name	Concentration (mg/mL)	Active substance	Medicament class	*Lc. lactis*				
				DF2Mi	DF3Mi	DF5Mi	DF6Mi	DF60Mi
				Inhibition zone (mm) [MIC (mg/mL)]				
Amoxil	100	Amoxicillin	β-Lactam antibiotic (Penicillin)	39 [<0.4]	41 [<0.4]	43 [0.4]	38 [0.4]	40 [<0.4]
Arotin	4	Paroxetine	selective serotonin reuptake inhibitor (SSRI) antidepressant	14 [1.0]	16 [1.0]	16 [1.0]	13 [1.0]	16 [1.0]
Atlansil	40	Amiodarone	Antiarrhythmic	12 [0.625]	15 [1.25]	15 [1.25]	14 [1.25]	18 [0.312]
Cataflam	10	Diclofenac potassium	Non-steroidal anti-inflammatory drug (NSAID)	12 [2.5]	14 [2.5]	9 [5.0]	10 [5.0]	12 [2.5]
Diclofenaco potassico[a]	10	Diclofenac potassium	NSAID	15 [5.0]	13 [10.0]	16 [5.0]	15 [5.0]	18 [2.5]
Diclofenaco potassico[a]	10	Diclofenac potassium	NSAID	16 [5.0]	10 [10.0]	17 [5.0]	15 [5.0]	16 [5.0]
Dorflex	10	Orphenadrine citrate, Metamizole sodium, Cafein	Analgesic	8 [10.0]	0	0	0	7 [10.0]
Fenergan	5	Promethazine hydrochloride	Antihistaminic	10 [5.0]	10 [5.0]	10 [2.5]	8 [5.0]	11 [2.5]
Spidufen	120	Ibuprofen arginine	NSAID	27 [15.0]	31 [30.0]	34 [30.0]	30 [15.0]	35 [30.0]
Urotrobel	80	Norfloxacin	Antibiotic	12 [10.0]	10 [10.0]	14 [5.0]	14 [5.0]	13 [10.0]

Following commercial drugs has no effect on the growth of *Lactococcus lactis* DF2Mi, DF3Mi, DF5Mi, DF6Mi and DF60Mi: AAS (Acetylsalicylic acid, Analgesic/Antipyretic at 20 mg/mL); Antak (Ranitidine hydrochloride, Histamine H2-receptor antagonist that inhibits stomach acid production (Proton pump inhibitor) at 30 mg/mL); Aspirina (Acetylsalicylic acid, Analgesic/Antipyretic at 100 mg/mL); Celebra (Celecoxib, NSAID at 40 mg/mL); Clorana (Hydrochlorothiazide, Diuretic at 5 mg/mL); Coristina R (Acetylsalicylic acid, Pheniramine maleate, Phenylephrine hydrochloride, Cafein, Analgesic/Antipyretic/Antihistaminic/Decongestant at 10 mg/mL); Doxuran (Doxazosin, Antihypertensive/Treatment of prostatic hyperplasia at 0.8 mg/mL); Dramin (Dimenhydrinate, Antiemetic at 20 mg/mL); Fluimucil (Acetylcysteine, Mucolitic agent at 8 mg/mL); Flutec (Fluconazole, Antifungal at 30 mg/mL); Higroton (Chlorthalidone, Thiazide diuretic at 10 mg/mL); Neosaldina (Metamizole sodium, isomethetene mucate, cafein, Analgesic at 60 mg/mL); Nimesulida (Nimesulide, NSAID at 20 mg/mL); Nisulid (Nimesulide, NSAID at 20 mg/mL); Omeprazol (Omeprazole, Proton pump inhibitor at 4 mg/mL); Redulip (Sibutramine hydrochloride monohydrate, Anorexiant/Sympathomimetic at 3 mg/mL); Seki (Cloperastine, Antitussives (central and periferic mode of action) at 3.54 mg/mL); Superhist (Acetylsalicylic acid, Pheniramine maleate, Phenylephrine hydrochloride, Analgesic/Antipyretic/Antihistaminic/Decongestant at 80 mg/mL); Tylenol (Paracetamol, Analgesic/Antipyretic at 150 mg/mL); Tylex (Paracetamol, Codein, Analgesic at 6 mg/mL); Yasmin (Ethinylestradiol, drospirenone, Contraceptive at 0.6 mg/mL); Zestril (Lisinopril, Antihypertensive (Angiotensin-converting enzyme (ACE) inhibitor) at 4 mg/mL); Zocor (Simvastatin, Hypolipidemic at 2 mg/mL); Zyrtec (Cetirizine hydrochloride, Antihistaminic at 2 mg/mL).

[a] Produced by two different companies.

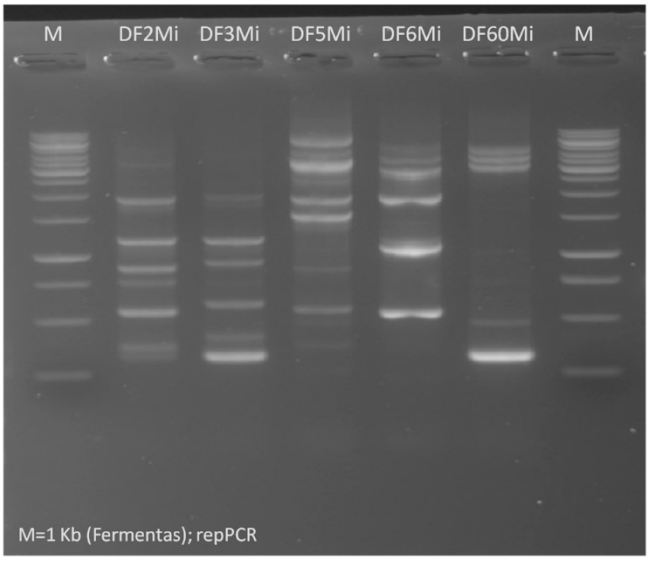

Fig. 1. repPCR profile of *Lc. lactis* DF2Mi, DF3Mi, DF5Mi, DF6Mi and DF60Mi strains; M: 1 kb ladder (Fermentas).

(produced by DF3Mi, DF5Mi and DF6Mi) were looses their activity after treatment with EDTA, but only when *L. monocytogenes* ScottA was used as test microorganisms (Table 3a). It is probable that interaction between EDTA and receptor for bacteriocin action on the surface of *L. monocytogenes* ScottA is involve in this process.

Bacteriocins/BLIS produced by DF2Mi and DF60Mi, were more sensitive to effect of the temperature compared to that produced by DF3Mi, DF5Mi and DF6Mi, and lose of activity was recorded after explosion to 100 °C for 60 and 120 min or 121 °C for 20 min (Table 3a). Thus can be an additional argument for the difference between bacteriocins/BLIS produced by DF2Mi and DF60Mi and these produced by DF3Mi, DF5Mi and DF6Mi. Exposure to pH 12.0 effected activity of bacteriocins/BLIS produced by DF2Mi, DF6Mi and DF60Mi. However, other levels of pH were not affecting stability of studied bacteriocins/BLIS (Table 3a). It is well known that different strains of *Lc. lactis* can express different variants of nisin, presenting different specificity, generally related to mutations in the N or C terminals of the antimicrobial peptide (Al Khatib, Lagedroste, Fey, et al., 2014; Al Khatib, Lagedroste, Zaschke, et al., 2014). The N-terminal of nisin is responsible for recognizing the receptor on the surface of the target bacteria, while the C-terminal is more responsible for bacteriocin mode of action and killing properties (Reiners et al., 2020).

The amount of bacteriocin/BLIS produced by the five studied strains when cultured for 24 h at 37 °C varied, being 1600 AU/mL, 3200 AU/mL, 3200 AU/mL, 6400 AU/mL and 1600 AU/mL for strains DF2Mi, DF3Mi, DF5Mi, DF6Mi and DF60Mi, respectively. Bacteriocin production is influenced by the environmental conditions, particularly the incubation temperature, as reported for plantaricin ST31, sakacin K, and a bacteriocin produced by *E. faecium* RZS C5 (LeRoy & De Vuyst, 1999; Todorov, Gotcheva, Dousset, Onno, & Ivanova, 2000). Also, production is related to the genetic background of the producer strain. Structural bacteriocin gene is part of the operon and can be located in the bacterial chromosome or is plasmid DNA (Ahn and Stiles, 1990), that can be

Table 3a
Effect of temperature, pH, chemicals on the activity of CFS containing bacteriocins/BLIS produced by *Lc. lactis* DF2Mi, DF3Mi, DF5Mi, DF6Mi and DF60Mi against *E. faecalis* ATCC 19443, *Lb. sakei* ATCC 15521 and *L. monocytogenes* ScottA. (+) = active (presence of inhibition zone >3 mm); (−) = inactive (inhibitionzone <3 mm or absent).

Bacteriocin producers →	*Lc. lactis* DF2Mi bacteriocin/BLIS			*Lc. lactis* DF3Mi bacteriocin/BLIS			*Lc. lactis* DF5Mi bacteriocin/BLIS			*Lc. lactis* DF6Mi bacteriocin/BLIS			*Lc. lactis* DF60Mi bacteriocin/BLIS		
Treatments ↓	Test organisms →														
	E. faecalis ATCC 19443	*Lb. sakei* ATCC 15521	*L. monocytogenes* ScottA	*E. faecalis* ATCC 19443	*Lb. sakei* ATCC 15521	*L. monocytogenes* ScottA	*E. faecalis* ATCC 19443	*Lb. sakei* ATCC 15521	*L. monocytogenes* ScottA	*E. faecalis* ATCC 19443	*Lb. sakei* ATCC 15521	*L. monocytogenes* ScottA	*E. faecalis* ATCC 19443	*Lb. sakei* ATCC 15521	*L. monocytogenes* ScottA
Effect of selected chemicals															
SDS, Tween 20, Tween 80, Urea, NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
EDTA	−	−	−	+	+	=	+	+	−	+	+	−	−	−	−
Effect of temperature															
30, 37, 45, 60 and 80 °C for 20 min, 1 and 2h; 100 °C for 20 min	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
100 °C for 1 and 2 h	−	−	−	+	+	+	+	+	+	+	+	+	−	−	−
121 °C for 20 min	−	−	−	+	+	+	+	+	+	+	+	+	−	−	−
Effect of pH															
2.0–10.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12.0	−	−	−	+	+	+	+	+	+	−	−	−	−	−	−

presented in multiple copies, reflecting on the levels of expression of some bacteriocins (Ahn & Stiles, 1990; van Belkum, Hayema, Geis, Kok, & Venema, 1989).

3.2. Mode of activity

Addition of CFS to the growing cultures (logarithmic phase) of *L. monocytogenes* ScottA, *Lb. sakei* ATCC 15521 or *E. faecium* ATCC 19434 resulted in the complete inhibition of bacterial growth (Fig. 2). In addition, less than 100 CFU/mL (detection limit) of *L. monocytogenes* ScottA or, *Lb. sakei* ATCC 15521 and *E. faecium* ATCC 19434 were recorded at 12 h from the cultures treated with bacteriocin/BLIS (data not shown). Bacteriocin activity against target microorganisms can be complete killing (bactericidal effect) or inhibition (bacteriostatic effect) mode of action. The bacteriostatic mode of action of bacteriocin is more difficult to be detected as the test organisms can recover after the effect is over or the bacteriocin is effective against only part of the microbial population and not against all cells. It must be considered that the bacteriostatic mode of action can be a potential mechanism for generation of bacteriocin resistant variants. The observed results of inhibition of the test cultures and no detection of viable cells in BHI or MRS after 12 h of activity suggest that the mode of action of studied bacteriocins/BLIS versus *L. monocytogenes* ScottA or, *Lb. sakei* ATCC 15521 and *E. faecium* ATCC 19434 is bactericidal. Similar observations occurred for other bacteriocins, including those produced by different *Lc. lactis* strains (Kruger et al., 2013; Reiners et al., 2020; Todorov et al., 2006).

Treatment of cells suspensions of *L. monocytogenes* ScottA, *Lb. sakei* ATCC 15521 and *E. faecium* ATCC 19434 with equal volumes of CFS containing bacteriocins/BLIS from the strains DF2MI, DF3Mi, DF5Mi, DF6Mi and DF60Mi resulted in complete inhibition of test organisms. After plating on BHI or MRS agar and appropriate incubation, no evidence for survival of *L. monocytogenes* ScottA and *Lb. sakei* ATCC 15521 was recorded. However, when *E. faecium* ATCC 19434 was applied as target microorganisms, effectiveness of inhibitory effect of CFS from strains DF3Mi, DF5Mi and DF6Mi was confirmed by absence of bacterial growth of the test microorganism, pointing bactericidal effect. On other side, low levels of *E. faecium* ATCC 19434 (2.3×10^2 CFU/mL and 6.1×10^2 CFU/mL) were recorded after initial cultures of 8.4×10^9 CFU/mL were exposed to CFS obtained from strains DF2Mi and DF60Mi. Differences between initial counts of *E. faecium* ATCC 19434 and that recorded after incubation in the presence of bacteriocins/BLIS produced by DF2Mi and DF60Mi indicate the bacteriostatic effect for both bacteriocins/BLIS. It is interesting to point that strains DF2Mi and DF60Mi produced less bacteriocin/BLIS if compared to strains DF3Mi, DF5Mi or DF6Mi. Bacteriocins produced by these three strains are most probably nisin, based on confirmation for presence of complete nisin operon. In

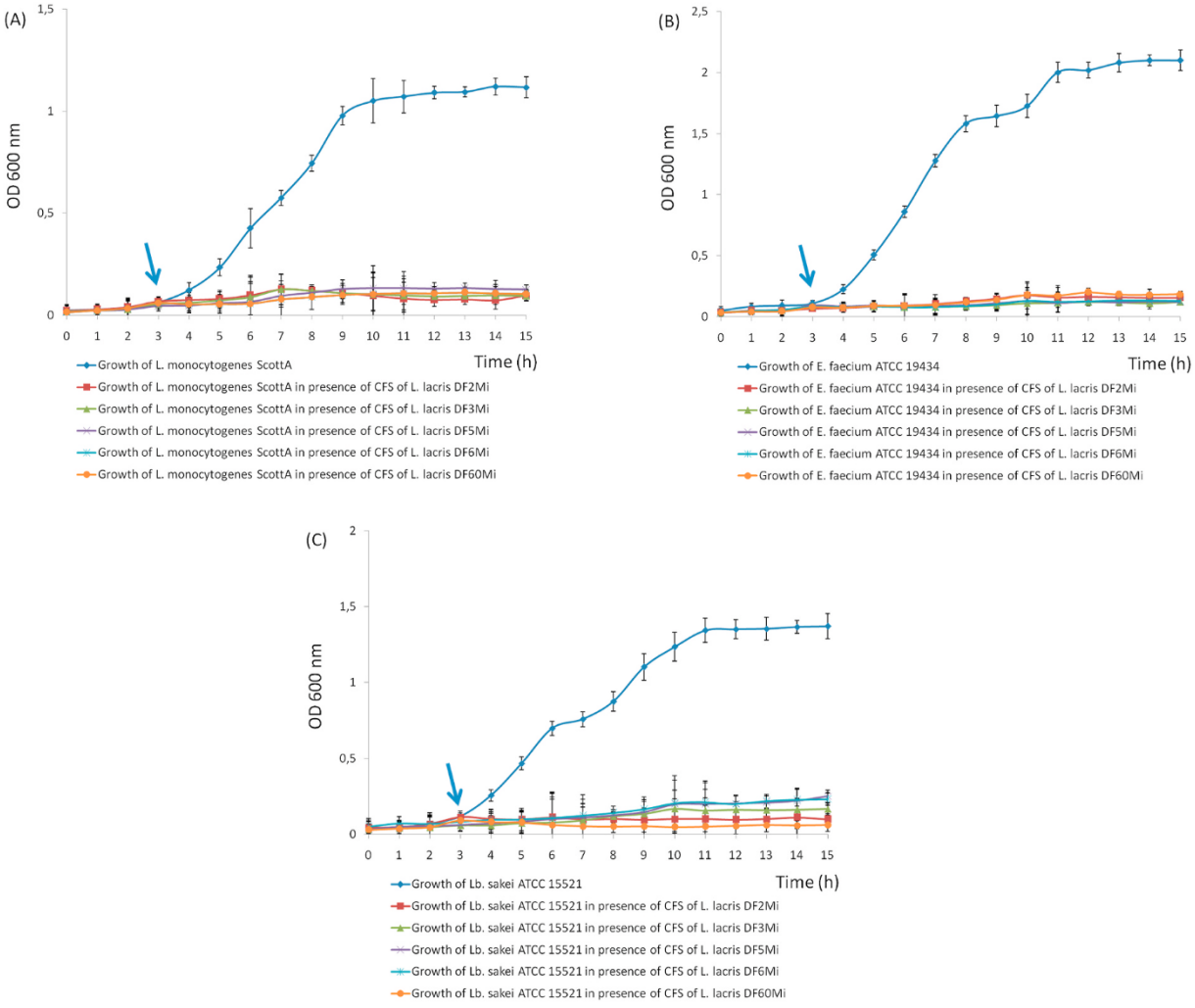

Fig. 2. Activity of the BLIS produced by *Lc. lactis* DF2Mi, DF3Mi, DF5Mi, DF6Mi and DF60Mi strains *versus* (A) *L. monocytogenes* ScottA, (B) *E. faecium* ATCC 19434 and (C) *Lb. sakei* ATCC 15521. (-◆-) represent growth of test microorganisms without addition of CFS containing BLIS. Symbols from (-■-) to (-●-) presents growth of the studied test organisms in presence of CFS containing BLIS produced by *Lc. lactis* DF2Mi, DF3Mi, DF5Mi, DF6Mi and DF60Mi strains. CFS containing BLIS were added 3 h after initial incubation of test organisms, indicated by arrow. Standard deviation bars are indicated.

strains DF2Mi and DF60Mi only an uncomplete operon for nisin production was recorded, pointing that the bacteriocin produced by these two strains is different from nisin and less effective against *E. faecium* ATCC 19434. Other possible hypothesis is the existence of resistant mutants in the *E. faecium* ATCC 19434 culture, which deserves additional attention and future evaluation, as a similar process can occur in consequence of improper use of antibiotics (Roche et al., 2016).

3.3. Adsorption of the BLIS to the target microorganisms and influence of temperature, pH and selected chemicals on activity

Different levels of adsorption of bacteriocin/BLIS produced by DF2MI, DF3Mi, DF5Mi, DF6Mi and DF60Mi to the test organisms were observed (Table 3b). Moreover, temperature, pH and selected chemicals have shown to affect bacteriocins/BLIS adsorption to the test organisms (Table 3b). Adsorption of the bacteriocin is the first step for activity against the test organisms, by mean of recognition of specific receptors on the cell surface, which in the case of nisin, is lipid II (Todorov, Cavicchioli, et al., 2019). The fact that environmental conditions, such as temperature, pH and presence of chemicals influence this adsorption process can be explored for a better efficacy for the control of spoilage and/or pathogenic organisms, particularly *L. monocytogenes,* potentially pathogenic psychotropic bacteria in dairy products kept under refrigeration. Pingitore et al. (2012) also reported that these factors affected the adsorption of bacteriocins produced by *E. mundtii* CRL35 and *E. faecium* ST88Ch to different strains of *L. monocytogenes* and pointed potential of strain CRL35 versus ST88Sh. Obtained results for effect of environmental factors on the adsorption of bacteriocins to the targeted test organisms can be interpreted and help to predict efficacy of studied bacteriocins in the future application processes for control of spoilage or pathogen microorganisms.

3.4. Adsorption of BLIS to the producer cells

Adsorption of bacteriocins/BLIS produced by DF2MI, DF3Mi, DF5Mi, DF6Mi and DF60Mi strains to the surface of the producer cells was very low, between 200 AU/mL and 400 AU/mL. Yang et al. (1992) suggested that the yield of the bacteriocins adsorbed to the cell surface can be regarded as way for recovery of bacteriocins, phenomena that can facilitate their purification when the adsorption levels are high. Similar observation of low levels of bacteriocins adsorbed to the cell surface of producers was reported for other bacteriocin producer strains, including *Lactococcus* spp. (Kruger et al., 2013; Todorov et al., 2006). In some cases, bacteriocins adsorbed to the cell surface can be related to auto-induction of bacteriocin production or to involvement in the stimulation of immune response against own bacteriocins (Perin, Miranda, Todorov, Franco, & Nero, 2016; Todorov, Cavicchioli, et al., 2019).

3.5. Screening for bacteriocin genes

The five tested strains resulted negative for the tested bacteriocins genes, except for nisin. Presence of genes related to bacteriocins produced by different from *Lactococcus* spp. microorganisms was not highly expected to be recorded in DNA from studied *Lc. lactis* strains. Such result is not surprising, since *Lactococcus* spp. are known nisin producers (Reiners et al., 2020). Even industrial production of nisin is based on use of *Lc. lactis* (Özel, Şimşek, Akçelik, & Saris, 2018). However, based in previous reports, pediocin PA-1 and his mutations were recorded in DNA from *Lb. plantarum* (Van Reenen, Chikindas, Van Zyl, & Dicks, 2003), or even coagulin A, an initially reported for *Bacillus coagulans* bacteriocin was reported to be produced by *Pediococcus pentosaceus* strain (Todorov, Cavicchioli, et al., 2019). Bacteriocins normally associated with different taxonomic groups can be cross detected. This can be results of previously occurs gene transfer. Moreover, according to Chikindas et al. (2018), bacteriocin production needs a broader evaluation, and not just as simple antimicrobial peptides, as bacteriocins can be actively involved in the "cross talk" between species and take part in *quorum sensing* processes and interaction with eukaryotic cells, besides more complex roles in the microbial universe. In this sense, the presence of a bacteriocin structural gene must not be regarded as the only evidence for expression of that specific bacteriocin, because production of bacteriocins is not a constant process. Bacteriocins production can be related to specific environmental factors and be induced if the specificity of the condition requires needs of production (Özel et al., 2018).

Table 3b
Adsorption of bacteriocins/BLIS produced by *Lc. lactis* DF2Mi, DF3Mi, DF5Mi, DF6Mi and DF60Mi to different test strains, and to *L. monocytogenes* ScottA in the presence of chemicals, at different pH and temperature.

Test organisms	adsorption (%) of the bacteriocin/BLIS produced by:									
	Lc. lactis DF2Mi		*Lc. lactis* DF3Mi		*Lc. lactis* DF5Mi		*Lc. lactis* DF6Mi		*Lc. lactis* DF60Mi	
L. monocytogenes ScottA	60		60		60		60		60	
L. monocytogenes 711	60			80	60		60		60	
L. monocytogenes 426	60			80	60		60			80
L. monocytogenes 603		80		80		80		80		80
L. monocytogenes 101	60		60		60		60			80
L. monocytogenes 211	60		60			80	60		60	
L. monocytogenes 703		80		80		80		80		80
E. faecalis ATCC 19434	40		40		60		60		40	
Lb. sakei ATCC 15521	60		60			80		80	60	
L. monocytogenes ScottA in presence of:										
NaCl (1%)		80		80		80		80		80
Tween 20 (1%)	40		40		40		40		40	
Tween 80 (1%)	40			60		60	40		40	
Glycerol (1%)	40		40		40		40		40	
SDS (1%)	60			60		60		60		60
at pH 4.0		80		80		80		80		80
at pH 6.0	60			60		60		60		60
at pH 8.0	60			60	40		40			60
at pH 10.0	40		40		40		40		40	
at 4 °C		80		80		80		80		80
at 25 °C	60			80		80	60			80
at 30 °C	60			60		60		60		60
at 37 °C	60			60		60		60		60

3.6. Nisin operon identification

Bacteriocin production and transportation out of the producer cells of the prepeptide or mature peptide is a complex process, involving different transporters and immunity mechanisms. Thus, pointing of the need of all genes from the active bacteriocin operon to be present and to be functional (Perin et al., 2016). Evaluation of the DNA from DF2MI, DF3Mi, DF5Mi, DF6Mi and DF60Mi indicated positive results for the structural genes for production of nisin. All genes required for nisin production, transmembrane expression and immunity were detected as well on strain specific basis (Table 1). However, the targeted genes were detected partially in the DNA of DF2Mi, and DF60Mi, suggesting that the bacteriocin(s) produced by these two strains differ from nisin. In order to produce and express effectively bacteriocin/s, producer strains supposed to carry "complete" set of genes, grouped in functional operon. Only expression of all genes, part of the bacteriocin operon will be resulting on effective expression and secretion of mature antimicrobial (Chikindas et al., 2018). By "convenience", in different research projects, authors only screening for presence of structural bacteriocin gene and by detection of it, presume that strain can be considered as bacteriocin producer. Moreover, suggesting that detected structural bacteriocin gene was expressed and *de facto* reported bacteriocin activity on physiological experiments are results of expression of recorded gene. Such as hypothesis needs to be considered with highly scientific scepticisms, since as was previously underline, all components of the bacteriocin operon needs be present in order to have successful bacteriocin production. Even more, strains can carry genetic determinants for expression of more than one bacteriocins, and express them based on specificity of culturing conditions and presence of additional factors. However, in our study, none of the other bacteriocin genes evaluated in the screening process were detected, reinforcing the hypothesis that DF2Mi and DF60Mi produce a novel antimicrobial peptide. However, the confirmation of this hypothesis requires further studies, as depends on appropriate bacteriocin purification, amino-acid sequences and mass spectrometry.

3.7. Assessment of the safety of isolates

The investigation of presence of genes related to virulence factors, antibiotic resistance and biogenic amines production is a relevant point for the evaluation of the safety of a particular strain for application in a food product or as probiotic strain. Such evaluations need to be performed on each particular strain and not relay on the GRAS status of the species. There are several evidences showing that representatives of GRAS species may possess virulence genes, compromising their safe application (Muñoz-Atienza et al., 2013). Despite requiring confirmation that detected virulence genes will be expressed or not, safety risks need to be taken very seriously.

The positivity for virulence genes, vancomycin resistance genes and genes related to biogenic amines, summarized in Table 1, was low: *Lc. lactis* DF2Mi was positive for *asa1* and *esp*, *Lc. lactis* DF3Mi for *asa1*, while *Lc. lactis* DF60Mi generated positive results for *asa1*, *efa* and *tdc*. It is difficult to conclude if such results are good or bad, since in general *Lc. lactis* strains are considered safe and not associated with clinical cases. Comparing these results to those reported by Perin, Miranda, Todorov, Franco, and Nero (2014), who detected a higher prevalence of virulence genes in *Lc. lactis* strains isolated from dairy products, we can conclude that the five strains pose a different degree of challenge for safe application, as DF5Mi and DF6Mi were free of virulence factors while DF2Mi, DF3Mi and DF60Mi strains were positive for some of them, particularly *asa*1, *efa*, *esp* and *tdc*. It can be hypothesized that these genes were detected in consequence of horizontal gene transfer from *Enterococcus* spp. strains, commonly found in the dairy environmental niche. Even if this is with a very low level of probability (Suvorov, 2020), such as scenario is possible. From another point of view, presence of the gene does not necessarily mean that the gene is expressed, as environmental conditions affect the expression process (Perin et al., 2014). Presence of virulence genes cannot be neglected and needs to be taken seriously in consideration in future plans for the potential application of the studied strains as probiotics, starter or biopreservatives cultures. Moreover, whole genome sequence and *in silico* screening for virulence associated genes needs to be regarded as future compulsory criteria in the safety evaluation of any beneficial strain intended to be applied as probiotic and/or starter culture.

3.8. Effect of selected drugs on the growth of studied Lc. lactis strains

Efficacy of application of beneficial bacterial strains as probiotics can be affected by different factors. The increased use of probiotics must be monitored by health professionals, as prescribed drugs may interfere in the desired effect (Carvalho et al., 2009). For the drugs with potential inhibitory effect on the studied *Lc. lactis* strains, the determined MIC (Table 2) indicated that most of the non-steroidal anti-inflammatory drug (NSAID), some selective serotonin reuptake inhibitor (SSRI) antidepressant (Arotine, cantaining paroxetine), analgesics (Dorflex, containing orphenadrine citrate, metamizole sodium, cafein), antiarrhythmic (Atlansile, containing amiodarone) or antihistaminic (Fenergan, containing promethazine hydrochloride) can be considered as not appropriate to be received in combination with studied *Lc. lactis* strains, since inhibitory effect of the drugs versus studied bacterial cultures were observed. Such results have valuable practical relevance, in subscription of specific probiotic strains in combination with commonly used drugs, as this combinations may have unexpected results, depending on the daily doses, frequency of application and history of accumulation in the GUT and blood. Special attention needs to be given to the drugs applied in the control of cardiovascular and neuroleptic diseases, cancer treatment, or other chronically clinical cases, since they are generally applied in long term and high doses, affecting negatively the viability of the probiotic cultures and compromise their role in health promotion. Interaction of probiotic cultures with the common drugs applied in therapeutically practice drugs (human and veterinary medicine) need a additional attention. Pharmaceutical sciences clearly evaluated and suggest possible interactions between different drugs, pointing on potential contraindications in combined applications between. However, probiotics are still considered only as additives and complementary agents, generally for prevention application. Solid evidences from last decade were pointed that probiotics can be play more strong role not only in preventive, but in active clinical therapy. All these pointing on need for better understanding of interaction between probiotics (as life microorganisms, performing their beneficial role for the host) and conventional drugs (as chemical agents, beneficial for treatment of specific clinical case) in order to have effective and even synergetic interactions and benefits for the host.

4. Conclusions

Isolation and characterization of new bacteriocinogenic strains become a routine practice and data base on new producer and new bacteriocins is constantly growing. However, in order to suggest new bacteriocin/s producer/s for an appropriate commercial application in biopreservation processes or medical (human and veterinary) fields, safety of the producing strains needs to be regarded as essential priority. Moreover, deeper knowledge on the bacteriocins, including their mode of action and specificity in interactions with target organisms will be always requested in order to tray to predict the safety of the antimicrobial peptides and efficacy of their inhibitory properties. In this study we have evaluated safety aspects of the five bacteriocinogenic *Lc. lactis* strains, isolated form dairy environment, showing evidences that they can be considered as different strains, and producing nisin variants. In addition interactions of the evaluated five *Lc. lactis* strains with commercial drugs from different groups was adding valuable information about potential application of that specific strains as potential beneficial

(probiotic) cultures for human and other animals application.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval

This article does not contain any studies with human or animal subjects.

CRediT authorship contribution statement

Danielle Nader Furtado: conducted experiments. **Bernadette Dora Gombossy de Melo Franco:** provided laboratory space and financial support, corrected the manuscript. All authors read and approved the manuscript. **Svetoslav Dimitrov Todorov:** wrote the manuscript, conceived, designed research, conducted experiments and analysed data, corrected the manuscript. All authors read and approved the manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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