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Effect of cheese-making and ripening processes on the anti-*Clostridium* activity of *Lactococcus* strains

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ABSTRACT

Lactic acid bacteria (LAB) can produce bacteriocins that exert an antimicrobial activity against *Clostridium* spp. responsible for late blowing defects (LBD) in cheese. This study aimed to evaluate the influence of cheese-making and ripening temperature and salt concentration on the LAB bacteriocin production to enable their effective use in dairy chain.

Five *Lactococcus* strains (*Lc. cremoris* FT27 and *Lc. lactis* N16, N26, SV77 and VC106) with anti-*Clostridium* activity were selected for their ability to produce bacteriocin at the optimal growth temperature (30 °C) and in temperature conditions resembling those of the uncooked and semi-cooked cheese-making. At optimal growth condition the bacteriocin titre resulted to be strain-dependent (from 2.4 to 3.3 $\log_{10}$ IU/mL), differently at temperatures applied during the cheese-making process only one strain (*Lc. lactis* VC106) was able to produce a high amount of bacteriocins (2.8 and 2.9 log10 IU/mL, respectively. During the period that simulated the ripening process at 10 °C, the bacteriocin titre of *Lc. lactis* VC106 declined progressively (1.6 and 1.9 $\log_{10}$ IU/mL after 170 days), but remained above the threshold required to inhibit the LBD. Our findings provide new evidence of how cheese-making and ripening processes adversely affect the bacteriocins synthesis and, consequently, the anti-*Clostridium* activity. *Lc. lactis* VC106 showed an antimicrobial activity comparable to that obtained at optimal growth temperature suggesting that this strain could provide a useful tool to inhibit spoilage bacteria in the dairy chain.

1. Introduction

Lactococcus lactis are Gram-positive, catalase-negative, nonmotile, mesophilic cocci that belong to the lactic acid bacteria (LAB) group. Despite these microorganisms are associated with the dairy environments, *Lc. lactis* strains were originally isolated from different plant material, such as corn, beans, cabbage, peas, wheat, grass and clover (Kazou, 2022). Initially *Lc. lactis* included four subspecies namely *lactis*, *cremoris*, *hordniae* and *tructae*, but recently *Lc. lactis* subsp. *cremoris* was elevated to the species level as *Lc. cremoris* (Li, Tian, & Gu, 2021). *Lc. lactis* and *Lc. cremoris* are generally employed as mesophilic starters in uncooked and semi-cooked cheese production. Due to their long history of safe use, these species have been granted the generally recognized as safe (GRAS) status and the qualified presumption of safety (QPS) status according to the Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA), respectively (Kazou, 2022). In addition to their essential role in milk fermentation and flavour compound production, the ability of some *Lactococcus* strains to produce bacteriocins is also considered an important technological trait to hamper the pathogenic and spoilage bacterial growth in cheeses and to improve the safety of dairy products (Mills et al., 2017). *In situ* bacteriocin production by starters or protective cultures is a particularly attractive option for the dairy sector, since they offer advantages such as an easy implementation without specific and/or costly equipment (Pujato et al., 2024). The addition of the QPS/GRAS strains in the cheese-making process has no regulatory limitations, and their use is less expensive than that of purified bacteriocins (Pujato et al., 2024). These strains could be a useful tool to avoid the cheese late blowing defect (LBD). This defect is caused

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by the contamination of milk by spore-forming bacteria belonging to the *Clostridium* genus. During ripening of cheese, the clostridial spores can germinate and produce butyric acid, acetic acid and high quantities of carbon dioxide as well as hydrogen. The pressure exerted by the gas production leads to textural defects, such as splits, cracks and irregular cheese eyes. Moreover, the high amounts of butanoic acid generated by *Clostridium* spp. strains cause strong rancid off-flavours that implicate an unfavourable economic impact on cheese production (Brändle, Domig, & Kneifel, 2016). Several studies have shown how Nisin, Pediocin and Reuterin were able to inhibit the growth of vegetative cells and spores of dairy-related *Clostridium* (*Clostridium beijerinckii*, *Cl. butyricum*, *Cl. sporogenes* and *Cl. tyrobutyricum*) (Ávila, Gómez-Torres, Hernández, & Garde, 2014; Meghrous, Lacroix, & Simard, 1999). Nisin has been reported to inhibit the vegetative forms by membrane pore formation that leds to the leakage of essential ions and consequently the cell death. Moreover, this bacteriocin showed sporostatic activity against *Clostridium* spores, preventing their post-germination swelling and subsequent outgrowth (Egan et al., 2016). Despite that, the application of protective cultures in cheese is currently a practice that is not always feasible with regard to late blowing because it is not always effective. Different studies investigated the effects of bacteriocin-producing *Lactococcus* strains on LBD, but conflicting results were reported. Havlíková, Kvasničková, Kavková, and Němečková (2018) showed that the use of *Lc. lactis* strains in the production of Dutch-type cheese reduce the LBD caused by *Cl. tyrobutyricum* without negatively affecting the physico-chemical and sensory properties of cheese. Moreover, Demirbaş, Arıcı, and Dertli (2024) detected a reduction in *Cl. sporogenes* spores (approximately 1 log) in white cheese obtained with protective cultures. Ávila, Gómez-Torres, Gaya, and Garde (2020) and Garde et al. (2020) highlighted that the nisin produced by *Lc. lactis* INIA 415 during the cheese ripening process delayed the LBD without completely arresting the *Cl. tyrobutyricum* activity. All the above-mentioned studies highlight the need to acquire new information and to deepen the knowledge on how the cheese-making influence the bacteriocin synthesis by LAB. In particular, it is necessary to understand how the temperature and salt concentration of the dairy productions affect the LAB antimicrobial capability against *Clostridium* strains. Therefore, the objective of this study was twofold. First, we aimed to evaluate the anti-*Clostridium* activity and bacteriocin production of five wild *Lactococcus* spp. strains isolated from raw milk and raw milk cheeses. The second objective was to determine, through the in vitro simulating cheese-making processes, the influence of temperature and salt concentration on the *Lactococcus* bacteriocin production, providing the basis for the application of these strains in dairy productions.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Four *Lactococcus lactis* strains N16, N26, SV77 and VC106 and the *Lc. cremoris* strain FT27, isolated from bovine raw milk and cow as well as goat raw milk cheeses, were considered in this study (Table 1). These strains were chosen based on their ability to produce antimicrobial compounds, including Nisin, upon different cheese ripening condition (Gazzola et al., 2024; Morandi, Silvetti, Battelli, & Brasca, 2019). Twenty-two clostridial strains were used as indicator microorganisms to evaluate the *Lactococcus* spp. antimicrobial activity. These strains were provided by different culture collections or isolated from cheeses with LBD (Table 1). Before each experiment, *Lactococcus* spp. strains were propagated in de Man, Rogosa and Sharpe (MRS) broth (Scharlab, Barcelona, Spain) at 30 °C for 24 h, while the *Clostridium* strains were grown anaerobically (AnaeroGen™, Thermo Fisher Scientific, Waltham, MA, U.S.A.) in Reinforced Clostridial Medium (RCM) broth (Scharlab) at 37 °C for 72 h. All strains were refreshed twice before testing.

Table 1
Characteristics of strains considered in this study.

Species	Strain	Source	Bacteriocin	
			Genes (+)	Proteins (+)
Lc. cremoris	FT27	Goat cheese	*nisA*	Nisin A
Lc. lactis	N16	Raw milk	*nisZ*	Nisin Z
	N26	Raw milk	*nisZ*	–
	SV77	Cow cheese	*nisZ*	Nisin Z
	VC106	Cow cheese	*nisZ*	Nisin Z
			lctA	Lacticin 481
			lcnB	–
			–	Lactococcin A
			–	Lactococcin 972 family
Cl. beijerinckii	DSM 791	DSM		
Cl. butyricum	DSM 10702	DSM		
Cl. sporogenes	ATCC 3584	ATCC		
	ATCC 10000	ATCC		
	CL14	Cow cheese		
	CL16	Cow cheese		
	CL219a	Cow cheese		
	CL219b	Cow cheese		
	CL13	Cow cheese		
	CL1	Cow cheese		
	CL16	Cow cheese		
	CL106	Cow cheese		
Cl. tyrobutyricum	DSM 2637	DSM		
	ATCC 8260	ATCC		
	CL10	Cow cheese		
	CL12	Cow cheese		
	CLoc1	Cow cheese		
	CLoc2	Cow cheese		
	CL11	Cow cheese		
	CL167	Cow cheese		
	CL6a	Cow cheese		
	IN15b	Cow cheese		

ATCC: American Type Culture Collection (Manassas, VA, U.S.A.).
DSM: German Collection of Microorganisms (Braunschweig, Germany).

2.2. Evaluation of Lactococcus spp. anti-Clostridium activity

Agar disk diffusion method was used to evaluate the *Lactococcus* spp. antimicrobial activity against *Clostridium* spp. strains according to Morandi, Silvetti, Miranda Lopez, and Brasca (2015). Overnight cultures of *Lactococcus* spp. strains were centrifuged at 9300*g* for 5 min (Benchtop centrifuge 5425, Eppendorf, Hamburg, Germany), the obtained cell-free supernatants (CFS) were neutralized (adjusting to pH 6.5) with sodium hydroxide solution (Carlo Erba Reagents, Cornaredo, Italy) and sterilized by filtration through the 0.20 μm pore size PDVF membrane filter (Minisart, Sartorius, Goettingen, Germany). Twenty microliters of CFS were spotted on sterilized filter paper disks placed onto RCM agar plates seeded with *Clostridium* spp. indicator strains (10^6 CFU/mL). The plates were incubated anaerobically at 37 °C for 72 h. Anticlostridial activity was expressed as the difference between the diameters of the inhibition zone and the discs used (9 mm).

2.3. Bacteriocin gene detection

DNA of the *Lactococcus* spp. strains was extracted from overnight cultures using the Microlysis kit (Microzone, Stourbridge, UK) following the manufacturer's instructions. The presence of nisin-encoding gene was studied by PCR amplification according to Ghrairi, Manai, Berjeaud, and Freère (2004). The obtained PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced by Macrogen Europe (Amsterdam, the Netherlands). The

sequence analysis to establish the Nisin variants was performed as described by Pisano et al. (2015). Moreover, genomic DNA was also used to detect by PCR the presence of genes encoding the following lactococcal bacteriocins: Lactococcin A (*lcnA*), Lactococcin B (*lcnB*), Lactococcin 513 (*lsbA*), Lactococcin 972 (*lclA*), Lacticin RM (*lacA*) and Lacticin 481 (*lctA*) (Dal Bello et al., 2010). To confirm the amplified products originated from bacteriocin genes, the amplicons were sequenced as described above and the resulting sequences compared to those in the GeneBank database using the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4. Bacteriocin production at different temperature conditions

Bacteriocins were produced in the MRS broth at optimal growth temperature and in temperature conditions resembling those of the uncooked and semi-cooked cheese-making. A laboratory scale bioreactor (MiniBio 500, Applikon Biotechnology, Delft, the Netherlands) equipped with a pH and pO_2 electrodes (L135, D8 M12 MiniBio 500, Applikon Biotechnology) was used to evaluate the growth and the bacteriocin production of the *Lactococcus* spp. strains.

Strains were inoculated (1 % *v*/v) in 300 mL of MRS broth with Tween 80 (0.20 % *w/v*) (Biolife Italiana, Milan, Italy) to obtain an initial concentration of 10^6–10^7 CFU/mL. Tween 80 was added to prevent any non-specific adsorption of bacteriocins on the pipette tips, tubes and containers' surfaces (Joosten & Nuñez, 1995). To evaluate the bacteriocin synthesis at optimal growth temperature the cultures were stirred in the bioreactor at 100 rpm, 30 °C for 24 h. Table 2 reports the incubation times and temperatures in the MRS medium applied to simulate the cheese-making process of uncooked and semi-cooked cheeses. To reproduce the ripening stage, all samples were stored at 10 °C for 170 days (Table 2). pH electrodes were calibrated using standard solutions with pH 4.0 and 7.0 (Scharlab). Before each analysis and throughout the experiment the pH values were automatically recorded by Lucullus software (Applikon Biotechnology) at 1-min intervals. From the collected data the ΔpH ($pH_{zero\ time}$ - $pH_{at\ time}$) was calculated after 2, 4, 6, 8 and 24 h (Morandi, Silvetti, Guerci, Tamburini, & Brasca, 2024). During the fermentation processes, aliquots of cultures were taken aseptically at different time points (2, 4, 6, 8 and 24 h) and used for further analysis (growth kinetics and bacteriocin activity). All experiments were performed in duplicate using two bioreactors. Finally, the fermentation broths obtained from the trials that simulated the cheese-making process were subsequently incubated at 10 °C for 90–170 days, and aliquots of these cultures were collected at 7, 15, 30, 60, 90 and 170 days to evaluate the bacteriocin activity. All experiments were performed in duplicate.

2.4.1. Lactococcus spp. growth kinetics

Growth kinetics of each *Lactococcus* spp. strain in the conditions described above, was investigated evaluating the Generation Time (GT), which is defined as the time needed to double the population of microorganisms in a culture medium. Optical density at 600 nm (OD_{600}) was determined at different time points (2, 4, 6, 8 and 24 h) using Uvidec 320 spectrophotometer (Jasco, Hachioji, Japan). The growth rate (r) was calculated for each strain using the eq. (1):

$$r = \ln (OD_2 - OD_1)/(t_2 - t_1) \quad (1).$$

where OD_2 and OD_1 are the OD_{600} values at the time t_1 and t_2, respectively. GT was calculated for each culture from the corresponding value of r ($GT = \ln 2/r$) (Huang et al., 2021).

Table 2
MiniBio 500 bioreactor (Applikon Biotechnology, Delft, the Netherlands) parameters applied to reproduce the conditions (incubation time and temperatures) applied in the cheese-making and ripening processes of uncooked and semi-cooked cheeses.

Uncooked cheese		Semi-cooked cheese		Processing steps
Time	Temperatures	Time	Temperatures	
0 min	18 °C	0 min	18 °C	
35 min	18 °C → 32 °C	35 min	18 °C → 32 °C	Heating
40 min	32 °C	40 min	32 °C	Coagulation and cutting
30 min	32 °C → 38 °C	40 min	32 °C → 45 °C	Heating
20 min	38 °C	20 min	45 °C	Cooking
80 min	38 °C → 18 °C	90 min	45 °C → 18 °C	Moulding
24 h	18 °C	24 h	18 °C	Salting
170 days	10 °C	170 days	10 °C	Ripening

2.4.2. Nisin activity bioassay

Nisin bioassay was performed after 2, 4, 6, 8 and 24 h of fermentation as described by Pongtharangkul and Demirci (2004, 2006) and Murat and Hakkı (2021). A Nisin A stock solution (900 IU/mL) was prepared by adding 0.025 g of commercial Nisin A 900 IU/mg (Sigma-Aldrich) into 25 mL of sterile diluent solution of 0.02 N HCl (Carlo Erba Reagents). Standard curves were constructed using Nisin A standard solutions (from 9 to 900 IU/mL) obtained from the stock solution. *Cl. tyrobutyricum* IN15b indicator strain (10^6 CFU/plate) was spread on RCM agar plates. Equidistant wells (Ø 7 mm) were punched in the plate and filled with 100 μL of Nisin A standard solutions. RCM plates were incubated in anaerobic conditions (AnaeroGen™) at 37 °C for 48–72 h. In all plates, the diameter of the inhibition zone around each well was measured horizontally and vertically and then averaged. All experiments were conducted in triplicate. Aliquots of *Lactococcus* spp. cultures were aseptically collected and immediately adjusted to pH 2.5 using 37 % HCl (Murat & Hakkı, 2021). After acidification, the samples were heated at 90 °C for 5 min to eliminate the possible protease activity in the broth (Pongtharangkul & Demirci, 2006). Subsequently, the samples were centrifuged at 9300 *g* for 5 min and the supernatants were filtered through a 0.20 μm pore size PDVF membrane filter and immediately utilized or stored at −18 °C until analysis. Nisin production was determined by agar well diffusion bioassay. The diameters of the inhibition halos were measured horizontally and vertically and the concentrations of Nisins produced by *Lc. lactis* strains were calculated using the equation that best fitted the standard curve, which should have the correlation coefficient of at least 0.98 (Pongtharangkul & Demirci, 2006). All experiments were conducted in triplicate.

2.5. Effect of osmotic stress on bacteriocin production

To investigate the influence of osmotic stress on bacteriocin production, overnight cultures of *Lactococcus* strains were inoculated (1 % *v*/v) in MRS broth supplemented with Tween 80 (0.20 % *w/v*) and different NaCl concentrations (0.5, 1.0 and 2.0 % w/v). All cultures were incubated at 30 °C for 24 h. Nisin bioassay was performed at the end of incubation as described above (Murat & Hakkı, 2021; Pongtharangkul & Demirci, 2004).

2.6. LC-MS analysis of unpurified cell-free supernatants

The unpurified CFSs, obtained after an overnight incubation at 30 °C, were filtered [0.20 μm pore size PDVF membrane filter (Minisart, Sartorius)] and analyzed by ultra performance liquid chromatography/high-resolution mass spectrometry (UPLC/HR-MS) on an Acquity UPLC chromatographic separation system (Waters, Milford, MA, USA) coupled to a high-resolution Orbitrap mass spectrometer (Q Exactive™, Thermo Fisher Scientific, San Jose, CA, USA) through an electrospray ionization source according to Malvisi et al. (2016) with some modifications. Briefly, samples were separated on an Aeris Widepore C4 column (2.1 × 150 mm, 3.6 μm; Phenomenex, Torrance, CA, USA) kept at 40 °C. The eluents were 0.1 % formic acid in MilliQ-treated water (solvent A) and 0.1 % formic acid in acetonitrile (solvent B). The UPLC separation was performed by using a linear elution gradient (10 % to 55 % of solvent B in 13.7 min) at a flow rate of 0.2 mL/min. Proteins in the eluate were

detected by MS using full scan analysis in the range of 300 to 2000 *m/z*. The MS data were processed using the Xcalibur version 3.0 software (Thermo Fisher Scientific), and protein mass deconvolution was performed using the embedded Qual Browser Xtract tool (Thermo Fisher Scientific). Nisin A was purchased from Sigma-Aldrich (St. Louis, MO, USA), and Nisin Z was from DBA Italia (Segrate, Italy). These standard proteins were used as controls.

2.7. Proteomic analysis of VC106 secretome by LC-MS/MS.

Lc. lactis VC106 was cultured overnight at 30 °C. An aliquot of its CFS (10 mL) was submitted to protein precipitation with trichloroacetic acid (TCA, final concentration 10 %) by incubating for 2 h at 4 °C. The protein pellet was recovered by centrifugation (16.000 *g* for 15 min at 4 °C), washed twice with 1 mL of 80 % acetone and solubilized in 0.1 mol/L Tris-HCL, 8 mol/L urea, pH 8.5.

An aliquot of protein sample was desalted by solid phase extraction using Pierce C18 Spin Columns (Thermo Fisher Scientific) and the manufacturer's instructions and directly analyzed by LC-MS/MS while another aliquot was analyzed by applying a shotgun proteomic approach using a Q Exactive™ mass spectrometer (Thermo Fisher Scientific) interfaced with an UltiMate 3000 RSLCnano LC system (Thermo Fisher Scientific). For the shotgun approach, protein reduction and alkylation, tryptic digestion, LC-MS/MS analyses, data acquisition and bioinformatic processing were carried out as already described in detail in Mazzeo et al. (2024). For both experiments, data acquisition was performed in the *m/z* range 250–1600 and in a data dependent mode Full MS/ddMS2, enabling the acquisition of MS/MS spectra for the ten most intense precursor ions (top ten) and dynamic exclusion of 10 s. Resolution was set to 70,000 for MS spectra acquisition and 17,500 for MS/MS spectra acquisition. The identification of the expressed bacteriocins was based on the measurement of an accurate monoisotopic mass of the intact proteins (also taking into account the already reported post-translational modifications) and verified by manual interpretation of fragmentation spectra (MS/MS spectra).

The Proteome Discoverer (PD) search engine platform (version 2.1. Thermo Fisher Scientific) was used for processing the MS raw files obtained from the shotgun proteomic analysis and to achieve protein identification by data-base searches in the *Lc. lactis* database (downloaded from UniProtKB on June 2024, https://www.uniprot.org/) and a contaminant protein data-base (provided by the manufacturer). The identification of bacteriocins was validated by visual inspection of MS/MS spectra.

2.8. Statistical analysis

All data related to acidifying activities, growth kinetics and bacteriocin production were presented as means ± standard deviations (SD). Significant differences ($P < 0.05$) among the data were calculated by one-way ANOVA using Minitab ver. 14.13 software (Minitab Inc., State College, PA, USA).

3. Results and discussion

3.1. Antimicrobial activity against Clostridium strains

In this study, we tested four *Lc. lactis* strains N16, N26, SV77 and VC106 and the *Lc. cremoris* FT27 strain for their antimicrobial activity against *Clostridium* species implicated in cheese LBD. As reported in Table 3, all tested strains exhibited an antimicrobial activity against different indicator microorganisms, with inhibition zone diameters ranging from 3.2 to 19.4 mm. *Clostridium* spp. susceptibility to the *Lactococcus*-produced antimicrobial compounds appeared strain and species-dependent. In particular, all *Cl. sporogenes* strains were more sensitive (mean value of inhibition halo was 10.0 ± 4.0 mm) than *Cl. tyrobutyricum* ones (7.4 ± 4.2 mm). These data confirmed previous observations by Ávila et al. (2014) and could explain the differences in clostridial inhibition reported by different authors (Ávila et al., 2020; Demirbaş et al., 2024; Havlíková et al., 2018).

3.2. Bacteriocin production by Lactococcus strains

To investigate the nature of the antimicrobial compounds produced by *Lactococcus* spp. strains, we carried out a polyphasic approach: at the genetic as well as at the protein level. Firstly, we investigated the presence of the Nisin-coding genes. All four *Lc. lactis* strains harboured the *nisZ* gene, while *Lc. cremoris* FT27 was positive for *nisA*. The presence of these genes in wild lactococci from dairy products has been extensively documented (Bravo, Rodríguez, & Medina, 2009; Murat & Hakkı, 2021). Nisin A and Z are two natural variants, and they differ in a single

Table 3
Antimicrobial activity of the *Lactococcus* strains grown at 30 °C in MRS broth with Tween 80 (0.20 % *w/v*) against *Clostridium* spp. indicator microorganisms.

Species	Strain	*Lactococcus* strains				
		FT27	N16	N26	SV77	VC106
Cl. beijerinckii	DSM 791	+	++	++	++	++
Cl. butyricum	DSM 10702	++++	++++	+++	++++	++++
Cl. sporogenes	ATCC 3584	+	+	+	+	+
	ATCC 10000	++	++	++	++	++
	CL14	+	++	++	++	++
	CL16	++	++	++	++	++
	CL219a	+++	+++	++++	+++	+++
	CL219b	+++	++++	++++	+++	++++
	CL13	+++	+++	+++	+++	+++
	CL1	++	++	++	++	++
	CL16	++++	+++	+++	+++	+++
	CL106	++	++	++	++	++
Cl. tyrobutyricum	DSM 2637	+	+	++	+	++
	ATCC 8260	+	+	+	+	+
	CL10	+	+	+	+	+
	CL12	++	+	++	++	++
	Coc1	++++	+	+++	+++	+++
	Coc2	+	+	+	+	+
	CL11	++	++	++	++	++
	CL167	+	++	++	++	++
	CL6a	++	+	+++	+++	+++
	IN15b	+++	+++	+	+++	+++

Diameter of the inhibition zone: +, 0–5 mm; ++, 6–10 mm; +++, 11–15; ++++, >15 mm.

amino acid residue at a position 27. Nisin A contains histidine and nisin Z harbours asparagine. This structural modification does not affect the antimicrobial activity, but it confers a higher rate of diffusion and solubility under neutral pH conditions to Nisin Z (Khelissa, Chihib, & Gharsallaoui, 2021). In addition to *nisZ*, *Lc. lactis* VC106 harboured the *lctA* and *lcnB* genes coding for the Lacticin 481 and Lactococcin B (Table 1). Similar results were reported by other authors that observed the presence of different bacteriocin-coding genes in the same *Lactococcus* spp. strain isolated from artisanal dairy products (Attar, Yavarmanesh, Mortazavi, Dovom, & Najafi, 2018; Bravo et al., 2009; Dal Bello et al., 2010; Garde, Rodríguez, Gaya, Medina, & Nuñez, 2001).

Secondly, we investigated the unpurified CFSs by LC-MS. A protein, the monoisotopic mass and the chromatographic retention time of which corresponded to the Nisin A (UniProt P13068), was identified in the *Lc. cremoris* FT27 fermented broth, while the *Lc. lactis* strains N16 and SV77 resulted to be producers of proteins the monoisotopic accurate masses of which corresponded to the Nisin Z (UniProt P29559) (Table 1 and Table S1).

In agreement with genotypic results, the LC-MS/MS analyses performed on the CFS of VC106 (intact proteins secreted by the *Lc. lactis* VC106 and purified by TCA precipitation) highlighted the presence of Nisin Z (monoisotopic mass 3328.565, UniProt P29559) and Lacticin 481 (monoisotopic mass 2899.272, UniProt P36499), as deduced from the measurement of their accurate mass and the interpretation of their fragmentation spectra (MS/MS spectra). On the other hand, the LC-MS and LC-MS/MS analyses of CFSs of *Lc. lactis* VC106 did not reveal the presence of Lactococcin B (UniProtKB P35518) or its truncated form (Vukotic et al., 2019) among the proteins secreted by *Lc. lactis* VC106 under the applied experimental condition (Table 1, Tables S1 and S2). In order to confirm the identity of the bacteriocins detected in the LC-MS and LC-MS/MS experiments and, more importantly, highlight the presence of other putative bacteriocins secreted by *Lc. lactis* VC106, an untargeted shotgun proteomic approach was applied. This study led to identify 218 secreted *Lc. lactis* proteins (Table S3), including other four unexpected bacteriocins (Table 1 and Table S4), in particular, Lactococcin A (UniProt P0A312, gene *lcnA*) whose gene was not detected by PCR experiments. We also confirmed the expression of Lactococcin A by the presence of signals originated by the intact bacteriocin in the LC-MS/MS data. In addition, Lactococcin 972 family bacteriocin (UniProt A0A0V8CSN1) and two Lactococcin family bacteriocins (UniProt A0AAD1NJ52 and A0A7L9LE58) were identified and their expression was also confirmed by the LC-MS/MS experiments on the intact proteins (Tables S2 and S4). Finally, Lactococcin A secretion protein LcnD (UniProt P0A3G5, gene *lcnD*) and Nisin immunity protein (UniProt P42708, gene *nisI*) were also identified (Table S4).

Discordances observed between phenotypic and genotypic results may be a consequence of different factors such as the culture media compositions, the protocols adopted for the bacterial cultivation and/or DNA/CFS purification and the non-induction of the expression of genes encoding bacteriocins (Moraes et al., 2010; Perez, Zendo, & Sonomoto, 2022; Rodi, Gadea, & Reginensi, 2020). For example, in the unpurified CFS from *Lc. lactis* N26, Nisin Z was not found although this strain harboured the *nisZ* gene and showed anticlostridial activity. It is possible that *Lc.lactis* N26 produced a small amount of Nisin Z and, the background noise obtained by ions originated from the proteins contained in the MRS medium did not allow the detection of this bacteriocin in its unpurified CFS. Instead, regarding for what concerns Lactococcin A synthetized by VC106 strain, the fact that *lcnA* gene was not detected by PCR assay could be linked to a gene sequence variation (change in a single nucleotide or gene deletion) or a mutation (Arlindo et al., 2006; Foudjing, Sarmast, Allahdad, Salmieri, & Lacroix, 2023). Multiple bacteriocin production showed by *Lc. lactis* VC106 represents an interesting feature in preventing the growth of undesired bacteria in the production of fermented foods. In the last decade genomic modification tools have been developed to study and improve the LAB antimicrobial properties (Xie, McAuliffe, Jin, & Miller, 2024). For example, Mills et al. (2017) used lactococcal conjugative plasmids to stack multiple bacteriocins into *Lactococcus* starter strains, while Feito et al. (2023) engineered a recombinant multi-bacteriocinogenic *Lc. cremoris* strain to synthesized three different bacteriocins: Garvicin A, Garvicin Q and Nisin Z. Considering the restrictive food legislation and poor consumer acceptance of genetically modified microorganisms, the application of these bacteria in the food industry is restricted. For this reason, the natural multiple bacteriocin producing strains represent an important tool for the control of the dairy spoilage and pathogenic bacteria.

3.3. Influence of the cheese-making temperature on the Lactococcus spp. growth and bacteriocin production

This study was designed to evaluate the ability of different *Lactococcus* spp. strains to synthesize bacteriocins during the cheese production. For this purpose, we cultivated the cultures at optimal temperature conditions (30 °C) and we simulated the temperatures of the uncooked and semi-cooked cheeses production and ripening (Table 2). All trials were performed without the pH regulation. The temperature decreases during the uncooked and semi-cooked cheese manufacture [from 38 to 45 °C (cooking) to 18 °C (moulding and salting)] led to a delay in the fermentation processes (Table 4). This effect was evident after 6 and 8 h, when ΔpH values detected at the optimal growth temperature condition differed significantly from those obtained during the simulation of cheese-making processes ($P < 0.05$) (Table 4). In this time lapse, also the GT values changed, the *Lactococcus* spp. growth rates slowed down with the achieving of the salting temperature (18 °C) for all tested strains (Table 4). On the contrary, after 24 h, we did not observe any noticeable differences among ΔpH and GT, indicating that *Lactococcus* spp. activities continued slowly at sub-optimal temperature (Table 4), as previously reported by Wouters et al. (2000).

The results of bacteriocins production at different fermentation processes were reported in Table 5. We detected the antimicrobial compounds at each sampling point, confirming that their production followed primary metabolite kinetics. For many years, it was believed that the Nisin synthesis occurred during the stationary growth phase, when nutrients were exhausted. However, different authors observed that this peptide is produced during the bacterial exponential growth phase reaching the maximum amount at the end of it (Liu et al., 2020; Pongtharangkul & Demirci, 2006).

In our experiments, at 30 °C, after 2 h of fermentation the Nisin A and Z accumulation increased rapidly and after 6–8 h, as previously described by Hugenholtz and De Veer (1991) the amount of produced bacteriocin was sufficient to prevent butyric acid fermentation (1.6 $\log_{10}$ IU/g), and after 24 h the five strains produced different quantities of Nisins (ranging from 2.4 ± 0.1 $\log_{10}$ IU/mL to 3.3 ± 0.2 $\log_{10}$ IU/mL) (Table 5). These data agreed with Khelissa et al. (2021) who highlighted that the Nisin production was strain-specific and it was related to the gene expression intensity and to the enzyme activity involved in the post-translational maturation process.

A different scenario was observed when we reproduced the times and temperatures applied during the cheese-making process of uncooked and semi-cooked cheeses. As reported in Table 5, in these conditions, the bacteriocins accumulation during the first 8 h of fermentations dramatically decreased (more than 95 %) and differed significantly from that produced at 30 °C ($P < 0.05$). Moreover, in this time lapse, none of the *Lactococcus* spp. strains was able to synthetize a quantity of Nisin A/Z sufficient to hamper the LBD (< 1.6 $\log_{10}$ IU/g). After 24 h of fermentation, only the *Lc. lactis* VC106 produced an amount of antimicrobial compounds comparable to those obtained at optimal growth conditions (2.8 ± 0.1 $\log_{10}$ IU/mL at temperatures of the uncooked cheese production and 2.9 ± 0.2 $\log_{10}$ IU/mL at temperature of the semi-cooked cheese production) (Table 5). Our findings support that the temperatures found in the cheese-making processes play a key role in the bacteriocin synthesis; the skills to produce antimicrobial compounds

Table 4
Acidification activity (ΔpH) and generation time (GT) of the *Lactococcus* spp. strains in MRS broth with Tween 80 (0.20 % w/v) during different fermentation processes resembling uncoocked (Uc) or semi-coocked (Scc) cheeses production in comparison with optimal growth temperature (Ogt). Data were expressed as means ± standard deviations.

Parameters	Strains		Time points				
			2 h	4 h	6 h	8 h	24 h
ΔpH	FT27	Ogt	0.1 ± 0.1	0.2 ± 0.1	0.6 ± 0.2 [a]	1.0 ± 0.3 [a]	1.6 ± 0.2
		Uc	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.2 [b]	0.2 ± 0.1 [b]	1.5 ± 0.2
		Scc	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1 [b]	0.2 ± 0.2 [b]	1.5 ± 0.3
	N16	Ogt	0.1 ± 0.1	0.4 ± 0.2	1.0 ± 0.3 [a]	1.3 ± 0.2 [a]	1.9 ± 0.2 [a]
		Uc	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1 [b]	0.2 ± 0.1 [b]	1.4 ± 0.3 [b]
		Scc	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1 [b]	0.2 ± 0.2 [b]	1.4 ± 0.2 [b]
	N26	Ogt	0.1 ± 0.1	0.2 ± 0.1	0.6 ± 0.1 [a]	1.0 ± 0.1 [a]	1.3 ± 0.3
		Uc	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1 [b]	0.3 ± 0.3 [b]	1.2 ± 0.1
		Scc	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1 [b]	0.2 ± 0.1 [b]	1.2 ± 0.0
	SV77	Ogt	0.1 ± 0.1	0.2 ± 0.1	0.6 ± 0.2 [a]	0.9 ± 0.2 [a]	1.3 ± 0.1
		Uc	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1 [b]	0.3 ± 0.2 [b]	1.3 ± 0.2
		Scc	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1 [b]	0.2 ± 0.1 [b]	1.4 ± 0.3
	VC106	Ogt	0.1 ± 0.1	0.2 ± 0.2	0.5 ± 0.2 [a]	0.8 ± 0.2 [a]	1.2 ± 0.1
		Uc	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1 [b]	0.2 ± 0.1 [b]	1.0 ± 0.2
		Scc	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1 [b]	0.1 ± 0.1 [b]	0.9 ± 0.2
GT (h)	FT27	Ogt	0.3 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.8 ± 0.0	2.2 ± 0.0
		Uc	0.3 ± 0.0	0.6 ± 0.0	0.8 ± 0.0	1.0 ± 0.0	2.2 ± 0.0
		Scc	0.3 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	1.0 ± 0.0	2.2 ± 0.0
	N16	Ogt	0.3 ± 0.0	0.6 ± 0.0	0.8 ± 0.0	1.1 ± 0.0	3.2 ± 0.0
		Uc	0.3 ± 0.0	0.6 ± 0.0	0.8 ± 0.0	1.1 ± 0.0	3.2 ± 0.0
		Scc	0.3 ± 0.0	0.6 ± 0.0	0.9 ± 0.0	1.1 ± 0.0	3.2 ± 0.0
	N26	Ogt	0.3 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.8 ± 0.0	2.1 ± 0.0
		Uc	0.3 ± 0.0	0.6 ± 0.0	0.8 ± 0.0	1.0 ± 0.0	2.2 ± 0.0
		Scc	0.3 ± 0.0	0.6 ± 0.0	0.8 ± 0.0	1.1 ± 0.0	2.3 ± 0.0
	SV77	Ogt	0.3 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.8 ± 0.0	2.1 ± 0.0
		Uc	0.3 ± 0.0	0.5 ± 0.0	0.8 ± 0.0	1.0 ± 0.0	2.2 ± 0.0
		Scc	0.3 ± 0.0	0.6 ± 0.0	0.8 ± 0.0	1.0 ± 0.0	2.2 ± 0.0
	VC106	Ogt	0.4 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	0.8 ± 0.0 [a]	2.3 ± 0.0
		Uc	0.0	0.6 ± 0.0	0.9 ± 0.0	1.0 ± 0.0 [a]	2.4 ± 0.0
		Scc	0.0	0.8 ± 0.0	1.1 ± 0.0	1.3 ± 0.0 [b]	2.4 ± 0.0

30.0 ± 0.4	37.6 ± 1.1	44.4 ± 0.3	18.0 ± 0.2

Means with different superscript letters within a column were significantly different ($P < 0.05$). Values without letters were not significantly different ($P > 0.05$).
ΔpH: $pH_{zero\ time}$ - $pH_{at\ time}$; GT: Generation time.
Colours refer to Temperatures (°C) of the fermentation processes at the different sampling time points:

under stress conditions are strain-dependent. Moreover, our data confirmed that the relationship between the cell number and the amount of produced Nisin is not linear, since at the same time points of different fermentation processes, and at similar GT values the same amount of the bacteriocin was not synthetized (Table 4 and Table 5). Similar results were obtained by Havlíková et al. (2018), who observed a different anti-*Clostridium* activity in Nisin producers *Lc. lactis* strains (CCDM 731 and CCDM 71) when used individually in experimental dairy productions.

As recently noted by Wörmann et al. (2024) the differences in antimicrobial activity can be related to the environmental parameters, such as temperature and pH that strongly influenced the antagonistic potential of LAB strains. Our findings agreed also with the observations of Chavez de Lima, de Moura Fernandes, and Cardarelli (2017), who showed that high lactococcal growth rate did not always result in a high bacteriocin titre, since the antimicrobial compounds production depended on the environmental conditions such as pH, aeration and temperature. For this reason, the bacteriocin production by LAB strains and their use in dairy sector remains a challenge. As previously described, *Lc. lactis* VC106 was able to produce similar bacteriocins amounts at optimal growth temperature as well as at the sub-optimal ones. This capability was recorded in other LAB strains belonging to the *Latilactobacillus sakei* species, and it was related to better utilization

Table 5
Bacteriocin production by *Lactococcus* spp. strains in MRS broth with Tween 80 (0.20 % w/v) at 30 °C (Optimal growth temperature; Ogt) and at temperatures resembling those used in uncooked (Uc) and semi-cooked (Scc) cheese production. *Clostridium tyrobutyricum* IN15b was used as an indicator microorganism. Data were expressed as $\log_{10}$ IU/mL, as means ± standard deviations.

Strains		Bacteriocin production ($\log_{10}$ IU/mL)										
		2 h	4 h	6 h	8 h	24 h	7 days	15 days	30 days	60 days	90 days	170 days
FT27	Ogt	0.3 ± 0.1	0.6 ± 0.1	1.5 ± 0.1 [a]	1.8 ± 0.1 [a]	3.0 ± 0.2 [a]	-	-	-	-	-	-
	Uc	-0.3 ± 0.1	-0.2 ± 0.1	0.1 ± 0.2 [b]	0.1 ± 0.1 [b]	0.1 ± 0.1 [b]	0.2 ± 0.1	0.2 ± 0.2	0.8 ± 0.2	1.0 ± 0.2	1.1 ± 0.2	-
	Scc	-0.4 ± 0.1	-0.1 ± 0.1	0.0 ± 0.1 [b]	0.0 ± 0.1 [b]	0.3 ± 0.2 [b]	0.3 ± 0.2	0.4 ± 0.2	0.7 ± 0.1	1.0 ± 0.2	1.1 ± 0.1	-
N16	Ogt	-0.3 ± 0.1	1.2 ± 0.2 [a]	1.9 ± 0.1 [a]	2.3 ± 0.2 [a]	3.3 ± 0.2 [a]	-	-	-	-	-	-
	Uc	-1.0 ± 0.1	-0.1 ± 0.1 [b]	-0.1 ± 0.1 [b]	-0.1 ± 0.1 [b]	0.2 ± 0.1 [b]	0.1 ± 0.1	0.4 ± 0.1	0.8 ± 0.2	0.8 ± 0.1	0.9 ± 0.2	-
	Scc	-1.0 ± 0.1	-0.3 ± 0.0 [b]	-0.1 ± 0.1 [b]	0.1 ± 0.1 [b]	0.5 ± 0.1 [b]	0.5 ± 0.2	0.5 ± 0.1	0.7 ± 0.1	0.8 ± 0.2	0.9 ± 0.1	-
N26	Ogt	0.6 ± 0.1 [a]	0.7 ± 0.1	1.9 ± 0.1 [a]	2.3 ± 0.1 [a]	2.4 ± 0.1 [a]	-	-	-	-	-	-
	Uc	0.0 ± 0.1 [b]	0.2 ± 0.1	0.3 ± 0.1 [b]	0.3 ± 0.1 [b]	0.7 ± 0.2 [b]	0.6 ± 0.2	0.7 ± 0.3	0.9 ± 0.1	0.9 ± 0.2	1.1 ± 0.1	-
	Scc	-0.1 ± 0.1 [b]	0.0 ± 0.1	0.1 ± 0.1 [b]	0.1 ± 0.2 [b]	0.5 ± 0.1 [b]	0.5 ± 0.2	0.5 ± 0.2	0.8 ± 0.2	0.9 ± 0.2	1.2 ± 0.2	-
SV77	Ogt	1.2 ± 0.1 [a]	1.3 ± 0.1 [a]	2.1 ± 0.1 [a]	2.5 ± 0.1 [a]	3.2 ± 0.2 [a]	-	-	-	-	-	-
	Uc	-0.2 ± 0.1 [b]	-0.3 ± 0.2 [b]	-0.2 ± 0.1 [b]	-0.2 ± 0.1 [b]	-0.1 ± 0.1 [b]	0.1 ± 0.1	0.3 ± 0.1	0.8 ± 0.2	1.1 ± 0.1	1.2 ± 0.1	-
	Scc	-0.4 ± 0.1 [b]	-0.4 ± 0.1 [b]	-0.3± 0.1 [b]	-0.2 ± 0.1 [b]	0.2 ± 0.1 [b]	0.1 ± 0.2	0.2 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	-
VC106	Ogt	0.1 ± 0.1	0.7 ± 0.1	2.0 ± 0.1 [a]	2.3 ± 0.1 [a]	3.2 ± 0.1	-	-	-	-	-	-
	Uc	0.1 ± 0.1	0.2 ± 0.1	0.4 ± 0.2 [b]	0.9 ± 0.1 [b]	2.8 ± 0.1	2.7 ± 0.1	2.6 ± 0.1	2.4 ± 0.1	2.3 ± 0.1	2.0 ± 0.1	1.6 ± 0.1
	Scc	0.0 ± 0.1	0.1 ± 0.1	0.3 ± 0.1 [b]	0.9 ± 0.1 [b]	2.9 ± 0.2	2.8 ± 0.1	2.7 ± 0.1	2.5 ± 0.1	2.2 ± 0.1	2.1 ± 0.1	1.9 ± 0.1
Temperatures (°C) of the fermentation processes at the different sampling time points:							30.0 ± 0.4	37.6 ± 1.1	44.4 ± 0.3	18.0 ± 0.2	10.0 ± 0.5	

Means with different superscript letters within a column were significantly different ($P < 0.05$). Values without letters were not significantly different ($P > 0.05$).
(-): not determined.

of essential metabolites during the bacteriocin production (Møretrø, Aasen, Storrø, & Axelsson, 2000). Comparing the data related to the bacteriocin synthesis by *Lc. lactis* VC106 during the simulations of cheese-making process, we did not observe any significant differences in antimicrobial compounds activity (Table 5) providing evidence that the time and temperature conditions applied during the cooking step (38 and 45 °C for 20 min, Table 2) did not influence the *Lactococcus* spp. activities.

3.4. Influence of the ripening temperature on the Lactococcus spp. growth and bacteriocin production

When simulating the cheese ripening (incubation at 10 °C) we noticed that the amount of Nisins produced by FT27, N16, N26 and SV77 strains, did not reach the titre needed to inhibit the butyric acid fermentation (1.6 $\log_{10}$ IU/g; Hugenholtz & De Veer, 1991). This means that when the first LBD symptoms arose (about 70 days of ripening; Morandi et al., 2021) these strains could not be able to hamper this defect. On the contrary, the titre of antimicrobial compounds produced by VC106 declined progressively during the 170 days at 10 °C, but it remained above the threshold necessary to prevent the blowing defect (1.6 and 1.9 $\log_{10}$ IU/mL) (Table 5). Similar results were reported by Garde et al. (2020) who observed a reduction in bacteriocin activity during the ripening period in model cheeses obtained with multiple-bacteriocin producing *Lc. lactis* strain. This trend could be linked to the action of proteolytic enzymes from LAB that act on the bacteriocins causing a degradation of their active forms (Bouksaim, Lacroix, Audet, & Simard, 2000). The progressive decrease of the antimicrobial compound amounts during the ripening period simulation could explain the results obtained by Ávila et al. (2020) and Garde et al. (2020) who showed that in cheeses obtained with bacteriocin-producing starter the activity of *Cl. tyrobutyricum* was delayed without the complete arrest of the blowing defects.

3.5. Influence of the osmotic stress on the Lactococcus spp. bacteriocins production

We also evaluated influence of the osmotic stress on the *Lactococcus* spp. bacteriocin production at 30 °C. We chose the NaCl concentrations (0.5, 1.0 and 2.0 % *w/v*) considering the salt content in semi-hard cheeses susceptible to LBD (Silvetti, Morandi, & Brasca, 2018). Bacteriocin production of *Lactococcus* spp. strains was reduced with increasing concentration of NaCl in a strain-dependent manner (Table 6). At lowest salt content (0.5 %) we observed a significant reduction in Nisin Z synthesis only in strain N26 strain (0.5 ± 0.1 log10 IU/mL), while an exposure to 2.0 % was necessary to affect the anti-clostridial activity of the FT27, SV77 and VC106 (Table 6). These findings agreed with those found by different authors who showed a variation in osmotic tolerance within the *Lactococcus* genus (Kristensen, Siegumfeldt, Larsen, & Jespersen, 2020) and that, high salt concentrations (> 2.5 % NaCl) are necessary to reduce the growth rate of these microorganisms and to inhibit their bacteriocins production (Sanders, Venema, & Kok, 1999; Simsek, Buzrul, Akkoc, Alpas, & Akcelik, 2009). Analogous observations were reported by Verluyten, Messens, and De Vuyst (2004) that studied the effects of different NaCl concentrations in MRS medium on the bacteriocin production of the *Latilactobacillus curvatus* LTH 1174. These authors highlighted as sodium chloride interfered both with the bacteriocin production, in particular, the antimicrobial compound synthesis decreased in the presence of increasing salt concentrations. Despite that, at the highest tested salt concentration (2.0 %) three out of five strains (FT27, N16 and SV77) produced an amount of

Table 6
Influence of different NaCl concentrations (% *w/v*) on bacteriocin production by *Lactococcus* strains in MRS broth with Tween 80 (0.20 % w/v). *Clostridium tyrobutyricum* IN15b was used as an indicator microorganism. Data were expressed as means ± standard deviations.

Strains	NaCl (%)			
	0.0	0.5	1.0	2.0
FT27	3.0 ± 0.2 [a]	2.6 ± 0.1 [a]	2.5 ± 0.0 [ab]	2.1 ± 0.1 [b]
N16	3.3 ± 0.2	3.2 ± 0.0	3.2 ± 0.0	2.9 ± 0.1
N26	2.4 ± 0.1 [a]	0.5 ± 0.1 [b]	0.5 ± 0.1 [b]	0.5 ± 0.1 [b]
SV77	3.2 ± 0.2 [a]	3.1 ± 0.0 [a]	2.9 ± 0.0 [a]	2.4 ± 0.0 [ab]
VC106	3.2 ± 0.2 [a]	2.4 ± 0.1 [ab]	2.2 ± 0.1 [ab]	1.3 ± 0.0 [b]

Bacteriocin production was reported as $\log_{10}$ IU/mL. Means with different superscript letters within a row were significantly different ($P < 0.05$). Values without letters were not significantly different ($P > 0.05$).

Nisin A/Z sufficient to prevent the LBD symptoms (Hugenholtz & De Veer, 1991). These results gain significance in view of the study of Oliveira et al. (2024) that showed, as a salt concentration of 1.25 % was sufficient to inhibit the growth of *Cl. sporogenes* in Requeijão cremoso cheese.

In this study we have evaluate the impact of the temperature and salt concentration on anti-*Clostridium* activity of wild *Lactococcus* strains. Our findings highlighted a different behaviour of the strains on varying the parameters considered, this suggest that is possible set up a tailor-made protective cultures taking into account the characteristics of each individual cheese-making process.

4. Conclusions

In this study, we investigated in vitro how the temperatures and salt concentrations applied in the cheese-making and ripening processes affect the *Lactococcus* bacteriocin production. Although the trials were conducted in MRS broth, and we did not consider some factors that can exert and inhibitory effect against the bacteriocins (additives/ingredients, enzymes inactivation, adsorption to dairy components, and starter cultures), our findings provide new evidences on factors suppressing the synthesis of bacteriocins and consequently the anti-*Clostridium* activity. Only the multi-bacteriocin producing *Lc. lactis* VC106 strain accumulated an amount of antimicrobial compounds sufficient to prevent the LBD symptoms, maintaining a consistent level over a period of 170 days. Further practical applications are needed to evaluate the antimicrobial activity of this strain and its interaction with starter LAB to develop more efficient tools in the LBD prevention. Moreover, our findings suggests that multi-bacteriocin producing strains could provide a flexible arsenal of antibacterial agents responding to different environmental conditions. Future research directions may involve the development of protection cultures, tailored to individual cheese-making process.

CRediT authorship contribution statement

Stefano Morandi: Validation, Investigation, Formal analysis, Conceptualization. **Tiziana Silvetti:** Writing – review & editing, Investigation. **Francesca Bonazza:** Writing – review & editing, Investigation. **Rosa Anna Siciliano:** Writing – review & editing, Investigation, Funding acquisition. **Maria Fiorella Mazzeo:** Writing – review & editing, Investigation. **Milda Stuknyte:** Writing – review & editing, Investigation. **Ivano De Noni:** Writing – review & editing, Investigation. **Milena Brasca:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2025.116239.

Data availability

Data will be made available on request.

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