Anti-*S. aureus* and Anti-*L. monocytogenes* Molecules Produced by Cheese-isolated Lactic Acid Bacteria

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Abstract

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Nine lactic acid bacteria from artisanal-made cheeses were investigated for their ability to inhibit *Listeria monocytogenes* and *Staphylococcus aureus*. Both extracellular and surface-bound bacteriocins were recovered. While *Lb. plantarum* molecule was present only extracellularly, all the other strains displayed interference in both compartments. Maximum bacteriocin production was observed at the end-logarithmic phase, with the exception of *Lb. plantarum* (late stationary) and *L. lactis* subsp. *cremoris* (very early exponential). *Lactobacillus* and *Lactococcus* strains inhibited both *List. monocytogenes* and *S. aureus.* On the contrary, both *E. faecium* strains were active only on *List. monocytogenes*, and the enterocin A amount was enhanced under oxygen stress. All *L. lactis* strains (including *L. lactis* subsp. *cremoris* EL3 generally producing nisin Z) biosynthesised nisin A, while *Lb. plantarum* caused interference because of its very high lactic acid production. All these results suggest that artisanal-made cheeses can contain promising strains for food biosafety: these strains can be employed *in toto* directly in the food matrix or the purified bacteriocins can be incorporated into food packaging.

Keywords: enterocin A; *Enterococcus faecium*; lactic acid; *Lactobacillus plantarum*; *Lactococcus lactis*; surface-bound bacteriocins

In spite of modern technologies and safety control interventions, the reported number of food-borne intoxications is still increasing (up to 9000 deaths annually). The most widespread bacterial pathogens responsible for these cases are *Listeria monocytogenes* and *Staphylococcus aureus* (Lanzas *et al.* 2011).

Until now, the main strategies to counteract food-borne diseases have been the cold chain, the use of salt, low pH and preservatives. More recently, the possibility to employ lactic acid bacteria (LAB) has been explored: bio-control can be achieved by both aspecific and specific mechanisms.

Nutrient competition is the best known aspecific type of antagonism. The specific antimicrobial mechanisms include the production of organic acids, H_2O_2, CO_2, and bacteriocins (Montalbàn-Lòpez *et al.* 2011). Bacteriocins are antimicrobial peptides or proteins active against bacteria. They differ from traditional antibiotics in their absence of toxicity and their direct synthesis at a ribosomal level (Cotter *et al.* 2005). Their mechanism of action is based upon dissipation of the membrane potential, enzymatic inhibition of peptidoglycan synthesis, and cell wall hydrolysis (Jin *et al.* 2010; Ross & Vederas 2011).

To achieve an efficient bio-control in food, two different strategies have been proposed: (*i*) the use of bacteriocin-producing LAB as bio-preservatives

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to be added directly in the food sample; (*ii*) the direct employment of partially purified bacteriocins either in the food matrix or in the food packaging after suitable immobilization (Jin *et al.* 2010).

Artisanal-made cheese is largely consumed but only limited information is available on the positive features associated with the microbiota contributing to final characteristics. The aim of the present study was to analyse different traditional Piedmont-cheese-isolated LAB for assessing their ability to produce *List. monocytogenes*- and *S. aureus*-active bacteriocins, to establish better conditions for maximum bacteriocin expression and to localise the inhibitory activity at a cellular level.

MATERIAL AND METHODS

Bacterial strains and culture conditions. Nine strains isolated from artisanal Piedmont cheeses were selected from the DIVAPRA bacterial culture collection. The inhibitory activity was evaluated against two *S. aureus* strains (ATCC 6538 and VET isolated from food) and four *List. monocytogenes* strains (NCTC 10527 serotype 4b, EDGe serotype 1/2a, #3 and #2 both isolated from food). All lactococci and enterococci were grown in M17 Broth (Sigma, Milan, Italy) at 37°C, *Lactobacillus plantarum* was grown in MRS Broth (Oxoid, Basingstoke Hampshire, UK) at 30°C, and the indicator strains were grown in BHI Broth (Sigma, Milan, Italy) at 37°C. All cultures were performed both in microaerophilic conditions and in the presence of O_2. Each culture was followed till the stationary phase by OD_{600nm} measuring.

Lactic acid determination. The amount of lactic acid produced was determined by D/L Lactate Measurement Kit (Megazyme, Bray, Co. Wicklow, Ireland) following the manufacturer's instructions.

Antibacterial molecule recovery. After strain propagation, 500 ml of the culture were centrifuged (15 000 *g* at 4°C for 20 min in a Beckman L8-60M ultracentrifuge; Beckman, Milan, Italy): the pellet of bacterial cells was tested for the Yang and co-workers protocol (Yang *et al.* 1992) while the cell free supernatant (CFS) was precipitated using 60% ammonium sulphate, dissolved in ammonium acetate 25mM pH 6.5 and then precipitated again using chloroform/methanol (purification step). The protocol of Yang and co-workers was based on pH dependent adsorption/desorption of bacteriocins from the bacterial cell surface. In the first step the pH of the culture was adjusted to 5.5 or 6.5 in order to obtain the maximum bacteriocin adsorption, then the pH was lowered to 2 (using NaCl 100mM with 5% phosphoric acid) in order to desorb them. Bacteriocins were then easily recovered by centrifugation (29 000 *g* at 4°C for 20 min).

Detection of antibacterial molecule activity. The inhibitory potential of bacteriocins was tested by agar well diffusion assay (Benkerroum *et al.* 1993) in line with both the recovery protocols (ammonium sulphate precipitation and pH dependent adsorption/desorption protocol). Each assay was performed in triplicate and the activity of bacteriocins was arbitrary determined by measuring the inhibition halo around the well. The arbitrary units (AU) of the bacteriocin were determined as the reciprocal of the highest dilution showing the inhibition of the indicator strain (Ten Brink *et al.* 1994).

Effect of proteinase K, heat treatments and pH on antibacterial activity. To confirm the proteinaceous nature of the antibacterial molecules, 4 µl of proteinase K (25 mg/ml; Sigma, Milan, Italy) were incubated with 60 µl of the sample (after ammonium sulphate precipitation and the pH dependent adsorption/desorption protocol) for two hours, according to the procedures of Todorov *et al*. (2010). The mixture was added into the well of a BHI Agar Petri dish inoculated with 1% of a 24-h culture of the indicator strain. After plate incubation at 37°C for 24 h, the presence of inhibition halos was evaluated.

The thermal stability of active compounds was determined by heating the samples (after ammonium sulphate precipitation and the pH dependent adsorption/desorption protocol) at 70, 80, and 90°C for 30 min, while the bacteriocin pH-dependent activity was tested by adjusting pH from 1 to 10 (Todorov *et al.* 2010).

Bacteriocin molecular size determination. The antibacterial molecules precipitated fraction was separated by Tricine-SDS-PAGE (Schägger 2006) using a MINI-PROTEAN 2 system (Biorad, Hercules, USA) and the gel was stained with colloidal Coomassie Blue. A duplicate of each gel was run in the same conditions but without reducing and denaturing treatments. The duplicate was used in an overlay assay (Millette *et al.* 2007) to identify the band with antibacterial activity against the target strain.

Bacteriocin purification and chromatographic analysis. Samples obtained as described in Section 2.2 were lyophilised (Heto mod. Drywinner 8 apparatus, London, UK) and re-suspended in MilliQ water. C8-SPE column (Supelclean LC8 3 ml; Supelco, Milan, Italy) was used for sample purification. The sample was applied into the column and eluted in

5 fractions of 12 ml of 0.1% trifluoroacetic acid in different water-acetonitrile mixtures (100 water, 75:25 water:ACN, 50:50 water:ACN, 20:80 water:ACN and 100 ACN, respectively). Each fraction was evaporated to dryness in Rotavapor and re-suspended in 500 µl of 0.1% TFA in 50:50 ACN:HPLC grade water. 50 µl of each fraction was analysed in a Shimadzu Class HPLC system, using Waters Spherisorb ODS 2 column (4.6 *i.d.*, 250 mm length) (Milan, Italy). The elution was done with pump A: 0.1% TFA and 5% ACN in HPLC-grade water, and pump B: 0.1% TFA and 5% water in ACN. Flow rate 1.0 ml/min. gradient pump B 5 – 100% in 45 min; detection UV 215 nm. For nisin quantification different weights of a commercial nisin formulation (Fluka, Milan, Italy) were purified by C8-SPE. The standard curve obtained showed a good linearity (R^2 = 0.9981). The LOD and LOQ of HPLC method were 1.7 ng and 5.5 ng of nisin, respectively.

Gene Targeting experiments. PCR was used to amplify the genes of nisin A and Z, lactococcin A, B, 513, 972, G, Q and lacticins RM and 481 in lactococci, and enterocins A and P, cytolysin and enterolysin A in enterococci, using the primers listed by Dal Bello *et al. (*2010).

RESULTS AND DISCUSSION

Antibacterial activity: cellular localization and spectrum of inhibition. Nine bacterial strains previously assessed to interfere with pathogen growth (Dal Bello *et al.* 2010) were investigated using as targets two *S. aureus* and four *List. monocytogenes* strains. The antibacterial compounds are generally secreted in the extracellular environment, but some reports indicate that they can be surface-bound as well (Yang *et al.* 1992). To get a better recovery of the produced molecules two different protocols have been developed and using the two combined methods eight of the nine bacterial strains displayed antibacterial activity in both districts whereas one strain exhibited inhibitory activity only in the extracellular precipitated pellets (Table 1). What concerns the inhibitory spectrum, all *Lactococcus* strains inhibited, although to a different extent, the four *List. monocytogenes* strains and the two *S. aureus* strains. They displayed the following activity against *List. monocytogenes* and *S. aureus* strains: for *L. lactis* 15, 150–166 AU/ml; for *L. lactis* 8A, 76–83 AU/ml; for *L. lactis* 8B, 75–86 AU/ml; for *L. lactis* 7, 30–33 AU/ml; for *L. lactis cremoris* EL3, not detectable – 12 AU/ml; for *L. lactis* 5, not detectable.

What concerns *E. faecium:* strain G6 was able to inhibit all the four strains of *List. monocytogenes* (32 AU/ml) whereas *E. faecium* G12 was active only towards three (NCTC 10527, EDGe, #2) of the four *List. monocytogenes* tested strains (30 AU/ml). Although the inhibitory action of *E. faecium* against *S. aureus* (Pinto *et al.* 2009) has been reported in the literature, nevertheless most reported studies

Table 1. Characterisation of antibacterial compounds produced by tested LAB

Strain	Spectrum of activity	Max production			Cellular localisation		Stability		Interfering molecule identification
		growth effect	O_2 effect		CFS	CS	temperature (90°C/30 min)	Proteinase K	
			$-O_2$	$+O_2$					
Lb. plantarum 37A	*List. monocyt* and *S. aureus*	LS (48 h)	+	–	+	-	yes	resistant	lactic acid
E. faecium G12	*List. monocytogenes*	LE (18 h)	+	++	+	+	yes	sensitive	Enterocin A
E. faecium G6	*List. monocytogenes*	LE (18 h)	+	++	+	+	yes	sensitive	Enterocin A
L. lactis 5	*List. monocyt* and *S. aureus*	LE (18 h)	+	–	+	+	yes	sensitive	Nisin A
L. lactis 7	*List. monocyt* and *S. aureus*	LE (18 h)	+	–	+	+	Yes	sensitive	Nisin A
L. lactis 8A	*List. monocyt* and *S. aureus*	ES (18 h)	+	–	+	+	Yes	sensitive	Nisin A
L. lactis 8B	*List. monocyt* and *S. aureus*	ES (18 h)	+	–	+	+	Yes	sensitive	Nisin A
L. lactis 15	*List. monocyt* and *S. aureus*	ES (18 h)	+	–	+	+	Yes	sensitive	Nisin A
L. lactis cremoris EL3	*List. monocyt* and *S. aureus*	EE (6 h)	+	–	+	+	Yes	sensitive	Nisin A

LS – late stationary; LE – late exponential; ES – early stationary; EE – early exponential; $-O_2$ – microaerophilic condition; $+O_2$– aerophilic condition; CFS – cell-free supernatant; CS – cell surface. + presence of antibacterial activity (measured as inhibitory zone halo in agar plate test); – no bacteriocin production (no inhibitory zone in agar plate test); ++ halo inhibitory zone about twice higher than the control condition

underline that the spectrum of *E. faecium* inhibition seldom includes *S. aureus* (Valenzuela *et al.* 2010). This is in partial agreement with our results and it suggests that the inhibition spectrum is rather strain-specific than species-related.

Oxygen stress effect on bacteriocin production. Some authors underlined the importance of stress for increasing the bacteriocin production (De Vuyst & Leroy 2007). Being all LAB microaerophilic, the enhancement of bacteriocin production by oxygen stress was tested: in contrast to what was expected (Table 1), for all strains, bacteriocin production was obtained only in optimal O_2 conditions with the exception of the *Enterococcus faecium* strains, which slightly increased the production during oxygen exposure. This could be explained either by a higher oxygen tolerance in enterococci (Winstedt *et al.* 2000) or by an oxygen stress-enhanced bacteriocin biosynthesis as referred by Neysens and De Vuyst (2005) for *Lb. amylovorus* DCE 417.

Growth phase effect on bacteriocin production. As shown in Table 1, most bacteria produced bacteriocins during the late exponential/early stationary growth phase. Bacteriocin production is under quorum sensing (QS) control (Di Cagno *et al.* 2011): it is therefore reasonable to detect the highest production of these molecules when the biomass has reached a threshold cell number. Two strains, on the contrary, produce maximum amounts of the antibacterial molecules in the very early exponential phase (*L. lactis* subsp. *cremoris* EL3) or in the very late stationary phase (*Lb. plantarum* 37A).

The early bacteriocin production in *L. lactis* subsp. *cremoris* EL3 is an interesting phenomenon already described in different bacterial species. In *E. faecium* a cell-density bacteriocin production switch-off was observed by Leroy and De Vuyst (2002) for enterocin RZSC5. Aasen *et al*. (2000) demonstrated in *Lb. sakei* that a low growth rate led to a gain of energy and nutrients, allowing higher bacteriocin synthesis. However, this is a transitional event since growth must proceed and hence energy and nutrients are soon directed towards biomass formation with a switch-off of bacteriocin production. Different behaviour of *L. lactis* subsp. *cremoris* EL3 concerning maximum bacteriocin biosynthesis suggests that its metabolism is quite different with respect to all the other *L. lactis* subs. *lactis* strains.

Characterisation of Lb. plantarum 37 A molecule. The interference molecule produced by *Lb. plantarum* 37 A was not damaged either by thermal stress [treatment at 90°C for 30 min (Table 1)] or by proteolytic digestion (proteinase K treatment) supporting its non-proteinaceous nature. Figure 1 shows the pH dependent inhibition over *List. monocytogenes*. The loss of activity for pH values above 4.5 indicates the presence of an organic acid: the purified molecule was identified as lactic acid. The quantification of lactic acid produced by *Lb. plantarum* 37A revealed an exceptionally high amount (about 38 g/l) as compared to the other LAB, which fully justifies its bacteriocidal effect over the target strains. Furthermore this finding can explain the maximum interference effect in the late stationary phase: actually a significant lactic acid accumulation has to occur before visible growth inhibition on the target cells.

Characterisation of** E. faecium **bacteriocin. The antibacterial molecules produced by *E. faecium* G12 and G6 were thermostable, sensitive to proteinase K (Table 1) and maintained their activity after each purification method and pH treatment, suggesting their proteinaceous nature. PCR amplification demonstrated the presence of a gene encoding a 4.829 kDa bacteriocin ascribable to enterocin A. Tricine SDS-PAGE was set up and an overlay test on *List. monocytogenes* was performed to verify its molecular size. Figure 2 shows the inhibition halos obtained: the molecular weight of the active band lies in the range of 4–5 kDa, consistent with the expected enterocin A. Generally, enterocin A has a narrow inhibitory spectrum, being selective especially against *List. monocytogenes* (Ennahar & Deschamps 2000) and this was confirmed in this study for the two enterococci both unable to inhibit *S. aureus*. Work is in progress to increase enterocin A yields and purity

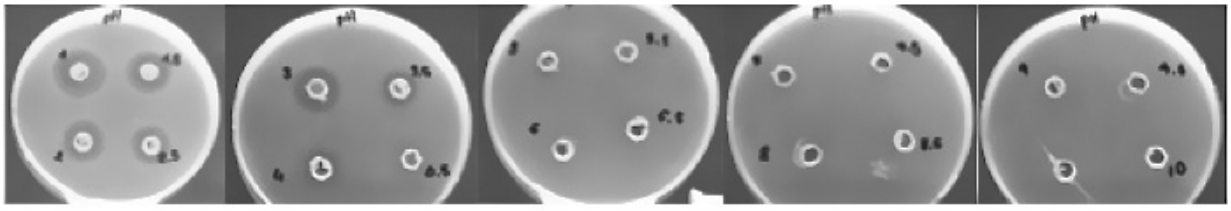
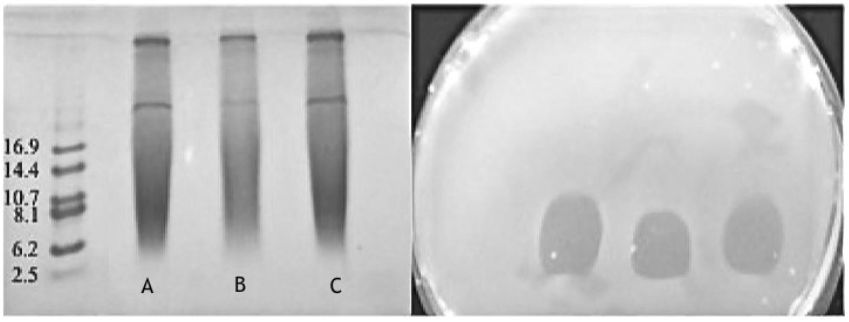


Figure 1. pH-dependent inhibition of *List. monocytogenes* by the *Lb. plantarum* 37A antimicrobial molecule: the interfering agent lost its activity at pH higher than 4.5



Lane A – CFS precipitated with 60% ammonium sulphate; lane B – CFS precipitated with 60% ammonium sulphate and purified with chloroform/methanol precipitation; lane C – sample obtained by pH dependent adsorption/desorption protocol

Figure 2. Tricine SDS-PAGE of ammonium sulphate precipitate of cell free supernatant of *E. faecium* G12 and overlay with *List. monocytogenes* EDGe serotype 1/2a

in order to immobilize it in the food packaging. This is of particular interest also because, at present, this enterocin A is not available in the market.

Characterisation of L. lactis bacteriocin. All the interfering molecules produced by *L. lactis* were thermostable at 90°C, proteinase K sensitive (Table 1) and resistant to all purification methods, also in this case confirming their peptide nature. Genetic investigations highlighted that all six *L. lactis* strains harbour the genes for *nisA* production. An interesting finding is that *L. lactis* subsp. *cremoris* EL3 strain also possesses a *nisA* gene unlike most *L. lactis* subsp. *cremoris* strains generally harbouring the *nisZ* gene (Dal Bello *et al.* 2010). A peculiar feature of this strain is also the early maximum production of this bacteriocin as referred before. These evidences underline that, being the molecule itself the same, the early expression of *nisA* in *L. lactis* subsp. *cremoris* is related rather to nutritional requirements of this subspecies than to the bacteriocin involved. To further characterise the produced nisin, SPE purification and HPLC analysis were applied to the broths and the solutions obtained by desorption method. The quantities of nisin produced by cells harvested at OD_{600} comparable (similar cell number) are reported in Table 2. A stronger producer is the strain *L. lactis* 15, followed by *L. lactis* 8A and *L. lactis* 8B. For these strains about 60–70% of nisin is released in the medium, the other part remaining bound to the cells. It is important to underline that also this "bound" activity contributes to the antibacterial potential of the strains and therefore its detection should be taken into consideration when evaluating bacteriocin production by LAB.

CONCLUSIONS

The detection of bacteriocin production by lactic acid bacteria isolated from artisanal-made cheeses underlines that traditional products can contain promising strains useful to be employed as bio-control agents to prevent food-borne diseases, caused by both *S. aureus* and *List. monocytogenes*. The present investigation has shown that the antibacterial activity should be searched both in the extracellular extracts and on the cell surface, since some bacteriocins are bound to the cell wall. Furthermore, it has to be considered that other interfering molecules (i.e. lactic acid), different from the common bacteriocins, although underexploited, can help in controlling food-related infections as well. It is now quite evident that strains naturally present in the cheese matrix, and evolving during ripening, cannot negatively interfere with starters, and this is an appreciated feature for possible application of these strains in industrial cheese production. Alternatively, the characterised bacteriocins can be further purified and immobilised into

Table 2. Nisin quantity present in culture broth and recovered after desorption procedure (expressed in μg from 500 ml of broth)

Strain	Nisin in broth	Nisin after desorption procedure	Total nisin
Lactococcus lactis 5	< LOQ	< LOD	–
Lactococcus lactis 7	0.50	< LOD	0.50
Lactococcus lactis 8A	2.25	1.11	3.36
Lactococcus lactis 8B	1.95	1.25	3.20
Lactococcus lactis 15	4.70	2.17	6.87
Lactococcus lactis cremoris EL3	< LOQ	0.13	0.13

the packaging polymers to ensure food safety without altering the microbial composition of each specific food. Experiments intended to enhance enterocin A yields (oxygen and other stressors) and purity are currently underway in our laboratories. The aim is to use the purified enterocin A and to immobilise it into plastic polymers because, at present, this molecule is not yet commercially available in its pure form. This is an innovative strategy since generally after immobilisation the interfering molecules retain all their antibacterial potential. Furthermore, this approach has also the advantage to avoid the use of enterococci whose safety is more and more questionable due to their intrinsic antibiotic resistance and virulence factor production (Pessione *et al.* 2012). Bacteriocin stability during processing and storage, their efficacy at low concentrations and their absence of deleterious effects on nutrients and on humans render them promising tools for the food safety control.

Finally, an important aspect is the probiotic potential of bacteriocins producing strains vehiculated by food. Actually after food ingestion, if these strains can survive to acidic gastric pH, to enzymatic inactivation by gastric proteases, to bile salt stress, then they can reach alive the gastro-intestinal tract and biosynthesise active compounds directly in the human gut, thus supporting *in vivo* interactions useful to control pathogenic competing bacteria. This potential can be useful to prevent diarrhoeas, to control gastrointestinal infections and to restore the lost microbiota balance after antibiotic treatment.

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