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ORIGINAL RESEARCH

Effect of nisin-producing *Lactococcus lactis* starter cultures on the inhibition of two pathogens in ripened cheeses

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The effect of Lactococcus lactis *nisin-producing strains, isolated from Italian fermented foods, on the survival of two foodborne pathogens namely* Listeria monocytogenes *and* Staphylococcus aureus *was investigated in experimental cheese production. One of the three* Lactobacillus lactis *nisin inn-oculated as starters,* Lactobacillus lactis *41FL1 lowered* S. aureus *count by 1.73 log colony-forming units (cfu)/g within the first 3 days, reaching the highest reduction, 3.54 log cfu/g, by the end of ripening period of 60 days. There was no effect on* L. monocytogenes. *The application of* L. lactis *41FL1 as bioprotective culture in controlling* S. aureus *shows considerable promise.*

Keywords *Lactococcus lactis*, nisin, *Staphylococcus aureus*, *Listeria monocytogenes*, Cheese.

INTRODUCTION

Lactic acid bacteria (LAB) are widely used for the fermentation and preservation of a wide range of milk, meat and vegetable foods. Among LAB strains, *Lactococcus lactis* is extensively used as starter culture in the dairy industry, and the potential applications of these strains in food production are well documented. In particular, in recent years, great interest was focused on the possible use of certain products of their metabolism, called bacteriocins, as potential biopreservatives in foods (O'Sullivan *et al.* 2002).

Bacteriocins are proteinaceous antimicrobial compounds ribosomially synthesised by bacteria and able to inhibit a high number of potential pathogens among which *Listeria monocytogenes* and *Staphylococcus aureus* (Cotter *et al.* 2005).

The current technologies employed to inactivate bacterial pathogens in foods are not always efficient and often represent particular treatments that may modify the fisical and chemical properties of the food products. Therefore, new approaches to combat pathogenic and spoilage microorganisms are needed. In the last few years, bacteriocins from LAB have been thoroughly characterised and tested in food systems as new food preservatives (Stiles 1996).

Although many bacteriocins have been characterised, two of the most widely used in food industry as biopreservative are nisin (Class I, lantibiotic) and pediocin (Class II). Nisin is the bacteriocin with the longest history of safe use in food industry. It has been shown to be effective for the microbial control in a number of dairy products and also widely assessed in cheese manufacturing (Ross *et al.* 2002). In most of cases, the bacteriocin is incorporated into the product as a dried concentrated powder (O'Sullivan *et al.* 2002), but currently, the use of nisin-producing starter or costarter cultures seems to be an alternative way of incorporating the bacteriocin (e.g. cheesemaking process) (Sobrino-López and Martőn-Belloso 2008).

Among the pathogenic bacteria potentially associated with dairy foods, *S. aureus* and *L. monocytogenes* are the cause of numerous

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food-poisoning outbreaks (De Buyser *et al.* 2001; Kousta *et al.* 2010).

Some *S. aureus* strains are able to produce staphylococcal enterotoxins (SEs) in food matrices and are responsible for food poisoning, one of the most common causes of gastroenteritis worldwide (Balaban and Rasooly 2000).

Contamination by *S. aureus* can come from raw material (e.g. mastitic milk), from the processing plant environment (e.g. biofilm on surfaces of processing plant) or from human activity (e.g. healthy carriage, sneeze, whitlow…) during food preparation and manipulation (Charlier *et al.* 2008). Among products, cooked meals and fermented milk are the most common types of food involved in *S. aureus* poisoning (Le Loir *et al.* 2003). In particular, an initial population of 3 log colony-forming units (cfu)/mL of *S. aureus* in milk may be sufficient for the production of enterotoxin A in cheese at detectable levels (Meyrand *et al.* 1998) with the possible risk of human intoxication.

L. monocytogenes and Listeria spp. are also widely distributed and abundant in nature as well as in food products especially ready to eat meats and dairy products (Santorum *et al.* 2012). *L. monocytogenes* is resistant to refrigeration, low pH as 3.5 and high salt concentration (Koutsoumanis and Sofos 2004), conditions for which it has been found in a variety of raw foods such as uncooked meats and vegetables (Miettinen and Wirtanen 2005; Lianou and Sofos 2007; Little *et al.* 2007) as well as milk products and various type of cheeses made of unpasteurised or pasteurised milk (Bille *et al.* 2005; Manfreda *et al.* 2005; Brito *et al.* 2008). Study published by Fox *et al.* (2009) showed also the relative high prevalence of *L. monocytogenes* in the dairy farm environment (water, soil, forage, milk, etc.) particularly in milking facilities due to its high ability of dispersion.

EU data for 2009 showed that *L. monocytogenes* was most often detected in soft and semisoft cheeses made from pasteurised milk (EFSA, 2011). Moreover, recent outbreaks due to contamination of listeria in cheeses made from pasteurised or heat-treated milk rather than raw milk were reported (Fretz *et al.* 2010; Koch *et al.* 2010; Kousta *et al.* 2010).

According to the EFSA Report, in EU, the number of confirmed listeriosis cases has increased from 2004 to 2006 with a slight decrease observed in 2010, and among cases, fatality rate was 17% (EFSA, 2012).

The ability of Listeria to inhabit a wide range of environments makes this pathogen particular difficult to control in food; therefore, new approaches to better control this foodborne pathogen are needed.

Several LAB bacteriocins offer potential applications in food preservation, and the use of bacteriocins in the food industry can help to reduce the addition of chemical preservatives as well as the intensity of heat treatments (Galvez *et al.* 2007).

Among the available approaches used to reduce the prevalence of Listeria and/or Staphylococcus in milk products, the application of bacteriocin and/or bacteriocin-producing cultures could be considered. The use of nisin-producing strains as biopreservative agents to control the foodborne *S. aureus* was successfully presented by Rodrőguez *et al.* (2001, 2005).

The objectives of the present study were: (i) to assess the technological potential as starter cultures of five *L. lactis* bacteriocin-producing strains in ripened cheese; (ii) to evaluate the potential application of some of these strains as bioprotective starter cultures in cheese productions inoculated with *S. aureus* and *L. monocytogenes*.

MATERIALS AND METHODS

Microorganisms and culture conditions

Four *L. lactis* nisin Z producers (44SGLL3, 29FL1, 28FL1 and 41FL1) and one *L. lactis* lacticin 481 producer (32FL3) (Dal Bello *et al.* 2010) were selected and used as starters in cheesemaking trials. Commercial lyophilised *L. lactis* Lyoto MO 540 nonbacteriocin producer (Sacco, Como, Italy) was used as control.

Five different cheesemakings were carried out on pasteurised cow milk. *L. lactis* strains were cultured twice in M17 broth (Oxoid, Milano, Italy) and incubated overnight at 30 °C. All revitalised lactococci were cultured in reconstituted skim milk at 30 °C for 24 h before use in cheesemaking.

Manufacture of cheese

Thermally treated whole milk (63 °C for 30 min) was transferred into a 5 L vat, cooled to 37 °C and maintained to this temperature in a water-bath before the inoculation with 8–9 log cfu/mL of *L. lactis* strains. Rennet (1:10000 of 75% chymosin–25% bovine pepsin) (Sacco) was added, and the milk held for 1 h to obtain a firm coagulum. After the coagulum was cut, the curd was left for 30 min at 37 °C. The curd was then put in the mould and turned upside down. After the moulding, the curd was salted in a salt saturated solution (20% w/w) for 15 h and then maintained in a ripening room at 8 °C and 90% of humidity for 60 days.

After the ripening time, the cheese was subjected to chemical and sensory analysis.

Chemical analyses of cheeses

Gross composition

Gross composition of cheese (moisture, pH, fat, ash, total nitrogen and soluble nitrogen) was determined using Official Methods of Analysis (AOAC 1990). The ripening index was also determined and calculated as percentage of water soluble nitrogen on total nitrogen. The analyses were performed in triplicate.

SDS-PAGE Electrophoresis

SDS-PAGE was performed according to the method of Laemmli (1970) using a Mini Protean III Dual Slab Cell

apparatus (Bio-Rad Laboratories S.r.l., Segrate, Italy). The analysis of proteins was carried out on the urea-soluble fraction of cheese obtained using a 6 M urea solution at pH 8.5. Gels were stained with Coomassie Blue R-250 to reveal protein bands. Fluor-S Multimager and Quantity One software (Bio-Rad) were used for gels analysis. Gel bands were expressed as relative percentage for each sample and used as parameters for statistical analysis.

Biogenic amines quantification

Biogenic amines extraction was performed by using 20 g of cheese added with 95 mL of pure HPLC water (Milli-Q, Millipore, Billerica, MA, USA): each sample was homogenised for 2 min. Then, 5 mL of trichloroacetic acid (TCA, 100% w/v) (Fluka, Buchs, Switzerland) was added. After centrifugation at 2000 rpm for 15 min, 50 mL of supernatant was extracted for three times with 15 mL of ether. The volume of aqueous solution (after removing traces of ether in a Rotavapor) was adjusted to 50 mL with HPLC water and filtered through a 0.22 μm syringe filter (Millipore, Bedford, MA, USA) before HPLC analyses as described elsewhere (Arlorio *et al.*1998).

Biogenic amines (tyramine, histamine, tryptamine and 2-phenylethylamine) and their precursor amino acids (tyrosine, histidine, tryptophan and phenylalanine) were determined using an ion-pair HPLC method already optimised (Coisson *et al.* 2004). Analyses were carried out on an Shimadzu Class VP HPLC system (Shimadzu, Milano, Italy) equipped with a temperature controller (Column Oven CTO-10AS), UV-VIS detector SPD-10A, using a ODS 2 column (4.6 I.D., 250 mm length) (Waters, Milano, Italy) and two pumps. The ion-pair reagent heptanesulphonate/phosphate was prepared by dissolving sodium heptanesulphonate (8.3 mM) and KH_2PO_4 (9.0 mM) in ultrapure water and adjusting the pH to 3.5 with phosphoric acid. 20 μL/L of octylamine wase added as second ion-pair reagent (Eluant 1). The solution was freshly prepared each second day, filtered (0.45 μm, Millipore type HA) and degassed before use.

Pump A: Eluant 1.

Pump B: Methyl alcohol HPLC-grade.

Gradient: 100% pump A for 1 min; pump B from 0 to 26% in 5.25 min; pump B from 26 to 5% in 9 min; pump B from 35 to 42% in 1.5 min; pump B at 42% for 24 min; pump A at 100% for 9.40 min.

Flow rate: 1.0 mL/min. Detection: (UV) 215 nm.

The column was kept at 27 °C during the analyses. Sample volume was injected 10 μL.

Sensory evaluation

A ranking test to evaluate the differences between the cheeses produced with commercial stater and nisin-producing strains was performed (EN ISO 2006a,b). An hedonic unipolar scale with values between 0 (dislike extremely) and 10 (like extremely) was used (EN ISO 2003). Assessors could also justify their assessment with a brief description of the product. The panel was made up of ten tasters (6 men and 4 women, between 30 and 42 years old, recruited according to EN ISO regulations) (EN ISO 1991, 2008). The sensory laboratory was designed according to EN ISO 8589 with separate booths (EN ISO 2007).

Statistical analysis

All statistical analyses were performed using the free statistical software R 2.8.1 version (Development Core and Team 2008). The PCA and the subsequent hierarchical classification were performed after a normalisation step to minimise the differences between the magnitude levels of the results. All the data were then previously mean centred and scaled to unit variance, dividing the variables mean by their respective standard deviations.

Evaluation of *S. aureus* and *L. monocytogenes* growth in ripened cheese made with nisin-producing starter cultures

Microorganisms and culture conditions

L. lactis nisin Z producers (44SGLL3, 29FL1 and 41FL1) were employed as starter cultures to manufacture cheese contaminated with *S. aureus* and *L. monocytogenes*.

Prior to cheese production, all revitalised lactococci were cultured in reconstituted skim milk at 30 °C for 24 h and indicator strains *L. monocytogenes* NCTC 10527 and *S. aureus* ATCC®6538™ propagated twice in brain heart infusion broth (BHI) (Oxoid) at 37 °C for 18 h.

Manufacture of cheese inoculated with L. monocytogenes *and* S. aureus

To validate the inhibition effect of the nisin-producing *L. lactis* against *L. monocytogenes* and *S. aureus*, a semi-industrial cheesemaking was carried out.

Pasteurised high quality milk kindly provided by ABIT (TreValli Cooperlat, Turin, Italy) was transferred into a 80 L vat and heated to 37 °C. A bacterial inoculum (7–8 log cfu/mL) of *L. lactis* strains, 44SGLL3, 29FL1, 41FL1 nisin Z producers and a nonbacteriocin producer *L. lactis* used as control, was added to milk in three separated trials. Each trial was separately inoculated only with *L. lactis* strains, with *L. lactis* and *S. aureus,* with *L. lactis* and *L. monocytogenes*. Pathogen concentration was approximately 6 log cfu/mL. *L. lactis* 44SGLL3, active only against *S. aureus*, was not employed in cheese spiked with *L. monocytogenes*. Cheese was manufactured as described above (see Manufacturing of cheese par.).

Microbiological analysis

The analyses on cheese inoculated with pathogenic strains were carried out after the cut of the curd (time 0), on curd

at 24 h, curd after brine and on cheeses at 3, 7, 15, 30, 45 and 60 days of ripening.

For enumeration of total lactococci, samples of cheese (10 g) were homogenised in ¼ Ringer's solution (Oxoid). Decimal dilutions were performed and plated on M17 agar (Oxoid). To assess the presence of the inoculated *L. lactis* bacteriocin-producing strains, after the enumeration, the count plates were covered with a soft BHI agar layer (8 g/L agar) containing about 5–6 log cfu/mL of the indicator strain (*L. monocytogenes* or *S. aureus*). The plates were kept at 30 °C for 24 h in aerobic condition, and after incubation, the bacteriocin-producing colonies were confirmed by observing an halo of inhibition. The pathogenic strains were also counted, *S. aureus* by using RPF medium (EN ISO 6888-2/1999) and *L. monocytogenes* by using ALOA agar (EN ISO 11290-2/1998) after incubation at 37 °C for 24–48 h.

RESULTS AND DISCUSSION

Chemical parameters of cheese innoculated with lactococcal bacteriocin-producing starter cultures

Gross composition

Table 1 reports the gross chemical composition of cheeses produced by inoculation of five *L. lactis* (44SGLL3, 29FL1, 41FL1, 28FL1 and 32FL3) bacteriocin-producing strains after a ripening period of 60 days.

In general, chemical parameters did not highlight particular differences among the cheeses. The differences in ashes, fat and protein could be attributed to variations in the chemical composition of the raw milk used in cheesemaking.

The lowest value of pH was observed in cheese made with *L. lactis* 32FL3 for which the highest value in proteolysis was detected (ripening index: 31.8%). In cheese made with *L. lactis* 41FL1, it was instead observed the lowest rate of proteolysis (ripening index: 23.4%).

Electrophoretic protein analysis

To evaluate the impact of the strains on the caseins primary proteolysis, electrophoretic analyses of urea-soluble protein extract were performed in SDS-PAGE (Figure 1). The electrophoretic profiles obtained for the cheeses analysed were similar concerning the molecular weight of the bands revealed, but their relative intensities were different, denoting variable proteolytic activities in the cheeses. The primary proteolysis was specially in charge to α_{s1}-casein (very evident for the strains 29FL1 and 32FL3) and could be attributed to residual chymosin activity, to some lactococcal cell envelope-associated proteinases (Sousa *et al.* 2001) and also to the effects of pH on the enzyme activities as indirect effects of the strains used as starter.

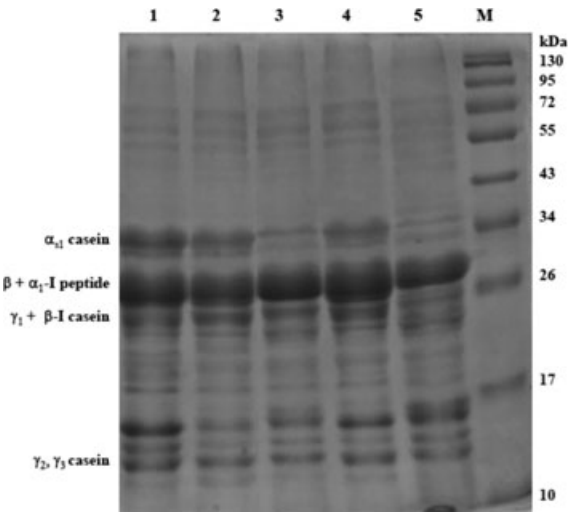

Figure 1 SDS-PAGE profiles of caseins extracted from ripened cheeses inoculated with: 1, 28FL1; 2, 44SGLL3; 3, 29FL1; 4, 41FL1; 5, 32FL3; M, Molecular marker.

Biogenic amines and related precursor amino acids

Biogenic amines (BA) production in cheese has been most extensively studied with respect to histamine and tyramine, probably the two most important BA of bacterial origin in food, due to their toxicological aspects (Stratton *et al.* 1991).

Data pertaining to free amino acid and biogenic amine concentrations detected in cheese inoculated with the five

Table 1 Gross chemical composition of cheese inoculated with *L. lactis* bacteriocin producers. Data are presented as mean ± SD for three replicate

L. lactis *strain*	*Moisture (%)*	*pH*	*Ashes (%, d.w.)*	*Fat (%, d.w)*	*Protein (%, d.w.)*	*R.I (%)*
44SGLL3	41.38 ± 0.02	5.81 ± 0.01	5.53 ± 0.01	45.06 ± 2.79	37.53 ± 1.41	30.6
29FL1	41.32 ± 0.31	5.69 ± 0.04	6.59 ± 0.05	42.94 ± 1.51	44.90 ± 0.46	30.5
41FL1	37.16 ± 0.31	5.54 ± 0.04	5.87 ± 0.02	43.90 ± 0.72	43.81 ± 0.43	23.4
28FL1	37.15 ± 0.13	5.58 ± 0.01	6.34 ± 0.01	39.14 ± 0.37	47.24 ± 1.13	31.7
32FL3	34.19 ± 0.12	5.42 ± 0.01	5.12 ± 0.03	55.74 ± 1.89	35.63 ± 0.62	31.8

R.I. – ripening index.

d.w.: dry weight.

Lact. lactis (44SGLL3, 29FL1, 41FL1, 28FL1 and 32FL3) bacteriocin-producing strains are reported in Table 2. Data are expressed as mg/Kg (mean ± SD) for three replicate analyses.

The concentration of the precursor amino acids quantified was low (<150 mg/Kg) for 4 cheeses (44SGLL3, 29FL1, 41FL1 and 32FL3), denoting a reduced secondary proteolysis. Only the cheese produced using the strain 28FL1 highlights a concentration of amino acids in the range of other commercial cheeses, such as Toma Piemontese, a PDO cheese produced with a similar technology and a ripening of 60 days (Arlorio *et al.* 2003).

Data on biogenic amines obtained showed that three of five strains applied as starters in cheesemaking did not show intense decarboxylase activity. For these strains, the BA concentration detected was quite low (<10 mg/Kg for each amine) in comparison with other cheeses after a similar (60 days) ripening time (Arlorio *et al.* 2003; Komprda *et al.* 2007). In contrast, one strain, *Lact. lactis* 28FL1, exhibited high tyramine production, reaching 136 mg/Kg for tyramine and another, *Lact. lactis* 32FL3, a medium level (>20 mg/Kg for tyramine).

Sensory evaluation

Sensory evaluations showed significant differences ($P < 0.05$) among cheeses inoculated with *L. lactis* strains and control cheeses. Cheeses obtained with 44SGLL3, 41FL1, 32FL3 strains were less appreciated than control for bitterness and sour-acid taste.

Instead, cheeses manufactured with 28FL1 and 29FL1 strains were more appreciated than control cheeses.

Statistical analysis on proteolysis data

To evaluate globally the impact of the strains on protein fraction, a statistical multivariate analysis was performed using all the data available related to proteolysis (N total and soluble, ripening index, electrophoretic patterns, free amino acids and biogenic amines, a total of 40 parameters).

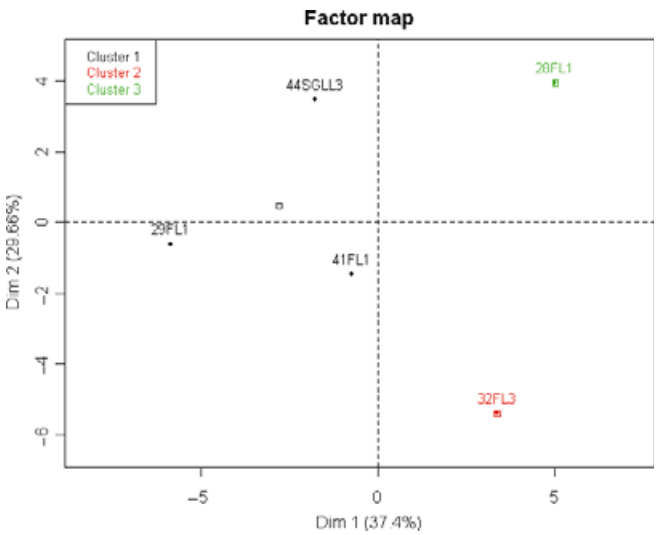

Figure 2 PCA and HCPC analysis score plot of the information obtained by proteolysis parameters (total and soluble N, ripening index, electrophoretic pattern, free amino acids and biogenic amines).

The analysis performed was a principal component analysis (PCA) followed by hierarchical classification on principal components (HCPC), a further unsupervised analysis to cluster the strains (Figure 2).

Principal component analysis yielded three principal components explaining about 85% of the total variance. The loadings values, which express the correlation degree between the new dimensions (i.e. the principal components, PCs) and the old variables, are presented in Table 3; significant correlation coefficients and the corresponding variables are highlighted using the boldface type. Among the parameters considered, only the electrophoretic protein fingerprint was useful for the samples discrimination; N total and soluble, ripening index, free amino acids and biogenic amines were not significant. Particularly, the first PC, explaining 37.4% of the total variance, negatively correlates with bands of molecular weight (MW) of 16 and 13 KDa, correspond-

Table 2 Free amino acids and biogenic amines concentration (mg/Kg) in cheese samples inoculated with five starters of *L. lactis* bacteriocin producers

L. lactis *strain*	*TYR*	*HIS*	*PHE*	*TRP*	*TYM*	*HIM*	*2-PHM*	*TRM*
44SGLL3	71.0 ± 1.5	28.9 ± 0.6	111.4 ± 0.7	10.9 ± 0.2	4.8 ± 0.7	4.3 ± 0.6	Tr.	0.9 ± 0.1
29FL1	31.6 ± 0.2	23.8 ± 0.1	60.3 ± 0.3	2.9 ± 0.1	4.7 ± 0.1	n.d.	n.d.	n.d.
41FL1	22.2 ± 0.6	11.2 ± 9.7	49.4 ± 1.3	2.4 ± 0.2	7.0 ± 0.4	5.0 ± 0.8	n.d.	Tr.
28FL1	27.7 ± 1.2	21.2 ± 0.6	409.7 ± 7.4	58.3 ± 0.8	136.8 ± 2.2	34.2 ± 3.8	20.6 ± 5.2	n.d.
32FL3	24.5 ± 0.4	21.1 ± 0.2	88.8 ± 2.1	4.6 ± 0.0	20.2 ± 1.3	Tr.	Tr.	1.6 ± 0.0

TYR, tyrosine; HIS, histidine; PHE, phenylalanine; TRP, tryptophan; TYM, tyramine; HIM, histamine; 2-PHM, 2-phenylethylamine; TRM, tryptamine.

Tr., <LOQ; N.D, <LOD.

n.d.: not detected.

Table 3 Principal component loadings (PC1, PC2 and PC3) related to Figure 2

	PC1	*PC2*	*PC3*
N soluble	0.5687	−0.0998	0.8079
N total	−0.4253	−0.0806	−0.3861
Ripening Index	0.5513	−0.0919	0.8190
Tyr	−0.3047	0.5592	0.1125
His	−0.2018	0.4069	0.7265
Phe	0.6783	0.6131	0.2409
Trp	0.6509	0.6449	0.2007
Tyramine	0.7182	0.4925	0.2064
Histamine	0.6859	0.6010	0.0641
2-Phenylethylamine	0.7365	0.6033	0.2092
Tryptamine	0.4328	−0.8422	0.1665
MW75	−0.8253	−0.5258	−0.1439
MW72	0.3053	−0.8022	−0.3218
MW64	0.1980	**−0.9543**	0.1564
MW56	0.6730	−0.5710	0.3112
MW50	0.8212	−0.2648	0.0530
MW48	−0.7338	0.4054	0.3007
MW47	0.0499	−0.6711	0.1967
MW43	−0.6077	0.5805	0.5389
MW41	0.5366	−0.7232	0.3562
MW38	0.5705	−0.2062	−0.7333
MW35	0.4217	**0.8807**	−0.2025
MW32	0.7944	0.2728	−0.3368
MW26.5	−0.6867	−0.4034	0.3521
MW24.5	0.4459	0.0744	−0.7636
MW24	−0.8228	0.1965	−0.2858
MW23	0.8059	−0.4178	0.4190
MW22	−0.7726	−0.4632	0.4322
MW21	−0.8080	−0.1198	0.5009
MW20	0.3689	**0.8977**	−0.0161
MW19	−0.8525	0.2880	0.2920
MW18.5	−0.2616	0.0807	0.7287
MW17.5	0.1014	0.8717	−0.0390
MW16	**−0.8821**	0.0663	0.3095
MW15	0.6823	−0.6386	0.0811
MW14	0.1763	−0.2290	**0.9539**
MW13	**−0.9399**	−0.1227	−0.3124
MW12	−0.8222	0.5194	−0.2280
MW10	−0.0500	0.6937	0.5324
MW9	0.3448	**0.8959**	0.0003
Variance (%)	37.40	29.66	17.95
Cumulative variance (%)	37.40	67.06	85.01

PC, principal component; Tyr, tyrosine; His, histidine; Phe, phenylalanine; Trp, tryptophan; MW, molecular weight.

ing respectively to the α_{s1}-casein f80-199 fragment and $\gamma_2+\gamma_3$ caseins. PC2 (27.7% of the total variance) was principally characterised by relative intensity of MW 64 band (significant negative correlation), while was positively associated to bands of MW 35, 20 and 9 (respectively intact α_{s1}-casein, an unknown peptide and α_{s1}-casein f121-199 fragment), confirming that the prevalence of proteolysis in charge to α_{s1}-casein. The band attributions were performed following literature data (Mooney *et al.* 1998; Gaiaschi *et al.* 2000).

Considering the results obtained from the HCPC analysis (Figure 2), the strains were classified in three groups: a group including 29FL1, 41FL1 and 44SGL3, and two separated strains. The strain 28FL1 was characterised by higher levels of free phenylalanine and biogenic amines; the strain 32FLC1 had the higher ripening index and the minimal levels of intact α_{s1}-casein.

Evaluation of anti-staphylococcal and anti-listerial activity of nisin-producing strains in cheese

LAB counts in cheese inoculated with L. monocytogenes and S. aureus.

To evaluate the antimicrobial activity of bacteriocin-producing strains against *S. aureus* and *L. monocytogenes*, three *L. lactis* nisin Z-producing strains (44SGLL3, 41FL1 and 29FL1), previously selected for their performance in the primary experimental cheese and minimal biogenic amines production, were employed as starter cultures in new experimental cheese productions. A commercial cheese starter *L. lactis* Lyoto MO 540 nonbacteriocin producer (Sacco) was also employed as control.

Figure 3 shows the viable counts of lactococcal strains added as starters during the ripening of cheese inoculated with *S. aureus* (panel a) and *L. monocytogenes* (panel b).

In the cheese made with *S. aureus,* an increase of ~2–3 log cfu/g in lactococcal population during the first 3 days from manufacture was observed both in control cheese inoculated with the nonbacteriocin producer and in cheese inoculated with the two nisin Z producers (41FL1 and 29FL1). With respect to the initial levels of inoculum (7–8 log cfu/g), *L. lactis* strains 44SGLL3 did not show a particular increase in counts from time 0 to day 3. From then, to the end of ripening period (60 days), a reduction of ~2 log cfu/g was observed for nonbacteriocin producer and 41FL1 and 29FL1 strains, while 44SGLL3 showed the highest growth after 7 days and rapidly decrease by ~3 log cfu/g to the end of ripening time.

In cheese spiked with *L. monocytogenes* (Figure 3b), *L. lactis* commercial strain and *L. lactis* nisin-producing strains, 29FL1 and 41FL1, showed good viability during all the ripening period (60 days). In particular, during the first 30 days of ripening, the viable count increased by 1–2 log cfu/g for *L. lactis* 29FL1 and 2–3 log cfu/g for *L. lactis* 41FL1 and the nonbacteriocin producer. From day 30 to day 60, a slight decrease of ~1 log cfu/g was detected for two nisin producers while the lactococcal count in control cheese remained almost unchanged.

In both cheese manufactures, *L. lactis* growth mainly occurred during the first 24 h maybe due to the physical retention of the cells in the coagulum which led their

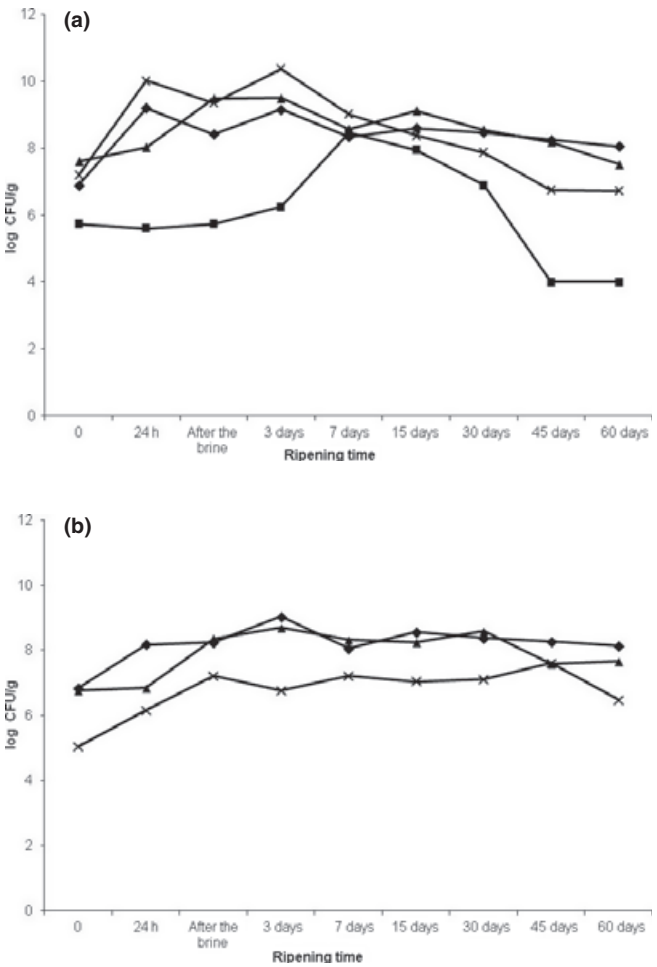

Figure 3 Growth of *L. lactis* commercial culture (♦); 44SGLL3 (■); 41FL1 (**x**); 29FL1 (▲) (log cfu/g) used as starter in ripened cheese contaminated with *S. aureus* (a) and *L. monocytogenes* (b).

multiplication during coagulation and whey drainage. In particular, in cheeses spiked with pathogens, both *L. lactis* 41FL1 and 29FL1 as well as commercial strain showed the highest increases during first 3 days maintaining unchanged their counts up to the end of ripening. Among two nisin strains, *L. lactis* 29FL1 showed similar trend compared to commercial starter, in particular in cheese spiked with *L. monocytogenes*. *L. lactis* 41FL1 count was lower in cheese spiked with *L. monocytogenes* maybe due to its initial inoculation level.

On the basis of the results obtained we can conclude that no great differences were shown in the counts of *L. lactis* nisin and nonbacteriocin-producing strains in presence of pathogens less than for *L. lactis* 44SGLL3 which showed a weak ability to grow when inoculated with *S. aureus*. In particular, *L. lactis* 44SGLL3 showed its highest increase from day 3 to day 7 leading to a rapid decrease from day 7 to the end of the ripening (Figure 3b).

The pH values of the cheese after manufacture and during the ripening gradually decreased from 6.9 (time 0) to values of 4.8–5 (24 h) to remain unchanged up to the end of ripening (60 days) (data not shown). No differences in pH value were detected both in cheeses made with the nonbacteriocin producer and in cheese made with nisin positive cultures.

Therefore, considering the evolution of the growth showed by the commercial starter culture and the pH value reached in cheese during 60 days of ripening, these three *L. lactis* nisin producers could be take into account for application as possible acidifying starter or co-starter cultures in cheese production.

Survival of S. aureus in cheese

Cheese is one of the principal ecosystem where LAB are the dominant microbiota and where *S. aureus* is prone to major public health problems. Recent studies reported the ability of *S. aureus* to grow both during acidification of milk (Charlier *et al.* 2008) and during the production of different type of cheeses productions (Meyrand *et al.* 1998; Lamprell 2003).

The survival of *S. aureus* in cheese manufactured with the *L. lactis* nonbacteriocin producer and nisin producers is shown in Table 4. The pathogen was able to grow in the presence of the nonbacteriocin-producing culture, increasing by ~1–1,50 log cfu/g within 30 days. In particular *S. aureus* showed the highest growth after the first 24 h, in particular in control cheese and in cheese inoculated with *L. lactis* 41FL1, maybe due to the concentration of the pathogen inside the curd after the draining (Charlier *et al.* 2008) to achieve little decrease by 1 log cfu/g at day 3 and day 15. These apparent decreases of *S. aureus* growth could be attributed to the lack of cells entrapped in the cheese.

At the end of ripening time (60 days), the viable counts of *S. aureus* slightly decreased by ~2 log cfu/g. Similar

Table 4 Survival of *S. aureus* (log cfu/g) in cheeses manufactured with commercial cultures (Control) and *L. lactis* nisin producers

L. lactis *strain*	*0*	*24 h*	*After the brine*	*3d*	*7d*	*15d*	*30d*	*45d*	*60d*
Control	6,57	7,75	7,48	6,85	7,23	6,23	7,67	5,08	5,57
44SGL L3	7,11	7,26	7,20	7,26	7,30	8,48	9,04	8,40	7,90
29FL1	7,95	7,38	7,81	7,48	7,54	7,91	7,91	5,45	6,23
41FL1	7,54	8,28	8,11	5,81	6,36	5,66	5,93	4,36	2.39

Table 5 Survival of *L. monocytogenes* (log cfu/g) in cheeses manufactured with commercial *L. lactis* nonbacteriocin producer (Control) and *L. lactis* nisin producers

L. lactis *strain*	*0*	*24 h*	*After the brine*	*3d*	*7d*	*15d*	*30d*	*45d*	*60d*
Control	7,04	7,88	7,41	8,08	5,00	5,89	5,92	6,90	4,85
29FL1	7,38	8,20	8,49	7,91	8,43	7,58	7,41	7,08	7,32
41FL1	7,11	8,20	8,11	8,66	8,68	8,41	8,49	8,94	7,72

results were also presented by Arqués *et al.* (2005), Erkmen (1995) and Meyrand *et al.* (1998) in three different types of ripened cheese, raw milk cheese, Feta and Camembert-type goat's cheese, respectively.

Among the three *L. lactis* nisin-producing strains, surprisingly, strain 41FL1 displayed the best antimicrobial activity and ability to reduce *S. aureus.* In particular, this nisin-producing strain was able to decrease *S. aureus* counts by 1.73 log cfu/g within the first 3 days. The values in *S. aureus* reduction observed in this study were higher than those reported by Rodrőguez *et al.* (2001, 2005). In that study, cheese manufactured with nisin-producing cultures reduced the pathogen counts by 1.02 log cfu/g during 30 days of cheese ripening. From days 3 to 30, *S. aureus* counts remained constant; however, between days 30 and 60, the highest inactivation and reduction in the growth of 3.54 log cfu/g was observed.

S. aureus counts in cheese inoculated with *L. lactis* strains 29FL1, and 44SGLL3 strains did not display important decreases for the entire ripening period. Among these, lowest value of *S. aureus* was only achieved in *L. lactis* strain 29FL1 after 45 day of ripening.

Survival of L. monocytogenes in cheese

Many studies reported the ability of *L. monocytogenes* to grow in different semiripened cheeses varieties such as Cheddar, Camembert and Manchego (Gahan *et al.* 1996; Nuñez *et al.* 1997; Linton *et al.* 2008), and several researches reported the efficacy with which actively growing nisin-producing starter cultures in cheeses inhibit *L. monocytogenes* (Rodrőguez *et al.* 2001, 2005; Benech *et al.* 2002).

The results obtained from this study (Table 5) showed that *L. monocytogenes* was not influenced by *L. lactis* employed during all ripening period. In cheese made with nonbacteriocin-producing culture, *L. monocytogenes* increased ~1 log cfu/g from curd (time 0) to day 3. A decrease of pathogen numbers of ~2 log cfu/g was instead observed from day 3 to the end of ripening period in agreement with previous studies in semihard cheese manufactured with commercial lactic culture (Rodrőguez *et al.* 2001, 2005). The application of nisin-producing *L. lactis* strains (29FL1 and 41FL1) had less effect in reducing *L. monocytogenes* counts than cheese manufactured with nonbacteriocin-producing culture. In particular, in cheese made with nisin-producing strains, *L. monocytogenes* counts increased about ~1–2 log cfu/g from curd (time 0) to the end of ripening period.

The data obtained in this study showed that the nisin-producing starter cultures selected in this study cannot be used to control *L. monocytogenes* in ripened cheese. Perhaps, the high inoculum of *L. monocytogenes* added to milk in present work (6 log cfu/mL) may be the reason why counts of pathogen in cheeses did not decrease throughout the ripening period.

CONCLUSIONS

Cheese made by using nisin-producing *L. lactis* 41FL1 as starter achieved reduction up to 3.54 log cfu/g for *S. aureus* in comparison with control cheese inoculated with a commercial starter. No inhibitory effect was observed in cheese spiked with *L. monocytogenes*, presumably for the higher level of pathogen inoculated.

From the results obtained, it can be concluded that in addition to a classical technological starter cultures usually employed in cheesemaking, *L. lactis* 41FL1 nisin Z producer could be considered a promising candidate as potential bioprotective starter or costarter culture to control and reduce *S. aureus* counts. New experimental approaches to evaluate the effective and potential use of this strain are required. Alternatively, the strain could be used for the production of crude nisin preparation for the direct application in foods with the aim in extending the biopreservation and shelf life of food as nisin is authorised as food additive by Directive 95/2/EC (EC 1995).

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