Anti-*Listeria* activity of lactic acid bacteria in two traditional Sicilian cheeses

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Abstract

Listeria monocytogenes is a pathogen frequently found in dairy products, and its growth is difficult to control. Bacteriocin-like inhibitory substances (BLIS), produced by lactic acid bacteria (LAB), having proven *in vitro* anti-*Listeria* activity, could provide an innovative approach to control *L. monocytogenes*; however, this application needs to be evaluated *in vivo*. In this study, twenty LAB strains isolated from different Sicilian dairy environments were tested for control of growth of *L. monocytogenes* in three different experimental trials. First, raw and UHT milk were inoculated with LAB strains alone, and LAB strains mixed with *L. monocytogenes*. Second, mini-cheeses containing LAB and/or *L. monocytogenes* were produced. Third, two traditional Sicilian cheeses inoculated with a multi-strain LAB mixture combined with *L. monocytogenes* were produced. The addition of BLIS produced by LAB to milk and in mini-cheese production was unable to inhibit the growth of *L. monocytogenes*. However, an anti-*Listeria* effect was observed in the *Pecorino Siciliano* cheeses, where, after 15 days of ripening, the cheeses with added LAB had fewer *L. monocytogenes* compared to the control cheeses with no added LAB, while in the *Vastedda della valle del Belice* cheeses, the multi-strain LAB mixture completely prevented the growth of *L. monocytogenes*.

Introduction

Foodborne diseases are among the most serious and costly public health concerns worldwide (WHO/FNU/FOS, 1995). *Listeria monocytogenes* is the causative agent of listeriosis, a foodborne disease that causes life-threatening infections in the elderly, pregnant women, newborn babies, and immunocompromised people. This pathogen is present in a range of foods, including dairy products (Fretz *et al.*, 2010; Koch *et al.*, 2010). The ability of *L. monocytogenes* to survive exposure to diverse adverse conditions, including acidic pH, low temperatures, and high sodium chloride concentrations, make this organism difficult to control in food (Farber and Peterkin, 1991). Bacteriocins produced by lactic acid bacteria (LAB) are able to prevent the growth of undesired bacteria, included *L. monocytogenes* (Scatassa *et al.*, 2015); hence, they have been extensively tested for food applications (Gálvez *et al.*, 2008). In this context, the use of bacteriocinogenic LAB strains as starter or co-cultures is a promising strategy to improve cheese hygiene conditions (Settanni and Moschetti, 2014). This interest is based on several properties that make them good candidates for food preservation provided their acceptance by food regulations. LAB bacteriocins are defined Generally Recognized As Safe (GRAS) substances, since they are produced by food-grade microorganisms that have been consumed for centuries (Cotter *et al.*, 2005). Furthermore, they show no activity or toxicity on eukaryotic cells and are inactivated by digestive proteases, ensuring little influence on gut microbiota (Bernbom *et al.*, 2006). Some studies have reported the use of bacteriocin produced by LAB for biopreservation of cheeses (Hernández *et al.*, 2005; O'Sullivan *et al.*, 2006). The products of LAB catabolism are not only useful for cheese preservation, but also contribute to its flavour, aroma, and texture, thereby helping to determine unique product characteristics (Monfredini *et al.*, 2012; Guarcello *et al.*, 2016).

This work was performed to characterize the bacteriocinogenic activities of LAB strains isolated from different Sicilian dairy environments, and to evaluate their anti-*Listeria* potential during the production of two traditional Sicilian cheeses: *Pecorino Siciliano* (PS) and *Vastedda della valle del Belice* (VB).

Materials and Methods

Antimicrobial activity assay

Twenty LAB strains (Table 1) were selected from a total of 37 strains isolated from different Sicilian dairy environments, based on their production of antimicrobial substances (Macaluso *et al.*, 2016), and thus their technological relevance in cheese making. The 20 strains were representative of different LAB species and isolation sources. The antimicrobial activity of these 20 strains was evaluated against eight strains of *L. monocytogenes* of food origin (LM 2011/2/3 - LM 2013/4/3 - LM 2013/6/4 - LM 2014/7/4 from smoked salmon; LM 2012/8/123 - LM 2012/3 /45 - LM 2014/5/43 from salami and LM 2013/1/3 from cheese) obtained from the culture collection of Institute for Experimental Veterinary Medicine of Sicily, Palermo, Italy. The antimicrobial activity of LAB strains was initially detected by the spot-on-the lawn method, and the strains showing positive results were subsequently tested by the well diffusion assay (WDA) (Schillinger and Lücke, 1989; Corsetti *et al.,* 2008), which is based on the diffusion of the antimicrobial substances in the culture medium, and indicates inhibitory activity against an indicator strain by the detection of an inhibition halo around the colony. The sensitivity of the active supernatants to proteolytic enzymes was tested by digesting them with proteinase K (12.5 $U.mg^{-1}$), protease B (45 $U.mg^{-1}$) and trypsin (10.6 $U.mg^{-1}$) at a final concentration of 1 $mg.mL^{-1}$ in phosphate buffer (pH 7.0). After

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incubating for 2 h at 37°C, the remaining activity was measured by a second WDA (Settanni *et al.*, 2005).

In vivo application

In order to evaluate the *in vivo* inhibitory activity of the LAB strains in cheese production, these strains were tested in single and multi-strain combinations with 'mix 1', containing *Lactococcus lactis* (992), *Lactobacillus rhamnosus* (623) and *Enterococcus faecium* (971), which were chosen for their potential to preserve the sensory profile and structural characteristics typical to each cheese (Mancuso *et al.*, 2016).

The anti-*Listeria* activity of the LAB strains was tested in three different experimental trials. In the first trial, raw and UHT milk was inoculated with LAB in single, and with *mix 1* in combination with *L. monocytogenes*. The milk was incubated at 37°C for 10-13 h until the value reached the range of 5.5. In the second trial, 10 mini-cheeses were produced with 2 L of pasteurised milk inoculated individually with combinations of LAB/*L. monocytogenes*. In the final trial, two traditional Sicilian cheeses (PS and VB) were produced with 10 L each of ewe's raw milk inoculated with a combination of *mix 1* and *L. monocytogenes* at experimental dairy level in standard conditions in order to keep all process variables constant. A control cheese of each type, inoculated with *L. monocytogenes* ATCC 7644 at about 10^4 CFU/mL, was included for comparison (denoted as PS1 and VB1).

All LAB were initially cultured in de Man-Rogosa-Sharpe (MRS) and M17 broth for 24 h at 30°C, while *L. monocytogenes* was refreshed in Brain Heart Infusion (BHI) broth at 37°C for 18-24 h, and thereafter streaked onto selective Agar *Listeria* acc. Ottaviani & Agosti (ALOA) plates to assess purity. After propagation in broth, both LAB and *L. monocytogenes* were centrifuged at 10,000 × g for 5 min, washed in Ringer's solution and re-suspended in the same solution to achieve an optical density (OD) of ca. 1.00, measured by 6400 Spectrophotometer (Jenway Ltd., Felsted Dunmow, UK) at 600 nm wavelength, which approximately corresponds to a concentration of 10^9 CFU/mL, to standardize bacterial inocula (Settanni *et al*., 2014). Cell suspensions were inoculated into milk during cheeses production (trial 2 and 3) at a final concentration of approximately 10^7 CFU/mL of LAB as a starter cultures, while *L. monocytogenes* ATCC 7644 was inoculated at about 10^4 CFU/mL to simulate massive contamination. In trial 1, raw and UHT milk were inoculated at about 10^2-10^4 CFU/mL of *L. monocytogenes* and approximately 10^7 CFU/mL of LAB.

Cheese production

The mini-cheeses were produced using 2 L of pasteurized milk, without cooking the curd under hot whey and dry salting in order to minimize the inhibition of *L. monocytogenes* due to biocompetition, heat treatment, and acid fermentation.

Cheese making trials were carried out in a dairy pilot plant (Institute for Experimental Veterinary Medicine of Sicily "Adelmo Mirri", Palermo, Italy) using the POLYFOOD mod. SI-050 (INVENTAGRI™, Modena, Italy). PS and VB cheeses were produced following traditional protocols (Figure 1), except for the use of wooden equipment. In both cheeses, raw ewe's milk was inoculated with a combination of *mix 1* and *L. monocytogenes*. The cheeses were produced in duplicate at one-month intervals in the spring and summer season (first week of June-first week of July) 2016.

Microbial analysis

Microbiological analysis was carried out to evaluate the concentrations of the combined LAB/*L. monocytogenes* culture in experimental cheese production (trial 2 and 3), and the concentration of *L. monocytogenes* in trial 1.

In trial 3, for the PS, curds were collected soon after transfer into perforated containers for analysis, while the cheeses were analysed after one day of drying following salting in saturated brine (t_0), and after 15 days of ripening (t_{15}) at 13°C.

In the case of the VB, curds were collected soon after transfer into perforated containers and before stretching (acidified curds). The final cheeses were analysed after one day of drying following salting in saturated brine (t_0), and after 15 days of refrigerated storage under vacuum (t_{15}). Cell suspensions of solid samples (25 g each of curd and cheese) were first homogenized in a stomacher (400 Circulator Bags; Seward, AK, USA) for 10 min at 260 rpm, and then serially diluted. Presumptive rod LAB were grown on MRS agar, acidified to pH 5.4 with lactic acid (5 mol L^{-1}), incubated anaerobically for 72 h at 30 and 44°C, respectively; while presumptive coccus LAB were grown on M17 agar, incubated aerobically for 48 h at 30 and 44°C, respectively. Detection and enumeration of *L. monocytogenes* was carried out on milk, 25 g of curd and cheese sample, on ALOA, following the UNI EN ISO 11290-1:2005 and UNI EN ISO 11290-2:2005, respectively (ISO, 2005a, 2005b).

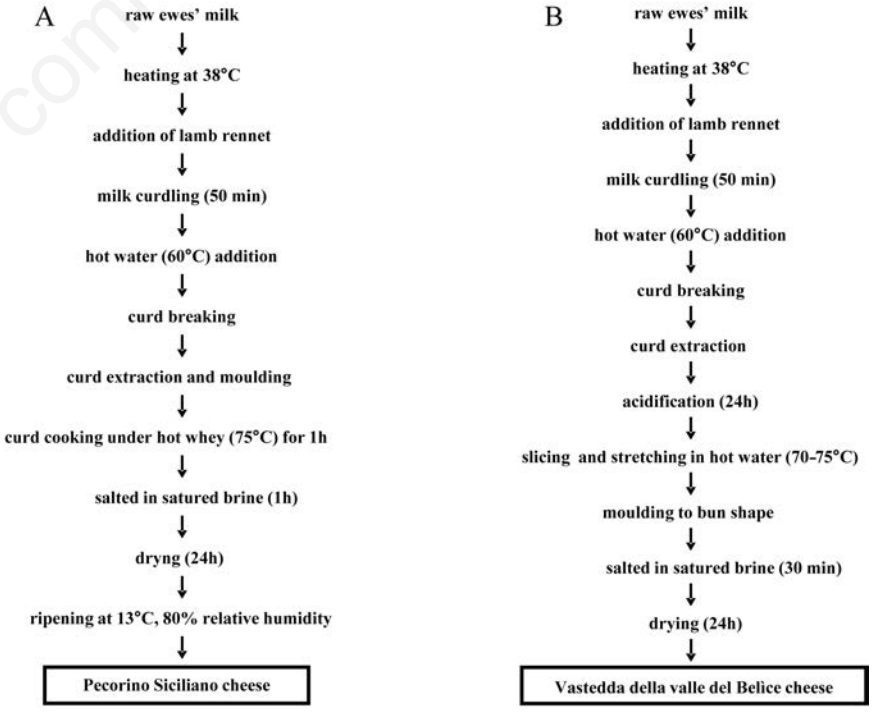

Figure 1. Flow diagram of *Pecorino Siciliano* cheese production (A) and protected designation of origin *Vastedda della valle del Belice* cheese production (B).

Results and Discussion

Antimicrobial activity assay

All 20 LAB strains exhibited similar antibacterial activity, inhibiting *L. monocytogenes* ATCC 7644 (Macaluso *et al.*, 2016) and eight *L. monocytogenes* of food origin, showing an inhibition halo between 5 and 7 mm. Treating the culture supernatants with proteolytic enzymes eliminated all inhibitory activity, confirming that the toxins were proteinaceous in nature. Because we did not characterize the amino acid and nucleotide sequences for these substances in this study, we will be referring to them generally as BLIS (Corsetti *et al.*, 2008). The capacity of our LAB strains to produce BLIS represents a positive attribute that ensures a competitive advantage in the control of undesirable microbial populations.

In vivo application and microbial analysis

Considering that *L. monocytogenes* can survive in cheeses during manufacture, ripening, and storage under refrigeration (Morgan *et al.*, 2001), the control of growth is a challenge for producers and consumers.

In accordance with previously published literature (Schillinger *et al.*, 1996), our results did not show a reduction in the growth of the pathogen in raw and UHT milk or in mini-cheeses, which demonstrates that the efficacy of bacteriocins in culture media is not always reproducible in food systems (*in vivo*).

To this purpose, the anti-*Listeria* activity of the LAB strains was tested in multi-stain combination for cheese production at pilot plant level as reported Callon *et al.* (2011). Changes in the levels of concentration of *L. monocytogenes*, mesophilic and thermophilic rod and coccus LAB during the two traditional production are reported in Table 2.

After inoculation, the raw ewe's milk had 7.32-7.48 log CFU/mL of LAB and 4.88 log CFU/mL of *L. monocytogenes*, whose concentration was affected by the addition of LAB and the pathogen. All cheeses had increased LAB concentrations of at least 1 log. Furthermore, all cheeses were dominated by growth of LAB and the differences between control and experimental cheeses were less evident in this population. However, the amount of *L. monocytogenes* in both experimental cheeses was reduced by approximately 3 log for PS, while it remained undetectable for VB. The addition of LAB strains caused a rapid decrease of the detectable pathogen in acidified curd, with 2.54 log CFU/g measured in VB, while 4.30 log CFU/g was detected in the control VB1.

A significant reduction in the CFU/g of *L. monocytogenes* caused by BLIS produced by LAB has been reported for camembert cheese (Maisnier-Patin *et al.*, 1992) and cheddar cheese (Buyong *et al.*, 1998), whereas no previous study has evaluated the inhibitory effect of selected

Table 1. Strains used in this study.

Strains	Species	Origin
150	*Lactobacillus casei*	*Pecorino* cheese
153	*Leuconostoc mesenteroides*	Raw ewe's milk
623	*Lactobacillus rhamnosus*	*Caciocavallo Palermitano* cheese
971	*Enterococcus faecium*	Wooden vat surfaces (cow's cheese)
979	*Enterococcus faecium*	Wooden vat surfaces (cow's cheese)
981	*Lactococcus lactis*	Wooden vat surfaces (cow's cheese)
983	*Streptococcus thermophilus*	Wooden vat surfaces (cow's cheese)
986	*Enterococcus faecium*	Wooden vat surfaces (ewe's cheese)
990	*Enterococcus faecium*	Wooden vat surfaces (ewe's cheese)
991	*Enterococcus faecium*	Wooden vat surfaces (ewe's cheese)
992	*Lactococcus lactis*	Wooden vat surfaces (ewe's cheese)
993	*Leuconostoc mesenteroides*	Wooden vat surfaces (cow's cheese)
995	*Leuconostoc mesenteroides*	Wooden vat surfaces (cow's cheese)
996	*Leuconostoc mesenteroides*	Wooden vat surfaces (cow's cheese)
997	*Leuconostoc pseudomesenteroides*	Wooden vat surfaces (cow's cheese)
999	*Lactobacillus delbrueckii*	Wooden vat surfaces (cow's cheese)
1000	*Streptococcus thermophilus*	Wooden vat surfaces (cow's cheese)
1001	*Enterococcus faecalis*	Wooden vat surfaces (cow's cheese)
1008	*Enterococcus faecium*	Wooden vat surfaces (cow's cheese)
1011	*Streptococcus thermophilus*	Wooden vat surfaces (cow's cheese)

Table 2. Microbial loads of samples collected through experimental cheese production.

Sample	Media				
	M17 30°C	M17 44°C	MRS 30°C	MRS 44°C	ALOA 37°C
PS					
Milk	7.36	7.38	7.48	7.32	4.88
Curd	ne	ne	ne	ne	4.00
PS T_0	8.60	8.86	8.64	8.62	1.00
PS T_{15}	9.04	8.64	9.30	9.20	1.00
PS1					
Milk	7.04	7.18	6.96	4.90	4.88
Curd	ne	ne	ne	ne	4.30
PS T_0	8.41	8.00	8.20	5.81	2.32
PS T_{15}	8.88	8.52	8.94	6.38	1.48
VB					
Milk	7.36	7.38	7.48	7.32	4.88
Curd	ne	ne	ne	ne	4.00
Curd before stretching	ne	ne	ne	ne	2.54
VB T_0	8.64	8.58	8.38	8.38	nd
VB T_{15}	9.04	8.96	8.75	8.89	nd
VB1					
Milk	7.04	7.18	6.96	4.90	4.88
Curd	ne	ne	ne	ne	4.30
Curd before stretching	ne	ne	ne	ne	4.30
VB T_0	8.59	8.54	7.30	7.34	1.00
VB T_{15}	8.85	8.49	8.66	7.85	1.70

M17, agar for coccus lactic acid bacteria (LAB); MRS, de Man-Rogosa-Sharpe agar for rod LAB; ALOA, Agar *Listeria* acc. to Ottaviani & Agosti for the detection of *L. monocytogenes*; PS, *Pecorino Siciliano* experimental production; PS1, *Pecorino Siciliano* control production; VB, *Vastedda della valle del Belice* experimental production; VB1, *Vastedda della valle del Belice* control production; ne, not evaluated; nd, not detected.

autochthonous LAB on the growth of this pathogen in traditional Sicilian cheeses.

In control cheeses at t_{15}, *L. monocytogenes* was detected at 1.6 log CFU/g and 1.7 log CFU/g, respectively, for the controls PS1 and VB1. This indicates that the stressful conditions experienced during curd cooking under hot whey for the PS, and curd acidification and stretching in hot water for VB, are not able to completely delete kill this pathogen. Furthermore, during the 15 days of refrigerated storage, *L. monocytogenes* in the VB1 cheese grew from 1.0 log CFU/g to 1.7 log CFU/g, which highlights the ability of this pathogen to grow at low temperatures (Walker *et al.*, 1990).

Conclusions

Sicilian dairy environments provide an appropriate ecological habitat for wild bacteriocin-producing LAB. The addition of BLIS produced by LAB to milk and mini-cheeses was not able to inhibit the growth of the pathogen *L. monocytogenes*. However, the addition of mixed LAB producers of BLIS (992 *L. lactis*, 623 *L. rhamnosus* and 971 *E. faecium*) to traditional PS and VB showed a broad spectrum of activity against *L. monocytogenes.* In particular, PS with added LAB showed a slight reduction in the number of CFU of the pathogen as compared to the control cheese, while in VB, the addition of LAB completely eliminated *L. monocytogenes*. This could be useful to prevent possible contamination of milk, cheese, or curd, and when producing cheese from raw milk. These results suggest that, with respect to PS and VB cheese production, the addition of autochthonous selected LAB is a good strategy to improve microbial quality, produce safer cheeses, and reduce risk to the consumers.

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