In vitro evaluation of bacteriocin-like inhibitory substances produced by lactic acid bacteria isolated during traditional Sicilian cheese making

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Abstract

Bacteriocins are antimicrobial proteins produced by bacteria that inhibit the growth of other bacteria with a bactericidal or bacteriostatic mode of action. Many lactic acid bacteria (LAB) produce a high diversity of different bacteriocins. Bacteriocinogenic LAB are generally recognised as safe (GRAS) and useful to control the frequent development of pathogens and spoilage microorganisms. For this reason they are commonly used as starter cultures in food fermentations. In this study, the authors describe the results of a screening on 699 LAB isolated from wooden vat surfaces, raw milk and traditional Sicilian cheeses, for the production of bacteriocin-like inhibitory substances, by comparing two alternative methods. The antagonistic activity of LAB and its proteinaceous nature were evaluated using the *spot-on-the-lawn* and the well-diffusion assay (WDA) and the sensitivity to proteolytic (proteinase K, protease B and trypsin), amylolytic (α-amylase) and lipolytic (lipase) enzymes. The indicator strains used were: *Listeria monocytogenes, Staphylococcus aureus, Escherichia coli, Salmonella enteritidis.* A total of 223 strains (belonging to the species *Enterococcus* spp., *Lactobacillus* spp., *Pediococcus* spp., *Streptococcus* spp., *Leuconostoc* spp. and *Lactococcus lactis*) were found to inhibit the growth of *Listeria monocytogenes* by using the *spot-on-the-lawn* method; only 37 of these were confirmed by using the WDA. The direct addition of bacteriocin-producing cultures into dairy products can be a more practical and economic option for the improvement of the safety and quality of the final product.

Introduction

Lactic acid bacteria (LAB) have been used in the fermentation of a variety of dairy products. The cheese microbiota, whose community structure evolves through a succession of different microbial groups, plays a central role in cheese making.

Traditional Sicilian cheeses are often manufactured with raw milk using wooden equipment without the addition of starter cultures. The source of desirable LAB is the natural milk microbiota and the microbial biofilms of the wooden equipment used during the cheese production (Lortal *et al.,* 2009).

Several studies, however, showed that the wooden vat surface host LAB (Lortal *et al.,* 2009; Didienne *et al.,* 2012), and some strains have persisted over time (Settanni *et al.,* 2012). High levels of LAB inhibiting sensitive strains by bacteriocin-like inhibitory substances (BLIS) were isolated on the wooden vat surfaces (Scatassa *et al*., 2015) confirming the safety of the wooden equipment as part of good manufacturing practices. Most recent studies focused on the ability of the cheese microbiota to control and/or prevent the growth of pathogen microorganisms in cheese (Guillier *et al*., 2008) as well as in the cheese-making environment (Maoz *et al*., 2003).

The preservative ability of LAB in foods is attributed to the production of antimicrobial metabolites including organic acids and bacteriocins. Since food safety has become an increasingly important international concern, the application of antimicrobial peptides from LAB that target food pathogens without toxic or other adverse effects has received great attention. The incorporation of bacteriocins as a biopreservative ingredient into model food systems has indeed been studied extensively and has been shown to be effective in the control of pathogenic and spoilage microorganisms.

Regarding the application of bacteriocin producing starters in food fermentation, the major problem is related to the *in situ* antimicrobial efficacy which can be negatively influenced by various factors, such as binding of the bacteriocins to food components, inactivation by proteases, changes in solubility and charge, changes in the cell envelope of the target bacteria (Aesen *et al.,* 2003; Gänzle *et al.,* 1999).

The study focused on BLIS production by LAB, both from wooden equipment and traditional Sicilian cheeses, by: i) evaluating the presence of BLIS-producing LAB isolated from wooden vat surfaces, raw milk and traditional Sicilian cheeses by comparing results from the *spot-on-the-lawn* and the well-diffusion assay (WDA); ii) investigating the antibacterial activities for their general behaviour with proteolytic, amylolytic and lipolytic enzymes.

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Materials and Methods

The 699 strains used in this study were isolated from 20 wooden vats used in 12 dairy factories located in western Sicily as previously reported (Scatassa *et al*., 2015), and during the production and ripening of traditional ovine (*Pecorino Siciliano* and *Vastedda della valle del Belice*) and bovine (*Caciocavallo Palermitano*) Sicilian cheeses.

Lactic acid bacteria were microbiologically investigated by using the ISTISAN 08/36 reports (ISTISAN, 2008): mesophilic and thermophilic cocci were plated on M17 Agar incubated at 30°C for 72 h and 44°C for 48 h, respectively; mesophilic lactobacilli were isolated by using MRS agar (pH 5.4) incubated anaerobically at 37°C for 72 h.

Genotypic identification of the LAB strains was carried out by *16S rRNA* gene sequencing as reported by Weisburg *et al.* (1991). The resulting DNA was sequenced by using the same primers employed for the polymerase chain reaction amplifications and an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). The sequences were compared with those available in the GenBank/EMBL/DDBJ (http://www.ncbi.nlm.nih.gov) (Altschul *et al.,* 1997).

For the *in vitro* antimicrobial activity assay, LAB isolates were tested using the *spot-on-the-lawn* method (ISTISAN 12/54 reports; ISTISAN, 2012) and the WDA (Schillinger and Lücke 1989, Corsetti *et al*., 2004), which are based on the diffusion of the antimicrobial substances in the culture media and show the inhibitory activity against indicator strains by the detection of an inhibition halo around the strain tested.

The 699 collected LAB cultures were submitted to an initial screening to verify the presence of antagonist activity using the *spot-on-the-lawn* method. They were revitalised in 5 mL of MRS broth at 37°C for 72 h in the presence of CO_2. Each Petri plate containing Tryptone Soya Agar (TSA) plus 0.5% of yeast extract was spotted with 2 μL of 5 LAB culture broths and incubated anaerobically overnight at 30°C. Brain Heart Infusion (BHI; Difco, Leeuwarden, The Netherlands) containing 1% agar was tempered to 45°C and seeded with 10^5-10^6 CFU/mL of each pathogen. The indicator strains used were *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* strains (ATCC 25923 and one milk isolated strain), *E. coli* ATCC 25922 and *Salmonella enteritidis* ATCC 13076 strains. The spotted plates were overlaid with 8 mL of the seeded BHI agar and then incubated at 30°C in anaerobiosis for 24 h. Inhibition was detected by a zone of clearing (>3 mm) around the producer colony as shown in Figure 1. For the WDA, agar base medium (1,5% agar) was overlaid with soft agar medium (1% agar) containing 3.0-3.3 MF (10^8 CFU/mL) of each indicator strain. In order to eliminate the inhibitory effect of lactic acid and/or H_2O_2, the supernatants were adjusted to pH 6.5 with 1 mol L^{-1} NaOH and treated with catalase (1 mg mL^{-1}), followed by filtration through a 0.22 μm pore size filter (Nucleopore; Costar Corporation, Cambridge, MA, USA). Five wells (0.7 mm in diameter) were cut into each agar and 120 μL of the cell-free supernatant was placed into each well. Prior to incubation for 24 h at the optimal growth temperature for the indicator strains, plates were refrigerated (4°C) for 4 h to allow the radial diffusion of the compounds contained in the supernatant (Corsetti *et al*., 2004) that produce a consisting inhibition halo as shown in Figure 2. The indicator strains used for WDA were the same ones used for the spot method. The assays were performed in duplicate.

Sensitivity to proteolytic enzymes of each supernatant containing antimicrobial activity was tested by treatment 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). The supernatants were examined for the sensitivity to lipolityc and amylolityc enzymes, by lipase (50 U mg^{-1}) and α-amylase (15 U mg^{-1}), respectively, at a final concentration of 1 mg mL^{-1} in phosphate buffer (pH 7.0). The supernatants were incubated with these enzymes at 37°C for 2 h after which remaining activity was determined by a WDA (Corsetti *et al*., 2004).

Results

A total of 223 strains (belonging to the species *Enterococcus* spp. *Lactobacillus* spp., *Pediococcus* spp., *Streptococcus* spp., *Leuconostoc* spp. and *Lactococcus lactis*) were found to inhibit the growth of *Listeria monocytogenes* ATCC 7644 by using the *spot-on-the-lawn* method.

The assays performed using the other indicator strains did not show any inhibition halo. In addition, the WDA method, performed on the 98 surnatants of the strains resulted positive to *spot-on-the-lawn* assay against *L. monocytogenes,* confirmed only 37 inhibiting strains isolated from pecorino cheeses (n=9), wooden vat surfaces (n=27), and from ovine milk (n=1). LAB positive strains belonged to the species: *Enterococcus faecium* (18), *Leuconostoc mesenteroides* (4), *Streptococcus thermophylus* (4), *Enterococcus faecalis* (3), *Leuconostoc pseudomesenteroides* (3), *Lactococcus lactis* (3), *Lactobacillus casei* (1) e *Lactobacillus deulbrueckii* (1). The cell-free supernatants derived from strains producing antimicrobial substances were assayed for their sensitivity to hydrolytic enzymes and all of them were inactivated by the 3 proteolytic enzymes tested, indicating that the inhibitory compounds are of proteinaceous nature, a general characteristic of bacteriocins. Only one antibacterial compound was sensitive to α-amylase and lipase treatments.

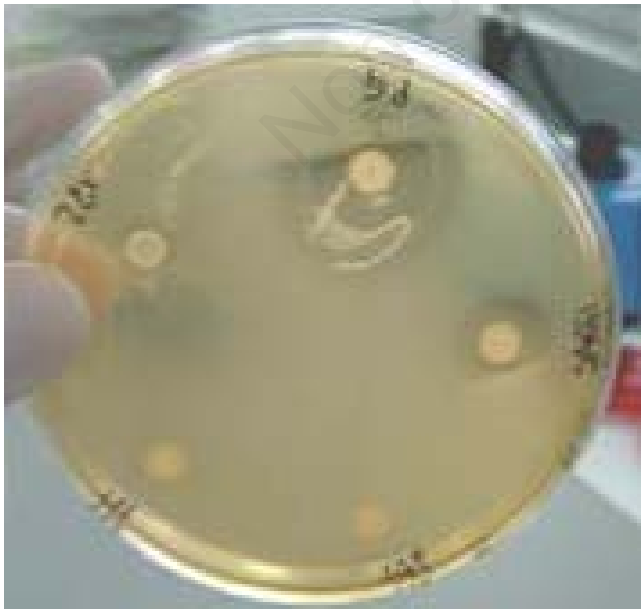

Figure 1. Inhibition halo: *spot-on-the-lawn.*

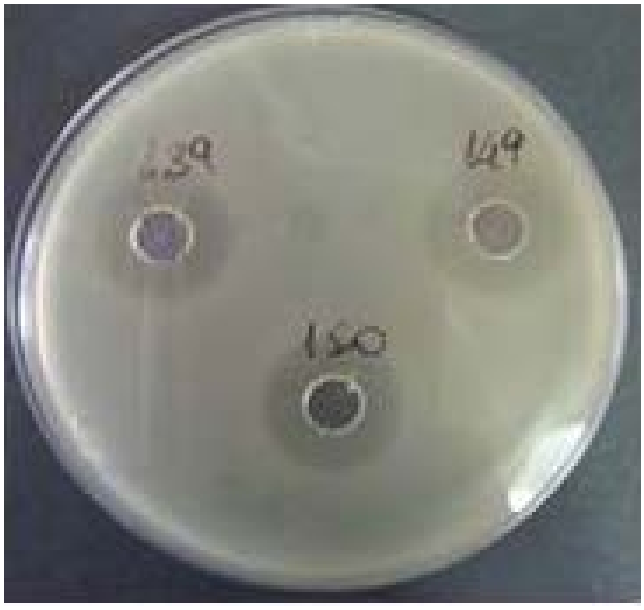

Figure 2. Inhibition halo: well diffusion assay.

Discussion

Bacteriocin-producing starter cultures have significant potential for improving the quality of different cheese types. Other authors (Maisnier-Patin *et al*., 1992) demonstrated that the nisin-producing starter for the inhibition of *L. monocytogene*s cheeses was composed of a pair of isogenic protease-positive and protease-negative strains of *L. lactis*.

According to Tagg *et al.* (1976) and Jack *et al.* (1995) the data obtained from the 37 antimicrobial compounds found in this study showed that the proteins detected against *L. monocytogenes* can be regarded as BLIS as they possess bacteriocin requisites, but they have not yet been characterised for amino acid and encoding nucleotide sequences. In particular, the strain showing the sensitivity to amilolytic and lipolytic treatments could preliminarily be regarded as a compound belonging to the fourth class of bacteriocins (Klaenhammer, 1993).

Conclusions

Of the 699 LAB strains isolated, 37 (representing the 5.29% of the strains tested) produced antibacterial compounds against the *L. monocytogenes* ATCC 7644. They were mainly isolated from wooden vat surfaces, the equipment used during dairy processing, demonstrating that high LAB percentages on the wooden vat surface are found to be BLIS producers.

All the antibacterial compounds lost their activities after treatment with proteolytic enzymes. Thus, they were proven to be proteins and, for this reason, indicated as BLIS.

The mechanism of inhibition is also observed in different foods, particularly those in which LAB dominate the population due to a growth rate advantage; the inhibition of *L. monocytogenes*, for example, has been identified by the natural biofilm microbiota on wooden shelves used in the ripening of a smear cheese. Indeed, the growth of *L. monocytogenes* stopped as soon as the biofilm microbiota entered in stationary phase. This effect was observed for two different inoculum levels of *L. monocytogenes* at two different times of inoculation in the biofilm (Guillier *et al.,* 2008).

Our results show that the correct maintenance of wooden vats and the good production practices of the traditional Sicilian cheeses promote the selection of a microbial flora able to play an active role in the achievement of the food safety objectives through the biocompetitive activity of LAB against spoilage and/or pathogenic bacteria, in particular *L. monocytogenes* which poses a serious problem during during the production and ripening of cheeses (O'Sullivan *et al.,* 2002).

The incorporation of bacteriocins as a biopreservative ingredient into model food systems has been shown to be effective in the control of pathogenic and spoilage microorganisms. The anti-*Listeria* effect found in this study might contribute to the safety of the microbial biofilms during cheese production. A more practical and economic option of incorporating bacteriocins into dairy products can be the direct addition of bacteriocin-producing cultures into food, for the improvement of the safety and quality of the final product.

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