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 method; only 37 of these were 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.

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) (Altshul 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 CO2. 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 105-106 CFU/mL of each pathogen. The indicator strains did not show any inhibition halo. In order to incubate 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 was placed into each well. Prior to incubation 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 supernatants 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 thermophilus (4), Enterococcus faecalis (3), Leuconostoc pseudomesenteroides (3), Lactococcus lactis (3), Lactobacillus casei (1) e Lactobacillus delbrueckii (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.
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. monocytogenes 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 amylolytic 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 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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