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Control of tyramine and histamine accumulation by lactic acid bacteria using bacteriocin forming lactococci

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ABSTRACT

The aim of this study was to evaluate the competitive effects of three bacteriocin producing strains of *Lactococcus lactis* subsp. *lactis* against two aminobiogenic lactic acid bacteria, i.e. the tyramine producing strain *Enterococcus faecalis* EF37 and the histamine producing strain *Streptococcus thermophilus* PRI60, inoculated at different initial concentrations (from 2 to 6 log cfu/ml). The results showed that the three *L. lactis* subsp. *lactis* strains were able to produce bacteriocins: in particular, *L. lactis* subsp. *lactis* VR84 and EG46 produced, respectively, nisin Z and lacticin 481, while for the strains CG27 the bacteriocin has not been yet identified, even if its peptidic nature has been demonstrated. The co-culture of *E. faecalis* EF37 in combination with lactococci significantly reduced the growth potential of this aminobiogenic strain, both in terms of growth rate and maximum cell concentration, depending on the initial inoculum level of *E. faecalis*. Tyramine accumulation was strongly reduced when *E. faecalis* EF37 was inoculated at 2 log cfu/ml and, to a lesser extent, at 3 log cfu/ml, as a result of a lower cell load of the aminobiogenic strain. All the lactococci were more efficient in inhibiting streptococci in comparison with *E. faecalis* EF37; in particular, *L. lactis* subsp. *lactis* VR84 induced the death of *S. thermophilus* PRI60 and allowed the detection of histamine traces only at higher streptococci inoculum levels (5–6 log cfu/ml). The other two lactococcal strains did not show a lethal action against *S. thermophilus* PRI60, but were able to reduce its growth extent and histamine accumulation, even if *L. lactis* subsp. *lactis* EG46 was less effective when the initial streptococci concentration was 5 and 6 log cfu/ml. This preliminary study has clarified some aspects regarding the ratio between bacteriocinogenic strains and aminobiogenic strains with respect to the possibility to accumulate BA and has also showed that different bacteriocins can have different effects on BA production on the same strain. This knowledge is essentially aimed to use bacteriocinogenic lactococci as a predictable strategy against aminobiogenic bacteria present in cheese or other fermented foods.

1. Introduction

Biopreservation refers to those processes in which the extension of food shelf life and safety improvement are obtained with the use of microorganisms, or their extracellular extracts, able to inhibit the growth of other bacteria, due to antimicrobial metabolite production (Papagianni, 2012). Such strategy is based upon the idea that some non-pathogenic bacteria can compete successfully with pathogenic and spoilage microorganisms avoiding their survival and growth in food (Stiles, 1996). This approach has been used for several centuries for the preservation of fermented food where the microbial population plays several roles, among which the inhibition of undesirable microbiota.

In recent years, particular interest has been posed on protective cultures, which are selected food-grade bacteria inoculated in food, due to their antagonistic properties rather than for their influence on the organoleptic or nutritional values. In fact, the microorganisms used as protective cultures should not affect the sensorial profile of the product (Rodgers, 2001). Lactic acid bacteria (LAB) are often used as biopreservation agents because they can produce a wide range of antimicrobial metabolites, such as organic acids, diacetyl, acetoin, hydrogen peroxide, antifungal peptides and bacteriocins without safety implications (Ghanbari et al., 2013).

Among the substances produced by bacteria with antimicrobial properties, bacteriocins have been deeply studied. Bacteriocins are defined as a heterologous group of ribosomally synthesized, extracellularly released bioactive peptides or proteins displaying antimicrobial activity against other bacteria (Klaenhammer, 1993; Nishie et al., 2012). In spite of the fact that several microbial groups (including Gram positive and Gram negative bacteria) can produce these

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molecules, the researchers' interest is focused mainly on LAB bacteriocins (Guinane et al., 2005; Parente and Ricciardi, 1999; Reis et al., 2012). This is due to the wide potential applications of these food-grade bacteria that open interesting perspectives for bacteriocin producing LAB (used as starter or protective cultures) or bacteriocin preparation in food (Beshkova and Frengova, 2012).

Lactococci are LAB ubiquitous in foods and they are widely present in dairy products because of their technological properties (Casalta and Montel, 2008). In fact, they are an important component of cheese microbiota, both during initial cheese-making steps, when they are often used as starter cultures, and during the ripening phase, when a complex microbiota determines transformations, which allow the obtaining of the peculiar cheese characteristics (Cogan et al., 2007). Moreover, they can have an important role as protective cultures in food preservation. In fact, they can exert important antimicrobial actions by synthesizing a variety of bacteriocins, such as nisins, lacticins and lactococcins (Beshkova and Frengova, 2012; Ghanbari et al., 2013; Leroy and de Vuyst, 2010; Stoyanova et al., 2012). In particular, nisin has a wide range of applications because of its broad bactericidal spectrum and its mode of action. Since it can be easily broken down by digestive proteases and it does not disturb gut biota, it is to date the first bacteriocin approved for commercial use (Guinane et al., 2005).

Due to the activities of ripening microbiota during cheese making and ripening, undesirable reactions can also take place, such as the formation of biogenic amines (BAs). These substances are accumulated through the microbial decarboxylation of amino acids. The most dangerous are histamine (produced from histidine) and tyramine (produced from tyrosine) (EFSA, 2011). Even if the selection of starter cultures is based on the absence of these features, the presence of aminobiogenic microorganisms in natural starter cultures or among ripening microbiota is often unavoidable (Linares et al., 2011; Novella-Rodríguez et al., 2002). Among LAB, many strains are endowed with high decarboxylating potential. For example, enterococci are known as the most efficient tyramine producers in fermented foods (Ladero et al., 2012; Marcobal et al., 2012). In addition, recently, the presence of efficient histaminogenic strains of *Streptococcus thermophilus* has been reported (Calles-Enríquez et al., 2010; Rossi et al., 2011; Tabanelli et al., 2012; Trip et al., 2011).

Even if some strains can produce tyramine (Buňková et al., 2011; de Llano et al., 1998), this feature in the genus *Lactococcus* is not widespread. For this reason, the selection of not tyraminogenic lactococci able to produce bacteriocins could represent an important tool to control BA accumulation in dairy products. In fact, the competition between two or more species in a habitat (such as cheese) affects both the partners in a negative way. However, it usually leads to an increase in the relative abundance of one of the interacting bacteria and to the possible exclusion or reduction of the other ones from the microbiota (Smid and Lacroix, 2013). Through this ability, selected lactococcal strains could contribute to reduce the risks of survival and multiplication of aminobiogenic microbiota during ripening and storage of fermented foods.

The aim of this research was the evaluation of the competitive effects against aminobiogenic LAB of three bacteriocin producing *Lactococcus lactis* strains isolated from raw cow milk. In particular, the tyramine producing strain *Enterococcus faecalis* EF37 (Gardini et al., 2001, 2008) and the histamine producing strain *S. thermophilus* PRI60 (Rossi et al., 2011; Tabanelli et al., 2012) were considered as target microorganisms. The effects of the inhibiting potential of *L. lactis* strains were evaluated in vitro and both the population dynamics and BA production were assessed.

2. Material and methods

2.1. Isolation and characterization of lactococci with antimicrobial activity

A total of 25 LAB was isolated from samples of raw cow milk from different local dairies. Colonies grown on M17 (Oxoid, Basingstoke, UK) plates incubated at 15 °C were randomly selected, purified and deposited in the laboratory culture collection. The isolates were subjected to a preliminary phenotypic characterization: cell morphology, Gram stain, gas production from glucose, catalase reaction, growth at 45 °C and with 6.5% (w/v) NaCl. *L. lactis* subsp. *lactis* LMG 6890^T and *L. lactis* subsp. *cremoris* LMG 6897^T served as control strains.

The presumptive lactococci isolated as described above were tested for their inhibiting potential towards the tyramine producer *E. faecalis* EF37 (Gardini et al., 2001) and the histamine producer *S. thermophilus* PRI60 (Rossi et al., 2011; Tabanelli et al., 2012). All strains were cultivated in M17 medium at 30 °C.

The antibacterial activity of lactococci was determined by using a deferred agar spot test under aerobic conditions (Schillinger and Lücke, 1989). M17 was used as bottom and upper layer medium. The production of antimicrobial substances was confirmed by well-diffusion agar assay using filter-sterilized and neutralized cell-free supernatants, as described by Aktypis et al. (1998). The presence of an inhibition zone greater than 5 mm around the well indicated a positive result. To evaluate the sensitivity of the inhibitory substances to proteolytic enzymes, the well-diffusion agar assay was repeated after treatment of the filter-sterilized cell-free supernatants with proteinase K (2 mg/ml), and pancreatin (1 mg/ml) at 37 °C for 4 h.

2.2. Molecular identification of lactococcal isolates

The presumptive lactococcal isolates showing the greater inhibition diameter in the deferred agar spot test (CG27, VR84 and EG46) were identified by 16S rDNA sequencing following by subspecies-specific PCR. Genomic DNA was extracted from pure cultures using the Instagene matrix (Bio-Rad Laboratories, Italy) according to the manufacturer's instructions.

In order to amplify the 16S rDNA gene the primers LpigF/LpigR (5′-TACGGGAGGCAGCAGTAG-3′ and 5′-CATGGTGTGACGGGCGGT-3′) (Eurofins MWG Operon, Germany) and the PCR conditions described by Di Cagno et al. (2011) were used. The resulting amplicons (each about 600 kb long) were purified with the QIAquick PCR Purification Kit (Qiagen, USA) and sequenced at the BMR Genomics sequencing facility (Padova, Italy) using the same primers used for amplification. Sequence similarity searches were performed using the BLAST network service (http://blast.ncbi.nlm.nih.gov/).

The primers Lhis5F/Lhis6R (5′-CTTCGTTATGATTTTACA-3′ and 5′-AATATCAACAATTCCATG-3′) and the conditions described by Beimfohr et al. (1997) were used for the distinction of the subspecies *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*. The expected sizes of amplification products for *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* were 1149 bp and 934 bp, respectively. To investigate the genetic diversity of the three lactococcal isolates, typing was carried out by randomly amplified polymorphic DNA (RAPD)-PCR with primer M13 according to Zapparoli et al. (1998).

2.3. Determination of the bacteriocin-encoding genes

A screening for structural genes encoding bacteriocins previously described for *L. lactis* was done using PCR of the genomic DNA from the three lactococcal strains CG27, VR84 and EG46, identified as *L. lactis* subsp. *lactis*.

PCRs were carried out with the primers listed in Table 1 and in a 20 μl reaction mixture containing 200 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 U of Taq polymerase (GoTaq, Promega, Italy) and 2.5 μM of each primer.

The amplification programmes comprised an initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation for 45 s at 94 °C, annealing for 60 s at the appropriate temperature, and a final extension at 72 °C for 60 s. All primers were used with an annealing temperature of 54 °C except for the primer pair LcnQZ-F/LcnQZ-R where a 53 °C annealing temperature was used. The generated PCR products were purified and sequenced with the specific primers, as previously described,