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Identification and functional traits of lactic acid bacteria isolated from Ciauscolo salami produced in Central Italy

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ABSTRACT

Lactic acid bacteria (LAB) from Ciauscolo salami produced in Marche Region of Central Italy, and LAB strains belonging to our laboratory collection were examined for their capability to survive at low pH and bile, to adhere to Caco-2 cells, and for antibiotic resistance. LAB from Ciauscolo were identified by ARDRA and RAPD-PCR. Our study showed that all LAB strains had good adaptation to gastric juice and moderate tolerance to bile. The adhesiveness was variable among strains but significantly lower in LAB from food. Antibiotic resistance was broadly spread among food strains, with level of resistance exceeding 15% for all the antibiotics tested. The resistance determinants *erm*(B) and *tet*(M) were found in nine strains of food origin (21.4%) while *tet*(L) in one strain of our collection (5%). Our work suggests that fermented foods are valuable sources of bacterial strains with functional traits of intestinal lactobacilli. These bacteria may be further studied for their use in probiotic applications.

1. Introduction

Lactic acid bacteria (LAB) used in food production are gaining increasing attention in the area of probiotics (Tannock, 2004). LAB associated with fermented foods include species of the genera *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus* and *Weissella*. Probiotics improve host's quality of life by providing beneficial effects in the gastro-intestinal tract (GIT). These effects occur at different levels, physiological, immunological and metabolic. Some articles suggest that probiotics can be used as supplements in major depressive disorder, for their ability to regulate systemic inflammatory cytokines, to lower oxidative stress and to improve nutritional status (Logan & Katzman, 2005). Given the fast growing demand for "healthy" foods and the increasing consumers' health consciousness, probiotic integrators are acquiring more and more attention in the food industry, with an expected further increase within the global market (Khan & Ansari, 2007). Microorganisms such as *Lactobacillus*, *Bifidobacterium*, and *Saccharomyces* are commonly included in probiotic pharmaceutical formula and not only in dairy products. These strains gained the "generally recognized as safe (GRAS)" status (Saarela, Mogensen, Fonden, Matto, & Mattila-Sandholm, 2000). "GRAS" strains are able to survive and proliferate in the target site, to resist gastric acid and bile salts, to adhere to the gastrointestinal tract, and to antagonize pathogenic bacteria such as *Salmonella typhimurium*, *Listeria monocytogenes*, *Helicobacter pylori*, and *Staphylococcus aureus* (Mercenier, Pavan, & Pot, 2002).

Therefore isolation and characterization of novel LAB strains from uninvestigated niches could have the twofold advantage of revealing taxonomic characteristics and obtaining strains with interesting functional traits that can be used in technological and/or probiotic applications as live microbial feed supplements that beneficially affect the host by improving its intestinal microbial balance (Fuller, 1988). Nutritious benefits of probiotic microorganisms have been most extensively investigated in dairy products (Meyer, Elmadfa, Herbacek, & Micksche, 2007) where they are the main responsible for fundamental technological properties; and it has also been observed that mesophilic strains, derived from some traditional food, can survive into the gastrointestinal tract (Vizoso Pinto, Franz, Schillinger, & Holzapfel, 2006). However, potential probiotic properties of LAB isolated from traditional Italian fermented foods have been poorly investigated (Aquilanti, Silvestri, et al., 2007). Our study is focused on Ciauscolo, a typical short-ripened Italian fermented sausage, traditionally manufactured in mountain towns of Marche and Umbria Regions, and recently also produced in other areas of Central Italy. Lately, Ciauscolo has been classified as Protected Geographical Indication (PGI), product based on European Economic Community (EEC) Regulation No. 510/06. Its peculiar traits are spreadability and the homogeneously rosy meat batter, due to a high fat content and to the particular conditions of its processing and ripening (Rea, Pacifici, Stocchi, Loschi, & Ceccarelli, 2003). The meat is mixed with salt, pepper, garlic and white wine, and for industrial production, potassium nitrate is usually added for preservation; the ripening of this sausage is 30–80 days. Together with raw materials and

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techniques employed, a great importance in the production of this fermented sausage is given by the composition and activity of the microbial population, which is responsible for sensory and biochemical characteristics (Trani et al., 2010); it is mainly composed of lactic acid bacteria (LAB), coagulase negative cocci (CNC), and to a small extent, yeasts and molds (Aquilanti, Santarelli, et al., 2007). Briefly, lactic acid bacteria release molecules with antimicrobial and organoleptic actions, such as bacteriocins and pyruvic acid (Beccaceci et al., 2006), while CNC are responsible for the final pigmentation of meat, lipolysis and proteolysis (Albano et al., 2009; Iacumin, Comi, Cantoni, & Cocolin, 2006).

The present research was aimed at investigating the LAB population of Ciauscolo salami produced in several mountain towns of Marche Region, without commercial starter adjuncts. We first tried to identify the LAB strains present in Ciauscolo both by molecular and phenotypic assays. Then, in order to determine their potential use as probiotics, the isolated LAB were examined for functional traits, such as acid resistance, bile tolerance, adhesion to Caco-2 cells and antibiotic resistance.

2. Materials and methods

2.1. Isolation of LAB from Ciauscolo salami

One hundred and twenty samples of Ciauscolo salami, produced without starter culture adjuncts, by 14 artisanal meat industries of Marche region (Central Italy) were examined for the isolation of LAB strains.

Ten grams of each matrix was homogenized in 90 ml of 0.1% (w/v) buffered peptone water (BPW) in a Stomacher apparatus (400 Circulator, International PBI, Milan, Italy) at 260 rpm for 2 min. Serial dilutions of the homogenates were performed in saline-peptone water and 100 μl was spread-plated onto: (i) MRS agar (Diagnostic International Distribution, Milan, Italy) incubated at 30 °C for 72 h under anaerobic conditions for isolation of presumptive lactobacilli, according to issued standards (ISO 15214:1998); and (ii) M17 agar (Biokar Diagnostics, Beauvois, France) at 42 °C for 48 h and 30 °C for 48 h under anaerobic conditions, for isolation of presumptive thermophilic cocci and mesophilic lactoccocci, respectively.

For each sample, 15–20 randomly selected colonies from M17 and MRS plates were subcultured until pure cultures were obtained, and identified by colony morphology, Gram staining and catalase reaction; only pure cultures of gram-positive and catalase-negative isolates were stored at −80 °C in a mixture of MRS broth and glycerol (3:2 v/v) until further molecular analyses.

2.2. DNA extraction

Genomic DNA was extracted from bacteria by a boiling method, a modification of the bacterial DNA extraction protocol described by Casey et al. (2006). A total of three colonies for each isolate were suspended in 100 μl of sterile double-distilled water and incubated at 100 °C for 12 min in Thermomixer Comfort (Eppendorf, Milan, Italy). The suspension was centrifuged at 15.294 ×*g* for 5 min and the supernatant was transferred into sterile 1.5 ml microcentrifuge tubes. The DNA was quantified spectrophometrically (OD_{260} nm) by Biophotometer (Eppendorf) and the samples were stored at −20 °C.

2.3. Identification by ARDRA and sequencing

The isolates were preliminarily identified by ARDRA amplifying the genomic DNA with primers Y1 (5′-TGGCTCAGAACGAACGCTGGCCCG-3′) and Y2 (5′-CCCACTGCTGCCTCCCGTAGGAGT-3′), as reported previously (Aquilanti, Silvestri, et al., 2007). The PCR was carried out in a Mastercycler 5333 (Eppendorf) and the size of the expected 360-bp portion of the 16S rRNA gene was checked in 1.5% agarose (w/v) (EuroClone, Milan, Italy) gel, using a 100-bp ladder (Amersham Biosciences, USA) as molecular weight standard.

The obtained amplicons were then digested at 37 °C for 12 h with *Alu*I, *Fok*I (BioLabs, New England) and *Hae*III (Amersham Biosciences). Restriction patterns were analyzed on 1.5% agarose gel, and digital images were captured using the UVsave Gel Documentation System (UVITEC, Cambridge, UK).

In order to perform the sequencing, the 16S rRNA gene amplicons of one or two isolates from each ARDRA group were purified by QIAquick PCR Purification Kit (Qiagen, Milan, Italy), according to the manufacturer instructions. The sequencing was performed using BigDye Terminator cycle v1.1 Sequencing Kit (Applied Biosystems, CA, USA) in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems), using 5 μl of purified DNA in a final volume of 20 μl, containing 8 μl of Ready Reaction Mix and 3.2 μl of each primer Y1 and Y2 (1 pmol μl^{-1}). Sequencing Analysis software v.3.7 (Applied Biosystems) was used for data analysis; the sequences were finally compared with those deposited in the GenBank DNA database using the BLASTn algorithm (http://www.ncbi.nlm.nih.gov/BLAST/). A phylogenetic tree based on 16S rRNA genes was also constructed to determine the closest bacterial species by the neighbor-joining method (Saitou & Nei, 1987), using MEGA5 (Tamura et al., 2011).

2.4. RAPD-PCR protocol

In this study, amplified polymorphic DNA (RAPD-PCR) analysis was performed using the primers D8635 (5′-GAGCGGCCA AAG GGAGCA GAC-3′) (Akopyanz, Bukanov, Westblom, Kresovich, & Berg, 1992) and Coc (5′-AGCAGCGTGG-3′) (Cocconcelli, Porro, Galandini, & Senini, 1995). Total genomic DNA, extracted as described above, was amplified in a 25 μl PCR mixture containing 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 1 μM of the primer, 2 U of Taq DNA polymerase (GoTaq, Promega, Milan, Italy) and 120 ng of DNA, for the primer D8635. For the second primer the following mixture was used: 0.2 mM dNTPs, 3 mM $MgCl_2$, 1 μM of primer, 2 U of Taq DNA polymerase (GoTaq, Promega), 40 ng of DNA.

Both the amplifications were performed with an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 42 °C for 1 min, elongation at 72 °C for 90 s, and a final extension at 70 °C for 10 min carried out in a Mastercycler 5333 (Eppendorf); the amplicons were analyzed on 1.5% agarose (w/v) (EuroClone) gels, using a 100-bp ladder (Amersham Biosciences) as molecular weight standard, in 0.5× TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA); gels were stained in ethidium bromide solution.

All the RAPD patterns were converted into a binary data matrix (0 = absence of a given band; 1 = presence of a given band). Pairwise similarity was calculated using the Dice coefficient (Dice, 1945). This similarity matrix was further used for the UPGMA cluster analysis (Sokal & Sneath, 1963) using the NTSYS-pc package version 1.8.

2.5. Determination of functional traits

Selected biotypes of the isolated LAB were examined for functional traits, such as acid resistance, bile tolerance, adhesion to Caco-2 cells and antibiotics resistance, in order to determine their potential use as probiotic strains. Moreover, 11 LAB strains obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and 9 LAB strains isolated from different sources (8 from human feces and one from milk) in the Division of Toxicological, Hygiene and Environmental Science (University of Urbino "Carlo Bo") (Table 3) were tested for the same properties comparing the obtained results with those of the strains isolated from Ciauscolo salami samples.

2.5.1. Acid and bile tolerance of lactic acid bacteria

Acid resistance of the isolated strains was examined in MRS broth (Oxoid, Milan, Italy) adjusted with hydrochloric acid (HCl) to a final pH of 2.5. Each strain was propagated in MRS broth for 24 h at 37 °C and harvested by centrifugation (1.145 ×*g*, for 10 min), washed twice in phosphate-buffered saline (PBS) pH 7.2, inoculated (10%) into