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A survey of non-starter lactic acid bacteria in traditional cheeses: Culture dependent identification and survival to simulated gastrointestinal transit

Annamaria Ricciardi [a, b, *], Angela Guidone [a], Rocco Gerardo Ianniello [a], Silvia Cioffi [a], Maria Aponte [b, c], Dimitrios Pavlidis [d], Effie Tsakalidou [d], Teresa Zotta [b], Eugenio Parente [a, b]

[a] Scuola di Scienze Agrarie, Forestali, Alimentari ed Ambientali, Università degli Studi della Basilicata, Potenza, Italy
[b] Istituto di Scienze dell'Alimentazione, Consiglio Nazionale delle Ricerche, Avellino, Italy
[c] Dipartimento di Agraria, Università degli Studi di Napoli "Federico II", Napoli, Italy
[d] Department of Food Science and Human Nutrition, Agricultural University of Athens, Athens, Greece

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ABSTRACT

Cultivable NSLAB in traditional Pasta filata and ewes' milk cheeses were studied by both PCR-DGGE of cells from Rogosa agar and by isolation and molecular identification after a simulated gastric juice (SGJ) treatment of the cheese. Two to six species were retrieved from each sample. The majority of isolates were identified as *Lactobacillus paracasei*, *Lactobacillus fermentum*, *Lactobacillus rhamnosus*, *Enterococcus* or *Pediococcus*. Bile tolerance and bile salt hydrolase (BSH) activity were tested on 88 strains: 64% were able to grow with ≥0.15% bile and 40% were BSH positive. The effect of simulated digestion was tested on 15 strains. Inactivation ranged from 0.15 to 2.93 log cycles; most of the lethality was associated with pancreatic juice treatment. Although SGJ treatment alone may not provide a correct estimate of tolerance to gastrointestinal transit, it allowed selection of strains with a high tolerance to gastric juice, which may be tested as probiotic candidates.

1. Introduction

A large variety of ripened and semi-ripened pasta filata (Aponte, Fusco, Andolfi, & Coppola, 2008; Cronin et al., 2007; Ercolini, Frisso, Mauriello, Salvatore, & Coppola, 2008; Gobbetti et al., 2002; Piraino, Zotta, Ricciardi, & Parente, 2005; Randazzo, Torriani, Akkermans, de Vos, & Vaughan, 2002) and ewes' milk cheeses (Albenzio et al., 2001; Aquilanti, Dell'Aquila, Zannini, Zocchetti, & Clementi, 2006; Bonomo & Salzano, 2012; Caridi, Micari, Caparra, Cufari, & Sarullo, 2003; Coda et al., 2010; Randazzo, Pitino, Ribbera, & Caggia, 2010) are produced in Southern Italy using raw milk and traditional cheese making recipes. As a result, a secondary microflora composed of wild non-starter lactic acid bacteria (NSLAB) develops and contributes to cheese ripening and quality (Settanni & Moschetti, 2010). The NSLAB species most frequently encountered in cheese include mesophilic and facultative heterofermentative lactobacilli, such as *Lactobacillus paracasei*, *Lactobacillus casei*, *Lactobacillus plantarum*, and *Lactobacillus rhamnosus*, but pediococci and obligate heterofermentative lactobacilli have also been isolated (Beresford & Williams, 2004).

NSLAB contribute to cheese ripening and quality by increased proteolysis and peptidolysis and production of aroma compounds (Beresford & Williams, 2004; Settanni & Moschetti, 2010), but may also provide a number of health benefits. Some species may produce bioactive peptides and γ-aminobutyric acid and/or may exert antigenotoxic activities (Settanni & Moschetti, 2010). NSLAB have frequently been screened with some degree of success for health-promoting properties (Caldini, Trotta, Corsetti, & Cenci, 2008; Guidone et al., 2014; Siragusa et al., 2007; Zago et al., 2011).

Cheese has been used as a carrier of probiotic microorganisms (Karimi, Mortazavian, & Da Cruz, 2011), because it affords better protection to their viability compared with fermented milks and probiotic bacteria in cheese are known to survive simulated gastrointestinal transit (SGT) (Mäkeläinen et al., 2009). Probiotic

* Corresponding author. Tel.: +39 0971205562.
E-mail address: annamaria.ricciardi@unibas.it (A. Ricciardi).

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cheese has been shown to exert positive effects on elderly humans by selectively altering lactobacilli and *Clostridium difficile* populations (Lahtinen et al., 2012). Consumption of cheese may also deliver a diverse set of microorganisms to the host, which may affect the immune response. In fact, dietary deprivation of fermented foods has been proven to adversely affect innate immune response of healthy human volunteers, which can be reinstated to normal conditions by providing either probiotic foods or yoghurt without probiotic microorganisms (Olivares et al., 2006). Consumption of Camembert has been shown to affect the composition of the colon microbiota in humans (Firmesse, Rabot, Bermãdez-Humarã, Corthier, & Furet, 2007), and a cheese containing diet has been shown to enhance anti-inflammatory responses and immune regulation in mice (Hosoya, Ogawa, Sakai, & Kadooka, 2012).

The objectives of this work were to (i) identify the NSLAB community associated with traditional pasta-filata and ewes' milk cheeses, (ii) evaluate if the cheeses were a potential source of LAB strains able to survive the gastric transit and (iii) confirm the survival ability of selected strain in a simulated gastrointestinal transit.

2. Materials and methods

2.1. Sampling, reagents and media

Twenty different cheese samples (labelled with letters from A to Z) were collected from cheese making plants in Basilicata, Southern Italy. The cheese samples were transferred to the laboratory under refrigerated conditions (4 °C) and analysed within 6 h (water activity, pH and microbiological analyses) or frozen at −80 °C (chlorides and moisture).

Unless otherwise indicated, all reagents were purchased from Sigma–Aldrich (Milan, Italy), and bacteriological media and ingredients from Oxoid (Thermo Fisher Scientific Italia SpA, Rodano, Italy).

2.2. Chemical and physico-chemical analysis

A spear tip electrode (Hamilton Bonaduz AG, Bonaduz, Switzerland) and a pH-meter (Orion 420A plus, Thermo Fisher Scientific, Rodano, Italy) were used to measure pH. Water activity (a_w) was measured on grated cheese using a HygroPalm (series 21/22/23/23-AW/TP22) with a HC2-AW sensor (Rotronic Italia Srl, Milano, Italy) at 25 °C. Moisture (%) was measured by oven drying (IDF, 1982) and chlorides by a potentiometric method (Fox, 1963).

2.3. Isolation and taxonomic characterisation of non-starter lactic acid bacteria

Cheese sample were homogenised (1:10) with 1% (w/v) trisodium citrate in a Stomacher (Lab-Blender 400, PBI International, Milano, Italy) for 2 min, and further dilutions were carried out in sterile quarter strength Ringer solution. Counts were performed by spread plating on Rogosa agar followed by incubation at 30 °C for 5 days under anaerobic conditions (GENbox anaer, bioMérieux Italia, Srl, Firenze, Italy). Furthermore, a selective treatment with simulated gastric juice (SGJ) was used to select for acid resistant LAB from cheese: a 10^{-2} dilution was mixed (1:10) with a simulated gastric fluid comprising 0.5% (w/v) NaCl and 3 g L^{-1} pepsin, pH 2.0, and incubated for 30 min at 37 °C. Further dilutions were carried out as described above and counts of the survivors were performed on mMRS-BPB (Lee & Lee, 2008) which was incubated at 30 °C for 48 h under anaerobic conditions as described above.

For each cheese, 15–20 colonies were randomly selected from mMRS-BPB agar plates using a Harrison disk (Harrigan & McCance, 1976) from plates containing 30–300 colonies. After further purification on mMRS-BPB, colony morphology (Lee & Lee, 2008), cell morphology, Gram stain, catalase and gas production from glucose (Sperber & Swan, 1976) were determined. Isolates were maintained as frozen stock in 20% (v/v) glycerol at −20 °C and routinely propagated 1% (v/v) in modified All Purpose Tween broth (APT; Sperber & Swan, 1976), pH 6.8, incubated at 30 °C for 16 h.

DNA was extracted using a salting-out method (Aponte et al., 2012). RAPD-PCR was carried out using primers Coc1 (Cocconcelli, Parisi, Senini, & Bottazzi, 1997) for cocci and M13 (Rossetti & Giraffa, 2005) for bacilli. The collection was de-duplicated by removing isolates whose profiles were not significantly different (the average similarity of replicate profiles for the same isolate was 87.5–90%) and 175 strains were identified by a combination of molecular methods: ITS-PCR for streptococci (Blaiotta et al., 2002), multiplex-PCR of the *recA* gene (Torriani, Felis, & Dellaglio, 2001) for strains whose colony morphology on mMRS-BPB was compatible with members of the *L. plantarum* group, multiplex-PCR of the *tuf* gene (Ventura, Canchaya, Meylan, Klaenhammer, & Zink, 2003) for strains whose colony morphology was compatible with members of the *L. casei* group and sequencing of the 16S rDNA using primers 27F and 1512R (Suzuki & Giovannoni, 1996) for all other strains or those with ambiguous identification results by other methods. Sequencing was performed by Genechron (Roma, Italy) and sequence identification was carried out using the Seqmatch tool of RDB (Wang, Garrity, Tiedje, & Cole, 2007); http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp.

2.4. Polymerase chain reaction-denaturing gradient gel electrophoresis of cultivable non-starter lactic acid bacteria

The species composition of the cultivable NSLAB community was determined by recovering cells grown on Rogosa agar plates in 2 mL sterile saline (0.85%, w/v, NaCl), followed by centrifugation (10,000 × *g*). The pellet was washed twice with the same solution and stored at −80 °C. DNA was extracted and purified as described above.

Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) of the V3 region of 16S ribosomal DNA was carried out as described by Ercolini, Mauriello, Blaiotta, Moschetti, and Coppola (2004) but staining was performed using 25 μL of SYBR Gold 10,000X (Invitrogen, Thermo Fisher Scientific, Rodano, Italy) in 250 mL of TAE 1X. Gel images were digitised using a GelDocXR apparatus with and XcitaBlue™ conversion screen and ChemiDoc™ XRS filter and Quantity One 1-D analysis software (Bio-Rad Laboratories, Hercules, CA, USA). For the identification of bands two procedures were used: two ladders including respectively amplified DNA from pure cultures of *L. plantarum*, *Pediococcus pentosaceus*, *Pediococcus acidilactici* (Ladder 1) or *Lactobacillus fermentum*, *Enterococcus faecium*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactococcus lactis* subsp. *lactis*, *Lactobacillus buchneri* and *L. paracasei* (Ladder 2) were included in all gels and bands which did not match with those of the ladder were eluted, re-amplified using the same primers, checked for purity and sequenced.

2.5. Evaluation of bile tolerance and survival to simulated gastrointestinal transit

Growth in the presence of bile (0.15, 0.3, 0.6, 1.2, and 3.6% (w/v) porcine bile salts in MRS agar, Pennacchia et al., 2004) and bile salt hydrolase (BSH) activity on sodium taurodeoxycholate (Zago et al., 2011) were tested on 88 isolates (1 *L. plantarum*, 66 *L. paracasei*, 21 *L. rhamnosus*; Supplementary Table S1).