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Short communication: Latin-style fresh cheese enhances lactic acid bacteria survival but not *Listeria monocytogenes* resistance under in vitro simulated gastrointestinal conditions

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ABSTRACT

Different studies in humans have provided evidence about the health benefits of probiotics. However, most probiotic strains do not maintain good viability in the harsh conditions of the gastrointestinal tract (GIT). In the present study, Latin-style fresh cheese produced with potential probiotic bacteria was tested to evaluate this cheese type as a food carrier for the delivery of viable microorganisms after exposure to simulated GIT conditions. The resistance of 28 lactic acid bacteria (LAB) strains and *Listeria monocytogenes* upon exposure to acidic conditions (pH 2.5) and bile and pancreatic enzymes (0.3% of bile salts and 0.1% of pancreatin) was evaluated in vitro. When compared with fresh cultures, fresh cheese greatly improved LAB survival to simulated GIT conditions, as no loss of viability was observed in either acidic conditions (pH 2.5) or bile salts and pancreatin environment over a 3-h period. In opposition, *L. monocytogenes* did not survive after 1 h under acidic conditions. These data demonstrated that Latin-style fresh cheese could play an important role in probiotic protection against gastrointestinal juices, enhancing delivery within the gut and thereby maximizing potential health benefits of LAB.
Key words: fresh cheese, lactic acid bacteria, probiotic, *Listeria monocytogenes*

Short Communication

Increasing evidence indicates that consumption of some lactic acid bacteria (**LAB**) strains reduces the risk of various diseases (Fijan, 2014). For this reason, interest in probiotic bacteria as dietary adjuncts has increased and new probiotic strains have been incorporated into numerous foods and beverages (Corbo et al., 2014). Nevertheless, to be effective and confer their beneficial effect on the host, probiotics must survive passage through the human gastrointestinal tract (**GIT**) and be present in sufficient numbers to colonize the human small and large intestine (Collins et al., 1998). To establish such a probiotic trait, in vitro simulators of digestion have been used for probiotic strain screening (Charteris et al., 1998; Gardiner et al., 1999; Cook et al., 2012; Burns et al., 2014).

Numerous reports have shown that bacteria from most probiotic products are not acid resistant and do not survive passage through the GIT (Ritter et al., 2009; Burns et al., 2014). Thus, to improve the survival of these bacteria in the human GIT several approaches have been investigated, including encapsulation or physical protection in the food system (Cook et al., 2012). Fermented milks and yogurts have been employed as delivery vehicles of probiotics, but some studies suggest that they may not be ideal food carriers for certain probiotic strains, due to the low pH and the high oxygen levels in the liquid matrix (Gardiner et al., 1999; Vinderola et al., 2000). In contrast, cheese has certain advantages as it creates a buffer against the highly acidic environment in the GIT. Moreover, the dense matrix and relatively high fat content of cheese may offer additional protection to probiotic bacteria in the stomach, enhancing probiotic survival throughout gastric transit (Sharp et al., 2008; Karimi et al., 2012). Nonetheless, survival of probiotic bacteria in cheese must be ensured, as many probiotic cultures are affected by cheese manufacture protocols and subsequent ripening. Latin-style fresh cheeses are traditionally made in Portugal and Spain from pasteurized goat or cow milk, without addition of a starter culture. They are ready for consumption immediately after production and are characterized by their soft paste and fresh mild flavor. These fresh cheeses may constitute an ideal food carrier for probiotic cultures because their pH is almost neutral, they have high moisture content, and no addition of starter or adjunct cultures that may compete with potential probiotic bacteria. Additionally, they have a simple manufacturing process, low cost, and good ac-

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ceptance by most consumers due to their mild taste (Evert-Arriagada et al., 2013). However, due to the relatively high pH and water activity, fresh cheeses are particularly sensitive to colonization by *Listeria monocytogenes* through postprocess contamination (Kabuki et al., 2004). Thus, the present study was aimed to investigate the effect of this type of fresh cheese on the protection of several LAB strains and a pathogen (*L. monocytogenes*) to adverse gastrointestinal conditions. Therefore, the resistance of 28 LAB strains and 1 *L. monocytogenes* strain to low pH, bile salts, and digestive enzymes was evaluated in vitro using both pure cultures and the inoculated cheese samples.

The LAB strains isolated from an artisanal cheese (Pico cheese) were identified by 16S rRNA sequencing analysis as indicated by Ribeiro et al. (2014). The 28 studied strains were identified as *Lactobacillus paracasei* (14), *Lactobacillus plantarum* (5), *Lactobacillus paraplantarum* (1), *Lactobacillus otakiensis* (1), *Lactococcus lactis* (2), *Leuconostoc mesenteroides* (4), and *Leuconostoc citreum* (1). Nucleotide sequence data have been assigned GenBank Accession numbers KF193424, KM079353-KM079358, KM079360, KM079361, KM096813-KM096826, KM096828, and KM103931-KM103934. All LAB strains were grown in de Man, Rogosa, Sharpe broth medium (BK089HA, Biokar Diagnostics, Beauvais, France) and stored with 20% glycerol (vol/vol; VWR, Fontenay-sous-Bois, France) at −80°C until use. *Listeria monocytogenes* (ATCC 7466) was also used to inoculate cheese to evaluate its resistance to simulated GIT conditions. *Listeria monocytogenes* kept at −80°C was revitalized and propagated in Nutrient Broth (AES, Bruz, France) for 18 h, at 37°C, before use.

To evaluate resistance to low pH, LAB strains and *L. monocytogenes* from overnight (18 h) cultures (cultured at 30°C for LAB or 37°C for *L. monocytogenes*) were harvested by centrifugation (10,000 × g, 5 min, 4°C), washed twice with PBS buffer (pH 7.2, Oxoid, Basingstoke, Hampshire, UK), before being resuspended in PBS solution and adjusted to pH 2.5. To simulate the small intestine conditions, bacterial cells were also resuspended in PBS solution (pH 7.2), containing 0.3% (wt/vol) bile salts (Fluka, Buchs, Switzerland) and 0.1% pancreatin (Sigma-Aldrich, St Louis, MO). Initial inoculum was corresponding to viable counts of approximately 10^{-7} cfu/mL. Resistance to both conditions was assessed in duplicate, in terms of viable colony counts after incubation at 37°C for 0, 1, 2, and 3 h, in a shaking incubator (Edmund Bühler GmbH, Hechingen, Germany) reflecting the time spent by food in the stomach and the small intestine. An aliquot of 1 mL was taken at each time point and serially diluted with buffered peptone water (AES). The LAB strains were enumerated in duplicate on de Man, Rogosa, Sharpe agar (Merck, Darmstadt, Germany) after incubation under aerobic conditions at 30°C for 72 h. *Listeria monocytogenes* counts were carried out by plating appropriate dilutions in duplicate on PALCAM-Listeria selective agar containing supplement (Biokar, Beauvais, France), followed by enumeration after incubation at 37°C for 48 h.

To evaluate Latin-style fresh cheese as food carrier of bacteria under the GIT conditions, cheeses without addition of starter cultures were made in a laboratory plant, following the procedure described in detail by Coelho et al. (2014). In each trial, pasteurized cow milk was distributed into 1-L vats and warmed at 32°C before each LAB culture (1%, vol/vol) or *L. monocytogenes* (1%, vol/vol) was individually inoculated. Initial inoculum corresponded to viable counts of approximately 10^{-7} cfu/mL. The milk was left for 20 min before the addition of rennet (LMF 1/15,000, 0.2 g/L, Lusocoalho, Lisbon, Portugal), calcium chloride (0.2 g/L), and NaCl (10 g/L). The curd was cut, molded into small cheeses (6.5 cm in diameter), and stored at 4°C until being tested. An aliquot (2 g) of fresh cheese (after 3 to 5 d of storage at 4°C), containing each LAB strain or *Listeria monocytogenes*, was added to each test tube containing 10 mL of acidic PBS (pH 2.5) or 10 mL of PBS (pH 7.2) containing 0.3% bile salts and 0.1% pancreatin. The contents were mixed well with the help of a sterile rod and all tubes were incubated in a shaking incubator at 37°C. Bacterial survival was determined at 0, 1, 2, and 3 h by removing 1 aliquot (1 mL) at each time point. Serial dilutions were performed in buffered peptone water and, subsequently, LAB or *L. monocytogenes* were enumerated as indicated above for pure cultures. Each experiment was conducted in quadruplicate.

Overall, the pure cultures showed poor resistance to gastric acidity in the absence of cheese matrix, as demonstrated by the rapid loss of viability after exposure to pH 2.5 (Figure 1A and B). After 2 h of incubation at this low pH, only 13 strains still persisted (classified as resistant; Figure 1A), although with reduced survival rate. Most of strains (15) were classified as sensitive to low pH, as they were not detected after 2 h of incubation at pH 2.5 (Figure 1B). Seven strains (6 lactobacili and 1 *Lactococcus*) were undetectable after 1 h at this low pH. Yet all the strains tested were undetectable (<1 log cfu/mL) after 3 h of exposure to pH 2.5 (Figure 1A and B). These results were not surprising, as susceptibility to gastric acidity can greatly diverge among species and strains. Nevertheless, several studies reported a considerable loss of LAB viability after ex-