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Identification and evaluation of the probiotic potential of *Lactobacillus paraplantarum* FT259, a bacteriocinogenic strain isolated from Brazilian semi-hard artisanal cheese

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ABSTRACT

This study aimed to identify a bacteriocinogenic *Lactobacillus* isolate (FT259) obtained from Brazilian semi-hard Minas type cheese and to evaluate its probiotic and antimicrobial potentials. The strain was identified by biochemical tests (at genus level), and by 16S rDNA sequencing combined with *rec*A gene amplification (for species). To determine the inhibitory spectrum towards food borne pathogens and lactic acid bacteria, the spot-on-the-lawn assay was carried out. Moreover, the proteinaceous nature of the antimicrobial compound produced was evaluated by susceptibility to degradation by proteolytic enzymes. The isolated strain was tested for survival in acidified culture media (pH 2.0, 2.5 and 3.5), *in vitro* tolerance to bile salts and viability under gastric conditions. Adhesion of *Lactobacillus paraplantarum* FT259 to Caco-2 cells was evaluated by surface plate count on De Man, Rogosa, and Sharpe (MRS) agar and also by FISH method (fluorescent *in situ* hybridization) with the aid of Eub338 probe for fluorescence microscopy analysis. The isolate was identified as *L. paraplantarum* FT259 and it produced bacteriocins that inhibited the growth of *Listeria monocytogenes*, *Listeria innocua* and several lactic acid bacteria. It was also observed that *L. paraplantarum* FT259 tolerated exposure to pH 3.5, and bile salts 0.3% for up to 180 min. In experiments with simulated gastric juice, viable cells of *L. paraplantarum* FT259 decreased from 8.6 log CFU/mL to 3.5 log CFU/mL after 180 min. For the same strain, in studies with Caco-2 cells, 74% of adhesion was observed through plate count and FISH assays. It was also demonstrated isolated FT259 was susceptible to the majority the antibiotics tested. Overall, the results indicated *L. paraplantarum* FT259 is a potential probiotic and the production of bacteriocin may be an interesting feature for food applications.

1. Introduction

The first observation of beneficial roles of bacteria was done by Élie Metchnikoff, the Nobel Prize winner in medicine, 1908. That researcher hypothesized that replacing or diminishing the number of 'putrefactive' bacteria in the gut with lactic acid bacteria could lead to a normalized bowel health and prolonged life [1–3]. The term "probiotic" means "for life" and it was first used by Lilly and Stillwell in 1965 [1,2]. The concept of probiotics changed over the years, but in 2001, a consensus definition of probiotics was adopted as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [1].

Some authors argue that genetically engineered and non-viable microbes should also be considered as probiotics, since in some cases, they may exhibit beneficial effects equal to live microbes [4–7]. Recently, Maeda et al. [4] examined the effect of the oral administration of heat-killed *Lactobacillus plantarum* L-137 (HK-LP) to avoid influenza virus infection in mice. Surprisingly, they found that in early stage of infection, the viral titers in the lung were significantly lower in mice treated with HK-LP than in controls.

Probiotics have been recognized to affect positively the treatment of several medical conditions such as intestinal inflammation, Crohn's disease, bacterial vaginosis, tooth decay, respiratory infections, antibiotic-related diarrhea, pregnancy-related and urinary tract infections, colorectal cancer, constipation and allergic diseases [3,8–11].

To qualify a microorganism as a probiotic, certain criteria have to be fulfilled: (i) identification at genus, species, and strain level, (ii) production of antimicrobial substances (e.g. bacteriocins), (iii) safety

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for food and clinical use, (iv) survival during intestinal passage, (v) adhesion to mucosal surfaces, (vi) colonization of the human intestine or vagina (at least temporarily), (vii) inhibition of pathogenic bacteria, (viii) clinically documented and validated health effect and (ix) stability during processing and storage [3,10–12].

Probiotics may produce bacteriocins, which are defined as ribosomally synthesized antimicrobial peptides, produced as a defense response and generally active against closely related bacteria [13]. These compounds have also the potential to be used as safe and natural food biopreservatives, as they are produced by beneficial microbes and are destructed by gastro-intestinal tract (GIT) proteolytic enzymes [14,15].

There is an increasing demand for foods containing probiotic bacteria and most of commercial probiotic food products have 10^6 CFU/g up to 10^{12} CFU/g [3,16,17]. Probiotic microorganisms are often incorporated into dairy products, but, there is also great interest for non-dairy-based products such as ice creams, nutrition bars, breakfast cereals, infant formulas, as well as supplements in the form of tablets, capsules and freeze-dried preparations [2,18,19]. To incorporate probiotics in foods, adequate technological process are needed to maintain viability under diverse conditions of acidity, oxygen level, presence of naturally or artificially added antimicrobial substances and nutrient availability [8,20].

Most probiotics belong to *Lactobacillus* and *Bifidobacteria* genera and, the former is the most abundant member of the group of lactic acid bacteria (LAB). Many lactobacilli are used as starter cultures for manufacturing cheeses, yoghurt, sourdough breads, silage, table olives, sauerkraut, fermented fish and sausages. Lactobacilli play a role as natural biopreservatives in non-fermented vegetables [21–23] and several studies have demonstrated the health benefits of lactobacilli strains as probiotics [8,16,19,22]. However, to our knowledge, there is only one report on the probiotic potential of a *Lactobacillus paraplantarum* strain [24]. *L. paraplantarum* is a facultative heterofermentative rod shaped Gram-positive bacterium that grows from 15 °C to 37 °C, with NaCl concentrations up to 8% and it is closely related to *L. plantarum* and *Lactobacillus pentosus* [25].

In this study, molecular biology techniques were employed to identify an isolate obtained from a Brazilian semi-hard Minas cheese and that presented antilisterial properties. Bacteriocin production and probiotic potential were also evaluated for the cheese isolate.

2. Materials and methods

2.1. Bacterial strains

FT259, a rod-shaped lactic acid bacterium, was selected among several Gram-positive and catalase negative bacteria previously isolated from Brazilian raw cow's milk and cheese, due to its antilisterial activity. The isolate obtained from Brazilian semi-hard Minas cheese was grown at 37 °C in MRS broth (De Man, Rogosa, Sharpe, Oxoid, UK). *Listeria monocytogenes* IAL 633 was grown at 37 °C for 24 h in BHI broth (Brain Heart Infusion, Oxoid), and it was used as an indicator strain for antagonistic tests. Other strains used to determine the spectrum of inhibitory activity are listed in Table 1. The strains were maintained at −80 °C in BHI or MRS broth (Oxoid) containing 20% (*v*/*v*) glycerol (Synth, Brazil).

2.2. Phenotypic identification of the isolate FT259

It was conducted according to Cogan et al. [26] and for that, gas production from glucose was tested with Durham tubes, using MRS broth (Oxoid), without citrate, at 25 °C for 48 h. Regular MRS broth was also used to evaluate the ability of isolate FT259 to grow in the presence of NaCl (Synth, Brazil) at concentrations of 20, 40 and 65 g/l and at 15 °C (5 days) and 45 °C (48 h).

2.3. Genotypic identification of the isolate FT259

The genomic DNA of isolate FT259 was extracted and purified with Illustra bacteria genomic Prep Mini Spin Kit (GE Life Sciences, Sweden) and used for molecular identification. Polymerase chain reaction (PCR) was carried out with 84 μl of Taq Platinum Blue (Invitrogen, USA), 30 pmol of each primer (forward and reverse) and, 300 ng of target DNA. 16S rDNA gene was amplified using the primers 27F and 1492R [27,28] (Invitrogen). The thermal cycling used was: denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min, for a total of 30 cycles. The PCR products were analyzed in 1% (*w*/*v*) agarose gel and purified using Illustra GFX PCR DNA and Gel Band Purification (GE Life Sciences). They were sequenced using ABI 3730 DNA Analyzer (Applied Biosystems, USA) with BigDye Terminator v3.1 Cycle Sequencing Reagent (Applied Biosystems) at The Human Genome Research Center (HGRC) of Universidade de São Paulo, São Paulo,

Table 1
Strains used during this study.

Organism	Source	Culture media	Incubation temperature
Carnobacterium maltaromaticum A9b-	Danish Institute for Fisheries Research[a]	BHI[e]	25 °C
Cronobacter sakazakii ATCC 29004	American Type Culture Collection[b]	BHI	37 °C
Enterobacter aerogenes CDC 1535	Centers for Disease Control and Prevention[c]	BHI	37 °C
Escherichia coli CDC 02A.2B	Centers for Disease Control and Prevention	BHI	37 °C
Klebsiella pneumoniae ATCC 10031	American Type Culture Collection	BHI	37 °C
Lactobacillus sakei ATCC 15521	American Type Culture Collection	MRS[f]	25 °C
Listeria innocua ATCC 3309	American Type Culture Collection	BHI	37 °C
Listeria monocytogenes IAL 633	Instituto Adolfo Lutz[d]	BHI	37 °C
Listeria monocytogenes ATCC 19115	American Type Culture Collection	BHI	37 °C
Proteus mirabilis CDC 305	Centers for Disease Control and Prevention	BHI	37 °C
Pseudomonas aeruginosa ATCC 14502	American Type Culture Collection	BHI	37 °C
Salmonella enterica subsp. Enterica ATCC 13076	American Type Culture Collection	BHI	37 °C
Staphylococcus aureus ATCC 29213	American Type Culture Collection	BHI	37 °C
Staphylococcus epidermidis ATCC14990	American Type Culture Collection	BHI	37 °C

[a] American Type Culture Collection, Manassas, USA.
[b] Danish Institute for Fisheries Research, Department of Seafood Research, Lyngby, Denmark.
[c] Centers for Disease Control and Prevention, Atlanta, USA.
[d] Instituto Adolfo Lutz, São Paulo, Brazil.
[e] BHI broth (Brain Heart Infusion).
[f] MRS broth (De Man, Rogosa, and Sharpe).