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Characterization and evaluation of lactic acid bacteria isolated from goat milk

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ABSTRACT

The aim of this study was to characterise and select Lactic Acid Bacteria (LAB) from goat milk with potential probiotic use and to evaluate the safety of these cultures in artisanal cheeses. The isolates of LAB were subjected to simulation of tolerance to the gastrointestinal tract, haemolytic test, antimicrobial susceptibility, antibacterial activity, EPS production, gas production, evaluation of proteolytic activity, diacetyl production and tolerance to NaCl. The genus and species of the selected LAB isolates were confirmed using molecular identification. Three goat cheeses (1, 2 and control) were manufactured to evaluate the inhibitory action of LAB against *Escherichia coli*. Subsequently, all cheese samples underwent bacterial enumeration and physical–chemical analyses. Statistical analysis was performed. UNIVASF CAP 14 and 20 were differentiated by survival up to pH 2 and pancreatin, resistance to NaCl and antibacterial activity against *Klebsiella pneumoniae*. UNIVASF CAP 4 and 29 were characterised by resistance to intestinal juice and antibacterial activity against *Salmonella* Typhi and *Listeria monocytogenes*. UNIVASF CAP 27, 38, 43 and 139 exhibited diacetyl production, antibacterial activity against *Bacillus cereus*, *Staphylococcus aureus* and *Enterococcus faecalis*. UNIVASF CAP 35 and 138 were characterised by proteolytic activity, EPS production, antibacterial activity to *E. coli* and *Shigella flexneri*. A cocktail of these 10 isolates with potential probiotic properties were inoculated in artisanal goat cheese and improved microbiological safety of product against *E. coli*.

1. Introduction

Lactic Acid Bacteria (LAB) are indigenous habitants of the human Gastro Intestinal Tract (GIT) and have a long history of use in foods and fermented products as starter cultures (Collado, Meriluoto, & Salminen, 2007). A variety of microorganisms, typically food grade lactic acid bacteria (LAB), have been evaluated for their probiotic potential and are applied as adjunct cultures in various types of food products or in therapeutic preparations (Rodgers, 2008; Zago et al., 2011). Numerous health benefits of LAB have made them promising probiotic candidates and being extensively studied to explore their safety and other desirable properties (Iranmanesh, Ezzatpanah, & Mojgani, 2014) and contribute to add value to products. New product categories, and thus novel and more complex raw materials with regard to probiotics technology, are certainly a key research and development area for the functional foods market (Coman et al., 2012).

Brazil has the world's 7th largest goat herd. The herd is concentrated in the northeast, in the semi-arid region, especially in the states of Bahia and Pernambuco (FAO, 2014; IBGE, 2011). The herding of goats is critical to the region. From an economic perspective, due to the growing consumer interest in functional food, goat milk has great potential because the product possesses unique biological properties, such as high digestibility, distinct alkalinity, high buffering capacity and therapeutic values in medicine (Park, 2009; Park & Haenlein, 2006). The second aspect that affects the demand for goat milk is the interest connoisseurs have shown in goat milk products, especially cheeses and yoghurt, in many countries (Haenlein, 2004). In the semi-arid region of Pernambuco, known nationally and internationally for its fine wines, the production of cheeses complements gastronomic tourism, supplementing the income of smallholders.

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However, to gain access to the market, new alternatives for the safety and processing of milk should be investigated. Goat milk and its derivatives are still associated with low microbiological and technological quality (Silva et al., 2013). Lactic Acid Bacteria, in addition to their probiotic properties, impede the growth of pathogenic and spoiling bacteria by competing for nutrients and starter-derived inhibitor compounds, such as lactic acid, hydrogen peroxide and bacteriocins (Stiles & Holzapfel, 1997) thereby technically improving the quality of the milk. Therefore, this study was conducted to characterise and select LAB from goat milk with potential probiotic use and to evaluate the safety of these cultures in artisanal cheeses.

2. Material and methods

2.1. Raw milk samples and microbiological analyses

Samples of raw goat milk were collected from extensively small-scale dairy farms with mixed-breed goats in six municipalities (Juazeiro, Uauá, Senhor do Bonfim, Curaçá, Jaguarari and Petrolina) of the San Francisco Valley, Northeast of Brazil; seven farms were randomly selected from each county for a total of 42 milk samples. Trained research personnel aseptically collected the milk samples (150 mL) from containers, in which the product was stored after the daily milking, using a stainless steel ladle (ethanol-sanitised) and sterile plastic bottles. Samples were collected in triplicate. Samples were stored at 4 °C until delivery to the laboratory for enumeration of Lactic Acid Bacteria (LAB) on the same day.

In microbiological analysis for LAB, 0.1 mL aliquots of goat milk and dilutions (10^{-1} to 10^{-8}) were transferred to plates with a specific medium, de Man, Rogosa and Sharpe (MRS, Himedia). The plates were incubated at 37 °C for 72–96 h in aerobic conditions described by Lima et al. (2009). Six colonies per sample were randomly picked from MRS agar plates, reaching a total of 252 colonies. Basic characterisation of the isolates was performed through Gram reaction, morphology, motility, catalase (H_2O_2, 3% vol/vol) and cytochrome–oxidase activities. From this, fifty isolates were selected for the tests described in the study.

2.2. Simulation of tolerance to the gastrointestinal tract (GIT)

To simulate survival in the GIT, the 50 pre-selected LAB isolates were tested in an *in vitro* model that chemically replicates physiological conditions. In the tolerance to low pH test, the pH of MRS broth was adjusted to 2.0 with 1 N hydrochloric acid. In the bile tolerance test, the medium was MRS broth supplemented with 2.0% bovine bile (Sigma-Aldrich). For the pancreatic fluid tolerance test, 150 mM $NaHCO_3$, 1.9 mg/mL pancreatin (Sigma-Aldrich) and pH 8 were used, as suggested by Rönkä et al. (2003). To test the tolerance to intestinal juice, in accordance with Bao et al. (2010), 0.1 g of trypsin (Sigma-Aldrich) and 1.8 g of bile salts were added to a sterile solution of 1.1 g of sodium bicarbonate and 0.2 g of sodium chloride in 100 mL distilled water. The pH of the solution was adjusted to 8.0 with 0.5 M sodium hydroxide and sterilised by filtering through a 0.45 μm membrane.

The strains for each test were initially cultured for 24 h in MRS broth at 37 °C. After this period, the strains were centrifuged for 5 min and washed 3 times in Phosphate Buffered Saline (PBS) pH 7.0. Individual tubes containing each strain and test medium were incubated for 3 h at 37 °C in a water bath. Viability was evaluated in duplicate at 0 and 3 h on MRS agar. Survival rates were calculated according to the following equation:

$$\text{Survival rate}(\%) = \frac{\log \text{CFU } N_1}{\log \text{CFU } N_0} \times 100$$

where N_1 represents the total viable count of strains at time 3 h, and N_0 represents the total viable count of strains at time 0 h.

2.3. Characterisation of virulence factors of microorganisms

The production of DNase was determined by addition of aliquots of 1 μl of the isolates in drops on the DNase test agar surface with toluidine blue at 0.1%, the plates were then incubated at 37 °C for 48 h. Positive result for the presence of DNase was indicated by the formation of rosy halos around the colonies.

For the coagulase test, 0.3 mL of each culture of isolates was transferred to sterile tubes containing 0.3 mL of rabbit plasma (plasma-Coagu LaborClin) and incubated at 36° C ± 1 °C for 6 h. The formation of a large and organised clot or total coagulation was considered a positive result for the test.

For hemolysis test, the LAB isolates were cultured in MRS broth at 37 °C for 15 h and then transferred onto blood agar (Himedia) plates supplemented with 5% defibrinated whole horse blood (Oxoid). After 48/72 h, the haemolytic reaction was evaluated by observing both the partial hydrolysis of red blood cells and the production of a green zone (α-hemolysis), as well as the total hydrolysis of red blood cells producing a clear zone around bacterial colony (β-hemolysis) or no reaction (γ-hemolysis).

In antimicrobial susceptibility testing, the antimicrobials chloramphenicol (30 μg/disc), oxacillin (1 μg/disc), vancomycin (30 μg/disc), tetracycline (30 μg/disc), ciprofloxacin (5 μg/disc) and penicillin G (UI/disc) were used according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2012). LAB isolates were grown on MRS agar for 24 h at 37 °C. The strains were inoculated in 4 mL of sterile distilled water to achieve the n° 0.5 McFarland turbidity standard (Probac, Brazil). A swab was used to spread the inoculum across the surface of Muller Hinton agar (Merck), and then antibiotic disks were applied to the plate. Antimicrobial susceptibility was assessed by measuring the zone of inhibition of bacterial growth after incubation for 24 h at 37 °C. *Escherichia coli* ATCC 25922 were used for quality control testing.

2.4. Agar disc diffusion – antibacterial activity

The inhibitory effect of different strains of LAB over pathogens was tested using the agar disc diffusion method. *E. coli* (ATCC 8739), *Salmonella* Typhi (ATCC 6539), *Listeria monocytogenes* (ATCC 7644), *Staphylococcus aureus* (ATCC n° 25923), *Shigella flexneri* (ATCC n° 12022), *Enterococcus faecalis* (ATCC n° 19433), *Bacillus cereus* (ATCC n° 11778) and *Klebsiella pneumoniae* (ATCC n° 13883) were grown in Tryptone Soya Agar (TSA, Himedia) supplemented with 0.6% yeast extract for 24 h at 37 °C. Each pathogen was suspended in 4 mL of sterile water and standardised to approximately 10^8 CFU/mL, comparable to the standard turbidity n° 0.5 of McFarland. A sterile swab was soaked in the suspension and applied to the surface of a plate with TSA agar. After the inoculum was added and allowed to absorb, and 6 mm sterile paper filter discs (Whatmann n°1) moistened with 20 μl of cell free supernatant obtained by centrifugation (2500 × g/10 min) from each isolate of LAB in exponential growth phase were added. The susceptibility of pathogens to the discs was assessed by measuring the zone of inhibition of bacterial growth around the discs (radius – mm) after incubation for 24 h at 37 °C. A clear zone of inhibition of at least 2 mm radius was recorded as positive. The experiment was performed in triplicate.