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Effect of indigenous lactic acid bacteria isolated from goat milk and cheeses on folate and riboflavin content of fermented goat milk

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ABSTRACT

The aim of the present study was to isolate riboflavin- and folate-producing lactic acid bacteria (LAB) from raw goat milk and cheeses, identify them and evaluate their capability to increase the content of these vitamins in fermented goat milk, envisaging potential application for development of novel bio-enriched goat milk products. From 179 LAB isolates obtained, 151 (84%) were capable to produce at least one of these vitamins. The average production of total folate and riboflavin in vitamin-free media was 138 ng/ml and 364 ng/ml, respectively. Based on RAPD-PCR and 16S rDNA sequencing, 19 different genetic profiles were obtained and 7 species were identified, with predominance of *Streptococcus thermophilus* (7), *Weissella paramensenteroides* (6), and *Lactococcus lactis* (4). Seven isolates that produced folate and riboflavin above the average were tested for vitamins production in UHT goat milk. Five isolates were capable to increase four to six fold the original amount of folate in the milk in 24 h. Folate content in milk fermented with *Lc. lactis* FP368 for 24 h was 313 ng/ml that could provide 19% of the recommended daily intake of this vitamin. In addition, *St. thermophilus* FP268 increased the folate concentration in the milk almost four fold in only 6 h.

1. Introduction

There is an increasing tendency worldwide for the consumption of goat-milk derived products because of their organoleptic and nutritional properties (Park, Juárez, Ramos, & Haenlein, 2007; Schirru et al., 2012). The contribution of goat milk to local economies and nutrition of certain populations is indisputable in various countries, especially in certain regions of the Mediterranean, Middle Orient, Oriental Europe and South America (Ribeiro & Ribeiro, 2010). The physical-chemical characteristics of goat milk allow their use in a wide range of products besides fluid milk, that can be consumed either raw, pasteurized or UHT, such as cheese, butter, yogurt, ice-cream and sweets, amongst others.

Lactic acid bacteria (LAB) are a group of Gram-positive, catalase-negative microorganisms with similar metabolic and physiological characteristics. In general, LAB are classified by FDA as being "generally recognized as safe" or by the EFSA as having the "qualified presumption of safety" designation because of their long history of use in the elaboration of fermented foods. LAB are frequently used as starter or adjunct cultures because of their capacity to increase the safety of foods, provide texture and flavor, and produce beneficial compounds such as organic acids and vitamins (Carr, Chill, & Maida, 2002; Salvetti, Torriani, & Felis, 2012).

Although most LAB are auxotrophic for vitamins, there is increasing evidence that certain strains have the capacity to produce specific water soluble vitamins such as those found in the B-group (Capozzi, Russo, Dueñas, López, & Spano, 2012; LeBlanc et al., 2011, 2014, 2013; LeBlanc, Savoy de Giori, Smid, Hugenholtz, & Sesma, 2007; LeBlanc, Taranto, Molina, & Sesma, 2010). The most studied vitamins that are produced by LAB are folates and riboflavin because of their importance in human health and the frequency of deficiencies found in populations world-wide even in countries where obligatory fortification programs exist (ENNyS, 2007).

Riboflavin (vitamin B2) is an essential component of basal cellular metabolism and is the precursor of the enzymes flavin mononucleotide (FAD) and flavin adenine dinucleotide (FMN) that

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are electron acceptors and involved in many oxidation–reduction reactions (Powers, 2003). According to the World Health Organization, the recommended dietary allowance (RDA) for humans is between 0.9 and 1.6 mg (FAO/WHO, 2002). Although present in a wide variety of foods, deficiency of riboflavin (arriboflavinosis) or sub-clinical deficiencies are present in both developing and highly industrialized countries (O'Brien et al., 2001). Folates (vitamin B9) are involved in many metabolic functions such as replication, repair and methylation of DNA and synthesis of nucleotides, amino acids and vitamins (LeBlanc et al., 2010). Because folate deficiencies can cause serious health problems, such as defects in the formation of the neural tube (NTD), megaloblastic anemias and are correlated with Alzheimer's and coronary diseases and colon and breast cancer, many countries have adopted mandatory fortification of staple foods with folic acid. However, many countries have not adopted national fortification programs because of possible unwanted side effects associated with an excess consumption of folic acid (pteroylglutamic acid) that can mask early hematological manifestations of vitamin B12 deficiency (Morris & Tangney, 2007). Since natural folates, such as 5-methyltetrahydrofolate, that are normally found in foods and sometimes produced by microorganisms do not mask B12 deficiency (Scott, 1999), this folate form would be a more efficient and secure alternative than supplementation with folic acid (Lamers, Prinz-Langenohl, Brämswig, & Pietrzik, 2006).

Previous studies have shown that the concentration of riboflavin and folates is frequently increased in certain fermented milk products such as yogurts, buttermilk and cheeses due to the production of these vitamins by indigenous LAB (LeBlanc et al., 2011). For this reason, these authors have proposed that the adequate selection of vitamin producing LAB could be an economically feasible alternative to chemical fortification and production of high vitamin containing products. The isolation of LAB from the ecological niche where these are to be used is logical since these microorganisms are adapted to the specific food matrix, increasing their capability to grow and produce beneficial compounds.

There are no published reports on the occurrence of vitamin-producing LAB in goat milk derived products. Thus the objective of the current study was to isolate folate and riboflavin producing LAB from raw goat milk and goat cheeses, identify them and test their capability to increase the content of these vitamins in fermented goat milk, envisaging their potential application for elaboration of novel goat milk products bioenriched with the natural form of these vitamins.

2. Materials and methods

2.1. Isolation of LAB from goat milk and cheeses

Raw goat milk samples (n = 47) were obtained from the School of Veterinary Medicine and Animal Science, University of Sao Paulo, Brazil, and from Sitio Rekantinho goat dairy farm, located in the city of Ibiuna, SP, Brazil. Goat cheese samples (n = 5) were obtained from commercial establishments in the city of Sao Paulo, Brazil.

To isolate potential vitamin producing lactic acid bacteria, the technique described by Schirru et al., 2012 was slightly modified. In the case of goat milk, samples were serially diluted with 0.1% (w/v) peptone water. For cheese samples, a 25 g portion was homogenized with 225 ml 0.1% (w/v) peptone water using a stomacher and serial dilutions were also made. Diluted samples were then plated on MRS (Oxoid, Basingstoke, UK) and M17 (Difco, NJ, USA) containing 10% (w/v) lactose agar plates and incubated at either 30, 37 or 42 °C during 48 h under anaerobic conditions, using Anaerogen sachets (Oxoid, Basingstoke, UK). Five colonies were randomly selected from each plate and inoculated in MRS broth at 37 °C during 24 h. These isolates were then plated again on MRS agar to ensure purity and presumable LAB isolates (Gram-positive, catalase-negative, non-motile rods and cocci) were selected for further characterization and identification. Isolates fulfilling these requirements were stored at −20 °C in glycerol (20% v/v) for further analysis.

2.2. Selection of presumptive folate producing LAB

After activation of the isolates in MRS broth at 37 °C for 24 h, the cultures were washed three times with saline solution (0.85% w/v NaCl), resuspended in this solution to the original culture volume, and used to inoculate 4% v/v folate-free Folic Acid Casei Medium (FACM, Difco, NJ, USA). The cultures were incubated without agitation at 37 °C for 24–72 h, and submitted to the same washing-resuspension procedure. The resulting solution was used to inoculate at 2% v/v fresh FACM. The cultures presenting growth, indicated by increased visible turbidity, were submitted to this last step seven times. Those strains that did not grow in FACM were tested again for growth in FACM added of vitamin, and those presenting growth in this medium were discarded. After the 7th passage, two samples of the cultures were taken after 24 h incubation to determine the concentration of extra- and intra-cellular folate, as described previously (Juarez del Valle, Laiño, Savoy de Giori, & LeBlanc, 2014; Laiño, LeBlanc, & Savoy de Giori, 2012). Briefly, samples of LAB-containing FACM (500 μL) were mixed with equal amount of the protecting buffer (0.1 mol/L phosphate buffer, pH 6.8, containing 1.5% (m/v) ascorbic acid to prevent vitamin oxidation and degradation), and immediately centrifuged at 5000× *g* for 5 min (Sigma 1–14k, Ostrode, Germany). The supernatant corresponded to extracellular folate sample and the pellet, resuspended in 500 μL of protecting buffer, corresponded to intracellular folate sample. Both samples were then boiled (100 °C) for 5 min, centrifuged for 6 min at 10 000× *g* (Sigma 1–14k, Ostrode, Germany), and stored at −70 °C until used for folate determinations.

In culture media samples, total vitamin concentrations were calculated summing the intra- and the extra-cellular vitamins concentrations. It is important to clarify that extra-cellular vitamin concentrations (from the supernatant) might be a consequence of cellular lysis or leakage and not necessarily secretion of the vitamins to the culture media.

2.3. Selection of presumptive riboflavin producing LAB

The procedure was the same described for folate, except that cultures were grown in riboflavin-free Riboflavin Assay medium (RAM, Difco). After washing, the resulting culture suspensions were used to inoculate (4% v/v) fresh RAM, and those cultures showing increased turbidity were submitted to the incubation and washing-resuspension steps four times. The strains that did not grow in RAM were discarded. After the forth incubation, two samples of the cultures were taken to determine the concentration of extra- and intra-cellular riboflavin, as described previously (Juarez del Valle et al., 2014; Laiño et al., 2012). The concentration of riboflavin in the RAM medium was determined mixing 500 μL of the cultures with 500 μL of 1% v/v acetic acid. The mixtures were immediately centrifuged for 5 min at 10 000× *g*. The supernatants were collected for determination of extracellular riboflavin and the pellets were resuspended in 500 μL of 1% v/v acetic acid for determination of intracellular riboflavin. Both samples were heated at 100 °C for 5 min, centrifuged for 6 min at 10 000× *g*, and stored at −70 °C.