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Influence of the combination of probiotic cultures during fermentation and storage of fermented milk

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ABSTRACT

The objective of this study was to evaluate the influence of different combinations of cultures, *Lactobacillus acidophilus* — La and *Bifidobacterium animalis* subsp. *lactis* — Bb as pure cultures or in co-culture with *Streptococcus thermophilus* — St, on fermented milk during fermentation, including changes to the acidification profile, organic acid production and lactose consumption and during 28-day storage at 4 °C, in terms of bacteria viability, syneresis, sensory properties, organic acid content and viability under simulated *in vitro* gastrointestinal conditions. La culture was showed the lowest acidification rate (V_{max}) values, whereas the pure St culture showed the highest V_{max} values. During fermentation, Bb produced the largest amount of acetic acid, and only La was able to metabolize citric acid. Syneresis decreased during storage for all treatments. Counts of *S. thermophilus* and *B. animalis* subsp. *lactis* remained stable during the storage period in all treatments, while the counts of *L. acidophilus* decreased over time only in the case of the La treatment. The simulation of probiotic resistance to gastrointestinal conditions indicated that bifidobacteria possess a greater tolerance to acid and bile than the lactobacilli strain. The La treatment resulted in lower scores for all attributes in both periods of sensory analysis. When lactic acid was present in smaller quantities and citric acid was present in larger amounts, the scores regarding flavor and overall acceptability attributes were higher. Depending on the combination of microorganisms used in fermented milk manufacturing, it had positive or negative impacts on the product's characteristics.

1. Introduction

Fermented milk is the result of milk acidification through the metabolic activity of lactic acid bacteria (LAB), which causes important physicochemical, sensory and microbiological changes in fermented milk products. In recent years, probiotic bacteria have been added to fermented milk in order to meet consumers demand. These bacteria promote beneficial health effects for the host, if administrated regularly and in adequate amounts (FAO/WHO, 2002). In order to exert their functional properties, probiotics need to be delivered to the desired sites in an active and viable form. Nevertheless, no general agreement has been reached regarding the recommended levels, and suggested levels have ranged from 10^6 CFU/mL to over 10^7 and 10^8 CFU/mL (Vasiljevic & Shah, 2008). Several beneficial health effects have been attributed to probiotic fermented milk consumption, including reduction of total cholesterol and LDL-c (Ejtahed et al., 2011), improvement of immune system during exhausting physical-exercise (Lollo et al., 2013), modulation of intestinal microbiota (Wang et al., 2012) and reduction of *Helicobacter pylori* loads (Yang & Sheu, 2012).

Lactobacillus acidophilus and *Bifidobacterium animalis* subsp. *lactis* are the lactic acid bacteria that are most frequently used as probiotics. These bacteria grow slowly in milk because they lack essential proteolytic activity and for this reason they are usually combined with *Streptococcus thermophilus*. Thus, the use of these combinations allows dairy processors to produce fermented dairy products with the desired technological characteristics, as well as with potential nutritional and health benefits (Candy, Heath, Lewis, & Thomas, 2008).

However, despite some fields concerning probiotic foods have been extensively explored, including the therapeutic properties (Sharma & Devi, 2014) and the effect of the ingredients on their viability (Marafon et al., 2011), only few studies have investigated the interactions among probiotic strains during fermentation and storage. Therefore, it is important to study the interactions, whether beneficial or unfavorable, between the species of probiotics most frequently used in the manufacturing of fermented milk, such as *L. acidophilus* and *B. animalis*, along with *S. thermophilus* (Saccaro, Tamime, Pilleggi, & Oliveira, 2009; Vinderola, Mocchiutti, & Reinheimer, 2002).

The sensory profile of fermented milk is also directly influenced by the metabolic activity of the bacteria, which interact strongly with the components of the media to convert certain metabolic products during

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the growth, particularly organic acids (Serra, Trujillo, Guamis, & Ferragut, 2009). Furthermore, the species of bacteria used in fermented milk manufacturing has an impact on the physical characteristics of the product. Gel formation is the most important functional property of fermented dairy products, and the rheological characteristics of their gel are affected by the starter culture selected. When the fermentation process does not occur properly, the formation of the protein network will be disorganized and the final product may have technological defects, such as low water holding capacity and syneresis (Lucey, 2004).

The possible interactions among the strains selected to manufacture a dairy product should be taken into account to select the best combination(s) and to optimize their technological performance in the process and their survival in the products during cold storage (Saccaro et al., 2009; Vinderola et al., 2002). Although previous researches have studied the combination of probiotic cultures to produce fermented milk (Oliveira, Perego, Converti, & Oliveira, 2009), in this study the aim was to evaluate the changes in fermented milk with different culture combinations during fermentation and 28-day storage at 4 °C, focusing in probiotic survival under gastrointestinal conditions and sensory acceptability.

2. Materials and methods

2.1. Inoculum preparation

Three commercial starter freeze-dried strains were used in this study — specifically, *S. thermophilus* TA040 (St) (Danisco, Sassenage, France), *B. animalis* subsp. *lactis* BB-12 (Bb) and *L. acidophilus* La-5 (La) (Chr. Hansen, Valinhos, Brazil). The inoculum of starter cultures was prepared adding 150, 800 or 1000 mg of the St, Bb and La pure freeze-dried strains, respectively, to 50 mL of sterilized skim milk (10% w/v), followed by activation at 42 °C for 30 min before use. One milliliter of each pre-activated culture was inoculated in 250 mL of milk according to the treatment, which allowed for initial counts of approximately 6 log CFU/mL after milk inoculation.

2.2. Fermentation and kinetic parameters

Skim powder milk (Nestlé, Araçatuba, Brazil) was reconstituted to 13% (w/w) in distilled water. Reconstituted milk was thermally treated at 90 °C for 10 min in a water bath and distributed in 500-mL sterile bottles inside a laminar flow chamber, and then stored at 4 °C for 24 h before use. On the day of fermentation, the milk bottles were warmed to 42 °C and the cultures were added (0.4%) according to the treatment. In addition, 40-mL aliquots of the inoculated milk were aseptically distributed in 50-mL sterile flasks. One flask was prepared for each sampling time and it was used for all analyses. The pH value, organic acids and lactose contents were determined in the incubated milk before fermentation, at pH values of 6.0, 5.5, 5.0 and 4.6 (end of fermentation).

Five treatments were conducted in three separate independent trials: St (composed of *S. thermophilus* TA040), La (composed of *L. acidophilus* La-5), Bb (composed of *B. animalis* subsp. *lactis* BB-12), StBb (composed of *S. thermophilus* TA040 + *B. animalis* subsp. *lactis* BB-12), and StLa (composed of *S. thermophilus* TA040 + *L. acidophilus* La-5). After inoculation, flask samples were transferred to a water bath that was connected to a CINAC (Cynetique d'acidificacion, Alliance Instruments, Frepillon, France) system that allows for the continuous measurement and recording of pH values, as well as the evaluation of the acidification kinetics throughout the run. Batch fermentations were performed at 42 °C up to a pH of 4.6, which was selected as the condition for stopping fermentation. Afterward, fermented milk was transferred to an ice bath and cooled down to 15 °C. The gel was broken using a stainless steel perforated disk with up and down movements for approximately 1 min. The product was put in 80-mL sterile plastic cups, and stored at 4 °C for 28 days.

From the data collected during fermentation, the acidification rate (V_{max}) was calculated as the time variation of the pH (dpH/dt) and expressed as 10^{-3} pH units/min. During the incubation period, the following kinetic parameters were also calculated: (i) t_{max} (h), time at which V_{max} was reached; (ii) $t_{pH5.0}$ (h), the time required to reach pH 5.0; and (iii) $t_{pH4.6}$ (h), the time required to reach pH 4.6 (*i.e.*, to complete fermentation).

2.3. Post-acidification and spontaneous whey separation

Fermented milk post-acidification was determined in triplicate 1 day after the fermentation was complete and after 14 and 28 day-storage at 4 °C using a pHmeter model PG1800 (Gehaka, São Paulo, Brazil). The susceptibility of fermented milk to syneresis was determined using a drainage method as described by Hassan, Frank, Schmidt, and Shalab (1996).

2.4. Microbiological analyses

Bacterial counts of each treatment were carried out in duplicate after 1, 14 and 28 days of storage. *S. thermophilus* colonies were enumerated in M17 agar (Himedia, Mumbai, India), whereas those of *L. acidophilus* were carried out in MRS agar (Acumedia, Lansing, USA) with a bile solution added (0.15%) (Sigma, St. Louis, USA), and those of *B. animalis* subsp. *lactis* in MRS agar (Acumedia, Michigan, USA) with 0.2 and 0.3% lithium chloride and sodium propionate (Sigma), respectively. The M17, MRS-bile and MRS-LP media were prepared according to IDF (1997), IDF (1995), and Vinderola and Reinheimer (1999), respectively. Plates of *S. thermophilus* and *L. acidophilus* were incubated under aerobic conditions at 37 °C for 72 h. Plates of *B. animalis* subsp. *lactis* were incubated under anaerobic conditions provided by Anaerobac (Probac, São Paulo, Brazil). Cell concentration was expressed as log CFU/mL of fermented milk.

Microbiological safety of fermented milk samples was examined before the sensory evaluation to evaluate the yeasts and molds using Yeast and Mold Compact Dry YM (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) incubated at 25 °C, as well as *Escherichia coli* and total coliforms using Compact Dry CF and EC (Nissui Pharmaceutical Co) incubated at 45 °C and 35 °C, respectively.

2.5. Survival of probiotics under simulated gastrointestinal conditions

The survival of probiotics under simulated gastric and enteric conditions was evaluated in duplicate after 1, 14 and 28 days of refrigerated storage, according to the method used by Buriti, Castro, and Saad (2010). In the gastric phase, fermented milk samples were diluted in a 0.5% NaCl solution, and 10 mL was transferred to 3 sterile flasks. The pH was adjusted to 1.4–1.9 with 1 N HCl, and pepsin (Sigma-Aldrich, St. Louis, USA) and lipase (Aldrich Chemical Company, Milwaukee, USA) solutions were added to samples to reach a concentration of 3 g/L and of 0.9 mg/L, respectively. Flasks were incubated at 37 °C, with an agitation rate of approximately 150 rpm (Metabolic Water Bath Dubnoff MA-095, Marconi, Piracicaba, Brazil) for 2 h. In the enteric phase I, the pH of the samples was increased to 4.3–5.2 using an alkaline solution containing bile (Sigma-Aldrich) and pancreatin (Sigma-Aldrich) at concentrations of 10 g/L and 1 g/L, respectively, followed by incubation at 37 °C for 2 h under agitation. In the enteric phase II, the pH was increased to 6.7–7.5 using an alkaline solution containing bile and pancreatin (the concentration were adjusted to maintain the same used in enteric phase I), and samples were incubated again at 37 °C for 2 h under agitation. Enumeration of probiotics was performed in aliquots (1 mL) collected from duplicate samples after 30 min, 120 min, 240 min and 360 min. The media employed were the same as those described previously.