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Co-culture fermentation of peanut-soy milk for the development of a novel functional beverage

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ABSTRACT

Most of the commercial probiotic products are dairy-based, and the development of non-dairy probiotic products could be an alternative for new functional products. The peanut-soy milk (PSM[1]) was inoculated with six different lactic acid bacteria (LAB), including probiotic strains and yeasts and fermentation was accomplished for 24 h at 37 °C and afterwards, another 24 h at ±4 °C. The *Pediococcus acidilactici* (UFLA BFFCX 27.1), *Lactococcus lactis* (CCT 0360), *Lactobacillus rhamnosus* (LR 32) probiotic LAB, and the *Lactobacillus delbrueckii* subsp. *bulgaricus* (LB 340) yogurt starter culture reached cell concentrations of about 8.3 log CFU/mL during fermentation. However, these strains were not able to acidify the substrate when inoculated as pure culture. The *Lactobacillus acidophilus* (LACA 4) probiotic produced significant amounts of lactic acid (3.35 g/L) and rapidly lowered the pH (4.6). *Saccharomyces cerevisiae* (UFLA YFFBM 18.03) did not completely consume the available sugars in PSM and consequently produced low amounts of ethanol (0.24 g/L). In pure culture, *S. cerevisiae* (UFLA YFFBM 18.03), *L. rhamnosus* (LR 32), *L. acidophilus* (LACA 4), and *P. acidilactici* (UFLA BFFCX 27.1) promoted the increase of total amino acids (48.02%, 47.32%, 46.21% and, 44.07%, respectively). However, when in co-cultured, the strains consumed the free amino acids favoring their growth, and reaching the population of 8 log CFU/mL in PSM. Lactic acid production increased, and 12 h was required to reach a pH value of 4.3. In general, the strains were more efficient in the use of available carbohydrates and release of metabolites in co-cultured than in single culture fermentations. An average of 58% and 78% of available carbohydrates was consumed when single and co-cultures were evaluated, respectively. Higher lactic acid contents were found in a binary culture of *P. acidilactici* (UFLA BFFCX 27.1) and *L. acidophilus* (LACA 4), and by co-culture of *P. acidilactici* (UFLA BFFCX 27.1), *L. acidophilus* (LACA 4) and *S. cerevisiae* (UFLA YFFBM 18.03) (9.03 and 8.51 g/L, respectively). The final content of ethanol was 0.03% (*v*/*v*) or less, which classified the final beverage as non-alcoholic.

1. Introduction

The demand for alternatives to dairy products is growing due to problems with lactose intolerance, cholesterol content, allergenic milk proteins and desire for vegetarian alternatives (Granato et al., 2010). These are the major drawbacks related to the intake of dairy products, which makes the development of new non-dairy probiotic essential foods (Heenan et al., 2005; Rivera-Espinoza and Gallardo-Navarro, 2010; Yoon et al., 2004). Probiotic food contains high populations of probiotic microorganisms in its formulation. According to FAO (2001), probiotics are live microorganisms that confer benefits to the health of the host when administered in adequate amounts.

Non-dairy probiotic products represent a huge growth potential for the food industry, and may be widely explored through the development of new ingredients, processes, and products. There are a wide variety of traditional non-dairy fermented beverages produced around the world. Much of them are non-alcoholic beverages manufactured with cereals as the main raw material (Prado et al., 2008). Nevertheless, fruit juices, desserts and legume based products can also be used featuring probiotics (Cargill, 2008).

Many studies indicated that soy is a good substrate for probiotic bacteria (Champagne et al., 2009; Coda et al., 2012; Farnworth et al., 2007; Hou et al., 2000; Wang et al., 2003). Peanuts have also been described as a potential substrate for this use (Schaffer and Beuchat, 1986; Mustafa et al., 2009). Over the years, peanut milk has been successfully converted into low cost edible products with high nutritional value (Diarra et al., 2005). In this area, researchers have focused on peanut milk fermented foods, such as yogurt, buttermilk, ripened cheese analogs, and curd or "tofu" (Beuchat and Nail, 1978; Diarra et al., 2005; Isanga and Zhang, 2009; Lee and Beuchat, 1991; Schaffer and Beuchat, 1986; Yadav et al., 2010).

The use of two substrates at once may enhance the nutrient availability for the microbial population and affect their growth and metabolism (Granato et al., 2010). Research related with cereals, fruits and

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[1] PSM—peanut-soy milk.

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vegetable fermentation for production of functional foods utilize mainly single substrates for potentially probiotic LAB growth (Angelov et al., 2006; Charalampopoulos et al., 2003; Helland et al., 2004).

It is known that the use of probiotic cultures requires a large fermentation period to reach low pH values. However, the food industries always prefer short fermentation periods in order to increase plant output and reduce microbial contamination. The potential solution to this problem is the use of mixed cultures or co-cultures (Macedo et al., 1998). Mixed culture fermentations provide complex growth patterns that can also considerably affect the organoleptic and functional properties of the food. No information is available regarding the growth of probiotic bacteria in mixed cultures and how mixed LAB, and yeasts with probiotics will behave in PSM.

The development of a fermented PSM containing probiotic microorganisms in co-culture requires a strain selection showing ability to grow in the substrate, ability to compete, and/or establish a synergistic growth between strains. The aim of this work was to study the growth and acidification of PSM by *Lactobacillus rhamnosus* (LR 32), *Lactobacillus acidophilus* (LACA 4), *Lactobacillus delbrueckii* subsp. *bulgaricus* (LB 340), *Pediococcus acidilactici* (UFLA BFFCX 27.1), *Lactococcus lactis* (CCT 0360), and *Saccharomyces cerevisiae* (UFLA YFFBM 18.03); to select strains better adapted and with best characteristics for the fermented mixed milk; and to describe the development of an inoculum that rapidly ferments peanut and soy maintaining significant probiotic yields after refrigerated storage.

2. Materials and methods

2.1. Peanut-soy milk preparation

Peanut milk was prepared as previously described (Salunkhe and Kadam, 1989) with minor modifications. The seeds were sorted to remove discolored grains and any foreign material. Selected peanut seeds were roasted at 130 °C for 20 min in a forced air circulation oven. The seeds were peeled and weighed before being soaked in 0.5 g/100 mL $NaHCO_3$ for at least 12 h. The shelled seeds were then washed with running water and mixed with distilled water in a ratio of 1:5 [peanuts (g):water (mL)] and transferred to a stainless steel blender (Cemaf, São Paulo, Brazil) for 5 min. The resulting paste was filtered through a double-layered cheese cloth.

For the soymilk preparation, 250 g of selected beans was initially soaked in 1 L of distilled water for 16 h at 25 °C. Subsequently, 190 g of these moistened beans was soaked in 500 mL water in a stainless steel blender and mixed for 3 min. Then, the resulting slurry was filtered through double-layered cheese cloth and boiled for 5 min (Champagne et al., 2009).

The peanut and soybean milk were mixed in the 2:1–peanut milk: soymilk ratio. The heat treatment was carried out by pasteurization at 90 °C/20 min, followed by immediate cooling to 4 °C.

2.2. Preparation of cultures

The commercial probiotic cultures *L. rhamnosus* (LR 32) and *L. acidophilus* (LACA 4) were acquired from Danisco (Flora Fit, USA) and from Danisco (Yo Mix, Deutschland), respectively. The *L. delbrueckii* subsp. *bulgaricus* (LB 340) yogurt starter culture was purchased from Danisco (Yo Mix, France). The *P. acidilactici* lactic acid bacteria (UFLA BFFCX 27.1) was isolated from *caxiri* indigenous beverage (Santos et al., 2012). The *S. cerevisiae* (UFLA YFFBM 18.03) yeast was isolated from cocoa beans fermentations (Pereira et al., 2012). These strains belong to the culture collection of Microbial Physiology Laboratory/ Department of Biology, Federal University of Lavras (UFLA), Brazil. *Lc. lactis* was isolated from cocoa fermentations, which belong to the André Toselo Tropical Culture Collection (Campinas, SP, Brazil).

The commercial cultures activation was performed according to the manufacturer's instructions. Stock cultures for bacteria were prepared by mixing culture medium Man Rogosa Sharp broth (MRS, Merck Whitehouse Station, USA) and 40% glycerol (*w*/*v*) in the 1:1 proportion placing 1 mL in cryovials and freezing at −20 °C. For yeast, YPD agar (10 g/L yeast extract (Merck, Darmstadt, Germany), 10 g/L peptone (Himedia, Mumbai, India), 20 g/L glucose (Merck, Darmstadt,Germany), and 20 g/L agar (Merck, Darmstadt, Germany) pH 3.5 were mixed with 40% glycerol (*w*/*v*) in the 1:1 proportion placing 1 mL in cryovials and freezing at −20 °C. The glycerol solution was previously sterilized at 121 °C for 15 min (Champagne et al., 2009).

The preparation of inoculum for single and co-culture fermentation was performed by culturing 100 μL of each strain separately in 5 mL of sterile MRS broth (bacteria) and YPD pH 3.5 (yeast), then incubating at 37 °C and 30 °C, respectively. After 16 h and 24 h, the supernatant was removed and the cells were transferred to 50 mL MRS and YPD, respectively. The flasks were again incubated for 16 h (bacteria) and 24 h (yeast), the cells were subsequently transferred to 500 mL of the respective medium and then incubated in the same conditions described above. The cells were centrifuged for 7 min at 7000 rpm at 4 °C and the supernatant was removed. Cells were washed two times with sterile distilled water and inoculated in PSM with a population of 7 log CFU/mL. To perform the inoculation, OD growth kinetics were constructed by plotting the OD (600 nm) of suspensions minus the OD of non-inoculated media vs the time of incubation (Ruiz-Moyano et al., 2008). To calibrate the ODs against the cell concentration of the cultures, the viable counts (VC) of a strain for each broth used were determined between readings, by the method of Miles et al. (1938).

2.2.1. Single fermentations

The washed cells were inoculated in 400 mL of the pasteurized peanut-soy milk (2:1). The suspension with inoculated cells was fractionated in 25 mL portions, placed into 50 mL conical tubes. These tubes were incubated at 37 °C for up to 24 h and afterwards, the fermented PSM remained for another 24 h at ±4 °C. The experiments were performed in three independent assays. For each repetition two samples were taken at each time (duplicate).

2.2.2. Co-culture fermentations

Four co-culture fermentations were performed with: (1) *S. cerevisiae* (UFLA YFFBM 18.03) and *P. acidilactici* (UFLA BFFCX 27.1); (2) *S. cerevisiae* (UFLA YFFBM 18.03) and *L. acidophilus* (LACA 4); (3) *P. acidilactici* (UFLA BFFCX 27.1) and *L. acidophilus* (LACA 4); and finally, (4) *S. cerevisiae* (UFLA YFFBM 18.03), *P. acidilactici* (UFLA BFFCX 27.1) and *L. acidophilus* (LACA 4) strains. An initial population of 7 log CFU/mL of each strain was inoculated in pasteurized PSM. The suspension with inoculated cells was fractionated in 25 mL portions, and placed into 50 mL conical tubes. These tubes were incubated at 37 °C for up to 24 h and afterwards, the fermented PSM remained for 24 h at ±4 °C. The experiments were performed in three independent assays. For each repetition two samples were taken at each time (duplicate).

2.3. Viable count of inoculated strains (VC)

A one milliliter sample was taken from each fermentation flask. Serial tenfold dilutions were prepared in a solution of 0.9% NaCl (*w*/*v*) and 0.1% (*w*/*v*) bacto peptone (Difco). Viable counts of LAB and probiotic bacteria in mixed cultures were obtained by spreading 0.1 mL sample of an appropriate dilution onto the surface of MRS plates containing 0.1% (*w*/*v*) cysteine-HCl and 0.01% (*w*/*v*) aniline blue under anaerobic conditions during incubation (37 ° C for 2 days) (Evangelista et al., 2012; Wang et al., 2002). The colonies presented different shades of color (white and blue) allowing differential count. Yeasts were grown on YPD agar pH 3.5 at 28 °C for 5 days. Colony forming units (CFU) were enumerated in plates containing 30 to 300 colonies, and cell concentration was expressed as log CFU/mL of fermented PSM.

2.4. Analytical methods

2.4.1. Determination of pH and titratable acidity

The pH of the fermenting PSM samples was measured with a pH meter (Tecnal, Tec-3MT, São Paulo, Brazil). Estimation of titratable acidity was performed by a previously reported method AOAC (2000), in which a sample of 5 mL was titrated against 0.1 or 0.01 M NaOH using phenolphthalein as indicator.

2.4.2. Organic acids, alcohol and carbohydrates

Samples were prepared by adding 3 mL of n-hexan (Sigma-Aldrich) to 3 mL of samples. The mixture was vortexed for 30 s and the non-hexanic phase was pipetted into another tube. The procedure was performed again. Subsequently, the samples were centrifuged two times at 7000 rpm for 10 min at 4 °C, and then supernatant was filtered through 0.22 μm filters, and analyzed. Organic acids (acetic, lactic, and succinic acid), alcohol (ethanol) and carbohydrates (glucose, sucrose, maltose, fructose, raffinose, and stachyose) were identified according to the methodology proposed by Duarte et al. (2010). The analyses were carried out using a high performance liquid chromatography system (HPLC) (Shimadzu, model LC-10A*i*, Shimadzu Corp., Japan), equipped with a dual detection system consisting of a UV–vis detector (SPD-10A*i*) and a refractive index detector (RID-10A*i*). A Shimadzu ion exclusion column (Shim-pack SCR-101 H, 7.9 mm × 30 cm) was used operating at 50 °C, using 100 Mm of perchloric acid as the eluent at a flow rate of 0.6 mL/min. The acids were detected via UV absorbance (210 nm), while alcohols were detected via RID. For carbohydrates, the Supelcosil LC-NH_2 column (4.6 mm × 25 cm) was used, operating at 30 °C with a

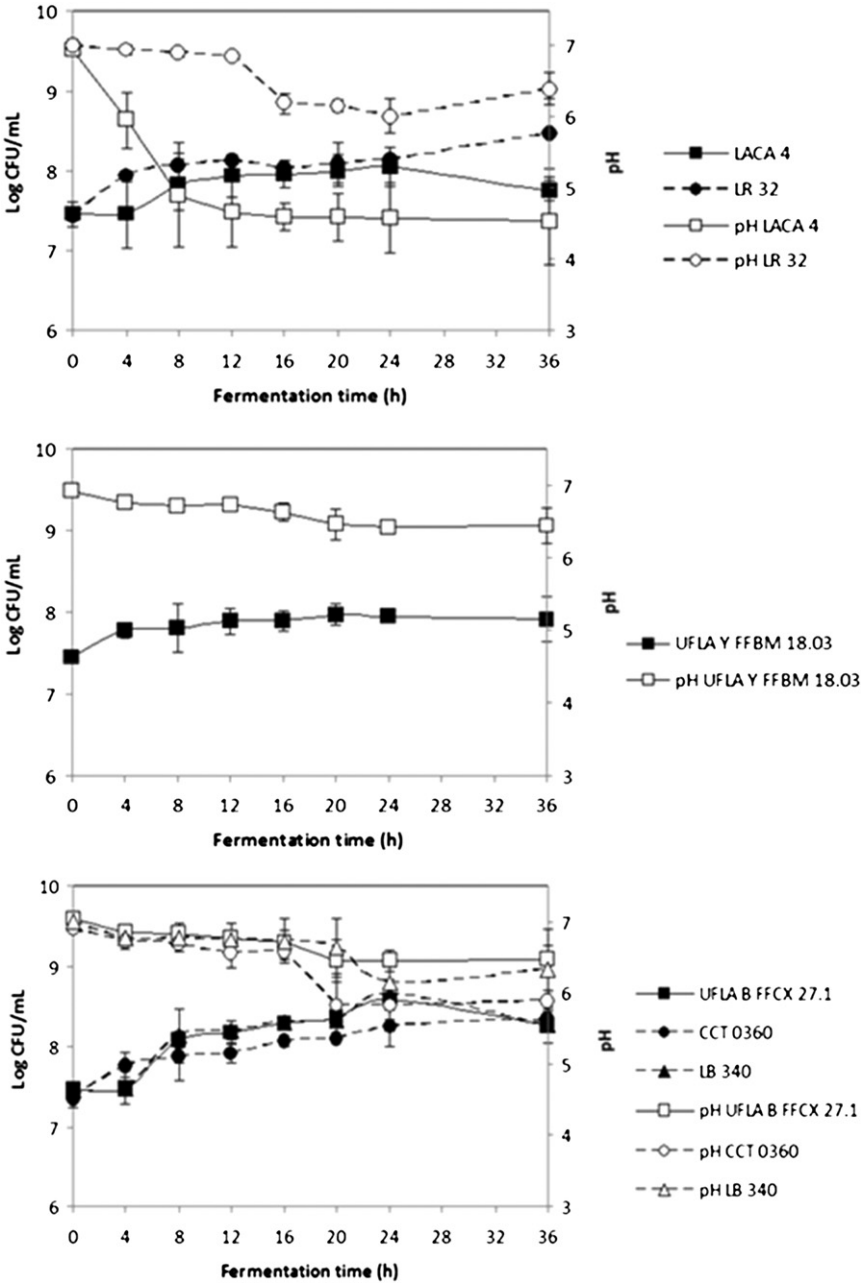

Fig. 1. Results of cell growth and acidification profiles of single cultures.

mobile phase (acetonitrile:water (75:25)) in a flow rate of 1 mL/min. The sugars were detected via RID. Individual compounds were identified based on the retention time of standards injected in the same conditions, and their concentrations were determined using the external calibration method. All samples were examined in triplicate.

2.4.3. Amino acids

The determinations of total amino acids were performed by High Performance Liquid Chromatography (HPLC) as described by White et al. (1986). After a 24 h hydrolysis in 6 M HCl at 100 °C, the amino acids were reacted with phenyl isothiocyanate, and the derivatives separated using a Luna C-18, 100 Å; 5 μ, 250 mm × 4.6 mm (00G-4252-E0, Torrance, CA, USA) column, at 50 °C. The detection was by ultraviolet absorption measurements using a fixed wavelength (254 nm) detector. Quantification was carried out by comparison with a standard mixture (Thermo Scientific, Rockford IL, USA) and DL-2-aminobutyric acid was used as an internal standard from Sigma-Aldrich Corp., St. Louis, MO (Hagen et al., 1989).

2.5. Data presentation and statistical analyses

All the results are expressed as means ± standard deviation (SD). In all analyses, the assumption of normality was tested using Shapiro and Wilk test. The assumption of homogeneity of variances was tested using Barlett test. As both assumptions of normality and homogeneity of variances were satisfied, parametric testing was performed. The data discussed are the averages from three independent assays and were subject to one-way analysis of variance (ANOVA), followed by Fisher's Least Significant Difference (LSD) test (Granato et al., 2014). Differences in values were considered significant when the *P* value was less than 0.05. The statistical analyses were performed using the Sisvar 5.3 software (Ferreira, 2010), except the Bartlett test, which was performed using the Assistat software (Silva and Azevedo, 2009).

3. Results and discussion

3.1. Growth and acidification profiles

The success of new probiotic formulations does not only rely on the ability to provide enough probiotic cells that may survive the human gastrointestinal tract. An appropriate selection of substrate composition and strains is necessary to efficiently control the distribution of the metabolic end products (De Vuyst, 2000). Santos et al. (unpublished results) developed a formulation using peanut milk mixed with soy milk, with high nutritional value, as a suitable novel substrate to fermentation. However, the present study is the first to report the behavior of different single and co-cultures, including probiotic ones in this novel PSM.

Results of cell growth and acidification profiles of single cultures are presented in Fig. 1. The *P. acidilactici* (UFLA BFFCX 27.1) LAB showed the highest increase in viable population ($p < 0.05$) during PSM fermentation, followed by *L. rhamnosus* (LR 32) probiotic bacteria and the *L. delbrueckii* subsp. *bulgaricus* (LB 340) yogurt starter, that do not differ statistically ($p > 0.05$). All these strains reached populations above 8 log CFU/mL. Although these bacteria were effective in growing, they did not acidify the PSM, and the pH remained above 6. This poor ability of lowering the pH of the soy-based substrates by *L. rhamnosus* and *L. delbrueckii* subsp. *bulgaricus* has also been reported by Champagne et al. (2009) and Farnworth et al. (2007).

A rapid pH value drop in the PSM substrate was observed by *L. acidophilus* (LACA 4), reaching 4.6 at 12 h of fermentation.

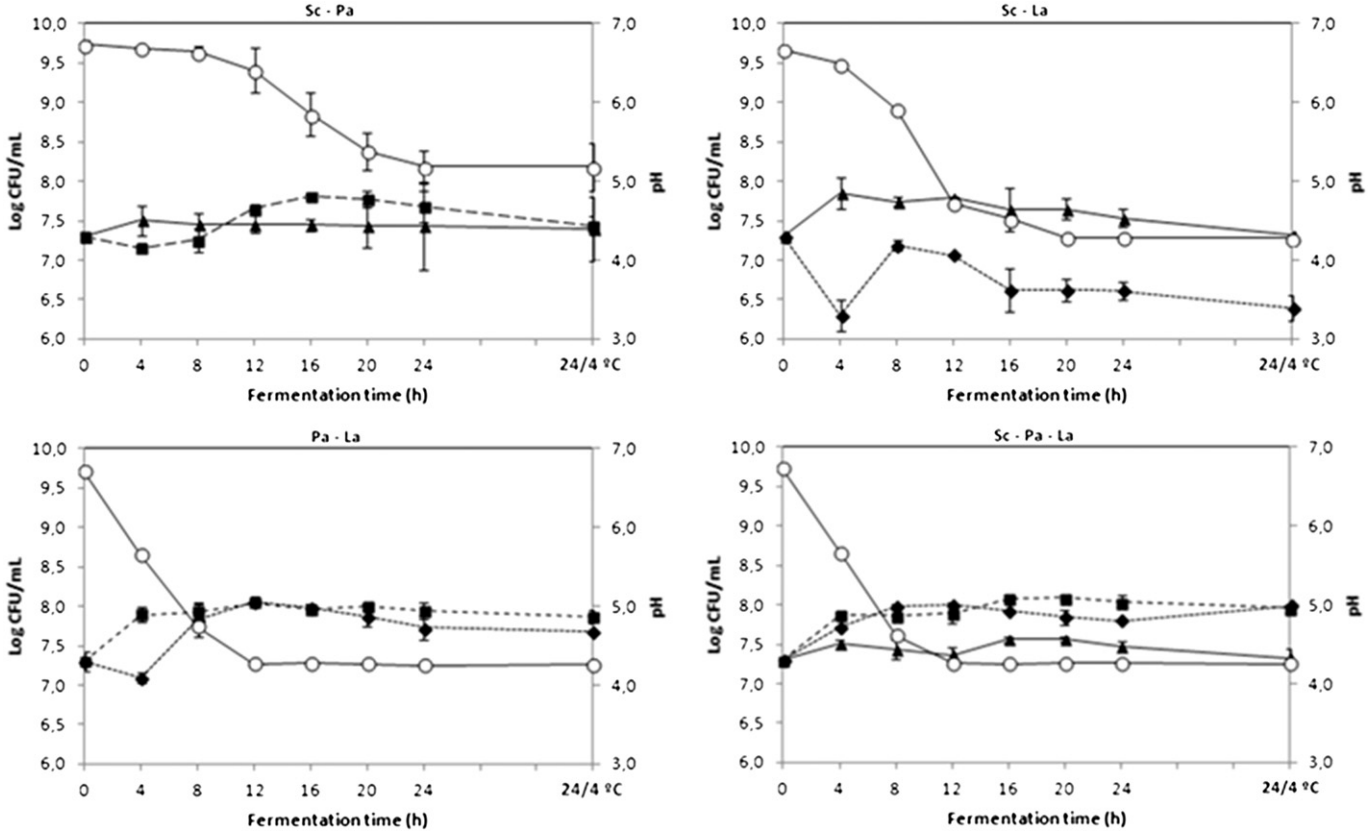

Fig. 2. Evolution of cell populations and acidification during co-culture fermentations of PSM.

L. acidophilus is an obligatory homofermentative bacterium that produces a single end product (lactic acid) from the glycolysis of carbohydrates (EM pathway) (Rathore et al., 2012). High viable counts are necessary to get the desired acid production and reduction in pH, which affects organoleptic properties and shelf-life, and prevents product contamination (Rathore et al., 2012). The cell populations of *L. acidophilus* (LACA 4) increased rapidly within the first 8 h of fermentation and remained stable at 7.9 log CFU/mL. After 24 h under refrigerated conditions the viable cell counts were about 7.75 log CFU/mL (Fig. 1).

The growth of *Lc. lactis* (CCT 0360) over 24 h was slow and constant reaching 8.25 log CFU/mL at 24 h. The slow growth reflected in the acidification curve showing an equally slow acidification rate in peanut-soy beverage, with a pH of 5.8 eventually reached after 24 h. Therefore, the PSM provided appropriate growth and fermentation conditions for the pure microbial cultures. *L. acidophilus* (LACA 4) and *P. acidilactici* (UFLA BFFCX 27.1) presented populations above 8 log CFU/mL and may preserve the low pH of the final beverage. The use of *S. cerevisiae* (UFLA YFFBM 18.03) may give rise to a new class of products, where the addition of yeast may serve as a source of protein and vitamin B (Steinkraus, 1996). Thus, these strains were selected to be co-cultured to obtain short fermentation periods and larger populations of the probiotic bacteria.

The evolution of cell populations and acidification during co-culture fermentations of PSM are shown in Fig. 2. It should be noted that fermentations carried out in co-culture were more efficient in substrate acidification. Twelve hours was required to reach a pH value of 4.3 in both fermentations with *L. acidophilus* (LACA 4) and *P. acidilactici* (UFLA BFFCX 27.1), and with *L. acidophilus* (LACA 4), *P. acidilactici* (UFLA BFFCX 27.1) and *S. cerevisiae* (UFLA YFFBM 18.03). The co-culture of *L. acidophilus* and the *S. cerevisiae* yeast also reached pH 4.3, but required a longer time of about 20 h.

The pH value reached was below 4.5 in all co-cultured fermentations, which demonstrated that 7 log CFU/mL of inoculum was sufficient to decrease the pH. The pH around 3.5–4.5 reported for food formulations aid the pH increase of the gastrointestinal tract, and thus enhances the stability and benefits of probiotic strains consumed (Kailasapathy and Chin, 2000).

It has been suggested that fermented products required probiotic bacteria at 7 log CFU/mL in order to give health effects in the gastrointestinal tract when consumed (Ouwehand and Salminen, 1998). Data from this study showed that high levels (8 log CFU/mL) of the *L. acidophilus* (LACA 4) probiotic could be reached when this strain is inoculated with *P. acidilactici* (UFLA BFFCX 27.1), and *S. cerevisiae* (UFLA YFFBM 18.03) at the beginning of fermentation of PSM. When these three selected strains were co-incubated, the probiotic bacteria had their growth favored reaching the population of 8 log CFU/mL within 8 h of fermentation. *P. acidilactici* populations remained above 8 log CFU/mL after 12 h of fermentation. *S. cerevisiae* population ranged from 7.3 to 7.5 log CFU/mL at 24 h. At the end of 24 h, the population decreased slightly, but it was not statistically significant ($p > 0.05$).

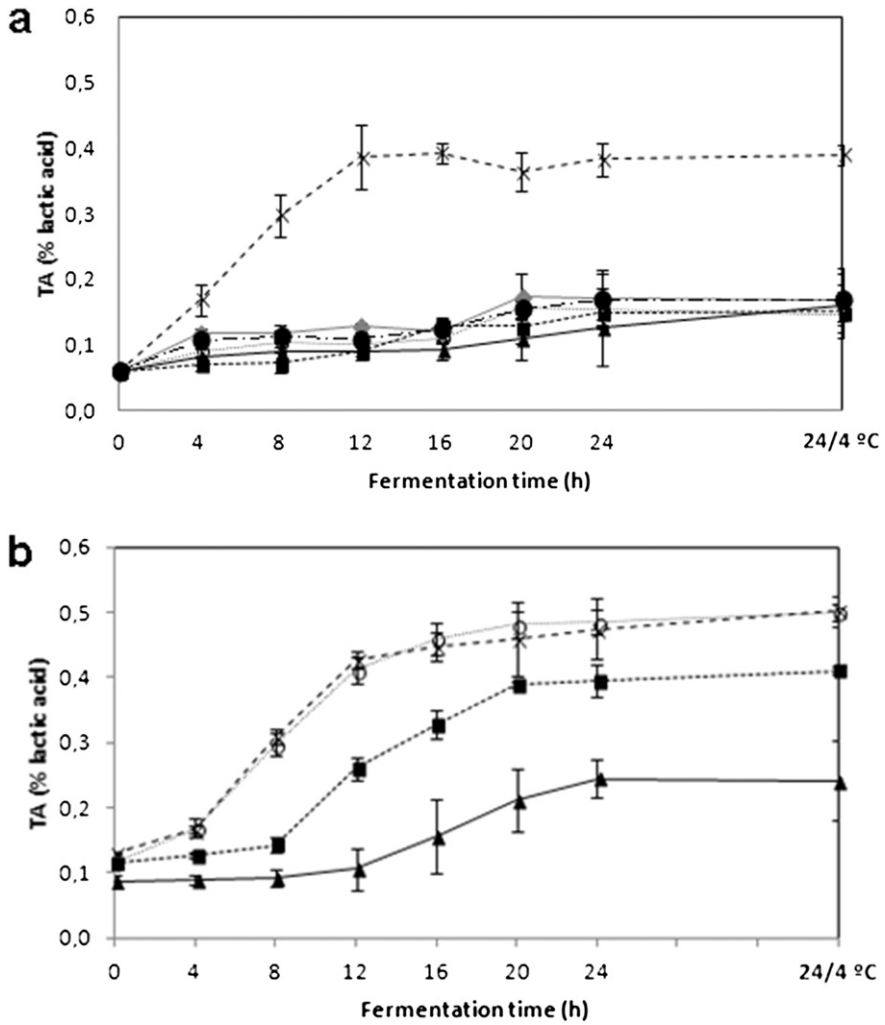

Fig. 3. *L. acidophilus* (LACA 4), in pure culture, as the only strain able to acidify significantly the substrate; efficient acidification profiles generally achieved in co-culture fermentation.

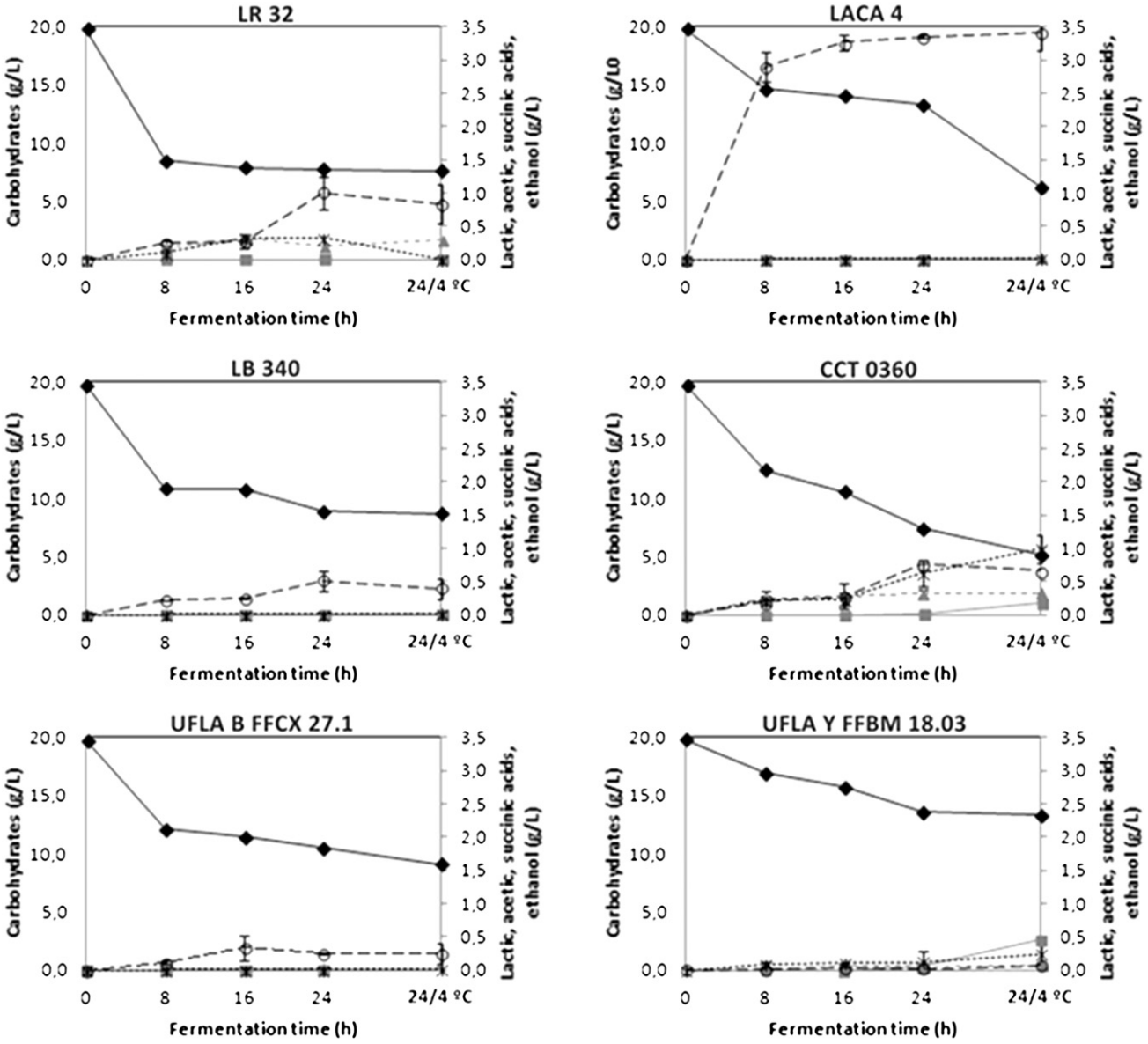

Fig. 4. Metabolic behaviors of the strains in pure cultures.

The co-cultured organism may compete for nutrients or may produce metabolic products that may stimulate or inhibit each other's growth. It has been reported that yeast may produce vitamins that enhance the growth of LAB (Marshall, 1987). Growth of lactic acid bacteria is believed to be promoted when co-cultured with yeasts, mainly due to the excretion of specific amino acids and small peptides by the latter, either during growth, and regardless of the antagonism for the main carbon source (Gobbetti et al., 1994) or as a consequence of an accelerated autolysis (Zambonelli et al., 2000).

The yeast when co-cultured with the *L. acidophilus* probiotic was more competitive than in mono culture, regarding the final population reached that showed an increase of 1 log CFU/mL. The yeast probably caused a significant ($p < 0.05$) reduction in the population of bacteria in 4 h of fermentation. After a reduction in the *L. acidophilus* (LACA 4) population, these LAB increased their population and stabilized at 6.6 log CFU/mL. In the co-culture of *S. cerevisiae* and *P. acidilactici*, both presented one log higher than when cultivated in pure culture. Despite the slow growth start, the LAB reached viable counts of about 8 log CFU/mL after 16 h, and remained constant ($p > 0.05$) until the end of the fermentative process.

The cultures showed a trend to decrease their populations when they were kept at 4 °C. Fermentations conducted with *L. acidophilus* (LACA 4) and *P. acidilactici* (UFLA BFFCX 27.1), and the fermentation involving all strains were the ones that showed smaller effect of low temperature on viable populations ($p < 0.05$). The fermentation involving all cultures led to an increase of *L. acidophilus* (LACA 4) viable count during 24 h under refrigeration.

In general, the results of the pH values followed an opposite trend to that observed for titratable acidity measurements, i.e., as the acidity increased, the pH decreased. Fig. 3a shows clearly that *L. acidophilus* (LACA 4), in pure culture, was the only strain able to acidify significantly the substrate ($p < 0.05$). Efficient acidification profiles were generally achieved in co-culture fermentation (Fig. 3b).

The titratable acidity of PSM increased from 0.06% to 0.48% after 24 h of fermentation with the co-cultivation of all microorganisms. After 24 h at ±4 °C, the titratable acidity increased to 0.50% of lactic acid with

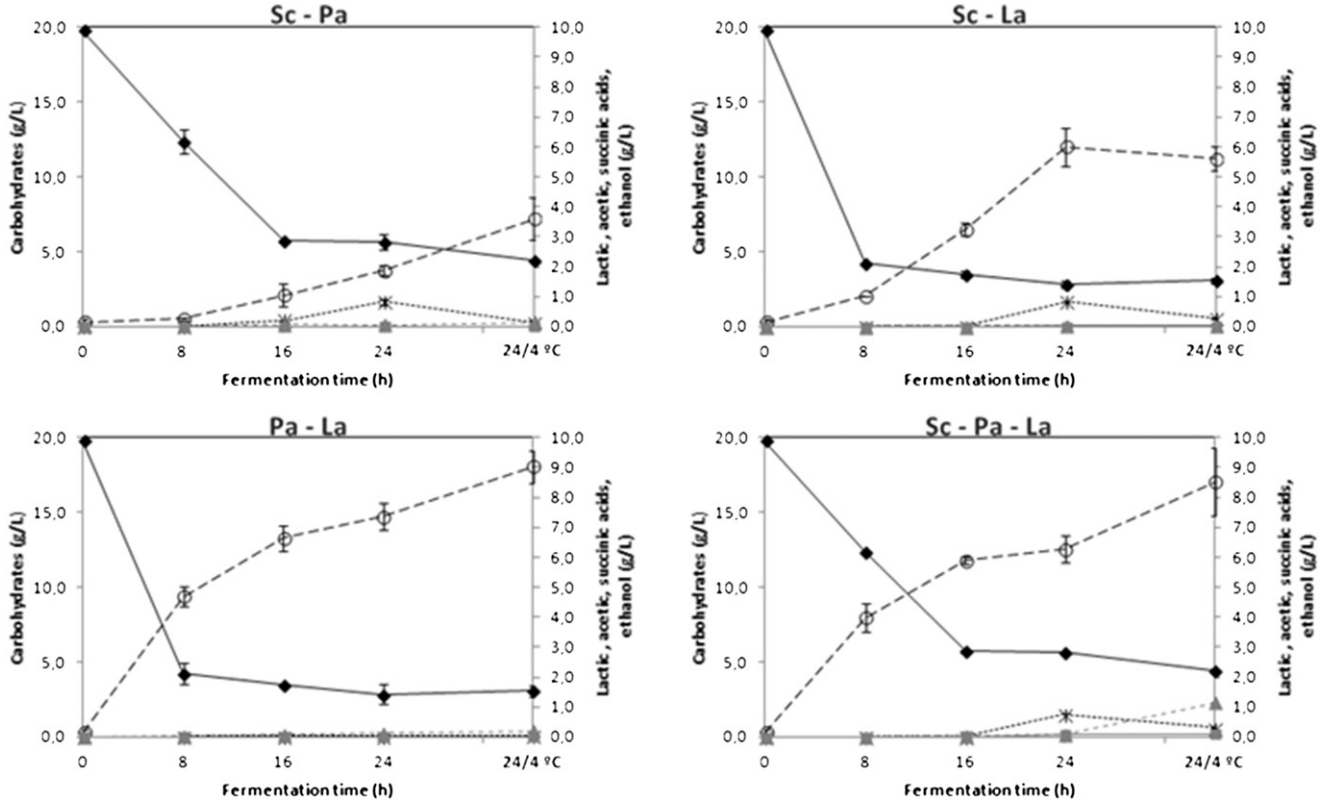

Fig. 5. Metabolic behaviors of the strains in co-cultures.

UFLA BFFCX 27.1 and LACA 4, and with the three mixed cultures. Isanga and Zhang (2007) developed fermented peanut milk with the mixed cultures of *Lactobacillus delbrueckii* ssp. *bulgaricus*, and *Streptococcus salivarius* ssp. *thermophilus*. In accordance, the authors reported that fermentation with combined cultures had higher titratable acidity values (0.39%) than with the single cultures of these strains.

3.2. Consumption of sugars and produced metabolites

Figs. 4 and 5 illustrate the metabolic behaviors of the strains either in pure cultures or in co-cultures. They were monitored in fermented PSM by determination of the main metabolic products (lactic, acetic, succinic acids, and ethanol) and consumed sugars. As shown in Fig. 4, the

Table 1
Carbohydrate content in fermented peanut-soy milk with different combinations of *L. acidophilus* (LACA 4), *P. acidilactici* (UFLA BFFCX 27.1), and *S. cerevisiae* (UFLA YFFBM 18.03).

Time (h)	Fructose	Glucose	Sucrose	Maltose	Raffinose	Stachyose
0	1.26 ± 0.04 [a]	Nd	12.16 ± 0.33[a]	Nd	0.77 ± 0.01[a]	5.52 ± 0.02[a]
L. acidophilus (LACA 4) + P. acidilactici (UFLA B FFCX 27.1)						
8	0.63 ± 0.02 [b]	0.43 ± 0.03[a]	1.08 ± 0.04 [b]	Nd	Nd	2.09 ± 0.74 [b]
16	0.43 ± 0.00 [c]	0.10 ± 0.04 [b]	Nd	0.94 ± 0.02[a]	Nd	2.00 ± 0.00 [b]
24	0.29 ± 0.00 [d]	Nd	Nd	0.46 ± 0.04 [b]	0.25 ± 0.09 [b]	1.84 ± 0.65 [b]
24 h/4 °C	0.74 ± 0.01 [b]	Nd	Nd	0.43 ± 0.03 [b]	0.80 ± 0.06[a]	1.10 ± 0.35 [b]
L. acidophilus (LACA 4) + S. cerevisiae (UFLA Y FFBM 18.03)						
8	0.70 ± 0.01 [bc]	0.64 ± 0.14[a]	6.87 ± 1.74 [b]	Nd	0.65 ± 0.00 [bc]	4.80 ± 0.02 [b]
16	0.43 ± 0.00 [d]	0.21 ± 0.01 [b]	2.21 ± 0.06 [c]	0.65 ± 0.01[a]	Nd	3.97 ± 0.22 [c]
24	0.76 ± 0.02 [b]	0.14 ± 0.00 [b]	0.44 ± 0.04 [d]	0.42 ± 0.00 [b]	0.61 ± 0.06 [bc]	3.76 ± 0.25 [c]
24 h/4 °C	0.49 ± 0.07 [cd]	0.12 ± 0.01 [b]	0.33 ± 0.01 [d]	0.39 ± 0.01 [b]	0.51 ± 0.00 [c]	3.61 ± 0.16 [c]
P. acidilactici (UFLA B FFCX 27.1) + S. cerevisiae (UFLA Y FFBM 18.03)						
8	0.84 ± 0.05 [c]	0.36 ± 0.05[a]	7.84 ± 0.48 [b]	Nd	0.41 ± 0.15 [ab]	2.89 ± 0.79 [b]
16	Nd	Nd	2.83 ± 0.78 [c]	0.64 ± 0.01[a]	Nd	2.25 ± 0.00 [b]
24	0.35 ± 0.00 [c]	0.19 ± 0.02 [b]	1.48 ± 0.29 [c]	0.62 ± 0.03[a]	0.86 ± 0.22[a]	2.19 ± 0.52 [b]
24 h/4 °C	0.31 ± 0.01 [c]	0.24 ± 0.03 [ab]	0.68 ± 0.15 [c]	0.74 ± 0.10[a]	0.47 ± 0.09 [ab]	2.00 ± 0.07 [b]
L. acidophilus (LACA 4) + P. acidilactici (UFLA B FFCX 27.1) + S. cerevisiae (UFLA Y FFBM18.03)						
8	0.63 ± 0.00 [c]	0.29 ± 0.00 [a]	5.43 ± 0.13 [b]	Nd	0.48 ± 0.03 [b]	3.78 ± 0.09 [b]
16	0.96 ± 0.08 [b]	Nd	Nd	Nd	0.02 ± 0.10 [c]	3.04 ± 0.10 [c]
24	0.58 ± 0.01 [c]	Nd	Nd	0.51 ± 0.18 [ab]	Nd	2.51 ± 0.03 [d]
24 h/4 °C	0.60 ± 0.02 [c]	0.26 ± 0.04 [a]	Nd	1.03 ± 0.24[a]	Nd	2.45 ± 0.15 [d]

Nd—not detected.
[a] Mean ± SD. Means followed by the same letters in each column do not differ significantly ($p > 0.05$).

L. acidophilus (LACA 4), *P. acidilactici* (UFLA BFFCX 27.1), and *S. cerevisiae* (UFLA YFFBM 18.03) strains in single cultures produced 3.26, 0.26 and 0.03 g/L, respectively, of lactic acid after 24 h of fermentation. The highest values (9.03 and 8.51 g/L) were found in fermentations after 24 h at 4 °C in a binary culture of *P. acidilactici* and *L. acidophilus*, and in combination of the three strains, respectively. The marked lactic acid increase in co-culture fermentations could be due to interactions between the different species used. These results highlighted the importance of the selection of the substrate composition and inocula in the development of the organoleptic properties of these fermented products.

Acetic acid was produced in three co-culture fermentations, and amounts of 1.41 g/L after 24 h at 4 °C were detected. The *S. cerevisiae* yeast (UFLA YFFBM 18.03) also produced succinic acid, reaching 0.46 g/L as the maximum content in pure culture. In co-culture, this acid was produced from 0.02 to 0.10 g/L after 24 h.

The *S. cerevisiae* yeast produced low ethanol content (0.24 g/L after 24 h under refrigeration) in pure culture. This value was smaller than those obtained for *L. rhamnosus* (0.32 g/L). It may be due to non-complete substrate adaptation, inhibition of growth by some medium components, or lack of required nutrients in the raw material. The highest ethanol (0.84 and 0.75 g/L) concentrations were found after 24 h of fermentation in the co-culture of *S. cerevisiae* and *L. acidophilus*, followed by *S. cerevisiae*, *P. acidilactici* and *L. acidophilus*, respectively. A decrease in these values was noticed during storage under refrigeration. It was probably due to the ethanol evaporation. The final content of ethanol was 0.03% (*v*/*v*) or less, which classifies the beverage as a non-alcoholic beverage (Brasil, 2009). It seems that *L. acidophilus*, *P. acidilactici*, and *S. cerevisiae* may influence each other's metabolism, which can lead to different profiles of important organoleptic compounds. There might also be other interactions among the produced metabolites, where a compound produced by one organism may be further metabolized by another (Axelsson, 1998; Rathore et al., 2012).

The content of sugars in PSM is due to raw materials and the breakdown of starch during the heat treatment. The available carbohydrates contributed to the cell viability and to the production of metabolites. In our work, sucrose and stachyose decreased during all fermentations (Table 1). Our results are in agreement with Beuchat and Nail (1978). These authors reported that sucrose is the major fermentable carbohydrate found in peanut milk. Champagne et al. (2009) stated that, for soy beverages, the available carbohydrates, by order of importance, are sucrose, stachyose, raffinose, glucose and fructose.

The reduction in the contents of raffinose and stachyose and the increase in the contents of monosaccharide may be attributed to the hydrolytic reaction catalyzed by α- and β-galactosidases produced by both LAB species studied here (Donkor et al., 2007). Raffinose and stachyose are α-galactosides of sucrose, comprising three and four monomeric units, respectively, and are non-digestible in the gut due to the absence of α-galactosidase in the human intestinal mucosa. Consequently, intact oligosaccharides pass directly into the lower intestine, where they are metabolized by bacteria that possess this enzyme, resulting in the production of gases (Tsangalis and Shah, 2004).

The α-galactosides are sources of carbon for the growth of *Lactobacillus* species, such as *L. acidophilus* (Scalabrini et al., 1998). Our data showed that binary co-culture of *L. acidophilus* and *P. acidilactici*, and co-culture of *L. acidophilus*, *P. acidilactici* and *S. cerevisiae* may be a practical approach to overcome the flatulence factor in PSM.

The utilization of sugars in PSM was different than the data reported by Farnworth et al. (2007), who showed that fructose was the most utilized sugar, while glucose, raffinose, and stachyose were much less used. These authors studied the growth of *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. johnsonii*, several *Bifidobacterium* sp. and *Streptococcus termophilus* in a soy-yogurt formulation. These discrepancies in the results suggested that the initial concentration of the carbohydrates may alter the dynamics of consumption thereof.

In general, when co-cultured, the strains were more efficient in the use of available carbohydrates and in the release of metabolites in the fermentation medium. In single cultures, the strains consumed an average of 58% and in co-cultures the consumption of available carbohydrates was approximately 78%. The fermentations carried out in binary culture of the probiotic bacteria *L. acidophilus* (LACA 4) and the LAB *P. acidilactici* (UFLA BFFCX 27.1), were the most efficient in the consumption of sugars. At the end of the fermentation, there was 3.07 g/L of available carbohydrates (15.58%).

3.3. Total amino acids

As shown in Table 2, the unfermented and fermented PSM contain almost all the essential amino acids and non-essential amino acids, though in varying amounts. According to Blandino et al. (2003) the

Table 2
Total amino acids (g/100 g of sample, on dry basis) in unfermented peanut-soy milk and after 24 h of fermentation (pure and co-cultures).

Amino acid	Unfermented										
	P-S milk	UFLA YFFBM 18.03	LACA 4	LR 32	LB 340	UFLA BFFCX 27.1	CCT 0360	Sc–Pa	Sc–La	Pa–La	Sc–Pa–La
Histidine (Hys)	1.12	1.32	1.15	1.2	1.09	1.31	1.07	1.21	1.16	1.07	0.97
Isoleucine (Ile)	1.78	2.10	1.97	2.03	1.77	1.84	1.82	1.81	1.74	1.67	1.71
Leucine (Leu)	2.90	3.27	3.13	3.01	2.88	2.92	2.87	2.90	2.78	2.72	2.77
Lysine (Lys)	1.75	2.10	2.09	1.82	1.90	1.82	1.81	1.84	1.72	1.65	1.78
Methionine (Met)	0.50	0.63	0.60	0.67	0.53	0.56	0.53	0.58	0.55	0.55	0.51
Cysteine (Cys)	0.67	0.72	0.76	0.8	0.20	0.65	0.67	0.49	0.38	0.45	0.54
Phenylalanine (Phe)	1.54	1.67	1.66	1.6	1.61	1.67	1.59	1.38	1.51	1.35	1.37
Tyrosine (Tyr)	1.61	1.80	1.67	1.67	1.57	1.67	1.60	1.55	1.48	1.50	1.45
Threonine (Thr)	1.71	1.92	1.89	1.97	1.66	1.71	1.61	1.65	1.60	1.52	1.57
Valine (Val)	2.01	2.31	2.12	2.42	2.00	2.07	2.00	2.05	1.94	1.89	1.90
Total essential amino acids	15.58	17.84	17.04	17.19	15.21	16.22	15.57	15.46	14.86	14.37	14.57
Arginine (Arg)	4.80	5.07	4.65	4.68	4.33	4.96	4.33	4.57	4.61	4.49	4.50
Alanine (Ala)	1.92	2.10	2.07	2.19	1.85	1.97	1.85	1.94	1.86	1.78	1.83
Aspartic acid (Asp)	5.13	5.62	5.41	5.52	5.06	5.10	5.00	5.00	4.87	4.78	4.77
Glutamic acid (Glu)	9.44	10.37	10.19	10.4	9.24	9.34	9.24	9.02	8.87	8.91	8.92
Glycine (Gly)	1.93	2.09	1.98	2.06	1.88	1.90	1.87	1.89	1.81	1.76	1.77
Proline (Pro)	2.40	2.61	2.64	2.65	2.35	2.44	2.27	2.35	2.26	2.16	2.21
Serine (Ser)	2.20	2.33	2.25	2.63	2.09	2.13	2.06	2.07	2.03	2.07	2.07
Total amino acids	43.37	48.02	46.21	47.32	42.01	44.07	42.19	42.30	41.15	40.31	40.64

P-S milk—peanut-soy milk. Pure cultures: UFLA Y FFBM 18.03—*S. cerevisiae*; LACA 4—*L. acidophilus*; LB 340—*L. rhamonsus*; LB 340—*L. delbrueckii* subsp. *delbrueckii*; UFLA B FFCX 27.1—*P. acidilactici*; and CCT 0360—*L. lactis*. Co-cultures: *S. cerevisiae*–*P. acidilactici* (Sc–Pa); *S. cerevisiae*–*L. acidophilus* (Sc–La); *P. adicilaciti*–*L. acidophilus* (Pa–La) and *S. cerevisiae*–*P. acidilactici*–*L. acidophilus* (Sc–Pa–La).

effect of fermentation on the protein and amino acids levels is a topic of controversy. It appears that the effect of fermentation on the nutritive value of foods is variable, although the evidence for improvements is substantial. Certain amino acids may be synthesized and the availability of B group vitamins may be improved. Yeast extract provides growth stimulants such as amino acids and nucleotides (Nagodawithana, 1992) for cheese starter which results in increased activity (Nsofor et al., 1996). In fact, the *S. cerevisiae* yeast (UFLA YFFBM 18.03) cultivated in pure culture was the strain that provided the greatest increase in the amounts of amino acids in PSM.

S. cerevisiae (UFLA YFFBM 18.03), *L. rhamnosus* (LR 32), *L. acidophilus* (LACA 4), and *P. acidilactici* (UFLA BFFCX 27.1) promoted the release of amino acids into the PSM. The percentages of total amino acids in PSM fermented for 24 h at 37 °C were about 48.02%, 47.32%, 46.21%, and 44.07%, respectively for the above pure cultures (Table 2). However, when co-cultured, the strains consumed the amino acids, this probably contributed to growth increase, higher cell viability, greater production of lactic acid, and consequent fast drop in pH value.

PSM, fermented with pure cultures, was found to be richer in some essential amino acids (such as threonine, histidine, phenylalanine, tyrosine, and lysine) than unfermented milk. However, unfermented milk was richer in other amino acids (like cysteine, glutamic acid, arginine, and serine) than in pure culture fermentations.

In general, the lysine content of PSM prepared in this study was close to the range observed in the similar studies referred to here on peanut-based beverages. The ranges of lysine content in experimental fermentations were 1.65–2.10% in PSM. According to Rubico et al. (1988), the amino acid profiles of various peanut beverage treatments indicated that raw peanut extracts had a lysine content of 4.31%; and the lysine content in homogenized peanut milk was within the range of 2.88–3.81%.

4. Conclusion

The main aim for the development of this product was the utilization of two important vegetable protein sources, peanut and soy, which are readily available in abundance and at a reasonable cost. Both peanut and soy protein are comparable with protein content from animal based food and it is suitable for all ages (Deshpande et al., 2008). This study is the first report of the potential of *L. acidophilus* as probiotics in fermented peanut-soy beverage in mixed cultures. This strain showed higher viability when cultivated with *P. acidilactici* and *S. cerevisiae* in PSM. Further studies are needed to evaluate the survival of these cultures over a longer period of post-acidification and also, the sensory acceptation of the final beverage.

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