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**SHORT COMMUNICATION**

# Metabolic capacity of probiotic mixed cultures formed by *Lactobacillus* and *Bifidobacterium* strains for use in functional fermented dairy foods

# Capacidad metabólica de cultivos mixtos probióticos formados por cepas de *Lactobacillus* y *Bifidobacterium* para uso en alimentos lácteos fermentados funcionales

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## ABSTRACT

The metabolic capacity of probiotic mixed cultures formed by *Lactobacillus* and *Bifidobacterium* strains was assessed through the determination of the acidification profile and the production of amino acids and volatile compounds, during the fermentation of ultra-pasteurized skim milk. Two mixed cultures formed by [*L. acidophilus* + *B. bifidum*] and [*L. acidophilus* + *B. animalis*] stood out in the production of essential amino acids: His, Ile, Leu, Met, Thr, Trp and Val. As well as increased production of volatile compounds such as: acetoin, 2-heptanone, 2-nonanone, acetic

acid, acid butanoic and hexanoic acid, in contrast to commercial fermented dairy products. Additionally, these bacterial mixed cultures were characterized by the production of distinctive volatile compounds: 1-heptanol, diisobutylcarninol, hemellitol, 1-dodecanol, acetone, 2-pentanone, 2-undecanone, ethyl acetate, benzaldehyde as well as valeric, acetyl valeric and isopropylpyruvic acids. Finally, the culture formed by [*L. acidophilus* + *B. bifidum]* presented a good acidification profile with a lactic acid production of 26.1 ± 0.1 g / L and pH 3.6 at the end of fermentation. This data suggests a great potential of these mixed cultures to improve the nutritional value and organoleptic characteristics of fermented dairy products, when added as starter or adjunct cultures in the fermentation process.

**Keywords:** amino acids, functional food, probiotic, mixed culture, volatile compound.

## RESUMEN

Se determinó la capacidad metabólica de cultivos mixtos probióticos formados por cepas de *Lactobacillus* y *Bifidobacterium,* a través del perfil de acidificación, producción de aminoácidos y compuestos volátiles, durante la fermentación de leche descremada ultrapasteurizada. Dos cultivos mixtos formados por [*L. acidophilus* + *B. bifidum]* y [*L. acidophilus* +*B. animalis],* destacaron en la producción de aminoácidos esenciales: His, Ile, Leu, Met, Thr, Trp y Val, así como en la producción de compuestos volátiles, tales como: acetoina, 2-heptanona, 2-nonanona, ácido acético, ácido butanoico y ácido hexanoico, en contraste con productos lácteos fermentados comerciales. Adicionalmente, estos cultivos mixtos bacterianos se caracterización por la producción de compuestos volátiles distintivos: 1-heptanol, diisobutil carbinol, hemelitol, 1-dodecanol, acetona, 2-pentanona, 2-undecanona, etil acetato, benzaldehído, ácido valérico, ácido acetil valérico y ácido isopropil pirúvico. Finalmente, el cultivo formado por [*L. acidophilus* + *B. bifidum]* presentó un buen perfil de acidificación con una producción de ácido láctico de 26.1 ± 0.1 g/L y un pH de 3.6 al término de la fermentación. Los resultados sugieren un gran potencial de los consorcios en la mejora del valor nutrimental y características organolépticas de los productos lácteos fermentados, al ser adicionados como cultivos iniciadores o adjuntos en el proceso de fermentación.

**Palabras clave:** alimento funcional, aminoácidos, compuestos volátiles, cultivo mixto, probiótico.

## 1. INTRODUCTION

Functional food is defined as food that is or is similar in appearance to a conventional normally consumed food that contains demonstrated physiological benefits and/or reduces the risk of chronic disease beyond basic nutritional function (Olagnero *et al*., 2007). Fermented dairy products possess diverse functional components ranging from bioactive peptides, free amino acids, organic acids, and vitamins to probiotic microorganisms (Tamang *et al*., 2016). Probiotics constitute one of the main functional components and represent an accessible option to the population of many countries due to the people´s consumption of fermented dairy foods (Chilton *et al.,* 2015).

According to FAO/OMS (2002), probiotics are defined as live microorganisms which confer a health benefit to the host when administered in adequate amounts. *Lactobacillus* and *Bifidobacterium* genera are considered safe for human consumption, and various clinical studies have shown their positive effect on the treatment and prevention of various physiological conditions, such as food intolerances, inflammation, diarrhea, cancer, diabetes, obesity, and HIV. Many of these illnesses are associated with an imbalance of the intestinal microbiota due to specific conditions of each pathology or nutritional modifications, generating immunological, physiological, and metabolic alterations of the host (Banan-Mwine & Lee, 2015; Wang *et al*., 2017).

The application of probiotic strains has been extended technologically to the production of fermented dairy foods with functional properties, where the release of free amino acids during the fermentation by proteolysis of milk protein is considered a very important biochemical process. These free amino acids serve as precursors for the synthesis of compounds that impart flavor and aroma and help increase the nutritional value of the food. This process is carried out by a complex strain-specific proteolytic system that comprises proteinases anchored to the cell wall, transporters of amino acids and peptides, as well as intracellular peptidases (Liu *et al*., 2010; Savijoki *et al.,* 2006). The qualitative and quantitative amino acids profile produced is strain specific, however, in some cases the metabolic complementation between strains in mixed cultures favors the availability of essential amino acids, improving the nutritional and sensorial value of the fermented dairy products (Akabanda *et al*., 2014; Chinellato *et al*., 2017; Tammam *et al*., 2000).

Despite the great diversity of *Lactobacillus* and *Bifidobacterium* strains publicly available and the commercialization of dairy products fermented by these and other probiotic strains, the studies that report the sensory and nutritional impact on dairy fermentations, determined by acidification, free amino acids, and volatile compounds profiles generated by mixed cultures during fermentation are limited to few clinically tested strains (Burns *et al*., 2012; Karimi *et al*., 2012; Ong *et al*., 2006).

In this study, we evaluated the metabolic capacity of mixed probiotic cultures formed by publicly available *Lactobacillus* and *Bifidobacterium* strains in skim milk fermentation by determining free amino acids, lactic acid, and volatile compounds. These experiments allowed us to evaluate the effect of mixed cultures on the nutritional value and sensory characteristics of skim milk fermentation in order to estimate their potential in the production of functional fermented dairy foods.

## 2. MATERIALS AND METHODS

### 2.1. Microorganisms

The probiotic strains used in this study were obtained from the American Type Culture Collection (ATCC). They included *Lactobacillus acidophilus* ATCC 4356 (La), *Bifidobacterium adolescentis* ATCC 15703 (Bado), *Bifidobacterium animalis* ATCC 27536 (Bani), *Bifidobacterium bifidum* ATCC 29521 (Bbi), *Bifidobacterium breve* ATCC

15700 (Bbr) and *Bifidobacterium longum subsp. infantis* ATCC 15702 (Bin). All strains were selected according to their potential clinical use (Atherosclerosis, Immunomodulatory effect, allergic airway disease, inhibitory activity on clinical *Helicobacter pylori strains* and Cholitis*)*. Furthermore, the metabolic capacity in a lactic fermentation of these strains has not been described. The strains were tested for compatibility prior to this study.

For all strains, a stock culture was made from a culture grown in an MRS broth medium (MRS; BD, USA) and re-suspended in the same medium containing 30% v/v glycerol (Sigma, USA) supplemented with 0.5% w/v of sodium thioglycollate (Sigma, USA). The stock culture was stored at -80°C.

## 2.2. Technological properties

### 2.2.1. Inoculum preparation

Precultures of axenic ATCC strains were prepared by inoculating 100 µL of stock culture in 10 mL of MRS broth, followed by incubation at 37 °C, under anaerobic conditions (BD BBL GasPack system, USA) for 18 h. Afterward, cells were washed twice with a PBS solution (pH 7.0) and resuspended in the same solution at the initial volume. The inoculum was adjusted to $10^6$ colony-forming units (CFU) divided into equal proportions between the strains that form the mixed culture. The composition of probiotic mixed cultures evaluated was the following: La-Bad, La-Ban, La-Bbi, La-Bbr, La-Bin, and La-Bado+Bani+Bbi+Bbr+Bin (CON). The *L. acidophilus* strain was included in all mixed cultures due to its proteolytic capacity in milk protein, in contrast to *Bifidobacterium* strains. The axenic culture of *L. acidophilus* and unfermented milk were used as controls.

### 2.2.2. Fermentation conditions

Fermentation was carried out by inoculating each mixed culture in 250 mL of sterile ultra-pasteurized (UHT) skim milk, followed by incubation at 37°C under anaerobic conditions (BD BBL GasPack system, USA) for 72 h. 1 mL of samples were aseptically withdrawn at 0, 24, 48, and 72 h of fermentation. Viable cells, pH, lactose, organic acids, free amino acids, and volatile compounds were determined. The experiments were performed in triplicate and the results were reported as the mean ± standard deviation.

### 2.2.3. Sample treatment

For the determination of lactose, organic acids, and free amino acids, it was necessary to prepare the samples. Control samples were composed of unfermented UHT skim milk. Samples and control (1 mL) were first treated with an equal volume of acetonitrile (Sigma, USA) for 12 h at 4 °C, centrifuged for 5 min at 13000 x g to remove proteins, fats, and cells and then extracted, as described by Polson *et al.* (2003). Subsequently, the samples were lyophilized and resuspended to the initial volume with ultra-pure water.

### 2.2.4. Determination of free amino acids

The samples were derivatized with a Pico Taq derivatization kit (Waters Corp., Milford, MA, USA), as recommended by the manufacturer. Phenylisothiocyanate derivatives of free amino acids were quantified by HPLC, as described by Bidlingmeyer *et al.* (1984). Amino acid content was evaluated using an Agilent Technologies 1200 chromatograph coupled to a UV detector. The column used was a C18 Pico-Tag (packed in 3.9 x 150 mm, 5 µm particle size, Waters, USA). The operating conditions were the following: flow rate 1.0 mL/min, solution A: 0.14 M acetic acid pH 6.51 (Sigma, USA) in ultra-pure water and solution B: acetonitrile in ultra-pure water (60:20 v/v). Elution was performed using 96% of solution A and 4% of solution B for 10 min. After this, a washing step was performed using 100% of solution B. The injection volume was 4 µL of a derivatized sample. As amino acids standard, H-standard (Waters, USA) was used. Tryptophan and asparagine were calibrated separately. Only glutamine was not measured. Absorbance was measured at 240 nm. The mean concentration was expressed in mg/L of the fermented substrate.

### 2.2.5. Change of pH and organic acids content

The pH of samples was measured with a pHmeter, and the organic acids content was quantified by HPLC, according to Al-Tamimi *et al.* (2006). 50 µL of samples were diluted in a proportion of 1:20 with ultra-pure water. Organic acids content was determined using an Agilent Technologies 1200 chromatograph coupled to a UV detector. The procedure was performed using an exchange ionic column Aminex HPX-87H (300 x 7.8 mm, 9 µm particle size, Bio-Rad, Watford, Herts, UK). The operating conditions were the following: flow rate 0.6 mL/min; mobile phase 5 mM $H_2SO_4$ performed at 50 °C. The injection volume was 10 µL. Standards of organic acids were used including lactic, acetic, propionic, and butyric (Sigma, USA). Calibration curves were made for each organic acid ranging from 1 to 100 mM. Absorbance was measured at 220 nm. The mean concentration was expressed in g/L of the fermented substrate.

### 2.2.6. Determination of lactose content

Lactose was quantified by HPLC, according to Joseph (2014). 50 µL of samples were diluted in a proportion of 1:20 with ultra-pure water. Lactose content was evaluated using an Agilent Technologies 1200 chromatograph coupled to a refractive index detector. The procedure was performed with a ZORBAX carbohydrate column (150 x 4.6 mm, 5.0 µm particle size, Santa Clara, USA). The operating conditions were the following: flow rate 1.0 ml/min, mobile phase 75% acetonitrile, temperature of 50 °C. The injection volume was 3.0 µL. A calibration curve of lactose was made from a range of 1 to 100 mM, and the concentration was expressed in g/L of the fermented substrate.

### 2.2.7. Determination of volatile compounds by Solid Phase Micro Extraction (SPME) and GC-MS

The volatile compounds were determined, according to Rosales *et al.* (2017) with some modifications. Briefly, 10 g of fermented skim milk by the mixed culture at 0, 24, and 72 h of fermentation were placed in headspace vials with 10 g of anhydrous sodium sulfate. Vials were sealed and heated in a water bath at 70 °C for 15 min. Later the septum was pierced with a sharp needle to allow the insertion of the SPME-syringe (Supelco, USA). Two centimeters of the divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) coated fiber was exposed to the volatiles in the vial headspace maintaining the temperature at 40 °C for 20 min. The fiber head was not allowed to have direct contact with the fermented milk. Samples were analyzed in a gas chromatograph (Agilent Technologies 7890A series) coupled to a mass selective detector with electron impact ionization (Agilent Technologies 5975 series). The fiber could desorb in the GC injection port for 15 min. Measurements were performed by triplicate in three independent experiments. The GC parameters were adjusted to obtain the best resolution of analytes. The injection temperature was 230 °C. The splitless injection mode was used. The initial oven temperature was 40 °C and kept constant for 3 min, then increased at a rate of 6 °C/min to 160 °C. Afterward, a second ramp of 10 °C/min until 240 °C was applied. A J&W DB-1MS Ultra Inert capillary column (60 m x 25 mm x 0.25 µm) was used. The electron impact mass spectrometer was operated in the scan mode, ranging between 25 and 450 m/z. The MS source and quadrupole temperatures were 230 °C and 150 °C, respectively. The collision energy was 70 eV. The data was collected with Mass Hunter software (Agilent Technologies, Inc.). Data analysis was done with Automated Mass Spectral Deconvolution and Identification System (AMDIS) software. For compounds identification, the Mass Spectra Library software and the Database NIST MS Search version 2.0 (National Institute of Standards and Technology, 2008) was used. The results were expressed as relative area (RA).

### 2.2.8. Measurement of viable cells in mixed culture

Samples (10 mL) of fermented skim milk by mixed cultures were taken at 0, 24, 48, and 72 h. Each sample was dissolved in 90 mL of PBS at pH 7.0 and stirred for 30 s. Serial 10-fold dilutions were made and spread in an MRS agar plate. The count of probiotic strains was carried out according to colony characteristics wherein *L. acidophilus* and *Bifidobacterium* strains were quantified in a mixed culture. For the consortium of La-Bado+Bani+Bbi+Bbr+Bin, *Bifidobacterium* strains were counted as a single strain due to their similar macroscopic characteristics. The incubation was carried out under anaerobic conditions at 37 °C for 24, 48, and 72 h, respectively. The results are expressed in CFU/mL of fermented milk.

### 2.2.9. Data presentation and statistical analyses

All experiments were conducted in triplicate and the results are expressed as the mean ± standard deviation. Normality was tested using the Shapiro's test. The assumption of the homogeneity of variances was tested using Levene's test. As both assumptions of normality and homogeneity of variances were satisfied, parametric testing was performed. The data were subjected to a two-way analysis of variance and significant differences at $p < 0.05$ were identified by Tukey's test, using statistical program R (Core Team 2013).

## 3. RESULTS

### 3.1. Production of free amino acids

La-Bbi and La-Bani were highlighted with 23-28% of total free amino acids production with respect to a *L. acidophilus* monoculture ($p < 0.05$) (Fig. 1a).

The amino acids profile produced by fermentation were grouped into three major groups. The first group contained Arg, Cys, His, Phe, Thr, and Trp. The second group contained Ala, Asn, Lys, and Ser. The third group contained Asp, Gly, Glu, Ile, Leu, Met, Pro, Tyr, and Val (Fig. 1b). La-Bbi and La-Bani cultures were the best producers of essential amino acids, including Ile (15.69 and 14.29 mg/L, respectively), Leu (43.60 and 43.10 mg/L, respectively), and Met (12.12 and 12.64 mg/l, respectively) ($p < 0.05$). However, La-Bbi showed the highest content of Trp (2.48 mg/L) and Val (4.54 mg/L), while La-Bani, La-Bado, and La-Bbr were the best producers for His (15.69 – 18.30 mg/l) and Thr (8.16 – 8.22 mg/L) ($p < 0.05$). The *L. acidophilus* monoculture was the best producer for Lys (35.85 – 38.84 mg/L) ($p < 0.05$). Finally, all cultures showed a significant reduction of Phe with respect to unfermented milk ($p < 0.05$) (Fig. 1b).

For non-essential amino acids, La-Bbi and La-Bin cultures showed the highest production of Glu (127.83 and 108.30 mg/L, respectively), Gly (13.75 and 10.43 mg/L, respectively), and Pro (66.92 and 71.94 mg/L, respectively) ($p < 0.05$). In addition, La-Bbi was the best producer for Tyr (13.99 mg/L). La, La-Bbr and La-Bani cultures stood out in the production of Ala (19.36 – 20.06 mg/L), Asn (3.05 – 3.62 mg/L), Cys (1.38 – 1.68 mg/L) and Ser (3.97 – 5.83 mg/L) ($p < 0.05$) (Fig. 1b).

### 3.2. Acidification profile, lactose, and viable cell content

The acidification profile was dependent on the culture and the fermentation time evaluated. La-Bbi, La-Bado, and La-CON showed higher efficiency in acidification profile with a lowest pH (3.5 – 3.6) and highest production of lactic acid (26.1±0.1 – 27.4±0.3 g/L) at the end of the process ($p < 0.05$) (Table 1). In addition, all mixed cultures showed similar acetic acid production at different fermentation times, highlighting La-Bani (3.8 g/L) at 72 h.

La-Bbi, La-Bin, and La-CON cultures stood out with the highest lactose consumption at the end of fermentation, ranging between 58.1 – 63.1% of residual lactose ($p < 0.05$) (Table 2). Our results show that the viability of *L. acidophilus* was reduced one logarithmic unit in all cultures, while the *Bifidobacterium* content was not affected and remained the same across all the mixed cultures ($p <0.05$) (Table 2).

### 3.3. Production of volatile compounds

We determined the amount (RA) and profile of volatiles produced during fermentation in UHT skim milk by mixed cultures and compared them to the volatiles present in

commercially fermented dairy products (*i.e.* yogurt, Emmental, and gouda cheese). A total of 52 volatile compounds were detected at both 24 and 72 h of fermentation, including 1.9% aldehydes, 7.7% esters, 9.6% alcohols, 15.4% ketones, 23.1% carboxylic acids, and 42.3% hydrocarbons (Fig. 2).

All mixed cultures showed a higher diversity of volatile compounds with respect to commercial products. La-Bbi (RA=10.42), La-Bado (RA=11.74), and La-Bin (RA=12.36) were the mixed cultures with the highest production of volatile compounds (61.5, 63, and 73% of the total volatile compounds determined, respectively).

The La-Bado profile comprised 2.55% aldehydes, 10.5% alcohols, 13.20% carboxylic acids, 18.4% ketones, and 55.3% hydrocarbons. The La-Bin profile comprised 3.3% aldehydes, 9.6% alcohols, 16.1% carboxylic acids, 19.4% ketones, and 51.6% hydrocarbons. Finally, the La-Bbi profile comprised 3.1% aldehydes and alcohols, 18.75% ketones, 21.8% carboxylic acids, and 53.1% hydrocarbons. The distinctive volatile compounds of La-Bin with respect to La-Bbi were as follows: 1-octanol, diisobutilcarbinol, hemellitol, and ethyl acetate. Meanwhile, 1-dodecanol and 2-undecanone were found distinctive in La-Bado with respect to La-Bbi. Also, acetyl valeric acid was only found in the La-Bado consortium, octanoic acid only detected in the La-Bbi and the La-Bado profiles, and 2-methyl-butyric acid in the La-Bbi profile.

The volatile profile produced by La-Bbi, La-Bin, and La-Bado showed distinctive volatile compounds with respect to the *L. acidophilus* monoculture, such as ethanol, diisobutilcarbinol, 1-heptanol, hemellitol, dodecanol, 2-pentanone, 2-undecanone, and octanoic acid. In addition, with respect to commercial products, the distinctive volatile compounds of these consortiums were 1-heptanol, diisobutilcarbinol, hemellitol, 1-dodecanol, acetone, 2-pentanone, 2-undecanone, isopropylpyruvic, isovaleric and acetyl valeric acids, benzaldehyde, and ethyl acetate. Also, these mixed cultures showed an increased amount of some volatile compounds with respect to commercial products, such as acetoin (1.9 to 14.1 times), 2-heptanone (2.1 to 10.5 times), 2-nonanone (1.6 to 9.0 times), acetic acid (14 to 75 times), butanoic acid (2.5 to 4.6 times) and hexanoic acid (1.5 to 3.2 times).

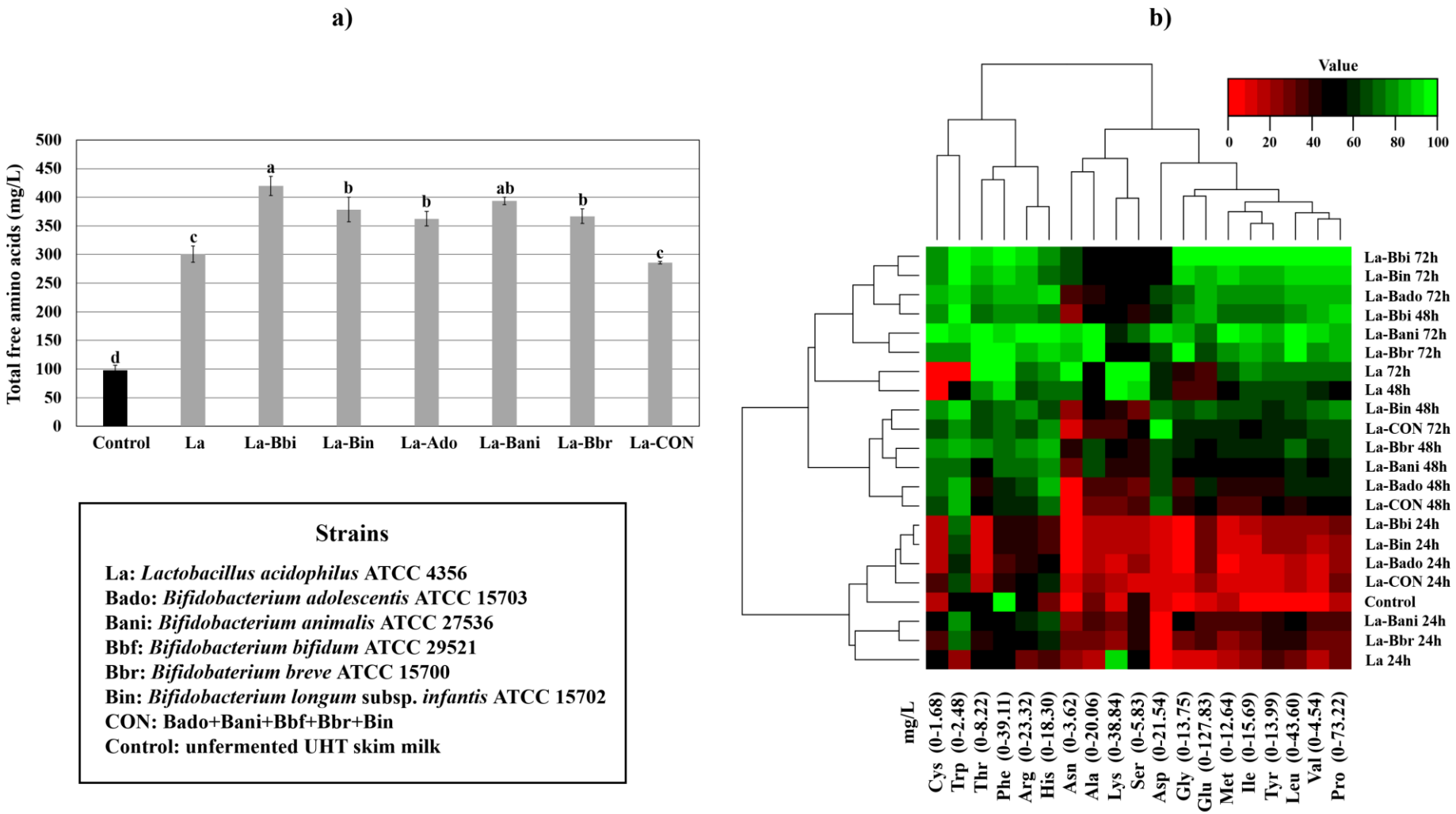


**Fig. 1** a) Content of total free amino acids produced during anaerobic fermentation of UHT skim milk at 37° C for 72 h by probiotic mixed cultures. The data shown represent the mean obtained from three independent experiments. Bars with a different superscript are significantly different after performing Tukey's test ($p< 0.05$). b) Heat-map of free amino acids profiles produced by probiotic mixed culture after anaerobic fermentation of UHT skim milk at 37°C for 24, 48, and 72 h. The values obtained indicate the total production of free amino acids (without subtracting the value of each control) and represent the mean obtained from three independent experiments. The total production values were normalized based on a 0-100 scale. The upper limit corresponds to the maximum value for each amino acid in mg/L.

**Table 1.** Evolution of pH and organic acids content during fermentation of UHT skim milk by probiotic mixed culture incubated under anaerobic conditions at 37° C for 72 h.

| Culture | pH | | | Organic acids (g/L) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Lactic acid | | | Acetic acid | | |
| | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h |
| La | 5.3±0.02$^{g}$ | 4.5±0.01$^{d}$ | 4.1±0.03$^{c}$ | 3.4±0.4$^{j}$ | 10.8±0.2$^{f}$ | 17.3±0.3$^{d}$ | 0.5±0.1$^{e}$ | 0.5±0.1$^{e}$ | 0.5±0.2$^{e}$ |
| La-Bbi | 4.9±0.03$^{e}$ | 3.6±0.03$^{a}$ | 3.6±0.02$^{a}$ | 11.4±0.1$^{ef}$ | 18.8±0.6$^{d}$ | 26.1±0.1$^{ab}$ | 2.8±0.1$^{cd}$ | 2.9±0.2$^{d}$ | 3.2±0.0$^{bc}$ |
| La-Bin | 5.1±0.02$^{f}$ | 3.6±0.02$^{a}$ | 3.5±0.03$^{a}$ | 8.0±0.1$^{h}$ | 20.9±0.5$^{c}$ | 22.2±0.4$^{c}$ | 3.3±0.8$^{bc}$ | 3.4±0.1$^{ab}$ | 2.9±0.1$^{cd}$ |
| La-Bado | 5.2±0.01$^{fg}$ | 3.8±0.02$^{b}$ | 3.6±0.01$^{a}$ | 9.9±0.7$^{fg}$ | 25.3±0.7$^{b}$ | 27.1±0.5$^{a}$ | 3.5±0.8$^{ab}$ | 3.4±0.1$^{ab}$ | 2.9±0.2$^{cd}$ |
| La-Bani | 4.9±0.01$^{e}$ | 4.5±0.02$^{d}$ | 4.2±0.01$^{c}$ | 6.8±0.3$^{i}$ | 10.7±0.1$^{fg}$ | 16.0±0.5$^{d}$ | 3.5±0.3$^{ab}$ | 3.4±0.2$^{ab}$ | 3.8±0.1$^{a}$ |
| La-Bbr | 5.1±0.03$^{f}$ | 4.0±0.03$^{c}$ | 3.8±0.03$^{b}$ | 8.1±0.5$^{h}$ | 21.5±0.1$^{c}$ | 25.1±0.9$^{b}$ | 2.9±0.4$^{cd}$ | 2.8±0.2$^{e}$ | 3.6±0.1$^{ab}$ |
| La-CON | 4.4±0.02$^{d}$ | 3.8±0.01$^{b}$ | 3.6±0.02$^{a}$ | 12.7±0.4$^{e}$ | 24.9±1.3$^{b}$ | 27.4±0.3$^{a}$ | 3.3±0.1$^{bc}$ | 3.4±0.1$^{ab}$ | 3.3±0.1$^{bc}$ |

The strains included the following mixed cultures: *Lactobacillus acidophilus* ATCC 4356 (La), *Bifidobacterium adolescentis* ATCC 15703 (Bado), *Bifidobacterium animalis* ATCC 27536 (Bani), *Bifidobacterium bifidum* ATCC 29521 (Bbi), *Bifidobacterium breve* ATCC 15700 (Bbr), *Bifidobacterium infantis* ATCC 15702 (Bin) and a consortium formed by La+ five *Bifidobacterium* strains. Unfermented skim milk was used as a control and the reference values: pH 6.5± 0.01, lactic acid 0.88±0.04, and acetic acid 0.054±0.001 g/L.
Data show the averages obtained from three independent experiments and their standard deviation. Different superscripts for each determined variable indicate significant differences after performing Tukey´s test ($p < 0.05$)

**Table 2.** Residual lactose and viable cells content in fermented UHT skim milk by mixed probiotic culture incubated under anaerobic conditions at 37° C for 72 h.

| Culture | Residual lactose (g/L) | | | Viable cells log (cfu/mL) 72h | |
|---|---|---|---|---|---|
| | 24h | 48h | 72h | La | Bifidobacteria |
| La | 41.2±1.1$^{gh}$ | 37.2±2.6$^{de}$ | 34.1±2.4$^{bc}$ | 8.25±0.03$^{a}$ | - |
| La-Bbi | 45.1±1.9$^{ij}$ | 36.4±1.5$^{cd}$ | 28.0±1.5$^{a}$ | 7.20±0.16$^{b}$ | 8.77±0.19$^{a}$ |
| La-Bin | 47.2±0.7$^{l}$ | 35.0±1.8$^{bcd}$ | 27.6±2.7$^{a}$ | 7.15±0.08$^{b}$ | 8.53±0.14$^{ab}$ |
| La-Bado | 46.8±0.1j$^{k}$ | 37.1±1.9$^{de}$ | 32.8±1.9$^{b}$ | 7.17±0.05$^{b}$ | 8.52±0.08$^{ab}$ |
| La-Bani | 43.3±0.4$^{hi}$ | 39.8±1.6$^{fg}$ | 34.5±0.7$^{bc}$ | 7.14±0.02$^{b}$ | 8.44±0.21$^{ab}$ |
| La-Bbr | 41.8±0.8$^{gh}$ | 38.7±0.8$^{fg}$ | 36.6±0.6$^{cd}$ | 7.13±0.06$^{b}$ | 7.56±0.13$^{ab}$ |
| La-CON | 40.3±0.3$^{fgh}$ | 36.7±1.2$^{cd}$ | 30.0±0.1$^{ab}$ | 6.90±0.03$^{b}$ | 8.80±0.07$^{a}$ |

The strains included the following mixed cultures: *Lactobacillus acidophilus* ATCC 4356 (La), *Bifidobacterium adolescentis* ATCC 15703 (Bado), *Bifidobacterium animalis* ATCC 27536 (Bani), *Bifidobacterium bifidum* ATCC 29521 (Bbi), *Bifidobacterium breve* ATCC 15700 (Bbr), *Bifidobacterium infantis* ATCC 15702 (Bin) and a consortium formed by La+ five *Bifidobacterium* strains. The initial inoculum was adjusted to $10^6$ CFU, divided into equal proportions between the strains that form the mixed culture.

The lactose content in unfermented skim milk (control) was 47.5±0.74 g/L.

Data show the averages obtained from three independent experiments and their standard deviation. Different superscripts for each determined variable indicate significant differences after performing Tukey´s test ($p < 0.05$).

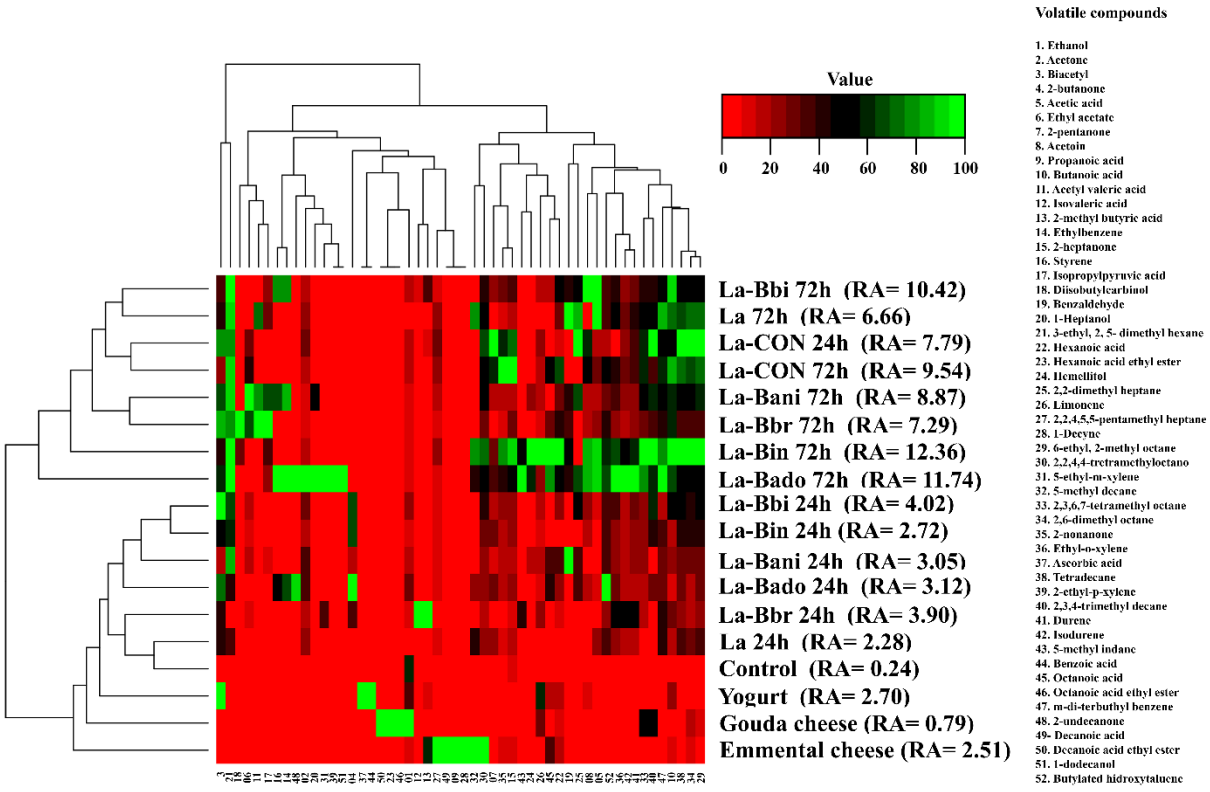

**Fig. 2** Heat map of volatiles produced by commercial dairy products and mixed probiotic culture after anaerobic fermentation of UHT skim milk at 37°C for 24 and 72 h. The strains are: *Lactobacillus acidophilus* (La), *Bifidobacterium adolescentis* ATCC 15703 (Bado), *Bifidobacterium animalis* ATCC 27536 (Bani), *Bifidobacterium bifidum* ATCC 29521 (Bbi), *Bifidobacterium breve* ATCC 15700 (Bbr), *Bifidobacterium infantis* ATCC 15707 (Bin) and a consortium formed by *L. acidophilus* + five *Bifidobacterium* strains (CON). The values represent the mean obtained from three independent experiments and were normalized based on a 0-100 scale. The upper limit is equivalent to the maximum value of Relative Area (RA) for each compound.

## 4. DISCUSSION

### 4.1. Acidification profile and cell viability of the mixed culture

All strains showed a high degree of cell viability at the end of fermentation, ranging between $10^6$ and $10^8$ CFU / mL, which corresponds to intake recommendations for the treatment of various intestinal infections (Olagnero *et al*., 2007). Although fermented dairy foods are subjected to different processing conditions that may influence cell viability, milk components increase the survival of *Lactobacillus* and *Bifidobacterium* in fermented dairy foods with high viability for a long time (Karimi *et al.,* 2012; Ong *et al*., 2016).

All mixed cultures showed a similar pattern of organic acid production and pH reduction at the end of fermentation. The content of acetic acid was reduced during the fermentation process as reported by De Sousa *et al*. (2012) for mixed cultures formed with *Streptococcus thermophilus* and *Bifidobacterium* strains. These results suggest the possible “bifidus” pathway inhibition for *Bifidobacterium* strains. With respect to commercial starter cultures, the mixed cultures evaluated in this study showed a low acidification rate. However, a low acidification rate in an adjunct culture used in cheese ripening is a desirable characteristic because excess of acidity negatively affects the sensory characteristics of the final product (Burns *et al*., 2012).

### 4.2. Production of free amino acids

It has been reported that the *Lactobacillus* genus has a more complete proteolytic system than *Bifidobacterium*, being the main difference the presence of extracellular proteinases (Boylston *et al.,* 2004). Probiotic species of *L. acidophilus* can express the necessary components to generate the cell wall-bound proteinases, prtP (precursor of the proteinase) and prtM (maturase), that allow for the ability to hydrolyze the milk protein (Azcarate-Peril, 2009). Something similar was found in this study, the *L. acidophilus* ATCC 4356 monoculture showed milk adaptability in contrast to the *Bifidobacterium* strains. The differences between the mixed cultures evaluated in free amino acid production are determined by the contribution of the proteolytic system of *Bifidobacterium* strains, resulting from the

secondary proteolysis of peptides released by *L. acidophilus,* as reported for other adjunct cultures in the manufacture of fermented dairy foods (Milesi *et al.,* 2009). Also, the increase in the concentration of free amino acids at the end of fermentation can be explained by bacterial autolysis and cytoplasmic peptidases released due to the unfavorable conditions resulting from fermentation (Savijoki *et al.*, 2006). Shihata & Sha (2000) reported that both *L. acidophilus* and *Bifidobacterium* spp. strains have high intracellular dipeptidase and aminopeptidase activity which could explain this behavior. Furthermore, *Lactobacillus* and *Bifidobacterium* strains show little growth under the unfavorable conditions produced during fermentation which implies low utilization of the amino acids in the medium for the synthesis of bacterial proteins. In addition, the amino acid availability in the medium will depend on the auxotrophies present in each strain which determine their nutritional requirements (Hayek & Ibrahim, 2013).

The availability of branched-chain (Ile, Leu, Val), aromatic (Trp, Phe, and Tyr), and sulfur amino acids (Met) in fermented dairy foods contribute directly to the flavor of these products and serve as precursors of aroma and flavor compounds (Ardö, 2006). These findings highlight the impact of LA-Bbi and LA-Bani cultures evaluate in this study. Additionally, LA-Bbi and LA-Bani cultures stood out in the production of Ala and Glu which contribute to the sweet and bitter taste of the dairy product, respectively (Pachlová *et al.,* 2013). Moreover, Glu availability stands out in importance because it contributes significantly to the catabolism of amino acids via transamination, resulting in a more efficient process of flavor compounds production (Kieronczyk *et al*., 2003). The amino acids profile obtained by La-Bbi and La-Bani in this study differs from those reported for other probiotic strains (*L. acidophilus, L. paracasei, L. rhamnosus, L. plantarum, L. rhamnosus, Bifidobacterium lactis*) used as adjunct cultures in the manufacture of soft and ripened cheese (Bergamini *et al.,* 2009; Burns *et al*., 2012; Milesi *et al.,* 2009). This could suggest different sensory and nutritional impacts on the final product.

The increased amount of free essential amino acids in fermented dairy products contributes significantly to the nutritional value of the food. The branched-chain amino acids (Ile, Leu, and Val) represent between 14-18% of muscle protein composition and Leu is one of the most important essential amino acids for the human diet and induces signaling pathways for muscle protein synthesis. This prevents its catabolism and favors the increase of muscle mass during physical activity and under various physiological stress conditions (van Loon, 2012). Aromatic amino acids (Trp, Tyr, and Fen) favor the central nervous system and serve as precursors for the synthesis of neurotransmitters with modulating effects in physiological and psychological processes (Jenkins *et al*., 2016). This highlights the metabolic capacity of mixed cultures formed by La-Bbi and La-Bani with increased production of aromatic and branched-chain amino acids.

### 4.3. Production of volatile compounds

The mixed cultures of La-Bado, La-Bani, and La-Bbi showed the highest complexity of volatile compounds profiles generated by fermentation compared to

commercial fermented dairy products. According to volatile compounds profiles reported for yogurt manufactured from commercial starter cultures formed by *Streptococcus thermophilus* and *L. delbrueckii spp. bulgaricus*, the mixed cultures La-Bado, La-Bani, and La-Bbi produced distinctive volatiles compounds, such as hemelitol, 1-dodecanol, isopropylpyrubic acid, and aliphatic and an aromatic hydrocarbons profile (Dan *et al.,* 2017). With respect to Parmesan, Emmental, and Beaten cheeses the distinctive volatile compounds detected were as follows: 1-heptanol, hemelitol, 1-dodecanol, diacetyl, acetoin, acetic, isobutanoic, acetyl valeric, isovaleric, isopropylpyruvic and 2-methyl-butyric acid, ethyl acetate, hexanoic acid ethyl ester, 2-undecanone, benzaldehyde, as well as hydrocarbon (Lee *et al*., 2003; Pillonel *et al.,* 2003; Sulejmani *et al*., 2014). In addition, monocultures of *L. acidophilus* and *Bifidobacterium* spp. strains have a little positive or null effect on the sensory properties of ripened cheeses. However, some mixed cultures formed by these strains have increased the consumer's acceptability of some milk products due to their impact on the sensory properties (Karimi *et al.,* 2012).

Our results suggest that the mixed cultures formed by La-Bani and La-Bbi have a potential application as starter or adjunct cultures in the production of fermented dairy foods since they would favor acidification and free amino acid profile, thereby increasing the nutritional food value. In addition, they would provide a distinctive sensory effect on the fermented dairy products through the production of aroma and flavor compounds.

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## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

## REFERENCES

Akabanda, F., Owusu-Kwarteng, J., Tano-Debrah, K., Parkouda, C. & Jespersen, L. 2014. The use of lactic acid bacteria starter culture in the production of Nunu, a spontaneously fermented milk products in Ghana. International Journal of Food Science. http://dx.doi.org/10.1115/2014/721067.

Al-Tamimi, M. A., Palframan, R. J., Cooper, J. M., Gibson, G. R., Rastal, R. A. 2006. *In vitro* fermentation of sugar beet Arabian and arabino-oligosaccharides by the human gut microflora. Journal of Applied Microbiology. 100: 407-414. DOI: 10.1111/j.1365-2672.2005.02780.x

Ardö, Y. 2006. Flavour formation by amino acid catabolism. Research review paper. Biotechnology Advances. 24: 238-242. https://doi.org/10.1016/j.biotechadv.2005.11.005

Azcarate-Peril, M. A., Tallon, R. & Klaenhammer, T. R. 2009. Temporal gene expression and probiotic attributes of *Lactobacillus acidophilus* during growth in milk. J. Dairy Sci. 92: 870–886. https://doi.org/10.3168/jds.2008-1457

Banan-Mwine, E. & Lee, B. H. 2015. New perspectives on probiotics in health and disease. Food Science and Human Wellness. 4: 56–65. http://dx.doi.org/10.1016/j.fshw.2015.06.002 2213-4530

Bergamini, C. V., Hynes, E. R., Palma, S. B., Sabbag, N. G., Zalazar, C. A. 2009. Proteolytic activity of three probiotic strains in semi-hard cheese as single and mixed cultures: *Lactobacillus acidophilus*, *Lactobacillus paracasei* and *Bifidobacterium lactis*. International Dairy Journal. 19: 467-475. https://doi.org/10.1016/j.idairyj.2009.02.008

Bidlingmeyer, B. A., Cohen, S. A., Tarvin, T. L.1984. Rapid analysis of amino acids using pre-column derivatization. Journal of Chromatography B: Biomedical Sciencias and Applications. 336: 93-104. https://doi.org/10.1016/S0378-4347(00)85133-6

Boylston, T. D., Vinderola, C. G., Ghoddusi, H. B., Reinheinmer, J. A. 2004. Incorporation of bifidobacteria into cheeses: Challenges and rewards. International Dairy Journal. 14: 375-387. https://doi.org/10.1016/j.idairyj.2003.08.008

Burns, P., Cuffia, F., Milesi, M., Vinderola, G., Meinardi, C., Sabbag, N. & Hynes, E. 2012. Technological and probiotic role of adjunct cultures of non-starter lactobacilli in soft cheese. Food Microbiology. 30: 45-50. https://doi: 10.1016/j.fm.2011.09.015.

Chilton, S. N., Burton, J. P. & Reid, G., 2015. Inclusion of fermented foods in food guides around the world. Nutrients. 7: 390-404. DOI: 10.3390/nu7010390

Chinellato de Azambuja, N., Moreno, I., Aparecida Gallina, D., Spadoti, L. M., Pettirossi Motta, E. M., Bertolodo Pacheco, M. T., Meneses de Queiroz, A. L. & Costa Antunes, A. E. 2017. Effect of adjunct culture *Lactobacillus heleveticus* (B02) on the composition, proteolysis, free amino acids release and sensory characteristics of Prato cheese. Food and Nutrition Science. 8(5): 512-525.DOI:10.4236/fns.2017.85035

Dan, T., Wang, D., Jin, R. L., Zhang, H. P., Zhou, T. T. & Sun, T. S. 2016. Characterization of volatile compounds in fermented milk using solid-phase microextraction methods coupled with gas chromatography-mass spectrometry.

Journal of Dairy Scicience. 100: 2488–2500. https://doi.org/10.3168/jds.2016-11528

De Sousa-Oliveira, R. P., Perego, P., Nogueira de Oliveira, M. & Converti, A. 2012. Growth, organic acids profile and sugar metabolism of *Bifidobacterium lactis* in co-culture with *Streptococcus thermophilus*: The inulin effect. Food Research International. 48: 21–27. https://doi.org/10.1016/j.foodres.2012.02.012

FAO/WHO. 2002. Guidelines for the Evaluation of Probiotics in Food. Report of a joint. Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food. London, Ontario, Canada. URL: Working Group Report21.PDF (who.int)

Hayek, S. A. & Ibrahim, S. A. 2013. Current Limitations and Challenges with Lactic Acid Bacteria: A Review. Food and Nutrition Sciences. 4: 73-87. DOI:10.4236/fns.2013.411A010

Jenkins, T. A., Nguyen, J. C. D., Polglaze, K. E. & Bertrand, P. P. 2016. Influence of tryptophan and serotonin on mood and cognition with a possible role of the gut-brain axis. Nutrients. https://doi.org/10.3390/nu8010056

Joseph, M. 2014. The Analysis of Milk Sugars with a Modern Refractive Index Detector and a Carbohydrate Column. Agilent Corporation. 4:1-2. URL: The analysis of Milk Sugars with a Modern Refractive Index Detector and a Carbohydrate Column (agilent.com)

Karimi, R., Sohrabvandi, S. & Mortazavian, A. M. 2012. Review Article: Sensory Characteristics of Probiotic Cheese. *Institute of* Food Technologists. 11: 437-452. https://doi.org/10.1111/j.1541-4337.2012.00194.x

Kieronczyk, A., Skeie, S., Langsrud, T. & Yvon, M. 2003. Cooperation between *Lactocaccus lactis* and nonstarter Lactobacilli in the formation of cheese aroma from amino acids. Applied and Environmental Microbiology. 69: 734-739. doi: 10.1128/AEM.69.2.734-739.2003

Lee, J. H., Diono, R., Kim, G.Y. & Min, D. B. 2003. Optimization of Solid Phase Microextraction Analysis for the Headspace Volatile Compounds of Parmesan Cheese. Journal of Agricultural and Food Chemistry. 51: 1136-1140. DOI: 10.1021/jf025910+

Liu, M., Bayjanov, J. R., Renckens, B., Nauta, A. & Siezen, R. J. 2010. The proteolytic system of lactic acid bacteria revisited: a genomic comparison. BMC Genomics. 11: 1-15. https://doi.org/10.1186/1471-2164-11-36

Milesi, M. M., Vinderola, G., Sabbag, N., Meinardi, C. A. & Hynes, E. 2009. Influence of cheese proteolysis and sensory characteristics of non-starter lactobacilli strains with probiotic potential. Food Research International. 42: 1186-1196. https://doi.org/10.1016/j.foodres.2009.06.005

Olagnero, G., Abad, A., Bendersky, S., Genevois, C., Granzella, C. & Montonati, M. 2007. Alimentos funcionales: fibra, prebióticos, probióticos y simbióticos. DIETA. 25: 20-33. URL: https://www.researchgate.net/publication/274072309_Alimentos_funcionales_fibra_prebioticos_probioticos_y_simbioticos_Functional_foods_Fiber_Prebiotics_Probiotics_and_Simbiotics

Ong, L., Henriksson, A. & Shah, N. P. 2006. Development of probiotic cheddar cheese containing *Lactobacillus acidophilus*, *Lb. casei*, *Lb. paracasei* and *Bifidobacterium* spp. and the influence of these bacteria on proteolytic patterns and production of organic acid. International Dairy Journal. 16: 446-456. https://doi.org/10.1016/j.idairyj.2005.05.008

Pachlová, V., Buňka, F., Chroměcková, M., Buňková, L., Bartak, P. & Pospíšil, P. 2013. The development of free amino acids and volatile compounds in cheese Oloumoucké tvaruzy' (PGI) during ripening. International Journal of Food Science & Technology. 48: 1868-1876. https://doi.org/10.1111/ijfs.12164

Pillonel, L., Ampuero, S., Tabacchi, R. & Bosset, J. O. 2003. Analytical methods for the determination of the geographic origin of emmental cheese: volatile compounds by GC/MS-FID and electronic nose. European Food Research and Technology. 216: 179–183. https://doi.org/10.1007/s00217-002-0629-4

Polson, C., Sarkar, P., Incledon, B., Raguvaran, V. & Grant, R. 2003. Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography-tandem mass spectrometry. Journal of Chromatography B: Analytical Technolgies in the Biomedical and Life Sciences. 785: 263-275. https://doi.org/10.1016/S1570-0232(02)00914-5

R: A language and environment for statistical computing. R foundation for Statistical Computing, Vienna, Austria. URL: http://www.R-project.org/.

Rosales-Bravo, H., Morales-Torres, H., Vázquez-Martínez, J., Molina-Torres, J., Olalde-Portugal, V and Partida-Martínez, L. 2017. Novel consortium of *Klebsiella* variicola and *Lactobacillus* species enhances the functional potential of fermented dairy products by increasing the availability of branched-chain amino acids and the amount of distinctive volátiles. Journal of Applied Microbiology. 123(5): 1237-1250. DOI: 10.1111/jam.13565

Savijoki, K., Ingmer, H. & Varmanen, P. 2006. Proteolytic systems of lactic acid bacteria. Applied Microbiology and Biotechnology. 71: 394-406. DOI: 10.1007/s00253-006-0427-1

Shihata, A. & Shah, N. P., 2000. Proteolytic profiles of yogurt and probiotic bacteria. International Dairy Journal. 10: 401-408.https://doi.org/10.1016/S0958-6946(00)00072-8

Sulejmani, E., Rafajlovska, V. & Guneser, O. 2014. Characterization of volatiles in beaten cheeses “Bieno sirenje” by SPME/GC-MS: Influence of geographical origin. Journal of Serbian Chemical Society. 79: 927–939. DOI:10.2298/JSC130917135S

Tamang, J. P., Shin, D.H., Jung, S. J. & Chae, S. W. 2016. Functional properties of microorganisms in fermented foods. Frontiers in Microbiology. 7: 578. doi: 10.3389/fmicb.2016.00578

Tammam, J. D., Williams, A. G., Noble, J. & Lloyd, D. 2000. Amino acid fermentation in non-starter *Lactobacillus* spp. Isolated from Cheddar cheese. Letters in Applied Microbiology. 30: 370-374. DOI: 10.1046/j.1472-765x.2000.00727.x

Van Loon, L. J. C. 2012. Leucine as pharmaconutrient in health and disease. Current Opinion of Clinical Nutrition and Metabolic Care. 15: 71-77. DOI: 10.1097/MCO.0b013e32834d617a

Wang, B., Yao, M., Lv, L., Ling, Z. & Li, L. 2017. The Human Microbiota in Health and Disease. Engineering 3: 71–82. https://doi.org/10.1016/J.ENG.2017.01.008