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ORIGINAL PAPER

Organic acids profiles in lactose-hydrolyzed yogurt with different matrix composition

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Abstract The composition of the dairy matrix used in yogurt manufacture could influence the metabolic activity of yogurt starter and the compounds produced during fermentation. In this way, the lactose hydrolysis with a β-galactosidase enzyme and the supplementation with dairy powders to obtain different types of delactosed yogurts for lactose-intolerant people modifies the milk base composition. In this work, we studied the influence of the addition of different doses of β-galactosidase and levels of dairy powders on organic acids profile during the manufacture and storage of two varieties of yogurt (natural and sweetened). Lactose, glucose, galactose, titratable acidity, fermentation time, and microbiological counts were also evaluated. The mean proportions of lactose/glucose/galactose in relation to the total sugars were 18:36:46 in hydrolyzed yogurts, while they were 81:4:15 in unhydrolyzed ones. In supplemented yogurts, the content of lactose, citric, orotic, and hippuric acids were significantly increased. The starter population was similar in all yogurts, but some changes in their activity were evidenced due to both factors studied. The fermentation time was slightly increased, and the lactic acid content and the titratable acidity were significantly increased due to fortification while they were decreased by enzyme addition. These results are probably due to the increase in the buffer properties of the milk base by the dairy powder addition and due to an inhibition of the starter activity caused by changes in the carbohydrate profile. The results demonstrated that the changes in the matrix composition of the yogurt affected the organic acids profile, above all the lactic acid content.

Keywords Lactose hydrolysis · β-galactosidase · Lactose-hydrolyzed yogurt · Lactose intolerance · Organic acids

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1 Introduction

Yogurt is a fermented dairy food made by the homofermentative lactic acid bacteria (LAB) *Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. These microorganisms in the final product must be active and at a high number (>10^7 CFU.g^{-1}) during its shelf-life (CAA 2010). The Argentinian Legislation also allows milk fortification/standardization with skim milk powder, milk protein concentrate, or condensed skim milk to get yogurts with different consistency (CAA 2010).

In the fermentation process of yogurt, the metabolic activity of the starter culture on lactose leads to deep changes in the chemical, physical, microbiological, and sensory attributes of milk. The lactic acid is the main compound produced, which gives the product the sharp and acidic taste. Other organic acids, such as acetic, butyric, pyruvic, and formic, can be also generated. In addition, the citric, uric, hippuric, and orotic acids have been found in yogurts, as they are natural compounds of milk. Some of them contribute to yogurt flavor directly or indirectly through the formation of intermediate compounds and act as preservatives (Fernandez-Garcia and McGregor 1994; Adhikari et al. 2002; Tamime and Robinson 2007). Despite all these changes, yogurt still contains significant and variable amounts of intact lactose depending on the milk formulation, as only a part of the lactose (~20–30%) is metabolized (Tamime and Robinson 2007). Thus, the intake of yogurt may cause difficulties to lactose-intolerant individuals. The problem of lactose intolerance is well-known and prevalent in more than half of the world population (Tuure 2007; Dekker and Daamen 2011). A wide variety of reduced-lactose or lactose-free dairy products have been developed and now are available in the market. In this regard, one of the technologies employed to produce delactosed yogurts is the enzymatic method. The β-galactosidase enzyme (EC 3.2.1.23.) catalyzes the hydrolysis of lactose into its constituent monosaccharides (glucose and galactose) (Shakeel-Ur-Rehman 2009). The changes in the carbohydrates profile of yogurt due to fortification with dairy ingredients and the inclusion of β-galactosidase could influence the metabolic activity of the starter and induce modifications in the compounds produced.

Some researchers have studied the profiles of organic acids in different formulations of yogurt in order to evaluate the metabolic activities of the starter culture and probiotic bacteria (Fernandez-Garcia and McGregor 1994; Adhikari et al. 2002; Oliveira et al. 2012). However, according to our knowledge, there are no data published in relation to organic acids profile in delactosed yogurts.

Therefore, the aim of this work was to evaluate the influence of the addition of β-galactosidase enzyme and dairy powder ingredients on the organic acids profiles during the manufacture and storage of two varieties of lactose-hydrolyzed yogurts (natural and sweetened).

2 Materials and methods

2.1 Preparation of milk base

Two varieties of yogurts, natural and sweetened, were selected for this study. Sweetened yogurts are very popular in Argentina and almost the only ones available

in the market since consumers prefer sweet and low-acid products. Until recently, natural yogurts were absent in our market, unlike what happens in the rest of the world where it is the second most consumed variety.

Bulk bovine milk standardized to 3%*w/v* fat content was supplied by a nearby dairy plant (Milkaut S.A., Argentina). Sucrose (Ingenio Ledesma S.A., Argentina) was used as a sweetener in the sweetened yogurts. The dairy ingredients employed included skim milk powder (SMP) and whey protein concentrate (WPC35), both kindly provided by the same supplier of the milk. The concentrations of protein/lactose in the supplements were 32.7/50.0 and 35.0/48.2 g 100 g^{-1} for SMP and WPC, respectively.

Each yogurt making day, 25 L of milk were distributed into five stainless steel vats (5 L each) operating simultaneously; the vats were immersed in a water bath in which the heat source was steam. At the cooling stage, the hot water was replaced by cold water. The milk was heated until it reached 40 °C, and then, the ingredients were incorporated in appropriate cases. Sucrose (8%*w/v*) was added to the milk in the sweetened yogurt variety, and SMP and WPC were also aggregated in different levels in order to obtain different contents of protein and lactose in the milk bases (Table 1). The ingredients were dissolved by manual agitation for 15 min. The milk bases was heated at 90±2 °C, stand for 5 min, and immediately cooled to 42±2 °C.

2.2 Yogurt making

Yogurts were made applying the classic method (Tamime and Robinson 2007) adapted to laboratory scale. Milk bases were inoculated with direct vat set lyophilized starter culture composed of *S. thermophilus* and *L. bulgaricus* (Chr. Hansen, Argentina) in the dosage suggested by the supplier. The commercial β-galactosidase enzyme YNL-2 from *Kluyveromyces lactis* (GODO, Shusei Company Limited, Japan) was added together with the starter at the following levels: 0.00, 0.25, and 0.40 g L^{-1}, according to Table 1. The incubation process was conducted at 42±2 °C until pH 4.70±0.10 was reached. Then, the set of yogurts were immediately cooled at 25 °C in an ice water bath, applying intermittent manual agitation. Then, the products were placed in 500-mL glass flasks with screw cap and stored at 5±1 °C for 28 days.

Table 1 Experimental design for natural and sweetened yogurt making

Yogurts	Factor 1: enzyme doses (E) (g.L^{-1})	Factor 2: powder levels (P) (SMP:WPC, % *w/v*)
E0P0	0 (0.00)	**0** (0.00:0.00)
E1P0	1 (0.25)	
E2P0	2 (0.40)	
E0PI	0 (0.00)	**I** (1.13:1.00)
E1PI	1 (0.25)	
E2PI	2 (0.40)	
E0PII	0 (0.00)	**II** (2.25:2.00)
E1PII	1 (0.25)	
E2PII	2 (0.40)	

SMP skim milk powder, *WPC* whey protein concentrate 35%

2.3 Physicochemical determinations and microbiological counts

The measurement of pH during fermentation and in the freshly made yogurts was done with a digital pH meter (Orion 3 star benchtop, Thermo Fisher Scientific Inc., USA). Titratable acidity (TA) of the milk bases and yogurts at 1 and 28 days of storage was determined by titration with 0.1 N NaOH detecting the end point at pH 8.3 with the pH meter (IDF International Dairy Federation 2012). The results were expressed as Dornic degree (1°D=100 mg lactic acid L^{-1}). Protein, total solid, and fat contents of the yogurts with 7 days of storage were analyzed according to standard methods (IDF 2001, 2005; Bradley et al. 1992, respectively).

Microbiological analyses were made in yogurt samples at the end of the storage period (28 days). For that, 1 g of each yogurt was decimally diluted in sterile peptone water 0.1%, and 0.1-mL aliquots dilutions were spread over the surfaces of plates of SMA (Birollo et al. 2000) to enumerate the total LAB from the yogurt starter cultures, and 1 mL was plated in yeast extract–glucose–chloranphenicol agar (YEGCA) to enumerate molds and yeasts (Frank et al. 1993). Plates were incubated at 37 °C–48 h for the counting of the total LAB and at 25 °C–5 days for the counting of molds and yeasts.

Analyses were performed in duplicate.

2.4 Organic acids and carbohydrates analyses by HPLC

The concentration of organic acids (lactic, citric, acetic, butyric, hippuric, and orotic) and carbohydrates (lactose, glucose, and galactose) were determined at different times during the manufacture and storage of yogurts. For organic acids, samples were taken at the start of process (milk bases), 150 min and in freshly made yogurts, and at 14 and 28 days of storage; in the case of carbohydrates, the analysis were performed in milk bases, fresh yogurts, and after 28 days. In particular, glucose and galactose were only quantified in natural yogurts because the presence of sucrose in the formulation of sweetened yogurts did not allow their quantification. In the chromatographic conditions used, acid medium and high temperature, the sucrose undergoes the reaction of inversion and fructose and glucose are formed. The peak of fructose is overlapping with galactose, and the peak of glucose is the result of the sum of the glucose produced during the manufacture of yogurts and those providing from the inversion of sucrose during the analysis of yogurt samples.

The simultaneous analyses were performed by high performance liquid chromatography (HPLC) method according to Zeppa et al. (2001), with some modifications. Chromatographic separation was carried out isocratically at 65 °C with a mobile phase of 10 mM H_2SO_4 at a flow rate of 0.6 mL.min^{-1} on an Aminex HPX-87H column (300×7.8 mm) equipped with a cation H^+ microguard cartridge (Bio-Rad Laboratories, USA). HPLC equipment consisted of a quaternary pump, an online degasser, a column oven, a UV–visible detector (all Series 200), and a refractive index detector thermostatized at 35 °C (Series Flexar) (Perkin Elmer, USA). The UV detector was set at 210 nm for the detection of organic acids, while the IR detector setting at 35 °C was used for the analyses of carbohydrates. Data were collected and processed on a computer with the software Chromera® (Perkin Elmer).

Briefly, 5 g of milk or yogurt were diluted with 10 mM H_2SO_4 to 50 mL. The suspension was homogenized and centrifuged at 15,000×*g*/20 min/4 °C. The supernatant was filtered through a 0.45-μm membrane (Millex, Millipore, Brazil) and injected into the chromatograph, using a loop of 60 μL. Quantification was achieved using the peak areas from external calibration with standard solutions (Sigma Aldrich, USA).

2.5 Statistical analyses

A 3×3 factorial experimental design, two factors (the addition of dairy ingredients and the β-galactosidase enzyme) at three levels (without addition, low and high levels), was assayed for each variety of yogurt. Thus, nine different types of yogurts were manufactured, which were performed in triplicate resulting in a total of 27 experimental units for each variety (Table 1). The distribution of yogurts making was randomized in successive weeks.

The results were analyzed by two-way analyses of variance (ANOVA) in order to detect differences according to the two factors studied. The Tukey test was used to compare the means when significant differences were detected (P=0.05). The SPSS version 10.0 software (SPSS Inc., Chicago, USA) was employed.

3 Results and discussion

3.1 Physicochemical parameters, fermentation time, and microbiological counts

The fermentation time necessary to reach pH 4.7 was higher (P<0.05) in supplemented yogurts in comparison to unsupplemented ones (Fig. 1). In effect, yogurts made with the addition of dairy ingredients had a delay of approximately 20 min, independently of the addition level, with respect to yogurts without powder addition; the mean processing time was 265 and 245 min, respectively. The presence of sucrose intensified weakly this effect. The manufacture of supplemented sweetened yogurts was approximately 35 min longer than that sweetened yogurts without powder addition. The influence of enzyme addition was insignificant, although in general, hydrolyzed yogurts experienced a slightly longer fermentation time than unhydrolyzed yogurts.

The results of titratable acidity of the milks, fresh yogurts, and yogurts after 28 days are shown in Table 2. The values for all products were between 62.0 and 99.8 °D, which are in accordance with the normal range established by the Argentinian Legislation (CAA 2010) (60–150 °D). There were differences in the acidity values depending on the composition of the fermentative environment. In particular, the influence of the enzyme β-galactosidase on the TA was only significant at 28 days of storage, being the values lower in hydrolyzed yogurts than in unhydrolyzed ones, for both yogurt varieties studied (natural and sweetened). In addition, the TA was significantly (P<0.05) higher in natural and sweetened yogurts supplemented with dairy ingredients in comparison to products without supplementation. In effect, the TA values of yogurts had the following order: E0P0, E1P0, E2P0<E0PI, E1PI, E2PI<E0PII, E1PII, E2PII, at 1 day, and E1P0, E2P0<E0P0<E1PI, E2PI<E0PI<E1PII, E2PII< E0PII, at 28 days.

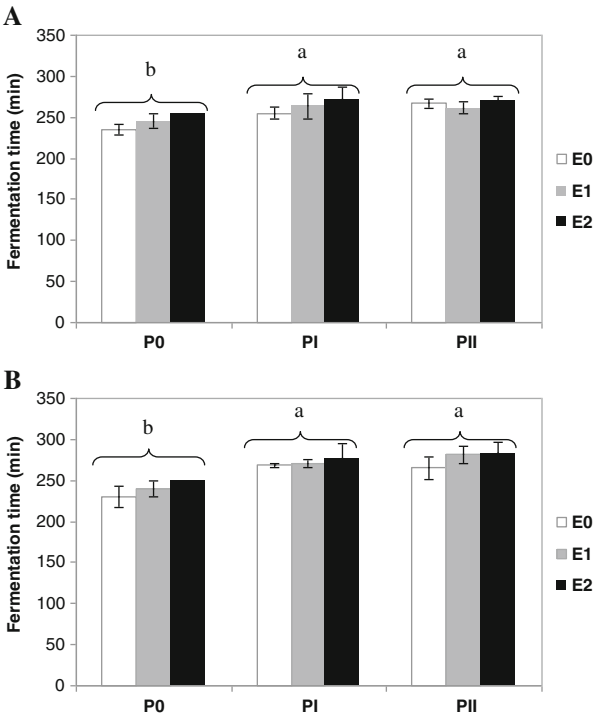

Fig. 1 Fermentation time (min) to reach the final pH of 4.7 of natural (**a**) and sweetened (**b**) yogurts. Mean values (n = 3) with different letters are significantly different ($P < 0.05$)

It is important to consider that the composition and properties of dairy powders used for the supplementation of milk base are very important in the fermentation process of yogurt because the decrease in pH depends on the buffering capacity of the matrix (Zare et al. 2012). Protein, total solids, and fat

Table 2 Titratable acidity (°D) of milk bases, fresh yogurts, and yogurts after 28 days of storage at 5 °C

Yogurts	Natural yogurt			Sweetened yogurt		
	Milk bases	1 day	28 days	Milk bases	1 day	28 days
E0P0	13.3±0.3^{c}	66.4±1.6^{c}	75.2±1.6^{e}	12.5±0.3^{c}	62.0±1.9^{c}	68.1±0.8^{e}
E1P0		66.3±0.7^{c}	73.6±1.5^{f}		62.5±1.7^{c}	66.7±1.0^{f}
E2P0		65.6±1.4^{c}	71.8±1.2^{f}		62.4±1.7^{c}	64.4±0.6^{f}
E0PI	16.4±0.3^{b}	75.2±2.9^{b}	89.2±2.8^{c}	15.3±0.2^{b}	70.7±3.0^{b}	84.0±3.0^{c}
E1PI		76.1±3.1^{b}	83.3±0.9^{d}		70.0±3.2^{b}	76.8±2.5^{d}
E2PI		72.9±3.0^{b}	83.2±1.2^{d}		70.7±1.8^{b}	75.4±0.9^{d}
E0PII	20.2±0.2^{a}	86.1±1.9^{a}	99.8±2.0^{a}	18.4±0.2^{a}	82.1±2.6^{a}	93.4±1.7^{a}
E1PII		86.6±3.0^{a}	94.5±0.5^{b}		79.9±0.9^{a}	87.9±1.8^{b}
E2PII		86.2±2.2^{a}	94.8±2.0^{b}		79.6±3.8^{a}	84.9±2.8^{b}

Mean values (n=3) with different superscripts within the same column are significantly different (P<0.05)

contents in natural and sweetened yogurts after 7 days are shown in the Table 3. The values of the yogurts made with the same level of supplementation but different doses of enzymes were averaged in this table because the enzyme addition did not affect the composition. By contrast, the supplementation increased significantly both parameters in the following order, P0<PI<PII, while the fat content was similar in all products.

The higher amount of proteins and other constituents such as phosphates and citrates, provided by the supplementation with dairy powders, increases the buffer capability of milk bases used in the yogurt manufacture (Tamime and Robinson 2007). In this way, Peng et al. (2009) have demonstrated an increase in the acid–base buffering properties of milk fortified with various types of protein ingredients, being the influence of micellar casein the highest, followed by SMP and milk protein isolates (MPIs). Meanwhile, Vasilean and Segal (2011) have observed that the acidity values in yogurts augmented with increasing the milk total solids content of the milk bases (from 8.5 to 14.5%). Likewise, the pH values in yogurts prepared with higher content of total solids were higher at the same fermentation time. According to the authors, these results are associated with the higher content of protein with buffering properties that favored the lactic acid production and limited the decrease in pH during fermentation. Similar results were found by Marafon et al. (2011) for titratable acidity in yogurts with supplementation of different dairy powders, while the time to reach the final pH of 4.5 was slightly affected (average increases of 31.5 min), although these changes were not significant. Damin et al. (2009) also found increases in the time to reach pH 4.6 with increases in SMP and WPC levels for yogurts supplemented. Meanwhile, Martins et al. (2012) reported processing time slightly higher for yogurts prepared from milk fortified with WPC.

The mean numbers of total LAB from yogurt culture in all yogurts were between 10^7 and 10^8 CFU.g^{-1}. The counts for hydrolyzed yogurts were, in general, slightly lower than for unhydrolyzed ones, although these differences were not significant. In addition, the results were similar ($P>0.05$) for supplemented and unsupplemented yogurts. Similar results were obtained by Damin et al. (2009) for yogurts with different levels of supplementation. On the other hand, the counts of molds and yeasts in all yogurts were lower than 10 CFU.mL^{-1}. The results of microbiological analyses were in agreement with the Argentinian Legislation for yogurt (LAB>10^7 CFU.mL^{-1}; molds and yeast<50 CFU.mL^{-1}) (CAA 2010).

Table 3 Content of protein, total solids and fat (g.100 g^{-1}) in yogurts after 7 days of storage at 5 °C

Level of supplementation	Natural yogurt			Sweetened yogurt		
	Protein	Total solids	Fat	Protein	Total solids	Fat
P0	3.26±0.18^{c}	11.77±0.10^{c}	3.0±0.1^{a}	3.08±0.18^{c}	17.88±0.15^{c}	2.8±0.1^{a}
PI	3.87±0.12^{b}	13.10±0.13^{b}	2.9±0.1^{a}	3.72±0.22^{b}	19.40±0.13^{b}	2.7±0.2^{a}
PII	4.50±0.15^{a}	14.80±0.37^{a}	2.9±0.2^{a}	4.25±0.13^{a}	20.90±0.21^{a}	2.6±0.1^{a}

Mean values (n=9) with different superscript within the same column are significantly different ($P<0.05$)

3.2 Carbohydrates profiles

The concentration of lactose, glucose, and galactose in natural yogurts at the end of manufacture and storage are shown in Fig. 2a–c, respectively). Likewise, the lactose concentration found in the sweetened yogurts is depicted in Fig. 3.

3.2.1 Lactose

The two factors studied affected significantly ($P<0.05$) the lactose concentration in similar way for natural and sweetened yogurts.

The lactose concentration in the milk bases increased significantly ($P<0.05$) due to the supplementation with dairy powders, as they provide lactose to the matrix. In fact, the SMP and WPC powders used for the supplementation contain 50.0 and 48.2 mg.100 g^{-1}, respectively. The values found in the milk bases without and with sucrose added were 4.67±0.09, 5.64±0.09, 6.68±0.04 and 4.56±0.04, 5.58±0.06, 6.46±0.10, respectively, depending on the level of dairy ingredients added. Important changes in the lactose content occurred during the preparation of hydrolyzed yogurts. The values decreased approximately 82% at the end of the fermentation process, reaching mean contents of 0.96±0.34 and 1.12±0.29 g.100 g^{-1} for natural and sweetened fresh yogurts, respectively. This diminution was produced mainly by the inclusion of β-galactosidase enzyme and also by the metabolic activity of starter. After manufacture, slight diminutions were observed during the refrigerated storage period, reaching values of 0.86±0.34 and 0.84±0.30 g.100 g^{-1} toward 28 days, for natural and sweetened yogurts, respectively. On the contrary, the changes in the lactose concentration during the manufacture of unhydrolyzed yogurts were relatively mild. The values decreased approximately 15 and 30% at the end of manufacture and at day 28 of storage, respectively, for both yogurt varieties. As well known, this fact is only due to the metabolic activity of the starter (Takano and Yamamoto 2011).

Hydrolyzed yogurts without powder addition (E1P0 and E2P0) had lower ($P<0.05$) levels of lactose in comparison to supplemented yogurts with the lower dose of enzyme (E1PI and E1PII), at the end of fermentation and at 28 days, for both varieties of yogurts. The other yogurt samples (E2PI and E2PII) had intermediate levels of lactose between these extreme groups.

The differences found among unhydrolyzed yogurts at the end of manufacture and at day 28 were the same as those observed for milk bases. In effect, the yogurts with the higher level of powders had significant higher concentrations ($P<0.05$) than yogurts with the lower level and than those without supplementation (E0PII>E0PI>E0P0).

3.2.2 Glucose and galactose

The two factors analyzed affected significantly ($P<0.05$) glucose and galactose concentrations in natural yogurts. Both monosaccharides were not found in milk bases, although they were detected in fresh yogurts and at day 28, due to the hydrolysis of lactose by the metabolic activity of the starter and also by the action of the β-galactosidase enzyme added. In hydrolyzed yogurts, the glucose and galactose concentrations sharply increased at the end of manufacture, reaching mean values of 1.89±0.30 and 2.44±0.31 g.100 g^{-1}, respectively, while a slight diminution was observed

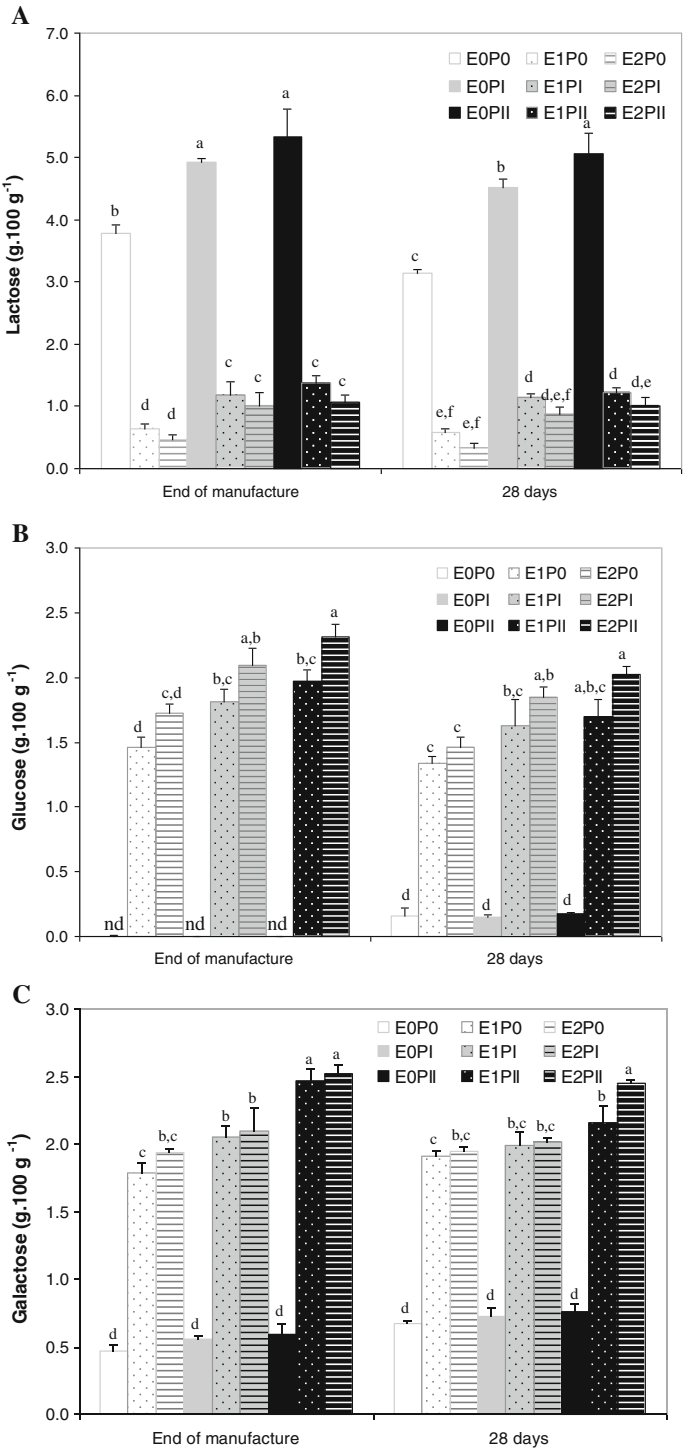

Fig. 2 Concentration of lactose (**a**), glucose (**b**), and galactose (**c**) (g.100 g^{-1}) for natural yogurts. Mean values (n = 3) with different letters are significantly different ($P < 0.05$) for each sampling time

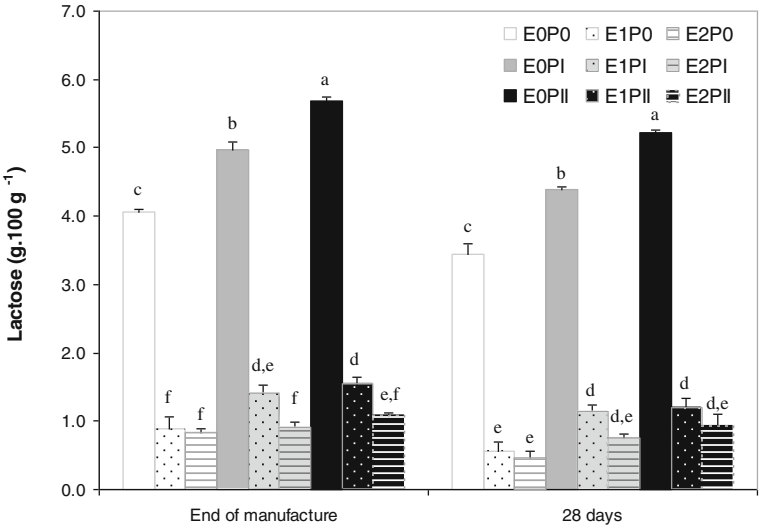

Fig. 3 Concentration of lactose (g.100 g^{-1}) for sweetened yogurts. Mean values (n = 3) with different letters are significantly different ($P < 0.05$) for each sampling time

toward the end of storage. Meanwhile, the glucose was not detected in unhydrolyzed fresh yogurts, but it was found at very low levels of about 0.18 g.100 g^{-1} at 28 days. It is well-known that the concentration of glucose is very low (not detectable) in traditional yogurts as LAB use rapidly the glucose to growth. On the contrary, in hydrolyzed yogurts, there was an overproduction of glucose that LAB could not fully use. Galactose was found at levels around 0.65 g.100 g^{-1} in unhydrolyzed freshly made yogurts, which slightly increased at the end of storage up to approximately 0.85 g.100 g^{-1}. This is due to the fact that galactose is not metabolized by the microorganisms of the yogurt starter, releasing this monosaccharide into the yogurt matrix (Gürakan and Altay 2010).

The hydrolyzed yogurts with the higher level of enzyme and dairy powders (E2PII) had higher level ($P<0.05$) of glucose and galactose than those without supplementation and with the lower level of enzyme added (E1P0) and than those unhydrolyzed yogurts with and without supplementation (E0P0, E0PI, and E0PII). The other yogurts samples (E1PII, E1PI, E2PI, and E2P0) were located between these extremes.

To sum up, the mean proportions of lactose/glucose/galactose in relation to the total sugars were 18:36:46 in natural hydrolyzed yogurts, while they were 81:4:15 in unhydrolyzed ones, at 28 days of storage. This fact could modify the metabolic activities of the starter culture and thus affect the production of organic acids.

The information available on delactosed yogurts obtained by enzymatic hydrolysis is not extensive. Despite this, the carbohydrates concentration reported are variable depending on the different factors such as types and doses of β-galactosidase enzyme, types of ingredients, and process conditions (type of starter, temperature, and time of incubation) employed for the yogurt manufacture. Toba et al. (1986) produced delactosed yogurts using a β-galactosidase enzyme from *A. Oryzae* on a milk base containing 11.5% of solids non-fat by adding SMP to whole milk, and a starter composed of *S. thermophilus*/*L. bulgaricus* (1:1). After 8 h of incubation at 41 °C, they achieved lactose values between 0.2 and 2.5 g.100 mL^{-1}, and values of glucose

and galactose between 1.0–3.0 and 1.6–3.8 g.100 mL^{-1}, respectively. In turn, most recently, Martins et al. (2012) obtained yogurts with 0.20 g.100 mL^{-1} of lactose using a milk base with 13.5% (*w*/*w*) of total solids and 9.1 g.100 mL^{-1} of lactose, prepared from whole milk powder and whey powder. The enzymatic catalysis was promoted by a mix of β-galactosidases from *K. lactis* and *A. niger*, and the lactic fermentation was carried out at 43 °C for approximately 3.50 h using a commercial starter containing the typical yogurt cultures together with two probiotic bacteria (*B. animalis* and *L. acidophilus*). On the other hand, higher concentration of lactose, from 1.83 to 2.93 g.100 mL^{-1}, and hence lower lactose conversion (only up to 50%), were reported by Ismail et al. (1983) for drinkable hydrolyzed yogurts made from whole milk. They tested two different commercial preparations of enzymes from *K. lactis*. The fermentation process was conducted at 43 °C/3.50 h using a starter composed of *S. thermophilus* and *L. bulgaricus* (1:1). Besides, they found galactose concentrations from 1.98 to 2.75 g.100 mL^{-1} for hydrolyzed yogurts, in comparison to 1.59 g.100 mL^{-1} for unhydrolyzed ones.

3.3 Organic acids profiles

Organic acids found in yogurts are the result of bacterial metabolism (lactic, acetic, propionic, pyruvic, and formic acids), normal bovine biochemical metabolism (citric, orotic, and uric acids), or the fat hydrolysis (butyric acid) (Tormo and Izco 2004). Regarding bacterial metabolism, the organic acids produced in fermented milk depend on the species and strain of LAB involved, composition of the medium, incubation, and storage conditions (Chick et al. 2001; Urbiene and Leskauskaite 2006; Ekinci and Gurel 2008). As far as we know, there are no data published in relation to organic acids profile in delactosed yogurts.

Table 4 shows the evolution of acetic, butyric, citric, hippuric, and orotic acid concentrations, and Fig. 4 shows the evolution of lactic acid concentration, during the manufacture and storage of natural and sweetened yogurts. In general, the values of organic acids in sweetened yogurts were lower than in natural ones.

3.3.1 Acetic acid

The concentration increased around 50% during the manufacture, and then they continued increasing slightly until the end of the storage period. The values found were similar ($P>0.05$) among unhydrolyzed and hydrolyzed yogurts, while significant increases ($P<0.05$) due to the dairy powder addition were observed for milk bases and for natural yogurts at 14 days of storage. However, the values reached at the end of the storage were low (lower than 10.50 mg.100 g^{-1}). Although the metabolism of typical starter cultures of yogurt is homofermentative, the production of trace/low levels of acetic acid during fermentation or the storage of traditional yogurt was reported (Fernandez-Garcia and McGregor 1994; Adhikari et al. 2002; Donkor et al. 2007). High values of acetic acid in yogurts are generally associated with the heterofermentative pathway of lactose produced by strains of bifidobacterias, as example. In this way, La Torre et al. (2003) reported a significant increase in acetic acid in fermented milks and yogurts due to the addition of probiotic bacteria (*L. acidophilus*, *B. bifidum, B. lactis*, *B. longun*, or/and *B. infantis*), being the values higher in fermented

Table 4 Content of organic acid (mg.100 g^{-1}) for natural and sweetened yogurts during manufacture process and storage at 5 °C

Organic acid	Dose of enzyme ($g.L^{-1}$)	Level of dairy powder	Natural yogurt					Sweetened yogurt				
			Milk base	150 minutes	End of manufacture	14 days	28 days	Milk base	150 minutes	End of manufacture	14 days	28 days
Acetic	**E0**	**P0**	3.30±0.14^{b}	5.14±0.09	7.87±1.00	7.82±0.36^{b}	8.28±0.40	3.40±0.14^{b}	4.75±0.10	6.87±0.16	8.59±0.27	8.08±1.16
	E1			5.41±0.89	7.45±0.40	8.25±1.51^{b}	8.58±1.09		4.49±0.71	6.80±0.88	8.22±0.76	7.05±0.33
	E2			5.39±0.01	7.17±0.87	9.37±0.02^{b}	8.36±0.30		4.79±0.44	6.11±0.10	7.86±0.13	6.37±0.67
	E0	**PI**	3.78±0.14^{a}	4.53±0.49	5.85±0.11	8.33±0.78^{b}	8.95±0.18	3.31±0.21^{b}	4.30±0.71	6.61±0.47	7.89±0.80	7.65±1.67
	E1			4.79±0.71	7.37±0.26	9.77±0.73^{b}	8.36±1.39		3.99±0.15	6.32±1.09	9.56±1.03	8.21±1.11
	E2			5.25±0.08	7.01±0.55	9.18±0.59^{b}	9.08±0.17		4.04±0.08	6.27±0.10	7.39±0.25	8.35±2.00
	E0	**PII**	3.84±0.07^{a}	5.28±0.83	7.14±1.03	9.93±0.34^{a}	10.21±0.51	3.79±0.27^{a}	4.19±0.28	6.88±0.09	7.47±2.09	8.00±0.52
	E1			5.03±0.20	7.86±1.00	9.87±0.22^{a}	8.83±1.66		3.94±0.21	6.24±0.07	8.64±0.76	10.41±1.36
	E2			5.34±0.08	6.96±0.91	10.44±0.2^{a}	9.20±1.13		4.01±0.24	5.89±0.78	8.58±0.25	8.91±2.38
Butyric	**E0**	**P0**	ND^{c}	2.54±0.50	11.88±4.71^{a}	27.54±3.20^{a}	34.41±3.18^{a}	ND^{c}	0.99±0.27	9.56±3.91^{a}	20.68±0.04^{a}	26.28±8.21^{a}
	E1			2.48±0.41	10.98±2.35^{a}	22.93±0.74^{a}	25.30±5.72^{a}		1.40±0.56	12.01±2.48^{a}	22.65±3.41^{a}	25.55±3.61^{a}
	E2			1.55±0.77	10.65±2.35^{a}	24.17±6.51^{a}	21.36±8.27^{a}		1.19±0.27	9.29±1.99^{a}	20.56±3.71^{a}	20.28±5.08^{a}
	E0	**PI**	0.80±0.00^{b}	1.69±0.85	8.00±2.66^{b}	18.96±3.08^{b}	19.53±3.08^{b}	0.25±0.21^{b}	1.00±0.42	7.34±0.53^{b}	16.51±1.98^{b}	17.00±2.70^{b}
	E1			1.45±0.64	7.55±2.67^{b}	16.45±1.31^{b}	15.17±1.44^{b}		1.10±0.00	7.30±1.15^{b}	14.23±2.04^{b}	16.72±2.12^{b}
	E2			2.00±0.99	5.68±1.01^{b}	16.45±4.46^{b}	17.61±2.81^{b}		2.64±1.62	6.51±2.28^{b}	11.26±2.10^{b}	13.38±0.71^{b}
	E0	**PII**	1.19±0.16^{a}	1.84±1.62	7.88±1.25^{b}	19.64±2.54^{b}	18.29±4.06^{b}	1.54±0.21^{a}	3.10±1.56	7.86±0.73^{b}	14.75±3.37^{b}	17.24±1.69^{b}
	E1			1.29±0.43	5.56±1.58^{b}	17.37±3.81^{b}	17.01±2.82^{b}		1.03±0.33	6.69±0.14^{b}	11.18±1.67^{b}	9.91±1.93^{b}
	E2			1.55±0.64	8.67±2.91^{b}	18.35±5.53^{b}	17.41±3.66^{b}		2.03±2.06	6.68±1.60^{b}	12.52±2.51^{b}	11.08±2.81^{b}
Citric	**E0**	**P0**	176.91±9.34^{c}	179.30±5.74^{c}	183.47±3.82^{c}	169.57±24.03^{c}	183.08±4.90^{c}	168.00±3.22^{c}	165.32±7.12^{c}	160.91±12.51^{c}	160.53±1.80^{c}	163.04±2.58^{c}
	E1			175.59±6.84^{c}	174.49±1.15^{c}	161.57±1.67^{c}	171.90±9.71^{c}		167.51±5.71^{c}	167.06±9.87^{c}	158.90±7.27^{c}	161.40±9.98^{c}
	E2			181.14±5.70^{c}	164.72±14.13^{c}	177.47±19.73^{c}	178.54±14.45^{c}		168.07±2.53^{c}	147.15±5.21^{c}	162.22±11.57^{c}	161.85±13.67^{c}
	E0	**PI**	221.04±1.36^{b}	218.68±2.46^{b}	211.17±0.92^{b}	216.15±1.25^{b}	210.00±0.40^{b}	199.65±8.41^{b}	203.38±10.53^{b}	203.71±8.84^{b}	198.44±4.02^{b}	194.17±2.71^{b}
	E1			220.82±3.42^{b}	218.39±5.36^{b}	212.91±8.20^{b}	213.70±1.10^{b}		198.37±11.45^{b}	191.28±19.49^{b}	195.96±3.33^{b}	196.19±1.22^{b}
	E2			220.95±1.68^{b}	219.05±1.45^{b}	210.42±2.32^{b}	203.62±5.12^{b}		200.01±3.16^{b}	202.73±3.62^{b}	195.32±3.77^{b}	195.66±7.41^{b}

Table 4 (continued)

Organic acid	Dose of enzyme ($g.L^{-1}$)	Level of dairy powder	Natural yogurt					Sweetened yogurt				
			Milk base	150 minutes	End of manufacture	14 days	28 days	Milk base	150 minutes	End of manufacture	14 days	28 days
	E0	**PII**	250.64±5.22^{a}	248.12±3.82^{a}	234.65±16.19^{a}	241.54±8.02^{a}	249.14±2.96^{a}	239.09±3.00^{a}	232.74±5.57^{a}	225.66±1.06^{a}	223.04±17.00^{a}	223.96±1.12^{a}
	E1			253.21±3.78^{a}	254.59±6.40^{a}	250.78±4.81^{a}	249.38±7.60^{a}		238.08±3.92^{a}	232.09±5.2^{a}	225.30±0.76^{a}	220.93±5.50^{a}
	E2			254.95±3.10^{a}	256.18±4.22^{a}	251.07±1.58^{a}	246.68±0.9^{a}		237.20±3.90^{a}	236.30±10.76^{a}	233.10±7.42^{a}	212.84±16.30^{a}
Hippuric	**E0**	**P0**	2.24±0.01^{c}	2.06±0.14^{c}	1.68±0.10^{c}	1.21±0.01^{b}	1.09±0.18^{c}	2.05±0.07^{c}	1.74±0.02^{c}	1.57±0.12^{c}	1.39±0.22^{c}	1.27±0.28^{b}
	E1			1.86±0.09^{c}	1.51±0.15^{c}	1.17±0.21^{b}	1.49±0.14^{c}		1.89±0.14^{c}	1.50±0.03^{c}	1.25±0.10^{c}	1.22±0.09^{b}
	E2			2.07±0.08^{c}	1.63±0.10^{c}	1.38±0.05^{a,b}	1.17±0.22^{c}		1.97±0.08^{c}	1.30±0.27^{c}	1.30±0.10^{c}	1.14±0.15^{b}
	E0	**PI**	2.79±0.07^{b}	2.68±0.08^{b}	2.04±0.04^{b}	1.74±0.11^{a,b}	1.53±0.12^{b}	2.57±0.18^{b}	2.44±0.08^{b}	1.87±0.03^{b}	1.61±0.21^{b}	1.41±0.34^{a,b}
	E1			2.57±0.27^{b}	2.02±0.14^{b}	1.81±0.05^{a}	1.62±0.03^{b}		2.38±0.19^{b}	1.99±0.22^{b}	1.68±0.11^{b}	1.45±0.14^{a,b}
	E2			2.64±0.07^{b}	1.92±0.09^{b}	1.66±0.08^{a,b}	1.56±0.08^{b}		2.51±0.05^{b}	1.78±0.03^{b}	1.63±0.17^{b}	1.54±0.11^{a,b}
	E0	**PII**	3.18±0.14^{a}	2.83±0.02^{a}	2.15±0.08^{a}	1.92±0.23^{a}	1.87±0.26^{a}	2.99±0.13^{a}	2.63±0.33^{a}	2.24±0.03^{a}	1.58±0.24^{a}	1.61±0.22^{a}
	E1			2.80±0.19^{a}	2.19±0.21^{a}	1.95±0.06^{a}	1.91±0.12^{a}		2.97±0.01^{a}	2.64±0.05^{a}	2.31±0.11^{a}	2.04±0.20^{a}
	E2			2.90±0.24^{a}	2.18±0.11^{a}	1.85±0.15^{a}	1.74±0.05^{a}		2.91±0.22^{a}	2.58±0.04^{a}	2.18±0.06^{a}	1.72±0.23^{a}
Orotic	**E0**	**P0**	8.65±0.19^{c}	9.59±0.29^{c}	9.41±0.15^{c}	8.68±1.36^{c}	9.45±0.09^{c}	8.24±0.48^{c}	9.00±0.21^{b}	9.33±0.22^{c}	8.89±0.99^{b}	9.36±0.49^{c}
	E1			9.05±0.46^{c}	8.70±0.12^{c}	9.40±0.08^{c}	9.18±0.31^{c}		8.40±0.47^{b}	8.87±0.54^{c}	8.21±0.89^{b}	8.94±0.24^{c}
	E2			9.09±0.89^{c}	8.92±0.29^{c}	9.47±0.48^{c}	9.33±0.32^{c}		8.79±0.89^{b}	7.32±1.63^{c}	9.07±0.26^{b}	8.91±0.43^{c}
	E0	**PI**	10.50±0.27^{b}	10.94±0.37^{b}	11.07±0.30^{b}	11.08±0.70^{b}	9.79±0.81^{b}	9.96±0.03^{b}	9.89±0.55^{b}	10.49±0.05^{b}	10.51±0.00^{a}	10.05±0.53^{b}
	E1			10.83±0.77^{b}	11.22±0.34^{b}	11.09±0.24^{b}	11.24±0.46^{b}		9.14±1.08^{b}	9.97±0.58^{b}	10.03±0.16^{a}	10.02±0.03^{b}
	E2			10.97±0.28^{b}	10.87±0.34^{b}	10.79±0.33^{b}	10.46±0.64^{b}		10.02±0.21^{b}	10.41±0.06^{b}	10.29±0.53^{a}	10.30±0.01^{b}
	E0	**PII**	12.13±0.59^{a}	12.23±0.26^{a}	11.92±0.62^{a}	11.91±0.00^{a}	12.19±0.16^{a}	11.56±0.56^{a}	11.06±1.43^{a}	11.87±0.18^{a}	10.32±2.40^{a}	11.43±0.51^{a}
	E1			12.31±0.14^{a}	12.11±0.08^{a}	12.21±0.33^{a}	12.24±0.35^{a}		11.75±0.30^{a}	12.03±0.18^{a}	11.90±0.31^{a}	11.59±0.01^{a}
	E2			12.18±0.03^{a}	12.16±0.07^{a}	12.25±0.04^{a}	12.13±0.05^{a}		11.46±0.62^{a}	12.10±0.11^{a}	11.99±0.19^{a}	10.98±1.28^{a}

Mean values (n=3) with different superscripts within the same column are significantly different (P<0.05) for each organic acid analyzed

ND not detected

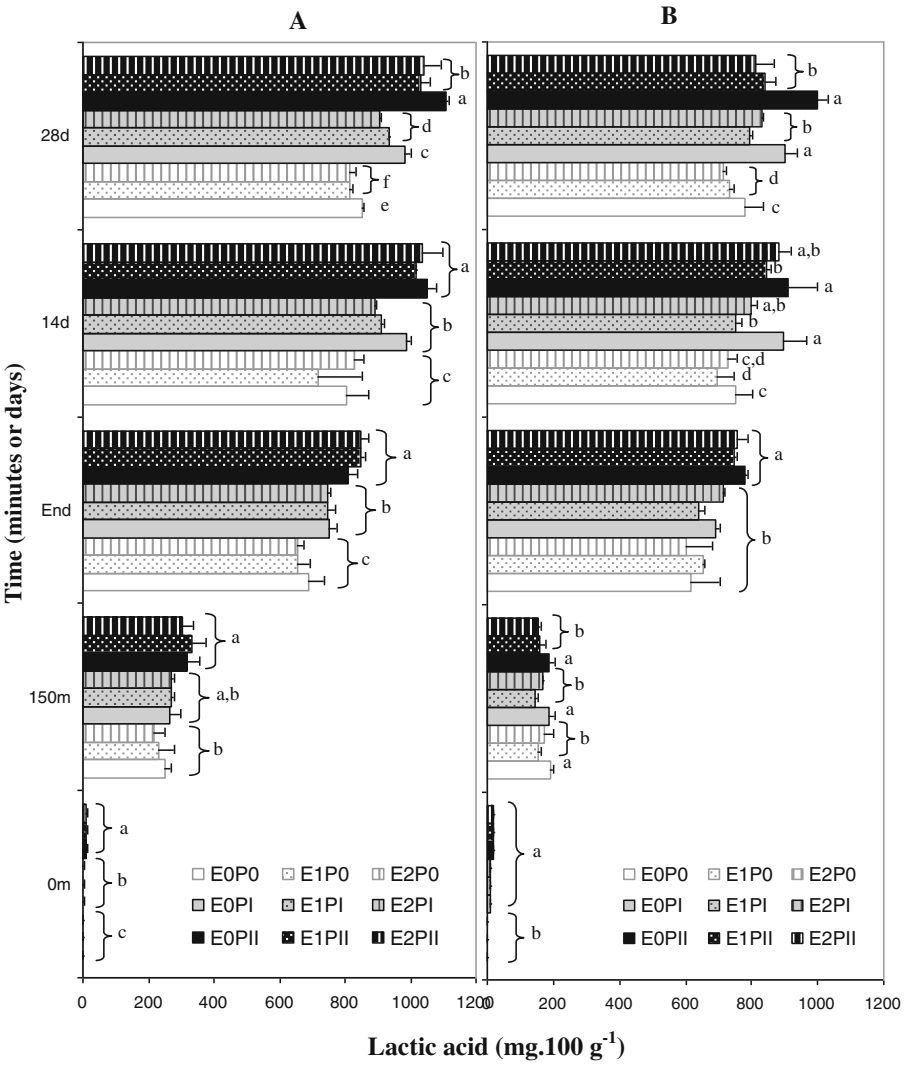

Fig. 4 Concentration of lactic acid (mg.100 g^{-1}) for natural (**a**) and sweetened yogurts (**b**) during manufacture process and storage at 5 °C. Mean values (n = 3) with different letters are significantly different ($P < 0.05$) for each sampling time

milks than in yogurts. In yogurts made only with the traditional starter, the acetic acid was also detected, although the values were lower than 7.6 mg.100 g^{-1}, similar to our results. However, Settachaimongkon et al. (2014) found comparable levels of acetic acid in traditional yogurt and in yogurt with the addition of *B. animalis* subsp. *lactis* BB12 during storage.

3.3.2 Citric acid

This acid remained almost unchanged during the manufacture and storage of yogurts. The levels were similar ($P>0.05$) among unhydrolyzed and hydrolyzed yogurts. As

expected, the fortification of milk with dairy powders resulted in a significant ($P<0.05$) increase, as this acid is a natural component of milk.

The values found in the present work are in agreement with those reported by Ekinci and Gurel (2008) (256 mg.100 g^{-1} at 15 days of storage). In general, bacteria of classical yogurt starter are not able to catabolize the citric acid, and, thus, our results are not surprising. However, citric acid could be metabolized by bacteria citrate (+) such as some strains of *Lactococci*, *Leuconostoc*, or strains of probiotic bacteria, which lead to the production of important flavor compounds such as diacetyl and acetoin. As for example, La Torre et al. (2003) obtained values of approximately 80 mg.100 g^{-1} in fresh yogurts or fermented milks with the addition of probiotic bacteria. The values found were lower than normal levels of citric acid in milk, which suggests a catabolism of this compound during fermentation or storage.

3.3.3 Hippuric acid

The concentrations decreased around 30% during the manufacture, reaching a decrease of approximately 50% at the end of the storage. The effect of the enzyme addition was found to be insignificant ($P>0.05$) at all sampling times. However, a significant ($P<0.05$) increase was observed with the addition of dairy powders. The values were higher for yogurts with level II of supplementation than for those without supplementation, while yogurts with level I of dairy powders had intermediate values.

The behavior observed in our results was comparable to that reported by Fernandez-Garcia and McGregor (1994) who found a great diminution of this acid during yogurt manufacture from 2.01 in milk to 0.13 mg.100 g^{-1} at 4 h of fermentation. In this sense, it has been demonstrated that the LAB can convert the hippuric acid, naturally present in milk, into benzoic acid, which may act as preservative and therefore enhance the shelf life of yogurt (Sieber et al. 1995). However, we did not analyze benzoic acids in yogurt samples. La Torre et al. (2003) found values of hippuric acid around 3 mg.100 g^{-1} in fresh fermented milk made with seven different starters and probiotics without observing changes during the storage; however, one of them, composed of *L. acidophilus*, *B. bifidum*, and *S. thermophilus*, was able to use this acid during fermentation, which reached values of 0.1 mg.100 g^{-1}.

3.3.4 Butyric acid

A steady increase in the concentration was observed in all yogurts; the milk samples had low concentration of this compound, but then the values increased sharply from 150 min to 14 days, and after that, they were maintained almost unchanged until 28 days. No effect for the enzyme factor was observed at each time analyzed, but significant differences ($P<0.05$) were found due to the addition of powders. Yogurts without powder addition had higher concentrations of butyric acid than those supplemented yogurts. Butyric acid is produced from milk fat, which was present at lower relative concentrations in yogurts with addition of dairy powders. In this sense, it is important to take into account that the addition of SMP and WPC and sucrose increases the total solids (Table 3), but they do not provide additional fat.

The concentrations reported for butyric acid are very variable among different authors. Adhikari et al. (2002) found values of about 200 mg.100 g^{-1} for yogurts

with 7 days of storage, while Güler and Park (2011) found a range from 3.6 to 14.7 μg.100 g^{-1} in different commercial set-type yogurts.

3.3.5 Orotic acid

The concentrations were not affected by enzyme addition but were significantly increased ($P<0.05$) with the powder level. In milk and fermented milk, the concentration of this acid is dependent on the amount of soluble whey solids and the conditions of the fermentation process (Larson and Hegarty 1979; Haggerty et al. 1984). In fact, orotic acid is an intermediate compound in the synthesis of nucleotides and may be used as growth factor by some LAB (González de Llano et al. 1996). In this way, significant decreases in orotic acid during the storage were detected by some authors in yogurt made with different starters (Haggerty et al. 1984; Fernandez-Garcia and McGregor 1994). However, in the present work, we found similar concentrations through the manufacture and storage in all yogurts, pointing out that the cultures used were not able to catabolize this compound. Similar results were found by La Torre et al. (2003) and Ekinci and Gurel (2008).

3.3.6 Lactic acid

The catabolism of lactose by yogurt starters results mainly in the production of lactic acid. In effect, this is the main organic acid found in all yogurts, representing approximately 75.7% of the total organic acids content in freshly made yogurts. In all yogurts, there was a sharp increase in the concentration from approximately 20 mg.100 g^{-1} in milk bases to 750±74 and 690±81 mg.100 g^{-1} at the end of manufacture, for natural and sweetened yogurts, respectively. Then, the concentrations continued growing weakly toward 28 days reaching values of 943±106 and 823±87 mg.100 g^{-1}, for natural and sweetened yogurts, respectively.

In the natural yogurts, the influence of the enzyme addition on the lactic acid formation was only detected at 28 days. In effect, the values for hydrolyzed yogurts with both levels of enzyme assayed (E1P0, E2P0, E1PI, E2PI, E1PII, and E2PII) were significantly lower ($P<0.05$), approximately 6%, than those obtained for unhydrolyzed ones (E0P0, E0PI, and E0PII). These values were in accordance with the results of titratable acidity. In relation to the supplementation with dairy powders, significant differences were observed among yogurts at all sampling times. The concentration significantly increased ($P<0.05$) with the addition of powders (E0PII, E1PII, E2PII> E0PI, E1PI, E2PI>E0P0, E1P0, and E2P0). In this sense, the probable increase in buffer properties of milk bases supplemented with powders led to a higher production of lactic acid by starter cultures in order to reach the final pH of 4.7, as was mentioned above. Likewise, the increase in lactic acid in supplemented yogurts may also be related to the higher levels of lactose, substrate for lactic acid formation, which are provided by dairy powders. On the other hand, these ingredients also supply peptides and free amino acids which can be favorable for the growth and metabolic activity of the starter culture (Sodini et al. 2002; Lucas et al. 2004; Zare et al. 2012). Amatayakul et al. (2006) also found higher levels of lactic acid in yogurts made with 14% of total solids (1250 mg.100 g^{-1}) than those with 9% (930 mg.100 g^{-1}), a fact that was attributed to a higher concentration of available nutrients.

In the case of the sweetened yogurts, the behavior of lactic acid was similar to that observed in natural yogurts, although the influence of enzyme addition was found earlier during fermentation. Indeed, hydrolyzed yogurts had significantly lower concentration ($P<0.05$) than unhydrolyzed ones at 150 min of manufacture and after 14 and 28 days. The values were approximately 12% lower at 28 days. The influence of the dairy powders addition was similar to those observed in natural yogurts. Overall, the content of lactic acid in sweetened yogurts was lower, approximately 11%, than those found in natural yogurts.

The lactic acid concentration reported for traditional yogurts is very variable. In fact, there were values of 965 mg.100 g^{-1} reported for fresh natural yogurts (Fernandez-Garcia and McGregor 1994) and 589 mg.100 g^{-1} (Adhikari et al. 2002), which reached up to 1,100 at 28 days and 631 mg.100 g^{-1} at 30 days of storage, respectively. In turn, La Torre et al. (2003) found levels around 1,140 mg.100 g^{-1} in freshly prepared yogurt, which was maintained constant during 20 days. Ekinci and Gurel (2008) reported higher content in yogurts of about 2,000 mg.100 g^{-1} at 7 days. However, Cruz et al. (2013) found levels of 128 mg.100 mL^{-1} on the first day, which increased to 306 mg.100 mL^{-1} at 28 days. In relation to delactosed yogurts obtained by the addition of probiotic bacteria, Cruz et al. (2012) found levels of 316 mg.100 mL^{-1} of lactic acid at 15 days.

To sum up, lactic acid was the only organic acid that experienced significant changes due to the inclusion of the β-galactosidase enzyme in the yogurt manufacture. In this way, it has been demonstrated that the acidification activity of some LAB is influenced by the sugars present in the growth medium. Baranowska (2006) found that milk enrichment with lactose and glucose for preparing yogurt significantly reduced the concentration of lactic acid. O'Leary and Woychik (1976) and Amoroso et al. (1989) found significant differences in the viability of pure and mixed cultures of *L. bulgaricus* and *S. thermophilus* incubated in broths containing different carbohydrates: glucose, galactose, lactose, and sucrose. Other authors reported that the effect of glucose, lactose, and sucrose on the viability of microorganisms is strain dependent. Hickey et al. (1986) found that the addition of glucose or galactose to lactobacilli strains grown on medium containing lactose caused an immediate repression of β-galactosidase synthesis. In effect, it has been demonstrated that a high concentration of galactose in the medium inhibits the lactose transport because this monosaccharide acts a competitive inhibitor (Zourari et al. 1992). Thus, in the present work, the higher concentration of galactose and glucose in natural hydrolyzed yogurts could inhibit the metabolism of lactose and the consequent production of lactic acid. This fact is deepened by the presence of sucrose in sweetened hydrolyzed yogurts, in which the lower production of lactic acid was observed since 150 min of fermentation. It has been reported an inhibitory effect of sucrose added at high level (>8%) on growth of yogurt starter due to an adverse osmotic effect and a low water activity (Zourari et al. 1992; Tamime and Robinson 2007). Likewise, this fact could also be due to differences in the buffering capacity of the milk bases. The addition of sucrose led to a decrease in the relative concentration of the different compounds, which have acid–base buffering properties. Thereby, the amount of lactic acid required to reach the final pH of 4.7 was lower in the sweetened yogurts in comparison to that of natural yogurts.

4 Conclusions

There were significant changes in the carbohydrate profiles due to the inclusion of the β-galactosidase enzyme during the manufacture of yogurts. The lactic acid was the only organic acid significantly affected by the modifications produced; hydrolyzed yogurts showed lower levels in comparison with unhydrolyzed ones. These results could be due to an inhibition of starter culture activity because of the high concentration of glucose and galactose in the medium by the activity of exogenous β-galactosidase. This effect was more marked in sweetened yogurts as sucrose could also inhibit the starter activity. On the other hand, the addition of dairy powders produced a significant increase in most organic acids and carbohydrates studied at all sampling times, which in part can be associated with the higher concentration of these compounds in the milk bases, provided by the dairy ingredients. In addition, the supplementation with skim milk powder and whey protein concentrate increased the buffering capability of the matrix, which led to a higher production of lactic acid (and consequently higher level of titratable acidity) and a slight delay of the fermentation process.

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