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Original article

Effect of reduction of lactose in yogurts by addition of β-galactosidase enzyme on volatile compound profile and quality parameters

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Summary A comparative study between reduced-lactose yogurts made with added β-galactosidase (E yogurts) and controls (C yogurts) was performed. The evolution of lactose content, pH, acidity and volatile compounds was measured during fermentation and storage at 5 °C. The hydrolysis percentages of lactose ranged from 75% to 78% in E yogurts and from 10% to 13% in C yogurts at the end of manufacture and stayed without changes throughout storage. There were no significant differences in pH and titratable acidity values among yogurts. A total of 22 volatile compounds were identified. The change in lactose level by the action of β-galactosidase influenced the production of some volatile compounds derived from this sugar. At the end of fermentation, minor differences in volatile composition were recorded among yogurt samples. During storage, acetaldehyde and diketone levels were always higher in hydrolysed yogurts than their respective controls.

Keywords Flavours, lactose, solid phase microextraction (SPME), yogurt.

Introduction

Yogurt is a fermented dairy product obtained with a specific starter culture consisting of a mix of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Tamime & Robinson, 2007). From nutritional viewpoint, it is an exceptional source of nutrients such as proteins, minerals and vitamins. Besides, beneficial health effects such as protection against cancer, enhancement of immune response and cholesterol reduction have been associated with yogurt intake (McKinley, 2005; Chandan & Kilara, 2008; Ebringer *et al*., 2008). These properties may be further improved by reducing the lactose content, which could be an excellent alternative for lactose-intolerant individuals. The problem of lactose intolerance is well known and prevalent in more than half of the world population (Messia *et al*., 2007; Adhikari *et al*., 2010). The main cause is the lack of activity of β-galactosidase enzyme, which splits the lactose into glucose and galactose, monosaccharides that are then absorbed in the small intestine and used as energy source by the organism (Ingram & Swallow, 2009; Dekker & Daamen, 2011). One of the strategies more assayed to obtain reduced-lactose yogurts is the lactose hydrolysis by the enzymatic *via* (Mlichová & Rosenberg, 2006). This practice changes the carbohydrate pattern, which could affect the rate of sugar consumption by starter culture and the production of derived compounds as well as the physicochemical and sensory characteristics of products. It is well known that yogurts made with β-galactosidase addition have a significantly lower level of lactose and consequently, a higher level of glucose and galactose than yogurts made without enzyme (Vénica *et al*., 2013).

Aroma is considered one of the most important sensory attributes of yogurts. It is basically the result of a delicate balance of both components initially present in milk and volatiles synthesised or increased during fermentation by biochemical action of cultures. Carbonyl compounds and volatile and nonvolatile acids have been reported among the most potent odorants of yogurt (Ott *et al*., 1997; Cheng, 2010). Lactose fermentation seems to be the major metabolic route for volatile compounds generation. Glucose produced from lactose hydrolysis can be converted into a variety of organic acids and volatile compounds such as lactic, acetic and formic acids, ethanol, acetaldehyde, acetoin, diacetyl and 2,3-butanediol (Georgala *et al*., 1995; Cheng, 2010) whereas galactose accumulates in the medium because the enzymes for its metabolism are present but at low activity.

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Reduced-lactose yogurts are still not available in Argentinian market. Recently, Vénica *et al.* (2013, 2014) studied the influence of some variables to hydrolyse the lactose (three levels of β-galactosidase and the time of its addition) and different matrix composition on carbohydrate and organic acids profiles during manufacture of drinkable and stirred yogurts. So far, according to our knowledge, no work has been performed to see the degree in which the bioformation of volatile compounds is affected by the change of sugar profile during manufacture of delactozed yogurts. The aims of this study were to evaluate the effect of the lactose reduction by exogenous β-galactosidase action on production of volatile compounds during fermentation and refrigerated storage as well as to compare the main quality parameters of traditional and delactozed yogurts.

Materials and methods

Preparation of milk base and yogurt making

Stirred type yogurts were made applying the traditional method (Tamime & Robinson, 2007) adapted to laboratory scale. Four stainless steel vats (5 L capacity) were operated simultaneously. Duplicate trials (trial 1 and trial 2) were made on different days.

Bulk bovine milk (pH 6.70 ± 0.05, titratable acidity 14 ± 1°D) standardised to 3% w/v fat content was provided by Milkaut S.A. (Santa Fe, Argentina). Sucrose at 8% w/v was used as sweetener. A mix of skim milk powder (SMP) and whey protein concentrate 35% (WPC 35%) (Milkaut S.A., Santa Fe, Argentina) were employed as dairy ingredients at the level of 4.4%. The ingredients were dissolved by manual agitation for 15 min. The milk base was heated to 90 ± 2 °C for 5 min and cooled to 42 ± 2 °C. Then, it was inoculated with commercial starter culture (YF-L811) composed by *S. thermophilus* and *L. bulgaricus* (Chr. Hansen, Buenos Aires, Argentina) in direct vat set form. The commercial β-galactosidase enzyme YNL-2 from *Kluyveromyces lactis* (GODO Shusei Company Limited, Tokyo, Japan) was added at the level of 0.25 g L^{-1} in the experimental (E) yogurts together with the starter; control (C) yogurts without enzyme addition were prepared simultaneously. The incubation was carried out at 42 ± 2 °C until the pH value reached 4.70 ± 0.10. Samples of each yogurt were obtained at different times: initial, 45 min, 150 min and at the end of fermentation (approximately 240 min). Yogurts were cooled in an ice water bath to 25 °C and transferred to glass containers with screw cap (1 L) and stored at 5 °C for 21 days. At each sampling time (1, 7, 14 and 21 days), approximately 100 mL of sample was homogenised and small aliquots were weighed according to each determination. Samples were kept in the freezer and thawed during 4 h at 5 °C prior to analysis.

Lactose and lactic acid analysis by HPLC

Lactose and acid lactic content in yogurts was determined during fermentation and storage by HPLC method according to Vénica *et al.* (2014). Analyses were performed in duplicate.

pH and titratable acidity and microbiological count

The measurement of pH during fermentation and storage was done using a digital pHmeter (Orion 3 star benchtop; Thermo Fisher Scientific Inc., Waltham, MA, USA). Titratable acidity (TA) was determined during storage by titration with 0.1 N NaOH until pH 8.3 using a pHmeter (IDF 150, 2012). Total lactic acid bacteria were enumerated according to the procedure described by Vénica *et al.* (2014). Results were expressed as Dornic degree (1°D = 100 mg lactic acid L^{-1}). Analyses were performed in duplicate.

Volatile compound analysis by SPME-GC/FID/MS

A mix of 5 g of yogurt and 5 g of a saturated NaCl solution was prepared in a 30-mL glass container and sealed hermetically with a Teflon-faced gray butyl septa and an aluminum seal. Vials were thermostatised at 45 °C for 10 min. Then, a CAR-PDMS fibre (Supelco Analytical, Bellefonte, PA, USA) was exposed into headspace of sample during 30 min.

Volatile compounds analysis was performed with a Perkin Elmer model 9000 gas chromatograph equipped with a flame ionisation detector (FID) and split/splitless injector. The compounds retained on the fibre were thermally desorbed at 250 °C for 5 min in the injector port (splitless mode). Separation was performed using a HP-Innowax capillary column (60 m × 0.25 mm ID × 0.25 μm film thickness), under the following conditions: 45 °C (4 min.), then increase at 8 °C min^{-1} to 150 °C (3 min.) and finally at 10 °C min^{-1} to 250 °C and held at 250 C for 5 min. Hydrogen carrier gas was used at a flow rate of 2.0 mL min^{-1}. Temperature of detector was maintained at 290 °C.

Peaks were tentatively identified by comparing their retention times with those of authentic standards (Sigma-Aldrich, Milan, Italy) (when available). A more reliable identification was performed by calculating linear retention indexes (LRI) according to the expression proposed by Van den Dool & Kratz (1963). In addition to GC-FID analysis, the samples were analysed by mass spectrometry (MS) employing a Varian CP-3800 gas chromatograph coupled with a Saturn 2000 ion trap mass detector. MS operating conditions were

as follows: transfer line at 250 °C; ionisation mode: electron impact (EI); ionisation voltage: 70 eV; mass acquisition range: from 40 to 350 amu; scan rate: 0.5 scans s^{-1}. Helium was used as carrier gas at flow rate of 1 mL min^{-1}. Mass spectra obtained for each compound was compared with library database provided by software [NIST 98 (Gaithersburg, MD, USA) and Wiley libraries (Hoboken, NJ, USA)]. The identified peaks were integrated, and the resulting area values were expressed in arbitrary units. Analyses were performed in triplicate.

Statistical analysis

Data from physicochemical composition and volatile profile were subjected to one-way ANOVA with a 95% confidence level to test the differences between both types of yogurt at each sampling time. As both trials were made on different days employing different milks, the comparison between C and E yogurts was performed for each trial. Statistical analysis was performed using the SPSS 10.0 software (Chicago, IL, USA).

Results and discussion

Lactose and acid lactic content

Results of lactose content for C and E yogurts are shown in Fig. S1. As can be seen, lactose levels decreased markedly during fermentation process in E yogurts. At 45 min, the hydrolysis percentages were higher than 50% in both trials. At 150 min, the greatest percentages were reached (from 75% to 78%), which remained approximately constant until the end of the manufacture. The percentage reduction in lactose was within the range established by Argentinian Legislation (CAA, 2013) for reduced-lactose foods (minimum value: 70%). In C yogurts, lactose showed a slow decrease during manufacture. The hydrolysis percentages at the end of process ranged from 10% to 13%. This fact is a consequence of lactose fermentation by β-galactosidase activity of starter cultures, which can metabolise up to 40% (Batista *et al*., 2008; Shapiro & Silanikove, 2010; Shah, 2013). As is well known, fermentation is inherently a variable phenomenon and thus, the degree of lactose utilisation and the rate of lactic acid formation by starter culture is dependent on multiple factors such as the genus and species of bacteria and their protocooperative effect, level of inoculum, conditions of fermentation, characteristics of medium, the presence of other sugars, among others (Tamime & Robinson, 2007; Sobowale *et al*., 2011). During storage for 21 days at 5 °C, lactose values exhibited a slight decrease in all samples.

Lactic acid was the only organic acid significantly affected by added β-galactosidase, at 21 days. Hydrolysed yogurts showed lower levels (797.3 mg 100 g^{-1} for E1 and 794.2 mg 100 g^{-1} for E2) in comparison with unhydrolysed ones (987.5 mg 100 g^{-1} for C1 and 986.4 mg 100 g^{-1} for C2). This aspect was recently discussed in depth by Vénica *et al*. (2014).

pH and titratable acidity and microbiological count

The pH value of the milk base was on average 6.40, which decreased in 1.80 units for both types of yogurt at the end of fermentation (Fig. S2). During storage, a slow decrease in pH was observed, which ranged from 0.27 to 0.43 units. As expected, the lactose consumption by microorganisms produces an increase of the lactic acid content, and the pH correspondingly decreases. Statistical differences in pH values were not detected among yogurt samples ($P > 0.05$) during fermentation and storage. In accordance with our results, Baranowska (2006) and Rodríguez *et al*. (2008) did not found differences in pH values between yogurts prepared from milk with different lactose contents and control yogurts. Recently, Vénica *et al*. (2014) studied the carbohydrate profiles in drinkable and stirred yogurts made with and without added β-galactosidase. They observed that starter cultures metabolise rapidly the glucose to growth as this sugar was not detected in unhydrolysed fresh yogurts, whereas in delactozed yogurts, there was an overproduction of glucose that lactic acid bacteria could not fully use. The results suggest that a higher level of glucose in hydrolysed yogurts than controls had not influence on the extension of its utilisation for producing lactic acid by starter. Concerning titratable acidity (TA), the values for all samples ranged from 80 to 95°D, which are within the normal range generally accepted for yogurts (Fig. S3). Differences were not detected ($P > 0.05$) between C and E yogurts during refrigerated storage, although E yogurts presented always lower TA values than their respective controls. A similar profile of acidity in yogurts made with and without β-galactosidase was reported by Özer & Atasoy (2002), suggesting that the presence of enzyme did not produce any significant effect on TA during storage.

As regards microbiological counts, the mean numbers of total lactic acid bacteria in all freshly made yogurts were 10^8 CFU g^{-1} and remained in the same order throughout storage. Viable lactic acid bacteria content during shelf-life of yogurts was in accordance with Argentinian Legislation, which establishes a minimum content of 10^7 CFU g^{-1}. To our knowledge, data on cell viability in delactozed yogurt during storage have not been reported. Instead, both *S. thermophilus* and *L. bulgaricus* have demonstrated to keep

counts higher than 10^7 CFU g^{-1} in different types of yogurt (Birollo *et al.*, 2000).

Changes in volatile profile during fermentation process

Twenty-two volatile compounds were detected during manufacture and storage of yogurts (Table S1). As expected, all compounds found in the samples have already been reported in different types of yogurt (Laye *et al.*, 1993; Güler *et al.*, 2009; Güler & Park, 2011). To simplify the interpretation of results, only those compounds that probably derive from lactose metabolism or those compounds reported as the main aroma contributors were considered in the discussion.

The area values of each compound in C and E yogurts for trials 1 and 2 during fermentation are shown in Tables S2 and S3, respectively. At the beginning of fermentation, statistically significant differences ($P < 0.05$) were found for almost all the volatile compounds identified. In particular, levels of acetaldehyde, methyl ketones, diacetyl and acids were higher in E than in C yogurts. A more deep change in volatile profile was recorded at 150 min with some differences depending on the trial. In both trials, acetaldehyde, 2,3-pentanedione and acetoin levels were significantly higher in C yogurts compared to E yogurts ($P < 0.05$), whereas acids had higher area values in yogurts made with added β-galactosidase. Levels of butanone and propanone were higher in E yogurts ($P < 0.05$) in trials 1 and 2, respectively. Finally, at the end of fermentation, the area values of the majority of compounds did not differ between samples. However, it was interesting to note that in both trials, hydrolysed yogurts had significantly higher levels of acetaldehyde and lower quantities of 2,3-pentanedione and hexanoic acid ($P < 0.05$) than their respective controls.

Acetaldehyde is the most typical aroma compound of natural or plain yogurt (Tamime & Robinson, 2007), being responsible for its fresh-fruity note. Three metabolic pathways can lead to its formation: glucose metabolism, DNA degradation and threonine catabolism (Zourari *et al.*, 1992). Ott *et al.* (2000b) reported that lactose conversion to glucose is the main reaction for acetaldehyde production during milk fermentation by starter culture. According to our results, the higher level of acetaldehyde at the end of fermentation in E than in C yogurts could be explained by the higher availability of glucose. This finding was in agreement with those reported by Özer & Atasoy (2002) who observed that the treatment of milk with β-galactosidase caused a significant increase in the acetaldehyde level in yogurt samples. On the other hand, Baranowska (2006) found differences in the acetaldehyde content among yogurts made from milk enriched with glucose, lactose or threonine and control samples. Among the additives assayed, only threonine had a significant effect on acetaldehyde production, whereas in the glucose-enriched samples, the level was lower than controls. Thus, available data suggest that starter cultures can use different substrates for acetaldehyde biosynthesis and the amounts found in fermented milks are mainly dependent on the profile of sugar in the medium and the presence of specific enzymatic activities in bacteria such as threonine aldolase. Besides, acetaldehyde is an intermediate that can be quickly converted to other volatiles, depleting the medium of this compound and thus avoiding its accumulation in the product (O'Learly & Woychik, 1976). Acetaldehyde is mostly reduced to ethanol by alcohol dehydrogenase enzyme (Tamime & Robinson, 2007). The evolution of acetaldehyde throughout the incubation period showed a strong increase after 45 min in both types of yogurt whereas ethanol underwent an early decrease and then remained almost without changes. The particular accumulation of acetaldehyde in all yogurts suggests that, in the assayed conditions, the specific enzymatic activities to form it are higher than those able to convert it to ethanol.

Diketones such as 2,3-butanedione (or diacetyl) and 2,3-pentanedione were also detected during manufacture. As a consequence of enzymatic reduction of diacetyl can be formed acetoin, a nonvolatile compound with minor relevance to yogurt flavour. Ott *et al.* (2000a) proposed that 2,3-butanedione and 2,3-pentanedione are produced by spontaneous decarboxylation of their precursors, 2-acetolactate and 2-acetohydroxybutyrate, respectively, which are intermediates derived from glucose for 2,3-butanedione, and both threonine and glucose for 2,3-pentanedione. Therefore, taking into account that glucose is the main substrate for generation of diketones, higher levels of diacetyl and 2,3-pentanedione in E than in C yogurts can be reasonably expected. In our study, this finding could not be verified. By contrast, the area value of 2,3-pentanedione was higher in C yogurts in both trials and the level of diacetyl was higher in E yogurts at the end of fermentation only in trial 2. The obtained results indicate that lactic acid bacteria can utilise both lactose and glucose to produce diketones. This assumption is in agreement with data reported by Baranowska (2006) who observed that yogurts enriched with lactose and glucose had a higher level of diacetyl than control samples; however, this difference was statistically significant only in some types of yogurt. Moreover, it should be considered that 2,3-butanedione can be also converted to acetoin by action of diacetyl reductase enzyme, depleting the medium of this compound. The evolution of diacetyl, acetoin and 2,3-pentanedione during fermentation revealed a similar behaviour in both types of yogurt; these compounds had an important increase after 45 min. On the other hand, the values of acetoin were similar in C

and E yogurts of both trials during almost all fermentation process. The fact that diacetyl and acetoin increased in parallel during fermentation demonstrates that a continuous replenishment of diacetyl could be achieved in yogurts.

Methyl ketones such as propanone and butanone are likely derived from β-oxidation of saturated fatty acids, and further decarboxylation of β-ketoacids; so, the presence of yogurt is associated with the lipolytic activity of bacteria (Cheng, 2010). Thus, the synthesis of these compounds should not be affected by the added enzyme. In fact, C and E yogurts had similar area values towards the end of fermentation.

Volatile acids originate from a number of sources, including the metabolism of lactose, citrate and amino acids by starter bacteria. The biosynthesis of fatty acids from milk fat is considered a pathway of minor importance in yogurt. At 45 and 150 min, the three acids had significantly higher levels in hydrolysed yogurts ($P < 0.05$), but at the end of process, this trend changed substantially. For both trials, differences were only observed for hexanoic acid, being the values reached in C yogurts significantly higher ($P < 0.05$) than those found in E yogurts. The amount of each volatile acid increased markedly after 150 min in all samples. Similar observations have been made by other authors (Fernández-García & McGregor, 1994; Tamime & Robinson, 2007).

Changes in volatile profile during refrigerated storage

Acetaldehyde decreased or remained almost constant during storage in all yogurts. In both trials, the levels were significantly higher ($P < 0.05$) in E yogurts than their respective controls at each sampling time (Fig. S4a). The trend observed over the storage period was in accordance with that indicated by some researchers (Gaafar, 1992; Laye *et al*., 1993; Güler *et al*., 2009), although other authors have also reported a different behaviour (Kaminarides *et al*., 2007; Pinto *et al*., 2009).

No significant differences were found between the controls and enzyme containing yogurts with regard to quantity of ethanol. Its level increased in E yogurts during the first days of storage and then remained stable, whereas in C yogurts stayed unchanged throughout the storage period. Baranowska (2006), Güler (2007) and Kaminarides *et al*. (2007) pointed out that the concentration of ethanol increased gradually during yogurt storage. It is interesting to notice that when acetaldehyde decreased in E yogurts (at 7 days) (Fig. S4a) the ethanol level increased in parallel (Fig. S4b). The same behaviour was reported by Georgala *et al*. (1995), Güler *et al*. (2009) and Pinto *et al*. (2009). The reduction in the acetaldehyde content and the increase in the ethanol level during yogurt storage have been attributed to the presence of alcohol dehydrogenase enzyme activity, which is more active at lower pH values (Güler *et al*., 2009).

Concerning diketones, diacetyl evidenced an important increase at 7 days and then remained unchanged in all the yogurts (Fig. S4c). Reduced-lactose yogurts had higher levels of diacetyl at each sampling time in trial 2. The evolution of 2,3-pentanedione showed a marked increase at 7 days for trial 2 and until 14 days for trial 1 (Fig. S4d). In both trials, levels of 2,3-pentanedione at 1, 7 and 14 days were significantly higher in hydrolysed yogurts. Other researchers have observed both increase and decrease in diketone contents with storage time (Gaafar, 1992; Laye *et al*., 1993; Ott *et al*., 1999; Baranowska, 2006; Pinto *et al*., 2009). Our results suggest that diketones can be synthesised during storage and at higher levels in those yogurts containing glucose. In fact, Ott *et al*. (1999, 2000a) have demonstrated that precursors accumulate in larger amounts during fermentation, being converted to the respective diketones during cold storage. Changes in the levels of acetoin in all samples over the entire storage period were minor, which was in agreement with those of Laye *et al*. (1993) and Kaminarides *et al*. (2007). Statistical differences ($P < 0.05$) between both types of yogurt were observed only at 7 and 14 days for trial 2.

Among methylketones, propanone evidenced a similar evolution that acetaldehyde whereas butanone remained almost unchange, with exception of C yogurts from trial 2, which decreased steadily after 7 days. Differences between yogurts were dependent on trial. For example, higher levels of propanone were detected in hydrolysed yogurts than controls at 7 and 14 days only for trial 1. The behaviour reported in literature for propanone indicate that increases (Kaminarides *et al*., 2007), decreases (Gaafar, 1992; Georgala *et al*., 1995) or maintenances (Laye *et al*., 1993; Georgala *et al*., 1995) can occur during storage.

Overall, all volatile acids showed a tendency to increase during storage. The same trend has also been pointed by other authors, in particular, the increase in acetic acid content (Gaafar, 1992; Fernández-García & McGregor, 1994; Adhikari *et al*., 2002; Güler, 2007; Kaminarides *et al*., 2007). As shown in Fig. S4e, levels of acetic acid were higher in C yogurts than hydrolysed yogurts at 7, 14 and 21 days only for trial 1.

Conclusions

The use of β-galactosidase enzyme in the manufacture of reduced-lactose dairy foods is nowadays an usual practice. The knowledge of the effect of its incorporation on different quality parameters such as pH, acidity, flavour, etc., is relevant to verify whether the obtained products have similar characteristics than

those traditional. As evidenced by this preliminary study, the hydrolysed yogurts presented similar characteristics regarding pH and titratable acidity in comparison with controls.

The presence of β-galactosidase did not alter the normal evolution of compounds during fermentation and storage. However, the obtained results suggest that changes in the carbohydrate pattern can affect the volatile compound production. The main differences in volatile profiles between both types of yogurt were observed at the beginning of incubation, stage in which the hydrolysed yogurts reached the maximum lactose conversion. At the end of fermentation, a similar volatile fraction was detected. During storage, the higher levels of acetaldehyde and diketones in hydrolysed yogurts compared to controls and the increase of diketones and acids were the most relevant findings. The effect of these changes on flavour of the products must be determined in further sensorial studies.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Lactose levels in control (C) and experimental (E) yogurts from trials 1 and 2 during fermentation and storage.

Figure S2. pH values in control (C) and experimental (E) yogurts from trials 1 and 2 during fermentation and storage.

Figure S3. Titratable acidity (°D) over 21 days of storage at 5°C in control (C) and experimental (E) yogurts from trials 1 and 2.

Figure S4. Evolution of acetaldehyde (A), ethanol (B), diacetyl (C), 2,3-pentanodione (D) and acetic acid (E) in control (C) and experimental (E) yogurts from trials 1 and 2 during storage.

Table S1. Volatile compounds identified in samples of yogurts during fermentation and storage at 5°C.

Table S2. Evolution of the main volatile compounds in control (C) and experimental (E) yogurts during fermentation process from trial 1.

Table S3. Evolution of the main volatile compounds in control (C) and experimental (E) yogurt during fermentation process from trial 2.