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Evolution of carbohydrate fraction in carbonated fermented milks as affected by β-galactosidase activity of starter strains

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Summary. The influence of carbonation on the evolution of lactose, galactose and glucose in fermented milks with added probiotic bacteria (*Lactobacillus casei*, *Lactobacillus acidophilus* and/or *Bifidobacterium bifidum*) was evaluated and related to β-galactosidase activity of starter strains. During incubation and first days of refrigeration, lactose hydrolysis resulting in the liberation of galactose and glucose occurred in CT (*Streptococcus thermophilus/Lb. casei*), AT (*Str. thermophilus/Lb. acidophilus*) and ABT fermented milks (*Str. thermophilus/Lb. acidophilus/Bifid. bifidum*). Levels of galactose were higher than those of glucose and could be related to the preferential consumption of glucose by actively growing bacteria. Through the incubation, lactose and monosaccharide levels were not affected by milk carbonation. However, during refrigerated storage the presence of this gas was associated with slightly lower content of lactose and higher levels of galactose and glucose in AT and ABT products but not in CT fermented milks. Through the refrigeration galactose was moderately utilised by *Lb. acidophilus* in AT products whereas the presence of *Bifid. bifidum* seems to prevent the consumption of this sugar in ABT fermented milks. Glucose remained constant, with minor variations in CT products but a continuous increase of this sugar occurred in carbonated AT and ABT fermented milks during storage. β-Galactosidase activity displayed by *Str. thermophilus* strains was similar at pH 6·5 (initial pH of non-carbonated samples) and pH 6·3 (initial pH of carbonated samples) whereas *Lb. acidophilus* LaA3 showed greater β-galactosidase activity at pH 6·3 than at higher pH values. Thus, the enhanced metabolic activity of *Lb. acidophilus* caused by the low initial pH of carbonated milk also promoted higher cellular β-galactosidase activity that could have released greater amounts of galactose and glucose from lactose in AT and ABT fermented milks through the refrigerated period. In CT fermented milks, similar β-galactosidase activity levels of *Str. thermophilus* at pH 6·5 and 6·3 together with the absence of β-galactosidase activity in *Lb. casei* could explain the lack of differences on glucose and galactose content between carbonated and non-carbonated samples.

Keywords: Carbohydrate, β-galactosidase, carbon dioxide, *Lactobacillus acidophilus*, fermented milk.

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The interest of people and the dairy industry in fermented milks with added probiotic bacteria has been increasing in recent years and a great variety of products containing probiotic *Lactobacillus* and *Bifidobacterium* strains have been formulated world-wide (Kneifel & Pacher, 1993). Main carbohydrates in yoghurt and fermented milks are lactose, galactose and glucose (Tamime & Deeth, 1980; Akalin *et al*. 1996). In lactic acid bacteria, lactose can enter the cell by two different pathways. In some lactococci and lactobacilli lactose is transported and phosphorylated by a phosphoenolpyruvate-dependent phosphotransferase system and the lactose phosphate is then cleaved by a β-phosphogalactosidase. In *Streptococcus thermophilus* and most lactobacilli, lactose is transported as such and then hydrolysed by a β-galactosidase (Jimeno *et al*. 1984; Hickey *et al*. 1986). The hydrolysis of lactose present in milk by the microbial β-galactosidase results in a subsequent increase of galactose and glucose in fermented milks. Galactose levels have been used as a measure of β-galactosidase activity (González-Andrada *et al*. 1996) since this sugar tends to accumulate in yogurt when glucose, the readily fermentable carbohydrate, is being consumed by actively growing bacteria.

One of the potential health benefits of fermented milks is that they are generally well tolerated by individuals who suffer symptoms of lactose intolerance (Mustapha *et al*. 1997). The increased tolerance for dairy products containing microorganisms is thought to be caused by intra-intestinal digestion of lactose by β-galactosidase released from the microorganisms during transit through, or colonisation of, the gut (Kilara & Shahani, 1976). On the other hand, milk and dairy products are important sources of carbohydrates for body development during the growth period in humans.

In spite of these considerations, there is scarce information on the evolution of lactose, galactose and glucose in fermented milks other than yogurt. In recent work (Vinderola *et al*. 2000) we showed that carbonation of pasteurised milk enhanced growth and metabolic activity of the starter during manufacture and slightly improved the sensory properties (mouth-feel, taste, acid acceptability and overall acceptability) of these products. Thus, carbonation of pasteurised milk could be a useful tool for the manufacture of AT (*Str. thermophilus/Lactobacillus acidophilus*) and ABT (*Str. thermophilus/Lb. acidophilus/Bifidobacterium bifidum*) fermented milks in order to reduce the incubation time of these products, with the subsequent reduction of economic costs for the dairy industry. However, the enhanced metabolic activity of microorganisms caused by the CO_2 could also modify some important chemical parameters of these products. Among them, glucose and galactose are of particular interest due to the origin of these monosaccharides from the lactose hydrolysis by microbial β-galactosidase, an enzyme that has been related to the improvement of lactose-intolerance symptoms. These considerations and our previous results led to the present work which is the first report concerning the effect of carbonation of pasteurised milk on the subsequent evolution of lactose, glucose and galactose during fermentation and cold storage of AT, ABT and CT (*Str. thermophilus/Lb. casei*) fermented milks. The influence of CO_2 on the β-galactosidase activity of starter strains was also analysed.

Materials and methods

Strains and culture conditions for fermented milk preparations

Str. thermophilus, strain St73, and a commercial strain of *Lb. acidophilus* (LaA3) were used as lactic starters for AT and ABT fermented milk preparations. A commercial strain of *Bifid. bifidum* (BBI) was used as probiotic adjunct in ABT

products. Two commercial strains, *Str. thermophilus* StJo7 and *Lb. casei* LcA13, were employed as lactic starters for the manufacture of CT fermented milk. All strains were obtained from the PROLAIN (Programa de Lactología Industrial, National University of Litoral, Argentina) collection. *Str. thermophilus* strains, *Lb. casei* LcA13 and *Lb. acidophilus* LaA3 were propagated in sterilised reconstituted skim milk (110 g/l) at 37 °C for 12 h. Each *Str. thermophilus* strain was then mixed with either *Lb. casei* or *Lb. acidophilus* (1 : 1 v/v) to inoculate pasteurised whole milk (carbonated and non-carbonated control) as further indicated. Cultures of *Bifid. bifidum* BBI were grown in MRS broth (Biokar, Beauvais, France) for 24 h at 37 °C using the Anaerocult A system (Merck, Darmstadt, Germany). These cultures were then centrifuged (10 min at 4340 ***g***) in a Sorvall RC-5B centrifuge (Du Pont Company, Wilmington, Delaware, USA), suspended in a solution of sucrose (80 g/l) and frozen at −80 °C. The preparation was then lyophilised at 0·16 mmHg for 18 h in an Alpha 1-4 freeze-dryer (B. Braun Biotech International, Melsungen, Germany). The lyophilised culture of *Bifid. bifidum* was used to inoculate pasteurised whole milk together with *Str. thermophilus* St73 and *Lb. acidophilus*.

Production and analysis of fermented milks

Raw milk (10 l) collected from one farm in Asturias (northern Spain) was supplemented with 20 g skim milk powder/l. The mix was pasteurised at 85 °C for 30 min, cooled to 4 °C, divided into two lots of 5 l each and held overnight with constant stirring (14 rev/min). During this time one lot was carbonated with food-grade CO_2 (Carburos Metálicos, Barcelona, Spain) to pH 6·3 as previously described (Ruas-Madiedo *et al.* 1996) and the other was kept as non-acidified control. Both lots were then inoculated (20 ml/l) with a mixture of *Str. thermophilus* StJo7 and *Lb. casei* LcA13 to produce CT fermented milk or with both *Str. thermophilus* StJ73 and *Lb. acidophilus* LaA3 to produce AT fermented milk. For the production of ABT fermented milk, in addition to AT inoculum a lyophilised culture of *Bifid. bifidum* BBI was added in order to give an initial cell count of *Bifid. bifidum* ranging from 10^6 to 10^7 cfu/ml. Inoculated carbonated and non-carbonated control milk were each distributed in sterile glass bottles (200 ml) and incubated without stirring at 42 °C. After pH 5·0 was reached, fermented milks were stored at 4 °C to avoid an excessive post-acidification during the first few days of refrigerated storage that could lead to a reduction of the viability of probiotic bacteria. Three trials of each type of fermented milk (CT, AT and ABT) were carried out.

Titratable acidity and pH of CT fermented milks were measured through the manufacture and refrigerated storage as previously described (Vinderola *et al.* 2000). Serial dilutions of CT fermented milks were made during fermentation and storage in a quarter-strength Ringer's solution (Oxoid, Unipath, Basingstoke, Hampshire, UK) and spread-plated in duplicate on M17 agar and MRS agar (Biokar) for counting *Str. thermophilus* and *Lb. casei*, respectively. These cultures were incubated aerobically at 37 °C for 3 d.

Lactose, galactose and glucose analysis in fermented milks

Lactose, galactose and glucose analyses through fermentation and cold storage of AT, ABT and CT fermented milks were done by means of gas chromatography of the trimethylsilyl derivatives of the free carbohydrate fraction using a 3 m × 1·0 mm stainless steel column (Chrompack, Middelburg, The Netherlands) packed with 2 % OV-17 on non-silanized 120/140 Volaspher A-2 (Merck), following the method described by Olano *et al.* (1986).

A milk (1 ml) sample was gently mixed with approximately 6 ml of methanol in a 10-ml volumetric flask, so that denatured protein particles would not get stuck above the volume mark, and filled to volume by adding additional methanol. After mixing again, the mixture was held for 24 h at room temperature. 1 ml of supernatant was mixed with 1 ml of phenyl-β-glucoside (1 g/l), evaporated under vacuum at room temperature and converted to trimethylsilyl derivatives using N-trimethylsilylimidazole.

Lactose, galactose and glucose fermentation capability of strains

Strains used for fermented milk preparations were characterised for their fermentation capability of lactose, galactose and glucose. This test was performed in 5 ml of a liquid basal medium (BM) (10 g tryptone/l, 5 g yeast extract/l, 1 ml Tween 80/l, 2 g dipotassium phosphate/l, 5 g sodium acetate/l, 2 g diammonium citrate/l, 0·2 g magnesium sulphate/l, 0·17 g bromocresol purple/l, pH 6·6) containing 20 g/l of the appropriate carbohydrate (Sigma Chemical Co., St. Louis, Missouri, USA). The development of a yellow colour after anaerobic incubation (Anaerocult A system, Merck) at 37 °C during 48 h for *Streptococcus* and *Lactobacillus* strains and after 3 d for *Bifid. bifidum* was considered a positive result. Assays were carried out in duplicate.

Effect of CO_2 on β-galactosidase activity

Washed cells of *Streptococcus* and *Lactobacillus* were assayed in buffer acidified with CO_2 or HCl.

β-Galactosidase activity was measured by the method of Noh and Gilliland (1993) with minor modifications. The substrate for the assay was 0·012 M-*o*-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma). Cultures of the different strains under study were separately grown overnight at 37 °C in BM plus 20 g lactose/l (BML). 1 ml of cell cultures were then washed and suspended in 3 ml of each of the following buffer lots: 0·03 M-sodium phosphate buffer (pH 7·0), the same buffer acidified to pH 6·6 with either CO_2 or HCl. Cold ONPG reagent (2 ml) was then added to each tube. The mixtures were incubated in a water-bath at 37 °C for 10 min. After incubation, 4 ml of cold 0·625 M-sodium carbonate was added to stop the reaction. Following mixing, the contents of each tube were centrifuged at 4340 ***g*** to remove the cells. The clear supernatant was recovered and the $A_{420\,nm}$ was read against a reagent blank in an Uvikon 930 spectrophotometer (Kontron Instruments, Zurich, Switzerland). The calculation of micrograms of *o*-nitrophenol (ONP) released was based on the relationship of the $A_{420\,nm}$ to a standard curve. Activity was expressed as μg ONP released/10 min of incubation. Assays were carried out in triplicate.

Influence of CO_2 on cellular integrity of strains

Ten millilitres of overnight cultures of *Streptococcus* and *Lactobacillus* strains in BML were washed and suspended in 1 ml of 0·03 M-sodium phosphate buffer. Sets of three test tubes were prepared for each culture: one with 10 ml of the same buffer (pH 7·0) and the other two with 10 ml of the same buffer acidified with either CO_2 or HCl (pH 6·6). Washed cell suspensions (100 μl) was added to each tube. The initial $A_{620\,nm}$ was read, and the mixtures were placed in a water bath at 37 °C for incubation. The A_{620nm} for each mixture was measured at 1-h intervals for 4 h. Any decrease of $A_{620\,nm}$ was attributed to the lysis of cells. Assays were made in triplicate.

Influence of CO_2 on β-galactosidase activity levels of actively growing strains

Measurement of enzyme activity was carried out in cells of *Streptococcus* and *Lactobacillus* grown in media acidified with CO_2 or HCl.

One set of BML was prepared and divided into five lots. One was maintained as a control (pH 7·0), another two lots were acidified to pH 6·5 with either HCl or CO_2 injection (Carburos Metálicos), and the remaining lots were acidified to pH 6·3 by either HCl addition or CO_2 injection. Sets of media were inoculated with fresh cultures (1/100 v/v) of each strain previously grown in BML, and incubated at 37 °C. When A_{620nm} of 0·5 was reached, 1 ml of each culture was washed with 0·03 M-sodium phosphate buffer (pH 7·0) and resuspended in 3 ml of the same buffer. β-Galactosidase activity was assayed as indicated above. Experiments were carried out in triplicate.

Statistical analysis

Statistical analysis was performed by using the SPSS-PC+ software (SPSS Inc., Chicago, IL, USA). Data of microbiological counts, pH, acidity and content of lactose, glucose and galactose during manufacture (time 0, 2 h and end of manufacture [time taken to reach a pH 5·0]) and cold storage (fermented milk of 2, 7, 28 and 49 d) of fermented milks were subjected to ANOVA using milk treatment as a factor with two categories: carbonated milk and non-carbonated milk.

β-Galactosidase activity and A_{620nm} on each set of data were also subjected to ANOVA test using the acidification as factor. Five categories were considered in experiments of cells grown in the presence of CO_2 and HCl: control (pH 7·0), acidified to pH 6·5 or 6·3 with either HCl or CO_2. Three categories were established for measurement of A_{620nm} and activity in buffer acidified with CO_2 and HCl: control (pH 7·0), acidified to pH 6·6 with either HCl or CO_2. The least significant difference test was applied for means comparison when appropriate (Snedecor & Cochran, 1980).

RESULTS AND DISCUSSION

Chemical and microbiological evolution of fermented milks

Chemical and microbiological evolution of AT and ABT fermented milk was published previously (Vinderola *et al*. 2000). Table 1 summarises the evolution of microbiological and chemical parameters as well as the manufacture time of CT fermented milks. *Str. thermophilus* increased in number through the incubation period whereas slight variations were detected on the population of *Lb. casei*. No remarkable differences were found between carbonated and non-carbonated samples ($P > 0·05$) for both *Str. thermophilus* and *Lb. casei*. In our previous study (Vinderola *et al*. 2000) we also reported an increase of *Str. thermophilus* and *Lb. acidophilus* counts in AT and ABT fermented milks during incubation although minor changes were detected on the population of *Bifid. bifidum* in ABT products. Slight growth stimulation of CO_2 on AT/ABT starter culture was also indicated in our previous work whereas this effect was not evident in CT cultures.

As previously indicated for AT and ABT fermented milks (Vinderola *et al*. 2000), in CT products the pH dropped and the acidity rose during manufacture of both non-carbonated and carbonated samples (Table 1) due to the metabolic activity of microorganisms. No differences in pH values attributable to the CO_2 were found ($P > 0·05$) in CT fermented milks through the cold storage. However, the acidity remained slightly higher during refrigeration when carbonated rather than non-carbonated milk was used. On the other hand, we have previously shown that the

Table 1. *Microbiological and chemical parameters and manufacture time* (*measured from the inoculum addition until a pH 5·0 was reached*) *of CT fermented milks* (Streptococcus thermophilus/Lactobacillus casei) *during milk fermentation and cold storage of the finished product*

(Values are mean ± SD for $n = 3$)

		Chemical parameters		Microbiological counts‡		
Time	Treatment	pH	Acidity†	*S. thermophilus*	*L. casei*	Fermentation time§
Fermentation 0 h	Non-carbonated	6·69 ± 0·04	0·18 ± 0·01	6·70 ± 0·81	6·65 ± 0·65	—
	Carbonated	6·30 ± 0·06	0·27 ± 0·03	6·70 ± 0·81	6·65 ± 0·65	—
		***	**			
Fermentation 2 h	Non-carbonated	6·24 ± 0·44	0·25 ± 0·07	7·90 ± 0·68	6·82 ± 0·77	—
	Carbonated	6·00 ± 0·34	0·36 ± 0·10	7·69 ± 0·90	6·66 ± 0·68	—
Fermentation end	Non-carbonated	4·93 ± 0·07	0·60 ± 0·05	8·83 ± 0·39	6·60 ± 0·82	209·00 ± 25·60
	Carbonated	4·98 ± 0·12	0·64 ± 0·05	9·02 ± 0·19	6·75 ± 0·48	185·00 ± 13·03
Cold storage 2 d	Non-carbonated	4·82 ± 0·14	0·67 ± 0·04	8·99 ± 0·19	6·83 ± 0·70	—
	Carbonated	4·80 ± 0·17	0·74 ± 0·05	8·96 ± 0·42	6·68 ± 1·02	—
Cold storage 7 d	Non-carbonated	4·68 ± 0·09	0·74 ± 0·03	9·15 ± 0·15	6·74 ± 0·44	—
	Carbonated	4·66 ± 0·23	0·79 ± 0·06	8·94 ± 0·24	6·73 ± 0·08	—
Cold storage 28 d	Non-carbonated	4·45 ± 0·09	0·82 ± 0·02	9·10 ± 0·20	6·94 ± 0·48	—
	Carbonated	4·43 ± 0·12	0·87 ± 0·06	8·77 ± 0·51	6·79 ± 0·64	—
Cold storage 49 d	Non-carbonated	4·43 ± 0·13	0·78 ± 0·04	8·76 ± 0·32	6·89 ± 0·43	—
	Carbonated	4·48 ± 0·18	0·81 ± 0·03	8·51 ± 0·33	6·93 ± 0·54	—

†Expressed as g lactic acid/100 ml. ‡Expressed as log cfu/ml. §Expressed in min. $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.

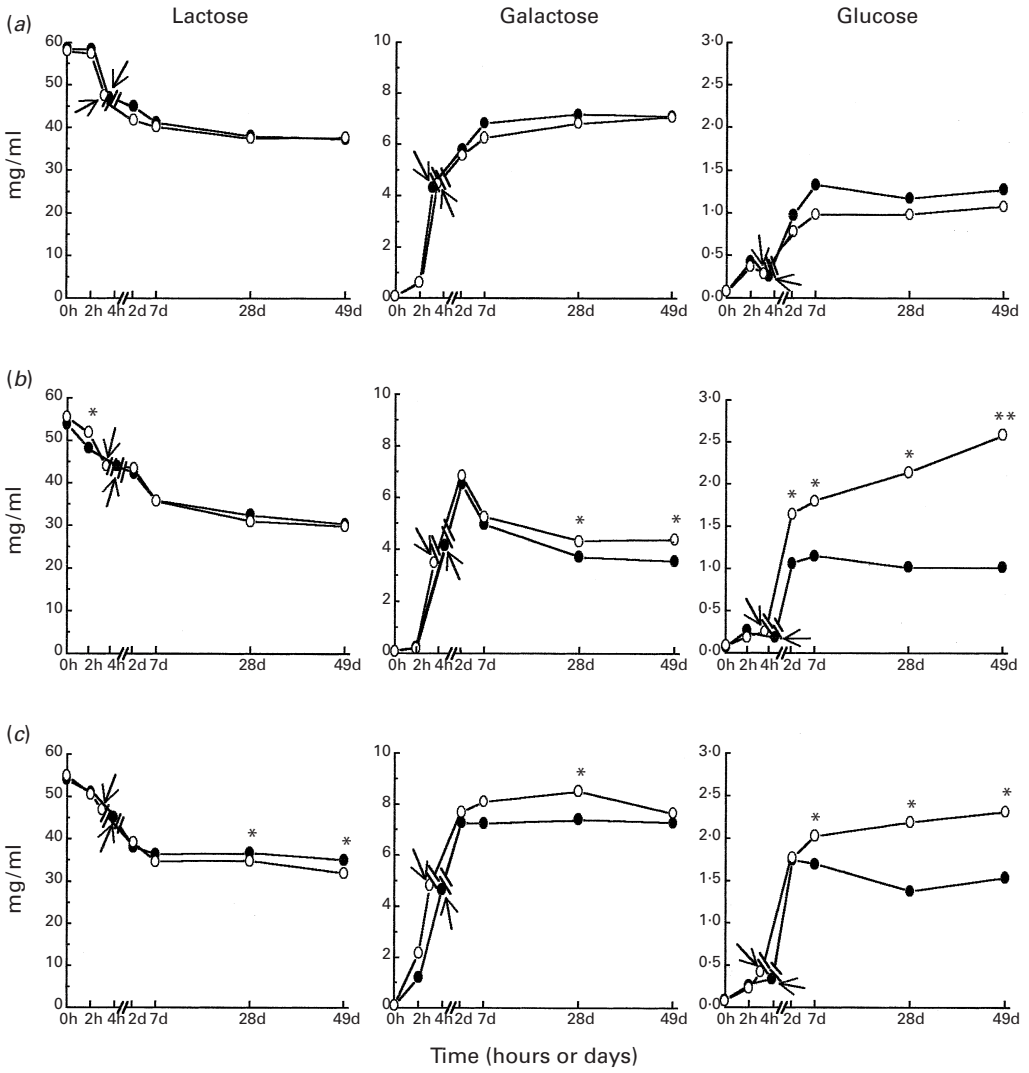

Fig. 1. Evolution of lactose, galactose and glucose during manufacture, h, and cold storage, d, in non-carbonated control (●) and carbonated samples (○) of (*a*) CT, (*b*) AT and (*c*) ABT fermented milks (*, ** and *** mean significant differences at levels of $P < 0{\cdot}05$, $P < 0{\cdot}01$ and $P < 0{\cdot}001$, respectively). Arrows indicate the end of fermentation (time taken to reach a pH 5·0).

enhanced metabolic activity of microorganisms caused by the initial lowered pH of carbonated samples promoted a reduction of the manufacture time in AT and ABT fermented milks (Vinderola *et al.* 2000). Contrary to that, in CT products the low initial pH of carbonated milk did not seem to exert any influence on the starter activity during fermentation and therefore the manufacture time was not subsequently reduced.

Lactose, glucose and galactose evolution in fermented milks

The evolution of lactose, galactose and glucose during the manufacture and cold storage of AT, ABT and CT fermented milks is shown in Fig. 1. As expected, lactose decreased during incubation and concentrations of 43·9–47·5 mg/ml of residual sugar were still present in fermented milks after manufacture. Throughout the cold storage

lactose slowly decreased to 29·7–30·3 mg/ml, 31·9–35·1 mg/ml and 37·3–37·7 mg/ml in AT, ABT and CT products, respectively. From the 7th to 49th day of refrigerated storage, levels of this carbohydrate remained lower in carbonated AT and ABT products than in the corresponding controls (significant differences in ABT fermented milks at 28 and 49 d) which indicated a greater consumption of lactose in the former samples. In CT fermented milks these differences were progressively reduced through the storage, reaching control samples at slightly lower levels of lactose than the carbonated ones after 49 d of refrigeration.

A moderate increase of galactose and glucose occurred until 2 h of incubation in all types of fermented milks. From this point to the end of fermentation, levels of glucose were slightly modified while those of galactose increased abruptly in all cases. Since the hydrolysis of lactose releases glucose and galactose in equal amounts, lower levels of glucose must be attributed to its subsequent consumption as a carbon source by the starter microorganisms actively growing in milk. On the other hand, no significant differences ($P > 0{\cdot}05$) attributable to the presence of CO_2 were detected in any product during fermentation.

Through the refrigerated storage galactose levels remained considerably higher than that of glucose in AT, ABT and CT fermented milks. The concentration of both monosaccharides in fermented milks augmented during the first 2 or 7 d of refrigeration as a result of the lactose hydrolysis by lactic acid bacteria. This increase was more pronounced for galactose than for glucose due to the greater accumulation rate of the former sugar with respect to the glucose. Behaviour of galactose and glucose during the remaining storage period varied depending on the type of product and previous CO_2 treatment of pasteurised milk.

During refrigerated storage, the CO_2 did not exert any influence on the evolution of monosaccharides in CT fermented milks but, remarkably, the presence of this gas was associated with higher levels of galactose (from the 28th d) and glucose ($P < 0{\cdot}05$) in AT and ABT products (Fig. 1). Throughout this period, the content of galactose in CT and ABT fermented milks (both carbonated and non-carbonated) suffer minor variations whereas a decrease of this sugar occurred in AT products. This fact will be further discussed. On the other hand, a slow and continuous increase of glucose occurred in carbonated AT and ABT samples during the refrigerated storage whereas the content of this sugar remained constant with minor variations or decreased slightly in CT fermented milks as well as in non-carbonated AT and ABT products. We have not found information in the literature about the effect of CO_2 on monosaccharide levels of fermented milks. However, the moderate increase of glucose in carbonated AT and ABT products could be related in our case to higher rates of lactose hydrolysis associated with a low consumption of glucose during storage.

Lactose, galactose and glucose fermentation capability of strains

Both strains of CT lactic starter, *Str. thermophilus* StJo7 and *Lb. casei* LcA13, were able to ferment lactose, galactose and glucose. With respect to the AT and ABT starter, glucose and lactose were fermented by *Str. thermophilus* St73 and *Lb. acidophilus* LaA3, whereas galactose was only utilised by *Lb. acidophilus*. Glucose was the sole carbohydrate fermented by *Bifid. bifidum*.

Since *Lb. acidophilus* was the sole strain able to metabolise galactose in AT and ABT fermented milks, the decrease of galactose content detected in AT products could be attributed to its utilisation by *Lb. acidophilus* during refrigeration. Contrary to that, in ABT fermented milks the presence of *Bifid. bifidum* BBI seems to act by

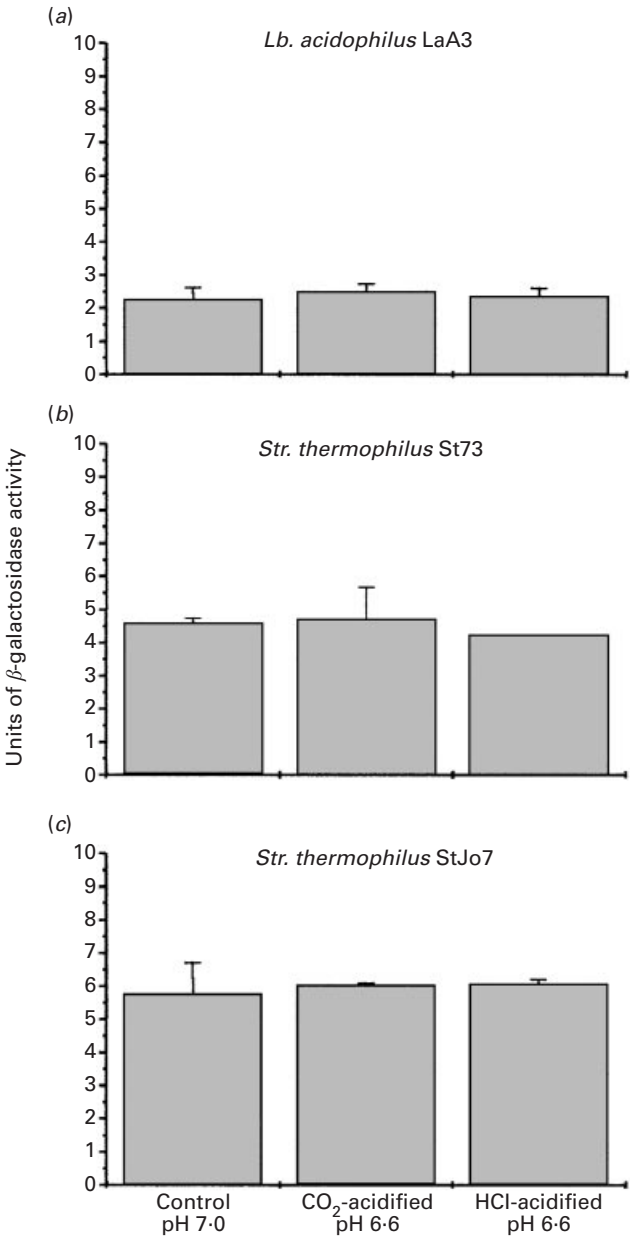

Fig. 2. *β*-Galactosidase activity of overnight cultures in basal medium plus lactose (20 g/l) of (*a*) *Lb. acidophilus* LaA3, (*b*) *Str. thermophilus* St73 and (*c*) *Str. thermophilus* Jo7 measured in phosphate buffer (pH 7·0) and phosphate buffer acidified to pH 6·6 with either HCl or CO_2. Vertical lines on the bars represent standard deviations. Bars with different superscripts are significantly different ($P < 0{\cdot}05$).

preventing the consumption of this sugar by *Lb. acidophilus* LaA3. In this respect, we recently reported a loss of viability for the same strain of *Lb. acidophilus* in carbonated ABT fermented milk which was associated with an interaction with *Bifid. bifidum* in the presence of CO_2 (Vinderola *et al.* 2000). Conversely, Heo & Yoon (1996) reported a reduction of galactose content in milk during prolonged incubation times of a mixture of two *Bifid. longum* and *Lb. acidophilus* strains.

Table 2. *Effect of CO_2 and HCl on cellular integrity of* Streptococcus thermophilus *and* Lactobacillus acidophilus

(Values are means ± SD of A_{620} for $n = 3$)

	Lb. acidophilus LaA3		
Time	Control	CO_2	HCl
0 h	0·233 ± 0·030	0·245 ± 0·006	0·245 ± 0·021
1 h	0·221 ± 0·032	0·232 ± 0·006	0·233 ± 0·022
2 h	0·207 ± 0·023	0·220 ± 0·023	0·220 ± 0·045
3 h	0·199 ± 0·012	0·207 ± 0·029	0·197 ± 0·042
4 h	0·202 ± 0·011	0·215 ± 0·013	0·195 ± 0·030
	Str. thermophilus St73		
Time	Control	CO_2	HCl
0 h	0·213 ± 0·037	0·220 ± 0·042	0·208 ± 0·039
1 h	0·186 ± 0·025	0·206 ± 0·043	0·192 ± 0·040
2 h	0·180 ± 0·041	0·198 ± 0·041	0·186 ± 0·039
3 h	0·180 ± 0·139	0·192 ± 0·045	0·179 ± 0·049
4 h	0·185 ± 0·036	0·190 ± 0·042	0·184 ± 0·036
	Str. thermophilus StJo7		
Time	Control	CO_2	HCl
0 h	0·395 ± 0·070	0·412 ± 0·093	0·383 ± 0·083
1 h	0·325 ± 0·060	0·330 ± 0·092	0·302 ± 0·055
2 h	0·291 ± 0·055	0·330 ± 0·051	0·315 ± 0·054
3 h	0·279 ± 0·061	0·309 ± 0·061	0·302 ± 0·058
4 h	0·274 ± 0·060	0·295 ± 0·060	0·296 ± 0·056

Effect of CO_2 on β-galactosidase activity

Assays of β-galactosidase in those strains able to ferment lactose (*Streptococcus* and *Lactobacillus*) were carried out in the presence of CO_2 or HCl in order to elucidate whether these compounds or the acidification that they promoted could have any effect on β-galactosidase activity.

Preliminary assays showed that β-galactosidase activity of *Lb. casei* was negligible, suggesting that lactose is probably hydrolysed by a β-phosphogalactosidase (Jimeno *et al.* 1984; Hickey *et al.* 1986). Thus, the activity of *Lb. casei* LcA13 was not analysed further.

As shown in Fig. 2, β-galactosidase activity of *Str. thermophilus* St73, *Str. thermophilus* StJo7 and *Lb. acidophilus* LaA3 showed similar values ($P > 0·05$) when measured in presence and absence of CO_2 and HCl, indicating that these two compounds did not have any influence on the activity. Thus, higher monosaccharide levels detected in carbonated fermented milk could not be attributed to a direct stimulation by CO_2 or acidity of the cellular β-galactosidase activity.

Influence of CO_2 on cellular integrity of strains

The effect of CO_2 and moderate acidity was measured by monitoring the A_{620} of cell suspensions of *Lb. acidophilus* and *Str. thermophilus* during incubation with and without added CO_2 or HCl (Table 2). All strains exhibited decreases in A_{620} as incubation time increased both in the presence and absence of CO_2 and HCl.

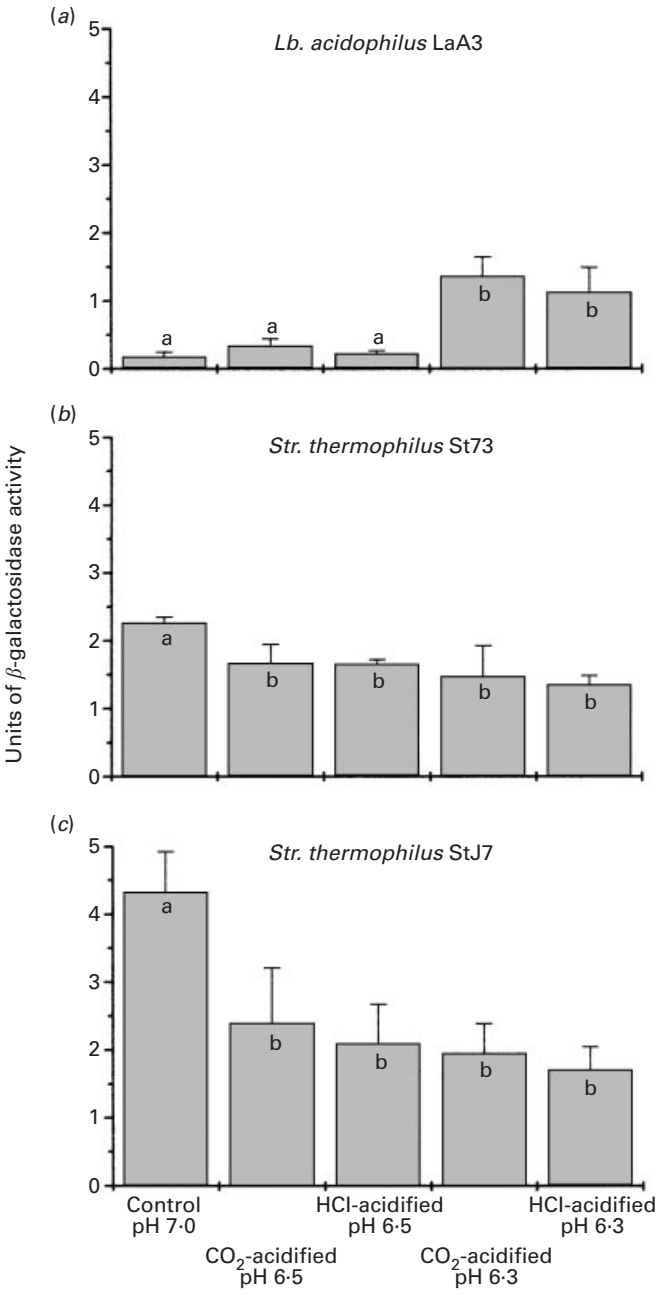

Fig. 3. *β*-Galactosidase activity measured in phosphate buffer (pH 7·0) of exponential cultures (A_{620} = 0·5) of (*a*) *Lb. acidophilus* LaA3, (*b*) *Str. thermophilus* St73 and (*c*) *Str. thermophilus* StJo7 grown in basal medium plus lactose (20 g/l) without acidification (pH 7·0) and acidified to either pH 6·5 or 6·3 with either HCl or CO_2. Vertical lines on the bars represent standard deviation. Bars with different letters are significantly different ($P < 0·05$).

However, no differences were significant among the decreases in turbidity between the three treatments for any of the strains ($P > 0·05$). Therefore, the presence of CO_2 or HCl did not enhance the cellular lysis of *Str. thermophilus* and *Lb. acidophilus* and

a subsequent release of enzyme that could lead to an increase of β-galactosidase activity did not occur.

Influence of CO_2 on β-galactosidase activity levels of actively growing strains

To elucidate whether the greater content of galactose and glucose in carbonated AT and ABT fermented milks could be related to higher cellular β-galactosidase levels, enzymatic activity was measured in *Lb. acidophilus* LaA3, *Str. thermophilus* St73 and *Str. thermophilus* StJo7 grown in media acidified with CO_2 or HCl.

β-Galactosidase activity levels attained by *Str. thermophilus* St73 and StJo7 strains grown in acidified media were significantly lower ($P < 0{\cdot}05$) than in non-acidified medium (pH 7·0) although no differences were found between the results obtained at pH 6·5 and 6·3 (Fig. 3). However, *Lb. acidophilus* LaA3 displayed significantly higher levels ($P < 0{\cdot}05$) of β-galactosidase activity at pH 6·3 than at pH 6·5 and 7·0. In addition, for each *Str. thermophilus* and *Lb. acidophilus* strain, activity levels obtained at a given pH were similar in media acidified with CO_2 or HCl. These results indicated that acidification of the growth medium (either by CO_2 or HCl) promoted greater β-galactosidase activity levels in *Lb. acidophilus* but caused a decrease of activity in *Str. thermophilus*.

Pasteurised milk used for fermentation had a pH 6·5–6·6 in control samples and 6·25-6·35 in carbonated ones. At these pH values, *Str. thermophilus* displayed similar β-galactosidase activity levels in both carbonated and non-carbonated milks whereas the activity of *Lb. acidophilus* was greater in carbonated milk (Fig. 3). Thus, higher monosaccharide amounts in carbonated AT and ABT fermented milks with respect to the non-carbonated milks could have been caused by the enhanced β-galactosidase activity of *Lb. acidophilus* LaA3 in the former samples. In CT fermented milks, similar levels of glucose and galactose could be due to the absence of β-galactosidase in *Lb. casei* together with similar activity levels displayed by *Str. thermophilus* in carbonated and non-carbonated samples. Interestingly, CO_2 promoted a reduction of fermentation times in AT and ABT fermented milks but not in CT products; higher acidity levels in AT and ABT fermented milks stimulate growth and metabolic activity of the starter during fermentation and cause a subsequent reduction of fermentation time (Vinderola *et al.* 2000). Thus, the enhanced metabolic activity of *Lb. acidophilus* LaA3 during growth could also promote greater levels of β-galactosidase in these strains. The enzyme could remain active, releasing greater amounts of galactose and glucose from lactose in AT and ABT fermented milks through the refrigerated storage.

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