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**Original article**

# Biochemical characteristics of fermented milk produced by mixed-cultures of lactic starters and bifidobacteria

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**Abstract** — Strains of bifidobacteria of human origin (*Bifidobacterium bifidum, B. breve, B. infantis* and *B. longum)* were inoculated simultaneously with mixed cultures of lactococci, citrate-fermenting lactococci and leuconostocs to determine their impact on biochemical characteristics of fermented dairy products. Fermentations were carried out under different conditions of temperature, milk protein concentration and ratio of acidifying/aromatic lactic acid bacteria. The survival or growth of bifidobacteria during production of fermented milk was not affected by the presence of lactic starters. On the other hand, the presence of bifidobacteria during the production of fermented milk did not influence the growth of total lactococci, citrate-fermenting lactococci and leuconostocs. The variation of the milk protein content did not modify the growth of bifidobacteria and mixed-cultures of lactic starters. However, the presence of bifidobacteria changed the final characteristic of the fermented milk. Residual lactose in fermented milks containing *B. breve*, *B. bifidum* and *B. infantis* was lower than that found in fermented milks without bifidobacteria and those containing *B. longum*. The fermented milks without bifidobacteria contained less acetaldehyde than those containing bifidobacteria whereas the concentration of diacetyl was less important in the presence of bifidobacteria. However, the ethanol concentration increased when bifidobacteria were used with mixed cultures of lactococci, citrate-fermenting lactococci and leuconostocs. A temperature of 35 °C seems to be desirable because it allows the growth of bifidobacteria without affecting in a drastic way the growth and activity of leuconostocs and other lactic starters.

**bifidobacteria / starter / leuconostoc / fermented milk / volatile compound**

**Résumé — Les caractéristiques biochimiques du lait fermenté par des cultures mixtes de ferments lactiques et de bifidobactéries.** Des souches de bifidobactéries d'origine humaine (*Bifidobacterium breve, B. longum, B. infantis* et *B. bifidum*) ont été inoculées simultanément avec des cultures mixtes de lactocoques acidifiants, des lactocoques aromatiques et de leuconostocs afin

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de déterminer leur impact sur les caractéristiques de produits laitiers fermentés. Les fermentations ont été réalisées sous différentes conditions de température, concentration protéique et rapports de bactéries lactiques acidifiantes et aromatiques. La croissance et la survie des bifidobactéries n'ont pas été affectées par la présence des bactéries lactiques. D'autre part, la présence de bifidobactéries n'influence pas la croissance des lactocoques acidifiants, des lactocoques aromatiques et des leuconostocs. La variation du contenu en protéines du lait n'affecte pas la croissance des bifidobactéries ou des cultures mixtes de levains mésophiles. Toutefois, la présence de bifidobactéries modifie les caractéristiques finales du lait fermenté. La teneur en lactose résiduel dans les laits fermentés contenant *B. breve, B. bifidum* et *B. infantis* est inférieure à celle retrouvée dans les laits fermentés sans la présence de bifidobactéries ou ceux contenant *B. longum*. Les laits fermentés produits en absence de bifidobactéries ont une teneur plus faible en acétaldéhyde que ceux obtenus en présence de bifidobactéries tandis que la concentration en diacétyle est moins importante en présence de bifidobactéries. Enfin, la concentration en éthanol augmente lorsque les bifidobactéries sont utilisées en même temps que des cultures mixtes de lactocoques acidifiants, de lactocoques aromatiques et de leuconostocs. Une température de fabrication de 35 °C semble désirable parce qu'elle permet la croissance des bifidobactéries sans affecter dramatiquement la croissance et l'activité des bactéries lactiques.

**bifidobactérie / levain lactique / leuconostoc / lait fermenté / composé volatil**

## 1. INTRODUCTION

During the last two or three decades, attempts have been made to improve the state of health by modulating the intestinal microflora with live microbial adjuncts called probiotics [12]. Indeed, different products containing probiotic bacteria have gained in popularity with consumers. Strains of *Lactobacillus acidophilus* and *Lactobacillus casei* complex are well represented in commercial probiotic products, followed by *Bifidobacterium* spp. (*B. animalis, B. bifidum, B. breve, B. infantis* and *B. longum*), some other lactic acid bacteria (lactococci, leuconostocs, enterococci) and non-lactic acid bacteria (propionibacteria, yeasts) [12, 15]. The consumption of these micro-organisms may affect the composition of indigenous microflora and may have several beneficial effects on human health such as the maintenance of a balanced flora, alleviation of lactose intolerance symptoms, resistance to enteric pathogens, immune system modulation, an antihypertensive effect as well as certain anti-carcinogenic effects [5, 15].

Selection of probiotics can be based on general microbiological criteria that refer to safety, technology, performance and health benefits [15]. The technological aspects for selection of strains should include the following criteria: strains claimed to be present in a product should survive in relatively high viable cell numbers, retain metabolic activity and provide desirable organoleptic qualities [12]. For instance, *Bifidobacterium bifidum*, *B. breve*, *B. infantis* and *B. longum* which are species isolated from humans can be used to produce fermented milks [12]. It has been established that *B. breve* and *B. longum* could survive up to 15 d at a level higher than $10^6$ cfu·g$^{-1}$ in fermented milk made with mixed cultures of *Lactococcus lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris* [22]. Similarly, *Bifidobacterium infantis* and *Bifidobacterium lactis* cell counts remained higher than $10^6$ cfu·g$^{-1}$ after 3 months ripening in Cheddar cheese [4] and goat cheese [11], respectively.

During production of fermented products, bifidobacteria may be simultaneously added to the lactic starters. It is well-known

that changes in the composition of the starter could disturb the ecological system and lead to changes in the characteristics of the product [20]. For instance, fermented milks containing bifidobacteria contained higher amounts of acetaldehyde, diacetyl and ethanol [18, 26]. From a general point of view, the impact of the addition of bifidobacteria on the kinetics of fermentation of lactic starters and their contribution to the organoleptic qualities of the resulting fermented product is little known in the literature.

The objective of the present experiment was to evaluate the impact of the presence of four species of bifidobacteria inoculated simultaneously with lactic starters on biochemical characteristics of fermented milk. The impact of bifidobacteria under different fermentation temperatures (30 °C and 35 °C to stimulate respectively lactic starters and bifidobacteria), milk protein concentrations (to increase cell growth due to protein buffer capacity) and ratio of acidifying/aromatic starters was also taken in account.

## 2. MATERIALS AND METHODS

### 2.1. Bacterial cultures

Lyophilized cultures of *Bifidobacterium bifidum* R071, *B. breve* R070, *B. infantis* R033 and *B. longum* R175 obtained from Institut Rosell Inc. (Montreal, QC, Canada) were used to produce fermented milk. The starter cultures used for fermented milk production were composed of acidifying (*Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris*) and aromatic (*Lactococcus lactis* ssp. *lactis* biovar *diacetylactis* and *Leuconostoc mesenteroides* ssp. *cremoris*) lactic acid bacteria (LAB). Commercial lyophilized preparations of two cheese starters (*Lactococcus lactis* ssp. *lactis* Strain I and *Lc. lactis* ssp. *cremoris* Strain II) were obtained from Institut Rosell Inc., and frozen liquid preparations of aromatic bacteria were obtained from Sanofi (Sanofi Bio-industries Inc, Waukesha, WI). An equal population of *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* was added to reach the population of acidifying bacteria needed for each ratio ($4 \times 10^7$ cfu·mL$^{-1}$ acidifying LAB for ratio 100:0%, $3 \times 10^7$ cfu·mL$^{-1}$ acidifying LAB and $1 \times 10^7$ cfu·mL$^{-1}$ aromatic LAB for ratio 75:25% and $2 \times 10^7$ cfu·mL$^{-1}$ acidifying LAB and $2 \times 10^7$ cfu·mL$^{-1}$ aromatic LAB for ratio 50:50%).

### 2.2. Fermented milk production

Skim milk was concentrated 3-fold by ultrafiltration (UF) and frozen at –20 °C. Native phosphocaseinate (PPCN) retentate was obtained by microfiltration of skim milk [9, 19] to a concentration factor of 4× using a pilot cross-flow microfiltration unit (Alfa-Laval MFS-7, Hojbjerg-Aarhus, Denmark) equipped with 0.1 μm pore size and 1.4 m$^2$ surface area ceramic membranes (Membralox membrane; Société des Céramiques Techniques, Tarbes, France) frozen at –20 °C until its use. Milk, UF retentate, PPCN and raw cream were analyzed to determine the percentage of total proteins and fat content of each component using a Dairylab 2 (Multispec Limited, Wheldrake, York, England). The milk was standardized to 15% milk fat and 4.5, 5.5 or 6.5% protein concentration. The standardized milk was heated to 50 °C and homogenized (Rannie homogenizer, Copenhagen, Denmark) under 300 bar. The milk was then pasteurized at 85 °C for 5 min in a culture incubator (Laboratorium Wiesby GmbH & Co., Niebüll, Germany) and rapidly cooled down to 4 °C until it was inoculated. Before inoculation, the milk was heated to 30 or 35 °C. The starter culture inoculum was adjusted to get a final level of approximately $4 \times 10^7$ cfu·g$^{-1}$ for LAB and $1 \times 10^8$ cfu·g$^{-1}$ for bifidobacteria. Five factors were studied: 2 temperatures (30 or 35 °C), 3 ratios of LAB as described previously and 3 rates of

milk protein concentration (4.5%; 5.5% and 6.5%). Five different fermented milks were prepared for each factor studied: a control, inoculated strictly with the starter cultures (according to 1 of the 3 ratio), and 4 experimental fermented milks inoculated with the starter cultures and 1 of the bifidobacteria strains (*B. bifidum*, *B. infantis*, *B. breve* or *B. longum*). The inoculated milks were then dispensed into 125 mL sterilized glass containers taken off for each of the sampling times (0, 2.5, 5, 7 and 9.5 h) and placed in an incubator set at 30 or 35 °C. The fermentation was allowed to take place for 9.5 h.

### 2.3. Analytical methods

Portions (10 g) of fermented milk were adjusted to 100 g by water containing 0.9% NaCl and 0.1% peptone and mixed with a Stomacher 400 laboratory blender (Seward Medical Ltd, London, England). Subsequent dilutions were made in deionized water containing 1 $g \cdot L^{-1}$ peptone and 9 $g \cdot L^{-1}$ NaCl. Enumeration of *B. infantis*, *B. breve* or *B. longum* was done using CAB medium (Columbia Agar Base, Becton Dickinson, Cockeysville, MD) supplemented with 5 $g \cdot L^{-1}$ raffinose (Difco Laboratories, Detroit, MI), 0.5 $g \cdot L^{-1}$ L-cysteine-HCl (Sigma Chemical Co., St-Louis, MO), 2 $g \cdot L^{-1}$ lithium chloride (Anachemia, Montréal, QC, Canada) and 3 $g \cdot L^{-1}$ sodium propionate (American Chemical, Montréal, QC, Canada) to improve the selectivity of the RAF 5.1 agar [22]. Enumeration of *B. bifidum* was also done using CAB medium supplemented with 5 $g \cdot L^{-1}$ lactose (BDH), 0.5 $g \cdot L^{-1}$ L-cysteine-HCl and the filter-sterilized NPNL solution ($L^{-1}$, Nalidixic acid, 15 mg; Neomycin sulphate, 100 mg; Paromomycin, 200 mg; LiCl, 3 g). The medium was adjusted to pH 5.1 and sterilized at 121 °C for 15 min. Viable counts of bifidobacteria were performed in duplicate by pour-plating 1 or 0.1 mL of appropriate dilutions on the selective medium RAF 5.1. Plates were incubated at 37 °C for 48 h under anaerobic conditions. Viable counts of lactococci were enumerated on M17 agar and enumeration of *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis* was carried out on KMK medium [14]. Plates were incubated at 30 °C for 48 h under anaerobic conditions. Enumeration of *Leuconostoc mesenteroides* ssp. *cremoris* was done on MRSV medium [16], which was composed of MRS added with 1.5% agar (sterilized at 121 °C for 15 min) and 20 $mg \cdot mL^{-1}$ vancomycin (Sigma Chemical Co, Saint-Louis), which was filter-sterilized (0.45 μm Filter, Millipore Bioprocess Division, Millipore Ltd, Ontario, Canada). Viable counts were done by spread plate, and the plates were incubated for 48 h at 30 °C under aerobic conditions.

The pH and titratable acidity of fermented milks were measured using a pH meter system (model PHM84 and titrator TTT80, Radiometer, Copenhagen, Denmark) by addition of 0.11 $mol \cdot L^{-1}$ NaOH. Titratable acidity was expressed as a percentage of lactic acid. Sugars and organic acids in fermented milks were measured by HPLC (Bio-Rad Laboratories, Richmond, CA) using a refractive index detector (Waters chromatography division, Mildford, MA) maintained at 31 °C, a UV detector (Waters) set at 210 nm and an Ion-300 column (Mandel Scientific Co., Rockwood, ON, Canada) operated at 25 °C and at a flow rate of 0.4 $mL \cdot min^{-1}$. The mobile phase was 0.02 $mol \cdot L^{-1}$ $H_2SO_4$. The HPLC system was calibrated between 0.75 and 15 $g \cdot L^{-1}$ for lactose, glucose, galactose, acetic acid and lactic acid. A sample (1 g) of fermented milk was blended with 3 mL of 0.08 $mol \cdot L^{-1}$ $H_2SO_4$ and vortexed. The slurry was centrifuged at 805 *g* for 30 min at 4 °C. The supernatant was then filtered through a 0.45 μm membrane filter (Millipore) into Eppendorf vials and stored at 4 °C in the Auto Injector (Bio-Rad).

Volatile compounds were determined in samples after adding 1 mL of 1 $mol \cdot L^{-1}$ sulfuric acid to 2 g of fermented milk into 8-mL

headspace vials stored at –40 °C. After thawing, 750 mg of $(NH_4)_2SO_4$ and 20 ppm of internal standard (1-propanol, Aldrich Chemical Company Inc., Milwaukee, WI) were added to each vial. Headspace analysis was carried out with a 19395A automatic head space sampler connected to a 5890 Series II gas chromatograph equipped with a flame ionization detector (Hewlett-Packard (Canada) Ltd, Kirkland, QC, Canada). Volatiles were separated with a fusedsilica capillary column (DB-Wax; 30 m × 0.32 mm i.d. × 0.5 μm film thickness; Chromatographic Specialties Inc., Brockville, ON, Canada). The column was conditioned with He at 200 °C for 18 h prior to use. Chromatographic conditions were the following: injector and detector temperatures at 250 °C; carrier gas: He at 2.0 $mL \cdot min^{-1}$; $N_2$ at 30 $mL \cdot min^{-1}$ and a column split of 1:10. The oven's temperature was programmed to rise gradually as follows: 35 °C for 12 min, and then rising to 150 °C at the rate of 10 $°C \cdot min^{-1}$, 150 °C for 5 min, rising to 200 °C at the rate of 25 $°C \cdot min^{-1}$ and remaining at the final temperature for 10 min. Calibration was carried out for acetaldehyde, acetone, ethanol and diacetyl, and estimated using an integrator (Hewlett-Packard).

### 2.4. Experimental design and statistical analysis

A split-split-plot design was used in our experiment. Five factors were studied: 2 temperatures, 3 ratios of lactic starters (acidifying vs. citrate-fermenting), 3 contents of milk proteins, 4 strains of bifidobacteria and 5 sampling times. The whole plots consisted of a factorial combination of 3 factors: temperature, ratio of lactic starters and content of milk proteins, which were fixed for a given day of the experiment. Control on a given day was thus performed with mixed-cultures of lactic starters (100% acidifying lactococci and 0% of citrate-fermenting lactococci and leuconostocs; 75:25% or 50:50%) without the presence of bifidobacteria at one temperature (30 or 35 °C) and one content of milk protein (4.5, 5.5 or 6.5%). The subplots were randomly assigned levels of the strains of bifidobacteria (*B. bifidum*, *B. breve*, *B. infantis* and *B. longum*) for a given day and the sub-subplots were sampled during production of fermented milk (0, 2.5, 5, 7, 9.5 h). Statistical analysis was carried out using the method of analysis of variance (ANOVA), and only significant interactions between the various factors already described are reported in this study. The experimental design was divided into 2 blocks (2 repetitions). For each day of production of fermented milk, one ratio of lactic starters, one temperature of fermentation and one content of milk proteins were studied. Multiple comparisons of LSD (least significant difference) were done by using the program Genstat (Genstat 5 Release 3.1 (VAX/VMS5), 1992, Lawes Agricultural Trust, Rothamsted Experimental Station, the U.K.). The statistical significance was $P = 0.05$.

## 3. RESULTS AND DISCUSSION

### 3.1. Growth of bifidobacteria and lactic starters

The growth of bifidobacteria during production of fermented milk was mainly affected by temperature (Fig.1). All the experimental fermented milks were inoculated simultaneously with lactic starters and bifidobacteria, except for control fermented milks (without bifidobacteria). In Figure 1, no control was shown because bifidobacteria could not be enumerated in fermented milks without bifidobacteria. Hence, each value corresponds to the mean of viable counts of each species of bifidobacteria obtained from those estimated at various ratios of lactic starters and content of milk proteins (Figs. 1A and 1B).When fermented milk production was carried out at 30 °C, no or slight increase of viable cells was

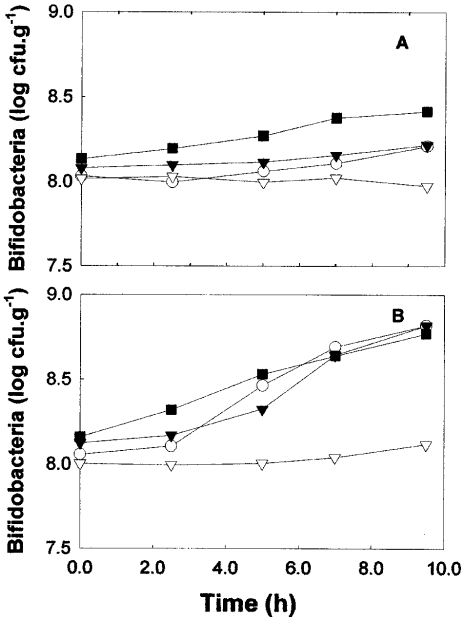


**Figure 1.** Growth of bifidobacteria during manufacture of fermented milks. (**A**) Fermented milk produced at 30 °C; (**B**) fermented milk produced at 35 °C; (○) fermented milk containing *Bifidobacterium breve*; (▼) fermented milk containing *Bifidobacterium longum*; (■) fermented milk containing *Bifidobacterium bifidum*; (▽) fermented milk containing *Bifidobacterium infantis*.

**Figure 1.** Croissance des bifidobactéries au cours de la fabrication de laits fermentés. (**A**) Lait fermenté fabriqué à 30 °C ; (**B**) lait fermenté fabriqué à 35 °C ; (○) lait fermenté contenant *Bifidobacterium breve* ; (▼) lait fermenté contenant *Bifidobacterium longum* ; (■) lait fermenté contenant *Bifidobacterium bifidum* ; (▽) lait fermenté contenant *Bifidobacterium infantis*.

observed (less than 0.5 $\log_{10}$ cfu·g$^{-1}$) for all species of bifidobacteria (Fig. 1A). On the other hand, during fermented milk production carried out at 35 °C, the number of viable cells increased by approximately one $\log_{10}$ for the strains *B. breve*, *B. longum* and *B. bifidum* whereas there was no growth observed for the *B. infantis* strain (Fig. 1B). No significant effect of milk protein content (by addition of PPCN) was observed for any strain of bifidobacteria or lactic starters (results not shown). It was also observed that the survival or growth of bifidobacteria during production of fermented milk was not affected by the presence of *Lactococcus lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris* in combination or not with *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis* and *Leuconostoc mesenteroides* ssp. *cremoris*.

Hence, the production temperature of fermented milk was the main factor affecting the growth of bifidobacteria. Since the optimum temperature of growth of bifidobacteria was 37 °C, a temperature of fermented milk production of 35 °C could stimulate the growth of these bacteria. The population of *B. bifidum*, *B. longum* and *B. breve* increased by one $\log_{10}$ cfu·g$^{-1}$ in agreement with previous results [7, 20]. The inhibition of the growth of *B. infantis* during fermentation carried out at 35 °C could be explained by the decrease of pH below 6 after 2.5 h of fermentation. Indeed, at a pH of 5.8, 8% of the acetate is present in a undissociated form, which has an antagonistic effect on the growth of bifidobacteria [17]. It is possible that the strain of *B. infantis* was more sensitive to the effect of pH than the other strains used in this study, which resulted in uncoupling between growth and acid production [8]. Similar results were observed during the growth of *B. infantis* ATCC 27920G (inoculated with a rate of $1 \times 10^8$ cfu·mL$^{-1}$) in a cream enriched with native phosphocaseinate [3].

Figure 2 shows that the presence of bifidobacteria during the production of fermented milk did not influence the growth of total lactococci (Fig. 2A) and citrate-fermenting lactococci (*Lc. lactis* ssp. *lactis* biovar d*iacetylactis*) (Fig. 2B) because there was no difference between the mean of viable counts estimated without the presence of bifidobacteria (control), for the various factors (ratio of lactic starters, content of milk proteins, temperature of

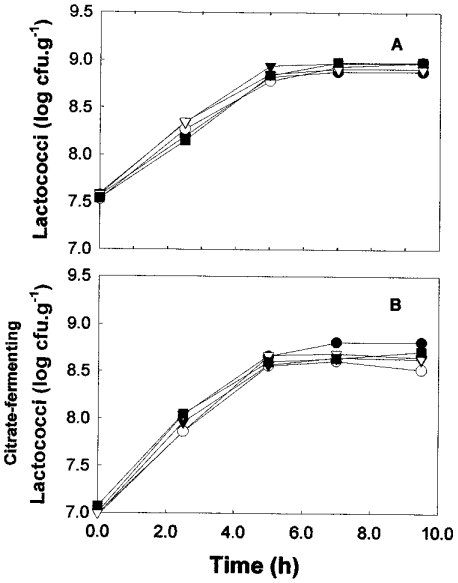


**Figure 2.** Growth of lactococci during manufacture of fermented milks. (**A**) Viable counts of total lactococci; (**B**) viable counts of citrate-fermenting lactococci; (●) control; (○) fermented milk containing *Bifidobacterium breve*; (▼) fermented milk containing *Bifidobacterium longum*; (■) fermented milk containing *Bifidobacterium bifidum*; (▽) fermented milk containing *Bifidobacterium infantis*.

**Figure 2.** Croissance des lactocoques au cours de la fabrication de laits fermentés. (**A**) Comptes viables des lactocoques totaux ; (**B**) comptes viables des lactocoques aromatiques ; (●) témoin ; (○) lait fermenté contenant *Bifidobacterium breve* ; (▼) lait fermenté contenant *Bifidobacterium longum* ; (■) lait fermenté contenant *Bifidobacterium bifidum* ; (▽) lait fermenté contenant *Bifidobacterium infantis*.

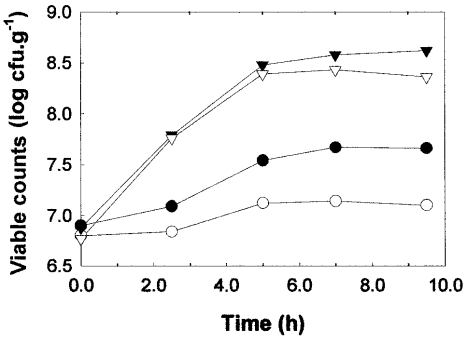


**Figure 3.** Growth of citrate-fermenting lactococci and leuconostocs during manufacture of fermented milks. (○) Leuconostocs in fermented milks made at 30 °C; (●) leuconostocs in fermented milks made at 35 °C; (▼) citrate-fermenting lactococci in fermented milks made at 30 °C; (▽) citrate-fermenting lactococci in fermented milks made at 35 °C.

**Figure 3.** Croissance des lactocoques aromatiques et des leuconostocs au cours de la fabrication de laits fermentés. (○) Leuconostocs dans les laits fermentés faits à 30 °C ; (●) leuconostocs dans les laits fermentés faits à 35 °C ; (▼) lactocoques aromatiques dans les laits fermentés faits à 30 °C ; (▽) lactocoques aromatiques dans les laits fermentés faits à 35 °C.

fermentation) studied in this experiment, and those in the presence of bifidobacteria. Finally, viable counts on M17 medium for estimated total lactococci (Fig. 2A) and those on KMK medium used for enumeration of citrate-fermenting lactococci showed that *Lc. lactis* ssp. *lactis* biovar *diacetylactis* (Fig. 2B) represented almost the totality of lactococci from the fifth hour of fermentation until the end of fermented milk production. These results indicate that this strain has a metabolic advantage as compared to the other lactococci tested.

The growth of leuconostocs was not influenced by the presence of bifidobacteria during fermented milk production because no significant interaction ($P = 0.958$) was found between the ratio of lactic starters and strains of bifidobacteria. Figure 3 shows that the growth of leuconostocs was influenced by the temperature of fermentation ($P < 0.001$). According to the mean values calculated from viable counts at various ratios of lactic starters, milk protein content in the presence or not of strains of bifidobacteria, the number of leuconostocs was lower for fermented milks made at 35 °C than those made at 30 °C whereas the temperature of fermentation did not affect the growth of citrate-fermenting lactococci (Fig. 3).

The increase in the population of leuconostocs during fermented milk production carried out at 30 °C could be possible because the optimum temperature of growth for this species is between 22–30 °C. Growth of leuconostocs was also observed during fermented milk production carried out at 35 °C but it was characterized by a lower growth rate as compared to fermented milk production carried out at 30 °C. Moreover, at temperatures higher than 30 °C the growth of leuconostocs could be affected by the presence of lactococci that grew more rapidly than the leuconostocs, as observed by several authors who noted a change in the ratio of leuconostocs vs. lactococci occurring at temperatures higher than 30 °C [2, 25].

### 3.2. Biochemical characteristics of fermented milk

During the production of fermented milks, significant differences were observed for the decrease in pH and development of titratable acidity between fermented milks containing bifidobacteria and those produced without bifidobacteria (Fig. 4). Figure 4A shows that the decrease in pH of fermented milks containing bifidobacteria (expressed as the mean values of pH determined in fermented milks made at different ratios of lactic starters, milk protein contents and temperatures of fermentation) was faster than the decrease in pH of the fermented milks produced without the presence of bifidobacteria ($P < 0.001$, LSD = 0.04). After 9.5 h of fermentation, the pH of all fermented milks ranged between 4.65 and 4.66. This was in agreement with the results obtained by Roy et al. [22], who did not observe any difference of pH during the storage of the control and fresh cheeses containing bifidobacteria. The difference in titratable acidity observed between the control fermented milks and fermented milks containing bifidobacteria (Fig. 4B) was due to the metabolic activity of bifidobacteria which produces acetate in addition to lactate [6]. Indeed, no difference was observed for the lactic acid concentration of various fermented milks whereas the acetic acid concentration of fermented milks containing bifidobacteria was higher than that for the control fermented milks (results not shown).

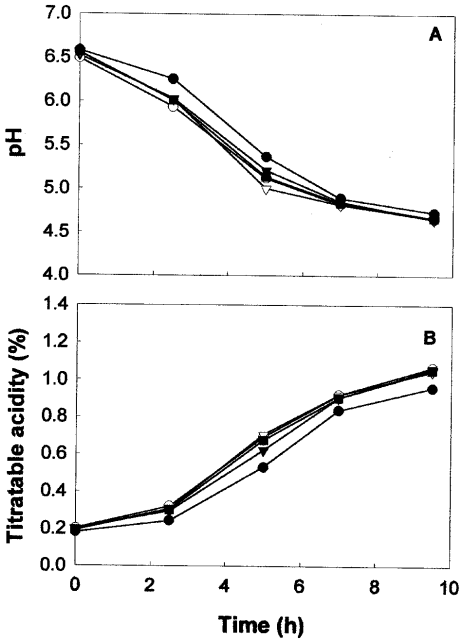


**Figure 4.** Evolution of pH (**A**) and titratable acidity (**B**) during manufacture of fermented milks. (●) Control; (○) fermented milk containing *Bifidobacterium breve*; (▼) fermented milk containing *Bifidobacterium longum*; (■) fermented milk containing *Bifidobacterium bifidum*; (▽) fermented milk containing *Bifidobacterium infantis*.

**Figure 4.** Évolution du pH (**A**) et de l'acidité titrable (**B**) au cours de la fabrication de laits fermentés. (●) Témoin ; (○) lait fermenté contenant *Bifidobacterium breve* ; (▼) lait fermenté contenant *Bifidobacterium longum* ; (■) lait fermenté contenant *Bifidobacterium bifidum* ; (▽) lait fermenté contenant *Bifidobacterium infantis*.

The final characteristics of the fermented milks produced with mixed-cultures of lactic acid bacteria and bifidobacteria were

**Table I**. Effect of bifidobacteria and ratio of lactic starters on residual lactose in fermented milk.

**Tableau I**. Effet des bifidobactéries et du ratio de ferments lactiques sur le lactose résiduel dans le lait fermenté.

| Species | Fermented milk with no bifidobacteria | Residual lactose (%) | | | |
|---|---|---|---|---|---|
| | | *B. breve* | *B. longum* | *B. infantis* | *B. bifidum* |
| Ratio (acidifying lactococci vs. citrate-fermenting lactococci and leuconostoc in %) | | | | | |
| 50:50 | 3.40* | 2.58 | 3.29 | 2.46 | 3.07 |
| 75:25 | 3.68 | 2.72 | 3.55 | 2.51 | 3.20 |
| 100:0 | 3.69 | 3.03 | 3.65 | 2.92 | 3.43 |

* Residual lactose obtained by calculating the mean value of lactose concentration estimated at different contents of milk protein (4.5, 5.5 and 6.5%), at both temperatures of manufacture (30, 35 °C) and at different times of fermentation (0, 2.5, 5, 7, 9.5 h).
LSD = 0.22.
LSD = 0.09 for the comparison of means with same level of ratio.

obtained by estimation of the metabolic products: residual sugars, acetaldehyde, diacetyl and ethanol. Significant differences were observed between ratio of lactic starters and strains of bifidobacteria for the mean values of lactose concentration ($P < 0.001$; Tab. I). Moreover, for fermented milks inoculated with the same species of bifidobacteria, lactose concentration was lower when the ratio of lactic acid bacteria (acidifying vs. citrate-fermenting) used was of 50:50%. The same phenomenon occurred in fermented milks made without the presence of bifidobacteria at various levels of milk protein and temperatures of fermentation. For fermented milks containing *B. breve*, *B. bifidum* and *B. infantis*, there was no significant difference between the 50:50% and 75:25% ratio (acidifying vs. citrate-fermenting) but these two ratios were different from the 100:0% ratio.

Figure 5A shows that the hydrolysis of lactose was different according to the species of bifidobacteria used ($P < 0.001$; LSD = 0.10). Indeed, the mean values of residual lactose (estimated from lactose concentration at various ratios of acidifying lactococci versus citrate-fermenting lactococci and leuconostocs, milk protein content of 4.5, 5.5 and 6.5%, and at both temperatures of fermentation) in fermented milks containing *B. breve*, *B. bifidum* and *B. infantis* was lower than that found in fermented milks without bifidobacteria (control) and those containing *B. longum*. This phenomenon was observed until the end of production of fermented milks. Moreover, after 5 h of fermentation, the hydrolysis of lactose was more important for fermented milks containing *B. infantis*.

Glucose was not detected in control fermented milks and the accumulation of glucose in fermented milks containing bifidobacteria was significantly affected ($P < 0.001$; LSD = 0.04) by strains and temperature. Figure 5B illustrates that in fermented milks containing *B. breve* and those containing *B. infantis*, the concentration of glucose was maximum after 2.5 h, and thereafter it decreased sharply. On the other hand, the concentration of glucose remained about the same throughout fermented milk production and was lower than 0.1% in fermented milks containing *B. longum* and *B. bifidum*. For fermented milks containing *B. breve*, *B. infantis* and *B. bifidum*, the galactose concentration increases throughout fermented milk production (Fig. 5C;

$P < 0.001$; LSD = 0.04). For fermented milks containing *B. longum*, a small quantity of galactose (less than 0.15%) was estimated at the end of fermented milk production whereas the concentration of galactose in fermented milk made without the presence of bifidobacteria was negligible and remained constant throughout fermented milk production.

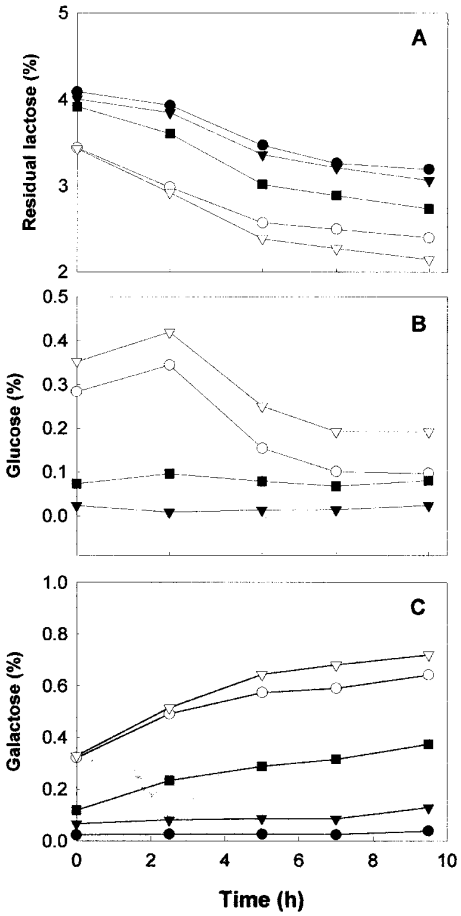


**Figure 5**. Biochemical changes observed during manufacture of fermented milks. (**A**) Residual lactose; (**B**) glucose; (**C**) galactose; (●) control; (○) fermented milk containing *Bifidobacterium breve*; (▼) fermented milk containing *Bifidobacterium longum*; (■) Fermented milk containing *Bifidobacterium bifidum*; (▽) fermented milk containing *Bifidobacterium infantis*.

**Figure 5.** Changements biochimiques observés au cours de la fabrication de laits fermentés. (**A**) Lactose résiduel ; (**B**) glucose ; (**C**) galactose ; (●) témoin ; (○) lait fermenté contenant *Bifidobacterium breve* ; (▼) lait fermenté contenant *Bifidobacterium longum* ; (■) lait fermenté contenant *Bifidobacterium bifidum* ; (▽) lait fermenté contenant *Bifidobacterium infantis*.

The residual lactose of various fermented milks was less important in fermented milks containing bifidobacteria, which indicated that the hydrolysis of lactose was stimulated by the presence of these bacteria. These results are in agreement with those observed by other authors [1, 10, 21]. Moreover, this hydrolysis was different according to the strain of bifidobacteria used, which could exhibit different β-galactosidase activity as observed by several authors [7, 13]. Fermented milks containing *B. infantis* and *B. breve* had lower concentrations of residual lactose, confirmed by the concentration of glucose and galactose which where in greater quantity in fermented milks produced with these species as compared with those containing *B. bifidum* and *B. longum*.

In fermented milks produced with a 100:0% (acidifying lactococci versus citrate-fermenting lactococci and leuconostocs) ratio of lactic starters, there was an accumulation of acetaldehyde (Fig. 6A) as compared to the ratios of 75:25% and 50:50% (Figs. 6B and 6C). The acetaldehyde concentration was maximum after 5 h of fermentation (Fig. 6A). Thereafter, the acetaldehyde concentration decreased slightly. The fermented milk with no bifidobacteria contained less acetaldehyde than those that contained bifidobacteria. No significant difference was observed for the acetaldehyde concentration found in fermented milks containing various species of bifidobacteria. The higher amount of acetaldehyde might be due to the metabolism of bifidobacteria. This result was in agreement with the study of Yuguchi et al. [26] who mentioned that the strains *B. longum*, *B. bifidum* and *B. breve* produced acetaldehyde. For fermented milks produced with the ratio of lactic starters of 75:25% and

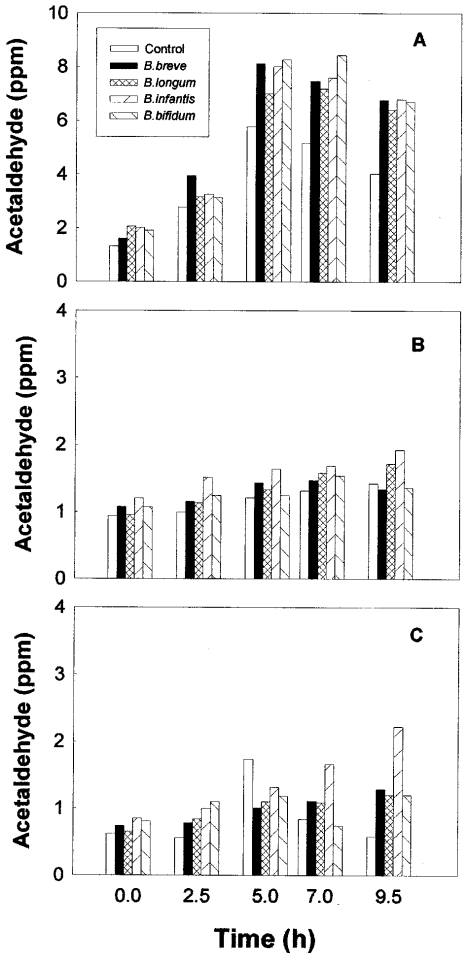


**Figure 6**. Evolution of acetaldehyde during manufacture of fermented milks. (**A**) Fermented milk produced with a ratio of lactic starters of 100:0% (acidifying vs. citrate-fermenting); (**B**) fermented milk produced with a ratio of lactic starters of 75:25% (acidifying vs. citrate-fermenting); (**C**) fermented milk produced with a ratio of lactic starters of 50:50% (acidifying vs. citrate-fermenting).

**Figure 6**. Évolution de l'acétaldehyde au cours de la fabrication de laits fermentés. (**A**) Lait fermenté fabriqué avec un ratio de bactéries lactiques de 100:0 % (acidifiant vs. aromatique) ; (**B**) lait fermenté fabriqué avec un ratio de bactéries lactiques de 75:25 % (acidifiant vs. aromatique) ; (**C**) lait fermenté fabriqué avec un ratio de bactéries lactiques de 75:25 % (acidifiant vs. aromatique).

50:50%, the acetaldehyde concentration remained similar throughout fermentation (Figs. 6B and 6C). The acetaldehyde could be converted into ethanol by the enzyme alcohol dehydrogenase present in the leuconostocs [24]. The production of diacetyl was also affected by the strains of bifidobacteria used ($P < 0.001$). The mean values of diacetyl calculated from diacetyl concentrations determined at different ratios, temperatures of fermentation, lactic starters, contents of milk proteins and sampling times were 3.73, 3.26, 2.50, 2.78 and 2.62 ppm (LSD = 0.53 ppm) for fermented milks made without the presence of bifidobacteria and those containing *B. breve*, *B. longum*, *B. infantis* and *B. bifidum*, respectively.

The ethanol concentration in various fermented milks increased throughout the fermentation (Fig. 7). There was no significant difference in the ethanol concentration for the various fermented milks at 0 and 2.5 h of fermentation. However, the quantity of ethanol found in fermented milks was different at 5 h of fermentation according to the ratio of lactic starters used. The estimated ethanol concentration increased with an increase of the proportion of citrate-fermenting lactococci and leuconostocs (Figs. 7B, 7C, 7E and 7F). Thus, the ethanol concentration in fermented milks produced with a ratio of lactic starters (acidifying lactococci vs. citrate-fermenting lactococci and leuconostocs) of 50:50% was higher than the ethanol concentration for fermented milks containing a ratio of 100:0%. Moreover, the temperature of fermented milk production influenced the quantity of ethanol for the ratio of lactic starters (acidifying vs. citrate-fermenting) of 75:25% and 50:50%. The ethanol concentrations found in fermented milks made at 30 °C (Figs. 7B and 7C) was higher than those found in fermented milks produced at 35 °C (Figs. 7E and 7F). In fermented milks produced with the ratios of lactic starters (acidifying vs. citrate-fermenting) of 75:25% and 50:50%, the presence of bifidobacteria resulted in an

increase of the ethanol concentration. The phenomenon was accentuated for a temperature of fermented milk production of 30 °C (Figs. 7B and 7C) as compared to 35 °C (Figs. 7E and 7F). The fermented milks produced with *B. longum* and *B. infantis* contained more ethanol than the other fermented milks.

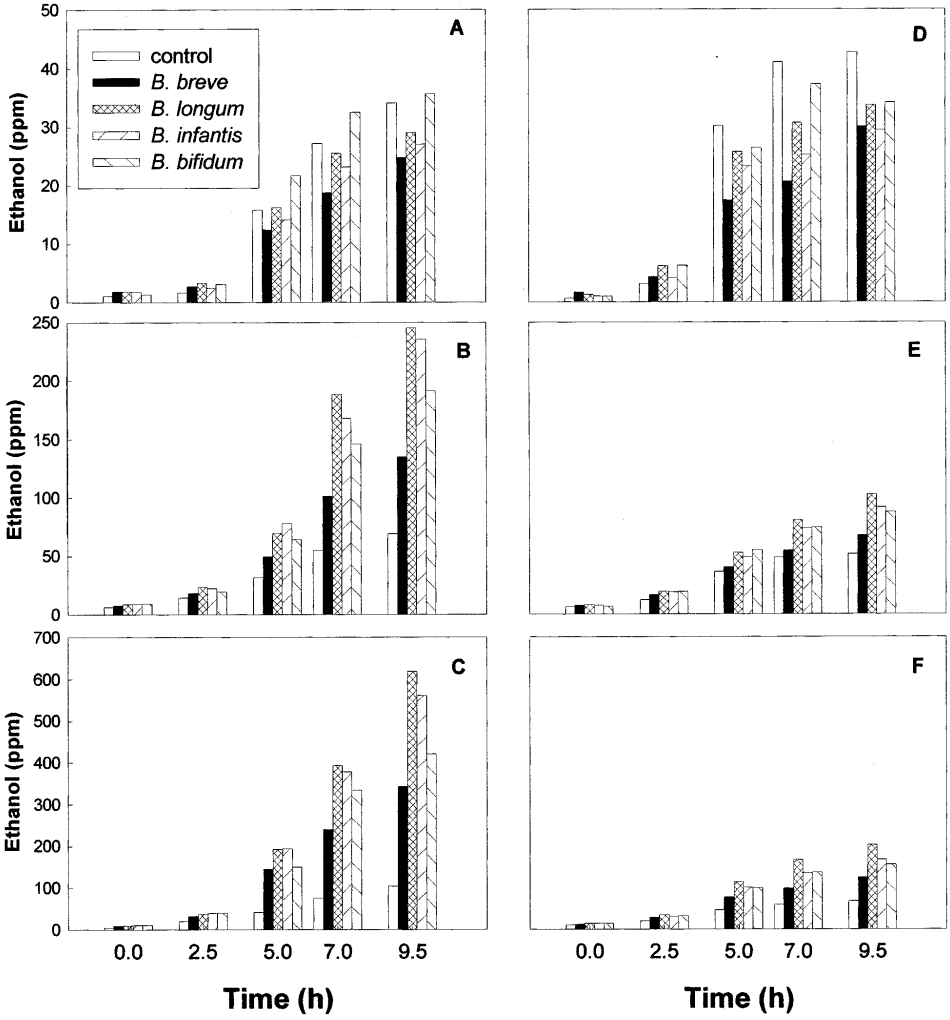


**Figure 7**. Effect of different factors of manufacture on the evolution of ethanol during manufacture of fermented milks. (**A** and **D**) Fermented milk produced with a ratio of lactic starters of 50:50% (acidifying vs. citrate-fermenting); (**B** and **E**) fermented milk produced with a ratio of lactic starters of 75:25% (acidifying vs. citrate-fermenting); (**C** and **F**) fermented milk produced with a ratio of lactic starters of 100:0% (acidifying vs. citrate-fermenting).

**Figure 7.** Effet de différents facteurs de fabrication sur l'évolution de l'éthanol au cours de la fabrication de laits fermentés. (**A** et **D**) Lait fermenté fabriqué avec un ratio de bactéries lactiques de 50:50 % (acidifiant vs. aromatique) ; (**B** et **E**) lait fermenté fabriqué avec un ratio de bactéries lactiques de 75:25 % (acidifiant vs. aromatique) ; (**C** et **F**) lait fermenté fabriqué avec un ratio de bactéries lactiques de 100:0 % (acidifiant vs. aromatique).

In the present study, it seems that the acetaldehyde was transformed into ethanol because the ethanol concentration was more important in fermented milks made with the ratio of lactic starters (acidifying vs. citrate-fermenting) of 75:25% and 50:50%. Moreover, the ethanol concentration was higher for a ratio of lactic starters (acidifying vs. citrate-fermenting) of 50:50% (Figs. 7C and 7F) as compared to a ratio of 75:25% (Figs. 7B and 7E). This higher concentration might be explained by the fact that the population of leuconostocs was more important in fermented milks containing a ratio of 50:50%, which allowed a larger acetaldehyde conversion. The same phenomenon also explains the higher ethanol concentration found in fermented milks made at 30 °C as compared with those produced at 35 °C (the population of leuconostocs was higher in fermented milks produced at 30 °C). The production of acetaldehyde by bifidobacteria and the transformation of this product into ethanol by the leuconostocs and their ability to transform lactose into pyruvate or ethanol [23] can also explain the greater ethanol concentration in fermented milks containing bifidobacteria as compared with the control fermented milks.

## 4. CONCLUSION

The survival or growth of bifidobacteria during production of fermented milk was not affected by the presence of *Lactococcus lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris* in combination or not with *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis* and *Leuconostoc mesenteroides* ssp. *cremoris*. The temperature of production of fermented milk was the main factor that affected the growth of bifidobacteria. On the other hand, the presence of bifidobacteria during the production of fermented milk did not influence the growth of total lactococci, citrate-fermenting lactococci and leuconostocs. The variation of milk protein content did not modify the growth of bifidobacteria and mixed-cultures of lactic starters. However, the presence of bifidobacteria changes the final characteristic of the fermented milk. Residual lactose in fermented milks containing *B. breve*, *B. bifidum* and *B. infantis* was lower than that found in fermented milks without bifidobacteria and those containing *B. longum*. The fermented milk without bifidobacteria contained less acetaldehyde than those that contained bifidobacteria. The concentration of diacetyl was less important in the presence of bifidobacteria, and the ethanol concentration increased when bifidobacteria were used in mixed cultures of lactococci, citrate-fermenting lactococci and leuconostocs.

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