Production of Crescenza Cheese by Incorporation of Bifidobacteria

M. GOBBETTI, A. CORSETTI, E. SMACCHI, A. ZOCCHETTI, and M. DE ANGELIS
Institute of Dairy Microbiology, Agriculture Faculty of Perugia, University of Perugia, Italy

ABSTRACT

Bifidobacterium bifidum, *Bifidobacterium infantis*, and *Bifidobacterium longum* were incorporated into Crescenza cheese, individually or as multispecies mixtures and as free cells or as cells immobilized in calcium alginate gel. The viability of the bifidobacteria differed in 14-d-old cheeses: when added individually, the cell counts of *B. bifidum*, *B. longum*, and *B. infantis* were $\log_{10}$ 8.05, 7.12, and 5.23 cfu/g, respectively. The multispecies mixtures only reached about $\log_{10}$ 5.0 cfu/g of cheese. The presence of bifidobacteria did not influence the aerobic microflora, the growth of *Streptococcus thermophilus* used as starter, or the gross composition of the cheese. No differences were found in the primary proteolysis with respect to the conventional production of Crescenza cheese, but higher levels of pH 4.6-soluble N and more pronounced activities of aminopeptidase, iminopeptidase, dipeptidase, and tripeptidase were detected in all of the cheeses with added bifidobacteria. Crescenza cheeses with added bifidobacteria contained only traces of lactose and had slightly higher concentrations of lactic and acetic acids than did the conventional cheese. The presence of bifidobacteria was also related to high α- and β-galactosidase activities in the cheese. Sensory evaluation of the cheeses with incorporated bifidobacteria was very similar to that of Crescenza cheese produced by the conventional method.

(**Key words**: bifidobacteria, Crescenza cheese, enzymes, ripening)

Abbreviation key: **IMC** = immobilized mixed culture, **MC** = mixed culture, ***p*-NA** = *p*-nitroanilide.

INTRODUCTION

Several health and nutritional benefits for humans have been ascribed to bifidobacteria: anticarcinogenic effects, increased immunocompetence, and antimicrobial activity (35). Moreover, dairy products containing bifidobacteria may be tolerated by individuals who have difficulty digesting lactose (5).

Despite the increased interest in probiotic dairy products, introducing bifidobacteria into the dairy food chain has proved to be difficult. The survival of bifidobacteria is conditioned by the metabolic interactions with lactic acid bacteria starters, fermentation conditions, and by the storage and preservation temperatures of the dairy products (31). The tolerance of lactic acid bacteria starters, the viability of bifidobacteria in fermented products of low pH over long storage periods at refrigeration temperatures (36, 38), and the sensitivity to oxygen (38) have been shown to be strain specific and have been considered as criteria for the selective use of bifidobacteria in probiotic products. The susceptibility of bifidobacteria to various antibiotics has been studied in the search for selective agents for enumerating viable cells in products containing mixed microflora and also in order to understand the alteration of normal intestinal microflora and the potential survival of bifidobacteria (30). Generally, to be effective, probiotic dairy products should probably contain $\geq\log_{10}$ 6.0 cfu/ml and should be consumed regularly (29). Milk products, such as cultured milk, cultured buttermilk, yogurt, and ice cream, have been shown to be promising for the delivery of viable bifidobacteria (20).

The use of bifidobacteria as additives to cheeses has recently been introduced. Freeze-dried and immobilized *Bifidobacterium bifidum* cells have been incorporated in Cheddar cheese (10), freeze-dried concentrates of *Bifidobacterium infantis* have been used to produce a cultured cottage cheese (4, 5), and *Bifidobacterium* spp. together with *Lactobacillus acidophilus* have been introduced in Gouda cheese production (18).

Crescenza is a soft, rindless, Italian cheese with a short ripening time that has been produced in Lombardy for many decades. Technology and starter selection of Crescenza cheese have been standardized and reviewed in depth (14, 32); cheese manufacturing takes place in various sizes of industrial plants. Crescenza is one of the favorite Italian cheeses, and

Received March 4, 1997.
Accepted August 1, 1997.

its appearance and high content of viable lactic acid bacteria fit well with the concept of probiotic dairy products.

In this paper, we studied the possibility of incorporating bifidobacteria in Crescenza cheese, the viability and enzymatic activity of those bacteria during ripening, and the effect on cheese biochemistry and flavor.

MATERIALS AND METHODS

Bifidobacteria Cultures and Immobilization

Lyophilized cultures of *B. bifidum*, *B. infantis*, and *Bifidobacterium longum* were kindly supplied by Chr. Hansen's (Horsholm, Denmark).

For immobilization, cultures of each strain were rehydrated in modified MRS (Difco Laboratories, Detroit, MI) containing 0.05% L-cysteine·HCl (Sigma Chemical Co., St. Louis, MO) and incubated for 24 h at 37°C. Cells were then cultured in the same conditions and harvested by centrifugation at $5000 \times g$ for 10 min. Equal amounts of the harvested cells from the three species were pooled and resuspended in a part of the residual culture broth; the cell slurry was immobilized in calcium alginate gels as reported by Champagne et al. (8). After freeze-drying, the material was ground and sifted to about 60 mesh size. The immobilized mixed culture (**IMC**) contained ca. $\log_{10}$ 7.0 cfu/g of beads showing a reduction of ca. 1.0 $\log_{10}$ cycle with respect to the fresh immobilized cultures. During cheese making, the IMC were added to the milk at the concentration of 10 g/L of milk.

Bifidobacteria species were inoculated individually or in a mixture (equal amounts of each species as were used to produce the mixed culture; **MC**), as direct-to-vat cultures, to attain concentrations of ca. $\log_{10}$ 6.0 cfu/ml of cheese milk.

Cheese Making

Six batches of Crescenza cheese were produced in triplicate, corresponding to the following conditions: addition of *B. bifidum*, *B. infantis*, *B. longum*, MC, and IMC or conventional production without bifidobacteria (control).

Whole bovine milk was pasteurized at 74°C for 30 s and cooled to 35°C. Direct-to-vat, freeze-dried *Streptococcus thermophilus* culture was added as starter at a concentration of $\log_{10}$ 6.0 cfu/ml of milk together with bifidobacteria at the concentrations just reported. Liquid calf rennet (25 ml/100 kg; Caglificio Clerici, Milan, Italy) was immediately added. The curd formed in approximately 25 min and was cut to a size of ca. 1.5 to 2.0 cm. After a 60-min holding period, the curd was then cut to a final size of ca. 0.5 to 1.0 cm and warmed at 35°C for ca. 150 min. The cheese was salted by immersion in 16 to 18% NaCl brine for 1 h at 15°C. The cheese was ripened for 10 d at 3 to 5°C and subsequently stored at 6°C for 4 d, which corresponded to commercial storage of Crescenza cheese.

The mean weight of the cheeses was about 2.5 kg, diameter was 20 cm, and height was 15 cm. The cheeses were sent to the laboratory under refrigeration (ca. 4°C) and were either analyzed immediately or frozen, depending on the assays. All of the analyses were conducted after 1, 6, and 14 d of ripening and storage.

Microbiological Analysis

For microbiological analysis, cheese samples (20 g) were diluted separately in 180 ml of 2% sodium citrate solution and homogenized (5 min) in a Stomacher Lab-Blender 400 (PBI International, Milan, Italy). Serial dilutions were made in quarter-strength Ringer's solution and plated on specific media for viable counts.

Total mesophilic bacteria (plate count agar), staphylococci plus micrococci (Baird Parker agar), coliforms (violet red bile agar), bifidobacteria under anaerobiosis NPNL (neomycin sulfate-paramomycin sulfate-nalidixic acid-lithium chloride) agar medium (40), thermophilic streptococci (M17 agar), lactobacilli under anaerobiosis (MRS agar), and yeasts and molds (Wort agar) were enumerated during ripening. Except for the NPNL medium, all agar media were from Difco Laboratories.

Analytical Methods

Moisture contents, NaCl contents, and pH were determined as described by the International Dairy Federation (22, 23, 24). Total N and pH 4.6-soluble N were determined by the micro-Kjeldahl method (16). Amounts of D- and L-lactic acids, acetic acid, lactose, and galactose were determined by enzymatic methods (Boehringer-Mannheim, Milan, Italy).

Assessment of Enzymatic Activity

Water-soluble extracts of the cheese, prepared according to methods of Kuchroo and Fox (28), were assayed for enzyme activity. The extracts were filtered prior to analysis (0.22-μm pore size, Syrfil

filter; Nucleopore, Costar Corporation, Cambridge, MA) to avoid interference from cellular activity.

Aminopeptidase (EC 3.4.11.11) and proline iminopeptidase (EC 3.4.11.9) activities were determined as described by Gobbetti et al. (17) using Leu-*p*-nitroanilide (***p*-NA**), Lys-*p*-NA, Ala-*p*-NA, Arg-*p*-NA, and Pro-*p*-NA as substrates, respectively. A unit of enzymatic activity was defined as the amount of enzyme that produced an increase in absorbance at 410 nm of 0.01/min at 37°C and pH 7.0. The pH and temperature used in this and subsequent assays were selected as optimal for the enzyme activities in ripened Crescenza cheese.

Carboxypeptidase (EC 3.4.16.1) activity was measured by the ninhydrin method, using N-carbobenzoxy-Leu as substrate (17). Dipeptidase (EC 3.4.13.11) and tripeptidase (EC 3.4.11.4) activities were determined by the cadmium-ninhydrin method of Doi et al. (11), using Leu-Leu and Leu-Leu-Leu, respectively, as substrate. A unit of activity was defined as the amount of enzyme that produced an increase in absorbance of 0.1/min at 570 (carboxypeptidase activity) or 505 nm.

Endopeptidase (EC 3.4.21.24) activity was measured on bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) as described by Gobbetti et al. (17). The reaction was stopped by diluting the mixture 1:1 (vol/vol) with 2 g/L trifluoroacetic acid. Activity was determined by reversed-phase HPLC (17), and a unit of enzymatic activity corresponded to 1% reduction per 10 min in the area of the bradykinin peak.

Proteinase activity was measured by fluorescent casein as substrate (17) after incubation at 30°C for 12 h. A unit of proteinase activity was expressed as an increase of 0.1 unit of fluorescence per 10 min.

Esterase (acetyl ester hydrolase, EC 3.1.1.6) and lipase (acylglycerol acylhydrolase, EC 3.1.1.3) activities were determined as described by Gobbetti et al. (17), using β-naphthyl butyrate (C_4), caproate (C_6), caprylate (C_8), myristate (C_{14}), and tributyrin as substrates. Esterase activity was expressed as the number of moles per 20 min of β-naphthol released, and lipase activity was calculated as the milliequivalents per 60 min of free fatty acids liberated from tributyrin.

The α- and β-galactosidase activities were measured by determining the rate of hydrolysis of *o*-nitrophenol-α-galactopyranoside and *o*-nitrophenol-β-galactopyranoside (Sigma Chemical Co.), respectively (21). In this case, the water-soluble extracts were not filtered prior to use. The release of *o*-nitrophenol was determined at 420 nm, and the quantity was measured by using a standard curve. One unit of enzyme activity was defined as the amount of enzyme that released 1 μM of *o*-nitrophenol/min.

Specific activity for all enzymes was determined as units of total activity per gram of total cheese.

Proteolysis and Lipolysis

For the different ripened cheeses, the N that was soluble at pH 4.6 and the N that was insoluble at pH 4.6 were analyzed by urea-PAGE according to the method of Andrews (1); gels were stained as described by Blakesley and Boezi (3).

Twenty grams of ripened cheese were diluted with 10 ml of distilled water, homogenized, and acidified to pH 3.0. The total free fatty acids were extracted after 2 h of incubation by a 60:40 (vol/vol) mixture of diethyl ether and light petroleum. Titration to pH 11.0 was made by a 0.02 *N* alcoholic KOH, and free fatty acids were expressed as milligrams of butyric acid liberated from 1 kg of cheese.

Microstructure

The microstructure of the immobilized bifidobacteria and Crescenza cheese were examined by scanning electron microscopy. Clean aluminum stubs were applied with double-sided scotch tape. Powder samples from immobilized bifidobacteria were applied to the sticky surface, and the excess was dusted off with a gentle air stream. Cheese samples were prepared according to Bottazzi et al. (7). Powder and cheese samples were sputter-coated under gold and palladium target to 20 nm thickness (SEM Coating Unit E 5100; Polaron Equipment, Watford, Herts, United Kingdom) and viewed on a scanning electron microscope (SEM XL30; Philips, Eindhoven, The Netherlands) operated at 10 kV. Samples were photographed on a type APX 25 Polaroid (Polaroid Corp., Cambridge, MA).

Sensory Evaluation

Using a 10-point hedonic scale (1 = poor to 10 = excellent), an experienced taste panel of 12 judges evaluated the Crescenza cheeses for flavor and texture after 10 d of ripening plus 4 d of storage.

Statistical Analysis

Data from microbiological, physicochemical, enzymatic, and sensory analyses were subjected to one-way ANOVA (37).

TABLE 1. Numbers of the principal microbial groups found in the samples of 14-d-old Crescenza cheese with and without added bifidobacteria.

Microbial group	Cheese sample[1]					
	BB	BI	BL	MC	IMC	C
	($\log_{10}$ cfu/g)					
Total mesophilic bacteria	7.94[a]	7.85[a]	7.76[a]	7.98[a]	8.03[a]	8.10[a]
Staphylococci plus micrococci	3.27[a]	3.07[a]	3.00[a]	3.30[a]	3.13[a]	3.04[a]
Coliforms	3.39[b]	4.14[a]	4.11[a]	4.25[a]	4.74[a]	4.68[a]
Thermophilic streptococci	8.95[a]	9.17[a]	9.07[a]	9.10[a]	9.27[a]	9.17[a]
Mesophilic lactobacilli	5.54[a]	5.32[a]	5.79[a]	5.55[a]	5.28[a]	5.67[a]
Bifidobacteria	8.05[a]	5.23[c]	7.12[b]	5.05[c]	5.23[c]	ND[2]
Enterococci	5.40[a]	4.77[b]	4.60[b]	4.51[b]	4.74[b]	4.68[b]
Yeasts	1.22[a]	1.55[a]	1.35[a]	1.45[a]	1.23[a]	1.12[a]

[a,b,c]Means within a row without a common superscript differ ($P < 0.05$).

[1]Cheese sample designations refer to the bifidobacteria added: BB, *Bifidobacterium bifidum*; BI, *Bifidobacterium infantis*; BL, *Bifidobacterium longum*; MC, mixed culture; and IMC, immobilized mixed culture. C = Control cheese without added bifidobacteria.

[2]Not detectable.

RESULTS

Microbiological Profile

The microstructure of the surface portion of the powder preparation, examined by scanning electron microscopy, showed the presence of bifidobacteria as fixed cells in the immobilized, freeze-dried preparation (Figure 1). Numbers of viable individual cells coated with calcium alginate were $\log_{10}$ 7.0 cfu/g.

The numbers of cells of the different microbial groups in Crescenza cheese made by the conventional procedure (Table 1) were within the limits reported by other researchers (32, 33). With few exceptions, no differences ($P < 0.05$) were found among the cheeses produced with added bifidobacteria. In all the cheeses, Micrococcaceae decreased by about 1 $\log_{10}$ cycle, and adventitious mesophilic lactobacilli sharply increased (ca. 1.5 $\log_{10}$) throughout cheese ripening (data not shown). *Streptococcus thermophilus* was at ca. $\log_{10}$ 9.0 cfu/g in all the cheeses. In the sample with *B. bifidum* added, coliforms decreased, and enterococci increased, by ca. 1 $\log_{10}$ cycle compared with values found for the other cheeses.

Cell concentrations of bifidobacteria at 14 d were varied. When added individually, *B. bifidum* and *B. longum* were at $\log_{10}$ 8.05 and 7.12 cfu/g, respectively, which were similar to numbers found at 1 d of ripening. *Bifidobacterium infantis* gradually decreased to a final cell number of $\log_{10}$ 5.23 cfu/g (data not shown). No differences were detected between MC and IMC, which had low values of ca. $\log_{10}$ 5.0 cfu/g.

Scanning electron microscopy of the cheese showed a cluster of *B. bifidum* cells contained in the cheese during ripening (Figure 2).

Cheese Composition

Contents of moisture, salt, protein, fat, and free fatty acids did not differ ($P < 0.05$) between the control cheese and cheeses with added bifidobacteria (Table 2). Only the cheese with *B. bifidum* added individually showed a slight difference in the pH from the high concentrations of lactic and acetic acids (Table 3). The data only refer to the cheeses containing the highest values of viable bifidobacteria cells; however, no differences were found in the other samples. All of the cheeses were within the limits reported for the gross composition of Crescenza cheese (6).

Lactic and Acetic Acid Production

All of the cheeses showed increased concentrations of lactic and acetic acids throughout ripening (Table

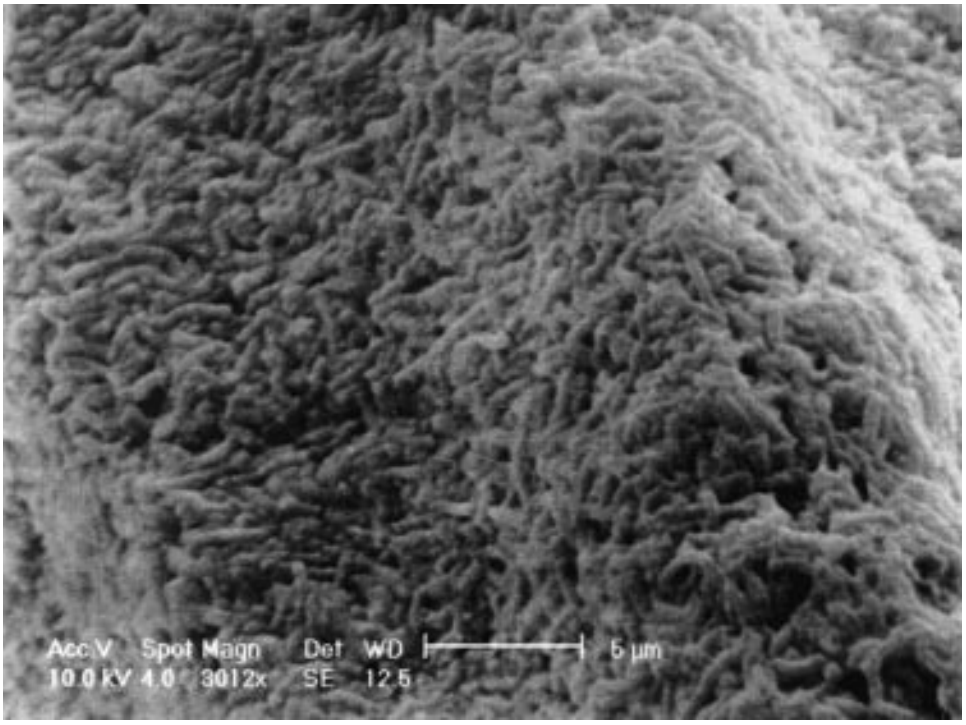
TABLE 2. Gross composition of samples of 14-d-old Crescenza cheese with and without added bifidobacteria.

Component	Cheese sample[1]		
	BB	BL	C
pH	5.23[b]	5.30[a]	5.35[a]
Moisture, %	61.7[a]	62.2[a]	63.0[a]
NaCl, %	0.68[a]	0.66[a]	0.63[a]
Protein, % of DM	35.2[a]	36.8[a]	35.3[a]
Fat, % of DM	57.2[a]	57.4[a]	56.0[a]
Free fatty acids, mg/kg	404[a]	422[a]	436[a]

[a,b]Means within a row without a common superscript differ ($P < 0.05$).

[1]Cheese sample designations refer to the bifidobacteria added: BB, *Bifidobacterium bifidum*, and BL, *Bifidobacterium longum*. C = Control cheese without added bifidobacteria.

3). The highest values were found in the cheeses with added bifidobacteria. On average, those cheeses had ca. 0.6 and 0.2 g/kg more lactic and acetic acids, respectively, than did the control. All of the cheeses contained <0.2 g/kg of D-lactic acid. The highest production of acetic acid (1.31 g/kg) was in the Crescenza cheese with the added *B. bifidum*. The presence of acetic acid in the control may be explained



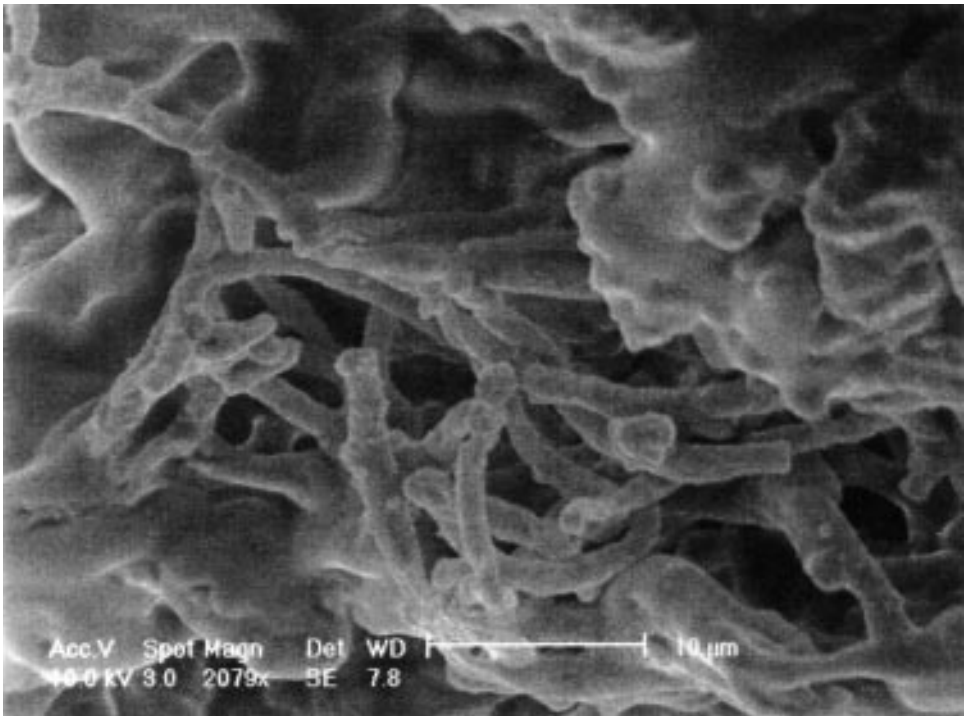


Figure 1. Scanning electron micrograph of a freeze-dried preparation of immobilized bifidobacteria.
Figure 2. Scanning electron micrograph showing a cluster of *Bifidobacterium bifidum* cells in 14-d-old Crescenza cheese.

TABLE 3. Amounts of pH 4.6-soluble N, lactic and acetic acids, lactose, and galactose in the 14-d-old Crescenza cheese samples with and without added bifidobacteria.

		Cheese sample[1]					
Component		BB	BI	BL	MC	IMC	C
pH 4.6-Soluble N, %	1 d	0.18[a]	0.20[a]	0.20[a]	0.19[a]	0.18[a]	0.19[a]
	6 d	0.27[a]	0.28[a]	0.27[a]	0.27[a]	0.25[a]	0.26[a]
	14 d	0.31[a]	0.32[a]	0.32[a]	0.32[a]	0.30[a]	0.25[b]
D- and L-Lactic acid, g/kg	1 d	5.48[b]	5.59[b]	5.54[b]	6.09[a]	5.12[b]	5.34[b]
	6 d	6.07[c]	7.00[a]	6.45[bc]	6.88[ab]	6.81[ab]	6.59[b]
	14 d	7.33[a]	7.02[b]	7.20[ab]	7.22[a]	7.17[ab]	6.44[c]
Acetic acid, g/kg	1 d	0.53[ab]	0.18[bc]	0.19[bc]	0.36[ab]	0.05[c]	0.14[bc]
	6 d	0.66[a]	0.18[c]	0.23[c]	0.44[b]	0.10[c]	0.34[bc]
	14 d	1.31[a]	0.60[b]	0.81[b]	0.88[b]	0.83[b]	0.51[c]
Lactose, mg/kg	1 d	0.12[d]	0.35[c]	0.15[d]	0.66[b]	0.43[c]	5.17[a]
	6 d	0.21[c]	0.45[b]	0.22[c]	0.43[b]	0.21[c]	4.76[a]
	14 d	0.05[d]	0.23[b]	0.08[cd]	0.22[b]	0.12[c]	4.07[a]
Galactose, mg/kg	1 d	8.45[b]	7.24[c]	8.58[a]	7.44[c]	8.12[b]	5.31[d]
	6 d	6.20[b]	5.12[c]	6.33[b]	6.29[b]	7.35[a]	4.48[d]
	14 d	5.27[a]	4.44[c]	5.65[ab]	5.55[ab]	5.18[b]	4.23[c]

[a,b,c,d]Means within a row without a common superscript differ ($P < 0.05$).

[1]Cheese sample designations refer to the bifidobacteria added: BB, *Bifidobacterium bifidum*; BI, *Bifidobacterium infantis*; BL, *Bifidobacterium longum*; MC, mixed culture; and IMC, immobilized mixed culture. C = Control cheese without added bifidobacteria.

by the activity of the contaminating microflora of cheese.

Traces of lactose and rather high amounts of galactose characterized the samples with added bifidobacteria. The decrease of galactose may be due to metabolism by bifidobacteria and endogenous microflora because production of acetic acid was higher in the Crescenza cheese with *B. bifidum* and

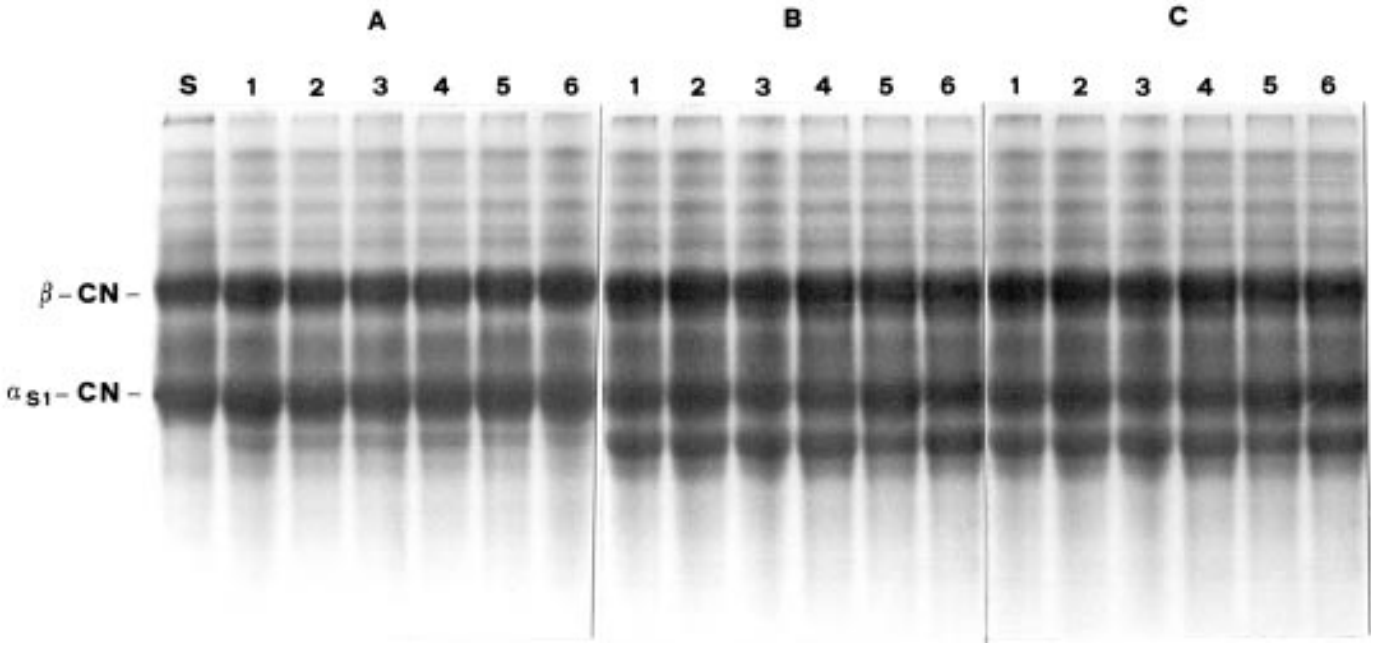


Figure 3. Urea-PAGE electrophoregrams of pH 4.6-insoluble N fraction from the control Crescenza cheese and samples with added bifidobacteria at 1 d (A), 6 d (B), and 14 d (C). Lane S, sodium caseinate standard; lanes 1 to 5, Crescenza cheese with added *Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Bifidobacterium longum*, mixed culture, and immobilized mixed culture, respectively; and lane 6, Crescenza cheese produced by the conventional method.

B. longum added than in the control. At 14 d, the residual lactose in the Crescenza cheese produced by conventional method was still 4.07 g/kg.

Proteolysis and Lipolysis

The urea-PAGE electrophoresis of the N that was insoluble or soluble at pH 4.6 at different periods of ripening and storage are shown in Figures 3 and 4. As expected, no detectable differences were found among the different cheeses. A progressive hydrolysis of α_{s1}-CN occurred throughout ripening and led to a rather complex profile for the pH 4.6-soluble peptides at 14 d. In soft cheeses produced without cooking, such as Crescenza, the chymosin activity on α_{s1}-CN persisted during ripening (13).

An increase of the pH 4.6-soluble N was found throughout ripening: at 14 d, differences ($P < 0.05$) were found between all samples that contained added bifidobacteria (ca. 0.31%) and the control sample (0.25%) (Table 3). The pH 4.6-soluble N of the control reached a plateau at 6 d.

Although ripened only briefly, Crescenza cheeses contained various proteolytic activities in the water-soluble extract (Table 4). The enzyme activities gradually increased during ripening and storage (data not shown). Cheeses with added bifidobacteria, particularly those containing *B. bifidum*, showed more pronounced aminopeptidase activity and, especially, imino-, di-, and tripeptidase activities. Endopeptidase activity was about the same, and proteinase activity was higher in the conventional Crescenza cheese. Carboxypeptidase activity was not found in any of the cheeses. This activity is extremely rare in lactic acid bacteria and bifidobacteria (39). As shown by the low concentrations of free fatty acids (Table 2), the lipolytic activity was very limited. Marked esterase activity on C_4, C_6 and C_8 β-naphthyl derivatives was detected but there were no differences among the cheeses (Table 4). Esterase activity decreased as chain length of the fatty acid derivatives increased, and activity on C_8 β-naphthyl was not detected.

α- and β-Galactosidase Activities

Except for IMC, all of the cheeses with added bifidobacteria showed higher α- and β-galactosidase activities than did the Crescenza cheese that was produced conventionally (Table 4). Those activities were also different ($P < 0.05$) in cheeses containing different bifidobacteria species or multispecies mix-

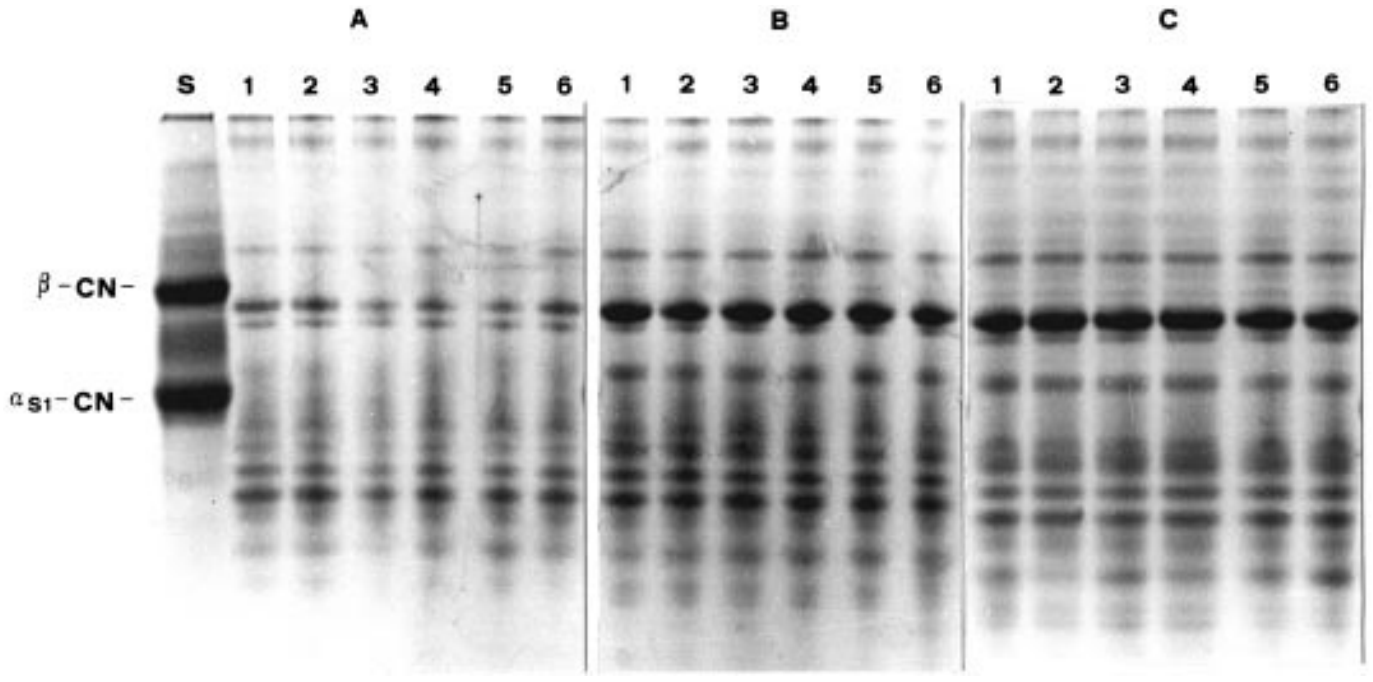


Figure 4. Urea-PAGE electrophoregrams of the pH 4.6-soluble N fraction from the control Crescenza cheese and samples with added bifidobacteria at 1 d (A), 6 d (B), and 14 d (C). Lane S, sodium caseinate standard; lanes 1 to 5, Crescenza cheese with added *Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Bifidobacterium longum*, mixed culture, and immobilized mixed culture, respectively; and lane 6, Crescenza cheese produced by the conventional method.

tures. α- and β-Galactosidase activities markedly increased throughout ripening (data not shown).

Sensory Evaluation

The addition of bifidobacteria to the Crescenza cheese affected the sensory evaluation very slightly (Table 5). No differences among the cheeses were found for body and texture. Only the cheese with *B. bifidum* added individually, which contained a higher amount of acetic acid, differed slightly (6.6 vs. 7.4) from the flavor score of the control cheese. The flavor intensity score of cheeses that had *B. bifidum* and *B. longum* added individually were slightly higher than that of the Crescenza cheese produced by conventional method, which was probably due to the combination of the higher concentrations of lactic and acetic acids and of free amino acids and soluble peptides.

DISCUSSION

The use of bifidobacteria as a starter adjunct to produce probiotic cheese has recently been applied in Gouda, Cheddar, and cottage cheeses (4, 5, 10, 18). However, cheese-making conditions, such as aerobiosis, the cooking procedure for hard or semi-hard cheeses, the presence of fast growing lactic acid bacteria starters, the rather low pH of the curd, and low temperatures during ripening and storage, make it difficult for bifidobacteria to survive.

This paper reports the incorporation and survival of bifidobacteria in Crescenza cheese, a soft Italian cheese that requires only a brief ripening period. To our knowledge, this paper also is the first report of a complete microbial and biochemical characterization of Crescenza cheese.

The viability of the bifidobacteria during cheese making and especially during ripening and storage of Crescenza cheese differed greatly. When added individually, *B. bifidum*, *B. longum*, and *B. infantis* were present at the same concentration in the milk ($\log_{10}$ 6.0 cfu/ml) but reached final cell numbers of $\log_{10}$ 8.05, 7.12, and 5.23 cfu/g, respectively. *Bifidobacterium bifidum* and *B. longum* adapted well to the cheese environment; the viable cell numbers of *B. infantis* decreased throughout ripening. *Bifidobacterium infantis* did not survive >10 to 15 d in creamed cottage cheese at pH 5.0 (5), and marked decreases of 1 to 2 $\log_{10}$ cycles are rather common for various bifidobacteria in fermented milks (21). Difficulties in the growth and survival of bifidobacteria were found when cocultured with *S. thermophilus*, which ex-

TABLE 4. Enzymatic specific activities in the water-soluble extract of the 14-d-old samples of Crescenza cheese with and without added bifidobacteria.

	Cheese sample[1]					
Enzymatic activity	BB	BI	BL	MC	IMC	C
	(units/g of cheese)					
Aminopeptidase						
Leu-*p*-NA[2]	8.5[a]	7.0[a]	6.7[a]	6.6[a]	7.0[a]	4.6[b]
Lys-*p*-NA	11.0[a]	8.6[b]	7.3[b]	8.7[b]	7.8[b]	5.1[c]
Arg-*p*-NA	2.9[a]	3.3[a]	2.8[a]	3.3[a]	3.5[a]	3.4[a]
Ala-*p*-NA	1.8[a]	2.5[a]	2.1[a]	2.3[a]	2.5[a]	2.5[a]
Iminopeptidase: Pro-*p*-NA	2.4[a]	1.3[b]	1.2[b]	1.3[b]	1.2[b]	0.4[c]
Dipeptidase: Leu-Leu	67.1[a]	47.3[c]	40.1[c]	56.4[b]	45.1[c]	25.2[d]
Tripeptidase: Leu-Leu-Leu	90.1[a]	56.3[b]	85.7[a]	56.4[b]	45.1[c]	45.2[c]
Endopeptidase: bradykinin	1.66[a]	1.18[a]	1.23[a]	1.44[a]	1.10[a]	1.34[a]
Proteinase: fluorescent casein	2.2[b]	3.0[b]	3.2[b]	3.0[b]	2.7[b]	4.5[a]
Esterase						
C_4-β-naphthyl	16.9[a]	16.3[a]	17.1[a]	18.0[a]	16.0[a]	16.7[a]
C_6-β-naphthyl	13.9[a]	13.7[a]	12.45[a]	11.2[a]	13.6[a]	14.2[a]
C_8-β-naphthyl	5.4[a]	5.3[a]	3.8[a]	5.5[a]	4.1[a]	4.9[a]
Lipase: tributyrin	0.9[bc]	1.6[a]	0.8[bc]	0.6[bc]	1.0[b]	0.4[c]
α-Galactosidase: α-ONPG[3]	6.0[a]	4.6[b]	6.4[a]	4.8[b]	2.5[c]	2.6[c]
β-Galactosidase: β-ONPG	68.3[a]	46.3[b]	63.2[a]	64.7[a]	23.5[d]	37.8[c]

[a,b,c,d]Means within a row without a common superscript differ ($P < 0.05$).

[1]Cheese sample designations refer to the bifidobacteria added: BB, *Bifidobacterium bifidum*; BI, *Bifidobacterium infantis*; BL, *Bifidobacterium longum*; MC, mixed culture; and IMC, immobilized mixed culture. C = Control cheese without added bifidobacteria.

[2]*p*-NA = *p*-Nitroanilide.

[3]ONPG = *o*-Nitrophenyl β-D-galactopyranoside.

TABLE 5. Sensory evaluation of the 14-d-old samples of Crescenza cheese with and without added bifidobacteria.

	Cheese sample[1]					
	BB	BI	BL	MC	IMC	C
Flavor[2]	6.6[c]	7.1[b]	7.3[ab]	7.1[ab]	7.9[a]	7.4[ab]
Flavor intensity[3]	7.5[a]	7.1[b]	7.8[a]	7.0[b]	7.0[b]	7.0[b]
Body and texture[2]	7.8[a]	7.8[a]	7.8[a]	7.9[a]	7.7[a]	7.6[a]

[a,b,c]Means within a row without a common superscript differ ($P < 0.05$).

[1]Cheese sample designations refer to the bifidobacteria added: BB, *Bifidobacterium bifidum*; BI, *Bifidobacterium infantis*; BL, *Bifidobacterium longum*; MC, mixed culture; and IMC, immobilized mixed culture. C = Control cheese without added bifidobacteria.

[2]Ten-point scale (1 = poor to 10 = excellent).

[3]Ten-point scale (1 = low intensity to 10 = high intensity).

hibited rapid acid production during fermentation of milk (26). Tolerance to cheese-making conditions of specific species or strains have also been reported (5). It has been claimed (29) that probiotic dairy products should contain $\geq \log_{10}$ 6.0 cfu/ml of bifidobacteria to be effective and should be consumed regularly (29); consumption of ≥100 g/d of product with $\log_{10}$ 5.0 to 6.0 cfu/ml of bacteria is recommended (19). Crescenza cheeses with sufficient numbers of added *B. bifidum* or *B. longum* satisfied this criterion. Bifidobacteria were also introduced as multispecies mixtures that were either MC or IMC in calcium alginate gels. Microencapsulation of bifidobacteria was used successfully by Rao et al. (34) to maintain greater viability during passage through the gastrointestinal tract. Although NPNL agar may selectively affect the recovery of bifidobacteria strains (15), metabolite and enzyme analyses were in agreement and indicated a low concentration of *B. infantis* in both individual or multispecies starters. Obviously, a mixture of *B. bifidum* and *B. longum* would be as good as the individual strains. The differences in cell numbers of bifidobacteria in the cheeses only partially influenced the metabolic activities detected in Crescenza cheese throughout ripening and storage.

The addition of bifidobacteria did not change the gross composition of the Crescenza cheese. The presence of bifidobacteria did not affect the normal areobic microflora of the Crescenza cheese, nor did it interfere with the growth and metabolic activity of *S. thermophilus* used as starter. The highest production of lactic and acetic acids in the presence of *B. bifidum* was probably responsible for the lower level of coliforms in the cheese.

Although no differences were found in the urea-PAGE electrophoretograms of the pH 4.6-soluble N and pH 4.6-insoluble N fractions, a higher concentration of the N that was soluble at pH 4.6 was found at 14 d in all the cheeses with added bifidobacteria. This finding was in agreement with the higher activities of aminopeptidase (on Lys-*p*-NA and Leu-*p*-NA), iminopeptidase, dipeptidase, and tripeptidase in the water-soluble extracts of these cheeses. *Bifidobacterium bifidum* had the highest peptidase activities; these differences should be related not only to the highest cell numbers but also to differences in activities between the strains. El-Soda et al. (12) showed that the peptide hydrolase system of *Bifidobacterium* spp. was comparable with that of lactic acid bacteria with respect to the presence of a general aminopeptidase, PepN (Lys-*p*-NA and Leu-*p*-NA were the best substrates), several dipeptidases, tripeptidases, and probably iminopeptidase. Those same researchers also found similarities between the activity of *B. longum* and *B. infantis*.

Cheeses with added bifidobacteria had higher concentrations of lactic and acetic acids than did the control cheese. The uncoupling of growth and acid production were observed by Desjardins et al. (9), who reported that the production of ca. 70 to 75% of total acids by bifidobacteria took place in the stationary phase of growth, which may explain the increase of lactic and acetic acids during the ripening and storage of Crescenza cheese and may suggest metabolic activity by injured cells, such as those of *B. infantis*, which were only partially recoverable by plating. Except for *B. bifidum*, which maintained a cell number of $\log_{10}$ 8.0 cfu/g, no great differences were found among the other cheeses with bifidobacteria added. Blanchette et al. (5) reached the same conclusion in a study of the behavior of *B. infantis* in creamed cottage cheese. The production of lactic and acetic acids was strongly related to the consumption of lactose and probably galactose during the ripening of Crescenza cheese. Compared with the control, all of the cheeses with bifidobacteria were almost lactose-

free and contained higher amounts of galactose, which gradually decreased during ripening and storage. Blanchette and Roy (4) also observed the uncoupling of growth and β-galactosidase activity in cultured cottage cheese dressing produced with bifidobacteria, which had low concentrations of lactose and high concentrations of galactose and glucose. With some differences among the species and except for the immobilized multispecies mixture, all of the Crescenza cheeses containing bifidobacteria showed higher activities of α- and β-galactosidases than did the cheese produced by conventional method. Contaminating bacteria may have produced α-galactosidase activity in the control. α-Galactosidase allows bacteria to hydrolyze polysaccharides that are not normally used by humans. These carbohydrates may constitute a significant source of carbon for the bifidobacteria colonizing the small intestine and may be a prerequisite for the competitive growth within the complex gastrointestinal microflora (2). Although α- and β-galactosidase activities are widely distributed among the bifidobacteria strains, the α-galactosidase activity was not maintained during 10 d of storage in fermented milk (4), which was probably due to the lower pH. The incorporation of bifidobacteria into Crescenza cheese not only provides a product that is free of lactose, but also contains microorganisms with a greater β-galactosidase activity than the conventional product which should result in a grteater tolerance for other dairy products by individuals who have difficulty digesting lactose. It has been shown that the increased tolerance for dairy products containing viable lactic acid bacteria is due to intraintestinal digestion of lactose by the β-galactosidase released from some bacterial species during transit through the gut or by colonization of the gut (25, 27).

The lack of extensive metabolic activities by bifidobacteria was also indicated by the sensory evaluation. Only slight differences were detected between the scores for cheeses with bifidobacteria and those for the conventional Crescenza cheese.

This study has shown that it is possible to produce Crescenza cheese that contains viable bifidobacteria. Both *B. bifidum* and *B. longum* showed the greatest adaptability to this cheese environment. The cheeses in which bifidobacteria had been incorporated were similar to the conventional Crescenza cheese with respect to flavor, appearance, and microbial and physicochemical features but contained a higher concentrations of probiotic enzymes and bacteria.

REFERENCES

1 Andrews, A. T. 1983. Proteinases in normal bovine milk and their action on caseins. J. Dairy Res. 50:45–55.

2 Bezkorovainy, A., and R. Miller-Catchpole. 1989. Biochemistry and Physiology of Bifidobacteria. CRC Press, Boca Raton, FL.

3 Blakesley, R. W., and J. A. Boezi. 1977. A new staining technique for proteins in polyacrylamide gels using Comassie brillant blue G250. Anal. Biochem. 82:580–582.

4 Blanchette, L., and D. Roy. 1995. Production of cultured cottage cheese dressing by bifidobacteria. J. Dairy Sci. 78:1421–1429.

5 Blanchette, L., D. Roy, G. Bélanger, and S. F. Gauthier. 1996. Production of cottage cheese using dressing fermented by bifidobacteria. J. Dairy Sci. 79:8–15.

6 Bottazzi, V. 1993. Formaggio Crescenza. Pages 236–239 *in* Microbiologia Lattiero-Casearia. Edagricole-Edizioni Agricole della Calderini s.r.l., Bologna, Italy.

7 Bottazzi, V., B. Battistotti, and F. Bianchi. 1982. The microscopic crystalline inclusions in Grana cheese and their X-ray microanalysis. Milchwissenschaft 37:578–580.

8 Champagne, C. P., F. Mondou, Y. Raymond, and E. Brochu. 1996. Effect of immobilization in alginate on the stability of freeze-dried *Bifidobacterium longum*. Biosci. Microflora 15(1): 9–15.

9 Desjardins, M. L., D. Roy, C. Toupin, and J. Goulet. 1990. Uncoupling of growth and acids production in *Bifidobacterium* ssp. J. Dairy Sci. 73:1478–1484.

10 Dinakar, P., and V. V. Mistry. 1994. Growth and viability of *Bifidobacterium bifidum* in Cheddar cheese. 1994. J. Dairy Sci. 77:2854–2864.

11 Doi, E., D. Shibata, and T. Matoba. 1981. Modified colorimetric ninhydrin methods for peptide assays. Anal. Biochem. 118: 173–182.

12 El-Soda, M., A. Macedo, and N. F. Olson. 1992. The peptide hydrolase system of *Bifidobacterium* species. Milchwissenschaft 47:87–90.

13 Foltmann, B. 1993. General and molecular aspects of rennets. Pages 37–68 *in* Cheese: Chemistry, Physics and Microbiology. Vol 1. 2nd ed. P. F. Fox, ed. Chapman & Hall, London, United Kingdom.

14 Ghitti, C., and G. Ottogalli. 1984. Il formaggio Crescenza (parte III). Sci. Tecn. Lattiero-Cas. 35:474–478.

15 Ghoddusi, H. B., and R. K. Robinson. 1996. Enumeration of starter cultures in fermented milks. J. Dairy Res. 63:151–158.

16 Gobbetti, M., L. Cossignani, M. S. Simonetti, and P. Damiani. 1995. Effect of the aminopeptidase from *Pseudomonas fluorescens* ATCC 948 on synthetic bitter peptides, bitter hydrolysate of UHT milk proteins and on the ripening of Italian Caciotta type cheese. Lait 75:169–179.

17 Gobbetti, M., A. Corsetti, E. Smacchi, M. De Angelis, and J. Rossi. 1997. Microbiology and biochemistry of Pecorino Umbro cheese during ripening. Ital. Food Sci. 2:111–126.

18 Gomes, A.M.P., F. X. Malcata, F.A.M. Klaver, and H. J. Grande. 1995. Incorporation and survival of *Bifidobacterium* sp. strain Bo and *Lactobacillus acidophilus* strain Ki in a cheese product. Neth. Milk Dairy J. 49:71–95.

19 Guyot, A. 1990. Milks cultured with bifidobacteria and acidophilic lactobacilli. Lait Nous 3:15–17.

20 Hoover, D. G. 1993. Bifidobacteria: activity and potential benefits. Food Technol. 47(6):120–124.

21 Hughes, D. B., and D. G. Hoover. 1995. Viability and enzymatic activity of bifidobacteria in milk. J. Dairy Sci. 78:268–276.

22 International Dairy Federation. 1970. Determination of dry matter content in whey cheese. IDF Stand. 58. Int. Dairy Fed., Brussels, Belgium.

23 International Dairy Federation. 1988. Determination of the salt content. IDF Stand. 12B. Int. Dairy Fed., Brussels, Belgium.

24 International Dairy Federation. 1989. Determination of pH. IDF Stand. 115A. Int. Dairy Fed., Brussels, Belgium.

25 Kilara, A., and K. M. Shahani. 1976. Lactase activity of cultured and acidified dairy products. J. Dairy Sci. 59:2031–2035.

26 Klaver, F.A.M., F. Kingma, and A. H. Weerkamp. 1993. Growth and survival of bifidobacteria in milk. Neth. Milk Dairy J. 47: 151–164.

27 Kolars, J. C., M. D. Levitt, D.A.G. Mostafa-Aouji, and D. A. Saviano. 1984. Yogurt—an autodigesting source of lactose. New England J. Med. 310:1–3.
28 Kuchroo, C. N., and P. F. Fox. 1982. Soluble nitrogen in Cheddar cheese: comparison of extraction procedures. Milchwissenschaft 37:331–335.
29 Kurmann, J. A. 1988. Starters with selected intestinal bacteria. Pages 41–45 *in* IDF Bull. 227. Int. Dairy Fed., Brussels, Belgium.
30 Lim, K. S., C. S. Huh, and Y. J. Baek. 1993. Antimicrobial susceptibility of bifidobacteria. J. Dairy Sci. 76:2168–2174.
31 Modler, H. W., R. C. McKellar, and M. Yaguchi. 1990. Bifidobacteria and bifidogenic factors. Can. Inst. Food Sci. Technol. J. 23:29.
32 Neviani, E., and G. Giraffa. 1993. Formaggi freschi e molli e loro differenziazione: le microflore lattiche. Il Latte 7:776–780.
33 Ottogalli, G., G. Rondinini, and C. Patano. 1985. Carica microbica di alcuni formaggi freschi: quali test e quali limiti? Il Latte 10:556–557.
34 Rao, A. V., N. Shiwnarain, and I. Maharaj. 1989. Survival of microencapsulated *Bifidobacterium pseudolongum* in simulated gastric and intestinal juices. Can. Inst. Food Sci. Technol. J. 22: 345–349.
35 Rasic, L. J., and J. A. Kurmann. 1983. Bifidobacteria and Their Role. Birkhauser Verlag, Bern, Switzerland.
36 Rogers, P. 1991. Banking on bifidus. Dairies see opportunities as research links bifidobacteria and good health. Dairy Foods 92(5):55–57.
37 SAS® User's Guide: Statistics, Version 6 Edition. 1989. SAS Inst., Inc., Cary, NC.
38 Shimamura, S., F. Abe, N. Ishibashi, H. Miyakawa, T. Yaeshima, T. Araya, and M. Tomita. 1992. Relationship between oxygen sensitivity and oxygen metabolism of *Bifidobacterium* species. J. Dairy Sci. 75:3296–3306.
39 Tan, P.S.T., B. Poolmann, and W. N. Konings. 1993. Proteolytic enzymes of *Lactococcus lactis*. J. Dairy Res. 60:269–286.
40 Teraguchi, S., M. Uehara, K. Ogasa, and T. Mitsuoka. 1978. Enumeration of bifidobacteria in dairy products. Jpn. J. Bacteriol. 33:753–761.