Available online at www.sciencedirect.com
SCIENCE DIRECT®
Food Chemistry 86 (2004) 263–267
Food Chemistry
www.elsevier.com/locate/foodchem

Caseinomacropeptide and whey protein concentrate enhance *Bifidobacterium lactis* growth in milk

C. Janer, C. Peláez, T. Requena *

Department of Dairy Science and Technology, Instituto del Frío (CSIC), José Antonio Novais 10, Ciudad Universitaria, 28040 Madrid, Spain

Received 2 July 2003; received in revised form 1 September 2003; accepted 1 September 2003

Abstract

Caseinomacropeptide (CMP), isolated from cow's or combined ewe's and goat's sweet cheese whey, and whey protein concentrate (WPC), enhanced growth of *Bifidobacterium lactis* in milk. Supplementation of milk at 2% CMP increased counts of *B. lactis* by 1.5 log cycles after 24 h incubation at 37 °C when compared with unsupplemented milk, showing no differences between the two CMPs. The highest bacterial growth (9.1 log cfu ml^{-1}) was obtained when milk was supplemented with 2% WPC. The growth-promoting activity shown by WPC could be due to its CMP and whey protein content. CMP and WPC could be utilised to complement milk in order to increase counts of bifidobacteria in probiotic fermented milks.

Keywords: *Bifidobacterium lactis*; Bifidogenic; Caseinomacropeptide; Whey protein concentrate

1. Introduction

Great interest has recently been focussed on the development of bioactive cultures and their incorporation in fermented dairy products. Within the various probiotic organisms, the genus *Bifidobacterium* has been subject to intense study, resulting in an extensive literature which describes the beneficial roles that these bacteria could produce for the host's health (De Roos & Katan, 2000; Rolfe, 2000; Sanders, 2000). The recently identified *B. lactis* has been claimed to benefit health by enhancement of the immune response (Chiang, Sheih, Wang, Liao, & Gill, 2000; Gill, Rutherfurd, Cross, & Gopal, 2001), reduction of diarrhoea associated with rotavirus and *Escherichia coli* (Shu, Qu, & Gill, 2001), antagonistic activity against enterotoxigenic *E. coli* (Gopal, Prasad, Smart, & Gill, 2001) and *Salmonella typhimurium* (Shu et al., 2000). Moreover, *B. lactis* shows interesting technological characteristics, such as certain oxygen and acid tolerances that render this microorganisms adequate for industrial utilisation in fermented milks as compared with other bifidobacteria species (Crittenden et al., 2001; Meile et al., 1997).

Because of bifidobacteria showing poor growth in milk, several substances have been studied for their potential growth-stimulating activity, with the aim of finding a suitable nutrient supplement to incorporate into culture media (Gomes, Malcata, & Klaver, 1998; Idota, Kawakami, & Nakajima, 1994; O'Sullivan, 1996; Proulx, Ward, Gauthier, & Roy, 1994). Among them, much attention has been paid to milk-derived components. Caseinomacropeptide (CMP), a hydrophilic glycopeptide derived from the action of chymosin on κ-casein during the milk-clotting process in cheese making, contains not only available nitrogen for bacterial growth but also amino-sugars, such as sialic acid and *N*-acetylgalactosamine, that could be fermented by bifidobacteria (Azuma, Yamauchi, & Mitsuoka, 1984). However, the literature on this particular subject is contradictory. Azuma et al. (1984) observed that human CMP promoted the growth of *B. infantis*. Poch and Bezkorovainy (1991) did not find this activity in CMP of cow's milk, whereas Idota et al. (1994) described a bifidus growth-promoting activity for bovine CMP. It has been proposed that the difference between the growth-enhancing activities of human and bovine CMP is due to

* Corresponding author. Tel.: +34-915-492-300; fax: +34-915-493-627.
E-mail address: trequena@if.csic.es (T. Requena).

0308-8146/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2003.09.034

amino-sugar content, which is three times lower in the latter (Modler, 1994). No literature has been found describing the bifidogenic activity of CMP derived from other sources, such as ewe's or goat's milk. Whey protein concentrate (WPC), which contains CMP as well as whey proteins, is a cheap and readily available additive, for which growth-enhancing activity on lactic acid bacteria has been described by Bury, Jelen, and Kimura (1998).

In this study we have analysed the effect of the addition of CMP and WPC on the growth and acid production of *B. lactis* in skimmed milk. The CMPs employed in the experiment were isolated in our laboratory out of bovine milk and a combination (50:50) of ovine and caprine milk. It is therefore a comparative study that also includes a characterisation of the isolated CMPs and the WPC.

2. Materials and methods

2.1. Caseinomacropeptide and whey protein concentrate samples

CMP was isolated from bovine milk and from combined (50:50) ovine and caprine milk by a method developed by Martín-Diana, Fraga, and Fontecha (2002). Briefly, whey, obtained by rennet clotting of milk, was adjusted to pH 3.5, heated to 90 °C for 1 h and centrifuged (5200*g*, 4 °C, 15 min) to remove residual casein and whey proteins. The supernatant was brought to neutral pH under vigorous stirring and ultrafiltered at 50 °C with a 10 kDa UF membrane. The retentate, which contained the CMP, was diafiltered to eliminate the excess of salts and lactose, concentrated and finally lyophilised. This yielded a product of concentration 58–75% CMP. Commercial CMP (Lacprodan CGMP-10, MD Foods Ingredients, Denmark) of 80% purity was also employed. WPC was kindly supplied by FERMO (Alcobendas, Madrid, Spain).

2.2. Growth of Bifidobacterium lactis

B. lactis DSM10140 was cultured under anaerobic conditions (Gas-pack, Anaerogen; Oxoid) for 24 h at 37 °C. Frozen stocks (−80 °C) were propagated in MRS broth (Panreac) and cultures used as inoculum for growth experiments were prepared in skimmed milk supplemented with 5 g glucose kg^{-1} and 5 g yeast extract kg^{-1} . To lower the redox potential, 10 g ascorbic acid kg^{-1} and 0.5 g cysteine kg^{-1} were added to all media. Growth medium consisted of skimmed milk, containing 10 g ascorbic acid kg^{-1} and 0.5 g cysteine kg^{-1}, supplemented with either lyophilised bovine CMP, combined ovine and caprine CMP or WPC at a concentration of 20 g kg^{-1}. The medium was heated to 80 °C for 30 min in a water bath, cooled to 37 °C and inoculated with *B. lactis* at 2%.

Unsupplemented skimmed milk was used as control. Cultures were anaerobically incubated at 37 °C for 0, 6 and 24 h and assayed for bacterial growth. Changes in pH were measured with a SCHOTT CG 837 pH-Meter. Cell turbidity was read at 480 nm in a Milton Roy Spectronic 20D spectrophotometer, after clarification with 0.2% EDTA at pH 12. Viable cell counts per ml (cfu ml^{-1}) were performed in MRS agar using the pour plate technique and inoculated plates were incubated at 37 °C under anaerobic conditions for 48 h. Results were means of at least three experiments analysed in duplicate. Results were studied using one-way analysis of variance to determine significant differences ($p < 0.05$), using the Statgraphics software (version 2.1; Statistical Graphics Co., Rockville, USA).

Free sialic acid, released to the medium during *B. lactis* growth, was estimated in the 15% trichloroacetic (TCA)-soluble fraction of samples at 0, 6 and 24 h incubation times by using a colorimetric assay (Roche Diagnostics). Possible interference by pyruvic acid in the method was estimated according to Schwimmer and Weston (1961).

2.3. WPC and CMP characterisation

2.3.1. Carbohydrate content

The concentration of sialic acid in the lyophilised CMPs and WPC was determined by using the Roche colorimetric assay. Lactose content was determined with the Lactose/D-Galactose enzymatic test kit (Roche Diagnostics).

2.3.2. Nitrogen content

Total nitrogen contents of the lyophilised CMPs and WPC were determined using a LECO FP2000 analyser (LECO Corporation). Non-protein nitrogen (NPN) was determined in the 12% TCA-soluble fraction of the samples and analysed in the LECO analyser after removal of TCA. The amino nitrogen (AN) content was determined in the NPN fraction by the *o*-phthaldialdehyde assay (Church, Swaisgood, Porter, & Catignani, 1983).

2.3.3. RP-HPLC analysis

RP-HPLC was employed to determine the CMP content in the isolated fractions of CMP and in WPC, following the method described by López-Fandiño, Acedo, and Ramos (1993) on a Jasco PU-1580 chromatograph equipped with a Jasco pump and UV detector and using a C_{18} Nucleosil column (300 Å, 250×4.6 mm), 5 μm particle size (Macherey Nagel Düren). Solvent A was 0.1% (v/v) trifluoroacetic acid (Merck) in double-distilled water while solvent B was 0.1% (v/v) trifluoroacetic acid in acetonitrile (Scharlau).

The gradient of B was increased from 21% to 37% over 40 min, then raised to 100%. Flow rate was set at 1 ml min^{-1}, temperature was 40 °C and detection was monitored at 214 nm. All samples were injected at a concentration of 1 mg ml^{-1} in double-distilled water except for WPC, which was diluted to 5 mg ml^{-1}. Prior to injection, all samples were filtered through 0.22 μm pore size acetate filters. Bovine CMP from Sigma was used as standard.

3. Results and discussion

The effect of the addition of CMP isolated from bovine and combined ovine and caprine milk and of WPC on the growth of *B. lactis* DSM10140 is shown in Table 1. It was observed that the growth of *B. lactis* in unsupplemented skimmed milk was poor; mean viable cell numbers, after a 24 h incubation time, increased less than one log cycle. On the other hand, the addition of WPC produced the highest increase ($p < 0.05$) in bacterial growth with counts that exceeded 9 log cfu ml^{-1} after 24 h. The growth-enhancing effect, on *B. lactis*, of bovine CMP and combined ovine and caprine CMP was intermediate, and no differences were found between them ($p > 0.05$). Maximum specific growth rates for milk with CMP ranged between 75% and 80% of that obtained with WPC, not exceeding 39% for unsupplemented milk. Thus, it may be accepted that CMP and WPC both exhibit a beneficial influence on the growth of *B. lactis* in skimmed milk.

Decrease of pH by *B. lactis* during incubation was in agreement with bacterial growth in all cultures (Table 1). Milk supplemented with WPC, which yielded the higher cell counts, reached the lowest ($p < 0.05$) pH values after 24 h of incubation time. Growth enhancement by bovine and combined ovine and caprine CMP also decreased the pH after 24 h to a larger extent ($p < 0.05$) than that of unsupplemented milk.

In order to elucidate if the amino-sugar content of the supplements could be related to differences in bifidobacterial growth, the release of free sialic acid, during growth of *B. lactis* in milk and milk supplemented with bovine CMP, combined ovine and caprine CMP or WPC, was also analysed. There was an increase of 0.1 mg free sialic acid ml^{-1} when milk was supplemented with either CMP or WPC. Table 2 shows the sialic acid content of bovine and combined ovine and caprine CMPs and of WPC. Nevertheless, the differences between sialic acid contents of the supplements did not agree with the differences observed in bacterial growth. Idota et al. (1994) reported an increase in bifidobacterial growth when their culture media was fortified with sialic acid and sialic acid-containing substances even at concentrations as low as 0.1 mg ml^{-1}. In the present study, enrichment of milk (at 20 g kg^{-1}) with bovine, combined ovine and caprine CMPs or WPC implies supplying growth media, respectively, with 0.7, 1.5 and 1.3 mg extra sialic acid ml^{-1}.

Table 2 also shows the nitrogen content of bovine CMP, combined ovine and caprine CMP and WPC. Total protein, as determined by applying a nitrogen to protein conversion factor of 6.38, was similar for bovine and combined CMPs (64.7% and 61.4%, respectively) whereas WPC had around 35% protein. NPN and AN contents were not higher in WPC than in CMPs. Therefore, the higher growth rate of *B. lactis* when milk was enriched with WPC cannot be explained as an outcome of culture supplementation with a directly available nitrogen source in the form of low molecular weight nitrogen. Another difference in the compositions of WPC and CMP derives from their content of lactose (Table 2). Addition of CMPs and of WPC to milk implies a lactose supplementation of 0.2% and 0.9%, respectively, in the culture media over its natural 4.5–5% content. However, Poch and Bezkorovainy (1988) described that no improvement was observed in the growth of *Bifidobacterium* spp. when lactose concen-

Table 2
Mean values (SD) for the nitrogen fractions and sialic acid and lactose contents, expressed as percentage (w/w), of the caseinomacropeptides isolated from bovine whey (bCMP) and combined ovine and caprine whey (cCMP) and of whey protein concentrate (WPC)

	bCMP	cCMP	WPC
Total protein	64.7 (0.41)	61.9 (0.96)	35.1 (0.05)
Non-protein nitrogen	1.98 (0.02)	3.28 (0.11)	1.14 (0.06)
Amino nitrogen	0.13 (0.01)	0.18 (0.01)	0.12 (0.02)
Sialic acid	0.37 (0.07)	0.75 (0.06)	0.67 (0.02)
Lactose	8.20 (1.00)	9.15 (2.05)	43.9 (7.46)

Table 1
Viable counts and pH decrease during the growth of *B. lactis* DSM10140 in skimmed milk (SM), skimmed milk supplemented with WPC, bovine CMP (bCMP), or combined ovine and caprine CMP (cCMP)

Sample	Counts (cfu ml^{-1}) × 10^7			pH		
	0 h	6 h	24 h	0 h	6 h	24 h
SM	1.6 (0.3)[a]	4.0 (0.3)[a]	9.4 (0.3)[a]	6.31 (0.01)[a]	6.13 (0.10)[a]	5.36 (0.08)[a]
bCMP	1.6 (0.1)[a]	6.4 (2.3)[a]	39.5 (5.7)[b]	6.26 (0.05)[a]	6.02 (0.07)[b]	5.13 (0.10)[b]
cCMP	1.7 (0.1)[a]	7.8 (2.3)[a]	38.0 (5.6)[b]	6.23 (0.04)[a]	5.91 (0.02)[c]	4.95 (0.09)[c]
WPC	1.8 (0.1)[a]	13.9 (4.7)[b]	118 (12.3)[c]	6.27 (0.10)[a]	5.95 (0.01)[bc]	4.58 (0.11)[d]

Means (SD) are averages from three independent trials. Different letters within columns indicate significant differences ($p < 0.05$).

tration of growth medium was increased above 3.5%, which suggests that the lactose increment is not responsible for the better growth of *B. lactis* on WPC-supplemented medium.

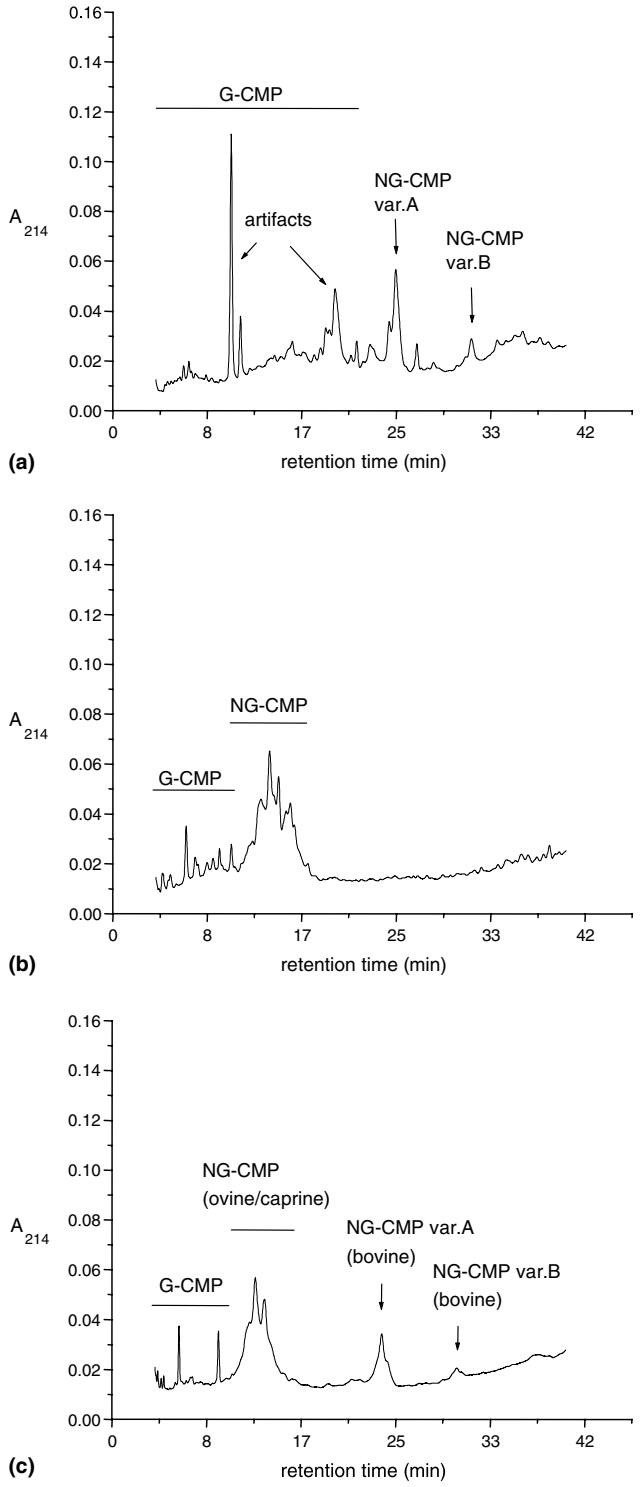

Fig. 1. RP-HPLC chromatographic patterns of (a) bovine CMP, (b) combined ovine and caprine CMP and (c) WPC. G-CMP: glycosylated CMP fraction. NG-CMP: non-glycosylated CMP fraction. Var A: genetic A variant of bovine CMP. Var B: genetic variant B of bovine CMP.

The CMP contents of bovine CMP, combined caprine and ovine CMP and WPC, expressed as percentage peak areas in the RP-HPLC chromatograms (Fig. 1), are shown in Table 3. Standard CMP of 99% purity was also analysed to verify the validity of the results obtained by this method. Table 3 also shows the glycosylation rate of the CMPs, as determined by their RP-HPLC elution profiles. The glycosylated fraction of CMP is constituted of *N*-acetylgalactosamine, galactose, and sialic acid, as either *N*-acetylneuraminic acid or *N*-glycolylneuraminic acid (Mollé & Léonil, 1995; Moreno, Recio, Olano, & López-Fandiño, 2000; Moreno, Recio, Olano, & López-Fandiño, 2001). Glycosylation rate of standard bovine CMP was in agreement with Vreeman, Visser, Slangen, and Van Riel (1986) who reported that 60% of bovine CMP was glycosylated. The resemblance between the elution patterns of ewe's and goat's CMPs is consistent with the similarity of their chemical sequences (Moreno et al., 2000, 2001) and permits their characterisation as a combined product. Both bovine CMP and combined CMP exhibited a lower glycosylation rate than that reported in the literature (Moreno et al., 2000, 2001; Vreeman et al., 1986), probably as a result of hydrolysis caused by heat treatment during the isolation procedure. As for the WPC, it contained around 20% CMP and presented a pattern indicating that was obtained from a mixture of cow's, ewe's and goat's whey (Fig. 1).

The 21% CMP content of the WPC (Table 3) could partially explain the increase of *B. lactis* growth. A noticeable difference in the composition of WPC, that can influence growth, is the content of whey proteins, mainly α-lactoalbumin and β-lactoglobulin, which were selectively removed during the isolation of the CMP fraction (Martín-Diana et al., 2002) but constitute most of the WPC protein content (De Wit, 1998). These proteins have been shown to stimulate the growth of *Bifidobacterium* spp. at a concentration as low as 3.5 $mg\,ml^{-1}$ (Ibrahim & Bezkorovainy, 1994; Petschow & Talbott, 1991) and could explain the higher growth-promoting activity of WPC than CMP.

CMP and WPC can stimulate the growth of bifidobacteria when added to milk as a supplement, the effect

Table 3
CMP content (SD) of bovine CMP (bCMP), combined caprine and ovine CMP (cCMP) and WPC, expressed as percentage peak areas in the RP-HPLC chromatograms (Fig. 1)

	Standard CMP	bCMP	cCMP	WPC
Purity	99.7	57.8 (4.09)	74.5 (1.99)	20.8 (2.80)
Glycosylated CMP	61.7	44.6 (3.26)	18.2 (1.33)	17.6 (1.13)
Non-glycosylated CMP	38.4	55.4 (3.26)	81.8 (1.33)	82.4 (1.13)

Pure bovine CMP was used as standard.

being higher in WPC than in CMP. Besides the CMP content, WPC includes whey proteins, which possibly have too high a molecular weight to be rendered available for direct bacterial uptake but could be enzymatically cleaved, leading to formation of bifidobacterial growth factors. The proteolytic and glycolytic activities of *B. lactis* on whey proteins and glycopeptides, such as CMP, are currently under study.

Acknowledgements

This research was financed by Projects ALI98-0969-C02-02 and AGL2000-0727-C03-02. C. Janer is the recipient of a scholarship from the Spanish Ministry of Science and Technology.

References

Azuma, N., Yamauchi, K., & Mitsuoka, T. (1984). Bifidus growth-promoting activity of a glycomacropeptide derived from human *k*-casein. *Agricultural and Biological Chemistry, 48*, 2159–2162.

Bury, D., Jelen, P., & Kimura, K. (1998). Whey protein concentrate as a nutrient supplement for lactic acid bacteria. *International Dairy Journal, 8*, 149–151.

Chiang, B. L., Sheih, Y. H., Wang, L. H., Liao, C. K., & Gill, H. S. (2000). Enhancing immunity by dietary consumption of a probiotic lactic acid bacteria (*Bifidobacterium lactis* HN019): optimization and definition of cellular immune responses. *European Journal of Clinical Nutrition, 54*, 849–855.

Church, F. C., Swaisgood, H. E., Porter, D. H., & Catignani, G. L. (1983). Spectrophotometric assay using *o*-phtaldialdehyde for determination of proteolysis in milk and isolated milk proteins. *Journal of Dairy Science, 66*, 1219–1227.

Crittenden, R. G., Morris, L. F., Harvey, M. L., Tran, L. T., Mitchell, H. L., & Playne, M. J. (2001). Selection of a *Bifidobacterium* strain to complement resistant starch in a symbiotic yoghurt. *Journal of Applied Microbiology, 90*, 268–278.

De Roos, N. M., & Katan, M. B. (2000). Effects of probiotic bacteria on diarrhoea, lipid metabolism, and carcinogenesis: a review of papers published between 1988 and 1998. *American Journal of Clinical Nutrition, 71*, 405–411.

De Wit, J. N. (1998). Nutritional and functional characteristics of whey proteins in food products. *Journal of Dairy Science, 81*, 597–608.

Gill, H. S., Rutherfurd, K. J., Cross, M. L., & Gopal, P. K. (2001). Enhancement of immunity in the elderly by dietary supplementation with the probiotic *Bifidobacterium lactis* HN019. *American Journal of Clinical Nutrition, 74*, 833–839.

Gomes, A. M. P., Malcata, F. X., & Klaver, F. A. M. (1998). Growth enhancement of *Bifidobacterium lactis* Bo and *Lactobacillus acidophilus* Ki by milk hydrolyzates. *Journal of Dairy Science, 81*, 2817–2825.

Gopal, P. K., Prasad, J., Smart, J., & Gill, H. S. (2001). In vitro adherence properties of *Lactobacillus rhamnusus* DR20 and *Bifidobacterium lactis* DR10 strains and their antagonistic activity against an enterotoxigenic *Escherichia coli*. *International Journal of Food Microbiology, 67*, 207–216.

Ibrahim, S. A., & Bezkorovainy, A. (1994). Growth-promoting factors for *Bifidobacterium longum*. *Journal of Food Science, 59*, 189–191.

Idota, T., Kawakami, H., & Nakajima, I. (1994). Growth-promoting effects of *N*-acetylneuraminic acid-containing substances on bifidobacteria. *Bioscience, Biotechnology and Biochemistry, 58*, 1720–1722.

López-Fandiño, R., Acedo, M. I., & Ramos, M. (1993). Comparative study by HPLC of caseinomacropeptides from cow's, ewe's and goat's milk. *Journal of Dairy Research, 60*, 117–121.

Martín-Diana, A. B., Fraga, M. J., & Fontecha, J. (2002). Isolation and characterisation of caseinomacropeptide from bovine, ovine and caprine cheese whey. *European Food Research International, 214*, 282–286.

Meile, L., Ludwig, W., Rueger, U., Gut, C., Kaufmann, P., Dasen, G., Wenger, S., & Teuber, M. (1997). *Bifidobacterium lactis* sp. nov, a moderately oxygen tolerant species isolated from fermented milk. *Systematic and Applied Microbiology, 20*, 57–64.

Modler, H. W. (1994). Bifidogenic factors – sources, metabolism and applications. *International Dairy Journal, 4*, 383–407.

Mollé, D., & Léonil, J. (1995). Heterogeneity of the bovine *k*-casein caseinomacropeptide, resolved by liquid chromatography on-line with electrospray ionization mass spectrometry. *Journal of Chromatography A, 708*, 223–230.

Moreno, F., Recio, I., Olano, A., & López-Fandiño, R. (2000). Chromatographic characterization of ovine *k*-casein macropeptide. *Journal of Dairy Research, 67*, 349–359.

Moreno, F., Recio, I., Olano, A., & López-Fandiño, R. (2001). Heterogeneity of caprine *k*-casein macropeptide. *Journal of Dairy Research, 68*, 197–208.

O' Sullivan, M. G. (1996). Metabolism of bifidogenic factors by gut flora – an overview. *Bulletin of the International Dairy Federation, 313*, 23–30.

Petschow, B. W., & Talbott, R. D. (1991). Response of *Bifidobacterium* species to growth promoters in human and cow milk. *Pediatric Research, 29*, 208–213.

Poch, M., & Bezkorovainy, A. (1988). Growth-enhancing supplements for various species of the genus *Bifidobacterium*. *Journal of Dairy Science, 71*, 3214–3221.

Poch, M., & Bezkorovainy, A. (1991). Bovine milk *k*-casein trypsin digest is a growth enhancer for the genus *Bifidobacterium*. *Journal of Agricultural and Food Chemistry, 39*, 73–77.

Proulx, M., Ward, P., Gauthier, S. F., & Roy, D. (1994). Comparison of bifidobacterial growth-promoting activity of ultrafiltered casein hydrolyzate fractions. *Lait, 74*, 139–152.

Rolfe, R. D. (2000). The role of probiotic cultures in the control of gastrointestinal health. *Journal of Nutrition, 130*, 396S–402S.

Sanders, M. E. (2000). Considerations for use of probiotic bacteria to modulate human health. *Journal of Nutrition, 130*, 384S–390S.

Schwimmer, S., & Weston, W. J. (1961). Enzymatic development of pyruvic acid in onions as a measure of pungency. *Journal of Agricultural and Food Chemistry, 9*, 301–304.

Shu, Q., Lin, H., Rutherfurd, K. J., Fenwick, S. G., Prasad, J., Gopal, P. K., & Gill, H. S. (2000). Dietary *Bifidobacterium lactis* (HN019) enhances resistance to oral *Salmonella typhimurium* infection in mice. *Microbiology and Immunology, 44*, 213–222.

Shu, Q., Qu, F., & Gill, H. S. (2001). Probiotic treatment using *Bifidobacterium lactis* HN019 reduces weanling diarrhea associated with rotavirus and *Escherichia coli* infection in a piglet model. *Journal of Pediatric Gastroenterology and Nutrition, 33*, 171–177.

Vreeman, H. J., Visser, S., Slangen, C. J., & Van Riel, J. A. (1986). Characterization of bovine *k*-casein fraction and the kinetics of chymosin-induced macropeptide release from carbohydrate-free and carbohydrate-containing fractions determined by high-performance gel-permeation chromatography. *Biochemical Journal, 240*, 87–97.