Growth Promotion of *Bifidobacterium* Species by Whey and Casein Fractions from Human and Bovine Milk

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An in vitro assay was used to study the growth-promotional activity of human milk (HM), cow's milk (CM), and whey and casein fractions of HM and CM for five strains of *Bifidobacterium* species isolated originally from stools of human infants. Whey- and casein-predominant CM-based infant formulas were studied as well. When compared on an equivalent protein basis, the growth promotion activity of HM was greater than that of CM for *Bifidobacterium bifidum* serovar pennsylvanicus and *Bifidobacterium longum* but comparable for *B. bifidum*, *Bifidobacterium infantis*, and *Bifidobacterium breve*. Pasteurization of HM and CM resulted in an increase of growth promotion activity for *B. bifidum* serovar pennsylvanicus and *B. bifidum*, a decrease for *B. infantis*, and no change for *B. longum* and *B. breve*. The growth promotion activity of HM whey was slightly higher than that of HM casein for four strains of bifidobacteria. When CM casein was a substrate, virtually no growth occurred for *B. bifidum* serovar pennsylvanicus, *B. bifidum*, *B. infantis*, and *B. longum*. The growth promotion activity of CM whey, however, was similar to that of HM whey. A similar trend was observed for CM-based infant formula. Whey-dominant formulas promoted better growth of *B. bifidum* serovar pennsylvanicus, *B. bifidum*, and *B. infantis* than casein-dominant formulas. The data suggest a direct relationship between amount of whey-specific factors and the ability to promote growth of clinically relevant strains of *Bifidobacterium* species by HM, CM, and CM-based infant formulas.

Epidemiological studies support the perception that breast-fed infants experience fewer episodes of diarrheal illness than infants ingesting cow's milk (CM) or infant formula (5–8, 15). Although the occurrence of intestinal infections in infants is most likely determined by a number of variables, including environmental hygiene and nutritional status, the anti-infective components of human milk (HM) are thought to play a major part in preventing diarrheal disease (13, 25). One mechanism that may contribute to the protection associated with breast feeding is based on the existence of factors in HM which promote the development of a favorable intestinal bacterial flora, which in turn discourages the proliferation of pathogenic microbes. Indeed, specific HM factors appear to promote the growth of *Bifidobacterium* spp. which have traditionally been associated with the favorable nature of stool flora in infants because of their potential role in resisting pathogen colonization through production and release of acetic and lactic acids. Recent studies have verified that high levels of fecal bifidobacteria exist in both breast-fed and formula-fed infants. However, stools from breast-fed infants typically have a lower pH and contain a lower proportion of less desirable, putrefactive-type bacteria than stools from infants fed formula (3, 19, 20, 27, 28).

The growth-promoting activity of HM factors for bifidobacteria has been measured most commonly by using *B. bifidum* serovar pennsylvanicus (4, 11, 12, 14, 21). This model strain, however, is uncommon in the intestinal tract of the human infant and is considered a mutant, since it requires exogenous *N*-acetylglucosamine for cell wall synthesis (9). Consequently, much of the growth promotion activity in HM has been attributed to *N*-acetylglucosamine-containing oligosaccharides and glycoproteins. Little information is available regarding growth promotion of major infant strains of bifidobacteria by HM and common HM

substitutes. Likewise, whether the lack of growth promotion activity in CM (4, 11) is due to the low oligosaccharide content relative to HM or other differences in HM and CM composition is not known.

The present study was undertaken to compare HM, CM, and various CM-based infant formulas for their ability to promote the growth of the major species of bifidobacteria found in stools of infants. The second objective was to determine the distribution of bifidobacterium growth promotion activity among whey and casein fractions of HM and CM.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains of *Bifidobacterium* species were purchased in lyophilized form from the American Type Culture Collection (Rockville, Md.). The five indicator strains used in this study, representing four different species, consisted of the following human infant fecal isolates: *B. bifidum* serovar pennsylvanicus ATCC 11863, *B. bifidum* ATCC 15696, *B. breve* ATCC 15700 (type strain), *B. infantis* ATCC 15697 (type strain), and *B. longum* ATCC 15708. Cultures were grown in anaerobic jars (GasPak; BBL Microbiology Systems, Cockeysville, Md.) in a prereduced buffered growth medium consisting of Reinforced Clostridial Medium (Difco Laboratories, Detroit, Mich.) supplemented with KH_2PO_4 (4.5 g/liter) and Na_2HPO_4 (6.0 g/liter). The phosphate-buffered Reinforced Clostridial Medium provided optimal growth during a 24-h incubation and minimized the decline in viability of cells which results from acidification of growth medium during growth.

Bifidobacterium growth promotion assay. The assay for measuring bifidobacterium growth promotion activity in various samples was based in part on the method of Bezko-

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rovainy et al. (4, 21). The test medium was the minimal growth medium of Norris (26) without added human milk. Briefly, 2.5 ml of sterile Norris medium (pH 6.8) was mixed with a specific volume of test sample (<1.4 ml). All volumes were adjusted to 3.9 ml with sterile saline and prereduced by anaerobic incubation at 37°C for at least 18 h. The standard bacterial inoculum was prepared from 24-h cultures by washing and adjusting cells by standard turbidimetric and plate counting methods to approximately 4×10^7 CFU/ml in sterile, prereduced anaerobe diluent (22). Assay tubes containing degassed test medium were inoculated with 0.1 ml of test bifidobacteria and incubated anaerobically at 37°C for 48 h. Following the 48-h incubation, A_{610}s were measured and cells were pelleted by centrifugation at $4,000 \times g$ for 10 min. Acidification of medium was measured as an index of bacterial growth by adding 2 ml of cell-free culture supernatant to 18 ml of distilled water and titrating the acid produced back to the starting pH of the growth medium (6.8) with 0.025 N NaOH. Results were expressed as units defined as the milliequivalents of NaOH required to neutralize the acid produced by the test strain per culture tube during a 48-h growth period. Control tubes were included in all experiments and consisted of 2.5 ml of Norris medium, 1.4 ml of saline, and 0.1 ml of test inoculum. Unless specifically indicated, all test samples were sterilized by membrane filtration (Gelman Sciences, Inc., Ann Arbor, Mich.) and tested in duplicate in at least two separate experiments.

Milk and infant formula samples. Samples of HM were obtained from the Mother's Milk Bank (Institute for Medical Research, San Jose, Calif.) and screened for growth promotion activity for *B. bifidum* serovar pennsylvanicus. Equal volumes of milk from three separate donors which showed high growth promotion activity were pooled and used to prepare casein and whey fractions or for comparison to CM or infant formulas in all assays, unless otherwise noted. Donors for this HM pool were in midlactation, not under any medication, and between 25 and 30 years of age at the time of donation. Bulk-tank raw CM used in all testing and fractionation procedures (not pasteurized unless otherwise indicated) was obtained from a commercial dairy establishment. All raw milk samples were kept frozen ($-75°C$) until needed. Samples of CM-based infant formulas, representing both whey-dominant (WF) and casein-dominant (CF) formulas, were obtained commercially or from the Mead Johnson Nutritional Group (Evansville, Ind.). All formula samples had a protein content of 1.5 g/dl and were sterilized by autoclaving before being tested in the growth promotion assay.

Milk fractionation procedures. Skim milk samples were prepared by centrifuging raw milk at $12,000 \times g$ for 30 min and filter sterilizing the resulting supernatant below the fat layer. Whey and casein fractions of HM and CM samples were prepared by conventional acid precipitation of casein as follows. The pH of skimmed milk was adjusted to 4.6 with 1.0 N HCl under constant stirring. The milk was permitted to stand at 37°C for 1 h before being centrifuged at $12,000 \times g$ for 30 min. The supernatant was readjusted to pH 6.8 and filter sterilized before testing. This fraction represented the acid whey fraction. The precipitated casein was suspended in saline with constant stirring for about 16 h. The acid casein solution was adjusted to a pH of 6.8 and filter sterilized. The whey fraction of HM and CM was also prepared by rennet (Chris Hansen's Laboratory, Inc., Milwaukee, Wis.) precipitation of casein. Human and cow skim milk were incubated with rennet (0.8 and 0.4 mg/dl, respectively) at 40°C for 2 h. Precipitated casein was pelleted at

$3,000 \times g$ for 30 min, and because this would not completely resolubilize, it was discarded. The supernatant was adjusted to pH 6.8, filter sterilized, and referred to as rennet whey. Bovine whey protein concentrate prepared by ultrafiltration (UF Whey) was purchased from Danmark Protein AS (Nr. Vium, Denmark). Electrodialyzed whey (ED Whey) and sodium caseinate were supplied by the Mead Johnson Nutritional Group (Evansville, Ind.). Both ED whey and UF whey were produced from rennet whey. Electrodialysis removed the majority of salts, and ultrafiltration reduced, primarily, the lactose content. Protein levels in samples of milk and milk fractions were determined by Kjeldahl nitrogen analysis.

Pasteurization of milk samples. Individual samples of HM or CM were skimmed as described above, filter sterilized, and pooled. Pooled milk samples were pasteurized by heating at 63°C for 30 min, followed by immersion in an ice bath. These conditions constitute minimal full pasteurization, which was expected to render HM or CM pathogen free with minimal loss in milk-borne host resistance factors (23).

Statistical methods. Growth promotion data were analyzed by a one-way analysis of variance by using the Statgraphics statistical software package (STSC, Inc., Rockville, Md.). Further comparisons among means were made by using a multiple range test based on confidence intervals.

RESULTS

Characterization of bifidobacterium growth response to HM. Incubation of *B. bifidum* serovar pennsylvanicus or *B. infantis* in Norris minimal medium containing HM revealed steady increases in both acid production and turbidity, which reached a plateau at about 48 h (Fig. 1). The pH of cell-free supernatants from these cultures decreased steadily to about 4.7 by 48 h and remained at this level for the remainder of the 72-h observation period. In contrast, viable-cell numbers increased to a maximum level by 24 h (0.5×10^9 to 1.0×10^9 CFU/ml) and steadily decreased for the remaining 48 h of incubation. Cell numbers of *B. infantis*, however, declined more rapidly than cell numbers of *B. bifidum* serovar pennsylvanicus between 24 and 72 h of incubation. In all subsequent experiments, acid production by 48 h was used as the marker of cell growth in the growth promotion assay.

Comparison of activities in HM and CM. Pooled HM obtained from three donors and bulk-tank CM obtained from a commercial source were tested for bifidobacterium growth promotion activity before and after pasteurization (Fig. 2). Sterile-filtered HM and CM samples were tested in the growth promotion assay at a final protein concentration of 2 mg/ml to allow a comparison of activity based on protein content, which is considerably higher in CM (3.5 g/dl [10]) than in HM (1.0 g/dl [17]). HM promoted good growth of *B. bifidum* serovar pennsylvanicus, while CM was inactive ($P < 0.05$). The growth promotion activity of HM was also greater than that of CM for *B. longum* but was comparable for *B. bifidum*, *B. infantis*, and *B. breve*. Pasteurization of HM and CM under conditions simulating routine pasteurization of banked HM resulted in statistically ($P < 0.05$) greater growth promotion activity for *B. bifidum* serovar pennsylvanicus and *B. bifidum* (Fig. 2). Pasteurization of CM, but not HM, also increased the growth of *B. longum*. Pasteurization reduced the activity of HM and CM for *B. infantis* ($P < 0.05$), while the growth of *B. breve* was not affected (Fig. 2).
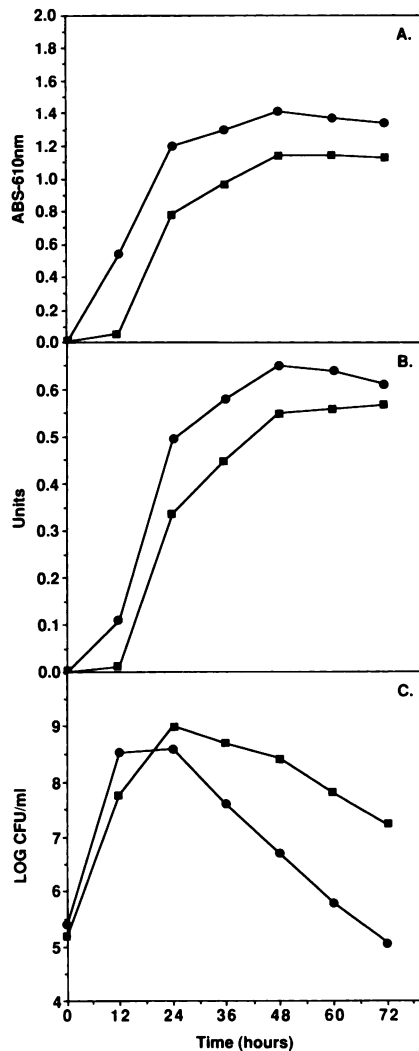
FIG. 1. In vitro growth promotion of *B. bifidum* serovar pennsylvanicus (■) and *B. infantis* (●) by HM, as determined by A_{610} (A), acid production in milliequivalents of NaOH (B), and viable-cell count in CFU per milliliter (C).
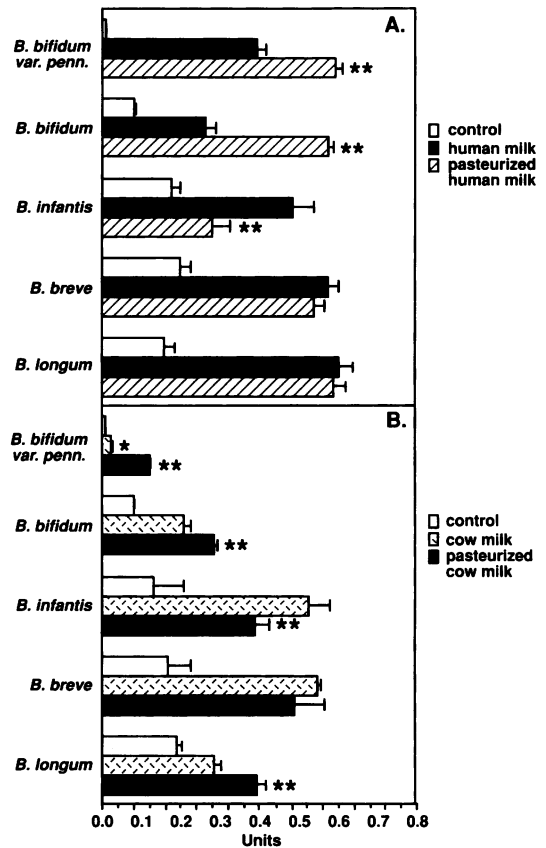

FIG. 2. Growth promotion activities of pasteurized and unpasteurized HM (A) and CM (B) for five human strains of *Bifidobacterium* species. Units are defined as milliequivalents of NaOH. The data shown represent the means ± standard deviations of at least quadruplicate samples. *, Not significantly different from saline control ($P > 0.05$); **, $P < 0.05$ compared with unpasteurized milk.

Distribution of activity among whey and casein fractions. Whey and casein fractions were prepared from HM and CM samples and tested for bifidobacterium growth promotion activity (Table 1). Growth promotion activity of HM casein was significantly greater ($P < 0.05$) than that of CM casein when compared on an equivalent protein basis for *B. bifidum* serovar pennsylvanicus, *B. bifidum*, *B. infantis*, and *B. breve*. The activity of CM casein was not significantly different ($P > 0.05$) from that of saline controls for four of the five test strains, while HM casein was inactive for only *B. longum*. Most of the growth promotion activity of HM and CM was associated with the whey fraction (Table 1). The activity of both rennet whey and acid whey from HM was greater than that of HM casein for three species of bifidobacteria. HM casein, however, promoted better growth of *B. bifidum* than HM whey ($P < 0.01$). CM whey promoted significantly greater ($P < 0.05$) growth of all five test strains of bifidobacteria than CM casein. HM whey was more active than CM whey for two strains ($P < 0.01$), equally active for two strains, and less active than CM whey ($P < 0.01$) for one strain of test bifidobacteria.

The relative ranking of activity for experimental CM whey and casein fractions (Table 1) was also found for two CM whey products and sodium caseinate obtained from commercial sources (Table 2). Sodium caseinate prepared by acid precipitation of casein from skim milk was inactive for four of five strains of bifidobacteria. ED whey and UF whey were significantly ($P < 0.01$) more active than sodium caseinate for four of five and three of five test strains of bifidobacteria, respectively, when compared on an equivalent protein basis. ED whey was more active than UF whey for *B. bifidum* serovar pennsylvanicus and *B. bifidum*.

Comparison of activities in CM-based infant formulas. WF and CF CM-based infant formulas were also compared for bifidobacterium growth promotion activity. HM was included as the whey protein-dominant reference standard in all formula comparisons. The results are shown in Fig. 3. WF formulas and HM were significantly more active ($P < 0.01$) than CF formulas in promoting the growth of *B. bifidum* serovar pennsylvanicus and *B. bifidum*. WF no. 2 formula was significantly more active ($P < 0.05$) than both the CF no. 1 and CF no. 2 formulas for *B. infantis*. The activities of HM and WF no. 1 formula for *B. infantis* were significantly greater ($P < 0.05$) than that of CF no. 2 formula, but not CF no. 1 formula. HM and all test formulas stimulated the growth of *B. breve* and *B. longum* equally.

TABLE 1. Growth promotion activity of whey and casein fractions from HM and CM for *Bifidobacterium* spp. isolated from human infants

Strain	Growth promotion activity (U)[a]						
	Saline	HM			CM		
		Acid casein	Rennet whey	Acid whey	Acid casein	Rennet whey	Acid whey
B. bifidum serovar pennsylvanicus	0.016 ± 0.001	0.366 ± 0.088[b]	0.602 ± 0.083[c,d]	0.495 ± 0.048[c,d]	0.014 ± 0.001[e]	0.414 ± 0.005[b]	0.442 ± 0.015[b]
B. bifidum	0.116 ± 0.009	0.472 ± 0.003[b]	0.014 ± 0.002[c,d]	0.227 ± 0.111[c,d]	0.167 ± 0.005[e]	0.670 ± 0.035[b]	0.574 ± 0.039[b]
B. infantis	0.067 ± 0.036	0.187 ± 0.066[f]	0.608 ± 0.006[c]	0.643 ± 0.014[c]	0.102 ± 0.031[e]	0.646 ± 0.036[b]	0.646 ± 0.062[b]
B. breve	0.366 ± 0.021	0.545 ± 0.009[b]	0.732 ± 0.005[c,d]	0.745 ± 0.004[c,d]	0.480 ± 0.009	0.685 ± 0.010[b]	0.702 ± 0.013[b]
B. longum	0.147 ± 0.089	0.219 ± 0.240[e]	0.525 ± 0.223	0.666 ± 0.106	0.122 ± 0.064[e]	0.574 ± 0.083[b]	0.540 ± 0.114[f]

[a] Units defined as the amount of NaOH required to neutralize the acid produced per 4-ml culture after a 48-h incubation with indicated sample at 2 mg/ml. Results are given as means ± standard deviations of at least quadruplicate samples.
[b] Greater than CM casein ($P < 0.01$).
[c] Different from HM casein ($P < 0.01$).
[d] Different from both rennet whey and acid whey from CM ($P < 0.05$).
[e] Not significantly different from saline control.
[f] Greater than CM casein ($P < 0.05$).

DISCUSSION

In this study, we found that both HM and CM are potent growth promoters for several species of bifidobacteria commonly found in stools of infants. Standard assay cultures showed increases in turbidity, viable-cell numbers, and accumulation of acid through 24 h of incubation. However, acid production by 48 h was chosen over increases in optical density as the assay indicator of cell growth, since certain test milk samples made the growth media highly turbid before incubation. Likewise, viable-cell number in incubated cultures was not a reliable indicator of growth promotion, since certain strains of bifidobacteria showed a more rapid decrease in CFU per milliliter after 24 h of incubation, which appeared to be due to a decrease in medium pH and not clumping or branching of individual cells (data not shown).

Results from this study and previous studies have shown that HM contains highly active growth promoters for *B. bifidum* serovar pennsylvanicus, while CM is inactive (4, 11). While the reason for this difference is still unclear, much of the activity of HM has been attributed to HM oligosaccharides that contain *N*-acetyl-D-glucosamine. This serovar, however, is a rare component of the stool of infants and may not accurately reflect the biochemical response of the genus *Bifidobacterium* to growth factors in milk or colostrum (26). Only recently have investigators used multiple strains of bifidobacteria from infants in the search for bifidobacterium growth promoters (1, 2, 24). The species of bifidobacteria most commonly found in previous studies to be the predominant strain in stools of breast-fed infants are *B. bifidum*, *B. infantis*, and *B. breve* (2, 3; reviewed in reference 26). We

found that HM and CM, when compared at equivalent protein levels, promoted the growth of *B. bifidum*, *B. infantis*, and *B. breve* equally well. HM was superior to CM in promoting the growth of *B. longum*, as well as *B. bifidum* serovar pennsylvanicus.

In testing whey and casein fractions from HM and CM for growth promotion activity, we found that HM whey and CM whey were both highly active, HM casein was only slightly active, and CM casein was nearly inactive, when compared on an equivalent-protein basis. Only *B. bifidum* showed a greater growth response to HM casein than HM whey. Poch and Bezkorovainy (24) also found that HM whey was not an exceptionally potent growth promoter for *B. bifidum*. Commercial whey and casein samples used in the manufacture of CM-based infant formulas also showed high activity in whey but not in casein protein samples. The whey-associated activity was not caused by the lactose content of whey from either HM or CM (data not shown). Virtually identical results were obtained with acid whey and rennet whey, suggesting that active factors either survived or were generated regardless of whether casein was precipitated by acid or rennet treatment. Azuma et al. reported that treatment of HM κ-casein with rennet or trypsin generated a glycomacropeptide with potent growth promotion activity for *B. infantis* (1).

Together, these data suggest that bifidobacterium growth promotion activity in HM was associated with both casein and whey constituents, while only whey factors were active in CM. However, it is possible that native CM casein is active but loses activity after acid precipitation. Bovine

TABLE 2. Bifidobacterium growth promotion activity of commercial CM whey and casein products

Strain	Growth promotion activity (U)[a]			
	Saline	Sodium caseinate	ED whey	UF whey
B. bifidum serovar pennsylvanicus	0.015 ± 0.002	0.011 ± 0.001[b]	0.442 ± 0.024[c,d]	0.025 ± 0.011
B. bifidum	0.121 ± 0.009	0.194 ± 0.007	0.657 ± 0.012[c,d]	0.616 ± 0.021[c]
B. infantis	0.141 ± 0.116	0.158 ± 0.039[b]	0.734 ± 0.003[c]	0.640 ± 0.039[c]
B. breve	0.196 ± 0.182	0.194 ± 0.026[b]	0.725 ± 0.009[c]	0.728 ± 0.032[c]
B. longum	0.179 ± 0.071	0.261 ± 0.050[b]	0.441 ± 0.099	0.391 ± 0.111

[a] Units are as defined in Table 1. Results are given as means ± standard deviations of at least quadruplicate samples.
[b] Not significantly different from saline control.
[c] Greater than sodium caseinate ($P < 0.01$).
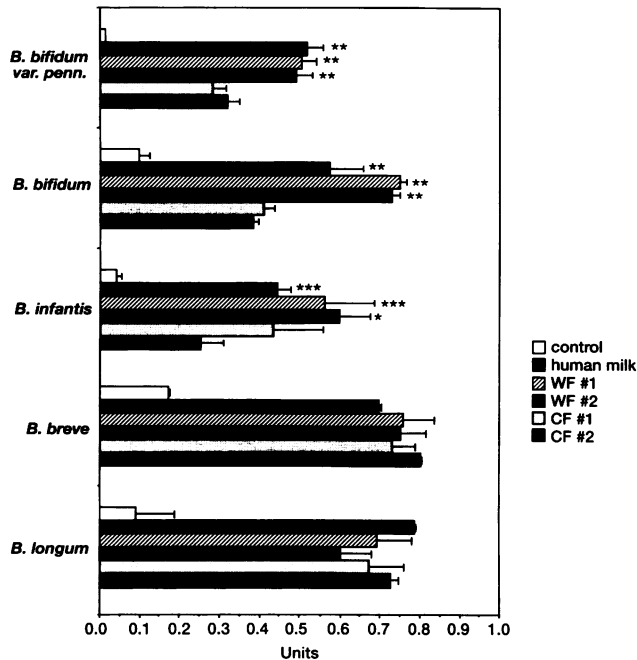[d] Greater than UF whey ($P < 0.01$)

FIG. 3. Comparison of growth promotion activities of HM and CM-based infant formulas for five strains of bifidobacteria. The whey-to-casein protein ratios of test samples of HM, WF (WF#1 and WF#2), and CF (CF#1 and CF#2) formulas were 70:30, 60:40, and 20:80, respectively. HM and formula samples were tested at equal volumes (1.0 ml) in the standard growth promotion assay. The data shown represent the means ± standard deviations of at least quadruplicate samples. *, $P < 0.05$ compared with CF no. 1 and CF no. 2 formulas; **, $P < 0.01$ compared with CF no. 1 and CF no. 2 formulas; ***, $P < 0.05$ compared with CF no. 2 formula.

casein has been reported to acquire growth promotion activity after extensive hydrolysis with proteolytic enzymes (14). We compared growth promotion activities of milks after normalizing for protein content, since protein levels were much higher in CM than HM. The lack of activity in CM, despite high activity in CM whey samples for *B. bifidum* serovar pennsylvanicus and *B. longum*, could be due to reduced factor levels as a result of CM dilution to normalize to HM protein levels. On the other hand, whey factors in CM were highly active at low concentrations for *B. bifidum*, *B. infantis*, and *B. breve*, since CM was as active as HM despite testing at about threefold-lower whey protein levels. It should also be recognized that these results were based on activity in pooled CM from many donors (bulk tank) and HM obtained from only three donors. The variability in activity of individual HM samples for multiple species of bifidobacteria has yet to be defined.

Higher growth promotion activity of CM whey over CM casein for infant strains of bifidobacteria was also found in a comparison of WF and CF CM-based infant formulas. Both HM and WF formulas promoted significantly better growth of *B. bifidum* serovar pennsylvanicus, *B. bifidum*, and *B. infantis* than CF formulas. Growth promoters in CM are heat stable, since infant formulas are subjected to numerous heat treatments during processing and samples were autoclaved before being tested in our growth promotion assay. Furthermore, growth promotion activity in HM and CM survived pasteurization for four of five test strains of bifidobacteria. Similar results have been shown by Beerens et al. (2). In other experiments not reported here, we found that auto-

claved HM and CM were inactive for *B. infantis*, slightly active for *B. bifidum* and *B. bifidum* serovar pennsylvanicus, and unchanged for *B. breve* and *B. longum*. The nature of the change in growth promotion activity in milks caused by heat is unknown.

Most studies agree that bifidobacteria dominate the fecal flora of most babies once the initial flora becomes established (3, 19, 20, 28). The composition of the relatively simple intestinal flora of infants, unlike that of adults, appears to be highly susceptible to alteration by diet. For example, introduction of CM or milk-based infant formula into the diet of an exclusively breast-fed infant usually results in a substantial increase in numbers of *Clostridium* spp., *Bacteroides* spp., and *Escherichia coli* (3, 16, 20, 27, 28). The unique intestinal flora of the breast-fed infant is characterized by a predominance of *Bifidobacterium* sp. and may represent a natural defense system that contributes to the lower incidence of intestinal infections in breast-fed infants (13, 18, 25). This report demonstrates that both HM and CM-based infant formulas contain heat-stable factors primarily associated with whey that promote the growth of strains of bifidobacteria commonly found in infants. Increasing the whey-to-casein ratio of CM-based formulas resulted in an increase of bifidobacterium growth promotion activity to levels found in HM. The identities of whey-associated factors responsible for the bifidobacterium growth promotion activity in HM and CM are as yet unknown.

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