

## **The effect of different milks and milk proteins on the growth of *Bifidobacterium infantis* ATCC 27920 *in vitro***

**Loh SP and Maznah 1**

*Department of Nutrition and Health Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor Darul Ehsan.*

### **ABSTRACT**

Bifidobacteria is a well known bacteria that is found in abundance in the intestine of infants which provides several health and nutritional benefits. Realizing the many benefits of bifidobacteria to human, this study has been conducted with the objective to determine the growth promotional effect of different types of milk and milk proteins on *Bifidobacterium* species. One strains of *Bifidobacterium* species that is *B. infantis* was used to study the growth promoting effect of human milk, cow's milk, goat's milk, milk based infant formula, soy-based infant formula, lactoferrin (1 mg/ml), lactoperoxidase (1p~g/ml), lysozyme (1 mg/ml) and the mixture of these three proteins. The growth promotion assay was done using the 96-well culture plates which consists of 200 (1 Trypticase-Peptone-Yeast extract (TPY) medium, 50  $\mu$ l sample and 10  $\mu$ l of bacteria inoculum. Control consists of PBS instead of the samples. The assay was incubated anaerobically at 37<sup>0</sup>C for 18 hours before being spread on the agar plate containing TPY medium with agar. Comparison was made between the mean count (log cfu/ml) of different types of milks, between infant formula and between milk proteins. From the results, Oneway ANOVA test at P<0.05 showed that there was significant differences in the mean counts (log cfu/ml) between the milks ( $P = 0.0000$ ). A similar trend was observed in the mean count (log cfu/ml) between the infant formulas ( $P = 0.0124$ ) and also between the milk proteins ( $P = 0.0005$ ). Duncan Multiple Range tests showed that there was significant differences between all the milks and control and among the milks themselves. There was however, no significant difference among the two types of infant formulas. The milk proteins also showed significant differences between the proteins and control and among themselves except for lysozyme which showed no significant differences with lactoferrin. This study showed that the growth of *B. infantis* could be promoted by different kinds of milks and milk proteins *in vitro*. Comparing the differences in growth promoting effect between samples and control indicated that human milk has the highest growth promoting effect

followed by cow's milk and the mixture of the three milk proteins. Lysozyme showed the lowest in terms of differences in percentage of growth promoting effect among all these samples. In conclusion the findings of this study supported that human milk is the best milk choice for infant in comparison to other types of milk in promoting the growth of bifidobacteria. In addition, this study also found that milk protein when used in combination may show better growth promoting effect than when used singly.

## INTRODUCTION

The type of milk fed to newborn infants greatly influences the physiochemical and microbiological condition of the infants' guts. Since the discovery of bifidobacteria by Tissier in 1899, there has been an almost general agreement that the faecal flora of breast-fed infants is dominated by Gram-positive non-spore forming rods, mainly bifidobacteria. Many studies have supported this statement and that breast-fed infants do experience fewer episodes of diarrheal illness than infants who are given cow's milk or infant formula (Heine *et al.*, 1992; Beerens *et al.*, 1980; Cunningham, 1979). These 'probiotic effects' are generally related to inhibition of pathogenic species (antibacterial actions), improve protein and vitamin synthesis especially vitamin B complex, antitumorigenic activity especially reducing the risk of colorectal cancer, increasing the immune response and lowering plasma cholesterol (Beeno *et al.*, 1984; Stark & Lee, 1982; Balmer & Wharton, 1989; Kleessen *et al.*, 1995). Although some of these effects do not seem to be important during infancy but may be beneficial in the long term wise. Studies using *B. bifidum* serovar *pennsylvanicus* in its growth promoting

factors have suggested the existence of bifidogenic growth promoter present in human milk which may largely be contributed by N-acetylglucosamine-containing oligosaccharides as well as glycoproteins.

Most of the studies were performed using infant formulas that consisted of cows' milk with minimal modification, whereas cows' milk is now extensively modified in the manufacture of an infant formula. There is also a lot of soy-based infant formula coming up in the market. So currently there have been countless efforts on the part of the formula industry to achieve a closer adaptation to the composition of human milk. This involves modification of the content of bovine milk protein, adjustment of fatty acid composition, addition of immunoglobulin concentrates, lactoferrin, lysozyme and including attempts to imitate its bifidogenic effect (Heine *et al.*, 1992; Dubey & Mistry, 1996). Besides that, dairy products especially cow's milk and goats' milk are on the rise in the market, however little information is known of their health benefits especially their growth promoting effects on bifidobacteria. In comparison to cow's milk, less is known about the presence of bifidogenic factors in goat's milk. This study attempts to

determine the growth promotion of *Bifidobacterium infantis* by different types of milk and whey proteins.

## MATERIALS AND METHOD

### Strains and cultivation

*Bifidobacterium infantis* ATCC 27920 used in this study was purchased from the American Type Culture Collection (ATCC, Rockville, MD). Working culture was propagated, by weekly transfer, in TPY medium containing (Per liter of distilled water, pH 6.5) trypticase peptone (BBL, Bectone Dickinson, Cockeysville, MD), 10.0 g; phytone peptone (BBL), 5.0 g; glucose (BDH Chemicals Ltd., Poole, England) 5.0 g; yeast extract (BBL), 2.5 g; Tween 80 (BDH), 1.0 ml; L-cysteine-HCl (Sigma, St. Louis, MO), 0.5 g; K<sub>2</sub>HPO<sub>4</sub> (BDH), 2.0 g; MgCl<sub>2</sub>·6H<sub>2</sub>O (BDH), 0.5 g; ZnSO<sub>4</sub>·7H<sub>2</sub>O (BDH), 0.25 g; CaCl<sub>2</sub> (BDH), 0.15 g and traces of FeCl<sub>3</sub> (AJAX). Active cultures were grown in anaerobic jars (GasPak; BBL) at 37°C for 24 h without agitation. For solid media, 15.0 g agar was added to the TPY medium. Inoculated plates were incubated anaerobically for 48 h at 37°C.

### Milks and milk proteins

Samples of mature human milk were obtained from a healthy donor, which were kept at - 20°C until the study was carried out. Powdered commercial infant formula of cow's milk-based and soy-based from the same manufacturer (Mead Johnsons, Indiana, USA) were used. Fresh goat's milk and cow's milk were obtained from the Faculty of Veterinary Medicine, Universiti Putra Malaysia. All milk

proteins (lactoferrin, lactoperoxidase and lysozyme) were purchased from Sigma. All of the proteins were sourced from human milk except for lactoperoxidase from bovine milk. All types of milk except for infant formula both of milk-based and soy-based were pasteurized before being used at 72°C for 20 seconds. After pasteurization, the milks were cooled and later kept at - 20°C until future analysis was conducted. The milk proteins namely lactoferrin, lactoperoxidase and lysozyme were mixed with Phosphate Buffered Saline (PBS) in order to obtain a concentration of 1 mg/ml, 1 µg/ml and 1 mg/ml respectively that is similar to the level present in mature human milk.

### Bifidobacterium growth promotion assay

The assay for measuring *bifidobacterium* growth promotion activity in various samples was based in part on the method of Nonnecke and Smith (1984). Bacterium inoculum and basal medium were prepared in an identical fashion for all studies. The physical support for the assay system was provided by a 120 x 80 mm tissue culture plate with 96 U-shaped wells (Nunc-Immuno Plate, MaxiSorp™, Nunc InterMed, Denmark). Of the 300 µl capacity of each well, 200 µl was allotted to growth medium (TPY medium), 50 µl for the addition of factors (human milk, cow's milk, goat's milk, infant formulas, lactoferrin, lactoperoxidase and lysozyme) to be determined for bacterial growth promotion and 10 µl for the bacterial inoculum which was obtained from the optimum dilution.

The *B. in* used in this experiment was an overnight culture and further diluted according to the optimum dilution obtained from the result of optimization of growth promotion assay. The concentration of lactoferrin, lactoperoxidase and lysozyme in the well as reported earlier were 1 mg/ml, 1 µg/ml and 1 mg/ml respectively. Controls consisted of 50 µl of diluent consisting of PBS. The prepared plate was covered with aluminum foil and incubated anaerobically according to the optimum incubation period of 37°C. After the incubation period, 200 µl was pipetted out and diluted in 1.8 ml of MRD. This was done in several dilutions, and for each dilution, 0.1 ml was pipetted and spread on the agar plate containing TPY medium with added agar. Each dilution was tested in duplicates. Inoculated plates were then incubated anaerobically for 48 hours at 37°C. After 48 hours of incubation, colonies were counted. Each of the samples was tested in duplicate in at least two separate experiments.

### Statistical methods

Colonies counted was converted to log cfu/ml. Data were analyzed using Statistical Packages for Social Sciences (SPSS Ver. 7.5). Oneway ANOVA was used to determine whether there were significant growth promotion by milks, infant formula and milk proteins at level of  $P < 0.05$ . Duncan Multiple Range Tests with significant level of 0.05 was used to test the significant differences of growth promotion among the milk samples, infant formulas and milk proteins.

## RESULTS

### Effect of different types of milk on the growth of *B. infantis* ATCC 27920

The number of colonies for the *B. infantis* was dependent on the type of milk used. For milks, the highest mean counts was human milk that was 9.80 log cfu/ml followed by cow's milk, 9.68 log cfu/ml and goat's milk, 9.23 log cfu/ml. The average mean count for infant formula milk-based was higher (9.94 log cfu/ml) than that of infant formula soy-based (9.86 log cfu/ml). The average mean count was highest for combination of the three proteins that was 10.14 log cfu/ml, followed by lactoferrin (9.92 log cfu/ml), lactoperoxidase (9.86 log cfu/ml) and lysozyme (9.66 log cfu/ml).

### Comparison of growth promoting effects of different types of milk

Oneway ANOVA was done ( $F < 0.05$ ) to compare the growth promotion effect between the different types of milk. The result showed that at the level of  $P < 0.05$ , there was significant difference in the mean count (log cfu/ml) of human milk, cow's milk, goat's milk and control. There were also significant differences in the mean count (log cfu/ml) of infant formula milk-based, infant formula soy-based and control. Significant differences were also found between lactoferrin, lactoperoxidase, lysozyme, combination of the three proteins and the control. Duncan's Multiple Range Tests indicated there were significant differences in the mean count (log cfu/ml) between the samples tested at significant level of 0.05 (Table 1).

**Table 1.** Result of Duncan's multiple range tests comparing different types of milk, infant formulas and milk proteins

| <i>Milks</i>     | <i>Infant Formulae</i> | <i>Milk Proteins</i> |
|------------------|------------------------|----------------------|
| HM <sup>a</sup>  | MB <sup>a</sup>        | LF <sup>a</sup>      |
| CM <sup>b</sup>  | SB <sup>a</sup>        | LP <sup>abc</sup>    |
| GM <sup>c</sup>  | CON <sup>b</sup>       | LYZ <sup>ce</sup>    |
| CON <sup>d</sup> |                        | COM <sup>d</sup>     |
|                  |                        | CON <sup>e</sup>     |

<sup>a,b,c,d,e</sup> Means in columns with no common superscripts within differ ( $P < 0.05$ )

Most of the samples showed significant differences between each other except for infant formula milk-based and soy-based, lactoferrin and lactoperoxidase and lysozyme and control.

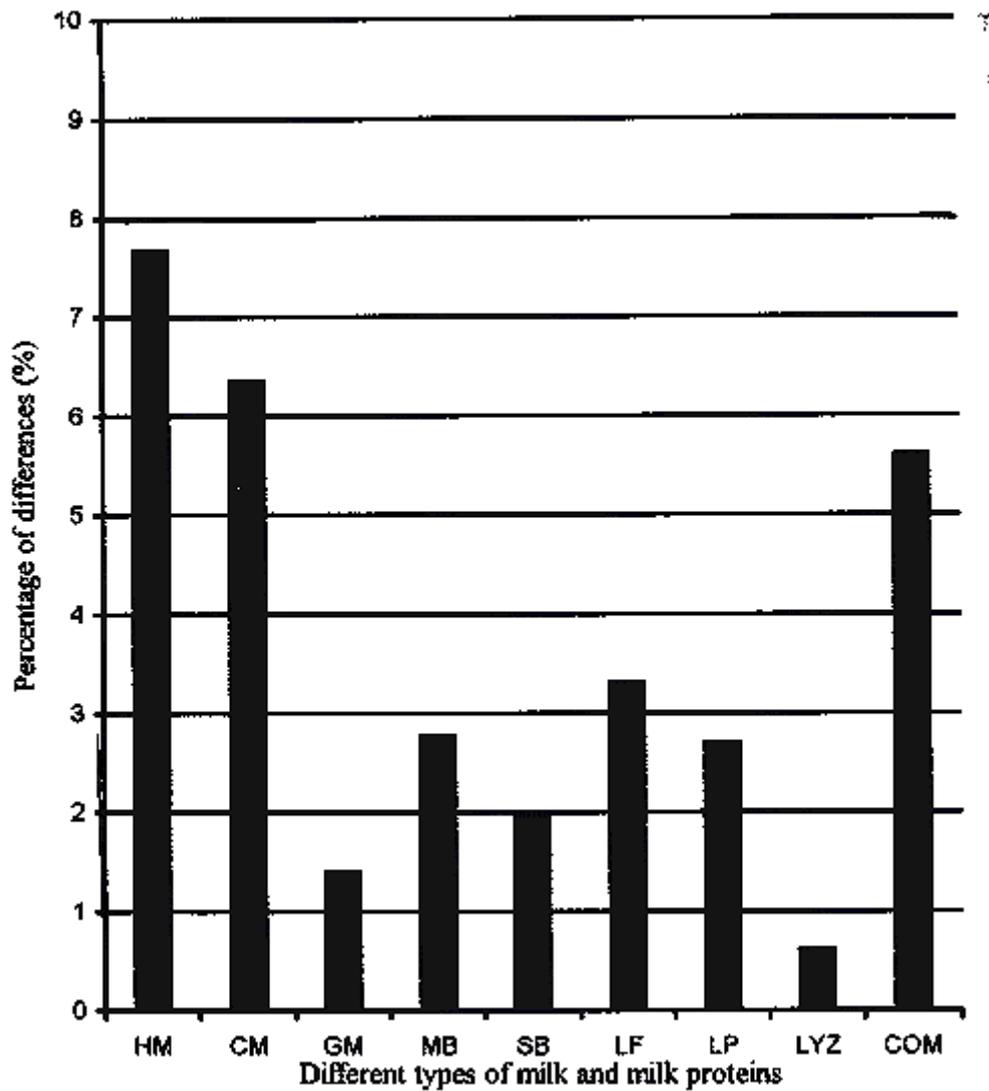
Comparing the differences in growth promoting effect between samples and control showed that human milk has the highest growth promoting effect (7.69%) followed by cow's milk (6.43%) and the mixture of the three proteins (5.63%). Lactoferrin showed a higher percentage of growth promoting effect (3.33%) compared to infant formula milk-based (2.79%) followed by lactoperoxidase and infant formula soy-based (1.97%). Lysozyme showed the lowest in term of differences in percentage of growth promoting effect (0.63%) among all these samples (Figure 1).

## DISCUSSION

All the different types of milk and milk proteins studied have a showed a significant promotive effect ( $P < 0.05$ ) on the growth of *B. infantis* ATCC 27920 compared with the control *except for lysozyme*. The reasons behind these

growth promotive effects may be attributed to certain compounds in these milks or milk proteins that can promote the growth of *B. infantis*. Growth of bifidobacteria is often thought to depend on the presence of growth factors called bifidus factors, whose presence in the intestine of breast-fed weanlings is held to be responsible for the predominance of bifidobacteria.

High growth promotive effects shown in this study by human milk and cow's milk is also consistent with the studies by Petschow and Talbott in 1990 which indicated that human milk and cow's milk are potent growth promoters for several species of bifidobacteria commonly found in stools of infants including *B. infantis*. Previous studies by several investigators have led to the conclusion that bifidobacteria growth promoters are present in human milk but absent in cow's milk, which consists of variety of NacGlu-containing oligosaccharides or glycoproteins (Beerens *et al.*, 1980; Poch & Bezkorovainy, 1988; Bezkorovainy & Topouzian, 1981). The reason for this difference is still unclear but much of the activity of



**Figure 1.** Percentage of differences in growth promoting effects of different types of milk and milk proteins compared with control.

HM = Human milk, CM = Cow's milk, GM = Goat's milk, MB = Infant formula milk-based, SB = Infant formula soy-based, LF = Lactoferrin, LP = Lactoperoxidase, LYZ = Lysozyme, and COM = Combination of the three proteins

human milk could be attributed to oligosaccharides in human milk that contains N-acetyl-D-glucosamine. However, conclusions made by Bezkorovainy and Topouzian, 1981; Poch and Bezkorovainy, 1988 were based primarily on the growth response of *B. bifidum* serovar *pennsylvanicus*. This species of bifidobacteria may not reflect accurately the biochemical response of genus *Bifidobacterium* on growth factors in milk or colostrum (Poupard *et al.*, 1973) because this model strain is unable to utilize glucose and requires D-glucosamine derivatives for cell wall synthesis.

The growth factors in human milk that promoted the growth of *B. infantis* in this study may not necessarily be due to only these oligosaccharides or glycoproteins. They may be due also to nonprotein nitrogen (NPN) factors (mol wt < 10 000) which comprised a heterogeneous group of low molecular weight, N-containing compounds (Petschow & Talbott, 1991). As for cow's milk, even though oligosaccharides that contain N-acetyl-D-glucosamine may not be found in cow's milk as indicated by Beerens *et al.*, (1980); Poch and Bezkorovainy, (1988), the result of this study however did show that cow's milk also promoted the growth of *B. infantis*.

Beerens *et al.*, (1980) also concluded from their studies that human milk factors favoring *B. longum* and *B. infantis* were destroyed by heat and their result were not in agreement with the result obtained from this study. Result from his study showed that even though human milk was pasteurized (72°C for 20 seconds)

however, the *B. infantis* was still able to grow. According to Packard, (1982), even minimal pasteurization or minimal heat treatment is believed to cause some loss in the component of milk. Some however, cites no loss due to pasteurization including the iron binding property of lactoferrin (Oria *et al.*, 1993). Thus, the end result of either pasteurization or even subminimal pasteurization treatment appears to be a mixed bag of surviving immune factors. As for the cow's milk, studies showed that only one strain of *B. infantis* was promoted by pasteurized cow's milk whereas other strains did not show any growth promoting effect (Beerens *et al.*, 1980). These results showed that the growth factors in cow's milk were stable to heat and storage at room temperature. In this study however, it is not known whether these proteins were destroyed because specific study was not done on them.

Goat's milk as found in this study could also significantly promote the growth of *B. infantis* and this is in agreement with the study done by Beerens *et al.*, 1980 and Krause *et al.*, 1996 which found that sheep and goat's milk possess growth-stimulating activity similar to cow's milk. Krause *et al.*, 1996 also found that human milk solids was at least sevenfold greater in growth promoting effect among rats versus all the other dietary treatment (cows and goats). They also found that cow's milk promoted the growth of bifidobacteria more than goat's milk. The result is in agreement with the result obtained in this study.

As for the infant formulas, the result of this study showed that both

types of infant formula namely milk-based and soy-based formula have significant difference ( $P < 0.05$ ) on the growth of *B. infantis*. However, there is no significant difference ( $P < 0.05$ ) among these two infant formulas meaning that both of them promoted the growth of *B. infantis* at the same level. It can be seen that milk-based infant formula have higher count compared with that obtained by Dubey and Mistry (1996) whereby the mean count for *B. infantis* was higher for soy-based compared with milk-based for the incubation period between 8 hours to 20 hours. The differences may be due to different brands or products used and therefore the component in the infant formula used could be different from one study to another. It may also be due to the protein level of soy-based used in this study which is lower than the level in milk-based. This may cause the result to be different from the study conducted by Dubey and Mistry (1996) which used soy-based formula with higher protein level than milk-based formula. Another study by Bullen et al., (1977) found that bifidobacteria grew better in milk that have lower protein and buffering capacity. So, in this case, if the results from Bullen's study could be applied then the result should show that milk-based could promote the growth more than soy-based formula.

All the milk proteins also showed a significant growth promotion effect for *B. infantis* ( $F < 0.005$ ) except for lysozyme. It has been shown that bovine lactoferrin is a potent growth promoter for different test strains bifidobacteria (Petschow and Talbott, 1991). Another evidence found by Hentges et al., (1992) *in vivo* and by

Roberts et al., (1992) is also in agreement with the result of this study. The addition of bovine lactoferrin, however, did not promote the growth of bifidobacteria in infants according to the study by Wharton and Balmer, (1992). They found that the addition of lactoferrin to the basic formula had little or no effect upon the faecal flora of babies fed both basic and added lactoferrin formula even though it has a very similar molecular structure to human lactoferrin which may have attracted different protein responses compared to bovine lactoferrin used in this study.

Lactoperoxidase showed a significant growth promotion ( $P < 0.05$ ) whereas lysozyme did not show a significant growth promotion of *B. infantis*. The active role of lactoperoxidase and lysozyme is not completely known. Lactoperoxidase and lysozyme are usually associated with antimicrobial system and have been suggested to exhibit bacteriostatic activities in the gastrointestinal tract of breast-fed babies. Their role as a growth promoter for bifidobacteria at present has not been studied. One *in vitro* experiment carried out by Heine et al., (1995) found that lysozyme of different origin have not exhibit any lysis of *Bifidobacterium* when compared to the decay of *Micrococcus luteus* affected by lysozyme. However, bacterial lysis became apparent after trypsin incubation of lysozyme-pretreated bifidobacteria.

As for the combination of the three proteins which showed the highest mean counts, the growth promotive effect of this might be due to the synergistic effect which was

achieved as these three proteins combine together. All of these proteins can be found in the whey component of the milk (Lonnerdal, 1985) and whey proteins are usually associated with growth promotion effect of human and cow's milk (Petschow & Talbott, 1991). The combination of these three proteins may collectively be responsible for the growth promotion of *B. infantis*.

In conclusion, this study has shown that human milk promoted the growth of *B. infantis* the most in comparison with control followed by cow's milk and the combination of the three proteins. Lysozyme on the other hand has the lowest promotive effect. As there have been studies done on the growth promotion effect of human milk (Petschow & Talbott, 1990; Balmer & Wharton, 1989) there is no doubt that human milk is more superior than other milks and milk proteins in its growth promotion effect. Results also showed that the result of the mixture of the three proteins is comparable to that of human milk and cow's milk. It is most like that the growth promotion factors of *B. infantis* in milks is due to the synergistic effect of a combination of certain component in the milk in particular these proteins. Despite extensive modification of cow's milk in the manufacture of modern infant formula, the faecal flora of bottle-fed babies' remains substantially different from that of breast fed babies. The effect of these could have been enhanced if the study was conducted in the environment that followed closely to that of the gastrointestinal environment.

## REFERENCES

- Balmer SE & Wharton BA (1989). Diet and faecal flora in the new born: breast milk and infant formula. *Arch Dis Child* 64:1672-1677
- Beerens H, Romond C & Neut C (1980). Influence of breast-feeding on the bifid flora of the newborn intestine. *Am J Clin Nutr* 33:2434-2439
- Beeno Y, Sawada K & Mitsuoka T (1984). The intestinal microflora of infants: Composition of faecal flora in breast-fed and bottle fed infants. *Microbiol Immunol* 28(9):975-986
- Bezkorovainy A & Topouzian N (1981). Bifidobacterium bifidus var pennsylvanicus growth promoting activity of human milk casein and its derivatives. *Int J Biochem* 13:585-590
- Bullen CL, Tearle PV & Stewart MG (1977). The effect of humanized' milks and supplemented breast feeding on the faecal flora of infants. *J Med Microbiol* 10(4):403-413
- Cunningham AS (1979). Morbidity in breast-fed and artificially fed infants II. *J pediatr* 95(5):685-689
- Dubey UK & Mistry VV (1996). Growth characteristics of bifidobacteria in infant formulas. *J Dairy Sci* 79:1146-1155
- Heine W, Braun OH, Mohr C & Leitzmann P (1995). Enhancement of Lysozyme Trypsin-mediated decay of intestinal bifidobacteria and lactobacilli. *J Pediatr*

*Gas troenterol Nutr* 21(1):54-58

Heine W, Mohr C & Wutzke KD (1992). Host-microflora correlations in infant nutrition. *Progress in Food and Nutrition Science* 16:18 1-197

Hentges Di, Marsh WW, Petschow BW, Thai WR & Carter MK (1992). Influence of infant diets on the ecology of the intestinal tract of human flora-associated mice. *J Ped tart Gas troenterol Nutr* 14:146-152

Kleessen B, Bunke H, Tovar K, Noack J & Sawatzki G (1995). Influence of two infant formulas and human milk on the development of the faecal flora in newborn infants. *Acta Faediatr* 84:1347-1356

Krause Li, Forsberg CW & O'Connor DL (1996). Feeding human milk to rats increases Bifidobacterium in the cecum and colon which correlates with enhanced folate status. *J Nutr* 126:1505-1511

Lonnerdal B (1985). Biochemistry and physiological function of human milk proteins. *Am J Clin Nutr* 42:1299-1317

Nonnecke BJ & Smith KL (1984). Inhibition of Mastitic bacteria by bovine milk Apo-Lactoferrin evaluated by in vitro microassay of bacterial growth. *J Dairy Sci* 67:606-613

Oria R, Maznah I, Sanchez L, Calco M & Brock JH (1993). Effect of heat treatment and other milk proteins on the interaction of lactoferrin with monocytes. *J Dairy Res* 60:363-369

Packard VS (1982). Human milk and infant formula. Academic Press, New York.

Petschow BW & Talbott RD (1990). Growth promotion of *Bifidobacterium* species by whey and casein fractions from human and bovine milk. *J Clin Microbiol* 28(2):287-292

Poch M & Bezkorovainy A (1988). Growth-enhancing supplements for various species of the genus *Bifidobacterium*. *J Dairy Sci* 71:32 14-322 1.

Poupard J, Husain I & Norris RF (1973). Biology of the Bifidobacteria. *Bacteriological Reviews* 37(2): 136-165.

Stark PL & Lee A (1982). The microbial ecology of the large bowel of breast-fed and formula-fed infants during the first year of life. *J Med Microbiol* 15:189-203

Wharton BA & Balmer SE (1992). Diet and faecal flora in the newborn. In *New Perspectives in Infant Nutrition*. B. Renner and G. Sawatzki (eds). Thieme medical Publishers, NY. Pg 89-94