Old Herborn University Seminar Monograph 4:
Host-microflora interactions in the first years after birth.
Editors: Peter J. Heidt, Rial D. Rolfe, Volker Rusch, and Dirk van der Waaij.
Institute for Microbiology and Biochemistry, Herborn-Dill, Germany: 33-40 (2002).

INFLUENCE OF INFANT DIETS ON THE ECOLOGY OF THE INTESTINAL TRACT OF HUMAN FLORA ASSOCIATED GNOTOBIOTIC MICE

DAVID J. HENTGES[1], WALLACE W. MARSH[1], BRYON W. PETSCHOW[2], WENDY R. THAL[1], and MELODY K. ADAMS[1]

[1]Department of Microbiology, Texas Tech University Health Sciences Center, Lubbock, TX 79430, USA, and [2]Bristol-Myers Company, U.S. Nutritional Group, Evansville, IN 47721, USA

SUMMARY

Germfree mice were associated orogastrically with predominant aerobic and anaerobic intestinal flora components isolated from the faeces of breast-fed human infants. The flora components colonised the intestines of mice and persisted at fixed population levels. Groups of flora associated mice received diets consisting of either human milk, cow's milk, Enfamil or Enfamil formula modifications exclusively for two weeks. The effects of the diets on small intestinal and caecal flora composition, caecal pH and resistance to intestinal colonisation with *Salmonella typhimurium* were then determined. Total populations of both aerobes and anaerobes were greatest in mice fed human milk. *Bifidobacterium* and *Bacteroides* were the predominant anaerobes and *Enterococcus* the predominant aerobe. Similarly, *Bifidobacterium* and *Enterococcus* predominated in the intestines of Enfamil-fed mice. Modification of the Enfamil formula did not have a significant effect on flora composition. In mice receiving cow's milk, however, *Bacteroides* and Gram-negative enteric bacteria were the predominant intestinal bacteria. The pH of caecal contents was lowest in mice consuming human milk and highest in mice consuming cow's milk. Titration of the diets demonstrated that human milk had the least buffering capacity and cow's milk the greatest. After orogastric challenge, mice consuming human milk were significantly more resistant to colonisation with *S. typhimurium* than mice consuming cow's milk, Enfamil or Enfamil formula modifications.

INTRODUCTION

A number of studies demonstrate that diet influences the composition of the intestinal flora of human infants. In breast-fed infants, bifidobacteria predominate whereas in formula-fed infants other anaerobes, in addition to bifidobacteria, and facultatively anaerobic bacteria are all present at high population levels (*Benno* et al., 1984; *Mevissen-Verhage* et al., 1985a, 1985b; *Stark* and *Lee*, 1982). The pH of the faeces of breast-fed infants, at a mean of 5.0 to 5.5, is considerably lower than the pH of the faeces of formula-fed infants, at a mean of 8.0 to 9.0 (*Bullen* et al., 1977; *Cooperstock* and *Zedd*, 1983;

Table 1: Composition of the inoculum given to mice

Bifidobacterium bifidum*	10^7
Bacteroides sp.	10^6
Clostridium bifermentans	10^4
Enterococcus faecalis	10^4
Escherichia coli	10^5
Staphylococcus epidermidis	10^2

*Streptomycin resistant variant

Willis et al., 1973).

The data indicate that human milk fa-vours multiplication of bifidobacteria in the intestine of infants. It contains a greater amount of lactose and has less buffering capacity than cow's milk (*Bullen* and *Willis*, 1971). The low intestinal pH that is induced by human milk, enhances multiplication of bifidobacteria (*Stark* and *Lee*, 1982) but specific growth promoting factors for bifidobacteria present in human milk may also be important (*Beerens* et al., 1980). Some researchers speculate that differences in intestinal flora composition account, in part, for the greater resistance of breast-fed infants to enteric infections compared to formula-fed infants (*Beerens* et al., 1980; *Kovar* et al., 1985). Bifidobacteria may contribute to this protective capacity by establishing an acetate buffer in the intestinal tract of breast-fed infants (*Bullen* and *Tearle*, 1976).

The purpose of this project is to examine the influence of various human infant diets on the ecology of the intestinal tract employing an experimental animal model. The composition of the intestinal flora of conventional animals is sufficiently different from that of the human infant to preclude the use of these animals for this purpose. We have therefore associated germfree mice with predominate flora components isolated from the faeces of breast-fed human infants and examined the influence of milk diets, infant formulas and formula modifications on the ecology of the intestinal tract.

MATERIALS AND METHODS

Flora analysis

Germfree BALB/c mice were housed in Trexler type isolators and were given sterile fortified rodent chow (Ralston Purina Co., St. Louis, MO) and sterile water *ad libitum*. Mice used in the experiments, weighing approximately 25 g, were randomly selected and transferred to another isolator where they were associated orogastrically with 0.1 ml of a suspension containing a mixture of flora components isolated from the faeces of breast-fed human infants (Table 1). They were then transferred to barrier isolators, which permitted exposure to laboratory personnel but not to other animals. In the barrier isolators the mice were separated into groups of five each, and were given sterile water and either human milk, cow's milk, Enfamil low iron formula (Mead Johnson Nutritionals, Evansville, IN) or a modification of the Enfamil formula exclusively for 14 days. Human milk was obtained from healthy volunteers who were not receiving antibiotics or other medications at the time of donation. Formula modification consisted of the addition of sterilised hog gastric mucin to

Table 2: Media used for isolation of intestinal anaerobic bacteria

Medium	Predominant organism isolated	Incubation time (days)
Brucella blood*	Total anaerobes	5
Bacteroides bile aesculin	Bacteroides	2
Cycloserine - mannose	Clostridium	2
Reinforced clostridial agar with streptomycin	Bifidobacterium	2

*Enriched with vitamin K

Enfamil at a concentration of 0.25 mg/ml (Enfamil with mucin) and the alteration of Enfamil to contain a high casein to whey protein ratio (Formula 3305) rather than the high whey protein to casein ratio present in Enfamil.

On day 14, the mice were removed from the barrier isolators, sacrificed by cervical dislocation, and introduced into an anaerobic chamber. Inside the chamber the small intestines and caeca of the mice were aseptically removed. The organs were weighed and then homogenised in 9 volumes of prereduced sterile 0.05% yeast extract. Serial 100-fold dilutions of the homogenates were plated on various prereduced selective anaerobic media (Table 2). The plates were incubated anaerobically at 37°C for 48 hours. The dilution series was removed from the anaerobic chamber and plated on various selective aerobic media (Table 3). These plates were incubated aerobically for 48 hours at 37°C. Colonies of aerobic and anaerobic organisms were counted and the organisms were identified by standard bacteriological procedures including Gram-staining and API analysis. Counts were reported as viable organisms per gram homogenate.

pH Analysis

On day 14 after flora association, mice were sacrificed by cervical dislocation and introduced into the anaerobic chamber. The caeca were exposed and a small incision made in the wall of each. A micro combination pH electrode (Microelectrodes Inc. Londonerry, NH) was inserted through the incision into the luminal contents. pH values were obtained using a Corning 125 Potentiometer.

Buffering Capacity

Twenty ml aliquots of human milk, cow's milk, Enfamil or Enfamil formula modifications were each titrated with 0.1N NaOH and 0.1N HCl. Titration curves were plotted, obtained by the addition of small increments of either acid or base. The pH values were determined using a combination electrode and a Corning 125 Potentiometer.

Table 3: Media used for isolation of intestinal aerobic bacteria

Medium	Predominant organism isolated	Incubation time (days)
Trypticase soy blood	Total aerobes	2
MacConkey	E. coli and other enterobacteria	1-2
Bile aesculin azide	Enterococcus	2

Table 4: Effect of diet on caecal flora composition

	Human milk	Cow's milk	Enfamil
Total anaerobes	10.65 ± 0.28[1]	10.03 ± 0.36	10.08 ± 0.21
Clostridium	4.79 ± 1.61 (4/5)[2]	4.33 ± 1.31 (4/5)	3.31 ± 0.21 (3/5)
Bacteroides	9.31 ± 0.54	8.71 ± 0.74	7.79 ± 1.15
Bifidobacterium	9.33 ± 0.50	7.89 ± 0.79	8.64 ± 0.61
Total aerobes	9.85 ± 0.19	9.06 ± 0.14[3]	9.12 ± 0.18[3]
Enterobacteriacae	8.74 ±0.32	8.69 ± 0.22	8.67 ± 0.21
Enterococcus	9.43 ± 0.21	7.89 ± 0.27[3]	8.79 ± 0.20

[1]Mean log 10 viable bacteria/gram ± SEM.
[2]Incidence of isolation of the organism from the mice; in all other cases it was 5/5.
[3]Statistically significant fewer counts ($p<0.05$) than from mice receiving human milk.

Colonisation Resistance

The effects of consumption of the various diets by the gnotobiotic mice on colonisation resistance against *Salmonella typhimurium* were determined. Fourteen days after association with human infant flora components (Table 1) five mice on each of the diets were challenged orogastrically with 0.1 ml of a suspension of 1.0×10^4 streptomycin resistant *S. typhimurium*. Three days after challenge, the mice were sacrificed by cervical dislocation and the small intestines and caeca were removed aseptically, were weighed and were homogenised individually in 9 volumes of 0.05% yeast extract. Serial 100-fold dilutions of the homogenates were plated on Mac Conkey's Agar containing 1mg/ml streptomycin sulphate, which is selective for the streptomycin-resistant *S. typhimurium* strain. The plates were incubated aerobically for 24 hours at 37°C. Colony counts were reported as viable organisms per gram homogenate. Each experiment was repeated twice.

Statistical Analysis

Statistical evaluations of the significance of the differences in viable bacterial counts obtained from intestinal homogenates and pH values of caecal contents were performed using Fisher's least significant difference test and the Duncan-Neuman-Keul test at the 95% confidence interval level.

RESULTS

Results of studies comparing the effects of exclusive consumption by the mice of human milk, cow's milk, Enfamil or Enfamil formula modifications on the ecology of the gastrointestinal tract will be described.

Initially, the effects of diets consisting of either human milk, cow's milk or Enfamil were examined. Flora analysis of caecal homogenates demonstrated that, with all diets, anaerobes outnumbered aerobes by a factor of approximately 10 to 1 and that bacterial populations were greater in mice consuming human milk than in mice consuming cow's milk or Enfamil (Table 4). In mice consuming human milk, *Bifidobacterium* and *Bacteroides* were the predominant anaerobes and *Enterococcus* was the predominant aerobe. Similarly, *Bifidobacterium* and *Enterococcus* predominated in the

Table 5: Effect of diet on small intestinal flora composition

	Human milk	Cow's milk	Enfamil
Total anaerobes	8.86 ± 0.10[1]	7.95 ± 0.43[3]	7.93 ± 0.12[3]
Clostridium	2.55 ± 1.25 (3/5)[2]	2.98 ± 1.04 (4/5)	1.96 ± 1.28 (3/5)
Bacteroides	7.39 ± 0.73	6.69 ± 0.43	5.68 ± 0.64
Bifidobacterium	7.17 ± 0.18	6.19 ± 0.52	6.85 ± 0.48
Total aerobes	8.39 ± 0.19	7.15 ± 0.16[3]	7.64 ± 0.10[3]
Enterobacteriacae	7.62 ± 0.18	6.59 ± 0.22[3]	6.80 ± 0.21[3]
Enterococcus	7.90 ± 0.15	6.56 ± 0.22[3]	7.14 ± 0.23[3]

[1]Mean log 10 viable bacteria/gram ± SEM.
[2]Incidence of isolation of the organism from the mice; in all other cases it was 5/5.
[3]Statistically significant fewer counts ($p<0.05$) than from mice receiving human milk.

intestinal tract of Enfamil-fed mice. *Bifidobacterium* populations were greatest in mice consuming human milk and smallest in mice consuming cow's milk, although the difference was not statistically significant. In mice consuming cow's milk, *Bacteroides* and Gram-negative enteric bacteria predominated. Similar results were obtained when small intestinal homogenates were analysed except that bacterial counts were approximately 100-fold lower than counts from caecal homogenates (Table 5). In several instances, counts obtained from mice consuming human milk were significantly greater than counts obtained from mice consuming either cow's milk or Enfamil.

Subsequent experiments were done examining the effects of modifications of the Enfamil formula on the composition of the intestinal flora of the mice. Controls with each experiment consisted of groups of mice consuming human milk and mice consuming Enfamil. Diets of Enfamil with mucin or Formula 3305 (high casein to whey protein ratio) had no effects on the composition of the flora. There were no statistically significant differences in counts of any of the bacteria isolated from the caecum or the small intestine of the mice consuming human milk, Enfamil, or Enfamil formula modifications in these experiments.

Table 6: Influence of diet on the pH of caecal contents

	Diet	pH
Study 1:	Human milk	5.99 ± 0.11
	Enfamil	6.88 ± 0.11[1]
	Cow's milk	7.16 ± 0.06[1]
Study 2:	Human milk	5.86 ± 0.09
	Enfamil	6.35 ± 0.14
	Enfamil with mucin	6.73 ± 0.23[1]
Study 3:	Human milk	6.20 ± 0.15
	Enfamil (liquid)	7.02 ± 0.11[1]
	Formula 3305	6.56 ± 0.22

[1]Statistically significantly greater values ($p<0.05$) than from mice consuming human milk.

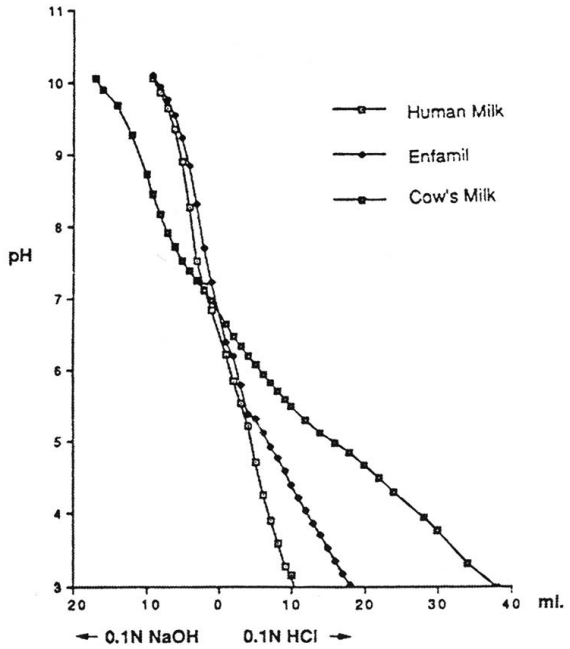

Figure 1: Titration curve of various diets.

The influence of the various diets on the pH of the caecal contents of the mice was also determined. Results are illustrated in Table 6. In all three studies, the mean pH of caecal contents of mice consuming human milk was lower than the pH of caecal contents of mice consuming the other diets. In several cases these differences were statistically significant. However, there were no significant differences in the pH of the contents between mice consuming cow's milk, Enfamil or Enfamil formula modifications. Contents of mice consuming cow's milk had the highest pH, which was more than one log10 greater than the pH of contents of mice consuming human milk.

Human milk had considerably less buffering capacity than cow's milk and somewhat less buffering capacity than Enfamil or Enfamil formula modifications. Figure 1 shows that approximately 7.0 ml of 0.1N HCl was required to lower the pH of human milk from 7.0 to 4.0 and 12.0 ml and 28.0 ml were required to lower than pH of Enfamil and cow's milk, respectively, to the same degree. Modifications of the Enfamil formula had no appreciable effect on its buffering capacity.

The colonisation resistance of the gnotobiotic mice on the various diets to challenge with *S. typhimurium* was determined next. Results are presented in Table 7. The table shows that there were significantly fewer *S. typhimurium* isolated from caecal homogenates of mice consuming human milk than mice consuming cow's milk, Enfamil or Enfamil formula modifications. However, counts obtained from mice consuming cow's milk were not significantly different from counts obtained from mice consuming Enfamil. Nor did modifications of the Enfamil formula significantly alter caecal *S. typhimurium* counts when compared with a diet of Enfamil.

Table 7: Influence of diet on population levels of *S. typhimurium* in the mouse caecum and the incidence of colonisation

	Diet	pH	
Study 1:	Human milk	5.48 ± 0.40	14/15
	Enfamil	6.55 ± 0.24[1]	15/15
	Cow's milk	6.71 ± 0.37[1]	15/15
Study 2:	Human milk	5.32 ± 0.44	13/13
	Enfamil	7.96 ± 0.39[1]	13/13
	Enfamil with mucin	7.60 ± 0.24[1]	15/15
Study 3:	Human milk	5.22 ± 0.52	4/5
	Enfamil (liquid)	8.45 ± 0.11[1]	5/5
	Formula 3305	8.83 ± 0.23[1]	5/5

[1]Statistically significantly greater values ($p<0.05$) than from mice consuming human milk.

DISCUSSION

These studies demonstrate that germfree mice can be associated successfully with intestinal flora components isolated from the faeces of breast-fed human infants. The flora components colonise the small and large intestines of the animals and persist at fixed population levels.

Dietary variations influenced the composition of the intestinal flora of the mice to a moderate degree, although differences in population levels of bacteria were usually not statistically significant. *Bifidobacterium*, generally, was the predominant anaerobe and *Enterococcus* the predominant aerobe isolated from mice consuming human milk or Enfamil. *Bacteroides* and Gram-negative enteric bacteria predominated, on the other hand, in the intestines of mice consuming cow's milk. These results are similar to those reported in other studies demonstrating the predominance of *Bifidobacterium* in the intestinal tract of human breast-fed infants (*Benno* et al., 1984; *Mevissen-Verhage* et al., 1985a, 1985b; *Stark* and *Lee*, 1982). When compared with a diet of Enfamil, Enfamil formula modifications had little effect on the composition of the intestinal flora of the mice.

A consistent finding in all of our studies was the lower pH of the caecal contents of mice consuming human milk than of mice consuming cow's milk, Enfamil or Enfamil formula modifications. This is in accord with the results of studies showing that the pH of the faeces of human breast-fed infants is lower than the pH of the faeces of formula-fed infants (*Bullen* et al., 1977; *Cooperstock* and *Zedd*, 1983; *Willis* et al., 1973). The reason for the lower pH of intestinal contents when human milk is consumed is unknown. However, we demonstrated in these studies that the buffering capacity of human milk is much less than the buffering capacity of cow's milk and somewhat less than the buffering capacity of the infant formulas. The pH of the intestinal contents may therefore be a reflection of the buffering capacities of the diets.

Human milk consumption by the mice provided significantly greater protection against colonisation with *S.*

typhimurium than consumption of cow's milk, Enfamil or Enfamil formula modifications. Greater protection may be a consequence of low intestinal pH which results in increased concentrations of undissociated fatty acid molecules that inhibit multiplication of *Salmonella* and other enteric pathogens (*Hentges*, 1983). Alternatively, it may be due to some other factor such as the presence of protective anti-*Salmonella* antibodies in human milk. Additional work needs to be done to determine the mechanisms responsible for the protection that is apparent when human milk is consumed.

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