Antimicrobial Activity of Breastmilk Against Common Pediatric Pathogens*

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INTRODUCTION

Infant feeding is an emotive subject and the slogan "Breast is Best" has been in use as a pious medical incantation for the past several decades. Advocates of breastfeeding have sometimes relied more on a personal belief in its naturalness, and therefore rightness, than on scientific evidence, and there has consequently been a widespread belief that artificial milk is just as good. Recently, however, evidence showing the unique character of human milk in infant nutrition has accumulated.[1]

Apart from biochemical and nutritional considerations, its anti-allergic properties, its effects on child spacing, economy and maternal-neonate bonding,[2,3] breastfeeding is advocated because of its anti infective properties, which are unavailable from other foodstuffs.[2]

Early epidemiologic studies reported that breastfed babies had lower incidence of both morbidity and mortality from infectious illnesses than did bottle-fed infants.[4,5] More recent reviews on breastfeeding in both developed and developing countries support these observations.[6-8] However, many of the studies showing health advantage for breastfed infants relied on anecdotal clinical experience, medical records and retrospective data.[9] The effect of feeding mode on infant health as measured by the number of illnesses appears not to be a simple cause-effect relationship. Definite proof of the protection from infection afforded by breast milk is lacking in some studies.[10-12] Taking into consideration confounding variables such as birth weight, maternal education, maternal smoking and social status, breastfeeding was found to have no significant association with rates of hospital admissions due to respiratory and gastrointestinal infections.[10-12] This was, however, challenged by other studies which demonstrated that breastfeeding reduces the incidence of hospital admissions for infection in infants 13 and offers a significant health advantage independent of socioeconomic status, family size, day-care exposure, infant birth weight, parental education and passive smoking.[14,15] Likewise, a study in Malaysia analyzed the protective effect of breastfeeding with correction of biases which could lead to overestimation of its benefits. When such factors were taken into account, the benefits from breastfeeding became stronger and a causal relationship between breastfeeding and improved survival of infants throughout the first year of life was established.[16]

The protective effect of breastfeeding takes a greater magnitude when its implication in reducing morbidity and mortality of infectious diseases, particularly in the Third World countries, is taken into consideration. Among the infectious diseases, diarrheal disease is one of the leading causes of morbidity and mortality causing an estimated five million deaths per year in children under five years of age, 80% of which occur in the first two years of life.[17] Enteropathogenic bacteria have been found to be inhibited by human milk when tested on in vitro assay systems.[4,18-22] However, the full spectrum of microbial agents inhibited by human milk remains to be defined.

This study was undertaken with the following objectives: (1) To demonstrate whether mature human milk exerts any inhibitory effect on bacterial growth using an in vitro assay system; and (2) To determine the extent of inhibition exerted by human milk against different enteric and non-enteric bacterial pathogens of infants and children using an in vitro assay system.

MATERIALS AND METHODS

After having secured informed written consent, samples of milk were collected from five nursing mothers beyond four weeks postpartum. All donors were in good health and had no clinical evidence of mastitis nor tuberculosis, delivered to healthy full-term infants, and with no intake of medications within one week prior to collection.

Following thorough hand washing and cleansing of the breast and nipple with soap and tap water, milk samples were expressed manually into sterile Erlenmeyer flasks, pooled, dispensed into sterile test tubes and frozen at -20^{O}C. A commercially prepared pre-modified cow's milk infant formula (Bonna; Wyeth-Suaco Laboratories) used as control was also dispensed in sterile test tubes properly labelled and frozen at -20°C. Milk samples were tested on in vitro system within the first 24 hours after collection. Prior to testing, each sample of milk was plated in culture media to rule out the presence of contamination.

A total of nine bacterial strains were obtained from the Bacteriology Section of our Pathology Laboratory. For the in vitro assay, organisms of an identified bacterial strain were harvested from a confluent!y streaked Petri dish and diluted in 5ml of brain heart infusion broth (or trypticase soy broth in the case of *Vibrio cholerae*) and allowed to stand for two hours until turbidity was noted. Using a calibrated wire loop (0.01 ml), 2 loopfuls from each of the broth was added to 1 ml of human milk or infant formula to make a concentration of 2×10^5 cfu per ml. Tenfold dilutions from both the milk and formula were made and 0.01 ml of such dilution was plated in duplicate onto pre-dried agar plates appropriate for growth of the test organisms, at 0 and after 4 h of incubation at 37^{O}C. The incubation period of 4 hours was chosen to approximate physiologic conditions in the feeding infant. The transit time through the small intestine is estimated to be between 1 and 5 h. Also, infants feed at 2 to 4 h intervals, providing the intestinal milieu with a continuous supply of milk or formula and their respective components. After 24 h of incubation, the colony count of each bacterial pathogen at the time of inoculation and at 4 h was determined using a Fisher Colony Counter. The colony count was converted to equivalent $\log_2$ values, and the number of bacterial doublings (or generation time, g) was calculated using the following formula: $g = \log N^4 - \log N_O/\log_2$ [34]

Human milk was deemed bactericidal when the 4 h colony count revealed a fourfold or greater decline (more than 2 negative doublings) as compared with the initial inoculum. It was deemed bacteriostatic if there was a less than fourfold decline (less than 2 negative doublings), but a less than fourfold increase (less than 2 positive doublings) at the 4 h count. If colony counts at 4 h demonstrated a greater than 4-fold increase (more than 2 positive doublings) of the initial inoculum, the medium was deemed non-inhibitory.

The in vitro assay test was repeated making a total of four determinations each for breast milk and infant formula at 0 and 4 h for each of the nine test organisms. The same sources of milk and the same bacterial species were used throughout the test.

The Wilcoxon rank sum test,[35] a non-parametric test, was used to determine whether there was a significant difference in the 4 h colony count of each bacteria in the 2 types of milk tested. A p value of less than or equal to 0.05 was considered significant.

RESULTS

C1inical isolates of nine bacterial species were tested, namely: *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa*, enteric isolates of *Escherichia coli* urinary isolates of *E. coli, Salmonella typhimurium, Enterobacter spp, Vibrio cholerae* Ogawa strain, and *Shigella flexneri* Except for *E. coli, Vibrio cholerae* and *Shigella flexneri*, the rest were isolated from the blood or cerebrospinal fluid.

Tables 1-2 show the mean colony count at 0 and at 4 h of the different test organisms in breast milk and infant formula. Applying the Wilcoxon rank sum test, there is a significant increase in the 4 h colony count of the following test organisms when applied in infant formula as against human milk: *P. aeruginosa*, enteric *E. coli*, salmonella, enterobacter, *Vibrio cholerae,* and shigella. Figures 1 and 2 summarize the results of the study.

Table 1. Mean Colony Count ($x10^2$) as indicated in parentheses at 0 hour and 4 hours incubation of the different organisms in human milk and infant formula

	First Trial			
	Breast		Formula	
Pathogen	0	4	0	4
1. S. aureus	20,64 (42)	22,46 (34)	35,43 (39)	24,25 (24)
2. S. epidermidis	44,44 (44)	33,51 (42)	77,69 (73)	123,94 (108)
3. P. aeruginosa	22,28 (25)	40,46 (43)	90,110 (100)	450,550(500)
4. E. coli (stool)	87,72 (79)	83,88 (85)	66,76 (71)	225,325 (275)
5. E. coli (urine)	170,180 (175)	200,210 (205)	120,200 (160)	600,700 (650)
6. Salmonella	150,110 (130)	140,200 (170)	100,150 (125)	450,572 (511)
7. Enterobacter	5,10 (7)	2,16 (9)	12,7 (9)	50,104 (77)
8. V. cholerae	1000,1000 (1000)	350,400 (375)	9,27 (18)	100,150 (125)
9. Shigella	45,42 (43)	9,12 (10)	65,70 (67)	350,260 (305)

Table 2. Mean Colony Counts (xl02) as indicated in parentheses at 0 hour and at 4 hours incubation of the different test organisms in human milk and infant formula

	First Trial			
	Breast		Formula	
Pathogen	0	4	0	4
1. S. aureus	145,230 (187)	163,150 (156)	102,85 (93)	151,81 (232)
2. S. epidermidis	103,151 (127)	94,82 (88)	97,60 (81)	400,600 (500)
3. P. aeruginosa	90,100 (95)	300,275 (287)	200,250 (225)	1000,1000 (1000)
4. E. coli (stool)	9,10 (9)	18,26 (22)	10,12 (11)	182,120 (151)
5. E. coli (urine)	10,8 (9)	400,450 (425)	58,56 (57)	450,500 (475)
6. Salmonella	350,80 (215)	62,300 (181)	170,190 (180)	600,800 (700)
7. Enterobacter	10,20 (15)	38,26 (32)	10,25 (17)	390,400 (395)
8. V. cholerae	1000,1000 (1000)	49,144 (96)	1000,1000 (1000)	3000,5000 (4000)
9. Shigella	75,72 (73)	41,15 (28)	70,72 (71)	450,500 (475)

It can be seen that human milk is uniformly bactericidal to *V. cholerae* among the bacterial species tested. Commercial milk is non-inhibitory to growth of vibrio. Shigella and salmonella were also inhibited uniformly by breast milk causing bacteriostasis. This effect was not demonstrated by commercial milk. *S. aureus* and *S. epidermidis* were inhibited by human milk although they occasionally showed static growth in commercial formula but t o lesser extent. Bacteriostasis in human milk but not in formula was noted among strains of gram-negative organisms isolated from blood like enterobacter and pseudomonas. Likewise, enteric isolates of *E. coli* were inhibited by breast milk in contrast to urinary tract isolates, which showed variable results.

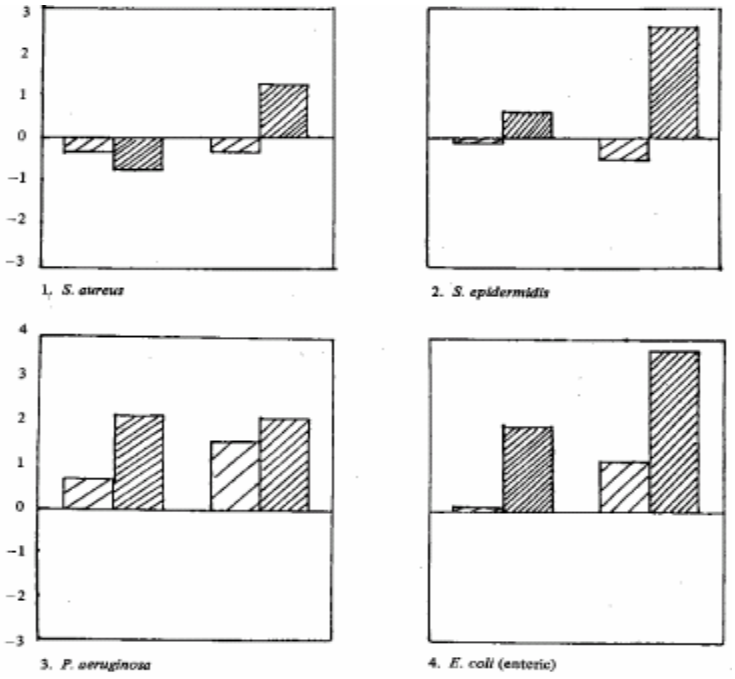

Figure 1. Growth (inhibition) of various potential pathogens in human milk (widely hatched columns) is compared with growth in a commercial infant feeding formula (narrowly hatched columns) and plotted as doublings (negative doublings = killings) per 4 hours of incubation.

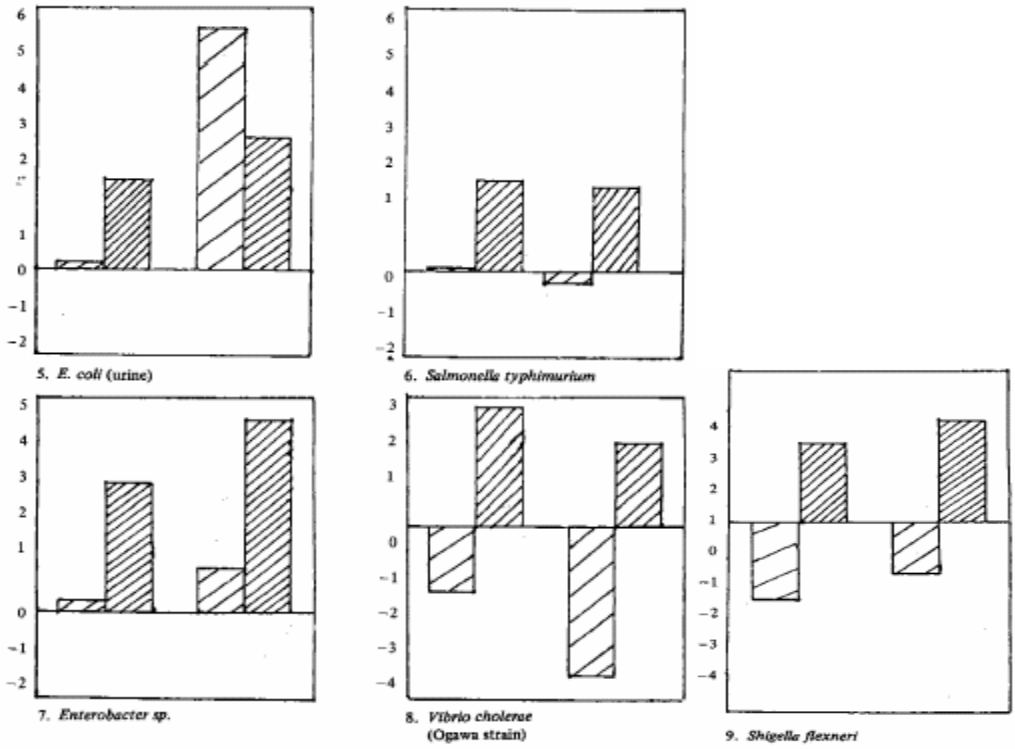

Figure 2. Growth (inhibition) of various potential pathogens in human milk (widely hatched columns) is compared with growth in a commercial infant feeding formula (narrowly hatched columns) and plotted as doublings (negative doublings = killings) per 4 hours of incubation at 37^{O}C.

Table 3. Generation time of the various test organisms expressed as number of doublings (negative doublings = killings) pet 4 hours of incubation at 37°C, in breast milk and infant formula.

	First Trial		Second Trial	
Pathogen	Breast	Formula	Breast	Formula
1. S. aureus	- 0.3	- 0.67	- 0.26	1.3
2. S. epidermidis	- 0.06	0.57	- 0.53	2.6
3. P. aeruginosa	0.78	2.3	1.6	2.159
4. E. coli (stool)	0.10	2.0	1.21	3.79
5. E. coli (urine)	0.22	2.02	5.5	3.06
6. Salmonella	0.116	2.1	- 0.2	2.07
7. Enterobacter	0.26	3.03	1.09	4.5
8. V. cholerae	- 1.42	2.8	- 3.38	2
9. Shigella	- 2.05	2.19	- 1.39	2.75

DISCUSSION

Our results showed that a broad spectrum of enteric and non-enteric bacterial pathogens was inhibited or killed by human milk in vitro. Both gram negative and positive organisms were inhibited in varying degrees.

Epidemiologic Considerations

All enteric pathogens were inhibited by human milk, proving the protective role of breast milk against gastrointestinal infections. This is confirmed by epidemiologic reports of decreased incidence and severity of diarrheal diseases among breastfed infants in the first six months of life. Enteric infections due to *E. coli* and shigella are rare in breast-fed infants unlike in artificially fed infants.[23,24] Svirsky and Groos suggested that this effect may be due not simply to a lower intake of bacterial pathogens, since there was evidence that *E. coli* enteritis could be treated successfully with human milk feedings.[23]

Despite poor hygienic conditions, shigellosis is rare in the first months of life in a semi-primitive breastfeeding culture of Central America. Mata and co-workers established that the relative lack of colonization of shigella and *E. coli* in the Mayan infants was due to breastfeeding. In the same Mayan culture, intestinal colonization of EPEC was low in breastfed infants despite the prevalence of carriers in the community and the presence of fecal bacteria in the maternal milk. In contrast, EPEC infections were frequent among urban infants of a similar ethnic background who were partly or wholly fed cow's milk.[23]

Likewise, in a case control study that assessed the effect of breastfeeding in reducing the severity of illness in shigellosis among Bangladeshi children, breastfeeding showed a high degree of protection in children up to 35 months of age, as well as for children at high risk for death due to severe malnutrition or measles.[25]

Host Resistance Factors

It is now recognized that there are many antibacterial factors in human milk that may be responsible for its protective function.[2, 23, 24, 26-32]

Secretory IgA

Specific antibodies against a variety of viruses, enterobacteria, and enterotoxins have been detected and are functionally effective in the gut lumen against the respective microorganisms and their products.[9,19] The chief immunoglobulin of breast milk is secretory IgA. Various investigators have demonstrated that the specificity of human milk's IgA depends on the

mother's antigenic exposure.[9,26] The mechanism responsible for the appearance of antibodies is only partially understood. Sensitized plasma cells are transported from the gastrointestinal and bronchotracheal-associated lymphatic tissues to multiple mucosal surfaces, including breast alveoli during lactation. Participation of the maternal urinary tract in this general response has also been suggested. During lactation, the "homing" of these cells to the breast appears to be activated by lactogenic hormone. This mechanism provides specific immunity to most mucosal surfaces.[26]

Secretory IgA has the ability to attach itself to mucosal epithelium and prevent the attachment and possible invasion of specific infectious agents.[2,26] This mechanism has been confirmed by studies on *E. coli* and *Vibrio cholerae* in the gut.[2,33]

S IgA may bind to an infant's buccal mucosa, providing a potential mechanism to act as a protective factor in the infant's hypopharynx. IgA is more resistant to acid conditions and to proteolytic activity of gut enzymes than in serum IgA. It therefore seems possible that it can act both in the gut and in some parts of the respiratory tract where it could be deposited during gurgling by the feeding infant.[2,23]

The ability of the mother to secrete antibodies directed against specific antigens that she and her infant encounter in the environment gives human milk an environmental specificity with significant protective potential.[26] Antibodies to many types of microorganisms have been demonstrated in human milk. In the review of Goldman and Smith, they have demonstrated the presence of antibodies to *Clostridium tetani, Corynebacterium diphtheriae, Streptococcus pneumoniae, E. coli*, salmonella, shigella, streptolysin and staphylolysin.[2,23] The principal immunoglobulin was IgA. Stoliar et al[19] have also reported that colostrum of Guatemalan women inhibited the pathogenic activity of *E. coli* and *V. cholerae* enterotoxins in experimental rabbits.

Complement

In addition to the immunoglobulin secreted in breast milk, the presence of nine components of complement have been demonstrated, though at low or very low levels.[2] It seems likely that human milk IgA and IgE activate C_3 through the alternate pathway of complement activation. Activated C_3 should be potentially important because of its known opsonic, anaphylatoxic and chemotactic properties.[23,28]

Leukocytes in Milk

Several investigators have noted the presence in breast milk of viable leukocytes.[2,32] These comprise macrophages, foamy macrophages, polymorphonuclear leukocytes and T and B lymphocytes.

The macrophages, foamy macrophages and neutrophils are capable of phagocytosis and have been shown to act against staphylococci, *E. coli* and *C. albicans*.[2,21] Milk lymphocytes were found to be stimulated by *E. coli* K1 but not by blood leukocytes, suggesting accumulation of particular lymphocyte clones in the breast and the localized nature of breast cell immunity.[2]

Lactobacillus bifidus growth factor

The gut of the breastfed infant is enriched with lactobacilli, whereas in others, the familiar pattern of commensal microorganisms is quickly established. The difference had been attributed to the high concentration in human milk of a carbohydrate growth factor necessary for the growth of L. bifidus, and to its high lactose concentration, low protein content, low bulk, and low buffering capacity.[2,28] Due to the production of acetic and lactic acid by the lactobacilli, the stool ph of breastfed infants is much lower than that of infants fed bovine milk. This acid environment inhibits the in vitro growth of shigella, *E. coli*, and yeast.[23,28]

Anti-staphylococcal factor

Since the pre-antibiotic era, human milk was found to have a therapeutic effect upon staphylococcal infection. Gyorgi et al have demonstrated a thermostable anti-staphylococcal factor in human milk and in vivo protection experiments with young mice. This factor appears to be a fatty acid C 18:2 distinct from linoleic acid. However, protection against staphylococcal infection by oral administration of this factor had not been tested.

Non-specific Protective Factors

In contrast to the highly specific protective proteins in human milk, there are a number of nonspecific factors that may play protective roles in vivo.

One of these is lactoferrin. This substance binds free iron in human milk avidly; presumably it also limits iron availability to potentially pathogenic flora by competing with bacterial enterochelin for iron.[26] Bacteria such as staphylococci and *E. coli* are inhibited by this mechanism. In that respect, it should be noted that lactoferrin in human milk is largely unsaturated and therefore could be a potential microbicidal agent.[23]

Lactoferrin alone exerts only a slightly inhibitory effect against $^{E.\ coli}$ due to the secretion by *E. coli* of the iron chelator, enterochelin, which ensures continuity of iron supply for the organism. However, in the presence of antibody and bicarbonate, lactoferrin exerts a strong bacteriostatic effect, probably by causing deformation in transfer RNA.[2,22]

Lysozyme, another nonspecific protective factor, catalyzes the hydrolysis of beta 1,4 glycosidic bonds in bacterial cell walls. In vitro, it acts in concert with IgA, to lyse *E. coli* and some salmonellae. IgA, peroxidase and ascorbate are all present in breast milk.[2,23,24]

A unique peroxidase that aids in the in vitro killing of streptococci is found in milk and saliva. This enzyme, lactoperoxidase, together with hydrogen peroxide and thiocyanate ions comprise an in vitro antibacterial system in milk. [2,23] It has been shown to exert a bactericidal effect against gram-negative bacteria including EPEC, *S. typhimurium* and *P. aeruginosa*.[2,18] The bactericidal effect closely relates to the oxidation of thiocyanate.[18]

Two general mechanisms have been proposed to explain the manner in which specific components in human milk may protect the infant from infection. One of these is the interaction between specific constituents in milk with epithelial surfaces or with specific substances in the gastrointestinal lumen during digestion and absorption of milk. The other mechanism is the possible modulation of the infant's immune system by protective factors in the milk, which results in selective production of immune factors in the infant.[26]

It has to be emphasized that current studies reveal that antibacterial defense factors n i milk are not influenced by the mother's nutritional status.[24] This finding is of considerable public health significance since a majority of the women in poor communities are undernourished.

SUMMARY

The spectrum of antimicrobial activity of breast milk was determined using nine common bacterial pathogens of infants and children on in vitro assays. Using a commercial milk formula as control, breast milk was found to exert bactericidal activity against *Vibrio cholerae*, and bacteriostasis for enteric pathogens like E. coli, salmonella, shigella and other gram negative bacteria as pseudomonas and enterobacter, as well as gram positive bacteria like staphylococci. The results of this study further confirm the protective effect of breastfeeding against infections, particularly in the first six months of life, which is substantiated by epidemiologic reports both in developed and developing countries. The host resistant factors, both specific and nonspecific in nature, interact in a dynamic manner to provide immunity and resistance to the breastfed infant not afforded by artificial feeding.

Recommendations

The distinct health advantage offered by breastfeeding to the nursing infant cannot be overemphasized. However, the full spectrum of antimicrobial activity of breast milk deserves further attention and research. Other organisms common in the pediatric group as *Streptococcus pneumoniae* and *Hemophilus influenzae* and others should be examined further. Otitis media has been seen more often in bottle-fed infants. This has been attributed to bottle propping causing positional otitis or otomastoiditis, and not to the absence of antibacterial effect of human milk.

Lastly efforts should be exerted to promote breastfeeding if only for its vast potential to control malnutrition and infection in the first six months of life, which is the critical period associated with the highest morbidity and mortality particularly in a developing country such as ours. This could be done through small-scale programs, which can either be hospital based or community based. Another way is through promotional campaigns and outreach programs which give due information and support to mothers and would-be mothers. Simple, low-cost modifications of pre-natal and puerperal care need to be implemented.

It can be said that in any part of the world, no single pediatric measure has such widespread and dramatic potential for child health as a return to breastfeeding,

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