

Effects of a Human Milk-Derived Human Milk Fortifier on the Antibacterial Actions of Human Milk

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ABSTRACT

Objectives: To compare the effects of a human breastmilk-derived fortifier on the antibacterial activity of milk obtained from lactating mothers delivering prematurely with the effects of a powdered fortifier on the same milk.

Study Design: Human milk samples were obtained after the first week of postnatal life from 10 lactating mothers, who had delivered prematurely. A bovine milk-based powdered fortifier and a human breastmilk-based frozen fortifier were evaluated. All mothers were healthy and they were not on any medications, although they were taking prenatal vitamins during lactation. The effects of each fortifier on the antimicrobial activity of milk toward *Enterobacter sakazaki* (ES), *Escherichia coli*, *Clostridium difficile* (CD), and *Shigella sonnei* (SS) were evaluated by both the filter paper method and the growth inhibition method.

Results: Human milk inhibited the growth of all of the test organisms. This antibacterial activity was almost totally inhibited by the addition of the bovine protein-based human milk fortifier, while it remained unaffected by the addition of the human breastmilk-based fortifier.

Conclusions: Breastmilk from women who have delivered preterm has antibacterial activity that can be affected by the addition of bovine-based fortifier, but not by the addition of a human breastmilk-based fortifier.

INTRODUCTION

HUMAN MILK IS THE OPTIMUM FOOD for the human infant. The American Academy of Pediatrics goes so far as to say that even in the neonatal intensive care unit (NICU), breastmilk is the optimal food, and in the event a mother cannot provide breastmilk then donor milk may be an adequate substitute.¹ Data published over the past few decades indicate that human milk has particular benefits for infants being treated in the NICU, particularly related to decreased incidences of sepsis and necrotizing enterocolitis (NEC).²⁻⁵ It has also been rec-

ognized that breastmilk alone is not sufficient to meet the higher nutritional needs of babies born significantly preterm.⁶ This realization has led to the widespread use of human milk fortifiers (HMF) to boost the nutritional content of the breastmilk fed to premature infants in the NICU.

Chan⁷ previously had demonstrated that the antibacterial activity inherent in human breastmilk could be inhibited through the addition of a bovine protein-based fortifier containing added iron. A bovine protein-based fortifier with smaller amounts of iron had similar antibacterial activity as unfortified milk. With the

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TABLE 1. MILK COMPOSITION USING THE TWO FORTIFIERS PER 100 mL

Nutrients	Enfamil® HMF	Prolact + 4
Energy	84	82
Protein, g	2.9	2.5
Fat, g	5	4.8
Carbohydrate, g	7.4	7.4
Vitamin A, IU	950	530
Vitamin D, IU	150	n/a
Vitamin E, IU	4.6	n/a
Calcium, mg	112	79
Phosphorus, mg	64	37
Iron, mg	1.5	0.2

availability of a human breastmilk-derived human milk fortifier, the authors investigated whether that product would interfere with milk's antibacterial activity in the same way as had been seen with some bovine fortifiers against four major organisms that can cause neonatal sepsis and NEC.

MATERIALS AND METHODS

Human milk samples were obtained from 10 lactating mothers who had delivered preterm infants. The mothers ranged from 7 to 134 days postpartum at the time of participation. The mean gestation was 32 weeks and the mean length of lactation was 4 weeks. All mothers were healthy and were taking standard vitamin supplements prenatally.

Milk samples were immediately frozen at -18°C until needed and all were used within 4 weeks of expression. Each frozen sample was allowed to thaw under tap water, and care was taken to ensure that the water never reached the level of the container cap in order to prevent contamination. All thawed milk was kept refrigerated, and all experiments were run using aliquots of milk from the same mother as control.

The human milk fortifiers evaluated were Enfamil (Mead Johnson, Evansville, IN) bovine protein-based fortifier with added iron (1.44 mg/100 mL) and Prolact + 4 [Pasteurized, Human] (Prolacta Bioscience, Monrovia, CA) breastmilk-based fortifier. Both fortifiers were mixed with mother's milk per manufacturer's directions. Enfamil was mixed at one packet

per 25 mL of milk. Prolact + 4 was thawed and then 5 mL were added to 20 mL of mother's milk (1:4 ratio). The milk composition after the addition of the two fortifiers is shown in Table 1.

Both the bacterial inhibition and bacterial growth studies were performed as described in an earlier publication.⁷ In the bacterial inhibition study, a disk of filter paper was soaked with the milk preparation and placed on a plate that had previously been inoculated with one of the test organisms. The organisms were clinical isolates from the microbiology laboratory at University of Utah Medical Center except for ES, which was obtained from the American Type Culture Collection (29544; Rockville, MD). After 24–36 hours of incubation, the zone of inhibition, if present, was measured.

Bacterial growth was measured by the technique described by Hernandez et al.⁸ One milliliter of bacterial suspension in normal saline with a bioburden of 10^5 – 10^7 CFU/mL was added to 1 mL of the milk preparation. After 3.5 hours of incubation at 37°C , triple aliquots were plated onto sheep blood agar plates and colonies were counted after incubation of the plates at 37°C for 24 hours.

Results were analyzed using the usual two-sample *t*-test with a two-sided significance level of 0.05.

The nutritional values for Prolact + 4 were determined by an outside laboratory using the following methodologies as promulgated by the Association of Analytical Communities' (AOAC) Official Methods Program: Fat (Mo-jonnier) AOAC 989.05, Vitamin A AOAC-HPLC-FDA, Calcium AOAC 984.27, Phosphorus AOAC 984.27, and Iron AOAC 984.27. Protein was measured by the Kjeldahl method; carbohydrates were calculated though lactose was measured by HPLC, and energy was calculated using Atwater factors. Nutritional values for the Enfamil fortifier were obtained from the product label.

RESULTS

Human milk (HM) inhibited the growth of all the organisms studied. When mixed with human breastmilk-based fortifier, there was no

TABLE 2. GROWTH INHIBITION DIAMETER OF ZONE OF INHIBITION (MM)

	Number of replicates	<i>E. sakazaki</i> (ES)	<i>E. coli</i>	<i>C. difficile</i> (CD)	<i>Shigella</i>
Human milk (HM)	29	23.6 ± 0.8	22.2 ± 4.5	22.9 ± 0.6	22.8 ± 0.6
HM + HMF	30	0.9 ± 4.5*	2.4 ± 6.9*	2.4 ± 6.9*	1.6 ± 5.9*
HM and Prolact + 4	29	23.3 ± 0.7	23.3 ± 0.7	22.7 ± 0.5	22.9 ± 0.5

* $p < 0.001$ compared with HM or HM and Prolact + 4, $p = \text{NS}$ HM compared with HM and Prolact + 4.

change seen in the level of growth inhibition as determined from the measurement of the diameter of the zones of inhibition. In contrast, addition of the bovine protein-based, iron-supplemented fortifier almost completely eliminated the inhibitory activity of the milk. These results are presented in Table 2.

Similarly, after 3.5 hours of incubation of a seeded aliquot in the human milk preparations, there was no difference in the rate of growth between milk alone and milk to which the human breastmilk-based fortifier had been added. By contrast, there was significantly greater growth, almost double ($p = 0.001$), when the seeded aliquot was mixed with the bovine protein-based, iron-supplemented fortifier. These results are presented in Figure 1.

DISCUSSION

In 2003, Chan⁷ published work looking at the effect of iron in a bovine protein-based fortifier on the antimicrobial effects of milk. In 2002, a new human breastmilk-based fortifier became available. Since this new fortifier did not contain additional iron, it was hypothesized that the addition of this fortifier with mother's milk would not decrease the antibacterial activity of the milk. The study, which compared Enfamil[®] Human Milk Fortifier, Mead Johnson Nutritionals, (Evansville, IN) with iron to Similac Ross Products (Columbus, OH) without extra iron, was carried out using the same methodology employed in the current work. The results of that experiment showed that the En-

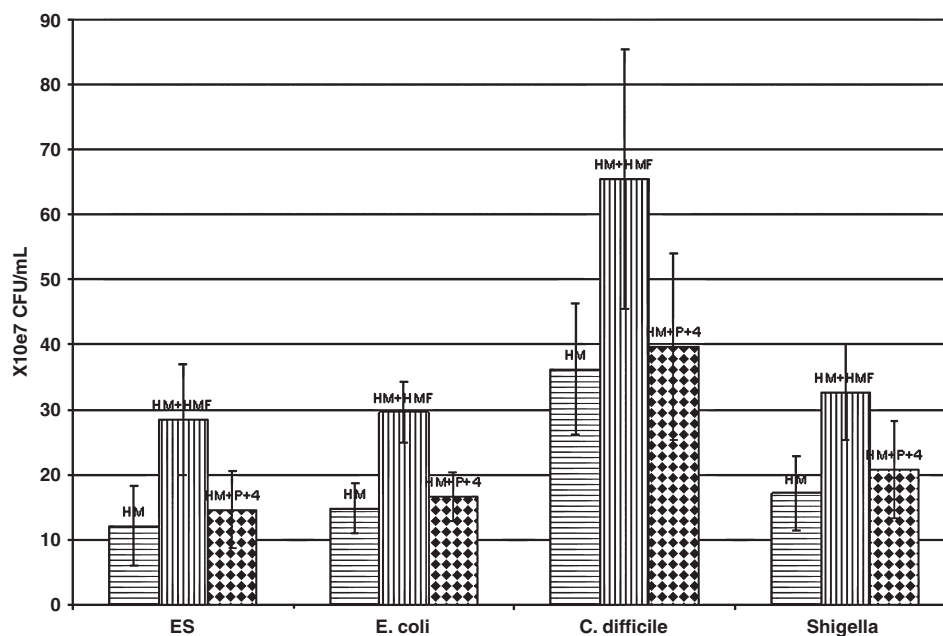


FIG. 1. Bacterial growth at 3.5 hours.

famil[®] Human Milk Fortifier, which contained supplemental iron, diminished the antibacterial activity of human milk toward the organisms *E. coli*, *Staphylococcus*, *ES*, and Group B *Streptococcus* while the other product did not.

The addition of iron to the bovine-based fortifier results in its chelation by lactoferrin found in the milk which may saturate the lactoferrin and prevent its iron-dependent antimicrobial activity. This is certainly a potential mechanism that may explain this phenomenon, as this is the third study to show a similar effect. The Chan study was followed by a paper by Ovali et al.,⁹ who obtained essentially the same results. Addition of a bovine fortifier which did not contain iron seemed to retain the antibacterial activity of milk, but one with added iron did not. Our current report is consistent with the findings of the preceding two papers.

Whether there is also some inhibition of the iron-independent bactericidal effect of lactoferrin,^{10–12} or of other antimicrobially active agents in milk by the presence of bovine protein cannot be definitively answered from the current experiment. It is known that an earlier experiment using a version of the same bovine-based fortifier with less iron also showed greater bacterial growth in fortified compared with unfortified milk. Whether this represents a dose-response effect or an alternative mechanism of inhibition remains to be elucidated. It also seems clear that the *in vivo* effects of milk are multifactorial, and that experiments such as ours are limited in explaining the mechanism of the antibacterial action of human milk.

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