Bovine Lactoferrin Supplementation for Prevention of Late-Onset Sepsis in Very Low-Birth-Weight Neonates

A Randomized Trial

Paolo Manzoni, MD
Matteo Rinaldi, MD
Silvia Cattani, MD
Lorenza Pugni, MD
Mario Giovanni Romeo, MD
Hubert Messner, MD
Ilaria Stolfi, MD
Lidia Decembrino, MD
Nicola Laforgia, MD
Federica Vagnarelli, MD
Luigi Memo, MD
Linda Bordignon, MD
Onofrio Sergio Saia, MD
Milena Maule, PhD, MSc, BSc
Elena Gallo, MD
Michael Mostert, MD
Cristiana Magnani, MD
Michele Quercia, MD
Lina Bollani, MD
Roberto Pedicino, MD
Livia Renzullo, MD
Pasqua Betta, MD
Fabio Mosca, MD, PhD
Fabrizio Ferrari, MD, PhD
Rosario Magaldi, MD
Mauro Stronati, MD
Daniele Farina, MD
for the Italian Task Force for the Study and Prevention of Neonatal Fungal Infections, the Italian Society of Neonatology

For editorial comment see p 1467.

Context Sepsis is a common and severe complication in premature neonates, particularly those with very low birth weight (VLBW) (<1500 g). Whether lactoferrin, a mammalian milk glycoprotein involved in innate immune host defenses, can reduce the incidence of sepsis is unknown. In animal models, the probiotic *Lactobacillus rhamnosus* GG (LGG) enhances the activity of lactoferrin but has not been studied in human infants.

Objective To establish whether bovine lactoferrin (BLF), alone or in combination with LGG, reduces the incidence of late-onset sepsis in VLBW neonates.

Design, Setting, and Patients Prospective, multicenter, double-blind, placebo-controlled, randomized trial conducted in 11 Italian tertiary neonatal intensive care units. Patients were 472 VLBW infants enrolled from October 1, 2007, through July 31, 2008, and assessed until discharge for development of sepsis.

Intervention Infants were randomly assigned to receive orally administered BLF (100 mg/d) alone (n=153), BLF plus LGG (6×10^9 colony-forming units/d) (n=151), or placebo (n=168) from birth until day 30 of life (day 45 for neonates <1000 g at birth).

Main Outcome Measure First episode of late-onset sepsis, ie, sepsis occurring more than 72 hours after birth with isolation of any pathogen from blood or from peritoneal or cerebrospinal fluid.

Results Demographic, clinical, and management characteristics of the 3 groups were similar, including type of feeding and intake of maternal milk. Incidence of late-onset sepsis was significantly lower in the BLF and BLF plus LGG groups (9/153 [5.9%] and 7/151 [4.6%], respectively) than in the control group receiving placebo (29/168 [17.3%]) (risk ratio, 0.34; 95% confidence interval, 0.17-0.70; $P=.002$ for BLF vs control and risk ratio, 0.27; 95% confidence interval, 0.12-0.60; $P<.001$ for BLF plus LGG vs control). The decrease occurred for both bacterial and fungal sepsis. No adverse effects or intolerances to treatment occurred.

Conclusion Compared with placebo, BLF supplementation alone or in combination with LGG reduced the incidence of a first episode of late-onset sepsis in VLBW neonates.

Trial Registration isrctn.org Identifier: ISRCTN53107700

JAMA. 2009;302(13):1421-1428 www.jama.com

INFECTIONS ARE THE MOST COMMON cause of death in premature infants and a major threat for poor outcomes.[1] Late-onset sepsis, ie, infections arising after the perinatal period, are mainly nosocomial and affect 21% of very low-birth-weight (VLBW) (<1500 g) neonates, with many more undergoing empirical antibiotic treatment.[2] In VLBW neonates, the digestive tract is a major site for colonization and systemic translocation by many pathogens. Also, prolonged

Author Affiliations and Task Force Members are listed at the end of this article.
Corresponding Author: Paolo Manzoni, MD, Neonatology and Neonatal Intensive Care Unit, S. Anna Hospital, Azienda Ospedaliera Regina Margherita-S. Anna, C. Spezia 60, 10126 Torino, Italy (paolomanzoni@hotmail.com).

maintenance of a central venous catheter (CVC) is a major risk factor.

Attempts to reduce incidence of late-onset sepsis are hampered by nonspecific clinical features, inadequate sensitivity of diagnostic tests, and late recognition. Furthermore, early diagnosis and successful treatment do not prevent prolonged stays in neonatal intensive care units, high costs, or late neurodevelopmental impairment.[3]

Lactoferrin is the major whey protein in mammalian milk and is important in innate immune host defenses. In human milk, its concentration peaks in colostrum and then decreases, the decrease being slower in the milk of mothers of premature neonates.[4] Bovine lactoferrin (BLF) and human lactoferrin (HLF) have high (77%) amino acid homology, with BLF exhibiting even higher in vitro antimicrobial activity than HLF.[5] Both lactoferrins resist proteolysis in the digestive tract,[6] bind to specific receptors on enterocytes,[7] and are poorly absorbed in the gut.[8]

Bovine lactoferrin anti-infective activities have been elucidated in vitro[9] and in animal models,[10] resulting either from iron sequestration or from effects on microbial cell membranes that cause their disintegration.[11-14] In animal models, BLF activity is enhanced by the probiotic *Lactobacillus rhamnosus* GG (LGG).[15]

Bovine lactoferrin has been granted GRAS (generally recognized as safe) status by the US Food and Drug Administration[16] and on this basis is added to infant formula by some manufacturers in Italy and Japan, with no reported adverse effects. Despite largely promising experimental data, clinical information on BLF in infants is scarce, with no studies investigating the effects of BLF supplementation in VLBW neonates.

This study was a prospective, multicenter, double-blind, placebo-controlled, randomized clinical trial examining whether oral supplementation with BLF alone or in combination with LGG reduces late-onset sepsis in VLBW neonates.

METHODS

Between October 1, 2007, and July 31, 2008, we enrolled VLBW neonates younger than 3 days at 11 tertiary Italian neonatal intensive care units. The study was approved by the ethics committees of the sponsoring Scientific and Charity Neonatology Foundation "Fondazione Crescere Insieme al Sant'Anna" and of each participating institution. Parents or guardians provided written informed consent.

The primary objective was to evaluate the effectiveness of BLF alone or BLF plus LGG in the prevention of the first episode of late-onset sepsis of bacterial or fungal origin. Secondary objectives were assessment of the incidence of gram-positive/gram-negative bacterial and fungal sepsis, mortality prior to discharge (overall and sepsis-attributable), incidence of urinary tract infections, fungal colonization, progression from fungal colonization to invasive fungal infection (IFI), stage 2 or greater necrotizing enterocolitis, threshold retinopathy of prematurity, severe (grade 3-4) intraventricular hemorrhage, bronchopulmonary dysplasia, alteration of liver function, and adverse effects or intolerance.

Exclusion criteria were parental consent lacking/refused, ongoing antifungal prophylaxis, early onset sepsis (before the third day of life), or liver failure (aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transferase, and direct bilirubin serum values 3-fold higher than reference range). All infants underwent follow-up until death or discharge.

Infants were randomly allocated to 1 of 3 groups in a 1:1:1 ratio. Randomization was stratified by center, and randomly permuted blocks of size 9, 12, and 15 were used. The random allocation sequence was generated using ralloc.ado version 3.2.5 in Stata 9.2 (StataCorp, College Station, Texas). The pharmacy at each center used these computer-generated randomization lists to form the 3 groups and prepared the drug doses. Clinical and research staff remained unaware of study group assignments during the study.

Infants received either BLF (100 mg/d) (LF100; Dicofarm SpA, Rome, Italy) alone or BLF plus LGG (6×10^9 colony-forming units/d) (Dicoflor60; Dicofarm SpA); the control group received placebo (2 mL of a 5% glucose solution). The dosage of LGG was taken from published data.[17] The dosage of BLF was based on the mean HLF intake that VLBW neonates ingest with mother's fresh milk in the first 2 weeks of life (30-150 mg/d). A single BLF lot with a projected stability of 2 years or longer was used. We decided not to have a fourth group receiving only supplementation with LGG, owing to the absence of evidence addressing LGG effectiveness on late-onset sepsis in VLBW infants. Treatment lasted 6 (birth weight <1000 g) or 4 (birth weight 1001-1500 g) weeks, unless neonates were discharged earlier. Different treatment durations were chosen because of the different durations of risk for sepsis. Drug administration began on the third day of life with 1 daily dose; all doses including placebo were diluted in prepared milk so as to maintain blinding.

Neonates not feeding in the first 48 hours received the drug(s) or placebo by orogastric tube. Nutritional and feeding policies followed a common protocol.[18] Administration of fresh, expressed maternal milk was encouraged. Each mother could supply milk only for her infant. When needed, feeding was supplemented with a formula for VLBW infants (PreAptamil; Milupa Italia, Milano, Italy) not supplemented with BLF.

Systematic surveillance of adverse events (eg, vomiting, feeding intolerance, skin rashes) was performed through daily infant examination until 2 days after end of treatment. Given the enhanced risk of preterm infants for nutrition-related cholestasis but also that BLF supplementation might improve liver function via antioxidative actions,[19] weekly surveillance of liver function was also performed.

Outcomes

Late-onset sepsis was defined as occurring more than 72 hours after birth and before discharge. This condition was based on the detection of clinical signs and symptoms by the physician in

charge, presence of laboratory findings consistent with sepsis, and isolation of a causative organism from blood (drawn from peripheral sites) or cerebrospinal or peritoneal fluid.[18] Diagnostic criteria were based on the existing literature, guidelines from international consensus documents,[20] and recommendations from the Italian Neonatology Society's Fungal Infections Task Force.[21] Presumed sepsis (clinical presentation consistent with sepsis but no microorganisms isolated) was not considered late-onset sepsis.

For *Staphylococcus* species, including coagulase-negative *Staphylococcus* species, diagnosis required 2 positive culture results from peripheral blood drawn within 48 hours or only 1 positive culture result accompanied by a concomitant positive culture result from the CVC (or blood drawn from the CVC) for the same organism. In the case of *Staphylococcus aureus*, only 1 positive result was required. For all microorganisms, isolation from specimens other than those listed above was considered colonization. Infants with an episode of late-onset sepsis continued to receive follow-up until discharge for secondary outcomes.

Severe bronchopulmonary dysplasia was defined as use of supplemental oxygen for 28 days plus 30% oxygen, positive pressure ventilation at 36 weeks' postmenstrual age, or both.[22] Necrotizing enterocolitis was defined as clinical signs with the presence of pneumatosis intestinalis on abdominal radiographs, according to the Bell criteria[23]; retinopathy of prematurity was defined according to the Early Treatment for Retinopathy of Prematurity study.[24] Urinary tract infections were diagnosed by isolation of a pathogen from urine collected by suprapubic puncture or bladder catheterization, with growth of more than 100 000 bacteria/mL or more than 10 000 fungi/mL. Sepsis-attributable mortality was defined as death within 5 days after the last positive culture result from any site without other causes or as isolation of pathogens at autopsy. Presence and grade of intraventricular hemorrhage were documented by the most negative ultrasound finding available; intraventricular hemorrhage was classified by the Papile criteria.[25] Fungal colonization was defined as growth of at least 1 fungal isolate from at least 1 surveillance culture.

Microorganism Isolation and Identification

To diagnose bacterial and fungal colonization, as well as progression from fungal colonization to infection, surveillance cultures were obtained from ear canal swabs and umbilical catheters at birth; in addition, at least 3 of 4 swabs (stool, gastric aspirate, rectal, pharyngeal) were obtained every week for 6 weeks. Cultures were also obtained from surgical devices after removal and from any sites at any time when ordered by the physician.

Standard laboratory methods were used to identify bacteria from cultures.[26,27] For *Candida* species, specimens were incubated on chromogen culture plates (Albicans ID; bioMérieux, Marcy l'Etoile, France) to identify *Candida albicans* colonies as blue stains after 48 hours at 37°C. Colonies were speciated biochemically (Vitec Yeast, bioMérieux).

Statistical Analysis

All primary and secondary outcomes were represented by dichotomous variables (presence/absence) and analyzed by intention-to-treat.

Categorical predictor variables were represented by percentages. Birth weight, gestational age, Apgar score, number of days receiving a given treatment, and daily amount of milk intake were represented by continuous variables. A complete list of the categorical and continuous variables considered is shown in **TABLE 1**.

The BLF and BLF plus LGG groups were separately compared with the control group, overall and by birth weight groups. Proportions and continuous variables were compared using the Fisher exact 2-tailed test and the *t* test, respectively. Risk ratios (RRs) and 95% confidence intervals (CIs) were calculated to compare cumulative between-group incidences using Stata version 9.2. A multilevel (random-intercept) logistic regression model[28] was fitted to investigate the effect of relevant risk factors, taking into account the center-level variance component. Covariates included in the model were chosen a priori on the basis of their clinical relevance and included treatment assignment (BLF/BLF plus LGG/placebo), sex (male/female), gestational age, birth weight, nutrition (maternal milk/formula/both formula and maternal milk/human nonmaternal milk), use of H_2 blockers, use of postnatal steroids, and daily milk intake.

The Wald test was used to assess the significance of the estimated coefficients. The likelihood ratio test was used to test the significance of the center-level variance component. Goodness-of-fit was evaluated through the log-likelihood of the fitted model. All tests were 2-tailed, and $P < .05$ was considered statistically significant. No adjustment was made for multiple comparisons.

Sample size analysis predicted that 114 patients would be needed for each group, based on 2-sided type I error rates of .05 or less and 80% power to detect a relative difference between treated and nontreated infants of at least 66% (decrease from 18% to 6%, given a pretrial incidence of 18%) for late-onset sepsis. A total of 153 infants in each group would be needed to reach a power of 90%. Incidence of late-onset sepsis was also compared in infants treated with BLF and with BLF plus LGG. Since there were no significant differences between the 2 treatment groups, a post hoc analysis was performed comparing the combined treatments with the control group, both overall and by birth weight groups. However, given the low incidence of late-onset sepsis in either treatment group, the study was underpowered to detect possible significant differences. Assuming an incidence of 6% in either treatment group, 749 infants in each group would have been needed to reach 80% power to detect a relative differ-

Table 1. Demographic and Nutritional Characteristics of Patients and Major Risk Factors for First Episode of Late-Onset Sepsis

Characteristic	BLF (n = 153)	BLF + LGG (n = 151)	Control (n = 168)	*P* Value[a] BLF vs Control	*P* Value[a] BLF + LGG vs Control
Demographic					
Birth weight, mean (SD) [range], g	1142 (244) [634-1495]	1138 (253) [550-1500]	1109 (269) [437-1500]	.25	.31
Gestational age, mean (SD) [range], wk	29.6 (2.5) [23-36]	29.8 (2.8) [23-35]	29.5 (3.2) [23-39]	.82	.39
Apgar score at 5 min, mean (SD)[b]	7.6 (1.4)	7.5 (1.6)	7.6 (1.5)	.57	.42
Male sex, No./total (%)	74/153 (48.4)	72/151 (47.7)	86/168 (51.2)	.66	.58
White race, No./total (%)[c]	138/153 (90.2)	133/151 (88.1)	153/168 (91.1)	.84	.46
Born at another facility, No./total (%)	19/153 (12.4)	30/121 (19.9)	27/168 (16.1)	.43	.38
Vaginal delivery, No./total (%)	29/153 (19.0)	32/151 (21.2)	34/168 (20.2)	.78	.89
Maternal preeclampsia, No./total (%)	37/153 (24.2)	33/151 (21.8)	41/168 (24.4)	.68	.68
Medication use, No./total (%)					
Antenatal corticosteroids	109/153 (71.2)	104/151 (68.9)	123/168 (73.2)	.80	.45
Antenatal antibiotics	119/153 (77.8)	113/151 (74.8)	129/168 (76.8)	.89	.69
Surfactant (at least once)	109/153 (71.2)	112/151 (74.1)	129/168 (76.8)	.30	.59
Risk factors for late-onset sepsis, mean (SD), days until discharge					
Use of TPN	20.2 (20.9)	17.8 (18.1)	18.5 (15.0)	.35	.57
Umbilical catheter positioned	5 (0.9)	5 (0.9)	5 (0.9)	.99	.99
Intubation	6.5 (6.3)	6.8 (6.4)	6.9 (10.2)	.31	.40
Medication use					
H_2 Blockers	3.1 (6.8)	3.0 (8.3)	2.7 (6.5)	.60	.73
Third-generation cephalosporins	0.7 (0.5)	0.7 (0.6)	0.9 (0.8)	.65	.88
Antibiotics	11.6 (8.5)	11.8 (9.5)	13.3 (10.5)	.12	.19
Postnatal steroids	1.0 (0.9)	1.1 (1.1)	0.9 (1.3)	.80	.62
Supplemental oxygen	14.0 (9.8)	14.3 (11.0)	14.4 (12.5)	.76	.77
Mean duration of stay in NICU					
Alive infants	54.2 (24.4)	54.7 (22.3)	55.1 (21.7)	.73	.87
Deceased infants	14 (5.6)	14 (7.0)	27.4 (21.6)	.25	.16
Central venous catheter(s) positioned	13.2 (10.3)	13.7 (10.2)	15.0 (11.5)	.13	.28
Early onset neutropenia, No./total (%)	15/153 (10.2)	13/151 (8.6)	12/168 (7.2)	.45	.76
Nutritional characteristics					
Time of initiation of oral feeding, mean (SD), DOL	2.2 (3.1)	2.1 (3.8)	2.4 (3.5)	.49	.34
Time of achievement of full feeding, mean (SD), DOL	12.5 (4.1)	13.4 (5.1)	14.8 (4.7)	.05	.21
Volume of feedings advanced daily, mean (SD), mL/d	10.0 (4.5)	11.0 (3.9)	10.6 (3.0)	.59	.68
Fed with only formula, No./total (%)	24/153 (15.7)	26/151 (17.2)	22/168 (13.1)	.53	.35
Fed with only maternal milk, No./total (%)	42/153 (27.4)	32/151 (21.2)	37/168 (22.1)	.30	.89
Fed with both formula and maternal milk, No./total (%)	87/153 (56.9)	93/151 (61.6)	109/168 (64.8)	.72	.84
Daily human fresh milk intake, mean (SD), mL/kg	69.3 (41.7)	65.7 (41.5)	66.8 (35.5)	.56	.79
Total days of human fresh milk feeding, mean (SD)	22.3 (13.6)	21.4 (13.7)	22.8 (12.6)	.71	.33

Abbreviations: BLF, bovine lactoferrin; DOL, days of life; LGG, *Lactobacillus rhamnosus* GG; NICU, neonatal intensive care unit; TPN, total parenteral nutrition.
[a] Calculated using the Fisher exact 2-tailed test for comparing proportions and the *t* test for comparing continuous variables (eg, birth weight).
[b] Score ranges from 0 to 10, with higher scores indicating better functioning.
[c] Race was determined by the investigators. Percentage refers to both parents.

ence of 50%, and 279 infants in each group would have been needed for a 75% difference.

Subgroup analyses of late-onset sepsis were carried out by type of nutrition (infants fed maternal and formula milk). The study was underpowered to detect statistically significant differences in this subgroup analysis.

Power calculations were performed using S-plus version 2000 (MathSoft, Cambridge, Massachussetts). Primary analyses were performed using Stata 9.2.

RESULTS

Patients

Four hundred ninety-four VLBW neonates survived 3 days or longer and were assessed for eligibility. Twenty-two were ineligible (FIGURE). Four hundred seventy-two neonates were randomized to the BLF (n=153), BLF plus LGG (n=151), or control (n=168) groups.

One infant in the BLF group discontinued treatment (receiving only 8 drug doses) but was included in the BLF group for the intention-to-treat analysis. Nine infants had incomplete data on some variables included in the protocol but not for the analysis of study outcomes.

There were no significant baseline differences between groups in risk factors for sepsis, treatment, or nutritional characteristics (Table 1).

Late-Onset Sepsis

Forty-five infants had a first episode of late-onset sepsis (TABLE 2), with 56 causative isolates (2 concomitantly in 11 episodes) (eTable 1, available at http://www.jama.com). Overall, late-onset sepsis occurred less frequently in the BLF and BLF plus LGG groups (9/153 [5.9%] and 7/151 [4.6%], respectively) than in the control group (29/168 [17.3%]) (RR, 0.34; 95% CI, 0.17-0.70; $P=.002$ for BLF vs control and RR, 0.27; 95% CI, 0.12-0.60; $P<.001$ for BLF plus LGG vs control) (Table 2). The decrease occurred for bacterial as well as fungal episodes but was not statistically significant for gram-negative bacteria in the BLF group and for fun-

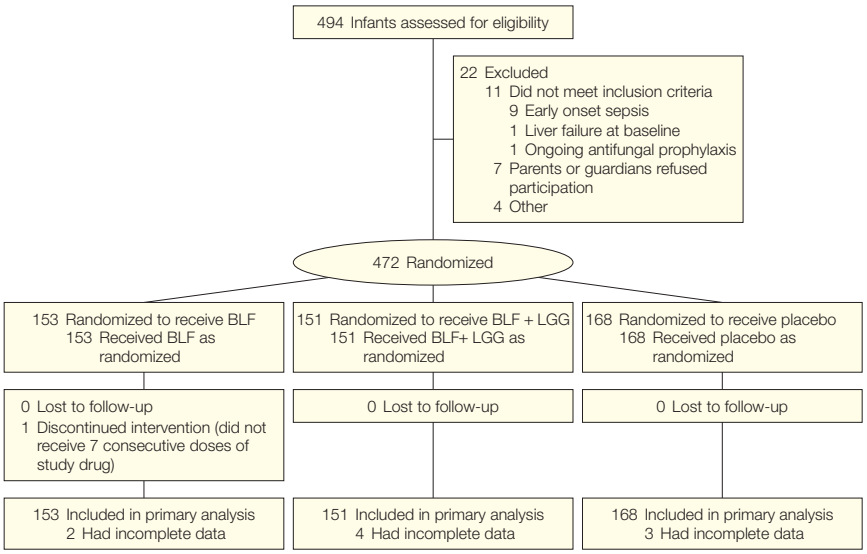
gal infections in the BLF plus LGG group. The distribution of infecting pathogens by type was not significantly different in treated and untreated groups (eTable 1).

In a post hoc analysis, no significant difference in the proportion of infants with late-onset sepsis was observed between the BLF and BLF plus LGG groups ($P > .99$), although the study was not powered to detect a difference between these groups.

When stratifying for birth weight, the decrease in late-onset sepsis was significant in extremely low-birthweight (ELBW) (<1000 g) neonates (RR, 0.31; 95% CI, 0.14-0.70; $P = .002$ for BLF vs control and RR, 0.30; 95% CI, 0.13-0.69; $P = .002$ for BLF plus LGG vs control), whereas it was not significant in neonates weighing 1001 to 1500 g (RR, 0.46; 95% CI, 0.12-1.74; $P = .34$ for BLF vs control and RR, 0.16; 95% CI, 0.02-1.27; $P = .07$ for BLF plus LGG vs control) (Table 2).

Analysis of the combined treatment groups (BLF and BLF + LGG) vs the control group showed a reduction of late-onset sepsis in treated infants (16/304 [5.3%] vs 29/168 [17.3%]) (RR, 0.26; 95% CI, 0.14-0.50;

Figure. Study Flow



BLF indicates bovine lactoferrin; LGG, *Lactobacillus rhamnosus* GG.

Table 2. Bacterial and Fungal Late-Onset Sepsis, Fungal Colonization, Progression From Colonization to Infection, Mortality in the Study Groups

Result	No./Total (%)			BLF vs Control		BLF + LGG vs Control	
	BLF (n = 153)	BLF + LGG (n = 151)	Control (n = 168)	RR (95% CI)	*P* Value[a]	RR (95% CI)	*P* Value[a]
Bacterial and fungal late-onset sepsis							
Total late-onset sepsis (total = 45)	9/153 (5.9)	7/151 (4.6)	29/168 (17.3)	0.34 (0.17-0.70)	.002	0.27 (0.12-0.60)	<.001
Day of onset, mean (SD), DOL	18.2 (4.2)	16.1 (8.0)	19.6 (12.7)		.76		.50
Gram-negative bacteria	7/153 (4.6)	5/151 (3.3)	17/168 (10.1)	0.45 (0.19-1.06)	.09	0.33 (0.12-0.87)	.03
Gram-positive bacteria	2/153 (1.3)	1/151 (0.7)	13/168 (7.7)	0.17 (0.04-0.74)	.007	0.09 (0.01-0.65)	.002
Gram-positive bacteria, including episodes diagnosed with ≥1 positive blood culture for CoNS	4/153 (2.6)	2/151 (1.3)	19/168 (11.3)	0.23 (0.08-0.66)	.002	0.12 (0.03-0.49)	<.001
ELBW neonates	6/53 (11.3)	6/54 (11.1)	22/60 (36.7)	0.31 (0.14-0.70)	.002	0.30 (0.13-0.69)	.002
Neonates weighing 1001-1500 g	3/100 (3.0)	1/97 (1.0)	7/108 (6.5)	0.46 (0.12-1.74)	.34	0.16 (0.02-1.27)	.07
Neonates weighing ≤750 g	0/9 (0)	2/12 (16.7)	11/18 (61.1)		.003	0.27 (0.07-1.02)	.03
Neonates ≤27 weeks' gestational age at birth	5/35 (14.3)	6/37 (16.2)	19/46 (41.3)	0.35 (0.14-0.84)	.01	0.39 (0.17-0.88)	.02
Invasive fungal infection							
Total	0/153 (0)	2/151 (1.3)	9/168 (5.4)		.004	0.25 (0.05-1.13)	.07
ELBW neonates	0/53 (0)	2/54 (3.7)	6/60 (10.0)		.03	0.37 (0.08-1.76)	.28
Neonates weighing 1001-1500 g	0/100 (0)	0/97 (0)	3/108 (2.8)		.25		.25
Progression from fungal colonization to fungal sepsis							
Overall fungal colonization (at least 1 site)	27/153 (17.6)	25/151 (16.6)	31/168 (18.5)	0.96 (0.60-1.53)	.89	0.90 (0.56-1.45)	.77
Progression rate colonization/invasive fungal infection (all neonates)	0/27 (0)	2/25 (8.0)	9/31 (29.0)		.002	0.28 (0.07-1.16)	.09
Mortality (prior to discharge)							
Overall (all causes)	4/153 (2.6)	6/151 (4.0)	12/168 (7.1)	0.37 (0.12-1.11)	.07	0.56 (0.21-1.45)	.24
Attributable to sepsis	0/153 (0)	1/151 (0.7)	8/168 (4.8)		.008	0.14 (0.02-1.09)	.04

Abbreviations: BLF, bovine lactoferrin; CI, confidence interval; CoNS, coagulase-negative *Staphylococcus* species; DOL, days of life; ELBW, extremely low birth weight; LGG, *Lactobacillus rhamnosus* GG; RR, risk ratio.
[a]Calculated using the Fisher exact 2-tailed test for comparing proportions.

$P<.001$). The decrease was significant both in ELBW neonates (12/107 [11.2%] vs 22/60 [36.7%]; $P<.001$) and in neonates weighing 1001 to 1500 g (4/197 [2.0%] vs 7/108 [6.5%]; $P=.05$).

Table 3. Multivariable Logistic Regression Analysis Controlling for the Most Important Risk Factors Possibly Associated With Late-Onset Sepsis[a]

Factor	OR (95% CI)[b]	*P* Value
Treatment[c]		
BLF	0.32 (0.14-0.77)	.01
BLF + LGG	0.21 (0.08-0.55)	.002
Sex[c]	1.91 (0.89-4.09)	.10
Gestational age, per week	0.71 (0.57-0.89)[d]	.002
Birth weight, per gram	1.00 (1.00-1.00)[d]	.35
Milk type[c]		
Mix	2.43 (0.50-11.75)	.48
Maternal	2.69 (0.50-14.63)	
Human (nonmaternal)	4.78 (0.64-35.94)	
Use of H_2 blockers, total days	1.04 (0.99-1.08)[d]	.09
Use of postnatal steroids, total days	1.10 (0.54-2.25)[d]	.79
Daily mean human fresh milk intake, mL/kg	0.99 (0.98-1.01)[d]	.29

Abbreviations: BLF, bovine lactoferrin; CI, confidence interval; LGG, *Lactobacillus rhamnosus* GG; OR, odds ratio.
[a] Within-center correlation = 3.4% ($P=.28$). Log likelihood of full model = −109.41775; Wald test (11 *df*) = 52.73 ($P<.001$).
[b] Adjusted for all other terms listed in the table.
[c] Referents were placebo for treatment, male for sex, and formula milk for milk type.
[d] OR for a 1-unit increase.

When risk factors were controlled for, both treatments conferred protection against late-onset sepsis (odds ratio, 0.32; 95% CI, 0.14-0.77 for BLF vs control and odds ratio, 0.21; 95% CI, 0.08-0.55 for BLF plus LGG vs control) (TABLE 3). The amount of residual variability explained by the center effect was low (intraclass correlation coefficient = 3.4%, $P=.28$). The other statistically significant predictor of late-onset sepsis was gestational age (odds ratio, 0.71; 95% CI, 0.57-0.89 for each additional gestational week).

Sepsis-attributable mortality was significantly lower in the 2 treatment groups (0/153 [0%] with BLF and 1/151 [0.7%] with BLF plus LGG, vs 8/168 [4.8%] with control; $P=.008$ and $P=.04$, respectively). Statistically significant differences in overall mortality were not found (Table 2).

Fungal colonization rates were similar in the 3 groups (Table 2). However, IFI occurred less frequently in the treatment groups (0/153 [0%] with BLF and 2/151 [1.3%] with BLF plus LGG, vs 9/168 [5.4%] with control; $P=.004$ and $P=.07$, respectively). The rate of progression from colonization to IFI was 0/27 (0%) with BLF and 2/25 (8.0%) with BLF plus LGG, vs 8/31 (25.8%) with control ($P=.005$ and $P=.16$, respectively).

Necrotizing enterocolitis of stage 2 or greater occurred less frequently with BLF plus LGG (0/151 [0%]) vs control (10/168 [6.0%]) ($P=.002$) but not with BLF (TABLE 4). Threshold retinopathy of prematurity occurred less frequently with BLF (6/153 [3.9%] vs 19/168 [11.3%]) ($P=.02$). All other secondary outcomes were not statistically significant.

In infants fed only maternal milk, late-onset sepsis occurred less frequently in treated than in control infants (1/42 [4.2%] and 2/32 [6.3%] with BLF and BLF plus LGG, respectively, vs 7/37 [18.9%] with control; $P=.02$ and $P=.16$, respectively). The same occurred in infants fed only formula milk (1/24 [4.2%] and 0/26 [0%] with BLF and BLF plus LGG, respectively, vs 4/22 [18.2%] with control; $P=.18$ and $P=.04$, respectively).

Safety Surveillance

No intolerances or adverse effects to BLF were recorded. *Lactobacillus rhamnosus* GG was never isolated from cultures. Drug administration was not discontinued because of presumed adverse effects, intolerance, or potentially dangerous interactions with other drugs. At age 4 weeks, liver enzyme values were in the reference ranges but were significantly lower in both treatment groups compared with the control group (eTable 2). The incidence of hyperbilirubinemia requiring phototherapy was similar in the 3 groups. No infants displayed signs of hepatotoxicity or cholestasis.

Table 4. Secondary End Points

	No./Total (%)			*P* Value[a]	
	BLF	BLF + LGG	Control	BLF vs Control	BLF + LGG vs Control
Threshold ROP requiring surgery[b]	6/153 (3.9)	13/151 (8.6)	19/168 (11.3)	.02	.46
Severe (grade 3-4) IVH[c]	6/153 (3.9)	4/151 (2.7)	2/168 (1.2)	.16	.43
Bronchopulmonary dysplasia[d]	4/153 (2.6)	4/151 (2.7)	6/168 (3.6)	.75	.75
Infants undergoing major surgery, including ligation of PDA	5/153 (3.3)	2/151 (1.3)	3/168 (1.8)	.49	.99
Death prior to hospital discharge, all causes	4/153 (2.6)	6/151 (4.0)	12/168 (7.1)	.08	.24
NEC ≥stage 2	3/153 (1.9)	0/151 (0)	10/168 (6.0)	.09	.002
Death or NEC ≥stage 2	7/153 (4.6)	7/151 (4.6)	18/168 (10.7)	.06	.06
Urinary tract infections	4/153 (2.6)	6/151 (4.0)	10/168 (6.0)	.18	.45

Abbreviations: BLF, bovine lactoferrin; BPD, bronchopulmonary dysplasia; IVH, intraventricular hemorrhage; LGG, *Lactobacillus rhamnosus* GG; NEC, necrotizing enterocolitis; PDA, patent ductus arteriosus; ROP, retinopathy of prematurity.
[a] Calculated using the Fisher exact 2-tailed test for comparing proportions.
[b] Defined according to Stoll et al.[3]
[c] Defined according to Jobe and Bancalari.[25]
[d] Defined according to Papile et al.[22]

COMMENT

In this study, oral BLF administered alone or in combination with LGG decreased the incidence of a first episode of late-onset sepsis in VLBW neonates. Bovine lactoferrin inhibits the growth of a wide variety of bacteria, fungi, viruses, and parasites.[10-14] The involved mechanisms include direct actions toward membrane components,[9,12,29] immunomodulatory effects,[30,31] and a synergistic action with anti-infective drugs.[32] Susceptibility of microorganisms to the inhibitory effect

of BLF is greater in certain growth stages[33]; this mechanism could explain why in our study BLF did not affect fungal colonization but rather progression from colonization to infection. In this study, gram-positive pathogens were mainly *Staphylococcus* species, including coagulase-negative *Staphylococcus* species. These pathogens contaminate CVCs at the skin entry site, while gram-negative pathogens and *Candida* species enter through the gut. The beneficial effects of BLF applied to all pathogens, since the distribution of infecting pathogens by type was not significantly different in treated and untreated groups. Further study is needed of the nonsignificantly greater reduction in gram-positive and fungal compared with gram-negative pathogens in the treated groups.

Given the high homology between HLF and BLF, it might be argued that supplemented BLF overlaps with maternal milk in protecting against sepsis. However, in untreated infants the incidence rates of late-onset sepsis were similar in those fed exclusively maternal milk vs exclusively formula; furthermore, the decrease in late-onset sepsis episodes in treated infants was comparable regardless of the type of milk feeding. Thus, maternal milk alone does not confer the benefits of BLF supplementation. This implies the need for additional lactoferrin, specifically to prevent late-onset sepsis.

The protective effect of BLF was clear in the infants weighing less than 1000 g, whereas it did not reach statistical significance in those weighing 1001 to 1500 g. This may be because ELBW infants received higher per-kilogram BLF doses and longer treatment courses. Of note, HLF concentration in milk from mothers of ELBW infants is significantly higher than in that of mothers of infants with higher birth weight.[34] Supplemental BLF administered at concentrations adjusted for the degree of prematurity may further improve outcomes. Alternatively, a higher dose of BLF may need to be been given for a longer time to the subgroup weighing 1001 to 1500 g.

Lactobacillus rhamnosus GG interacts with BLF to boost the defenses of an immature intestine.[15] Although incidence of late-onset sepsis in the treatment groups was too low to allow comparisons between BLF alone and BLF plus LGG, we did not observe any synergistic effect of LGG with BLF in preventing sepsis, in contrast with studies in mice.[15] It is possible that LGG and BLF target the same mechanism(s) in humans, thereby excluding a cumulative effect. Alternatively, since BLF accelerates intestinal maturation in preterm infants,[5] this might override the reported LGG effects and be responsible for a reduction of sepsis related to germ translocation.

Beneficial effects of LGG on fungal enteric colonization in preterm humans have been described[17] but were not seen in our study. Wide intercenter variations in *Candida* gastrointestinal tract colonization have been reported.[1] It is possible that LGG plays a role additional to that of BLF when these rates are high. In the study cited, LGG decreased fungal colonization from 46% to 23%,[16] whereas in our study colonization rate was 18.5% in controls.

Our study has some limitations. Generalizability is a potential limitation; in addition, the trial was underpowered to detect adverse events or to compare the 2 treatment groups. We administered a BLF dose based on the mean HLF intake that 1000-g infants usually ingest with mother's milk in their first weeks of life. Dose-finding studies should show whether different dosages or schedules fit better. No adverse effects were observed in the treated groups, in line with the considerable homology between BLF and HLF. However, although BLF is not reported as a milk protein responsible for nutritional intolerances or allergies, such events may become clinically evident after months or even years. Our study was limited to assessing infants at discharge. Since BLF has a mitigating effect on inflammation,[35] a primary cause of long-term complications of late-onset sepsis, BLF administration may, in turn, have long-term beneficial effects. Longer-term studies may be warranted.

Sepsis is the primary cause of death and long-term neurologic impairment in preterm VLBW neonates.[2,3,17] Prevention of neonatal sepsis relies on hygiene measures, cautious use of invasive procedures, medication stewardship, administration of fresh maternal milk, and early diagnosis. Nevertheless, none of these interventions is fully effective in decreasing the burden of the disease and overall have not been subjected to randomized controlled trials. This study has demonstrated that supplemental BLF, either alone or in combination with LGG, reduces first episodes of late-onset sepsis in VLBW infants.

Author Affiliations: Neonatal Intensive Care Unit (NICU), S. Anna Hospital, Torino (Drs Manzoni, Gallo, and Farina); NICU, Ospedali Riuniti, Foggia (Drs Rinaldi and Magaldi); NICU, University of Modena, Modena (Drs Cattani and Ferrari); NICU, IRCCS Mangiagalli Hospital, Milano (Drs Pugni and Mosca); NICU, Azienda Ospedaliera Universitaria Policlinico, Catania (Drs Romeo and Betta); NICU, Ospedale Regionale, Bolzano (Drs Messner and Renzullo); NICU, Policlinico Umberto I, Rome (Drs Stolfi and Pedicino); NICU, IRCCS San Matteo, Pavia (Drs Decembrino, Bollani, and Stronati); NICU, Policlinico University Hospital, Bari (Drs Laforgia and Quercia); NICU, Arcispedale S. M. Nuova, Reggio Emilia (Drs Vagnarelli and Magnani); NICU, Ca' Foncello Hospital, Treviso (Drs Memo, Bordignon, and Saia); Departments of Biomedical Sciences and Human Oncology, Cancer Epidemiology Unit, University of Torino, Torino (Dr Maule); and Department of Pediatrics, University of Torino, Torino (Dr Mostert), Italy.

Author Contributions: Drs Manzoni and Maule had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.
Study concept and design: Manzoni, Cattani, Stolfi, Decembrino, Vagnarelli, Maule, Mostert, Pedicino, Mosca, Ferrari, Stronati, Farina.
Acquisition of data: Manzoni, Rinaldi, Cattani, Pugni, Romeo, Messner, Stolfi, Decembrino, Laforgia, Vagnarelli, Memo, Bordignon, Saia, Maule, Gallo, Mostert, Magnani, Quercia, Bollani, Pedicino, Renzullo, Betta, Mosca, Ferrari, Magaldi, Stronati, Farina.
Analysis and interpretation of data: Manzoni, Maule, Mosca, Stronati, Farina.
Drafting of the manuscript: Manzoni, Rinaldi, Cattani, Pugni, Romeo, Messner, Stolfi, Decembrino, Laforgia, Vagnarelli, Memo, Bordignon, Saia, Maule, Gallo, Mostert, Magnani, Quercia, Bollani, Pedicino, Renzullo, Betta, Mosca, Ferrari, Magaldi, Stronati.
Critical revision of the manuscript for important intellectual content: Manzoni, Mostert, Mosca, Stronati, Farina.
Statistical analysis: Manzoni, Rinaldi, Maule, Betta.
Obtained funding: Manzoni, Vagnarelli, Stronati, Farina.
Administrative, technical, or material support: Cattani, Pugni, Laforgia, Bordignon, Gallo, Renzullo.
Study supervision: Manzoni, Romeo, Messner, Stolfi, Decembrino, Saia, Mostert, Magnani, Quercia, Pedicino, Mosca, Ferrari, Magaldi, Stronati.

Financial Disclosures: Dr Manzoni reported receiving 2 travel grants (in 2008 and 2009) from Dicofarm SpA. No other authors reported disclosures.
Funding/Support: Dicofarm SpA supported this study with a grant and supplied the drugs and placebo used in the study.
Role of the Sponsor: Dicofarm SpA had no role in the study design and enrollment of patients; the collection, analysis, and interpretation of the data; or the preparation and submission of the manuscript.
Independent Statistical Analysis: The independent statistical analysis was performed by Dr Maule, Department of Biomedical Sciences and Human Oncology, Cancer Epidemiology Unit, University of Torino, Italy. Dr Maule received no compensation or funding for conducting the analysis.
Italian Task Force for the Study and Prevention of Neonatal Fungal Infections; the Italian Society of Neonatology: In addition to the bylined authors, the task force includes the following persons: Amelia Di Comite, Alessandro Borghesi, Chryssoula Tzialla (Neonatal Intensive Care Unit [NICU], IRCCS S. Matteo, Pavia); Giovanni Agriesti, Riccardo Arisio, Giovanna Gomirato, Chiara Giovannozzi (NICU, S. Anna Hospital, Torino); Domenico Cipolla (NICU, University of Palermo); Fabio Natale (NICU, La Sapienza University, Roma); Giuseppina Corona (NICU, University of Messina); Francesco Tumminelli (NICU, Umberto I Hospital, Enna); Gennaro Vetrano (NICU, Fatebenefratelli Hospital, Benevento); Elisabetta Tridapalli (NICU, University of Bologna); Stefano Vitale (Neonatology Policlinico Casilino, Roma); Elio Caliendo (NICU, "San Giovanni di Dio e Ruggi d'Aragona" Hospital, Salerno).
Previous Presentation: Some data from this study were presented in abstract form as preliminary data at the Pediatric Academic Societies Meetings (May 2-5, 2008; Honolulu, Hawaii; and May 2-4, 2009; Baltimore, Maryland); at the First International Congress of the Union of European Neonatal and Perinatal Societies (November 17-19, 2008; Rome, Italy); and at the 48th ICAAC–46th IDSA Conference (October 24-28, 2008; Washington, DC).
Additional Information: eTables 1 and 2 are available at http://www.jama.com.

REFERENCES

1. Kaufman D, Fairchild KD. Clinical microbiology of bacterial and fungal sepsis in very-low-birth-weight infants. *Clin Microbiol Rev*. 2004;17(3):638-680.
2. Stoll BJ, Hansen N, Fanaroff AA, et al. Late-onset sepsis in very-low-birth-weight neonates: the experience of the NICHD Neonatal Research Network. *Pediatrics*. 2002;110(2, pt 1):285-291.
3. Stoll BJ, Hansen NI, Adams-Chapman I, et al; National Institute of Child Health and Human Development Neonatal Research Network. Neurodevelopmental and growth impairment among extremely low-birth-weight infants with neonatal infection. *JAMA*. 2004;292(19):2357-2365.
4. Hirai Y, Kawakata N, Satoh K, et al. Concentrations of lactoferrin and iron in human milk at different stages of lactation. *J Nutr Sci Vitaminol (Tokyo)*. 1990;36(6):531-544.
5. Buccigrossi V, de Marco G, Bruzzese E, et al. Lactoferrin induces concentration-dependent functional modulation of intestinal proliferation and differentiation. *Pediatr Res*. 2007;61(4):410-414.
6. Davidson LA, Lönnerdal B. Persistence of human milk proteins in the breastfed infant. *Acta Paediatr Scand*. 1987;76(5):733-740.
7. Kawakami H, Lönnerdal B. Isolation and function of a receptor for human lactoferrin in human fetal intestinal brush-border membranes. *Am J Physiol*. 1991;261(5, pt 1):G841-G846.
8. Lönnerdal B. Nutritional and physiologic significance of human milk proteins. *Am J Clin Nutr*. 2003;77(6):1537S-1543S.
9. Kuipers ME, de Vries HG, Eikelboom MC, Meijer DK, Swart PJ. Synergistic fungistatic effects of lactoferrin in combination with antifungal drugs against clinical *Candida* isolates. *Antimicrob Agents Chemother*. 1999;43(11):2635-2641.
10. Venkatesh MP, Pham D, Kong L, Weisman LE. Prophylaxis with lactoferrin, a novel antimicrobial agent, in a neonatal rat model of coinfection. *Adv Ther*. 2007;24(5):941-954.
11. Ward PP, Uribe-Luna S, Conneely OM. Lactoferrin and host defense. *Biochem Cell Biol*. 2002;80:95-102.
12. Orsi N. The antimicrobial activity of lactoferrin: current status and perspectives. *Biometals*. 2004;17(3):189-196.
13. Farnaud S, Evans RW. Lactoferrin—a multifunctional protein with antimicrobial properties. *Mol Immunol*. 2003;40(7):395-405.
14. Gifford JL, Hunter HN, Vogel HJ. Lactoferricin: a lactoferrin-derived peptide with antimicrobial, antiviral, antitumor and immunological properties. *Cell Mol Life Sci*. 2005;62(22):2588-2598.
15. Sherman MP, Bennett SH, Hwang FF, Yu C. Neonatal small bowel epithelia: enhancing anti-bacterial defense with lactoferrin and *Lactobacillus* GG. *Biometals*. 2004;17(3):285-289.
16. CFSAN/Office of Food Additive Safety. Agency response letter: GRAS notice No. GRN 000077. US Food and Drug Administration Web site. http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/GRASListings/ucm154188.htm. August 14, 2001. Accessed May 21, 2009.
17. Manzoni P, Mostert M, Leonessa ML, et al. Oral supplementation with *Lactobacillus casei* subspecies *rhamnosus* prevents enteric colonization by *Candida* species in preterm neonates: a randomized study. *Clin Infect Dis*. 2006;42(12):1735-1742.
18. Schanler RJ, Lau C, Hurst NM, Smith EO. Randomized trial of donor human milk versus preterm formula as substitutes for mothers' own milk in the feeding of extremely premature infants. *Pediatrics*. 2005;116(2):400-406.
19. Tsubota A, Yoshikawa T, Nariai K, et al. Bovine lactoferrin potently inhibits liver mitochondrial 8-OHdG levels and retrieves hepatic OGG1 activities in Long-Evans Cinnamon rats. *J Hepatol*. 2008;48(3):486-493.
20. Ascioglu S, Rex JH, de Pauw B, et al; Invasive Fungal Infections Cooperative Group of the European Organization for Research and Treatment of Cancer; Mycoses Study Group of the National Institute of Allergy and Infectious Diseases. Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. *Clin Infect Dis*. 2002;34(1):7-14.
21. Manzoni P, Pedicino R, Stolfi I, et al; Task Force per le infezioni fungine neonatali del GSIN; Socieà Italiana di Neonatologia. Criteria for the diagnosis of systemic fungal infections in newborns: a report from the task force on neonatal fungal infections of the GSIN [in Italian]. *Pediatr Med Chir*. 2004;26(2):89-95.
22. Jobe AH, Bancalari E. Bronchopulmonary dysplasia. *Am J Respir Crit Care Med*. 2001;163(7):1723-1729.
23. Bell MJ, Ternberg JL, Feigin RD, et al. Neonatal necrotizing enterocolitis: therapeutic decisions based upon clinical staging. *Ann Surg*. 1978;187(1):1-7.
24. Good WV, Hardy RJ, Dobson V, et al; Early Treatment for Retinopathy of Prematurity Cooperative Group. The incidence and course of retinopathy of prematurity: findings from the Early Treatment for Retinopathy of Prematurity study. *Pediatrics*. 2005;116(1):15-23.
25. Papile LA, Burstein J, Burstein R, Koffler H. Incidence and evolution of subependymal and intraventricular haemorrhage: a study of infants with birth weights less than 1,500 gm. *J Pediatr*. 1978;92(4):529-534.
26. Murray PR, Baron EJ, Jorgenson JH, Pfaller MA, Yoken RH, eds. *Manual of Clinical Microbiology*. 8th ed. Washington, DC: American Society for Microbiology; 2003:2113.
27. National Committee for Clinical Laboratory Standards (NCCLS). *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts: Approved Standard M27-A*. Wayne, PA: NCCLS; 1997.
28. Goldstein H. *Multilevel Statistical Models*. 3rd ed. New York, NY: Oxford University Press; 2003.
29. Bhimani RS, Vendrov Y, Furmanski P. Influence of lactoferrin feeding and injection against systemic staphylococcal infections in mice. *J Appl Microbiol*. 1999;86(1):135-144.
30. Wakabayashi H, Takakura N, Yamauchi K, Tamura Y. Modulation of immunity-related gene expression in small intestines of mice by oral administration of lactoferrin. *Clin Vaccine Immunol*. 2006;13(2):239-245.
31. Lupetti A, Paulusma-Annema A, Welling MM, et al. Synergistic activity of the N-terminal peptide of human lactoferrin and fluconazole against *Candida* species. *Antimicrob Agents Chemother*. 2003;47(1):262-267.
32. Zuccotti GV, Vigano A, Borelli M, Saresella M, Giacomet V, Clerici M. Modulation of innate and adaptive immunity by lactoferrin in human immunodeficiency virus (HIV)-infected, antiretroviral therapy-naïve children. *Int J Antimicrob Agents*. 2007;29(3):353-355.
33. Bortner CA, Arnold RR, Miller RD. Bactericidal effect of lactoferrin on *Legionella pneumophila*: effect of the physiological state of the organism. *Can J Microbiol*. 1989;35(11):1048-1051.
34. Ronayne de Ferrer PA, Baroni A, Sambucetti ME, López NE, Cerian Cernadas JM. Lactoferrin levels in term and preterm milk. *J Am Coll Nutr*. 2000;19(3):370-373.
35. Berlutti F, Schippa S, Morea C, et al. Lactoferrin downregulates pro-inflammatory cytokines upexpressed in intestinal epithelial cells infected with invasive or noninvasive *Escherichia coli* strains. *Biochem Cell Biol*. 2006;84(3):351-357.

