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ARTICLE

Systemic and mucosal levels of lactoferrin in very low birth weight infants supplemented with bovine lactoferrin[1]

Hannah L. Itell, Andrew Berenz, Riley J. Mangan, Sallie R. Permar, and David A. Kaufman

Abstract: Lactoferrin supplementation may help prevent infections in preterm infants, but the efficacy has varied with different doses and products. We assessed the absorption and excretion of bovine lactoferrin (bLF) in 31 infants receiving 100, 200, or 300 mg·kg^{-1}·day^{-1} of enteral bLF for 30 days. bLF and human lactoferrin (hLF) in infant saliva, blood, urine, and stool, as well as expressed (EBM) or donor breast milk (DBM) that were collected (*i*) before the treatment was initiated, (*ii*) at study day 22, and (*iii*) one week after treatment cessation, were measured using ELISA. During treatment, bLF was absorbed from the gastrointestinal tract and detected in plasma, saliva, and urine, as well as excreted in stool. Levels of bLF in the saliva and stool began to decline within 12 h after dosing, and bLF was undetectable in all samples one week after treatment. The concentrations of hLF exceeded those of bLF across sample types and time-points. Infants receiving EBM demonstrated higher levels of hLF in the saliva and stool than those receiving DBM. Neither bLF nor hLF levels varied by patient characteristics, bLF dosage, or infection status. This is the first study demonstrating bLF absorption into the bloodstream and distribution to saliva and urine in preterm infants. Future studies should further explore LF pharmacokinetics because higher and more frequent dosing may improve the clinical benefit of LF supplementation.

Key words: lactoferrin, preterm infants, bovine, supplementation, postnatal infection.

Résumé : La supplémentation en lactoferrine peut aider à prévenir les infections chez les prématurés, mais son efficacité varie en fonction du dosage et des produits. Les auteurs ont évalué l'absorption et l'excrétion de la lactoferrine bovine (bLF) chez trente et un nourrissons recevant 100, 200 ou 300 mg·kg^{-1}·jour^{-1} de bLF entérale pendant 30 jours. Les niveaux de bLF et de lactoferrine humaine (hLF) dans la salive, le sang, l'urine et les selles des nourrissons et dans le lait maternel exprimé ou de donneuses (LME, LMD), prélevés avant le début du traitement, le 22e jour de l'étude et une semaine après la fin du traitement ont été mesurés par ELISA. Pendant le traitement, la bLF était absorbée par le tractus gastro-intestinal, détectée dans le plasma, la salive et l'urine, et excrétée dans les selles. Les niveaux de bLF dans la salive et les selles commençaient à diminuer dans les 12 h suivant l'administration et la bLF était indétectable dans tous les échantillons une semaine après le traitement. Les concentrations de hLF dépassaient celles de bLF dans tous les types d'échantillons et à tous les moments. Les nourrissons qui ont reçu du LME présentaient des niveaux de hLF dans la salive et les selles plus élevés que les patients nourris au LMD. Ni les niveaux de bLF ni ceux de hLF ne variaient en fonction des caractéristiques du patient, de la dose de bLF ou du statut de l'infection. Il s'agit de la première étude qui démontre l'absorption de la bLF dans la circulation sanguine et sa distribution dans la salive et l'urine chez les prématurés. Les études futures devraient explorer plus avant la pharmacocinétique de la LF, car un dosage plus élevé et plus fréquent pourrait améliorer le bénéfice clinique de la supplémentation en LF. [Traduit par la Rédaction]

Mots-clés : lactoferrine, prématurés, bovins, supplémentation, infection postnatale.

Introduction

Lactoferrin (LF) supplementation has emerged as a strategy to prevent late-onset infections and associated complications in preterm, very low birth weight (<1500 g at birth, VLBW) infants. As an iron-binding protein with a basic N-terminal domain, LF competes with pathogens for nutrients and can directly bind microbes to enhance cell lysis or modulate immune responses (Valenti and Antonini 2005; Legrand 2016). In the acidic environment of the stomach, the potent antimicrobial peptide lactoferricin is released from LF and can bind endotoxins on bacterial membranes to control inflammation and initiate bacterial cell death (Gifford et al. 2005; Legrand 2016). LF also contributes to anti-inflammatory responses by downregulating the production of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α; Otsuki et al. 2005; Drago-Serrano et al. 2017). Owing to these antimicrobial and anti-inflammatory properties, LF may help combat infection-related morbidity and mortality in VLBW infants.

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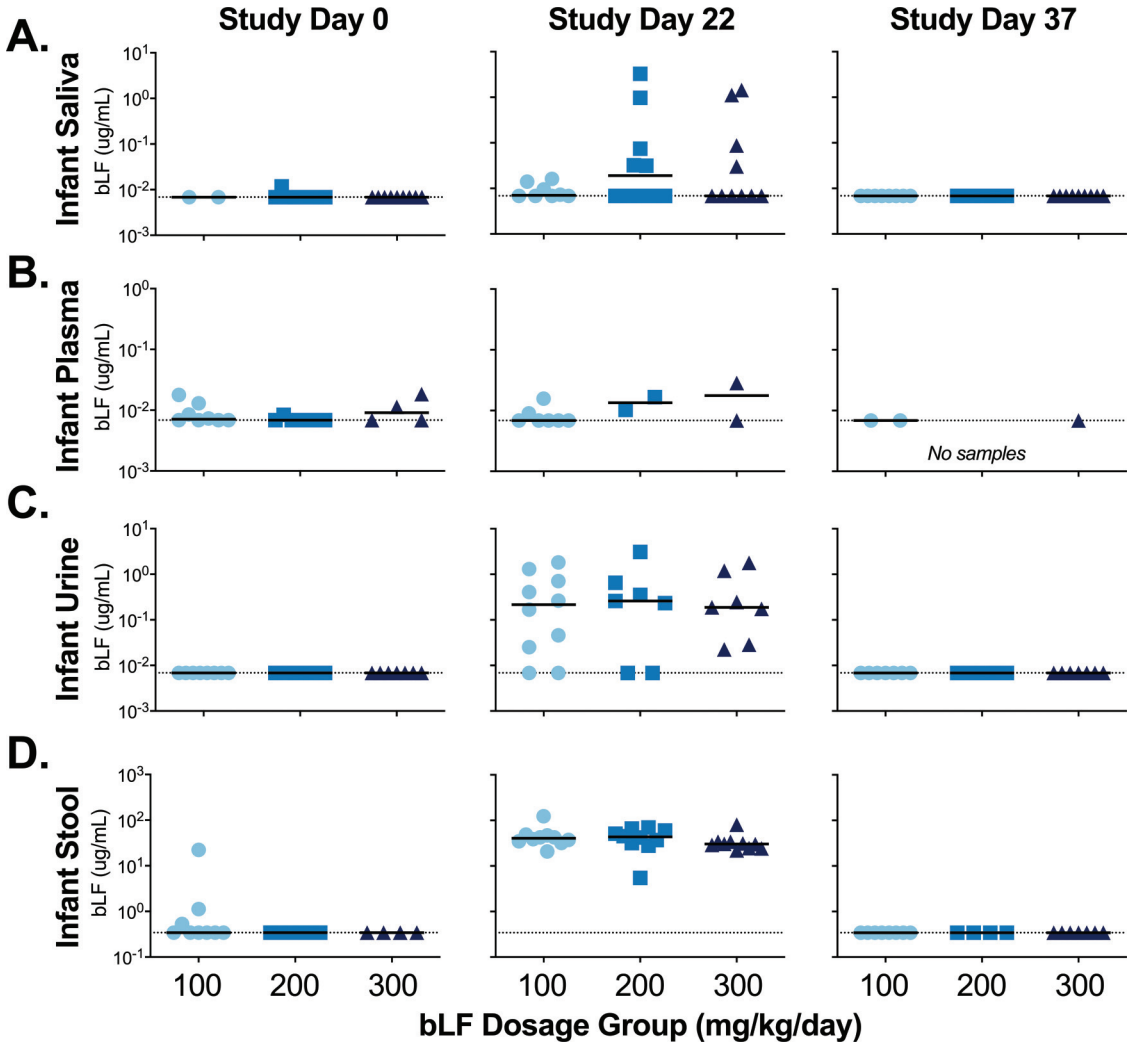
Fig. 1. Levels of bovine lactoferrin (bLF) in infants with very low birth weight did not vary by dose. The levels of bLF were measured in samples of infant saliva, plasma, urine, and stool (A–D) at three time-points. All of the sample types had undetectable levels of bLF 7 days after the final dose was administered (day 37). Levels of bLF did not vary significantly among the different bLF dosage groups within each time-point ($p > 0.05$, Kruskal–Wallis test). The number of samples varies by types, owing to sample criteria, availability, and volume restrictions. Dotted lines indicate the assay limits of quantification. Solid lines indicate median concentrations. [Colour online.]



Although LF is found in high concentrations in human colostrum and milk (Mastromarino et al. 2014), VLBW infants receive inadequate amounts of the protein due to their limited food tolerance. Therefore, studies have investigated the safety, tolerability, and benefits of human and bovine LF (hLF, bLF) supplementation in preterm infants during the first weeks of life (Manzoni et al. 2009, 2014; Akin et al. 2014; Kaur and Gathwala 2015; Ochoa et al. 2015; Barrington et al. 2016; The ELFIN Trial Investigators Group 2019; Tarnow-Mordi et al. 2020). Importantly, bLF shares 77% amino acid homology with hLF (Vorland et al. 1998), has anti-inflammatory properties similar to hLF (Berlutti et al. 2006), and has demonstrated higher antimicrobial activity than hLF (Vorland et al. 1998; Buccigrossi et al. 2007). A systematic review of studies investigating enteral hLF or bLF supplementation in preterm infants found that, overall, the interventions decreased late-onset sepsis and necrotizing enterocolitis without adverse effects (Pammi and Suresh 2017, 2020). However, discrepancies between studies remain due to the use of different LF products, doses, and treatment durations.

To inform the optimal dose of bLF for preterm infants, we analyzed the absorption and excretion of bLF and hLF for three escalating regimens of enteral bLF (100, 200, 300 mg·kg^{-1}·day^{-1}) in VLBW infants, and these doses were found to be safe and well tolerated, as described in our accompanying report in this issue (Kaufman et al. 2020). While hLF levels have been examined extensively in maternal human milk, there have only been a few pharmacodynamic studies of hLF, and none of bLF, in preterm infants (Decembrino et al. 2017; Weimer et al. 2020). We also evaluated whether systemic and mucosal levels of LF varied by postnatal infection status or by patient characteristics such as birth weight, gestational age, and nutritional source. Our goal for this pilot study was to provide insights for pharmacokinetics and dosage for future LF supplementation studies.

Materials and methods

Study population

Signed informed consent was received from the parents or legal guardians within 14 days after birth. Product administration was started thereafter and continued for up to 30 days.

Fig. 2. Saliva levels of bovine lactoferrin (bLF) demonstrate log–linear decay within 24 h of administration. The levels of bLF in the infants on study day 22 (D22) were assessed according to sample collection time in hours after the most recent dose of bLF (A–D). After $\log_{10}$ transformation, bLF levels from samples collected up to 24 h post-treatment were modeled using a linear regression. The R^2 model for linear fit is indicated with gray lines. [Colour online.]

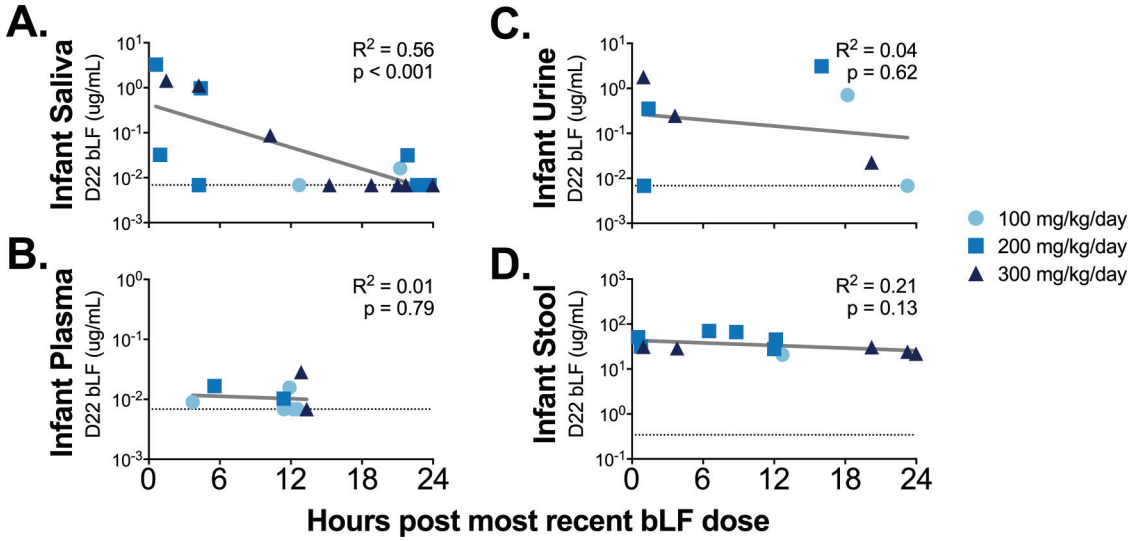
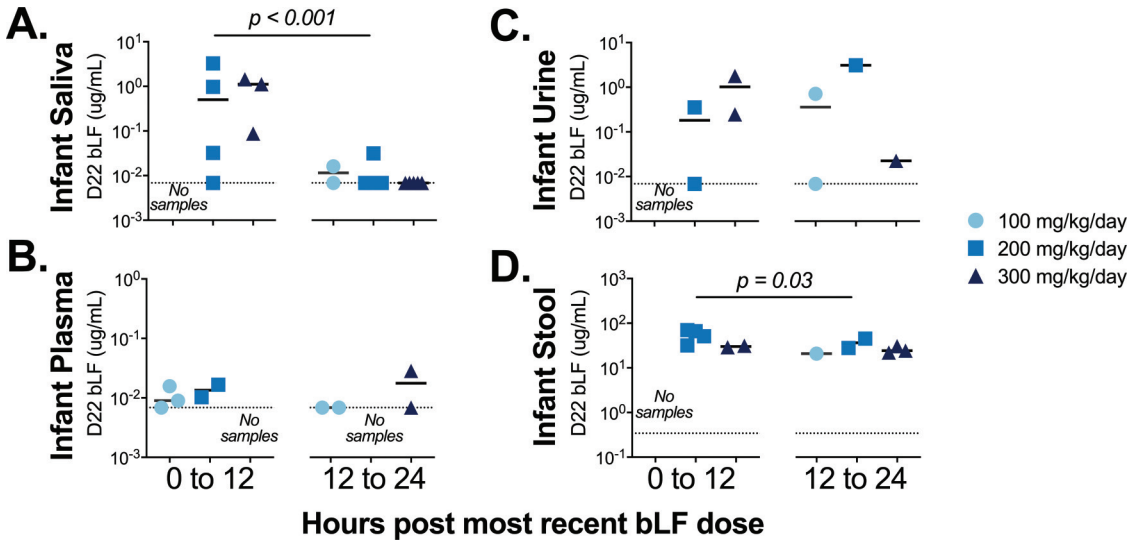


Fig. 3. The levels of bovine lactoferrin (bLF) in both the saliva and stool samples declined after 12 h post-administration. The levels of bLF on study day 22 (D22) in each infant compartment (saliva, plasma, urine, and stool) were stratified according to whether samples were collected within 12 h or 12–24 h after bLF treatment (A–D). The difference between stratified samples, combined across supplementation groups, was evaluated using the Mann–Whitney *U* tests (*p* values indicated in the figure). The number of samples varied by compartment due to sample criteria, availability, and volume restrictions. [Colour online.]



Over a 10 month period, 31 VLBW infants at the University of Virginia's (UVA) neonatal intensive care unit (NICU) were assigned to receive enteral bLF for 30 days (Supplementary data, Fig. S1[1]; ClinicalTrials.gov Identifier: NCT02731092). VLBW infants born at or transferred to the UVA NICU within 7 days of birth were eligible for enrollment. Demographic feeding information and infection-related events were collected from study participants' medical records.

Study intervention

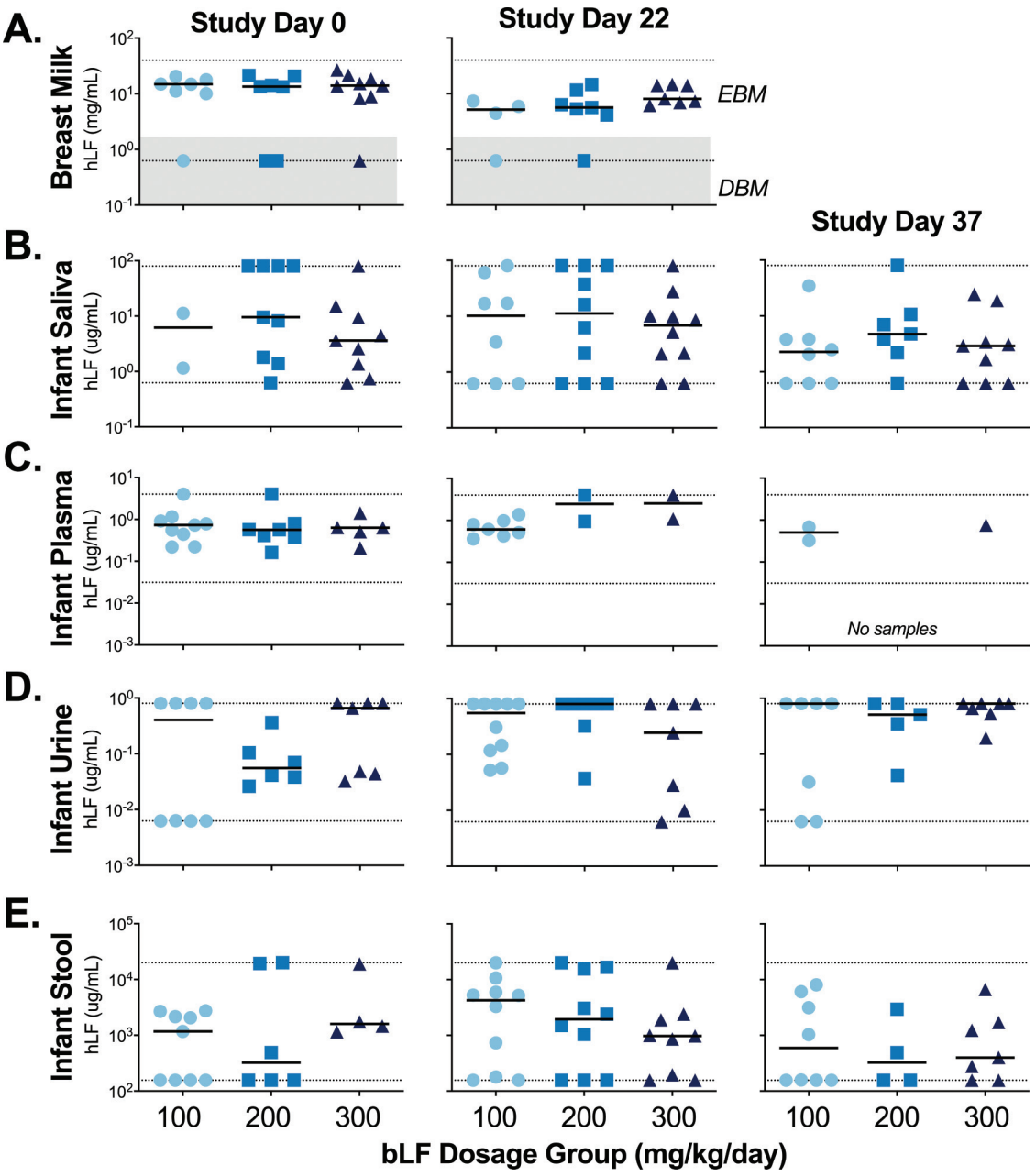
The details of the study are available in our accompanying report, also in this issue (Kaufman et al. 2020). In brief, study participants were enrolled sequentially into one of three escalating bLF supplementation groups: 100 (n = 10), 200 (n = 10), or 300 mg·kg^{-1}·day^{-1} (n = 11). bLF (Glanbia Nutritionals, Gooding, Idaho, USA) was prepared at the UVA Investigational Pharmacy by dissolving the product powder in sterile water under aseptic conditions, and the mixture was administered enterally, daily.

Sample collection and processing

Samples of infant saliva, blood, urine, and stool, as well as expressed (EBM) or donor breast milk, (DBM) were collected (*i*) prior to the first LF supplement (study day 0), (*ii*) 22 days into

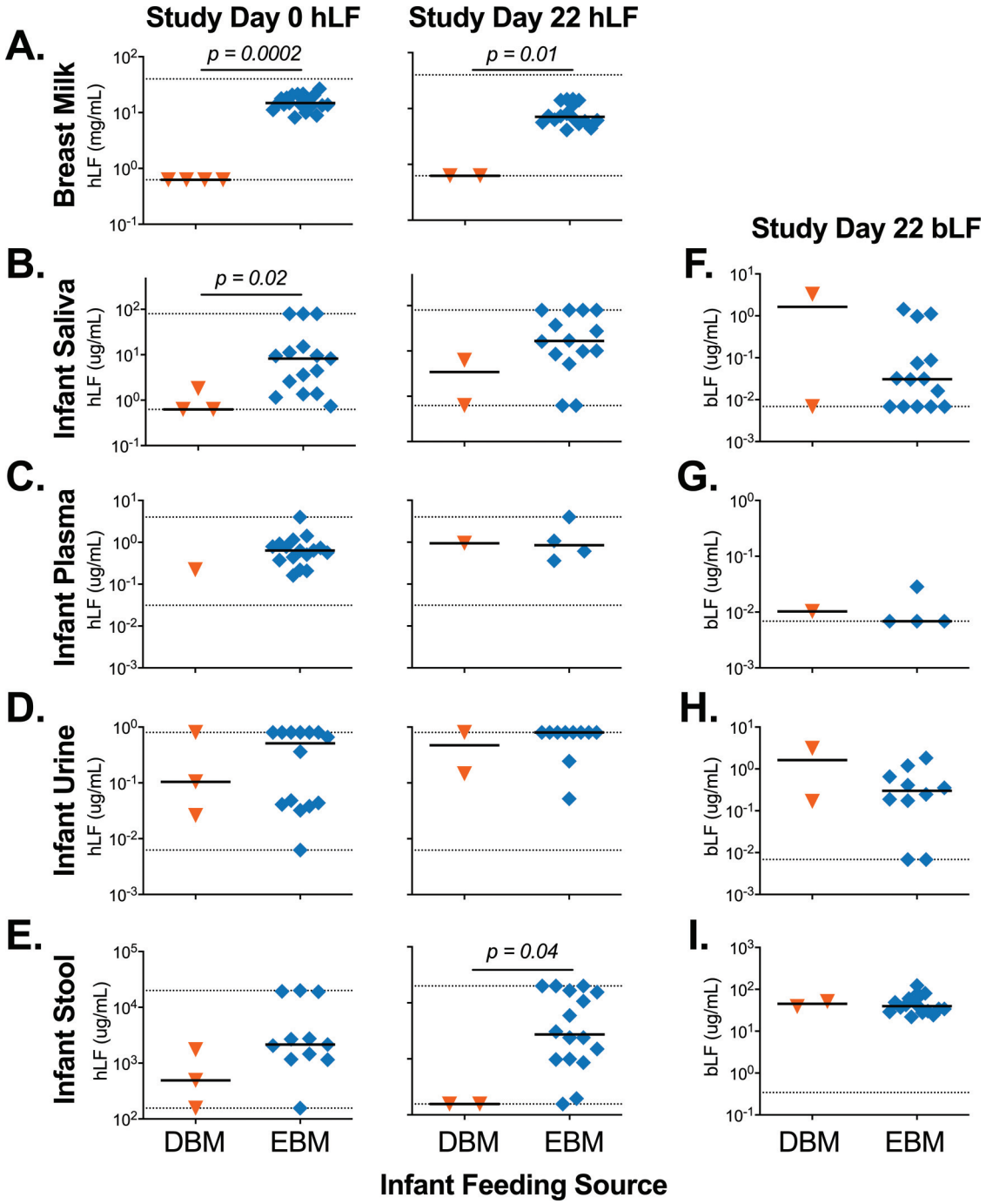
[1]Supplementary data are available with the article at https://doi.org/10.1139/bcb-2020-0238.

Fig. 4. Human lactoferrin (hLF) was detectable in the majority of samples. (A) The levels of hLF were measured in maternal expressed breast milk (EBM) and donor breast milk (DBM; data points in shaded region), and (B–E) in samples of infant saliva, plasma, urine, and stool, on study days 0, 22, and 37. The levels of hLF did not vary significantly among the bovine LF groups (100, 200, and 300 mg·kg^{-1}·day^{-1}) within each time-point ($p > 0.05$, Kruskal–Wallis test). [Colour online.]



supplementation (study day 22), and (*iii*) 7 days after the last administration of lactoferrin (study day 37). Unstimulated saliva was collected using two swabs: (*i*) sterile polyester tipped swab (VWR, Radnor, Pennsylvania, USA) to screen for cytomegalovirus (CMV), and (*ii*) sterile Weck-Cel swab (Beaver-Visitec International, Waltham, Massachusetts, USA) to quantify LF. The polyester-tipped swabs were stored at room temperature, and the Weck-Cel swabs were stored at −80 °C before being transported to the laboratory in batches for processing. Scavenged blood samples, which refers to residual blood from laboratory samples collected for clinical care, were collected in EDTA tubes, refrigerated, and then processed by centrifugation within 7 days of collection to isolate plasma. All of the specimens were stored at −80 °C until further processing by lab personnel, at which time they were thawed and kept at 4 °C. Upon thawing, the saliva swabs were rehydrated with 200 μL of sterile water, incubated for 20 min at room temperature, and spun down in a 0.22 μm Spin-X cellulose acetate filter tube (Corning, Corning, New York, USA) to extract the contents of the swab. The stool samples were weighed and reconstituted in sterile water at a ratio of 1 mg of stool to 4 μL of water. Breast milk and reconstituted stool specimens were spun at 14 000 rpm for 20 min at 4 °C to remove lipids and cell debris. All laboratory personnel performing specimen processing and assays followed a blind protocol with respect to the dose of bLF being administered.

Fig. 5. Infants receiving maternal expressed breast milk (EBM) have higher levels of human lactoferrin (hLF) in their saliva and stool, compared with infants fed donor breast milk (DBM). No hLF was detected in the DBM breast milk samples. Infant feeding types were recorded on study days 0 and 22. Levels of hLF in breast milk (DBM, ▼; EBM, ♦), and the levels of hLF and bLF in infant saliva, plasma, urine, and stool (A–E and F–I, respectively) on study days 0 and 22 were compared between infants fed EBM or DBM. All comparisons were evaluated with a Mann–Whitney *U* test (*p* values indicated in the figure). [Colour online.]



Assessment of CMV

Postnatal CMV infection was determined by assessing infant saliva collected on study days 0 and 37 for the presence of CMV DNA using quantitative polymerase chain reaction (qPCR), as previously described (Bialas et al. 2016). Prior to qPCR, the saliva samples were incubated at 95 °C for 5 min to denature any protein present. A sample was considered CMV-positive (by qPCR) if at least 2 of the 6 within-assay replicates had detectable CMV DNA. Infants were considered to have postnatally acquired CMV if they tested negative by saliva qPCR at study day 0 but positive at study day 37.

Lactoferrin measurements

The processed saliva, plasma, urine, stool, and breast milk specimens from the three collection time-points were assayed for bLF and hLF using commercial enzyme-linked immunosorbent

Table 1. The levels of human and bovine lactoferrin (LF) did not vary by infant gestational age or birth weight.

		Gestational age		Birth weight	
	N	Spearman *R*	*P*	Spearman *R*	*P*
Study day 0, human LF					
EBM	19	−0.07	0.77	−0.05	0.84
Saliva	20	−0.24	0.31	−0.03	0.90
Plasma	21	−0.01	0.96	−0.002	>0.99
Urine	21	−0.34	0.13	−0.29	0.21
Stool	19	−0.07	0.78	−0.09	0.72
Study day 22, human LF					
EBM	16	−0.34	0.19	−0.38	0.15
Saliva	28	−0.03	0.88	0.08	0.68
Plasma	11	0.19	0.56	0.12	0.72
Urine	24	**−0.56**	**0.01***	**−0.56**	**0.004***
Stool	29	0.01	0.96	0.05	0.82
Study day 22, bovine LF					
Saliva	28	**−0.41**	**0.03***	**−0.39**	**0.04***
Plasma	11	0.04	0.90	0.18	0.59
Urine	24	−0.34	0.10	−0.23	0.27
Stool	30	0.03	0.87	0.11	0.56
Study day 37, human LF					
Saliva	24	−0.30	0.15	−0.28	0.19
Plasma	3	0.87	0.67	0.50	>0.99
Urine	19	−0.38	0.11	−0.19	0.43
Stool	19	−0.36	0.13	**−0.58**	**0.01***

Note: *, $p < 0.05$; Spearman.

assay (ELISA) kits, in accordance with the manufacturer's instructions (Bovine Lactoferrin ELISA Kit, Bethyl Laboratories, Montgomery, Texas, USA; Human Lactoferrin ELISA Kit, AssayPro, St. Charles, Missouri, USA). Prior to being assayed, the samples were spun-down to pellet any residual debris. Point dilutions by volume were determined for each specimen type and ELISA to maximize the number of samples within range of the standard curve (bLF in saliva, plasma, and urine, 1:10; bLF stool, 1:1000; hLF saliva, 1:1000; hLF plasma, 1:50; hLF urine, 1:10; hLF stool, 1:50 000; hLF breast milk, 1:500 000). All of the specimens were tested in duplicate. Plates were read on a SpectraMax Plus Plate Reader (Molecular Devices, San Jose, California, USA) to determine optical density at 450 nm. SoftMax Pro 6.3 software (Molecular Devices) was used to interpolate concentrations from standard curves. The upper and lower limits of quantification for each assay equal the highest and lowest concentration of the standard, respectively, multiplied by the sample dilution factor. Samples outside of this range were assigned the concentration value of the quantification limit.

Importantly, product datasheets for the human and bovine LF ELISA kits we selected demonstrate that the assays yielded undetectable levels of activity (0%) for bLF and hLF, respectively, indicating the lack of antigen cross-reactivity for both assays. To verify this with our study samples, we compared the levels of hLF and bLF on study day 0 because we expected hLF at this time-point to be very high and bLF levels to be low or undetectable. Importantly, the hLF and bLF levels on study day 0 did not correlate, indicating that high levels of hLF do not yield a cross-reactive bLF signal (Supplementary data, Fig. S2[1]). Therefore, assay cross-reactivity was not appreciable in our hLF or bLF observations.

Statistical analysis

All statistical analyses were conducted using GraphPad Prism 8.0 software (GraphPad, San Diego, Calif.). Nonparametric tests were used to analyze the results because the data were not normally distributed. The Kruskal–Wallis test with Dunn's test for multiple comparisons was used to evaluate LF levels between the bLF dosage groups. All of the *p* values associated with these tests are multiplicity-adjusted. Two-tailed Mann-Whitney *U* tests were applied to data stratified into two groups, such as LF levels by sample collection time, infant feeding source, and postnatal infections. The two-tailed Spearman–Rank test was used to evaluate correlations between LF levels and infant characteristics, including gestational age and birth weight. Finally, a log–linear regression model was used to assess the relationship between bLF levels on study day 22 and sample collection timing relative to bLF administration.

Results

Study population characteristics

Thirty-one preterm, VLBW infants were enrolled for this study. The infants were sequentially assigned to receive enteral bLF supplementation of increasing dosage: 100 ($n = 10$), 200 ($n = 10$), or 300 ($n = 11$) mg·kg^{-1}·day^{-1}. The median start day after birth was day 7 (range: 2–15) (Supplementary data, Fig. S1[1]) and treatment continued for 30 days. The gestational age and birth weight of the study participants ranged from 24.1 to 34 weeks, and 540–1480 g, respectively (Supplementary data, Table S1[1]). Importantly, gestational age and birth weight did not statistically vary between bLF dosage groups (gestational age, $p = 0.18$; birth weight, $p = 0.07$; Kruskal–Wallis test). During the study period, one infant in each group acquired a bloodstream infection (BSI), caused by coagulase-negative staphylococci (CoNS), methicillin-susceptible *Staphylococcus aureus*, and polymicrobial organisms (CoNS + *Enterococcus fecalis*) on study days 9, 11, and 14, respectively by dosage group. There were no urinary tract infections or necrotizing enterocolitis in any of the test groups. Additionally, two infants (twins) in the 200 mg·kg^{-1}·day^{-1} group, demonstrated evidence of postnatal CMV acquisition by testing negative for CMV (by qPCR) on study day 0 (day 3 after birth) but positive on study day 37 (day 40 after birth). These twins received human milk only (93% EBM, 7% DBM) during the study period. The majority of infants were fed EBM on study day 0, but feeding sources diversified over the study period (Supplementary data, Fig. S3[1]).

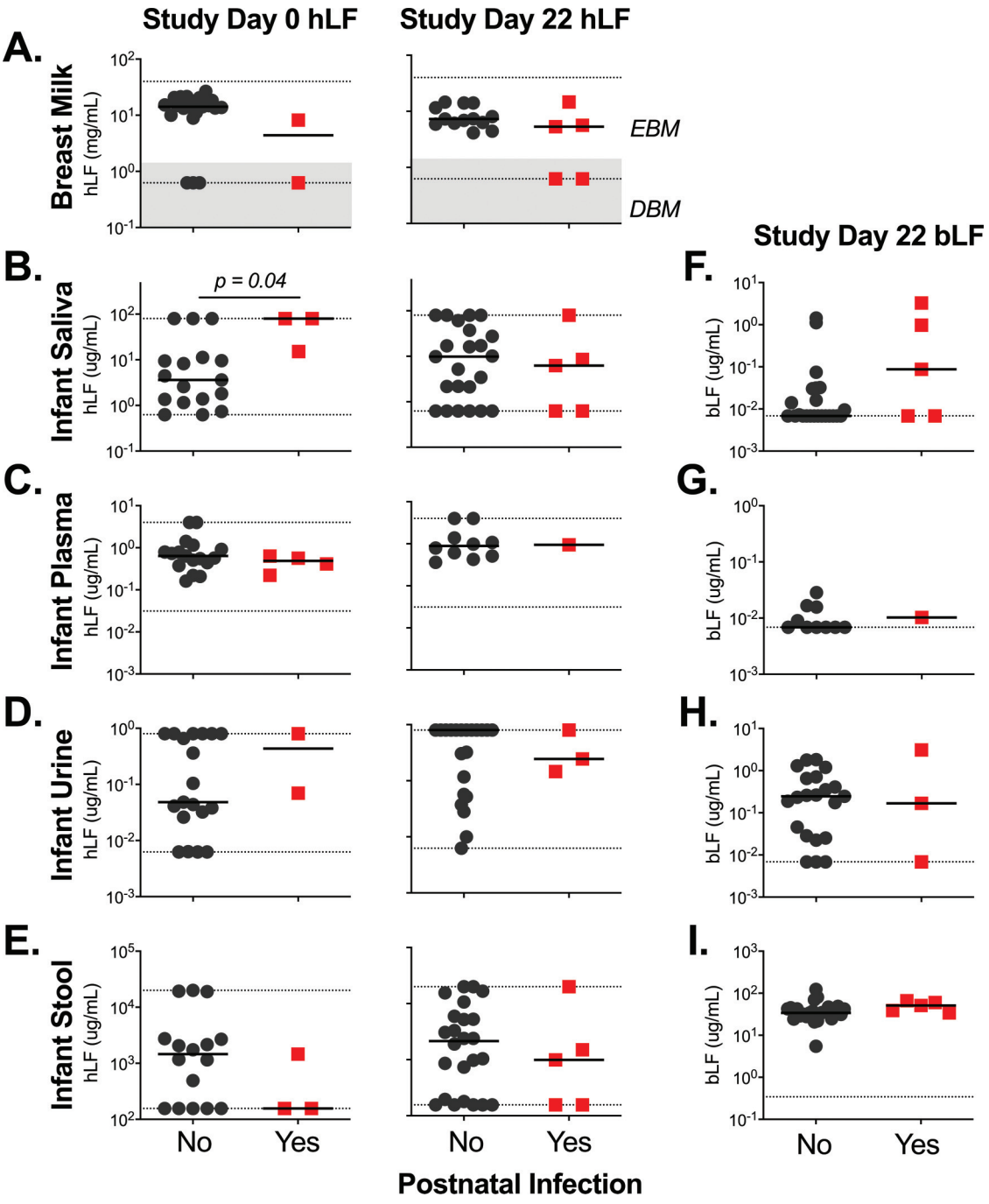
bLF levels in VLBW infants

Systemic and mucosal levels of bLF were detectable in VLBW infants during the 30-day treatment period, as indicated by the levels on study day 22 (Fig. 1). The highest increases from baseline and the highest concentrations of bLF overall occurred in infant urine and stool, suggesting the importance of LF in mucosal compartments. The levels of bLF in the different treatment groups were not statistically different for any sample type, and bLF had completely cleared from the infants one week after the last dose. To understand bLF clearance from the various anatomical compartments, we applied a log–linear regression model to evaluate the relationship between the levels of bLF on study day 22 and sample collection time, relative to the most recent bLF dose (Fig. 2). The bLF levels in the saliva samples, but not in other sample types, were consistent with log–linear decay within 24 h after bLF was administered ($R^2 = 0.56$, $p < 0.001$). Accordingly, saliva levels also peaked a few hours after enteral administration in some of the patients in the 200 and 300 mg·kg^{-1}·day^{-1} groups, whereas bLF in the other sample types did not appear to substantially fluctuate with time. To further investigate the impact of sample collection time on bLF levels, we stratified the bLF levels in the samples on study day 22 according to whether they were collected within 12 h or 12–24 hours after bLF administration (Fig. 3). The results show that the bLF levels in the saliva and stool samples were significantly reduced in the samples collected 12–24 hours after administration ($p < 0.001$ and $p = 0.03$ respectively, Mann–Whitney *U* test).

hLF levels in VLBW infants

hLF was detected in most of the infant stool, saliva, plasma, and urine samples from all of the time-points and exceeded bLF levels in every sample type and at every time-point. We found no differences in hLF concentrations among the different bLF

Fig. 6. The levels of lactoferrin (LF) in infants with very low birth weight were not associated with infection status. (A) The levels of human LF (hLF) in maternal expressed breast milk (EBM) and donor breast milk (DBM; data points are in the shaded region) and (B–E) in samples of infant saliva, plasma, urine, and stool from study days 0 and 22 were compared between the infants without postnatal infection and those that acquired a postnatal infection during the study period. (F–I) The levels of bovine LF (bLF) in samples of infant saliva, plasma, urine, and stool on study day 22 were also compared between these groups. All comparisons were evaluated with the Mann–Whitney *U* test (*p* values are indicated in the figure). [Colour online.]



dosage groups at any time-point for any of the samples (Fig. 4), although we did see large variations in hLF within sample types. As expected, maternal EBM from study day 0, which was primarily composed of colostrum or transitional breast milk, demonstrated the highest levels of hLF (median: 14.8 mg/mL; range: 8.2–26.7 mg/mL), whereas maternal EBM from study day 22, which was composed of transitional and mature breast milk, had slightly decreased levels from baseline (median: 7.1 mg/mL; range: 4.1–14.7). In contrast to the high concentration of hLF measured in EBM, there was no measurable hLF in any DBM samples (Figs. 4 and 5A).

Among the infant sample types we assessed, infant stool contained the highest concentration of hLF, with levels reaching 20 mg/mL at

Table 2. The levels of human and bovine lactoferrin (LF) do not explain the risk of infection for infants with a very low birth weight.

	No infections (n = 26)	Postnatal infections		
		CMV (n = 2)	BSI (n = 3)	Combined (n = 5)
Study day 0, human LF				
EBM (mg/mL)	14.88 (8.89–26.69)	No samples	8.2 (8.2–8.2)	8.2 (8.2–8.2)
Saliva (μg/mL)	**3.62 (0.63–80)***	80 (80–80)	15.19 (15.19)	**80 (15.19–80)***
Plasma (μg/mL)	0.64 (0.16–4)	0.56 (0.56–0.56)	0.41 (0.22–0.63)	0.49 (0.22–0.63)
Urine (μg/mL)	0.05 (0.01–0.80)	No samples	0.43 (0.07–0.80)	0.43 (0.07–0.80)
Stool (μg/mL)	1459 (156.3–20000)	156.3 (156.3–156.3)	1461 (1461)	156.3 (156.3–1461)
Study day 22, human LF				
EBM (mg/mL)	7.32 (4.13–14.55)	5.48 (5.34–5.63)	14.69 (14.69–14.69)	5.63 (5.34–14.69)
Saliva (μg/mL)	9.82 (0.63–80)	40.3 (0.63–80)	6.26 (0.63–8.56)	6.26 (0.63–80)
Plasma (μg/mL)	0.89 (0.36–4)	No samples	0.95 (0.95)	0.95 (0.95)
Urine (μg/mL)	0.8 (0.01–0.80)	0.24 (0.24)	0.47 (0.15–0.80)	0.24 (0.15–0.80)
Stool (μg/mL)	2142 (156.3–20000)	10749 (1497–20000)	156.3 (156.3–992.9)	992.9 (156.3–20000)
Study day 22, bovine LF				
Saliva (μg/mL)	0.01 (0.01–1.44)	0.49 (0.01–0.97)	0.09 (0.01–3.27)	0.09 (0.01–3.27)
Plasma (μg/mL)	0.01 (0.01–0.03)	No samples	0.01 (0.01)	0.01 (0.01)
Urine (μg/mL)	0.25 (0.01–1.83)	0.01 (0.01)	1.63 (0.17–3.10)	0.17 (0.01–3.10)
Stool (μg/mL)	34.11 (5.48–123.7)	63.27 (60.09–66.45)	38.85 (34.11–51.28)	51.28 (34.11–66.45)

Note: CMV, cytomegalovirus; BSI, bloodstream infection; EBM, maternal expressed breast milk. Values presented are the median (range); *, $p < 0.05$ for no infections vs. combined postnatal infections; Mann–Whitney U test.

both study days 0 and 22 (Fig. 4). Despite the higher concentration of hLF in EBM at study day 0 compared with day 22, the levels of hLF in infant samples remained similar across the study period.

LF levels by patient characteristics and feeding type

The levels of hLF and bLF did not strongly correlate with infant gestational age or birth weight (Table 1). Moreover, these characteristics did not differ between the dosage groups, and therefore cannot explain the lack of difference in bLF levels between groups (Supplementary data, Table S1[1]). We did observe differences in the levels of hLF, but not bLF, between the infants fed EBM and those fed DBM (Fig. 5). As previously noted, the samples of EBM had consistently high levels of hLF whereas the samples of DBM had no detectable hLF (Figs. 4 and 5A). Accordingly, infants receiving EBM tended to have higher hLF levels across compartments than infants receiving DBM. The levels of hLF in the infant saliva and stool samples were most influenced by the intake of EBM compared with DBM, whereas the levels in the infant plasma and urine samples varied less by hLF intake. Specifically, the infants fed EBM had statistically higher levels of hLF in the saliva samples from study day 0 and stool samples from study day 22 levels compared with the infants fed DBM (p = 0.02 and 0.04, respectively, Mann–Whitney U Test).

LF levels by postnatal infection status

During the study period, one infant from each bLF dosage group acquired a BSI, and two infants (twins) in the 200 mg·kg^{-1}·day^{-1} bLF group showed evidence of postnatal CMV acquisition (Supplementary data, Table S1[1]). We found no significant differences in hLF and bLF levels between infants with or without postnatal infections, combining both postnatal CMV and BSI cases (Fig. 6; Table 2), except for the elevated baseline levels of hLF in the saliva of infants with postnatal infections (p = 0.04, Mann–Whitney U Test).

Discussion

This study is the first report of the absorption, secretion, and excretion of bLF in preterm infants. While previous studies have administered hLF or bLF to infants (Manzoni et al. 2009, 2014; Akin et al. 2014; Kaur and Gathwala 2015; Ochoa et al. 2015, 2020; Barrington et al. 2016; Sherman et al. 2016; The ELFIN Trial Investigators Group 2019; Asztalos et al. 2020; Tarnow-Mordi et al. 2020), few have measured the resulting levels of hLF, and none, to our knowledge, have evaluated the levels of bLF in multiple anatomical compartments. Our study demonstrates that bLF and hLF can be detected in infant saliva, plasma, urine, and stool using commercially available ELISA kits, without cross-reactivity. The bLF in infant saliva and stool appears to decline within 24 h after administration, whereas urine and plasma levels remain constant. These findings imply that additional pharmacokinetic studies of dose, frequency of administration, route of administration, and study duration may potentially influence levels found as well as the clinical outcomes.

In light of the recent findings from the ELFIN, LIFT, and NEOLACTO studies, indicating that enteral supplementation with bLF at doses of 150 and 200 mg·kg^{-1}·day^{-1} did not reduce the risk of late-onset infections in preterm infants (The ELFIN Trial Investigators Group 2019; Asztalos et al. 2020; Ochoa et al. 2020), it is particularly important to evaluate LF levels to optimize supplementation dose and duration. Compared with the results from the 150 mg·kg^{-1}·day^{-1} group, which did not differ with respect to acquisition of BSI, in the two studies using 200 mg·kg^{-1}·day^{-1} the rate of BSI trended to being decreased (by 17% and 25%). In the NEOLACTO study, which administered 200 mg·kg^{-1}·day^{-1} divided between three doses a day, the results showed a trend to 55% reduction in BSI in the subgroup of infants weighing <1000 g. Additionally, the one study using hLF administered at 300 mg·kg^{-1}·day^{-1} divided between two doses a day, reported a significant decrease in hospital-acquired infections ($p < 0.04$), with a 40% decrease in BSI compared with the placebo group (p = 0.52) (Sherman et al. 2016).

The administration of bLF at doses of 100, 200, and 300 mg·kg^{-1}·day^{-1} in our small study did not significantly affect the bLF levels in the different infant sample types over the study period, and infant GA, BW, and feeding type likely did not affect this outcome. While this result may be misleading due to the small study size, sampling time, and limited infant sample availability for some sample types, it could also indicate that excretion occurs rapidly, or that the maximum levels of bLF uptake are achieved by all three dosage groups after three weeks of treatment. However, a few observations in this small study suggest that higher doses of bLF may influence the resulting levels in infants. For instance, saliva samples from infants receiving 200 and 300 mg·kg^{-1}·day^{-1} of bLF had higher levels of bLF at study day 22 (30% in each group had greater than 0.05 μg/mL) compared with those

receiving 100 mg·kg^{-1}·day^{-1} (none reached 0.05 μg/mL). This trend also applied to bLF levels in the plasma samples at this time-point, with the maximum bLF levels (15.8, 16.6, 28.4 ng/mL, respectively, by treatment group) being dose-dependent. Additionally, 100% of the infants in the 300 mg·kg^{-1}·day^{-1} dosage group had detectable levels of bLF in their urine, compared with around 75% of the infants in the 100 and 200 mg·kg^{-1}·day^{-1} dosage groups. Another observation is that excretion via the urine and stool was relatively constant, which may imply that higher doses could lead to an enhanced local gastrointestinal effect as well as absorption. Additional studies on bLF dosage could inform whether escalating doses impact uptake dynamics.

In addition to observing differences in bLF levels across dosage groups, we also found that bLF levels in the samples rapidly declined with time, with decreased levels in the saliva and stool samples just 12 h after administration. bLF was completely undetectable in the different infant sample types only one week after the final dose. Because bLF is quickly cleared from infant systemic and mucosal compartments, more frequent dosing may be needed to sustain levels in the saliva and other mucosal sites. Additionally, if a supplementation regimen is found to be beneficial, it will likely need to continue while its desired effects are observed.

During the study period, only two infants (twins) acquired postnatal CMV, and three developed bloodstream infections. It is possible that the two CMV infections were perinatally acquired, as these twins were tested just 3 days after birth (their time of enrollment) and postnatal acquisition was obtained on day 40 after birth (study day 37). A sample on day 30 after birth would have more definitively established the presence or absence of perinatal infection. Timing of saliva sampling for CMV detection is also important, because it is possible that samples were taken after feedings and could be affected if CMV was present in maternal milk. Evaluating the urine for postnatal CMV infection would add additional identification and confirmation in some cases, especially in preterm infants. While we did not observe an association between LF levels and protection against late-onset infections, the scarce number of infection cases, timing of sample collection, and incomplete sample availability for some compartments likely limited our ability to effectively evaluate this relationship. A larger cohort, which would consequently have a higher incidence of postnatal infections, may be necessary to determine the relationship between LF levels and risk of infection.

Regarding the levels of hLF, although we observed slightly higher levels of hLF in colostrum and transitional milk compared with some previous reports (Hirai et al. 1990; Ronayne de Ferrer et al. 2000; Mastromarino et al. 2014; Yang et al. 2018), our findings are very similar to those found in a large clinical trial in Peru involving 346 mothers of low birth weight infants (Villavicencio et al. 2017). Also similar to other studies (Paulaviciene et al. 2020), we did not detect any measurable amount of hLF in DBM (Fig. 5). The presence of hLF in saliva, plasma, urine, and stool samples from infants not receiving EBM suggests endogenous production of hLF in these preterm infants. Moreover, it appears that the levels of hLF in the saliva and stool samples, but not the plasma and urine samples, are affected by hLF intake, because the levels in saliva and stool corresponded with whether infants received EBM or DBM. This may suggest that plasma and urine levels of hLF are mostly influenced by local production, whereas saliva and stool levels may result from both local production and secretion of hLF acquired through feedings. Gastroesophageal reflux events may also affect saliva levels.

Our study is also limited in that we only administered the bLF once a day, primarily administered via a nasogastric or orogastric tube. Given our findings of decreased levels of bLF in the saliva and stool samples just 12 h after treatment, with saliva levels demonstrating log–linear decay, dosing multiple times a day may meaningfully affect the resultant bLF levels and clinical outcomes. The NEOLACTO trial administered bLF three times a day, compared with once daily, and observed promising trends, which were noted above. Future studies with more frequent collection time-points will need to examine LF pharmacokinetics in more detail to inform optimal dosing regimens. Data from animal studies also suggests that absorption may be improved by administering part of the LF dose via the sublingual route (Hayashi et al. 2017). The levels of hLF detected in the infants fed DBM on the day of testing may have been affected in some cases by EBM received prior to the testing day.

Together, our data highlight both the feasibility of quantifying the levels of bLF and hLF in the systemic and mucosal compartments of VLBW preterm infants, and also the insights that these measurements provide with respect to designing supplementation protocols. We are the first study to demonstrate that supplemental bLF can be absorbed by the gastrointestinal tract into the blood, secreted into saliva and urine, and potentially cleared more quickly from the saliva and stool than blood and urine. Quantification of the levels of bLF and hLF will hopefully allow future studies to determine optimal dosing and to elucidate whether treatment with LF is protective against late-onset infections and other neonatal outcomes.

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