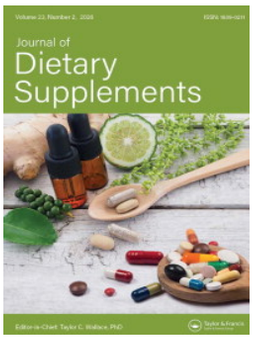

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RESEARCH ARTICLE

Effects of Human Lactoferrin (Effera®) at Two Doses Versus Bovine Lactoferrin on the Adult Gut Microbiome and Fecal Short-Chain Fatty Acids: a Randomized, Double-Blind Trial

Ross D. Peterson[a], Julian van der Made[a], Sharon M. Donovan[b], Mei Wang[b], Ryan N. Dilger[c], Nicole Kaplan[a] and Anthony J. Clark[a]

[a]Helaina Inc, New York, NY, USA; [b]Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, Urbana, IL, USA; [c]Department of Animal Sciences, University of Illinois at Urbana-Champaign, Urbana, IL, USA

ABSTRACT

Human lactoferrin (hLF) is a glycoprotein of commercial interest as a food ingredient for gut health. We conducted an exploratory analysis comparing the effects of Helaina hLF (effera®), produced by *Komagataella phaffii*, and bovine LF (bLF) on the adult gut microbiome and fecal metabolites. In a randomized, double-blind, parallel-arm, controlled trial, 66 healthy adults received either high-dose (HD) effera® (3.4 g/day), low-dose (LD) effera® (0.34 g/day), or bLF (3.4 g/day) supplementation for 28 days. Fecal samples collected at Days 0 (baseline), 28, 56, and 84 were analyzed for microbial diversity, taxonomic shifts, and volatile fatty acids (VFA). Alpha-diversity remained stable across all groups. Beta-diversity showed no main effect of treatment; however, bLF was associated with significant visit-related shifts, as assessed by weighted UniFrac. At the phylum level, significant changes associated with effera® were observed, including decreases in *Bacillota* (LD) and *Verrucomicrobiota* (HD), and notable genus-level increases in *Lachnospira*, *Paraprevotella*, and *Faecalibacterium* (HD), while bLF was associated with an increase *Bacteroidota* at the phylum level and with an increase in the genus *Roseburia*. Both effera® and bLF were associated with decreases in *Blautia* and *Dorea*. VFA analysis revealed that bLF increased absolute concentrations of total short-chain fatty acids (SCFAs) and branched-chain fatty acids (BCFAs); both effera® treatments produced proportional changes in SCFAs as a percent of total VFA, acetate, and individual BCFAs. In healthy adults, effera® supplementation promoted a proportional increase in acetate and supported potentially beneficial taxa while maintaining microbial diversity, without disrupting community structure. As a pre-specified exploratory outcome, microbiome and VFA findings should be interpreted as hypothesis-generating. (clinicaltrials.gov: NCT06012669).

KEYWORDS

Human lactoferrin; effera®, microbiome; short-chain fatty acids; taxonomy; diversity; gut health

CONTACT Ross Peterson ross@myhelaina.com Helaina Inc., 345 Park Ave, South, New York, NY 10010, USA

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Introduction

Human lactoferrin (hLF) is a single polypeptide chain of approximately 80 kDa, consisting of around 690–691 amino acid residues with three glycosylation sites (Lu et al. 2024). The tertiary structure of hLF is characterized by two homologous globular domains, the N- and C-terminal lobes, which consist of α-helices, β-pleated sheets, and turns (Anderson et al. 1987). Each lobe contains a deep cleft capable of binding one ferric iron ion (Baker and Baker 2009). hLF has a reversible iron-binding property that gives rise to three forms: iron-depleted (apoLF), a monoferric form, and iron-saturated (holoLF) (Baker and Baker 2009). LF has been shown to play a role in various physiological processes, including support of gut barrier function (Zhao et al. 2019; Troost et al. 2003), immune development (Donovan 2019), iron homeostasis (Rosa et al. 2017), and the gut microbiome (Ceroni et al. 2025; Ruiz-Rico et al. 2025).

LF exerts multifaceted effects to influence the gut microbiome (Conesa et al. 2023). The bacteriostatic effects of LF can affect the growth of pathogenic bacteria, including *Escherichia coli*, *Salmonella spp.*, and *Staphylococcus aureus*. In addition, bovine lactoferrin (bLF) has been shown to support beneficial taxa, such as *Bifidobacterium* and *Lactobacillus* spp (Kim et al. 2004; Petschow et al. 1999). The growth-promoting activity of LF can be strain-dependent and influenced by the degree of iron saturation. For example, holo-bLF enhances the growth of *Lactobacillus acidophilus*, whereas the bifidogenic activity appears less dependent on saturation state (W.-S. Kim et al. 2004). However, studies show that bLF and hLF each support distinct *Bifidobacterium* strains (Petschow et al. 1999). Moreover, proteolytic fragments of LF, such as lactoferricin, not only display direct antimicrobial effects but also can contribute to the enrichment of beneficial taxa (Vega-Bautista et al. 2019). In one study, holo-bLF supported the growth of commensals, such as *Alistipes putredinis* (Ruiz-Rico et al. 2025). *In vivo* animal models have further confirmed LF's ability to shape microbial ecology, with supplementation increasing bacterial richness and the abundance of *Bifidobacterium* and *Lactobacillus spp.*, while reducing opportunistic pathogens, including *Escherichia-Shigella*, *Veillonella*, and *Leptotrichia* (Hu et al. 2019; Dierick et al. 2024).

In neonates, higher LF levels in breast milk have correlated positively with *Bifidobacterium* and *Lactobacillus* abundance (Chong et al. 2022). These genera have been known to be adapted to low-iron environments, and due to the high abundance of these genera, acetate has been the primary short-chain fatty acid (SCFA) reported in infant stool (Tsukuda et al. 2021). Recombinant hLF (talactoferrin) was shown to modulate *Enterobacteriaceae* in very-low-birth-weight infants (Conesa et al. 2023). However, the overall clinical impact on infant gut microbiota has been modest, with some studies reporting only small reductions in opportunistic pathogens, such as *Staphylococcus*, and no significant shifts in diversity (Embleton et al. 2021; González et al. 2023). Despite compelling preclinical evidence and modest effects observed in infants, clinical studies directly evaluating LF's impact on the adult gut microbiome remain scarce, representing a key gap in understanding its translational relevance to adult populations. Notably, early-life LF biology also offers a mechanistic frame for interpreting LF-associated microbiome signals detected in adults (Villavicencio et al. 2017). LF exposure in early life is both quantitatively substantial and developmentally patterned, with concentrations highest in colostrum and declining across lactation,

consistent with a role in shaping microbial succession during a period of heightened ecological plasticity (Villavicencio et al. 2017).

LF can also modulate epithelial and immune programs, thereby altering the selective pressures that govern colonization at the mucosal interface (Donovan 2016). In parallel, infant-adapted *Bifidobacteria* encode enzymes that release and metabolize complex N-glycans from human milk glycoproteins, supporting a selection in the milk-fed intestine (Newburg and Morelli 2015). Consistent with this framework, in a cohort of breastfed mother–infant pairs that included preterm infants, higher early fecal LF concentrations were positively associated with fecal *Bifidobacterium* and *Lactobacillus*, consistent with a potential role for luminal LF in supporting the establishment of beneficial early-life taxa (Mastromarino et al. 2014). Together, these considerations support evaluating whether recombinant hLF preparations can elicit functionally coherent yet non-disruptive microbiome responses in adults, which may be informative for future work across life stages.

Due to the significant interest in LF supplementation, alternative sources beyond animal milk have been developed, including human-equivalent LF alpha. Helaina human lactoferrin (effera®) is a sprayed dried powder expressed in the yeast, *Komagataella phaffii* (*K. phaffii*), and is produced at a commercial scale. Helaina effera® powder possesses an amino acid sequence identical to native human milk lactoferrin (hmLF) (Lu et al. 2024), and its secondary structure, as revealed by microfluidic modulation spectroscopy, mirrors that of hmLF with globular structures closely akin to hmLF (Lu et al. 2024). Glycosylation of effera® is consistent with its production in *K. phaffii* and contains N-linked glycans that are predominantly oligomannose structures (M5–M9), which have also been detected in the human milk glycobiome (Goonatilleke et al. 2019) and in bLF (Valk-Weeber et al. 2020). Low levels of oligomannose glycans have been detected at the same known glycosylation sites as those of hmLF (Lu et al. 2024). Helaina effera® has been developed as a food ingredient and dietary supplement and has met the Generally Recognized As Safe (GRAS) standard of general acceptance and general availability supported by safety studies in adults (Anaya et al. 2024; Kim et al. 2024; Lu et al. 2024; Malinczak et al. 2024; Vishwanath-Deutsch et al. 2024; Peterson et al. 2025a, 2025b). In this study, effera® was given as a powdered drink mix that was reconstituted at the time of consumption. Nutrition information on the drink mix may be found in Peterson et al. study (R. D. Peterson et al. 2025)

bLF has been manufactured on an industrial scale and is widely used in consumer products, such as infant formula, fortified yogurts, and nutritional supplements. In the United States, bLF was granted numerous letters of no objection from the FDA regarding its GRAS status for various intended uses, including infant formula, functional foods, ice cream, and powdered milk, among others (US FDA 2017, 2014a, 2014b, 2001a, 2001b). The existing literature highlights the beneficial effects of LF on the gut, but comprehensive evidence on its impact on the healthy adult gut remains limited, with most research focusing on preclinical models or vulnerable populations (e.g. preterm infants) (Embleton et al. 2021; Conesa et al. 2023; Peterson et al. 2025a).

To our knowledge, no study has evaluated the impact of orally consumed fermentation-derived hLF on the healthy adult gut microbiome. The current study presents exploratory analyses of the fecal microbiota composition and volatile fatty acid (VFA) nested within a randomized, double-blind, controlled, parallel-arm trial.

Microbiome diversity, taxonomic composition, and VFA concentrations were designated as exploratory outcome variables in the parent protocol and were not primary or secondary endpoints. Accordingly, all microbiome-related findings in this report are intended to be hypothesis-generating rather than confirmatory and are not adjusted for multiple comparisons across all exploratory endpoints.

Materials and methods

Sample collection

The original study, which enrolled healthy adults (18–45 years), was designed to assess the safety and immunogenicity/alloimmunization potential of Helaina effera®(Peterson et al. 2025a). Participants in the original study received one of three treatments: high-dose (HD) effera® (3.4 g/d, 1.7 g twice daily), low-dose (LD) effera® (0.34 g/d, 0.17 g twice daily), or bLF as the active control (3.4 g/d, 1.7 g twice daily) over a 28-day dietary intervention period (Peterson et al. 2025a). Helaina effera® had an iron saturation of ~50%, whereas bLF exhibited <10% saturation. Fecal samples ($n = 195$) were collected at four time points during the clinical study (Figure 1, Table 1). Participants were provided with a sample collection kit and instructed to collect a freshly voided fecal sample during the 72 h prior to the baseline visit (Day 0) and 24 h prior to their subsequent visits on Day 28, Day 56, and Day 84. Results of additional analyses of microbiome data from follow-up visits (i.e. Day 56 and Day 84) are available as supplementary figures and tables. All samples were placed in separate sterile tubes, and frozen at −20 °C until delivered to the clinical site during a clinical visit. The samples were immediately placed in a −80 °C freezer for storage

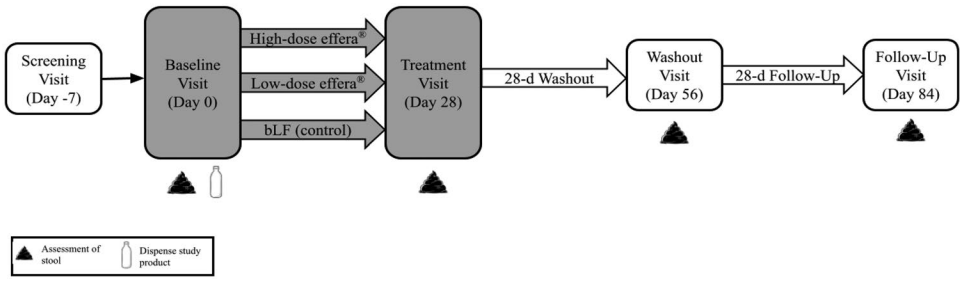

Figure 1. Study design schematic for microbiome sample fecal collection. Assessments on fecal microbiome included alpha- and beta-diversity, taxonomy, and VFA concentrations, with comparisons between baseline (Day 0) and treatment visit (Day 28). Abbreviation: bLF, bovine lactoferrin. Grayed boxes indicate the treatment phase of the study.

Table 1. Number of fecal samples collected from healthy adults at baseline, treatment visit, washout visit, and follow-up visit.

Time point	Number of samples
Baseline visit, Day 0	56
Treatment visit, Day 28	49
Washout visit, Day 56	45
Follow-up visit, Day 84	45

until shipped on dry ice to the University of Illinois for analysis. The primary analysis presented in this paper compares baseline (Day 0) to Day 28 across the three treatments.

DNA isolation

DNA was extracted from ~200 mg of feces using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Valencia, CA) in combination with bead beating on the FastPrep-24 System (MP Biomedicals, Carlsbad, CA) as previously described (Reznikov et al. 2018). DNA concentration was measured on a Qubit 3.0 Fluorometer using the Qubit 1X dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA), and its quality was checked on a 1% agarose gel.

Library preparation and sequencing of full-length 16S rRNA genes

Construction of full-length 16S rRNA gene libraries and sequencing on the PacBio REVIO were performed at the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. The full-length 16S rRNA amplicons were generated from 2.5 ng of DNA with the barcoded Kinnex primers (forward primer: 5′-AGRGTTYGATY MTGGCTCAG-3′ and the reverse primer: 5′-RGYTACCTTGTTACGACTT-3′) following the protocol from PacBio (PacBio, Menlo Park, CA). The individually barcoded amplicons were pooled and converted to a library with the SMRTBell Template Prep kit 3.0 (PacBio). The library was quantified using a Qubit 3.0 (Thermo Fisher Scientific) and run on a Fragment Analyzer (Agilent Technologies, Santa Clara, CA) to confirm the presence of DNA fragments of the expected size. The library was sequenced with SPRQ chemistry on a SMRT cell in the PacBio REVIO instrument with a 30-h movie time. Circular consensus sequence (CCS) analysis and demultiplexing were performed using SMRTlink 25.1, applying the following parameters: ccs –min-passes 3 –min-rq 0.999.

Sequence processing

Sequences were processed using the DADA2 R package and the QIIME 2 pipeline (Bolyen et al. 2019; Callahan et al. 2019). Demultiplexed reads obtained from the sequencing facility were primer removed, dereplicated, denoised, and chimera checked using DADA2 (version 1.36.0) (Callahan et al. 2019). The amplicon sequence variant (ASV) table and representative sequences generated from DADA2 were imported and further analyzed in QIIME 2 (version 2025.4). The representative sequences were aligned, and a phylogenetic tree was constructed from the filtered alignment as previously described (Smith et al. 2020). Alpha-diversity (observed features, Shannon entropy, Pielou's evenness, and Faith's phylogenetic diversity) and UniFrac distance metrics were computed using the plugin diversity with the core metrics-phylogenetic method on the ASV table and phylogenetic tree. Samples were rarefied to an equal number of reads (13,900) for calculating alpha-diversity and UniFrac distance metrics. Taxonomic assignments were performed using the plugin feature-classifier with the classify sklearn method (Bolyen et al. 2019), in which a pre-trained Naïve-Bayes

classifier with Greengenes2 2024.09 full-length sequences was used (https://library.qiime2.org/data-resources). Taxonomic bar plots were generated using the plugin taxa with the taxa barplot method. The bar plots were visualized using QIMME 2 View,('QIIME 2 View', n.d.) and the count tables at the phylum and genus levels were downloaded for differential abundance analysis.

Volatile fatty acid concentrations

The concentrations of SCFA (acetate, butyrate, and propionate) and branch-chain fatty acids (BCFA; isobutyrate, isovalerate, and valerate) were analyzed by gas chromatography (model 5890 A series 99; Hewlett-Packard, Palo Alto, CA) using a glass capillary column (100 $m \times 0.25$ mm i.d. × 0.2 µm film thickness; model SP-2560; Supelco, Bellefonte, PA).

Oven temperature, detector temperature, and injector temperature were 125 °C, 175 °C, and 180 °C, respectively. All VFA concentrations (µmol/g) were expressed on a dry matter basis.

Statistical analysis

Alpha-diversity was analyzed using the lmer function of the lme4 R package (version 4.5.1) (Bokulich et al. 2018). The effect of treatment and visit on beta-diversity was evaluated with principal coordinate analysis (PCoA) and permutational multivariate analysis of variance (PERMANOVA) based on UniFrac distances. The statistical model included treatment, visit, and the interaction between treatment and visit as fixed effects, and participant identification as a random effect. Total fiber intake was included in the model as a covariate. PCoA plots were generated using QIIME2. PERMANOVA was performed using the adonis2 function of the R package vegan 2.7-1.QIIME2 plugin q2-longitudinal (pairwise-distances method) was applied to compare the within-subject distance between baseline visit (Day 0) and Day 28 for each treatment (Bokulich et al. 2018). Statistical significance was set at $p \leq 0.05$.

Multivariate analysis by linear models (MaAsLin 3.0) (Nickols et al. 2024) was used to determine associations between visit and the relative abundance of bacterial taxa within each treatment. The relative abundance data were log2-transformed; only the taxa with relative abundances ≥ 0.1% in more than 10% of the samples were included in the analyses. All *P*-values were corrected for multiple testing by the Benjamini-Hochberg false discovery rate (FDR), and *P*-values ≤ 0.05 were considered statistically significant. and $0.05 < p \leq 0.10$ were reported as trends toward significance.

The proportions of SCFA and BCFA in total fatty acids were calculated from gas chromatography data for each participant at each study visit. Additionally, the proportion of each fatty acid was first expressed as a fraction of its respective class (SCFA or BCFA). Data from the baseline visit (Day 0), the end of the 28-day dietary intervention period, and follow-up visits at Days 56 and 84 were used in the analysis. Participants with any missing data were removed, and one participant from the HD effera® group was excluded due to an outlier value of butyrate in the 28-day sample. Due to the high degree of dietary record variability collected across participants, including inter-individual variation in fiber intake, no dietary covariates were

incorporated into the VFA statistical models. Dietary intake was assessed using 3-day diet records (2 weekdays and 1 weekend day or holiday) collected prior to visits 2 and 4 (Days 0 and 28) and analyzed using Food Processor SQL Nutrition Analysis and Fitness software. For alpha- and beta-diversity analyses, total fiber intake (mean of 6 diet record days per participant) was included as a covariate to partially account for dietary influence on microbial community composition.

For each participant, the percentage change in VFA proportion relative to the baseline visit was computed for each metabolite. Group-level summary statistics were then calculated by visit, protocol population, and metabolite. Specifically, the median, 25th and 75th percentiles, and standard deviation of both raw proportions and percent changes were computed.

To assess whether post-treatment changes differed significantly from zero, a Wilcoxon signed-rank test was performed on the percentage change values within each treatment group and metabolite, excluding missing data. To evaluate whether the magnitude of percent change differed significantly between treatment groups, Mann–Whitney U tests were conducted pairwise between protocol populations for each metabolite and visit. Resulting *P*-values were merged into the summary table for each visit and metabolite. All analyses were performed in Python using pandas and scipy.stats libraries.

Results

Microbiome alpha and beta-diversity measures

Alpha-diversity measures

The alpha-diversity measures of observed features, Shannon entropy, evenness, and Faith's phylogenetic diversity (Supplementary Table A1) remained stable across treatments and visits, and no interaction effects were identified. These results demonstrate that both HD and LD effera® supplementation maintained the microbiome diversity of healthy adults after the 28-day dietary intervention period.

Beta-diversity measures

The QIIME2 plugin q2-longitudinal (pairwise-distances method) was applied to compare the within-subject distances between baseline (Day 0) and Day 28 for each treatment (Figure 2). When weighted UniFrac distances were used, within-subject distances between the two visits were similar among the treatment groups ($p > 0.05$) (Figure 2A). Based on unweighted Unifrac distances, HD effera® had a smaller within-subject distance than bLF or LD effera® treatments ($p = 0.009$ and $p = 0.005$, respectively) (Figure 2B).

PERMANOVA indicated that neither treatment nor interaction between the treatment and visit impacted beta-diversity ($p > 0.05$ for both weighted and unweighted UniFrac distances). However, there was a global effect of visit based on both weighted and unweighted UniFrac distances ($p = 0.011$ and $p = 0.020$, respectively). To determine which treatment contributed to the visit effect between baseline (Day 0) and the end of the 28-day intervention (Day 28), data were stratified by treatment. bLF supplementation affected visit-related shifts in beta-diversity based on weighted UniFrac

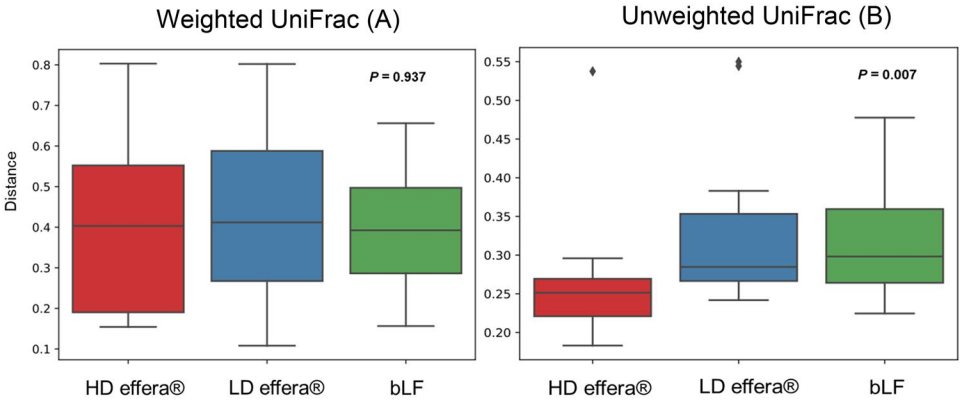

Figure 2. Effects of lactoferrin supplementation on beta-diversity. Within-subject comparisons of weighted (A) and unweighted (B) UniFrac distances between day 0 to day 28 across treatment groups were evaluated. Samples were collected from each participant at two time points: baseline (Day 0) and day 28. The total participants analyzed for HD effera® (3.4 g/d) were $n = 14$, LD effera® (0.34 g/d) were $n = 15$, bLF (3.4 g/d) were $n = 14$. Abbreviations: HD, high-dose effera®; LD, low-dose effera®; bLF, bovine lactoferrin.

distances ($p = 0.027$) but not on unweighted UniFrac distances ($p > 0.05$). There were no visit effects for both the HD and LD effera® treatments ($p > 0.05$).

Microbial compositional shifts in response to effera® and bLF interventions

Phylum level

Shifts in microbiome taxonomy were observed at the phylum level for bLF and HD and LD effera®. Taxonomic stacked bar plots identified various phyla affected by LF supplementation (Figure 3). *Bacteroidota* had a trend increase in median relative abundance in the bLF group (Table 2). In response to LD effera®, *Bacillota_I* significantly decreased and Verrucomicrobiota significantly decreased in the HD effera® (Table 2). Taxonomic stacked bar plots for each treatment showing the mean phyla relative abundance from baseline (Day 0) through Day 84 may be found in Supplementary Figure A1.

Genus level

Taxonomic stacked bar plots visually show various genera affected by LF supplementation (Figure 4). In response to the 28-day dietary intervention, several predominant and low-abundance gut bacterial genera exhibited significant, or trend shifts in relative abundance in response to LF supplementation (Table 3). Many shifts were consistent across multiple treatment groups. Exclusive to both effera® groups, significant increases were observed in *Lachnospira, Paraprevotella,* and *Sutterella.* Specific to both the LD effera® and bLF groups significant or trend decreases were measured in *Dorea* (*Dorea_A*), Blautia (*Blautia_A_141781*), and *Fimenecus*; *Dysosmobacter* showed a trend increase in both groups. *Prevotella* was significantly increased in the HD effera® and bLF groups; *Ruminococcus_B* significantly decreased in both groups. Taxonomic stacked bar plots for each treatment showing the mean genera relative abundance from baseline (Day 0) through Day 84 may be found in Supplementary Figure A2.

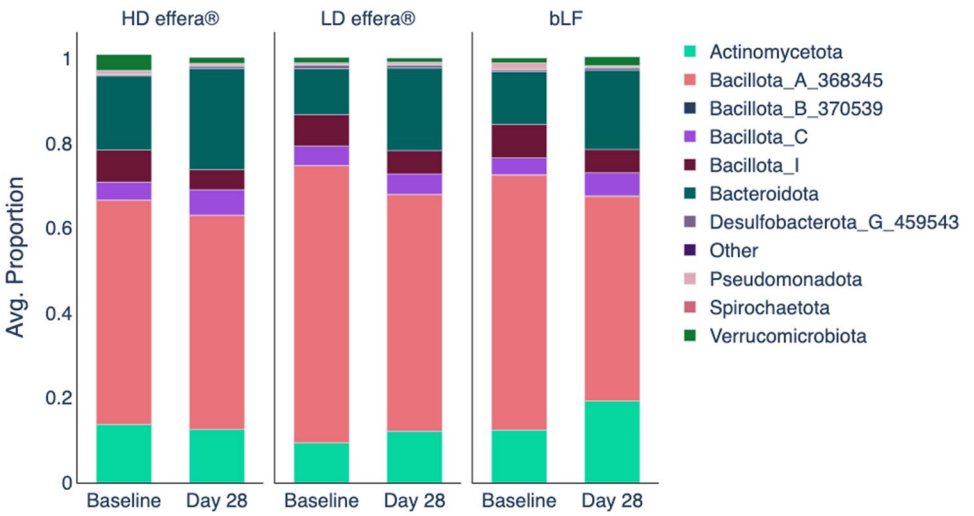

Figure 3. Taxonomic stacked bar plots showing the mean relative abundance of phyla at baseline through supplementation (Day 0 and day 28) for each treatment group. Counts were normalized to their library size; the 10 most abundant phyla were plotted; 'other' phyla included all remaining phyla not in the top 10. Average proportions were individual relative abundances, averaged across the treatment group. The total participants analyzed for HD effera® (3.4 g/d) were $n = 14$, LD effera® (0.34 g/d) were $n = 16$, bLF (3.4 g/d) were $n = 15$. Abbreviations: HD, high-dose effera®; LD, low-dose effera®; bLF, bovine lactoferrin.

Certain microbial changes were treatment-group specific. LD effera® produced significant or trend reduction in *Fusicatenibacter, Howardella, Intestinibacter, Limiplasma, Mogibacterium_A,Turicibacter* and increases in *Acidaminococcus, Adlercreutzia_A_404257 Coprococcus (Coprococcus_A_121497), Eubacterium (Eubacterium_F), G11, Lawsonibacter, Parasutterella_564865, Porcincola, UBA6382,* and *Wujia*. The HD effera® was associated with decreases in *Akkermansia, Angelakisella, Clostridium (Clostridium_AP_143938),* and *Ruthenibacterium;* increases were measured in *Agathobacter (Agathobacter_164117), Alistipes (Alistipes_A_871404), Allisonella, Bilophila, Butyricimonas Catenibacterium, Enterocloster,* and *Faecalibacterium*. bLF supplementation led to a significant or trend reduction in relative abundance of *Anaerobutyricum*, *Clostridium* (*Clostridium_AQ and _T*), *Dorea* (*Dorea_D_146760) Eubacterium (Eubacterium_J), Lachnoclostridium (Lachnoclostridium_B) Fusicatenibacter, Limisoma, PeH17, Romboutsia (Romboutsia_B), Schaedlerella* and *Terrisporobacter.* Conversely, bLF significantly increased *Alloprevotella, Bacteroides (Bacteroides_H_857956), Clostridium (Clostridium_A_51961), Cryptobacteroides, Eisenbergiella, Hominicoprocola, Megasphaera_A_38685,* and *Roseburia (Roseburia_C).*

Volatile fatty acid concentrations

Bovine LF supplementation produced no significant within-group proportional changes in stool VFA composition (Table 4). On an absolute change basis, bLF led to significant or had near-significant median increases of total SCFA (53.9 mmol/kg), propionate (23.1 mmol/kg), BCFA (4.4 mmol/kg), isobutyrate (1.6 mmol/kg), and valerate (2.6 mmol/kg).

Table 2. Directional shifts in phyla from baseline (Day 0) to Day 28 in response to high-dose effera® (3.4 g/d), low-dose effera® (0.34 g/d), and bovine lactoferrin (3.4 g/d) treatments in healthy adults. Data are presented as median (25th percentile to 75th percentile).

	High-dose effera® (3.4 g/d) ($n = 14^{\dagger}$)			Low-dose effera® (0.34 g/d) ($n = 15^{\dagger}$)			Bovine lactoferrin (3.4 g/d) ($n = 14^{\dagger}$)		
Phylum	Δ 28 days	Directional shift	within *P*-value[‡]	Δ 28 days	Directional shift	within *P*-value[‡]	Δ 28 days	Directional shift	within *P*-value[‡]
Bacillota_I				−1.1 (−3.3 to 0.1)	↓	≤0.05			
Bacteroidota							5.9 (0.6 to 17.2)	↑	0.09
Verrucomicrobiota	−1.1 (−2.5 to −0.1)	↓	≤0.05						

[†]Samples were collected from each participant at two time points: baseline (day 0) and day 28.

[‡]Multivariate analysis by linear models (MaAsLin 3.0)38 was used to determine associations between visit and the relative abundance of bacterial taxa within each treatment. The relative abundance data were log2-transformed; only the taxa with relative abundances ≥ 0.1% in more than 10% of the samples were included in the analyses. All *P*-values were corrected for multiple testing by the Benjamini-Hochberg false discovery rate (FDR). *P*-values ≤ 0.05 were considered statistically significant; $0.05 < p \leq 0.10$ were reported as trends toward significance.

Abbreviations: g/d, grams per day; ↑ and ↓, directional shift in a phylum at day 28 compared to day 0.

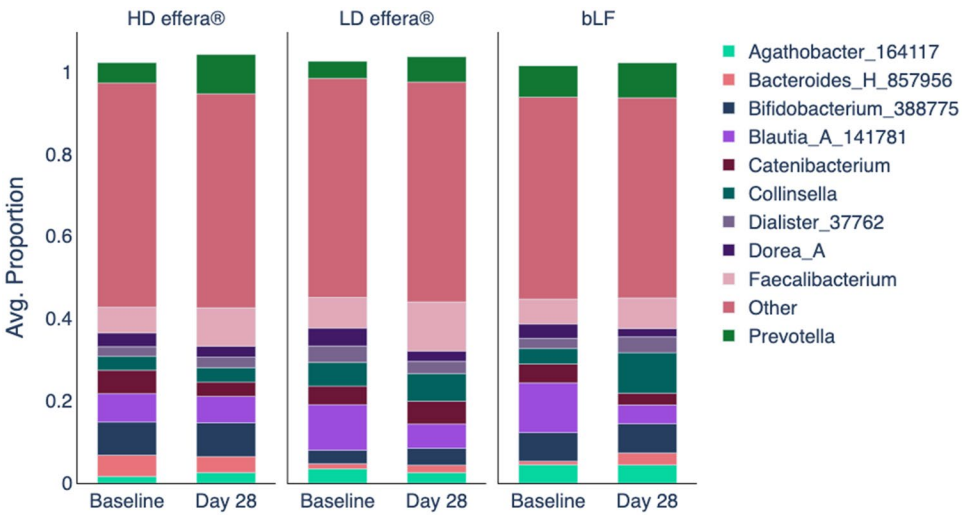

Figure 4. Taxonomic stacked bar plots showing the mean relative abundance of genera at baseline through supplementation (Day 0 and day 28) for each treatment group. Counts were normalized to their library size; the 10 most abundant genera were plotted; 'other' genera included all remaining genera not in the top 10. Average proportions were individual relative abundances, averaged across the treatment group. The total participants analyzed for HD effera® (3.4 g/d) were $n = 14$, LD effera® (0.34 g/d) were $n = 16$, bLF (3.4 g/d) were $n = 15$. Abbreviations: HD, high-dose effera®; LD, low-dose effera®; bLF, bovine lactoferrin.

In contrast to the bLF group, both effera® groups produced no significant within-group absolute changes in stool VFA composition. Significant or near-significant proportional changes, however, were observed for numerous VFA in both effera® groups (Table 4). The proportion of total SCFAs (of total VFA) also had a trend increase in the HD effera® group ($p < 0.10$) (Supplementary Figure A3). In the HD and LD effera® groups, fecal acetate increased by 7.7% and 4.7%, respectively, relative to baseline ($p < 0.10$). Proportionally, fecal butyrate in the LD effera® group decreased by 2.0% ($p < 0.05$); however, no absolute butyrate changes were observed (Supplementary Figure A4). The HD effera® group showed trend proportional increases in overall BCFA and isobutyrate. Isovalerate, on the other hand, showed a proportional decrease.

Between-group analysis showed that the proportional increase in stool acetate was greater in both effera® groups than in the bLF group ($p < 0.05$ for HD effera® vs bLF; $p < 0.05$ for HD effera® vs bLF). In addition, between-group differences were observed in the absolute total BCFA and individual BCFA including valerate, isovalerate, and isobutyrate between bLF and HD effera®. A significant difference between the LD effera® and bLF group was found for valerate (Table 4).

The trend of increasing acetate after the 28-day treatment appeared to shift concentrations back toward baseline at both follow-up visits at Day 56 and Day 84 (Supplementary Figure A5). The increased acetate production in the LD and HD effera® was not dose-responsive; however, among females, acetate was more consistently elevated after the 28-day supplementation compared to males (Supplementary Figure A6). We did not observe similar changes among males and females in either butyrate or propionate concentrations (Supplementary Figure A6).

Table 3. Directional shifts in genera in response to high-dose effera® (3.4 g/d), low-dose effera® (0.34 g/d), and bovine lactoferrin (3.4 g/d) treatments in healthy adults. Data are presented as median (25th percentile to 75th percentile).

	High-dose effera® (3.4 g/d) ($n = 14^{\dagger}$)			Low-dose effera® (0.34 g/d) ($n = 15^{\dagger}$)			Bovine lactoferrin (3.4 g/d) ($n = 14^{\dagger}$)		
Genus	Δ 28 days*	Directional shift	Within *P*-value‡	Δ 28 days*	Directional shift	Within *P*-value‡	Δ 28 days*	Directional shift	Within *P*-value‡
Acidaminococcus				0.8 (0.2 to 1)	↑	0.06			
Adlercreutzia_A_404257				0.1 (0 to 0.6)	↑	0.06			
Agathobacter_164117	0 (−0.6 to 3)	↑	≤0.05						
Akkermansia	−1.4 (−5.8 to −0.8)	↓	≤0.05						
Alistipes_A_871404	0.04 (0 to 0.3)	↑	≤0.05						
Allisonella	0.02 (0.01 to 0.03)	↑	≤0.05						
Alloprevotella							2.1 (0.6 to 3.3)	↑	0.07
Anaerobutyricum							−0.3 (−0.9 to 0.2)	↓	0.09
Anaerostipes							−0.3 (−0.9 to 0)	↓	0.1
Angelakisella	−0.1 (−0.9 to 0)	↓	≤0.05						
Bacteroides_H_857956							0.2 (0 to 1.3)	↑	0.1
Bilophila	0.1 (0 to 0.3)	↑	0.1						
Blautia_A_141781				−8 (−10.4 to 1.7)	↓	≤0.05	−2.8 (−5.7 to −0.8)	↓	≤0.05
Butyricimonas	0.1 (0 to 0.1)	↑	≤0.05						
Catenibacterium	−0.3 (−0.6 to 1.7)	↓	0.1				−0.6 (−2.9 to 1.9)	↓	
Clostridium_A_51961							0.1 (0 to 0.2)	↑	≤0.05
Clostridium_AP_143938	−0.1 (−0.2 to −0.1)	↓	0.1						
Clostridium_AQ							−0.1 (−0.4 to 0)	↓	0.08
Clostridium_T							−0.2 (−0.9 to 0.5)	↓	0.06
Coprococcus_A_121497				0.1 (0.1 to 0.4)	↑	0.09			
Cryptobacteroides							0.6 (0.1 to 1.2)	↑	≤0.05
Dorea_A				−2.1 (−3.4 to 0.5)	↓	0.06	−2 (−3 to −0.4)	↓	≤0.05
Dorea_D_146760							−0.2 (−0.4 to 0)	↓	0.06
Dysosmobacter				0 (0 to 0.8)	↑	0.1	0.1 (0 to 0.2)	↑	0.1
Eisenbergiella							0.1 (0 to 1.2)	↑	≤0.05
Enterocloster	0 (0 to 0.1)	↑	0.07						
Eubacterium_F				0.1 (0.1 to 0.3)	↑	0.09			
Eubacterium_J							0 (−0.2 to 0.1)	↑	0.1
Faecalibacterium	3.2 (−0.8 to 6.2)	↑	0.08						
Fimenecus				−0.1 (−0.6 to 0.2)	↓	0.1	−0.6 (−1.1 to −0.2)	↓	0.06
Fusicatenibacter							−0.6 (−0.8 to 0)	↓	0.06
G11				0.3 (0.1 to 0.5)	↑	0.06			
Hominicoprocola							1.1 (0.2 to 1.9)	↑	0.1
Howardella				−0.1 (−0.1 to −0.1)	↓	≤0.05			
Intestinibacter				0 (−0.1 to 0)	↓	0.06			

(*Continued*)

Table 3. Continued.

Genus	High-dose effera® (3.4 g/d) ($n = 14^{\dagger}$)			Low-dose effera® (0.34 g/d) ($n = 15^{\dagger}$)			Bovine lactoferrin (3.4 g/d) ($n = 14^{\dagger}$)		
	Δ 28 days*	Directional shift	Within *P*-value‡	Δ 28 days*	Directional shift	Within *P*-value‡	Δ 28 days*	Directional shift	Within *P*-value‡
Lachnoclostridium_B							0 (−0.2 to 0)	↑	0.09
Lachnospira	0 (0 to 0.1)	↑	≤0.05	0 (0 to 0.1)	↑	0.1			
Lawsonibacter				0 (0 to 0.1)	↑	0.1			
Limiplasma				−0.1 (−0.1 to 0)	↓	≤0.05			
Limisoma							0.4 (0.1 to 0.5)	↑	0.07
Megasphaera_A_38685							1.2 (1 to 3)	↑	0.07
Mogibacterium_A				−0.9 (−1.1 to −0.6)	↓	≤0.05			
Paraprevotella	0.1 (0.1 to 0.1)	↑	≤0.05	0.2 (0.1 to 0.4)	↑	≤0.05			
Parasutterella_564865				−0.1 (−0.1 to 0.2)	↑	0.08			
PeH17							−0.1 (−0.1 to 0.2)	↓	0.07
Porcincola				0 (0 to 0.1)	↑	≤0.05			
Prevotella	0.3 (−0.5 to 2.6)	↑	≤0.05				0.3 (0 to 4.2)	↑	≤0.05
Romboutsia_B							0 (−0.5 to 0.2)	↑	0.05
Roseburia_C							0.2 (0 to 0.6)	↑	0.08
Ruminococcus_B	0 (−0.1 to 0)	↓	≤0.05				−3.5 (−4.5 to −2.2)	↓	≤0.05
Ruthenibacterium	−0.1 (−0.1 to 0)	↓	≤0.05						
Schaedlerella							0 (−0.1 to 0)	↑	0.1
Sutterella	0.1 (0.1 to 0.2)	↑	≤0.05	0 (−0.1 to 0.5)	↑	≤0.05			
Terrisporobacter							0 (−0.1 to 0)	↑	≤0.05
Turicibacter				0 (−0.1 to 0)	↑	0.07			
UBA6382				0 (0 to 0.2)	↑	0.09			
Wujia				0.1 (0 to 0.3)	↑	0.09			

*Percent change in relative abundance for each taxon from baseline (day 0) to day 28. For each participant, the percentage increase or decrease was calculated as the difference between day 28 and day 0 values.

†Samples were collected from each participant at two time points: baseline (day 0) and day 28. The total participant analyzed for HD effera® were $n = 14$, LD effera® were $n = 15$, bLF were $n = 14$.

‡Multivariate analysis by linear models (MaAsLin 3.0)38 was used to determine associations between visit and the relative abundance of bacterial taxa within each treatment. The relative abundance data were log2-transformed; only the taxa with relative abundances ≥ 0.1% in more than 10% of the samples were included in the analyses. All *P*-values were corrected for multiple testing by the Benjamini-Hochberg false discovery rate (FDR). *P*-values ≤ 0.05 were considered statistically significant; $0.05 < p \leq 0.10$ were reported as trends toward significance.

Abbreviations: g/d, grams per day; ↑ and ↓, directional shifts in a genus at day 28 compared to day 0.

Table 4. Effects of 28 days of lactoferrin supplementation on changes in proportional fecal volatile fatty acid concentrations of healthy adults.

	High-dose effera®† (3.4 g/d) ($n = 10$)			Low-dose effera®† (0.34 g/d) ($n = 12$)			Bovine lactoferrin† (3.4 g/d) ($n = 15$)					
Metabolite	Baseline (Day 0)	Δ 28 days	Within *P*-value*	Baseline (Day 0)	Δ 28 days	Within *P*-value*	Baseline (Day 0)	Δ 28 days	Within *P*-value*	LD vs. bLF *P*-value**	HD vs. bLF *P*-value**	HD vs. LD *P*-value**
SCFA (mmol/kg)	203.4 (133.1 to 247.8)	59.7 (−25.0 to 138.1)	0.32	223.5 (149.3 to 382.5)	49.4 (−56.0 to 131.0)	0.57	222.0 (144.1 to 360.1)	53.9 (−1.9 to 164.9)	**0.07**	0.44	0.54	0.79
SCFA, % of total VFA	86.3 (83.8 to 91.2)	2.9 (1.8 to 4.3)	**0.08**	86.6 (83.1 to 90.9)	3.4 (−1.2 to 7.4)	0.11	93.6 (87.3 to 94.4)	−0.84 (−3.5 to 4.3)	0.72	0.23	0.13	0.98
Acetate (mmol/kg)	103.9 (80.6 to 158.3)	50.8 (−5.8 to 83.4)	0.16	117.9 (81.9 to 222.3)	36.1 (−20.6 to 80.55)	0.42	116.1 (89.7 to 207.9)	21.4 (−6.44 to 87.8)	0.11	0.82	0.77	0.98
% Acetate	58.8 (50.7 to 61.8)	7.7 (−0.9 to 9.4)	**0.06**	55.3 (53.7 to 58.4)	4.7 (0.6 to 7.1)	**0.08**	60 (55.8 to 64.0)	−0.9 (−6.4 to 1.0)	0.39	**0.03**	**0.04**	0.73
Propionate (mmol/kg)	47.8 (40.2 to 50.7)	17.3 (−10.3 to 37.1)	0.38	61.3 (27.8 to 112.6)	5.4 (−17.7 to 38.6)	0.79	65.3 (28.0 to 98.0)	23.1 (−9.3 to 68.7)	**0.07**	0.41	0.51	0.86
% Propionate	23.7 (21.8 to 29.0)	−0.1 (−3.8 to 1.8)	0.55	25.4 (20.2 to 27.2)	−0.2 (−3.7 to 2.3)	0.73	25.9 (19.6 to 27.9)	−0.5 (−3.0 to 3.6)	0.80	0.38	0.26	0.75
Butyrate (mmol/kg)	40.3 (18.1 to 52.6)	−4.1 (−13.9 to 20.0	0.85	52.4 (30.2 to 73.5)	6.2 (−19.5 to 16.1)	0.97	34.2 (24.6 to 55.3)	5.5 (−4.4 to 26.9)	0.19	0.27	0.42	0.63
% Butyrate	15.5 (13.7 to 17.5)	−3.9 (−6.7 to 1.3)	0.13	17.9 (15.7 to 23.3)	−2.0 (−7.1 to −0.4)	**0.03**	15.1 (12.1 to 19.2)	1.8 (−4.9 to 4.1)	0.93	0.16	0.31	0.90
BCFA (mmol/kg)	25.1 (14.4 to 36.3)	0.04 (−8.9 to 12.2)	0.85	24.5 (19.5 to 33.8)	4.7 (−6.5 to 12.2)	0.62	20.7 (16.9 to 26.3)	4.4 (−1.5 to 17.7)	**0.07**	0.50	**0.06**	0.27
BCFA, % of total VFA	13.7 (8.8 to 16.2)	−2.9 (−4.3 to −1.8)	**0.08**	13.4 (9.0 to 16.9)	−3.4 (−7.4 to 1.2)	0.11	6.4 (5.6 to 12.7)	0.8 (−4.3 to 3.5)	0.72	0.23	0.13	0.98
Isobutyrate (mmol/kg)	6.8 (5.6 to 9.2)	−0.2 (−2.7 to 2.4)	1.0	5.4 (4.7 to 8.2)	1.2 (−1.3 to 4.4)	0.52	4.9 (3.7 to 6.3)	1.6 (0.3 to 4.7)	**0.06**	0.80	**0.03**	**0.07**
% Isobutyrate	24.2 (21.8 to 25.4)	1.7 (0.01 to 3.5)	**0.10**	24.0 (22.7 to 27.6)	2.1 (−0.4 to 3.6)	0.18	24.9 (23.2 to 26.1)	2.5 (−0.2 to 4.5)	0.30	0.98	0.56	0.57
Isovalerate (mmol/kg)	12.0 (6.5 to 15.2)	−1.1 (−4.6 to 3.7)	0.70	11.1 (8.0 to 15.9)	3.0 (−2.2 to 6.0)	0.57	7.2 (4.8 to 11.3)	3.0 (−2.8 to 6.5)	0.30	0.83	**0.05**	**0.10**
% Isovalerate	43.0 (41.1 to 45.4)	−2.2 (−5.5 to −1.2)	**0.10**	43.5 (36.7 to 47.0)	−0.4 (−6.0 to 5.9)	1.0	40.5 (32.4 to 50.1)	0.8 (−15.1 to 7.0)	0.76	0.32	0.97	0.26
Valerate (mmol/kg)	8.6 (5.3 to 12.7)	−0.4 (−2.1 to 2.6)	1.0	8.4 (6.9 to 10.0)	0.8 (−4.5 to 1.6)	0.73	6.5 (4.8 to 10.1)	2.6 (−1.0 to 5.0	**0.07**	**0.01**	**0.01**	0.89
% Valerate	34.7 (30.4 to 35.8)	1.2 (−3.1 to 3.0)	0.77	34 (27.4 to 40.8)	−4.5 (−7.4 to 7.1)	0.73	29.6 (24.6 to 45.0)	−2.0 (−9.1 to 17.5)	0.89	0.15	0.81	0.18

Significant differences ($p < 0.05$) and trend differences ($.0.05 < p \leq 0.10$) in bold.

*Derived from the wilcoxon signed-rank test, which assesses whether the median percent change from baseline of each metabolite within each treatment group differs significantly from zero.

**derived from the Mann–Whitney U test, which compares the distributions of percent change from baseline between independent treatment groups for each metabolite and visit.

†Data are presented as median (25th percentile – 75th percentile. Participants with any missing data were removed, and one participant from the HD effera® group was excluded due to an outlier value of butyrate in the 28-day sample. Due to the high degree of dietary record variability, no covariates (e.g. fiber) were used in the analysis.

Abbreviations: HD: High-dose effera®; LD: Low-dose effera®; bLF: bovine lactoferrin; SCFA: short-chain fatty acids; VFA: volatile fatty acids; BCFA: branched-chain fatty acids.

Discussion

In this exploratory microbiome study conducted within a randomized controlled trial primarily designed to assess immune function outcomes using fecal samples collected from healthy adults consuming free-living diets, supplementation with effera® or bLF for four weeks did not induce large-scale restructuring of the gut microbiome, as assessed by alpha and beta-diversity metrics.

Alpha-diversity analyses showed no significant changes in richness, evenness, or phylogenetic diversity over time and no differences between bLF or HD effera® and LD effera®. The stability of alpha-diversity observed in a healthy adult population mirrors findings from other human intervention trials involving LF. In a study of healthy elderly women supplemented with 1 g/day of bLF for three weeks, Konstanti et al. reported no significant differences in alpha-diversity (utilizing Faith's PD and Shannon index) (Konstanti et al. 2022). Similarly, a randomized trial in toddlers (12–18 months old) receiving 1 g/day of bLF for six months found no significant differences in alpha-diversity metrics between the treatment and placebo groups (González et al. 2023). These data suggest that among participants with a relatively stable or established microbiome, LF supplementation preserves community richness and evenness. From a safety and tolerability standpoint, the absence of marked reductions in alpha-diversity reemphasises LF as a safe dietary protein that maintains microbiome diversity metrics over a 28-day dietary intervention.

Analysis of beta-diversity revealed that supplementation with effera® maintains the gut microbial community in healthy adults. PERMANOVA results indicated no significant main effect of treatment or treatment-by-visit interaction for either weighted or unweighted UniFrac distances, suggesting that neither LD effera® nor HD effera® induced disruptions to the gut microbiota. These findings align with previous investigations in healthy populations, such as the study by Konstanti et al. which found no significant differences in beta-diversity between healthy elderly women supplemented with either bLF or a placebo (Konstanti et al. 2022). Similarly, a trial with toddlers supplemented with bLF found no significant clustering by treatment using Aitchison distance (González et al. 2023). In preterm infants, targeted 16S analyses have reported that bLF supplementation does not overtly disrupt microbiota development supporting a 'selective modulation without destabilization' model (Grzywacz et al. 2020). Together, these infant data support the idea that LF might act as a context-dependent modulator, most evidently at the level of specific taxa and functions, rather than as the primary driver of widespread community remodeling, which aligns with the overall stability observed here in adults (Donovan 2016).

Against this backdrop of overall stability, an effect of visit was observed, indicating temporal shifts, and stratification by treatment revealed distinct patterns of stability. Supplementation with bLF was associated with significant visit-related shifts in beta diversity, as measured by weighted UniFrac distances. Weighted UniFrac accounts for the relative abundance of taxa, suggesting that bLF may have induced shifts in the proportions of specific microbial populations over the 28-day treatment period. This is consistent with findings of Chichlowski et al. who reported subtle but significant differences in beta-diversity (Jaccard distance) among infants fed bLF-enriched formula, driven by changes in low-abundance species (Chichlowski et al. 2021). Similarly, Embleton et al. reported small but significant differences in community composition between bLF and placebo groups in preterm infants (Embleton et al. 2021).

At the phylum level, the general shift toward a higher relative abundance of *Bacteroidota* is important for host health and aligns with the previously reported modulatory effects of LF on intestinal microbiota (Koropatkin et al. 2012; Hu et al. 2019; Connell et al. 2021; Wang et al. 2024). Moreover, this increase in *Bacteroidota* corresponded with a significant decrease of *Bacillota_I* in the LD effera® groups and a non-significant decrease in HD effera®. Although the changes did not always reach significance, shifts in these major microbiome phyla have been observed in previous animal and human studies (Li et al. 2022; Wang et al. 2024; Ruiz-Rico et al. 2025).

Despite a broad relative abundance decrease in the *Bacillota* phyla, effera® induced increases in several butyrate-producing genera, which support a healthy gut environment. Both effera® doses increased the abundance of *Lachnospira*, a key genus known for fermenting dietary fiber into butyrate and acetate (Vinelli et al. 2022). The positive correlation of effera® with *Lachnospira* may be related to the ability of effera® to bind iron, as *Lachnospira*'s abundance is associated with low iron intake (Matsumoto et al. 2021). Overall, *Lachnospira* contributes to gut health by producing fermentation metabolites (butyrate/acetate) that nourish colonocytes and support the intestinal barrier (Arrieta et al. 2015). These observations are notable in light of longitudinal infant cohorts in which early-life depletion of specific fermentative lineages, including *Lachnospira*, has been associated with increased later risk of wheeze/asthma, implicating early functional capacity as a potential determinant of immune trajectories (Arrieta et al. 2015). However, infant microbiome assembly is constrained by human-milk glycans, rapid ecological succession, and strong clinical confounding attributes, such that adult-directionality cannot be assumed to translate across life stages. Accordingly, infant-focused translation of LF should prioritize integrated endpoints that couple composition with function (absolute SCFAs, lactate, pH, and cross-feeding signatures) and, where feasible, host readouts of barrier and immune status (Roager et al. 2023).

Paraprevotella also rose significantly in both effera® groups. *Paraprevotella* ferments non-digestible complex carbohydrates and produces SCFAs (notably succinate and acetate) (Morotomi et al. 2009). Higher abundance of *Paraprevotella* has been associated with adherence to fiber-rich diets (Vázquez-Cuesta et al. 2024). Consistent increases in *Paraprevotella* across both the effera® groups signals a shift favoring *Bacteroidetes* (as was corroborated in the phylum data). These changes support the idea that LF supplementation can modulate the microbiome in a prebiotic-like manner.

Similarly, the LD effera® increased the genus *Eubacterium* which is known to produce butyrate and acetate and promote colonocyte health and dampen inflammation (Matsumoto et al. 2021). *Eubacterium* helps maintain a eubiotic state and has been associated with protection against conditions inflammatory bowel disease and insulin resistance (Mukherjee et al. 2020; Xiao et al. 2024). The enhancement of SCFA producers such as *Lachnospira* and *Eubacterium* by effera® could thus have positive implications, potentially contributing to improved gut barrier integrity and immune balance.

Faecalibacterium abundance showed a trend increase in HD effera® and a near trend increase in the LD effera® after 28 days of treatment. This major butyrate producer is well known for supporting a healthy inflammatory response (Chow et al. 2011; Leylabadlo et al. 2020; Qin et al. 2025) and enhancing the intestinal epithelial barrier and maintaining gut homeostasis (W. Li et al. 2024). This finding is encouraging as

effera® supported the increase in the relative abundance of a genera that contains *F. prausnitzii*, a next-generation probiotic species (Leylabadlo et al. 2020).

We report the HD effera® group was significantly associated with an increase in *Agathobacter*, which includes the species *A. rectalis. A. rectalis* is one of the most abundant butyrate-producing commensals in healthy adults and has recently been shown to alleviate intestinal and even neurological inflammation in experimental settings through its production of butyrate (Lv et al. 2024). An effera®-driven rise in *Agathobacter* is again consistent with enriching for beneficial fiber fermenters. HD effera® also significantly increased *Alistipes*. This finding was in agreement with a study that showed how different degrees of LF iron saturation supported the growth of commensals (Ruiz-Rico et al. 2025). Another study in a preclinical obese mouse model showed bLF increased *Alistipes*, which was associated with a healthy inflammatory response in obese mice (Wang et al. 2024).

Interestingly, we found that both bLF and effera® (particularly LD effera®) consistently suppressed several genera in the family *Lachnospiraceae*, including *Dorea* and *Blautia*, which are normal commensals in a balanced gut ecosystem (Ozato et al. 2019; Vacca et al. 2020). However, an overabundance of *Dorea* has been linked to gut dysbiosis and adverse host phenotypes, including type 2 diabetes (Carrizales-Sánchez et al. 2023). *Blautia*, on the other hand, is often considered beneficial. It is a major butyrate producer in the gut of healthy individuals, and has been inversely associated with visceral fat accumulation and metabolic disease risk (Ozato et al. 2019). The mechanism by which *Blautia* may have been reduced is through iron sequestration by LF. *Blautia* is known to prefer iron-rich growth environments (Pereira et al. 2024; Welsh et al. 2025), which may have been impeded by LF's ability to sequester iron.

HD effera® and bLF were significantly associated with *Prevotella. Prevotella* is known to be associated with plant-based diets and has the potential for protection against food allergy development when observed in the pregnant woman (Vuillermin et al. 2020). We found that bLF, while sharing some microbiome-modulation effects with effera®, had distinct impacts on the healthy adult gut microbiome. bLF caused a significant increase of a major butyrate producer *Roseburia*. In a piglet model, Hu et al. showed significant increases of *Roseburia* (Hu et al. 2019).

BLF significantly increased beneficial genera including *Bacteroides, Cryptobacteroides*, and *Eisenbergiella. Eisenbergiella* is a new genus but it has been identified as a butyrate producer that may support gut barrier function and support a healthy inflammatory response (Amir et al. 2014). Overall, the genus *Bacteroides* are commensal organisms, however there are scenarios where they act as opportunistic pathogens in specific scenarios where the immune system is compromised (Shin et al. 2024).

On the metabolite side, a trend toward increased acetate proportion after 28-day treatment with HD and LD effera® supplementation and a significant increase, compared to bLF, suggest a potentially meaningful shift in microbial metabolic activity. Acetate is the most abundant SCFA in the colon, constituting more than 50% of the total SCFAs in our study (Parada Venegas et al. 2019). Acetate is found in high amounts in infants fed human milk compared to those fed infant formula, due to the higher abundance of Bifidobacteria strains in human milk-fed infants (Edwards et al. 1994; Fusco et al. 2023; Łoniewska et al. 2023). The trend in acetate levels appeared consistent with our reported multiple genus shifts rather than a single genus. The

directional change was consistent with the capacity of hLF to exert an effect on the human gut microbiota, which is of interest given that acetate is the predominant SCFA in human milk-fed infants (Fusco et al. 2023).

It is also noteworthy that in our exploratory study, sex-linked acetate production trend was found among female participants, who appeared to respond to HD and LD effera® with greater increases in acetate, whereas men showed smaller or no changes. While our sample size was limited, sex hormones and other physiological differences between females and males are known to shape the gut ecosystem across the lifespan (Valeri and Endres 2021). Further studies on controlled dietary conditions could further resolve these observations on both effera® doses compared to the bLF cohort. Although this study was conducted in healthy adults, the distinct metabolic profiles observed between the LF groups—where bLF drove absolute increases in total SCFA and BCFAs while effera® drove proportional shifts toward acetate—mirror the metabolic divergence observed between formula-fed and human milk-fed infants (Bridgman et al. 2017). From a translational perspective, acetate is a compelling functional endpoint because it typically dominates fecal SCFAs in milk-fed infancy, and acetate-enriched profiles are repeatedly associated with breastfeeding-associated fermentation networks (Łoniewski et al. 2022). Future infant studies with effera® would therefore benefit from pairing taxonomic profiling with quantitative metabolomics (absolute SCFAs, lactate, succinate, where feasible) and linking these to barrier/immune readouts to determine whether LF preferentially tunes microbial function rather than global community structure.

The significant absolute increases in total BCFAs and two individual BCFAs (i.e. isobutyrate and valerate) in the bLF group act as a biomarker of proteolytic fermentation, a process driven by the microbial catabolism of branched-chain amino acids such as valine, leucine, and isoleucine (Rios-Covian et al. 2015). In contrast, the effera® groups exhibited a metabolic profile analogous to the human milk-fed infant, defined by significantly higher relative proportion of acetate. This distinction may be physiologically relevant because, while carbohydrate fermentation is generally beneficial, the fermentation of proteins yields a more diverse range of metabolites, including ammonia, BCFAs, and phenolic compounds (Rodríguez-Romero et al. 2022). Some of these compounds can be deleterious to the colonic epithelium at high concentrations (Beaumont et al. 2017; Rodríguez-Romero et al. 2022).

The SCFA and BCFA shifts observed across the LF groups were modest in magnitude and should be interpreted within the context of intra-individual variability that characterizes fecal SCFA quantification in healthy adults (de la Cuesta-Zuluaga et al. 2018). The proportional increases in acetate with effera®, in the absence of corresponding absolute changes, are consistent with the established physiological roles of acetate in colonic barrier homeostasis and immune regulation (Macia et al. 2015; Parada Venegas et al. 2019). The observed magnitudes, however, fall below thresholds at which clinically meaningful effects have been demonstrated in healthy adults (Rios-Covian et al. 2015). Similarly, the absolute increases in total SCFA, propionate, and BCFA in the bLF group reflect a modest expansion of overall fermentative activity that is unlikely to be clinically meaningful in a healthy adult population (Windey et al. 2012; Beaumont et al. 2017). Overall, these findings represent small compositional shifts within normal physiological ranges, and larger, placebo-controlled studies are needed to determine whether these shifts carry clinically-relevant significance.

Taken together, these compositional and metabolite-level observations highlight both the overlapping as well as distinct effects of hLF produced by *K. phaffii* and bLF on the human gut microbiome and fecal VFA. Some similarities are probably due to the evolutionary conservation of LF across species (Lambert et al. 2005; Zlatina and Galuska 2021). However, species-specific structural features probably explain the observed differences. In the original study, in which this primary analysis is based, Peterson et al. showed that bLF induced anti-bLF antibodies in numerous participants, whereas both doses of effera® did not show any increase in antibodies at any of the post-treatment follow-up visits (Peterson et al. 2025a). These observations suggest that, despite 69% amino acid sequence homology, the immune system can distinguish between bLF and effera®. (R. D. Peterson et al. 2025)

In conclusion, this hypothesis-generating study provides evidence that LD and HD effera® maintained a diverse microbiome as measured by alpha- and beta-diversity, promoted proportional increases in SCFA production (particularly acetate), and enriched numerous potentially beneficial genera in healthy adults. Future adult clinical studies should consider employing more controlled dietary conditions and a placebo group that would strengthen the causal link of effera® on the microbiome composition and microbial metabolism. In addition, studies should look at larger participant cohorts (larger 'n') to corroborate these reported findings on taxa shifts and VFA profiles. Lastly, the format in this study was a drink mix. Given that a previous study demonstrated that approximately 25–30% of effera® hLF remained intact through the simulated gastric phase (B. J. Kim et al. 2024), a future study examining effera® hLF delivered in an enterically-coated capsule may demonstrate a greater effect on the gut microbiome.In addition, investigating effera® hLF in infant populations may further clarify its potential to help close the compositional gap between formula-fed and breastfed infants, at both metabolite and microbial levels.

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Author contributions

CRediT: **Ross D. Peterson**: Conceptualization, Project administration, Resources, Supervision, Visualization, Writing – original draft; **Julian van der Made**: Formal analysis, Methodology, Writing – review & editing; **Sharon M. Donovan**: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing; **Mei Wang**: Conceptualization, Formal analysis, Writing – review & editing; **Ryan N. Dilger**: Formal analysis, Methodology, Writing – review & editing; **Nicole Kaplan**: Conceptualization; **Anthony J. Clark**: Conceptualization, Resources, Supervision, Writing – review & editing.

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Ethics approval statement: These studies were approved by the WIRB-Copernicus Group Institutional Review Board (protocol: MB-2305, 8/16/2023).

Informed consent statement

Written informed consent was obtained from each participant prior to testing.

Disclosure statement

Julian van der Made, Nicole Kaplan, Ross Peterson, and Anthony Clark are employees of Helaina Inc. and receive equity or stocks in the company. Sharon Donovan and Ryan Dilger received funding from Helaina Inc. to perform the microbiome and VFA analyses, respectively.

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Notes on contributors

Dr. Ross Peterson (PhD) is Vice President of Regulatory and Scientific Affairs at Helaina Inc., where he leads regulatory strategy and safety and efficacy science for effera® (recombinant human lactoferrin) produced via precision fermentation. He holds a Ph.D. in Food Science and Human Nutrition from the University of Illinois at Urbana-Champaign and has over a decade of experience in novel ingredient regulatory affairs and GRAS qualification.

Dr. Julian van der Made is the Lead Fermentation Engineer at Helaina Inc., New York, NY, where he leads fermentation process development and scale-up for recombinant protein production. His work integrates bioprocess engineering, fermentation optimization, and transcriptomic data analysis to support strain development and industrial biomanufacturing.

Dr. Sharon Donovan (PhD) is the Melissa M. Noel Endowed Professor at the University of Illinois, Urbana-Champaign. Her research focuses on how postnatal nutrition, including human milk, infant formula, and human milk bioactive components, influences infant development.

Dr Mei Wang (PhD) is a Senior Research Specialist in Nutrition at the University of Illinois, Urbana-Champaign. Her research focuses on how early life nutrition (including breastmilk, infant formula, components of human milk, pre- and probiotics) and other environmental factors shape the development and maturation of human gut microbiome.

Dr Ryan N. Dilger (PhD) is a Professor of Animal Sciences at the University of Illinois Urbana-Champaign and Chair of the Institutional Animal Care and Use Committee. His research interests integrate nutrition, immunology, and neuroscience, with a focus on early-life nutrition, growth, immunity, tissue development, and the use of the pig as a translational model for human health.

Dr. Nicole Kaplan is the Senior Lead of the Immunology and Bioassays team at Helaina Inc., New York, NY, where she directs research focused on the safety, efficacy, and mechanistic characterization of novel bioactive proteins. Her work integrates immunology, functional assay development, and hypothesis-driven in vitro studies to evaluate biological activity, immunogenicity, and mechanism of action.

Dr. Anthony Clark (PhD) is Chief Technology Officer at Helaina Inc., where he leads the development of proprietary precision fermentation technology for producing human milk proteins. He brings over 25 years of biotechnology and R&D leadership experience across companies including MycoTechnology, Intrexon, IFF, PepsiCo, and Firmenich.

ORCID

Ross D. Peterson http://orcid.org/0009-0002-7405-4602
Julian van der Made http://orcid.org/0000-0002-6704-0854
Sharon M. Donovan http://orcid.org/0000-0002-9785-4189
Mei Wang http://orcid.org/0000-0003-0379-6633
Ryan N. Dilger http://orcid.org/0000-0003-2538-2845

Data availability statement

The datasets generated during and analyzed during the current studies are available from the corresponding author on reasonable request.

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