Original Research Article

Impact of formula protein quantity and source on infant metabolism: serum, urine, and fecal metabolomes of a randomized controlled study

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ABSTRACT

Background: Human milk offers significant health benefits for infants; however, when not feasible, infant formula serves as an alternative. The higher protein content in infant formula is thought to contribute to the distinct metabolic profiles observed in formula-fed infants compared with those fed human milk.

Objectives: This study investigates the impact of formula protein quantity and whey protein types on the serum, urine, and fecal metabolomes of infants.

Methods: A secondary analysis was performed on a random subset of 200 well-characterized per-protocol infants who completed a prospective, randomized, double-blind controlled trial. Infants were randomly assigned to 1 of 3 groups: standard formula, protein-reduced formula with α-lactalbumin-enriched whey, or protein-reduced formula with casein glycomacropeptide-reduced whey, along with an observational reference group of exclusively breastfed infants. Serum, urine, and fecal metabolites were quantified using ^{1}H nuclear magnetic resonance spectroscopy at baseline (1–2 mo), 4, and 6 mo of age. Dietary intake was assessed monthly ≤6 mo of age.

Results: Formula protein content and type of whey protein used significantly influenced the amino acid profile and associated catabolic markers in serum and urine but had minimal impact on the fecal metabolome. Reduced protein formulas yielded metabolome profiles closer to those of breastfed infants compared with standard formula. Despite these improvements, infants fed human milk still demonstrated enhanced branched-chain amino acid (BCAA) oxidation and a greater capacity to eliminate catabolic waste products from BCAA metabolism over infants consuming protein-reduced formulas.

Conclusions: Comprehensive metabolomics profiling of serum, urine, and feces captures molecular-level changes and informs potential strategies for formula optimization. Both the quantity and source of protein significantly influenced the metabolic profiles of formula-fed infants. However, modifications in protein alone cannot fully resolve the metabolic differences between formula-fed and breastfed infants, highlighting the complexity of mimicking the human milk feeding-associated metabolic profile.

This study was registered at clinicaltrials.gov as NCT02410057.

Keywords: human milk, infant formula, metabolomics, α-lactalbumin, glycomacropeptide-reduced whey, branched-chain amino acids, urine osmolality, hydration status, gut microbiota, protein content

Introduction

Human milk offers demonstrable benefits to newborns and infants by reducing risk of developing health problems later in life such as overweight/obesity and type 2 diabetes [1–5]; however, the precise mechanism underlying these protective effects is not fully understood. Although human milk is universally recognized as the optimal choice for the nutrition of developing infants, circumstances may arise when its use is not feasible, leading to the use of infant formula as an alternative. Notably, infant formula contains a higher protein content than human milk, and in formula-fed infants, essential amino acids derived from the diet remain elevated in the circulation for 2–3 h postprandially [6,7], and do not decline because of frequent feedings that are required to accommodate the small gastric volume of infants.

The growing field of metabolomics promises unique opportunities to elucidate the link between diet and health through comprehensive assessment of metabolite markers of immediate and long-term response to food intake [8,9]. To deepen our understanding of diet-driven metabolic development in early life, we performed a post-hoc secondary analysis of serum, urine, and fecal metabolic profiles of 200 per-protocol infants randomly selected from the ALFoNS (ALFa-laktalbumin Och Nutrition till Spädbarn) cohort, before enrollment, during, and at the end of the intervention. This cohort consists of 328 well-characterized healthy, full-term infants enrolled in a double-blinded, randomized controlled, prospective intervention trial conducted from 1–2 to 6 mo of age. The primary objective of this study was to evaluate the growth and metabolic outcome of infants who consumed a protein-reduced α-lactalbumin-enriched or glycomacropeptide-reduced whey formula, compared with those receiving a standard formula (SF) and a breastfed reference group. The primary outcome of the study was the effect of the intervention on growth, results of which have been previously reported [10]. Prespecified secondary outcomes include measurements of gastrointestinal tolerance, incidences of infection, metabolomics, hematological, inflammatory and hormonal markers, and behavioral outcomes such as crying duration and time to fall asleep.

Methods

Participants and study design

The AlFoNS study is a prospective, double-blind, randomized controlled trial with a parallel design, which was conducted at designated centers in Malmö/Lund and Umeå, Sweden from December 2014 to March 2020. The trial compared 3 infant formula groups: a SF containing 2.2 g of protein per 100 kcal, a low-protein formula enriched with α-lactalbumin (LP αLAC-EW) containing 1.75 g of protein per 100 kcal, and a low-protein formula with glycomacropeptide-reduced whey (LP CGMP-RW) containing 1.76 g of protein per 100 kcal. Infants were fed their assigned diets from baseline (1–2) to 6 mo of age. Formula-fed infants were randomly assigned. A non-randomized group of exclusively human breastfed infants was included as an observational reference group [human milk (HM)]. Parents were instructed to adhere strictly to the assigned formula or continue exclusive breastfeeding until ≥4 mo of age and to limit complementary foods to taste portions until 6 mo of age. The ALFoNS study evaluated 1 primary outcome, the effect of the intervention on growth [10]. This analysis focuses on metabolomics, which has not been previously reported. Other secondary outcomes have been detailed in prior publications [10,11].

Methodology details, including inclusions/exclusions, sample size estimation, randomization, and blinding procedures, have been previously reported [10]. In brief, formula-fed infants were stratified by sex and randomly assigned to consume 1 of the 3 formulas using a computerized tool in blocks of 6 or 12. Eligible participants were healthy, term infants under 2 mo old, born at 37–42 wk gestation, with a birth weight between 2.5 and 4.5 kg, and without chronic illness or complications interfering with normal feeding or growth. Exclusion criteria included infants delivered via cesarean section or those who had received antibiotic treatment before enrollment.

In total, 328 infants were initially recruited for the trial. Of these, 285 infants remained in the study until 6 mo of age and 270 infants successfully completed the feeding intervention according to the protocol requirements (per-protocol). A subset of 200 per-protocol infants, with complete dietary records and available serum, urine, and fecal samples at baseline, months 4 and 6 was randomly selected for metabolomics analysis (Supplemental Figure 1).

The trial was initially powered based on the primary outcome of growth, resulting in a sample size of $n = 80$ infants (40 males and 40 females) to detect significant weight differences at 6 mo. Given that the metabolomics profiling is exploratory, it was included as a secondary outcome based on the per-protocol population, and thus, no separate power calculation for metabolomics was performed.

Ethical considerations

The study was reviewed and approved by the Regional Ethical Review Board in Lund. Before participation, parents/legal guardians of the infants were provided with oral and written informed consent. This study was registered at clinicaltrials.gov under the identifier NCT02410057. All procedures and protocols followed the Declaration of Helsinki and Good Clinical Practice guidelines.

Study formulas

The SF provided 2.20 g protein per 100 kcal, comprising 60% whey and 40% casein, including 10% of the total protein as α-lactalbumin and 9% of the total protein as casein glycomacropeptide (CGMP)-reduced whey. The LP αLac-EW contained 1.75 g of protein per 100 kcal, comprising 70% whey and 30% casein, including 27% α-lactalbumin and 19% CGMP in its protein composition. The LP CGMP-RW formula provided 1.76 g of protein per 100 kcal, comprising 70% whey and 30% casein, with 14% protein as α-lactalbumin and 0% protein as CGMP.

Parents received all formulas (in powder, ready-to-feed forms) with detailed instructions for preparation. Specifically, infant formulas were prepared by dissolving 12.9 g of dry powder in 100 mL of water to reach the nutrient concentrations presented in Supplemental Table 1. Detailed nutrient content of these formulas has been previously reported [10]. All study formulas were produced at Laiterie de Montaigu, Le Planty, France. The whey protein fractions used in the study formulas (Lacprodan ALPHA-10, Lacprodan DI-8090, and Lacprodan DI-8095) were supplied by Arla Foods Ingredients Group P/S, based in Viby, Denmark.

Estimation of dietary intake

Compliance with study feeding, formula intake, and complementary food intake was assessed via 3-d food diaries at 3, 4, 5, and 6 mo of age. All food and drink that an infant consumed were measured either by weight (grams) or volume (milliliters, deciliters, teaspoons, or tablespoons) by parents using household measures and kitchen utensils. Daily macronutrient and energy intake from complementary foods

were calculated by a dietician using Dietist Net Pro (Kost och Näringsdata AB), which references the Swedish Food Composition Database alongside nutritional labels provided by food manufacturers. The mean dietary intake over 3 d was calculated for each time point. Although the continuation of feeding with mother's own milk was documented, the specific amount of milk consumed was not measured.

Sample collection

Venous blood samples were collected at baseline (1–2), 4, and 6 mo of age after a minimum 2 h fast and 1 h post application of local anesthetic cream to minimize discomfort. Serum was obtained through centrifuging blood samples at $1300 \times g$ for 10 min. After separation, serum samples were immediately frozen. Stool and urine samples were collected by parents at home shortly before scheduled visits or at the study center (at baseline, 4, and 6 mo). Stool samples were collected from the diaper and ≥ 2 mL was placed into 2 freezer-safe vials. Spot urine samples were collected in pediatric urine collection bags, and transferred to 2 freezer-safe vials with ≥ 1 mL in each vial. Samples collected at home were stored at $-20°C$ for ≤ 1 wk and subsequently transported to the study center on freezer packs. All samples were placed in $-80°C$ freezer for long-term storage until they were ready for nuclear magnetic resonance (NMR) metabolomics analysis.

NMR-based metabolomics of infant serum, urine, and feces

Serum sample preparation

To eliminate proteins and lipids, serum samples underwent filtration using an ultracentrifugal filter with a 3000 molecular weight cut-off (Amicon Ultra-0.5 mL, Millipore). The volume of each filtrate was precisely measured. The appropriate amount of Milli-Q water (Millipore) was added to each filtrate to ensure a final volume of 199 µL. Subsequently, 8 µL of potassium phosphate buffer (1M, pH 6.1) and 23 µL of internal standard (4.47 mM DSS-d6 (3-(trimethylsilyl)-1-propanesulfonic acid-d6 sodium salt) in 99.8% D_2O containing 0.2% NaN_3) were added. Sample pH was adjusted to 6.8 ± 0.1 using small amounts of NaOH or HCl. A blank sample was included in each extraction batch. Final concentrations were corrected for dilution.

Urine sample preparation

Urine samples were centrifuged to remove insoluble salt crystals. To the resulting supernatant (207 µL), 23 µL of internal standard (4.48 mM DSS-d6 in 99.8% D_2O with 0.2% NaN_3) was added. The pH was measured and adjusted to 6.8 ± 0.1. A blank sample was included in each extraction batch. Final concentrations were corrected for dilution.

Fecal metabolite extraction and sample preparation

Approximately 250 mg homogenized fecal material was weighed and extracted with 1.5 mL of ice-cold potassium phosphate buffer (40 mM, pH 6.7) and centrifuged. The supernatant was subsequently filtered through a 0.22 µm syringe filter (Millex-GP, Millipore) followed by an ultracentrifugal filter with a 3000 molecular weight cut-off (Amicon, Millipore). To 207 µL of filtrate, 23 µL of internal standard (4.48 mM DSS-d6 containing 99.8% D_2O with 0.2% NaN_3) was added. The pH of each sample was adjusted to 6.8 ± 0.1. A blank sample was included in each extraction batch.

Fecal dry weight determination

A fecal sample separate from the one used for metabolomics was lyophilized (Labconco FreeZone 4.5L Freeze Dry System, Labconco,

Kansas City, MO) and the percentage of fecal water calculated as *[(wet weight – dry weight)/wet weight] × 100*.

NMR data acquisition and spectral analysis

Targeted metabolomics via ^{1}H-NMR demonstrates high reproducibility and provides accurate and precise measurements, with an accuracy of $\pm 1\%$ and a measurement uncertainty of $< 0.1\%$. The upper quantifiable limit is in the molar range, and the limit of detection is in the low micromolar range (~1–5 µM) [12,13]. All ^{1}H-NMR spectra were acquired at $25°C$ using the noseypr1d pulse sequence on a Bruker Avance 600 MHz spectrometer (Bruker) as described [14]. Spectra were Fourier transformed, and manually phased and baseline corrected using Chenomx NMR Suite (version 8.6, Chenomx). A total of 68 serum metabolites, 88 urinary metabolites, and 84 fecal metabolites were quantified using targeted ^{1}H NMR-based metabolomics. Metabolite quantification was verified by ≥ 2 researchers. Metabolites (excluding bile acids) from serum and fecal samples were expressed in µmol/L. Fecal bile acids were quantified via spectral binning between 0.65 and 0.75 ppm with internal reference area normalization and dilution correction. Urine metabolite concentrations were normalized to effective osmolality and expressed in µM/Osm. Urine osmolality was measured in duplicate using a VAPRO vapor pressure osmometer (ELITech) and expressed in mmol/kg of water. Although urinary urea contributes to measured urine osmolality, because it freely crosses cell membranes, it does not contribute to tonicity. The urinary effective osmolality was determined as measured osmolality minus the osmolarity contribution of urea.

Sample exclusions and handling of missing data

No imputation was performed for missing data in this study. Details of sample selection and exclusion for the secondary analysis of serum, urine, and fecal metabolome are reported in Supplemental Figure 1. Specifically, 13 serum samples and 72 fecal samples were excluded because of insufficient volume or low spectral quality caused by heavy dilution. Five fecal samples, showing high urea concentrations, were suspected of urine contamination and subsequently were excluded from statistical analysis. One urine sample exhibited an elevated protein level, and another urine sample presented a slightly brown hue along with significantly elevated levels of acetate, succinate, and butyrate. These two samples were suspected of fecal contamination and thus excluded from statistical analysis. Additionally, 1 infant from the SF group discontinued the assigned study formula at month 6 according to dietary records; therefore, the corresponding serum, urine, and fecal metabolome data at that timepoint were excluded because of nonadherence.

Statistical analyses

Statistical analyses for baseline characteristics were conducted using independent samples t-tests or 1-way analysis of variance (ANOVA) for continuous variables, with post-hoc pairwise comparisons performed using t-tests with Bonferroni correction. Categorical variables were assessed using chi-square tests or Fisher's exact tests, depending on cell counts. All subsequent statistical analyses were performed in R (version 4.2.2), with data visualization generated using the *ggplot2* and *ggpubr* packages.

To approximate data normality, metabolite concentrations were generalized log-transformed (defined as *log(y + sqrt(y² + lambda) where lambda is 1)*. To explore the overall structure of the metabolomics data and visually inspect patterns among groups, principal component analysis was computed using the *pca* function from the

mixOmics package, where each variable was centered to their means *(center = True),* but not scaled by the SD *(scale = False).* To evaluate significant differences in the overall metabolomic composition between groups, adonis, a nonparametric implementation of a permutational analysis of variance (PERMANOVA), was computed using the *adonis2* function from the *vegan* package with the Canberra distance as the metric for comparison and 999 permutations (nperm = 999). To validate the assumption of homogeneity of dispersion, the *betadisper* function from the *vegan* package was used, followed by Tukey's Honestly Significant Difference (HSD) post-hoc tests to assess pairwise differences in dispersion among groups.

Pairwise differences between dietary groups were assessed using a linear mixed model via the *lmer* function from the *lme4* package and the *ls_means* function from the *lmerTest* package. Group differences were evaluated using repeated measures analysis of covariance using data collected at 4 and 6 mo of age. This model included fixed effects for dietary group (HM, SF, LP αLAC-EW, or LP CGMP-RW), time-point (Month 4 or 6), baseline concentration values, and caloric contribution from complementary food intake (in kcal per day). The dependent variable in this model is the metabolite concentration, although the covariate "baseline concentration" refers to the same metabolite measured at the baseline timepoint. The model was formatted as follows: *lmer (Measurement ~ Group + Time + Baseline + Complementary_food_energy + (1|Subject_ID)).*

Effect size was evaluated using partial omega squared, calculated using the *omega_squared* function *(partial = TRUE)* from the *effect-size* package. Assumptions for the linear mixed-effect model were verified using the *simulateResiduals* function from the *DHARMa* package.

Significant group differences in formula intake, essential amino acid intake, urine effective osmolality, metabolite differences at baseline, ratio of serum branched-chain α-ketoacids (BCKA) to branched-chain amino acids (BCAAs), and fecal water percentage were evaluated using the Mann–Whitney U test. The significance of the interaction effect between formula-fed groups and time on formula intake was assessed using aligned rank transform ANOVA. This analysis was performed using the *art* function from the *ARTool* package, followed by the *anova* function from the base R package. Pearson's correlation coefficient *r* was used to evaluate the strength of correlation. False discovery rate from multiple hypothesis tests was controlled using the Benjamini-Hochberg procedure, with an overall significance level set at $P < 0.05$.

Results

To understand the impact of lowering protein content and modulating the amino acid composition of infant formula, we evaluated the serum, urine, and fecal metabolomes of a random subset of 200 per-protocol full-term infants who completed the ALFoNS study. All infants were, based on parental reports, exclusively fed mother's own milk or infant formula before enrollment and consumed the assigned diet from baseline (1–2) to 6 mo of age. The flow diagram illustrating participant randomization, group allocation, and the sample selection process is presented in Supplemental Figure 1. Baseline demographics and characteristics are reported in Supplemental Table 2; there were no group differences in gestational age, birth weight, mode of delivery, child sex, and maternal ancestral/geographical origin. Notably, mothers of breastfed infants had lower BMI at enrollment than those of formula-fed infants, and the use of probiotics before inclusion was less common in breastfed infants. Among formula-fed groups, significant differences were noted only in maternal BMI and paternal education level. Mothers

of infants receiving the LP CGMP-RW formula had a lower BMI at enrollment compared with those receiving SF. Fathers in the LP CGMP-RW group were more likely to have higher education levels compared with the SF group. All formulas were well tolerated, with only minimal adverse events reported [10]. All infants grew well according to WHO Child Growth Standards.

In this random selection of 200 per-protocol infants, the initial analysis was conducted using the aligned rank transform ANOVA, a robust nonparametric method for assessing interaction effects. This analysis revealed no statistically significant interaction between formula group and time ($P = 0.18$). A subsequent cross-sectional evaluation showed that those fed the 2 low-protein formulas had a significantly lower formula intake compared with those receiving the SF at 4 mo of age (Figure 1A). This finding is consistent with our previous results using the entire cohort [10]. Following guidelines from the Swedish National Food Agency, a taste portion of complementary foods was introduced starting from 4 mo of age in some infants. By 6 mo, 31% of infants fed human milk and 36% of those fed formula consumed >2 tablespoons of complementary food per day. Only 105 infants in this random subset were still exclusively fed study formula or human milk without exposure to complementary food. Although a significant difference in complementary food intake was not observed between dietary groups, intake was highly variable within groups, which introduced diversity in the type and amount of food consumed and impacted the amount of formula consumed. Notably, for infants who had a record of complementary food consumption at 6 mo of age, increased energy intake from complementary foods corresponded to decreased consumption of study formula (Figure 1B), which increased the complexity of evaluating the dietary effect introduced by the protein level and the type of whey protein in the formula on the serum, urine, and fecal metabolomes. Therefore, to investigate the age-dependent response of each metabolite, and to account for the influence of complementary food and any possible confounding elements derived from differences at enrollment linked to initial feeding choice, a linear mixed model accounting for timepoint, complementary food intake, and baseline values was applied to evaluate intervention-induced metabolomics-level differences at 4 and 6 mo of age.

Protein quantity and source are key drivers of circulating amino acids

To provide a comprehensive view of metabolic phenotype before, during, and after the dietary intervention, ^{1}H NMR-based metabolic profiling was conducted on samples collected at multiple timepoints. Overall, diet profoundly affected the serum, urine, and fecal metabolomes throughout the early feeding period (Figure 1C and Supplemental Figure 2). No differences were observed between formula-fed groups at baseline; however, significant differences in the serum and urine metabolomes among infants receiving different formulas were more pronounced at 4 mo of age and became less apparent by 6 mo. Notably, the metabolomes of breastfed infants were already distinct from those of formula-fed infants at baseline and remained distinct throughout the early feeding period. By 6 mo of age, a profound impact of complementary food intake was also noted in the serum, urine, and fecal metabolomes (Supplemental Figure 2).

As we previously reported [6,14–19], the serum metabolome of breastfed infants was characterized by elevated levels of ketogenic metabolites, including 3-hydroxybutyrate, acetone, acetoacetate, as well as tricarboxylic acid cycle (TCA) intermediates (citrate, succinate, and fumarate) and short-chain fatty acids (acetate and formate), which may originate from gut microbial fermentation, intracellular
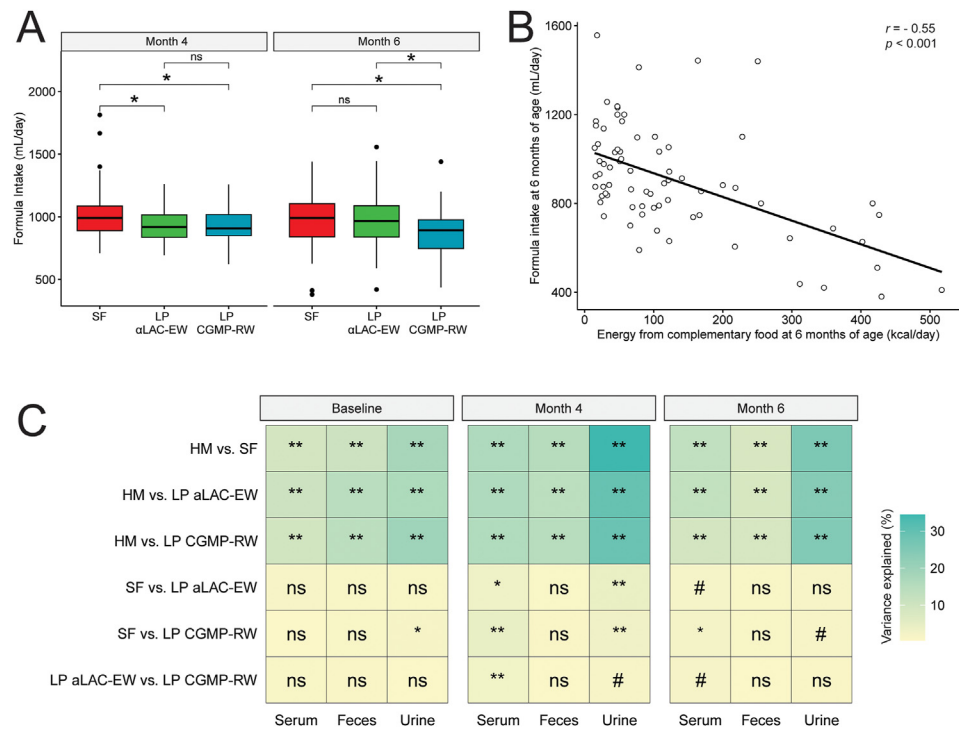
FIGURE 1. Impact of dietary intervention on formula intake and infant metabolome during the exclusive feeding period. (A) Formula intake at 4 and 6 mo of age. Group differences were evaluated cross-sectionally using the Mann–Whitney U test, focusing on intervention effects at each time point independently. The interaction between group and time was analyzed using aligned rank transform ANOVA; the interaction term was not statistically significant ($P = 0.18$). *$P <$ 0.05; ns, not significant. (B) Scatter plot demonstrates a negative correlation between infant formula intake and complementary food intake among formula-fed infants who reported complementary food intake at 6 mo of age. Pearson's correlation was used to evaluate the relationship. (C) Permutational multivariate analysis of variance (PERMANOVA) was performed to assess the difference in serum, fecal, and urine metabolomes among infants receiving the standard formula (SF), the low-protein α-lactalbumin-enriched whey formula (LP αLac-EW), the low-protein casein glycomacropeptide-reduced whey formula (LP CGMP-RW) and human milk (HM) at baseline (1–2 mo), 4, and 6 mo of age. Analysis was performed using the adonis function with Canberra distance on generalized log-transformed metabolome data. Urine metabolome data were adjusted for effective osmolality. The homogeneity of dispersion assumption was validated using the *betadisper* function. The results of these tests are presented in Supplementary Materials, specifically within the section "Supplementary Note on PERMANOVA Assumption Validation and Justification." This section contains Supplementary Note Figures 1, 2, and 3 as well as Supplementary Note Tables 1 and 2. P values from PERMANOVA were adjusted for multiple testing using the Benjamini-Hochberg FDR procedure. ** Adjusted P value < 0.01, * adjusted P value < 0.05, # unadjusted P value < 0.05 (not significant after adjustment), ns, not significant. The corresponding visualization via principal component analysis (PCA) is in Supplemental Figure 2. ANOVA, analysis of variance; FDR, false discovery rate.

deacetylation reactions, 1-carbon metabolism, or fatty acid α-oxidation. In contrast, formula-fed infants exhibited higher levels of protein catabolism markers, such as the nitrogen waste product urea, essential amino acids (leucine, isoleucine, valine, tyrosine, methionine, and phenylalanine), and a few amino acid catabolism intermediates (2-aminobutyrate, 2-hydroxybutyrate, 2-ketoisocaproate, 2-ketoisovalerate, 2-ketomethylvalerate, and 3-hydroxyisobutyrate; summarized in Supplemental Figure 3).

During the intervention, the serum metabolome differed significantly between infants consuming the SF and the 2 low-protein formulas (Figure 1C and Supplemental Figure 3). Reduction of formula protein content from 14.8 g/L to 11.9 g/L resulted in a notable reduction in the serum urea level (mean fold change: 39%, Figure 2); yet, the circulating urea level in the 2 low-protein formula groups was still significantly higher than that observed in the breastfed reference group (~10.3 g/L protein in human milk [20]). Because protein from infant formula is the primary source of essential amino acids in formula-fed infants during exclusive feeding, modulating the protein content and the type of whey protein in the infant formula directly impacted amino acid intake (Supplemental Figure 4), which in turn was reflected in the essential amino acid profile in the bloodstream (Figure 2). Reducing

protein content in the formula effectively lowered the concentration of several amino acids closer to the levels seen in the breastfed reference group. However, this did not alter the breastfeeding-specific metabolic features related to ketogenesis and TCA cycle intermediates (Supplemental Figure 3). A subsequent analysis was performed to evaluate the pairwise relationship between essential amino acid intake from formula-feeding with serum and urine concentrations of the essential amino acids and their downstream catabolites. A clear directional link between intake and serum concentrations of these metabolites was observed; however, the association between intake and urine concentrations of these metabolites and their catabolic byproducts was absent or relatively weak, with the notable exception of dietary tryptophan (Figure 3 and Supplemental Figure 5). This discrepancy led us to perform a more detailed evaluation of the effect of urine osmolality on the urine metabolome.

The influence of urine osmolality on metabolite profiles across different feeding regimens

Urine specimens consist of a high concentration of food-derived metabolites with wider dynamic ranges than those found in blood,
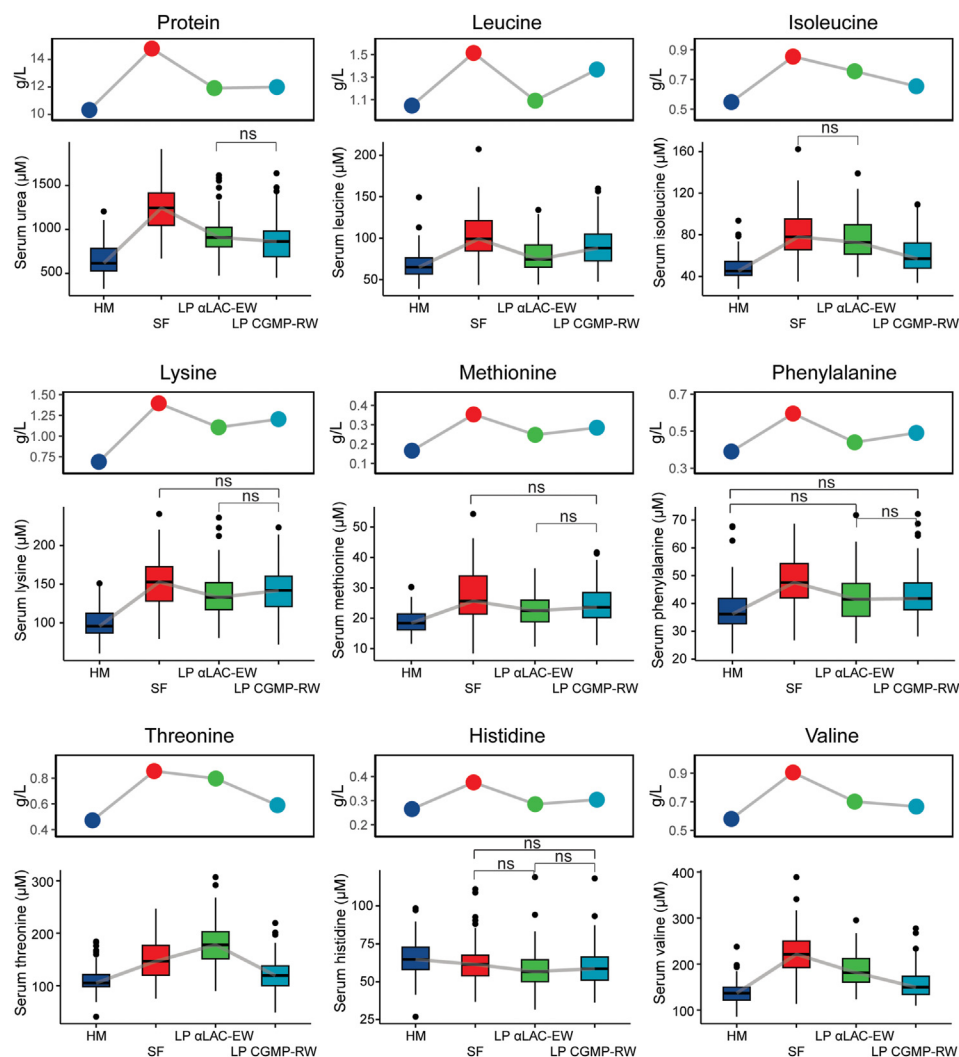
FIGURE 2. Dietary protein and essential amino acids are reflected in the circulating levels of urea and essential amino acids in infants during the exclusive feeding period. Visual representation of the dietary protein and essential amino acid levels in human milk and study formulas (top) and circulating level of urea and the corresponding essential amino acids in infants at 4 and 6 mo of age (bottom). The protein content and amino acid composition of each diet are presented in Supplemental Table 1. The significant pairwise difference was assessed using age-, baseline- and complementary food intake-adjusted repeated measures ANCOVA (details described in Methods). Statistical significance was determined with a cut-off of Benjamini and Hochberg FDR-adjusted *P* value <0.05. Only pairs that are not significantly different are labeled (ns, not significant). ANCOVA, analysis of covariance; FDR, false discovery rate; HM, human milk; LP αLac-EW, low-protein α-lactalbumin-enriched whey formula; LP CGMP-RW, low-protein casein glycomacropeptide-reduced whey formula; ns, not significant; SF, standard formula.

offering an objective assessment of diet. Unlike blood, variations in urine volume and metabolite concentration are linked to an individual's hydration status. In this study, urine osmolality, a representation of total solute content in a urine sample was measured through the measurement of vapor pressure depression. Urine osmolality was found to be strongly correlated with urine creatinine levels (Supplemental Figure 6). However, unlike creatinine levels, urine osmolality is not influenced by diurnal rhythms, dietary creatine levels, activity, gender, age, stress, and health [21], and therefore provides a more robust method for normalizing urine metabolite concentrations. Furthermore, although urine urea affects urine osmolality, it does not contribute to tonicity (water balance) because it freely crosses cell membranes, thus effective osmolality was calculated by subtracting the osmolality contribution of urea from the measured urine osmolality.

In general, urine effective osmolality gradually increased with age, reflecting changes in water homeostasis. Formula-fed infants exhibited more concentrated urine with higher urine effective osmolality compared with infants in the breastfed reference group, suggesting differences in hydration status and urine volume. Notably, at 4 mo, infants fed the low-protein formulas demonstrated significantly lower urine effective osmolality compared with those provided the SF, aligning more closely with levels observed in the breastfed reference group (Figure 4A).

Urine effective osmolality had a substantial impact on the infant urine metabolome. Metabolites that significantly differed between dietary groups are summarized in Supplemental Figure 7. Before adjusting for hydration status, in comparison to breastfed infants, 15 and 25 urine metabolites were significantly elevated or reduced respectively in SF infants. After hydration status adjustment, 10 metabolites remained
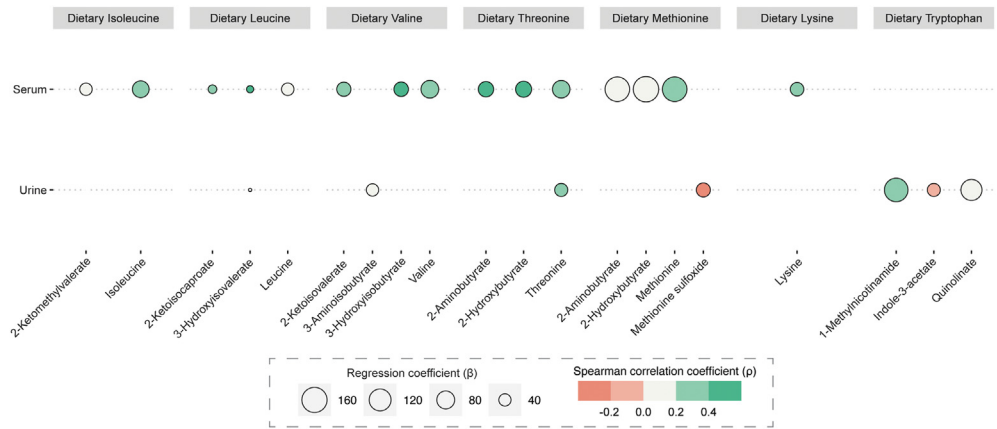
FIGURE 3. Relationship between dietary essential amino acid intake, and corresponding serum and urine metabolites. Pairwise associations between intake of dietary essential amino acids, their corresponding serum and urine levels, and their metabolic intermediates or byproducts were evaluated using Spearman's rank correlation coefficient to measure the strength of the relationships and linear regression β coefficients to assess effect sizes. Data from all formula-fed groups were used for the analysis. Significance was determined using Benjamini and Hochberg FDR-adjusted P value <0.05, and only statistically significant associations are illustrated. Information on which specific pairs were evaluated can be found in Supplemental Figure 5. FDR, false discovery rate.
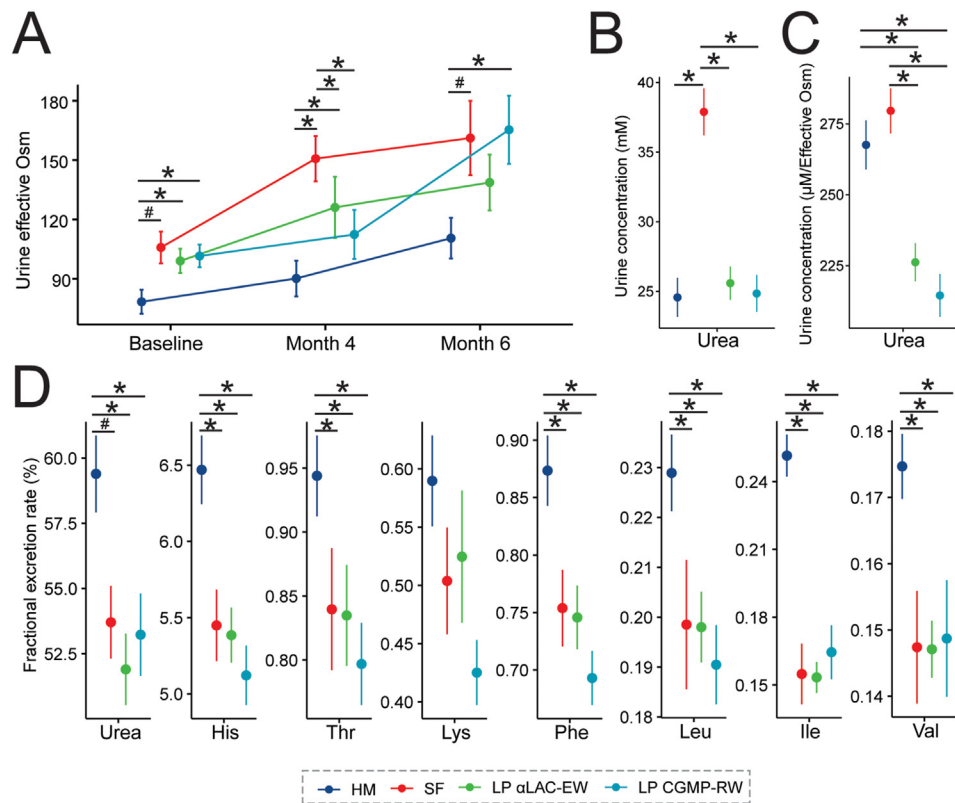
FIGURE 4. Impact of diet on the infant urine metabolome. (A) Trajectory of urine effective osmolality over time. The difference between dietary groups was assessed cross-sectionally using the Mann–Whitney U test. (B, C) Urine urea concentrations, uncorrected and corrected for hydration status, respectively. Effective osmolality was determined by subtracting the molar contribution of urinary urea from measured osmolality. (D) Fractional excretion rate of urea and essential amino acids. The fractional excretion rate (%) was calculated using the formula: ([urine metabolite] × [serum creatinine]/[serum metabolite]/[urine creatinine]) × 100%. (B–D) Significant differences were evaluated using repeated measures ANCOVA, accounting for timepoint, complementary food intake, and baseline values as covariates. Data are presented as mean ± SEM. * Benjamini and Hochberg FDR corrected P value < 0.05, # unadjusted P value <0.05. FDR, false discovery rate; HM, human milk; LP αLac-EW, low-protein α-lactalbumin-enriched whey formula; LP CGMP-RW, low-protein casein glycomacropeptide-reduced whey formula; ns, not significant; SF, standard formula.

significantly higher, whereas 55 metabolites were significantly lower in the SF group. This suggests that urine metabolite profiles associated with formula-feeding are strongly mediated by variations in urine effective osmolality, leading to greater differences based on diet.

Without considering hydration status, reducing protein content in the formula significantly reduced urea concentrations in urine to levels found in breastfed infants (Figure 4B). However, after accounting for hydration status, urinary urea excretion in the SF group was comparable with that in breastfed infants, despite the high protein content in SF compared with human milk (Figure 4C). Additionally, several essential amino acids were significantly lower in the urine of formula-fed infants compared with those in the breastfed reference group (Supplemental Figure 8). Formula-fed infants also showed lower fractional excretion rates of essential amino acids compared with breastfed infants, indicating less efficient elimination of excess dietary amino acids from circulation (Figure 4D).

Diet-dependent effect on BCAA catabolism

Under homeostatic conditions, a precise balance is maintained between amino acid intake and loss. Amino acids not incorporated into protein synthesis must be oxidized and disposed of to maintain homeostasis. Interestingly, most catabolites from essential amino acids, particularly from BCAAs, are more concentrated in urine than in serum (Supplemental Table 3), suggesting that the urinary levels are better indicators of the extent of essential amino acid catabolism.

BCAAs are a significant component of the diet and body, and their metabolism is closely tied to the rates of whole-body protein metabolism [22,23]. BCAA oxidation predominantly occurs in the mitochondrial matrix, and only a few catabolites that escape are detectable in serum and urine (Figure 5). To explore the dietary effects on BCAA catabolism, we examined the levels of BCAA intermediates and side products, including 2-hydroxyisovalerate, 2-ketoisocaproate, 2-ketoisovalerate, 2-ketomethylvalerate, 3-aminoisobutyrate, 3-hydroxy-3-methylglutarate, 3-hydroxyisobutyrate, and 3-hydroxyisovalerate.

Generally, serum levels of BCKA, specifically 2-ketoisocaproate, 2-ketomethylvalerate, 2-ketoisovalerate, are closely reflective of dietary BCAA levels. For example, breastfed infants showed lower BCKA levels compared with those fed with SF. Among the formula-fed groups, the LP αLAC-EW group, which had the formula with the lowest leucine content, also exhibited the lowest serum 2-ketoisocaproate level. Similarly, the LP CGMP-RW group, which was provided a formula containing the lowest levels of isoleucine and valine, had the lowest circulating levels of 2-ketomethylvalerate and 2-ketoisovalerate (Figure 6A). Despite the lower protein content in human milk compared with infant formulas, breastfed infants displayed significantly higher ratios of BCKAs to BCAAs before and during the study intervention (Figure 6B). This suggests that the rate of BCAA transamination does not directly correlate with the BCAA intake level, and factors beyond BCAA intake contribute to the elevated BCAA metabotype typically seen in formula-fed infants.

Interestingly, considering hydration status, urinary levels of BCAA catabolites also do not correspond directly to the dietary BCAA level. Infants from the breastfed reference group exhibited significantly higher urinary levels of leucine catabolites, such as 3-hydroxyisovalerate and 3-hydroxy-3-methylglutarate, as well as valine catabolites 3-hydroxyisobutyrate, both before and during the study intervention (Figure 6C). Additionally, these infants showed a significantly higher level of the urinary valine catabolite 3-aminoisobutyrate during the intervention. Furthermore, infants in the breastfed reference group had higher fractional excretion rates of 3-hydroxyisobutyrate and 3-hydroxyisovalerate compared with formula-fed infants (Figure 6D). Together, these results suggest breastfed infants possess enhanced capabilities of BCAA oxidation, along with a greater capacity to process and eliminate catabolic waste products from BCAA metabolism.

The impact of early diet on the fecal metabolome

To evaluate potential diet-induced functional changes in intestinal microbial fermentation capacity, longitudinal assessments of the fecal metabolome were performed. We observed a significantly higher
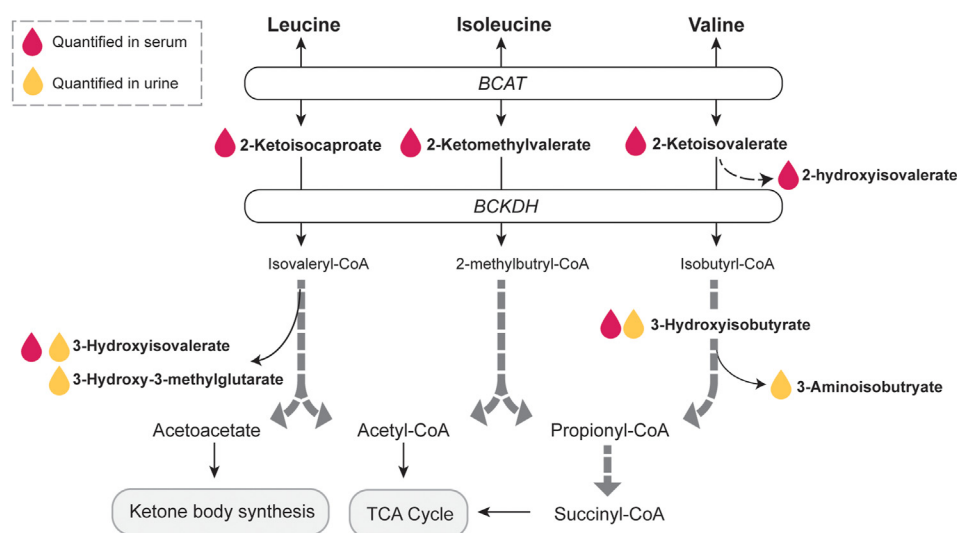
FIGURE 5. BCAA catabolism pathway. BCAA, branched-chain amino acids; BCAT, branched-chain amino transferase; BCKDH, branched-chain α-ketoacid dehydrogenase; CoA; coenzyme A; TCA, tricarboxylic acid. Commonly used synonyms: 2-ketoisocapraote (α-ketoisocaproate, 2-oxoisocaproate, ketoleucine, 4-methyl-2-oxovalerate), 2-ketomethylvalerate (α-ketomethylvalerate, ketoisoleucine, 3-methyl-2-oxovalerate), 2-ketoisovalerate (α-ketoisovalerate, ketovaline, 2-oxoisovalerate, 3-methyl-2-oxobutyrate), 2-hydroxyisovalerate (α-hydroxyisovalerate, 2-hydroxy-3-methylbutyrate), 3-hydroxyisovalerate (β-hydroxyisovalerate, 3-hydroxy-3-methylbutyrate), 3-hydroxy-3-methylglutarate (β-hydroxy-β-methylglutarate), 3-hydroxyisobutyrate (β-hydroxyisobutyrate), 3-aminoisobutyrate (β-aminoisobutyrate).
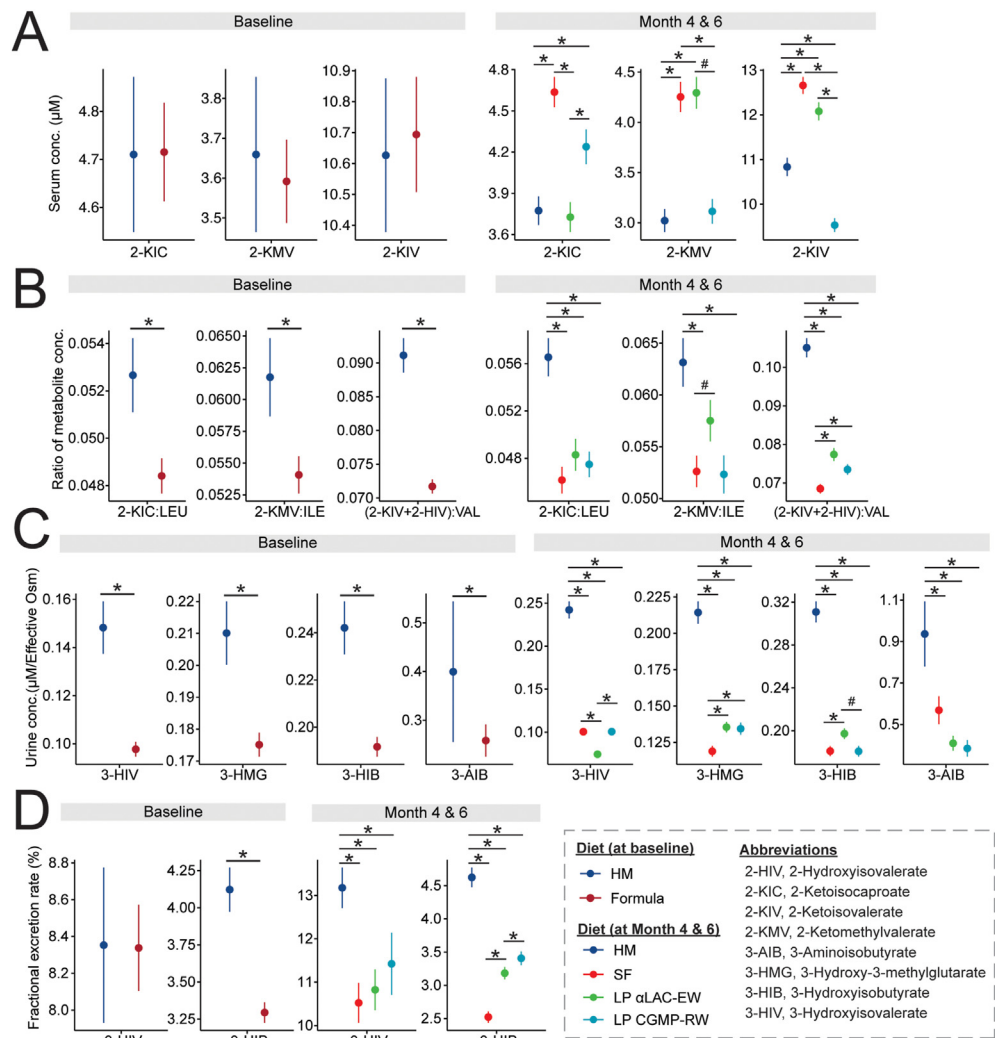
FIGURE 6. Dietary effects on markers of BCAA catabolism. (A) Serum levels of the branched-chain α-ketoacids (BCKA) 2-ketoisocaproate, 2-ketomethylvalerate, 2-ketoisovalerate. (B) Ratio of BCKA and BCAA in serum. For valine, the concentrations of the BCKA 2-KIV and the α-hydroxy branched-chain acid 2-HIV were summed because both are breakdown products of valine (see Figure 5). (C) Concentrations of urinary BCAA catabolites. (D) Fractional excretion rates of BCAA catabolites. (A–D) Significant differences at baseline were evaluated using Mann–Whitney *U* test. During the intervention (at months 4 and 6), significant differences were assessed using repeated measures ANCOVA accounting for complementary food intake and baseline values as covariates. (B) Significant differences at baseline and during intervention (at months 4 and 6) were evaluated using Mann–Whitney *U* test. Data are presented as mean ± SEM. * Benjamini and Hochberg FDR corrected *P* value <0.05, # unadjusted *P* value <0.05. ANCOVA, analysis of covariance; BCAA, branched-chain amino acids; HM, human milk; LP αLac-EW, low-protein α-lactalbumin-enriched whey formula; LP CGMP-RW, low-protein casein glycomacropeptide-reduced whey formula; ns, not significant; SF, standard formula; 2-HIV, 2-hydroxyisovalerate; 2-KIC, 2-ketoisocaproate; 2-KIV, 2-ketoisovalerate; 2-KMV, 2-ketomethylvalerate; 3-AIB, 3-aminoisobutyrate; 3-HMG, 3-hydroxy-3-methylglutarate; 3-HIB, 3-hydroxyisobutyrate; 3-HIV, 3-hydroxyisovalerate; FDR, false discovery rate.

percentage of fecal water in breastfed infants compared with formula-fed infants (Supplemental Figure 9), consistent with our earlier findings of increased stool frequency and softer/looser stool consistency in breastfed infants from the entire cohort [10]. From baseline (1–2 mo) through 6 mo, a distinct difference in the fecal metabolome between breastfed and formula-fed infants persisted. Stools from breastfed infants were enriched with metabolic markers indicative of microbial carbohydrate utilization, including elevated levels of sugar monomers (N-acetylneuraminate, fucose, galactose, and glucose), pyruvate, and 1, 2-propanediol. In contrast, stools from infants who consumed the SF were characterized by higher levels of butyrate, valerate, isobutyrate, isovalerate, and 4-hydroxyphenylacetate, which have been linked to microbial degradation of peptides and amino acids [24,25].

Modification of formula protein content and the type of whey isolate used did not significantly alter fecal water percentage, the overall composition of fecal metabolome, or the levels of nitrogen-containing metabolite markers of microbial fermentation of proteins and peptides. A detailed list of fecal metabolites that differed significantly between groups is presented in Supplemental Figure 10.

Discussion

Infant formula is the only safe alternative to human milk and is formulated to contain higher protein than human milk to ensure adequate intake of essential amino acids. The addition of metabolomics

data as part of clinical studies provides a more accurate reflection of dietary impact on health status. Consistent with numerous prior studies [6,14–19,26], significant differences were observed in the serum, urine, and fecal metabolomes of formula-fed infants compared with breastfed infants. Breastfed infants showed higher circulating levels of markers of ketogenesis and TCA cycle intermediates whereas formula-fed infants showed higher levels of urea, essential amino acids, and amino acid catabolism byproducts.

Modification of protein content or composition of infant formulas did not significantly influence the fecal metabolome because the fecal metabolome profile in infants is largely attributed to the influence of human milk oligosaccharides on gut microbial colonization. This indicates a minimal effect of the formula protein content and source on intestinal microbial metabolism, likely because the high digestibility of milk proteins enables rapid absorption of small peptides and amino acids from proteolytic action in the upper gastrointestinal tract, leaving little substrate for microbial fermentation. These findings align with our previous observation that there was no significant difference in the number or consistency of stools among the formula-fed groups [10]. This is further supported by a prior study demonstrating large individual variations within the formula-fed groups and no significant impact of formulas enriched with either α-lactalbumin or CGMP on key intestinal microorganisms [27].

One current working hypothesis to explain the development of long-term risk of negative health outcomes of children and adults fed predominantly formula as infants has been linked to excess protein intake during infancy which overstimulates mammalian target of rapamycin (mTORC1) signaling that leads to increased adipogenesis, decreased ketogenesis, rapid weight gain, and increased risk of childhood obesity ("Early Protein Hypothesis" [28]). We, therefore, speculate that reducing protein content and modifying amino acid composition in infant formula, particularly by lowering levels of mTORC activators like leucine, can suppress the upstream activators of mTOR signaling and, consequently, mitigate mTORC hyper-activation, a phenotype driving obesity. Our study demonstrates that lowering the protein content in the formula directly reduced both the intake and bloodstream levels of essential amino acids, bringing them closer to the levels observed in breastfed infants (Figures 2 and 3 [10]). These findings align with our previous research on rhesus monkeys, which showed similar effects when fed a low-protein formula [17]. Moreover, the protein source of the formula directly reflects the circulating amino acid profile and the associated catabolism markers. For instance, infants consuming the LP αLAC formula, which contains the least leucine among other formulas, correspondingly showed the lowest levels of serum leucine and its catabolites, 2-ketoisocaproate, and 3-hydroxyisovalerate in serum and urine, compared with those consuming the other formulas (Figure 2 and Figure 6A).

In addition to the dietary protein level, the rate of amino acid oxidation also plays a crucial role in whole-body amino acid disposal. Although formula-fed infants had higher levels of BCKA, the rate of conversion is not proportional to the BCAA intake. Notably, BCKAs can activate mTORC1[29,30] and serve as biomarkers of insulin resistance in adolescents with obesity [31]. This reduced BCAA oxidation and subsequent accumulation of intracellular levels of BCAA and BCKA may lead to greater stimulation of mTORC signaling in formula-fed infants regardless of composition.

Essential amino acids not utilized for protein synthesis undergo oxidation to maintain homeostasis, with their rate of disposal matching intake levels, thereby influencing the overall circulating amino acid levels. Although only a small proportion of essential amino acids are excreted in urine, the majority are metabolized, with their intermediate- or byproducts also disposed of in the urine. In this study, we observed significantly higher concentrations and fractional excretion rates of essential amino acids and their catabolites in breastfed compared with formula-fed infants, suggesting that breastfed infants can more efficiently eliminate excess amino acids and their catabolism byproducts. Furthermore, the metabolic discrepancies observed in the urine profiles of formula-fed and breastfed infants may be partially related to the higher urinary effective osmolality in formula-fed infants (Figure 4A), which could reflect differences in protein and osmolyte load between typical infant formulas and human milk [32,33]. Taken together, these results suggest that dietary modification of protein content alone cannot fully account for the metabolic discrepancies between formula-fed and breastfed infants. Human milk is a complex matrix containing numerous bioactive components, including milk fat globule membrane, oligosaccharides, exosomes, functional proteins, and microorganisms, all of which collectively support infant development [34]. This further highlights the complexity of attempting to achieve the metabolic profile of human milk feeding by solely modifying protein in infant formula.

This study has several notable strengths, including its double-blind, randomized design among formula-fed infants and a collection of detailed prospective dietary and comprehensive metabolomics data. However, the comparisons between formula-fed and breastfed infants should be interpreted cautiously, as these groups were not randomly assigned, and potential confounding factors related to feeding choice cannot be excluded. One limitation of the study is the absence of data regarding the volume and composition of mother's milk consumed, as well as detailed information on feeding frequency and human milk feeding styles, such as exclusive latching, pumping and bottle-feeding, or combo feeding. Additionally, ~40% of infants assigned to formula-fed groups consumed human milk for some duration before the study enrollment within the first months of life, as recruiting infants who were exclusively formula-fed from birth is logistically challenging. Lastly, the ALFoNS cohort predominantly consisted of participants of European descent, with ~85% identified as Nordic. Therefore, the generalizability of our findings to healthy-term infants from other diverse racial backgrounds remains uncertain.

This secondary analysis of a randomized controlled trial provides proof-of-concept evidence that dietary essential amino acids from milk protein are effectively absorbed and significantly contribute to the circulating amino acid levels in developing infants. Strategies such as reducing protein content or modifying the type of whey protein prove effective in aligning the serum metabolic profiles of formula-fed infants more closely with those of breastfed infants. Furthermore, markers of amino acid catabolic elimination in urine should be considered as an important outcome for future studies on metabolic response to dietary protein. Differences in hydration status may impact the efficient urinary elimination of amino acid metabolic waste products, which should be considered. The insights gained from this study offer practical implications for infant formula optimization and further shed light on protein-dependent effects on shaping infant metabolic health.

Acknowledgments

Author contributions

The author's responsibilities were as follows – XH: performed statistical analysis, created visualizations, and wrote the manuscript; UTN, PKÅ: coordinated the clinical trial and contributed significantly to the clinical data curation; XH, DOM: generated the metabolomics data; BL, OH, CMS, PKÅ: conceived, designed, and managed the study; CMS: provided guidance on the metabolomics data analysis and result interpretation and holds primary responsibility for the manuscript's final content; UTN, DOM, OH, BL, LNJ, ASK, MLH, CMS, PKÅ: each critically reviewed the manuscript; and all authors: have read and approved the final manuscript.

Conflict of interest

The study was funded by Arla Foods Ingredients Group P/S, Viby J, Denmark, which also supplied protein ingredients for the infant formulas used. MLH, LNJ, and ASK are employees of Arla Foods Ingredients and contributed to project logistics and funding communication. CMS, PKÅ, BL, and OH have received research grants from Arla Foods Ingredients. BL, OH, and PKÅ have received research honorariums from Arla Foods Ingredients. The authors declare no other conflict of interest.

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Data availability

Data described in the manuscript, code book, and analytical code are available on reasonable request pending application and approval.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajcnut.2025.02.002.

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