Supplementary Information

The role of protein and free amino acids on intake, metabolism and gut microbiome: a comparison between breast-fed and formula-fed rhesus monkey infants

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• **References**
### SI Table 1. Nutrient composition of each dietary treatment.

|                        | Regular formula | Regular formula plus free AAs | Reduced protein formula | Reduced protein formula plus free AAs | Reference formula [a] | Rhesus milk reference |
|------------------------|-----------------|-------------------------------|-------------------------|--------------------------------------|-----------------------|-----------------------|
| **Protein (g/L)**      | 14.4            | 14.6                          | 12.3                    | 12.6                                 | 18.4                  | 16.6 ± 2.2 [b]        |
| **Fat (g/L)**          | 36.6            | 36.6                          | 36.5                    | 36.5                                 | 36.7                  | 50.7 ± 23.6 [c]       |
| **Carbohydrate (g/L)** | 75.2            | 75.2                          | 77.6                    | 77.6                                 | 77.7                  | 78.3 ± 3.1 [d]        |
| **Lactose (g/L)**      |                 |                               |                         |                                      |                       | 81.6 ± 2.1 [f]        |
| **Amino acids (µM)**   |                 |                               |                         |                                      |                       |                       |
| Ala                    | 7870            | 8099                          | 6746                    | 6953                                 | 9260                  | 7452.3 ± 372.6 [g]    |
| Arg                    | 2161            | 2161                          | 1852                    | 1852                                 | 3474                  | 4478.8 ± 381.2        |
| Asp/Asn                | 11353           | 11353                         | 9731                    | 9731                                 | 13883                 | 4569.4 ± 500.8        |
| Cys                    | 2483            | 2483                          | 2129                    | 2129                                 | 1526                  | 1643.6 ± 410.9        |
| Glu/Gln                | 18877           | 20360                         | 16180                   | 17677                                | 25222                 | 21620.2 ± 566.0       |
| Gly                    | 3836            | 3836                          | 3288                    | 3288                                 | 4886                  | 3094.5 ± 221.0        |
| His                    | 2071            | 2071                          | 1775                    | 1775                                 | 2837                  | 2139.2 ± 213.9        |
| Ile                    | 6746            | 6746                          | 5783                    | 5783                                 | 9089                  | 7211.9 ± 379.6        |
| Leu                    | 12144           | 12144                         | 10409                   | 10409                                | 15100                 | 14044.2 ± 379.6       |
| Lys                    | 8382            | 8382                          | 7184                    | 7184                                 | 11684                 | 8175.1 ± 681.3        |
| Met                    | 2126            | 2126                          | 1822                    | 1822                                 | 3319                  | 2781.5 ± 222.5        |
| Phe                    | 3358            | 3358                          | 2879                    | 2879                                 | 4774                  | 4421.3 ± 100.5        |
| Pro                    | 9105            | 9105                          | 7805                    | 7805                                 | 13859                 | 16152.9 ± 576.9       |
| Ser                    | 7514            | 7514                          | 6440                    | 6440                                 | 9952                  | 7581.4 ± 473.8        |
| Thr                    | 7886            | 7886                          | 6760                    | 6760                                 | 9700                  | 5575.1 ± 278.8        |
| Trp                    | 1247            | 1247                          | 1068                    | 1068                                 | 1527                  | ND                    |
| Tyr                    | 2359            | 2359                          | 2022                    | 2022                                 | 4454                  | 3756.1 ± 91.6         |
| Val                    | 7670            | 7670                          | 6574                    | 6574                                 | 10192                 | 7371.5 ± 283.5        |

[a] Values for the reference formula is adapted from (1)
b] Value is adapted from the true protein measurement of mature rhesus milk (2). True protein was estimated using (nitrogen in whole milk – non-protein nitrogen) X 6.25. Nitrogen was estimated by micro-Kjeldahl analysis.
c] Value is adapted from measurement of rhesus milk collected after 36 days of lactation (3). Total lipid was measured by a colorimetric method through sulfo-phospho-vanillin reaction.
d] Value is adapted from measurement of milk collected at 1 month of lactation (4). Total lipid was measured by a micromodification of Rose-Gottleib procedure.
e] Value is adapted from measurement of milk collected at 1 month of lactation (4). Total carbohydrate was determined by phenol-sulfuric acid method.
f] Value is adapted from measurement of milk collected after 36 days of lactation (3). Lactose was measured using an enzymatic approach involving lactase and glucose oxidase.
g] The amino acid composition was converted using measurements adapted from (5) and corrected using true protein measurement adapted from (2). Amino acid concentration was measured spectrophotometrically after HCl digestion. However, tryptophan was destroyed by acid hydrolysis therefore not included in the total amino acid concentration. Due to this limitation of excluding tryptophan as a part of total amino acid, the concentration for each amino acid present in the table is over-estimated.

Data on rhesus milk are presented from literature as Mean ± SD. For unit conversion, rhesus milk density is conventionally assumed as 1.03 g/mL. ND: not determined.
**SI Table 2.** Differentiating OTUs between the breast-fed and the formula-fed rhesus infants. ● High in breast-fed, ○ High in the formula-fed. Significance is evaluated using Analysis of Composition of Microbiomes (ANCOM) with p < 0.05 after FDR correction.

| Taxa (Phylum)       | Taxa (family genus)  | Age (weeks) |
|---------------------|----------------------|-------------|
|                     |                      | 2  | 4  | 6  | 8  | 10 | 12 | 14 | 16 |
| Actinobacterium     | Corynebacteriaceae   | ●  | ●  | ●  | ●  |     |     |     |     |
|                     | Corynebacterium      | ●  | ●  | ●  | ●  |     |     |     |     |
| Firmicutes          | Lactobacillaceae     | ●  | ●  | ●  |     |     |     |     |     |
|                     | Lactobacillus        | ●  |     |     |     |     |     |     |     |
| Firmicutes          | Lachnospiraceae      | ●  |     |     |     |     |     |     |     |
|                     | Lachnospira          | ●  | ●  | ●  | ●  |     |     |     |     |
| Firmicutes          | Clostridiaceae       | ●  |     |     |     |     |     |     |     |
|                     | SMB53                | ●  |     |     |     |     |     |     |     |
| Firmicutes          | Streptococcaceae     | ○  | ○  | ○  | ○  |     |     |     |     |
|                     | Lactococcus          | ○  | ○  | ○  | ○  |     |     |     |     |
| Firmicutes          | Veillonellaceae      | ○  | ○  | ○  | ○  | ○  | ○  | ○  | ○  |
|                     | Anaerovibrio         | ○  | ○  | ○  | ○  | ○  | ○  | ○  | ○  |
| Bacteroidetes       | Paraprevotellaceae   | ○  | ○  | ○  |     |     |     |     |     |
|                     | Prevotella           | ○  | ○  | ○  |     |     |     |     |     |
| Firmicutes          | Veillonellaceae      | ○  |     |     |     |     |     |     |     |
|                     | Megasphaera          | ○  |     |     |     |     |     |     |     |
SI Figure 1. Comparison of serum urea, blood urea nitrogen (BUN) and ammonia between breast-fed (black) and formula-fed rhesus infants (red, orange, green, blue). Serum urea, BUN and ammonia were quantified using NMR, biochemical assay and AAA, respectively. Data are presented as mean ± SEM.
SI Figure 2. Comparison of serum creatinine between breast-fed (black) and formula-fed rhesus infants (red, orange, green, blue). Data were quantified using NMR (left) and biochemical assay (right). Data are presented as mean ± SEM.
SI Figure 3. Comparison of urinary creatinine level between breast-fed (BF) and formula-fed (FF) rhesus infants from the current study and from the previous work ((1), BF ref, FF ref). Data are presented as mean ± SEM.
**SI Figure 4.** Comparison of serum pancreatic polypeptide between breast-fed (black) and formula-fed rhesus infants (red, orange, green, blue). Data are presented as mean ± SEM.
**SI Figure 5.** Comparison of serum total carbon dioxide (TCO2), anion gap, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALK PHOS) and chloride between breast-fed (black) and formula-fed rhesus infants (red, orange, green, blue). Data are presented as mean ± SEM.
SI Figure 6. Comparison of serum glucose, galactose and myo-inositol between breast-fed (black) and formula-fed rhesus infants (red, orange, green, blue). Measurement of glucose was conducted using NMR (top left) and biochemical assay (top right). Data are presented as mean ± SEM.
**SI Figure 7.** Comparison of serum hemoglobin, hematocrit and mean corpuscular hemoglobin concentration (MCHC) between breast-fed (black) and formula-fed rhesus infants (red, orange, green, blue). Data are presented as mean ± SEM.
**SI Figure 8.** Comparison of serum branched chain amino acids (isoleucine, leucine, valine) between breast-fed (black) and formula-fed rhesus infants (red, orange, green, blue). Data were quantified using NMR (left) and AAA (right). Data are presented as mean ± SEM.
SI Figure 9. Comparison of serum lysine, methionine and threonine between breast-fed (black) and formula-fed rhesus infants (red, orange, green, blue). Data were quantified using NMR (left) and AAA (right). Data are presented as mean ± SEM.
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**Si Figure 12.** Comparison of serum ketone bodies (acetoacetate and 3-hydroxybutyrate) between breast-fed (black) and formula-fed rhesus infants (red, orange, green, blue). Data are presented as mean ± SEM.
SI Figure 13. Serum Phenylalanine and serine concentration that were not statistically significant between the formula-fed (FF) and breast-fed (BF) rhesus infants in the present study but significantly different in our previous work ([1], BF ref, FF ref). Statistical difference was evaluated using repeated measures ANCOVA. Data are presented as mean ± SEM.
SI Figure 14. The fold change (%) of average daily protein intake from consuming the reduced protein formula. The smooth curve was fitted using loess regression and the 95% confidence interval was constructed using a t-based approximation.
SI Figure 15. Serum metabolites at 4 weeks of age that are higher in the formula-fed rhesus infants who consumed reduced protein formulas in comparison to those who consumed the regular formulas. (A). 4-hydroxyproline, (B). hydroxylysine, (C). glycine, (D). serine, (E). homocysteine, and (F). ethanolamine were significant (p<0.05) after adjustment of multiple comparison using FDR.(G). aspartate shows an increasing trend in those who consumed the reduced protein formulas (p<0.05 before FDR correction). Statistical significance was evaluated using multiple 2-way ANOVAs.
SI Figure 16. Boxplots of urine metabolites before 1 month of age. The data presented here is the combination of measurements at 2 and 4 weeks of age. Essential AAs (lysine, phenylalanine, valine), non-essential AAs (tyrosine, ornithine), AA metabolism intermediate products and nitrogen waste products (3-hydroxyisobutyrate, 3-hydroxyisovalerate, allantoin, dimethylglycine, sarcosine, urea), and products from microbial degradation of protein or host-microbe co-metabolism (2-hydroxyisobutyrate, 3-indoxylsulfate, 4-hydroxyphenylacetate, dimethyl sulfone, phenylacetate, phenylacetylglucose) were significantly lower with reduced protein in infant formula (p<0.05 after FDR correction, two-way ANOVA). Urinary level of histidine, threonine, isoleucine and glycine showed a trend toward lower concentrations in reduced protein formula (p<0.05 before FDR correction, two-way ANOVA).

**Essential AAs:**

**Non-essential AAs:**

**AA metabolism intermediate products and nitrogenous waste products:**

**Products from microbial degradation of protein or host-microbe co-metabolism:**
**SI Figure 17.** The influence of adding free amino acids to infant formula on metabolism. In urine, amino acids (aspartate, proline, threonine), amino acid derivatives (hydroxyproline, dimethylglycine, cadaverine) were significantly higher in the urine of infants fed formulas with the addition of free amino acids (p<0.05 after FDR correction, repeated measures ANOVA). Urinary asparagine, betaine, glycine isoleucine and serine showed a tendency to be higher in the urine those infants receiving formula with the addition of free amino acids (p<0.05 before FDR correction, repeated measures ANCOVA). Serum betaine showed a trend toward lower levels in infants fed formula with the addition of free amino acids (p<0.05 before FDR correction, repeated measures ANOVA). Data are presented as mean ± SEM.

**Serum:**

**Urine:**

![Graphs showing the concentration of various amino acids and derivatives in urine over different ages for infants fed different formulas.](image-url)
SI Figure 18. Relative abundance of bacterial genera in feces of rhesus infants consuming rhesus milk, regular formula, regular formula plus free amino acids, reduced protein formula, or reduced protein formula plus free amino acids from 2 to 16 weeks of age.
SI Figure 19. Principal coordinate analysis of weighted and log transformed unifrac distances (A) over time. (B) Shifting of centroids over time. The centroids are calculated using the average of PC1 and PC2 within each cluster.
SI Figure 20. Relative abundance of *Bifidobacterium*, *Dorea* and *Ruminococcus* (from Ruminococcaceae family) from data collected at and before 1 month of age (2 weeks and 4 weeks).
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