Metabolomics insight into the gut microbiome of infants with cow's milk allergy

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Chapter V

Exploring the fecal metabolome in infants with cow's milk allergy: The distinct impacts of cow's milk protein tolerance acquisition and of synbiotic supplementation

Based on:

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Abstract

Scope: Cow's milk allergy (CMA) is one of the most prevalent food allergies in early childhood, often treated via elimination diets including standard amino acid-based formula or amino acid-based formula supplemented with synbiotics (AAF or AAF-S). This work aimed to assess the effect of cow's milk (CM) tolerance acquisition and synbiotic (inulin, oligofructose, *Bifidobacterium breve* M-16 V) supplementation on the fecal metabolome in infants with IgE-mediated CMA

Methods and results: The CMA-allergic infants received AAF or AAF-S for a year during which fecal samples were collected. The samples were subjected to metabolomics analyses covering gut microbial metabolites including SCFAs, tryptophan metabolites, and bile acids. Longitudinal data analysis suggested amino acids, bile acids, and branched SCFAs alterations in infants who outgrew CMA during the intervention. Synbiotic supplementation significantly modified the fecal metabolome after six months of intervention, including altered purine, bile acid, and unsaturated fatty acid levels, and increased metabolites of infant-type *Bifidobacterium* species: indolelactic acid and 4-hydroxyphenyllactic acid.

Conclusion: This study offers no clear conclusion on the impact of CM-tolerance acquisition on the fecal metabolome. However, our results show that six months of synbiotic supplementation successfully altered fecal metabolome and suggest induced bifidobacteria activity, which subsequently declined after 12 months of intervention.

1. Introduction

Cow's milk allergy (CMA), characterized by an immune-mediated response to cow's milk protein(s), is one of the major food allergies in early life.[1,2] Over the past decades, the estimated CMA prevalence in children of developed countries is approximately 0.5–3%.[3,4] The allergic symptoms typically occur in the first year of life, whereas the resolution age varies and is related to the type of CMA.[5] Based on symptoms and pathophysiology, CMA is categorized into immunoglobin E (IgE)-mediated, non-IgE mediated, and mixed IgE CMA.[6] Subjects with IgE-mediated CMA, constituting approximately 60% of all CMA cases,[3] require longer time for tolerance acquisition to CM than non-IgE mediated CMA subjects.[7,8] In recent decades, the relevance of the gut microbiome (GM) in CMA has been highlighted, and studies show that compared to healthy counterparts, children with IgE-mediated CMA exhibit a reduction in bifidobacteria.[9]

Bifidobacteria, the prototypical health-promoting bacteria, are dominant inhabitants in a breast-fed infants gut[10] and play a pivotal role in GM development in early life.[11,12] As co-evolved bacteria, bifidobacteria possess unique glycosidases to digest complex host-derived glycans, particularly the human milk oligosaccharides (HMOs).[13,14] The oligosaccharide fermentation products not only satisfy the energy and carbon demands of bifidobacteria but also benefit other bacteria through cross-feeding activities, thereby contributing to maintaining the GM homeostasis in early life.[10,11]

Thus, bifidobacteria-related probiotics and HMO-mimicked prebiotics have gained popularity in the management of CMA in early-life, alongside the conventional interventions with extensively hydrolyzed formula or amino acids-based formula (AAF).[15] Indigestible oligosaccharides, such as fructooligosaccharides (FOS) and galactooligosaccharides, are used as prebiotics due to their bifidogenic effect on the GM.[16] *Bifidobacterium* species, including *B. bifidum*,[17] *B. longum*,[18] and particularly *B. breve*.[18–21] are widely used probiotics for IgE-mediated CMA management in infants. These bifidobacteria have key immunomodulatory roles in the cross-talk between GM and host immune system: *B. bifidum*, for example, can induce the expression of FoxP3

in the regulatory T (T_{reg}) cells through cell surface polysaccharides,[22] while *B. longum* in neonatal microbiota can alleviate the risk of allergy by promoting the T_{reg} maturation;[23] *B. breve*, particularly the *B. breve* M-16V, can trigger the anti-allergic process in early infancy by regulating the intestinal microbiota, intestinal epithelial barrier, and immune system.[24] Overall, bifidobacteria with HMO-utilization genes are found to induce intestinal IFN-β and silence Th2 and Th17 cytokines, thereby regulating the systemic immune balance in infants.[25] Additionally, by breaking down HMOs, bifidobacteria can indirectly enhance the production of butyrate[26] which is essential for the interplay between GM and systemic immunity,[27] possibly through epigenetics mechanisms.[28] Bifidobacteria-derived indolelactic acid also actively engages in the immunoregulation during infancy.[25,29] However, despite these findings and the wide application of bifidobacteria-related interventions for IgE-mediated CMA,[17–21] none of the studies have reported comprehensive metabolome exploration.

In this study, we investigated longitudinal fecal metabolome changes of infants with IgE-mediated CMA undergoing dietary management with AAF, with and without synbiotics (*Bifidobacterium breve* M-16V; FOS: oligofructose, inulin). By applying linear mixed models (LMMs) and repeated measures analysis of variance simultaneous component analysis+ (RM-ASCA+), we compared the longitudinal fecal metabolome of infants with persistent CMA to those who developed CM-tolerance, and identified key metabolic changes associated with the synbiotic intervention.

2. Experimental section

2.1 Study design and dosage information

This study arises from a multicenter, randomized, double-blind, controlled clinical study PRESTO (registered as NTR3725 in Netherlands Trial Register). Detailed information on ethics committees, institutional review boards, and regulatory authorities that approved the study was previously published.[30]

PRESTO enrolled infants diagnosed with IgE-mediated CMA who then received either amino acid formula (AAF, produced by Nutricia, Liverpool, United Kingdom) or AAF with synbiotic (AAF-S) to manage their CMA. The synbiotic blend consisted of chicory-

derived neutral FOS: oligofructose and inulin in a 9:1 ratio (total concentration of 0.63g/100 ml formula, BENEO-Orafti SA, Oreye, Belgium) and *Bifidobacterium breve* M-16V (1.47×10^9 cfu/100 ml formula, Morinaga Milk Industry, Tokyo, Japan). Caretakers were instructed to provided subjects with a minimum daily dose of 450mL, 350mL, and 250mL for infants aged 0 to 8 months, 9 to 18 months, and older than 18 months, respectively.[19] After 12 months of intervention, the allergy status was re-evaluated through double-blind, placebo-controlled food challenge (DBPCFC) with CM. Detailed information on the diagnosis and reassessment was previously published.[19] Out of the 169 participants enrolled in PRESTO, 40 subjects (aged 3-13 months) were selected for this study based on sample availability. One subject was excluded due to unclear allergy status after 12 months.[30] Of the 16 AAF and 23 AAF-S participants, 10 and 14 infants, respectively, outgrew CMA within 12 months. Stool samples were available at 0 (baseline, TP0), 6 (TP1), and 12 months (TP2) after the start of the intervention, resulting in a total of 117 samples.

2.2 Sample collection and storage

The sample collection procedure has been described previously.[30] In short, fecal samples were collected at home and immediately stored in freezers, then transferred on ice to the participant hospitals and stored at -80°C until transfer to Danone Research & Innovation (Utrecht, the Netherlands) for wet sample aliquoting and SCFAs and lactic acid analysis. Sample aliquots for LC-MS metabolomics analysis were transferred on dry-ice to Leiden University and stored at -80°C until analysis.

2.3 Metabolomic analysis

2.3.1 SCFAs and lactic acid analysis

Quantitative SCFAs, including branched SCFAs (BSCFAs) analysis was performed using GC coupled to flame ionization detector and lactic acid was measured using lactic acid assay kit (Megazyme, Wicklow, Ireland) as previously described.[31]

2.3.2 LC-MS metabolomics analysis

The wet sample aliquots were lyophilized at 4 mbar and -110°C for 20h (Martin Christ Gefriertrocknungsanlagen GmbH, Germany), weighed (20±0.2mg), and stored at -80°C until extraction. Liquid-liquid extraction was performed as described by Hosseinkhani *et al.*[32] with adjusted sample amount and doubled solvent-to-feces ratio. Detailed information on the chemicals, the sample preparation, and the quality control (QC) is available in supplementary materials.

Polar to semi-polar metabolites, including acetylcarnitines, amines, benzenoids, organic acids, indoles, nucleosides, and nucleotides, were analyzed using reverse phase LC coupled with quadrupole (Q)-TOF-MS operated in full-scan positive and negative ionization modes, as described previously[33] and in the supplementary material. Bile and fatty acids were measured using reverse phase LC separation using Q-TOF-MS operated in full scan negative ionization mode, as described in the supplementary material.

Targeted peak integration was performed using SCIEX OS (version 2.1.6., SCIEX) with a maximum mass error of 10 ppm. The retention times were verified against authentic standards. In case of coelution, the targets were reported using the name or abbreviation of one of the targets followed by a "#". Details on the abbreviations used are listed in Table S2. For the polar to semi-polar metabolites, peak area was used for further data analysis, whereas for the bile and fatty acids, the area ratio of compounds to stable isotopically labelled standards (Table S1) was used. Data quality inspection was performed using an in-house quality assurance software performing between batch correction and removal of metabolites with high technical variance (RSD of QC>30%).

2.3.3 Data analysis

Data handling and statistical analyses were performed in R (version 4.3.2). Metabolites with missingness above 20% and with median signal of the samples less than five times the mean signal of the procedure blanks were removed, leaving 166 metabolites. To identify group bias in missingness, Fisher's exact test was performed for metabolites with missingness above 20% at each time point after grouping the subjects by intervention or CM-tolerance status, and the results are summarized in Table S2. Ratios of secondary to primary and unconjugated to conjugated bile acids (BAs) were added,

resulting in a total of 177 variables. A list of the reported metabolites and their abbreviations can be found in Table S3. The raw data were normalized by dry weight and subsequently log_2-transformed. Missing values were imputed per metabolite using the quantile regression imputation of left-censored (QRILC) method.[34] Available clinical characteristics that potentially associated with CM-tolerance status at TP2 or intervention were analyzed with the two-sided Mann-Whitney U-test for numeric variables and the Fisher's exact test for binary variables as reported previously.[30,35]

To assess the change from TP0 to TP1 and TP2, LMMs were built using the lme4 package in R. Prior to building the model, the data was scaled by the standard deviation of all baseline samples. The metabolites were modelled as response variables with group and time as fixed effects and subject ID as a random effect. After grouping the subject by either their CM-tolerance status at TP2 (CM-allergic versus CM-tolerant) or intervention (AAF versus AAF-S), two models were built, namely tolerance-allergy and intervention. For the tolerance-allergy model (*Metabolite ~ time + CM-tolerance_status + time:CM-tolerance_status + (1|ID))*, TP0 and the CM-allergic group were used as references. Pairwise comparisons between groups at each time point and within a group between the time points were performed using the emmeans package in R. For the intervention model (*Metabolite ~ time + time:intervention + (1|ID)*), TP0 and the AAF group were used as references. The main effect of the intervention was removed from the model but its interaction with time was kept ensuring the groups are equal at baseline. The p-values were calculated to assess a change from baseline with the Satterthwaite's degrees of freedom method using the lmerTest package within the ALASCA package.[36] In this study, the combined CM-tolerance status–intervention model was not performed because CM-tolerance acquisition as investigated in the parent study did not differ between the interventions at TP2 and aligned with natural rates of CMA outgrowth in infants.[19] For most metabolites, the addition of age as a covariate to models led to no improvement of the performance based on akaike information criterion (Tables S4 and S5). Therefore, age was not used as a covariate in the LMMs. Multiple testing correction was performed using the Benjamini-Hochberg method where $Q<0.1$ was considered as statistically significant.

Visualization of the longitudinal metabolomic alterations was achieved using RM-ASCA+ with ALASCA package,[36] as detailed in the supplementary materials. Performances of the analysis was validated using nonparametric bootstrapping, and the 95% confidence intervals (CI) were estimated based on 1000 resampling iterations.

2.4 16S rRNA gene sequencing and pre-processing

Extraction of DNA from stool samples and the subsequent gut microbiota profiling by 16S rRNA gene sequencing was performed as described previously.[30] Correlations between the changes in metabolites and the relative abundance of *Bifidobacterium* were examined using Spearman's rank correlation analysis. Relative abundance comparisons of *Bifidobacterium* between and within the AAF and AAF-S groups were evaluated with two-side unpaired t-tests.

3. Results

3.1 Patient characteristics

The statistical results of important clinical characteristics are summarized in Table S6-S7. When grouping the subjects by the CM-tolerance status at TP2, the father allergy occurrence and the SCORing Atopic Dermatitis (SCORAD) at baseline were significantly higher in the CM-allergic group than in the CM-tolerant group (Table S6). None of the clinical characteristics were significantly different between AAF and AAF-S groups (Table S7).

3.2 More pronounced fecal metabolome changes in the CM-tolerant group

Firstly, RM-ASCA+ was used to examine the longitudinal metabolome alterations within and between infants that remained allergic and those that acquired tolerance to CM by TP2 (CM-allergic vs CM-tolerant). The PC1 score plot (Figure 1A) describes the direction of maximum variance in the modeled data, whereas the loadings plot (Figure 1B) highlights the top metabolites contributing to PC1. Metabolites with positive loadings follow the trend described by the score, whereas the opposite holds for metabolites with negative loadings. Figure 1B shows that almost half of the variation (47%) described by the fixed effects of the tolerance-allergy model was explained by

PC1 (Figure 1A). The scores and loading for PC1 showed that over time ferulic acid, desaminotyrosine, pipecolic acid, 3-hydroxybenzoic acid increased, whereas dodecanoylcarnitine, pregnenolone sulfate, betaine, pyruvate decreased (Figure 1). Few BAs also showed overall change with time. The primary BAs cholic acid (CA), chenodeoxycholic acid (CDCA), and hyocholic acid (HCA) declined over time. In contrast, the secondary BAs deoxycholic acid (DCA) and the ratios of secondary to primary BAs, including DCA/CA, lithocholic acid (LCA)/CDCA, increased. Although with overlapped CIs between the two groups, those changes were more pronounced for the CM-tolerant group where the PC1 score declined more sharply than the CM-allergy group and for which the CI between the time points were separated, suggesting a significant time effect in this group.

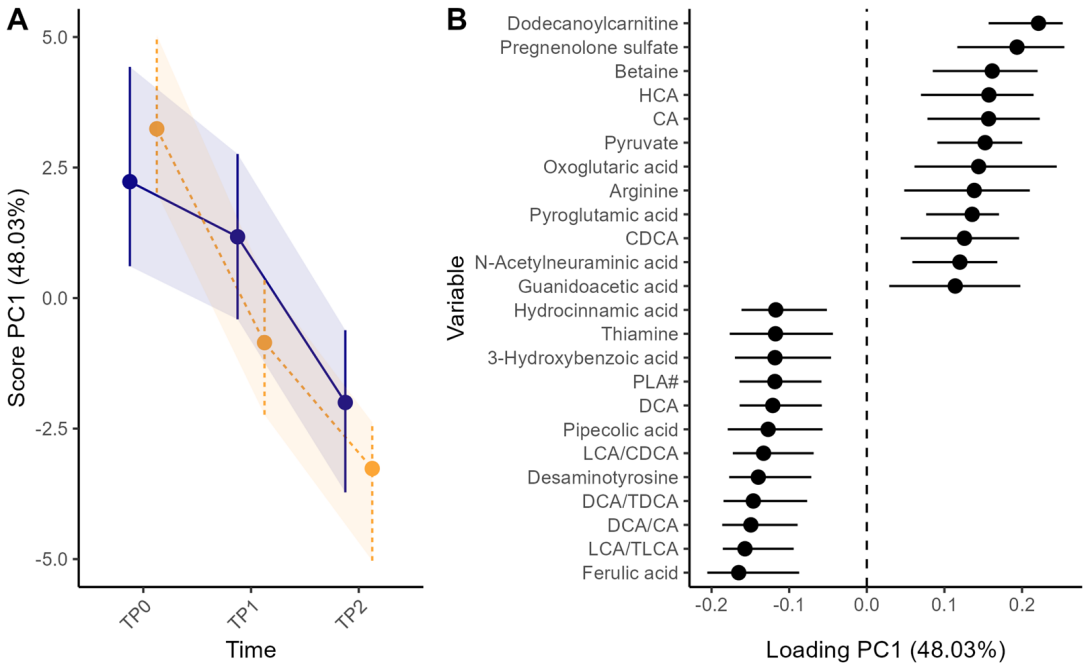

Figure 1. RM-ASCA+ combined effect matrix showing the common metabolome development throughout the study for the CM-allergic (blue solid line, n=15) and CM-tolerant (orange dashed line, n=24) groups as scores (A) and loadings (B). Only the metabolites with 12 highest and 12 lowest loadings are shown in the plot. Error bars representing 95% CI were estimated based nonparametric bootstrapping.

Univariate marginal means comparison showed that around five times more metabolites were significantly altered over time in infants that acquired CM-tolerance versus those

that remained CM-allergic (TP0-TP1: 9 metabolites in CM-tolerant vs 2 metabolites in CM-allergic; TP0-TP2: 30 metabolites in CM-tolerant and 7 in CM-allergic; Figure S1 and Table S8). Pregnenolone sulfate, pyroglutamic acid, pyruvate, oxoglutaric acid, and ferulic acid were significantly affected by time for both groups and follow comparable time-development trends (Figure S1). Similarly, arginine decreased, whereas 3-hydroxybenzoic acid, hydrocinnamic acid, LCA, DCA increased simultaneously in both groups, but significantly only in the CM-tolerant group (Figure S1). Pipecolic acid levels increased over time in both groups, but the rise was steeper and significant only in the CM-tolerant group. Dodecanoylcarnitine followed the trend described by PC1 of the combined effect matrix (Figure 1A) with a decline in time at both TP1 and TP2 significant only in the CM-tolerant group. The rest of the significantly altered metabolites showed dissimilar longitudinal profiles between the groups (Figure S1). Butyric acid, PLA#, desaminotyrosine, and phenylacetic acid were significantly increased, whereas 5-hydroxytryptophan and the primary BAs CA and CDCA showed significant decreases in the CM-tolerant group only. In contrast, threonine#, and tryptophan significantly increased over time only in the CM-allergic group.

Next, the RM-ASCA+ interaction effect matrix was examined to focus on the alterations associated with CM-tolerance acquisition. The PC1 scores and loading of the interaction matrix, Figure 2, suggest that compared to the CM-allergic group, the CM-tolerant group showed overall alterations in amino acid metabolism with an increase in citrulline, lysine, N-acetyltyrosine, phenylacetic acid, gamma-aminobutyric acid (GABA#), glutamate, orotate, ornithine and a decrease in 5-hydroxytryptophan and serotonin. The BAs metabolism was also altered: decline in CDCA, CA, glycochenodeoxycholic acid (GCDCA), tauroursodeoxycholic acid (TUDCA), taurochenodeoxycholic acid (TCDCA) and increase in LCA/CDCA for the CM-tolerant group. The BSCFAs, isobutyrate and isovalerate, also contributed to PC1, showing higher levels in the CM-tolerant group. However, only citrulline and lysine were found significantly different at TP2 between the two groups univariately (Table S6, Figure S2).

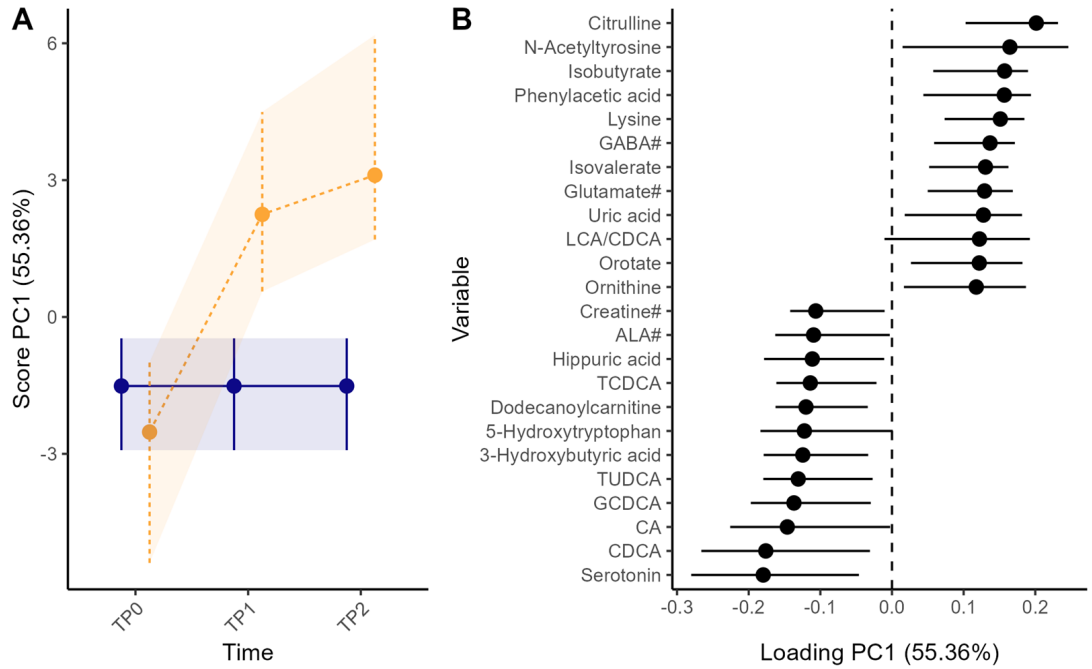

Figure 2. RM-ASCA+ interaction effect matrix showing the metabolome differences between the CM-allergic (blue solid line, n=15) and CM-tolerant group (orange dashed line, n=24) over time as scores (A) and loadings (B). Only the metabolites with 12 highest and 12 lowest loadings are shown in the plot. Error bars representing 95% CI were estimated based nonparametric bootstrapping.

3.3 Synbiotic supplementation altered fecal metabolome after six months of intervention

The longitudinal alterations of the fecal metabolome between the AAF and AAF-S group were studied to understand the effect of the synbiotic supplementation. As shown in Figure 3, clear group separation was observed in PC1 of the RM-ASCA+ interaction effect matrix, especially at TP1.

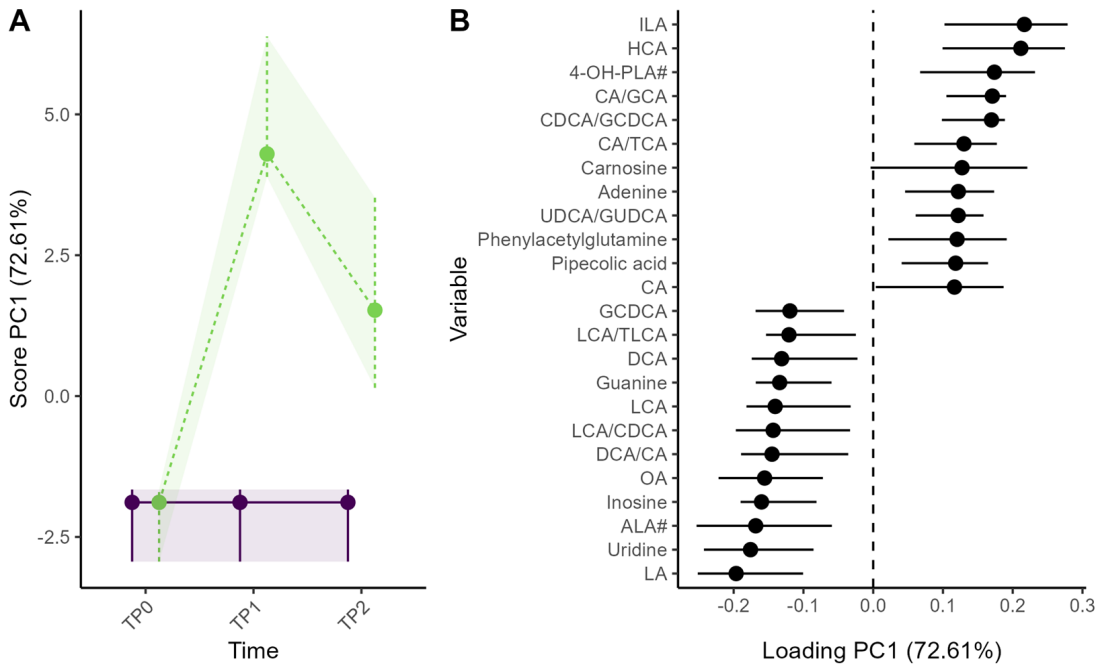

Figure 3. RM-ASCA+ interaction effect matrix showing the metabolome differences between the AAF (purple solid line, n=16) and AAF-S (green dashed line, n=23) group over time as scores (A) and loadings (B). Only the metabolites with 12 highest and 12 lowest loadings are shown in the plot. Error bars representing 95% CI were estimated based nonparametric bootstrapping.

Among all the metabolites, 12 metabolites and three BA ratios were found to be statistically different between the AAF and AAF-S groups at TP1, and only inosine at TP2 (Figure S3, Table S8). The estimated marginal means plot of those analytes can be found in Figure S3. The synbiotic supplementation led to an increase of gut microbial metabolites indolelactic acid (ILA) and 4-hydoxyphenyllactic acid (4-OH-PLA#) and a decline in the fatty acids linoleic acid (LA), alpha-linolenic acid (ALA#), and oleic acid (OA) at TP1 (Figure 4). Amino acid glutamine was also decreased in the AAF-S group at TP1. Three purine metabolites inosine, guanine, and adenine as well as the pyrimidine uridine were also affected by the intervention. While adenine was higher upon the synbiotic addition, the opposite was true for inosine, guanine, and uridine. HCA and CDCA/GCDCA, CA/glycocholic acid (GCA), ursodeoxycholic acid (UDCA)/glycoursodeoxycholic acid (GUDCA) were all significantly higher in the AAF-S than in the AAF group at TP1, whereas GCDCA was significantly lower (Figure

4). A few other BAs were found to be among the main contributors to PC1 of the interaction matrix (Figure 3) or to have significant interaction coefficient at TP1 prior to multiple testing correction (Figure 4), namely, the glyco-conjugated BAs GCA and GUDCA and the secondary BAs and their ratio to primary BAs: LCA, DCA, DCA/CA, and LCA/CDCA.

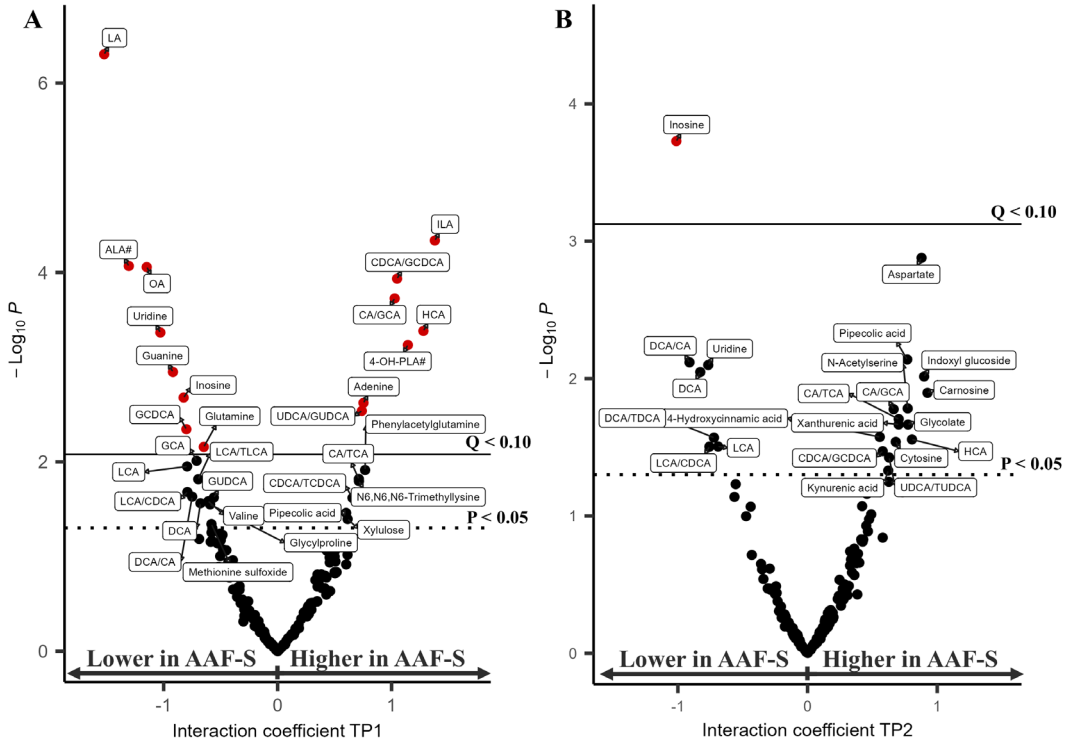

Figure 4. Volcano plot showing the resulting p-value of the interaction coefficient for TP1 (left) and TP2 (right) in intervention LMM, dashed (p = 0.05), solid line (Q = 0.1) for TP1 (A) and TP2 (B). Red symbols indicate metabolites with Q<0.1 after Benjamini-Hochberg procedure.

3.4 Association between changes in *Bifidobacterium* and metabolites significantly altered by the synbiotic

The synbiotic supplementation significantly increased the relative abundance of *Bifidobacterium* in the AAF-S group from baseline to TP1 and TP2 compared to the AAF group (Figure S4).[35] To determine whether these increases were associated with the significantly changed metabolites, Spearman's rank correlation analysis was performed between the changes in metabolite levels and *Bifidobacterium*'s relative

abundance from baseline to TP1 (TP1-TP0) and TP2 (TP2-TP0), respectively (Table S9). In the AAF-S group, changes in ILA and 4-OH-PLA# from TP0 to later time points were positively correlated with those of *Bifidobacterium* ($r > 0.6$, $p < 0.005$), while changes in glutamine were negatively correlated ($r \leq -0.5$, $p < 0.05$) (Figure 5). The changes in *Bifidobacterium* were positively correlated with those of adenine at TP1 and TP2 in both groups ($r > 0.5$, $p < 0.05$), and with CDCA/GCDCA and CA/GCA only at TP1 in the AAF-S group ($r > 0.4$, $p < 0.05$). *Bifidobacterium* also showed negative correlations with GCDCA and inosine in changes from TP0 to TP1 only in the AAF-S group ($r < -0.4$, $p < 0.05$) (Figure S5).

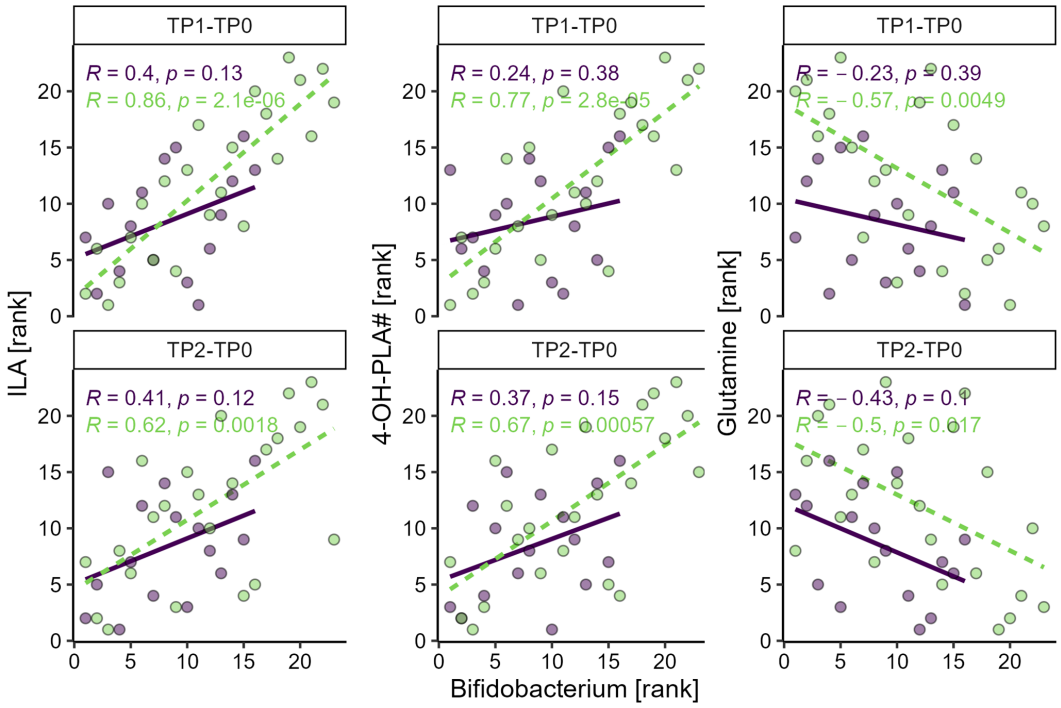

Figure 5. Spearman's rank correlations between the changes in Bifidobacterium and ILA, 4-OH-PLA#, glutamine in AAF (purple solid line, n=16) and AAF-S (green dashed line, n=23) groups from baseline to TP1 (TP1-TP0) and TP2 (TP2-TP0). The rank of the changes in metabolite response and relative abundance of Bifidobacterium within each group were used for plotting. The figure shows p values; the Q values after Benjamini-Hochberg procedure are provided in Table S9.

4. Discussion

In this study we followed the fecal metabolome alterations in infants with IgE-mediated CMA who received AAF with or without synbiotics for a year. Firstly, we examined the effect of CM-tolerance acquisition on the fecal metabolome over time. Time, reflecting growth and diet diversification, had a more pronounced impact on the metabolome than CM-tolerance acquisition (Figure 1, Figure S1). The diet enrichment was evidenced by the overall increase of the phenolic acids which are ubiquitously produced in plants,[37] including ferulic acid, 3-hydroxybenzoic acid, and hydrocinnamic acid. The decrease in the steroid hormone (pregnenolone sulfate), energy metabolites (pyruvate, oxoglutaric acid, dodecanoylcarnitine), and the altered amino acids and derivatives (pyroglutamic acid, arginine, pipecolic acid) suggest metabolome modification associated with somatic growth.[38,39]

The multivariate RM-ASCA+ analysis showed an association of CM-tolerance acquisition status with alterations in amino acids, BAs, and (B)SCFAs (Figure 2). Compared to infants with persistent CMA, citrulline and lysine were significantly higher in the infants who developed CM-tolerance at TP2 (Figure S2). Lower plasma citrulline levels are known marker of increased gut permeability,[40] which can raise the chance of allergen(s) passing the intestinal barrier and triggering the immune system.[41] The increase in fecal citrulline in the CM-tolerant group in this study might suggest improved gut barrier function and gut health. Although not significantly different between the two groups, the amino acids GABA#, glutamate#, threonine#, and ornithine were also higher in the CM-tolerant group compared to the CM-allergic group (Figure S1-S2). Lower fecal threonine levels have previously been reported in infants with IgE-mediated CMA compared to healthy controls.[42] Interestingly, although not significant, 5-hydroxytryptophan and serotonin were higher in the CM-allergic group at TP1 and TP2 (Figure 2), while their precursor tryptophan significantly declined only from TP0 to TP2 in this group (Figure S1). As serotonin is involved in intestinal epithelial proliferation[43] and plays an essential role in regulating intestinal inflammation,[44] the upregulated tryptophan-serotonin metabolism in the CM-allergic group may reflect an inflammatory state of the intestine in the CMA infants.

Children who outgrew CMA showed differences in their BAs profile. The primary BAs (CA, CDCA) significantly decreased, while the secondary BAs (DCA, LCA) and the secondary/primary BAs ratios (DCA/CA, LCA/CDCA) significantly increased from TP0 to TP2 only in the CM-tolerant group (Figure S1). A recent study found that, compared to healthy children, children with IgE-mediated CMA had lower ratios of fecal secondary/primary BAs from the CA pathway, with DCA and other oxidized keto BAs included in the calculation.[45] Secondary BAs from the CDCA pathway, including LCA, were reported lower in children with food allergy compared to healthy controls as well.[46] Although the secondary BAs and secondary/primary BAs ratios were not significantly different between the two groups in our study, the altered BAs profiles in the CMA-tolerant group likely indicate a more mature GM for secondary BAs production. This may contribute to improved intestinal functions in infants outgrowing CMA, as LCA is known to attenuate disruption in the intestinal barrier.[47]

(B)SCFAs were also altered during the CMA tolerance acquisition process. Butyrate significantly increased from TP0 to TP2 only in the CM-tolerant group (Figure S1). Isobutyrate and isovalerate tended to have group separation at TP1, with a continuous elevation in the CM-tolerant group over time, and a decrease at TP1 in the CM-allergic group (Figure S2). Consistent with our finding, those (B)SCFAs, specifically butyrate, are known for their anti-inflammatory effects,[27,48] and are generally observed to be lower in feces of children with IgE-mediated food allergy.[42,48] Additionally, phenylalanine, phenyllactic acid (PLA#), and desaminotyrosine, which are GM metabolites from amino acids and dietary polyphenols,[49–51] were significantly increased from TP0 and TP2 only in the CM-tolerant group (Figure S1). The significant elevations of these metabolites may promote CM-tolerance acquisition, especially considering the recently recognized anti-inflammatory property of desaminotyrosine.[52,53]

The synbiotic (*B. breve* M-16V, FOS: inulin, oligofructose) significantly altered the levels of aromatic lactic acids, purine metabolites as well as fatty acids and BAs, particularly after six months of intervention. The intervention enhanced ILA and 4-OH-

PLA levels (Figure S3), and their increases from baseline to TP1and TP2 were positively correlated with those of bifidobacteria (Figure 5). This finding aligns with reports that ILA and 4-OH-PLA are metabolites of tryptophan[29,54,55] and tyrosine[29] produced by infant-type *Bifidobacterium* species, including *B. breve*. Earlier published microbiome and metaproteomics analysis of stool samples from the same clinical trial revealed that the synbiotic raised the level of bifidobacteria,[19,35] as well as bifidobacterial Carbohydrate-Active enZymes,[35] known to metabolize FOS.[56] Although the proportion of *Bifidobacterium* was significantly higher in the AAF-S group compared to the AAF group at both time points (Figure S4),[19,35] the increases in ILA and 4-OH-PLA# were significantly higher in the AAF-S group only at TP1. These results suggest that the synbiotic promoted the growth and/or the activity of aromatic lactic acids producers, e.g., infant-type *Bifidobacterium* species, especially at TP1. This can be evidenced by stronger positive correlations between changes in the two aromatic lactic acids and bifidobacteria from baseline to TP1 than to TP2 in the AAF-S group (Figure 5). To validate our observations, *Bifidobacterium* species should be quantified. Alternatively, aromatic lactate dehydrogenase reported to convert tryptophan and tyrosine to respectively ILA and 4-OH-PLA in infant-type *Bifidobacterium* species should be analyzed.[29] The possibility that the ILA and 4-OH-PLA# were produced by some lactic acid bacteria should not be ignored neither.[57,58] Overall, the increased ILA and 4-OH-PLA# levels in the AAF-S group suggest enhanced abundance or activity of infant-type bifidobacteria, supporting the successful synbiotic supplementation together with the microbiome and metaproteomics findings.[19,35] Although the parent study found that the CM-tolerance acquisition after 12 (TP2) and 24 months of synbiotic intervention aligned with natural outgrowth,[19] our findings, along with the reported anti-inflammatory effect of ILA,[25,29,55,59] suggest that the synbiotic intervention may pose beneficial effects on infants' immune system. Further metabolomics studies on larger cohorts are required to verify this hypothesis.

In addition to the increase in ILA and 4-OH-PLA, the synbiotic lowered inosine, guanine, and uridine and raised adenine levels. The same purine-pyrimidine trend was observed in conventionally raised and core microbiota-colonized mice in comparison to

germ-free mice,[60] indicating the importance of the GM in purine and pyrimidine metabolism.[60] A decline of inosine and uridine has also been reported in co-culture of *B. breve* with small intestinal-like epithelial cells.[61] *Lactobacillus brevis,* belonging to the *Lactobacillaceae* family, was found to be elevated in the AAF-S group for the same set of samples[35] and was also reported to have inosine degradation capabilities.[62] To link the purine-pyrimidine metabolism to the gut microbiome, and the role of *Bifidobacterium* spp. and *Lactobacillaceae* spp. herein, more research is required.

The AAF-S intervention lowered LA, ALA#, and OA levels, suggesting high consumption of these fatty acids by gut bacteria. This may be a result of hydration by bacteria of the *Lactobacillus* and *Bifidobacterium* genera[63] or production of conjugated fatty acids.[64–68] *Bifidobacterium* strains, especially *B. breve*, are among the best producers of conjugated linoleic acids[66,67] and conjugated linolenic acids.[66,68]

The synbiotic enhanced the deconjugation of BAs, especially at TP1, where significantly decreased GCDCA and increased CDCA/GCDCA, CA/GCA, and UDCA/GUDCA were observed in the AAF-S compared to AAF group (Figure 4). *Bifidobacterium*, in general, are active bile salt hydrolase (BSH) producers,[69] which perform preferred deconjugation activity on glyco-conjugated BAs.[70] This aligns with our results showing that *Bifidobacterium* changes from baseline correlated negatively with those of GCDCA, and positively with those of CA/GCA and CDCA/GCDCA at TP1 in the AAF-S (Figure S5). These correlations in changes disappeared at TP2, possibly due to increased GM diversity. Compared to TP0, families from other phyla, including Bacteroidetes, Firmicutes, and Proteobacteria, were more abundant at later timepoints in both groups, especially at TP2.[35] These bacteria have also been identified as active BSH producers,[71] thus might eliminate the correlation between the activity of BAs deconjugation and *Bifidobacterium*. Unexpectedly, the increased deconjugation activity of BAs failed to promote the production DCA and LCA. In contrast, although not significant, their levels and ratios to precursors (DCA/CA, LCA/CDCA) were lower in the AAF-S than the AAF group (Figure 4). Considering that the conversion of primary BAs to secondary ones is highly conserved in bacteria with the *bai* operon,[72] and that the host liver can further hydroxylate secondary BAs to tertiary BAs after gut-liver

circulation,[73] it is likely that more complex mechanisms underlie the host-gut metabolism of BAs during the intervention.

Our study has several limitations, including the wide age range of the participants at baseline of 3-13 (9.00 ± 2.90) months. Considering the rapid development of the GM in the first two years of life,[39] the wide age range may obscure the observation of fecal metabolome alterations related to CM-tolerance acquisition and the effect of intervention. Another limitation is the lack of information on the CM-tolerance status at TP1. Knowing the status at TP1 could have aided in the interpretation of CM-tolerance acquisition results. The research carried out for this paper is exploratory due to the small samples size (39 subjects). Increasing the sample size is necessary to verify these findings and would also allow to build LMM and RM-ASCA+ models following the intervention and CM-tolerance acquisition simultaneously. In addition, the parent study concluded that the synbiotic supplementation did not significantly affect CMA-resolution. Thus, in this study we cannot draw any conclusions regarding the clinical benefits of the synbiotic supplementation on CM-tolerance acquisition based on fecal metabolome alterations. Despite those limitations, our study revealed several fecal metabolome pathway alterations which may contribute to CMA outgrowth. Most importantly, we found that the AAF-S significantly altered the fecal metabolome after six months of the intervention, not after 12 months, suggesting that early intervention is required to maximize the effect of synbiotics. These findings aid in understanding the link between IgE-mediated CMA-tolerance acquisition, GM, and synbiotics intervention.

List of abbreviations

CMA: cow's milk allergy; CM: cow's milk; GM: gut microbiome; IgE: immunoglobin E; HMO: human milk oligosaccharide; AAF: amino acid-based formula; FOS: fructooligosaccharides; T_{reg}: regulatory T cell; *B.*: *Bifidobacterium*; LMMs: linear mixed models; RM-ASCA+: repeated measures analysis of variance simultaneous component analysis+; AAF-S: amino acid-based formula with synbiotic; DBPCFC: double-blind, placebo-controlled food challenge; BSCFAs: branched short-chain fatty

acids; QC: quality control; CI: confidence intervals; SCORAD: scoring of atopic dermatitis; BSH: bile salt hydrolase

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Conflict of interest statement

Harm Wopereis is an employee of Danone Research & Innovation. The project is part of a partnership programme between NWO-TTW and Danone Research & Innovation. The other authors declare that they have no known conflicts of interest.

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Supplementary Material

Chemicals

Methyl tert-butyl ether (MTBE, ≥99.8%) and ammonium formate (≥99.0%) were purchased from Sigma Aldrich (St. Louis, United States). LC-MS-grade methanol (MeOH), isopropanol and formic acid (FA) were purchased from Biosolve B.V. (Valkenswaard, Netherlands). LC-MS grade acetonitrile was purchased from Actu-all chemicals (Randmeer, The Netherlands) and Biosolve B.V. (Valkenswaard, Netherlacnjugnds). Purified water was obtained from a Milli-Q PF Plus system (Merck Millipore, Burlington, United States). List of the isotopically labelled standards (SILs), including supplier details, can be found in Table S1.

Sample preparation

Briefly, 72 µL of water and 216 µL MeOH, containing stable isotopically labelled standards (SILs) (Table S1), were added to the 20 mg dry-weight fecal sample. After a 3-minute vortex mixing (Marshall Scientific, Cambridge, UK) 120 µL ice-cold MTBE was added, followed by another 3-minute vortex mixing. Following a brief centrifugation (30s, 100g, 4 °C), 200 µL of water and 168 µL of MTBE were added. The samples were vortex mixed for another 3 min, incubated at 4°C for 10 minutes until centrifugation (20 min, 16 000g, 4°C) inducing aqueous and organic layer separation. All solvents used during the LLE were ice-cold and vortex mixing was always at maximum speed. Following layer separation, each layer was transferred to an Eppendorf tube, followed by 5 and 2.5 minutes of centrifugation (16000g, 4°C) for aqueous and organic layers respectively. After extraction, 150 µL of the aqueous layer was aliquoted for polar to semi-polar metabolites analysis, while 48.8 µL of aqueous and 28.8 µL of organic layer was combined for the bile and fatty acids analysis. The aliquots were dried in a Speedvac (Labcono, USA) and stored at -80°C. Prior to LC-MS analysis, the extracts were reconstituted in 50 µL of 0.1% FA in water for polar to semi-polar metabolites analysis, and 200 µL of MeOH for the bile and fatty acids analysis. The reconstitution solvents contained different SILs (Table S1).

Quality Control

Samples were randomized into two batches, with those from the same subject prepared and measured in the same batch. For the preparation of the quality control sample, 30 study samples were weighed and extracted. After the extraction, equal volumes of each layer were taken from each sample and pooled, resulting in pooled QC aqueous and organic layers. Those pooled layers were used to prepare QC samples for each platform. The LLE and aliquoting steps were performed as described in Sample preparation.

LC-MS analysis of polar to semi polar metabolites

Analysis of polar to semi-polar metabolites were performed with a Shimadzu Nexera X2 LC system coupled to a TripleTOF 6600 mass spectrometer (SCIEX, Foster City, CA, USA), as described previously. Briefly, the LC separation was carried out at 40 °C using a Waters Acquity UPLC HSS T3 column (1.8 µm, 2.1 mm × 100 mm) with pre-column in-line stainless steel filter (0.3 µm, Agilent Technologies, Waldbronn, Germany). The mobile phase A was 0.1% FA in water, and the mobile phase B was 0.1% FA in ACN (Actu-all chemicals). With a flow rate of 0.4 mL min^{-1} and 1 µL of injection volume, the gradient starts at 100% A; 0–0.5 min 80% A; 0.5–2.5 min 2% A; 2.5–7.5 min 2% A; 7.5–12 min 2% A; 12 – 15 100% A. The data were acquired under full scan mode over the *m/z* range of 60-800 Da with Analyst TF software 1.7.1 (SCIEX) in negative and positive ionization modes. The preferred ionization mode for metabolites detectable in both polarities was chosen based on lower RSD% and higher signal-to-noise ratio of the QC samples.

LC-MS analysis of bile acid and fatty acids

Analysis of bile and fatty acids was performed on an UPLC-TOF/MS system consisting of ExionLC™ AC UHPLC system and SCIEX ZenoTOF 7600 system (Darmstadt, Germany) equipped with an IonDrive™ Turbo V Source, operated in negative ESI mode. The ion source conditions were as follows: spray voltage of 4.5 kV, capillary temperature of 550°C, ion source gas 1 50 psi, ion source gas 2 50 psi, curtain gas 35 psi, CAD gas 7 psi. The MS data was acquired under full scan mode over the m/z range of 200-900 Da. Accumulation time was set to 0.25 s, delustering potential to -70V and collision energy to -10eV. Chromatographic separation was performed on a Waters Acquity UPLC HSS T3 column (1.8 µm, 2.1 mm × 100 mm) with pre-column in-line stainless

steel filter (0.3 µm, Agilent Technologies, Waldbronn, Germany). The flow rate was set at 0.4 ml min^{-1}, the column was kept at 45 °C, injection volume at 2 µL. Mobile phase A consisted of 10 mM ammonium formate in water/ACN (Biosolve B.V) (95:5, v:v), while mobile phase B was 10 mM ammonium formate in MeOH/water (99:1, v:v). The gradient was as follows: starting at 0% B; 0–0.2 min 70% B; 0.2–7.5 min 100% B; 7.5–11.5 min 100% B; 11.5–11.6 min 0% B; 11.6 – 15 0% B. Isopropanol was used as an external rinsing solution (2 s sip time + rinse port). The flow was directed to waste in the first minute of the run. The autosampler temperature was set at 10 °C. Data acquisition was carried out on SCIEX OS 2.1.6.

Visualization RM-ASCA+

Visualization of the longitudinal metabolomic alterations was achieved using RM-ASCA+, which is an extension of LMMs for multivariate data. In the first step, LMMs are used to decompose the response matrix into effect matrices. The effect matrices are then analyzed using principal component analysis (PCA), and the results are summarized into PCA scores and loadings. The LMMs used for RM-ASCA+ were the LMMs used for the univariate analysis. The visualized effect matrices included the time effect matrix (‘time’) which shows time development of the reference group over time. The interaction matrix (‘time:group’) and the group-interaction matrix (‘group + time:group’) both show the deviations of the study group compared to the reference group over time with the latter also displaying the baseline differences. Lastly, the combined matrix (‘time + time:group’ or ‘time + group + time:group’) shows the time development of both the study and the reference group.

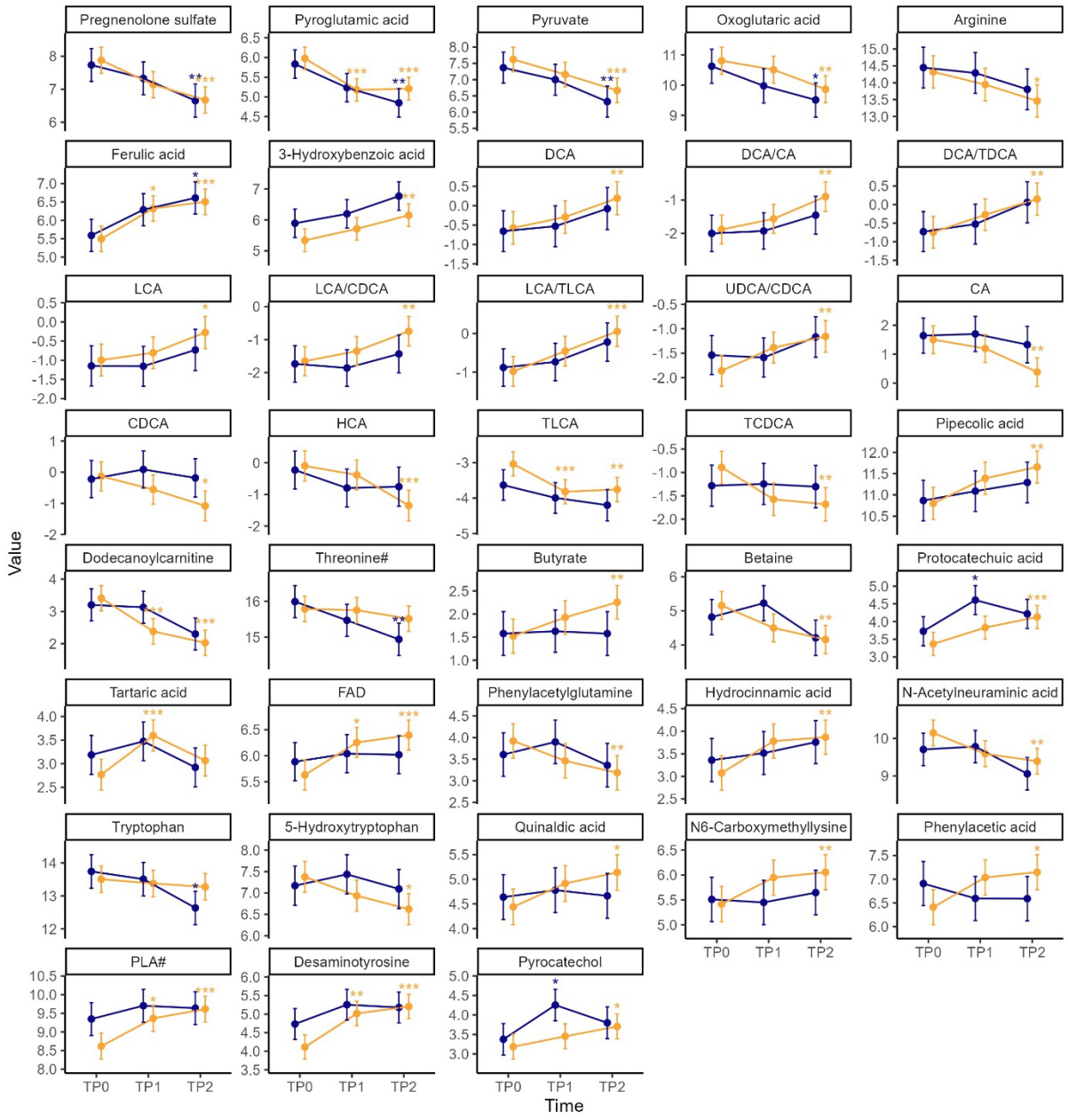

Figure S1. Marginal means estimated from the LMMs for participants who acquired tolerance (CM-tolerant, orange) and those that remained allergic (CM-allergic, blue). Only the metabolites for which pairwise comparison in time was found significant are plotted. The q-values are based on the marginal mean comparison to TP0 for each group, $q < 0.01$ (***), $q < 0.05$ (**), $q < 0.1$ (*).

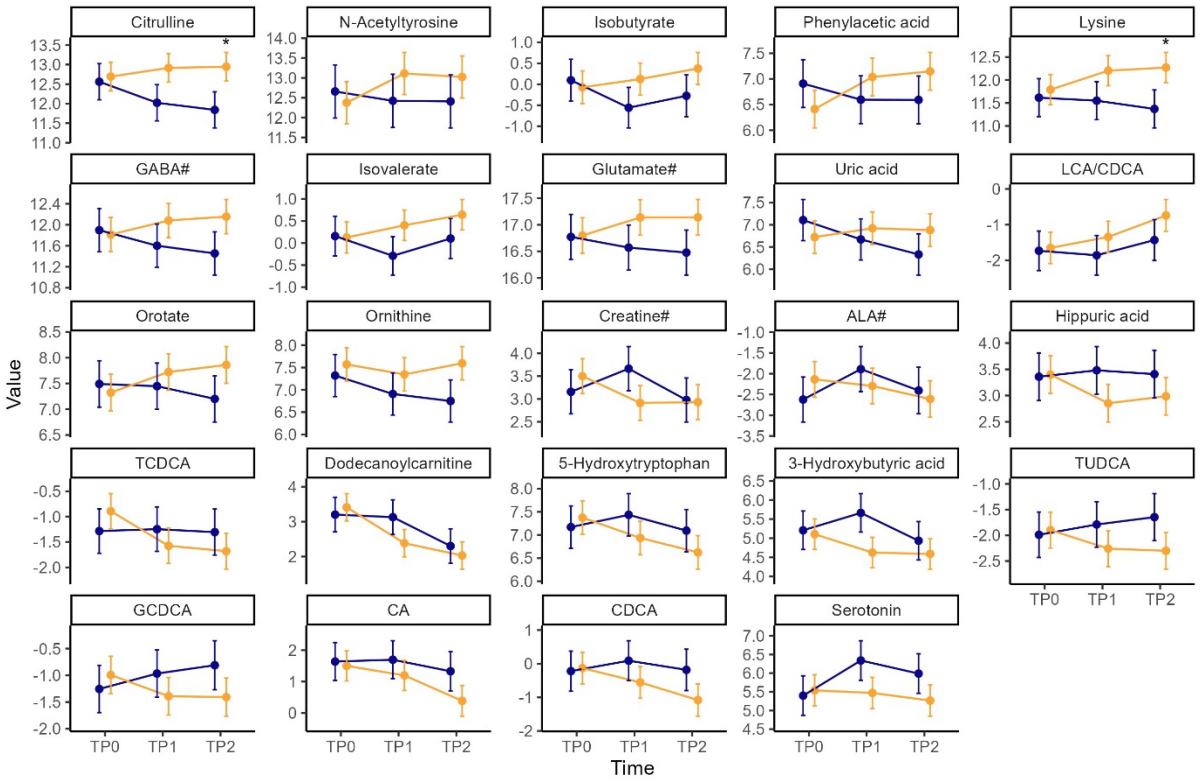

Figure S2. Marginal means estimated from the LMMs for participants who acquired tolerance (CM-tolerant) and those that remained allergic (CM-allergic). The metabolites with top loadings in PC1 of the RM-ASCA+ interaction matrix are plotted. The q-values are based on the marginal mean comparison between the groups at each time point, q <0.1 (*).

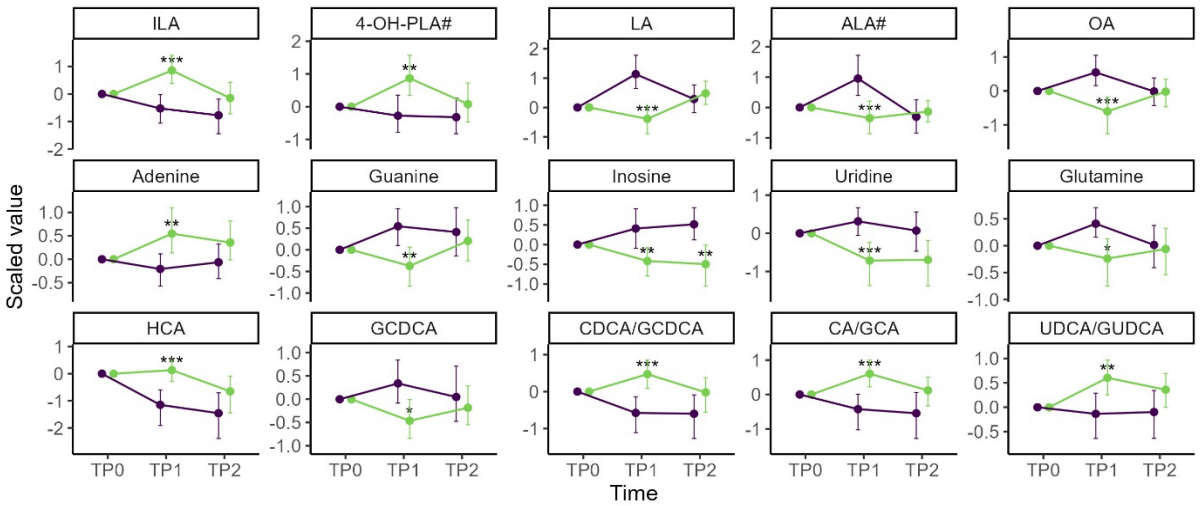

Figure S3. Marginal means estimated from the LMMs for AAF and AAF-S group. Only the metabolites for which an interaction coefficient was found significant are plotted. The response has been scaled. The q-values are based on/denote the significant

between-group change in the within-group change from baseline. q < 0.01 (***), q < 0.05 (**), q <0.1 (*)

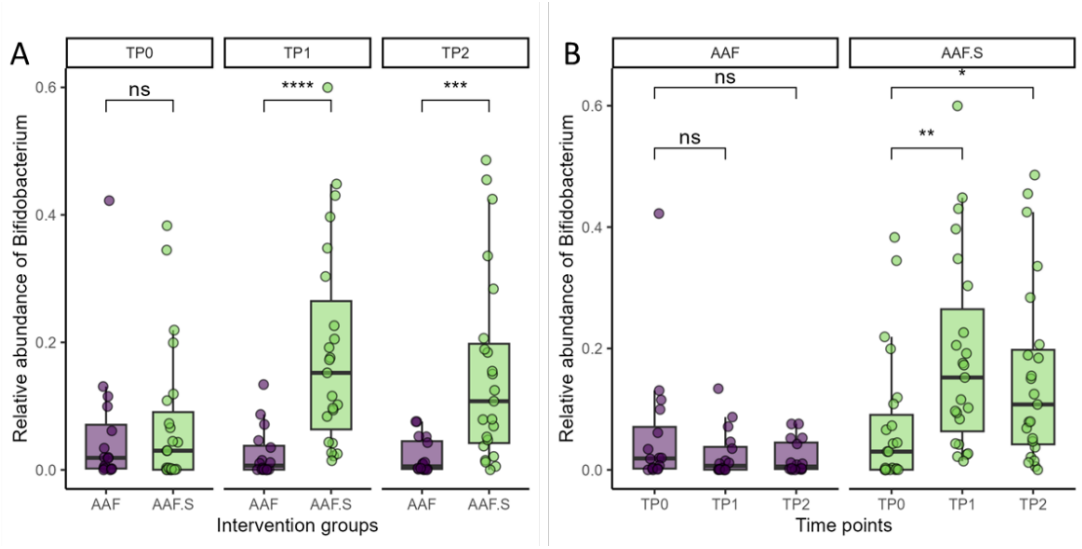

Figure S4. Relative abundance of *Bifidobacterium* comparisons between AAF and AAF-S groups at each time point (A), and between time points in each group (B). Statistical significance was evaluated with two-side unpaired t-tests; p > 0.05 (ns), p ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***), p ≤ 0.0001 (****).

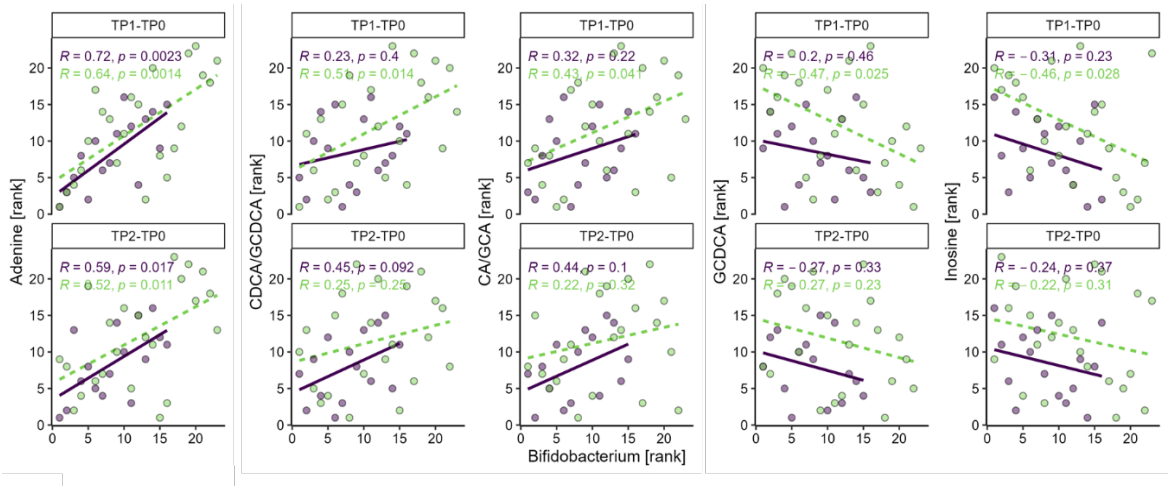

Figure S5. Spearman's rank correlations between the changes in *Bifidobacterium* and adenine, CDCA/GCDCA, CA/GCA. GCDCA, inosine in AAF (purple solid line) and AAF-S (green dashed line) groups from baseline to TP1 (TP1-TP0) and TP2 (TP2-TP0). The rank of the changes in metabolite response and relative abundance of *Bifidobacterium* within each group were used for plotting.

Table S1: General information and solution preparation for all the stable isotopically labeled standards (SILs)

Compound Name	Compound Formula	Supplier	product number	Spiked concentration (µM)	Usage
choline-d_4	C5H9D4NO	CDN	D-2464	16.00	Polar to semi-polar metabolites platform IS
cytidine-$^{15}N_3$	C9H13[15N]3O5	cambridge Isotope laboratories	NLM-3797-50	64.50	Polar to semi-polar metabolites platform IS
DL-leucine-d3	C6H10D3NO2	CDN	D-2400	56.00	Polar to semi-polar metabolites platform IS
DL-proline-d_7	C5H2D7NO2	cambridge Isotope laboratories	DLM-2657-0	58.00	Polar to semi-polar metabolites platform IS
hippuric acid-d_5	C9H4D5NO3	chem Cruz	sc-490158	42.00	Polar to semi-polar metabolites platform IS
hypoxanthine-d_3	C5D3HN4O	cambridge Isotope laboratories	DLM-2923-0.1	12.00	Polar to semi-polar metabolites platform IS
indole-d_5-3-acetic acid	C10H4D5NO2	TRC	I577344	44.00	Polar to semi-polar metabolites platform IS
L-tryptophan-d_3	C11H9D3N2O2	CDN	D-7419	20.00	Polar to semi-polar metabolites platform IS
L-tyrosine-$^{13}C_9$-^{15}N	[13C]9H11[15N]O3	cambridge Isotope laboratories	CNLM-439-H-0.1	26.00	Polar to semi-polar metabolites platform IS
octanoyl-l-carnitine-d_3	C15H26D3NO4	CDN	D-6651	0.40	Polar to semi-polar metabolites platform IS
propionyl-L-carnitine-(n-methyl-d_3)	C10H16D3NO4	CDN	D-6651	4.00	Polar to semi-polar metabolites platform IS
quinaldic acid-d_6	C10HD6NO2	CDN	D-6514	10.00	Polar to semi-polar metabolites platform IS
u-^{15}N-guanosine	C10H13[15N]5O5	Silantes	125303603	114.00	Polar to semi-polar metabolites platform IS
4-hydroxyphenylactic acid-d_6	C8H2D6O3	TRC	H949062	97.78	Polar to semi-polar metabolites platform spiked in reconstitution solution
fludrocortisone-d_5	C21H24D5FO5	TRC	F428102	0.76	Polar to semi-polar metabolites platform spiked in reconstitution solution
caffeine-d_9	C8HD9N4O2	TRC	C080102	2.77	Polar to semi-polar metabolites platform spiked in reconstitution solution
valine-d_8	C5H3D8NO2	cambridge Isotope laboratories	DLM-488	42.12	Polar to semi-polar metabolites platform spiked in reconstitution solution
Lithocholic acid-d_4 LCA-d_4	C24H36D4O3	CDN Isotopes	u501p49	200	Bile and fatty acids platform
cholic acid-d_4 (CA-d_4)	C24H36D4O5	CDN Isotopes	z75p40	65	Bile and fatty acids platform
Deoxycholic acid-d_4 (DCA-d_4)	C24H36D4O4	CDN Isotopes	w133p40	100	Bile and fatty acids platform
Ursodeoxycholic acid-d_4 (UDCA-d_4)	C24H36D4O4	CDN Isotopes	v275p43	100	Bile and fatty acids platform
Glycocholic acid-d_4 (GCA-d_4)	C26H39D4NO6	Cayman Chemical	21889	37.5	Bile and fatty acids platform
Glycoursodeoxycholic Acid-d_4 (GUDCA-d_4)	C26H39D4NO5	Cayman Chemical	21890	37.5	Bile and fatty acids platform
Tauroursodeoxycholic acid-d_5 (TUDCA-d_5)	C26H40D5NO6S	Santa-Cruz Biotechnology	sc-220192	10	Bile and fatty acids platform
Arachidonic Acid-d8 (AA-d_8)	C20H24D8O2	Cayman Chemical	390010	500	Bile and fatty acids platform
Oleic Acid-d_{17} (OA-d_{17})	C18H17D17O2	Cayman Chemical	9000432	1	Bile and fatty acids platform spiked in reconstitution solution
12-[[(cyclohexylamino)carbonyl]amino]-dodecanoic acid (CUDA)	C19H36N2O3	Cayman Chemical	10007923	0.5	Bile and fatty acids platform spiked in reconstitution solution

Table S2: Fisher's exact test results for the metabolites with missingness above 20% in the tolerant-allergy and the treatment groups

Compound name	Time point	CM-Allergic NA(%)	CM-Tolerant NA(%)	P values	model type
Vanillic acid	6	0.0	33.3	0.01457	CM tolerance-allergy
Valerate	6	73.3	33.3	0.02248	CM tolerance-allergy
2-Methylglutaric acid	6	80.0	41.7	0.02441	CM tolerance-allergy
Vanillactic acid	6	13.3	50.0	0.03785	CM tolerance-allergy
Agmatine	6	13.3	50.0	0.03785	CM tolerance-allergy
Creatinine	6	26.7	62.5	0.04837	CM tolerance-allergy
Agmatine	12	20.0	62.5	0.01950	CM tolerance-allergy
Asparagine	12	80.0	41.7	0.02441	CM tolerance-allergy

Compound name	Time point	AAF NA(%)	AAF-S NA(%)	P values	model type
L-Acetylcarnitine	0	62.5	21.7	0.0184	intervention
1,7-Dimethyluric acid	6	62.5	8.7	0.0009	intervention
Xanthosine	6	6.3	52.2	0.0047	intervention
3-Methylindole	6	50.0	8.7	0.0073	intervention
3-Methylhistidine	6	50.0	8.7	0.0073	intervention
GLCA	6	43.8	82.6	0.0172	intervention
2-Ketobutyric acid	6	0.0	30.4	0.0287	intervention
Saccharopine	6	31.3	4.3	0.0332	intervention
Dopamine	6	12.5	47.8	0.0371	intervention
Guanidinosuccinic acid	12	56.3	17.4	0.0172	intervention
Xanthosine	12	6.3	39.1	0.0279	intervention
GLCA	12	20.0	59.1	0.0409	intervention
TLCA-3S	12	26.7	63.6	0.0448	intervention

Table S3: Target list and abbreviations for final data ananlysis

Platform (ionization mode)	Compound_name_reported	abbreviations
Polar to semi polar (negative)	1-Methyluric acid	1-Methyluric acid
Polar to semi polar (negative)	2,5-Furandicarboxylic acid	2,5-Furandicarboxylic acid
Polar to semi polar (negative)	Deoxyinosine	Deoxyinosine
Polar to semi polar (negative)	Deoxyuridine	Deoxyuridine
Polar to semi polar (negative)	ortho-Hydroxyphenylacetic acid	ortho-Hydroxyphenylacetic acid
Polar to semi polar (negative)	Protocatechuic acid	Protocatechuic acid
Polar to semi polar (negative)	Dihydrocaffeic acid/3-hydroxy-3-(3-hydroxyphenyl)propanoic acid/Hydroxyphenyllactic acid	4-OH-PLA#
Polar to semi polar (negative)	3-Hydroxybenzoic acid	3-Hydroxybenzoic acid
Polar to semi polar (negative)	3-Hydroxybutyric acid	3-Hydroxybutyric acid
Polar to semi polar (negative)	3-Methyl-2-oxovaleric acid	3-Methyl-2-oxovaleric acid
Polar to semi polar (negative)	3-Methylxanthine/1-Methylxanthine/ 7-Methylxanthine	3-Methylxanthine/1-Methylxanthine/7-Methylxanthine
Polar to semi polar (negative)	Phenyllactic acid/3-(3-Hydroxyphenyl)propanoic acid	PLA#
Polar to semi polar (negative)	Hydrocinnamic acid	Hydrocinnamic acid
Polar to semi polar (negative)	4-Hydroxybenzoic acid	4-Hydroxybenzoic acid
Polar to semi polar (negative)	4-Hydroxycinnamic acid	4-Hydroxycinnamic acid
Polar to semi polar (negative)	p-Hydroxyphenylacetic acid/Mandelic acid	p-Hydroxyphenylacetic acid#
Polar to semi polar (negative)	Desaminotyrosine	Desaminotyrosine
Polar to semi polar (negative)	4-Pyridoxic acid	4-Pyridoxic acid
Polar to semi polar (negative)	Pyroglutamic acid	Pyroglutamic acid
Polar to semi polar (negative)	alpha-Aminobutyric acid/gamma-Aminobutyric acid/3-Aminoisobutanoic acid/Dimethylglycine	GABA#
Polar to semi polar (negative)	Argininosuccinic acid	Argininosuccinic acid
Polar to semi polar (negative)	Ascorbic acid	Ascorbate
Polar to semi polar (negative)	Carnosine	Carnosine
Polar to semi polar (negative)	Citric acid	Citrate
Polar to semi polar (negative)	Gluconic acid	Gluconate
Polar to semi polar (negative)	Flavin adenine dinucleotide	FAD
Polar to semi polar (negative)	Glutamine	Glutamine
Polar to semi polar (negative)	Glycine	Glycine
Polar to semi polar (negative)	Glycolic acid	Glycolate
Polar to semi polar (negative)	Guanine	Guanine
Polar to semi polar (negative)	Hippuric acid	Hippuric acid
Polar to semi polar (negative)	Histidine	Histidine
Polar to semi polar (negative)	Indolelactic acid	ILA
Polar to semi polar (negative)	Indoxyl glucoside	Indoxyl glucoside
Polar to semi polar (negative)	2-Hydroxyethanesulfonate	2-Hydroxyethanesulfonate
Polar to semi polar (negative)	Isobutyrylglycine	Isobutyrylglycine

Polar to semi polar (negative)	Oxoglutaric acid	Oxoglutaric acid
Polar to semi polar (negative)	Lysine	Lysine
Polar to semi polar (negative)	Malic acid	Malate
Polar to semi polar (negative)	Methionine.sulfoxide	Methionine sulfoxide
Polar to semi polar (negative)	myo-Inositol/ Galactose/ Fructose	Fructose#
Polar to semi polar (negative)	N-alpha-Acetylarginine	N-alpha-Acetylarginine
Polar to semi polar (negative)	N-Acetylglutamine	N-Acetylglutamine
Polar to semi polar (negative)	N-Acetylneuraminic acid	N-Acetylneuraminic acid
Polar to semi polar (negative)	N-Acetylserine	N-Acetylserine
Polar to semi polar (negative)	N-Acetyltryptophan	N-Acetyltryptophan
Polar to semi polar (negative)	N2-gamma-Glutamylglutamine	N2-gamma-Glutamylglutamine
Polar to semi polar (negative)	N6-Carboxymethyllysine	N6-Carboxymethyllysine
Polar to semi polar (negative)	O-Acetylserine/Glutamic acid	Glutamate#
Polar to semi polar (negative)	Orotate	Orotate
Polar to semi polar (negative)	p-Cresol	p-Cresol
Polar to semi polar (negative)	p-Cresol sulfate	p-Cresol sulfate
Polar to semi polar (negative)	Pantothenic acid	Pantothenic acid
Polar to semi polar (negative)	Phenylacetic acid	Phenylacetic acid
Polar to semi polar (negative)	Phenylacetylglutamine	Phenylacetylglutamine
Polar to semi polar (negative)	Phenylpropionylglycine	Phenylpropionylglycine
Polar to semi polar (negative)	Pregnenolone sulfate	Pregnenolone sulfate
Polar to semi polar (negative)	Pseudouridine	Pseudouridine
Polar to semi polar (negative)	Pyrocatechol	Pyrocatechol
Polar to semi polar (negative)	Pyruvate	Pyruvate
Polar to semi polar (negative)	Serine	Serine
Polar to semi polar (negative)	Syringic acid	Syringic acid
Polar to semi polar (negative)	Tartaric acid	Tartaric acid
Polar to semi polar (negative)	Taurine	Taurine
Polar to semi polar (negative)	Thymidine	Thymidine
Polar to semi polar (negative)	trans-Aconitic acid	trans-Aconitic acid
Polar to semi polar (negative)	Ferulic acid	Ferulic acid
Polar to semi polar (negative)	Tryptophan	Tryptophan
Polar to semi polar (negative)	Uric acid	Uric acid
Polar to semi polar (negative)	Uridine	Uridine
Polar to semi polar (negative)	Valine	Valine
Polar to semi polar (negative)	Xanthine	Xanthine
Polar to semi polar (negative)	Xylulose	Xylulose
Polar to semi polar (positive)	1-Methyladenosine/N6-Methyladenosine/2'-O-Methyladenosine	1-Methyladenosine#

Polar to semi polar (positive)	4-Guanidinobutanoic acid	4-Guanidinobutanoic acid
Polar to semi polar (positive)	Dihydrouracil	Dihydrouracil
Polar to semi polar (positive)	5-Aminolevulinic acid/4-Hydroxyproline	5-Aminolevulinic acid#
Polar to semi polar (positive)	5-Aminopentanoic acid	5-Aminopentanoic acid
Polar to semi polar (positive)	5-Hydroxytryptophan	5-Hydroxytryptophan
Polar to semi polar (positive)	Adenine	Adenine
Polar to semi polar (positive)	Adenosine/Deoxyguanosine	Adenosine#
Polar to semi polar (positive)	Alanine/beta-Alanine/Sarcosine	Alanine#
Polar to semi polar (positive)	Aminoadipic acid	Aminoadipic acid
Polar to semi polar (positive)	Arginine	Arginine
Polar to semi polar (positive)	Aspartic acid	Aspartate
Polar to semi polar (positive)	Betaine	Betaine
Polar to semi polar (positive)	Biotin	Biotin
Polar to semi polar (positive)	Cadaverine	Cadaverine
Polar to semi polar (positive)	Carnitine	Carnitine
Polar to semi polar (positive)	Choline	Choline
Polar to semi polar (positive)	Citrulline	Citrulline
Polar to semi polar (positive)	Creatine/Beta-Guanidinopropionic acid	Creatine#
Polar to semi polar (positive)	Cytidine	Cytidine
Polar to semi polar (positive)	Cytosine	Cytosine
Polar to semi polar (positive)	Ethanolamine	Ethanolamine
Polar to semi polar (positive)	Glycerophosphocholine	Glycerophosphocholine
Polar to semi polar (positive)	Glycylproline	Glycylproline
Polar to semi polar (positive)	Guanidoacetic acid	Guanidoacetic acid
Polar to semi polar (positive)	Hypoxanthine	Hypoxanthine
Polar to semi polar (positive)	Indoleacetic acid	Indoleacetic acid
Polar to semi polar (positive)	Inosine	Inosine
Polar to semi polar (positive)	Isoleucine	Isoleucine
Polar to semi polar (positive)	Kynurenic acid	Kynurenic acid
Polar to semi polar (positive)	Feature_mz_130.086	Feature_mz_130.086
Polar to semi polar (positive)	Dodecanoylcarnitine	Dodecanoylcarnitine
Polar to semi polar (positive)	Leucine	Leucine
Polar to semi polar (positive)	Methionine	Methionine
Polar to semi polar (positive)	N-Acetylcadaverine	N-Acetylcadaverine
Polar to semi polar (positive)	N-Acetylputrescine	N-Acetylputrescine
Polar to semi polar (positive)	N-Acetyltyrosine	N-Acetyltyrosine
Polar to semi polar (positive)	Targinine/Homoarginine	Homoarginine#
Polar to semi polar (positive)	N1-Methyl-4-pyridone-3-carboxamide/Nudifloramide	Nudifloramide#

Polar to semi polar (positive)	N2,N2-Dimethylguanosine	N2,N2-Dimethylguanosine
Polar to semi polar (positive)	N6,N6,N6-Trimethyllysine	N6,N6,N6-Trimethyllysine
Polar to semi polar (positive)	Nicotinic acid	Nicotinic acid
Polar to semi polar (positive)	Ornithine	Ornithine
Polar to semi polar (positive)	Phenylalanine	Phenylalanine
Polar to semi polar (positive)	Phenylethylamine	Phenylethylamine
Polar to semi polar (positive)	Picolinic acid	Picolinic acid
Polar to semi polar (positive)	Pipecolic acid	Pipecolic acid
Polar to semi polar (positive)	Proline	Proline
Polar to semi polar (positive)	Pyridoxal	Pyridoxal
Polar to semi polar (positive)	Quinaldic acid	Quinaldic acid
Polar to semi polar (positive)	Riboflavin	Riboflavin
Polar to semi polar (positive)	Serotonin	Serotonin
Polar to semi polar (positive)	Spermidine	Spermidine
Polar to semi polar (positive)	Sphinganine	Sphinganine
Polar to semi polar (positive)	Sphingosine	Sphingosine
Polar to semi polar (positive)	Symmetric dimethylarginine/Asymmetric dimethylarginine	SDMA#
Polar to semi polar (positive)	Thiamine	Thiamine
Polar to semi polar (positive)	Threonine/Homoserine	Threonine#
Polar to semi polar (positive)	Thymine	Thymine
Polar to semi polar (positive)	Trimethylamine	Trimethylamine
Polar to semi polar (positive)	Tryptamine	Tryptamine
Polar to semi polar (positive)	Tyramine	Tyramine
Polar to semi polar (positive)	Tyrosine	Tyrosine
Polar to semi polar (positive)	Uracil	Uracil
Polar to semi polar (positive)	Urocanic acid	Urocanic acid
Polar to semi polar (positive)	Xanthurenic acid	Xanthurenic acid
Bile and fatty acids	Cholic acid	CA
Bile and fatty acids	Chenodeoxycholic acid	CDCA
Bile and fatty acids	Deoxycholic acid	DCA
Bile and fatty acids	Oleic acid	OA
Bile and fatty acids	Linoleic acid	LA
Bile and fatty acids	alpha-Linolenic acid/gamma-Linolenic acid	ALA#
Bile and fatty acids	Dihomo-gamma-linolenic acid/Dihomo-alpha-linolenic acid	DGLA
Bile and fatty acids	Arachidonic acid	AA
Bile and fatty acids	Eicosapentaenoic acid	EPA
Bile and fatty acids	4,8,12,15,19-Docosapentaenoic acid	DPA
Bile and fatty acids	Docosahexaenoic acid	DHA
Bile and fatty acids	Glycocholic acid	GCA
Bile and fatty acids	Glycochenodeoxycholic acid	GCDCA
Bile and fatty acids	Glycoursodeoxycholic acid	GUDCA
Bile and fatty acids	Hyocholic acid	HCA
Bile and fatty acids	Lithocholic acid	LCA
Bile and fatty acids	Taurocholic acid	TCA
Bile and fatty acids	Taurochenodesoxycholic acid	TCDCA
Bile and fatty acids	Taurodeoxycholic acid	TDCA
Bile and fatty acids	Tauroursodeoxycholic acid	TUDCA
Bile and fatty acids	Taurolithocholic acid	TLCA
Bile and fatty acids	Ursodeoxycholic acid	UDCA

SCFA	Acetate	Acetate
SCFA	Butyrate	Butyrate
SCFA	Isobutyrate	Isobutyrate
SCFA	Isovalerate	Isovalerate
SCFA	Propionate	Propionate

Table S4: AIC comparison of model fitting with and without age as a covariate

metabolite	CM tolerance-allergy model		intervention model	
	without age	with age	without age	with age
1-Methyladenosine#	345	351	345	352
4-Guanidinobutanoic acid	345	352	343	350
Dihydrouracil	315	321	314	321
5-Aminolevulinic acid#	328	334	328	334
5-Aminopentanoic acid	328	334	325	332
5-Hydroxytryptophan	324	330	329	335
Adenine	296	302	286	292
Adenosine#	306	313	307	314
Alanine#	319	320	322	323
Aminoadipic acid	279	286	280	287
Arginine	381	386	379	384
Aspartate	314	319	306	310
Betaine	351	356	353	359
Biotin	347	353	345	351
Cadaverine	310	317	313	319
Carnitine	353	358	352	357
Choline	300	306	300	306
Citrulline	322	328	336	340
Creatine#	336	343	341	348
Cytidine	326	333	323	330
Cytosine	340	346	335	341
Ethanolamine	327	325	325	322
Glycerophosphocholine	301	308	301	309
Glycylproline	307	308	305	306
Guanidoacetic acid	364	364	364	366
Hypoxanthine	302	308	301	308
Indoleacetic acid	304	311	304	311
Inosine	320	326	300	307
Isoleucine	309	307	309	306
Kynurenic acid	341	348	340	346
Dodecanoylcarnitine	334	341	338	344
Leucine	306	303	305	300
Methionine	320	319	321	318
N-Acetylcadaverine	316	323	316	323
N-Acetylputrescine	368	375	368	375
N-Acetyltyrosine	407	411	410	413
Homoarginine#	328	334	329	335
Nudifloramide#	301	306	303	308
N2,N2-Dimethylguanosine	330	337	332	339
N6,N6,N6-Trimethyllysine	325	332	325	332
Nicotinic acid	305	312	305	312
Ornithine	329	328	334	332
Phenylalanine	316	315	314	311
Phenylethylamine	325	331	327	333
Picolinic acid	347	348	350	349
Pipecolic acid	325	332	318	325
Proline	333	327	334	327
Pyridoxal	278	285	280	286
Quinaldic acid	322	329	321	328
Riboflavin	357	363	355	361
Serotonin	352	356	359	363
Spermidine	335	341	333	338
Sphinganine	312	313	313	313
Sphingosine	318	324	315	322
SDMA#	350	352	351	354
Thiamine	354	359	353	358
Threonine#	308	309	314	314
Thymine	299	305	294	300
Trimethylamine	319	324	324	329
Tryptamine	308	315	311	318
Tyramine	334	339	333	339
Tyrosine	295	299	291	294
Uracil	332	337	328	333
Urocanic acid	328	332	327	330
Xanthurenic acid	336	343	334	341
1-Methyluric acid	323	329	324	331
2,5-Furandicarboxylic acid	320	327	318	325
Deoxyinosine	315	321	310	317
Deoxyuridine	302	308	296	303
ortho-Hydroxyphenylacetic acid	293	300	295	302
Protocatechuic acid	282	286	289	292
4-OH-PLA#	357	364	348	355
3-Hydroxybenzoic acid	321	325	322	325
3-Hydroxybutyric acid	345	350	354	358
3-Methyl-2-oxovaleric acid	347	347	347	347
Methylxanthine isomers	289	296	289	295
PLA#	312	319	320	326

Hydrocinnamic acid	329	335	329	335
4-Hydroxybenzoic acid	338	344	343	349
4-Hydroxycinnamic acid	296	303	291	297
p-Hydroxyphenylacetic acid#	350	356	351	357
Desaminotyrosine	300	306	304	310
4-Pyridoxic acid	318	320	316	317
Pyroglutamic acid	268	267	270	269
GABA#	296	300	304	307
Argininosuccinic acid	303	309	302	307
Ascorbate	317	323	312	319
Carnosine	383	389	376	382
Citrate	291	296	289	293
Gluconate	333	340	338	345
FAD	272	271	276	276
Glutamine	283	289	275	280
Glycine	321	321	326	324
Glycolate	359	365	355	361
Guanine	320	327	310	317
Hippuric acid	321	328	326	333
Histidine	282	289	284	291
ILA	364	370	350	356
Indoxyl glucoside	366	369	360	364
2-Hydroxyethanesulfonate	353	360	352	358
Isobutyrylglycine	318	325	317	324
Oxoglutaric acid	360	358	362	359
Lysine	299	303	312	315
Malate	355	359	357	361
AGN_mandelic.acid	347	354	348	354
Methionine sulfoxide	324	329	320	325
Fructose#	301	307	311	316
N-alpha-Acetylarginine	296	303	295	302
N-Acetylglutamine	320	327	317	324
N-Acetylneuraminic acid	307	314	310	316
N-Acetylserine	346	348	341	342
N-Acetyltryptophan	324	331	323	330
N2-gamma-Glutamylglutamine	327	333	325	332
N6-Carboxymethyllysine	307	313	309	316
Glutamate#	303	305	310	311
Orotate	318	325	322	329
p-Cresol	315	322	316	323
p-Cresol.sulfate	322	328	322	329
Pantothenic acid	303	309	299	305
Phenylacetic acid	328	335	332	339
Phenylacetylglutamine	336	340	332	337
Phenylpropionylglycine	371	377	374	379
Pregnenolone sulfate	331	328	330	328
Pseudouridine	313	320	309	316
Pyrocatechol	283	289	289	295
Pyruvate	318	319	316	317
Serine	344	344	344	344
Syringic acid	297	304	298	305
Tartaric acid	285	290	288	293
Taurine	379	384	379	385
Thymidine	322	328	320	327
trans-Aconitic acid	304	306	309	312
Ferulic acid	314	321	312	320
Tryptophan	343	346	344	348
Uric acid	321	326	327	331
Uridine	334	341	319	326
Valine	309	312	308	310
Xanthine	285	293	287	295
Xylulose	330	337	326	333
CA	379	385	380	387
CDCA	375	381	379	385
DCA	338	344	329	336
OA	328	332	313	315
LA	341	345	313	315
ALA#	352	357	334	338
DGLA#	311	317	315	321
AA	309	314	315	320
EPA	331	336	335	340
DPA	340	346	343	348
DHA	332	333	339	338
GCA	306	313	303	310
GCDCA	309	316	307	314
GUDCA	281	287	281	287
HCA	371	375	360	364
LCA	339	345	333	339
TCA	304	311	304	311
TCDCA	307	314	311	318
TDCA	333	339	329	334
TUDCA	310	317	316	322
TLCA	287	290	286	288
UDCA	310	317	314	320
Acetate	304	311	305	312
Butyrate	310	314	315	320
Isobutyrate	325	332	333	340
Isovalerate	304	311	312	320
Propionate	304	311	303	310

DCA/CA	352	358	345	351
UDCA/CDCA	289	294	290	296
LCA/CDCA	356	362	352	359
CA/GCA	318	325	299	306
CDCA/GCDCA	314	320	297	303
UDCA/GUDCA	291	298	276	283
CA/TCA	327	334	317	325
CDCA/TCDCA	321	327	316	323
DCA/TDCA	343	348	335	341
UDCA/TUDCA	322	326	320	324
LCA/TLCA	320	323	313	317

Table S5: Clinical characteristics associated with outgrowth of cow's milk allergy

characteristics	**Allergic (n=15)**	**Tolerant (n=24)**	**P_values**
egg_allergy : N	10 (67%)	15 (62%)	1.000
egg_allergy : Y	5 (33%)	9 (38%)	
sibling : N	5 (33%)	6 (25%)	0.718
sibling : Y	10 (67%)	18 (75%)	
allergy_father : N	6 (40%)	18 (75%)	**0.044**
allergy_father : Y	9 (60%)	6 (25%)	
allergy_mother : N	5 (33%)	15 (62%)	0.105
allergy_mother : Y	10 (67%)	9 (38%)	
delivery : Caesarean	8 (53%)	18 (75%)	0.185
delivery : Vaginal	7 (47%)	6 (25%)	
race : Asian	12 (80%)	16 (67%)	0.617
race : Caucasian / White	3 (20%)	6 (25%)	
race : Combination of above / Other	0 (0%)	2 (8%)	
sex : F	3 (20%)	8 (33%)	0.477
sex : M	12 (80%)	16 (67%)	
Daily.Formula.Intake.g : TP1	96.6± 34.38	89.75± 31.45	0.664
Daily.Formula.Intake.g : TP2	85.27± 47.37	85.58± 35.27	
Daily.Formula.Intake.mL : TP1	658± 275.95	601.25± 258.58	0.761
Daily.Formula.Intake.mL : TP2	577.33± 352.65	586.46± 281.46	
SCORAD.index : TP1	8.13± 9.67	5.46± 8.32	0.338
SCORAD.index : TP2	10.37± 8.77	6.77± 8.25	0.266
SCORAD.index : TP0	16.27± 13.24	8.98± 14.41	**0.036**
breastfeding duration until study entry (days)	206.87± 116.53	182.33± 107.6	0.453
age : TP1	15.59± 2.54	14.62± 3.02	0.427
age : TP2	21.88± 3.01	20.84± 3.05	0.411
age : TP0	9.68± 2.63	8.57± 3.04	0.254
AAF	6 (40%)	10 (42%)	1.000
AAF-S	9 (60%)	14 (58%)	

bottle.feeding.type until study entry	**Allergic (n=15)**	**Tolerant (n=24)**
Amino Acid Formula	6 (40%)	4 (18%)
Hydrolysate	0 (0%)	2 (9%)
Hydrolysate;Amino Acid Formula	4 (27%)	1 (5%)
Whole protein (milk / soy)	0 (0%)	1 (5%)
Whole protein (milk / soy);Amino Acid Formula	1 (7%)	2 (9%)
Whole protein (milk / soy);Hydrolysate	1 (7%)	5 (23%)
Whole protein (milk / soy);Hydrolysate;Amino Acid Formula	3 (20%)	7 (32%)
missing	0	2

Numeric variables are presented as mean ± standard deviation; categorical variable are presented as number (%)

Table S6: Clinical characteristics associated with interventions

characteristics	AAF (n=16)	AAF-S (n=23)	P_values
egg_allergy : N	12 (75%)	13 (57%)	0.32
egg_allergy : Y	4 (25%)	10 (43%)	
sibling : N	5 (31%)	6 (26%)	0.73
sibling : Y	11 (69%)	17 (74%)	
allergy_father : N	11 (69%)	13 (57%)	0.52
allergy_father : Y	5 (31%)	10 (43%)	
allergy_mother : N	9 (56%)	11 (48%)	0.75
allergy_mother : Y	7 (44%)	12 (52%)	
delivery : Caesarean	9 (56%)	17 (74%)	0.31
delivery : Vaginal	7 (44%)	6 (26%)	
race : Asian	10 (62%)	18 (78%)	0.62
race : Caucasian / White	5 (31%)	4 (17%)	
race : Combination of above / Other	1 (6%)	1 (4%)	
sex : F	6 (38%)	5 (22%)	0.31
sex : M	10 (62%)	18 (78%)	
Daily.Formula.Intake.g : TP1	91.44± 32.93	93.04± 32.64	0.86
Daily.Formula.Intake.g : TP2	72± 30.23	94.83± 43.38	0.07
Daily.Formula.Intake.mL : TP1	596.25± 285.61	641.74± 251.41	0.68
Daily.Formula.Intake.mL : TP2	475.62± 254.16	657.61± 322.09	0.07
SCORAD.index : TP1	8.03± 10.27	5.41± 7.74	0.24
SCORAD.index : TP2	8.75± 7.9	7.74± 9.08	0.54
SCORAD.index : TP0	13.34± 16.06	10.7± 13.12	0.69
breastfeding duration until study entry (days)	217.25± 105.31	174.04± 112.4	0.20
age : TP1	15.06± 2.88	14.95± 2.89	0.83
age : TP2	21.24± 2.84	21.24± 3.23	0.99
age : TP0	9.09± 2.91	8.93± 2.96	0.91
Allergic: TP2	6 (38%)	9 (39%)	1.00
Tolerant: TP2	10 (62%)	14 (61%)	

bottle.feeding.type until study entry	AAF (n=16)	AAF-S (n=23)
Amino Acid Formula	3 (20%)	7 (32%)
Hydrolysate	0 (0%)	2 (9%)
Hydrolysate;Amino Acid Formula	2 (13%)	3 (14%)
Whole protein (milk / soy)	0 (0%)	1 (5%)
Whole protein (milk / soy);Amino Acid Formula	3 (20%)	0 (0%)
Whole protein (milk / soy);Hydrolysate	3 (20%)	3 (14%)
Whole protein (milk / soy);Hydrolysate;Amino Acid Formula	4 (27%)	6 (27%)
missing	1	1

Numeric variables are presented as mean ± standard deviation; categorical variable are presented as number (%)

Table S7: Significantly altered metabolites in CM-allergic and CM-tolerant groups from marginal means comparison

CM-Allergic				
Metabolite	TP0	TP1	P value	Q value
Protocatechuic acid	3.727 (3.317, 4.137)	4.607 (4.197, 5.017)	0.0006	0.0674
Pyrocatechol	3.374 (2.969, 3.778)	4.252 (3.847, 4.656)	0.0008	0.0674
CM-Allergic				
Metabolite	TP0	TP2	P value	Q value
Pyroglutamic acid	5.833 (5.472, 6.194)	4.849 (4.489, 5.21)	0.0002	0.0328
Threonine#	15.992 (15.544, 16.44)	14.939 (14.491, 15.387)	0.0004	0.0340
Pyruvic acid	7.365 (6.89, 7.841)	6.322 (5.847, 6.798)	0.0006	0.0347
Pregnenolone sulfate	7.735 (7.236, 8.234)	6.658 (6.159, 7.157)	0.0011	0.0460
Tryptophan	13.74 (13.233, 14.247)	12.636 (12.129, 13.142)	0.0030	0.0806
Oxoglutaric acid	10.624 (10.057, 11.191)	9.508 (8.941, 10.076)	0.0033	0.0806
Ferulic acid	5.59 (5.151, 6.029)	6.61 (6.171, 7.049)	0.0034	0.0806
CM-Tolerant				
Metabolite	TP0	TP1	P value	Q value
Tartaric acid	2.769 (2.443, 3.094)	3.597 (3.271, 3.922)	0.0001	0.0082
Pyroglutamic acid	5.977 (5.691, 6.262)	5.178 (4.893, 5.463)	0.0001	0.0082
Dodecanoylcarnitine	3.411 (3.02, 3.802)	2.383 (1.992, 2.774)	0.0002	0.0082
TLCA	-3.038 (-3.378, -2.698)	-3.819 (-4.159, -3.479)	0.0002	0.0082
Desaminotyrosine	4.112 (3.785, 4.44)	5.017 (4.689, 5.344)	0.0003	0.0095
Ferulic acid	5.495 (5.148, 5.842)	6.323 (5.976, 6.67)	0.0026	0.0707
PLA#	8.617 (8.268, 8.965)	9.364 (9.015, 9.712)	0.0042	0.0920
FAD	5.631 (5.342, 5.92)	6.255 (5.966, 6.544)	0.0044	0.0920
Pregnenolone sulfate	7.88 (7.486, 8.275)	7.141 (6.746, 7.535)	0.0051	0.0945
CM-Tolerant				
Metabolite	TP0	TP2	P value	Q value
Dodecanoylcarnitine	3.411 (3.02, 3.802)	2.03 (1.639, 2.421)	0.000001	0.0001
Pregnenolone sulfate	7.88 (7.486, 8.275)	6.675 (6.281, 7.07)	0.000004	0.0003
Desaminotyrosine	4.112 (3.785, 4.44)	5.204 (4.877, 5.532)	0.000012	0.0007
LCA/TLCA	-0.98 (-1.363, -0.598)	0.054 (-0.335, 0.443)	0.0001	0.0025
Pyruvate	7.619 (7.243, 7.995)	6.664 (6.288, 7.04)	0.0001	0.0025
PLA#	8.617 (8.268, 8.965)	9.615 (9.267, 9.963)	0.0001	0.0030
Protocatechuic acid	3.367 (3.043, 3.691)	4.128 (3.804, 4.452)	0.0002	0.0040
HCA	-0.1 (-0.574, 0.373)	-1.354 (-1.836, -0.871)	0.0002	0.0040
Ferulic acid	5.495 (5.148, 5.842)	6.505 (6.158, 6.852)	0.0002	0.0040
Pyroglutamic acid	5.977 (5.691, 6.262)	5.21 (4.924, 5.495)	0.0002	0.0043
FAD	5.631 (5.342, 5.92)	6.396 (6.107, 6.685)	0.0004	0.0064
TLCA	-3.038 (-3.378, -2.698)	-3.756 (-4.101, -3.41)	0.0008	0.0113
Pipecolic acid	10.801 (10.423, 11.178)	11.656 (11.279, 12.034)	0.0010	0.0141
DCA/CA	-1.888 (-2.322, -1.454)	-0.899 (-1.341, -0.456)	0.0015	0.0188
Oxoglutaric acid	10.807 (10.359, 11.256)	9.865 (9.417, 10.314)	0.0016	0.0188
DCA/TDCA	-0.745 (-1.17, -0.321)	0.149 (-0.283, 0.582)	0.0019	0.0209
3-Hydroxybenzoic acid	5.341 (4.976, 5.706)	6.151 (5.786, 6.516)	0.0023	0.0234
Betaine	5.159 (4.751, 5.567)	4.16 (3.752, 4.568)	0.0027	0.0262
TCDCA	-0.893 (-1.24, -0.547)	-1.681 (-2.035, -1.327)	0.0030	0.0278
N-Acetylneuraminic acid	10.142 (9.802, 10.483)	9.392 (9.051, 9.732)	0.0032	0.0279
CA	1.501 (1.022, 1.979)	0.386 (-0.104, 0.875)	0.0040	0.0327
Hydrocinnamic acid	3.076 (2.698, 3.454)	3.869 (3.491, 4.246)	0.0041	0.0327
UDCA/CDCA	-1.858 (-2.175, -1.542)	-1.156 (-1.479, -0.832)	0.0054	0.0392
LCA/CDCA	-1.654 (-2.092, -1.216)	-0.744 (-1.191, -0.297)	0.0054	0.0392
Butyrate	1.522 (1.153, 1.891)	2.255 (1.893, 2.617)	0.0055	0.0392
Phenylacetylglutamine	3.922 (3.524, 4.321)	3.185 (2.786, 3.583)	0.0073	0.0481
DCA	-0.571 (-0.988, -0.155)	0.192 (-0.232, 0.616)	0.0076	0.0481
N6-Carboxymethyllysine	5.415 (5.064, 5.767)	6.055 (5.704, 6.406)	0.0076	0.0481
5-Hydroxytryptophan	7.375 (7.013, 7.737)	6.623 (6.261, 6.985)	0.0131	0.0802
CDCA	-0.131 (-0.601, 0.339)	-1.081 (-1.561, -0.601)	0.0139	0.0809
Quinaldic acid	4.44 (4.08, 4.8)	5.138 (4.777, 5.498)	0.014754102	0.080889787
Arginine	14.321 (13.842, 14.799)	13.461 (12.983, 13.94)	0.015285229	0.080889787
Pyrocatechol	3.184 (2.864, 3.504)	3.703 (3.383, 4.023)	0.015426353	0.080889787
Phenylacetic acid	6.412 (6.044, 6.78)	7.148 (6.78, 7.516)	0.015766331	0.080889787
LCA	-0.995 (-1.408, -0.583)	-0.276 (-0.696, 0.145)	0.015995156	0.080889787
TP2				
Metabolite	Allergic	Tolerant	P value	Q value
Citrulline	11.841 (11.378, 12.303)	12.946 (12.58, 13.311)	0.0003	0.0537
Lysine	11.371 (10.957, 11.785)	12.273 (11.946, 12.601)	0.0010	0.0823

Table S8: Spearman's rank correlation between the changes of *bifidobacterium* and metabolites/ratios which are significantly altered in the AAF-S group

Compound	Rho	P value	time points	Intervention	Q value
ILA	0.858695652	2.13E-06	TP1-TP0	AAF-S	0
4-OH-PLA#	0.768774704	2.81E-05	TP1-TP0	AAF-S	2.00E-04
Adenine	0.637351779	0.001379994	TP1-TP0	AAF-S	0.0069
Glutamine	-0.57312253	0.004939677	TP1-TP0	AAF-S	0.0185
Adenine	0.720588235	0.002305841	TP1-TP0	AAF	0.0346
CDCA/GCDCA	0.507905138	0.014441199	TP1-TP0	AAF-S	0.0433
Inosine	-0.462450593	0.027527897	TP1-TP0	AAF-S	0.059
GCDCA	-0.468379447	0.025413081	TP1-TP0	AAF-S	0.059
CA/GCA	0.43083004	0.041340241	TP1-TP0	AAF-S	0.0775
Guanine	-0.31027668	0.149497361	TP1-TP0	AAF-S	0.2492
Uridine	-0.244071146	0.260522306	TP1-TP0	AAF-S	0.3908
UDCA/GUDCA	-0.185770751	0.394330031	TP1-TP0	AAF-S	0.5377
Inosine	-0.314705882	0.234711639	TP1-TP0	AAF	0.5423
4-OH-PLA#	0.235294118	0.379021393	TP1-TP0	AAF	0.5423
Glutamine	-0.229411765	0.391370422	TP1-TP0	AAF	0.5423
Guanine	-0.267647059	0.315146037	TP1-TP0	AAF	0.5423
ILA	0.397058824	0.128882583	TP1-TP0	AAF	0.5423
OA	0.208823529	0.436322033	TP1-TP0	AAF	0.5423
ALA	0.247058824	0.354996461	TP1-TP0	AAF	0.5423
GCDCA	-0.197058824	0.463185494	TP1-TP0	AAF	0.5423
HCA	-0.194117647	0.470031308	TP1-TP0	AAF	0.5423
CA/GCA	0.323529412	0.221281258	TP1-TP0	AAF	0.5423
CDCA/GCDCA	0.226470588	0.397628203	TP1-TP0	AAF	0.5423
UDCA/GUDCA	-0.294117647	0.268071938	TP1-TP0	AAF	0.5423
Uridine	-0.164705882	0.541223723	TP1-TP0	AAF	0.5799
ALA	0.136363636	0.533413277	TP1-TP0	AAF-S	0.6668
HCA	0.12055336	0.582372852	TP1-TP0	AAF-S	0.672
OA	0.032608696	0.883337839	TP1-TP0	AAF-S	0.9119
LA	0.024703557	0.911925712	TP1-TP0	AAF-S	0.9119
LA	0	1	TP1-TP0	AAF	1
4-OH-PLA#	0.674901186	0.000570581	TP2-TP0	AAF-S	0.0086
ILA	0.624505929	0.001818976	TP2-TP0	AAF-S	0.0136
Adenine	0.523715415	0.011326837	TP2-TP0	AAF-S	0.0566
Glutamine	-0.497035573	0.016967834	TP2-TP0	AAF-S	0.0636
Adenine	0.594117647	0.017246545	TP2-TP0	AAF	0.2147
Uridine	-0.467391304	0.025756077	TP2-TP0	AAF-S	0.0773
LA	-0.571428571	0.028623176	TP2-TP0	AAF	0.2147
OA	-0.521428571	0.048830208	TP2-TP0	AAF	0.2442
Guanine	-0.407114625	0.05494236	TP2-TP0	AAF-S	0.1374
CDCA/GCDCA	0.453571429	0.091529268	TP2-TP0	AAF	0.2572
Glutamine	-0.426470588	0.101056074	TP2-TP0	AAF	0.2572
CA/GCA	0.439285714	0.103199216	TP2-TP0	AAF	0.2572
ILA	0.405882353	0.120020814	TP2-TP0	AAF	0.2572
4-OH-PLA#	0.373529412	0.154767766	TP2-TP0	AAF	0.2902
Guanine	-0.35	0.184066376	TP2-TP0	AAF	0.3068
LA	-0.29079616	0.188679939	TP2-TP0	AAF-S	0.4043
GCDCA	-0.267080745	0.228602156	TP2-TP0	AAF-S	0.423
CDCA/GCDCA	0.25352908	0.253790419	TP2-TP0	AAF-S	0.423
Inosine	-0.219367589	0.313053704	TP2-TP0	AAF-S	0.4424
CA/GCA	0.219649915	0.324461463	TP2-TP0	AAF-S	0.4424
GCDCA	-0.267857143	0.333445517	TP2-TP0	AAF	0.5002
Inosine	-0.241176471	0.366896119	TP2-TP0	AAF	0.5003
Uridine	-0.208823529	0.436322033	TP2-TP0	AAF	0.5454
UDCA/GUDCA	0.185714286	0.506673971	TP2-TP0	AAF	0.5519
HCA	0.182142857	0.515060927	TP2-TP0	AAF	0.5519
UDCA/GUDCA	-0.119141728	0.596160223	TP2-TP0	AAF-S	0.7452
ALA	0.128571429	0.648201799	TP2-TP0	AAF	0.6482
OA	-0.086391869	0.701645989	TP2-TP0	AAF-S	0.7598
ALA	0.084133258	0.709154443	TP2-TP0	AAF-S	0.7598
HCA	-0.049124788	0.828554014	TP2-TP0	AAF-S	0.8286