

RESEARCH PAPER

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An observational investigation of the faecal microbiota and metabonome of gastrostomy fed children, on blended and formula diets

Julian R. Marchesi^a, Sophie Allen^b, Emma Scott^a, Huw Jenkins^c, Claire Sadlier^d, and Sian Thomas^e

^aDepartment of Metabolism, Digestion and Reproduction, St Mary's Hospital, Imperial College London, London, UK; ^bSchool of Biosciences, Cardiff University, Cardiff UK; ^cDepartment of Paediatric Gastroenterology, Noah's Ark Children's Hospital for Wales, Cardiff, UK; ^dDepartment of Child Health, University Hospital of Wales, Cardiff UK; ^eAneurin Bevan University Health Board, Royal Gwent Hospital, Newport, UK

ABSTRACT

Gastrostomy fed children traditionally have a Formulae diet (FD), which fulfills nutritional requirements; however, many families are adopting Blended diets (BD), which are what the whole family would eat. We undertook an observational investigation of the colonic microbiota and metabonome in a small group of gastrostomy fed children, who were either on an FD or BD, and compared, where possible to their siblings (17 FD, 28 BD, 19 HS). There was no increase in complications in tube blockage or infection rates, but a significant improvement in the prevalence of bowel problems, a reduction in medication and an increase in quality of life. Metataxonomic analysis showed that the FD group was significantly different to the Sibling group, and that families did not cluster together. Whole sample metabonomics showed no differences between groups; however, univariate analysis of biologically important metabolites did differ. Changing to a BD resulted in no increase in complications or risks, but improved the overall quality of life for the children and families.

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Introduction


The gut microbiota plays a key role in determining the features of the gut microbiome, and one aspect which defines the gut microbiota is diet.^{1–4} Over the last two decades, we have steadily developed a better understanding of the roles that the gut microbiota plays in the gut and extra-intestinally in the host.^{5,6} Furthermore, we can map the relationship between the host and its microbiota in more detail in rodents raised in a sterile environment, and we see how wide the impact of many subsystems within the host is felt. Therefore, voluntary or involuntary changes to our dietary intake not only affect the host but it impacts the gut microbiota which can disturb its regular functions. Thus, impacts of dietary interventions now need to be measured in a much more holistic fashion and not simply from the point of view of the host's metabolism and physiology.

For one group of individuals, namely gastrostomy fed children, diets are usually nutritionally very well controlled. These children are unable to swallow safely, or take sufficient diet orally, due to,

in the main part, an underlying condition such as a neurological impairment. They are fed directly into their stomach via a surgically placed tube. The gastrostomy tube has a narrow internal diameter; therefore, the food being administered needs to be given as a liquid. Specialized 'formulae feeds', or diets, have been developed by commercial companies to give the exact nutritional requirements for a gastrostomy fed child. As well as being nutritionally complete, the formula diets are convenient, portable and can be administered to a regular feeding regimen. Historically they have been endorsed and prescribed by clinicians as they can calculate the exact amount of micronutrients and calories required by each individual child.

However, it has been reported by several families that this method and approach to feeding can separate the child from the rest of the family during mealtimes.⁷ Some clinicians and patients are now questioning the impact of formula diets on, not only the health and mental well-being of the patients and their families, but also on the digestive system of the patients too.⁸ An alternative approach

CONTACT Julian R. Marchesi  j.marchesi@imperial.ac.uk  Division of Digestive Diseases, Department of Metabolism, Digestion and Reproduction, St Mary's Hospital, Imperial College London, South Wharf Road, London W2 1NY UK

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which uses blended “home foods” has started to gain attention in families with gastrostomy fed children; however, only one study has been undertaken to assess to what extent the switch to a blended diet affects the child’s gut microbiota.⁹ This study reported changes in Proteobacteria and Firmicutes, but did not undertake an in-depth analysis of the microbiota over time or its associated metabonome. In comparison to the formula diets, which have a controlled nutritional content, these blended diets can vary in their nutritional content and there is a concern that to achieve the correct consistency to administer via gastrostomy, blended diets need to be diluted, which may reduce nutritional quality, also there are concerns about the risk of infection due to the way the feeds are prepared.¹⁰

Hence, we undertook an observational study of families with gastrostomy fed children in the South Wales region between 2017 and 2019 and collected stool samples from children on blended and formula diets, and where possible, their siblings too. Using these samples, we pursued a metataxonomic, metabonomic, limited culturomics, QoL questionnaire and exo-proteome analysis to measure features in the three cohorts.

Results

Study cohort and questionnaire results

Most gastrostomy-fed participants had feeding issues from birth and the mean length of time that participants were tube-fed was 7.5 years. Tube-feeding was the sole source of nutrition for most of the children in both FD and BD groups, with only seven parents stating that their child consumed some small amounts of food orally. All participants initially received a formula diet, but some moved to a blended diet after experiencing gastrointestinal disturbances. The participants receiving a blended diet did so for an average of 2.1 years at the time of sampling. The contents of the blended diets varied between participants, depending on what was being eaten at home, and a variety of different formula diets were used by the subjects of this study. The demographics for the study group were analyzed, where the data were available. No significant differences were seen between age (95% CI -2.28 to 2.43 , $P = .9707$)

and gender ($P = .7530$), with a median age of 6 years and 6.5 years for the BD and FD groups, respectively, and a gender split of 15:13 M:F for the BD group and 10:6 M:F for the FD group.

From the questionnaire, it was clear that the blended diet was not statistically different to the formulae fed in 3 of the 7 categories that we measured, namely gastrostomy tube blockage, tube damage/leakage and infection rates (Figure 1). Bowel disturbances were significantly reduced in the blended diet (BD) compared to the formulae diet (FD) (6.3% vs 70%, $P = .009$), respectively, as was total vomiting/retching (BD 12.5% v FD 50%, $P < .001$). Both general health and mood were also reported to have significantly improved in the BD vs FD groups. More details from the questionnaire are shown in supplementary figures S1-S6.

Metataxonomic analysis of the three groups, formulae fed children show the largest differences

Metataxonomic analysis showed significant differences between the community structure of the Formulae fed vs the Sibling group (Figure 2a. PERMANOVA analysis F Model 5.72, $R^2 = 0.006$), but not between the bacterial communities of the Blended vs Formulae, or Blended vs Sibling groups. The PCA of the samples shows no clustering according to the family from which they originated (Figure 2b), for example in family B (denoted by ■), the two healthy siblings were much closer to each other, than they were to the sibling being given a Formulae diet. For the NMDS plots of the Weighted Unifrac distance (Supplemental Figure S7) the Formulae group appears to be more diverse than the Blended or Sibling groups, the ratio of the areas of the 95% CI ellipses was approximately 1:0.43:0.26 for the Formulae:Blended:Sibling groups in Supp Fig S7. This difference shows that the stool samples in the Blended and Sibling groups were more like each other, in their respective group, and showed less diversity, as measured using a β -diversity index, than the Formulae group. qPCR of the 16S rRNA gene load for each group (Supplemental Fig S8) led us to conclude that between the Blended and Sibling groups the overall abundance was significantly lower in the former (-377881 , 95% CI -696867 to -58895 ; $P = .023$). There was no

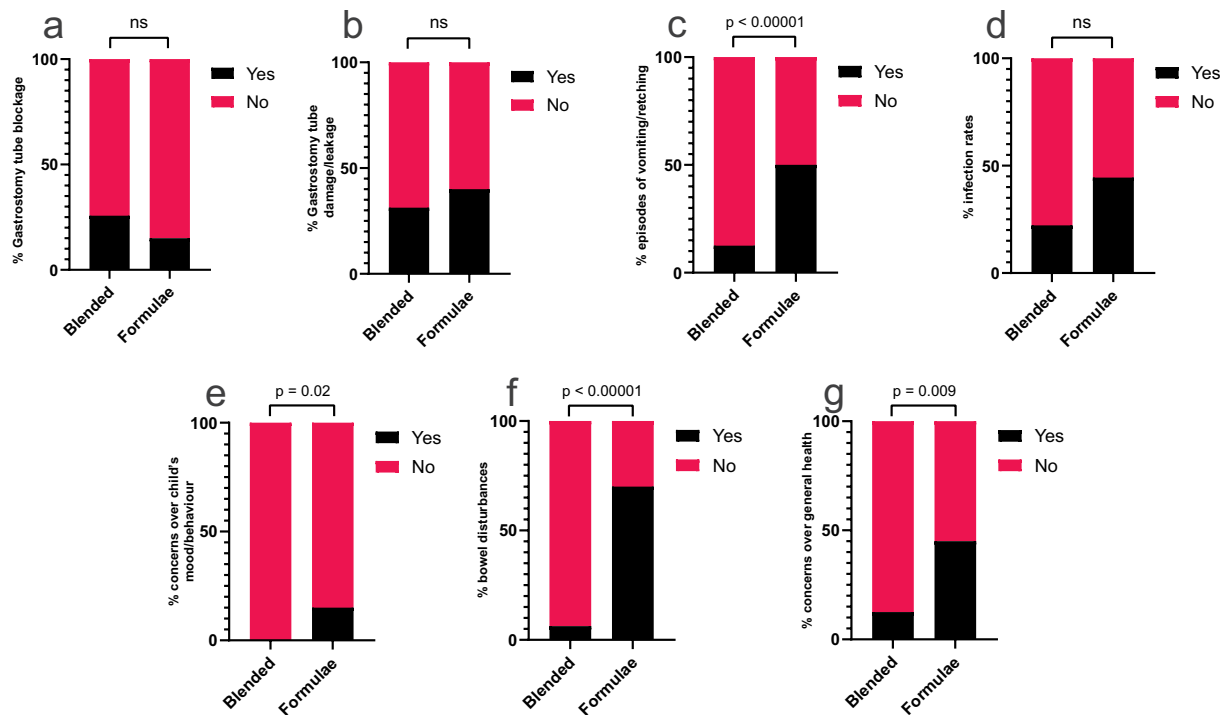


Figure 1. Health outcome measures taken from the questionnaire (see supplementary documents for the semi-structured interview schedule) data analyzed using GraphPad Prism and analyzed using Fisher's exact test. Each figure represents the responses to the following questions (a) "Have you experienced any difference with tube blockage – Yes/No?"; (b) "Have you experienced any difference with damage to the gastrostomy/leakage Yes/No?"; (c) "Any episodes of being sick (vomiting) or wanting to be sick (retching) – Yes/No?"; (d) "Difference with infections e.g., stomach, chest, skin – Yes/No?"; (e) "Have you noticed any – Mood/behaviour – Yes/No?"; (f) "Do you think using a blended diet has made any difference to your child in the following areas: a) Stools/bowel?" and (g) "Have you noticed any – General health – Yes/No?".

significant difference between the 16S rRNA gene counts in the Sibling and Formulae group or Blended and Formulae, however there was a trend of increasing 16S rRNA genes in the groups: $2.30 \times 10^5 \pm 5.03 \times 10^5$ vs $6.06 \times 10^6 \pm 1.07 \times 10^6$ vs $6.08 \times 10^5 \pm 5.76 \times 10^5$ for the Blended vs Formulae vs Sibling groups, respectively. While the qPCR data trends toward higher bacterial load, the variation is too large to determine if this difference was significant. The qPCR adjusted phylum bar plots are shown in Figure S8 and reflect the overall similar abundance of the phyla between the three groups. The alpha diversity for each group is shown in Figure 3, and there was a clear reduction of the alpha diversity in both diet groups when compared to the Sibling group (Figures 3A and 3b), but not between the feeding regimes. When comparing siblings from the same family (Figures 3C and 3d) it was clear that there was no consistent pattern, with some siblings on defined diets having either higher or lower alpha diversity than their healthy comparator.

When the communities were analyzed in more detail, significant differences were observed for a wide range of genera (Figure 4). There were more genera found to be different in the analysis of the Formulae vs Sibling groups (78 features after correcting for multiple testing and 18 showing an effect size >1%), compared to the comparison of the Blended vs Sibling (64 features after correcting for multiple testing, and 7 showing an effect size >1%) and Blended vs Formulae (55 features after correcting for multiple testing, and 6 showing an effect size >1%). The largest changes were seen in ASVs from the *Anaerostipes* and *Bifidobacterium*, with the former enriched, 14.1%, and the latter depleted, 14.2%, in the Formulae fed children, compared to the Sibling group. In the Blended diet group, there were modest enrichments, between 1% and 3%, of the *Enterococcus*, *Erysipelatoclostridium*, *Lachnoclostridium* and *Klebsiella* genera, while *Dialister*, an ASV called *Ruminococcus_2* and *Subdoligranulum* genera were depleted compared to the Sibling group. Between the Blended and Formulae groups, the main change

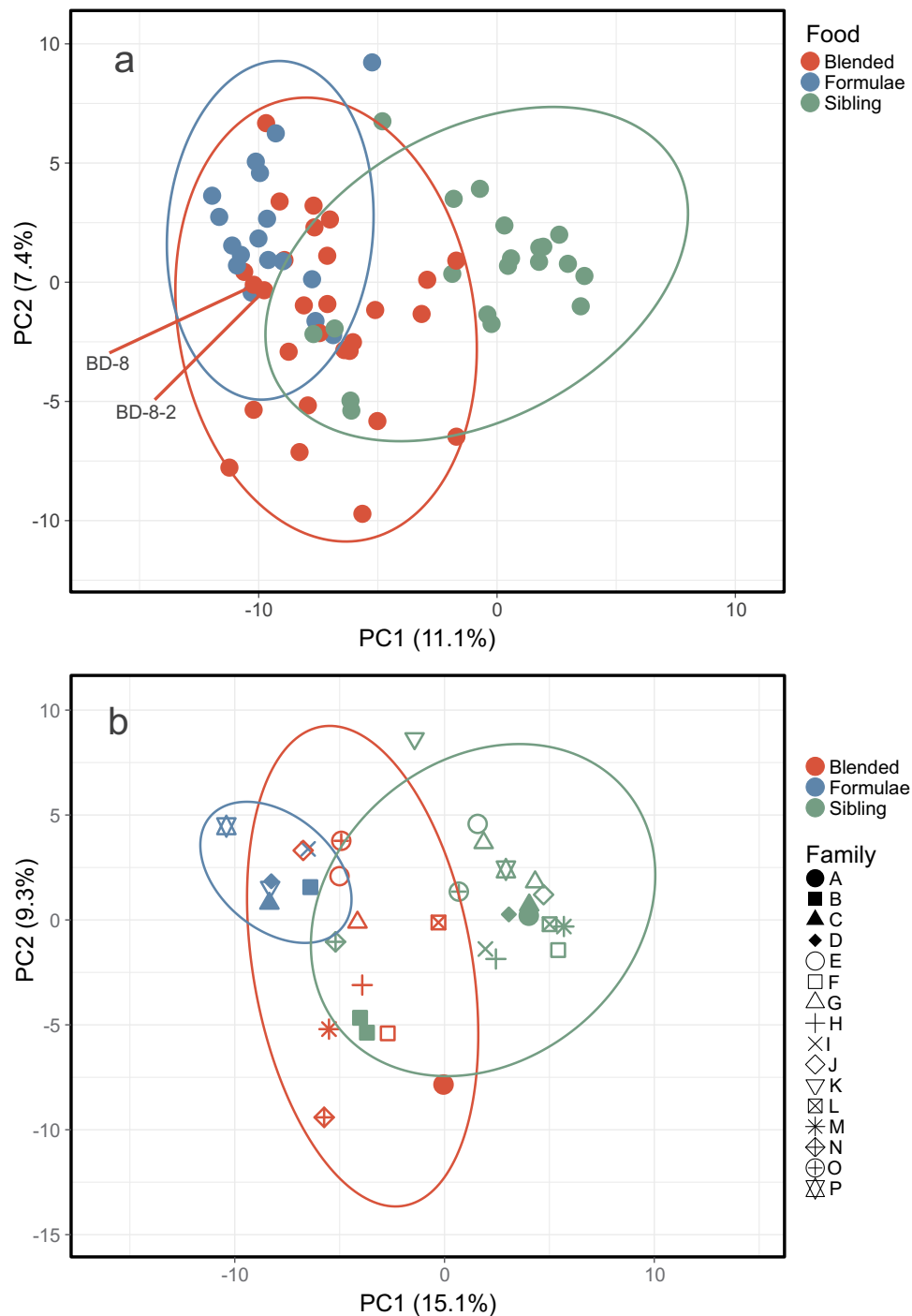


Figure 2. PCA plot of 16S rRNA gene read data at the genus level. Panel (a) shows the individual samples color coded according to the diet of the volunteer and status i.e. sibling while panel (b) adds familial information to the plot, to show how samples clustered when they come from the same household. Original values are $\ln(x + 1)$ -transformed. Unit variance scaling is applied to rows; SVD with imputation is used to calculate principal components. Prediction ellipses are such that with probability 0.95, a new observation from the same group will fall inside the ellipse. $N = 65$ data points for panel A and $N = 34$ data points for panel B. Duplicate sample from child BD-8 (BD-8 and BD-8-2) have been highlighted to show the reproducible nature of the metaxonomic analysis.

was an enrichment of the *Anaerostipes* ASVs in the Formulae group, 13.3%. In fact, this change was the main feature of the formulae group that there was a significant increase in the ASVs from the genus

Anaerostipes. Additionally, Tukey-Kramer post hoc test results, comparing all three groups, identified that the Formulae group had significantly more *Holdemania* than both the Blended diet ($P < .001$)

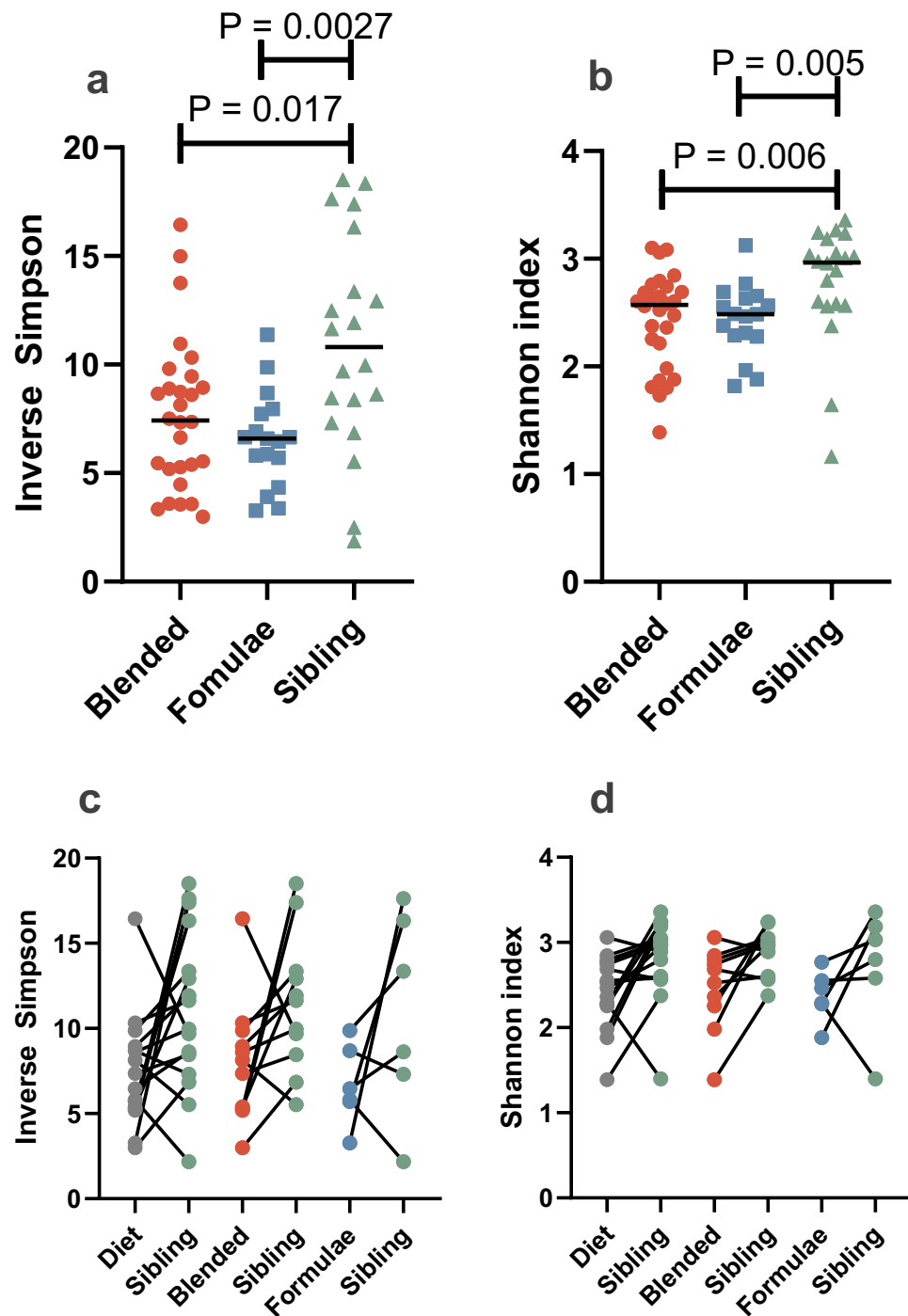


Figure 3. Alpha diversity of the 3 dietary groups. Panel A: Inverse Simpson's diversity of the 3 dietary groups; Panel B: Shannon diversity of the 3 dietary groups, statistical differences were tested using the Kruskal–Wallis test with Dunn's multiple comparisons test. Panel C: Ladder plot of the all the gastrostomy sibling (□) to their matched healthy sibling's Inverse Simpson's (□), Inverse Simpson scores of the blended diet children (□) to their matched sibling (□) and Inverse Simpson scores of the formulae diet children (□) to their matched sibling (□); Panel D: Is the same as Panel C, but using the Shannon index instead of the inverse Simpson index.

and healthy Sibling groups ($P < .01$). Significantly more *Lachnospirillum* was present in both the Formulae and Blended diet groups than in the Sibling group ($P = .0015$)

There were 16 sibling pairs with matched samples, 10 Blended diet and 6 Formulae diet. Examining the phylum-level taxonomic profiles of each sibling pair showed some general trends (Fig. S10). Children on

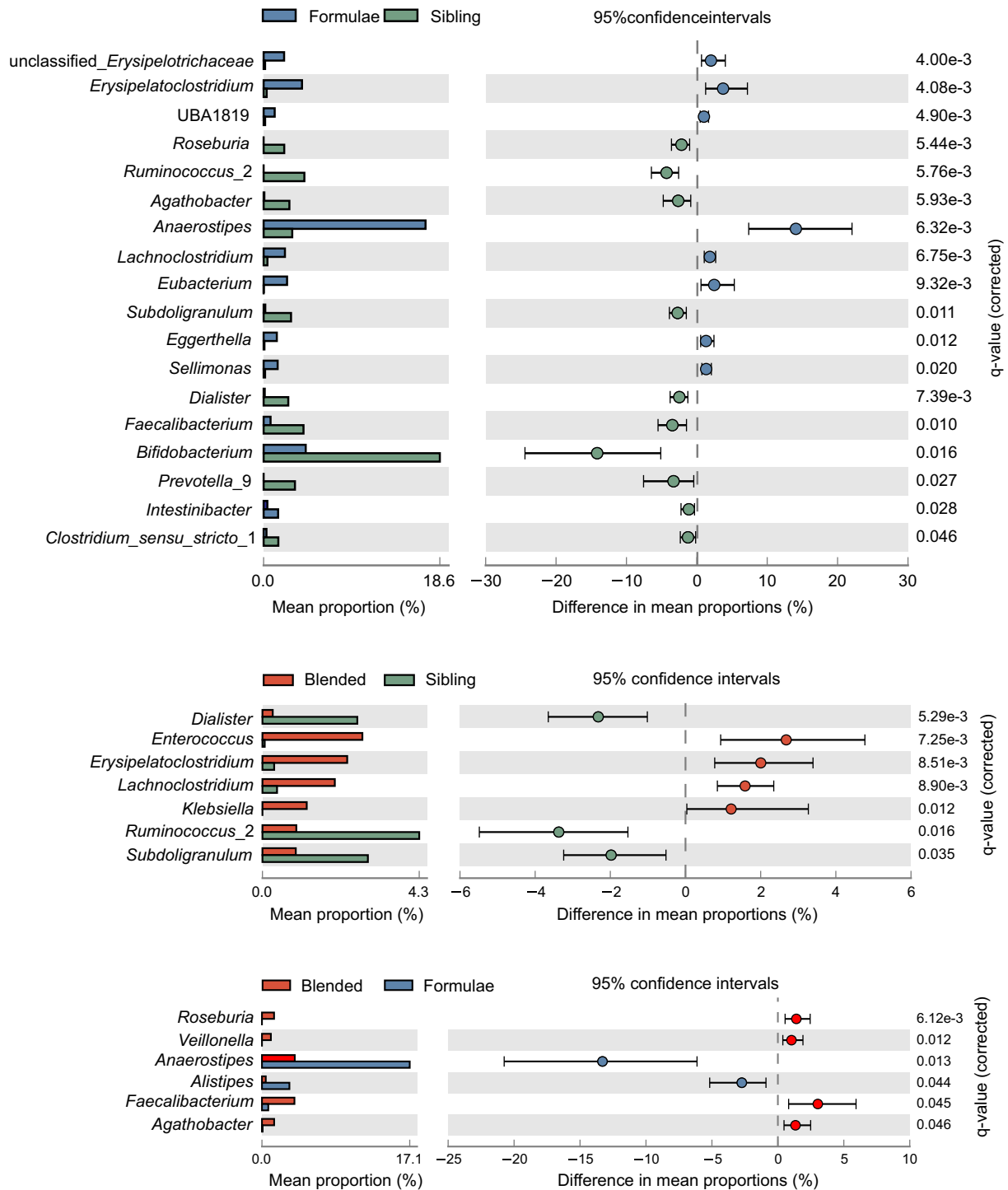


Figure 4. Extended error bar plots showing statistically significant differences between genera in a two-group analysis, between the different groups, e.g. blended diet vs formula diet. All reported P value are adjusted for multiple testing using the Benjamini-Hochberg FDR method, and an effect size filter of 1% was applied to leave only changes that were > 1% between groups. The genera differences between groups was tested using White’s non-parametric t-test.

both Blended and Formula diets showed greater enrichment of members of the *Firmicutes* than their healthy sibling counterparts, 11/16. Blended diet samples generally contained more *Gammaproteobacteria*, *Bacilli*, *Erysipelotrichia* and

Coriobacteria than their siblings, while the siblings contained more *Bacteroidia*. Formulae diet samples generally contained more members of the class *Bacteroidia*, *Clostridia* and *Erysipelotrichia* than their sibling counterparts, while the siblings

contained more *Actinobacteria*. Analyzing the individual sibling pairs further down the taxonomic levels showed similar results in the overall dataset. The sibling group possessed more *Ruminococcus* ASVs than both their blended diet and formula diet counterparts and more of the beneficial *Bifidobacterium* than the formula diet group. Children from the blended diet group contained more of the beneficial bacteria *Lactobacillus* than their siblings and the siblings contained more *Roseburia*, *Dialister*, *Anaerostipes*, *Prevotella* and *Faecalibacterium*

Inferred metabolic pathways using Piphillin

Three metabolic pathways showed statistically significant differences between the Formulae and Blended diet groups: BioCyc annotation RXN-14277, RXN-14274 and RXN-17782 (Data not shown). The differences in mean proportion of the pathways between these two groups, although statistically significant, were very small, <0.007%. These three reaction pathways were identified using the MetaCyc database and were found to be involved in fatty acid oxidation.¹¹ All three of these fatty acid oxidation pathways were significantly more prevalent in samples from children fed with a Blended diet than with a Formulae diet.

Analysis of functions in stool samples using culture-based approaches and enzyme assays

Two functions were screened using a culture-based approach which shows the phenotype of the growing colony on solid media. Numbers of CFU of protease producing bacteria, where determined using lactose-free skimmed milk agar (LF-SM).¹² Eighteen random samples were selected, six from each test group. For all samples tested, only the lowest dilutions in the dilution series showed any protease activity through their ability to produce a clear zone on the LF-SM agar, and no clear colonies exhibiting the phenotype were observed at higher dilutions. Therefore, these highest dilutions were spread as a lawn, and incubated anaerobically, on new plates with the aim being to identify individual colonies exhibiting the phenotype of interest. However, no single proteolytic colonies

were seen, and we were unable to identify and isolate any colonies of interest.

β -glucuronidase producing bacteria were assessed by an enzyme activity assay in 30 samples, 10 from each test group (Figs S11A and S11B). After adjusting the enzyme activity using the qPCR data, we observed that there was no difference between any of the diets. However, while not significant, the formulae feed group also had a higher median value for adjusted β -glucuronidase activity in the stool sample.

Global metabonomic analysis of fecal samples does not separate the dietary groups

The PCA analysis of the full NMR spectra for each group (Figure 5a) shows that there was no clear separation of the three groups, with the Blended diet group showing a larger and more diverse metabolic space compared to the Formulae fed and Sibling groups. The 95% confidence ellipse showed that for the BD diet the metabolic space in the first 2 dimension was much larger than that for the FD and siblings. OPLS-DA was used to identify the components that best differentiate the predefined classes of samples in each of the blended, formula and sibling groups. The OPLS-DA analysis confirmed that there were no discriminatory features between the metabolic profiles of the samples for comparisons between the three groups (data not shown). This analysis indicated that the global metabolic profiles between each of the Blended, Formulae and Sibling groups was not statistically different. There was a significant difference between the three short chain fatty acids, acetate, butyrate, and propionate (Figure 5b) in the blended and formulae feeds when compared to the sibling group, but not between the blended and formulae feed groups.

Discussion

The gut microbiota has, over the last decade, gained more importance as it has been shown time and time again to be a significant factor in host health.^{13–17} Furthermore, the gut microbial community, mainly bacterial in mass, sits between the host and its diet.^{18–20} It has adapted to use components of the diet that are not available to the host,

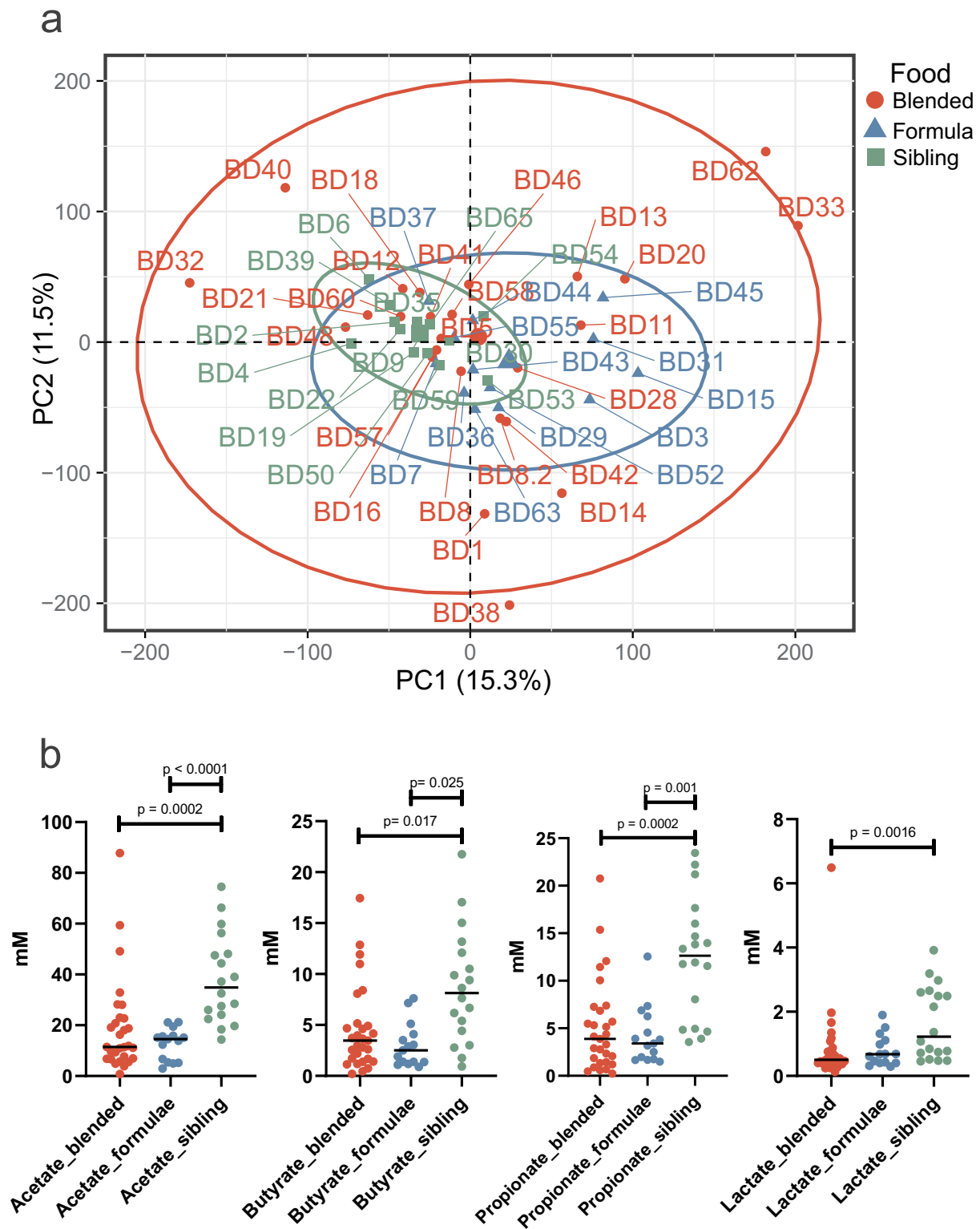


Figure 5. Global and specific metabolite plots for blended versus formulae feeds. (a): PCA plot of the global metabolic profiles of the fecal water samples as measured by $^1\text{H-NMR}$, (b): Difference of the concentrations of the 3 main short chain fatty acids as measured by NMR. The median values were plotted, and statistical differences tested using the Kruskal–Wallis test with Dunn’s multiple comparisons test.

for example, resistant starches^{21,22} and can produce a wide range of metabolites on which the host relies, for example short chain fatty acids.¹³ Therefore, diet is a key driver of the composition, structure, and metabolic output of the gut microbiome, influencing gut barrier function,²³ epigenetic status²⁴ and inflammation.²⁵ In gastrostomy fed children the diet is commonly a prescribed formulae feed, which, fulfills the nutritional requirement of the child. More recently blended diets have started to become more common as an alternative to the standardized commercial formulae feed. A blended diet is simply a meal, prepared at home and liquidized to a suitable consistency which allows it to be administered via the gastrostomy tube into the child's stomach. However, there is currently a feeling amongst professionals that the blended diet is not safe and is substandard,⁷ while among parents of the gastrostomy fed child, report that commercial feeds separate the child from the family meal and focus only on the medical nature of nutrition.²⁶ There is also an ongoing debate, among professionals, about the use and safety of blended diets for gastrostomy fed children, leading to ambiguity, which can create tension between the clinicians responsible for the child, and the parents of the child. Moreover, there are reports of parents saying that their child is physically better when on a BD. Hence, there is a clear need to understand whether a blended diet shows a significant impact on the gut microbiota and metabolome of gastrostomy fed children.

At the time of review of this work, there were no studies which had combined metataxonomic with metabolomics to investigate the microbiota and metabolite profiles of gastrostomy fed children, on blended or formulae feeds, with a comparison to their siblings. In undertaking this study, we wanted to determine the impact of the two dietary regimes on the structure and composition of the colonic microbiome and the metabolites therein. Moreover, we also undertook a questionnaire to assess a range of important lifestyle parameters in both cohorts of children. The results from the questionnaire showed that in many instances the BD was not inferior to the FD and was superior to the FD in important parameters such as vomiting and retching. This finding is in keeping with recent review of the literature^{7,27,28} also showing that

blended diets are not inferior to a formulae diet from a QoL perspective. Recent studies of blended enteral diets^{9,29,30} have also concluded that they lead to improved clinical outcomes and symptom scores.

The analysis of the microbiota in the stool samples showed that the BD was not creating bacterial communities that were significantly different to the siblings and that the FD gut communities were significantly more different to the siblings. The child's status i.e., gastrostomy fed was most probably one of the strongest drivers of community structure, which is shown in [Figure 2b](#) where the samples do not cluster based on family, but rather on diet. In two families who had two siblings, it can be seen that the siblings stool community structures were much more closely related than they were to their sibling who was gastrostomy fed, e.g., Families B and G. This observation again highlights the impact of the feeding modality rather than the diet. The bacterial diversity was lower in both FD and BD diets, with a lower diversity seen in FD, but this was not significant. There is no clear explanation as to why they may have lower diversity, but it may be due to higher load of prescribed antibiotics that these children are taking with nearly 70% having them in the last 6 months. In the study of Gallagher and colleagues⁹ in which they intervened with a BD diet in FD fed cohort, there was a clear increase in the diversity of the stool microbiota over time on a BD diet. Minimal taxonomic data was provided, with a reduction of OTUs in the Proteobacteria, and an increase in OTUs from the Firmicutes being reported. There were also enrichments of two OTUs related to *Eubacterium dolichum* and a *Lachnospira* sp. which did not match ASVs there were different between Blended and Formulae in our study. However, it might be too difficult to reconcile the two cohorts due to a different geographical location, i.e., Canada and the interventional style of their study.

At a genus level, there were clear and significant differences between the levels of bacteria. Most notable was the much lower levels of bifidobacteria in the FD group when compared to the sibling group, and much higher levels of *Anaerostipes*. The *Anaerostipes* were also much higher in the FD compared to the BD and appear to be an enriched feature of the FD group. Modest changes were seen

between the BD and sibling groups with enrichment of enterococci and depletion of ruminococci in the BD group. The depletion of the bifidobacteria is a point of concern as this genus is a very well-established probiotic, with documented roles in gut barrier function,³¹ atopic diseases,³² IBS,^{33,34} IBD³⁵ and several other non-communicable diseases.³⁶ Moreover, it has been tested as an intervention in a wide range of conditions, such as IBS, anxiety, NEC, and IBD³⁶ and more recently in obesity³⁷ and upper respiratory infections.³⁸ Hence, any changes in the levels of this genus should be noted and a possible intervention with a probiotic, in this group, be considered for further investigation. The other notable genus which changed was *Anaerostipes*, this genus is a lactate and acetate utilizer, and produces butyrate as a by-product of cross-feeding,^{39–41} however, there was no increase in butyrate in the FD group (*vide supra*). A more modest change in the genus *Eubacterium* was also seen in the FD group, again this a recognized producer of butyrate.⁴² The most parsimonious explanation for an increase in these genera would be the reduction of bifidogenic components in the FD group's diet, for example short-chain galactooligosaccharides and long-chain fructooligosaccharides,⁴³ which provides an opportunity for both *Anaerostipes* and *Eubacterium* to flourish. From the questionnaire (data not shown) we did note that in both groups, the majority of children were not receiving any probiotics (BD 68.7% and FD 75%), which again may be an area for intervention in both groups. The FD may not contain these prebiotic elements which would be found in a blended diet that includes a more complex mixture of fiber such as inulin.⁸ However, both these genera are also known to feed on the lactate and acetate,⁴⁴ and bifidobacteria produce these acids, but there was no significant reduction in the levels of lactate and acetate in the FD compared to the BD groups, which leads us to conclude that a loss of bifidobacteria does not impact the production of these acids. The enrichment of *Anaerostipes* and *Eubacterium* may indicate that these genera are competing with bifidobacteria for other resources and may not be cross-feeding on the lactate or acetate, e.g., glucose or fructose.^{44–47} The BD and FD also impacted the alpha diversity and total bacterial load in both these groups. Interesting,

while not significant, the median values of diversity were higher in the BD compared to FD groups, while the trend in the bacterial load was in the opposite direction, with higher median value for the bacterial load in the FD compared to BD. These trends may reflect the nature and diversity of the diets being used but may also reflect other underlying issues with respect to disease and quantities of food being consumed, features which were not captured in this study.

Another aspect of the gut microbiota that we measured was the metabolic landscape of the fecal environment. Global profiles of the three different groups were not statistically different and showed that the BD and FD were not separating from each other in metabolic space, and we concluded from this analysis that the different diets were not translating into significantly different metabolotypes. For the limited analysis of specific metabolites, *viz* short chain fatty acids, both the FD and BD showed significant reductions in concentrations of these important metabolites. These metabolites are important to the host in a wide range of physiological and immunological settings¹³ and any changes in the levels of these metabolites need to be considered for remedial intervention. For example, there are dietary products that can deliver propionate to the gut,⁴⁸ which was depleted in both BD and FD groups, and should be considered in these cohorts of children and supplements in their diet. Other options may again include probiotic supplements which may, with an appropriate prebiotic (aka a symbiotic), increase the levels of SCFA in the colon,⁴⁹ while also addressing the low levels of "beneficial" bacterial such as the bifidobacteria. A more detailed and targeted profiling of the colonic metatome would be valuable, for example bile acids and more SCFA, to assess the impact of the diet on this niche. Moreover, if we were able to obtain blood and urine, we would be able to explore the co-metabolic landscape in more detail, and map this to immunological data too, e.g. T cell types and abundance.

There were limitations to this study, which included the limited numbers in the study, the lack of a longitudinal aspect, more detail on the disease phenotypes and detailed data on the diet itself, its components and how much was consumed. We were also not able to collect dietary

data to determine the children's nutritional status or weight-for-age, which would be important aspects to collect for future studies. However, the study does, for the first time, show that a blended diet does not make a dramatic change to either the diversity of the colonic bacteria and the metabolic space therein, when compared to a formulae diet. In fact, we have shown that the FD group did significantly impact some groups of bacteria that would be beneficial to the host, i.e., bifidobacteria. Moreover, from this study, we can conclude that more needs to be undertaken to improve the diets for this group of children, and this may be in the form of addressing the fiber content and the use of probiotics, prebiotics or synbiotics.

Materials and methods

Cohort and ethics

This study was undertaken following approval by Southwest – Plymouth & Cornwall Research Ethics Committee (REF 17/SW/0049), and informed consent was obtained from all subjects, or their guardians, prior to sampling. Gastrostomy-fed children, fed with either a formula diet ($n = 17$) or a blended diet ($n = 28$), due to medical reasons, were recruited along with healthy siblings ($n = 19$) in a multi-center collaboration across five Health Boards in South Wales, led by Aneurin Bevan University Health Board. See supplementary methods for more details.

Sample preparation

The 66 frozen fecal samples were kept on ice, and frozen, and sub-sampled using a sterile 4 mm disposable biopsy punch (Kai Medical) to create a pellet, ~100 mg, which was dispensed into a sterile 1.5 mL microcentrifuge tube and stored at -80°C until required for DNA extraction or metabonomics (Dr Laura Osborne pers. comm.).

DNA extraction from stool, quantification, and qPCR of 16S rRNA genes

Sub-sampled fecal punches were thawed on ice, and DNA extraction was carried out using the DNeasy

PowerLyzer PowerSoil Kit (Qiagen, UK) with 0.1 mm silica glass beads and following the protocol's instructions with a few optimization steps for fecal samples, as follows. See supplementary methods for more details.

Metataxonomics of stool DNA

Next-Generation Sequencing (NGS) of the hyper-variable V1-V2 region of the 16S rRNA gene, using the Illumina MiSeq platform (Illumina Inc.), was performed in collaboration with the Genome Sequencing Hub at Cardiff University. See supplementary methods for more details.

Processing of stool metataxonomic data

The 16S rRNA gene sequencing data was processed using the DADA2 pipeline as amplicon sequence variants (ASV).⁵⁰ The raw FASTQ files obtained from sequencing were unzipped and uploaded to a CLIMB server.⁵¹ See supplementary methods for more details.

Analysis of metataxonomic data

Statistical analysis was carried out using the STAMP statistical software to compare the taxonomic reads between the different test groups at different taxonomic levels,⁵² and ANOVA were performed to compare differences between all three diet groups with a Turkey-Kramer post hoc test set at a 95% confidence limit and with Benjamini-Hochberg FDR multiple test correction. White's non-parametric two-sided t-tests were used to compare between two specific groups with bootstrapping set at a 95% confidence limit and Benjamini-Hochberg FDR multiple test correction. PCA plots of Hellinger transformed ASV data and PERMANOVA analysis of Weighted Unifrac distances were also created and tested in R using the packages BiodiversityR, vegan, and MASS. Alpha diversity indices were calculated using vegan in R on normalized data. Abundance counts of bacterial groups were determined by using the qPCR data to adjust the reads in each sample.⁵³ Duplicate samples were not included in the statistical analysis here or for other tests outlined below, but one duplicate sample from child

BD8 was included in the PCA plot to assess variation between duplicate stool samples (see Figure 2a).

Protease activity of cultured isolates

The fecal samples were prepared as an anaerobic slurry using 8% v/v DMSO in PBS and performed in an anaerobic chamber. Mixed bacteria in the fecal samples were grown anaerobically at 37°C on YCFA medium supplemented with lactose-free skimmed milk (LF-SM) to observe the protease activity.¹² See supplementary methods for more details.

β -Glucuronidase activity assay

β -glucuronidase enzyme activity was measured, as previously described by Kim and Jin⁵⁴ to for the fecal samples and to determine if there were differences between the three test groups. See supplementary methods for more details.

Statistical analysis for culture and enzyme activity

The differences between the three test groups were checked for statistical significance using a one-way ANOVA and a Tukey HSD test, which performs multiple comparisons of means, in R statistical software (R Core Team 2018).

Sample preparation for ^1H NMR spectroscopy

Sub-sampled fecal samples were thoroughly thawed at room temperature and weighed. Two portions of H_2O were added to each fecal sample, vortexed for 5 seconds and centrifuged at 14,000 g at 4°C for 10 minutes. See supplementary methods for more details.

^1H NMR spectral acquisition and experimental parameters

^1H NMR spectra were obtained using a Bruker 800 MHz spectrometer (Bruker, Rheinstetten, Germany), at a temperature of 300 K running a one dimensional (1D) standard experiment.

See supplementary methods for more details.

Spectral processing and metabolite identification

TOPSPIN 3.1 software (Bruker) was used for Fourier transformation, phasing and baseline correction. The TSP signal for each spectrum was visually assessed. See supplementary methods for more details.

Statistical analyses of NMR data

All multivariate statistical analyses, including Principal Components Analysis (PCA) and Orthogonal Partial Least Squares – Discriminant Analysis (OPLS-DA) were performed using MATLAB R2017a (MathWorks Inc., Natick, USA). R2Y (OPLS-DA) and R2X and Q2 (PCA and OPLS-DA) values were used to assess the robustness of the models. For univariate analyses, statistical significance was determined using Kruskal–Wallis test with Dunn’s multiple comparisons test. The level of significance was set at $P < .05$.

Quality of life questionnaire

A survey was developed to capture the views and experiences of children receiving a blended diet (group A; BD) and parents of children in receipt of a commercial formulae diet (group B; FD). See supplementary methods for more details and supplementary documents D1 and D2 for the two questionnaires used in this study.

Accession numbers:

The data that support the findings of this study are openly available at the EBI’s ENA: PRJEB48405 and MetaboLights: MTBLS3692.

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ORCID

Julian R. Marchesi  <http://orcid.org/0000-0002-7994-5239>

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