Modelling the gut microbiota of children with malnutrition: *in vitro* models reveal differences in fermentability of widely consumed carbohydrates

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Abstract

There is increasing evidence in children suffering from Severe Acute Malnutrition (SAM) that there is disruption of the gut microbiome and low gut microbiota diversity, which may be contributing factors to poor outcomes during nutritional treatment and recovery. The gut microbiome of children with SAM has been demonstrated to have a lower production of beneficial short chain fatty acids, which may contribute to impaired gut barrier function. Recently, several microbiota-directed therapies have been tested in clinical trials in children with SAM. Among them we hypothesized that feeds containing fermentable carbohydrates from various sources (legumes, chicory, milk oligosaccharides) would be fermented to produce beneficial microbial metabolites by the microbiota of children with SAM. In this study we used an *in vitro* model system inoculated with stool from children with SAM to investigate the fermentability of four substrates; inulin (a chicory-derived fructan), two milk powders (one supplemented with a human milk oligosaccharide) and a chickpea enriched feed. We demonstrated that while the milk powders and chickpea feed were fermented to produce short chain fatty acids, inulin was only fermented to a very limited degree. Through 16S rRNA sequencing we demonstrated that the samples inoculated with inulin had low microbial diversity and linked this to the limited ability to metabolise inulin. Through revealing the fermentability of different complementary feeds, the findings of this study will be of use for the design of future therapeutic feeds for treatment of SAM.

Importance

Malnutrition is a major contributor to childhood mortality globally and is a major public health problem primarily affecting Lower- and Middle-Income Countries. Despite the development of nutritional recovery therapies, for those with the severe and complicated form of malnutrition

(SAM), mortality and relapse rates remain high. Emerging evidence suggests a role for the gut microbiome in these poor outcomes, which is known to be significantly altered in children in SAM, compared to healthy age matched controls. To aid in recovery from SAM, nutritional interventions should be designed to support the gut microbiome, using a range of ingredients targeted for colonic fermentation. It is important to understand the fermentation capacity of the gut microbiome of children with SAM, to design future nutritional interventions. In this work, we demonstrate that inulin, a widely used chicory-derived prebiotic, is not a suitable fermentation substrate for the gut microbiome of SAM children, while legume-based formulations and milk oligosaccharides result in increased production of beneficial metabolites.

Introduction

Undernutrition is still a widely prevalent problem in many Lower- and Middle-Income Countries (LIMCs), affecting almost 25% children under the age of 5 years globally, and being implicated in almost half of all childhood deaths (1, 2). Childhood undernutrition can be broadly split into two categories, namely stunting (length-for-age Z-score [LAZ] ≤2) and wasting (weight-for-height Z-score ≤2) (1). Although less prevalent than stunting, the most severe form of wasting, Severe Acute Malnutrition (SAM) is associated with higher mortality rates. For children hospitalised with SAM up to 20% die , with a high proportion of deaths occurring during the first week of hospitalisation (3). Nutritional rehabilitation alongside supportive treatments has been the cornerstone of SAM treatment. The feeding regimes have been developed to deliver optimum nutrition to children hospitalized with SAM. The current WHO recommendation for nutritional treatment of acutely sick children hospitalized with SAM are mainly milk-based feeds (called F75 and F100 indicating their calorie content/100ml) for inpatient management followed by Ready to Use Therapeutic Feeds (RUTF) as a paste for rehabilitation. This treatment regime leads to weight gain and recovery of appetite, as well as improvement in other indicators such as glycaemic control (4). Despite this, nutritional recovery (as well as recovery of anthropomorphic indicators) in SAM is a poor indicator of long term outcomes (5, 6), indicating that the damage caused by SAM is far more complex and long-lasting than simple nutritional deficiencies.

The human gut microbiota has recently become an area of intense research interest, driven by advances in metagenomic sequencing technologies (7, 8), but also a greater understanding that the gut microbiota influences many physiological processes including; nutrient acquisition (9); growth hormone signalling and appetite control (mediated through production of Short Chain Fatty Acids (SCFA)) (10); and immune regulation (11). During long term recovery from SAM

there is evidence of systematic dysregulation of many of these processes, leading to altered appetite regulation impaired acquisition of key nutrients and increased susceptibility to a range of infections (12). This points to the potential for a key role for the microbiota in the pathogenesis and rehabilitation of SAM.

16S based amplicon sequencing studies of the stool microbiota of infants suffering from SAM have revealed that there are dramatic alterations in the gut microbiota (13-17). Twins studies on infants discordant for SAM have demonstrated that distinct microbiota changes occur as a result of SAM, independent of host genetics, environment and background diet (13). These changes include a general reduction in α and β diversity, as well as increases in potentially pathogenic bacteria such as *Klebsiella* and Enterobacteriaceae, and a reduction in beneficial saccharolytic bacteria in *Clostridium* clusters IV and XIVa (such as *Blautia*, Lachnospiraceae, Ruminoccoaceae and *Faecalibacterium prausnitzii*), *Bacteroides* species and *Lactobacillus* (15, 18). It has been hypothesised that some aspects of the microbiome of children with SAM represent a failure of the gut microbiota to fully mature (15, 16), although there is also strong evidence that there is significant overgrowth of potential enteropathogens (17, 18), and risk of invasive bacterial infection (3).

Emerging evidence indicates that reversing the gut microbiota changes that occur as a result of SAM have the potential to improve outcomes (19), although gut microbiome targeted therapies have to be carefully designed as interventions using probiotics and synbiotics have had limited success (18, 20). The most promising approach is the use of so called ‘microbiota directed foods’ (21, 22), in which food ingredients rich in carbohydrates that are fermentable by the gut microbiota of children with SAM are supplemented conventional RUTFs. The selection of

fermentable carbohydrate is crucial as the damage to the gut microbiota diversity that occurs during SAM has the potential to restrict the range of substrates which are accessible to the microbiota. By successfully identifying substrates that support the metabolism of the broadest range of microbial species possible, an increase in SCFAs may be achieved.

In this study, we aimed to directly determine the fermentability of potential microbiota directed foods by the gut microbiota sampled from children with SAM by employing and *in vitro* batch fermentation model seeded with stool samples collected from infants hospitalised with SAM. We investigated the fermentation of 4 different substrates. Infant formula, similar in composition to the F75/F100 formula was selected as a baseline for the standard SAM recovery formula. Inulin, a chicory-root derive fructan oligosaccharide, was selected as it is a widely used prebiotic carbohydrate (23) which is highly fermentable by the gut microbiota of healthy infants (24), but conversely in animal models of the SAM gut microbiota it was shown to not to support growth (25). The same animal study demonstrated that milk oligosaccharides similar to those found in human milk did support weight gain (25), so we selected a milk powder supplemented with 2'-Fucosyllactose to reflect this. Finally, we used a legume enriched feed designed to support microbial recovery in SAM (26, 27) formulated with chickpea and high in resistant starch (28, 29). Chickpea has been successfully used as a feed supplement to support microbiota recovery in moderate acute malnutrition (19), and the formulation used here has been specially formulated for use in intervention trials to support recovery of infants with SAM (26). Each of these foods was used as a substrate for *in vitro* batch fermentation in models seeded with stool samples from children hospitalized with SAM. Metabolomic analysis of the fermentation media using ^{1}H NMR was conducted to indicate substrate fermentability through production of microbial metabolites during the time course of fermentation. Samples were also taken for 16S metataxonomic analysis

148 to determine the effects of each of the substrates on microbial diversity and potential pathogen
149 burden.
150
151 **Results**
152 *Cohort demographics and baseline microbiome composition*
153 The demographics of the cohort are shown in Table 1. The average age of the cohort is 2.4 years,
154 with an age range of 0.9 years to 4.5 years. All children included in this study met the criteria for
155 SAM with an average MUAC of 11.1 cm. Clinical data from this cohort was collected from
156 February to April 2016 from day of admission to day 7 of hospitalization.
157 **Table 1. Demographics of the study cohort. Values are given as mean ± standard deviation.**

Demographic	Total (n=10)	Female (n=5)	Male (n=5)
Demographic (mean ± SEM)			
Age (years)	2.2 ± 0.4 [0.8 - 4.5]	2.1 ± 0.6 [0.8 - 3.9]	2.4 ± 0.6 [1.1 - 4.5]
MUAC (cm)	11.1 ± 0.5 [9.0 - 13.2]	10. 4 ± 0.6 [9.0 - 12.3]	11.8 ± 0.8 [9.1 – 13.2]
Height (cm)	74.9 ± 4.0	71.2 ± 4.8	78.6 ± 6.6
Weight (kg)	7.3 ± 0.9	6.0 ± 0.8	8.7 ± 1.5
Weight-for-height/length z score	-3.2 ± 0.5	-3.9 ± 0.6	-2.5 ± 0.8
Oedema at admission: ≥3 grade	6/10	3/5	3/5
Diarrhoea	3/10	2/5	1/5
Breast feeding	1/10	none	1/5
Days receiving F75 feed up to day 7	5.0 ± 2.3	6.0 ± 2.2	4.0 ± 2.1
Days receiving F100 feed up to day 7	2.0 ± 2.3	1.0 ± 2.2	3.0 ± 2.1
F75 feeding	5/10	4/10	1/10
F100 feeding	5/10	1/10	4/10
Clinical Features (mean ± SEM) Day 7 after admission			

Pulse (beats/minute)	131 ± 6 [94 - 180]	133 ± 8 [102 - 180]	130 ± 8 [94 - 175]
Resp Rate (breaths/minute)	30 ± 1 [94 - 180]	31 ± 2 [94 - 180]	30 ± 1 [94 - 180]
Temperature (Celsius)	36.5 ± 0.2 [35.0 – 38.0]	36.8 ± 0.2 [35.5 – 38.0]	36.2± 0.2 [35.0 – 37.3]
Mean blood glucose over 72hrs following admission (mmol/L)	5.2 ± 0.4 [2.8- 9.5]	5.1 ± 0.6 [2.8- 9.1]	5.4 ± 0.5 [3.8- 9.5]

*MUAC: Mid-upper arm circumference.

158

159 The baseline microbiome composition determined by 16S rRNA sequencing of the samples is
160 depicted in Fig. 1, showing large inter-individual differences in microbiome composition. All the
161 microbiomes sampled contained a high proportion of Proteobacteria, particularly *Escherichia*
162 *coli*, which represents up to 80% of the total microbial abundance in some samples. In addition
163 to Proteobacteria, the samples were dominated by bacteria from Actinobacteriota, a phylum
164 which is primarily represented by the genus *Bifidobacterium* in the human gut, and Firmicutes as
165 would be expected in developing children microbiomes (30). Conversely, very low abundances
166 of Bacteroidota were observed in these samples.

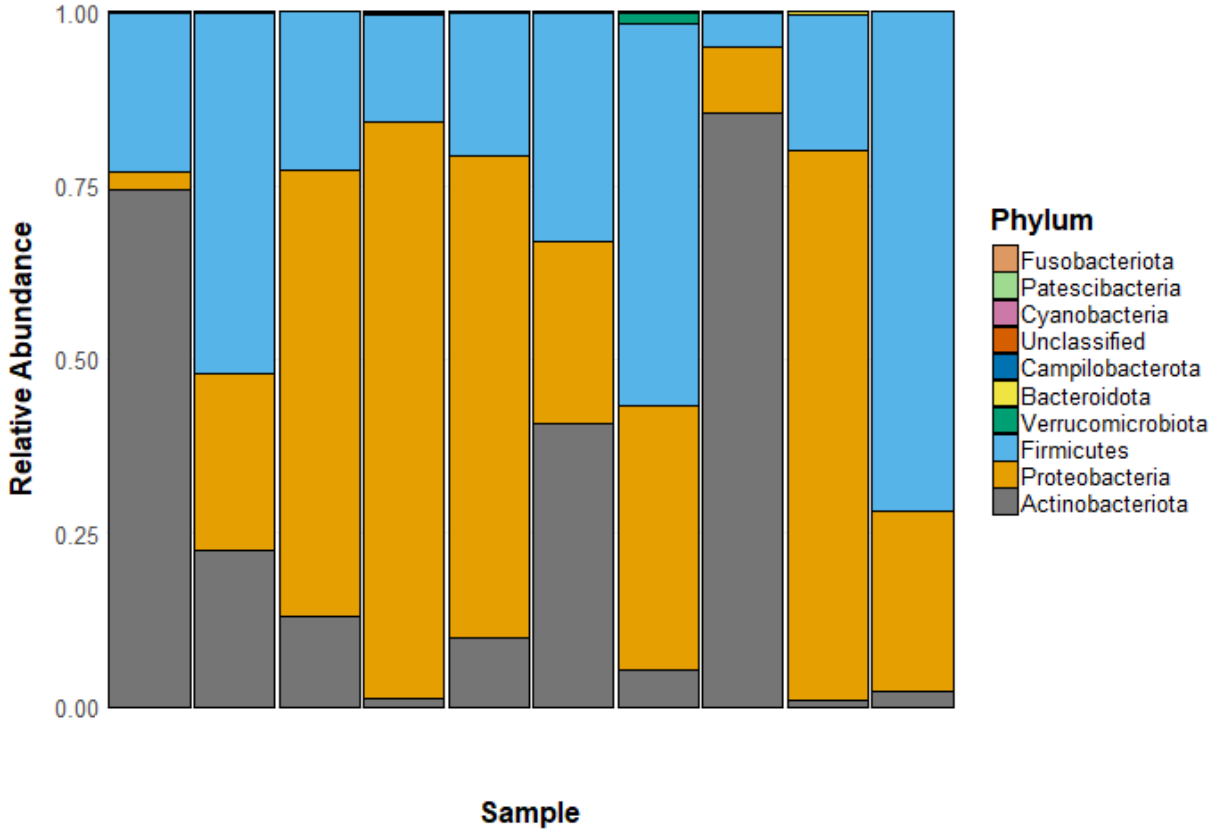


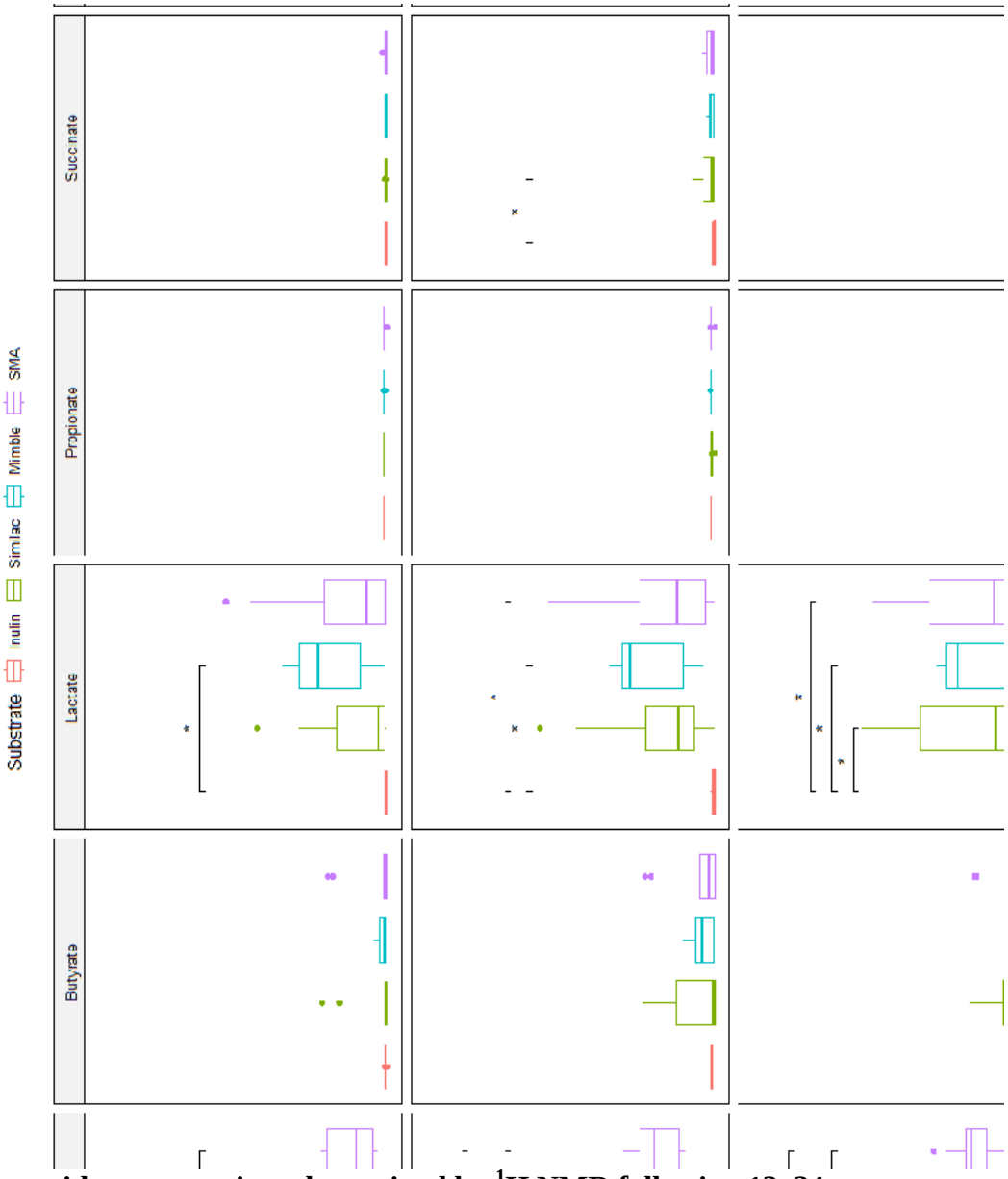
Figure 1. Participant-level phylogeny profile showing the Phylum level abundances of bacterial taxa determined by 16S sequencing at the start of the experiment (time = 0), prior to *in vitro* fermentation.

In vitro fermentation reveals substrate driven differences in SCFA production

In vitro fermentation experiments were established using a batch model colon fermentation system seeded with stool samples from each of the 10 SAM trial participants who were on standard nutritional and supportive treatments. Each of the fermentation vessels included either inulin, MIMBLE feed, SMA or Similac milk powders as substrates. Samples were taken at defined timepoints (with baseline samples, time = 0, taken prior to fermentation), and microbial metabolites were quantified using ^{1}H-NMR (Fig. 2, Table S1). Following up to 36 hours of

179 fermentation, all the substrates tested gave rise to production of SCFA's (p value = 0.007) and

180 other microbial metabolites, with significant differences identified between substrates.



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182 **Figure 2. Short chain fatty acid concentrations determined by ^{1}H NMR following 12, 24**

183 **and 36 hours of *in vitro* fermentation.** Metabolite differences were tested using ANOVA with a

184 post-hoc Tukey's test. Statistically significant differences are indicated with * p-value < 0.05, **

185 p-value < 0.01, *** p-value < 0.001, **** p-value < 0.0001. All SCFA concentrations are indicated in

186 mM.

The total SCFA production from the inulin substrate was significantly lower than from the other three substrates after 24h of fermentation (Fig. 2). The differences in total SCFA were mainly driven by acetate and butyrate production. Acetate was highest for the two milk powders, slightly lower for the MIMBLE feed and significantly lower for the inulin. Butyrate levels were similar between the MIMBLE, SMA and Similac substrates, but lower in the vessels with inulin. Propionate production was not significantly different across all substrates and timepoints, although it should be noted that propionate production was low in all the fermentations, and only modest increases from baseline were seen with the MIMBLE and SMA substrates. The clearest differences observed between the substrates were in the metabolic intermediates lactate and succinate. Lactate was produced in response to MIMBLE, Similac and SMA substrates from 12 h of fermentation onwards, while succinate showed delayed kinetics of formation, with the peak of metabolite production occurring between 12 and 24 h (Fig. 2, Table S1). Neither lactate nor succinate were produced in significant quantities from the fermentation of inulin. The absence of these metabolic intermediates would hinder the production of SCFA end products and may be reflected in the lower butyrate and acetate concentrations observed for inulin compared to the other substrates tested in this study (9, 31). It is likely that the low production of microbial metabolic intermediates reflects limited breakdown of inulin by the microbiota inoculated into these models. Evidence for limited breakdown of inulin can be derived from the ^{1}H NMR spectra of the fermentation media following 36h of fermentation (**Fig 3**). Several peaks are observed only in the inulin supplemented media which can be assigned to inulin, including the peaks at 4.3 and 4.1ppm which arise from protons in the fructose ring of inulin (32). These inulin peaks remain invariant at all the timepoints, suggesting that the inoculum used in these fermentations is

unable to degrade and utilize inulin as a substrate and it remains unmetabolized throughout the course of the fermentation experiments, unlike the three other substrates tested which were fermented at similar rates.

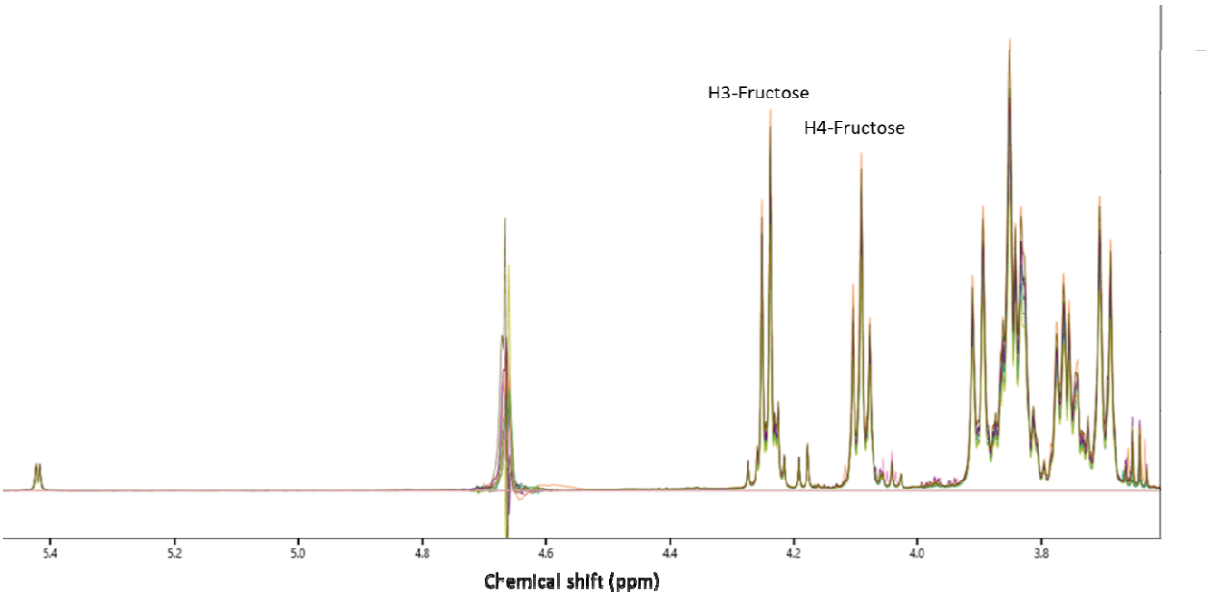

Figure 3. ^{1}H NMR spectra for fermentation media sampled from each of the inulin substrate fermentation vessels following 36h of fermentation. Peak assignments from Caleffi et al.(32) for the H3 and H4 protons in the fructose ring.

Substrates drive differences in microbiome composition

The microbial composition within the fermentation vessels (Fig. 4) changed over the time course of the experiment, depending on both time and substrate. Immediately following inoculation, the microbial community composition for each of the substrates was near identical (Fig. 4), with the 0 h timepoints, sampled immediately following inoculation, clustering very closely together on a PCoA plot (Fig. 4) reflecting the individual microbial composition of each donor. Following sampling at subsequent time points the microbiome compositions deviated from the composition at 0h, depending both on the substrate and the time point and by 48 h the microbial community

composition has moved away from that seen at baseline, although there were not significant differences observed between the substrates.

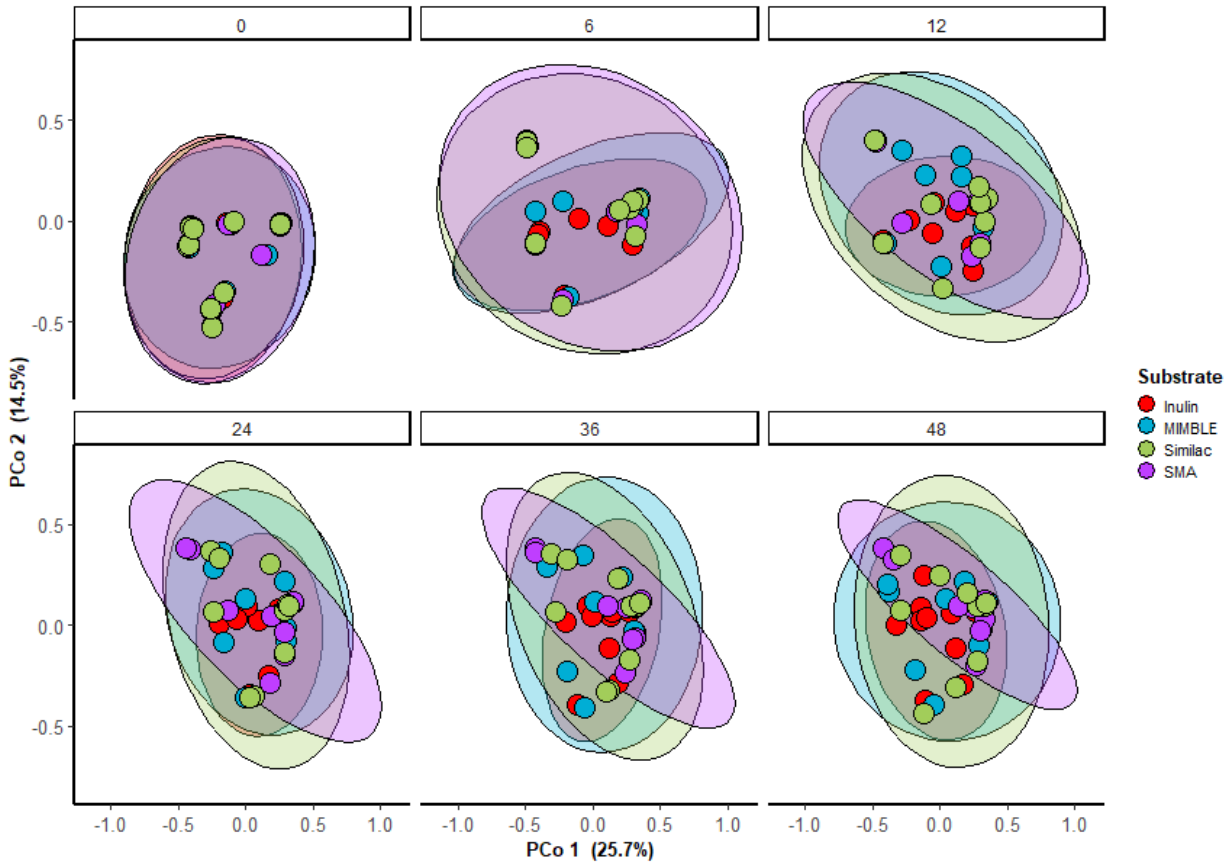

Figure 4. PCoA plot based on Bray-Curtis dissimilarity at ASV level for microbial communities coloured by substrate at 0, 6, 12, 24, 36 and 48 hours of fermentation.

Across all the substrates there was reduction in microbial diversity (Shannon diversity) observed over time in the models, although this did recover slightly at later time points (Fig. 5a). The greatest reduction in microbial diversity was observed in the Similac and SMA milk powders at the 12h time point. In contrast, the Shannon diversity in vessels inoculated with inulin was less reduced at 12h, and at subsequent time points, compared to the other substrates. The changes in

239 microbial diversity are reflected in the phylogeny profiles (**Fig 5b**), where the inulin remains
240 similar to baseline after 24 h fermentation, whereas the other three substrates see reductions in
241 Actinobacteriota and Proteobacteria, and increased Firmicutes abundance.

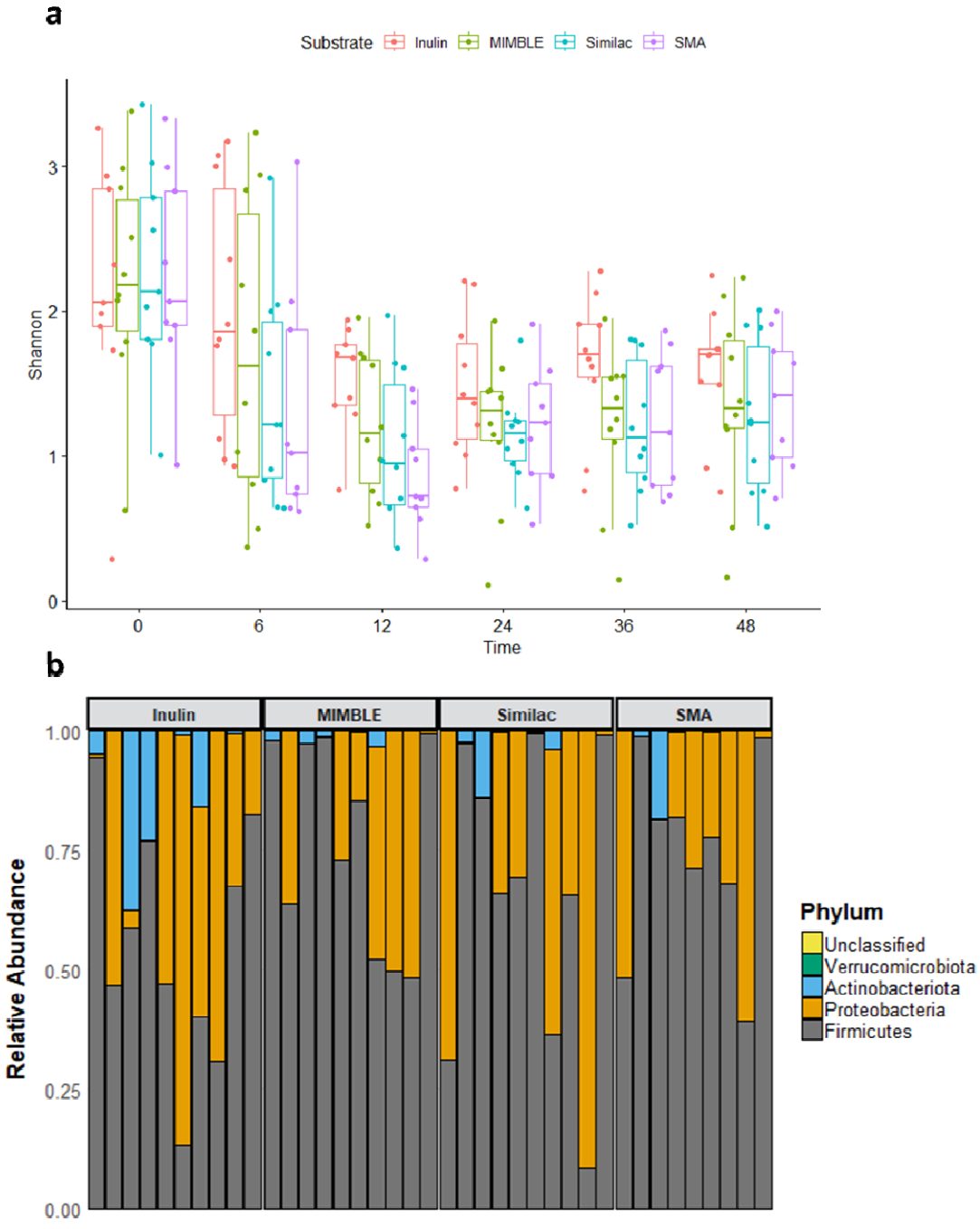

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Figure 5. Microbiome changes during fermentation. a. Changes in alpha-diversity (Shannon index) over time for each of the substrates during fermentation. b. Phylogeny plot showing phylum level taxa abundances for each participant fermenting each individual substrate following 24h of fermentation.

Discussion

The gut microbiome has recently emerged as an important target in the treatment of SAM, due to a series of studies indicating that SAM is associated with gut microbiome immaturity (15, 16, 33), leading to reduced production of beneficial microbial metabolites such as SCFA which are protective of the gut endothelium and are crucial to healthy gut function. In a clinical setting, the gut microbiome can be further damaged by the extensive use of antibiotics in treatment regimes. In several studies, reduced stool SCFA has been associated with increased mortality in children with SAM (27, 34). As a result, dietary interventions building on the standard WHO recommendations to include ingredients which specifically target the gut microbiome, such as chicory inulin and legume based ingredients have been developed and trialled in interventions with SAM patients (21, 26, 27, 35, 36).

In this paper we have adapted an *in vitro* model colon protocol to mimic the gut microbiome of SAM patients. The basal media used is a minimal media with only key vitamins and minerals for microbial growth, but no additional carbon sources reflecting the restricted diet of SAM patients. To establish the gut microbiota of the SAM patients in vitro, stool samples collected from the patients are seeded into the vessels. The stool samples were selected from patients who had been in clinical treatment for SAM for 7 days following a standard WHO treatment pathway, and therefore reflected the impact of SAM, standard WHO milk-based nutritional feeds and antibiotic treatment on the gut microbiota of SAM patients (27). This was reflected in the microbial composition of the samples which were inoculated into the model, which showed high

levels of Proteobacteria and very low levels of Bacteroidetes. The high abundance of Proteobacteria was also observed in the larger cohort that these samples were obtained from (27), where Proteobacteria levels were found to peak at 7 days following hospital admission, the point at which the samples in our study were taken. Low relative abundances of Bacteroidetes (<5% in all samples) were observed in these samples, consistent with observations in previous studies of infant malnutrition (15, 37).

Using this model, we demonstrate that inulin is almost completely unfermentable by the gut microbiota sampled from infants with SAM. In previous studies investigating *in vitro* fermentation of inulin by stool sampled from infants, there was a clear age dependent effect where the gut microbiome of very young infants is unable to ferment inulin, while older infants can partially ferment lower molecular weight inulin (24, 38). These studies, however, investigated much younger infants than the current study (median age 2.2 years). This may reflect the immaturity of the gut microbiome of children with SAM. In adults, short chain inulin's are fermented preferentially by species of the genus *Bifidobacteria* while longer chain inulin's are fermented preferentially by *Bacteroides* (39). The low levels of Bacteroidetes in the samples analysed in this study, and in children with SAM, may limit the fermentability of inulin in these groups. Recent animal model studies and an intervention study in children with SAM found similar results, with inulin failing to increase faecal SCFA or increase weight gain (25, 27). The mechanism underpinning this is not clear from the data presented in this study.

Bifidobacteria were observed to be more abundant in the fermentation vessels containing inulin compared to the other substrates, although the difference was not statistically significant (Fig. S1). However, *Bacteroides* species were not observed to increase in abundance in the inulin fermentation vessels (Fig. S2). The reasons for the lack of growth of *Bacteroides* species are not

known. In contrast, the 'MIMBLE' feed, which is enriched with legumes, and milk powders both with and without human milk oligosaccharides, were found to be fermentable. Animal studies have demonstrated that the human milk oligosaccharide (HMO) 2'-FL contributes to increased SCFA output and supported weight gain in a model of SAM (25). In the present study the fermentability of the two milk formulas tested was very similar, and the SCFA output of both was high. In contrast to HMO's, legumes are cheap and widely produced, therefore making an excellent candidate for addition to supplementary feeds for use in the treatment of SAM. In the present study we tested the MIMBLE feed, containing 10% chickpea flour, which was found to be fermented by the gut microbiota of children with SAM in an *in vitro* model, yielding a range of SCFAs. The chickpea enriched feed also resulted in a greater reduction in Proteobacteria abundance during fermentation than the other substrates tested. Several recent studies have identified that feeds containing chickpea flour have the potential to act as a food source for the gut microbiota of children with SAM, due to the diverse range of polysaccharides including pectins and arabinoxylans present in the cell wall polysaccharides of chickpea. These polysaccharides can be used to modulate the gut microbiome and promote the growth of beneficial bacteria in the immature gut microbiota of SAM patients (19, 35, 36). These results indicate the potential for the use of locally sourced plant-based foods, such as chickpeas, for use in recovery foods which can support SCFA production in the colon as effectively as HMO's (21, 35, 40).

In conclusion, in this study we have used an *in vitro* model of the gut microbiome of children with SAM to test the fermentability of four different substrates. Sequencing revealed that the microbiome of the stool samples had low diversity with high Proteobacteria and low Bacteroidetes abundance. We demonstrated that, while two milk powders and a legume-based

feed were fermentable and produced SCFA in the model, inulin was not fermented to a significant degree. This may reflect the limited microbial diversity, in particular Bacteroidetes needed to ferment longer chain inulin's. These results demonstrate that assumptions cannot be made regarding the fermentability of carbohydrates by the gut microbiota of children with SAM, and that results obtained in healthy childhood cohorts are not translatable to children with SAM. This is, therefore, an urgent need for future studies screening the fermentability of carbohydrates by the gut microbiota of children with SAM. The results presented in this paper provide insights useful for the development of therapeutic and complimentary feeds for use during treatment and recovery from SAM.

Materials and Methods

Substrates

Chicory inulin (catalogue no. I2255) was purchased from Sigma-Aldrich, (Gillingham, UK). Similac Pro-Advance with HMO was purchased from Amazon (UK) and SMA Pro 3 Toddler milk was purchased from Boots Pharmacists (UK). MIMBLE feed was prepared by Campden BRI as described in Walsh et al.(26) Each substrate (0.500 ± 0.005g, dry weight) was weighed into sterilized fermentation bottles (100mL) prior to start of experiment. Nutrient composition (Table S2) was approximated using Nutritics software (Ireland) or using manufacturer provided information.

Inoculum collection and preparation

Modifying Intestinal Integrity and Microbiome in Malnutrition with Legume-Based Feeds [MIMBLE] was a single centre (Mbale Regional Referral Hospital) randomised comparator trial

evaluating safety and feasibility of three feeding strategies (registered on https://pactr.samrc.ac.za as PACTR201805003381361) (26, 27). The protocol was approved by the ethics committees of Imperial College London (15IC3006) and Mbale Regional Referral Hospital (UG-IRC-012). Following parental written consent children were enrolled on day 1 post-admission following consent and followed for 28 days. The trial was conducted to the standards of ICH GCP. Children hospitalised with SAM (n=69) were screened for eligibility (one or more of mid-upper arm circumference (MUAC) <11.5cm, weight-for-height Z-score (WHZ) < -3 or Kwashiorkor) and randomized to either standard milk-feed F75 (n=18); inulin-supplemented standard feeds (InF: n=20) to cowpea supplemented standard feed CPF: n=20). Faecal samples were provided at 7 days following admission to hospital, from trial participants receiving the standard WHO F75/F100 feeding regime and other supportive therapies including antibiotics. Stools from children receiving legume-based feeds were not recruited to this study. Stool samples were frozen and stored at -80°C prior to use. Stool sample was diluted 1:10 with pre-warmed, anaerobic, sterile phosphate buffer saline (0.1M, pH 7.4) in a double meshed stomacher bag (500 mL, Seward, Worthing, UK) and homogenized using a Stomacher 400 (Seward, Worthing, UK) at 200 rpm for 2 cycles at 60 minutes each.

Batch fermentation

Fermentation experiments were performed with media adapted from Warren and colleagues (2018) (41). In brief, fermentation vessels (100 mL) each contained an aliquot (3.0 mL) of filtered faecal slurry, 82mL of sterilized growth medium, and substrate. The growth medium contained 76mL of basal solution, 5 mL vitamin phosphate and sodium carbonate solution, and 1 mL reducing agent. The composition of the solutions used in the preparation of the growth

360 medium is described in detail in Ravi et al. (42). A single stock (7 litres) of growth medium was
361 used for all vessels prepared for this experiment. Vessel fermentations were pH controlled and
362 maintained at pH 6.8 to 7.2 using 1N NaOH and 1N HCl regulated by a Fermac 260 (Electrolab
363 Biotech, Tewkesbury, UK). A circulating water jacket-maintained vessel temperature at 37°C. A
364 magnetic stirrer was used to keep mixture homogenous and the vessels were continuously
365 sparged with nitrogen (99% purity) maintaining anaerobic conditions. Media with no inoculum
366 was used as a blank whereas, chicory inulin, and SMA, Similac and chickpea supplemented feed
367 with inocula were the experimental conditions evaluated. Samples were collected at 0 (~5 min
368 post inoculation), 6, 12, 24, 36 and 48 hours after inoculation. The biomass from 1.8 mL aliquot
369 of sample was concentrated by refrigerated centrifugation (4°C; 10,000 g for 10 min),
370 supernatant removed, and then stored at -80°C until DNA extraction. The supernatants were
371 removed to a fresh tube and stored separately at -20°C for ^{1}H NMR metabolomic analysis.
372
373 *DNA extraction*
374 Concentrated biomass pellets were resuspended in 500 µL (samples collected at 0 and 6 hr) and
375 650 µL (samples collected at 12 and 24 hr) with sterile, nuclease-free water (Sigma-Aldrich,
376 Gillingham, UK) which was chilled (4°C). The resuspensions were frozen overnight at-80°C,
377 thawed on ice and an aliquot (400uL) was used for bacterial genomic DNA extraction.
378 FastDNA® Spin Kit for Soil (MP Biomedical, Solon, US) was used according to manufacturer's
379 instructions which included two bead-beating steps of 60s at a speed of 6.0m/s (FastPrep24, MP
380 Biomedical, Solon, US). DNA concentration was determined using the Quant-iT™ dsDNA
381 Assay Kit, high sensitivity kit (Invitrogen, Loughborough, UK) and quantified on a FLUOstar
382 Optima plate reader (BMG Labtech, Aylesbury, UK).

383

384 *Library preparation and 16S rRNA sequencing*

385 Extracted genomic DNA was normalised to 5ng/µl with elution buffer (10mM Tris-HCl). A PCR
386 master mix was made up using 4 ul kapa2G buffer, 0.4 µl dNTP's, 0.08 µl Polymerase, 0.4 µl 10
387 µM forward tailed specific primer, 0.4 µl 10 µM reverse tailed specific primer and 12.72 µl PCR
388 grade water (contained in the Kap2G Robust PCR kit Sigma Catalogue No. KK5005) per sample
389 and 18 µl added to each well to be used in a 96-well plate followed by 2 µl of DNA and mixed.
390 Specific PCR conditions were 95□C for 5 minutes, 30 cycles of 95□C for 30s, 55□C for 30s
391 and 72□C for 30 seconds followed by a final 72□C for 5 minutes. Following PCR, a 0.7X SPRI
392 clean-up was performed using KAPA Pure Beads (Roche Catalogue No. 07983298001) eluting
393 the DNA in 20ul of water. A second PCR master mix was made up using 4 ul kapa2G buffer, 0.4
394 µl dNTP's, 0.08 µl Polymerase, and 6.52 µl PCR grade water per sample and 11 µl added to
395 each well to be used in a 96-well plate. 2 µl of each P7 and P5 of Nextera XT Index Kit v2 index
396 primers (Illumina Catalogue No. FC-131-2001 to 2004) were added to each well. Finally, the 5
397 µl of the clean specific PCR was added and mixed. The second PCR was run using 95□C for 5
398 minutes, 10 cycles of 95□C for 30s, 55□C for 30s and 72□C for 30 seconds followed by a final
399 72□C for 5 minutes. Final libraries were quantified by Qubit and equimolar pooled together. A
400 single 0.7X SPRI clean-up was performed on the pool. A final Qubit and sizing on High
401 Sensitivity D1000 Screen Tape (Agilent Catalogue No. 5067-5579) using the Agilent
402 Tapestation 4200 was done to calculate the final library pool molarity. The pool was run at a
403 final concentration of 10pM on an Illumina MiSeq instrument using MiSeq® Reagent Kit v3
404 (600 cycle) (Illumina Catalogue FC-102-3003) following the Illumina recommended
405 denaturation and loading recommendations which included a 20% PhiX spike in (PhiX Control

v3 Illumina Catalogue FC-110-3001). The raw data was analysed locally on the MiSeq using MiSeq reporter.

Bioinformatics analysis

Raw demultiplexed forward and reverse reads were processed using the methods implemented in QIIME2 version 2020.11 with default parameters unless otherwise stated (43). DADA2 was used for paired-end joining, quality filtering, denoising and calling Amplicon Sequence Variants (ASV's) using the QIIME dada2 denoise-paired method (44). The first 13 bp and the final 50 bp were trimmed before merging due to lower quality scores. The ASV's were aligned and used to calculate a phylogenetic tree using the QIIME function phylogeny align to tree (45, 46). Taxonomic assignment of the ASV's was performed using the QIIME naïve Bayesian classify Scikit-learn using the Silva 99% OTU database (47-49).

The raw sequencing data had a median of 43,909 reads per sample. Following filtering, denoising and merging this was reduced to 37,185 reads per sample. After the removal of chimeric sequences this was further reduced to 32,378 reads per sample. A total of 855 ASV's were identified across all the samples. Samples with fewer than 1000 reads were excluded from subsequent analysis and the data were rarefied to 17,599 reads. Downstream analysis and visualization was carried out using the PhyloSeq (50) and PhyloSmith (51) packages in the R software package (version 4.0.3), including alpha and beta diversity metrics.

^{1}H NMR metabolomics

The samples containing the supernatant from the fermentation media were centrifuged (3,000 x g, 3 min) and 400-µL aliquots were pipetted directly into NMR tubes (Norell® Standard

Series™, 5 mm), followed by the addition of 200 µL of phosphate buffer (NaH_2PO_4 (21.7 mM), K_2HPO_4 (82.7 mM), NaN_3 (8.6 mM), 3-(trimethylsilyl)-propionate-d_4 (TMSP, 1.0 mM), prepared in D_2O). Spectra were collected on a Bruker NEO 600 MHz spectrometer equipped with a cryoprobe, at a ^1H frequency of 600 MHz. All experiments were acquired at room temperature, using Bruker's 'noesygppr1d' pulse sequence, with a minimum of 64 scans. A 90° pulse length of 11.09 µs was set for all samples with a mixing time of 0.01 s, acquisition time of 2.62 s, relaxation delay of 4 s, featuring selective pre-saturation (1.0 ms) on the residual H_2O peak frequency during relaxation delay and mixing time for effective solvent suppression. Spectra were referenced using the TMSP peak (0.0 ppm). The metabolites were quantified using the NMR Suite v7.6 Profiler (Chenomx®, Edmonton, Canada).

Statistical analysis

Statistical analysis of the metabolite data was carried out in R (version 4.2.0) and SPSS. ANOVA test with post-hoc Tukey's HSD was used to test for significant differences in metabolite concentrations between substrates and over time.

Data availability

The raw sequencing data used in this manuscript can be accessed through the NCBI SRA project number PRJNA1080518.

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