The gut microbiome of 7-37 month old children from The Gambia shows the development of a distinct non-industrial Prevotella-based trophic network

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The gut microbiome of 7-37 month old children from The Gambia shows the development of a distinct non-industrial *Prevotella*-based trophic network

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ABSTRACT

Distinctive bacterial trophic networks exist in the gut microbiota of individuals in industrialized and non- industrialized countries. To study the development of these networks, we investigated the gut microbiota of 7-37 month old children living in rural Gambia (616 children, 1407 stool samples, stratified by 3-month age groups). We found that child age was the largest discriminating factor between samples, and that anthropometric indices WAZ, HAZ, and WHZ, collection timepoints, and iron supplementation did not significantly influence the gut microbiome in this data set. *Prevotella copri*, *Faecalibacterium prausnitzii*, and *Prevotella stercorea* were the most abundant species (35%, 11%, and 7%, respectively). Distinct bacterial trophic network clusters were identified, centered around *Prevotella stercorea* and *Faecalibacterium prausnitzii*, which were found to develop steadily as the gut microbiome matured. The *Prevotella stercorea* trophic network cluster is distinct from those found in individuals in industrialized countries and therefore this dataset, set within a critical developmental timeframe, provides an excellent opportunity to understand the influence of a high fiber, low-protein diet on the development of a *Prevotella*-enriched gut microbiome.

- 41 **Abbreviations:**
- 42 16S V1V2 = bacterial 16S ribosomal RNA variable region 1 and 2
- 43 CSS = cumulative-sum scaling
- 44 DNA = deoxyribonucleic acid
- 45 mths = months
- 46 PCoA = Principal Coordinate Analysis
- 47 TSS = Total Sum Scaling
- 48 PERMANOVA = **PER**mutational **M**ultivariate **AN**alysis **Of** **VA**riance
- 49 PCA = Principal Component Analysis
- 50 WAZ = Weight-for-Age Z Score
- 51 HAZ = Height-for-Age Z Score
- 52 WHZ = Weight-for-Height Z-score
- 53 FDR = False Discovery Rate (correction method for multiple testing)

54 INTRODUCTION

55 One of the largest characterizations of bacterial species present in stool samples to date was
56 from a study from 531 humans representing healthy Amerindians from the Amazonas of
57 Venezuela, residents of rural Malawian communities, and inhabitants of metropolitan areas
58 in the USA ¹. This study was of particular value because it included infants, children,
59 teenagers and adults. Striking differences were observed between USA city residents and
60 rural populations from the Amazonas and Malawi. Species belonging to the genus *Prevotella*
61 were one of the most dominant differences in the USA versus non-USA comparisons. The
62 importance of *Prevotella* species as a discriminatory taxon between the gut microbiota of
63 people living in industrialized vs. non-industrialized societies was also highlighted in a study
64 comparing children in West Africa (Burkina Faso) and Europe (Italy) ². In this study, the Italian
65 children's gut samples were dominated by *Bacteroides* and the children's gut samples from
66 Burkina Faso were again dominated by *Prevotella*.

67 The term enterotype has been proposed to describe distinctive bacteriological patterns of
68 human gut microbiota. Several studies have highlighted distinctive enterotypes ^{3,4}, one
69 dominated by *Bacteroides* or *Clostridiales* species, which is present more commonly in
70 industrialized countries (such as America and Europe) and one dominated by *Prevotella*
71 species, which is more commonly detected in countries with non-industrialized lifestyles ^{5 6}.

72 In the original paper describing enterotypes the human gut microbiome was classified into
73 three groups based on its composition ⁵. However, the concept of enterotypes has been
74 critically re-appraised in the last few years. For example, a combined analysis of five studies
75 with 747 samples across five continents could not confirm the enterotypes hypothesis as
76 originally proposed ⁷. The same group concluded that the abundances of

77 *Prevotella* and *Bacteroides* do not represent consistent microbial communities within the

“enterotypes”, and that “The gut microbiomes of Americans and Europeans having a Western diet tend to be dominated by *Bacteroides* and Clostridiales, while rural populations with a high fiber, low-protein diet tend to be dominated by *Prevotella*”. Other groups have also re-analysed the enterotypes hypothesis^{8,9} and these detailed cluster and statistical analyses concluded that only two bacterial communities constitute our gut microbiota, one dominated by *Prevotella* and one by *Bacteroides* or species of the *Firmicutes*^{9–13}. In the current study we utilised data from an iron intervention trial in The Gambia in West Africa¹⁴. Consisting of 616 children, this is the largest paediatric cohort studied thus far in a critical time window for gut microbiome development (7 to 37 months) in a non-industrialized environment. This exploratory study provides important new insights into the development of trophic networks, including those centred on *Prevotella* species. Such analyses have to date been mostly performed on *Prevotella*-poor cohorts from industrialized countries using combinations of adults and children, or to some degree, on smaller paediatric groups in non-industrialized countries¹⁵.

Study setting for the Iron Intervention Trial in The Gambia in Africa

The study was sponsored by the London School of Hygiene and Tropical Medicine (LSHTM) and conducted at the Medical Research Council (MRC) Unit The Gambia at LSHTM. The Upper River Region (URR) from which the cohort was recruited has an approximate population of 200,000, and only one major town, Basse, and is otherwise typical of rural sub-Saharan Africa. The URR has the highest under-5 mortality rate in the country (92 deaths per 1000 livebirths), the highest percentage of severely malnourished children (7-11%), and the highest prevalence of malaria and anaemia in children under 5 years (4.5% and 82.5% respectively)¹⁶. The study area included 45 villages in the Wuli and Sandu districts, situated in the north bank of the river Gambia, approximately 400 km east of the capital Banjul, with a population of approximately

2800 children aged 6-35 months. All communities have access to borehole tap water at central places. Study specimen samples were collected at one of the study health clinics: Yorrobawol Health Centre, Darsilami Community Health Post, Konkuba Community Health Post, Taibatu Health Post and Chamo Health Centre.

Nutritional and diet information

The Gambia is a low-income country in West Africa, where food availability and nutritional status in rural areas are poor. In rural areas, food availability and nutritional status are strongly influenced by seasonality, and a chronically marginal diet is exacerbated by a “hungry season” (July-September), when food stocks from the previous harvest season are depleted. Infants in rural Gambia are breast-fed to 2 years of age, with fewer than half of infants being exclusively breast-fed to 6-months of age as per WHO recommendation¹⁷. The first foods introduced from 3 months of age are thin gruels made from only cereal, water (occasionally cow milk is added), salt, and sugar, and are of a low energy and fat content. A thicker porridge made from rice and pounded groundnuts is sometimes administered. Cow milk alone is infrequently given to infants < 1 year of age; only 57% of infants receive it more than once a week, although it is provided often to children in the second year of life. From 6 months, infants start to share the family food bowl, the most common meals consisting of boiled rice and a sauce made from groundnuts or leaves. Dried fish may be added to sauces in very small quantities, but fresh fish is not given to infants before 9 months¹⁸.

RESULTS

Sampling framework and sample characteristics

We performed 16S rDNA amplification and sequencing on a total of 1546 samples of which after quality filtering, oligotyping, contaminant removal, and taxonomic classification, 1407 samples from 616 patients were available for detailed analysis. A description of the patient and sampling framework is available in the supplementary Materials and Methods. The samples were collected at three different timepoints during an iron intervention trial in The Gambia ¹⁴ and were used for detailed developmental and bacterial trophic network analysis. The children were initially split into three age groups (7 - <12 mths, 12 - <24 mths, and 24 – 37 mths at the time of enrolment) and subsequently analyzed in detail in 11 age groups. The mean age at enrolment was 22 months (7 – 37 months) and 48% of the children were female (**Table 1**). At the end of study some children were up to 40 months old due to a day 85 stool collection after initial enrolment. Children with severe malnutrition (N=88, 6% of children who were screened) (z-scores for length/height-for-age (HAZ), weight-for-age (WAZ), weight-for-length/height (WHZ) ≤ -3 standard deviations (SD) were excluded from the trial. Mean z-scores for the included children were around -1 (**Table 1**).

Table 1. Child cohort available for microbiome analysis

Patient characteristics		All age group	youngest age 7 - <12 mths*	middle age 12 - <24 mths*	oldest age 24 - 37 mths*
Children number		633	87	266	280
Age min-max (mean)		7-37 (22)	7-11 (9)	12-23 (17)	24-37 (30)
Female		305 (48%)	38 (44%)	139 (52%)	128 (46%)
Weight (kg), mean (SD)		10 (1.7)	7.9 (1.0)	9.5 (6.1)	10.7 (2.0)
Height (cm), mean (SD)		81.6 (6.2)	70.7 (3.8)	75.85 (11.8)	83.3 (14.3)
WAZ, mean (SD) (N = 1373)		-0.95 (0.85)	-0.47 (0.88)	-0.94 (0.83)	-1.09 (0.80)
HAZ, mean (SD) (N = 1373)		-0.85 (0.94)	-0.38 (0.90)	-0.71 (0.93)	-1.1 (0.89)
WHZ, mean (SD)n (N = 1376)		-0.77 (0.85)	-0.62 (0.90)	-0.88 (0.84)	-0.72 (0.84)
Sample characteristics		1407	159	608	640
Timepoint	Day 1	522 (37.1%)	72	238	212
	Day 15	413 (29.4%)	55	177	181
	Day 85	472 (33.5%)	32	193	247
Treatment	Treatment	950 (67.5%)	106	408	436
	Placebo	457 (32.5%)	53	200	204
Geographic location of health facility	Chamoi	156 (11.1%)	24	86	46
	Darsilami	254 (18.1%)	28	99	127
	Kuwonkuba	133 (9.5%)	18	62	53
	Taibatu	416 (29.6%)	37	163	216
	Yorrobawol	448 (31.8%)	52	198	198

Abbreviations: min: minimum, max: maximum, mths = months, HAZ: height-for-age Z score, WAZ: weight-for-age Z score, WHZ: weight-for-height Z score, SD: standard deviation

* age at enrolment

In this large study only 15/633 patients (2.4%) had received antibiotics within the collection time frame. The reason for receiving antibiotics and the time frame is explained in detail in the online supplementary section 4.8. We performed multivariate PCoA analysis for the middle age group (12 - <24 mths) (**Supplementary Figure 1.a**) and old age group (24 – 40 mths) (**Supplementary Figure 1.b**) patients comparing the microbiome from the antibiotic treatment samples with the non-antibiotic samples from the day 85 collection time points. PCoA analysis revealed that all antibiotic treatment samples clustered within the expected range of the other samples and did not present as outliers. ANCOM testing¹⁹ revealed no

significant differences in microbial composition between antibiotic positive and negative samples in the middle age group (data not shown). In the old age group, only one species was statistically significantly depleted in the antibiotic treated group. *Holdemanella bififormis*, with an overall abundance of 0.416 % in all 1407 samples, were detected at a mean abundance of 0.069 in the antibiotic positive group and at a mean abundance of 0.65 in the antibiotic negative group, **Supplementary Figure 1.c** (ANOVA uncorrected P value 7.5e-06). As antibiotic use had not caused obvious microbiome composition distortions the analysis was continued with all samples included. The limited or absent effect of antibiotics was most likely due to the large time frame (min 23 days, max 65 days, mean 41 days) between antibiotics being given and the day 85 stool collection.

Supplementary Figure 1. Multivariate PCoA did not identify different clustering of patients treated with antibiotics

1.a Middle age groups

1.a Old age groups

1.c Species difference in the old age groups between children on antibiotics (yes) and non antibiotics (no)

Supplementary Figure 1. Multivariate PCoA did not identify different clustering of patients treated with antibiotics

The day 85 samples from the middle age group (**Sup Fig 1.a**) and from the old age group (**Sup Fig 1.b**) were analysed by PCoA to expose whether children on antibiotics treatment clustered differently from the children without antibiotics. *Holdemanella biformis* was the only significantly different species between antibiotic treated and non-treated children at the old age group (**Sup Fig 1.c**)

As a first step we conducted multivariate and univariate analysis to identify whether children from the treatment arm (iron supplementation) and from the placebo group could be analysed together (**Supplementary Figure 2**). Multidimensional scaling using Principal Coordinates Analysis (PCoA) did not cluster children differently based on iron supplement treatment or placebo (**Supplementary Figure 2.a**). Volcano Plot analysis identified only one species which was statistically different between the two sample groups (red dot in **Supplementary Figure 2.b**). This single species was subsequently identified by the Kruskal-Wallis Rank test as *Megamonas funiforms* which was detected at higher abundance in the treatment group with a false discovery rate (FDR) corrected P value of 0.0099 (**Supplementary Figure 2.c**). For a second univariate statistical test we used the ALDEx2 test. With the ALDEx2 test no species were identified as statistically significantly different between the treatment and placebo group. The top species was again *Megamonas funiforms* with a Benjamini Hochberg corrected P value of 0.18 for the Welch's t-test and 0.32 for the Wilcoxon test (**Supplementary Figure 2**, ALDEx2 table). In addition, a Permutational Multivariate Analysis of Variance (PERMANOVA) test did not identify a statistically different beta diversity between the treatment and placebo groups (data not shown).

Supplementary Figure 2. Multivariate PCoA and univariate statistic did not identify a different microbiome between children on iron supplement treatment and placebo

Kruskal-Wallis Rank Test

Taxa (N = 219)	P (rank test)	Adjusted P (Bonferroni)	FDR	treatment mean	Placebo mean	treatment median	Placebo median	Fold Change
Megamonas_funiformis_0.308	0.000045	0.0099	0.0099	0.38	0.14	0	0	2.635
Phascolarctobacterium_succinatutens_0.284	0.0015	0.33	0.13	0.34	0.21	0	0	1.617
Lactobacillus_delbrueckii_0.016	0.002	0.44	0.13	0.022	0.0036	0	0	6.211
Coprococcus_sp.HPP0074_0.029	0.0023	0.5	0.13	0.033	0.025	0	0	1.302
Lactobacillus_mucosae_0.1	0.0049	1	0.18	0.065	0.16	0	0	-2.512
Cetobacterium_somerae_0.152	0.005	1	0.18	0.19	0.09	0	0	2.07
Lactobacillus_gasseri_0.072	0.0095	1	0.29	0.021	0.17	0	0	-8.397
Erysipelatoclostridium_ramosum_0.092	0.012	1	0.29	0.1	0.075	0.005	0	1.354
Allisonella_histaminiformans_0.421	0.012	1	0.29	0.4	0.45	0.06	0.13	-1.129
Anaerostipes_sp.5_0.27	0.014	1	0.3	0.29	0.24	0.14	0.1	1.2

ALDEx2 test

Taxa (N = 219)	we.ep	we.eBH	wi.ep	wi.eBH	Treatment.median	Placebo.median
Megamonas_funiformis_0.308	0.003671115	0.188101982	0.01316173	0.320446849	0	0
Phascolarctobacterium_succinatutens_0.284	0.022874477	0.33851409	0.021533292	0.359167159	0	0
Allisonella_histaminiformans_0.421	0.012571362	0.341245546	0.011244605	0.379086281	11	24
Anaerostipes_sp.5_0.27	0.087207799	0.568860721	0.015656061	0.43363021	25	19
Escherichia_coli_3.505	0.084030385	0.60753828	0.047735245	0.533137991	125.5	174
Lactobacillus_mucosae_0.1	0.106601785	0.433416708	0.154709086	0.546906955	0	0
Lactobacillus_salivarius_0.077	0.099588445	0.442231554	0.142775321	0.52718634	0	0
Lactobacillus_fermentum_0.03	0.11175917	0.486656866	0.13400698	0.557791069	0	0
Coprococcus_sp.HPP0074_0.029	0.100757862	0.518303646	0.058450184	0.492780291	0	0
Weissella_confusa_0.097	0.06207127	0.518328589	0.057128628	0.522893692	0	1

Multidimensional scaling using Principal Coordinates Analysis (PCoA) did not cluster children differently based on iron supplement treatment and placebo (Sup fig 2.a). Volcano Plot only identified one species which was statistically different between the two sample groups (red dot in Sup fig 2.b). The only statistically significant species identified by the Kruskal-Wallis Rank test was *Megamonas funiformis* which was detected at higher abundance in the treatment group with a false discovery rate (FDR) corrected P value of 0.0099 (Sup fig 2.c). For the second univariate statistical test we used the ALDEx2 test. The ALDEx2 test used Bayesian methods to infer technical and statistical error. The ALDEx2 test generated the following output:

we.ep = the expected P value of the Welch's t-test, we.eBH = the expected value of the Benjamini Hochberg corrected P value for the Welch's t-test, wi.ep = the expected P value of the Wilcoxon test, wi.eBH = the expected value of the Benjamini Hochberg corrected P value of the Wilcoxon test. No taxa were statistically significantly implicated in either of the two sample groups with a Benjamini Hochberg corrected P value < 0.05.

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Because of the highly similar bacterial compositions of the treatment and the placebo groups we considered the entire cohort to be highly homogenous and thus all samples were subsequently analysed together as one group. Thus, while this gut microbiome data set does not inform on the case/control aspects of the original study, it does represent an excellent opportunity for understanding the gut microbiome development over time in these African children within this critical gut microbial developmental timeframe.

Bacterial changes were observed between the earliest and latest sampling timepoints within individuals.

We used Mixed Effect Regression modelling (MERM) to analyze samples from paired repeated timepoints. 682 paired samples were available from 341 patients with a repeated sample collection from D1 and D15, 758 paired samples were available from 379 patients with a repeated sample collection from D1 and D85, and 624 paired samples were available from 312 patients with a repeated sample collection from D15 and D85. (Table 2).

Table 2. Longitudinal repeat sample analysis

Number of paired samples (patients)		Number of significant species with FDR P value < 0.05	MERM with diversity indexes FDR P value reported			
			Richness	Chao1	Fisher's Alpha	Simpson
D1 + D85	758 (379)	8	0.0017	6.00e-05	0.0014	1
D15 + D85	624 (312)	2	0.15	0.0069	0.64	1
D1 + D15	682 (341)	0	0.29	0.056	1	1

The largest differences were observed between D1 and D85 sampling timepoints for which eight species were statistically significantly different between the two timepoints with FDR corrected P value < 0.05. Six of the eight species increased in abundance from D1 to D85. These species and total cohort abundance in % were *Erysipelotrichaceae sp.* (0.32%), *Ruminococcaceae sp.* (0.23%), *Ruminococcus lactaris* (0.09%), *Streptococcus equinus* (0.9%),

Ruminococcus torques (0.41%) and *Pseudobutyrvibrio ruminis* (0.57%) (Supplementary Figure 3 a to 3). Two of the eight species decreased from D1 to D85 with *Bifidobacterium* being the most abundant species (4.12) followed by *Allisonella histaminiformans* (0.42%) (Supplementary Figure 3 g and h). The alpha diversity indexes for Richness, Chao1, and Fisher's Alpha were also significantly higher in the D85 samples compare to the D1 samples (Supplementary Figure 3 i to l).

Significant differences between the young, middle, and old age group samples

Beta diversity analysis (between group analysis) was conducted to identify if the microbial composition between the young, middle, and old groups differs from each other. The Bonferroni corrected P-value from the PERMANOVA test between the three different age groups was 0.0003 and the calculated F statistic was between 35 and 125 for the species taxon, between 46 and 163 for the genus taxon, and between 62 and 238 for the family taxon (Supplementary Table 1). For all three taxonomic levels, the F statistic was highest between the young and old age groups.

Supplementary Table 1. Pairwise PERMANOVA for taxon species, genera and family with age group variables

species and age group			genus and age group			family and age group		
Bonferroni corrected P value	Young	Middle	Bonferroni corrected P value	Young	Middle	Bonferroni corrected P value	Young	Middle
Old	0.0003	0.0003	Old	0.0003	0.0003	Old	0.0003	0.0003
Young		0.0003	Young		0.0003	Young		0.0003
F statistic	Young	Middle	F statistic	Young	Middle	F statistic	Old	Middle
Old	125.3	35.28	Old	163.3	46.05	Old	237.6	84.54
Young		53.11	Young		65.01	Young		62.27

Supplementary Table 1. Pairwise PERMANOVA for taxon species, genera and family with age group variables for young, middle, and old age groups

The PERMANOVA test-confirmed differences between young, middle, and old age were also analysed using PCoA which shows a different microbial population between each of the three

age groups at the species (**Figure 1.a**), genus (**Figure 1.b**), and family (**Figure 1.c**) level. As expected, the young and old age groups were separated the furthest apart from one another while the middle age group clustered between them. It also showed that the greatest separation was observed at the lowest (species) taxonomic resolution. We also plotted all species, genera, and families with a minimum abundance of 0.5% across all samples to show which taxa were most responsible for the separation. At the species level, these included 22/491 species encompassing 82% of all bacterial 16S reads (**Figure 1.a**). At the genus level these included 26/216 genera which included 89% of all reads (**Figure 1.b**) and at the family level these include 18/71 families which included 97% of all reads (**Figure 1.c**). In our analysis, age was the only contributing factor which differentiated the gut microbiomes of samples in this cohort. Further detailed analysis was therefore conducted to investigate the maturation of the gut microbiome in this large rural community from The Gambia in West Africa during the first three years of life based on age, split into 11 age groups (3-month intervals).

Figure 1. PCoA clustering for species, genera, and family separated into 3 age groups

Figure 1. PCoA clustering for species, genera, and family separated into 3 age groups

Principal co-ordinate analysis (PCoA) was conducted with all 1407 samples and highlights a

clear separation of samples based on three age groups at the species taxonomic level (Figure

1.a), the genus taxonomic level (**Figure 1.b**), and the family taxonomic level (**Figure 1.c**). The column graphs next to the PCoA plots show the relative abundance of the top species with a minimum abundance of 0.5% for the species taxon (n=22), for the genus taxon (n=26), and for the family taxon (n=18). All *Bacteroides* species and all *Bifidobacterium* species were combined.

Significant changes in the bacterial alpha diversity over time from 7 to 40 months of age

To identify changes over time in the whole data set, we split the data into 11 age groups, separated by three-month intervals, and analyzed alpha diversity. The Fisher's alpha parameter indicated a statistically significant increase in alpha diversity from the youngest age group (7-9 months) to the 34-36 months age group (Kruskal-Wallis $P < 0.0001$) (**Figure 2.a**). The Simpson's index was also statistically significantly different between the 11 age groups, but an upwards trend as seen by the Fisher's alpha was not observed (Kruskal-Wallis $P = 0.0002$) (**Figure 2.b**). Both the richness estimator index Chao1 (**Figure 2.c**) and the observed richness (**Figure 2.d**) increased over time and this increase was highly significant for both tests (Kruskal-Wallis $P < 0.0001$ for both).

Figure 2. Alpha diversity indexes for 11 age groups

Figure 2. Alpha diversity indexes for 11 age groups

Alpha diversity was measured by two different diversity indexes and by two different richness indexes. **Figure 2.a** The Fisher's alpha diversity test, **Figure 2.b** Simpson's diversity test, **Figure 2.c**, Chao1 estimated richness, **Figure 2.d** Observed richness

Maturation of the gut microbiome in a rural community from The Gambia during the first three years of life

We used the non-parametric Kruskal-Wallis rank test to identify significantly differentially abundant bacterial taxa in the 11 age groups (3-month intervals), (**Supplementary Table 2**). From the Kruskal-Wallis rank test we report the uncorrected and the false discovery rate (FDR) corrected P value, as well as the mean and median abundancies for the different taxa. In the 11-age-group comparison 301/488 taxa were statistically different between the age groups of which a large majority (231/301) had a low abundance (<0.1%). The top 70 significantly different species with a minimum abundance of 0.1% include 92.5% of all sequence reads. *Prevotella copri* was the most abundant species, with an average abundance in all samples of 35.2% of all reads. The second and third most abundant species were *Faecalibacterium prausnitzii* (11.4%) and *Prevotella stercorea* (7.1%), respectively. These three species accounted for a total of 53.6% of all reads (**Supplementary Tables 2**, first three species entries in column "Taxa"). All *Prevotella* species combined accounted for 27%, 44%, and 48% of all reads, in the young, middle, and old age groups respectively. The maturation pattern over time of the top ten bacterial taxa, shown either at the species or genus level, with a minimum abundance of 1%, which covers 73.2% of all bacterial 16S reads, are shown in **Figure 3**. These ten bacterial groups are from four major bacterial phyla namely,

310 **Bacteroidetes**; *Prevotella copri* (35.2%), *Prevotella stercorea* (7.6%), *Bacteroides* (4.6%),
 311 *Paraprevotella xylaniphila* (2%); **Firmicutes**; *Faecalibacterium prausnitzii* (11.4%),
 312 *Streptococcus salivarius* (1.1%); **Actinobacteria**; *Bifidobacterium* (4.1%); and **Proteobacteria**;
 313 *Escherichia coli* (3.5%), *Succinivibrio dextrinosolvens* (2.4%), *Sutterella wadsworthensis* (1.8%).
 314 The maturation pattern was investigated with two different normalized datasets.
 315 The abundance of *Faecalibacterium prausnitzii*, *Prevotella stercorea*, *Succinivibrio*
 316 *dextrinosolvens*, and *Paraprevotella xylaniphila* increased steadily over time which can be
 317 seen clearly in the 11 age groups dataset. However, *Bacteroides*, *Bifidobacterium*, *Escherichia*
 318 *coli*, *Sutterella wadsworthensis*, and *Streptococcus salivarius* show the reverse pattern.
 319 *Prevotella copri*, the most abundant bacterium, reached a very high abundance level (~40%
 320 soon after the age of 12 months and maintained its dominance after that. An interesting
 321 observation was the very far from normal distribution within development periods of
 322 *Prevotella stercorea*, *Succinivibrio dextrinosolvens* and *Paraprevotella xylaniphila*. These
 323 species co-developed in a distanced bivariate distribution compared to the other dominant
 324 species presented in **Figure 3**, suggesting separate development of high- and low-abundance
 325 populations. These 3 species showed a fast increase in abundance up to 18th months of age,
 326 followed by a steady state and then again, an increase in abundance between the age of 30
 327 months and 36 months followed with a slight drop in abundance after 3 years of age.
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Figure 3. Abundance of the top 10 species with a minimum abundance of 1% over time

329

330 Figure 3. Top 10 species with a minimum abundance of 1%, significantly associated in the 11

331 age groups

Figure 3.a shows the significantly differentially abundant bacterial taxa using relative (total sum scaling (TSS) abundance. **Figure 3.b** shows the same dataset further transformed using cumulative sum scaling (CSS) +log 2 transformation.

A direct comparison of taxa negatively and positively associated with age is presented in **Figure 4**. **Figure 4.a** shows the top 11 negatively associated taxa with age (Spearman's rho min -0.3 and min abundance of 0.1%) and **Figure 4.b** shows the top 20 positively associated taxa with age (Spearman's rho min 0.3 and min abundance of 0.1%). The largest upward and downward shifts occurred within the first ~26 months of life.

Figure 4. Top significant taxa associated with age development between the age of 7 months and 40 months

4.a Negative correlated taxa with age with a Spearman Rho index of min -0.3 (-0.3 to -0.69) and a Spearman FDR P < 0.05 and a minimum abundance of 0.1% (N=11 taxa)

4.b Positive correlated with age with a Spearman Rho index of min 0.3 (0.3 to 0.56) and a Spearman FDR P < 0.05 and a minimum abundance of 0.1% (N=20)

346

347 Figure 4. Top significant taxa associated with age development between the age of 7 months

348 and 40 months

All taxa (N=11) associated with a decreasing abundance over time with a minimum Spearman's rho index of -0.3 from the minimum 0.1% abundance taxa dataset are presented in **Figure 4.a**. And, all taxa (N=20) associated with an increasing abundance over time with a minimum Spearman's rho index of 0.3 from the minimum 0.1% abundance taxa dataset are presented in **Figure 4.b**.

Correlation analysis identifies distinct clusters representing bacterial trophic networks

Microbial populations form clusters based on nutrients available ²⁰, i.e., at the trophic level a microbial population constitutes a food web of metabolically interdependent organisms ²¹.

To identify these trophic networks, we first generated a heatmap of the Spearman Rho correlation coefficients between the different bacterial species including the top 51 species with a minimum abundance of 0.2% in all samples to visualize distinct clusters of bacteria (**Figure 5.a**). The most distinct and visually striking cluster that was identified was the cluster, indicated with a green box, that includes the third most dominant species, *Prevotella stercorea*, and consists of species generally not found in the microbiota of individuals from industrialised countries. *Succinivibrio dextrinosolvens* and *Paraprevotella xylaniphila*, which showed the same non-normal distribution development pattern as *P. stercorea* (Figure 3) are part of this cluster, highlighting the codependence of these species. A second cluster, indicated with a light blue box, which contains many species found in typically industrialised microbiome compositions, also contained the second most abundant species, *Faecalibacterium prausnitzii*. A third apparent cluster, indicated in black and containing the *Bifidobacterium* genus, is the result of species associating with one another due to being prevalent mainly or only within young subjects. Finally, a cluster of bacteria typically found in

the small intestine (like *Veillonella dispar* and *Streptococcus salivarius*) is indicated with a purple box. The most dominant species, *P. copri* (* in heatmap Figure 5.a), was not strongly associated with any of the clusters but is clearly negatively correlated with *Bacteroides* (** in heatmap Figure 5.a). A summary of the correlations between the main groups of organisms found in this study is shown in Figure 5.b.

The green box (*Prevotella stercorea* & co) and the blue box (*Faecalibacterium prausnitzii* & co) most likely represent trophic networks of interdependent species which develop steadily over time at cluster specific rates. The *F. prausnitzii* & co cluster takes about 2 years to mature and reach a stable level (Supplementary Figure 5.a) while the *P. stercorea* & co cluster takes about 2.5 years to reach a stable state (Supplementary Figure 5.a). *Prevotella copri* on the other hand is not dependent on any other bacteria as it rapidly becomes the dominant species within 12 months (Figure 3.a and Supplementary Figure 4).

Supplementary Figure 4. Development of bacterial trophic network species in 11 age groups (three-months interval)

4.a Development of bacterial trophic network in the *Prevotella stercorea* cluster together with *Prevotella copri*

4.b Development of bacterial trophic network in the *Faecalibacterium prausnitzii* cluster together with *Prevotella copri*

387

388 Supplementary Figure 4. Development of bacterial trophic network species over time

389 The mean abundances of the individual trophic network species are shown as a stacked line

390 graph for the *Prevotella stercorea* network (Sup Fig 4.a) and for the *Faecalibacterium*

391 *prausnitzii* network (**Sub Fig 4.b**) for the 11 three- months age groups. The mean abundances
392 in % were calculated from total sum scaling (TSS) normalised absolute read counts which
393 were subsequently transformed using cumulative sum scaling (CSS)+log2.

394

395

396 However, the *Faecalibacterium* & co. cluster does seem to benefit from a high abundance of
397 *P. copri* (**Figure 6.a**) whilst the *Prevotella stercorea* & co. cluster does not seem to have a
398 distinct interaction with *P. copri* (**Figure 6.b**). Several of the species associated with the
399 *Prevotella stercorea* & co. and the *Faecalibacterium* & co. clusters seem to be shared
400 between both clusters to some degree, having high positive correlation coefficients with
401 members from both clusters (green and blue clusters in **Figure 5.a and b**).

402 The steady build-up of *F. prausnitzii* cluster over time appears to mirror the development of a
403 trophic network seen in children in industrialised counties ¹⁵, where the build-up of a trophic
404 network of fibre degraders, acetate and lactate producers combined with butyrate producers
405 appears to be essential for a healthy immune system development. However, the addition or
406 expansion of the *P. stercorea* & co trophic network in our African cohort (alongside *P. copri*)
407 likely helps explain why higher levels of short-chain-fatty-acid production levels are seen in
408 Africans with non-industrialized gut microbiomes. The apparent positive association of
409 *Bifidobacterium* with various other bacteria (black box, **Figure 5.a**) is most likely an indirect
410 effect caused by being abundant in the young age group. These positive associations do not
411 seem to represent a trophic network except possibly for the association of *Bifidobacterium*
412 (which produces lactate) with *Megasphaera elsdenii* (which metabolizes lactate and produces
413 butyrate). We also note that *Megasphaera micronuciformis* is positively correlated with
414 *Bifidobacterium* (**Figure 5.a**).

Figure 5. Species association and trophic networks

5.a Heatmap analysis for trophic networks of the top 51 species with minimum 0.2% abundance

5.b Bacterial network - Summary

Figure 5. Species association and trophic networks

Figure 5.a A network heatmap was generated using the top 51 species with a minimum abundance of 0.2% in all samples. The most dominant clusters identified in the bacterial trophic network correlation analysis are highlighted by different coloured boxes. The

Prevotella stercorea network is highlighted in green, the *Faecalibacterium prausnitzii* network in blue, the *Bifidobacterium* network in grey, and the small intestinal microbiome network in purple. The red heatmap colour indicates strong positive correlation and the blue heatmap colour indicates strong negative correlation. There are additional species correlated outside the obvious labelled clusters, which are labelled by lines. A bacterial network summary is presented in **Figure 5.b**. Spearman correlation coefficient analysis was used to identify a bacterial trophic network with the strongest self-correlations. The leading species in each network is highlighted. A positive correlation is highlighted by green lines, a negative correlation is highlighted by red lines, and intermediate correlation is highlighted by grey lines

Principal Component Analysis (PCA) combined with Spearman rho correlation analysis was performed to analyse the maturation process in more detail. Principal component 1 (PC1) represents the maturation process as the accumulation of *Prevotella copri* and both the *F. prausnitzii* and *P. stercorea* trophic networks over time vs. the decline of the *Bacteroides* genus. The build-up of the trophic networks over time is furthermore especially well represented by PC3 (**Figure 6.d**). PC2 mainly demonstrates how *P. copri* and the *P. stercorea* network are negatively correlated with *Bacteroides* whilst the *F. prausnitzii* network is positively correlated with *Bacteroides* (**Figure 6.e**). PC4 highlights that *P. copri* and the *F. prausnitzii* network are positively correlated with one another (**Figure 6.f**) but that this positive correlation does not extend to the *Prevotella stercorea* network.

Figure 6. Maturation process analysed by Principle Component Analysis combined with Spearman rho correlation

Figure 6. Maturation process analysed by Principal Component Analysis combined with Spearman rho correlation

A Principal Component Analysis (PCA) combined with Spearman rho correlation analysis was performed to analyse the maturation process in more detail for different dominant taxa and bacterial networks. The correlations between *F. prausnitzii* and *P. copri* are shown in Figure

6.a. **Figure 6.b** shows the correlation between *P. copri* and *P. stercorea* & co cluster. Principal component 1 (PC1) in **Figure 6.c** represents 73.5% of the variation in the data and indicates the maturation process to be the accumulation of *Prevotella copri* and both the *F. prausnitzii* and *P. stercorea* trophic networks over time. The build-up of the trophic networks over time is furthermore well described by PC3 in **Figure 6.d**. PC2 in **Figure 6.e** indicates how *P. copri* and the *P. stercorea* network are negatively correlated with *Bacteroides* whilst the *F. prausnitzii* network is positively correlated with *Bacteroides*. PC4 in **Figure 6.f** indicates that *P. copri* and the *F. prausnitzii* network are positively correlated with one another

The gut microbiome was stable across Weight-for-Age Z Score (WAZ), Height-for-Age Z Score (HAZ), and Weight-for-Height z-score (WHZ)

Our study population in terms of WAZ, HAZ, and WHZ had a mean Z score of -0.95, -0.85, and -0.77, respectively and a standard deviation between 0.85 and 0.94 for the three Z scores (**Table 1**). In order to investigate whether different bacterial taxa were associated with these three Z scores values we used multiple linear regression (MLR) for the identification of an association between bacterial taxa and the clinical factors WAZ, HAZ, and WHZ. The association between clinical factors and bacterial taxa was controlled for age. A small number of bacterial taxa were significantly associated with WAZ (N = 1), HAZ (N = 7), and WHZ (N = 3) (**Supplementary Table 3**, columns WAZ.p, HAZ.p, WHZ.p, yellow box) but none of the associations remained significant after correction for multiple testing by the FDR method (**Supplementary Table 3**, columns WAZ.p.fdr, HAZ.p.fdr, and WHZ.p.fdr). We also tested for an association between microbial diversity indexes and our three multiple explanatory variables for WAZ, HAZ, and WHZ, controlled for age (**Supplementary Figure 5**). For this

472 analysis the same alpha diversity indexes were used as shown in **Figure 1**. Fisher's Alpha
473 (**Supp Fig 5.a**), Richness index (**Supp Fig 5.c**), and Chao1 (**Supp Fig 5.d**) were all highly
474 significantly associated with age. However, none of the alpha community diversities (i.e.,
475 Fisher's alpha, Simpson's index, Richness, and Chao1) remained significantly associated with
476 WAZ, HAZ, and WHZ after controlling for age (**Supplementary Figures 5.a to 5.d**).

477

478

Supplementary Table 3. Multivariate linear regression (MLR) did not identify an association between bacterial taxa and WAZ, HAZ, and WHZ

Taxa	age.p	WAZ.p	HAZ.p	WHZ.p	(Intercept).c	age.c	WAZ.c	HAZ.c	WHZ.c	age.p.fdr	WAZ.p.fdr	HAZ.p.fdr	WHZ.p.fdr
Prevotella_copri_35.153	0	0.356	0.56	0.701	25.75	0.361	-0.851	-0.388	-0.283	0	0.94184	0.97604	0.96837
Faecalibacterium_prausnitzii_11.383	0	0.763	0.835	0.155	5.397	0.266	0.112	0.056	-0.425	0	0.94184	0.97604	0.68333
Prevotella_stercorea_7.054	0	0.775	0.509	0.879	3.061	0.182	0.109	-0.181	-0.046	0	0.94184	0.97604	0.96837
Bacteroides_4.636	0	0.15	0.802	0.66	9.53	-0.198	0.661	0.083	-0.162	0	0.94184	0.97604	0.96837
Bifidobacterium_4.117	0	0.823	0.23	0.144	16.033	-0.537	0.084	-0.325	0.441	0	0.94184	0.71176	0.68333
Escherichia_coli_3.505	0	0.488	0.098	0.423	8.359	-0.206	-0.261	0.45	0.242	0	0.94184	0.49	0.81346
Succinivibrio_dextrinosolvens_2.424	0	0.905	0.925	0.802	-0.48	0.131	0.034	-0.019	-0.057	0	0.94184	0.97604	0.96837
Paraprevotella_xylaniphila_2.02	0	0.376	0.655	0.946	-0.446	0.11	-0.159	0.058	0.01	0	0.94184	0.97604	0.96837
Sutterella_wadsworthensis_1.751	0	0.793	0.469	0.122	3.486	-0.078	-0.035	-0.069	0.164	0	0.94184	0.97604	0.67778
Streptococcus_salivarius_1.137	0	0.322	0.037	0.823	1.947	-0.041	0.121	-0.185	-0.022	0	0.94184	0.28571	0.96837
Megasphaera_elsdenii_0.9	0	0.488	0.859	0.453	3.726	-0.122	0.091	0.017	0.079	0	0.94184	0.97604	0.68333
Ruminococcaceae_UCG002_0.716	0	0.923	0.591	0.463	-0.366	0.052	-0.008	0.03	0.046	0	0.94184	0.97604	0.82679
Thalassospira_0.707	0	0.419	0.886	0.184	-0.087	0.037	0.067	0.009	-0.088	0	0.94184	0.97604	0.68333
Eubacterium_rectale_0.673	0	0.373	0.162	0.055	-0.165	0.042	-0.067	0.076	0.116	0	0.94184	0.675	0.475
Prevotella_ruminicola_0.615	0	0.302	0.451	0.921	0.25	0.021	0.065	0.034	0.005	0	0.94184	0.97604	0.96837
Pseudobutyrvibrio_ruminis_0.568	0	0.892	0.028	0.292	-0.252	0.043	-0.008	0.095	0.051	0	0.94184	0.28571	0.68333
Lactobacillus_ruminis_0.544	0	0.865	0.264	0.24	1.877	-0.055	-0.019	0.088	0.103	0	0.94184	0.73333	0.68333
Roseburia_inulinivorans_0.465	0	0.49	0.91	0.882	-0.058	0.023	-0.033	-0.004	0.006	0	0.94184	0.97604	0.96837
Holdemanella_biformis_0.416	0	0.732	0.585	0.512	-0.067	0.023	0.015	-0.018	0.024	0	0.94184	0.97604	0.85333
Allisonella_histaminiformans_0.421	0	0.093	0.001	0.949	0.895	-0.023	0.07	-0.1	0.002	0	0.94184	0.05	0.96837
Collinsella_aerofaciens_0.396	0	0.989	0.458	0.705	1.194	-0.037	0.001	-0.023	0.013	0	0.989	0.97604	0.96837
Erysipelotrichaceae_sp_0.318	0	0.421	0.071	0.51	-0.014	0.015	-0.028	0.046	-0.019	0	0.94184	0.44375	0.85333
Veillonella_dispar_0.314	0	0.67	0.209	0.276	0.578	-0.015	0.017	-0.036	-0.035	0	0.94184	0.69667	0.68333
Megamonas_funiformis_0.308	0	0.445	0.022	0.988	0.937	-0.031	0.054	-0.118	0.001	0	0.94184	0.28571	0.988
Phascolarctobacterium_succinatutens_0.284	0	0.537	0.717	0.896	-0.032	0.016	0.043	-0.018	0.007	0	0.94184	0.97604	0.96837
Veillonella_ratti_0.302	0	0.569	0.685	0.265	1.476	-0.052	-0.045	0.023	0.07	0	0.94184	0.97604	0.68333
Lachnospiraceae_sp_0.295	0	0.817	0.636	0.02	0.45	-0.008	0.004	-0.005	-0.029	0	0.94184	0.97604	0.33333
Eubacterium_coprostanoligenes_0.278	0	0.225	0.843	0.281	-0.232	0.024	0.03	0.003	-0.021	0	0.94184	0.97604	0.68333
Pasteurella_multocida_0.249	0	0.86	0.983	0.71	-0.275	0.024	0.012	0.001	-0.021	0	0.94184	0.983	0.96837
Ruminococcaceae_sp_0.231	0	0.539	0.977	0.837	0.004	0.011	0.016	0.001	-0.004	0	0.94184	0.983	0.96837
Olsenella_sp.SK9K4_0.238	0	0.78	0.87	0.328	1.019	-0.034	0.016	-0.007	0.045	0	0.94184	0.97604	0.68333
Megasphaera_micronuciformis_0.233	0	0.28	0.03	0.003	1.098	-0.034	0.076	-0.11	0.165	0	0.94184	0.28571	0.125
Lachnospiraceae_sp_0.215	0	0.108	0.04	0.605	0.069	0.007	-0.019	0.018	0.005	0	0.94184	0.28571	0.96837
Parabacteroides_merdae_0.215	0	0.593	0.242	0.323	0.664	-0.019	0.028	-0.044	0.041	0	0.94184	0.71176	0.68333
Oscillibacter_sp_0.202	0	0.735	0.576	0.634	-0.012	0.01	-0.008	0.01	0.009	0	0.94184	0.97604	0.96837
Prevotella_nanceiensis_0.285	0.005	0.359	0.198	0.725	-0.048	0.015	0.073	-0.074	-0.022	0.0069444	0.94184	0.69667	0.96837
Streptococcus_equinus_0.895	0.015	0.874	0.763	0.743	1.43	-0.029	-0.028	-0.039	-0.047	0.02027	0.94184	0.97604	0.96837
Ruminococcus_torques_0.411	0.038	0.158	0.852	0.057	0.308	0.005	0.05	-0.005	-0.054	0.05	0.94184	0.97604	0.475
Ruminococcus_gnavus_0.311	0.046	0.646	0.639	0.209	0.421	-0.007	-0.025	0.019	-0.056	0.058974	0.94184	0.97604	0.68333
Sutterella_sp.YIT_0.82	0.052	0.765	0.346	0.844	0.691	0.009	0.021	0.047	0.011	0.063415	0.94184	0.9025	0.96837
Dorea_sp_0.214	0.051	0.134	0.111	0.398	0.178	0.002	0.018	-0.014	-0.008	0.063415	0.94184	0.50455	0.796
Catenibacterium_mitsuokai_0.294	0.081	0.699	0.779	0.086	0.102	0.007	0.022	-0.012	-0.079	0.096429	0.94184	0.97604	0.5625
Parabacteroides_distasonis_0.474	0.125	0.336	0.207	0.09	0.647	-0.005	-0.052	0.049	0.073	0.14535	0.94184	0.69667	0.5625
Roseburia_faecis_0.213	0.229	0.085	0.512	0.005	0.129	0.003	0.067	-0.018	-0.088	0.26023	0.94184	0.97604	0.125
Fusobacterium_mortiferum_0.643	0.263	0.04	0.092	0.298	0.869	-0.013	-0.347	0.205	0.141	0.29222	0.94184	0.49	0.68333
Anaerostipes_sp.5_0.27	0.375	0.573	0.937	0.245	0.203	0.001	-0.014	-0.001	-0.023	0.40761	0.94184	0.97604	0.68333
Klebsiella_pneumoniae_0.691	0.41	0.902	0.022	0.056	0.87	-0.007	-0.017	0.223	-0.207	0.43617	0.94184	0.28571	0.475
Clostridium_celatum_0.207	0.471	0.899	0.361	0.201	0.162	0.002	0.005	0.025	-0.039	0.49062	0.94184	0.9025	0.68333
Blautia_sp_0.352	0.593	0.887	0.897	0.797	0.377	-0.002	-0.009	-0.006	-0.013	0.6051	0.94184	0.97604	0.96837
Haemophilus_parainfluenzae_0.89	0.706	0.866	0.857	0.325	0.862	-0.003	-0.018	0.014	-0.083	0.706	0.94184	0.97604	0.68333

MLR was used to identify an association between community composition (219/488 bacterial taxa with a minimum abundance of 0.01%) and multiple explanatory variables for age, Weight-for-Age Z Score (WAZ), Height-for-Age Z Score (HAZ), and Weight-for-Height z-score (WHZ). Bacterial taxa were used as the dependent (response) variables and the clinical factors for age and score for WAZ, HAZ, and WHZ, were used as independent variables. The table was sorted by the false discovery rate (fdr) corrected p-values for age. The table shows the uncorrected p-values the coefficient ("c") value and the corrected p-values. MLR model was performed in Calypso Web Portal (version 8.72). The top 50 taxa are shown. Statistically significantly associated taxa with an uncorrected p-value < 0.05 are highlighted in yellow and statistically significantly associated taxa with a FDR corrected p-value < 0.05 are highlighted in red.

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DISCUSSION

In this study we utilised a very large cohort of children from an iron intervention trial in a rural region of The Gambia. This large study gave us an excellent opportunity to analyse in detail the microbiome in children in the first 40 months of life, a critical time frame in the development of the gut microbiome of humans both in industrialised and non-industrialised societies¹. Our analyses showed that age was the main factor that determined the overall bacterial composition in these samples and highlighted the particular importance of the *Prevotella* genus. It has previously been established that the *Prevotella* genus can be used as the most important discriminatory taxon to differentiate between rural Africans and people from industrialised countries and that this separation defined by *Prevotella* is not just limited to Africa^{1,2,22}. This study however shows how these *Prevotella*-rich gut microbiome compositions develop over time; it shows that *Prevotella copri* rises to dominance rapidly in the first 12 months and remains the most abundant species during the remainder of early childhood (~35% of all reads) and in addition shows the steady development of a trophic network of species centered around *Prevotella stercorea* in addition to the trophic network centered around *Faecalibacterium prausnitzii*. At the age of 30 months and onwards these two trophic networks together account for ~38% of all reads (20% + 18% approximately, respectively).

F. prausnitzii is well known to be of importance in industrialised settings and is often associated with health; it is typically absent before the age of 6 months but then appears and its numbers steadily increase over time once weaning commences^{15,23}. The *F. prausnitzii* trophic network development seen in this study mirrors the *F. prausnitzii* trophic network development seen in a study with Finnish and Estonian children, though perhaps reached a

stable level about 1 year earlier. The reasons for this are that the *F. prausnitzii* trophic network in Finnish/Estonian children can be said to be slightly more complex and as a whole more abundant in terms of the percentage of reads, and that the *F. prausnitzii* trophic network in children from The Gambia appears to utilize the metabolic activity of *P. copri*, a species which independently reaches a very high stable prevalence already at the age of one year. *Prevotella copri*, the most dominant species in this dataset, is expected to be negatively correlated with most bacterial groups, even in the absence of clear antagonism, when normalizing the abundances of species to 100% of all reads, due to its high abundance (~35%) yet appears to have a somewhat positive association with the *F. prausnitzii* trophic network (Figures 6.a and 6.f) and a non-antagonistic yet numerically expected negative association with the *Prevotella stercorea* trophic network when looking at the bulk of the samples (**Figures 6.b and 6.f**).

The trophic network centred around *Prevotella stercorea* is typically not found in gut microbiomes from individuals in industrialized countries. Many of the species associated in this trophic network are not found in microbiota in industrialized countries, or in only low numbers^{1,2,15,24}. As a result, they have often not been studied sufficiently (or at all) which makes it difficult to understand or explain, with any degree of certainty, what their roles are within this trophic network. The *P. stercorea* trophic network, as described here in the Gambian context, appears more diverse and self-contained than the *F. prausnitzii* network as it does not depend on *P. copri* and is typically strongly negatively correlated with all other main bacterial groups, apart from *P. copri* and the *F. prausnitzii* trophic network (**Figure 5.b**). Whilst not all species within this network have been studied in detail, it is clear, from what is

529 known about many of these species, that the assemblage should have a very wide
530 assortment of metabolic capabilities covering everything needed to form a self-contained
531 trophic network, leading (for example) to an enhanced production of short chain fatty acids,
532 as compared to microbiome compositions lacking this trophic network.

533 A particularly strong and important factor in the composition of the gut microbiome in these
534 children from The Gambia is the clear antagonistic relationship between the *Prevotella* and
535 *Bacteroides* genera (**Figures 5 and 6**). The *Prevotella copri* and the *Prevotella stercorea*
536 network are both very strongly negatively correlated with the abundance of *Bacteroides*
537 (Spearman rho = -0.36 and -0.32, respectively). The *F. prausnitzii* network however is not
538 negatively correlated with *Bacteroides*, similar to the association commonly found between
539 *F. prausnitzii* and *Bacteroides* in gut microbiome compositions from industrialized countries
540 where it is often found to be positive.

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542 Similar to infant microbiotas in industrialized countries, the *Bifidobacterium* genus is the
543 most important and dominant group early in infancy. As infants are weaned, the dominance
544 of *Bifidobacteria* wanes and they are rapidly replaced by bacteria associated with a more
545 adult-like “African” microbiome composition, especially in the case of these Gambian
546 children ². *Bifidobacterium* levels at 3 years of age are much lower in children from The
547 Gambia than in European children, possibly because the lactate- and acetate-producing
548 *Bifidobacteria* become part of the trophic networks found in Europe whereas they are
549 replaced by other species with a similar function (acetate and lactate production) in The
550 Gambia. The higher abundance of *Bifidobacterium* in European children may also be a result
551 of earlier and longer formula-feeding in those children ^{25,26}.

A decrease was also seen in the abundance of several potentially pathogenic species (e.g., *E. coli* and *Sutterella*), the *Bacteroides* genus, and several other infant/baby-associated microorganisms.

The high abundance of *P. copri* in the The Gambia cohort emphasises the function of *P. copri* and their metabolomic pathways in fermenting complex polysaccharides, which is important for people with a high fibre diet. It has also been shown in other studies that *Prevotella* species play an important role in glucose metabolism improvements after the consumption of barley kernel-based bread ²⁷. Metatranscriptomic RNA sequencing has provided detailed information showing that *P. copri* encodes all the enzymes necessary for the Embden-Meyerhof-Parnas (EMP) pathway which is the most recognised glycolytic pathway ²⁸ and is based on glycolysis and succinate production from fumarate. In addition, transcriptomic profiling has further revealed that it can degrade pyruvate to acetate and formate ²⁹. Another study looking at sports athletes associated *Prevotella* with a number of amino acid and carbohydrate metabolism pathways including lysine biosynthesis, alanine, aspartate and glutamate metabolism, and D-glutamine and D-glutamate metabolism. *Prevotella* is also known to be important in multiple pathways involved in drug metabolism, carbohydrate metabolism, and metabolism of cofactors and vitamins, including vitamin B6 metabolism ³⁰.

In conclusion, we have analysed a stool collection from children between the age of 7 and 40 months of the upper region of The Gambia with a high fiber diet; the largest African paediatric microbiome collection to date. Detailed analysis of the gut microbiome has provided insights into the development of a *Prevotella*-rich gut microbiome. We observed similar changes to those described in paediatric cohorts from industrialised countries, such as

576 the shift from Bifidobacteria to the development of trophic network centered around *F.*
577 *prausnitzii*. However, in addition we were able to describe the steady development of a
578 trophic network centered around *P. stercorea* and the rapid rise to dominance of *P. copri*.
579 The high abundance of *Prevotella* in non-industrialized countries makes this genus the most
580 discriminating taxon between a healthy high-fiber diet and an unhealthy low-fiber diet seen
581 in industrialized countries. In industrialized countries, the increase of various diseases of
582 affluence, like diabetes, is typically associated with the (partial) loss of the last trophic
583 network (indicated by lower *F. prausnitzii* numbers) and an increase of Bacteroides and/or
584 Proteobacteria. The parallel loss of *P. copri* and the *P. stercorea* trophic network, whose
585 development is described here, indicates the likely importance of the metabolic pathways
586 and capabilities of *Prevotella* and species associated with them, both for maintaining a higher
587 fermentative capacity (SCFA production), a healthier gut microbiome environment and for
588 keeping opportunistic pathogens at bay.

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600 This study is registered at Clinical Trials. gov as NCT02941081.

601 **Competing interests**

602 D.I.A.P. is one of the inventors of the IHAT iron supplementation technology for which she
603 could receive future awards to inventors through the MRC Awards to Inventor scheme.

604 D.I.A.P. has served as a consultant for Vifor Pharma UK, Shield Therapeutics, Entia Ltd,
605 Danone Nutritia, UN Food and Agriculture Organization (FAO) and Nemysis Ltd. D.I.A.P. has
606 since moved to full employment with Vifor Pharma UK, but all work pertaining to this study
607 was conducted independently of Vifor Pharma.

608 Notwithstanding, the authors declare no potential conflicts of interest with respect to the
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610

611 **Contributors**

612 Josef Wagner, data analysis and original draft preparation

613 Marcus De Goffau, data analysis and manuscript review & editing

614 Julian Parkhill, involved in initial study set up and critical review of the manuscript

615 Dora I.A. Pereira, conceptualization, data curation, funding acquisition, methodology, project
616 administration, supervision, visualization, writing – review & editing

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619 editing

620 Andrew M. Prentice, conceptualization, funding acquisition, supervision, writing – review &
621 editing

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633

634 **Ethics approval**

635 The study protocol and any subsequent amendments have been reviewed and approved by
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712 **FIGURE legends**

713 **Supplementary Figure 3. Eight species were statistically significantly different between the**

714 **D1 and D85 timepoints**

715 Mixed Effect Regression modelling (MERM) was used to analyse samples from paired
716 repeated timepoints. Six species significantly increased in the paired sample analysis from D1
717 to Day 85 (**Sup Fig 3 a – f**) and two species significant decreased in the paired sample analysis
718 from D1 to Day 85 (**Sup Fig 3 g-h**). MERM was also used to look at the changes in alpha
719 diversity indexes in the D1/D85 paired samples set. Three alpha diversity indexes significantly
720 increased from D1 to D85 which were Richness, Chao1, and Fisher's Alpha, whereas
721 Simpson's index did not change (**Sup Fig 3 i-l**).

722 **Supplementary Figure 5. Multivariate linear regression (MLR) analysis for alpha diversity**

723 **indexes with age, WAZ, HAZ, and WHZ**

724 MLR was performed to identify associations between bacterial taxa, community alpha
725 indexes and clinical factors for WAZ, HAZ, and WHZ. The different diversity indexes were
726 controlled for age. **Sup Fig 5.a** shows the MLR model for Fisher's Alpha $\sim$ age + WAZ + HAZ +
727 WHZ. **Sup Fig 5.b** shows the MLR model for Simpson's index $\sim$ age + WAZ + HAZ + WHZ. **Sup**
728 **Fig 5.c** shows the MLR model for Richness index $\sim$ age + WAZ + HAZ + WHZ, and **Sup Fig 5.a**
729 shows the MLR model for Chao1 index $\sim$ age + WAZ + HAZ + WHZ.

730

731 **Supplementary Table 2. Non-parametric Kruskal-Wallis rank test to identify significantly**

732 **differentially abundant bacterial taxon in the 11 three-months age groups**

733 The non-parametric rank test (Kruskal-Wallis) was used to identify significantly differentially
734 abundant bacterial taxon in the 11 age groups. The first column shows all 488 species present
735 in the entire datasets. The second column shows the relative abundance (%). The third

column shows the P value from the rank test. The fourth columns show the False Discovery Rate (FDR) corrected P value. The next 11 columns show the mean abundance value in the 11 age groups (three months interval) and the last 11 columns show the median abundance value for the same 11 age groups. The table is sorted from highest abundance species to the lowest abundance species. All FDR significant species are highlighted in grey. A p value $< 1e-20$ are shown as "0". 301/488 taxa were statistically significantly different between the 11 age groups. However, a large majority of them (231/301) were very low abundance taxa with an abundance below 0.1%. The top 70 statistically significant species with a minimum abundance of 0.1% include 92.5% of all sequence reads.

Supplementary Table 3. Multivariate linear regression did not identify an association between bacterial taxa and WAZ, HAZ, and WHZ

MLR was used to identify an association between community composition (219/488 bacterial taxa with a minimum abundance of 0.01%) and multiple explanatory variables for age, Weight-for-Age Z Score (WAZ), Height-for-Age Z Score (HAZ), and Weight-for-Height z-score (WHZ). Bacterial taxa were used as the dependent (response) variables and the clinical factors for age and score for WAZ, HAZ, and WHZ, were used as independent variables. The table was sorted by the false discovery rate (fdr) corrected p-values for age. The table shows the uncorrected p-values the coefficient ("c") value and the corrected p-values. MRL model was performed in Calypso Web Portal (version 8.72). The top 50 taxa are shown. Statistically significantly associated taxa with an uncorrected p-value < 0.05 are highlighted in yellow and statistically significantly associated taxa with a FDR corrected p-value < 0.05 are highlighted in red.

Figures

1.a PCoA plot for Species taxon

1.b PCoA plot for genus taxon

1.c PCoA plot for family taxon

Figure 1

PCoA clustering for species, genera, and family separated into 3 age groups Principal co-ordinate analysis (PCoA) was conducted with all 1407 samples and highlights a clear separation of samples based on three age groups at the species taxonomic level (Figure 1.a), the genus taxonomic level (Figure

1.b), and the family taxonomic level (Figure 1.c). The column graphs next to the PCoA plots show the relative abundance of the top species with a minimum abundance of 0.5% for the species taxon (n=22), for the genus taxon (n=26), and for the family taxon (n=18). All *Bacteroides* species and all *Bifidobacterium* species were combined.

Figure 2

Alpha diversity indexes for 11 age groups Alpha diversity was measured by two different diversity indexes and by two different richness indexes. Figure 2.a The Fisher's alpha diversity test, Figure 2.b Simpson's diversity test, Figure 2.c, Chao1 estimated richness, Figure 2.d Observed richness

Figure 3

Top 10 species with a minimum abundance of 1%, significantly associated in the 11 age groups Figure 3.a shows the significantly differentially abundant bacterial taxa using relative (total sum scaling (TSS)

abundance. Figure 3.b shows the same dataset further transformed using cumulative sum scaling (CSS) +log 2 transformation.

Figure 4

Top significant taxa associated with age development between the age of 7 months and 40 months. All taxa (N=11) associated with a decreasing abundance over time with a minimum Spearman's rho index of -0.3 from the minimum 0.1% abundance taxa dataset are presented in Figure 4.a. And, all taxa (N=20)

associated with an increasing abundance over time with a minimum Spearman's rho index of 0.3 from the minimum 0.1% abundance taxa dataset are presented in Figure 4.b.

5.b Bacterial network - Summary

Figure 5

Species association and trophic networks Figure 5.a A network heatmap was generated using the top 51 species with a minimum abundance of 0.2% in all samples. The most dominant clusters identified in the bacterial trophic network correlation analysis are highlighted by different coloured boxes. The *Prevotella stercora* network is highlighted in green, the *Faecalibacterium prausnitzii* network in blue, the

Bifidobacterium network in grey, and the small intestinal microbiome network in purple. The red heatmap colour indicates strong positive correlation and the blue heatmap colour indicates strong negative correlation. There are additional species correlated outside the obvious labelled clusters, which are labelled by lines. A bacterial network summary is presented in Figure 5.b. Spearman correlation coefficient analysis was used to identify a bacterial trophic network with the strongest self-correlations. The leading species in each network is highlighted. A positive correlation is highlighted by green lines, a negative correlation is highlighted by red lines, and intermediate correlation is highlighted by grey lines

Figure 6

Maturation process analysed by Principal Component Analysis combined with Spearman rho correlation A Principal Component Analysis (PCA) combined with Spearman rho correlation analysis was performed to analyse the maturation process in more detail for different dominant taxa and bacterial networks. The correlations between *F. prausnitzii* and *P. copri* are shown in Figure 6.a. Figure 6.b shows the correlation between *P. copri* and *P. stercorea* & co cluster. Principal component 1 (PC1) in Figure 6.c represents 73.5% of the variation in the data and indicates the maturation process to be the accumulation of *Prevotella copri* and both the *F. prausnitzii* and *P. stercorea* trophic networks over time. The build-up of the trophic networks over time is furthermore well described by PC3 in Figure 6.d. PC2 in Figure 6.e indicates how *P. copri* and the *P. stercorea* network are negatively correlated with *Bacteroides* whilst the *F. prausnitzii* network is positively correlated with *Bacteroides*. PC4 in Figure 6.f indicates that *P. copri* and the *F. prausnitzii* network are positively correlated with one another

Supplementary Files

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- [SupplementaryFigure5MLRAlphaindexwithageWAZHAZWHZ.pdf](#)