Stunted childhood growth is associated with decompartmentalization of the gastrointestinal tract and overgrowth of oropharyngeal taxa

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Linear growth delay (stunting) affects roughly 155 million children under the age of 5 years worldwide. Treatment has been limited by a lack of understanding of the underlying pathophysiological mechanisms. Stunting is most likely associated with changes in the microbial community of the small intestine, a compartment vital for digestion and nutrient absorption. Efforts to better understand the pathophysiology have been hampered by difficulty of access to small intestinal fluids. Here, we describe the microbial community found in the upper gastrointestinal tract of stunted children aged 2–5 y living in sub-Saharan Africa. We studied 46 duodenal and 57 gastric samples from stunted children, as well as 404 fecal samples from stunted and nonstunted children living in Bangui, Central African Republic, and in Antananarivo, Madagascar, using 16S Illumina Amplicon sequencing and semiquantitative culture methods. The vast majority of the stunted children showed small intestinal bacterial overgrowth dominated by bacteria that normally reside in the oropharyngeal cavity. There was an overrepresentation of oral bacteria in fecal samples of stunted children, opening the way for developing noninvasive diagnostic markers. In addition, *Escherichia coli/Shigella* sp. and *Campylobacter* sp. were found to be more prevalent in stunted children, while *Clostridia*, well-known butyrate producers, were reduced. Our data suggest that stunting is associated with a microbiome "decompartmentalization" of the gastrointestinal tract characterized by an increased presence of oropharyngeal bacteria from the stomach to the colon, hence challenging the current view of stunting arising solely as a consequence of small intestine overstimulation through recurrent infections by enteric pathogens.

stunting | sub-Saharan Africa | microbiota | oropharyngeal taxa | decompartmentalization

To date, one-fourth of children under 5 y suffer from linear growth delay (stunting) (1). Stunting is defined by the World Health Organization (WHO) as a height-for-age *z*-score of more than two SDs below the mean of a reference cohort (2). It is a consequence of chronic undernutrition, including macro- and micronutrient deficiencies, and thought to be initiated or exacerbated by repeated enteric infections and by poor hygiene in general (3, 4). Chronic malnutrition is associated with important pathophysiological disturbances, including an increased susceptibility to disease, diminished response to oral vaccines (5), and delayed or diminished psychomotor development (3, 6, 7). The consequences of chronic malnutrition extend into adulthood and are associated with decreased economic output (8). However, the pathophysiological mechanisms remain largely unknown and we still lack effective preventive or therapeutic solutions. In recent years, a syndrome called pediatric environmental enteropathy (PEE) has

Significance

Stunting globally affects an estimated 155 million children under 5 years of age, representing about 25% of children worldwide. Due to poor understanding of the underlying pathophysiology, therapeutic interventions to efficiently correct for linear growth delay or associated pathophysiological disturbances are still lacking. Here, we describe the microbial composition of duodenal fluids from stunted children. We show that these children are affected by small intestinal bacterial overgrowth and harbor a characteristic microbial community composed mainly of oropharyngeal bacteria. This microbial signature is also reflected in their feces and conserved between countries. Stunting is traditionally considered to arise from recurrent enteric infections. This study shows that oropharyngeal taxa are associated with stunting, suggesting that alternative pathophysiological mechanisms are involved.

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Data deposition: Sequence reads have been deposited in the European Nucleotide Archive, https://www.ebi.ac.uk/ena/ [accession nos. PRJEB27868 and ERP110005 (ERS2620873–ERS2621413)].

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been proposed as an underlying cause of stunting (9–11). PEE is a chronic inflammation of the small intestine, characterized by villus blunting and increased permeability (reviewed in ref. 12). PEE is associated with undernutrition as well as with continuous exposure to fecal–oral contamination (6, 13–15) and repeated enteric infections (4, 16–18), suggesting a vicious cycle between undernutrition and infection. Small intestinal bacterial overgrowth (SIBO) may also contribute to PEE and, therefore, to undernutrition. The small intestine is normally only sparsely populated by microbes [10^3–10^4 colony-forming units (CFU)/mL small intestinal fluid] (19). In contrast, SIBO is defined as an overgrowth to $>10^5$ CFU/mL. This overgrowth is hypothesized to drive local inflammation and lead to a "leaky" small intestinal barrier, as well as to impaired digestive and absorptive functions (20–24).

SIBO is prevalent in children living in difficult sanitary and economic situations, where they are often exposed to sewage and waste material. Studies of children living in shantytowns in South America and Asia have detected SIBO at a prevalence of 16–61% (23, 25–28). SIBO has also been described in children affected by PEE (23) and in undernourished children living in Gambia (29), Indonesia (30), and Nigeria (31). Across these studies, SIBO was negatively correlated with linear growth. However, previous studies have been limited. Most of them used the hydrogen or lactulose breath test to diagnose the presence of SIBO (in this test, bacterial fermentation of an ingested sugar leads to increased hydrogen in the breath); however, this approach does not provide any information on the community composition of SIBO (25–28). Furthermore, these studies included only a limited number of participants or were performed before next-generation sequencing methods became available. Hence, these early studies document only a fraction of the actual bacterial community associated with SIBO. The bacterial species putatively causing the pathology in PEE-associated SIBO have therefore remained elusive. A few studies to date have examined the intestinal microbiota and stunting and have found inconsistent differences in α-diversity and in the abundance of specific taxa (32, 33). However, these studies have been limited by small sample sizes. In addition, they have all been performed on fecal samples, which likely do not reflect the composition of the microbiota at the site of PEE in the upper small intestine (24). Indeed, growing evidence from comparative studies (19, 34, 35) suggests that the small intestinal microbiota is profoundly different from fecal microbiota, reflecting a microbial compartmentalization along the intestinal tract.

In conclusion, today we still lack a clear picture of the intestinal microbiota associated with stunting and PEE. The Central African Republic (CAR) and Madagascar are two of the countries with the highest prevalence of stunting, with roughly half of all children under age 5 y affected; however, these countries have never before been included in an analysis of PEE (36, 37). Here, we compare gastric, duodenal, and fecal samples from stunted children and healthy control subjects in both countries, using 16S-based metataxonomics and culture techniques. We identify SIBO in more than 80% of stunted children and show SIBO to be dominated by bacteria that normally reside in the oropharyngeal cavity. The overrepresentation of oral bacteria is reflected in fecal samples of stunted children. This study describes SIBO composition in stunted children and suggests that oropharyngeal taxa may be an important contributing factor to the pathophysiology of PEE.

Materials and Methods

Study Set-Up, Recruitment of Participants, and Sample Collection. This transversal study was carried out in children (aged 2–5 y) living in Bangui, CAR, or Antananarivo, Madagascar. The study population comprises the first 404 children recruited in the community in the context of the AFRIBIOTA project. The study protocol for AFRIBIOTA has been approved by the institutional review board of the Institut Pasteur (2016-06/IRB) and the national ethics review boards of Madagascar (55/MSANP/CE, May 19, 2015) and CAR (173/UB/FACSS/CSCVPER/16). All participants received oral and written information about the study, and the legal representatives of the children provided written consent to participate in the study. The detailed inclusion and exclusion criteria and recruitment procedures are described elsewhere (38). The children were classified according to the median height of the WHO reference population (2, 39) in three groups: severe stunting (height-for-age *z*-score ≤ -3 SD), moderate stunting (height-for-age *z*-score between -3 SD and -2 SD), and not stunted (height-for-age *z*-score ≥ -2 SD). Caregivers were instructed to collect the feces in the morning before coming to the hospital and to note/look up the time of emission. Gastric and duodenal samples were collected using a pediatric nasogastric tube (Vygon) and, due to ethical concerns, were only collected for stunted children. Once the gastric, duodenal, or fecal samples were collected, they were aliquoted, frozen at −20 °C, and transferred on the same day to a −80 °C freezer (Bangui) or directly snap-frozen in liquid nitrogen and then transferred to a −80 °C freezer (Antananarivo). Next, 100 μL of fresh duodenal samples were inoculated directly in 0.9 mL of Robertson's Cooked Meat (RCM) medium and processed for culture.

Culture of Duodenal Aspirates and Identification of Colonies. RCM-diluted duodenal aspirations were diluted and streaked on plates according to the protocol described in Chandra et al. (40). Reisolated colonies were identified either by MALDI-TOF mass spectrometry (Bruker Biotyper; Bruker Daltonics) or by classic microbiology. Cultures were considered positive for SIBO if the total bacterial count was $\geq 10^5$ CFU/mL duodenal fluid (40).

DNA Extraction and Sequencing. Samples were extracted by commercial kits (QiaAmp cador Pathogen Mini or cador Pathogen 96 QIAcube HT Kit; Qiagen) following the manufacturer's recommendations, with an additional bead-beating step to increase mechanical disruption. Samples were stored at −80 °C until sequencing. Extracted DNA samples were shipped to a commercial provider where library generation and sequencing were performed (Microbiome Insights). Library preparation was performed as recommended by Kozich et al. (41) using primers v4.SA501–v4.SA508 and v4.SA701–v4.SA712. The amplicon library was sequenced on a MiSeq using the MiSEq 500 Cycle V2 Reagent Kit (250 × 2).

Bioinformatic and Biostatistic Analysis. Retrieved sequences were demultiplexed in QIIME v1.9 (42) and then trimmed, clipped, and quality-filtered using the Fastx Toolkit (hannonlab.cshl.edu/fastx_toolkit) to 245 bp with a minimum quality threshold of Q19. Filtered R1 reads were processed into operational taxonomic units (OTUs) using minimum entropy decomposition (MED) (43) with the minimum substantive abundance (-m) parameter set to 250, yielding 2,246 unique OTUs. Taxonomy was then assigned to the representative sequence for each MED node by matching it to the SILVA 128 (44, 45) database using QIIME. Singlets, mitochondrial, and cholorplast reads were filtered out. The final filtered OTU table consisted of 2,029 unique sequences and 9,155,211 reads. The stunted vs. nonstunted groups were compared using Pearson's χ^2 test or Fisher's exact test for qualitative variables and the student *t* test or the Mann–Whitney *U* test for quantitative variables. Statistical analyses and visualizations of the microbial data were conducted in R v3.4.1 using PhylosEq (46), vegan (47), randomForest (48, 49), DeSeq2 (50–52), and ggplot2 (53) R packages. α-Diversity was quantified using a measure of richness [Chao1 index (54)] and a measure of evenness (Simpson's diversity index = 1 − Simpson's index) while β-diversity was quantified using the Bray–Curtis dissimilarity index (55). Tests of differences in α-diversity between samples were performed using nonparametric multivariate analysis of variance (PERMANOVA) with the function "adonis" in the R package vegan (47, 56) or linear-mixed models (α value of 0.05). *P* values were Benjamini–Hochberg-corrected. Multivariate analyses of differentially abundant taxa were performed on pooled samples from both countries as well as on data from each country independently. Multivariate models were corrected for gender, age (in months), as well as country of origin and stratified on sample type, and then on country of origin. Picrust analysis (57) was performed on the Galaxy server of the Langille group (galaxy.morganlangille.com). The gene counts were categorized by function and rarefied to 2,000,000 gene counts. The differential gene count was analyzed by linear-mixed models correcting for gender and age (in months), as well as country of origin. The metadata, OTU table, taxonomy table, R code, and a detailed description of the methods can be found in *SI Appendix*.

Results

Description of Study Population. Of the total study population, 38% of the samples came from Antananarivo, Madagascar and 62% came from Bangui, CAR (Table 1). Gender was evenly distributed between CAR and Madagascar (Pearson's χ^2 test, $P = 0.823$), and age was equally distributed between samples from stunted and nonstunted children (Pearson's χ^2 test, $P = 0.381$). There were significantly more samples from stunted children from Madagascar and more samples from nonstunted children from CAR in the study population (Pearson's χ^2 test, $P < 0.0001$). General characteristics of the study population are given in Table 1. Of the duodenal samples analyzed, 12 were from Madagascar and 34 from CAR, totaling 46 samples (*SI Appendix*, Table S1). Of the 57 gastric samples analyzed, 10 were from Madagascar and 47 from CAR (*SI Appendix*, Table S2). After rarefaction (see *SI Appendix*, Fig. S2 for rarefaction curves), a total of 343 fecal, 46 duodenal, and 50 gastric samples remained (*SI Appendix*, Fig. S3). Gastric and duodenal pH was significantly different (Mann–Whitney U test, $P < 0.001$). However, within respective compartments, pH did not change between moderately and severely stunted children (*SI Appendix*, Fig. S4*A*). Illumina sequencing targeting the V4 region of the 16S rRNA gene of 404 fecal samples, 57 gastric samples, and 46 duodenal samples resulted in 9,155,211 total reads and a mean sequencing depth of 18,057 sequences per sample (*SI Appendix*, Figs. S1 and S4*B*).

Community Composition Differs Between Duodenal and Fecal Samples. We compared bacterial community composition between gastric, duodenal, and fecal samples using the Bray–Curtis dissimilarity metric and visualized this using a principle coordinates analysis (PCoA) plot (Fig. 1*A*). Because gastric and duodenal samples were only available from stunted children, we only included stunted children for this analysis. Gastric and duodenal samples were clustered together and were separated from the fecal samples (Fig. 1*A*), and overall bacterial composition was significantly different between sampling sites (PERMANOVA, $P = 2e\text{-}04$, $P = 0.001$ in a multivariate model) (*SI Appendix*, Table S5). A few gastric and duodenal samples fell outside of the main cluster of duodenal and gastric samples in the PCoA plot. These outlier samples clustered according to country of origin (*SI Appendix*, Fig. S5*A*) and were predominantly from younger children (*SI Appendix*, Fig. S5*B*). Overall phylum abundance of the individual samples was variable and differed for the fecal samples between Madagascar and CAR (*SI Appendix*, Fig. S6 and Dataset S2) [PERMANOVA on phylum distribution in between the two countries, correcting for nutritional status, gender, age (in months): $P = 0.017$]. The most abundant phyla (i.e., having the highest count) in the duodenal samples were *Proteobacteria* (32.4%), *Bacteroidetes* (29.6%), and *Firmicutes* (25.6%), followed by *Fusobacteria* (9.2%) and *Actinobacteria* (1.7%). The most prevalent phyla (i.e., being present in the most samples) were *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, and *Proteobacteria*, which were present in all duodenal samples analyzed. Other phyla included the *Absconditabacteria* (0.8% abundance, 88.5% prevalence), *Tenericutes* (0.35% abundance, 92.3% prevalence), *Spirochaetae* (0.25% abundance, 92.3% prevalence), *Verrucomicrobia* (0.005% abundance, 34.6% prevalence), and *Cyanobacteria* (abundance 0.05%, 49.6% prevalence). In very low abundance (<0.005%) and prevalence (<10%), there were also members of the phyla *Elusimicrobia*, *Lentisphaerae*, and *Euryarchaeota* present in the duodenal samples. *Absconditabacteria* were prevalent in duodenal (88.5%) and gastric (92.6%) samples and very rare in fecal samples (6.9%) (Dataset S1). Richness [as measured by the Chao1 index (54)] of the gastric, duodenal, and fecal samples at the OTU level was roughly the same (*SI Appendix*, Fig. S7). However, evenness (as measured by Simpson's diversity index) of the samples was significantly lower (linear-mixed model, $P = 2.6e\text{-}09$) in fecal samples compared with the gastric and duodenal samples, indicating that there are dominant taxa in the feces while there is a more even distribution of taxa in the duodenum and stomach (*SI Appendix*, Fig. S7). The core microbiota (i.e., taxa present in at least 90% of all samples and with a relative abundance of least 0.01% in each sample) displayed more taxa in the gastric and duodenal samples compared with fecal samples, suggesting that the upper gastrointestinal tract might show a more conserved structure at the OTU level than the lower intestinal tract. Gastric and duodenal samples shared 43 OTUs, while the upper gastrointestinal samples (stomach, duodenum) shared only three OTUs, *Veillonella* sp. ICM51a, *Haemophilus influenza*, and *Prevotella copri*, with the lower gastrointestinal tract (colon) (Fig. 1*B*). The same phenotype was also observed when sample count was equilibrated in between the different compartments by randomly subsampling the fecal samples to 50 (*SI Appendix*, Fig. S8). In summary, these data show that the bacterial microbiota of the upper and lower gastrointestinal tracts are distinct.

α-Diversity in Gastric, Duodenal, and Fecal Communities Is Not Affected by Stunting but Is Dependent on Age and Country of Origin. Richness and evenness were compared between stunted (moderately or severely) and nonstunted children for each sample type (gastric, duodenal, or fecal). Richness of gastric, duodenal, or fecal samples was not affected by nutritional status (Fig. 1*C* and *SI Appendix*, Fig. S9 *C* and *D*). We further investigated if country of origin of the children or their age might have an effect on the community structures. Indeed, older children had a significantly richer and more even fecal microbiota compared with younger children (*SI Appendix*, Fig. S9*A*). Country of origin had no overall significant effect on community evenness. However, there was a small trend visible for higher community richness in samples from Madagascar (*SI Appendix*, Fig. S9*B*). This trend became significant in children aged 3 y or older if samples were stratified according to age (*SI Appendix*, Fig. S10). The contribution of both country and age to overall richness, and of sample type to overall evenness, was confirmed in linear-mixed models (*SI Appendix*, Tables S3 and S4). Fecal samples were collected at home. The time the fecal samples spent at room temperature before freezing therefore differed for each sample. To assess for a possible cofounding of results by this covariable, we analyzed the effect of time to freezing on community diversity in multivariate models correcting for gender, age (in months), and country of origin. The time to freezing had no significant influence on either of these

Table 1. Description of the study population ($n = 404$)

Description	Prevalence
Country	
Madagascar	38% (153/404)
Central African Republic	62% (251/404)
Gender	
Female	52% (211/404)
Male	48% (193/404)
Age	
Median (interquartile range)	41 mo (32/49 mo)
2–3 y	35% (141/404)
3–4 y	36% (145/404)
4–5 y	29% (118/404)
Nutritional status	
Normal height	58.8% (236/404)
Moderately stunted	23.3% (94/404)
Severely stunted	18.3% (74/404)
SIBO in stunted children*	91.3% (42/46)
Moderately stunted with SIBO	96% (24/25)
Severely stunted with SIBO	85.7% (18/21)

*No data available for nonstunted children.

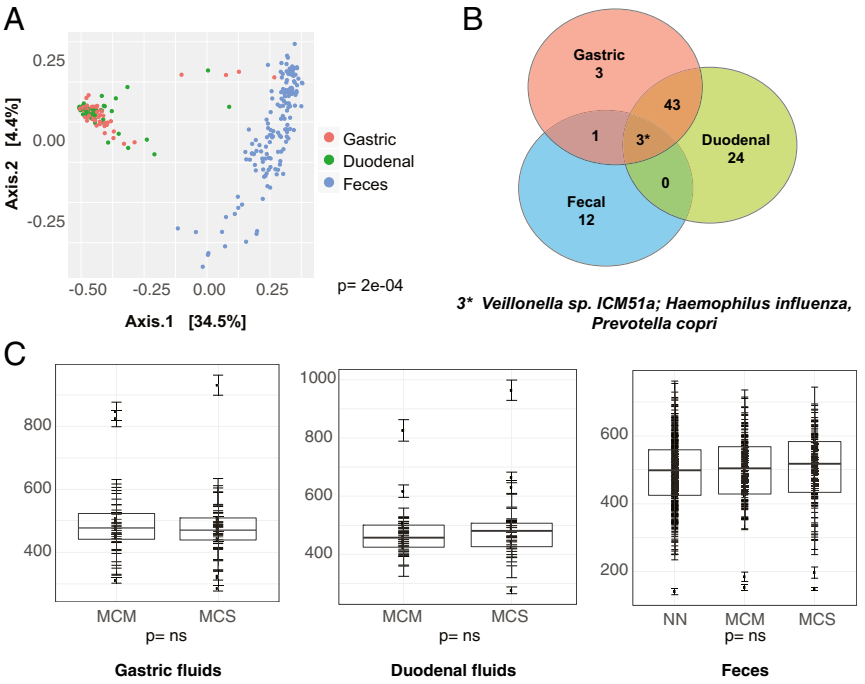

Fig. 1. Overall microbiota composition of gastrointestinal samples. (*A*) PCoA plot of the Bray–Curtis dissimilarity index of the samples rarefied to 10,000 sequences. Sample count: gastric aspirates: $n = 50$; duodenal aspirates: $n = 46$; feces: $n = 343$. Gastric samples are colored in red, duodenal samples in green, and fecal samples in blue. PERMANOVA test based on sample type yielded a P value of 2×10^{-4}. (*B*) Venn-diagram depicting the number of taxa constituting the core microbiota of gastric (red), duodenal (green), and fecal (blue) samples. The core microbiota was defined as taxa with a relative abundance of at least 0.01% in each sample and a prevalence of 90% in the corresponding sample group. The number of shared core taxa between the different compartments is indicated in the overlapping regions. (*C*) Richness (as measured by the Chao1 index) according to nutritional status in gastric (*Left*, Student's t test, $P = 0.60$), duodenal (*Center*, Student's t test, $P = 0.53$), and fecal (*Right*, ANOVA, $P = 0.70$) samples. Fecal samples compared: 200 from nonstunted, 80 from moderately stunted, and 63 from severely stunted children. Duodenal samples compared: 25 from moderately stunted and 21 from severely stunted children. Gastric samples compared: 30 from moderately stunted and 27 from severely stunted children. Analysis performed on samples rarefied to 10,000 sequences. MCM, moderately stunted; MCS, severely stunted; NN, nonstunted.

measurements if analyzed as a continuous variable in minutes, if recoded as a binary variable of more or less than half an hour spent at room temperature before freezing or if recoded as a categorical variable (<30 min, 0.5–1 h, 1–2 h, 2–3 h, 3–4 h and so forth, spent at room temperature before freezing) [PERMANOVA; Chao1: $P = 0.79$ (continuous), $P = 0.89$ (binary), $P = 0.84$ (categorical); Simpson: $P = 0.82$ (continuous), $P = 0.98$ (binary), $P = 0.76$ (categorical); Bray–Curtis: $P = 0.2$ (binary), $P = 0.6$ (categorical)]. In conclusion, our data suggest that fecal α-diversity is dependent on country of origin and age of the children but independent of their stunting status.

Stunted Children Suffer from SIBO with Oropharyngeal Taxa. Among the duodenal samples collected ($n = 46$), 44 (96%) gave rise to colonies on the different selective media. SIBO (CFU > 10^5/mL of aspirated fluid) was present in 12 of 12 samples from Madagascar (100%) and in 30 of 34 samples from CAR (88%). Per aspirate, between two and six different morphotypes were randomly chosen and identified. No exhaustive characterization of all colonies was performed. In total, 47 different species were isolated in either CAR or Madagascar. *Streptococcus*, *Staphylococcus*, *Haemophilus*, *Neisseria*, *Moraxella*, and *Rothia* were the most predominant genera recovered (Table 2). Analyzing the duodenal samples with 16S amplicon sequencing, we confirmed the presence of the bacterial taxa identified by culture and the high prevalence of bacterial genera normally found in the oropharyngeal cavity (Fig. 2 and Dataset S3). The overall community structure and especially the most prevalent taxa were remarkably similar in the two countries at the genus level as well as the species level (Fig. 2 and *SI Appendix*, Fig. S11). They were also very similar between moderately and severely stunted children (Dataset S4). The 20 most prevalent taxa contributed to more than half of the total abundance of taxa in the community (58%). They included several taxa of nasopharyngeal/oral origin (see Table 2 and Dataset S3 for a list of oropharyngeal bacteria), including four different species of *Haemophilus* (*H. influenzae*, *H. parahaemolyticus*, *H. aegyptius*, uncultured *Haemophilus*), three different *Neisseria* species (*N. cinerea*, *N. lactamica*, uncultured *Neisseria*), *Streptococcus oralis*, two species of *Veillonella* (ICM51a and oral taxon 780), an uncultured *Porphyromonas*, and *Moraxella catharralis.* Three of the 20 most-abundant taxa belong to the genus *Prevotella* (*P. melaninogenica*, *P. nanceiensis*, and *P. bacterium* Marseille−P2826) (*SI Appendix*, Fig. S11 and Dataset S3). Using a likelihood-ratio test (LRT) model and correcting for gender, age, and country of origin, we did not detect any taxon to be overrepresented in duodenal aspirates of severely compared with moderately stunted children. However, we saw a significant decrease in two members of the *Haemophilus* genus in the duodenal aspirates of severely compared with moderately stunted children (*SI Appendix*, Fig. S13*A*). The SIBO composition described in duodenal samples was mirrored by a surprisingly similar community in gastric samples: gastric and duodenal samples showed a very similar composition (Fig. 1*A*) and shared a large fraction of the core microbiota (Fig. 1*B* and *SI Appendix*, Fig. S8). For both, the driving factors shaping microbiome composition were country of origin and age of the children (*SI Appendix*, Tables S6 and S7). Like duodenal samples, the gastric samples were mainly composed of oropharyngeal taxa (*SI Appendix*, Fig. S12 and Datasets S5 and S6). In CAR, *Helicobacter* was among the most prevalent taxa, reaching as much as 70% for some of the subspecies of *Helicobacter pylori*. In Madagascar, prevalence of the most prevalent *Helicobacter* taxon (annotated as *H. pylori* SA170A) was similar (70%) but significantly lower for the other *Helicobacter* taxa detected (Dataset S5). When stratified on the country of origin there were no major differences in the sample composition between duodenal and gastric samples (*SI Appendix*, Fig. S13*B*). This was unexpected, as the pH of the two aspirates significantly differs (pH 6.7 for duodenal aspirates; pH 2.7 for gastric aspirates, Mann–Whitney U test, $P < 0.0001$). Overall, these results suggest that stunted children suffer from overgrowth of bacteria in the upper gastrointestinal tract composed mainly of oropharyngeal taxa.

Stunted Children Show a Distinct Signature in Their Feces with Overrepresentation of Oral Bacteria and Enteropathogens and Underrepresentation of Butyrate Producers. The factors contributing most to microbiome composition in fecal samples were age, stunting, and country of origin (*SI Appendix*, Table S8). Therefore,

Table 2. Genera and species cultivated from duodenal fluids

Genus	No. isolates CAR*	Species CAR	No. isolates Madagascar*	Species Madagascar
Streptococcus	27	*Streptococcus mitis* ($n = 6$);† *S. salivarius* ($n = 3$);† *Streptococcus pneumoniae* ($n = 13$);† *Streptococcus intermedius* ($n = 5$) (94)†	23	*S. mitis* ($n = 8$);† *S. salivarius* ($n = 5$);† *S. oralis* ($n = 4$);† *Streptococcus parasanguinis* ($n = 3$);† *S. pneumoniae* ($n = 2$) (94);† *Streptococcus cristatus* ($n = 1$) (95)†
Staphylococcus	19	*Staphylococcus hominis* ($n = 1$); *Staphylococcus haemolyticus* ($n = 3$); *Staphylococcus aureus* (n= 14); *Staphylococcus* sp. ($n = 1$)	3	*Staphylococcus epidermidis* ($n = 2$); *Staphylococcus oralis* ($n = 1$) (96)†
Haemophilus	13	*H. influenzae* ($n = 8$);† *Haemophilus parainfluenzae* ($n = 2$) (97);†,‡ *Haemophilus haemolysans* ($n = 1$); *Haemophilus* sp. (n= 2)	1	*Haemophilus parahaemolitycus* ($n = 1$) (97)†
Moraxella	8	*Branhamella/M. catharrhalis* ($n = 1$) (98, 99);† *Moraxella* sp. ($n = 7$)	3	*Branhamella catarrhalis* ($n = 3$) (98, 99)†
Neisseria	8	*Neisseria sicca* ($n = 4$);† *Neisseria mucosa* ($n = 2$);† *Neisseria sicca mucosa* ($n = 2$) (100)†	14	*Neisseria flavescens* ($n = 8$);† *Neisseria macacae* ($n = 1$);† *N. mucosa* ($n = 1$);† *Neisseria perflava* ($n = 1$);† *Neisseria subflava* ($n = 1$);† *Neisseria* spp. ($n = 2$) (100, 101)†
Rothia	0		6	*Rothia dentocariosa* ($n = 5$);† *Rothia mucilaginosa* ($n = 1$);†,‡
Kocuria	5	*Kocuria varians* ($n = 5$)	1	*Kocuria marina* ($n = 1$)
Lactococcus	5	*Lactococcus lactis* ($n = 4$);†,‡ *Lactococcus* sp. ($n = 1$)	0	
Leuconostoc	4	*Leuconostoc* sp. ($n = 4$)†,‡	0	
Actinomyces	0		3	*Actinomyces naeslundii* ($n = 2$);† *Actinomyces oris* ($n = 1$) (101)†,‡
Aerococcus	2	*Aerococcus viridans* ($n = 2$)	0	
Gemella	2	*Gemella haemolysans* ($n = 2$)†,‡	1	*Gemella haemolysans* ($n = 1$)†,‡
Pasteurella	1	*Pasteurella pneumotropica* ($n = 1$)	0	
Kingella	0		1	*Kingella denitrificans* ($n = 1$)†,‡

Genera overrepresented in the fecal samples of stunted children are underlined.
*The 34 subjects for CAR and 12 subjects for Madagascar included in the study.
†Oropharyngeal species.
‡CORE (microbiome.osu.edu/) and ref. 102.

all models were corrected for age and country of origin or stratified accordingly. We also corrected for gender, a variable described in the literature to be associated with microbiota composition (58). Fecal samples of stunted and nonstunted children were compared using the program DeSeq2 using the LRT model. Several taxa changed in their prevalence or abundance according to stunting phenotype (Fig. 3). These included several taxa normally found in the oropharyngeal cavities (marked with a green star in Fig. 3; see Table 2 and *SI Appendix*, Table S3 for a list of putative oral bacteria and references thereof): for example, members of the genera *Streptococcus*, *Porphyromonas*, *Neisseria*, *Fusobacterium*, *Veillonella*, *Gemella*, or *Actinobacillus*. Several of these taxa were the same as those contributing to SIBO (Fig. 2, Table 2, and Dataset S8). Resolution to the species level allowed identifying several of the taxa annotated to "oropharyngeal species" to be more abundant in stunted children compared with their nonstunted controls. These included *Lactobacillus salivarius* (fold-change: 333.9), *Prevotella histicola* (fold-change: 43.0), *Porphyromonas assacharolytica* (fold-change: 26.2), *P. melaninogenica* (fold-change: 19.7), *N. cinerea* (fold-change: 18.1), *Fusobacterium periodonticum* (fold-change: 17.5), *Lactobacillus mucosae* (fold-change: 4.6), *Prevotella nigrescens* (fold-change: 4.0), *Veillonella atypica* (fold-change: 3.7), *H. aegypticus* (fold-change: 3.7), and *H. parahaemolyticus* (fold-change: 3.2) among others (Table 3 and Dataset S13). Members of two genera of potentially enteropathogenic microorganisms were also more prevalent in stunted children compared with nonstunted controls. These were *Escherichia coli*/*Shigella* and *Campylobacter*, which were resolved to the species level as *Shigella flexneri*/*E. coli* (not distinguishable by 16S, fold-change: 7.6) and *Campylobacter concisus* (fold-change: 3.2). Other species found to be more abundant in stunted children compared with their healthy

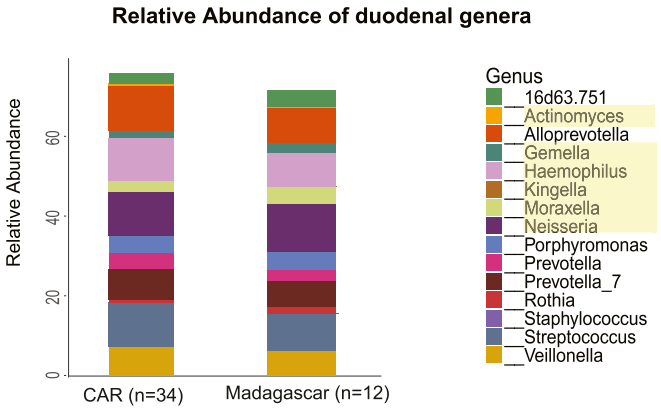

Fig. 2. Relative abundance of the 10 most-abundant genera in the duodenum as well as genera cultured in the samples. The analysis was performed on 12 samples from Madagascar and 34 samples from CAR. Data are stratified on country of origin of the duodenal samples. The color code for the different genera is given on the right. Genera highlighted in yellow are also identified by culture techniques.

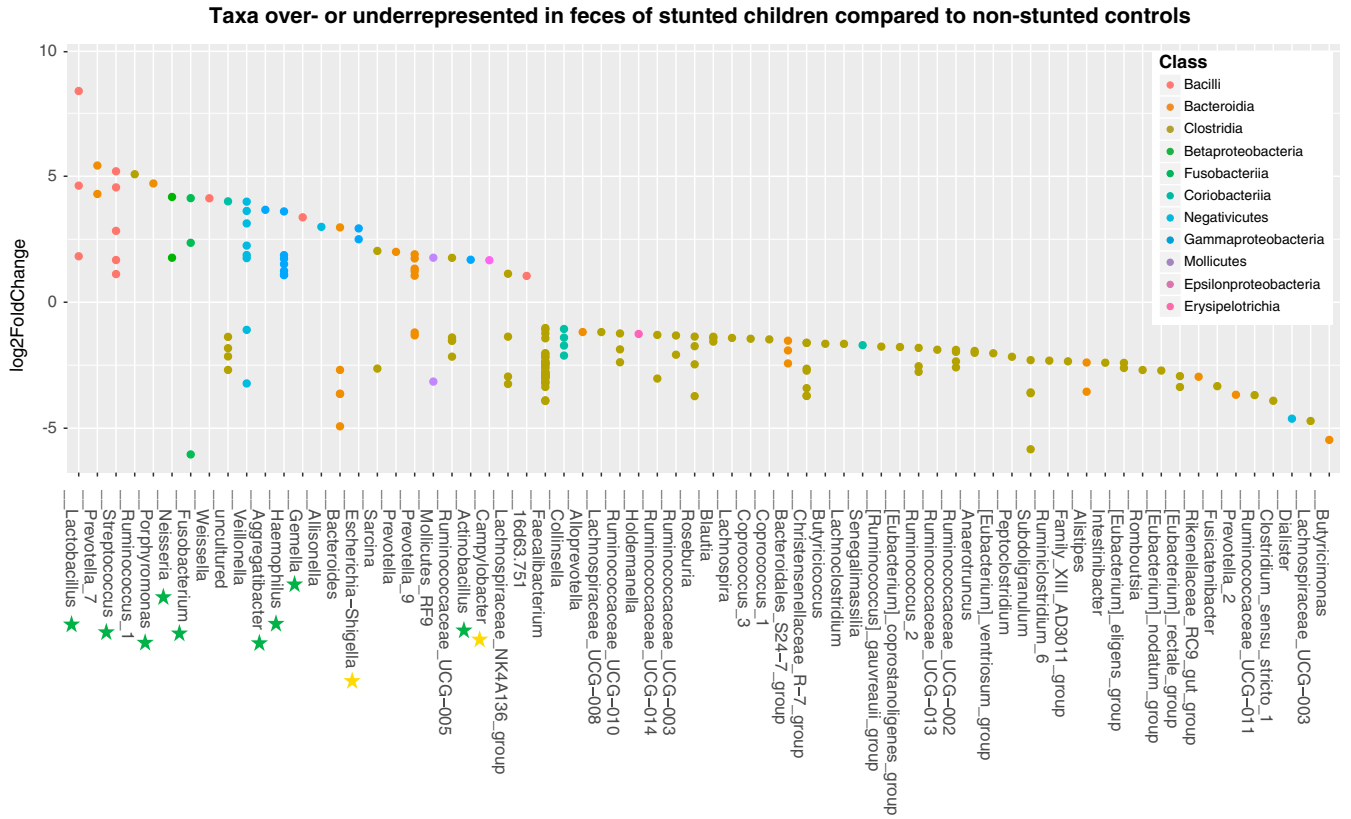

Fig. 3. Taxa with differential relative abundance in fecal samples of stunted children compared with nonstunted controls (DeSeq2 analysis). Analysis was performed on 404 fecal samples, of which 236 were nonstunted and 168 were stunted. Taxa are ordered according to $\log_2$ fold-change. Each individual OTU affected for a given genus is indicated with a dot. Dots are colored according to their class according to the color code given on the right of the graph. Only taxa with at least $\log_2$ fold-change and which are significantly differentially abundant (LRT, $P \leq 0.01$) are indicated on the graph/in the table. Differentially abundant OTU are grouped according to genera. Genera with species mainly found in the oropharyngeal cavities are indicated with a green star. Genera, which represent enteropathogens, are indicated with a yellow star.

control subjects included *Allisonella histaminiformans* (fold-change: 8.0) and *Morococcus cerebrosus* (fold-change: 3.4); interestingly, *M. cerebrosus* was also one of the most-abundant species in the duodenum (*SI Appendix*, Fig. S11).

Several taxa were less abundant in stunted children compared with their nonstunted control subjects. These include many members of the butyrate-producing *Clostridia* (Fig. 3). To see if the phenotype was conserved between both countries independently, we then analyzed the data stratified on country of origin (*SI Appendix*, Fig. S14). Overrepresentation of oropharyngeal taxa and enteropathogens, as well as underrepresentation of butyrate producers, was observed in stunted children from both Madagascar and CAR independently. Members of several genera were overrepresented in stunted children in both countries, including members of the *Fusobacterium*, *Streptococcus*, *Veillonella*, and *Actinobacillus* genus. Members of the genus *Campylobacter* were overrepresented in both countries, while *Shigella*/*E. coli* was found overrepresented only in CAR. In both countries, *A. histaminiformans* was overrepresented in the stunted children compared with their nonstunted control subjects (Table 3 and *SI Appendix*, Fig. S13). Detailed species identification was available for some of the overrepresented taxa and is given in Table 3. Overrepresentation of oropharyngeal taxa was also independently found when nonstunted children were compared with either moderately stunted or severely stunted children, either for a pooled dataset from both countries or stratified for the country of origin (*SI Appendix*, Fig. S15). Several oropharyngeal taxa as well as butyrate producers were also found to be the most-discriminatory taxa between stunted and nonstunted children using randomForest modeling (*SI Appendix*, Fig. S16).

Prevalence of specific oral species in fecal samples varied with prevalences between 0.5 and 40%. For *H. influenzae*, one taxon reached more than 96% prevalence in stunted and nonstunted children (Dataset S7). Oral taxa identified to species level with a high prevalence in fecal samples include *Streptococcus salivarius* (~90%), followed by *N. cinerea* (~53%), *Actinomyces odontolyticus* (50%), and *S. oralis* (~46%) (Datasets S7 and S9). The prevalence of these same taxa was much higher in the duodenum, reaching prevalences of >90% of many of the oral taxa (Dataset S10). Prevalences of given species and substrains slightly differed between countries. *Fusobacterium equinum*, as an example, was only present in the CAR (prevalence of 8%) but absent in Madagascar, and *Fusobacterium necrophorum* was present in Madagascar (42%) but absent from the CAR (Dataset S11). This indicates that while there are some country-specific signatures in the duodenum, there is consistently high prevalence of oropharyngeal taxa in the duodenum in both countries and, at lower prevalence, in feces of stunted and nonstunted children ("bottleneck effect"). Prevalence of enteropathogens in feces was also high, with 41.2% of Malagasy children and 15.5% of Central African children showing the most prevalent *Campylobacter* taxon (*Campylobacter jejuni* ssp. *jejuni*) (Dataset S12), several others, including *C. concisus* and *Campylobacter hominis* were also present. Different species of *Campylobacter* were also found in the duodenum, including *C. concisus* and *Campylobacter rectus* (>90% prevalence in both countries), *C. jejuni* (prevalence: 3% in CAR, 25% in Madagascar for the most prevalent

Table 3. Summary of taxa differentially overrepresented in feces from stunted children with species level identification

Genus	Species	Overrepresented in the following dataset
Lactobacillus	*L. salivarius,* L. mucosae,* L. ruminis*	Merged dataset
Porphyromonas	*P. assacharolytica**	Merged dataset
Fusobacterium	*F. periodonticum**	CAR, merged dataset
	*F. nucleatum**	CAR
	*F. mortiferum**	Madagascar
Neisseria	*N. cinerea**	CAR, merged dataset
Haemophilus	*H. aegypticus,* H. parahaemolyticus**	CAR, merged dataset
	*H. paraphrohaemolyticus**	Madagascar
Streptococcus	*S. salivarius,* S. oralis**	CAR
Veillonella	*V. atypica*	CAR, Madagascar
Aggregatibacter	*A. aphrophilus**	CAR
Allisonella	*A. histaminiformans*	CAR, Madagascar, merged dataset
Campylobacter	*C. jejuni*	Madagascar
	*C. concisus**	CAR, merged dataset
Shigella	*S. flexneri*	CAR, merged dataset
Prevotella	*P. melaninogenica**	CAR, merged dataset
	*P. nigrescens**	CAR, merged dataset
Morococcus	*M. cerebrosus*	CAR, merged dataset

CAR, dataset comprising only samples for the Central African Republic; Madagascar, dataset comprising only samples for Madagascar; merged dataset, dataset comprising all fecal samples.
*Members of the oropharyngeal core microbiota.

taxon), *Campylobacter showae* (prevalence of 53% in CAR, 8% in Madagascar), and *C. hominis* (only present in Madagascar; prevalence = 8%) (Dataset S11).

We next used Picrust analysis to predict the functional content of these microbiota datasets. Genes implicated in butyrate (also called butanoate) metabolism tended to be less prevalent in feces from stunted children compared with nonstunted control subjects; however, this was not statistically significant (linear-mixed model, $P = 0.179$). The abundance of butyrate-producing bacteria differed most strongly by stunting in CAR; we therefore stratified our Picrust analysis on fecal samples from CAR, and noted that the trend in butyrate metabolism was stronger in this population (linear-mixed model correcting for gender and age, $P = 0.067$) (*SI Appendix*, Fig. S17). This suggests that differences in butyrate-producing microbes might be mirrored by changes in butanoate metabolism, albeit to a lesser degree. In conclusion, our data describe a conserved signature in feces of stunted children, consisting of the increased presence of oropharyngeal taxa and of enteropathogens, reflecting the community composition of small intestinal fluids from the same children.

Discussion

Our study highlights three major findings: (*i*) the composition of SIBO in the context of stunting, characterized by oropharyngeal species; (*ii*) a similar microbiota in both the stomach and duodenum of stunted children; and (*iii*) a clear microbiological signature of stunting in the feces, characterized by an overrepresentation of pathobionts and of many oropharyngeal species found in SIBO. Taken together, we propose the following model: that stunted children experience a decompartmentalization of the gastrointestinal tract, such that microbial taxa in the oral cavity, or introduced through a fecal–oral contamination, can more easily colonize the stomach and duodenum. These oropharyngeal species and pathobionts are carried through the gastrointestinal tract from stomach to colon and are ultimately detectable in the feces as a signature of SIBO and of stunting (Fig. 4). Importantly, the overgrowth of oropharyngeal bacteria in the small intestine may be responsible for the small intestinal inflammation and damage that is characteristic of PEE, a hypothesis that warrants further investigation.

The main oral taxa described in the literature are *Streptococci* (including *S. oralis*, *S. salivarius*, *S. mitis*, *S. pneumoniae*) and *Veillonella* sp. Other taxa present in lower abundance include members of the genera *Lactobacillus*, *Prevotella*, *Gemella*, *Neisseria*, *Haemophilus*, *Prophyromonas*, *Fusobacterium*, and *Actinomyces*, among others (59, 60). We found several members of these genera, which belong to the oral core microbiota (see CORE, microbiome.osu.edu/), to be highly present in the duodenum and overrepresented in the feces of stunted children compared with their nonstunted controls. In healthy subjects, the gastric microbiota is dominated by members of the genus *Lactobacillus* and, to a lesser extent, *Streptococcus*, *Staphylococcus*, *Propionibacterium*, *Actinomyces*, *Bacteroides*, *Neisseria*, and *Fusobacterium* (19, 61, 62), but exact composition seems to greatly vary between subjects (reviewed in refs. 61 and 62). Due to difficulties in sampling, the duodenal microbiota is less well described and essentially corresponds to diseased subjects or subjects with disease suspicion. In healthy adults, the most prevalent genera include members of *Streptococcus*, *Prevotella*, *Staphylococcus*, *Actinomyces*, *Veillonella*, and *Neisseria* (34, 35, 40, 63). This was also observed in children (64, 65), but large numbers of *H. influenzae* were also detected in children (65). Large interindividual differences in the duodenal community structure have additionally been reported (64).

The aspirates collected for this project show a similar composition as those described in the literature. However, instead of the described dominance of *Lactobacilli* in the stomach and *Streptococci* in the duodenum, and despite the drastic pH difference observed between the two compartments, stunted children in this study displayed community homogenization between the stomach and the duodenum. Furthermore, children in this study had a more diverse small intestinal community than those described so far in the literature. Although for ethical reasons we could not obtain small intestinal samples from nonstunted control children, we could show that oropharyngeal taxa were overrepresented in the feces of stunted compared with control children. This suggests a previously unrecognized decompartmentalization of the microbiota along the oral/gastrointestinal tract in stunted children. In the future, gastric samples could therefore be used as a proxy for

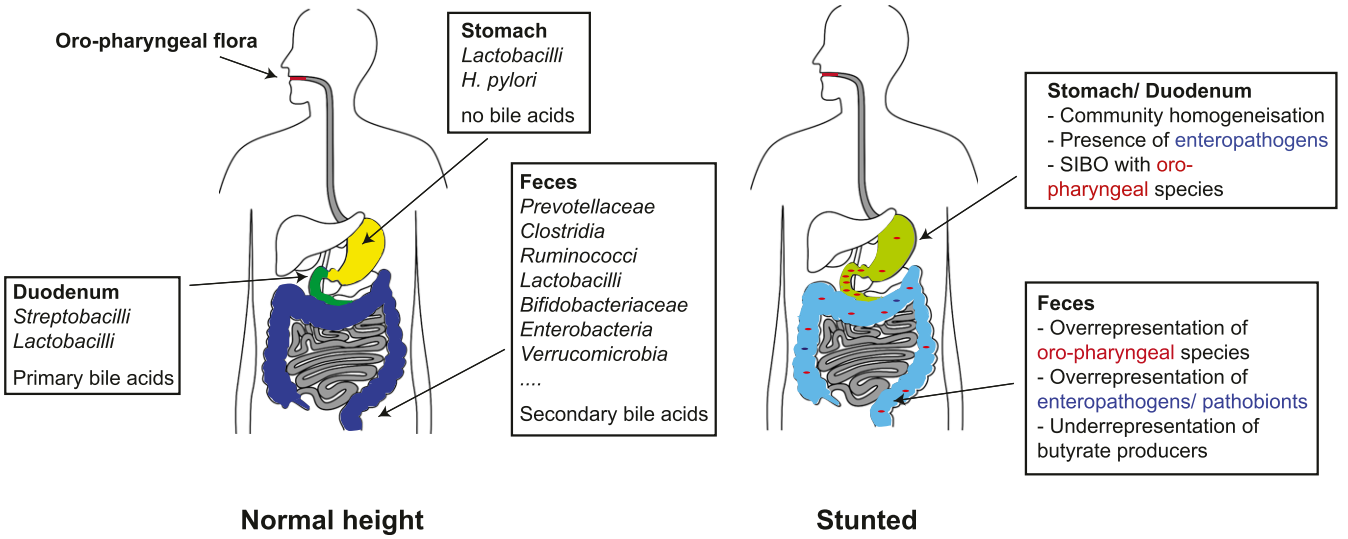

Fig. 4. Schema of the decompartimentalization of the gastrointestinal tract observed in the context of stunting. (*Left*) Gastrointestinal compartmentalization is indicated as described in the literature for nonstunted children. (*Right*) The observed decompartimentalization with associated changes in stunted children are depicted.

the duodenal microbiota composition, as these samples are more readily accessible.

The observed overgrowth of oropharyngeal taxa in the small intestine could be favored by several factors: massive delivery of oropharyngeal microorganisms due to poor oral hygiene and recurrent/chronic rhino-pharyngeal infections; a hypochloric environment in the stomach, alleviating the natural barrier of stomach acidity; changes in other bactericidal factors, which are poorly described in the stomach, but which could include bile acids, pancreatic enzymes, or antimicrobial peptides in the upper small intestine; or alterations in motility, leading to prolonged stagnation of chyme and associated bacteria within the small intestine. In earlier work, it was shown that undernourished children had decreased gastrin levels (66), a phenotype that was also observed in an animal model of undernutrition (67). In our study, we did not detect any significant difference in the gastric pH of moderately and severely stunted children. While nonstunted control subjects are needed to conclude whether stunted children experience dyspepsia, the low gastric pH observed in our samples suggests that stunting is not associated with a significant decrease in gastric acidity. It therefore seems likely that the SIBO phenotype is associated with other factors. More research is needed to understand the underlying mechanisms leading to the observed decompartmentalization of the gastrointestinal tract in stunted children. The reduction in butyrate-producing *Clostridia* and the overrepresentation of enteropathogenic taxa observed in fecal samples of stunted compared with nonstunted children constitute a bona fide dysbiosis (68) and signature of stunting. Interestingly, despite the high prevalence of potentially enteropathogenic taxa, none of the children was suffering from severe diarrhea at the time point of inclusion (asymptomatic carriage).

Butyrate is a calorie-rich nutrient and a potent regulator of host metabolism. Its chronic depletion may therefore contribute to undernutrition. Furthermore, butyrate is an effector of colonization resistance to facultative anaerobic enteropathogens, such as *Salmonella* Typhimurium (69). Depletion of butyrate-producing *Clostridia* was shown to facilitate outgrowth of facultative anaerobic bacteria in a mouse model of colitis (69). The reduction in butyrate could therefore promote the overrepresentation (facultative) of aerobic *Streptococcus*, *Neisseria*, *Staphylococcus*, *Haemophilus*, *Campylobacter*, and *Escherichia*/*Shigella* genera that was observed in our data. Reduced butyrate might also explain the increased frequency and severity of gastrointestinal diseases in undernourished children compared with normally nourished control subjects, which has been described in earlier studies (70, 71). In turn, afflux of oropharyngeal bacteria (pathobionts) could also directly or indirectly affect clostridial populations, hence leading to a vicious cycle.

In addition to the overrepresentation of oral taxa, *C. concisus* and *C. jejuni* were increased in feces of stunted children compared with nonstunted control subjects. This supports observations from a longitudinal pilot study of stunting in India, which found members of the Campylobacterales order to be more prevalent in stunted children compared with nonstunted control subjects (33). Enteric pathogen carriage and associated inflammation might contribute directly to stunting (e.g., via Igf1); moreover, this could be an important factor in shaping the local microbiota and hence nutrient degradation. Interestingly, *C. concisus* has been described both as an oral taxon leading to periodontitis and as an enteropathogen leading to diarrhea and associated with ulcerative colitis (reviewed in ref. 72). Several inflammatory diseases have been previously associated with small intestinal dysbiosis. These include liver cirrhosis (73, 74), as well as adult (63) and pediatric celiac disease (75). Dysbiois in these diseases was characterized by the presence of *Proteobacteria*, as well as certain oral taxa, such as *Veillonella* (63, 64, 74, 75). Furthermore, in liver cirrhosis, the intestinal abundance of *Haemophilus* was decreased (73). Although we did not see any taxa that were more abundant in duodenal aspirates of severely compared with moderately stunted children, we saw a significant decrease in two members of the *Haemophilus* genus. Furthermore, we saw a higher prevalence of *Proteobacteria* (*Shigella*, *Campylobacter*) and of *Veillonella* and *Prevotella* in the feces of stunted children. The presence of members of the oral microbiome at distant sites also relates to other inflammatory diseases, including gastric, pancreatic, and colorectal cancers (76–78); inflammatory bowel disease (79–82); primary sclerosing cholangitis (83), rheumatoid arthritis (84); diabetes (85–87); and cardiovascular diseases (reviewed in refs. 88 and 89). Furthermore, many members of the oral microbiota have been shown to induce inflammation in vitro and in vivo (90–93). Administration of *Porphyromonas gingivalis* to mice led to systemic inflammation, impaired barrier function, and changes in the small intestinal microbiota (92). Most recently, oral bacteria from patients with Crohn's disease

were shown to induce T_H1 inflammatory responses in mice (93). Together with our study, these data support the hypothesis that decompartmentalization of the gastrointestinal tract and presence of oral bacteria in the intestine might play a major role in inflammation and gastrointestinal disease.

This study has a few limitations. Because stunting is a chronic syndrome, a longitudinal rather than cross-sectional approach would have allowed us to assess a causal relationship between oral bacteria and stunting. Furthermore, the sequencing depth of the present study was relatively low, and therefore the analyses performed on microbiota α-diversity in different groups might be confounded by the fact that very low abundance taxa were not captured by the approach. Detailed analysis of the α-diversity of different communities not being the main objective of this study, we opted for analyzing more samples rather than achieving a deeper sequencing depth within each sample. In contrast, the large sample number allows for properly taking into account the natural biological variability in microbiota composition across subjects. The full dataset to be established in the context of the AFRIBIOTA Consortium, comprising almost 1,000 children, will allow us to consolidate the obtained results. Furthermore, as the presence of oral bacteria in the lower gastrointestinal tract was not anticipated, we did not collect oral samples from the same children. The taxa identified as "oral" species are therefore taxa known in the literature to be part of the core oral microbiota rather than proven oral taxa from the corresponding individual. This point should be addressed in more detail in future clinical studies as to clearly demonstrate that the same strains are found in the oropharyngeal cavity and the lower intestinal tract of stunted children.

Here, we report a study comparing duodenal, gastric, and fecal samples of stunted children. In the past, enteropathogens were considered the main taxa driving inflammation in undernutrition. Our study confirms the suspected overrepresentation of enteropathogenic bacteria in the duodenum of stunted children but extends the current picture by demonstrating a previously unknown microbial decompartmentalization of the oropharyngeal to gastrointestinal tract. While our study does not provide mechanistic details about the role of these oropharyngeal bacteria in inflammation, we show the overrepresentation of such taxa in stunted children is a conserved phenotype in two geographically, genetically, and nutritionally divergent populations. This suggests that overrepresentation of oral bacteria and pathobionts in the small intestine and in the colon, in addition to a reduction in butyrate-producing *Clostridiales*, could be a general hallmark of stunting and is likely to play a major role in its pathophysiology. If we can confirm the predicted role of oropharyngeal bacteria in driving intestinal inflammation, this will lead to a shift in understanding the pathophysiology of stunting and may lead to major changes in the way the syndrome is treated.

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