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Gut microbiota analysis reveals a marked shift to bifidobacteria by a starter infant formula containing a synbiotic of bovine milk-derived oligosaccharides and *Bifidobacterium animalis* subsp. *lactis* CNCM I-3446

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Summary

Non-digestible milk oligosaccharides were proposed as receptor decoys for pathogens and as nutrients for beneficial gut commensals like bifidobacteria. Bovine milk contains oligosaccharides, some of which are structurally identical or similar to those found in human milk. In a controlled, randomized double-blinded clinical trial we tested the effect of feeding a formula supplemented with a mixture of bovine milk-derived oligosaccharides (BMOS) generated from whey permeate, containing galacto-oligosaccharides and 3'- and 6'-sialyllactose, and the probiotic *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) strain CNCM I-3446. Breastfed infants served as reference group. Compared with a non-supplemented control formula, the test formula showed a similar tolerability and supported a similar growth in healthy newborns followed for 12 weeks. The control, but not the test group, differed from the breast-fed reference group by a higher faecal pH and a significantly higher diversity of the faecal microbiota. In the test group the probiotic *B. lactis* increased by 100-fold in the stool and was detected in all supplemented infants. BMOS stimulated a marked shift to a bifidobacterium-dominated faecal microbiota *via* increases in endogenous bifidobacteria (*B. longum*, *B. breve*, *B. bifidum*, *B. pseudocatenulatum*).

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Introduction

After deciphering the sequence of the human genome, the scientific community 'discovered' our second genome: that of microbes colonizing our body (Ding and Schloss, 2014; Human Microbiome Project Consortium, 2012a,b). Advances in sequencing technologies have allowed a deep look into this world of microbial symbionts. Most research went into the gut microbiota revealing its compositional complexity (Arumugam *et al.*, 2011), its maturation in infants (Yatsunenko *et al.*, 2012) and its association with human health and disease states (Gordon *et al.*, 2012; Saavedra and Dattilo, 2012; Buccigrossi *et al.*, 2013; Subramanian *et al.*, 2014). What is far less clear are ways how to change a microbiota composition from a disease- to a health-associated state (Brüssow, 2015a,b). Recent data document substantial success with the transplantation of processed whole faeces from healthy donors into the gut of patients who suffer from *Clostridium difficile* infection (van Nood *et al.*, 2013; McCune *et al.*, 2014). Because the standardization of such preparations as 'bacteriotherapy' is problematic from a regulatory viewpoint, more research has been conducted to develop defined oral supplements such as

probiotics and prebiotics, which beneficially influence the gut microbiota composition (Preidis and Versalovic, 2009; Kumar *et al*., 2014).

Probiotics are defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host'. Although this definition does not specify the microorganisms, most data come from bifidobacteria and lactobacilli used against gastrointestinal inflammations, infections and allergic conditions. Prebiotics on the other hand are defined as 'nonviable food components that confer a health benefit on the host associated with the modulation of the microbiota'. Although the chemical nature of the food component is again not specified in this definition, most research has been done with fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) in the context of gastrointestinal and allergic diseases (Mugambi *et al*., 2012a; Osborn and Sinn, 2013; van Hoffen *et al*., 2009). Human milk oligosaccharides (HMOs) are natural prebiotics because they represent the bifidogenic factors that favour the growth of bifidobacteria, which typically dominate the gut microbiota of breastfed infants (Garrido *et al*., 2013; Smilowitz *et al*., 2014). Current research suggests that HMOs may represent a 'glycan code', which the mother uses to influence the colonization of the gut in breastfed infants with beneficial microbes (Zivkovic *et al*., 2011). Because HMOs synthesis at large scale is still challenging, milk oligosaccharides from cow's milk – although present at lower concentration and with distinct chemical specificity – represent an alternative source for a milk-derived prebiotic in infant formula (Chichlowski *et al*., 2011; Zivkovic and Barile, 2011; Barile and Rastall, 2013).

Probiotic and prebiotic approaches have their problems. Introducing probiotics into the gut already populated by a large number of indigenous bacteria meets colonization resistance from the resident gut microbiota. Prebiotics, on the other hand, can only affect the growth and metabolic activities of bacteria that are already present in the gut, not those that are missing. A possible solution for these problems is a combination of probiotics and prebiotics, called a 'synbiotic', where the chosen prebiotic selectively increases the chance of the ingested probiotic to gain a foothold in the gut (Mugambi *et al*., 2012b).

In the study presented here we investigated the impact of a synbiotic formula on gut microbiota composition in healthy infants in a double-blinded placebo-controlled clinical trial. The test formula consisted of BMOS combined with *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) strain CNCM I-3446 (Steenhout *et al*., 2009; Philippe *et al*., 2011). The same formula without addition of the prebiotic–probiotic blend was used in the control group. Breastfed infants served as reference group. The test formula with the pre- and probiotic was well tolerated, and a strong bifidogenic effect was observed, mainly driven by the prebiotic effect on the indigenous bifidobacteria.

Results

Nutritional intervention trial

At a mean age of 5 days, 115 healthy full-term infants were enrolled into a nutritional intervention trial. Infants from mothers who decided not to breastfeed were randomized to either a starter infant formula (control formula C, $n = 37$, 1.8 g protein/100 kcal; whey/casein ratio 70:30) or the same formula supplemented with a prebiotic (BMOS) at a total oligosaccharide concentration of 5.7 ± 1.0 g/100 g of powder formula (8 g l^{-1} in the reconstituted formula) and a probiotic (*B. lactis* strain CNCM I-3446 with 1×10^7 cfu g^{-1} of powder formula) (test formula T, $n = 39$) for a 12-week feeding period. Infants from mothers who decided to exclusively breastfeed were enrolled in the breastfed group (group B, $n = 39$), which served as physiological reference group. Figure 1 presents the flowchart for infants who were enrolled, lost to follow-up and analysed for the different outcomes. At baseline and over the 12-week observation period of the intention to treat (ITT) population ($n = 115$ infants), no significant difference was seen between the three groups for anthropometric measurements (Tables S1 and S2).

Stool characteristics and infant behaviour

In the per protocol analysis, the number of stools decreased from 4.9 to 2.4 stools per day over the observation period, with no difference between the groups ($P > 0.4$). During the feeding trial, infants from the T, but not the C group, showed a proportion of yellowish versus greenish stools equivalent to B infants (Fig. S1). Infants in the T group showed during intervention more liquid stools than infants in the C group; liquid stools were the dominant observation in B infants (Fig. S2). At 6 weeks, infants in the T and B groups showed a similar stool pH, whereas C infants showed a significantly higher stool pH (Tables S3 and S4). Infants from the T and C groups did not differ in 'spitting up', vomiting, crying, colic, flatulence and irritability (data not shown).

Stool microbiology analysis: quantitative real-time polymerase chain reaction (PCR)

Stool samples were collected from infants before first product application and at 6 and 12 weeks of age when on the allotted feeding regime. In the per protocol analysis, stools from 18 to 23 infants were available per group and

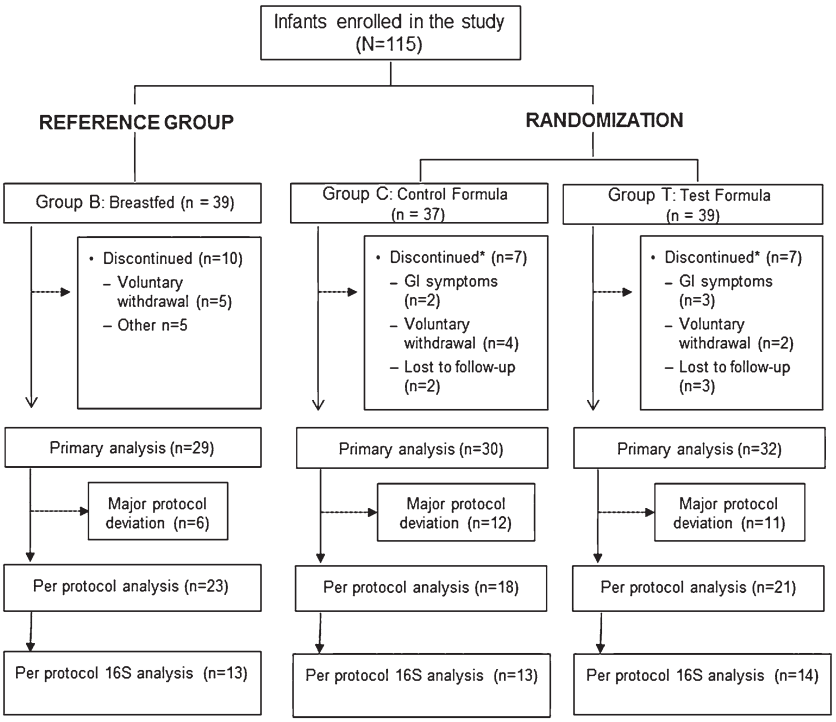

Fig. 1. Flowchart of the enrolled infants. *Several reasons could be reported for the same subject. Note: due to technical reasons, 36 subjects from the study site in Poland had no primary outcome measured and were excluded from the ITT population (http://www.ema.europa.eu).

time point. When using a universal 16S rRNA gene primer pair, the nutritional intervention slightly increased the total bacterial count (Fig. 2 top, left). In average, we obtained median numbers of 5×10^{10} to 10^{11} bacterial cells g^{-1} stool for all feeding groups and all time points. Subsequently we used 16S rRNA gene primers allowing taxonomic differentiation. Total counts of bifidobacteria increased in all three groups between enrolment and 6 weeks of age,

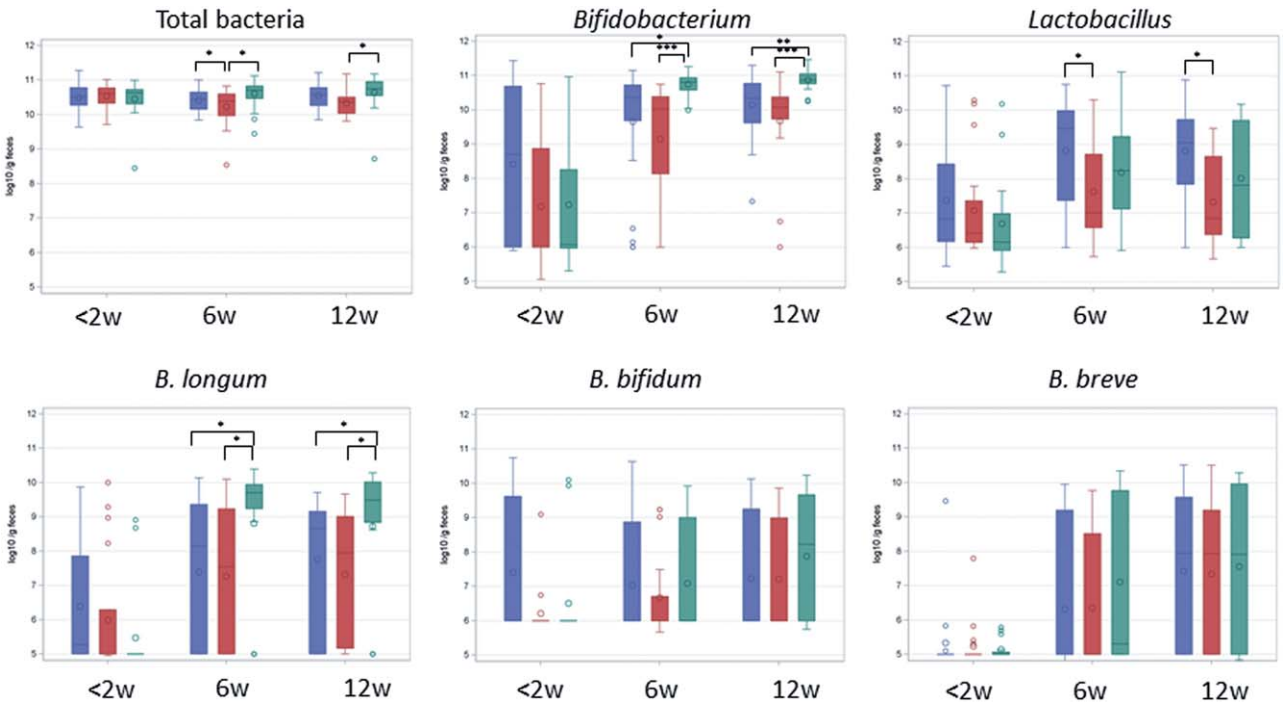

Fig. 2. Bacterial counts measured by qPCR in the faeces of infants receiving the specified formula (red = control; green = supplemented test formula) or who were breastfed (blue) at enrolment (< 2w) and at six (6w) and twelve (12w) weeks of age. qPCR results are given as boxplots around median of bacterial cells per gram of stool. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

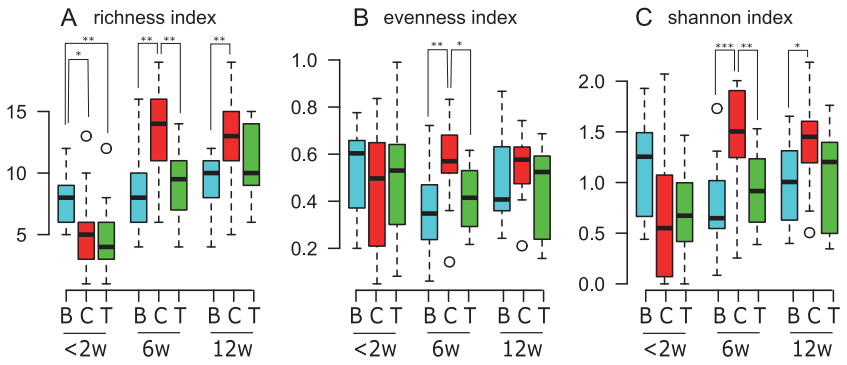

Fig. 3. Boxplots of alpha-diversity analyses at three time points for the three feeding groups using three different indices (panels A–C). *$P < 0.05$; **, $P < 0.01$; ***$P < 0.001$.

after which it remained constant until 12 weeks (Fig. 2 top, middle). Compared with infants in the C group, infants in the T group showed a more pronounced increase in the *Bifidobacterium* titre by 0.8 log. In the T group, the final faecal titre of bifidobacteria was with 0.8×10^{10} bacteria g^{-1} stool significantly higher than in the C and in the B group. *Bacteroides* and lactobacilli showed a 10-fold higher and 10-fold lower stool number, respectively, in infants from the C group compared with those from the T group (data not shown and Fig. 2 top, right).

To achieve further resolution in the *Bifidobacterium* genus, we used a qPCR set of primers based on the *groEL* gene that allowed the detection of 12 *Bifidobacterium* species (Junick and Blaut, 2012). *Bifidobacterium adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. dentium*, *B. longum* and *B. pseudocatenulatum* were detected (Fig. 2 bottom, and data not shown). *Bifidobacterium longum* and *B. bifidum* were the dominant bifidobacteria in the test group, whereas *B. bifidum* and *B. breve* dominated the microbiota in the breastfed and in the control group.

Detection of the added probiotic

Stools positive for *B. animalis* (to which the subspecies *lactis* belongs) by qPCR increased from 6% to 95% after feeding the test formula and titres increased from $10^{7.1}$ to $10^{9.4}$ cells g^{-1} stool, yet *B. lactis* remained a minor fraction of the total bifidobacteria. In contrast, only 13% of the control formula recipients and 5% of breastfed infants showed *B. lactis* in their stools.

Microbiota composition: diversity index

The qPCR analysis allows estimating absolute bacterial counts in the stool for a defined bacterium but does not give an untargeted view on the faecal microbiota. Therefore, the microbiota analysis was complemented by 16S rRNA gene pyrosequencing. After analysis and exclusion of subjects with incomplete data sets, we obtained faecal microbiota compositions for all three time points from at least 13 infants per group. Figure 3 compares the α-diversity for the three groups at baseline and after 6 and 12 weeks of feeding. At baseline the three feeding groups did not differ significantly in microbial diversity (Shannon index). At 6 weeks of age, infants in the C group showed a higher diversity index, due to an increase in both richness and evenness when compared with infants in the T group and the B group. At 12 weeks of age, the C group but not the T group differed from the B group with respect to diversity and richness (Fig. 3).

Microbiota composition: biplot

The bacterial communities of the three groups were analysed by ordination based on the weighted UniFrac distance (Fig. 4). The coordinates of the six most abundant bacterial genera (weighted average of the relative abun-

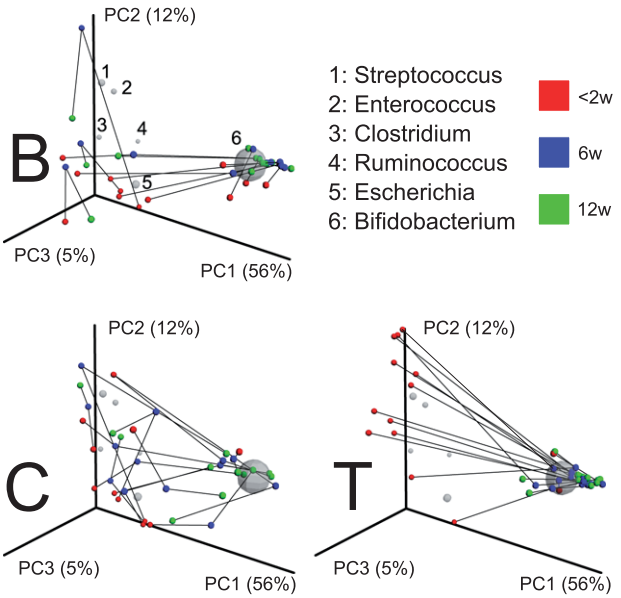

Fig. 4. 3D Principal Coordinate Biplot of Weighted UniFrac distances. Lines connect the three time points (see colour code) of each individual. The six most abundant genera are shown as grey bubbles (see code numbers).

dance in all samples) is indicated by grey bubbles. All infants in the T group showed from baseline to 6 weeks of age a shift to the position of bifidobacteria and remained there until at least 12 weeks of age. No clear development towards a *Bifidobacterium*-dominated faecal microbiota was seen in the C group at 6 weeks of age. Even at 12 weeks of age not all these children had moved to the *Bifidobacterium* coordinates. In the B group, about one third of the infants did not shift to a *Bifidobacterium*-dominated faecal microbiota.

Microbiota composition: taxonomic affiliation of bacteria

Based on 16S rRNA gene sequencing, the microbiota composition of the stool samples for all individual infants in each feeding group is displayed in a bubble plot (Fig. 5, Fig. S3). Bifidobacteria are increased in all three feeding groups, most prominently in the T and B groups and substantially less so in the C group. *Bifidobacterium pseudocatenulatum* became dominant in B and T, but not in C group. Two other bifidobacterial species, which were prominent in all three feeding groups, are *B. longum* and *B. breve*. However, only one *Bifidobacterium* species dominated the stool of infants at a given time point. *Bifidobacterium animalis* was nearly absent in the B and C stools, whereas it was detected in practically all faecal samples of the T group, although with low abundance (median = 0.7%) ($P < 0.001$). *Escherichia* is prominent at baseline in all three feeding groups and decreases substantially at 6 weeks and 12 weeks. The decrease is less prominent in the C group ($P < 0.01$ compared with other groups). In the T group *Streptococcus* is prominent at baseline and disappears practically at 6 weeks and 12 weeks, whereas it is maintained in C and B infants. *Ruminococcus gnavus*, a typical human gut commensal, dominates some C infants at 6 and 12 weeks of age. *Clostridium perfringens* is found with high titres in three B infants at 6 weeks of age, but not in the two formula groups, whereas *Peptostreptococcaceae* were significantly more abundant in group C than in group B and T infants ($P < 0.05$).

Discussion

The synbiotic intervention had a marked effect on the development of the gut microbiota. Compared with the control formula, the supplemented formula induced a shift in all treated infants towards a *Bifidobacterium*-dominated faecal microbiota, which was already apparent after 6 weeks of feeding the test formula. The treatment effect became also evident when using other common parameters in microbiota analyses. For example, at 6 weeks of age the treated infants showed a significantly lower diversity index than infants fed the control formula. For these microbiota parameters infants from the synbiotic treatment group resembled the breastfed infants who likewise – albeit less markedly – showed a shift to a *Bifidobacterium*-dominated gut microbiota as is commonly observed in breastfed infants. This comparison should be interpreted with caution because the breastfed group was self-selected, and only the subjects from the two formula groups were randomized at enrolment, thus allowing direct comparisons only between them. However, overall the infants from the three feeding groups matched well in many parameters except that breastfed infants had a higher gestational age, less caesarean sections and more siblings than formula-fed infants (data not shown).

What might be the reason for the higher microbiota diversity in the control group? In recent publications (Bäckhed *et al*., 2015; Dogra *et al*., 2015a) and a review (Dogra *et al*., 2015b) three stages of gut colonization were distinguished in infants during the first year of life: a relatively diverse *Proteobacteria*-dominated microbiota in the neonatal period, followed by an *Actinobacteria*-dominated more uniform microbiota induced by breastfeeding, which is then replaced by a more diverse, more adult-like *Firmicutes* and *Bacteroidetes*-dominated microbiota induced more by the cessation of breastfeeding than by the introduction of formula feeding or solid-food supplementation. Breastfeeding and as demonstrated in the current communication also milk oligosaccharide feeding shifts the gut microbiota to the less diverse *Actinobacteria*/*Bifidobacterium*-dominated stage, whereas infants fed with a formula initially prolong the more diverse *Proteobacteria*-dominated profile and then develop earlier into an adult-type, more diverse microbiota.

As we compared a formula containing a combined pre- and probiotic supplement with an un-supplemented control formula, we cannot deduce what compound of the supplement mediated the strong *Bifidobacterium*-stimulating effect in the test group. However, the microbiota analysis provided a clear answer. When using a species-specific PCR for *B. animalis* (to which the supplemented probiotic belongs), we observed a robust increase in the faecal representation of the probiotic, which was not observed in the control and the breastfed group indicating a successful introduction of the probiotic in the gut of the infants in the test group. Mean titres of *B. lactis* were with $10^{9.4}$ cells g^{-1} stool relatively high. However, compared with the overall stimulation of bifidobacteria observed with the synbiotic supplement, the added probiotic could only account for a small percentage of the *Bifidobacterium*-stimulating effect. The major contributor to this stimulatory effect was clearly the prebiotic supplement and its effect on the indigenous bifidobacteria. Our observation concurs with other data on probiotic supplementation. Larsen and colleagues (2011)

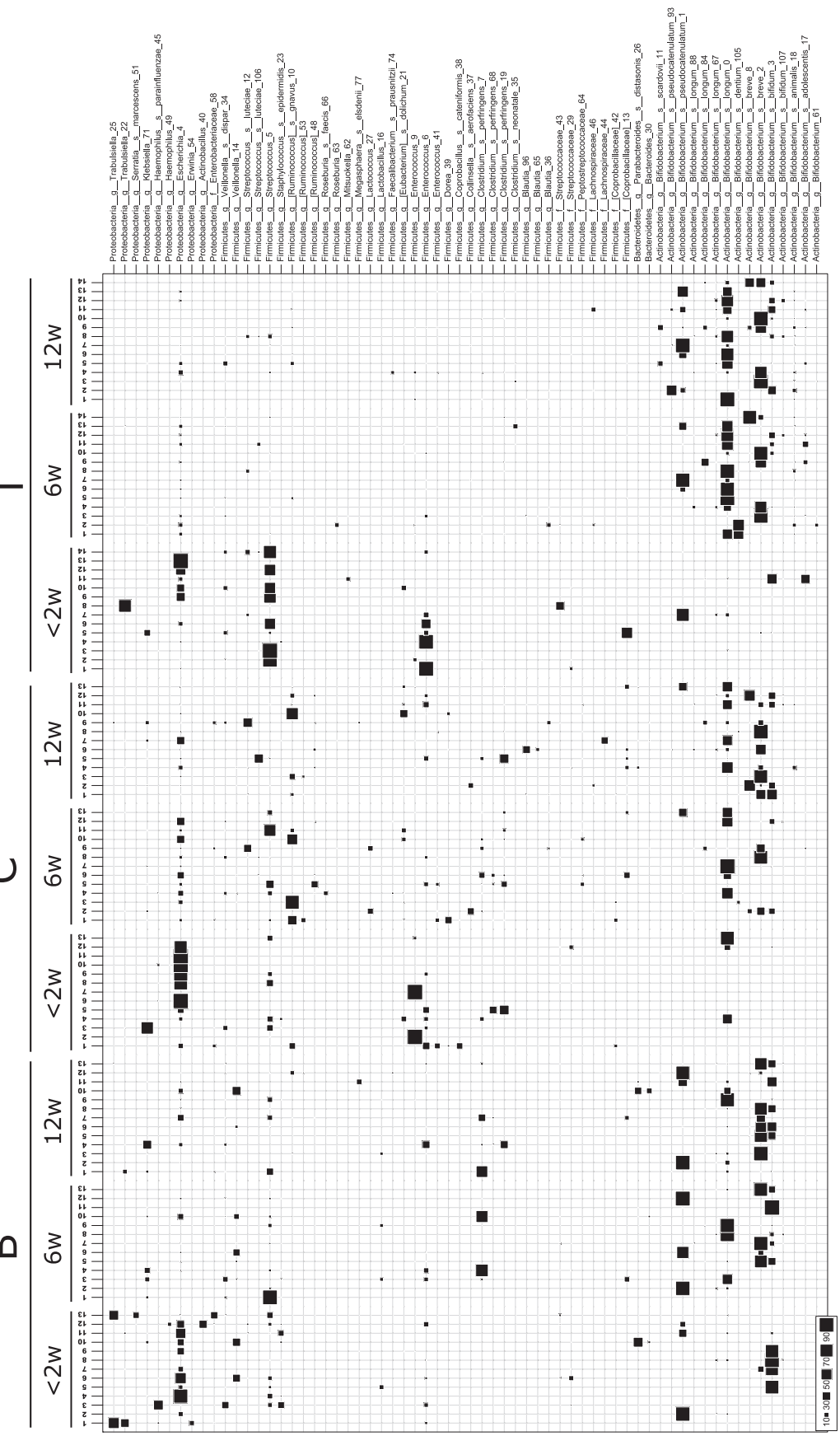

Fig. 5. Distribution of OTUs in all stool samples in the specified feeding groups (B: breastfed reference; C: control formula; T: test formula). Each column describes one sample. Numbers correspond to individual infants in each feeding group at the three specified ages. The size of the squares depicts the percentage abundance (bottom left code). OTUs are shown for each row at the right as best classified at family (f), genus (g) or species (s) level, with terminal ID number. Only OTUs present at > 5% in at least one sample are shown.

tested another *B. animalis* subsp. *lactis* strain in infants at a daily dose of 10^{10} bacteria, and did not observe an increase in total faecal bifidobacteria compared with that in controls and pre-intervention, but the probiotic strain increased from non-detection to $10^{7.6}$ cells g^{-1} stool by qPCR. No impact on bacterial population diversity was seen. Infants supplemented with 10^6 bifidobacteria g^{-1} formula showed an increase in prevalence, but not in titre of bifidobacteria compared with that in placebo recipients (Langhendries *et al*., 1995). Likewise feeding *B. animalis* subsp. *lactis* at 6×10^{10} cells l^{-1} formula to full-term infants did not lead to an increased *Bifidobacterium* cell count over the control formula (Bakker-Zierikzee *et al*., 2005).

How does the stimulatory effect of BMOS prebiotic compare with other bifidogenic prebiotics? In the literature only GOS/FOS prebiotics have so far been investigated in infants for stimulatory effects on faecal bifidobacteria (Veereman, 2007; Piirainen *et al*., 2008). Although a stimulation of faecal bifidobacteria by these prebiotics has consistently been observed, the degree of stimulation differs substantially, with some groups reporting only small or only transient effects. Preterm infants receiving a formula containing 10 g l^{-1} GOS/FOS (90:10%) showed a 50-fold higher *Bifidobacterium* viable stool titre over preterm infants receiving the same formula without prebiotic supplements. The difference was significant after 4 weeks, but not 2 weeks of feeding (Boehm *et al*., 2002). A 50-fold increase in the titre of viable *Bifidobacterium* was seen in term infants fed with a formula supplemented with 4 and 8 g l^{-1} GOS/FOS compared with that in control formula (Moro *et al*., 2002). However, another group observed with the same GOS/FOS mixture only a moderate increase in bifidobacteria, namely from 50% to 60% of total faecal bacteria, but no increase in faecal *Bifidobacterium* counts compared with that in standard formula (Knol *et al*., 2005). A transient increase of bifidobacteria was seen in children between 4 and 8 weeks of age with formula supplemented with 6 g of GOS/FOS l^{-1} compared with that in standard formula (70% vs 50% bifidobacteria of total faecal bacteria) (Bakker-Zierikzee *et al*., 2005).

BMOS thus shows a robust bifidobacteria-stimulating effect that is as high or even higher than FOS/GOS. The test formula was faster and more efficient (absence of non-responder) in bringing the infant microbiota to a bifidobacteria-dominated community, even when compared with the breastfed group. Recent data suggest that in addition to the composition of the gut microbiota, its rate of acquisition is important (Bäckhed *et al*., 2015; Dogra *et al*., 2015a). Therefore, the strong and rapid stimulation of bifidobacteria by the test formula may have a positive effect on infants. Some authors have suggested that a general bifidobacteria-stimulating effect is not enough to achieve health benefits, but that a species-specific *Bifidobacterium* stimulation is needed for alleviating for example allergy-related symptoms (Ouwehand *et al*., 2001). The prebiotic supplement stimulated *B. pseudocatenulatum*, which was not observed in the control group. Interestingly, *B. pseudocatenulatum* is also a prominent *Bifidobacterium* species in breastfed infants strengthening its link to potential health effects. The fact that *specific* indigenous bifidobacterial species can be stimulated by BMOS *in vivo* offers interesting possibilities for targeted gut microbiota 'engineering' by nutritional interventions in the future. However, we need to know more about the relative health-promoting properties of the different bifidobacterial species to fully exploit the bifidogenic properties of oligosaccharides. As some probiotic effects are strain-specific, the synbiotic approach might allow the targeted stimulation of a given strain by a specific prebiotic. Because of the design of our clinical trial we cannot decide whether the increase in *B. lactis* is the result of its oral supplementation or whether its presence was further boosted by the probiotic supplement. From a biotechnological perspective, it will be important to explore whether specific prebiotics can be designed that favour the outgrowth of a specific bifidobacterial strain tested in competition with other gut-derived bifidobacteria by *in vitro* tests and whether this specific stimulatory activity is translated into the *in vivo* situation.

Our study was powered to demonstrate non-inferiority for the test formula compared with the control formula for growth. This goal was achieved. All anthropometric parameters (weight gain, length and head circumference) did not differ between groups from inclusion until 3 months of age. The study was not powered to detect differences in health outcomes. In fact, only three infections were observed and distributed evenly across the three groups. Were there observations suggesting potential health benefits of the formula supplementation? A major contribution of *Bacteroides* (40% of total), staphylococci and *E. coli* was described in bottle-fed babies (Harmsen *et al*., 2000). We observed indeed 10-fold higher *Bacteroidetes* titres in infants in control than on test formula or in breastfed infants. Likewise *Ruminococcus gnavus*, a common faecal isolate from humans and ruminants, was increased in control infants. *Escherichia coli* was high at enrolment but decreased significantly in abundance in all feeding groups. Similar to the breastfed group, the test formula compared with the control formula showed a lower abundance of *Peptostreptococcaceae*, a family to which the undesired *C. difficile* belongs (Yutin and Galperin, 2013). The test formula induced a significant decrease in faecal streptococci, which could be interesting because the specific group of *S. lutetiensis* identified in our study was associated in Chinese children

with diarrhoea for which no known aetiological agent could be identified (Jin *et al*., 2013).

Differences were seen in stool properties. Infants in the test group produced stool with a consistency and stool colour more comparable with that of the breastfed infants than to the control group. Although the first property is an important parameter of gut comfort of the infant, the physiological importance of the second parameter is less clear. One might suspect an impact of gut microbes on the catabolism of tetrapyrrolic bile pigments as an end-product of haem catabolism (stercobilin, biliverdin), but not much is known about potential health implications of these changes in stool colour. More interesting is the difference in stool pH. At 6 weeks of age, infants who received the test formula had a significantly lower stool pH than those receiving the control formula. Apparently, the intestinal microbiota from infants on the test formula produced – like breastfed infants – more acidic metabolites than the microbiota of the control groups. Whether this observation is associated with the higher faecal titre of bifidobacteria cannot be decided from the available data. One might suspect that the lower pH is due to the production of lactic acid and short chain fatty acids. These are formed by intestinal bacteria from non-digestible polysaccharides and oligosaccharides and may have numerous physiological effects (Brüssow and Parkinson, 2014). Further investigations into the chemical identity of these compounds conferring a lower faecal pH are clearly indicated in future studies.

Whatever the health implications of these observations are, the current study demonstrates that the gut microbiota can be modulated by a nutritional intervention in such a way that the diversity and composition of the microbiota becomes closer to those of breastfed infants. More intervention studies exploring the diversity of milk oligosaccharide chemistry (Tannock *et al*., 2013) will help transform microbiota research from a descriptive branch of microbiology into one of applied nutrition to the benefit of human health.

Material and methods

Trial design and population

This study was registered at ClinicalTrials.gov under the number NCT01983072 where eligibility criteria and locations, among other information, are referred. The two study formulae contained proteins, carbohydrates, fats, vitamins and minerals in amounts intended for full nutritional support of infants from birth to 4 months of age. Both were whey-predominant (whey/casein ratio of 70/30) low-protein (1.8 g/100 kcal) formulas. The study formulas were manufactured at the Nestlé Product Technology Center (Konolfingen, Switzerland) and were packaged in identical cans. Neither parents/caregivers nor investigators and clinical project managers knew the identity of the formulas. Infants were exclusively fed their assigned formula or were fully breastfed *ad libitum* from enrolment until 3 months of age.

Faecal DNA extraction

Stool samples were collected for each time point, refrigerated at 4°C for a maximum of 10 h after defecation and kept frozen at –80°C until the microbiota analysis was carried out. Total DNA was extracted using the QIAamp DNA Stool Mini Kit (QIAGEN), following the manufacturer's instructions, except for the addition of a series of mechanical disruption steps (11×45 s) using a FastPrep apparatus and Lysing Matrix B tubes (MP Biochemicals) (Junick and Blaut, 2012).

Quantitative PCR

Total bacterial cell counts (Nadkarni *et al*., 2002), *Bifidobacterium* (Matsuki *et al*., 2002), *Lactobacillus* (Walter *et al*., 2001; Heilig *et al*., 2002) and counts for individual species of bifidobacteria (Junick and Blaut, 2012) were determined per gram of faeces as previously described. Bacterial counts were log transformed, and summary statistics as well as the frequencies of undetectable bacterial counts were recorded. Values below the detection limit were replaced by 5 log g^{-1} for *B. longum* and *B. breve*, and by 6 log g^{-1} for the others. The Wilcoxon rank-sum test was used to compare the groups.

Pyrosequencing and analysis of 16S genes

The 16S variable regions V1–V3 was PCR amplified and sequenced on Roche 454 GS-FLX-Titanium Sequencer as previously described (Sanchez *et al*., 2014). Raw sequence data were deposited in the GenBank Short Read Archive (Accession number: SRP064888) and analysed using MOTHUR v.1.33.0 (Schloss *et al*., 2009) and QIIME v.1.8 (Caporaso *et al*., 2010b) software packages. Pyrosequencing reads were denoised with the MOTHUR implementation of PyroNoise (Quince *et al*., 2009) according to the 454 SOP described in Schloss and Westcott (2011). Chimeras were identified using usearch61 in QIIME (Edgar *et al*., 2011). The sequences were then trimmed as described in the MOTHUR 454 SOP to keep sequences overlapping the same 16S region. Subsequent analytical steps were performed in QIIME. Operational taxonomic units (OTUs) *de novo* picking at 97% identity was performed using UCLUST (Edgar, 2010). Taxonomy assignment of OTU representative sequences used the RDP Classifier with confidence threshold of 0.6 (Wang *et al*., 2007) on the Greengenes reference database v.13.8 (McDonald *et al*., 2012). The same sequences were

aligned using PyNAST (Caporaso *et al*., 2010a) on the Greengenes core reference alignment (DeSantis *et al*., 2006). The resulting multiple alignments was then filtered and used to build a phylogenetic tree with FastTree (Price *et al*., 2010). After quality filtering (Bokulich *et al*., 2013), phylogenetic distances between all samples were computed as UniFrac distances (Lozupone and Knight, 2005). Diversity analyses based on the Unifrac distances were performed in QIIME, whereas the analyses based on taxonomy assignments used the websites Calypso at http://bioinfo.qimr.edu.au/calypso.

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References

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D.R., *et al*. (2011) Enterotypes of the human gut microbiome. *Nature* **473:** 174–180.

Bakker-Zierikzee, A.M., Alles, M.S., Knol, J., Kok, F.J., Tolboom, J.J., and Bindels, J.G. (2005) Effects of infant formula containing a mixture of galacto- and fructo-oligosaccharides or viable *Bifidobacterium animalis* on the intestinal microflora during the first 4 months of life. *Br J Nutr* **94:** 783–790.

Barile, D., and Rastall, R.A. (2013) Human milk and related oligosaccharides as prebiotics. *Curr Opin Biotechnol* **24:** 214–219.

Bäckhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., *et al*. (2015) Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host Microbe* **17:** 690–703.

Boehm, G., Lidestri, M., Casetta, P., Jelinek, J., Negretti, F., Stahl, B., *et al*. (2002) Supplementation of a bovine milk formula with an oligosaccharide mixture increases counts of faecal bifidobacteria in preterm infants. *Arch Dis Child Fetal Neonatal Ed* **86:** F178–F181.

Bokulich, N.A., Subramanian, S., Faith, J.J., Gevers, D., Gordon, J.I., Knight, R., *et al*. (2013) Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat Methods* **10:** 57–59.

Brüssow, H. (2015a) Microbiota and the human nature: know thyself. *Environ Microbiol* **17:** 10–15.

Brüssow, H. (2015b) Human microbiota: 'The philosophers have only interpreted the world in various ways. The point, however, is to change it'. *Microb Biotechnol* **8:** 11–12.

Brüssow, H., and Parkinson, S.J. (2014) You are what you eat. *Nat Biotechnol* **32:** 243–245.

Buccigrossi, V., Nicastro, E., and Guarino, A. (2013) Functions of intestinal microflora in children. *Curr Opin Gastroenterol* **29:** 31–38.

Caporaso, J.G., Bittinger, K., Bushman, F.D., DeSantis, T.Z., Andersen, G.L., and Knight, R. (2010a) PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* **26:** 266–267.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., *et al*. (2010b) QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7:** 335–336.

Chichlowski, M., German, J.B., Lebrilla, C.B., and Mills, D.A. (2011) The influence of milk oligosaccharides on microbiota of infants: opportunities for formulas. *Annu Rev Food Sci Technol* **2:** 331–351.

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., *et al*. (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* **72:** 5069–5072.

Ding, T., and Schloss, P.D. (2014) Dynamics and associations of microbial community types across the human body. *Nature* **509:** 357–360.

Dogra, S., Sakwinska, O., Soh, S., Ngom-Bru, C., Brück, W.M., Berger, B., *et al*. (2015a) Dynamics of infant gut microbiota are influenced by delivery mode and gestational duration and are associated with subsequent adiposity. *mBio* **6:** e02419–14.

Dogra, S., Sakwinska, O., Soh, S., Ngom-Bru, C., Brück, W.M., Berger, B., *et al*. (2015b) Rate of establishing the gut microbiota in infancy has consequences for future health. *Gut Microbes* **6:** 321–325.

Edgar, R.C. (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26:** 2460–2461.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., and Knight, R. (2011) UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27:** 2194–2200.

Garrido, D., Dallas, D.C., and Mills, D.A. (2013) Consumption of human milk glycoconjugates by infant-associated bifidobacteria: mechanisms and implications. *Microbiology* **159:** 649–664.

Gordon, J.I., Dewey, K.G., Mills, D.A., and Medzhitov, R.M. (2012) The human gut microbiota and undernutrition. *Sci Transl Med* **4:** 137ps12.

Harmsen, H.J., Wildeboer-Veloo, A.C., Raangs, G.C., Wagendorp, A.A., Klijn, N., Bindels, J.G., *et al*. (2000) Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. *J Pediatr Gastroenterol Nutr* **30:** 61–67.

Heilig, H.G., Zoetendal, E.G., Vaughan, E.E., Marteau, P., Akkermans, A.D., and de Vos, W.M. (2002) Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. *Appl Environ Microbiol* **68:** 114–123.

van Hoffen, E., Ruiter, B., Faber, J., M'Rabet, L., Knol, E.F., Stahl, B., *et al*. (2009) A specific mixture of short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides induces a beneficial immunoglobulin profile in infants at high risk for allergy. *Allergy* **64:** 484–487.

Human Microbiome Project Consortium (2012a) Structure, function and diversity of the healthy human microbiome. *Nature* **486:** 207–214.

Human Microbiome Project Consortium (2012b) A framework for human microbiome research. *Nature* **486:** 215–221.

Jin, D., Chen, C., Li, L., Lu, S., Li, Z., Zhou, Z., *et al*. (2013) Dynamics of fecal microbial communities in children with diarrhea of unknown etiology and genomic analysis of associated Streptococcus lutetiensis. *BMC Microbiol* **13:** e141.

Junick, J., and Blaut, M. (2012) Quantification of human fecal bifidobacterium species by use of quantitative real-time PCR analysis targeting the groEL gene. *Appl Environ Microbiol* **78:** 2613–2622.

Knol, J., Scholtens, P., Kafka, C., Steenbakkers, J., Gro, S., Helm, K., *et al*. (2005) Colon microflora in infants fed formula with galacto- and fructo-oligosaccharides: more like breast-fed infants. *J Pediatr Gastroenterol Nutr* **40:** 36–42.

Kumar, H., Salminen, S., Verhagen, H., Rowland, I., Heimbach, J., Bañares, S., *et al*. (2014) Novel probiotics and prebiotics: road to the market. *Curr Opin Biotechnol* **32:** 99–103.

Langhendries, J.P., Van Hees, J., Lamboray, J.M., Darimont, J., Mozin, M.J., Secretin, M.C., *et al*. (1995) Effect of a fermented infant formula containing viable bifidobacteria on the fecal flora composition and pH of healthy full-term infants. *J Pediatr Gastroenterol Nutr* **21:** 177–181.

Larsen, N., Vogensen, F.K., Gøbel, R., Michaelsen, K.F., Abu Al-Soud, W., Sørensen, S.J., *et al*. (2011) Predominant genera of fecal microbiota in children with atopic dermatitis are not altered by intake of probiotic bacteria *Lactobacillus acidophilus* NCFM and *Bifidobacterium animalis* subsp. *lactis* Bi-07. *FEMS Microbiol Ecol* **75:** 482–496.

Lozupone, C., and Knight, R. (2005) UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* **71:** 8228–8235.

McCune, V.L., Struthers, J.K., and Hawkey, P.M. (2014) Faecal transplantation for the treatment of *Clostridium difficile* infection: a review. *Int J Antimicrob Agents* **43:** 201–206.

McDonald, D., Price, M.N., Goodrich, J., Nawrocki, E.P., DeSantis, T.Z., Probst, A., *et al*. (2012) An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J* **6:** 610–618.

Matsuki, T., Watanabe, K., Fujimoto, J., Miyamoto, Y., Takada, T., Matsumoto, K., *et al*. (2002) Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. *Appl Environ Microbiol* **68:** 5445–5451.

Moro, G., Minoli, I., Mosca, M., Fanaro, S., Jelinek, J., Stahl, B., *et al*. (2002) Dosage-related bifidogenic effects of galacto- and fructooligosaccharides in formula-fed term infants. *J Pediatr Gastroenterol Nutr* **34:** 291–295.

Mugambi, M.N., Musekiwa, A., Lombard, M., Young, T., and Blaauw, R. (2012a) Probiotics, prebiotics infant formula use in preterm or low birth weight infants: a systematic review. *Nutr J* **11:** e58.

Mugambi, M.N., Musekiwa, A., Lombard, M., Young, T., and Blaauw, R. (2012b) Synbiotics, probiotics or prebiotics in infant formula for full term infants: a systematic review. *Nutr J* **11:** e81.

Nadkarni, M.A., Martin, F.E., Jacques, N.A., and Hunter, N. (2002) Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology* **148:** 257–266.

van Nood, E., Vrieze, A., Nieuwdorp, M., Fuentes, S., Zoetendal, E.G., de Vos, W.M., *et al*. (2013) Duodenal infusion of donor feces for recurrent *Clostridium difficile*. *N Engl J Med* **368:** 407–415.

Osborn, D.A., and Sinn, J.K. (2013) Prebiotics in infants for prevention of allergy. *Cochrane Database Syst Rev* **3:** CD006474.

Ouwehand, A.C., Isolauri, E., He, F., Hashimoto, H., Benno, Y., and Salminen, S. (2001) Differences in *Bifidobacterium flora* composition in allergic and healthy infants. *J Allergy Clin Immunol* **108:** 144–145.

Philippe, D., Favre, L., Foata, F., Adolfsson, O., Perruisseau-Carrier, G., Vidal, K., *et al*. (2011) *Bifidobacterium lactis* attenuates onset of inflammation in a murine model of colitis. *World J Gastroenterol* **17:** 459–469.

Piirainen, L., Kekkonen, R.A., Kajander, K., Ahlroos, T., Tynkkynen, S., Nevala, R., *et al*. (2008) In school-aged children a combination of galacto-oligosaccharides and *Lactobacillus* GG increases bifidobacteria more than *Lactobacillus* GG on its own. *Ann Nutr Metab* **52:** 204–208.

Preidis, G.A., and Versalovic, J. (2009) Targeting the human microbiome with antibiotics, probiotics, and prebiotics: gastroenterology enters the metagenomics era. *Gastroenterology* **136:** 2015–2031.

Price, M.N., Dehal, P.S., and Arkin, A.P. (2010) FastTree 2 – approximately maximum-likelihood trees for large alignments. *PLoS ONE* **5:** e9490.

Quince, C., Lanzen, A., Curtis, T.P., Davenport, R.J., Hall, N., Head, I.M., *et al*. (2009) Accurate determination of microbial diversity from 454 pyrosequencing data. *Nat Methods* **6:** 639–641.

Saavedra, J.M., and Dattilo, A.M. (2012) Early development of intestinal microbiota: implications for future health. *Gastroenterol Clin North Am* **41:** 717–731.

Sanchez, M., Darimont, C., Drapeau, V., Emady-Azar, S., Lepage, M., Rezzonico, E., *et al*. (2014) Effect of *Lactobacillus rhamnosus* CGMCC1.3724 supplementation on weight loss and maintenance in obese men and women. *Br J Nutr* **111:** 1507–1519.

Schloss, P.D., and Westcott, S.L. (2011) Assessing and improving methods used in operational taxonomic unit-based approaches for 16S rRNA gene sequence analysis. *Appl Environ Microbiol* **77:** 3219–3226.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., *et al*. (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* **75:** 7537–7541.

Smilowitz, J.T., Lebrilla, C.B., Mills, D.A., German, J.B., and Freeman, S.L. (2014) Breast milk oligosaccharides: structure–function relationships in the neonate. *Annu Rev Nutr* **34:** 143–169.

Steenhout, P.G., Rochat, F., and Hager, C. (2009) The effect of *Bifidobacterium lactis* on the growth of infants: a pooled analysis of randomized controlled studies. *Ann Nutr Metab* **55:** 334–340.

Subramanian, S., Huq, S., Yatsunenko, T., Haque, R., Mahfuz, M., Alam, M.A., *et al*. (2014) Persistent gut microbiota immaturity in malnourished Bangladeshi children. *Nature* **510:** 417–421.

Tannock, G.W., Lawley, B., Munro, K., Gowri Pathmanathan, S., Zhou, S.J., Makrides, M., *et al*. (2013) Comparison of the compositions of the stool microbiotas of infants fed goat milk formula, cow milk-based formula, or breast milk. *Appl Environ Microbiol* **79:** 3040–3048.

Veereman, G. (2007) Pediatric applications of inulin and oligofructose. *J Nutr* **137:** 2585S–2589S.

Walter, J., Hertel, C., Tannock, G.W., Lis, C.M., Munro, K., and Hammes, W.P. (2001) Detection of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. *Appl Environ Microbiol* **67:** 2578–2585.

Wang, Q., Garrity, G.M., Tiedje, J.M., and Cole, J.R. (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* **73:** 5261–5267.

Yatsunenko, T., Rey, F.E., Manary, M.J., Trehan, I., Dominguez-Bello, M.G., and Contreras, M. (2012) Human gut microbiome viewed across age and geography. *Nature* **486:** 222–227.

Yutin, N., and Galperin, M.Y. (2013) A genomic update on clostridial phylogeny: Gram-negative spore formers and other misplaced clostridia. *Environ Microbiol* **15:** 2631–2641.

Zivkovic, A.M., and Barile, D. (2011) Bovine milk as a source of functional oligosaccharides for improving human health. *Adv Nutr* **2:** 284–289.

Zivkovic, A.M., German, J.B., Lebrilla, C.B., and Mills, D.A. (2011) Human milk glycobiome and its impact on the infant gastrointestinal microbiota. *Proc Natl Acad Sci USA* **108:** 4653–4658.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Proportion of infants in the three feeding groups specified at the bottom showing stools with the colour defined on the abscissa at < 2 weeks (left), 6 weeks (centre) and 12 weeks of age (right panel).

Fig. S2. Proportion of infants in the three feeding groups specified at the bottom showing stools with the consistency defined on the abscissa at < 2 weeks (left), 6 weeks (centre) and 12 weeks of age (right panel).

Fig. S3. Heat map representation of the bubble plot data represented in Fig. 5.

Table S1. Anthropometric measurements at 12 weeks. sd, standard deviation.

Table S2. Comparison of anthropometric measurements at 12 weeks. CI, confidence interval.

Table S3. Stool pH. sd, standard deviation.

Table S4. Comparison of stool pH. CI, confidence interval; *P*-values corrected according to Tukey.