Non-digestible fibers increase the capacity to produce short-chain fatty acids in stool of very preterm infants

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Abstract

Introduction: Preterm infants have a delayed intestinal colonization. Supplementation of immune modulatory fibers to their diet can increase the production of beneficial short-chain-fatty acids profiles (SCFA) by intestinal microbes. The aims of this ex-vivo study were to determine: (1) capability of stool microbiota of premature infants to produce SCFA and its time course and (2) the influence of dietary fibers on the SCFA production and the SCFA profile after fermentation using feces from these infants.

Methods: in vitro fermentation using feces of preterm infants (GA<30 weeks) at 4 different time points (first stool, 40 ml/kg/day and 140 ml/kg/day enteral feeding, 4 weeks) with either blank or 2 modulatory fibers [short chain galactose oligosaccharide/ long chain fructose oligosaccharide (scGOS/lcFOS) and corn arabinoxylon (CAX)].

Results: Stool of 7 infants (27.8 ± 1.2 weeks, birth weight of 1101± 190 g, of which 3/7 infants were born by Caesarean section) have been analyzed. Total SCFA production was very low using the first stool (mean 4.4 ± 1.5 days) even when using both tested fibers. It increased at time point 2 (mean 12.0 days ± 2.6 days) only after the addition of scGOS/lcFOS (p= 0.03), and further increased at time point 3 (mean 19.3 ± 3.9 days) for scGOS/lcFOS (p<0.01) as well as CAX (p=0.02). Highest SFCA were detected at time point 4 (mean 29.1 ± 0.5 days days) for scGOS/lcFOS (p<0.01) as well as CAX (p=0.04). These increases were due especially to an increase in acetate. SCFA production was positively influenced by vaginal delivery and negatively influenced by longer initial antibiotic course.

Conclusion: The fecal microbiome in preterm infants generates very low amounts of SCFAs during the first weeks of life, but increases after approximately two weeks, when dietary fibers, especially scGOS/lcFOS was added. The increase was mainly due to acetate. C-section delays the ability of intestinal microbiota to produce SCFA even in the presence of dietary fibers.
Introduction

The gut microbiome is essential for the development of a healthy mucosal barrier function. Mucosal integrity, mucosal immunity and even systemic immune responses highly depend on a healthy intestinal microbiome. The initial colonization of the gut is an important event in the adjustment of the newborn to the extra uterine environment. The microbiota plays a pivotal role in the development of both the mucosal as well as systemic immune system. The composition of the gut microbiota is very complex and essential for human health, as the bacteria can influence the immune system directly via among others TLRs but also indirectly by the secretion of unique immune modulating factors (metabolites produced by the microbiota). The major microbial metabolites produced by gut bacteria upon dietary fiber fermentation are short-chain fatty acids (SCFA). The most studied SCFAs are acetate, propionate and butyrate, as these are the most common SCFAs in the human intestine. SCFAs are known to reduce colonic pH, thereby inhibiting the growth of opportunistic, pathogenic bacteria, decreasing the activity of co-carcinogenic enzymes such as glucuronidases, glycosidases and 7α-hydroxylases, maintain normal intestinal barrier function and mucosal integrity and promote immune tolerance and an anti-inflammatory milieu in the intestine. Furthermore SCFA are an important energy source for the body and a preferred source of energy for the intestinal epithelial cells. Butyrate is the most desirable SCFA, at least in adults, because it has both anti-inflammatory activities and it seemed to be a main energy source for colonocytes. Butyrate is a potent modulator of epigenetic processes through its HDAC inhibition as well. Vaginal delivered-breastfed infants show acetate as the most common SCFA and limited butyrate production. Therefore the relevance of butyrate during infancy for promising gut health, mucosal immune maturation and tolerance remains to be determined.

It is known that in preterm infants the colonization of the gut, especially by beneficial bacteria, such as Bifidobacteria, is delayed. Several factors play a role in the delayed colonization of the gut in preterm infants. The infants have a higher likelihood of being delivered by Caesarean section (c-section), lacking the contact with the maternal vaginal and colonic microbiota, less exposure to maternal skin microbiota due to intensive care treatment, non-homogenous feeding pattern and more antibiotics. All these factors may negatively influence the microbiome composition and function, potentially leading to a less differentiated microbiome, a relatively unstable microbiome profile, with less ability to produce an optimal SCFA profile and delayed acquisition of “adult-type” microbiome profiles. Several non-digestible fibers are known to stimulate colonization of the intestine with beneficial bacteria, thereby also influencing the SCFA production. This might be particularly important in preterm infants with an immature gut and delayed colonization, although it is unknown whether premature infants with a delayed colonization can respond to fiber supplements to produce desirable SCFA profile in the intestine.
The aim of this ex-vivo study was to fill this gap in our knowledge and to determine the influence of dietary fibers on the SCFA production after fermentation by fecal microbes of preterm infants. The secondary aim was to determine possible other perinatal factors that might influence the SCFAs production in these preterm infants.

Methods

Patients
This was a non-randomized, longitudinal prospective cohort study of 7 preterm infants admitted to the Rush University Medical Center NICU between February and November 2010. Infants were eligible if the gestational age was < 35 weeks, the birth weight was < 2000 grams, and in the absence of significant congenital anomaly or chromosomal abnormality. This study was approved by the Rush University Institutional Review Board. Signed informed consent was obtained from a parent/guardian of all enrolled subjects. Perinatal and infant demographic, feeding and clinical data were collected prospectively. Infant feedings were per NICU protocol and attending neonatologist’s discretion and were not influenced by study participation. Infants were preferentially fed maternal human milk if available, which was fortified with bovine human milk fortifier once feedings reached 140ml/kg/d. If maternal human milk was unavailable, then subjects received preterm infant formula. Infant stool samples were collected during the first 30 days of life. The samples were placed in sterile cryovials and snap frozen in liquid nitrogen at the time of collection. The samples were stored in a -70 °C freezer until time of analysis. One gram of stool was used for fermentation analysis at the following time points: T1: first stool; T2: 48 hours after reaching 40 ml/kg/day of enteral feeding; T3: 48 hours after reaching 140 ml/kg/day of enteral feeding; and T4: last stool collected (around 30 days of life).

Dietary fibers
Purified lactosefree short chain galactose-oligosaccharides [scGOS] was obtained from FrieslandCampina foods (Amersfoort, the Netherlands). Long chain fructose oligosaccharide [lcFOS] was obtained from Nutricia Danone (Utrecht, the Netherlands). Corn arabinoxylon (CAX) was generously provided by professor Hamaker and extracted as previously described by Rose et al. in the lab of Purdue University (West Lafayette, Indiana).

In vitro fermentation
In vitro fermentation was performed as previously described with some minor adaptations. In short: feces were defrosted. Combining stool from several days after the selected day was necessary in some subjects to reach a minimal measurable amount (1 gram). The amounts used from each individual subject were not equal due to different amounts of stool.
collected. In an anaerobic cabinet, the feces of a subject was combined and homogenized with 6 volumes of diluted standard efflux medium. A blank sample (without fibers) was collected at 0h. Each sample was fermented for 24 hours with 50 μg of a combination of scGOS:lcFOS 9:1, 50 μg of CAX or no fibers as the control (24h blank). After 0 hours (blank) and 24 hours (blank 24 hours, scGOS/lcFOS and CAX) the dialysate of the samples was collected. Fermentation experiments were performed in triplicate.

**SCFA determination**

For SCFA determination, dialysate samples were centrifuged (12 000 rpm, 5 min). A mixture of formic acid (20 %), methanol and 2-ethyl butyric acid (internal standard, 2 mg/ml in methanol) was added to the supernatant. 4 ul sample was injected onto a Nukol capillary column (length 30 m, inner diameter 0·25 mm ID,0.25um bonded phase Supelco Bellefonte PA) in a Agilent 6890 GC under the following conditions: injector temperature 240°C; detector temperature 230°C; detector, FID; initial oven temperature, 100°C; temperature program 8°C/min to 192°C, with hold for 10 min at final oven temperature; carrier gas, helium at 1 ml/min.

**Data analysis**

Data were analyzed with SPSS software using ANOVA. Fisher’s least significant different test was used to determine significant differences among supplement combinations and the different time points. Multivariate analysis was used to determine the influence of GA, mode of delivery and duration of initial course of antibiotics on SCFA production.

**Results**

The subjects had a mean gestational age of 27.8 ± 1.2 weeks, birth weight of 1101± 190 g, with 1/7 (14%) male and 3/7 (42%) born by C-section. Initiation of enteral feedings occurred at a median of 3 days (IQR 2-5) with all infants receiving own mother’s milk mostly. In total 3/7 were exclusive maternal human milk fed during the 30 days. The remaining 4 received some formula feedings in addition to own mother’s milk, starting at days 8, 12, 14 and 24 of life. All mothers received antibiotics before delivery and all infants received antibiotics starting on the first day of life (mean course 6.8 ± 3.2 days). Infants with birth weight <1000g received prophylactic fluconazole while central catheters were in place per NICU protocol (n=2).

Without the addition of any fiber the cumulative SCFA production generated in the stool of preterm infants was low and did not change during the first 4 weeks of life (p>0.05) (Figure 11.1). There was an increase in propionate (p<0.01) and butyrate (p=0.04) production around time point 4 compared to the first two time points.
The cumulative production of SCFA in the first stool (T1, mean 4.4 ± 1.5 days) was very low and fermenting the stool with scGOS/lcFOS or CAX did not change the SCFA profiles at this time point.

At the second time point (T2, mean 12.0 ± 2.6 days) SCFA production increased in the stools with the addition of scGOS/lcFOS (p=0.03), but not with CAX or without fibers.

At time point 3 (T3, mean 19.3 ± 3.9 days) cumulative SCFA production increased with the addition of scGOS/lcFOS (p<0.01) and with CAX (p=0.02). This increase was due to an increase in acetate, as there were no differences in the butyrate and propionate levels.

At time point 4 (T4, mean 29.1 ± 0.5 days), the highest production of SCFAs was generated by scGOS/lcFOS (p<0.01), but CAX increased SCFAs production also (p=0.04). Only at time point 4, butyrate production was significantly increased by scGOS/lcFOS (p=0.04), for CAX this was only a trend (p=0.09).

Overall, with the addition of fibers, there seemed to be an increase in the generation of SCFA over time, with the highest increase between time point 2 and 3 (Figure 11.1). This increase is primarily due to increase in acetate production.

**Influence of infant’s characteristics on SCFA profiles after fermentation**

In a multivariate analysis the mode of delivery influenced the production of cumulative SCFAs after fermentation only with scGOS/lcFOS and only at time point 3 (p=0.04) (Figure 11.2), with higher levels seen after vaginal delivery. All other time points did not show any influence on SCFA production with or without fibers. Our data show that C-section delays production of SCFA by stool microbiota even in the presence of dietary fiber. More days of initial antibiotic course decreased the production of cumulative SCFAs only at time point 3, in the blank 24h and with CAX (p=0.02 and p=0.04 respectively), but not with scGOS/lcFOS (p=0.69). No association was found at any of the other time points. Gestational age was not associated with SCFA production with or without fibers in this group of very preterm infants.
Discussion

This study shows that the microbiota in preterm infants stool generate low amounts of SCFAs during the first 4 weeks of life without dietary fibers. With addition of dietary fibers, especially scGOS/lcFOS, the SCFA production increases after approximately two weeks, especially the production of acetate. The fecal microbiota in the stool showed low potential to generate butyrate, a desirable SCFA, during the first weeks of life. scGOS/lcFOS did increase butyrate at the last time point only. In this current study a positive influence of vaginal delivery and a negative influence of longer duration of the initial antibiotic courses on the level of SCFA production was found; however, this was only evident at the third time point.

The well-reported delayed intestinal colonization in preterm infants might play a pivotal role in very low SCFA producing capacity of preterm infants. After birth, maternal breast milk promotes the colonization and maturation of the infant gut microbiome. scGOS/lcFOS aimed to mimic human milk oligosaccharides as a microbiome promoter. The addition of fibers only starts increasing the ability to produce SCFAs after 2-3 weeks, which is in line with previous studies of intestinal colonization in preterm infants.

Microbiome analysis in a single C-section born preterm infant during week three of life supports the impact of prematurity and mode of delivery on time course of intestinal mi-
crobiota colonization. The microbial community undergoes a compositional shift, in which obligate anaerobes (fermenters) overtake Escherichia coli as the most abundant species. This raises the question whether a supplementation with dietary fibers can increase the production of SCFA in preterm infants having a delayed colonization and therefore lacking the presence of anaerobes.

The two different dietary fibers were chosen because of their previously demonstrated influence on the microbiome, thereby potentially promoting an increase in generation of SCFA. scGOS/lcFOS is a combination of non-human oligosaccharides that aim to mimic human milk oligosaccharides, known for their influence on the (developing) immune system in infants and increasing acetate production. Acetate is the fermentation product of bifidobacteria and lactobacillus species which are known to have beneficial properties in newborn stool. The fecal bacteria of preterm infants do not demonstrate any potential to produce butyrate during the first three weeks of life. This changes around four weeks of age, when there seemed to be an increase in butyrate production, especially after addition of scGOS/lcFOS. This suggests a shift towards productivity of another subset of bacteria, the ones that have the required enzyme butyryl-CoA: acetate CoA-transferase, which utilizes acetate as a co-substrate to produce butyrate. It is known, that Bifidobacteria are not able to produce butyrate, but are able to ferment fibers and produce acetate and lactate. These SCFA are used as a substrate for other bacteria to grow including butyrate producing bacteria. There is a wide range of representatives of clostridial cluster XIVa and some cluster IV and XVI strains that are known to be able to produce butyrate. This raises the question where the butyrate-producing bacteria might come from or whether they might be present since the birth of the infant, but start to flourish later in life. However, there might be another step in the developing microbiome necessary, as Koenig et al. showed a positive correlation between Bacteroides and the butyrate producing potential of the microbiome. This group of bacteria seems to be necessary to break down polysaccharides, to give the butyrate-producing bacteria the opportunity to use these fibers in their production of butyrate. Around weaning, with the introduction of fruit and vegetables and the complex carbohydrates, the growth of bacteroides is further stimulated resulting in higher butyrate production than during the neonatal period.

The addition of scGOS/lcFOS influences mainly the acetate- and lactate-producing bacteria, and thereby increases the “fuel” to produce butyrate. There is some evidence however that FOS also might promote butyrate-producing bacteria in adults. In infants the lack of butyrate producers may explain that this effect is not found yet. It remains to be determined whether the limited number of butyrate-producing bacteria available in the intestine of preterm infants needs to be increased, as in term infants receiving human milk the microbiota is primarily bifidogenic, an acetate producer. Our ex-vivo study suggest that increasing butyrate-producing bacteria in preterm infants currently appears not possible In the first 2 weeks by using prebiotics. The administration of probiotics in adults results in higher butyr-
SCFA production, by cross-feeding acetate producers and thereby providing the substrate to butyrate-producing bacteria. However, increasing butyrate in preterm infants appears to be not practical as there are no safe strains of bacteria that can increase butyrate production in infants. Alternatively, one could also consider the direct administration of SCFA. However, it is not clear whether butyrate is the preferred SCFA in infants and the immature gut of preterm infants might be overwhelmed by this method in spite of compelling evidence that butyrate is the desirable SCFA with anti-inflammatory and tolerogenic effects in adults.

CAX is a slow fermenting fiber that aimed to target mostly the butyrate and propionate producing potential of the microbiome in the colon. It is reported to be water soluble, with a low viscosity and prebiotic and antioxidant properties. Our experience is that the solubility of CAX was less than of scGOS/LcFOS, which makes its clinical use less favorable. Although CAX increased SCFA levels in the stool of preterm infants, the increase was lower than scGOS/LcFOS. One of the reasons that CAX might have less influence on the SCFA producing potential of the stool is because the structure of this prebiotic is more complex than scGOS/LcFOS and therefore harder to break down by the bacteria in preterm stool. The neonatal microbiome during the first few weeks of life may not be far enough in its succession to have reasonable amounts of Bacteroidetes that are specialized in the breakdown of the more complex plant polysaccharides. This would make CAX a less useful prebiotic in the developing gut of preterm infants.

The data of our in vitro experiment are showing comparable results to the in vivo study in preterm infants of Westerbeek et al. showing low SCFA amounts during the first weeks of life, increasing with supplementation of scGOS/LcFOS/pAOS. This suggests that our in vitro model is a good screening system for the effect of dietary fibers as a SCFA promotor.

The fecal microbiota of infants who were delivered vaginally showed an increased response to the addition of scGOS/LcFOS sooner [at time point 3] than those delivered by C-section [at time point 4]. It has been described that mode of delivery influences the composition of the microbiota in term infants. Although not significant, Figures 11.1 and 11.2 suggest that the influence might start earlier in certain infants, as at time point 2 increased production of SCFA in 2 vaginal delivered infants was found, but not in any of the infants delivered by C-section. Studies with a larger group of preterm infants need to be done to confirm that the shift in the microbiome towards fermenters may be earlier in vaginal delivered preterm infants than in C-section preterm infants. Positive effects of vaginal delivery in term infants might be diminished in preterm infants, as it is known that diversity of the vaginal microbiome correlates with preterm birth because of changes during pregnancy towards a preferable microbiome at the end of the third trimester. It has been shown in term infants that a suboptimal maternal vaginal microbiome can influence the infant’s intestinal microbiome after vaginal delivery.

Antibiotics are known to alter the colonization of the intestines, by both diminishing the bacterial counts and delaying colonization. All preterm infants in this study group
received broad-spectrum antibiotics directly after birth. Previous studies have shown that the effect of antibiotics on the gut microbiota extends far after the supplementation of the antibiotics,\textsuperscript{44,225,227} which might explain why the influence of the duration of the initial antibiotic course does not influence the changes in fermentation potential as much as we expected. Only at the third time point an influence of the initial course length was demonstrated. In this group no preterm infants that did not receive any antibiotics directly after birth to compare with were available. Interestingly it seems that the addition of scGOS/lcFOS stimulates the growth of bacteria and the recovery of the microbiota starts earlier even in case of antibiotic use.

Little is known about the maturation of SCFA receptors in (preterm) infants and their sensitivity for the different SCFA during the neonatal period. SCFA receptors are suggested to have an influence in preterm birth as fetal membrane expression of GPR43 was significantly higher in women delivering prematurely with evidence of infection.\textsuperscript{295} Furthermore it is suggested that SCFA receptors might offer therapeutic opportunities later in life.\textsuperscript{296} Further investigation in the maturation of SCFA receptors is therefore needed.

A limitation of this study was that in this small group of infants formula feeding, although not the preferred way of feeding, was introduced in 4 out of the infants, because of insufficient availability of mother’s milk, thereby possibly changing the infants microbiome.\textsuperscript{297}

In conclusion, the fecal microbiome in preterm infants produced low SCFA during the first weeks of life, although this production increased after approximately two weeks, when dietary fibers, especially scGOS/lcFOS were added to the test system. These fibers seem to increase the production of cumulative SCFA after fermentation in vitro. The fecal microbiota showed low potential to generate butyrate or propionate during the first weeks of life. scGOS/lcFOS only increased butyrate production after 4 weeks. C-section and longer initial antibiotic course diminish the production of SCFA. The explorative research data suggests that feeding supplementation within first 2-3 weeks of delivery in preterm infants and especially those born by C-section might not provide the desired outcome such as increased production of SCFA. Thus, this study suggests that one needs to carefully consider the desired end points of feeding supplementation in premature infant in order to appropriately select the timing and type of supplements.

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