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Combining galacto-oligosaccharides and 2′-fucosyllactose alters their fermentation kinetics by infant fecal microbiota and influences AhR-receptor dependent cytokine responses in immature dendritic cells†

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Galacto-oligosaccharides (GOS) and 2′-fucosyllactose (2′-FL) are non-digestible carbohydrates (NDCs) that are often added to infant formula to replace the functionalities of human milk oligosaccharides (HMOs). It is not known if combining GOS and 2′-FL will affect their fermentation kinetics and subsequent immune-modulatory effects such as AhR-receptor stimulation. Here, we used an *in vitro* set-up for the fermentation of 2′-FL and GOS, either individually or combined, by fecal microbiota of 8-week-old infants. We found that GOS was fermented two times faster by the infant fecal microbiota when combined with 2′-FL, while the combination of GOS and 2′-FL did not result in a complete degradation of 2′-FL. Fermentation of both GOS and 2′-FL increased the relative abundance of *Bifidobacterium*, which coincided with the production of acetate and lactate. Digesta of the fermentations influenced dendritic cell cytokine secretion differently under normal conditions and in the presence of the AhR-receptor blocker CH223191. We show that, combining GOS and 2′-FL accelerates GOS fermentation by the infant fecal microbiota of 8-week-old infants. In addition, we show that the fermentation digesta of GOS and 2′-FL, either fermented individually or combined, can attenuate DC cytokine responses in a similar and in an AhR-receptor dependent way.

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Introduction

Infant formulas are often supplemented with non-digestible carbohydrates (NDCs) or enzymatically produced human milk oligosaccharide (HMO) to mimic some HMO functions from human milk (HM).[1] This includes stimulation of gut microbiota colonization and guidance and development of balanced gut immune responses.[2] Galacto-oligosaccharides (GOS) and the HMO analog 2-fucosyllactose (2′-FL) are commonly applied in infant formula,[3] either individually, or in case of GOS in combination with long-chain fructo-oligosaccharides (lcFOS) in a typical ratio of 9 : 1.[4] To date, there are only a few other combinations of NDCs and/or HMOs known that are used in infant formulas, while the list of NDCs that are allowed for their application in infant formula is growing.

GOS is very broadly applied in infant formula and comprised of a mixture of galactose chains with a terminal galactose or glucose unit.[5] The degree of polymerization (DP) of GOS molecules usually ranges from 2–8, with a large variation in glycosidic linkages and level of branching between different structures. A recent study by Logtenberg *et al.* identified over 100 different structures in commercially available Vivinal GOS.[6] In various studies, supplementation of GOS to infant formulas was shown to increase the abundance of *Bifidobacterium* in infants of different ages when compared to infants who received formula without GOS.[7,8] The utilization of GOS by specific bacterial species might also influence gut immune responses, as GOS fermentation leads to the formation of short chain fatty acids (SCFAs), such as propionate, butyrate and acetate, which exert immuno-regulatory properties.[9,10] However, there are also studies showing direct immune-regulatory effects of GOS. For example, it was shown that GOS could attenuate pro-inflammatory responses in dendritic cells induced by co-cultured epithelial cells.[11]

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Since a few years, the HMO 2′-FL is also applied in infant formulas, as it can now be produced as a fermentation product of genetically engineered microorganisms such as *E. coli*[12] or yeast.[13] This makes it possible to produce 2′-FL in sufficient quantities and in a cost-effective way to allow its application in infant formula. In contrast to GOS, 2′-FL is applied as an individual molecule in infant formulas. 2′-FL is composed of a lactose molecule decorated with an α(1–2)-linked fucose unit. In HM, 2′-FL is the most prevalent HMO that makes up around 30% of all HMOs.[14] Like GOS, 2′-FL has been shown to have immune regulating effects. Supplementation of infant formula with 2′-FL restored the level of inflammatory cytokines in healthy term formula-fed infants, to more similar levels as in breastfed children.[15]

Next to immune effects, 2′-FL is considered to play an important role in the colonization of bifidobacteria as infants fed with HM of non-secretor mothers who lack α1–2-fucosylated HMOs like 2′-FL, were found to have difficulties in acquiring bifidobacterial species.[16] 2′-FL indeed has been shown to stimulate *Bifidobacterium adolescentis* and butyrate-producing bacteria in a validated *in vitro* gut model using microbiota of 6-month-old formula-fed infants.[17] However, in contrast to GOS, 2′-FL appears to be more selectively fermented by specific infant gut microbiota as illustrated by the differentiation of infant fecal inocula into 'slow and fast 2′-FL fermenters'.[18] The 'slow 2′-FL fermenters' might benefit from a combination of 2′-FL with other more widely fermented oligosaccharides such as GOS. In addition, the combination of NDCs and/or HMOs with their unique structure-specific immune effects[19,20] could result in novel supplements for the application in tailored infant formula to meet the demands of specific groups of infants of different ages or health statuses.

The immune effects resulting from NDC fermentation are most often attributed to the SCFAs that are formed during the fermentation. However, nowadays more evidence becomes available that NDC fermentation might also influence tryptophan metabolism and thereby the formation of indole-derivatives.[21] Microbial tryptophan metabolites resulting from proteolysis can influence host health by binding to aryl hydrocarbon receptors (AhR) and thereby exert anti-inflammatory effects.[21] In addition, there are studies suggesting that indole-derivatives can improve the gut barrier function.[22,23] Furthermore, indole-derivatives also serve as important signaling molecules within the microbial community in the gut.[24] However, to date, minor knowledge is available on the effect of indole-formation and AhR activation on immune responses in early life and how this is influenced by the fermentation of NDCs and HMOs in infants.

In the present study we used an *in vitro* fermentation set-up with pooled fecal inoculum of 8-week-old infants to investigate whether combining the commonly used NDC GOS with the HMO 2′-FL influenced the fermentation kinetics of specific oligosaccharides. GOS and 2′-FL were fermented either individually or combined at a ratio of 4 : 1, with 2′-FL representing the amount of DP3 molecules in GOS. The degradation of GOS and 2′-FL by the infant fecal microbiota was studied as well as the microbiota compositions and the production of SCFAs during fermentation. Fermentation digesta collected at different timepoints were incubated with immature dendritic cells under normal cell-culture conditions or in presence of the AhR-receptor blocker CH223191 to study the effect of the fermentation products on dendritic cell cytokine responses.

Materials and methods

Substrates

2′-FL and purified Vivinal GOS (<3% monomers and lactose (w/w dry matter)) were provided by FrieslandCampina Ingredients (Amersfoort, The Netherlands). The purified Vivinal GOS was obtained from Vivinal GOS through the removal of lactose and monomers by lactase treatment and nanofiltration.

Fermentation of GOS and 2′-FL by infant fecal inoculum

Pooled fecal inoculum. Pooled infant fecal inoculum was prepared from the fecal material of 4 vaginally born infants (4 × 0.1 gram) as described elsewhere.[19] The bacterial functionality of the pooled fecal inoculum has been shown to resemble the bacterial functionality of the individual fecal inocula.[24] At an age of 8 weeks, the fecal material was collected directly after defecation from the diaper, transferred to tubes and stored at −80 °C. Until the day of the fecal collection, the infants only received human milk. In addition, the infants did not receive antibiotic treatment, neither did they have health issues.

***In vitro* fermentation.** The *in vitro* fermentations were performed in duplicate in an anaerobic chamber (gas phase: 81% N_2, 15% CO_2 and 4% H_2) (Bactron 300, Sheldon Manufacturing, Cornelius, USA) as described elsewhere.[25] In short, the standard ileal efflux medium (SIEM) containing GOS and/or 2′-FL were combined with the pooled fecal inoculum in sterile fermentation flasks in a ratio of 1 : 10 (v/v) with a total volume of 54 ml. GOS and 2′-FL were fermented in combination in a ratio of 4 : 1 (v/v) and individually. In all the fermentations, the final concentration of the substrates in the fermentation liquid was 10 mg ml^{-1}. Samples were collected with a syringe in triplicate at the start and after 12, 18, 24 and 36 h of fermentation. To preserve the bacteria for further analysis, one sample was immediately frozen in liquid nitrogen and stored at −80 °C. Samples for other analysis were heated for 5 min in a boiling water bath to inactivate all enzymes. Subsequently, the samples were stored at −20 °C until further analysis. The following control fermentations were included: (1) GOS and 2′-FL without inoculum to monitor contamination, (2) inoculum without GOS and 2′-FL to monitor background fermentation.

Fate of GOS and 2′-FL during fermentation

Ultra-high-performance liquid chromatography coupled to mass spectrometry. The degradation of GOS, individually and in combination with 2′-FL, as well as 2′-FL in combination

with GOS, by infant fecal microbiota was monitored by ultra-high-performance liquid chromatography coupled to mass spectrometry (UHPLC-MS) as described elsewhere with minor modification of the elution gradient.[6] Mobile phase A (ULC-MS water + 0.1% (v/v) formic acid) and B (acetonitrile + 0.1% (v/v) formic acid) were eluted as follows: 0–2 min, 3% B, 2–51.7 min, 3–11% B, 51.7–58.6 min, 11–15% B, 58.6–60.2 min, 15–100% B, 60.2–68.0 min, 100% B, 68.0–69.5, 100–3% B and 69.5–77.3 min, 3% B. Porous graphitic carbon (PGC) (Hypercarb, 3 μm particle size, 2.1 mm × 150 mm, Thermo Scientific, San Jose, CA, USA) was applied as stationary phase. To prevent the anomerization of oligosaccharides, prior to analysis fermentation samples were reduced using sodium borohydride as described in a previous study.[26] Subsequently, the reduced samples were purified using solid phase extraction.[26] The relative abundance of the oligomers with specific DP was determined by the selection of the specific mass range followed by the integration of the peaks. The data were analyzed using Chromeleon 7.0 (Thermo Scientific).

High-performance-anion-exchange-chromatography. The degradation of 2′-FL during the individual fermentations by infant fecal microbiota was monitored by high performance anion exchange chromatography (HPAEC) as described previously with minor modification of the elution gradient.[20] Mobile phase A (0.1 M sodium hydroxide) and B (1 M sodium acetate in 0.1 m sodium hydroxide) were eluted as follows: 0–15 min, 0–15% B, 15–16 min, 15–100% B, 16–21 min, washing step with 100% B, 21–22 min, 100–0% B, 22–37 min, equilibration with 100% A.

Microbial composition analysis

To determine the impact of the fermentation of GOS and/or 2′-FL on the microbiota composition, 16S rRNA gene amplicon sequencing was performed as described previously.[19] Subsequent to DNA extraction, the V5–V6 region of 16S ribosomal RNA (rRNA) genes were amplified by polymerase chain reactions (PCR) in triplicate with the unique barcoded primer pair BSF784 (RGGATTAGATACCC) and R1064 (CGACRRCCATGCANACCT). For samples collected at the start of the fermentation, 10 μl of DNA template was used in PCR reactions. The PCR reactions of samples collected at later time points were performed with 0.7 μl of DNA template. Two synthetic communities with known composition were included as positive controls.[27]

The PCR products were purified and prepared for sequencing as reported elsewhere.[19] In short, an amplicon pool with a volume of 40 μl was formed by combining 200 ng of each uniquely barcoded sample followed by a concentration step using the HighPrep PCR kit (MagBio Genomics, Alphen aan den Rijn, The Netherlands). The library was sent for adapter ligation and sequencing with Illumina Hiseq2500 (GATC-Biotech, Konstanz, Germany).

The 16S rRNA gene amplicon sequencing data were processed and analyzed using the NG-Tax 2.0 pipeline, with default settings and R version 3.5.0.[28] SILVA database release 132 [29] was used for taxonomic classification.

Production of SCFAs and other acids during fermentation

The production of acetate, propionate and butyrate during fermentation of GOS and 2′-FL was monitored by gas chromatography (GC) as described in previous study.[19] High performance liquid chromatography (HPLC) was applied for the monitoring of lactate and succinate production during the fermentation.[30]

Stimulation of DCs with fermentation digesta

Dendritic cell culture and stimulation under normal conditions or in presence of CH223191. Immature DCs generated from umbilical cord blood CD34+ progenitor cells (hematopoietic stem cells) were purchased from MatTek Corporation (Ashland, MA, USA). DCs were freshly thawed and seeded into 96-well plates at a density of 7×10^4 cells per well and cultured for 24 h under normal conditions (37 °C, 21% O_2 and 5% CO_2) according to manufacturer's instructions.

After 24 h, cells were attached to the plate and pre-incubated with normal DC-MM culture medium or medium containing 20 μM CH223191 for 1 h. Before replacing the medium, by medium containing fermentation digesta, bacteria were removed from the fermentation digesta by centrifugation (10 min, RT, 12000*g*). Subsequently, supernatants were filtered through a 0.2 μm filter and the digesta was diluted in DC-MM culture medium (MatTek Corporation, Ashland, MA, USA) containing Polymyxin-B (50 ng ml^{-1}) (Invivogen) or in DC-MM culture medium containing Polymyxin-B (50 ng ml^{-1}) and 20 μM CH223191 at a ratio of 1 : 10. The pH was set at 7.4 by the addition of 2N NaOH. DCs were then incubated with 200 μl per well medium containing the fermentation samples for 48 h. After incubation supernatants were collected and stored at −20 °C until further analysis. Experiments were repeated 6 times.

Assessment of cytokine expression. The levels of MCP-1/CCL2, MIP-1α/CCL3, IL-1β, IL-6, IL-10 and TNFα in the supernatant of DCs were quantified using a magnetic Luminex® Assay (R&D systems, Biotechne, Minneapolis, USA) according to manufacturer's protocol. Briefly, cytokine standards were re-suspended and serial dilutions were prepared. An antibody magnetic bead mix was added to a 96-well plate. Standards and samples were added and incubated overnight at 4 °C while shaking. After washing, detection antibodies were added and the plate was incubated for 30 min at RT while shaking. After incubation, the plate was washed again and incubated with streptavidin-PE for 30 min at RT while shaking. Finally, the plates were washed again and 100 μl of wash buffer was added to each well. The plates were analyzed using a Luminex 200 System. The data obtained was analyzed using the Luminex xPONENT software.

Statistical analysis. Statistical tests were performed using Prism 8 software (GraphPad, San Diego, CA, USA). Outliers were removed after testing using a Grubbs outlier test (alpha = 0.05). Data are shown as averages and error bars represent standard error means. Data was distributed normally and analyzed using a mixed-effects model (REML) to test for differ-

ences between groups, followed by a Tukey's multiple comparisons test to compare the induced cytokine responses of the digesta of different substrates with each other. To test for differences between cytokine responses in untreated DCs *versus* CH223191-treated DCs a mixed-effects model (REML) was used followed by a Sidak's posttest.

Results

Combination of GOS and 2′-FL alters the fermentation kinetics by infant fecal microbiota

The fermentation digesta of both the individual and combined fermentations were analyzed by UHPLC-PGC-MS and HPAEC to determine if the combination of GOS and 2′-FL altered the fermentation behaviour of infant fecal microbiota. When GOS was fermented individually, the fecal microbiota of 8-week-old infants preferably fermented GOS oligomers with a lower degree of polymerization (DP) (Fig. 1A). After 36 h of fermentation, only 5 ± 0.6% of the total GOS preparation was remaining, while still more than 20% of the DP5 and 6 oligomers present in the GOS preparation were remaining.

Interestingly, GOS was fermented two times faster by the infant fecal microbiota when combined with 2′-FL (Fig. 1B). In addition, the size-dependency as observed during the individual fermentation was absent. After 36 h of fermentation, DP6 oligomers (8.5 ± 0.4%) were less abundant than oligomers with DP 3–5 present in the GOS preparation, respectively 12.1 ± 0.5, 11.3 ± 0.3 and 22 ± 0.7%.

The fecal microbiota of 8-week-old infants degraded 2′-FL completely between 24 and 36 h of fermentation (Fig. 2A). However, whereas 2′-FL remained intact in the first 24 h of fermentation of 2′-FL alone, more than 20% of 2′-FL was degraded after 24 h of fermentation when fermented in combination with GOS (Fig. 2B). Nevertheless, the combination of 2′-FL and GOS did not result in the complete degradation of 2′-FL. After 36 h of fermentation, 70 ± 2.7% of 2′-FL was still remaining.

Increase in *Bifidobacterium* during fermentation of GOS and 2′-FL by infant fecal microbiota

To investigate the changes in microbiota composition upon fermentation, the digesta collected during the fermentation of GOS and/or 2′-FL by fecal microbiota of 8-week-old infants were analyzed by 16S rRNA gene amplicon sequencing. A fermentation without added GOS and/or 2′-FL served as control and showed an enrichment in bacteria belonging to the genus *Escherichia-Shigella* and *Enterococcus*, which are suggested to be stimulated by the protein-rich SIEM (Fig. S1†).[31]

The fermentation of GOS increased the relative abundance of *Bifidobacterium*, from 45% at the start of the fermentation to 57% after 36 h of fermentation (Fig. 3). The decrease in relative abundance in the first 18 h of fermentation can be attributed to the background fermentation on SIEM constituents by bacteria belonging to the genus *Escherichia-Shigella*. This background fermentation resulted in an increase in 16S rRNA gene copy numbers in the initial stage of the fermentation.[20] In comparison to GOS, the fermentation of 2′-FL by infant fecal microbiota increased the relative abundance of *Bifidobacterium* to a larger degree (Fig. 3). The relative abundance of *Bifidobacterium* increased from 45% at the start of the fermentation to 64% after 36 h of fermentation. Next to the increase in *Bifidobacterium*, an increase in the abundance of *Escherichia-Shigella* and *Enterococcus* was observed which was likely a result of background fermentation of SIEM constituents.

The fermentation of the combination of GOS and 2′-FL by fecal microbiota of 8-week-old infants slightly enhanced the bifidogenic effect (Fig. 3). At the start of the fermentation, 44% of the bacteria belonged to the genus *Bifidobacterium*. This increased up to 68% after 36 h of fermentation which is 4% higher than with 2′-FL alone and 11% higher than with GOS. Similar to the individual fermentations, bacteria belonging to the genus *Escherichia-Shigella* were also enriched upon fermentation.

Organic acid production during fermentation of GOS and 2′-FL by infant fecal microbiota

The fermentation digesta were analyzed by GC and HPLC to monitor the production of immune-active SCFAs, lactate and succinate[32,33] during fermentation of GOS and/or 2′-FL by fecal microbiota of 8-week-old infants. A control fermentation without added GOS and/or 2′-FL resulted in minor production of organic acids with a total level of 2.3 ± 0.1 μmol mg^{-1} after 36 h of fermentation (Fig. S2†). Butyrate and branched-chain fatty acids were not detected in any of the fermentation digesta.

The fermentation of GOS by fecal microbiota of 8-week-old infants resulted in a higher total production of organic acids acetate, lactate, succinate and propionate compared to the control fermentation (Fig. 4). The total organic acid concentration measured 8.5 μmol mg^{-1} after 18 h of fermentation, which further increased up to 13.4 ± 0.3 μmol mg^{-1} after 36 h of fermentation. Acetate, lactate, succinate and propionate were present in a ratio of 76 : 21 : 2 : 1. In comparison to GOS, the organic acid concentration in fermentation digesta of 2′-FL increased only after 24 h of fermentation with a lower total organic acid level (Fig. 4). The total organic acid concentration measured 2.1 μmol mg^{-1} after 18 h of fermentation, which further increased up to 11.9 ± 0.1 μmol mg^{-1} after 36 h of fermentation. Acetate, lactate, succinate and propionate were present in a ratio of 65 : 29 : 4 : 2.

The fermentation of the combination of GOS and 2′-FL by infant fecal microbiota resulted in similar organic acid production as observed with the fermentation of GOS alone (Fig. 4). The total organic acid concentration measured 8.4 ± 0.1 μmol mg^{-1} after 18 h of fermentation, which further increased up to 11.2 ± 0.1 μmol mg^{-1} after 36 h of fermentation. Acetate, lactate, succinate and propionate were present in a ratio of 74 : 23 : 2 : 1.

Cytokine responses induced by fermentation digesta are AhR-receptor dependent

In vivo, DCs are abundantly present under the epithelial lining of the infant's gut and play an important role in the initiation of mucosal immune responses.[34] To investigate the effect of

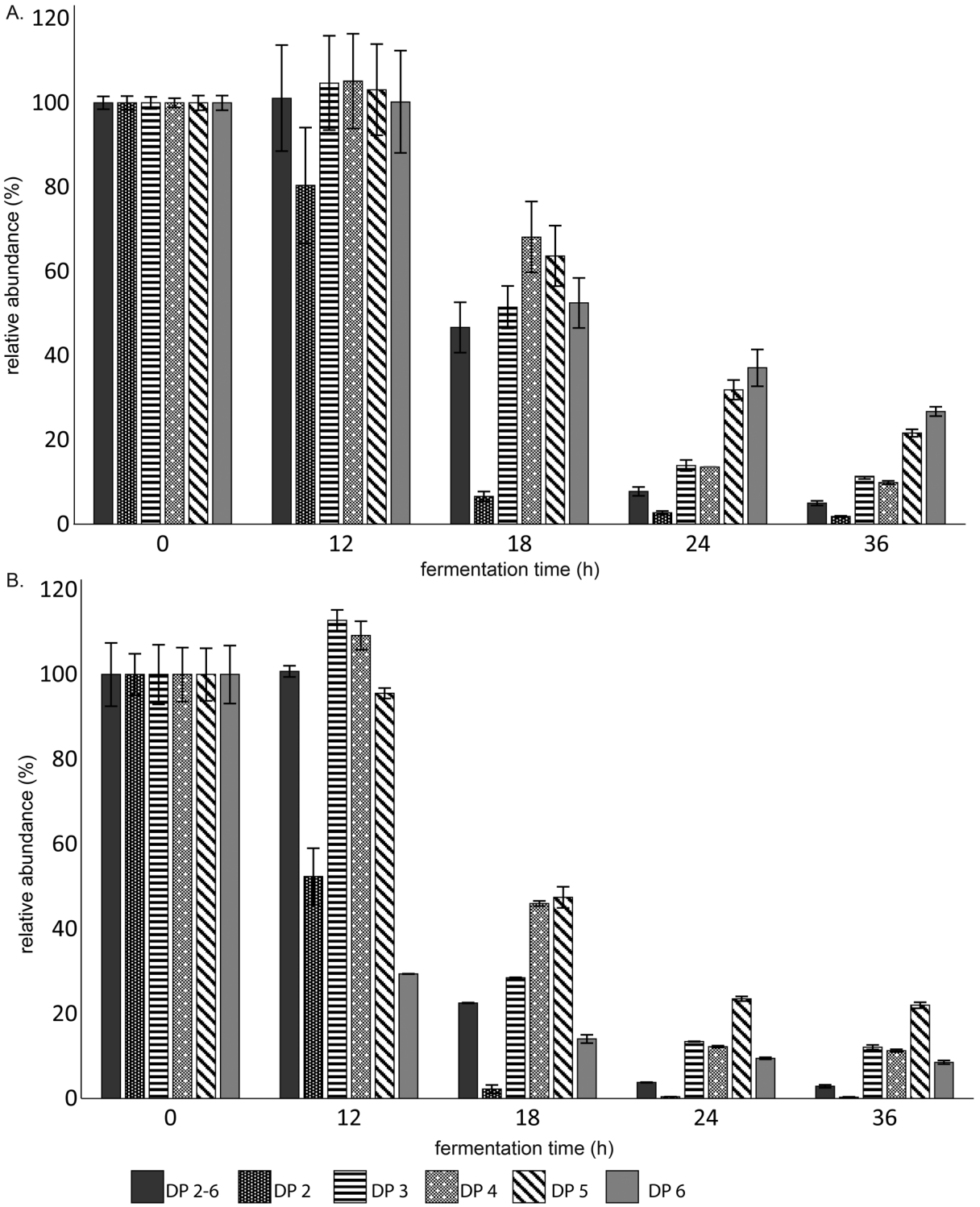

Fig. 1 Relative abundance of oligomers present in GOS, individual (A) and in combination with 2′-FL (B), at the start and after 12, 18, 24 and 36 h of fermentation using fecal inoculum of 8-week-old infants. Analysis was performed by UHPLC-PGC-MS. Concentrations per DP in the initial GOS preparation were set to 100%. Data are shown as average with standard deviation of the duplicate fermentation digesta.

the digesta of 2′-FL, GOS and the combination of 2′-FL/GOS fermented by fecal inoculum of 8-week-old infants on DC cytokine responses, DCs were incubated with digesta taken at different timepoints during the fermentation. The digesta of the blank fermentation increased cytokine production in DCs in a fermentation time dependent manner (Fig. 5).

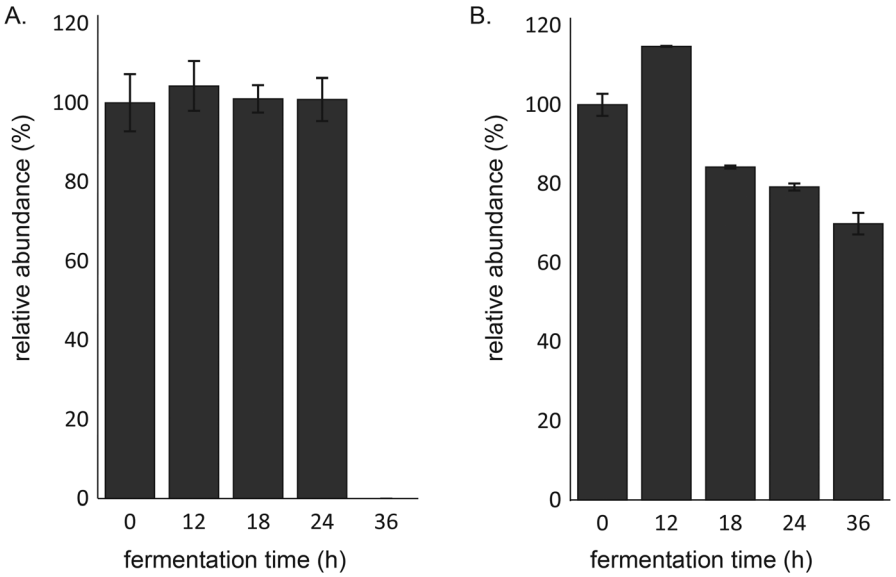

Fig. 2 Relative abundance of 2′-FL, individual (A) and in combination with GOS (B) at the start and after 12, 18, 24 and 36 of *in vitro* fermentation using pooled fecal inoculum of 8-week-old infants. Analysis was performed by HPAEC-PAD. Data are shown as average with standard deviation of the duplicate fermentation digesta.

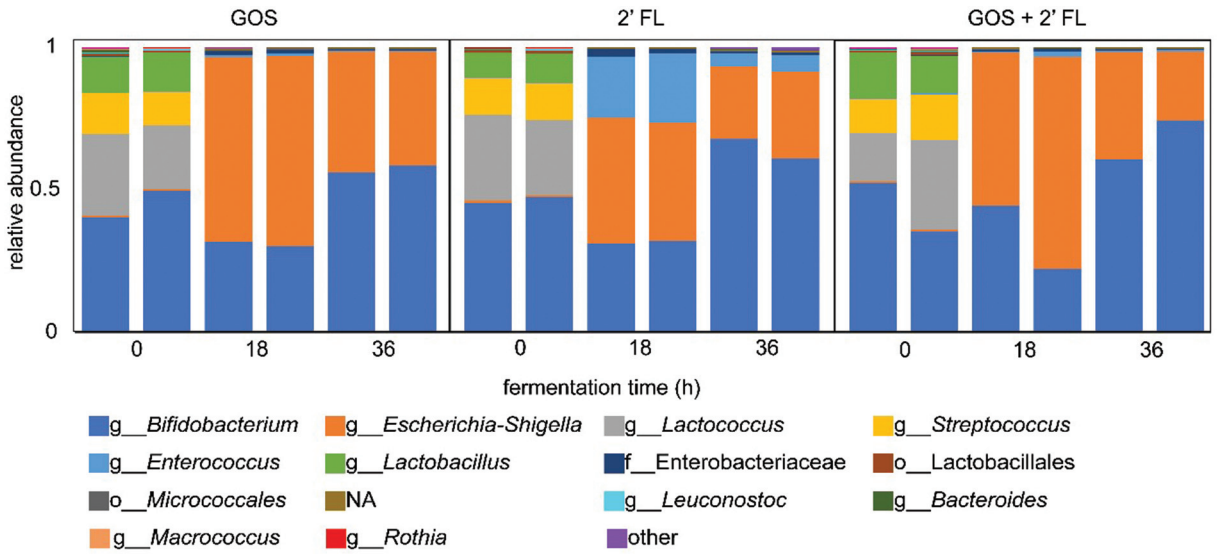

Fig. 3 Relative abundance of bacteria at the highest classified taxonomy in duplicate fermentation digesta collected at the start and after 18 and 36 h of fermentation by pooled fecal inoculum of 8-week-old infants of GOS and 2′-FL, individually and combined.

Incubation of DCs with both the $t = 18$ and $t = 36$ digesta from the 2′-FL fermentation resulted in a significant lower TNFα production when compared to the digesta of the blank fermentation. Incubation with the $t = 18$ digesta of the GOS fermentation resulted in significant lower secretion of MIP-1α/CCL3 and incubation of DCs with the $t = 18$ of the 2′-FL/GOS combination resulted in significant lower levels of MCP-1/CCL2 and MIP-1α/CCL3 when compared to the digesta of the blank control.

To investigate the influence of indole-derivatives present in the digesta on DC cytokine responses, DCs were also incubated with fermentation digesta in presence of the specific AhR-receptor antagonist CH223191.[35] This also resulted in differences in cytokine secretion patterns between the digesta of the blank and the NDC fermentations. Incubation of the DCs with the $t = 18$ digesta of the 2′-FL fermentation resulted in a significant higher production of TNFα, whereas incubation with the $t = 36$ digesta of GOS resulted in a significant lower production of IL-10 when compared to the corresponding digesta of the blank fermentations. Incubation of DCs with CH223191 alone had no effect on cytokine responses when compared to DCs cultured in normal cell culture (Fig. S3†).

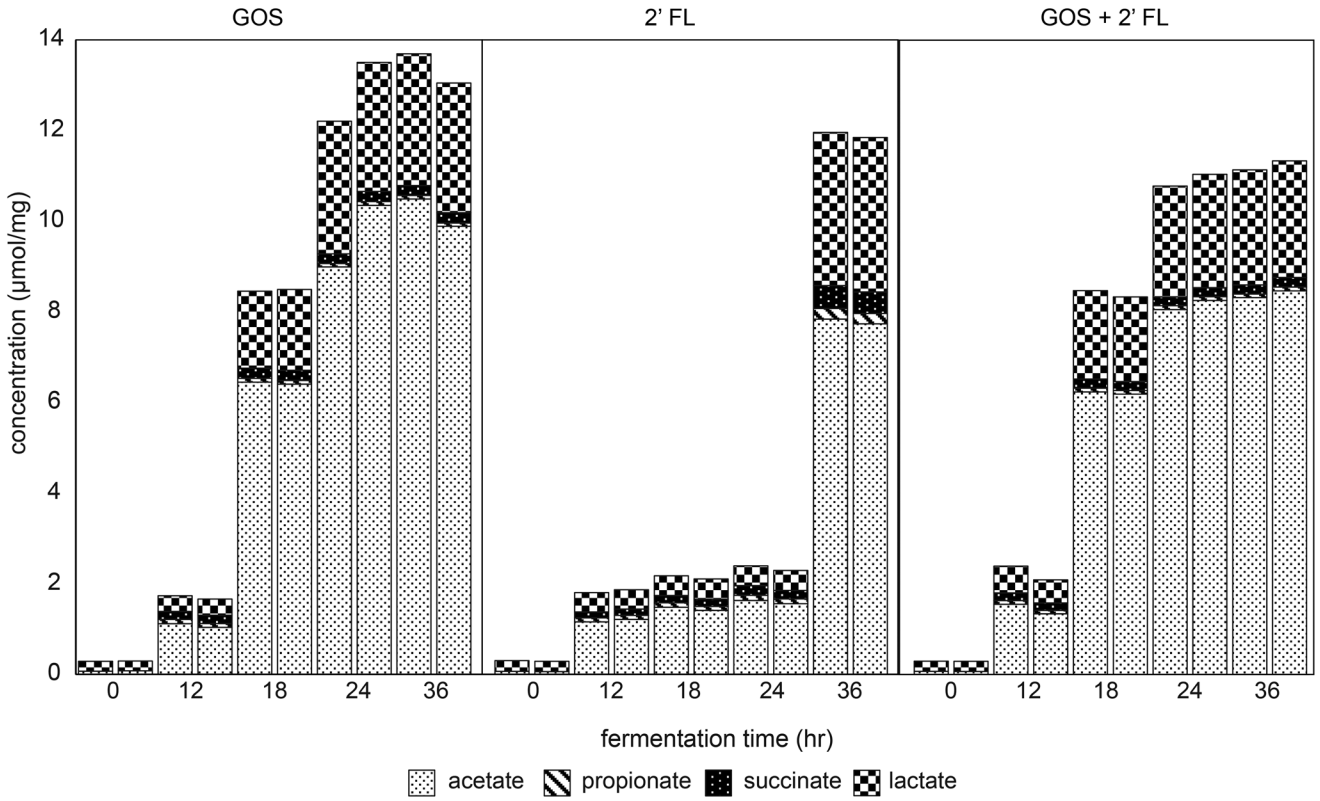

Fig. 4 Concentration of acetate, propionate, succinate and lactate in duplicate fermentation digesta collected at the start and after 12, 18, 24 and 36 h from *in vitro* fermentation by pooled fecal inoculum of 8-week-old infants of GOS and 2′-FL, alone and combined.

In addition, cytokine responses of DCs incubated with the fermentation digesta under normal circumstances or in presence of the specific AhR-receptor antagonist CH223191 were compared. Incubation of DCs with the t = 18 digesta of the blank fermentation resulted in significant higher secretion of IL-10 when incubated in presence of CH223191 compared to normal conditions. The t = 36 digesta of the blank induced higher secretion of both IL-10 and TNFα when incubated in presence of CH223191. Incubation of DCs with the t = 18 digesta of 2′-FL in presence of CH223191 resulted in a significant higher secretion of MCP-1/CCL2, MIP-1α/CCL3, IL-6, IL-10 and TNFα, while the incubation with the t = 36 digesta of the 2′-FL fermentation resulted in significant higher levels of IL-1β, IL-6 and TNFα when compared to DCs incubated with the digesta under normal conditions.

Incubation of DCs with the t = 18 digesta of the GOS fermentation resulted in significant higher levels of MCP-1/CCL2, MIP-1α/CCL3, IL-6 and TNFα, when compared to DCs incubated with the t = 18 GOS digesta under normal conditions. Incubation with the t = 18 digesta of the 2′-FL/GOS combination fermentation resulted in higher levels of MCP-1/CCL2 when compared to DCs incubated under normal conditions.

Discussion

Nutrition plays an important role in early life as it guides the colonization of the gut microbiota and it contributes to the maturation of the gut mucosal immune system.[3] Nowadays, NDCs are added to infant nutrition to mimic some HMO functions, including their prebiotic properties and their immune regulating effects.[2,36] GOS and the HMO analog 2′-FL are both added to infant formulas. However, it is unknown whether combining these NDCs impacts these beneficial health effects and whether fermentation of these NDCs has an effect on AhR activation and immune responses in early life. For this reason, we fermented GOS and 2′-FL, both individually and combined, using infant fecal inoculum and investigated the effect of the digesta on DC cytokine responses under normal conditions and in presence of the AhR antagonist CH223191.

GOS was fermented in a size-specific fashion by the fecal microbiota of 8-week-old infants, which is in line with the observations in our previous study.[25] Compared to GOS, the infant fecal microbiota required more time to adapt to the fermentation of 2′-FL as this substrate remained intact during the first 24 h of fermentation. After this adaptation period, 2′-FL was completely degraded between 24 and 36 h of fermentation. Fermentation of GOS in combination with 2′-FL expedited the fermentation of both GOS and 2′-FL, *i.e.* GOS was fermented two times faster and the fermentation of 2′-FL started earlier. The early onset of 2′-FL degradation could be explained by the immediate stimulation of GOS-degrading bacteria which are also capable of expressing enzymes needed to break down 2′-FL, like *Bifidobacterium longum* subsp. *infantis* expressing both β-galactosidases for the breakdown of GOS[37] and 1,2-α-L-fucosi-

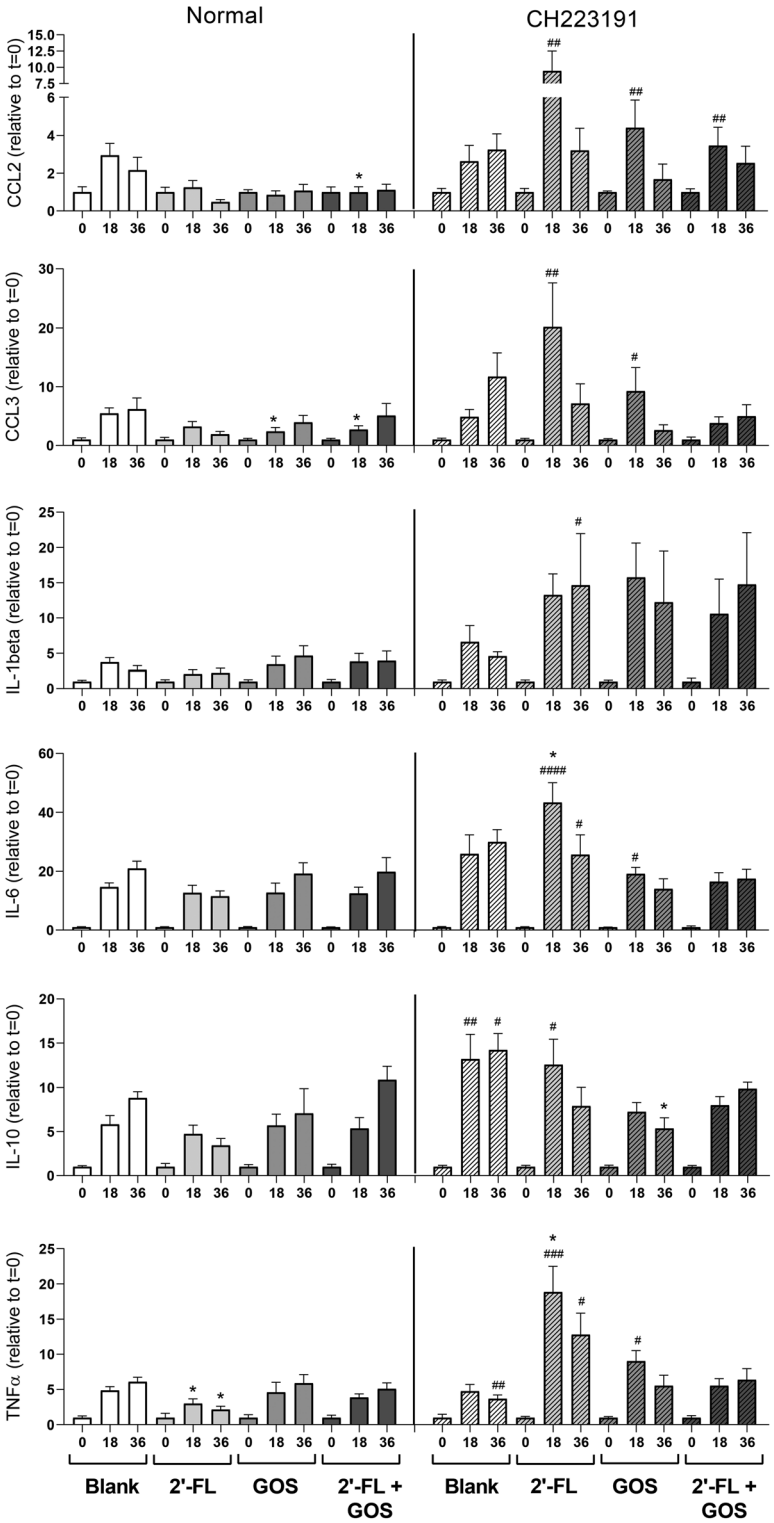

Fig. 5 Secretion of cytokines by DCs incubated with digesta of blank fermentation and fermentations of 2′-FL, GOS and a combination of 2′-FL/GOS. Stars above bars represent statistical different response compared to the corresponding blank samples (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Data is expressed as mean with SEM. Statistical differences between cytokine responses induced under normal conditions or in presence of CH223191 are indicated on the right side of the graph (#p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001).

dases for the breakdown of 2′-FL.[38] Nevertheless, GOS degradation remained preferred over 2′-FL degradation as illustrated by an incomplete degradation of 2′-FL when combined with GOS. In addition, the expedited GOS degradation confirms the preference of GOS over 2′-FL by the infant fecal microbiota.

The fermentation of both GOS and 2′-FL by the fecal microbiota of 8-week-old infants increased the relative abundance of *Bifidobacterium*. This coincided with the production of both lactate and acetate, which are important end products of the bifid shunt, a specific metabolic pathway in bifidobacteria for the degradation of hexose carbohydrates.[39] The bifidogenic effect of GOS *in vitro* is supported by many studies[40,41] as well as by various *in vivo* studies as reviewed by Vandenplas *et al.*[42] For 2′-FL there are also multiple studies showing bifidogenic effects *in vitro*[43,44] and *in vivo.*[16] The combination of GOS and 2′-FL did not result in significant synergistic bifidogenic effects on genus level, which might be explained by the capacity of several *Bifidobacterium* species to degrade both GOS and 2′-FL.[37,38] In addition, background fermentation occurred in the protein-rich SIEM medium as illustrated by an increased relative abundance of the protein-degrading bacteria *Escherichia-Shigella* in each fermentation.[31] The protein-degrading bacteria *Enterococcus* was only enhanced during the individual 2′-FL fermentation and the control fermentation without added substrate.

The digesta of the fermentations of GOS, 2′-FL and the GOS/2′-FL combination all induced cytokine responses in immature dendritic cells under normal conditions. We observed significant differences between the cytokine responses induced by the digesta of these fermentations when compared to the cytokine responses induced by the digesta of the blank fermentation. This might be related to the formation of different bacterial metabolites upon the fermentation of the different NDCs, like the SCFAs acetate and lactate or other molecules expressed and produced by bacterial species that were specifically increased in abundance *i.e.*, *Bifidobacterium*, during the NDC fermentations. Lactate has been shown to support the development of immunosuppressive phenotypes in DCs through the attenuation of cell maturation.[45] Acetate was also reported to have slight effects on chemokine secretion patterns in DCs before. In a study by Nastasi *et al.* incubation with acetate reduced the release of MIP-1α/CCL3.[33] In addition, metabolites and surface molecules produced by specific bacterial strains might influence the cytokine secretion patterns as well. For example, *Bifidobacterium*, which increased in abundance in all NDC fermentation groups but not in the control fermentation, is known for the production of immune regulating exopolysaccharides.[46] Also, other bacterial products formed upon fermentation, including ATP, lipoteichoic acid, polysaccharide A, nucleic acid, and peptidoglycan are likely present in the digesta and can interact with pattern recognition receptors (PRRs) like Toll-like receptors (TLRs) and C-type lectins expressed on the cell surface of DCs.[47]

Incubation of DCs with the digesta of the different fermentations in presence of the AhR-receptor blocker CH223191 resulted in significant different cytokine secretion patterns when compared to incubation under normal conditions. Especially incubation with the different $t = 18$ samples resulted in the secretion of significant higher cytokine levels. The effect was most pronounced for the NDC fermentation samples rather than for the blank fermentation samples. This might indicate that stimulation of the AhR-receptor by certain bacterial metabolites present in the digesta of the NDC fermentations has an immune attenuating effect in DCs. There are currently many different substances identified as agonist for the AhR-receptor, including synthetic compounds[48] and naturally occurring derivatives of tryptophan including indole, indole-3-acetate and tryptamine.[49]

The gut microbiota metabolizes tryptophan, which is abundantly present in the protein-rich SIEM medium, into derivatives *via* the action of the enzyme tryptophanase (TnaA), which is expressed in many Gram-negative, as well as Gram-positive bacterial species including *Escherichia coli*, Clostridium spp. and Bacteroides spp.[50] A recent study by Laursen *et al.*[51] reported that *Bifidobacterium* species also have the ability to convert aromatic amino acids (tryptophan, phenylalanine and tyrosine) into aromatic lactic acids, respectively indolelactate, phenyllactate and 4-hydroxyphenyllactate.[51] Considering the composition of the microbiota, with a high relative abundance of *Bifidobacterium* species in the fermentations of GOS, 2′-FL and GOS/2′-FL, it is likely that AhR ligands were formed during the NDC fermentations.

Stimulation of AhR-receptors is associated with immunoregulatory effects as, in DCs, AhR-receptors control both the differentiation and function of the cells.[52] A study by Kado *et al.* showed that AhR-receptor stimulation also influences TLR responses in DCs. They found that TLR stimulated DCs express higher levels of AhR and are more sensitive to AhR ligands and that AhR stimulation in these DCs decreased the expression of surface markers and TLR induced cytokine expression.[53] In addition, there are studies showing that decreased stimulation of the AhR-receptor is associated with pro-inflammatory responses as Lamas *et al.* showed for intestinal diseases like celiac disease.[54] Our results also show that AhR-receptor stimulation by metabolites present in the fermentation digesta of GOS and/or 2′-FL contribute partially to the attenuation of DC cytokine responses by NDC fermentation digesta.

Conclusions

Our study provides insight in the effect of combining the NDCs GOS and 2′-FL that are currently applied separately in infant formulas. Our data shows that combining GOS and 2′-FL accelerates GOS fermentation by the infant fecal microbiota of 8-week-old infants. In addition, we show that the fermentation digesta of GOS and 2′-FL, either fermented individually or combined, can attenuate DC cytokine responses in an AhR-receptor dependent way.

Author contributions

R. A., M. J. L, H. A. S. and P. D. V. designed the study. M. J. L performed the fermentation experiments. R. A. and M. B. performed the cell-based experiments. M. J. L performed the microbiota analysis, supported by E. Z., M. M. F. and B. J. D. H. assisted with the cell-based experiments. R. A., M. J. L., H. A. S. and P. D. V. wrote the manuscript. All authors have revised and improved the manuscript.

Conflicts of interest

There are no conflicts to declare.

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