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## Distinct fermentation of human milk oligosaccharides 3-FL and LNT2 and GOS/inulin by infant gut microbiota and impact on adhesion of *Lactobacillus plantarum* WCFS1 to gut epithelial cells†

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Human milk oligosaccharides (hMOs) are unique bioactive components in human milk. 3-Fucosyllactose (3-FL) is an abundantly present hMO that can be produced in sufficient amounts to allow application in infant formula. Lacto-*N*-triaose II (LNT2) can be obtained by acid hydrolysis of lacto-*N*-neotetraose (LNnT). Both 3-FL and LNT2 have been shown to have health benefits, but their impact on infant microbiota composition and microbial metabolic products such as short-chain fatty acids (SCFAs) is unknown. To gain more insight in fermentability, we performed *in vitro* fermentation studies of 3-FL and LNT2 using pooled fecal microbiota from 12-week-old infants. The commonly investigated galacto-oligosaccharides (GOS)/inulin (9 : 1) served as control. Compared to GOS/inulin, we observed a delayed utilization of 3-FL, which was utilized at 60.3% after 36 h of fermentation, and induced the gradual production of acetic acid and lactic acid. 3-FL specifically enriched bacteria of *Bacteroides* and *Enterococcus* genus. LNT2 was fermented much faster. After 14 h of fermentation, 90.1% was already utilized, and production of acetic acid, succinic acid, lactic acid and butyric acid was observed. LNT2 specifically increased the abundance of *Collinsella*, as well as *Bifidobacterium*. The GOS present in the GOS/inulin mixture was completely fermented after 14 h, while for inulin, only low DP was rapidly utilized after 14 h. To determine whether the fermentation might lead to enhanced colonization of commensal bacteria to gut epithelial cells, we investigated adhesion of the commensal *Lactobacillus plantarum* WCFS1 to Caco-2 cells. The fermentation digesta of LNT2 collected after 14 h, 24 h, and 36 h, and GOS/inulin after 24 h of fermentation significantly increased the adhesion of *L. plantarum* WCFS1 to Caco-2 cells, while 3-FL had no such effect. Our findings illustrate that fermentation of hMOs is very structure-dependent and different from the commonly applied GOS/inulin, which might lead to differential potencies to stimulate adhesion of commensal cells to gut epithelium and consequent microbial colonization. This knowledge might contribute to the design of tailored infant formulas containing specific hMO molecules to meet the need of infants during the transition from breastfeeding to formula.

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## 1. Introduction

Human milk (HM) is the gold standard for early life nutrition and promotes life-long health.<sup>1,2</sup> An important component in HM is the human milk oligosaccharides (hMOs). HMOs are the third most abundant component in HM and exert many health-promoting effects, such as modulating the gut microbiota composition,<sup>3</sup> reducing pathogen infection,<sup>4</sup> and regulating the gut immune barrier functions.<sup>5</sup> The World Health Organization recommends exclusively breastfeeding for up to six months of age. However, about 50% of infants rely on formula feeding even in the first month of life.<sup>6</sup> Most of the



infant formulas available on the market are based on cow milk. Cow milk lacks the unique oligosaccharides that are present in HM, *i.e.*, hMOs.<sup>7</sup>

Improving the nutritional quality of infant formula is essential to prevent the development of health issues in later life, especially in allergy-prone children.<sup>8</sup> HMO analogs are considered to be an important family of molecules for preventing these health issues. Until now, only 2'-fucosyllactose (2'-FL) and lacto-*N*-neotetraose (LNnT) are used in infant formula, while there are over 200 different hMO structures identified in HM.<sup>9,10</sup> To mimic some functions of hMOs, other non-digestible carbohydrates (NDCs) such as galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS), and inulin have also been tested and added in infant formula.<sup>11,12</sup> In recent years, hMOs have been produced microbiologically in a cost-effective way, such as 3-FL, a highly abundant hMO in HM, and lacto-*N*-triose II (LNT2), which are of interest to be applied in infant formula as well.<sup>10,13</sup>

One important function of hMOs and NDCs in infant formula is to guide the colonization of the gut microbiota.<sup>3,14</sup> HMOs and NDCs are resistant to human enzymes in the upper gastrointestinal tract, and cannot be directly utilized by the host. Therefore, they reach the colon intact where they can serve as substrates for bacterial fermentation.<sup>15,16</sup> In this way, hMOs and NDCs can specifically enrich beneficial species such as *Bifidobacterium* and *Lactobacillus*,<sup>17,18</sup> and lower the abundance of pathogenic species belonging to *Clostridia*, *Campylobacter*, and *stable toxin of enterotoxigenic E. coli*.<sup>19</sup> These enriched bacterial species may ferment hMOs and NDCs, which results in the production of short chain fatty acids (SCFAs) and other organic acids.<sup>5,20</sup> These breakdown products are not only a primary energy source for the gut epithelial cells, but are also important for tolerogenic immune responses<sup>21</sup> and in controlling undesired inflammatory responses.<sup>22</sup> Moreover, SCFAs and other organic acids produced during fermentation serve as nutrients for cross-feeding between gut bacteria, which might influence colonization and adherence of commensal bacteria to the host epithelium.<sup>23</sup>

Despite these proposed benefits, it still remains unknown how the currently developed hMO molecules for infant formula influence the gut microbiota composition as individual molecules. It is also not known whether they can be fermented by microbiota of babies, and whether they stimulate the production of SCFAs and other accessible organic acids. It is also still unknown how these hMOs influence, *e.g.*, adhesion of commensal bacteria to gut epithelium. To investigate this, we performed *in vitro* fermentation of NDCs and hMOs using the pooled fecal inoculum of 12-week-old infants. GOS/inulin, which has been widely investigated for infant formula, was taken as a reference. We tested fermentation of 3-FL, which has a similar structure as 2'-FL, the most abundant hMOs in mother's milk, and LNT2. LNT2 can be obtained by acid hydrolysis of LNnT. LNnT has shown health benefits and can be produced in sufficient amounts for supplementation in infant formula.<sup>24-26</sup> During the fermentation, we collected digesta at 0 (T0), 14 (T14), 24 (T24), and 36 (T36) hours to

study glycosidic breakdown, microbiota changes and SCFA formation. In addition, we investigated the effect of these digesta on the adhesion of a commensal bacterium *L. plantarum* WCFS1 to the intestine epithelial Caco-2 cells.

## 2. Materials and methods

### 2.1 Carbohydrates

The human milk oligosaccharides (hMOs) 3-FL and LNT2 were provided by Elicityl, France, and Frutafit TEX! inulin with oligomers in the range of degree of polymerization. DP8-DP50 was provided by Sensus, Roosendaal, the Netherlands. An overview of the structures of the carbohydrates is listed in Table 1.

### 2.2 Fermentation by infant fecal samples

**2.2.1 Fermentation medium.** The modified standard ileal efflux medium (SIEM) was applied and prepared, as described previously, with minor modifications.<sup>27</sup> The SIEM contained 40% BCO medium (v/v, Tritium Microbiology, Veldhoven, the Netherlands), 1.6% physiological salt solution (v/v, Tritium Microbiology), 0.8% MgSO<sub>4</sub> solution (v/v, 50 g L<sup>-1</sup>, Sigma, Zwijndrecht, the Netherlands), 0.4% cysteine hydrochloride (v/v, 40 g L<sup>-1</sup>, Sigma), 0.08% vitamin mixture solution (v/v, Tritium Microbiology), 0.4% carbohydrate mixture (v/v, Sigma), and 10% MES buffer (v/v, 1 M, pH 6.0, Sigma) in water. The specific carbohydrate mixture was added to SIEM to mimic the infant ileal environment, while also monitoring the background fermentation. The carbohydrate mixture contained pectin, xylan, amylopectin, and starch in the same ratio with a final concentration of only 0.24 g L<sup>-1</sup>. The pH was adjusted to 5.8 with MES buffer.

**2.2.2 Infant fecal inoculum.** Four infants who were vaginally delivered, exclusively breast-fed, without health issues, and without antibiotic treatment were recruited. At the age of 12 weeks, just before weaning, fecal samples were collected from the diaper immediately after defecation and stored at -20 °C, and subsequently stored at -80 °C until use. We chose

**Table 1** Overview of the structure of the selected carbohydrates

Carbohydrates <sup>a</sup>	Chemical composition	Structure <sup>b</sup>
GOS	Gal-(Gal) <sub>n</sub> -Glc	
Inulin	Fru-(Fru) <sub>n</sub> -Glc	
3-FL	Galβ1-4GlcFucα1-3	
LNT2	GlcNAcβ1-3Galβ1-4Glc	

<sup>a</sup> GOS, galacto-oligosaccharide; FL, fucosyllactose; LNT2, lacto-*N*-triose II.

<sup>b</sup>



12-week-old infants because 12 weeks is a common time point for infants to switch to infant formula.<sup>28</sup> The fecal inoculum for fermentation was prepared, as described elsewhere, with minor modifications.<sup>29</sup> After defrosting, fecal samples collected from the four infants were pooled (0.1 g × 4) and dissolved in 24 mL of sterilized NaCl solution (0.9%, w/v) in an anaerobic workstation (gas phase: 80% N<sub>2</sub>, 10% CO<sub>2</sub> and 10% H<sub>2</sub>; Whitley A35 HEPA Anaerobic Workstation, Brussels, Belgium). After homogenization, the fecal solution was combined with the fermentation medium in a ratio of 5 : 82 (v/v).<sup>20</sup>

**2.2.3 Anaerobic fermentation with carbohydrates.** 3-FL, LNT2, and GOS/inulin (9 : 1) were combined with the prepared infant fecal inoculum in a ratio of 1 : 10 (v/v) with a final concentration of 10 mg mL<sup>-1</sup> in sterile glass fermentation bottles. The infant fecal inoculum without the tested carbohydrates served as the control. The fermentation bottles were closed with a rubber stopper and sealed with a metal lid to guarantee an anaerobic condition. Fermentations were performed in duplicate in an incubator shaker at 37 °C, 100 rpm.

Fermentation samples were collected at the start (T0), and after 14 (T14), 24 (T24) and 36 h (T36). Three samples were collected from each group, one of which was centrifuged at 3000g for 10 min, snap frozen in liquid nitrogen and stored at -80 °C for microbial analysis. The other two samples were collected in a safe-lock Eppendorf tube to determine carbohydrate degradation, short chains fatty acids (SCFAs) and organic acids. These two samples were heated at 100 °C in a water bath for 10 min to inactivate the enzymes produced by the bacteria before storing at -80 °C.<sup>27</sup> Safe-lock Eppendorf tubes were applied to avoid evaporation of the SCFAs during heating. The cap of the tube was only removed when the sample was at room temperature. The method of inactivating/heating to stop fermentation and enzyme action did not influence SCFA analysis as validated using standards, as well as fermentation samples (ESI Fig. 1-4†).

### 2.3 Carbohydrates degradation

High performance anion exchange chromatography (HPAEC) was used to study the degradation of the carbohydrates in the infant fecal sample fermentation, as an indicator of the utilization of the carbohydrates by the gut microbiome.<sup>20,30</sup> Samples were diluted to 313 µg mL<sup>-1</sup> (GOS/inulin) or 10 µg mL<sup>-1</sup> (3-FL and LNT2) and centrifuged at 19 000g for 10 min. Ten µL of sample was injected to an ICS 5000 HPAEC system (Dionex, Sunnyvale, USA), in combination with a CarboPac PA-1 (250 mm × 2 mm ID) column and a CarboPac PA guard column (25 mm × 2 mm ID) (Dionex). The samples were eluted using solvent A (0.1 M NaOH solution) and solvent B (1 M NaOAc in 0.1 M NaOH) at a flow of 0.3 mL min<sup>-1</sup> through an electrochemical pulsed amperometric detector (Dionex) to detect the elution of the oligosaccharides. Two different gradients were applied, *i.e.*, for inulin, 0–50% B (0–50 min), 50–100% (50–55 min), 100% B (55–60 min), 0% B (60–75 min), and for GOS, 3-FL, and LNT2, 0–20% B (0–20 min), 100% B (20–26 min), 0% B (26–41 min).

### 2.4 Microbiological analysis

**2.4.1 DNA isolation.** DNA isolation of the fermentation samples was performed with a QIAamp PowerFecal DNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

**2.4.2 16S ribosomal RNA (rRNA) gene sequencing.** The bacterial 16S rRNA gene (V3–V4 region) of the isolated DNA was amplified using the primer pair 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGTATCTAAT-3'). All PCR procedures were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). In total, 20 samples were sequenced on an Illumina Novaseq6000 platform (PE250 strategy) at Novogene (Cambridge, London).

### 2.5 Short chain fatty acids and other organic acids production

High performance liquid chromatography (HPLC) was used to measure the production of SCFAs and other organic acids during the fermentation, as an indicator of the activity of the gut microbiome.<sup>20,31</sup> Samples were centrifuged at 19 000g for 10 min after five-fold dilution with Milli-Q water (Arium mini essential UV Ultrapure water filter, Sartorius, Göttingen, Germany). Ten µL of the diluent was injected to an Ultimate 3000 HPLC system (Dionex), with an Aminex HPX-87H column (Bio-Rad laboratories Inc., Hercules, USA). The SCFAs acetic acid, propionic acid, butyric acid, and the organic acids lactic acid and succinic acid were detected with a refractive index detector (RI-101, Shodex, Yokohama, Japan) and an UV detector at 210 nm. The samples were eluted in 50 mM sulphuric acid at a flow rate of 0.5 mL min<sup>-1</sup> at 50 °C. Standard curves in a range of 0.05–2 mg mL<sup>-1</sup> of acetate (VWR chemicals), propionate (Sigma Aldrich), butyrate (Sigma Aldrich), lactate (Alfa Aesar) and succinate (VWR chemicals) were used for quantification. The production was calculated based on each corresponding standard curves and the dilution factors. Results were presented as µmol mg<sup>-1</sup> of the fermentation substrate.

### 2.6 Bacteria adhesion to intestine epithelial Caco-2 cells with the fermentation samples

**2.6.1 Cell culture.** Human colorectal adenocarcinoma Caco-2 cells were cultured in DMEM (Lonza, Verviers, Belgium), supplemented with 10% heat deactivated fetal calf serum (Invitrogen), 1% nonessential amino acid (100×, Sigma), 50 µg mL<sup>-1</sup> penicillin-streptomycin, and 10 mM HEPES (Sigma), in a 5% CO<sub>2</sub> incubator at 37 °C. A number of 15 000 cells were seeded in 24-well plates per well, and were cultured for 21 days before use.

**2.6.2 Bacteria culture.** *L. plantarum* WCFS1 was recovered from glycerol stock at -80 °C and aerobically cultured in De Man, Rogosa and Sharpe (MRS) broth (Merck, Darmstadt, Germany) at 37 °C. After recovery, *L. plantarum* WCFS1 was plated on MRS agar to get single bacteria colonies. The single colony was then inoculated to MRS broth for the other overnight culture at 37 °C aerobically before the adhesion assay.



**2.6.3 *L. plantarum* WCFS1 adhesion assay.** *L. plantarum* WCFS1 was collected after overnight culture by centrifugation at 3000g for 10 min, adjusted to  $OD_{540} = 0.6$  in PBS, and re-suspended with half the volume of the fermentation sample. The fermentation samples were prepared by centrifugation at 3000g for 10 min once collected from Control, GOS/inulin, 3-FL, and LNT2 fermentation at T0, T14, T24, and T36, and filtered through a 0.2  $\mu$ m filter to remove the bacteria. The fermentation samples were diluted in Caco-2 cell culture medium without antibiotics at 10 times dilution and with a final pH of 7.4. *L. plantarum* WCFS1 was pre-incubated with the fermentation samples for 2 h at 37 °C, and then was brought to Caco-2 cells with the fermentation samples for another 2 h incubation at 37 °C. The group with the fermentation sample from SIEM fermentation was taken as control here. The non-adherent bacteria were removed by washing three times with PBS, and the adherent bacteria were released with 200  $\mu$ L of 0.1% Triton-X100. Serial dilutions and the drop-plate method were applied to determine the CFUs of the adherent bacteria on MRS agar plates.

## 2.7 Statistical analysis

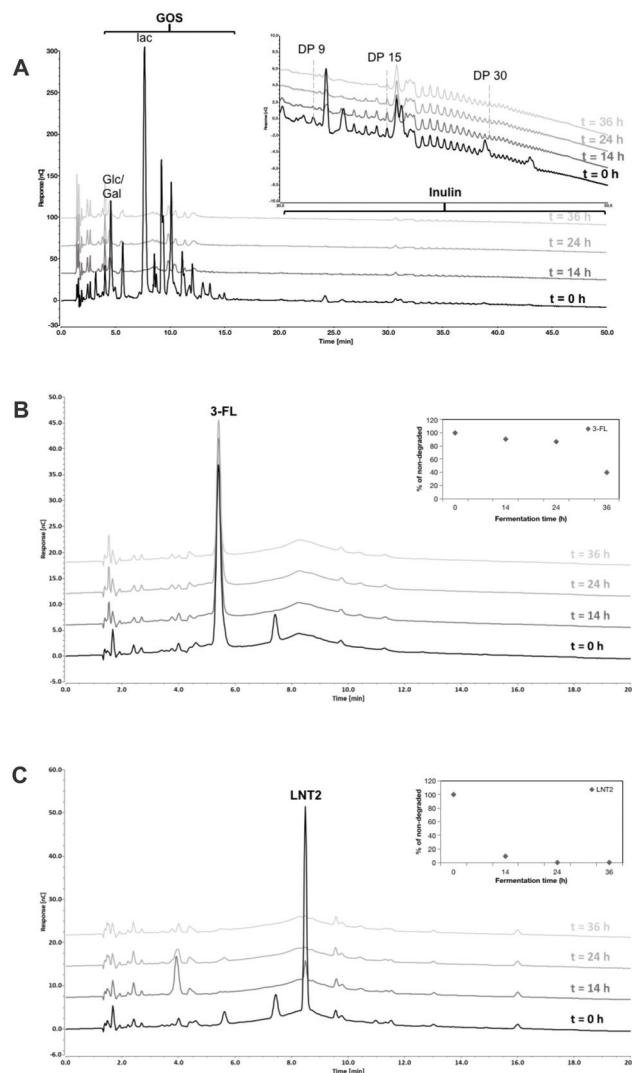
The carbohydrate degradation, and the SCFAs and other organic acids production were analyzed with ChromeleonTM 7.2.6 (Thermo Fisher Scientific, Massachusetts, USA). For the gut microbiome composition analysis, the high-quality clean reads were obtained from the sequencing by merging raw reads with FLASH (v1.2.7), filtering with QIIME (v1.7.0), and removal of chimera sequences detected with UCHIME algorithm. The clean reads were then analyzed with Uparse software (v7.0.1001). Sequences with similarities of  $\geq 97\%$  were assigned to the same operational taxonomic units (OTUs). Principal coordinate analysis (PCoA) was performed with R software by the WGCNA package, stat packages and ggplot2 package (v2.15.3). Data analysis of the bacteria adhesion assay was performed with GraphPad Prism 8 (v8.4.2, GraphPad Software LLC.). Results were shown as the mean  $\pm$  SD. Normal distribution of the data was confirmed with a Shapiro-Wilk test. Statistical differences were evaluated using a two-way ANOVA, followed by a Dunnet's multiple comparisons test. A  $p$ -value of  $<0.05$  was considered as a significant difference (\*,  $\#p < 0.05$ ; \*\*,  $\#\#p < 0.01$ ).

## 3. Results

### 3.1 Delayed fermentation of 3-FL compared to GOS/inulin and LNT2

We first determined the fermentability of the hMOS 3-FL and LNT2 by microbiota of 12-week-old infants, and compared it with the fermentability of GOS/inulin (9 : 1) as a reference.<sup>12,32</sup> We studied fermentability at 0 (T0), 14 (T14), 24 (T24) and 36 (T36) hours.

In the GOS/inulin fermentation, GOS was completely fermented at T14 by the fecal microbiome of the 12 weeks-old infants (Fig. 1A). This was different for inulin present in the



**Fig. 1** High performance anion exchange chromatography (HPC) profiles of GOS/inulin (A), 3-FL (B), and LNT2 (C) at the start (T0), and after 14 h (T14), 24 h (T24), and 36 h (T36) of fermentation with pooled fecal samples of 12-week-old infants. The percentages of 3-FL and LNT2 through the fermentation were quantified, and are presented in B and C.

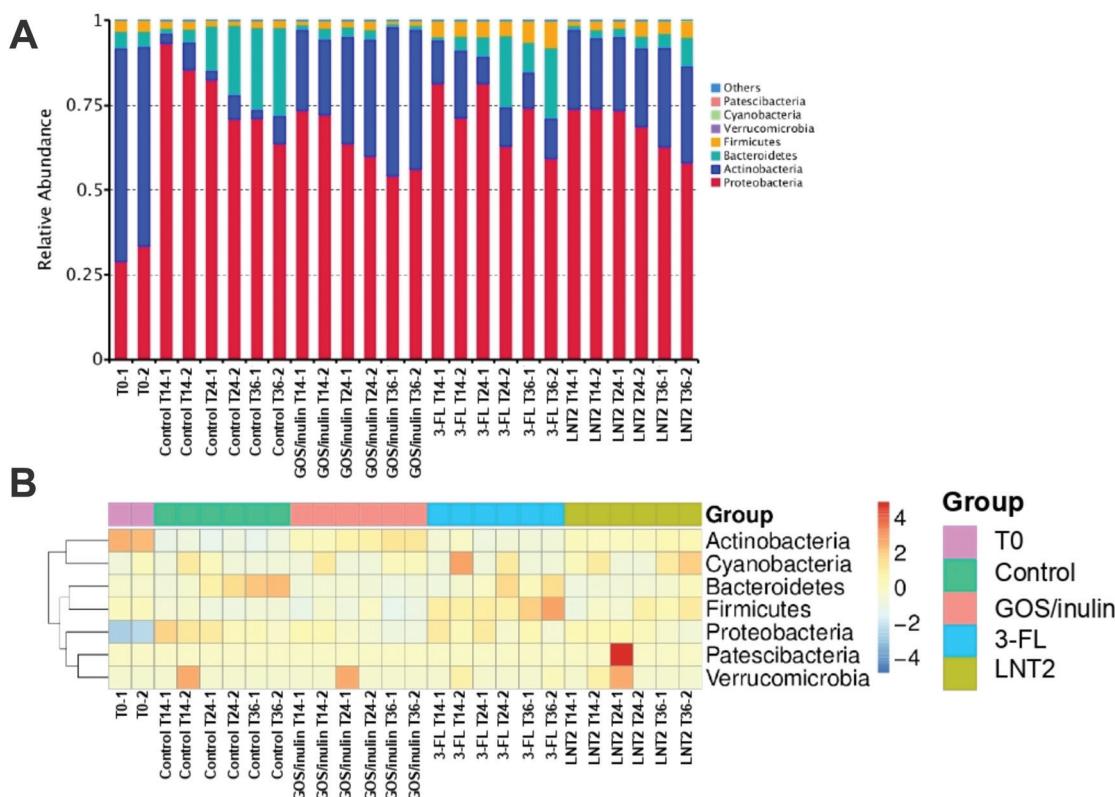
GOS/inulin mixture, *i.e.*, only the inulin oligomers eluting between retention time of 25–30 min (DP8–14) were fermented slowly over time until completely utilized at T36.

Fermentation of 3-FL was delayed when compared to GOS/inulin. At T14 and T24, only 9.5% and 13.4% was fermented but at T36, 60.3% of the 3-FL was utilized (Fig. 1B). LNT2 was more readily fermented and was already degraded for 90.1% at T14, and further fermented by 99.4% at T24 (Fig. 1C).

### 3.2 GOS/inulin and LNT2 increase abundance of Actinobacteria, and 3-FL specifically increases the relative abundance of Bacteroidetes at the phylum level

Next, we investigated which groups of gut bacteria were changed during the fermentation of SIEM medium, GOS/





**Fig. 2** Relative abundance of the top taxons in the phylum level by the fermentation of GOS/inulin, 3-FL, and LNT2 with pooled fecal samples of the 12-week-old infants. (A) Relative abundance of each phylum by the fermentation of GOS/inulin, 3-FL, and LNT2 at T0, T14, T24, and T36. (B) Taxonomic abundance heatmap showing the relative abundance distribution of the dominant phyla by GOS/inulin, 3-FL, and LNT2 fermentation, at T0, T14, T24, and T36. Control is set without GOS/inulin, 3-FL, or LNT2.

inulin, 3-FL, or LNT2, and contributed to the diversity differences. To this end, the relative abundance of different phyla during fermentation at T0, T14, T24, and T36 were first determined.

At the start of the fermentation, the gut microbiome communities were dominated by 60.8% of Actinobacteria, 31.3% of Proteobacteria, 4.9% Bacteroidetes, and 3.1% of Firmicutes. This microbiome composition changed during fermentation (Fig. 2A). In the control fermentation of the SIEM medium, which only contained pectin, xylan, amylopectin, and starch as a carbohydrate source, but without GOS/inulin or hMO, Actinobacteria was decreased from 60.8% at T0 to 5% at T14, and the level remained about 5.3% until T36. Proteobacteria, however, was nearly triplicated to 89.5% at T14, and the relative abundance was 67.5% at T36 of fermentation. This reduction might be induced by the increase of Bacteroidetes, which was increased from 2.8% (T14) to 25.1% (T36).

The microbiota composition was clearly influenced by GOS/inulin and the two tested hMOs. For the GOS/inulin fermentation at T14, we observed already a high level of Actinobacteria of 23.0% (Fig. 2A), and this abundance increased to 32.9% (T24) and 42.5% (T36) during the fermentation. The relative abundance of Proteobacteria was doubled to 72.9% at T14 of fermentation, but lowered to 55.3% at T36. GOS/inulin fermenta-

tion resulted in a relative low level of both Bacteroidetes and Firmicutes of around 2% during the fermentation.

With 3-FL, we observed a relative abundance of Actinobacteria of 16.1% at T14 (Fig. 2A), and the level decreased to 9.6% (T24) and 11.7% (T36) during the fermentation. Despite its low fermentability, the relative abundance of Proteobacteria was doubled at T14 of fermentation compared to T0, and it decreased to 66.8% at T36 of fermentation. The relative abundance of Bacteroidetes was decreased from 4.9% at T0 to 2.7% at T14, but it kept increasing to 14.8% at T36.

Results were different with LNT2, which induced a high abundance of Actinobacteria of 22% at T14 (Fig. 2A), and 22% at T24 and 28.8% at T36. The relative abundance of Proteobacteria at T14 was 74.1%, with LNT2 fermentation, the level decreased to 60.4% at T36.

Fig. 2B shows a heat map directly illustrating the differences of each phylum in different groups.

### 3.3 GOS/inulin and LNT2 fermentation increase the relative abundance of *Bifidobacterium*, and LNT2 specifically increases the relative abundance of *Collinsella* at the genus level

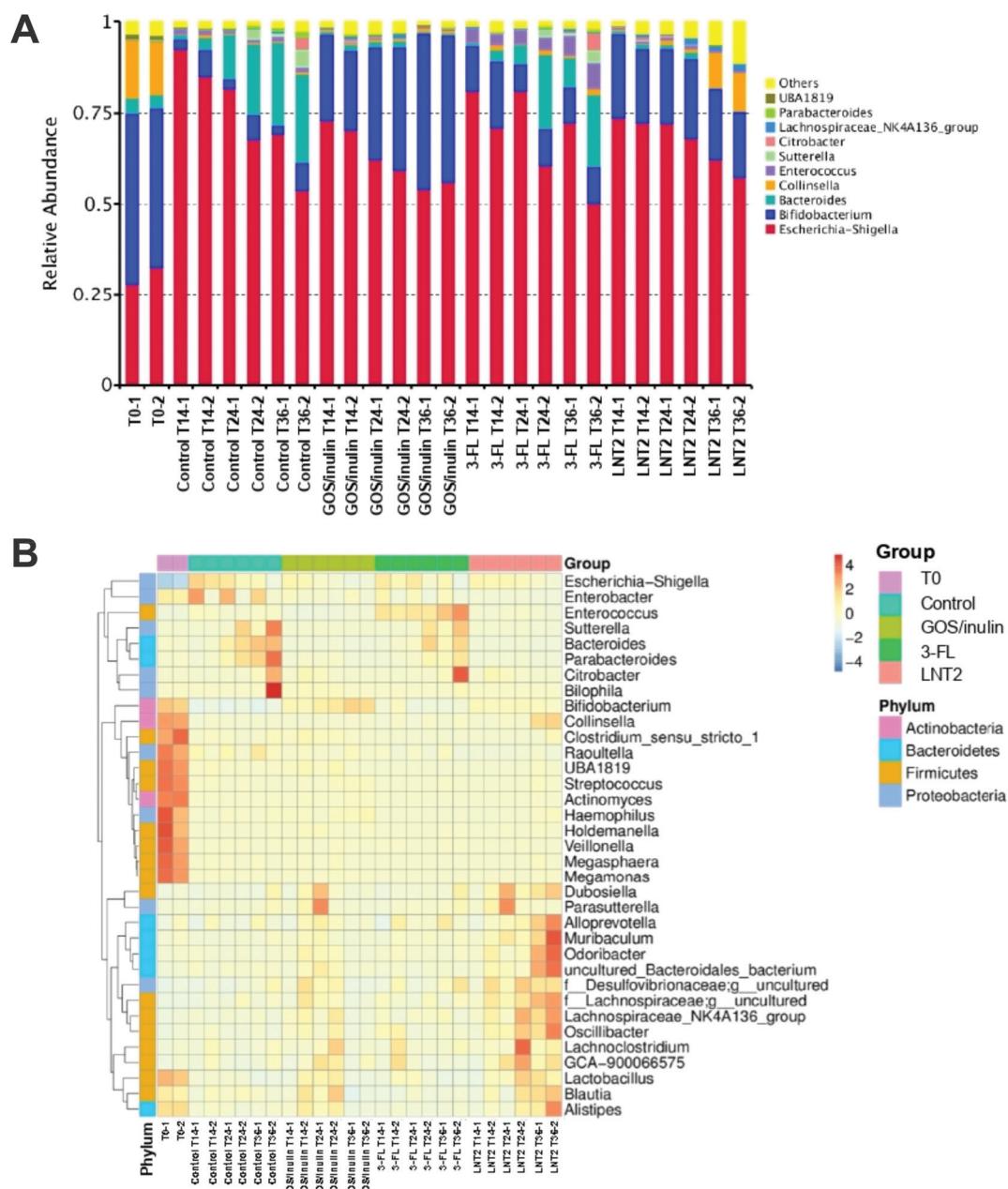
Then, the relative abundance of different genera during the fermentation at T0, T14, T24, and T36 were determined. At the start of fermentation (T0), the microbiome compositions in

the fecal samples were dominated by *Bifidobacterium* (45.2%), *Escherichia-Shigella* (30.4%), *Collinsella* (15.2%), and *Bacteroides* (3.9%).

In the SIEM control without added GOS/inulin or hMO, the relative abundance of *Bifidobacterium* was dramatically decreased from 45.2% at T0 to 4.9% at T14, and remained around 5% at T24 and T36 (Fig. 3A). However, the relative abundance of *Escherichia-Shigella* belonging to Proteobacteria was nearly triplicated to 88.9% at T14, and kept decreasing to

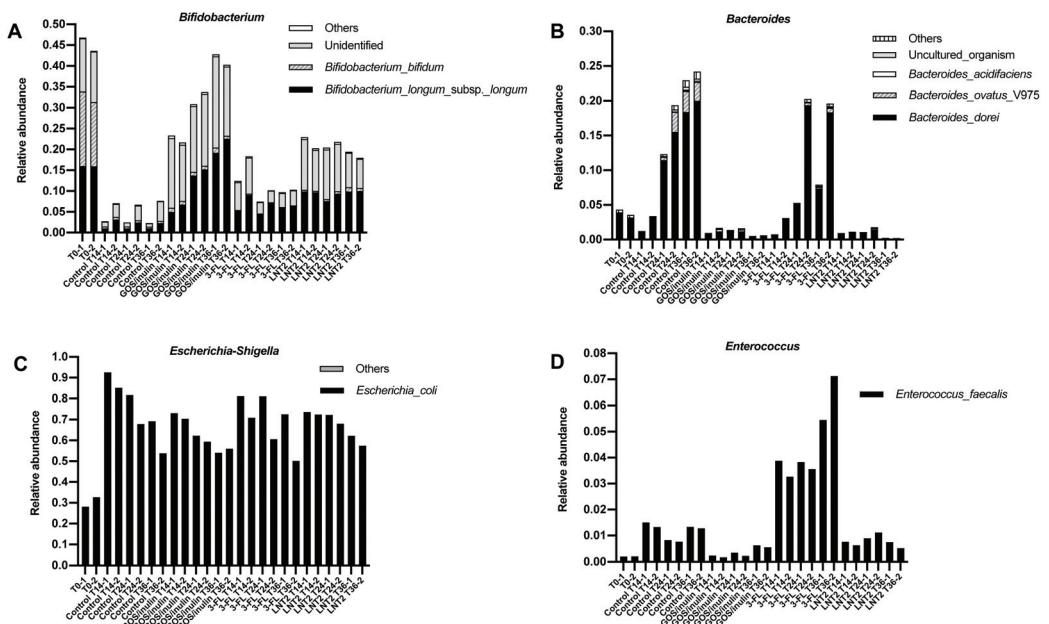
61.5% at T36 of fermentation. The relative abundance of *Bacteroides* at T14 was 2.3%, but it kept increasing to 23.6% (T36). There were 16 genera belonging to Firmicutes phylum of the top 35 (Fig. 3B), but they were all of low abundance.

GOS/inulin induced a relative abundance of 11.5% of *Bifidobacterium* at T14 (Fig. 3A), which was increased to 32.3% at T24, and finally achieved a level of 41.5% at T36. Although GOS/inulin induced a high abundance of *Escherichia-Shigella* of 71.8% at T14, the relative abundance kept decreasing to



**Fig. 3** Relative abundance of the top taxons in the genus level by the fermentation of GOS/inulin, 3-FL, and LNT2 with pooled fecal samples of the 12-week-old infants. (A) Relative abundance of each genus by the fermentation of GOS/inulin, 3-FL, and LNT2 at T0, T14, T24, and T36. (B) Taxonomic abundance heatmap showing the relative abundance distribution of the dominant genera by GOS/inulin, 3-FL, and LNT2 fermentation, at T0, T14, T24, and T36. Control is set without GOS/inulin, 3-FL, or LNT2.





**Fig. 4** Relative abundance of the species belonging to the genus of *Bifidobacterium* (A), *Bacteroides* (B), *Escherichia-Shigella* (C), *Enterococcus* (D) induced by the fermentation of GOS/inulin, 3-FL, and LNT2 at T0, T14, T24, and T36, with pooled fecal samples of 12-week-old infants. Control is set without GOS/inulin, 3-FL, or LNT2.

60.9% (T24) and 55.0% (T36). The change of *Escherichia-Shigella* can also be recognized from the heat map, which gradually changed from dark blue at T0 to shallow orange at T14–T36 (Fig. 3B).

Despite its low fermentability, 3-FL also induced a high relative abundance of *Bifidobacterium* of 15.41% at T14 (Fig. 3A), but it decreased during fermentation to 8.8% (T24) and 10.0% (T36). The lowering of *Bifidobacterium* might be induced by the increase in relative abundance of *Bacteroides* and *Enterococcus* during the fermentation, as *Bacteroides* increased from 1.9% at T14, to 12.8% at T24, and reached a level of 13.8% at T36. The relative abundance of *Bacteroides* was as low as 0.2% at T0, but it increased to 3.6% at T14, 3.7% at T24, and reached a level of 6.3% at T36 (Fig. 3B). Even though the relative abundance of *Escherichia-Shigella* was increased to 76.0% at T14, it kept decreasing to 70.8% (T24) and 61.3% (T36) with 3-FL (Fig. 3A).

LNT2 could have already induced a relative abundance of *Bifidobacterium* with 21.6% at T14 (Fig. 3A), but decreased slightly to 21.3% at T24 and 18.7% at T36. This might be due to the LNT2 effect on the increase of the relative abundance of *Collinsella*, which belonged to the same phylum of Actinobacteria as *Bifidobacterium*, by reaching a level of 10.1% at T36. The increase of *Collinsella* was unique for LNT2, because this effect was not observed with GOS/inulin or 3-FL. LNT2 also decreased the relative abundance of *Escherichia-Shigella* from 73.1% at T14 to 59.8% at T36.

#### 3.4 GOS/inulin, 3-FL, and LNT2 fermentation increase the relative abundance of *Bifidobacterium\_longum* subsp. *longum*, and 3-FL specifically increases *Bacteroides\_dorei*

As we observed such a different effect on the microbiome compositions during the fermentation of GOS/inulin, 3-FL, and

LNT2, especially on *Bifidobacterium* and *Escherichia-Shigella*, we next investigated which specific species contributed the most to the dominant genera including *Bifidobacterium*, *Bacteroides*, *Escherichia-Shigella*, and *Enterococcus*. To do so, a SIMPER test was performed. The species that got a contribution of >1% in microbiome compositions is shown in ESI Tables 3–6.†

At the start of fermentation at T0 (Fig. 4A), the relative abundance of *Bifidobacterium* genus was 45.2%, which mainly consisted of two species, *i.e.*, *Bifidobacterium\_bifidum*, with a relative abundance of 16.7% and *Bifidobacterium\_longum* subsp. *longum*, with a relative abundance of 16.0. However, there was still a high abundance of unidentified species, with a relative abundance of 12.4%. The *Bacteroides* genus was dominated by the species of *Bacteroides\_dorei* (Fig. 4B), which was 3.8% compared to the total relative abundance of 4.3%. Of the relative abundance of 30.4% of the *Escherichia-Shigella* genus in the gut microbiome, the species of *Escherichia\_coli* was the most prevalent (Fig. 4C), with a relative abundance of 30.4%. *Enterococcus\_faecalis* was the only species present in the 12-week-old baby microbiome belonging to the genus of *Enterococcus* (Fig. 4D).

Control fermentation in SIEM without GOS/inulin or hMO led to a dramatic decrease in the relative abundance of species of *Bifidobacterium\_bifidum* from 16.7% to 0.56% at T14 (Fig. 4A), and remained below 0.5% during the fermentation. *Bifidobacterium\_longum* subsp. *longum* and other identified species were around 2%. During the fermentation in SIEM, the increased species belonging to *Bacteroides* genus (23.6% at T36) were dominated by *Bacteroides\_dorei* and *Bacteroides\_ovatus\_V975* (Fig. 4B), of which, the relative abundance of *Bacteroides\_dorei* was increased up to 19.2%, and

*Bacteroides ovatus* V975 was up to 2.8% at T36. The increased effects on *Escherichia-Shigella* and *Enterococcus* genera were attributed to *Escherichia coli* and *Enterococcus faecalis* (Fig. 4C and D).

The enhancing effect of GOS/inulin during fermentation on the *Bifidobacterium* genus was achieved through increasing the relative abundance of *Bifidobacterium longum* subsp. *longum* and other unidentified species, but not *Bifidobacterium bifidum* (Fig. 4A). The relative abundance of *Bifidobacterium longum* subsp. *longum* was 14.5% at T24 and 20.9% at T36, which was even higher than T0, which was 16.0%. The effect of GOS/inulin on the unidentified species cannot be ignored, as the relative abundance was already up to 15.1% at T14, and reached a level of 19.2% at T36. Limited effects were observed on *Bifidobacterium bifidum*, as the relative abundance during the fermentation was kept stable at 0.9% (T14), 0.9% (T24) and 0.1% (T36).

3-FL fermentation induced a relative abundance at T14 of *Bifidobacterium* genus of 15.4%, and it was dominated by *Bifidobacterium longum* subsp. *longum* and the unidentified species, with a relative abundance of 7.1% and 7.7% (Fig. 4A). During 3FL fermentation, the unidentified species of *Bifidobacterium* was reduced to 2.8% at T24, and 3.5% at T36, but the relative abundance of *Bifidobacterium longum* subsp. *longum* was stable, which was 6.2% at T36. 3-FL failed to impact *Bifidobacterium bifidum*, and the relative abundance was only of 0.3% (T14), 0.1% (T24), and 0.1% (T36) during the fermentation. Meanwhile, the increasing effect of 3-FL on the *Bacteroides* genus was demonstrated as by increasing *Bacteroides dorei* (Fig. 4B). The relative abundance of *Bacteroides dorei* was up to 12.7% at T36, which was 93.3% of the relative abundance of *Bacteroides* genus (a level of 13.8% at T36).

LNT2 had a strong effect on the *Bifidobacterium* genus, which was 21.6% at T14 (Fig. 4A), and was dominated by *Bifidobacterium longum* subsp. *longum* and the unidentified species, with a level of 9.8% and 11.0%. The relative abundance of *Bifidobacterium bifidum* was only 0.4% at T14. During fermentation, LNT2 induced a relatively stable abundance of *Bifidobacterium longum* subsp. *longum*, which was of 10.0% at T36, and slightly increased the relative abundance of *Bifidobacterium bifidum* to 0.9% at T36, but the unidentified species was reduced to 7.6% at T36. As we explained in section 3.4, the reduction of the *Bifidobacterium* genus might be attributed to the increase of the *Collinsella* genus, but the species belonging to *Collinsella* was unidentified.

### 3.5 3-FL fermentation stimulates acetic acid, butyric acid and lactic acid production, and LNT2 also specifically induces succinic acid production

Next, we determined the production of SCFAs and organic acids by microbiota of 12-week-old babies during fermentation of GOS/inulin, 3-FL, and LNT2 at T14, T24, and T36. We took fermentation in the SIEM medium without added GOS/inulin, 3-FL, or LNT2 as a control. The fermentation of the control only resulted in a low amount of SCFAs and organic acids

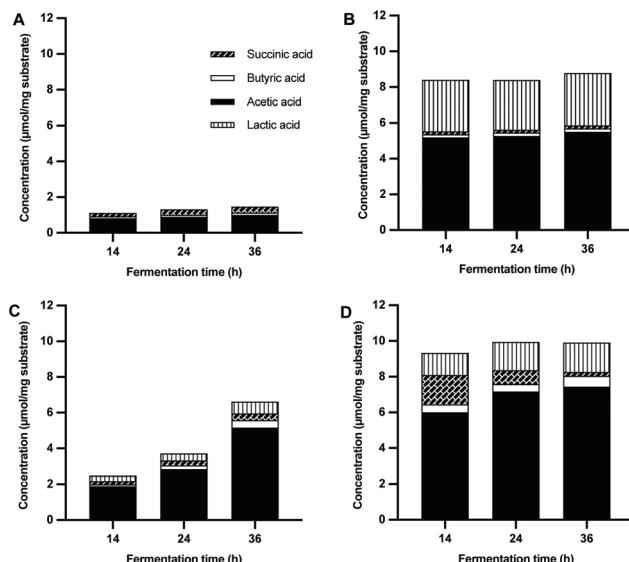


Fig. 5 Short chain fatty acids (acetic acid, butyric acid) and other organic acids (lactic acid, succinic acid) by the fermentation of Control (A), GOS/inulin (B), 3-FL (C), and LNT2 (D) at T14, T24, and T36 with pooled fecal samples of 12-week-old infants.

during the fermentation, *i.e.*, 1.1–1.5  $\mu\text{mol mg}^{-1}$  (Fig. 5A). Propionic acid was undetectable in the fermentation samples (data not shown).

During the fermentation of GOS/inulin, we observed a total SCFAs and organic acid production of 7.3  $\mu\text{mol mg}^{-1}$  at T14, which remained stable throughout the whole fermentation (Fig. 5B). SCFAs formed by GOS/inulin was dominated by acetic acid, with a level of 4.4  $\mu\text{mol mg}^{-1}$  at T14, and remained stable until T36. GOS/inulin fermentation only led to a production of low amounts of butyric acid, with less than 0.1  $\mu\text{mol mg}^{-1}$ . Organic acid of lactic acid, but not succinic acid, was formed during fermentation of GOS/inulin, with a level of 2.9  $\mu\text{mol mg}^{-1}$  at T14, which remained stable until T36.

During the fermentation of 3-FL, only minor amounts of acetic acid and lactic acid were produced (Fig. 5C). At T14, there was only 1.1  $\mu\text{mol mg}^{-1}$  of acetic acid and 0.3  $\mu\text{mol mg}^{-1}$  of lactic acid produced by fermentation of 3-FL. The acetic acid increased to 2.0  $\mu\text{mol mg}^{-1}$  at T24. At this time point, we also observed 0.1  $\mu\text{mol mg}^{-1}$  of butyric acid and 0.4  $\mu\text{mol mg}^{-1}$  of lactic acid, which formed a total SCFAs and organic acid production of 2.4  $\mu\text{mol mg}^{-1}$ . The total SCFAs and organic acid production kept increasing to 5.1  $\mu\text{mol mg}^{-1}$  at T36, with 4.2  $\mu\text{mol mg}^{-1}$  of acetic acid, 0.3  $\mu\text{mol mg}^{-1}$  of butyric acid, and 0.7  $\mu\text{mol mg}^{-1}$  of lactic acid.

Fermentation of LNT2 resulted in a higher production of total SCFAs and organic acids (Fig. 5D). At T14, a level of 8.2  $\mu\text{mol mg}^{-1}$  was reached, which consisted of acetic acid (5.2  $\mu\text{mol mg}^{-1}$ ), butyric acid (0.3  $\mu\text{mol mg}^{-1}$ ), succinic acid (1.5  $\mu\text{mol mg}^{-1}$ ), and lactic acid (1.3  $\mu\text{mol mg}^{-1}$ ). The production of succinic acid was distinct for LNT2, as it was not observed during fermentation of 3-FL. The total SCFAs and



organic acids production was stable throughout the fermentation, but the composition did change. The succinic acid production decreased to  $0.5 \mu\text{mol mg}^{-1}$  at T24, and was non-detectable at T36. At the same time, the production of acetic acid and lactic acid at T24 increased to  $6.3 \mu\text{mol mg}^{-1}$  and  $1.6 \mu\text{mol mg}^{-1}$ . At T36, the production of acetic acid, butyric acid, and lactic acid increased to  $6.4 \mu\text{mol mg}^{-1}$ ,  $0.5 \mu\text{mol mg}^{-1}$ , and  $1.6 \mu\text{mol mg}^{-1}$ , respectively, with a ratio of 75.41 : 5.38 : 19.21.

### 3.6 GOS/inulin and LNT2 fermentation products exposure enhance the adhesion of *L. plantarum* WCFS1 to intestine epithelial Caco-2 cells

To investigate whether the fermentation digesta of GOS/inulin, 3-FL, and LNT2 would have any impact on adhesion of a commensal bacterium to gut epithelial cells, we studied adhesion of *L. plantarum* WCFS1 to Caco-2 cells in the presence of the fermentation digesta. Digesta of the fermentation with only SIEM medium was taken as the control.

Compared to the fermentation products of the control, exposure to the T24 digesta of GOS/inulin significantly increased the adhesion of *L. plantarum* WCFS1, with an increase of 68.7% ( $p < 0.01$ , Fig. 6). Compared to the control, the fermentation digesta of 3-FL did not influence the adhesion of *L. plantarum* WCFS1, but the T14, T24 and T36 digesta of the LNT2 fermentation significantly increased the adhesion of *L. plantarum* WCFS1 to Caco-2 cells, with 52.6% ( $p < 0.05$ ), 87.7% ( $p < 0.01$ ), and 1.0 ( $p < 0.05$ ) fold, respectively (Fig. 6).

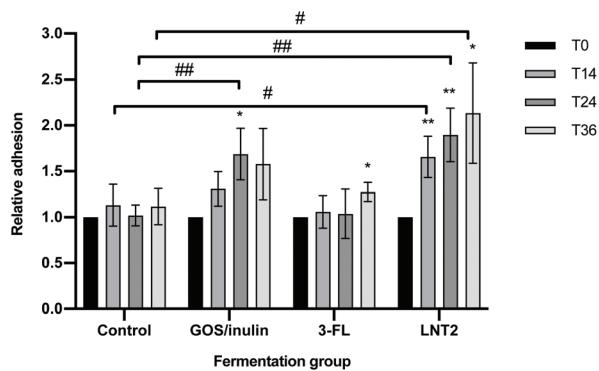
Within the fermentation of different carbohydrates, the effect on *L. plantarum* WCFS1 adhesion changed over time. Exposure to the T14, T24, and T36 digesta of the control fermentation did not influence the adhesion of *L. plantarum* WCFS1 on Caco-2 cells compared to T0 (Fig. 6). However, exposure to the T14 digesta of GOS/inulin showed the ten-

dency to increase *L. plantarum* WCFS1 adhesion by 30.9% ( $p = 0.085$ ) compared to T0. In addition, we found that the T24 digesta of this fermentation significantly increased the adhesion of *L. plantarum* WCFS1 with 68.7% ( $p < 0.05$ ), and the T36 digesta reached a tendency by 57.9% ( $p = 0.108$ ) compared to T0 digesta (Fig. 6). The T36 fermentation digesta of 3-FL also significantly increased the adhesion of *L. plantarum* WCFS1 ( $p < 0.05$ ), compared to the T0 digesta (Fig. 6). The digesta taken at earlier time points during this fermentation did not change adherence. The T14 digesta of LNT2 could also significantly increase the adhesion of *L. plantarum* WCFS1 ( $p < 0.01$ ), and the significant increase was also observed for the T24 digesta ( $p < 0.01$ ) and the T36 digesta ( $p < 0.05$ ), with an increase of 89.6% and 1.1-fold compared to T0 (Fig. 6).

## 4. Discussion

Human milk oligosaccharides (hMOs) and non-digestible carbohydrates (NDCs) in infant formula are considered to guide colonization by the gut microbiota and to support microbial metabolism.<sup>3,14</sup> A well-established gut microbiota is crucial for gastrointestinal health in early life and prevents disease at later age.<sup>2</sup> The NDCs GOS/inulin, and the hMOs 2'-FL and LNnT have been commonly applied in infant formula to mimic the functions of hMOs in mother's milk.<sup>12,33</sup> In recent years, 3-FL and LNT2 have also been proposed for supplementation in infant formula because of the specific benefits they might have.<sup>25</sup> Similar to 2'-FL, 3-FL has lactose as the core structure, and only differs in the fucosylation of fucose to lactose.<sup>26</sup> LNT2 is the acid hydrolysate of LNnT that might be formed *in vivo* to some extent after passage through the stomach.<sup>34</sup> 3-FL and LNT2 have been shown to have even stronger beneficial effects than 2'-FL in many *in vitro* studies, such as on enhancing commensal bacteria adhesion to the gut epithelium,<sup>35</sup> reducing inflammation,<sup>36</sup> and enhancing intestinal epithelial barrier function.<sup>25</sup>

Our data show that 3-FL was not as readily fermented by 12-week-old baby microbiota as LNT2 and GOS/inulin, but resulted in microbiota with a more diverse composition. Besides an increased relative abundance of the *Bifidobacterium* genus of the Actinobacteria phylum, the *Enterococcus* genus of the Firmicutes phylum was also increased compared to the control fermentation. The *Bifidobacterium* genus, dominated by *B. longum* subspecies *longum*, was stable during the full duration of the fermentation study. This may illustrate that *B. longum* subsp. *longum* cannot ferment 3-FL, which was also observed in a recent study.<sup>37</sup> The fermentation of fucosylated hMOs by bifidobacteria is subspecies and strain specific, *i.e.*, they were commonly utilized by *B. longum* subsp. *infantis*, *B. longum* subsp. *bifidum*, and *B. breve* strains,<sup>37-39</sup> while only certain *B. longum* subsp. *longum* strain such as *B. longum* subsp. *longum* SC596 can take 2'-FL and 3-FL as a growth substrate.<sup>40</sup> These strains might be responsible together with other species for the stimulation of the production of acetic acid and lactic acid.<sup>41,42</sup> With longer fermentation time, the



**Fig. 6** Exposure of the fermentation products of GOS/inulin, 3-FL, and LNT2 at T0, T14, T24, and T36 on the adhesion of *Lactobacillus plantarum* WCFS1 to intestine epithelial Caco-2 cells. Control is set without GOS/inulin, 3-FL, or LNT2. Values are shown as the mean  $\pm$  SDs,  $n = 5$ . \* indicates different from T0 in each fermentation group: \* $p < 0.05$ , \*\* $p < 0.01$ ; # indicates different from control at the same fermentation time: # $p < 0.05$ , ## $p < 0.01$ .



relative abundance of *Bacteroides*, which was specifically dominated by *B. dorei*, a species specifically present in vaginally born infants,<sup>43</sup> was increased. The increase of *B. dorei* positively correlates with the degradation of 3-FL and with enhanced production of acetic acid, butyric acid, and lactic acid. This might be attributed to the  $\alpha$ -1-fucosidase produced by both *Bifidobacterium* and *Bacteroides*, which is responsible for the degradation of 3-FL.<sup>44</sup>

The breakdown products produced by *Bifidobacterium* and *Bacteroides* after 3-FL fermentation may cross feed species from *Enterococcus* genus, which in this study was dominated by only one species, *i.e.*, *E. faecalis*. This suggestion is supported by a study by Wang *et al.* who showed that *Enterococcus* is not able to directly degrade hMO, and is dependent on breakdown products from hMO degrading *Bifidobacterium* and *Bacteroides* spp.<sup>45</sup> This cross feeding might contribute to the establishment of an adult-like gut microbiome, which helps infants to get through the transition from breastfeeding to infant formula or solid food.<sup>3,45</sup> The results may imply that the need for 3-FL increases with age during gut microbiota maturation, which is corroborated with the observation that 3-FL increases in mother's milk as lactation progresses.<sup>3</sup>

LNT2 was more readily fermented than 3-FL and induced a similar composition of acetic acid as induced by GOS/inulin after 14 h. At this time point, we also observed a high production of succinic acid and butyric acid besides the production of acetic acid and lactic acid, as observed during fermentation of GOS/inulin and 3-FL. There is only limited research available on the degradation of LNT2. Recently, a *Bifidobacterium* strain, *i.e.*, *B. longum* subsp. *infantis* belonging to the phylum of Actinobacteria was reported to grow on LNT2, resulting in the production of acetic acid,<sup>37</sup> which corroborates our observation that fermentation of LNT2 induces a high relative abundance of species belonging to the *Bifidobacterium* genus, as well as continuous production of acetic acid. However, we only observed enhanced enrichment of *B. longum* subsp. *longum* in this study, and a high relative abundance of unidentified *Bifidobacterium* species. Studies towards LNT2 fermentation and impact on microbiota are urgently needed as it is formed when LNnT, which is already applied in infant formula, passes the stomach.

The production of succinic acid during the fermentation of LNT2 may suggest a potential anti-inflammatory effect of LNT2.<sup>25</sup> Succinic acid formation is associated with an increased number of intestinal tuft cells, which can trigger anti-inflammatory immune responses.<sup>46</sup> Succinate was gradually consumed at later stages of fermentation, and correlated with an increased abundance of *Collinsella*.<sup>47</sup> A high abundance of *Collinsella* was also found in the T0 samples. *Collinsella* has been reported to be a commensal with ability to produce butyrate,<sup>48</sup> which is the most bioactive SCFA in controlling inflammatory responses.<sup>22</sup> LNT2 might also confer the anti-inflammatory effects through lowering the abundance of *Escherichia-Shigella*, a genus that is closely linked to acute necrotizing pancreatitis and other systemic inflammation.<sup>49</sup> Thus, the addition of LNT2 to infant formula might benefi-

cially influence microbiota compositions and strengthen the immune system.

Fermentation of GOS/inulin was more complex. We found that GOS was completely fermented after 14 h, while for inulin, DP8-DP14 was predominantly used by the fecal microbiota of 12-week-old infants. It supported the growth of *Bifidobacterium*, which is in line with previous findings.<sup>5,31</sup> *Bifidobacterium* is a signature organism of the gut microbiota in early life of breastfed infants. It is considered to provide substantial health benefits, which is the reason why enhancement of *Bifidobacterium* is a hallmark for efficacy of NDCs and hMOs in infant formula.<sup>31</sup> Our results confirm a bifidogenic effect of GOS/inulin,<sup>12</sup> as well as of 3-FL and LNT2, which all stimulated *B. longum* subsp. *longum*. *B. longum* subspecies express enzymes including  $\alpha$ -fucosidases,  $\beta$ -galactosidases, and  $\beta$ -hexosaminidase. These enzymes are essential for the utilization of hMOs and NDCs.<sup>50</sup>  $\beta$ -Galactosidases can degrade the  $\beta$ 1-4 linkages within GOS structures, leading to the formation of acetic acid and lactic acid during the subsequent fermentation.<sup>31,50</sup> Acetic acid is the most abundant SCFA and may serve as the intermediate product for the synthesis of butyrate.<sup>51</sup> Together with lactic acid, butyrate will promote a lower pH environment and support the growth of *Bifidobacterium*.<sup>52</sup> The production of acetic acid and lactic acid is also important for inhibition of, *e.g.*, *E. coli* infections and for improving gut barrier function.<sup>53,54</sup> We found experimental proof as we observed a reduction in the relative abundance of *Escherichia coli* belonging to the *Escherichia-Shigella* genus. The delayed degradation of inulin may have contributed to a continuous level of acetic acid and lactic acid. Inulin needs a longer time to be degraded and may stay in the colon for an average duration of 30–40 h,<sup>55</sup> and serve as a stock to guarantee a continuously supply of dietary fibers for the gut microbiota.

Not much is known about how the fermentation digesta of hMOs, and NDCs contribute to adhesion and colonization of commensal bacteria to the gut epithelium, which is essential to confer health effects.<sup>56</sup> We demonstrated that the fermentation digesta of the different tested NDCs could modulate the adhesion of the commensal bacterium *L. plantarum* WCFS1 to intestine epithelial Caco-2 cells, but that the efficacy is highly dependent on the fermented hMO or GOS/inulin. Compared to the control, the fermentation digesta of LNT2 at T14 could already significantly increase the adhesion of *L. plantarum* WCFS1 to Caco-2 cells, which might be explained by the high concentration of different SCFAs and other organic acids. The T24 and T36 fermentation digesta of LNT2 also increased the adhesion of *L. plantarum* WCFS1 to Caco-2 cells, but this seems to correlate with the continuous production of SCFAs and other organic acids during the fermentation of LNT2. The fermentation digesta of 3-FL did not increase the adhesion of *L. plantarum* WCFS1. This could possibly be explained by the relatively low fermentability, and consequently lower production of SCFAs and other organic acids. The fermentation digesta of the reference, GOS/inulin at T24, increased the adhesion of *L. plantarum* WCFS1 to Caco-2 cells.



The enhanced adhesion of commensal bacterium *L. plantarum* WCFS1 by the fermentation products of LNT2 and GOS/inulin might be induced in multiple ways. It is possible that SCFAs, lactic acid, and succinic acid formed during the fermentation increased the expression of adhesion molecules, such as lectin-binding adhesin or glyceraldehyde-3-phosphate dehydrogenase, on the bacteria cell membrane.<sup>56,57</sup> It is also possible that the SCFAs, lactic acid, and succinic acid modulate the glycosylation of the intestine epithelial cells to support bacteria adhesion.<sup>58</sup> The enhanced commensal bacteria adhesion to the intestinal epithelial cells is important for many processes, but also *eg.* to exclude pathogens adhesion and infection.<sup>59,60</sup>

## 5. Conclusions

In summary, we show here that the hMO molecules 3-FL and LNT2 were fermented by fecal microbiota of 12-week-old infants. However, the degradation pattern, gut microbiota changes, and SCFAs production were hMO structure specific. Especially, LNT2 was rapidly utilized, and increased the relative abundance of *Bifidobacterium* and *Collinsella*, as well as the production of SCFAs. The LNT2 fermentation digesta significantly enhanced the adhesion of the commensal bacterium *L. plantarum* WCFS1 to intestinal epithelial cells. In addition, fermentation digesta of GOS/inulin enhanced adhesion of *L. plantarum* WCFS1. 3-FL was gradually degraded, and specifically increased bacteria of *Bacteroides* and *Enterococcus* genus. Our findings demonstrate positive effects of 3-FL and LNT2 on gut microbiome microbial metabolic products. This knowledge may contribute to the design of tailored infant formula containing specific hMO molecules to meet the healthy development of infants during the transition from breastfeeding to formula or solid food feeding.

## Author contributions

C. K., R. A., and P. d. V. conceived and designed the experiments. C. K., R. A., C. E. K., M. B., and M. M. P. O. performed the experiments. C. K., C. E. K., and M. B. analyzed data. C. K., H. A. S., and P. d. V. wrote the paper.

## Conflicts of interest

There are no conflicts of interest to declare.

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