Supplementation of fructooligosaccharides to suckling piglets affects intestinal microbiota colonization and immune development[1]

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ABSTRACT: Emerging knowledge shows the importance of early life events in programming the intestinal mucosal immune system and development of the intestinal barrier function. These processes depend heavily on close interactions between gut microbiota and host cells in the intestinal mucosa. In turn, development of the intestinal microbiota is largely dependent on available nutrients required for the specific microbial community structures to expand. It is currently not known what the specificities are of intestinal microbial community structures in relation to the programming of the intestinal mucosal immune system and development of the intestinal barrier function. The objective of the present study was to investigate the effects of a nutritional intervention on intestinal development of suckling piglets by daily oral administration of fructooligosaccharides (**FOS**) over a period of 12 d (days 2–14 of age). At the microbiota community level, a clear "bifidogenic" effect of the FOS administration was observed in the colon digesta at day 14. The former, however, did not translate into significant changes of local gene expression in the colonic mucosa. In the jejunum, significant changes were observed for microbiota composition at day 14, and microbiota diversity at day 25. In addition, significant differentially expressed gene sets in mucosal tissues of the jejunum were identified at both days 14 and 25 of age. At the age of 14 d, a lower activity of cell cycle–related processes and a higher activity of extracellular matrix processes were observed in the jejunal mucosa of piglets supplemented with FOS compared with control piglets. At day 25, the lower activity of immune-related processes in jejunal tissue was seen in piglets supplemented with FOS. Villi height and crypt depth in the jejunum were significantly different at day 25 between the experimental and control groups, where piglets supplemented with FOS had greater villi and deeper crypts. We conclude that oral FOS administration during the early suckling period of piglets had significant bifidogenic effects on the microbiota in the colon and on gene expression in the jejunal mucosa by thus far unknown mechanisms.

Key words: development, early life, immune system, intestinal microbiota, nutrition, pigs

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INTRODUCTION

Emerging knowledge shows the importance of early life conditions, including nutrition, in the development and programming of the gut. It has been shown in multiple species that early life conditions affect microbial colonization in the gut and local immune system and barrier function development (Mulder et al., 2011; Arrieta et al., 2014; Francino, 2014; Schokker et al., 2015a; Gomez de Aguero et al., 2016). It might therefore be possible to influence the development and programming of the gut by nutritional factors that affect the early life microbial community structures, as has been demonstrated in young infants (Rijkers et al., 2010; Jacobi and Odle, 2012). In humans, it is widely appreciated that oligosaccharides (**OS**) exert beneficial health effects, by increasing the number of lactobacilli and bifidobacteria and subsequently higher butyrate production in the gut. The underlying mechanisms, however, are not yet fully elucidated. In pigs, it has been shown that fructooligosaccharides (**FOS**) supplementation affected the intestinal microbiota composition (Shim et al., 2005a), or the immune development in suckling piglets in a beneficial way (Le Bourgot et al., 2014), and induced an increased immune response to influenza vaccination when fed directly to post weaning piglets (Le Bourgot et al., 2016). The objective of the present study was to investigate the effects of administrating orally FOS on the gut microbiota colonization and gut development in suckling piglets. As our prime interest was on effects on small intestinal development, including morphology, and on metabolic and immunological processes in gut tissue, as well as on the cross-talk between microbiota and immune cells, which mainly occurs in the small intestine, in-depth analyses were performed on intestinal microbiota and intestinal tissue (transcriptomics and morphology). Because studies with FOS in adult animals showed changes in the microbiota composition of the colon, we also analyzed colonic digesta (microbiota) and colon tissue (transcriptomics). Here, we present results that contribute to the mode of action of dietary supplementation of FOS on the gut system.

MATERIALS AND METHODS

Ethics Statement

This animal experiment was approved by the institutional animal experiment committee "Dier Experimenten Commissie (DEC) Lelystad" (accession number 2013108.d), in accordance with the Dutch regulations on animal experiments.

Housing and Diet

Four Topigs20 sows of third and fourth parity were used. From gestation day 109 until weaning sows were individually housed in conventional farrowing rooms. During this period, water was ad libitum available. Sows were fed a conventional commercial diet and each sow had a customized feeding schedule, based on their BW, back fat thickness, and parity number. All sows had at least 14 piglets born alive in their litter. Size of the litters was limited to 14 piglets by cross fostering piglets to other sows. Piglets were not given access to creep feed or a milk replacer during the lactation period.

The dietary intervention was administered from days 2 to 14 of age, during which piglets in each litter got twice a day oral administration of either 15-mL water (control; $n = 8$) or 15 mL of a solution containing 5 g FOX ($n = 6$) by oral gavage. Due to observed incidental vomiting of the piglets in the first week, the total volume of oral administration was changed from day 7 onwards from 15 to 6 mL, but the dosage of FOS was still 5 g.

The FOS preparation used for this experiment was Frutafit TEX! (lcFOS) and Frutalose OFP (scFOS) from SENSUS. Both scFOS and lcFOS are from chicory and a polydispersed mixture of linear fructose polymers with mostly a terminal glucose unit, coupled by means of β(2-1) bonds. The number of units (degree of polymerization) varies between 2–60 (lcFOS) and 3–10 (scFOS). The mixture used in this study was based upon the recommendation for use in babies (Boehm et al., 2003), being 9:1 (wt/wt) for scFOS and lcFOS.

Sampling

On days 2, 14, and 25 of age, piglets were killed to extract intestinal tissue samples and intestinal digesta. At day 2 after parturition, 2 piglets per litter (1 control and 1 intervention, note that the supplementation with FOS has not yet started) were euthanized, and at days 14 and 25 after parturition, 4 piglets per litter (2 control and 2 intervention per time point) were euthanized. Before the start of the intervention, each piglet in a litter was ranked based on their order in birth weight, the heaviest being number 1, and the lightest being number 14. Based upon this ranking, all piglets per litter were allocated into either the control or the intervention treatment and allocated to the day of

dissection (Supplementary Table S1). Besides BW, the following biological samples were extracted: approximately 2 cm of jejunal tissue for histological measurements, as well as mucosal scrapings from the mid-jejunum and mid-colon for transcriptomics analyses, and digesta from the jejunum and colon for microbiota analyses.

Microbial DNA Extraction

Jejunal and colon digesta were snap frozen in liquid nitrogen and stored at −80 °C. For the microbial DNA extraction, the following protocol was used. Jejunal digesta was mixed 1:1 with PBS and vortexed; subsequently, it is centrifuged for 5 min (300 × *g*) at 4 °C. The supernatant was transferred to a new tube and spun for 10 min (9,000 × *g*) at 4 °C, and thereafter supernatant was removed. Deoxyribonucleic acid was extracted by using the QIAamp DNA Stool Mini Kit protocol as described by the manufacturer. The samples were eluted in 100 µL of the (provided) elute buffer and afterwards an optical density measurement to check the quality was performed on the Nanodrop (Agilent Technologies).

Microbiota Sequence Analysis and Bioinformatics (QIIME)

A PCR was used to amplify the 16S rDNA V3 fragment using forward primer V3_F (CCTACGGGAGGCAGCAG) and reverse primer V3_R (ATTACCGCGGCTGCTGG). Polymerase chain reaction conditions were as follows: 2 min at 98 °C, 15× (10 s at 98 °C, 30 s at 55 °C, and 10 s at 72 °C), and 7 min at 72 °C. Polymerase chain reaction efficiency was checked on agarose gel by visual inspection. Samples were sequenced by targeted-amplicon 16S sequencing using the MiSeq sequencer (Illumina) and analyzed for taxonomy profile per sample with clustering by profile by using QIIME (Caporaso et al., 2010). Standard assembly based on amplicon, with primer removal, was performed. For quality filtration, the following settings were used: 1) >Q20 and 2) amplicons >100 bases. For the data analysis, pseudoreads were clustered into operational taxonomic units (OTUs) per sample at 97% similarity and OTU-representative sequences were aligned against the aligned Greengenes core set (13_8 release; DeSantis et al., 2006; McDonald et al., 2012). Furthermore, chimeras were removed with Chimeraslayer (Haas et al., 2011).

Microbiota Statistical Analysis

Different statistical approaches were performed to identify differences between the intervention and control groups, similar to previous studies in piglets (Han et al., 2016; Fan et al., 2017). The microbial biodiversity was calculated by the *vegan* package (http://cran.r-project.org/web/packages/vegan/, accessed May 2, 2018) within the R environment, by using the Shannon diversity index. The redundancy analysis (**RDA**) was also performed by using the *vegan* package; the following model was run on the family level microbiota data: y = time + treatment + time × treatment + error. Furthermore, statistical significance testing for over- and under-representation of the bacterial groups was made at the family level by performing the Wilcoxon signed-rank test, and *P*-values were converted to false discovery rate (**FDR**) values to correct for multiple testing. All calculations were performed by using the relative abundance of microbiota groups.

Host RNA Extraction from Tissue

Total RNA was extracted from 50- to 100-mg jejunum or colon tissue. All samples were homogenized using the TisuPrep Homogenizer Omni TP (TH220P) in TRizol reagent (Life Technologies) as recommended by the manufacturer with minor modifications. The homogenized tissue samples were dissolved in 5 mL of TRizol reagent. After centrifugation, the supernatant was transferred to a fresh tube. Subsequently, Direct-zol RNA MiniPrep Kit by Zymo Research was used as described by the manufacturer. The RNA was quantified by absorbance measurements at 260 nm on the Nanodrop (Agilent Technologies). Quality Control was performed by using the Bioanalyzer in combination with the RNA 6000 Nano Kit, for each sample the RNA Integritry Number (RIN) was defined and assessed.

Labeling, Hybridization, Scanning, and Feature Extraction of Microarrays

Labeling of RNA was done as recommended by Agilent Technologies using the One-Color Microarray-Based Gene Expression Analysis Low input Quick Amp Labelling. The input was 10 ng of total RNA, and 600 ng of labeled complementary RNA was used on the 8-pack array. Hybridization was performed as described in the One-Color Microarray-Based Gene Expression Analysis Low input Quick Amp Labelling protocol from Agilent in the hybridization oven (G2545A hybridization Oven Agilent Technologies). The hybridization temperature is 65 °C with rotation speed 10 rpm for 17 h. After 17 h, the arrays were

washed as described in the One-Color Microarray-Based Gene Expression Analysis Low input Quick Amp Labelling protocol from Agilent. The arrays were scanned using the DNA microarray scanner with SureScan High-Resolution Technology from Agilent Technologies. Agilent Scan Control with resolution of 5 µm, 16 bits, and PMT of 100%. Feature extraction was performed using protocol 10.7.3.1 (version 10.7) for 1-color gene expression.

Transcriptomic Data Analysis

The data were analyzed by using R (version 3.0.2) by executing different packages, including LIMMA (Smyth, 2005) and arrayQualityMetrics (Kauffmann et al., 2009). The data were read in and background corrected (method = "normexp" and offset = 1) with functions from the R package LIMMA (Smyth, 2005) from Bioconductor (Gentleman et al., 2004). Quantile normalization of the data was performed between arrays. The duplicate probes mapping to the same gene were averaged (*avereps*), and subsequently, the lower percentile of probes were removed in a 3-step procedure: 1) get the highest of the dark spots to get a base value, 2) multiply by 1.1, and 3) the gene/probe must be expressed in each of the samples in the experimental condition.

Statistical and Functional Genomics Analysis

To evaluate the differences in results regarding the gene expression between the experimental groups (control and FOS) on both days 14 and 25, the following contrasts were generated: day 14 piglets supplemented with FOS vs. day 14 control piglets and day 25 piglets supplemented with FOS vs. day 25 control piglets, within the LIMMA package (Smyth, 2005). In addition, gene set enrichment analysis (**GSEA**) was also performed for the former contrasts (Subramanian et al., 2005).

Histological Tissue Handling

Tissue samples of the jejunum (n = 40 piglets), which were preserved in ethyl alcohol (**EtOH**) 70%, were cut to approximately 1.2 cm and subsequently transferred to the Leica tissue processor, where the following protocol was used: Step 1) EtOH 70% for 240 s, 2) EtOH 80% for 90 s, 3) EtOH 90% for 60 s, 4) EtOH 96% for 45 s, 5) 2× EtOH 100% for 30 s, 6) 3× xylene 100% for 60 s, and 7) 2× paraffin 100% for 60 s. Thereafter, the paraffin-embedded tissues were sectioned in coupes of 5 µm and transferred to coated Superfrost slides (coated with glycerin). The slides were kept overnight in an oven at 38 °C followed by dewaxing and staining with the Micron staining machine by using the Crossman procedure. The dewaxed and stained slides were mounted in DePeX and subsequently these were stored overnight in an oven at 38 °C. After drying in the oven, the villi height and crypts depth were measured. These measurements were performed by using analySIS^D (FIVE) software via a Nikon Microphot_FXA microscope with an Olympus DP50 video camera. In total, per piglet the height of 10 villi and their corresponding crypts depth per slide were measured.

Histological Statistical Analysis

Data on villus height and crypt depth of the jejunum were analyzed by using IBM SPSS Statistics 20. The statistical analysis was carried out on the mean value for each individual piglet for either villi height or crypt depth, that is, the mean of the 10 measurements.

RESULTS

Performance

The average BW at weaning (day 26) of was 7.0 kg for control piglets compared with 7.7 kg for piglets supplemented with FOS. There was no significant effect of treatment on the ADG (days 0–2, 2–7, 7–14, and 14–21; Supplementary Figure S1 [see online supplementary material for a color version of this figure]) or average BW (days 0, 3, 7, 14, 21, and 27; Supplementary Figure S2). However, the average BW of piglets supplemented with FOS was numerically greater at each time point (days 7, 14, 21, and 27).

Colon

Microbiota. To investigate whether the intervention with FOS had the anticipated effect of stimulating the growth of butyrate producing bacteria in the colon, the microbiota composition, diversity, and top lists of microbiota at the genus/species level were analyzed. The microbiota composition of colon digesta at day 14 of age differed (P-value is 0.01) between piglets supplemented with FOS and control piglets (Figure 1). However, no bacterial groups at the genus level could be identified that differed significantly. However, by targeting specifically on the Bifidobacteriaceae other ($P < 0.001$), *Lactobacilli* ($P = 0.05$),

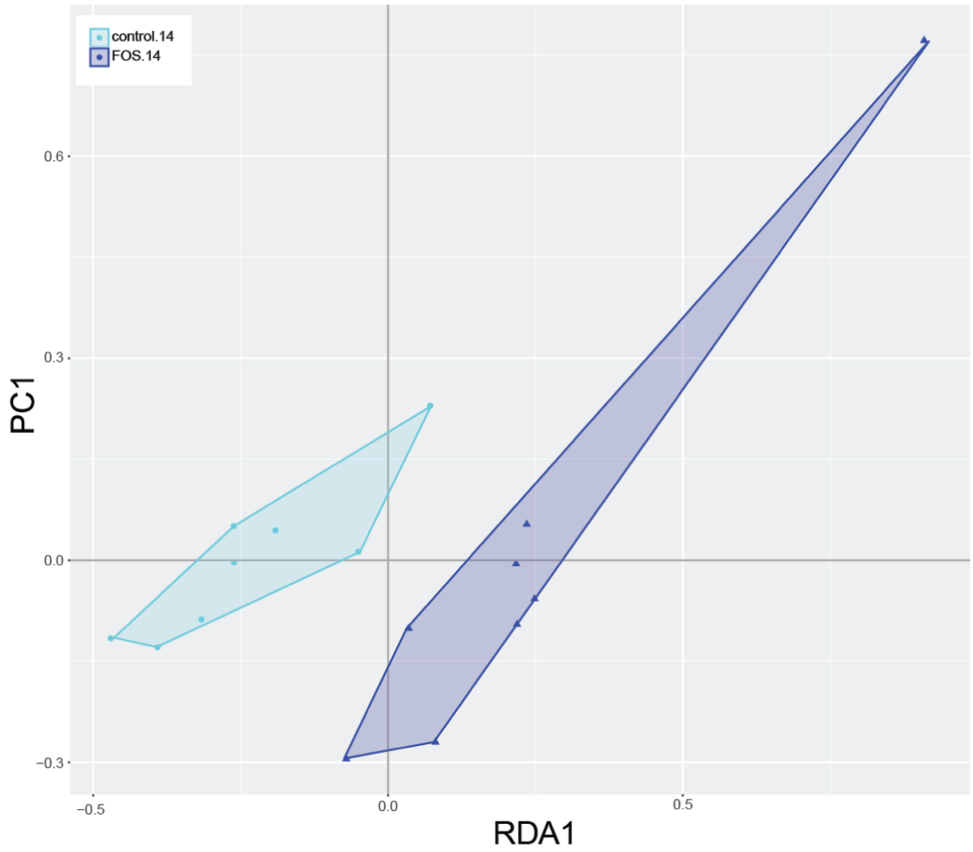

Figure 1. Redundancy analysis (RDA) of family-level microbiota in the pig colon. The x-axis depicts explanatory axis 1 (RDA1) and the y-axis depicts explanatory axis 2 (principal component 1 [PC1]). Each condition is represented by a different color, that is, day 14 controls are cyan (n = 8) and piglets supplemented with fructooligosaccharides are blue (n = 8). The following model was used as an input for the RDA, y = time + treatment + time × treatment + error (P = 0.01 by permutation test). Control.14 = day 14 control piglets; FOS.14 = day 14 piglets supplemented with fructooligosaccharides.

Bifidobacterium (P = 0.08), and Lactobacillaceae other (P = 0.11), an effect of FOS supplementation was observed (Figure 2). The microbiota diversity, as measured by the Shannon index, was not significantly different between piglets supplemented with FOS (3.16 [SEM 0.06]) and control piglets (3.12 [SEM 0.06]).

For each experimental group, top-10 lists of most abundant bacterial groups were generated (Table 1; including genus and/or species name). The greatest average relative contribution (**ARC**) of microbiota in control pigs were *Bacteroidia_Other*, *Bacteroidia*, and *Bacteroidia S24-7*. In piglets supplemented with FOS, the greatest change in ARC was observed for the species *Bacteroidia S24-7* followed by *Lactobacillus reuteri* and *Prevotella stercorea*.

Colonic gene expression. We investigated the overall gene expression patterns of all colonic mucosal samples at day 14. Principal component analysis did not reveal a clear separation of treatments (Figure 3). Also LIMMA statistical testing did not result in any significant differentially expressed genes between both treatments. Furthermore, GSEA was performed on the colonic data sets to test for differences in the expression of gene sets, including metabolic, cell cycle, and immunological processes, between control and piglets supplemented with FOS. This GSEA approach did not result in any differentially expressed gene sets (FDR < 1%) in colon tissue.

Jejunum

Microbiota. We investigated whether FOS supplementation had an effect on the microbiota composition in the jejunum, for which we analyzed luminal microbiota at days 2, 14, and 25 in both control piglets and piglets supplemented with FOS. An overview of the ARC of microbial species is given in Table 2. The data show that *Lactobacilli* were the most abundant throughout the suckling period. At day 2, the ARC of *Escherichia coli* was up to 9% in the jejunum, which decreased in the controls at days 14 and 25 to 1.9 and 2.5%, respectively. In piglets supplemented with FOS, the abundance of *E. coli* was relatively high: 8.0% at day 14 and

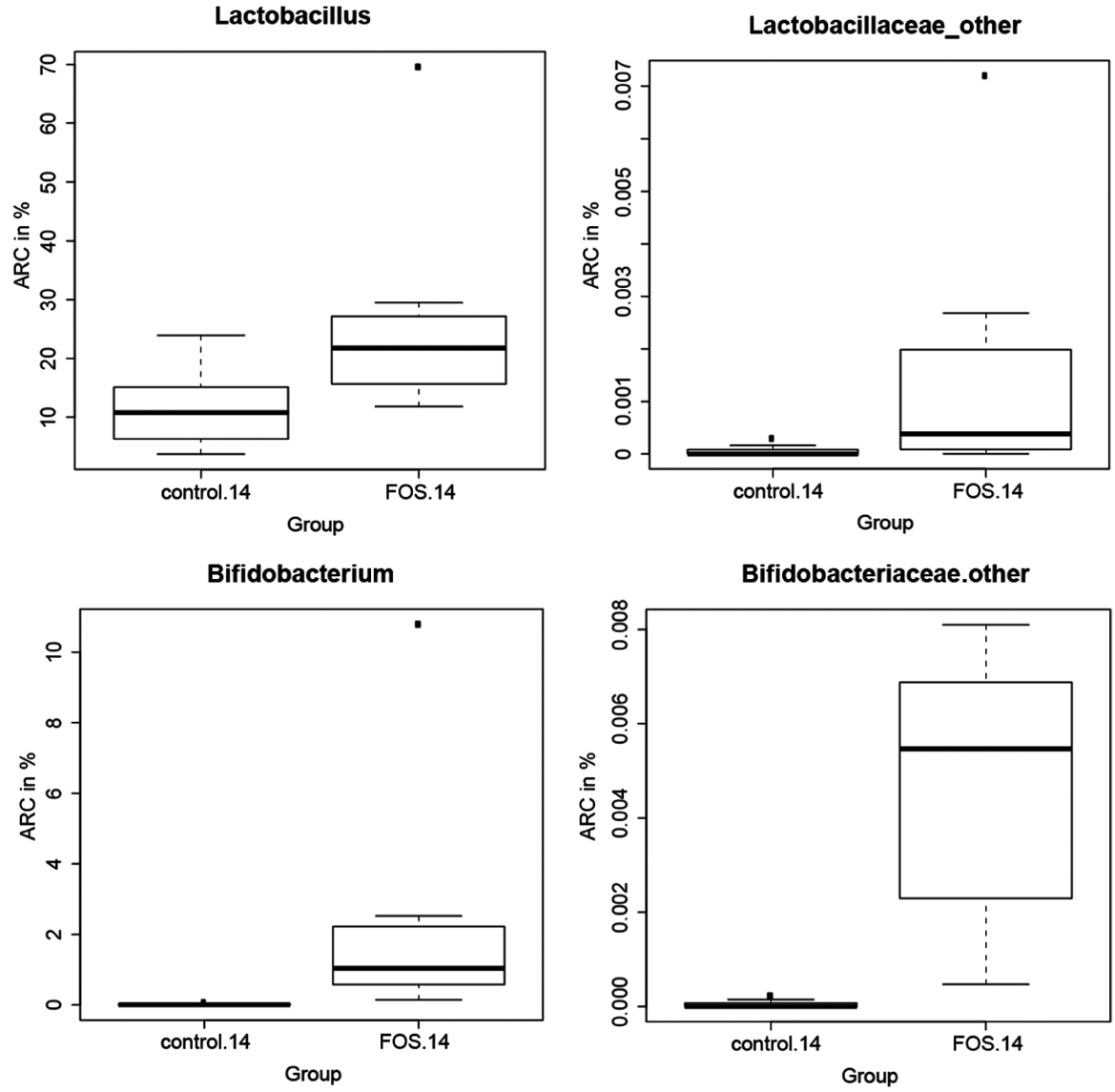

Figure 2. Bacterial species that exert a "bifidogenic effect" in colon digesta at day 14. The x-axis depicts control piglets (n = 8) or piglets supplemented with fructooligosaccharides (FOS) (n = 8) at day 14, whereas the y-axis depicts the average relative contribution (ARC) in percentage. Top left: *Lactobacilli* (P = 0.05); top right: Lactobacillaceae other (P = 0.11); bottom left: *Bifidobacterium* (P = 0.08); bottom right: Bifidobacteriaceae other (P < 0.001). P-values were calculated by a Student's t-test. Control.14 = day 14 control piglets; FOS.14 = day 14 piglets supplemented with fructooligosaccharides.

5.9% at day 25. At days 14 and 25, *Turicibacter* was also dominant in piglets supplemented with FOS, with values ranging from 5.6% to 8.6%. In time, the average microbial diversity, as measured by the Shannon index, increases from approximately 2.14 to 2.45 in piglets supplemented with FOS, whereas control piglets show a slight decrease from 2.14 to 1.92 (Table 3). To further evaluate the microbiota composition in the jejunal digesta, a RDA was performed, which showed a high overlap of all groups, both with respect to days (2, 14, and 25) and treatment (piglets supplemented with FOS vs. control piglets; Figure 4). Multiple samples in both FOS-supplemented piglets at days 14 and 25 in the RDA plot of Figure 4, however, shifted away from the respective controls.

Jejunal gene expression. First, we investigated the overall gene expression of all samples at all time points together (days 2, 14, and 25). This resulted in a clear separation in time of the samples (Figure 5). However, piglets supplemented with FOS did not significantly differ from the control group at either time point, but only showed a trend for a difference on day 14. The second step was to identify differentially expressed genes and/or processes between the piglets supplemented with FOS and control piglets. Statistical analyses did not result in the identification of differentially expressed genes. However, GSEA, including metabolic, cell cycle, and immunological processes, of the day 14 samples resulted in 26 significantly depleted gene sets (FDR < 1%) and nine significantly enriched gene sets for the

Table 1. Overview of the most abundant microbiota genus/species (%) in colon digesta at day 14 in control and fructooligosaccharide (FOS)-supplemented piglets

Taxon		Treatment[1,2]	
Genus	Species	Control.14	FOS.14
Bacteroidia		**11.9**	**3.9**
[*Prevotella*]		**3.3**	0.2
Bacteroides		**4.2**	1.8
Bacteroidia p-2534-18B5		**3.3**	0.3
Prevotella		0.4	**2.6**
Prevotella	*stercorea*	**4.7**	**6.9**
Bacteroidia S24-7		**10.1**	**8.3**
Bacteroidia, other		**12.5**	**6.0**
Lactobacillus	other	3.0	**5.8**
Lactobacillus		**3.2**	**6.4**
Lactobacillus	*agilis*	0.0	**2.6**
Lactobacillus	*reuteri*	**5.2**	**8.1**
Megasphaera		0.1	**4.7**
Sphaerochaeta		**3.1**	0.3
Other bacteria		35.0	42.1

[1]Average relative contribution (%). Control.14 = day 14 control piglets (n = 8); FOS.14 = day 14 piglets supplemented with FOS (n = 8).

[2]Bold depicts the top 10 most abundant species/genus per treatment.

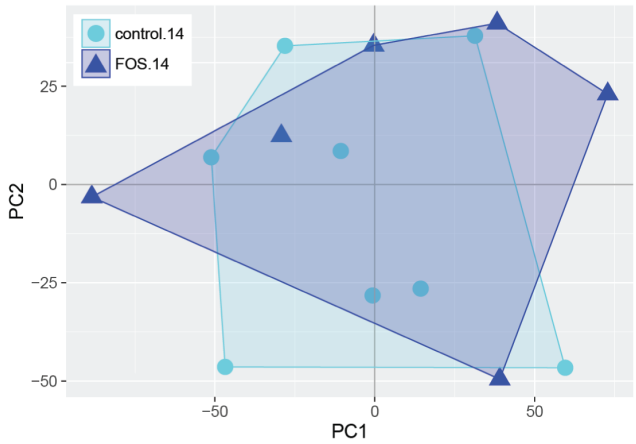

Figure 3. Principal component analysis of colon transcriptomics data at day 14. The x-axis depicts principal component 1 (PC1) and y-axis depicts principal component 2 (PC2). Fructooligosaccharide treatments are filled blue circles (n = 6), whereas the controls are cyan triangles (n = 8). Control.14 = day 14 control piglets; FOS.14 = day 14 piglets supplemented with fructooligosaccharides.

FOS treatment compared with control (Table 4). At day 25, 8 significantly depleted gene sets were observed and 1 enriched gene set for the FOS treatment compared with the control (Table 5). Gene sets are predefined sets of genes with a common denominator, including, for example, "cell cycle," "receptor activity," or "locomotory behavior." The depleted gene sets in the piglets supplemented with FOS at day 14 were all associated with cell cycle–related processes, whereas piglets supplemented with FOS showed enrichment of processes related to the extracellular matrix (**ECM**). This means that genes belonging to "cell cycle" were lower expressed in piglets supplemented with FOS compared with control piglets, whereas higher expression of genes involved in ECM was seen in piglets supplemented with FOS. At day 25, 3 out of the 8 significantly depleted gene sets were involved in chemokine/cytokine signaling and thus were lower expressed in piglets supplemented with FOS compared with control piglets. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE101147 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE101147, accessed May 2, 2018).

Histology. We investigated whether FOS supplementation had an effect on the morphology in the small intestine, that is, the jejunum. Villus height, crypt depth, and villus to crypt ratio were measured (Table 6). No significant differences were observed when comparing piglets supplemented with FOS with the respective controls. However, a trend was observed at day 25, where piglets supplemented with FOS had deeper crypts compared with control piglets (P = 0.06). Piglets supplemented with FOS had numerically greater villi and deeper crypts.

DISCUSSION

Mother's milk contains important nutritional factors for newborns, contributing to the energy requirement of the infant as well as providing adequate protection against harmful invaders (Kunz et al., 2000; Newburg, 2009). Components of mother's milk and/or colostrum were identified, especially OS, which specifically induce the expansion of specific bacterial species (Kunz and Rudloff, 1993; Verdonk et al., 2005; Penders et al., 2006; Boehm and Stahl, 2007; Boehm and Moro, 2008; Adlerberth and Wold, 2009; Bertino et al., 2012; Fernandez et al., 2013). It has been shown that short-chain (**sc**) FOS can be utilized by many different *Bifidobacterium* species (Pokusaeva et al., 2011). Long-chain (**lc**) FOS can be utilized by specific *Bifidobacterium* species, but only when degradation is initiated by other species of the microbial community in the gut (Biedrzycka and Bielecka, 2004; Meyer and Stasse-Wolthuis, 2009). For human neonates, the supplementation of formula milk with a combination of sc and lc OS showed an increase of the number of *Bifidobacteria* in colon digesta and feces, approximating the colonic

Table 2. Top 10 abundant microbiota genus/species in jejunal digesta at days 2, 14, and 25 for control piglets and piglets supplemented with fructooligosaccharides (FOS)

Taxon		Treatment[1,2]				
Genus	Species	Control.02	Control.14	FOS.14	Control.25	FOS.25
Lactobacillus		**33.1**	**35.1**	**29.1**	**25.4**	**17.9**
Lactobacillus	*reuteri*	**25.0**	**23.7**	**22.3**	**12.9**	**7.3**
Lactobacillus	*salivarius*	0.2	**1.8**	1.1	3.0	3.9
Streptococcus	other	**2.8**	0.9	1.2	**5.1**	**7.1**
Streptococcus		**2.5**	0.1	0.4	**4.5**	**5.7**
Streptococcus	*luteciae*	2.2	1.1	**2.6**	2.3	2.3
Turicibacter		**2.6**	**5.8**	**6.8**	**5.6**	**8.6**
Clostridium	*perfringens*	**2.3**	0.4	0.2	0.1	0.3
Veillonella	other	**4.4**	**2.8**	**2.0**	**3.2**	1.6
Escherichia	*coli*	**9.0**	**1.9**	**8.0**	2.5	**5.9**
Actinobacillus	other	1.2	**2.7**	**2.8**	**4.8**	**4.0**
Actinobacillus		2.2	**2.5**	**2.7**	**4.1**	**4.0**
Pasteurellaceae, other		**2.3**	**5.6**	**5.5**	**5.9**	3.6
Bacteria, other		1.2	1.5	1.4	1.5	**4.0**
Unclassified, other		**3.1**	**6.2**	**6.2**	**4.4**	**8.3**
Other bacteria		5.9	7.9	7.7	14.7	15.5

[1]Average relative contribution per group. Control.02 = day 2 control piglets (n = 8); Control.14 = day 14 control piglets (n = 8); FOS.14 = day 14 piglets supplemented with FOS (n = 8); Control.25 = day 25 control piglets (n = 8); FOS.25 = day 25 piglets supplemented with FOS (n = 8).

[2]Bold depicts the top 10 per treatment.

Table 3. Microbial diversity in jejunal digesta for piglets in both experimental treatments at different time points

Treatment[1]	Diversity[2]	P-value[3]
Control.02	2.14 (0.17)	
Control.14	2.28 (0.21)	0.98
FOS.14	2.29 (0.14)	
Control.25	1.92 (0.09)[a]	<0.001
FOS.25	2.45 (0.09)[b]	

[1]Control.02 = day 2 control piglets (n = 8); Control.14 = day 14 control piglets (n = 8); FOS.14 = day 14 piglets supplemented with fructooligosaccharides (n = 8); Control.25 = day 25 control piglets (n = 8); FOS.25 = day 25 piglets supplemented with fructooligosaccharides (n = 8).

[2]Calculated by the Shannon index. SEM in parentheses.

[3]Student's t-test comparing piglets supplemented with fructooligosaccharides vs. control piglets at a specific time point.

microbial composition in breast-fed babies (Boehm et al., 2003, 2005; Haarman and Knol, 2005; Sela and Mills, 2010). These data show that OS can be metabolized by different *Bifidobacterium* strains and can represent potential candidates to act as prebiotics in neonates. Increase in the number of *Lactobacilli* and *Bifidobacteria*, the so-called bifidogenic effect, leads to a higher synthesis of butyrate. Butyrate is a preferred energy substrate for colonocytes and promotes cell differentiation and proliferation (Blottiere et al., 1999). Furthermore, these short-chain fatty acids (**SCFA**) regulate sodium and water absorption (Ruppin et al., 1980), enhance calcium and mineral absorption (Scholz-Ahrens et al., 2001), and lower the pH in the colon. A low pH is important for inhibition of the growth of pathobionts and also stimulates the growth of butyrate producers. In addition to these differences in regulation of absorption and lowering of the pH, immunological effects of increased butyrate production have also been observed in different studies, ranging from an increase in regulator T cells locally in colonic tissue and systemically in spleen and lymph nodes (Arpaia et al., 2013; Smith et al., 2013), increased activity of Natural Killer cells (reviewed by Brouns et al. [2002] and Delzenne [2003]), and decreased inflammation in the colon (Chang et al., 2014; Singh et al., 2014). In mice, it has been demonstrated that the combination of scOS and lcOS stimulated the Th1 immune signaling pathways and downregulated Th2 immune response pathways as measured in blood (Vos et al., 2004). In pigs, a study showed an effect of maternal FOS supplementation to the offspring (Le Bourgot et al., 2014) and another study showed an effect of FOS on post-weaning piglets, whereby an increased immune response to influenza vaccination was observed (Le Bourgot et al., 2016).

In the present study, the effects of administrating FOS during days 2 till 14 of life were measured in suckling piglets on the microbiota colonization and intestinal development, with emphasis on the local immune system and the barrier function of the intestine. To this end, we monitored gut-related

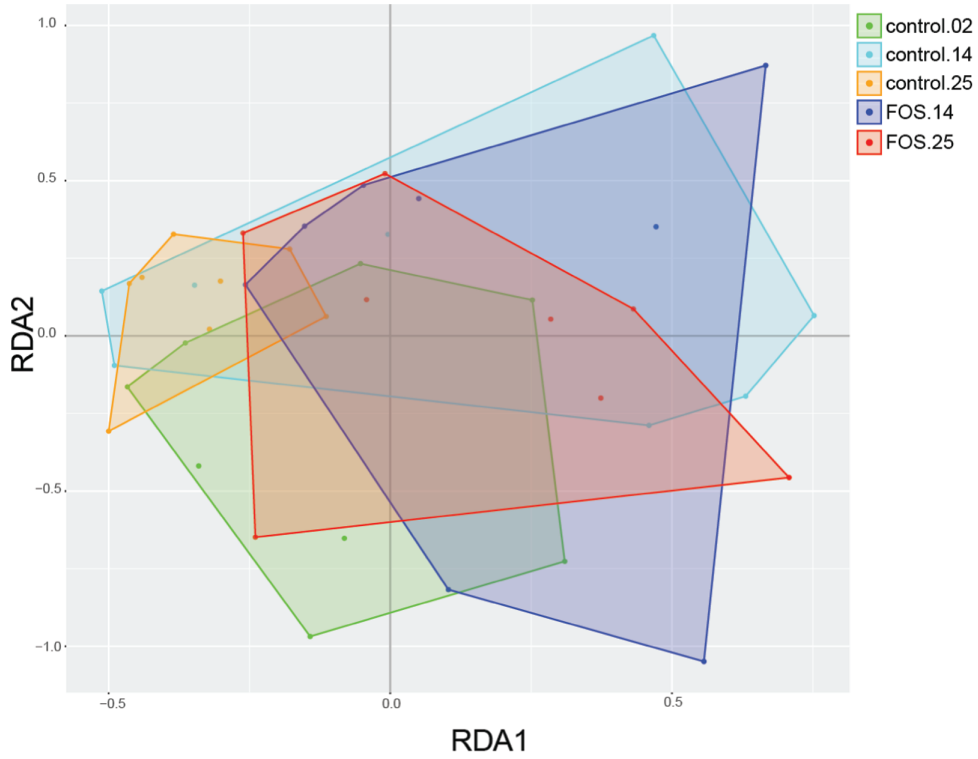

Figure 4. Redundancy analysis (RDA) of family-level microbiota in the pig jejunum. The x-axis depicts explanatory axis 1 (RDA1) and y-axis depicts explanatory axis 2 (RDA2). Each condition is represented by a different color, that is, day 2 is green (n = 8), day 14 control is cyan (n = 8) and day 14 fructooligosaccharides (FOS) are blue (n = 8), and day 25 control is orange (n = 8) and day 25 FOS is red (n = 8). The following model was used as an input for the RDA: y = time + treatment + time × treatment + error. Control.02 = day 2 control piglets; Control.14 = day 14 control piglets; FOS.14 = day 14 piglets supplemented with FOS; Control.25 = day 25 control piglets; FOS.25 = day 25 piglets supplemented with FOS.

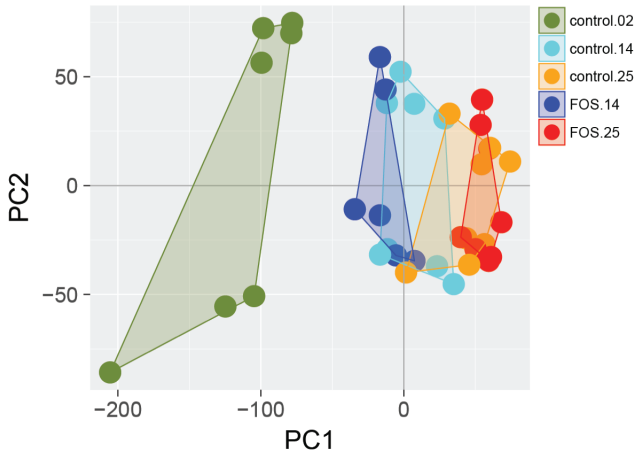

Figure 5. Principal component analysis of jejunal transcriptomics data. The x-axis depicts principal component 1 (PC1) and y-axis depicts principal component 2 (PC2). Each dot represented by a different symbol; day 2 is green (control; n = 8), day 14 is cyan (control; n = 6) and blue (fructooligosaccharides [FOS]; n = 8), and day 25 is orange (control; n = 8) and red (FOS; n = 8). Control.02 = day 2 control piglets; Control.14 = day 14 control piglets; FOS.14 = day 14 piglets supplemented with FOS; Control.25 = day 25 control piglets; FOS.25 = day 25 piglets supplemented with FOS.

parameters for both control piglets (days 2, 14, and 25 of age) and FOS-supplemented piglets (days 14 and 25). The timing of the intervention, days 2 until 14, was chosen because we hypothesized that this would have the greatest impact on the intestinal development and programming. Furthermore, evidence is accumulating that early life events can induce long-lasting effects, for example, early antibiotic treatment affected the intestinal immune status and microbiota composition in pigs (Schokker et al., 2015b), as has also been shown in human studies (Foliaki et al., 2009; Hoskin-Parr et al., 2013).

Colon digesta and tissue samples obtained on day 14 were analyzed to verify the expected bifidogenic effect of FOS (Meyer and Stasse-Wolthuis, 2009) and to investigate the eventual effects of a hypothesized increase in butyrate production and concomitantly changes in colonic gene expression. The oral administration of FOS to suckling piglets indeed resulted in the expected increase in the abundance of *Lactobacillus* and *Bifidobacterium* in the colon at day 14. Based on this, we presume a higher production of butyrate in the colon. However, the observed microbial bifidogenic effect in the colon did not result in changes in gene expression of the local colon tissue. Apparently, claimed health effects of increased abundances of *Lactobacillus* and *Bifidobacterium* in the colon (Rivière et al., 2016) and the presumed raise in local butyrate production do not modulate the gene expression in colon mucosal tissue at a detectable

Table 4. Gene set enrichment analysis of the pig jejunum at day 14

Name	SIZE[1]	Direction[2]	NES[3]	FDR[4]
Chromosome organization and biogenesis	66	↓	2.47	0
Establishment and or maintenance of chromatin architecture	35	↓	2.44	0
Chromosome	63	↓	2.33	0
Helicase activity	29	↓	2.31	0
Cell cycle process	101	↓	2.3	0
Cell cycle phase	88	↓	2.29	0
Chromosomal part	50	↓	2.27	0
Chromatin modification	28	↓	2.23	1.5×10^{-4}
M phase	58	↓	2.21	2.6×10^{-4}
Mitotic cell cycle	81	↓	2.19	3.5×10^{-4}
M phase of mitotic cell cycle	44	↓	2.19	3.2×10^{-4}
Cell cycle	161	↓	2.13	1.3×10^{-3}
Mitosis	42	↓	2.11	1.8×10^{-3}
Proteinaceous ECM[5]	33	↑	2.09	2.7×10^{-3}
Extracellular matrix	34	↑	2.07	2.3×10^{-3}
Spliceosome[5]	63	↓	2.06	2.9×10^{-3}
DNA replication	47	↓	2.03	3.0×10^{-3}
Transmembrane receptor activity	129	↑	2.03	2.4×10^{-3}
ATP dependent helicase activity	16	↓	2.02	3.3×10^{-3}
DNA dependent DNA replication	26	↓	2.01	3.8×10^{-3}
Systemic lupus erythematosus[6]	52	↓	2	4.3×10^{-3}
ECM receptor interaction	42	↑	1.99	5.2×10^{-3}
Chromatin	18	↓	1.98	5.5×10^{-3}
Extracellular matrix part	22	↑	1.98	7.1×10^{-3}
RNA helicase activity	15	↓	1.97	5.8×10^{-3}
Centrosome	35	↓	1.97	5.9×10^{-3}
Nuclear chromosome	24	↓	1.96	6.6×10^{-3}
Receptor activity	203	↑	1.96	7.2×10^{-3}
G protein coupled receptor activity	42	↑	1.95	6.9×10^{-3}
Extracellular region	161	↑	1.95	6.0×10^{-3}
Extracellular region part	124	↑	1.94	6.9×10^{-3}
Nuclear part	284	↓	1.93	8.5×10^{-3}
Spindle	24	↓	1.93	8.4×10^{-3}
Microtubule cytoskeleton	81	↓	1.92	9.2×10^{-3}
Base excision repair	17	↓	1.92	9.3×10^{-3}

[1]SIZE = number of genes in gene set.

[2]Enriched (↑) and depleted (↓) gene sets in piglets supplemented with fructooligosaccharides ($n = 6$) compared to control piglets ($n = 8$).

[3]NES = normalized enrichment score. The NES is the primary statistic for examining gene set enrichment results. By normalizing the enrichment score, gene set enrichment analysis accounts for differences in gene set size and in correlations between gene sets and the expression data set; therefore, the NES can be used to compare analysis results across gene sets.

[4]FDR = false discovery rate. The FDR is the estimated probability that a gene set with a given NES represents a false positive finding. For example, an FDR of 5% indicates that the result is likely to be valid 19 out of 20 times.

[5]ECM = extracellular matrix.

[6]Kyoto Encyclopedia of Genes and Genomes pathways.

level, at least in suckling piglets to which FOS were administered over a period of 12 d.

Linking Host Gene Expression to Biological Function

To monitor the potential FOS-induced changes in the cross-talk between luminal microbiota and cells in the mucosa of the small intestine, jejunal digesta and tissue samples were analyzed at days 2, 14, and 25. Transcriptional profiling was performed on the entire gene collection of intestinal tissue to identify differences in biological processes, including metabolic, cell cycle, and immunological processes. Differences in jejunum gene expression were observed at day 14, mainly in gene expression associated with ECM and cell cycle. Such ECM and cell cycle processes potentially relate to the barrier function of the gut, which has an important role in maintaining homeostasis in the gut. Disturbance

Table 5. Gene set enrichment analysis of jejunal mucosa of piglets at day 25

Name	SIZE[1]	Direction[2]	NES[3]	FDR[4]
Chemokine activity	21	↓	2.41	0
Chemokine receptor binding	22	↓	2.37	0
G protein coupled receptor binding	27	↓	2.31	0
Locomotory behavior	42	↓	2.22	0
Cytokine–cytokine receptor interaction[5]	103	↓	2.20	2.1×10^{-4}
Retinol metabolism	15	↑	2.12	8.4×10^{-3}
Behavior	63	↓	2.06	2.8×10^{-3}
Carbohydrate binding	28	↓	2.05	2.5×10^{-3}
ECM[6] receptor interaction	42	↓	2.00	8.2×10^{-3}

[1]SIZE = number of genes in gene set.

[2]Enriched (↑) and depleted (↓) gene sets in piglets supplemented with fructooligosaccharides (n = 8) compared to control piglets (n = 8).

[3]NES = normalized enrichment score.

[4]FDR = false discovery rate.

[5]Kyoto Encyclopedia of Genes and Genomes pathways.

[6]ECM = extracellular matrix.

Table 6. Villus height, crypt depth, and villus height:crypt depth (V:C) ratio in the jejunum in piglets in both experimental treatments at different time points

Treatment	Day	Villi height[1]	P-value[2]	Crypt depth[1]	P-value	V:C ratio	P-value
Control	2	719 (25)		159 (8)		5.3 (0.4)	
Control	14	672 (23)	0.72	168 (7)	0.87	4.8 (0.4)	0.81
FOS[3]	14	706 (23)		171 (7)		5.1 (0.4)	
Control	25	423[a] (12)	0.15	144[a] (8)	0.06	3.9 (0.3)	0.85
FOS	25	504[b] (12)		179[b] (8)		3.9 (0.3)	

[a,b]Values within a column within time point with different superscripts tend to differ ($0.05 < P < 0.10$).

[1]μ in micrometers (SEM) per treatment.

[2]Student's t-test comparing FOS (n = 8) vs. control (n = 8) at a specific time point.

[3]FOS = fructooligosaccharides.

of homeostasis may lead to a leaky gut, which may result in an increased translocation of microbes or microbe-derived products that evoke inflammatory responses. Ultimately, this may have severe negative effects on growth performance and health parameters of the animal (Bjornsson et al., 2005). However, it remains unclear by which signaling mechanism these jejunal gene expression changes are established. The signaling mechanism could originate from the jejunal mucosal gene expression and the local cross-talk between mucosal cells and luminal microbiota; however, it could also originate from the changes in microbiota composition of the colon. The latter is more plausible because the jejunal microbiota composition and diversity at day 14 were almost not affected by the FOS supplementation.

At day 25, FOS induced changes in jejunal gene expression that were associated with innate immunity and ECM. At the same time, we observed differences in several histological parameters, that is, a tendency of higher villi and deeper crypts, which may be related to the observed differences in gene expression. Furthermore, on day 25, we observed significant differences in the diversity of luminal microbiota in the jejunum. In principle, these effects could result from the "bifodogenic" changes and the presumed higher production of butyrate (Meyer and Stasse-Wolthuis, 2009) in the colon by unknown mechanisms. In this study, we also observe a direct effect of FOS administration on the diversity of microbiota in the jejunum, a significant increase in the microbiota diversity of piglets supplemented with FOS compared with control piglets, values being 2.45 vs. 1.92, respectively. At this age, a greater microbiota diversity is regarded as beneficial for the host (Lozupone et al., 2012). This may be another mechanism by which FOS exerts its beneficial health effects. A greater microbial diversity makes the gut (eco)system more resilient and new invasions or the outgrowth of pathobionts will be more difficult in a diverse and established (eco) system (Lozupone et al., 2012). This aspect of the potential beneficial health effects of FOS has not been described yet elsewhere, probably because in human studies often the focus is on colonic digesta or feces and not on effects in the small intestine.

Moreover, these gene expression data suggest that the turnover of epithelial cells is lower in piglets supplemented with FOS. It is probable that the higher gene expression of ECM processes features the tight junctions and barrier function of the intestinal mucosa (Bosman and Stamenkovic, 2003; Couchman, 2010; Frantz et al., 2010; Lu et al., 2011). At day 25, piglets supplemented with FOS showed lower activity of immune-related processes. Mainly chemokine-related processes were affected. Chemokines function as activators of pro-inflammatory pathways and homing of immune cells (leukocytes; Mortier et al., 2012). This lower activity suggests lower inflammatory responses by the host or less homing of immune cells, which could be beneficial in terms of growth performance of the host by not immunologically overreacting towards the resident microbiota. However, the immune system is a complex system operating at different biological sites (gut, blood, and skin) and scales, and therefore, it is difficult to draw a singular conclusion on the observed differences in the present study in which piglets were not subjected to a pathogenic or immunological challenge.

It should be noted that the FOS supplementation was given to the piglets on top of their normal intake of sow's milk. However, control and FOS-supplemented piglets both received a similar oral gavage, which may have resulted in short moments of stress, which were therefore experienced by all piglets in the study. Similar effects related to oral gavage have been shown in rats (Brown et al., 2000) and pigs (personal communication with Astrid de Greeff et al.). This stress, due to handling of the animals, may have led to an increased diarrhea incidence in "study" animals compared with other "non-study" piglets that did not receive oral gavage. The differences observed in the present study are due to the experimental treatment; however, temporary stress–related oral gavage could have influenced the responses measured in both control and FOS-supplemented piglets. It has already been established in rats and humans that stress may affect various components of the intestinal barrier function (Soderholm and Perdue, 2001). The structure of the tight junctions can change due to stress resulting in an increased permeability. Another possible reason for this higher incidence of diarrhea in piglets in the present study could be the dosage of FOS administered daily (Shim et al., 2005b). In turn, this rapid fermentation may lead to malabsorption of SCFA because of rapid transit of digesta out of the colon (Kien, 1996). This phenomenon has been reported in weaned pigs (Shim et al., 2005) and growing pigs (Xu et al., 2002). However, the former is unlikely because both the control and FOS-supplemented piglets received similar oral gavage. In conclusion, the observed increase in diarrhea incidence during the period of oral gavage could have induced a generic effect on the gut homeostasis. Nevertheless, both groups received a similar gavage treatment and FOS-supplemented piglets did show a bifidogenic effect under the circumstances described in the present experiment, which is in line with the expected mode of action of FOS after oral administration.

Systems Behavior of the Gut

The gut consists of multiple biological components, including the hosts' gene expression, microbiota, and diet, the sum of these aspects, and their interactions translate to a complex systems behavior. Here, we have shown major changes in gene expression in the jejunum, related to processes involved in health and barrier function, concomitant occurrence with minor changes in local microbiota. Furthermore, these changes in the jejunum are in line with major changes observed in the microbiota in the colon (bifidogenic effect). The colon might communicate with the jejunum as a result of changes in concentrations of nutrients and metabolites in the colonic lumen via unknown feedback loops. Such communication could progress via the vagus nerve by suppressing cytokine production (de Jonge, 2013) and/or via the lymphatic system (Macpherson et al., 2005). Another signaling route could be effectuated by butyrate modulating macrophages (Chang et al., 2014). Therefore, changes occurring at day 14 in the large intestine could trigger stimuli that may communicate the colonic status towards the small intestine, i.e., regarding the environment (i.e., luminal content). In the present study, we observed the hypothesized outcome of the bifidogenic effect in relation to the communication for the gut system. The modulation of the gut system via supplementation of FOS may not lead to changes in the systems behavior as a result in which singular factors being affected, but may cause a cooperative response of multiple aspects altering the systems behavior in the gut (Lau et al., 2012).

CONCLUSIONS

The present study shows that it is possible to modulate the early life intestinal colonization of neonatal piglets by the administration of FOS. The modulation affects the expression of major

immune-related parameters and barrier function in mucosal tissues. Supplementing FOS in suckling piglets modulates the bacterial colonization of the gut and the intestinal (immune) development of the host. FOS induce a bifidogenic effect in the colon of piglets as well as changes in mucosal gene expression profiles in the jejunum relating to intestinal barrier function and immunity. The precise underlying mechanisms of modulation of intestinal (immune) development by FOS during early life of piglets need to be elucidated further, but FOS supplementation might be beneficial for their health and performance.

SUPPLEMENTARY DATA

Supplementary data are available at *Animal Frontiers* online.

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