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LIVESTOCK RESEARCH
WAGENINGEN UR

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Foreword

Feed4Foodure is a public-private partnership between the Dutch Ministry of Economic Affairs, a consortium of various organizations within the animal production chain and Wageningen UR Livestock Research. Feed4Foodure aims to contribute to sustainable and healthy livestock farming in the Netherlands, simultaneously strengthening our competitive position on the global market. The Feed4Foodure program line “Nutrition, Intestinal Health, and Immunity”, aims to contribute to a reduction in the use of antibiotics in livestock farming by increasing general health and disease resistance. The main goals are to develop innovative measuring techniques and to evaluate new feeding concepts, feed ingredients and additives to improve gut health and immunity.

The current report describes a first model intervention in suckling piglets to investigate the effect of early life modulation of the gut microbiota (via fructooligosaccharides) and the (in)direct effects on the functioning and (immune) development of the gut tissue. Experiments were performed within the frame work of the Feed4Foodure program line “Nutrition, Intestinal Health, and Immunity”.

For the current study, scientist of Wageningen UR Livestock Research and Central Veterinary Institute worked together with representatives from the various private partners, including Agrifirm, ForFarmers BV, Nutreco, De Heus, Denkavit, van Drie, MSD, and Darling Ingredients International. The authors thank the industry partners of the project team for their worthwhile input.

Prof. Dr. Mari Smits, leader Feed4Foodure program line “Nutrition, Intestinal Health, and Immunity”.

Summary

Gut microbial colonization and immune competence development are affected by early-life environmental and dietary interventions. The interplay between microbiota in the intestinal tract and the gut mucosal surfaces of the host is critical for the development of an accurate immune competence. In the present study we intervened during early life of suckling piglets by a daily oral administration of fructooligosaccharides (FOS solution) from day 2 – 14 and investigated the effects on intestinal microbiota composition (by 16S rDNA sequencing) and biological processes of the intestinal mucosal tissue (by genome-wide intestinal gene expression analysis) during the suckling phase.

The results did not show significant effects of administration of FOS on the crypt depth, villi height, or villus-crypt ratio in the jejunum and the ileum at either day 14 or 25. No significant changes in microbial colonization (composition) were observed in the luminal associated bacteria in jejunal mucosa at days 14 and 25, although a higher microbiota diversity was observed at day 25 in piglets that received FOS. However, in the colon a clear “bifidogenic” effect could be observed. The gene expression patterns of mucosal tissue in jejunum and ileum differed between control piglets and piglets receiving FOS. At day 14 after birth, lower activity of cell cycle related processes and a higher activity of extracellular matrix processes were observed in piglets receiving FOS compared to control piglets. At day 25, lower activity of immune-related processes in jejunal tissue were seen in piglets receiving FOS. The observed ‘bifidogenic’ effect on the microbiota composition did not translate into effects on gene expression in tissue of the colon itself. These data suggest that the gene expression effects displayed in the jejunum and ileum may originate from signals expressed in the colon. A good candidate for this signalling is butyrate which is known to have multiple beneficial effects in the gut and which is produced by bifidobacteria and lactobacilli in the colon. Butyrate not only provides energy to cells, it also has been shown to have effects on cell proliferation, cell maturation and gut integrity. Butyrate also has a role as an anti-inflammatory agent, primarily via inhibition of nuclear factor kB (NF-kB) activation.

In conclusion, oral FOS administration during the postnatal period of piglets had significant effects on the microbiota in the colon and a limited effect on the microbiota in ileum and jejunum. FOS administration did not result in detectable morphological changes in jejunum and ileum. FOS affected the microbiota in the colon and the expression of genes involved in cell proliferation, extracellular matrix formation and immune related processes in jejunum and ileum. It is hypothesized that the FOS treatment results in a higher butyrate production in the colon due to the increase in bifidobacteria and lactobacilli. Higher levels of butyrate result in beneficial gene expression changes in the ileum and jejunum by thus far (partly) unknown mechanisms.

The observed changes in gene expression, cellular processes and in microbiota composition and diversity will help to define parameters critical for an accurate immune competence.

1 Background

1.1 Overall aim of VDI3

Aim of VDI3 'Neonatal models' is the development and application of neonatal models with predictive value to study the impact and underlying mechanisms of nutritional- and (indirect) microbial interventions in the gut. Furthermore, VDI-3 also aims to detect indicators associated with gut development in terms of immune competence and functionality. It is known that during the neonatal period a life-long influence can be exerted on the development and competence of the immune system. The challenge is to identify the early life physiological parameters that are associated with an accurate immune competence later in life.

1.2 Introduction

This study forms part of the basis for the development of a test model with predictive value for nutritional and microbial interventions on immune competence. Since it is known that during the neonatal period a life-long influence can be exerted on the development and competence of the immune system later in life, it is expected that important indicators for immune competence are displayed during this early period of life.

1.2.1 Choice of intervention ~ oligosaccharides

To gain more insight into underlying biological mechanisms and processes with respect to a dietary intervention such as oligosaccharides, it is important to induce a contrast in the gut in terms of the dietary compound being studied. Here, we investigated the effect of an oral dietary intervention that was provided in the suckling period of piglets.
In literature, it is described that human galactooligosaccharides (GOS), and related products, such as fructooligosaccharides (FOS) lead to changes in the composition, diversity and activity of the microbiota in different parts of the gastrointestinal tract [1-3]. GOS is based on milk oligosaccharides and FOS is derived from plant products. Studies have shown that inulin-type fructans (FOS) have "positive" effects on the microbiota, gut integrity, and on production performance (of pigs, poultry, calves) such as weight gain and feed efficiency [4]. However, the results with respect to the effects of FOS are not always consistent and there are also studies that show only a limited effect of these prebiotics. The degree of polymerization of fructans can have influence on the effects in rats [5], however, the chain length of the inulin-type fructans in infants appears to have no effect and clear dose-response relationships have not (yet) been observed [6]. Another unexplored aspect is the effect of FOS on different immune competence parameters, including microbiota composition and diversity, when administered during the neonatal period on top of the GOS already present in the milk. Oligosaccharides are also found in (human) breast milk and have been referred to as immuno-active ingredients, in the concentration of approximately 0.5 mg/ml. Quantitative information on the concentrations of these particular sugars in sow milk was lacking at the start of this experiment. Most available data in literature on sow milk are on global parameters, such fat percentage, protein percentage, and gross energy [7]. However, during the course of the experiment more detailed information on the composition of sow milk became available (8).These authors also concluded that porcine milk proteins promote growth and immune system development [8]. Another study showed that porcine milk is most similar to the human milk oligosaccharide composition or structures, when comparing milk of multiple domestic animals to humans [9]. Furthermore, the latter study showed that porcine milk has approximately 20% neutral oligosaccharides and the highest variation in monosialylated and disialylated large oligosaccharides [9]. Contrary to individual sugars from which they are derived, GOS and FOS are well-known prebiotics for humans. The differences between GOS and FOS are in both composition of the sugar moieties and in the degree of polymerization. Where GOS consists mainly of oligo-galactose (85%) and an additional glucose and lactose, FOS mainly consists of β (2-1) fructans. The degree of polymerization of GOS is between 2 and 9 and for FOS from 2 to 60. For short chain FOS (scFOS), the degree of polymerization is 2-10 or 3-5, depending on the manufacturing method [10]. It has also been shown that oligosaccharides may interfere with the adhesion of certain pathogens and toxins in mucous membranes [11]. In addition to the human studies, also studies were performed with (preterm or) caesarean born piglets [12]. However, studies

investigating the effect of providing prebiotics during the suckling period of piglets under ‘practical’ circumstances are lacking. Nevertheless, in human literature studies have been described investigating the effect of the combined administration of GOS and FOS to babies. Two similar studies in human babies (age 28-90 days) with GOS/FOS prebiotics (formula-fed) showed an increase of the number of Bifidobacteria in colon/faeces and that the composition of Bifidobacteria species were more similar to breast-fed babies [13, 14].
For the experiment as described here we wanted to create a contrast between the control and the prebiotics treatment. For that reason it was suggested to use both short and long-chain oligosaccharides. From human literature an 'optimal' ratio of nine short chain GOS and one long chain FOS (lcFOS) has been described to have a ‘bifidogenic’ effect [15]. Because only limited evidence is available that (human) breast milk contains long chain GOS (≥ 4), often in these human studies short-chain GOS is used [16]. As opposed to human experiments in which a prebiotic 'formula-fed’ group and the breast-fed control group are often compared, in the present experiment we only used a single mixture of short and long chain FOS (commercial products; see Appendix 1 and 2 for specifications) with the above-mentioned ratio of 9:1. In mice, studies have demonstrated that combined short-term and long-chain oligosaccharides stimulated Th1 immune signalling pathways and down-regulated Th2 immune response pathways [17]. Furthermore, children fed with the combination of short-term and long-chain oligosaccharides display a lower incidence of recurrent respiratory infections and diarrhoea [18]. This shows that oligosaccharides can be metabolised by different Bifidobacterium strains, and represent potential candidates to act as biologically active molecules (prebiotics) in neonates.
In conclusion, oligosaccharides, both plant or animal derived, are relatively well established to show a bifidogenic effect in the colon, at young age as well as in the adult stage. Different chain lengths of these oligosaccharides have effect on the microbiota composition, because Bifidobacterium species may possess different carbohydrate utilizing abilities.

1.2.2 Dosage of prebiotics

The used dosage of 10 g/day in the present study is based on both literature and contacts with various suppliers of prebiotics. We extrapolated the dosage used in the present experiment from research in weaned piglets, because there is little to no information available on the potential required dosages in neonatal piglets. It is known that a relatively small amount of FOS (5-15 g/day) in human babies and weaned piglets can induce bifidogenic effects [6]. For short chain GOS, there is also a consensus on the bifidogenic effects [19], where a minimum dose of 5 g/day showed a significant shift in the intestinal microbiota in healthy adults [20]. Studies in babies often use an average dosage of about 6 g of a prebiotic per day (e.g. 8.0 g/L (formula) milk [21]). The drinking moments of babies are spread throughout the day (on average 6 times per day). Expressed in grams of FOS per day, this equals 6 g FOS assuming an average milk consumption of 750 mL. In studies with weaned piglets, dosages are often larger, i.e. 40 g inulin per kg feed [22]. Another pig experiment showed a dose of 40 g/kg in the diet, however only a small but significant effect on the growth performance, feed conversion ratio, general health and faecal consistency score was observed (personal information FOS producer), however it could still be possible that subtle changes in the gut occurred. The latter is equivalent to 8.7 g of FOS per animal per day. Piglets in this study received FOS for a period of two weeks after weaning. According to this manufacturer, higher doses of up to 22 g FOS per day might result in more significant effects on the above-mentioned parameters. Thus, mainly based on human infant and weaned piglet studies, the dosage was set to 10g/day per piglet (2 oral dosages of 5g).

1.2.3 Effects of FOS

FOS escapes enzymatic digestion in the small intestine because of the β-linkages between the fructose monomers. These β-linkages cannot be hydrolysed by enzymes of endogenous origin and therefore FOS forms a substrate for the gastrointestinal microbiota [23, 24]. In adult humans, it has been shown that FOS inhibits *Escherichia coli* in the large intestine and has a positive effect on the growth of lactobacillus and bifidobacteria [25, 26]. FOS is fermented in the large intestine into SCFAs, lactate and carbon dioxide [27]. FOS selectively increases lactic acid bacteria, such as Lactobacilli and Bifidobacteria [28] and increases n-butyrate concentration in colon in humans [28]. These bacteria are able to compete with pathogens [29]. N-butyrate is the major source of energy for epithelial cells of the large intestine and stimulates the proliferation of cells and the water absorption from the lumen [27]. According to these authors, the effect of FOS on the large intestine physiology should be realized by an increased concentration of luminal n-butyrate. Feeding FOS to neonatal pigs tended to enhance the bifidobacteria population and prevent colonic epithelial mucosa atrophy [30].
Houdijk et al. [31] observed a decreased pH and increased Volatile Fatty Acid concentrations in the caecum and colon by an addition of non-digestible oligosaccharides (NDOs) to the diet because of the

fermentation of FOS in the hindgut. There is also an increased osmotic pressure in the stomach and small intestine when soluble NDOs such as FOS or TOSs are included in the diet [32]. When the pH in the stomach decreases, fermentation of dietary NDO had taken place [33]. The microbiota in the stomach is able to ferment NDOs because of the presence of Lactobacilli [31]. NDOs that escape the fermentation process in the stomach are fermented in or prior to the caecum [31]. scFOS is rapidly fermentable and highly effective at inducing morphological changes (i.e. villus height) in ileum, and in both jejunum and colon transport activity was higher [12]. In mice, it has been shown that combined scFOS and lcFOS stimulated the Th1 immune reaction and down regulated the Th2 pathway [17], have the ability to improve the intestinal morphology [24], and lower the incidence of respiratory infections and diarrhoea in children [34]. Cummings et al. [35] concluded that molecules with longer chain lengths (for instance lcFOS) are fermented more slowly. In conclusion, FOS is known to stimulate growth of butyrate producing bacteria, creating an unfavourable environment for pathobionts. Furthermore, links to immune cells have been shown in mice models.

1.3 Objective

The aim of this study was to investigate the effect of an oral administration (d 2-14) of fructooligosaccharides (FOS) on intestinal of neonatal piglets in the pre-weaning (suckling) phase. It was hypothesized that an oral administration of FOS to neonatal piglets increases the immune competence via a bifidogenic effect in the colon. The study also aimed to detect indicators associated with gut development in terms of immune competence and functionality. It is known that during the neonatal period a life-long influence can be exerted on the development and competence of the immune system. The challenge is to identify the early life physiological parameters that are associated with an accurate immune competence later in life.

2 Material and Methods

2.1 Experimental design

2.1.1 Housing and diet

The present experiment was performed on a commercial farm. Four Topigs20 sows with parity number 3 or 4 were used. During gestation the sows were group housed and from day 109 the sows were individually housed in conventional farrowing rooms until weaning. Water was available ad libitum during the gestation and lactation period. Sows were fed a conventional diet. A feeding schedule per sow was used which was based on back fat thickness, bodyweight and parity number of the sow. Piglets were not given access to creep feed or a milk replacer during lactation.
Each sow had at least 14 piglets born alive and if there were more than 14 piglets it was reduced to 14 piglets. Of each litter, ten piglets were finally euthanized during the course of the study for sample collection and four piglets remained alive during the study, unless used as reserve piglets for dissection. Two or three days after parturition each litter was split into two groups (see Table 2). The test group consisted of six piglets and the control group of eight piglets.

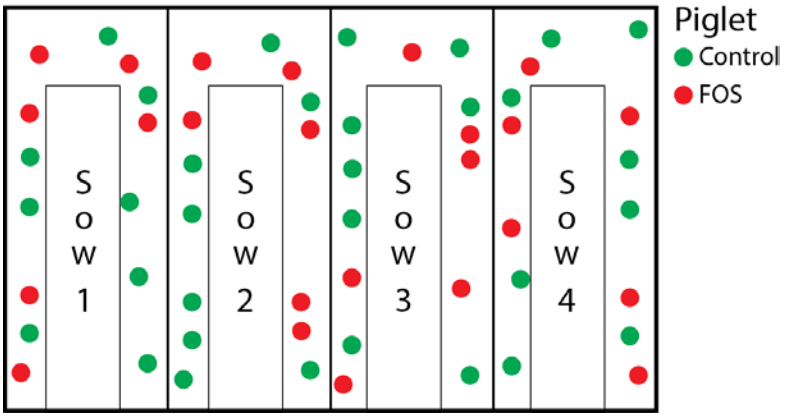

Figure 1. Overview of the experimental design.

2.1.2 FOS intervention

From day 2 to 14, the piglets in the intervention group (piglets receiving FOS) got twice a day an oral administration of fructooligosaccharides dissolved in water. The piglets in the control group were orally administered with the same volume of water at the same time. All piglets in each litter, six piglets receiving FOS and eight control piglets, were reared by their own mother (see Figure 1).The way of oral administration of FOS was changed during the course of the study. Because piglets were vomiting after the administration of either water or water solubilized FOS, the volume was reduced at day 7 (five days after the start of the experiment (d2; see below). The FOS administration procedure used in this study caused stress in the piglets, leading to an increased infection pressure in the stable (personal communication with personnel in stable). At day 7 the FOS mixture was dissolved in water to a total volume of 6 ml instead of 15 ml as used during the first days of the study. The FOS used for this experiment was Frutafit® TEX! (lcFOS) and Frutalose® OFP (scFOS) from SENSUS. Product specification is attached in Appendix 1 and 2 respectively. The mixture used was based on 10% lcFOS and 90% scFOS, as is recommended for human babies [15]. In short, the specifications of the FOS mix:
1) Sensus-Frutalose® OFP, this oligofructose from chicory is a polydispersed mixture of linear fructose oligomers partly ended by a glucose molecule, coupled by means of β(2-1) bonds. The number of units (degree of polymerization) varies mainly between 3 and 10.
2) Sensus-Frutafit® TEX!, this inulin from chicory is 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) can vary between 2 and 60.

2.2 Measurements

2.2.1 Sows

When sows were moved into the farrowing room, measurements on body weight and back-fat started and the final body weights and thickness of back-fat were measured at the day of weaning. Normally on this commercial farm, sows gave birth between Tuesday and Sunday. So from Tuesday morning till the moment that the last sow of these four sows had farrowed there was observation for 24 hours a day to determine the birth order, birth weight and the first suckling moment for all piglets in each of the four litters. Body weight was measured at day 109 of gestation and at weaning by using a scale. Back-fat was measured at day 109 of gestation and at weaning by using an ultrasonic device according to the P2 method. This method describes that the thickness of the back-fat has to be measured at the level of the last rib, 65 mm from the midline. Feed intake of sows was also registered daily.

2.2.2 Piglets

At the moment of birth of each piglet, the following measurements were made: time of birth, birth weight, vitality score (used Table 1), and sex. Each piglet got an unique ear tag number. The first born piglet got the lowest number, the last born piglet got the highest number.

Table 1.

Piglet vitality scores as described by [36].

Vitality score	Description
0	No movement, no breathing after 15 s.
1	No movement after 15 s, piglet is breathing or attempting to breathe (coughing, spluttering, clearing its lungs).
2	Piglet shows some movement within 15 s, breathing or attempting to breathe.
3	Good movement, good breathing, piglet attempts to stand within 15 s.

First suckling moment: the first time that the piglet was suckling. Body weight: The piglets were weighted at birth, 24 hours after the first piglet of the litter was born, and at day 2 (start of the intervention), 7, 14, 21 and at weaning. At day two after birth the piglets were renumbered. The piglet born alive with the lowest birth weight got number 1 and the piglet with the highest birth weight got number 14. Renumbering of piglets was necessary to distribute the body weight per treatment, control piglets or piglets receiving FOS, and this was coupled to the moment to euthanize the piglet (Table 2).

Table 2.

Allocation of piglets in each litter to the experimental treatments.

Weight	Piglet	Ear tag number		Group	Day
		before renumbering	**after renumbering**		
Heaviest	1	…	…	Control	14
	2	…	…	Prebiotic	14
	3	…	…	Control	25
	4	…	…	Prebiotic	25
	5	…	…	Control	Reserve piglets
	6	…	…	Prebiotic	Reserve piglets
	7	…	…	Control	2
	8	…	…	Control	2
	9	…	…	Control	Reserve piglets
	10	…	…	Prebiotic	Reserve piglets
	11	…	…	Control	25
	12	…	…	Prebiotic	25
	13	…	…	Control	14
Lightest	14	…	…	Prebiotic	14

At three different time-points, day 2, 14, and 25, piglets were sacrificed to extract samples of intestinal digesta, intestinal tissue and blood. At day 2 after parturition two piglets per litter were euthanized and at day 14 and 25 after parturition four piglets (two control piglets and two piglets receiving FOS) per litter were euthanized. In total, ten piglets per litter were euthanized for sample collection. The following biological samples were extracted; digesta of the stomach, jejunum and ileum, multiple tissue samples of jejunum and ileum (histology and mucosal scrapings for transcriptomics), and blood. Besides the biological samples, mortality during farrowing, body weight at death, reason of death and the date of death were recorded if possible.

2.3 Performance

2.3.1 Colostrum intake

The total intake of colostrum during the first 24 h of life was calculated by using the formula given below [37].

$Cl = -217{,}4 + 0{,}217 * t + 1861019 * BW/t + BW_B * (54{,}80 - 1861019/t) * (0{,}9985 - 3{,}7 * 10^{-4} * t_{FS} + 6{,}1 * 10^{-7} * t^2_{FS})$

- CL: Colostrum intake (gram)
- BW_B: Birth weight (kg)
- $BW_{to}BW_B$: Weight gain (kg) [body weight at 24 hours minus body weight at birth in kg]
- t: Age (minutes)
- t_{FS}: Interval between birth and first suckling moment (min)

For one of the piglets of the control group a negative value was calculated for the consumption of colostrum. This value was omitted in the further evaluation of colostrum ingestion in the study. From day 2 until day 7 most of the piglets of the control and test group had diarrhoea. It was not possible to evaluate or score occurrence of diarrhoea in detail for individual piglets. Piglets were weaned on average at 27.5 days.

2.3.2 Body weight gain

The average body weight gain (BWG) in a period was calculated by subtracting the end weight of the period by the start weight of the period over which the BWG was calculated and subsequently divided by the number of days of that period.

2.4 Histology data

2.4.1 Measurements

Tissue samples of jejunum and ileum of all 40 piglets were cut to approximately 1.2 cm and put into the Leica tissue processor by using the protocol (see Appendix 3 for details) and embedded in paraffin. Thereafter, the paraffin embedded tissues were sectioned in coupes of 5 µm and transferred to coated Superfrost slides (coated with glycerin). The slides were overnight in an oven at 38 degrees of Celsius, followed by dewaxing and staining with the Micron staining machine by using the protocol (see Appendix 4 for details). Mounting the dewaxed and stained slides in depex and again stored overnight in an oven at 38 degrees of Celsius. After drying in the oven, the villi height and crypts depth were measured.
These measurements were made by using analySIS^D (FIVE) software via a Nikon Microphot_FXA microscope with an Olympus DP50 (5 mega pixels) video camera. In total, per piglet the height of ten villi and their corresponding crypts depth of both jejunum and ileum were measured.

2.4.2 Statistical analysis

Data of villus height and crypt depth of the jejunum and ileum, data about the body weight of the individual piglets at certain moments, growth rates, vitality, first suckling moment, birth order and the

colostrum uptake is analysed by using IBM SPSS Statistics 20. Minimum, maximum and mean values and standard deviations were analysed by using descriptive statistics. Normality of the residues was checked and was assumed when values of Skewness and Kurtosis were between -2 and 2. Outcomes smaller than $p<0.05$ were considered as significant. P-values lower than 0.10 indicates a statistical tendency. The statistical analysis is carried out in two steps. The effect of time and the effect of the FOS intervention were analysed. For each individual piglet ten measurements were performed for either villi height or crypt depth. In the second step, only the average values of the villus height and crypt depth of the jejunum and ileum were used. After the statistical analyses were finished, graphs and figures were made using Excel 2010. The following abbreviations are used: VHJ, villus height jejunum; CDJ, crypt depth jejunum; VCRJ, villus crypt ratio jejunum; VHI, villus height ileum; CDI, crypt depth ileum; VCRI, villus crypt ratio ileum.

2.4.3 Effect of time

For this statistical analysis two different univariate general linear models were used. To calculate the effect of time, the following statistical model was used:

Y = α + Group + Time + E

Y =	dependent variable: VHJ, CDJ, VCRJ, VHI, CDI and VCRI
α =	intercept
Group = FOS or control =	fixed effect
Time =	day 2, 14 or 25
E =	residual error

This univariate general linear model corrected for the effect of the dietary intervention (control piglets of piglets receiving FOS). This model was used to calculate the (significant) differences between the three different time-points of the intestinal health parameters (table 5). There has not been a correction for the sow effect or the effect of different age of piglets at t=0, because piglets of the four different sows were evenly spread over the control piglets and piglets receiving FOS.

2.4.4 Effect of dietary intervention

Another univariate general linear model was used to calculate the effect of the FOS intervention, where the following statistical model was used:

Y = α + Sow + Group + Group*Sow + Group*Piglet + E

Y =	dependent variable : VHJ, CDJ, VCRJ, VHI, CDI and VCRI
α =	intercept
Sow = one of the four sows =	fixed effect
Group = FOS or control =	fixed effect
Group*Sow =	interaction effect
Group*Piglet =	interaction effect
E =	residual error

By using this univariate general linear model there is corrected for the effect of time (day 2, 14, or 25). This model was used to calculate the (significant) differences between the control piglets and piglets receiving FOS of the intestinal health parameters.

2.4.5 Other relations in the dataset

In the first step the effect of the dietary intervention and the effect of time on the villus height, crypt depth and villus:crypt ratio in jejunum and ileum were investigated. In the second step only the average villus height, average crypt depth and the average villus:crypt depth ratio of the jejunum and ileum of each piglet were used. In this step the effect of the vitality at birth, colostrum uptake and growth performances on the AVHJ, ACDJ, ACVRJ, AVHI, ACDI and ACVRI (intestinal health parameters) were investigated.

2.5 Microbiota data

2.5.1 DNA Extraction

Jejunal and colon content was snap frozen in liquid nitrogen and stored at -80°C. For the microbial DNA extraction the following protocol was used. Jejunal content was mixed 1:1 with phosphate buffered saline (PBS) and vortexed, subsequently it is centrifuged for 5 minutes (300g) at 4°C. The supernatant was transferred to a new tube and spun for 10 minutes (9000g) at 4°C, thereafter supernatant was removed. DNA 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 Nanodrop (Agilent Technologies).

2.5.2 Amplification of 16S rDNA (V3-PCR)

PCR was used to amplify the 16S rDNA V3 fragment using forward primer V3_F (CCTACGGGAGGCAGCAG) and reverse primer V3_R (ATTACCGCGGCTGCTGG). PCR conditions were as follows: 2 m at 98°C, 15 x (10s at 98°C, 30 s at 55°C, 10 s at 72°C), 7 min at 72°C. PCR efficiency was checked on agarose gel by visual inspection.

2.5.3 Sequence analysis and bioinformatics (QIIME)

Samples were sequenced by targeted-amplicon 16S sequencing using the MiSeq sequencer (Illumina) and analysed for taxonomy profile per sample with clustering by profile by using QIIME [38]. 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) [39, 40]. Furthermore chimeras were removed with Chimeraslayer [41].

2.5.4 Statistical analysis

The biodiversity was calculated by the vegan package (http://cran.r-project.org/web/packages/vegan/) within the R environment, by employing the Shannon diversity index. The Redundancy analysis (RDA) was also performed by using the vegan package, the following model was ran 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.
Absolute values of microbiota can be calculated by using a conversion factor (Appendix 3), however because no statistical significance was observed between the groups here we only show the relative abundance.

The following abbreviations are used for treatment groups: control.02, day 2 control piglets; control.14, day 14 control piglets; control.02, day 14 piglets receiving FOS; control.25, day 22 control piglets; FOS.25, day 25 piglets receiving FOS.

2.6 Transcriptomics data

2.6.1 RNA Extraction Tissue

Total RNA was extracted from 50 to 100 mg jejunum or colon tissue. All samples were homogenised using the TisuPrep Homogenizer Omni TP TH220P) in TRizol reagent (Life Technologies) as recommended by the manufacturer with minor modifications. The homogenised 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. Quality Control was performed by Agilent Bioanalyser.

2.6.2 Labelling, Hybridization, Scanning and Feature Extraction

Labelling 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 labelled cRNA is used on the eight 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 hours. After 17 hours the arrays are washed as described in the One-Color Microarray-Based Gene Expression Analysis Low input Quick Amp Labelling protocol from Agilent. The arrays where 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 (v10.7) for 1 colour gene expression.

2.6.3 Data Analysis

The data were analysed by using R (v3.0.2) by executing different packages, including LIMMA [42] and arrayQualityMetrics [43]. The data were read in and background corrected (method="normexp" and offset=1) with functions from the R package LIMMA [42] from Bioconductor [44]. Quantile normalisation of the data was done between arrays. The duplicate probes mapping to the same gene were averaged (‘avereps’) and subsequently the lower percentile of probes were removed in a three-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.

The following abbreviations are used for treatment groups: control.02, day 2 control piglets; control.14, day 14 control piglets; control.02, day 14 piglets receiving FOS; control.25, day 22 control piglets; FOS.25, day 25 piglets receiving FOS.

2.6.4 Statistical and Functional Genomics Analysis

To test the differences between the experimental groups (control and FOS) on both day 14 and 25, the following contrasts were generated, FOS.14-Control.14 and FOS.25-Control.25, within the LIMMA package [42]**.** DAVID was used to perform Functional Annotation Clustering (FAC) for the two different contrasts, i.e. FOS.14-Control.14 and FOS.25-Control.25. However, the up- and down-regulated genes were separately analysed.

3 Results

3.1 Performance

The average daily gain (ADG) of the piglets in the control and test group is shown in Figure 2. There was no significant effect of treatment on body weight gain.

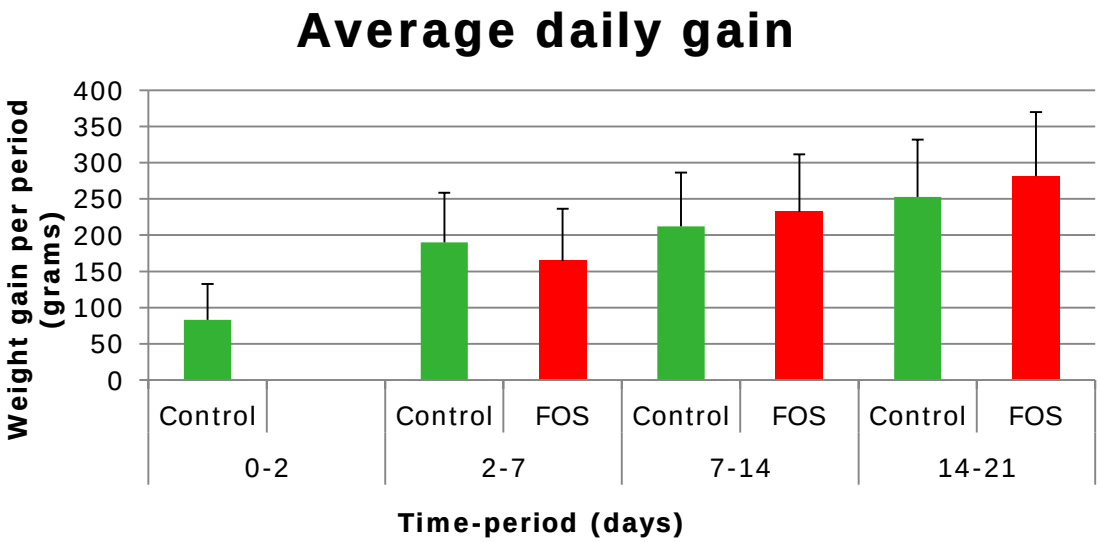

Figure 2. Average daily gain of the piglets.
The x-axis depicts different time slots and the y-axis depicts the average daily gain in grams. Error bars represent the standard deviation. All piglets of this study, 0-2: n=40, 2-7: n=32, 7-14: n=32, 14 until 21: n=16.

The ADG of the piglets of the control piglets and piglets receiving FOS were not significantly different ($p>0.05$), but as shown in Figure 3 the average body weight (ABW) of piglets receiving FOS was numerically higher at every time-point. The average body weight at weaning of the control piglets was 7,016 g compared to 7,678 g for piglets receiving FOS.

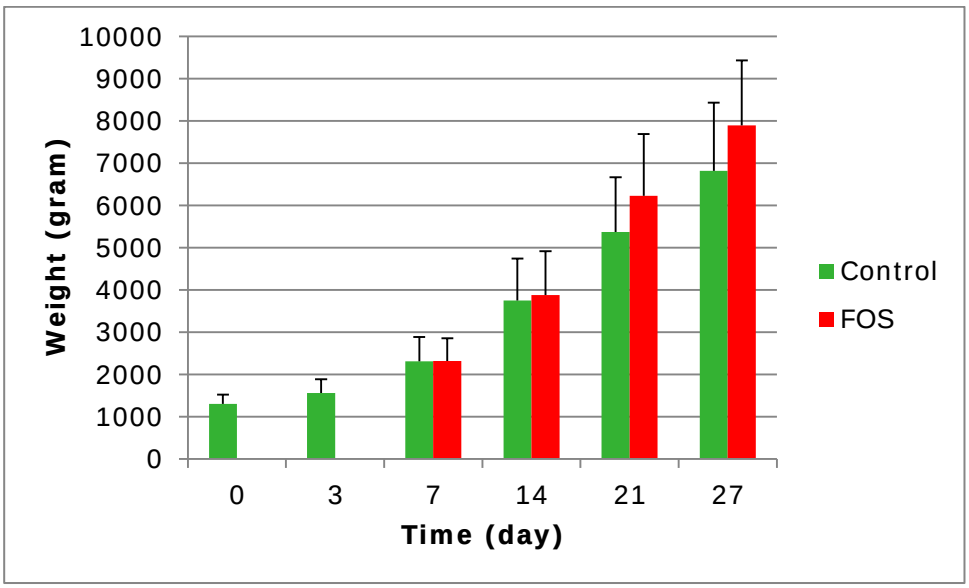

Figure 3. Average body weight of piglets.
The x-axis depicts different time-points and the y-axis depicts the average body weight in grams. Error bars depict the standard deviation. *All piglets of this study are included, day 0 (birth, n=40), 3 (n=40), 7 (n=32), 14 (n=32), 21 (n=16), and day27 (weaning, n=16).*

The average birth weight of all 40 piglets was 1,300 g, the average vitality score at birth was 2.05, and the colostrum intake 242 gram (Table 3). Retrospectively, on average piglets receiving FOS had a higher birth weight (approximately 80 gram) compared to control piglets. However, due to high variation in these measurements, birth weight, vitality score, and colostrum intake, differences between treatment groups were not statistically significant. No effect for sow/litter was considered as piglets within a litter were evenly distributed over both treatment groups.

Table 3.

Birth characteristics per treatment group (retrospective).

Group	n	Birth weight[1]		Vitality score[2]		Colostrum intake[1]	
		Mean	**SEM[3]**	**Mean**	**SEM**	**Mean**	**SEM**
All	40	1,300	35	2.05	0.1	242	14
Control.2	8	1,240	54	2.38	0.2	226	17
Control.14	8	1,300	113	2.00	0.2	222	46
FOS.14	8	1,380	867	1.75	0.3	206	29
Control.25	8	1,250	57	1.88	0.3	271	22
FOS.25	8	1,360	73	2.25	0.3	284	37

[1]in grams, [2]Vitality score is 0, 1, 2, or 3 (detailed explanation of score in section 2.2.2), [3]Standard error of the mean

3.2 Histology

3.2.1 Effect of time

Villus height, crypt depth and the villus:crypt ratio in both jejunum and ileum were measured at three different time-points (Table 4). The results shown in Table 4 show the effect of time (i.e. development) irrespective of the dietary intervention. Except for the villus:crypt ratio in ileum, there were no significant differences between day 2 and day 14. At day 25, the villus height of jejunum and ileum were significantly lower compared to day 2 or day 14. The crypt depth of ileum was significantly deeper at day 25 compared to day 2 and 14. Both the jejunal and ileal villus:crypt ratio significantly differed between day 25 and day 14 (as well as day 25 vs. day 2).

Table 4.

Time effect on the villus height, crypt depth and villus:crypt ratio in the jejunum and ileum. Data are expressed as least squared means ± standard error.

	Jejunum			Ileum		
Day	**Villus height**	**Crypt depth**	**V:C[1] ratio**	**Villus height**	**Crypt depth**	**V:C[1] ratio**
2	719^{a} ± 24.8	159 ± 8.1	5.27^{a} ± 0.4	937^{a} ± 31.9	144^{a} ± 8.8	8.11^{a} ± 0.6
14	689^{a} ± 17.5	170 ± 5.7	4.92^{a} ± 0.3	889^{a} ± 22.5	163^{a} ± 6.2	6.60^{b} ± 0.4
25	463^{b} ± 17.5	162 ± 5.7	3.92^{b} ± 0.3	624^{b} ± 22.5	182^{b} ± 6.2	5.42^{c} ± 0.4

[1] V:C; villus:crypt

$^{a, b, c}$: Different superscripts within a column indicate a statistical significance at the level $p<0.05$

3.2.2 Effect of dietary intervention

Villus height, crypt depth and the villus:crypt ratio in jejunum and ileum of the control piglets and piglets receiving FOS at two different time-points are summarized in (Table 5). Both at day 14 and at day 25 the piglets that received FOS had a differences in villus height in the ileum compared to control piglets. At day 14 higher ilium villi were observed in control piglets, whereas at day 25 the contrary was observed. Also, Jejunal villi height of piglets receiving FOS was significantly higher at day 25 compared to the control piglets, furthermore the crypt depth for jejunum was significantly deeper compared to control piglets. In ileum opposite observations were made, in which crypt depths of

control piglets were significantly deeper compared to piglets receiving FOS. For villus:crypt ratios no significant observation were made for either jejunum or ileum.

Table 5.

Effect of dietary intervention on the villus height, crypt depth and villus:crypt ratio in the jejunum and ileum at two different measurement points.

	Jejunum			Ileum		
Treatment	**Villus height**	**Crypt depth**	**VC[1] ratio**	**Villus height**	**Crypt depth**	**VC ratio**
Control.14	672 ± 22.7	168 ± 7.4	4.7 ± 0.4	942[a] ± 28.1	170 ± 6.1	6.3 ± 0.5
FOS.14	706 ± 22.7	171 ± 7.4	5.1 ± 0.4	836[b] ± 28.1	156 ± 6.1	6.9 ± 0.5
Control.25	423[a] ± 11.5	144[a] ± 7.6	3.9 ± 0.3	584[a] ± 20.6	199[a] ± 8.4	5.0 ± 0.6
FOS.25	504[b] ± 11.5	179[b] ± 7.6	3.9 ± 0.3	663[b] ± 20.6	164[b] ± 8.4	5.9 ± 0.6

[1] VC; villus:crypt

* Data were expressed as least squared means ± standard deviation

a, b Different subscripts within a column indicate a statistical significance at the level $p<0.05$

3.3 Microbiota analyses

3.3.1 Colon

To investigate the differences between the treatments regarding the microbiota, three analyses were performed. 1) microbiota composition, 2) microbiota diversity, and 3) top lists of microbiota genus/species.

The microbiota composition differed between piglets receiving FOS and control piglets (Figure 4). To investigate which species contribute to this different composition we focused on the microbial species that are often named having a ‘bifidogenic effect’, i.e. Lactobacillus and Bifidobacterium (Figure 5).

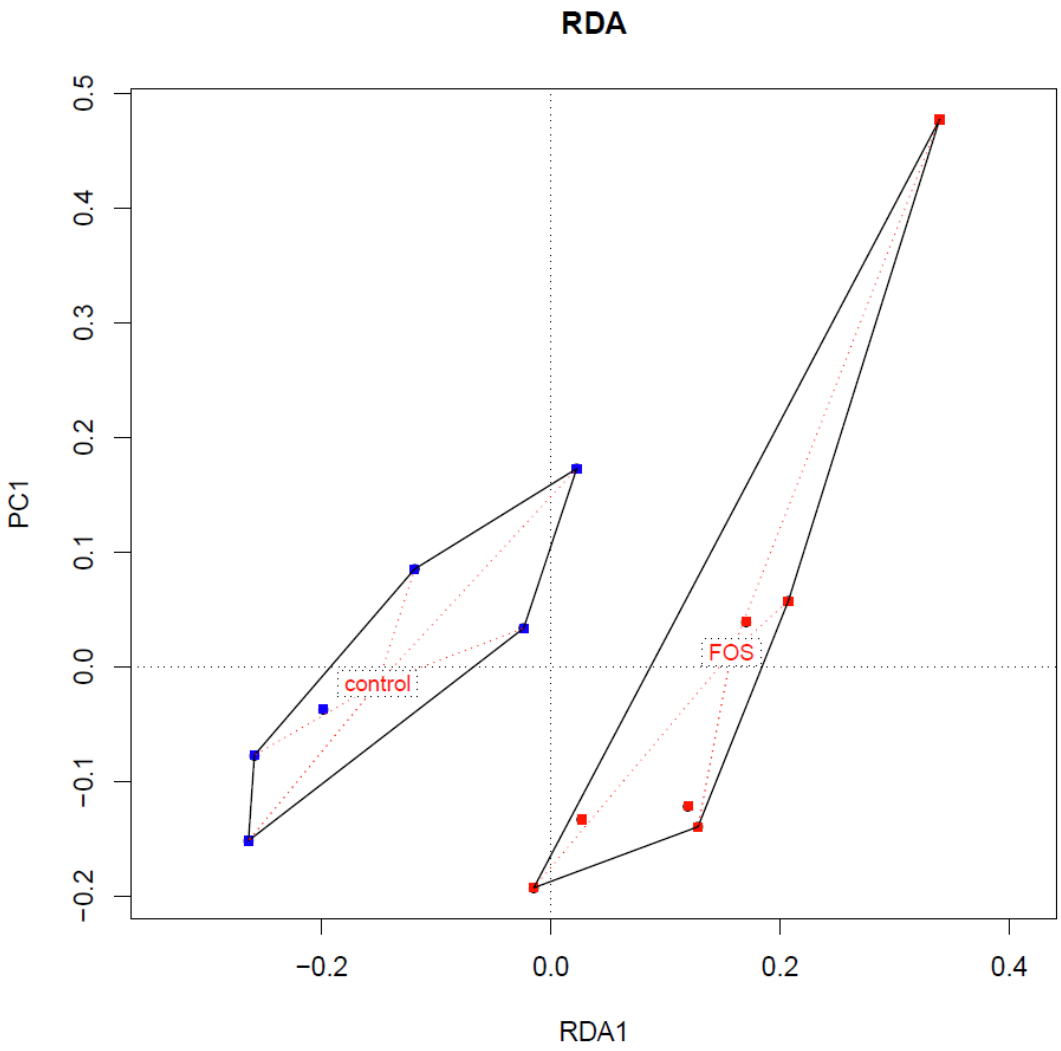

Figure 4. Redundancy analysis (RDA) of family level microbiota in pig colon.
*The x-axis depicts explanatory axis 1 (RDA1) and y-axis depicts explanatory axis 2 (RDA2). Each condition is represented by a different colour, i.e. day 14 controls is blue and piglets receiving FOS is red. The following model was used as input for the RDA: y = Time + Treatment + Time * Treatment + error, and was found significant p-value is 0.01.*

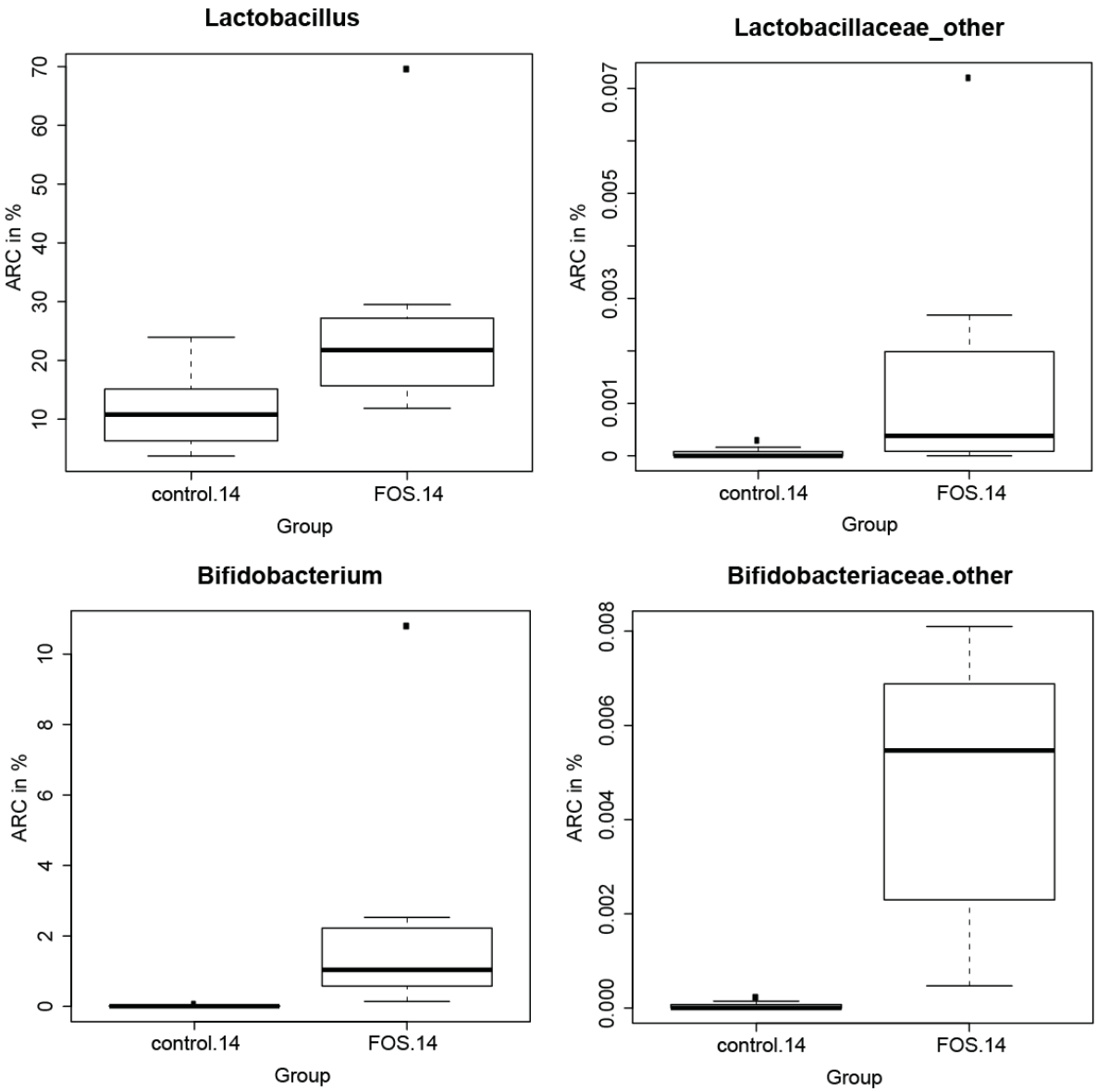

Figure 5. Differences in specific bacterial species that exert a 'bifidogenic effect' in colon at day 14. *The x-axis depicts control piglets or piglets receiving FOS at day 14, whereas the y-axis depicts the average relative contribution (ARC) in percentage. Top left Lactobacilli are depicted (p=0.05), top right Lactobacillaceae_other (p=0.11), bottom left Bifidobacterium (p=0.08), and bottom right Bifidobacteriaceae.other (p<0.001). p-values were calculated by a Student's T-test.*

The microbiota diversity, measured by the Shannon index, is for both piglets receiving FOS and control piglets approximately 3.1 (Table 6).

Table 6.

Diversity calculations of luminal microbiota in colon at day 14 for controls and piglets receiving FOS.

Treatment	Diversity[1]
control.14	3.12 ± 0.06[2]
FOS.14	3.16 ± 0.06[2]

[1] Calculated by the Shannon index
[2] Standard error of the mean

To investigate the colon microbiota immediately after the period of FOS treatment (day 14) we listed the top 10 most abundant microbial species for both the control and piglets receiving FOS (Table 7). The highest average relative contribution (ARC) of microbiota in control pigs are Bacteroidia_Other, Bacteroidia, and Bacteroidia_S24-7 (Note that all these Bacteroida were not assigned genus or species names). Whereas in piglets receiving FOS the highest ARC of microbiota are Bacteroidia_S24-7 followed by *Lactobacillus reuteri* and *Prevotella stercorea*.

Table 7.

Top 10 abundant microbiota genus/species (%) in pig colon at day 14 for controls and piglets receiving FOS.

Genus	Species	Control.14	FOS.14
*Bacteroidia_*[1]		**11.9**[2,3]	**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
All other bacteria combined		29.5	44.8
	Sum	100	100

1) Italics are family or class names, 2) Average relative contribution, (%) 3) Bold depicts the top 10 per treatment

3.3.2 Jejunum

We investigated if FOS had an effect on the microbiota composition in the small intestine, for which we sampled luminal microbiota at days 2, 14, and 25 in both controls and piglets receiving FOS. An overview of the average relative abundance of microbial species is given in Table 8. The data show that Lactobacilli were the most abundant throughout the suckling period. At day 2 *E. coli* was present up to 9% (average relative abundance) in jejunum, which decreased in the controls at day 14 and 25, 1.9 and 2.5% respectively. In piglets receiving FOS, *E. coli* was relatively high 8.0% at day 14 and 5.9% at day 25. At day 14 and 25 Turicibacter is also dominant; values ranging from 5.6 to 8.6%.

Table 8.

Top 10 abundant microbiota genus/species in pig jejunal digesta at day 2, 14, and 25 for control and FOS.

Genus	Species	Control.02	Control.14	FOS.14	Control.25	FOS.25
Lactobacillus		**33.1**[1,2]	**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[3]		**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**
All other bacteria combined		13.0	11.8	12.1	24.	27.1
	Sum	**100**	**100**	**100**	**100**	**100**

1) Average relative contribution, 2) Bold depicts the top 10 per treatment, and 3) Italics are family or even class names

The diversity was measured by the Shannon index, in time from day 2 to 25 the average Shannon index increases from approximately 2.14 to 2.45 in piglets receiving FOS, whereas control piglets show a slight decrease from 2.14 to 1.92 (Table 9). Due to the high variation in the relative abundance of microbiota genus/species among individual piglets within treatments no differences were observed when comparing piglets receiving FOS versus the controls.

Table 9.

Microbial diversity in jejunum for the different experimental treatments and time points.

Treatment	Diversity[1]
control.02	2.14 ± 0.17[2]
control.14	2.28 ± 0.21
FOS.14	2.29 ± 0.14
control.25	1.92 ± 0.09[a]
FOS.25	2.45 ± 0.09[a]

[1] Calculated by the Shannon index
[2] Standard error of the mean
[a] Different subscripts within a column indicate a statistical significance at the level $p<0.001$ (Students T-test)

To further evaluate the microbiota composition in the jejunum samples, a redundancy analysis was performed, which shows a high overlap of all different treatment groups, both day (2, 14, and 25) and treatment (piglets receiving FOS vs. control piglets) (Figure 6). Although multiple samples in both FOS.14 and FOS.25 seem to shift towards the bottom-right, away from the respective controls.

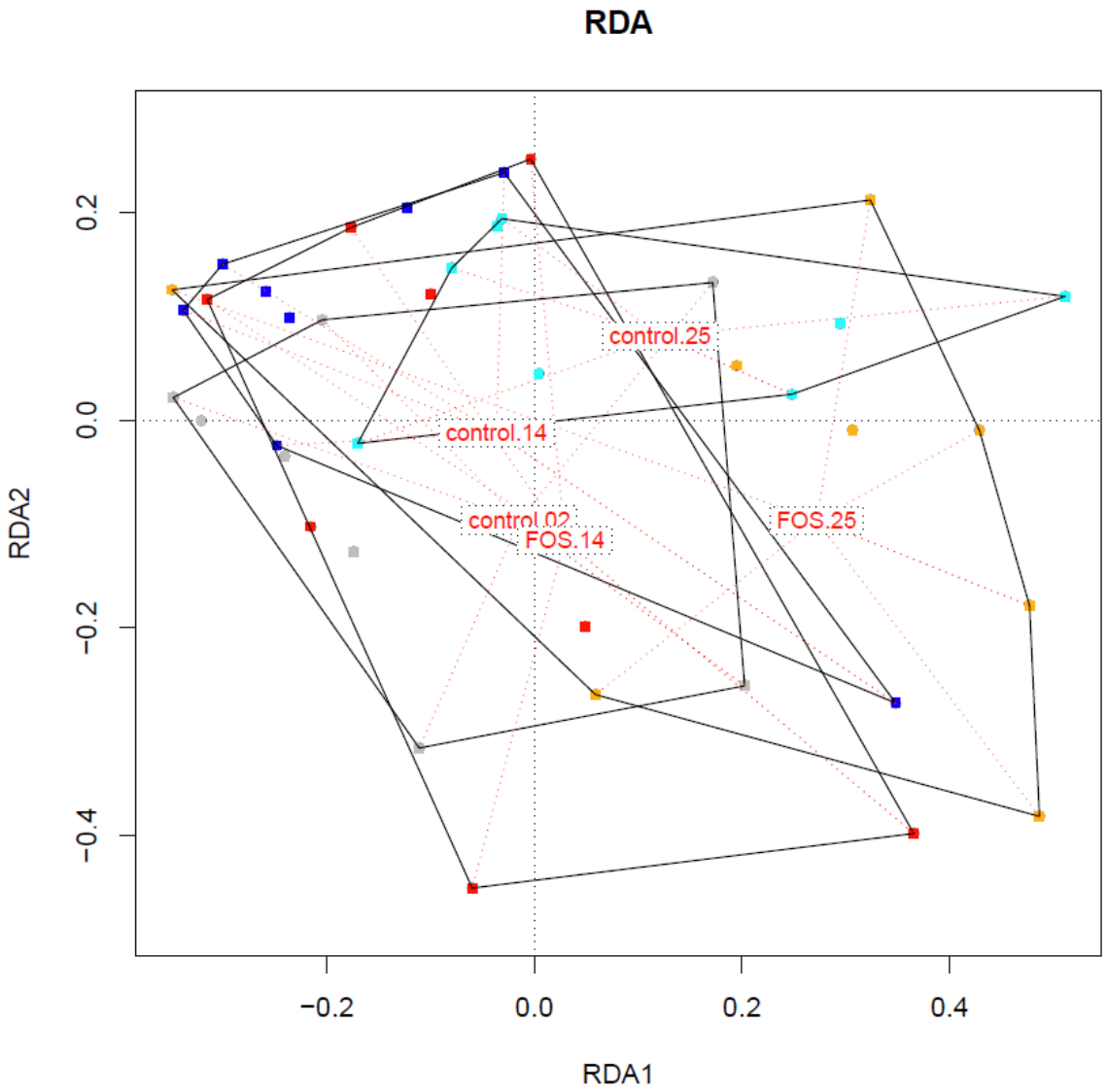

Figure 6. Redundancy analysis (RDA) of family level microbiota in pig jejunum.
*The x-axis depicts explanatory axis 1 (RDA1) and y-axis depicts explanatory axis 2 (RDA2). Each condition is represented by a different colour, i.e. day 2, grey; day 14 control is blue and FOS is cyan; and day 25 control is red and FOS is orange. The following model was used as input for the RDA: y = Time + Treatment + Time * Treatment + error.*

3.4 Transcriptomic analyses

3.4.1 Colon

First we investigated the overall gene expression of all samples at day 14. This did not result in a clear separation of treatments (Figure 7). Secondly, LIMMA statistical testing did not result in any significantly expressed genes between treatments. Also GSEA was performed in order to test for differences in expression of gene sets between control and piglets receiving FOS. This analysis did not result in the identification of gene sets differentially expressed (FDR<1%).

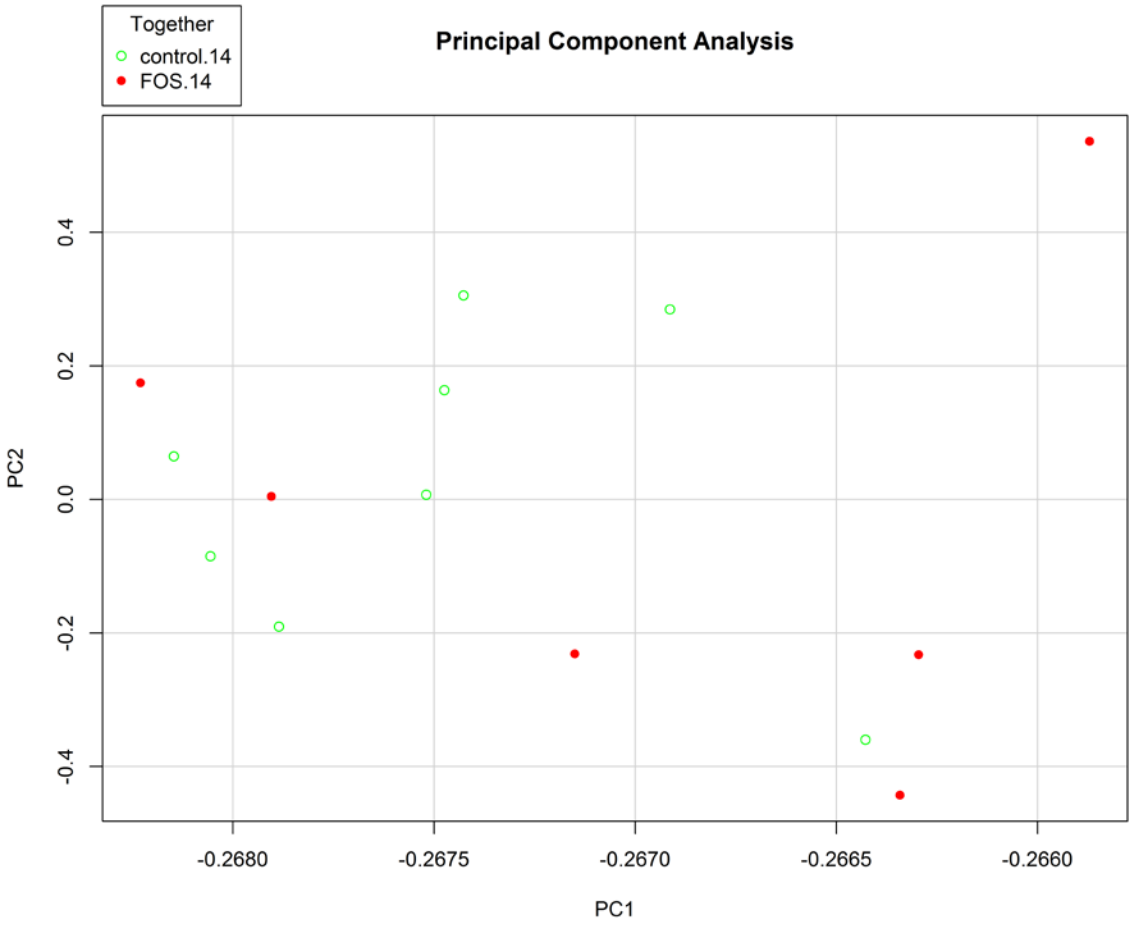

Figure 7. Principal Component Analysis (PCA) of colon transcriptomics data at day 14.
The x-axis depicts principal component 1 (PC1) and y-axis depicts principal component 1 (PC2). FOS treatment are filled red circles, whereas the controls are open green circles. Abbreviations: FOS; Fructooligosaccharides.

3.4.2 Jejunum

First we investigated the overall gene expression of all samples at all time-points together (day 2, 14, and 25). In other words, all values for gene activity are taken into account (no up- or down-regulation is measured yet, because no comparisons are made yet). This resulted in a clear separation in time of the samples (Figure 8). However, piglets receiving FOS did not distinct significantly from the control group at either time-point, but only showed a trend on day 14.

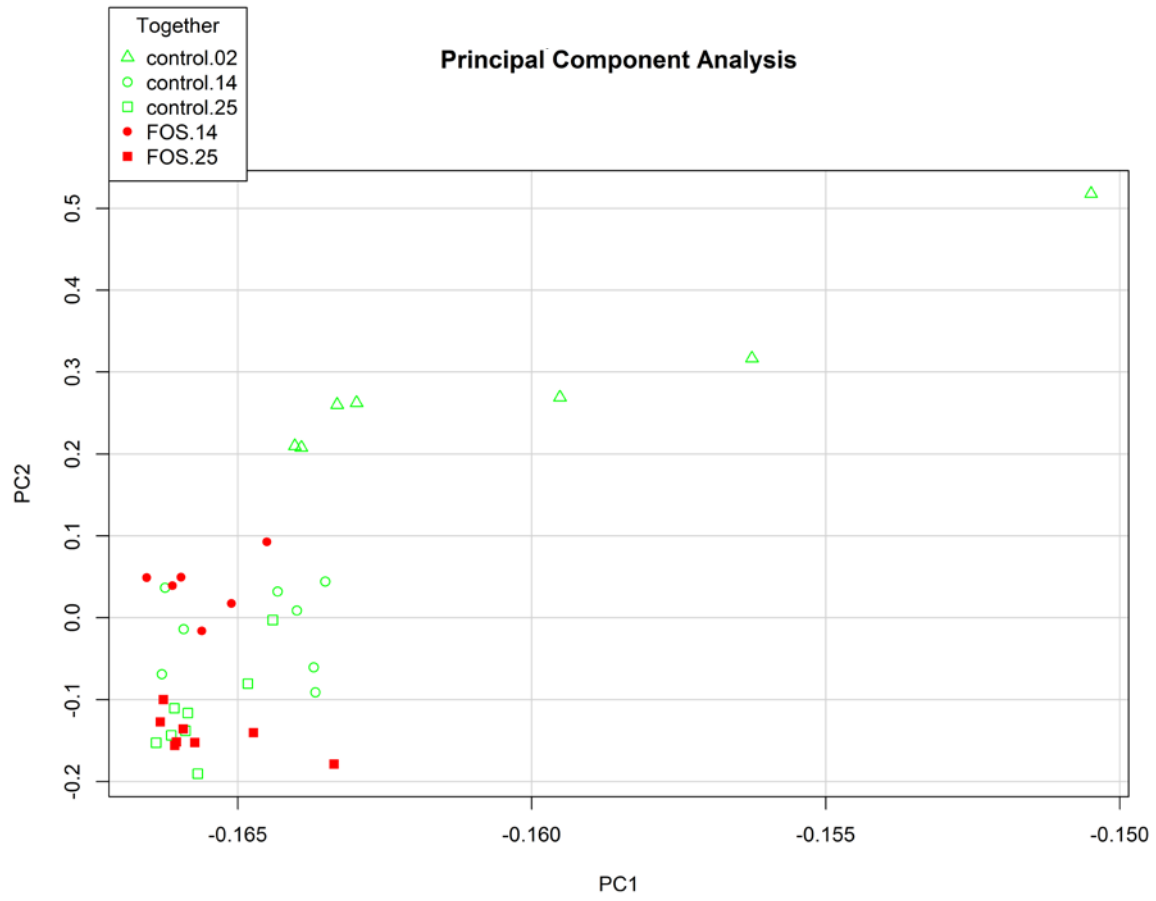

Figure 8. Principal Component Analysis (PCA) of jejunal transcriptomics data.
The x-axis depicts principal component 1 (PC1) and y-axis depicts principal component 1 (PC2). Each day is represented by a different symbol (day 2, triangle; day 14, circle; and day 25, square). The FOS treatment are filled red symbols, whereas the controls are open green symbols. Abbreviations: FOS; Fructooligosaccharides.

The second step was to identify differential expressed genes and/or processes between the piglets receiving FOS and control piglets. Statistical analyses did not result in differential expressed genes, however gene set enrichment analysis (GSEA) resulted in 26 significant enriched gene sets (FDR<1%) for control compared to FOS treatment and 9 significant gene sets for the FOS treatment compared to control on day 14 (Table 10). At day 25 for the controls 8 significant gene sets were observed and 1 for the FOS treatment (Table 11). Gene sets are predefined sets of genes with a common denominator, including 'cell cycle', 'receptor activity', or 'locomotory behaviour'. The enriched gene sets in control piglets at day 14 were all associated to cell cycle related processes, whereas piglets receiving FOS showed processes related to the extra cellular matrix (ECM). This means that genes belonging to 'cell cycle' were lower expressed in piglets receiving FOS compared to control piglets, whereas higher expression in piglets receiving FOS was observed for 'ECM'. At day 25, three out of the eight significant gene sets were involved in chemokine/cytokine signalling and were lower expressed in piglets receiving FOS compared to control piglets.

Table 10.

Gene Set Enrichment Analysis of pig jejunum at day 14.

Enriched Gene sets in control piglets			
NAME	**SIZE**	**NES[1]**	**FDR[2]**
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	-2.30	0
Cell cycle phase	88	-2.29	0
Chromosomal part	50	-2.27	0
Chromatin modification	28	-2.23	1.5E-04
M phase	58	-2.21	2.6E-04
Mitotic cell cycle	81	-2.19	3.5E-04
M phase of mitotic cell cycle	44	-2.19	3.2E-04
Cell cycle	161	-2.13	1.3E-03
Mitosis	42	-2.11	1.8E-03
Spliceosome[3]	63	-2.06	2.9E-03
DNA replication	47	-2.03	3.0E-03
ATP dependent helicase activity	16	-2.02	3.3E-03
DNA dependent DNA replication	26	-2.01	3.8E-03
Systemic lupus erythematosus	52	-2.00	4.3E-03
Chromatin	18	-1.98	5.5E-03
RNA helicase activity	15	-1.97	5.8E-03
Centrosome	35	-1.97	5.9E-03
Nuclear chromosome	24	-1.96	6.6E-03
Nuclear part	284	-1.93	8.5E-03
Spindle	24	-1.93	8.4E-03
Microtubule cytoskeleton	81	-1.92	9.2E-03
Base excision repair	17	-1.92	9.3E-03
Enriched Gene sets in piglets receiving FOS			
Name	**SIZE**	**NES**	**FDR**
Proteinaceous ECM	33	2.09	2.7E-03
Extracellular matrix	34	2.07	2.3E-03
Transmembrane receptor activity	129	2.03	2.4E-03
ECM receptor interaction	42	1.99	5.2E-03
Extracellular matrix part	22	1.98	7.1E-03
Receptor activity	203	1.96	7.2E-03
G protein coupled receptor activity	42	1.95	6.9E-03
Extracellular region	161	1.95	6.0E-03
Extracellular region part	124	1.94	6.9E-03

[1]Normalized Enrichment Score, The normalized enrichment score (NES) is the primary statistic for examining gene set enrichment results. By normalizing the enrichment score, GSEA accounts for differences in gene set size and in correlations between gene sets and the expression dataset; therefore, the normalized enrichment scores (NES) can be used to compare analysis results across gene sets.

[2]False Discovery Rate, The false discovery rate (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.

[3]Italic gene sets represent KEGG pathways

Table 11.

Gene Set Enrichment Analysis of pig jejunum at day 25.

Enriched Gene sets in control piglets - day 25			
Name	**SIZE**	**NES[1]**	**FDR[2]**
Chemokine activity	21	-2.41	0
Chemokine receptor binding	22	-2.37	0
G protein coupled receptor binding	27	-2.31	0
Locomotory behaviour	42	-2.22	0
Cytokine-cytokine receptor interaction[3]	103	-2.20	2.1E-04
Behaviour	63	-2.06	2.8E-03
Carbohydrate binding	28	-2.05	2.5E-03
ECM receptor interaction	42	-2.00	8.2E-03
Enriched Gene sets in piglets receiving FOS - day 25			
Name	**SIZE**	**NES**	**FDR**
Retinol metabolism	15	2.12	8.4E-03

[1]Normalized Enrichment Score, [2]False Discovery Rate, [3]Italic gene sets represent KEGG pathways

4 Discussion and conclusions

The aim of this study was to investigate the effect of an oral administration (d 2-14) of fructooligosaccharides (FOS) on intestinal health-related parameters of neonatal piglets in the pre-weaning (suckling) phase. It was hypothesized that an oral administration of FOS to neonatal piglets increases the immune competence via a bifidogenic effect in the colon. The study also aimed to detect indicators associated with gut development in terms of immune competence and functionality. It is known that during the neonatal period a life-long influence can be exerted on the development and competence of the immune system. The challenge is to identify the early life physiological parameters that are associated with an accurate immune competence later in life.
In the neonatal piglets we identified potential indicators for immune competence, i.e. higher abundance of 'bifidogenic' bacteria in the colon and expression of sets of genes encoding for 'cell cycle', 'extracellular matrix', and 'chemokine/cytokine activity'. Below we discuss the limitations of the experiment, and effects of postnatal oral administration of FOS on performance, intestinal histology, microbiota composition in the small and large intestine and intestinal gene expression is mucosal tissues.

4.1 Limitations of the test model

Stress
During the oral administration period of FOS (day 2-14) some piglets vomited after administration of FOS. This could have influenced the outcome of the experiment with respect to the pig growth performance and intestinal immune parameters. The former could be due to the volume of fluid administered, the handling of the piglet itself during the administration, or separation from the sows one hour prior to administration. Each of these factors can induce stress. It has already been established that stress may impact various components of the intestinal barrier function in rats and humans [45]. The structure of the tight junctions may change and permeability may increase. Stress stimulates the secretion of water, IgA, mucus and ions into the lumen. This increased intestinal permeability caused by stress is restored after one or two weeks in male rat pups (neonates) [46]. The rat pups were daily separated from their mother for three hours between day 2 and 12 after birth and this early life stress altered the faecal microbiota and increased the systemic immune response [46]. In conclusion, we cannot rule out the role and impact of stress of oral administration of fluid in the postnatal phase as part of the intervention evaluated on the results in the present experiment. It should be indicated that the fluid was given to both the FOS experimental group as well as the control group. Therefore, stress was not a variable in the experimental design.

Diarrhoea
Most of the piglets, irrespective of control or FOS group, had diarrhoea between day 2 to 7. This is most probably due to the stress, handling and oral administration of water or FOS solution (15 ml) twice a day. Due to these observations the protocol was changed at day 7 to an oral administration of 6 ml in control piglets and 6 ml containing the same quantity of FOS in the treatment group. This reduced volume resulted in less diarrhoea. In theory, this reduction in diarrhoea could also be affected by the supplementation of FOS, because high dosage of FOS could stimulate hyper fermentation in the large intestine [47]. This rapid fermentation leads to malabsorption of short chain fatty acids (SCFAs) because of rapid transit out of the colon [48]. In weaned pigs an inclusion level of 3% FOS could lead to diarrhoea [47]. Another study also reported a significant increased risk on diarrhoea incidence of piglets after addition of FOS [24]. Because irrespective of the experimental treatment piglets had diarrhoea, it is assumed that the diarrhoea was mainly caused by the stress imposed by the fluid administration. In conclusion, the results of the current study may be influenced by the stress and diarrhoea caused by the handling of the piglets and not by the intervention itself. Therefore it is still possible to evaluate the differences between the intervention and the control.

4.2 Performance and histology

This study was not designed to evaluate growth performance properly (to little power), however there still were significant changes and/or trends worth of mentioning. In the present study, the average

daily gain of the piglets over the period of day 2-21 was not significantly different between treatment groups. However, piglets receiving FOS had a higher average body weight (7.7 kg) at weaning compared to controls (7.0 kg), which is in agreement with observations in suckling piglets by Grela *et al.* [49] and in weaned piglets by Houdijk *et al.* [50], i.e. increased body weight after FOS treatment. A meta-analysis study showed that mannan oligosaccharides (a different group of NDOs) also have the potential of a growth promotor in nursery piglets [51]. A significant increase in body weight was observed in weaned piglets when 4 to 6 g FOS per kg diet was added [52]. Another study on suckling piglets also showed a significant increase of the average daily weight gain in the FOS supplemented group [29]. The observations made in these studies together, was that compared to the (weaned) control pigs, pigs receiving supplemented feed had an improved body weight gain and feed conversion. The precise mechanism underlying these observations are not yet known, but it is likely that the microbiota play an important role [53, 54]. Growth rate was improved by 4.1% when including mannan oligosaccharides (derived from specific yeast strains) compared to pigs of which the diet was not supplemented with mannan oligosaccharides [51]. Due to low number of piglets and high variation between piglets in our study, it is difficult to draw firm conclusions regarding the effects of FOS administration in the postnatal period on the growth performance.
In time, the jejunum and ileum developed during the course of the experiment and this is reflected in our data by a significantly decrease in the average villus height (Table 5). An earlier study [12] concluded that the villus height in the jejunum and ileum were increased by FOS supplementation at day 7 after birth. However, in the latter experiment neonatal piglets were used in combination with a jugular catheter and an 80% jejunoileal resection. Furthermore, those piglets had no access to sow's milk and got 10 g/L scFOS. The use of the catheter may cause less stress in those piglets compared to the oral administration of fluids in our study.On the other hand, the observed decrease in villus height in our data (ileum day 14) are in correspondence to the findings of Skrzypek *et al.* [55]. They measured the villus height in the mid-jejunum of suckling piglets at day 0, 3, 7 and 14 of life and the villus height decreased significantly from 980 µm (± 304 µm) on day 3 to 390 µm (± 152µm) on day 14. They did observe an increase in villus height between birth (441 ± 162 µm) and day 3 (980 ± 304 µm) [55], presumably because solid feed was introduced which triggers development and differentiation of the gut tissue. In our data the average crypt depths of both jejunum and ileum were deeper at later time-points (day 14 and 25) compared to day 2. This coincides with the height of the villi, because the crypts are the base from which enterocytes proliferate and migrate to the villi [29] and our measured crypt depths were in similar order of magnitude to Skrzypek *et al.* [55]. Lastly, in our study the villus to crypt ratio significantly decrease in time, this may be reflected by the aging and increasing amounts of digesta passing. In our data, the average villus height and average crypt depth of jejunum at day 25 were both significantly higher in piglets receiving FOS compared to control piglets, this increased villus height and deeper crypts suggest a higher turnover of epithelial cells and a larger absorptive capacity. This was less clear in ileum at day 25, because the average villus length was significantly higher in piglets receiving FOS, the average crypt depth was significantly lower.

4.3 Microbiota and gene expression

Microbiota
Microbiota composition in jejunum was not found different between FOS supplemented and control piglets. Significant differences in microbiota composition/diversity were observed in the colon. This is in agreement with information in the literature which indicates that FOS mainly impacts the microbiota in the colon. Still, we observed that the diversity of microbiota (Shannon index) in the jejunum was significantly increased ($P<0.001$) at day 25 in piglets receiving FOS compared to control piglets, 2.45 vs. 1.92 respectively.
In colon a clear effect of FOS is observed in the microbiota composition, where an increase of lactobacilli and bifidobacteria was observed in piglets receiving FOS. Such an effect is often called a 'bifidogenic effect', leading to a higher synthesis of butyrate. Butyrate is fuel for colonocytes and promotes cell differentiation and proliferation [56]. Furthermore, these short chain fatty acids regulate sodium and water absorption [57], enhance calcium and mineral uptake [58], and lower the pH. The latter is important for inhibition of the growth of pathobionts and also stimulates the growth of butyrate producers (such as lactobacilli and bifidobacteria). Studies, investigating the effect of the colon microbiota and its metabolites, have also observed an increase in the number of colonic immune regulator T cells [59, 60], a dampening of the production of pro-inflammatory cytokines by macrophages [61, 62], neutrophils, and higher activity of natural killer (NK) cells (reviewed by [63, 64]). The observed change in colonic microbiota may also lead to modulation of mucin production and reduced risk of gastrointestinal diseases [65], as well as protecting against enteric pathogens (in mice) [66]. All these before mentioned processes occur in the colon, however, it is also possible that

the colon communicates with the small intestine, via feedback loops, that are not yet elucidated. It may be possible that colon communicates with the small intestine via the vagus nerve by transmitting signals to suppress cytokine production [67] and/or via the lymphatic system [68]. Another signalling could be effectuated by luminal diffusion of butyrate or by butyrate signalling towards specific immune cells (mobile macrophages) [69].
The observed intimate interplay between host and microbiota results in complex interactions and systems behaviour in the gut. Understanding different aspects of the gut system will help uncover the intricate relations between host, microbiota, and environment, including the diet. Perturbing single aspects of the (gut) system does not lead to a linear systems behaviour, but causes a cooperative response of multiple aspects altering the systems behaviour in the gut [70]. In other words, when adding a single aspect for example FOS, not only a bifidogenic effect is observed in the colon, but changes in the small intestine can occur simultaneously.

Transcriptomics
Initially we targeted the jejunum for the gene expression studies, because the number and activity of immune cells in this part of the gut is much higher compared to the colon. On day 14 we identified clear differences in gene expression patterns between the two experimental groups. These differences in gene expression result in lower activity of cell cycle processes and higher activity of extracellular matrix (ECM) processes in piglets receiving FOS compared to control piglets. ECM processes include maintenance of cell and tissue structure and function. This lower activity of cell cycle processes in piglets receiving FOS at day 14 did not lead to lower villi or less deep crypts. Because no significant differences were observed in microbiota parameters at day 14 in this part of the gut, this is probably not due to a direct microbiota to host cell cross-talk. These data suggest that the turn-over of epithelial cells is lower in piglets receiving FOS. Probably, the higher gene expression of ECM processes feature the tight junctions/barrier function.
Lower activity of immune related processes was observed at day 25 in piglets receiving FOS. This lower activity suggests lower inflammatory responses by the host, which could be beneficial for the host under certain circumstances. However, this remains speculation because we did not ran the experiment until after weaning.

4.4 Overall conclusion

Fructooligosaccharides (FOS) supplementation to suckling piglets has a bifidogenic effect in the colon on day 14 after birth and an increased diversity of microbiota in jejunum. Both of these parameters can be used as potential indicators for immune competence. Furthermore, the jejunal gene expression on day 14 showed lower activity of cell cycle related processes, whereas extracellular matrix processes showed higher activity in piglets receiving FOS compared to the respective controls. At day 25, jejunal gene expression of piglets receiving FOS showed lower activity of immunological genes compared to the controls, suggesting lower inflammation that may be used as a potential marker for immune competence at young age.

4.5 Potential impact for F4F-VDI industry partners

The work described here shows that it is possible to modulate the intestinal (immune) development by affecting the intestinal microbiota at early age. This experimental animal model intervention for neonatal piglets showed that after supplementation of the piglet diets with FOS, higher butyrate producing bacteria were observed in the colon and lower activity of immune related processes in jejunal tissue. The next step in follow-up research within VDI will be to improve the protocols of dietary interventions in suckling piglets and to investigate (intestinal) immune competence development by the use of other “health-promoting” functional ingredients or specific additives.
From this study we could identify potential indicators for improved immune competence. In colon we observed a bifidogenic effect of FOS, i.e. higher relative abundance of both Lactobacilli and Bifidobacteria. Also, jejunal gene expression at day 25 showed lower activity of genes related to inflammation suppression in piglets receiving FOS, compared to control piglet. This suggests that inflammation suppression may be regarded as a “healthy” trait. However, it is not clear yet whether this is true for all environmental conditions, for example an environment with a high pathogenic load.

References

1. Adlerberth, I. and A.E. Wold, *Establishment of the gut microbiota in Western infants.* Acta Paediatr, 2009. **98**(2): p. 229-38.
2. Boehm, G. and G. Moro, *Structural and functional aspects of prebiotics used in infant nutrition.* Journal of Nutrition, 2008. **138**(9): p. 1818S-1828S.
3. Penders, J., et al., *Factors influencing the composition of the intestinal microbiota in early infancy.* Pediatrics, 2006. **118**(2): p. 511-21.
4. Verdonk, J.M.A.J., et al., *Application of inulin-type fructans in animal feed and pet food.* British Journal of Nutrition, 2005. **93**: p. S125-S138.
5. Biedrzycka, E. and M. Bielecka, *Prebiotic effectiveness of fructans of different degrees of polymerization.* Trends in Food Science & Technology, 2004. **15**(3-4): p. 170-175.
6. Meyer, D. and M. Stasse-Wolthuis, *The bifidogenic effect of inulin and oligofructose and its consequences for gut health.* European Journal of Clinical Nutrition, 2009. **63**(11): p. 1277-1289.
7. Kim, S.W., *Sow Milk*, in *Milk and Dairy Products in Human Nutrition*. 2013, John Wiley & Sons. p. 614-626.
8. Ogawa, S., et al., *Shotgun proteomic analysis of porcine colostrum and mature milk.* Animal Science Journal, 2014. **85**(4): p. 440-448.
9. Albrecht, S., et al., *A comparative study of free oligosaccharides in the milk of domestic animals.* British Journal of Nutrition, 2014. **111**(7): p. 1313-1328.
10. Macfarlane, S., G.T. Macfarlane, and J.H. Cummings, *Review article: prebiotics in the gastrointestinal tract.* Aliment Pharmacol Ther, 2006. **24**(5): p. 701-14.
11. Goldman, A.S., *Evolution of the mammary gland defense system and the ontogeny of the immune system.* J Mammary Gland Biol Neoplasia, 2002. **7**(3): p. 277-89.
12. Barnes, J.L., et al., *Intestinal adaptation is stimulated by partial enteral nutrition supplemented with the prebiotic short-chain fructooligosaccharide in a neonatal intestinal failure piglet model.* JPEN J Parenter Enteral Nutr, 2012. **36**(5): p. 524-37.
13. Boehm, G., et al., *Prebiotic carbohydrates in human milk and formulas.* Acta Paediatr Suppl, 2005. **94**(449): p. 18-21.
14. Haarman, M. and J. Knol, *Quantitative real-time PCR assays to identify and quantify fecal Bifidobacterium species in infants receiving a prebiotic infant formula.* Appl Environ Microbiol, 2005. **71**(5): p. 2318-24.
15. Boehm, G., et al., *Prebiotic concept for infant nutrition.* Acta Paediatr, 2003. **92**: p. 64-67.
16. Sela, D.A. and D.A. Mills, *Nursing our microbiota: molecular linkages between bifidobacteria and milk oligosaccharides.* Trends Microbiol, 2010. **18**(7): p. 298-307.
17. *A mixture of galacto and fructo oligosaccharides shows prebiotic effects and enhances systemic cellular immune responsiveness in mice.* Immunobiology, 2004. **209**(4-6): p. 403-403.
18. Bruzzese, E., et al., *Early Administration of Gos/Fos Prevents Intestinal and Respiratory Infections in Infants.* Journal of Pediatric Gastroenterology and Nutrition, 2006. **42**(5): p. E95.
19. Onishi, N., A. Yamashiro, and K. Yokozeki, *Production of Galacto-Oligosaccharide from Lactose by Sterigmatomyces-Elviae Cbs8119.* Applied and Environmental Microbiology, 1995. **61**(11): p. 4022-4025.
20. Davis, L.M.G., et al., *A dose dependent impact of prebiotic galactooligosaccharides on the intestinal microbiota of healthy adults.* International Journal of Food Microbiology, 2010. **144**(2): p. 285-292.
21. Moro, G., et al., *Dosage-related bifidogenic effects of galacto- and fructooligosaccharides in formula-fed term infants.* J Pediatr Gastroenterol Nutr, 2002. **34**(3): p. 291-295.
22. Patterson, J.K., et al., *Supplemental dietary inulin of variable chain lengths alters intestinal bacterial populations in young pigs.* Journal of Nutrition, 2010. **140**(12): p. 2158-61.
23. Tokunaga, T., T. Oku, and N. Hosoya, *Utilization and excretion of a new sweetener, fructooligosaccharide (Neosugar), in rats.* J Nutr, 1989. **119**(4): p. 553-9.
24. Xu, Z.R., et al., *Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora and morphology of growing pigs.* Asian-Australasian Journal of Animal Sciences, 2002. **15**(12): p. 1784-1789.
25. Hidaka, H., et al., *The effects of undigestible fructooligosaccharides on intestinal microflora and various physiological functions on human health.* Adv Exp Med Biol, 1990. **270**: p. 105-17.
26. Hidaka, H. and M. Hirayama, *Useful characteristics and commercial applications of fructo-oligosaccharides.* Biochem Soc Trans, 1991. **19**(3): p. 561-5.

27. Tsukahara, T., et al., *Stimulation of butyrate production in the large intestine of weaning piglets by dietary fructooligosaccharides and its influence on the histological variables of the large intestinal mucosa.* J Nutr Sci Vitaminol (Tokyo), 2003. **49**(6): p. 414-21.
28. Gibson, G.R., et al., *Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin.* Gastroenterology, 1995. **108**(4): p. 975-82.
29. Shim, S.B., et al., *Effects of feeding antibiotic-free creep feed supplemented with oligofructose, probiotics or synbiotics to suckling piglets increases the preweaning weight gain and composition of intestinal microbiota.* Archives of Animal Nutrition, 2005. **59**(6): p. 419-427.
30. Howard, M.D., et al., *Effects of dietary supplementation with fructooligosaccharides on colonic microbiota populations and epithelial cell proliferation in neonatal pigs.* J Pediatr Gastroenterol Nutr, 1995. **21**(3): p. 297-303.
31. Houdijk, J.G., et al., *Effects of dietary non-digestible oligosaccharides on microbial characteristics of ileal chyme and faeces in weaner pigs.* Arch Tierernahr, 2002. **56**(4): p. 297-307.
32. Wiggins, H.S., *Nutritional value of sugars and related compounds undigested in the small gut.* Proc Nutr Soc, 1984. **43**(1): p. 69-75.
33. Henriksson, A., L. Andre, and P.L. Conway, *Distribution of Lactobacilli in the Porcine Gastrointestinal-Tract.* Fems Microbiology Ecology, 1995. **16**(1): p. 55-60.
34. Bruzzese, E., et al., *Impact of prebiotics on human health.* Dig Liver Dis, 2006. **38 Suppl 2**: p. S283-7.
35. Cummings, J.H., G.T. Macfarlane, and H.N. Englyst, *Prebiotic digestion and fermentation.* Am J Clin Nutr, 2001. **73**(2 Suppl): p. 415S-420S.
36. Baxter, E.M., et al., *Investigating the behavioural and physiological indicators of neonatal survival in pigs.* Theriogenology, 2008. **69**(6): p. 773-783.
37. Devillers, N., et al., *Variability of colostrum yield and colostrum intake in pigs.* Animal, 2007. **1**(7): p. 1033-1041.
38. Caporaso, J.G., et al., *QIIME allows analysis of high-throughput community sequencing data.* Nat Methods, 2010. **7**(5): p. 335-6.
39. McDonald, D., et al., *An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea.* ISME J, 2012. **6**(3): p. 610-8.
40. DeSantis, T.Z., et al., *Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB.* Appl Environ Microbiol, 2006. **72**(7): p. 5069-72.
41. Haas, B.J., et al., *Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons.* Genome Res, 2011. **21**(3): p. 494-504.
42. Gentleman, R., et al., *limma: Linear Models for Microarray Data*, in *Bioinformatics and Computational Biology Solutions Using R and Bioconductor*. 2005, Springer New York. p. 397-420.
43. Kauffmann, A., R. Gentleman, and W. Huber, *arrayQualityMetrics--a bioconductor package for quality assessment of microarray data.* Bioinformatics, 2009. **25**(3): p. 415-6.
44. Gentleman, R.C., et al., *Bioconductor: open software development for computational biology and bioinformatics.* Genome biology, 2004. **5**(10).
45. Soderholm, J.D. and M.H. Perdue, *Stress and gastrointestinal tract. II. Stress and intestinal barrier function.* Am J Physiol Gastrointest Liver Physiol, 2001. **280**(1): p. G7-G13.
46. O'Mahony, S.M., et al., *Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses.* Biol Psychiatry, 2009. **65**(3): p. 263-7.
47. Shim, S.B., I.H. Williams, and M.W.A. Verstegen, *Effects of dietary fructo-oligosaccharide on villous height and disaccharidase activity of the small intestine, pH, VFA and ammonia concentrations in the large intestine of weaned pigs.* Acta Agriculturae Scandinavica Section a-Animal Science, 2005. **55**(2-3): p. 91-97.
48. Kien, C.L., *Digestion, absorption, and fermentation of carbohydrates in the newborn.* Clin Perinatol, 1996. **23**(2): p. 211-28.
49. Grela, E.R., V. Semeniuk, and A. Czech, *Efficacy of fructooligosaccharides and mannanoligosaccharides in piglet diets.* Medycyna Weterynaryjna, 2006. **62**(7): p. 762-765.
50. Houdijk, J.G.M., et al., *Effects of dietary oligosaccharides on the growth performance and faecal characteristics of young growing pigs.* Animal Feed Science and Technology, 1998. **71**(1-2): p. 35-48.
51. Miguel, J.C., S.L. Rodriguez-Zas, and J.E. Pettigrew, *Efficacy of a mannan oligosaccharide (Bio-Mos((R))) for improving nursery pig performance.* Journal of Swine Health and Production, 2004. **12**(6): p. 296-307.
52. Xu, C.L., et al., *Study of the application of fructooligosaccharides in piglets.* Asian-Australasian Journal of Animal Sciences, 2005. **18**(7): p. 1011-1016.
53. Million, M., et al., *Comparative meta-analysis of the effect of Lactobacillus species on weight gain in humans and animals.* Microb Pathog, 2012. **53**(2): p. 100-8.

54. Stanley, D., et al., *Intestinal microbiota associated with differential feed conversion efficiency in chickens.* Appl Microbiol Biotechnol, 2012. **96**(5): p. 1361-9.
55. Skrzypek, T., et al., *Changes in pig small intestinal absorptive area during the first 14 days of life.* Livestock Science, 2010. **133**(1-3): p. 53-56.
56. Blottiere, H.M., et al., *Production and digestive effects of short chain fatty acids.* Sciences Des Aliments, 1999. **19**(3-4): p. 269-290.
57. Ruppin, H., et al., *Absorption of Short-Chain Fatty-Acids by the Colon.* Gastroenterology, 1980. **78**(6): p. 1500-1507.
58. Scholz-Ahrens, K.E., et al., *Effects of prebiotics on mineral metabolism.* American Journal of Clinical Nutrition, 2001. **73**(2): p. 459s-464s.
59. Smith, P.M., et al., *The Microbial Metabolites, Short-Chain Fatty Acids, Regulate Colonic T-reg Cell Homeostasis.* Science, 2013. **341**(6145): p. 569-573.
60. Arpaia, N., et al., *Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation.* Nature, 2013. **504**(7480): p. 451-+.
61. Singh, N., et al., *Activation of Gpr109a, Receptor for Niacin and the Commensal Metabolite Butyrate, Suppresses Colonic Inflammation and Carcinogenesis.* Immunity, 2014. **40**(1): p. 128-139.
62. Chang, P.V., et al., *The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition.* Proceedings of the National Academy of Sciences of the United States of America, 2014. **111**(6): p. 2247-2252.
63. Brouns, F., B. Kettlitz, and E. Arrigoni, *Resistant starch and "the butyrate revolution".* Trends in Food Science & Technology, 2002. **13**(8): p. 251-261.
64. Delzenne, N.M., *Oligosaccharides: state of the art.* Proceedings of the Nutrition Society, 2003. **62**(1): p. 177-182.
65. Cherbut, C., *Inulin and oligofructose in the dietary fibre concept.* British Journal of Nutrition, 2002. **87**: p. S159-S162.
66. Buddington, K.K., J.B. Donahoo, and R.K. Buddington, *Dietary oligofructose and inulin protect mice from enteric and systemic pathogens and tumor inducers.* Journal of Nutrition, 2002. **132**(3): p. 472-477.
67. de Jonge, W.J., *The Gut's Little Brain in Control of Intestinal Immunity.* ISRN Gastroenterol, 2013. **2013**: p. 630159.
68. Macpherson, A.J., M.B. Geuking, and K.D. McCoy, *Immune responses that adapt the intestinal mucosa to commensal intestinal bacteria.* Immunology, 2005. **115**(2): p. 153-62.
69. Chang, P.V., et al., *The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition.* Proc Natl Acad Sci U S A, 2014. **111**(6): p. 2247-52.
70. Lau, K.S., et al., *Multi-Scale In Vivo Systems Analysis Reveals the Influence of Immune Cells on TNF-alpha-Induced Apoptosis in the Intestinal Epithelium.* Plos Biology, 2012. **10**(9).

Appendix 1 Product specification Frutafit® TEX!

Parameter	Specification	Actual values[1]	Unit	Method
Physical aspects				
Dry matter content	95-99	95.7	%	ICUMSA GS2/1/3/9-15(2007)
Composition on dry matter				
Carbohydrates	≥ 99.5	> 99.5	%	
Inulin	≥ 99.5	99.8	%	AV_029a[2]
Fructose, glucose, sucrose	≤ 0.5	0.2	%	ICUMSA GS7/4/8-23(2002)
Average chain length (monomers)	≥ 22	22	-	AV_003[2]
Ash	≤ 0.2	< 0.2	%	ICUMSA GS8-7(1998)
Microbiology				
Aerobic plate count (30^0 C)	≤ 1000	< 100	CFU/gram	ISO 4833:2003
Aerobic plate count (55^0 C)	≤ 1000	100	CFU/gram	ISO 4833:2003 (at 55^0 C)
Yeasts	≤ 20	< 10	CFU/gram	ISO 6611:2004
Moulds	≤ 20	< 10	CFU/gram	ISO 6611:2004
Bacillus cereus	≤ 100	< 100	CFU/gram	ISO7932:2004
Enterobacteriaceae	Absent	Absent	/gram	ISO 21528-1:2004
Staphylococcus aureus	Absent	Absent	/gram	ISO 6888-3:2003
Salmonella	Absent	Absent	/400 grams	ISO 6579:2002
Other information				
Production date		5-11-2012	dd/mm/yyyy	
Expiration date		5-11-2017	dd/mm/yyyy	

[1] printed values are mean values for this batch
[2] internal method, no international standard available

Appendix 2 Product specification Frutalose® OFP

Parameter	Specification	Actual values[1]	Unit	Method
Physical aspects				
Dry matter content	96-99	97.8	%	ICUMSA GS2/1/3/9-15(2007)
Composition on dry matter				
Carbohydrates	≥ 99.5	> 99.5	%	
Oligofructose	99 ± 2	93	%	AV_029a[2]
Fructose, glucose, sucrose	8 ± 2	7	%	ICUMSA GS7/4/8-23(2002)
Ash	≤ 0.2	< 0.2	-	ICUMSA GS8-7(1998)
			%	
Microbiology				
Aerobic plate count (30^0 C)	≤ 1000	< 100		ISO 4833:2003
Aerobic plate count (55^0 C)	≤ 1000	< 100	CFU/gram	ISO 4833:2003 (at 55^0 C)
Yeasts	≤ 20	< 10	CFU/gram	ISO 6611:2004
Moulds	≤ 20	< 10	CFU/gram	ISO 6611:2004
Bacillus cereus	≤ 100	< 100	CFU/gram	ISO7932:2004
Enterobacteriaceae	Absent	Absent	CFU/gram	ISO 21528-1:2004
Staphylococcus aureus	Absent	Absent	/gram	ISO 6888-3:2003
Salmonella	Absent	Absent	/gram	ISO 6579:2002
			/400 grams	
Other information				
Production date		24-3-2013		
Expiration date		24-3-2018	dd/mm/yyyy	
			dd/mm/yyyy	

[1] printed values are mean values for this batch
[2] internal method, no international standard available

Appendix 3 Protocol: Tissue processor

From EtOH to paraffin

Step	Description	Time
1	EtOH 70%	240
2	EtOH 80%	90
3	EtOH 90%	60
4	EtOH 96%	45
5	EtOH 100%	30
6	EtOH 100%	30
7	Xylene 100%	60
8	Xylene 100%	60
9	Xylene 100%	60
10	Paraffin 100%	60
11	Paraffin 100%	60

Before starting with the tissue processor, the intestinal tissue was preserved in EtOH 70%

Appendix 4 Protocol: Crossmon light green staining

Dewaxing

Step	Description	Time
1	Xylene 100%	2
2	Xylene 100%	2
3	Xylene 100%	2
4	EtOH 100%	2
5	EtOH 96%	2
6	EtOH 90%	2
7	EtOH 80%	2
8	EtOH 70%	2
9	Demineralized water	5
10	Demineralized water	5

Staining

Step	Description	Time
1	Haemaluin[1]	5
2	Rinse in running tap water	15
3	Acid fuchsine / orange G[2]	0.17
4	Demineralized water	2
5	Phosphotungstic acid[3]	2
6	Demineralized water	2
7	Light green[4] 1%	4
8	Demineralized water	1
9	Demineralized water	2
10	EtOH 100%	1
11	EtOH 100%	3
12	EtOH 100%	3
13	Xylene 100%	5
14	Xylene 100%	5

[1] Haemaluin-Eosin-staining by Mayer
[2] 1.3 gr acid fuchsine and 1.0 gr Orange G dissolved in 500 ml demineralized water. After shaking, add 5 ml glacial acetic acid and 0.33 gr thymol
[3] 25 gr phosphotungstic acid dissolved in 500 ml demineralized water
[4] 10 gr light green dissolved in 10 ml glacial acetic acid and 1000 ml demineralized water

To explore the potential of nature to improve the quality of life

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Together with our clients, we integrate scientific know-how and practical experience to develop livestock concepts for the 21st century. With our expertise on innovative livestock systems, nutrition, welfare, genetics and environmental impact of livestock farming and our state-of-the art research facilities, such as Dairy Campus and Swine Innovation Centre Sterksel, we support our customers to find solutions for current and future challenges.

The mission of Wageningen UR (University & Research centre) is 'To explore the potential of nature to improve the quality of life'. Within Wageningen UR, nine specialised research institutes of the DLO Foundation have joined forces with Wageningen University to help answer the most important questions in the domain of healthy food and living environment. With approximately 30 locations, 6,000 members of staff and 9,000 students, Wageningen UR is one of the leading organisations in its domain worldwide. The integral approach to problems and the cooperation between the various disciplines are at the heart of the unique Wageningen Approach.