Effects of a high level of dietary zinc over different post weaning periods on intestinal microbiota and mucosal gene expression in piglets

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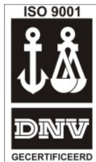

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Summary

The aim of the present study was to evaluate the effects of provision of a diet with a high concentration of zinc (Zn, 2790 mg/kg), as a model intervention for manipulation of immune competence of piglets, over different periods of time (d 0-14, 0-23 or 14-23 post weaning (pw)) on the composition of the small intestinal microbiota and on gene expression in the intestinal mucosa on d 14 and 22/23 pw. Growth performance was measured over 35 d pw. Provision of a high Zn diet was compared with feeding a reference weaner and starter diet with a regular Zn concentration (121 and 142 mg/kg, respectively). The study involved four experimental treatments (1 to 4, dietary zinc concentration low – low, low – high, high – low and high – high over d 0-14 and d 14-23 pw, respectively).

Over d 0-14, feed intake and body weight gain was significantly higher in the high zinc treatments compared to the control, low zinc treatment ($P<0.05$), while the feed conversion ratio did not differ among treatments. Over the periods of d 14-23, 23-35 and over the complete experimental period (d 0-35) feed intake, body weight gain and feed conversion ratio did not differ between treatments.
On d 14 and d 22/23, after provision of a high zinc diet higher plasma concentrations of zinc were found compared to after feeding a diet with a normal zinc concentration. On d 34/35 pw, 12 d after termination of the dietary treatments, no significant differences among treatments in plasma zinc concentration were observed. Based on the complete microbiota pattern at different taxa levels, no clear clustering of samples based on time (d14 or d22/23 pw) or dietary treatment (low of high zinc over different periods of time) was observed.
Considering the Chao 1 index, there was no treatment effect on microbial diversity in jejunal digesta but a significant time x treatment interaction in ileal digesta. On d 14, microbial diversity increased due to provision of a high zinc diet over the previous period, while the diversity tended to reduce when a high zinc diet was provided during d 14-22/23 and a low zinc diet was given over d 0-14. When providing a high zinc diet over d 0-14, there was no effect on microbial diversity on d 22/23, independent of feeding a low or high zinc diet over d 14-22/23. A similar result and interaction was found for the microbial diversity based on the Shannon index.

In both jejunal and ileal mucosal tissue, genes/probes were found which were differentially expressed as affected by age (d 14 or 22/23 pw) and dietary treatment (low or high dietary zinc). The largest numbers were found for the samples of d 14 for the treatment groups receiving either a low or a high zinc diet and for the comparison between jejunal tissue samples of pigs of the low zinc treatments on d 14 (treatments 1 and 2) and treatment 1 (low zinc over d 0-22/23) on d 22/23. A number of significantly enriched gene sets in the jejunal mucosa on d 14 by the high zinc intervention from d 0-14 is involved in processes related to signalling processes and peptides (jejunum and ileum) and innate defence responses (ileum). When comparing the gene expression on d 22/23 with the expression observed on d 14 on the low zinc dietary treatments, a relatively large number of genes was differentially expressed, related to a variety of processes, of which part relate to the (innate) immune system. Such a time effect, however, was not observed for the high zinc treatments.

It can be concluded from the present study that provision of a diet with a high concentration of zinc as zinc oxide (2730 mg/kg) in the immediate post weaning period
(d 0-14 pw) and/or during a day 14-23 pw (2850 mg/kg) induced some changes in intestinal microbiota composition, in particular in the ileum, and on the expression of genes in the jejunal and ileal mucosa, in part related to processes involved in the innate immune system. Effects were most pronounced after the immediate post weaning period (d 14 pw).

Effects of the present and a previous study, as well as data presented in the literature suggest that high zinc diets in the post weaning period interfere with development of the piglet's immune system and can be used as a model intervention for studying possibilities to modify immune competence. Further studies using piglets subjected to specific immune challenges can provide a more complete view on the biological and functional meaning of the observed changes induced by high zinc diets in the post-weaning period.

1. Introduction

The health status of farm animals is in part determined by the competence and responses of the immune system. Both determine the resilience of animals towards environmental stressors and to challenge by pathogenic micro-organisms. Responses of the immune system need to be expressed at the proper location in the animal and balanced in time. Modulation of the development of the immune system and its competence to express proper, well balanced responses later in life via dietary interventions is an expanding area of research in both the human and animal domain. Via the development and application of innovative dietary interventions and feeding strategies support can be given to the development of the gastrointestinal tract in pigs and poultry with regard to both its digestive and barrier function. The latter includes competence of the local immune system in the gut. A proper functioning of the immune system largely contributes to the support of animal health and their productive performance under a range of environmental conditions.

The research programme Nutrition, Gut Health and Immunity (VDI), as part of the public private partnership Feed4Foodure (F4F), is focussed on the development and validation of in vitro, ex vivo and in vivo models to study the effects of dietary interventions in pigs, poultry and veal calves on the development of immune competence. One of the research lines within the programme has the aim to develop models for studying the effects of dietary interventions in piglets on the development of immune competence and to identify indicators for function, health and mucosal immunity of/in the gut. These indicators will be useful for the evaluation of dietary interventions on gut health and function in future research.

High dietary concentrations of zinc were shown to change the composition, diversity and activity of the intestinal microbiota in piglets in the immediate post-weaning period (Vahjen et al., 2010, 2011; Hojberg et al., 2005) and can contribute to maintaining gut and animal health in the critical post weaning phase. It was also shown that a high concentration of ZnO in the diet influences the activity of intestinal brush border bound alkaline phosphatase in a dose-dependent manner, however, did not affect the activity of other brush border enzymes involved in nutrient digestion (Martin et al., 2013). In addition, Sargeant et al. (2010) showed that a high concentration of dietary zinc reduced the expression of immune related genes involved in inflammatory processes in gut tissue after challenge with a pathogenic *E. coli* (ETEC). In another study, a similar high zinc intervention via the diet induced changes in the intestinal expression of proteins involved in oxidative stress, cell differentiation and apoptosis (programmed cell death) (Wang et al., 2009).

From a first study in the framework of the current VDI project with piglets using a high Zn diet as model intervention (Jansman et al., 2016), it was concluded that provision of a diet with a high concentration of zinc as zinc oxide during a short period of time (9 days) to piglets in the post weaning period (day 14-23 pw) induced differences intestinal microbiota composition, in particular in the ileum and on the intestinal expression of genes, in part related to the functioning of the local innate immune system. The high dietary zinc intervention can therefore be considered as a suitable model for studying relationships between dietary interventions, intestinal microbiota composition and development of immune competence in post weaning piglets.

The aim of the present study was to evaluate the effects of provision of a diet with a high concentration of zinc (Zn, 2790 mg/kg) as a model intervention over different periods of time (d 0-14, 0-23 or 14-23 pw), compared to a control treatment, in which piglets were fed a weaner and starter a diet with a regular Zn concentration (132 mg/kg), on the composition of the small intestinal microbiota and on gene expression in the intestinal mucosa on d 14 and 22/23 pw. Also the zootechnical performance of the piglets in the four treatment groups was measured over the period of d 0-35 pw.

2. Material & methods

2.1 Experimental treatments

In the study four experimental treatments were evaluated (Table 1).

Table 1. *Overview of the experimental dietary treatments.*

Trm	Day 0-14 d	Day 14-23	Day 23-35
1	Low Zn	Low Zn	Low Zn
2	Low Zn	High Zn	Low Zn
3	High Zn	Low Zn	Low Zn
4	High Zn	High Zn	Low Zn

The piglets in the treatment groups received a weaning diet from d 0-14 with a low or high zinc concentration and a starter piglet diet with a low or high zinc concentration over the period of d 14-23. All piglets received a similar low Zn starter diet over the period of d 23-35 post weaning.

The study was performed with two pens, each containing 12 piglets, for each of the treatments, in total comprising 96 piglets.

2.2 Piglets, housing and feeding

The study was performed in the experimental facilities of Agrifirm "Laverdonk" in Heeswijk Dinther, The Netherlands.

The piglets were of the Tempo x Topigs 20 genotype. At the start of the study the piglets were divided per pen in a way that the mean body weight and its variation per pen were similar.

The piglets were weaned at a mean age of 28 ± 1.2 d. The piglets received creep feed during the suckling period. The mean body weight of the piglets one day prior to the start of the study was 7.6 kg.

The piglets were housed in floor pens (1.75 x 3.00 m) with 12 piglets per pen. Male (boars) and female piglets were equally distributed over pens. Litter mates were spread equally over the available pens as far as possible. The piglets were fed ad libitum using a dry feed dispenser. The diets were provided as pellets. The animals had free access to water via an automatic drinking device.

The ingredient and calculated nutrient composition of the low zinc weaner and starter diets are given in Tables 2 and 3. The diets were formulated to be nutritionally adequate using data on the composition and nutritional value of feed ingredients according to CVB (2011).

After weaning, the piglets were fed a weaning experimental diet with a low or high Zn concentration during the period of d 0 till 14. From day 14 till 23, piglets in the four treatment groups received an experimental starter diet with a low or high Zn concentration. The contrast in Zn concentration in the experimental diets was realised by supplementing zinc oxide (ZnO) to the low zinc diets. From day 23 till 35, the remaining piglets in each of the four treatment groups received the same starter diet with a low Zn concentration (analysed 142 mg Zn per kg).

Table 2. *Calculated ingredient and nutrient composition of the low zinc weaner diet and the low zinc starter diet (g/kg).*

	Weaning		Starter[1]	
Wheat	150.2		203.5	
Barley	300.0		300.0	
Maize	200.0		200.0	
Whey powder	75.0			
Soybean meal	95.0		195.0	
Soya concentrate	30.0			
Potato protein	20.0			
Sunflower meal	30.0		30.0	
Linseed	30.0		10.0	
Soya oil	30.0		21.5	
Premix[2]	5.0		5.0	
Chalk	6.5		14.2	
Monocalcium phosphate	8.0		8.5	
NaCl	4.0		5.5	
Sodium bicarbonate	1.3			
Natuphos 5000G	0.1		0.1	
Ca-formiate	7.5			
L-Lysine HCl	4.2		4.0	
DL-Methionine	1.5		1.2	
L-Threonine	1.1		1.1	
L-Tryptophan	0.6		0.4	
Dry matter	890		879	
Crude protein	177		181	
Crude fat (EE)	59		45	
Ash	52		55	
Crude fibre	35		37	
Starch (Ewers)	377		412	
Sugars	79		35	
NE (MJ/kg)	10.06		9.63	
EW *100	114		110	
Calcium	7.7		8.0	
P, total	5.7		5.7	
P dig.	3.7		3.5	
Sodium	2.5		2.2	
Potassium	7.9		7.9	
Chloride	5.2		4.5	
Zn[4]	27		30	
Base-excess (meq/kg)	164		168	
Amino acids	Total	AID[3]	Total	AID[3]
Lysine	11.9	10.5	11.5	10.0
Methionine	4.4	4.0	4.0	3.6
Cysteine	3.1	2.4	3.2	2.4
Methionine + Cysteine	7.5	6.4	7.1	6.0
Threonine	7.8	6.3	7.5	5.9
Tryptophan	2.7	2.2	2.5	2.1
Isoleucine	7.3	6.1	7.2	5.9
Valine	8.5	6.9	8.3	6.7

[1]The high zinc weaner and starter diets were formulated by additionally supplementing Zn as ZnO to obtain diets with 2730 and 2850 mg Zn per kg, respectively.

[2]Both the weaning and the starter diets contained 20 mg/kg supplemented Cu. The vitamin-mineral premix supplied per kg feed: 10,000 IU vitamin A, 2,000 IU vitamin D3, 40 mg vit E, 1.5 mg vitamin K, 1.0 mg vitamin B1, 4 mg vitamin B2, 15 mg d-pantothenic acid, 30 mg niacine, 50 µg biotine, 20 µg vitamin B12, 0.4 folium acid, 1.5 mg vitamin B6, 150 mg choline chloride, 100 mg Fe, 20 mg Cu, 70 mg Zn, 30 mg Mn, 0.7 mg I, 0.25 mg Se, 125 mg Oxytrap PXN (antioxidant).

[3]AID: apparent ileal digestible.

[4]Zn contribution from the feed ingredients, excluding the contribution of Zn in the premix (70 mg/kg)

Table 3. *Analysed nutrient composition and pellet hardness of the experimental weaning (d 0-14) and starter diets (d 14-35).*

	Speen		Start		
	C	High Zn	C	High Zn	Unit
Dry matter	892	894	885	885	g/kg
Calcium	10.6	10.4	11.4	11.3	g/kg
Phosphorus	6.0	5.9	6.0	5.9	g/kg
Sodium	3.2	3.2	3.2	3.3	g/kg
Ash	59	62	64	68	g/kg
Crude protein	173	174	183	184	g/kg
Crude fat	64	63	51	49	g/kg
Crude fibre	35	35	36	39	g/kg
Total sugars (glucose equiv.)	64	65	40	39	g/kg
Starch (Ewers)	374	372	408	402	g/kg
Zinc	121	2730	142	2850	mg/kg
Pellet hardness	16	17	9	10	N

2.3 Observations, measurements and sampling

- The experimental weaner and starter diets were analysed on the concentrations of dry matter, ash, N (crude protein), crude fat (EE), crude fibre, starch [Ewers] and sugars, calcium, phosphorus, sodium and zinc using standard methods. In addition, pellet hardness was measured.
- The piglets were weighed on d -1, 14, 23 en 35 post weaning.
- The feed intake per pen was registered over the periods of d 0-14, 14-23 and 23-35.
- The mean faeces consistency per pen was scored by visual judgement using a scale of 1 to 3, three times per week during the experimental period (d 0-35).
- On day 14, 22/23 and 34/35 per pen one (d 14) or two piglets per pen (d 22/23 and 34/35) were euthanized to obtain samples of intestinal digesta (duodenum, jejunum, ileum, en colon) for analysing microbiota composition (optional), intestinal mucosa (jejunum and ileum) for gene expression measurements, intestinal tissue (jejunum and ileum) for optional immuno-histochemical analyses, blood (for analysing Zn concentration in blood plasma and concentrations of cytokines and haptoglobin). Finally it was decided that jejunal and ileal digesta samples were analysed on microbiota composition and the corresponding sections of jejunal and ileal mucosa tissues were analysed on gene expression as further described in paragraph 2.4.
- Plasma samples obtained at dissection of piglets on d 14 and 22/23 were analysed on concentrations haptoglobin, IL6 en TNF-α using commercial ELISA kits according to instructions of the manufacturer (MT-diagnostics Etten-Leur, The Netherlands).

2.4 Microbiota and gene expression analysis

2.4.1 Microbiome analysis

<u>DNA Extraction</u>
Samples of jejunal and ileal digesta were snap frozen in liquid nitrogen and stored at -80 °C. For the microbial DNA extraction the following protocol was handled:

1) Digesta was mixed 1:1 with PBS
2) 10 glass beads were added
3) Vortex
4) Spin for 5 min 300g 4 °C
5) Supernatant to new tube
6) Spin for 10 min 9000g 4 °C
7) Remove supernatant
8) Follow QIAamp DNA Stool Mini Kit protocol as described

9) Elute in 100 µl of (provided) elute buffer
10) Optical Density measurement on Nanodrop (Agilent Technologies) to check quality

V3 PCR
Oligo V3_F, with sequence ; CCTACGGGAGGCAGCAG and oligo V3_R with sequence; ATTACCGCGGCTGCTGG

The following PCR program was run:
1) 98 °C 2min
2) 98 °C 10s
3) 55 °C 30s
4) 72 °C 10s
5) 72 °C 7 min

Where step 2-4 were repeated for 15 times. All PCR products were subjected to quality control by running 5 µl of CPR product on a 2% E-Gel® Agarose Gel Electrophoresis System (Life Technologies).

QIIME
Samples were sequenced by targeted-amplicon 16S-V3-V4 sequencing and taxonomy summaries per sample were generated using QIIME [12]. 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) [13, 14]. Furthermore chimeras were removed with Chimeraslayer [15].

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 and Chao-1 diversity indices. 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 (day) + Treatment + Time (day) * 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.

2.4.2 Transcriptome analysis

RNA Extraction Tissue
Total RNA was extracted from 50 to 100 mg tissue mucosal scrapings of jejunum and ileum. The jejunum and ileum 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 a phase separation with chloroform was performed as described by the manufacturer Life Technologies. The RNA was precipitated and dissolved and quantified by absorbance measurements at 260 nm. Quality Control was performed by Agilent Bioanalyser.

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 was 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 was 65°C with rotation speed 10 rpm for 17 hours. After 17 hours 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 (v10.7) for one colour gene expression.

Data analysis
The data were analysed by using R (v3.0.2) by executing different packages, including LIMMA and arrayQualityMetrics. The data were read in and background corrected (method="normexp" and offset=1) with functions from the R package LIMMA [1] from Bioconductor [2]. Quantile normalisation 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 was 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 an experimental condition (e.g. in ileum day 14 control).

2.5 Statistical analysis

The results for body weight gain, daily feed intake, feed conversion ratio and plasma concentrations of zinc, cytokines and haptoglobin were statistically analysed by ANOVA (Genstat 5, release 3; Payne et al., 1993). The data were analysed using "dietary treatment" as main experimental factor in the statistical model.

An effect of experimental treatment or time in the statistical evaluation was considered to be significant when the probability of having no effect was less than 5% ($P<0.05$). Differences between treatment means were evaluated further using the Least Significant Difference test (Snedecor and Cochran, 1980).

Details regarding the statistical evaluation of data on the microbiota composition in digesta and gene expression in intestinal mucosa tissues are provided in paragraph 2.4 and in the Chapter 3.

3. Results

3.1 Course of the study and zootechnical performance

The study generally went well. No specific health problems were encountered during the study.

Data on the development in body weight and the zootechnical performance of the piglets per experimental group over the different sub periods of the study are provided in Tables 4 - 6. They were measured for each of the parameters over two pens per experimental treatment (n=2). The mean body weight of the piglets at the start of the study in each treatment group was similar. Over d 0-14, feed intake and body weight gain was significantly higher in the high zinc treatments compared to the control, low zinc treatments (P<0.05; Table 4), while the feed conversion ratio did not differ among treatments. Over the periods of d 14-23, 23-35 and over the complete experimental period (d 0-35) (Tables 5-7) feed intake, body weight gain and feed conversion ratio did not differ between treatments. Also body weight at d 35 was not different between treatment groups.

Table 4. *Body weight (BW) on d 0 and 14, feed intake, body weight gain (BWG), and feed conversion ratio[1] over the period d 0-14 of the study.*

Treatment	BW day 0 (kg)	BW day 14 (kg)	Feed intake (g/d)	BWG (g/d)	FCR (g/g)
1. L-L-L	7.55	10.5[a]	296[a]	210[a]	1.411[b]
2. L-H-L	7.55	10.8[ab]	298[a]	233[ab]	1.275[a]
3. H-L-L	7.55	11.1[bc]	342[b]	253[bc]	1.349[b]
4. H-H-L	7.55	11.3[c]	345[b]	271[c]	1.273[a]
P-value	0.54	0.006	0.02	0.006	0.005
LSD	0.003	0.41	37.0	29.4	0.072
Low-Zn	7.55	10.6	297[a]	221[a]	1.343
High-Zn	7.55	11.2	344[b]	262[b]	1.311
P-value	1.00	0.33	0.04	0.01	0.44
LSD	0.89	1.2	45	31	0.09

[abc]Values with a different superscript per column differ at P<0.05.

Overall the faecal consistency score in all treatment groups was close to 1 throughout the study suggesting no deviations from normal consistency and absence of diarrhoea in each of the experimental groups (Table 8).

Table 5. *Body weight (BW) on d 14 and 23, feed intake, body weight gain (BWG), and feed conversion ratio of the pigs over the period d 14-23 of the study.*

Treatment	BW day 14 (kg)	BW day 23 (kg)	Feed intake (g/d)	BWG (g/d)	FCR (g/g)
1. L-L-L	10.5[a]	14.3	532	429	1.245
2. L-H-L	10.8[ab]	14.4	553	417	1.332
3. H-L-L	11.1[bc]	14.4	512	383	1.339
4. H-H-L	11.3[c]	15.2	580	444	1.308
P-value	<0.01	0.11	0.33	0.38	0.27
LSD	0.41	0.87	80	76	0.111

[abc]Values with a different superscript per column differ at P<0.05.

Table 6. *Body weight (BW) on d 23 and 35, feed intake, body weight gain (BWG), and feed conversion ratio of the pigs over the period d 23-35 of the study.*

Treatment	BW day 23 (kg)	BW day 35 (kg)	Feed intake (g/d)	BWG (g/d)	FCR (g/g)
1. L-L-L	14.3	20.1	806	495	1.630
2. L-H-L	14.4	20.5	793	523	1.523
3. H-L-L	14.4	20.6	803	515	1.590
4. H-H-L	15.2	21.0	772	490	1.573
P-value	0.11	0.82	0.95	0.88	0.45
LSD	0.87	2.2	142	108	0.146

Table 7. *Feed intake, body weight gain and feed conversion ratio over the period d 0-35 of the study.*

Treatment	Feed intake (g/d)	BWG (g/d)	FCR (g/g)
1. L-L-L	532	364	1.461
2. L-H-L	533	380	1.404
3. H-L-L	544	377	1.451
4. H-H-L	552	391	1.412
P-value	0.93	0.78	0.14
LSD	79	58	0.058

Table 8. *Faecal consistency score[1] of the pigs per treatment group during the course of the study.*

Treatment	Day 0-14	Day 14-23	Day 23-35
1. L-L-L	1.03	1.05	1.00
2. L-H-L	1.04	1.00	1.00
3. H-L-L	1.01	1.00	1.00
4. H-H-L	1.01	1.00	1.00

[1]scored per pen on a scale of 1 (normal faecal consistency) to 3 (diarrhoea).

3.2 Zinc and copper concentrations in plasma

The plasma concentrations of Cu and Zn were measured in the piglets on d 14, 22/23 and 34/35. Results are presented in Table 9. On d 22/23, plasma Cu concentration in piglets in treatment 1 was significantly lower compared to concentrations in treatments 2, 3 and 4. Cu concentrations did not differ between treatments at d 14 and 34/35.
Zn concentration in blood plasma was significantly affected by treatment on d 14 and d 22/23. Provision of high zinc diets resulted in higher plasma concentrations of zinc compared to feeding diets with a normal zinc concentration. On d 34/35, 12 d after termination of the dietary treatments, no significant differences among treatments in plasma zinc concentration were observed.

Table 9. *Effect of the experimental treatments on the concentrations of Zn and Cu in plasma (µmol/L) on d 14, 22/23 and 34/35.*

Treatment	Cu d 14	Cu d 22/23	Cu d 34/35	Zn d 14	Zn d 22/23	Zn d 34/35
1. L-L-L	24.8	17.8[a]	25.3	10.9[a]	16.9[a]	15.3
2. L-H-L	25.2	22.3[b]	23.5	9.8[a]	23.1[b]	16.0
3. H-L-L	23.4	22.4[b]	22.7	22.2[b]	17.6[a]	15.9
4. H-H-L	23.8	20.6[b]	23.9	23.5[b]	27.2[c]	18.3
P-value	0.42	0.02	0.21	<0.001	0.006	0.19
LSD	2.4	2.7	2.4	4.1	6.0	2.9
Low-Zn	24.9			10.3[a]		
High-Zn	23.6			22.8[b]		
P-value	0.15			<0.001		
LSD	1.8			2.8		

[abc]Values with a different superscript differ at P<0.05.

3.3 Haptoglobin and cytokines in plasma

The concentrations of IL-6, TNF-α and haptoglobin in plasma of piglets on d 14 and d 22/23 are given in Table 10 and 11, respectively. The plasma concentrations of these cytokines and the acute phase protein were not affected by dietary treatment.

Table 10. *Concentration of cytokines and haptoglobin in plasma of piglets on d 14 post weaning.*

Treatment	IL-6[1] (pg/ml)	TNF-α (pg/ml)	Haptoglobin (µg/ml)
1/2. L	4.6	70.7	248
3/4. H	3.8	80.4	121
P-value	0.83	0.33	0.20
LSD	8.2	21.0	204

[1]63% of the samples showed a concentration below the lowest detection limit of the assay (0.5 pg/ml).

Table 11. *Concentration of cytokines and haptoglobin in plasma of piglets on d 22/23 post weaning.*

Treatment	IL-6[1] (pg/ml)	TNF-α (pg/ml)	Haptoglobin (μg/ml)
1. L-L-L	23.2	92	79
2. L-H-L	7.2	97	431[2]
3. H-L-L	8.0	132	232
4. H-H-L	19.3	106	110
P-value	0.48	0.36	0.08
LSD	25.2	49	293

[1]38% of the samples showed a concentration below the lowest detection limit of the assay (0.5 pg/ml).

[2]Average value excluding one sample with a concentration of 1332 μg/ml is 302 μg/ml.

3.4 Microbiota composition in digesta

On d 14 and 22/23 the microbiota composition in jejunal and ileal digesta of piglets of the treatment groups was determined. In Figure 2 results of a clustering analysis are presented over all digesta samples analysed. The analysis showed that no clear discrimination could be made at genus level between jejunal and ileal digesta samples. Dominant taxa at genus level over time points in jejunal digesta are Lactobacillaceae, Enterobacteriaceae, and Veillonellaceae and in ileal digesta are Lactobacillaceae, Streptococcaceae, Enterobacteriaceae and Pasteurellaceae.

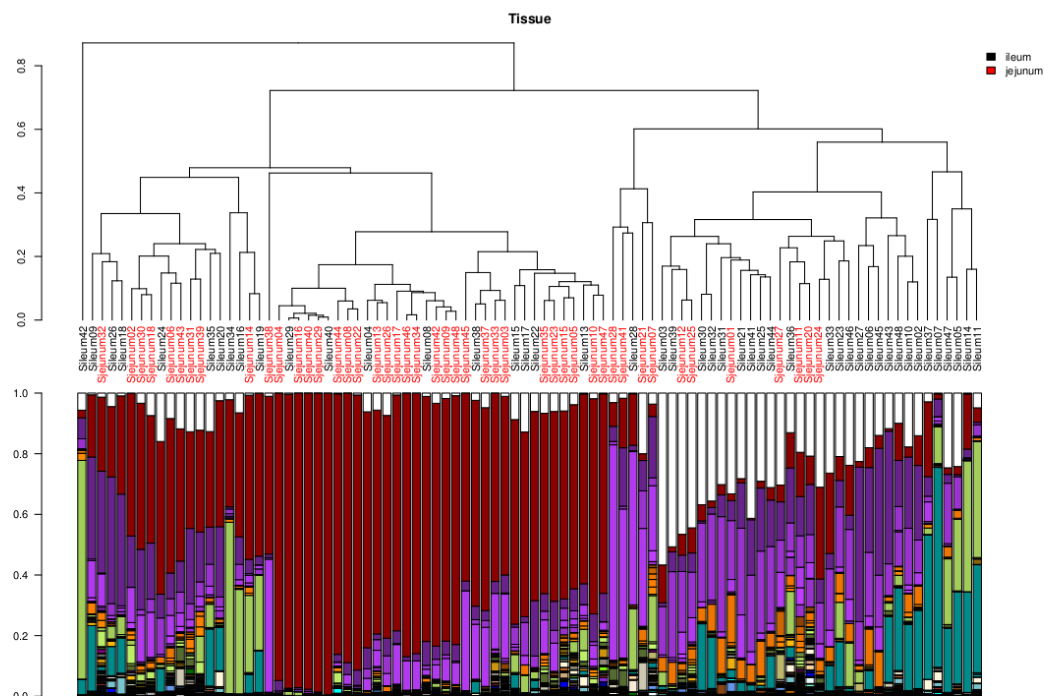

Figure 2. *Microbiota composition at genus level in jejunal and ileal digesta over the experimental treatments and time points of measurement (d 14 and 22/23).*

A clustering analysis over sampling time points and experimental treatments (high zinc vs. normal zinc) was made at phylum, class, family, genus and species level in jejunal and ileal digesta. Results are presented in Figures 3 to 7, respectively.

In both jejunal and ileal digesta, Firmicutes and Proteobacteria are the most dominant phyla, independent of dietary Zn treatment.

Overall, from family to species level there appears to be a difference in the microbiota composition between jejunal and ileal digesta for the most dominant taxa. At family level, e.g. in ileum, the Pasteurellaceae, Lactobacillaceae, Enterobacteriaceae and Turicibacteraceae appeared to be more dominant in all samples at both time points independent of dietary treatment, while the

Lactobacillaceae, Pasteurellaceae, and Veilonellaceae were more predominant in the jejunal digesta samples. This pattern was basically maintained at lower taxa levels (genus and species).

In jejunum digesta samples, d22/23_1, d22/23_3 and d14_12 seemed most related in the clustering analysis. In ileum digesta samples, d22/23_1 and d22/23_4 were most related digesta samples throughout the clustering at different taxa levels. The animals in these treatment groups all received a low zinc diet prior to digesta sampling.

Based on the complete microbiota pattern at different taxa levels no clear clustering of samples based on time d14 or d22/23 or dietary treatment (low of high zinc over different periods of time) was found.

Further data on the microbiota composition per time point (age) and dietary treatment in jejunal and ileal digesta are provided in Appendices 1-6.

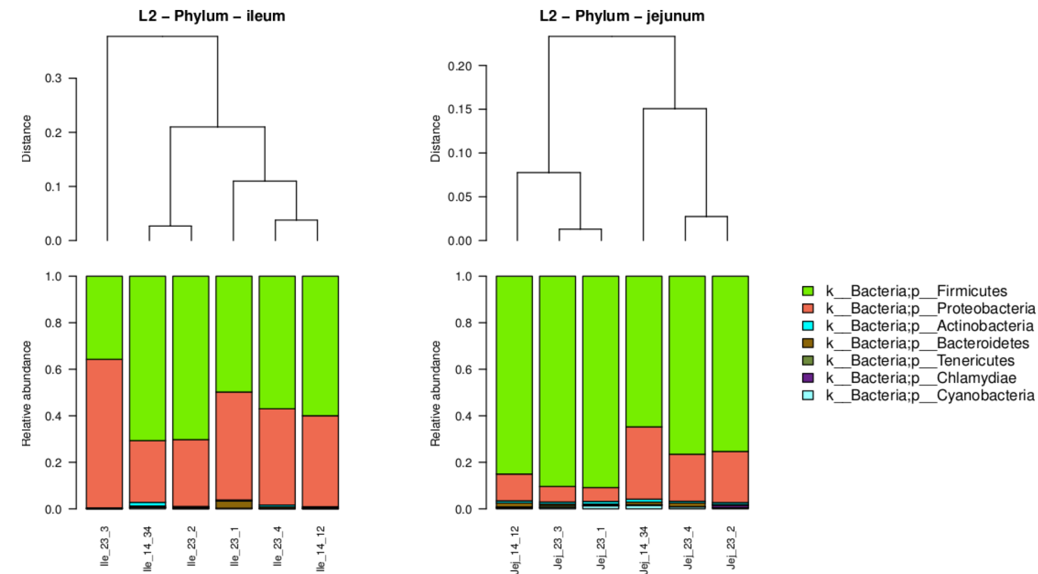

Figure 3. *Cluster analysis of microbiota composition at phylum level as analysed in jejunal (right panel) and ileal digesta (left panel) and affected by treatment (control – LO; high zinc – HI) and time point of measurement (d 14, and 22/23).*

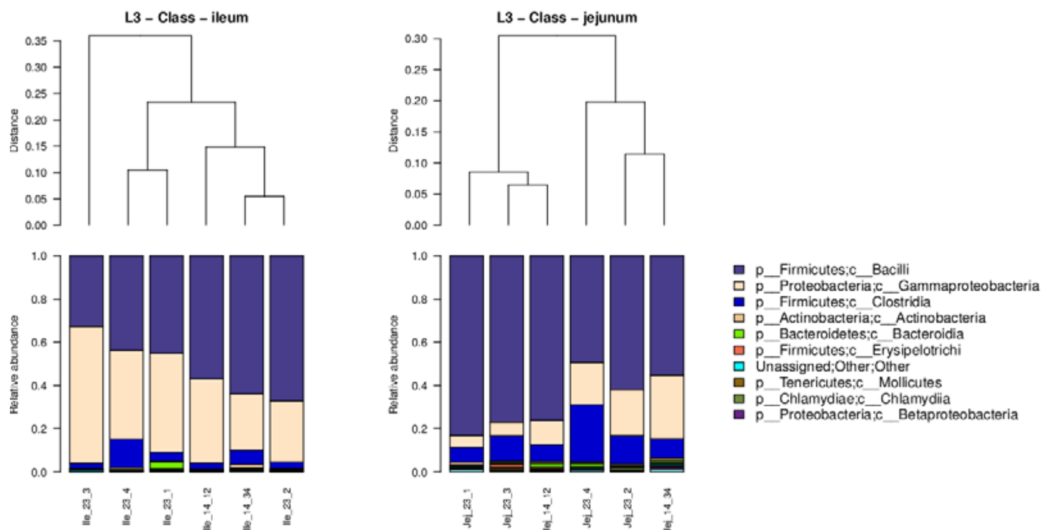

Figure 4. *Cluster analysis of microbiota composition at class level as analysed in jejunal (right panel) and ileal digesta (left panel) and affected by treatment (control – LO; high zinc – HI) and time point of measurement (d 14 and 22/23).*

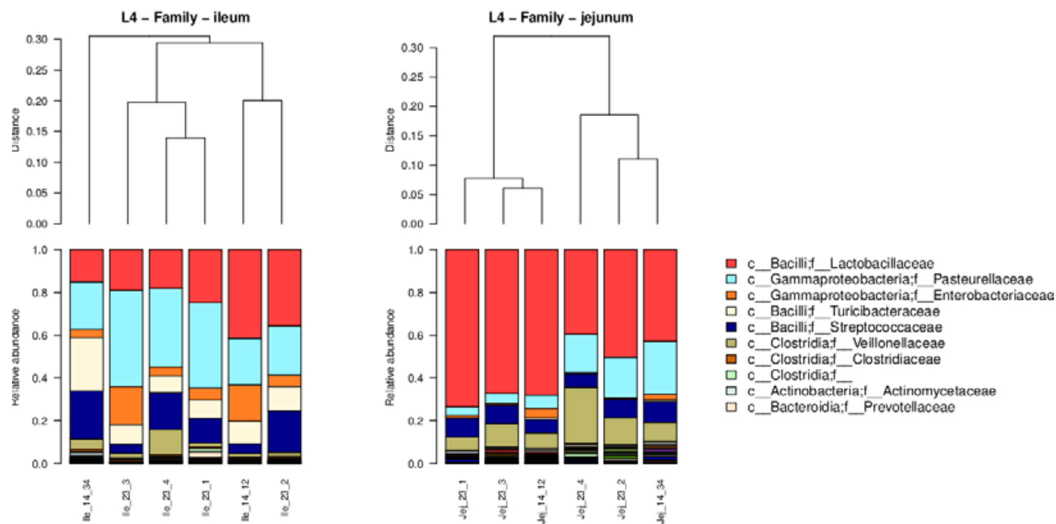

Figure 5. *Cluster analysis of microbiota composition at family level as analysed in jejunal (right panel) and ileal digesta (left panel) and affected by treatment (control – LO; high zinc – HI) and time point of measurement (d 14 and 22/23).*

A further analysis of the microbiota composition in jejunal and ileal digesta was made by calculating different indices for microbiota diversity in the samples. In Figures 8 and 9, data are provided on the α-diversity according to the Chao 1 index and the Shannon index and, respectively. Considering the Chao 1 index, there was no treatment effect on microbial diversity in jejunal digesta but a significant time x treatment interaction in ileal digesta. On d 14, microbial diversity increased due to provision of a high zinc diet over the previous period, while the diversity tended to reduce when a high zinc diet was provided during d 14-22/23 and a low zinc diet was given over d 0-14. When providing a high zinc diet over d 0-14, there was no effect on microbial diversity on d 22/23, independent of the dietary zinc treatment over d 14-22/23. A similar result and interaction was found for the microbial diversity based on the Shannon index.

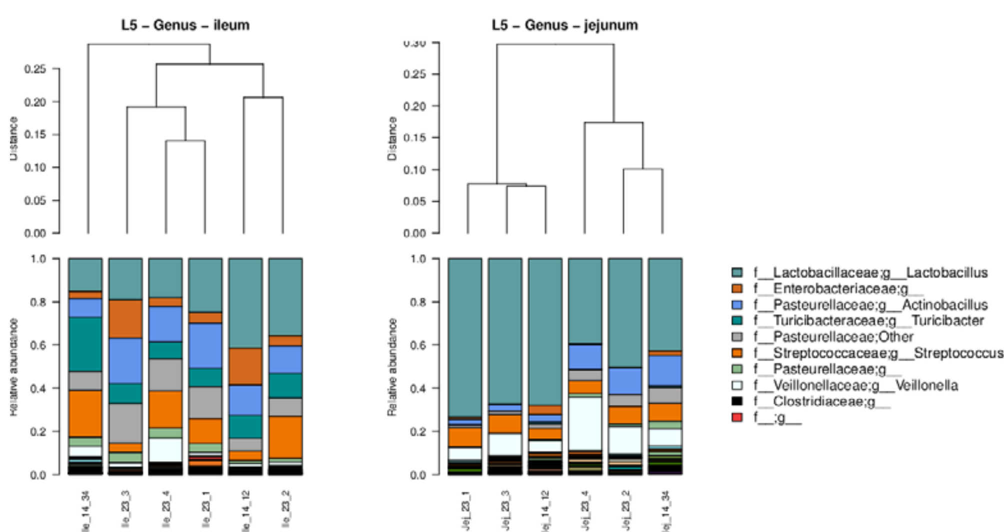

Figure 6. *Cluster analysis of microbiota composition at genus level as analysed in jejunal (right panel) and ileal digesta (left panel) and affected by treatment (control – LO; high zinc – HI) and time point of measurement (d 14 and 22/23).*

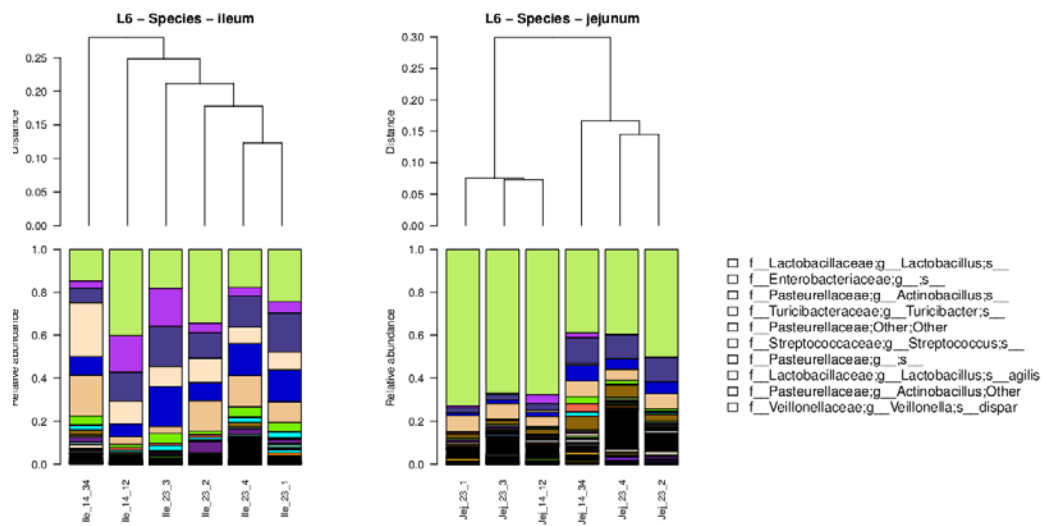

Figure 7. *Cluster analysis of microbiota composition at species level as analysed in jejunal (right panel) and ileal digesta (left panel) and affected by treatment (control – LO; high zinc – HI) and time point of measurement (d 14 and 22/23).*

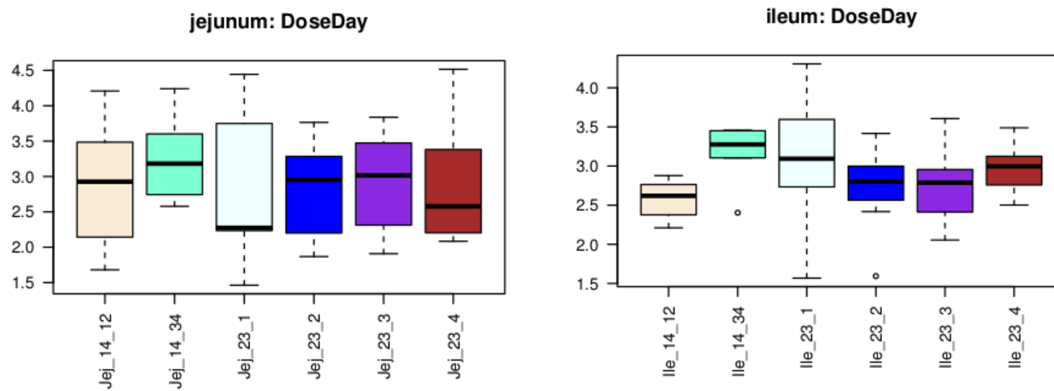

Figure 8. *Chao-1 diversity of the microbiota composition in the jejunum (left panel) and the ileum (right panel).*

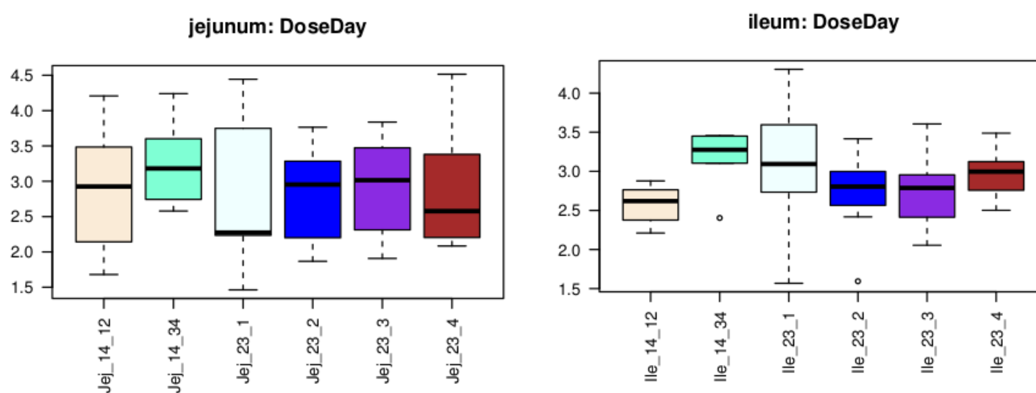

Figure 9. *Shannon diversity of the microbiota composition in the jejunum (left panel) and the ileum (right panel).*

In addition, the microbiota composition data were also analysed on the ß-diversity using a taxonomy summary and a PCA Unifrac approach. The ß-diversity of microbiota composition relates to changes in diversity as effected by "environmental gradients" such as the intestinal location (jejunum and ileum) and dietary treatment x time/age in the present study. It provides indications of changes in composition in terms of gain or loss of microbial species in a complex system. In Figure 10, based on differences in ß-diversity using the taxonomy summary approach, jejunum and ileal digesta samples can be discriminated rather well at the different phylogenetic levels via Principal Component Analysis (PCA), independent of age/time of sampling and dietary treatment.

Unifrac distances are based on the phylogenetic distances between the samples based on the sequence content rather than on the taxonomic diversity. It can be calculated 'unweighted' (not weighing the relative abundance) and 'weighted' (weighing the relative abundance). The unweighted analysis resulted in a very good separation of digesta samples from either jejunal or ileal origin (Figure 11).

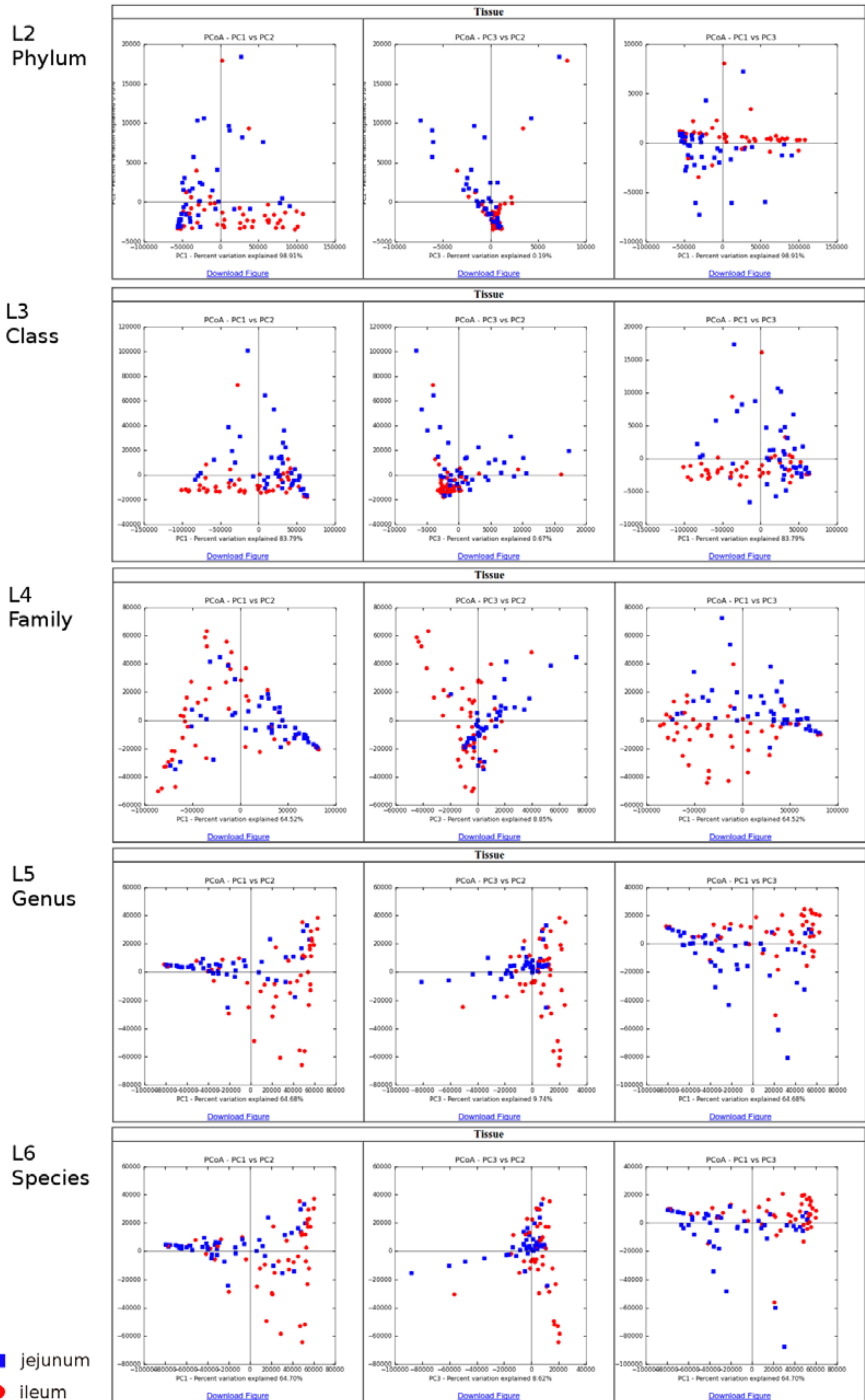

Figure 10. *PCA plots of β-diversity based on taxonomic profile distances to discriminate jejunal (blue) and ileal (red) digesta samples.*

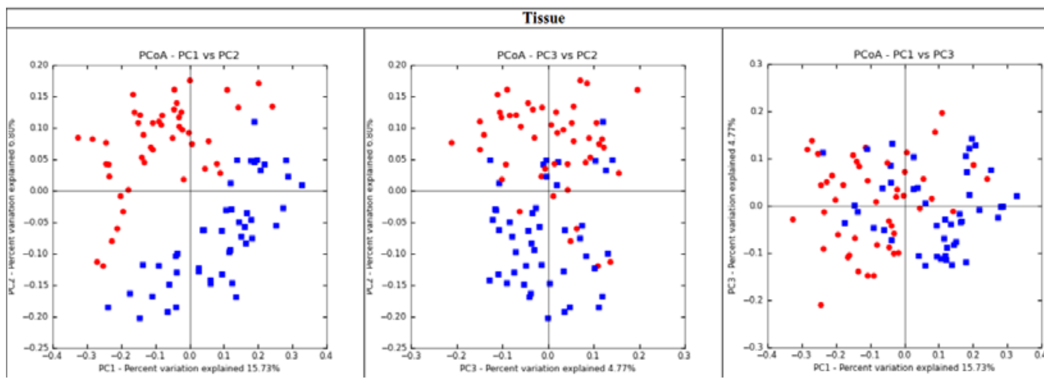

Figure 11. *PCA plots of β-diversity based on unweighted Unifrac distances to discriminate between origin of samples (jejunal vs. ileal digesta samples).*

Jejunum Ileum

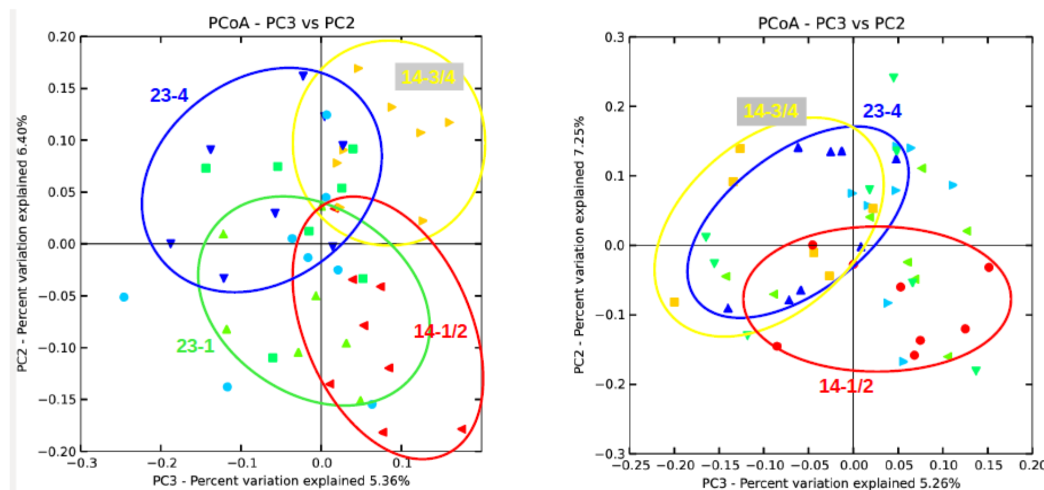

Figure 12. *PCA plots of β diversity based on taxonomic profile distances in jejunal and ileal digesta to discriminate effects of time/age or the dietary treatment x time/age.*

PCA plots of β diversity based on taxonomic profile distances as affected by age/time (day) and treatment (group) x day show separation of samples based on these factors in jejunal and ileal digesta (Figure 12). On d 14, in jejunal and ileal digesta, samples were relatively well discriminated based dietary zinc concentration in the previous period. On d 22/23, at jejunal level samples of treatment 1 and 4 were discriminated based on β diversity, while ileal samples of treatment 4 (high zinc provision over the period of d 0-22/23) overlapped with the samples of treatments 3 and 4 on d 14 (high zinc over d 0-14). The former indicates that at both time points the dietary zinc intervention had profound effects on the β diversity of the intestinal microbiota.

Finally, an ANOVA analysis was performed to evaluate whether there were differences in relative contribution at phylum, class, family, genus and species level of microbiota between samples obtained on d 14 and 22/23 and affected by the dietary treatments. From family down to species level there were five to six taxa showing a statistical difference in relative contribution at $P<0.05$, with the constraint that their overall contribution was at least 1% of the total microbiota. An overview of the taxa concerned is given in Appendix 1.

3.5 Gene expression in intestinal tissue

To explore the whole genome expression data in intestinal mucosal tissue, including the effects of different time-points of sampling, dietary Zn treatments, and intestinal tissue location, a Principal Component Analysis was performed (Figures 13, 14 and 15). This resulted in a clear distinction between samples of jejunal and ileal tissue, whereas the effects of time and dietary treatment on the gene expression in both intestinal segments was less clear.

Differences in expression of specific genes as affected by dietary treatment (high zinc intervention) were explored at both time-points within a tissue (d 14 and 22/23 in jejunum and ileum) (Table 12 and Appendix 7). In both jejunum and ileum, genes/probes were found which were differentially expressed as affected by time/age and dietary treatment. The largest numbers were found for the samples of d 14 for the treatment groups receiving either a low or zinc diet and for the comparison between jejunal digesta samples of the low zinc treatments on d 14 (treatments 1 and 2) and the treatment 1 (low zinc over d 0-22/23) on d 23.

Probes in samples of d 14 and 22/23 that were differently expressed and which had an functional annotation were subsequently used as input for further functional analysis.

Figure 16 shows a Venn diagrams with the number of genes/probes significantly regulated (up- or down) in the mucosa of the jejunum and ileum on d 14 and 22/23, as affected by dietary treatment and time. The diagram shows that most of the genes that were differentially expressed were unique for that specific contrast.

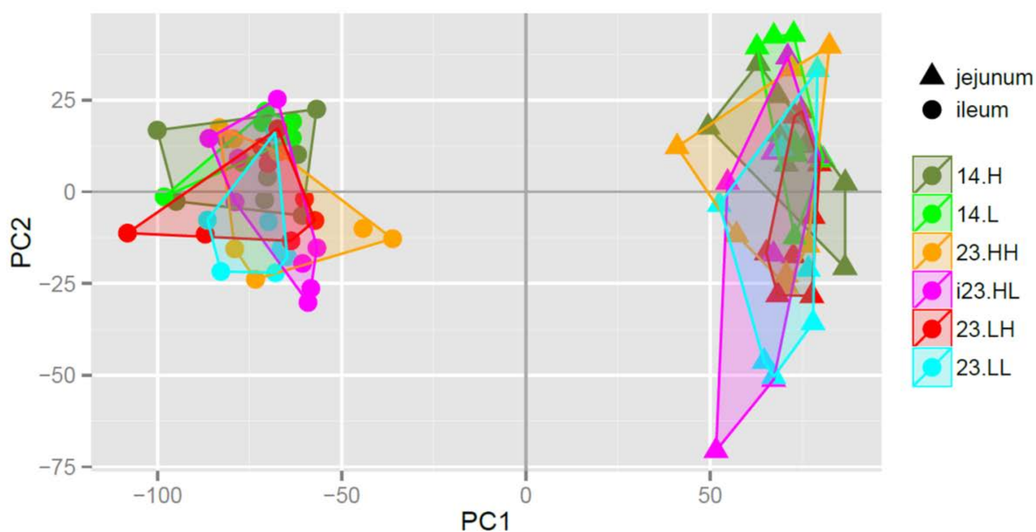

Figure 13. *Principal Component Analysis of gene expression data of jejunal and ileal mucosal tissue of piglets of different treatments on day 14 and 22/23 post weaning.*

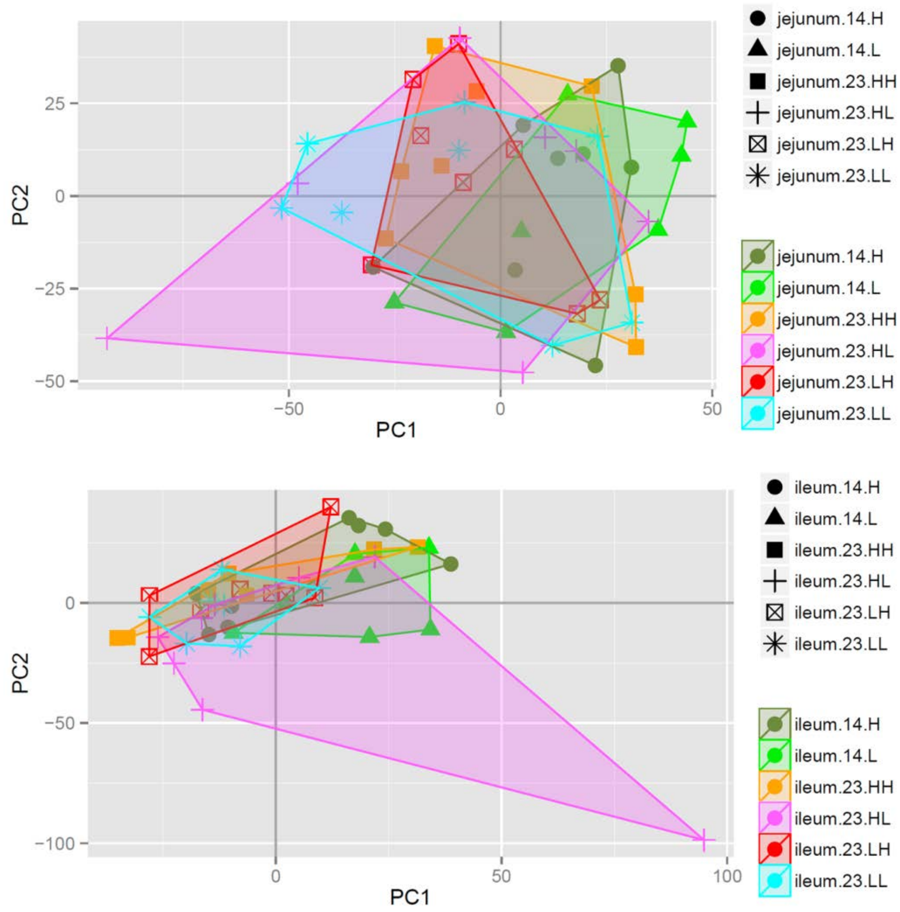

Figure 14. *Principal Component Analysis of gene expression data of jejunal (upper panel) and ileal (lower panel) mucosal tissue of piglets of different treatments on day 14 and 22/23 post weaning.*

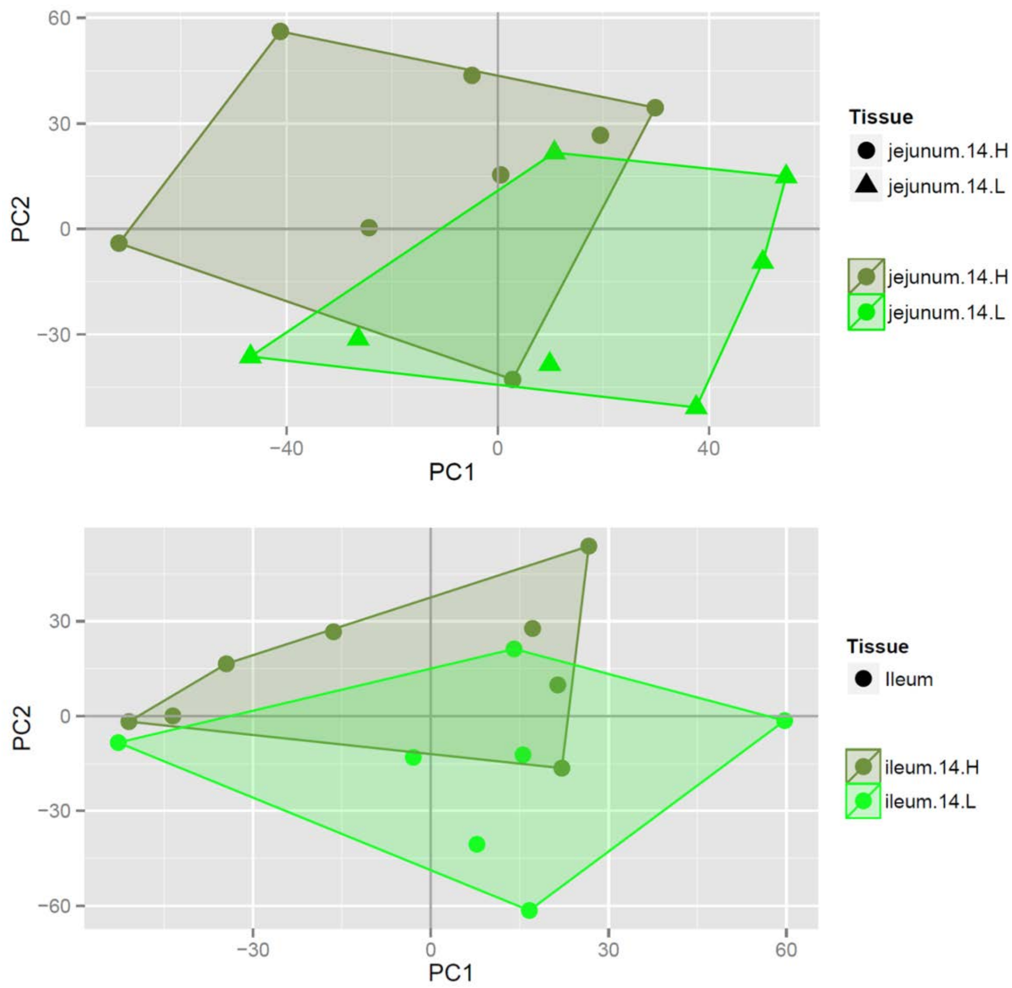

Figure 15. *Principal Component Analysis of gene expression data of jejunal (upper panel) and ileal (lower panel) mucosal tissue of piglets of different treatments on day 14 post weaning.*

Table 12. *Number of genes/probes significantly[1] up- or down regulated in the mucosa of the jejunum and ileum on day 14 and 22/23 as affected by dietary treatment and time.*

Contrast	Jejunum	Ileum
14.H-14.L	53	20
23.HH-23.LL	3	2
23.LH-23.LL	0	0
23.HL-23.LL	0	0
23.HH-23.LH	0	0
23.HH-23.HL	3	8
23.LH-23.HL	1	0
Time	Jejunum	Ileum
23.LL-14.L	110	3
23.HH-14.H	1	0

[1]Adjusted P value < 0.05 and log Fold Change > |1.0|

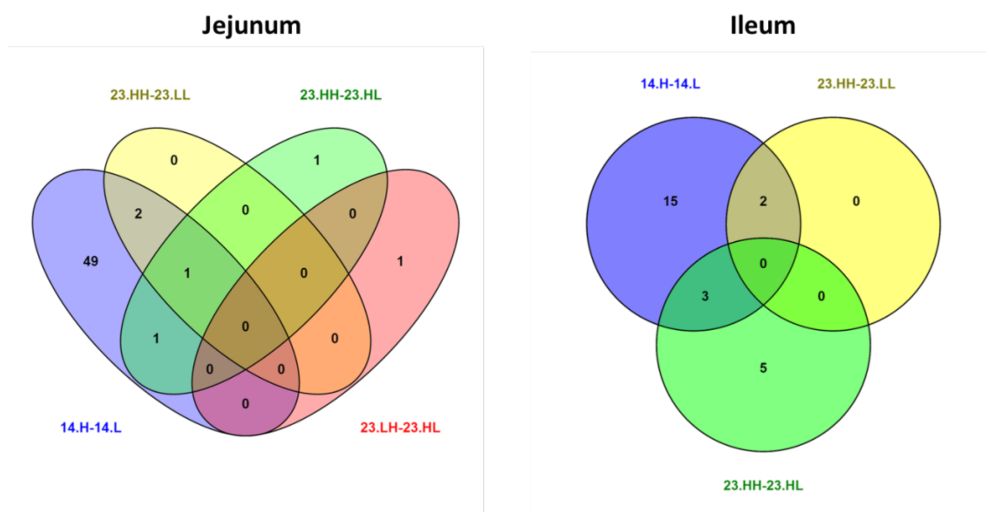

Figure 16. *Venn diagrams showing the number of genes/probes significantly up- or down regulated in the mucosa of the jejunum (left panel) and ileum (right panel) on day 14 and 22/23 as affected by dietary treatment and time.*

Functional analysis was performed by using the web-based tool DAVID. In DAVID it is possible to generate functional annotation clusters, in which different data repositories are queried and aggregated to 'functional clusters'. Tables 13 and 14 show a summary of the results (gene sets in which the differently expressed genes are involved) of separate runs using as input up- or down-regulated genes in jejunal and ileal mucosal tissue for the comparison low vs. high zinc on d 14 (treatment 1/2 vs. 3/4) on the one hand and treatment 1/2 on d 14 vs. treatment 1 on d 22/23 on the other hand. It should also be noted that hardly any genes were significantly differentially expressed when comparing other treatments/time points.

Table 13 shows that a number of significant enriched gene sets in the jejunal mucosa on d 14 by the high zinc intervention from d 0-14 is involved in processes related to signalling processes and signalling peptides (jejunum and ileum) and innate defence responses (ileum). Table 14 shows that, when comparing the low zinc treatment on
d 23 and 14, a larger number of genes related to a variety of processes, of which part relate to the (innate) immune system, are differentially expressed in the intestinal mucosa of piglets in the low zinc treatment

Table 13. *Significantly enriched gene sets in the jejunal and ileal mucosa of piglets of the high zinc treatment as compared to the control dietary treatment on d 14 (14.H versus 14.L) as determined via DAVID Functional Annotation Clustering.*

Term/pathway	**ES[1]**	**Genes**
Jejunum		
Secreted / extracellular / signal peptide	1.64	TFR2, SERPINA6, ANG, DUOXA2, SFTPD, MUC20, REG3G, CCL28, EFNA4, WFDC3, GDF15, SLC39A4
Secreted / signal peptide	2.20	ANG, SERPINA6, MUC20, SFTPD, REG3G, EFNA4, GDF15, SLC39A4, CCL28, WFDC3
Ileum		
Secreted / extracellular / signal peptide	1.77	MUC20, SFTPD, C4BPB, REG3G, SLC39A4, DMBT1, SPP1
Defence response (innate) / signal / cytoplasm	1.50	MUC20, SFTPD, C4BPB, REG3G, SLC39A4, DMBT1, SPP1

[1]ES, enrichment score; Values >1.3 are statistically significant.

Table 14. *Significantly enriched gene sets in the jejunal mucosa of piglets of the low zinc treatment on d 23 as compared to the low zinc control dietary treatment on d 14 (23.LL vs. 14.L) as determined via DAVID Functional Annotation Clustering.*

Term	**ES[1]**	**Genes**
Signal peptide / glycoprotein / extracellular / disulfide bond	5.01	VIP, CPO, LPL, EDN3, RNASE1, C9, CLCA1, CLCA4, CTRB1, FRZB, LGALS9, TOR2A, LGALS3BP, NTS, BTN1A1, FCN1, SFTPD, GHRL, C2, SEPP1, PLA2G3, FN1, SPP1, ABI3BP, SEMA5A, FCGR1A, DPEP1, CUBN, CA12, CTLA4, PTPRR, SUCNR1, BST2, DUOXA2
Hormone activity	2.24	VIP, TOR2A, EDN3, NTS, GHRL
Defence response / inflammation	1.94	LPL, C9, FCN1, SEPP1, C2, FN1, LGALS3BP, FCGR1A, SFTPD, SPP1, CYP1A1
Negative regulation of response to stimulus	1.93	GPX2, CTLA4, GHRL, SPP1, EDN3
Carbohydrate binding / lectin	1.77	LPL, LGALS9C, FCN1, SFTPD, LGALS9, FN1
Von Willebrand factor, type A	1.54	CLCA1, CLCA4, C2
PPAR signaling pathway / fatty acid biosynthetic process	1.48	LPL, FABP3, SCD5, ACSL3, CYP1A1,
Innate immune response	1.41	C9, FCGR1A, SFTPD, C2, LGALS3BP, SPP1, FN1, BST2, FCN1, CTLA4
Von Willebrand factor, type A	1.54	CLCA1, CLCA4, C2
PPAR signalling pathway/fatty acid biosynthetic process	1.48	LPL, FABP3, SCD5, ACSL3, CYP1A1
Innate immune response	1.34	C9, FCGR1A, SFTPD, C2

[1]ES, enrichment score. Values >1.3 are statistically significant.

4. Discussion

The present study was carried out to evaluate whether provision of a high zinc diet for a short period of time during (d 0-14 post-weaning) and/or after the immediate post-weaning phase of piglets (day 14-23 post weaning) as a "model intervention" would influence intestinal microbiota composition and intestinal mucosal gene expression (day 14 and 22/23 post weaning). The study is a follow up of a previous study in the framework of the same project in which the effects were studied of providing either or not a high zinc diet over a period of d 14-23 post-weaning as model intervention (Jansman et al., 2016).

The study should provide further information on whether a short high zinc dietary treatment, as model intervention could potentially interfere with the development of immune competence in pigs. It is well known that intestinal microbiota composition and activity interferes with the development and functionality of the local immune system in the gut in different animal species and in man (Round & Mazmanian, 2009; Hooper et al., 2012). Diet ingredient composition and inclusion of particular functional ingredients, such as pre- and probiotics and substances and extracts of plant origin with antimicrobial properties, can modulate the intestinal microbiota composition in various compartments of the intestinal tract (van Krimpen et al., 2014; Haenen et al., 2013; Taras et al., 2007; Jensen, 1998). There is an intimate contact between the luminal and gut wall associated microbiota and the intestinal mucosa. This interplay indicates that changes in intestinal microbiota composition can also influence responses of gut tissue towards these changes, including changes of responses of the immune system present in the mucosa, submucosa and lamina propria of the gut. It is estimated that about 70% of the immune cells present in the body are located in intestinal tissues (Vighi et al., 2008). Therefore, a change of the intestinal microbiota could also interfere with delicate crosstalk between the intestinal microbiota and intestinal tissue of the host. Given the diversity of inflammatory or immune responses that can be mounted by the intestinal epithelium, association of e.g. probiotics with epithelial cells might be sufficient to trigger signalling pathways that ultimately activate underlying immune cells in the lamina propria. Alternatively, probiotics or other dietary constituents may also release or contain factors that themselves trigger signalling cascades at the level of the epithelium or associated immune system (Corthésy et al., 2007).

Various studies have highlighted how the microbiota elicit innate and adaptive immune mechanisms that cooperate to protect the host and maintain intestinal homeostasis. Epithelial cells are a central component of the immune system of the gut. In a similar manner to immune cells, epithelial cells express receptors for microbial-associated molecular patterns (MAMPs). These receptors activate signalling cascades that finely tune epithelial cell production of antimicrobial products and chemokines, depending on the signals that are delivered by intestinal microbiota (Cerf-Bensussan & Gaboriau-Routhiau, 2010). Thus, gut epithelial cells form a potent and inducible physico-chemical barrier, which limits microbial growth and access to the gut surface. They can also recruit leukocytes to complement their barrier function or to participate in the activation of gut adaptive immune responses. In mammals, the development of gut-associated lymphoid tissues (GALT) is initiated before birth by a genetic programme. However, GALT maturation and the recruitment of IgA-secreting plasma cells and activated T cells to mucosal sites only occurs after birth and is strictly dependent on microbiota-derived signals. These signals influence the crosstalk between epithelial cells and gut dendritic cells (DCs), thereby modulating the nature and intensity of intestinal B and T cell responses (Cerf-Bensussan & Gaboriau-Routhiau, 2010).

Given the former, dietary constituents and specific interventions, such as a high concentration of dietary zinc in the present and previous study, could exert both direct and indirect effects on the intestinal mucosa and its residing immune system. Therefore, dietary interventions at young age could interfere with the development of immune competence and responsiveness of the immune system at later age.

The previous study (Jansman et al., 2016) showed that a short term intervention with a high zinc diet (2500 mg/kg) using ZnO as source of zinc, had a significant influence on microbiota composition and its diversity in small intestinal digesta, in particular in the ileum. On d 35, twelve days after feeding the control diet with a regular Zn concentration (100 mg/kg) similar to the control group, the

composition and diversity was still different compared to the control group. In the present study, the effects of the dietary treatments on the taxonomic intestinal microbiota composition were somewhat less pronounced compared to the previous study. At jejunal level, the composition of the microbiota was most similar in animals that had received a
diet with a regular Zn concentration prior to sampling at both time points (d 14 and 22/23). Post-hoc analysis on the composition at different taxonomic levels (from phylum to species) revealed a limited number of differences in microbiota composition among treatments and/or time points of sampling. However, when considering diversity of the intestinal microbiota, we found an increased diversity in the ileum, but not in the jejunum, on d 14 after a high zinc dietary treatment, similar as observed in the previous study after a high zinc intervention over the period of d 14 -23 post weaning. In the present study, microbiota α-diversity was neither affected in the jejunum nor in the ileum on d 22/23 of age by the experimental treatments. Based on the β-diversity, microbiota composition was rather distinct between low and high zinc treatments on d 14 in the jejunum and ileum, and on d 22/23, the diversity in jejunal digesta between treatments 1 (L-L) and 4 (H-H) being most distinct. Although effects on microbiota composition differed to some extent between both studies, the present study shows that the effects of a high zinc dietary treatment on intestinal microbiota composition can also be dependent of the timing of the provision of the high zinc treatment in the post weaning period. From the present study it can be derived that the intestinal microbiota is more subject to changes by dietary zinc concentration in the immediate post weaning period (d 0-14) compared to the period of d 14-23 post weaning. This is in agreement with a study of Starke et al. (2014) in which diets were fed to weaned piglets containing 57 (low) or 2425 (high) mg/kg for a period of 5 weeks. Intestinal digesta was sampled in weekly intervals and analysed for microbiota composition. The most prominent effects of high dietary zinc were observed one week after weaning in the stomach and small intestine. Pronounced reductions were observed for Enterobacteriaceae and the Escherichia group as well as for Lactobacillus spp. and for some Lactobacillus species. The impact of high dietary zinc diminished for enterobacteria with increasing age, but was permanent for Lactobacillus species.

Pieper et al. (2012) also observed effects of dietary zinc oxide concentrations (50, 150, 250, 1000 and 2500 mg zinc/kg) on microbiota composition in ileal digesta of piglets over a period of two weeks (one to three weeks after weaning). Ileal bacterial community profiles were analysed by denaturing gradient gel electrophoresis and selected bacterial groups quantified by real-time PCR. Species richness, Shannon diversity and evenness were significantly higher at high ZnO levels. Quantitative PCR revealed lowest total bacterial counts in the 50 mg/kg group. Increasing ZnO levels led to an increase in Enterobacteria from log 4.0 CFU/g digesta (50 mg/kg) to log 6.7 CFU/g digesta (2500 mg/kg). The increase in microbiota diversity in the gut by a high zinc intervention is in line with observations in the present study.

It is well established that dietary zinc oxide at pharmacological level helps to prevent post-weaning diarrhoea and improve pig performance, but the underlying mechanisms are still not clear. Besides the influence of high zinc doses on digestive function, immune system, muscle growth, transcription, apoptosis and hormone status, changes in intestinal microbial community composition could explain the reduction in diarrhoea in piglets (Suttle, 2010). In fact, the results presented by Liu et al. (2014) and the present study support previous findings showing that some bacterial groups are suppressed by high levels of dietary zinc oxide, whereas others increase in abundance at high zinc doses (Katouli et al., 1999; Højberg et al., 2005; Vahjen et al., 2010). An increased bacterial diversity in the digestive tract could be an indicator for a more stable bacterial ecosystem, thus resisting perturbations that could lead to impaired function and health of the gut.

The previous study also revealed effects of the high zinc intervention on gene expression in the jejunal and ileal mucosa, in particular on d 23, at the end of the intervention period (Jansman et al., 2016). No effects on intestinal gene expression were observed on day 35 post weaning, twelve days after termination of the dietary intervention. Both up and down regulation of genes was observed as affected by the dietary Zn intervention. The changes in gene expression were in part related to genes (e.g. MT1A and MT1F) encoding for the synthesis of metallothioneins,
involved in mineral absorption, intracellular transport and regulation of intracellular mineral concentrations. Metallothionein has been shown to control the amount of zinc available in cells and participates in the transfer of essential metal ions to critical stress response proteins and transcription factors such as zinc-finger proteins, apoenzymes and other metalloproteins (Wintergerst et al., 2007). In line, also the expression of SLC39A4 was affected in the jejunal and ileal mucosa in the present study. This gene encodes for a zinc transporter and plays an important role in cellular zinc homeostasis. In the present study, also effects of the dietary zinc treatments on gene expression of the jejunal and ileal mucosa were observed, in particular at

d 14 post weaning. In addition, an time x Zn treatment interaction was found for the gene expression in the jejunal mucosa, where expression of more than 100 genes differed between
d 14 and 22/23 in the normal zinc treatment (23.LL vs 14.L), while such a time effect was not observed in piglets receiving a high zinc diet over the first 14 d post weaning (treatments 3 and 4) or a high zinc diet over only d 14-22/23 (treatment 2). The concerned genes are involved in processes related to signalling processes and to the development of the innate immune system. The absence of such effects in piglets of the other treatment groups could suggest that dietary

provision of high levels of zinc as ZnO could accelerate some intestinal developmental processes in the jejunal mucosa. These effects can potentially be either caused by a direct effect of Zn or ZnO or by indirect effects via (subtle) changes in the jejunal microbiota composition induced by Zn or ZnO.

In the previous study (Jansman et al., 2016) at both the jejunal and the ileal level, the expression of the gene encoding for metallothioneins (MT) was increased. MT are small, cysteine-rich proteins with also significant immune modulatory activity. They have been shown to play a critical role in important cellular mechanisms including heavy metal detoxification, essential metal management and inflammatory response. MT production can be induced by a number of cellular stressors and acts to lessen the harmful effects of oxidizing agents and heavy metal exposure such as zinc (Roy-O'Reilly, 2012). There is evidence that the level of metallothioneins in man may have significant effects on the adaptive immune response. Roy-O'Reilly (2012) showed in research with mice that variation in gene expression for MT affects the speed and degree of the lymphocyte proliferative response. It has been hypothesized that the lack of functional MT reduces the ability to handle elevated levels of reactive oxygen species (ROS). This increase in intracellular ROS leads to activation of transcription factors like NF-kB, which augments the expression of immunomodulatory cytokines (Crowthers et al., 2000). A link between the expression of genes encoding for metallothionein 1A and IL-6 was observed in airway tissue of sheep stressed by physical injury (Yahaya et al., 2013.) In essence, a lack of endogenous MT creates a primed immune response that could potentially lead to autoimmune reactions in man under stressful conditions. Metallothioneins have also been found to be an essential component of the pathogen induced immune response, in particular in the early innate immune response to a Listeria infection in mice. Both mice with increased levels of MT and mice with decreased levels of were shown to be more resistant to early Listeria infection (Emeny et al., 2009). It has also been suggested that under inflammatory conditions, metallothioneins in the extracellular environment may support the movement of leukocytes to the site of inflammation (Yin et al., 2005). In pigs, zinc supplementation was associated with a marked decrease in expression of immune response genes concerned with inflammation, possibly related to the stage of infection (Sargeant et al., 2010). The key finding from their study was the differential expression of a number of genes associated with immunity and inflammation between dietary treatments. In the intestinal mucosa, expression of e.g. Peptidase Inhibitor 3 (PI3) (a gene that encodes an elastase-specific inhibitor that functions as an antimicrobial peptide against Gram-positive and Gram-negative bacteria), Bactericidal/Permeability-Increasing Protein (BPI) (a gene encoding a lipopolysaccharide binding protein) and Dual Oxidase 2 (DUOX2), each encoding antimicrobial molecules, and PSPD, encoding an opsonin, being a molecule that enhances phagocytosis, was lower in piglets provided with ZnO supplementation. They concluded that zinc oxide supplementation in post weaning ETEC challenged piglets may improve piglet performance, at least in part, by decreasing the level of intestinal inflammation caused by ETEC challenge, reducing disruption of normal intestinal morphology and function. Zinc oxide may also influence the severity of infection through altered expression of pathogen receptors (Sargeant et al., 2010). In the first study, also expression of the CXCL13 gene was affected in jejunal mucosal tissue by the Zn intervention. This gene codes for a protein that has chemotactic properties for B-lymphocytes, but not for T-lymphocytes, monocytes and neutrophils. It promotes homing B-cells from the periphery to Peyer's patches. This shows another link between the zinc intervention and changes in responses of the immune system.

Some other genes in intestinal mucosal tissues were up- or down regulated by the dietary zinc intervention in the present study, which were in part also related to pathways involved in the immune system (e.g. signalling pathways and cytokine receptor interactions).

The further biological consequences of these changes, in particular for the functioning of the immune system of pigs are not clear yet. The present data do also not reveal whether these effects are a direct effect related to the differences in dietary zinc supply and absorption or are indirect effects related to changes in the intestinal microbiota composition as induced by dietary supplementation of ZnO at pharmaceutical levels.

Zhang and Guo (2009) evaluated the pharmacological effect of Zn (2000 mg Zn/kg) on occurrence of diarrhoea in relation to intestinal permeability of post weaning piglets. Expression of mucosal tight junction protein was measured at RNA and protein level. Inclusion of tetrabasic zinc chloride (TBZC) or ZnO in the diet significantly increased average daily gain and average daily feed intake, while leading to reduced feed conversion ratio and faecal scores. TBZC reduced urinary lactulose : mannitol ratios of weaning piglets, while dietary supplementation with ZnO tended to reduce urinary lactulose : mannitol ratios. ZnO or TBZC significantly enhanced the mRNA and protein expression of occludin and zonula occludens protein-1 (ZO-1) as important tight junction proteins in the ileal mucosa. Piglets fed the TBZC-supplemented diet had a higher level of occludin than pigs fed the ZnO-supplemented diet. The results indicates that Zn supplementation decreased faecal scores and the reduction was accompanied by reduced intestinal permeability, which was evident from the reduced urinary lactulose : mannitol ratios and increased expression of occludin and ZO-1. Therefore, the protective effect of pharmacological levels of dietary Zn in reducing diarrhoea might, at least partly, also be associated with improved gut integrity.

Liu et al. (2014a) studied the effects of increasing levels of dietary Zn on mRNA expression levels of mucin (MUC) 1, 2, 13, 20, toll-like receptor (TLR) 2, 4, interleukin (IL)-1ß, 8, 10, interferon-c (IFN-γ) and transforming growth factor-ß (TGF-ß). The colonic crypt area increased in an age-depending manner, and the greatest area was found with medium concentration of dietary zinc. With the high concentration of dietary zinc, the number of goblet cells containing mixed neutral-acidic mucins and total mucins increased. The mRNA expression of TLR4 and the pro-inflammatory cytokine IL-8 were down-regulated with high dietary zinc treatment, while piglets fed with medium dietary zinc had the highest expression. It was concluded that dietary zinc level had a clear impact on colonic morphology, mucin profiles and immunological traits in piglets after weaning. These changes might support local defence mechanisms and affect colonic physiology and contribute to the reported reduction of post-weaning diarrhoea. Similarly, in our study the expression of INHBB was affected by the Zn intervention. INHBB codes for inhibins/
activins involved in regulating a number of diverse functions such as hypothalamic and pituitary hormone secretion, gonadal hormone secretion, germ cell development and maturation, erythroid differentiation, insulin secretion, nerve cell survival, embryonic axial development or bone growth, depending on their subunit composition. Inhibins appear to oppose the functions of activins. The link between the dietary zinc treatment and the energy metabolism at cellular level in the intestinal mucosa (expression of INHBB and PKLR (the protein encoded by this gene is a pyruvate kinase that catalyses the transphosphorylation of phohsphoenol pyruvate into pyruvate and ATP, which is the rate-limiting step of glycolysis) and could be related to a change a shift in energy metabolism from aerobe to anaerobe metabolism in cells exposed to high Zn conditions and to high intracellular oxidant concentrations (i.c. Zn) (Jansman et al., 2016).

Liu et al. (2014b) studied the effects of different dietary Zn concentrations in post weaning piglets on intestinal mucosal morphology and the number of goblet cells producing neutral, acidic, sulfated, and sialylated mucins, intraepithelial lymphocytes and gene expression of mucin 2 (MUC2), mucin 20 (MUC20), ß-defensin 3, and trefoil factor 3 (TFF3). Villus height and crypt depth were not affected by dietary Zn concentration. The mucin types were modified mainly by age, and dietary Zn had no effect in the proximal jejunum. In the distal jejunum, Zn concentration had effects on the mucin types found. A high dietary ZnO reduced the sulfomucins and increased the sialomucins in the crypts. High dietary Zn treatment led to a reduced abundance of CD8+ γδT-cells. The expression of MUC2 and MUC20 was not influenced by age or dietary Zn concentration. A high Zn intake resulted in a reduced gene expression of ß-defensin 3, but did not affect the expression of TFF3. It was concluded that dietary Zn concentration supplied as ZnO have specific effects on both the innate and adaptive gut associated immune system of piglets.

Hulst et al. (2015) evaluated the direct effects of incubation of cultured Intestinal Porcine Epithelial Cells (IPEC-J2), either or not challenged with Salmonella, with ZnO and judged the capability of this assay to predict enterocyte-specific physiological and immunological processes. Bioinformatics analysis revealed that the most important biological process regulated in IPEC-J2 cells by ZnO was "oxidative stress" in the presence as well as in the absence of Salmonella. IPEC-J2 cells responded to oxidative stress mainly by regulation of expression of genes part of the HIF1-signaling pathway and by regulation of genes that sense free radicals and/or sense DNA-damage. The fact that expression of most of these genes were not, or only weakly, regulated by Salmonella alone indicated that HIF1-signaling was a generic pathway by which IPEC-J2 reacted to an "oxidative stress" situation. Down-regulation of genes involved in sugar metabolism (HK) suggests that oxidative stress was also induced by a shortage of glucose as energy source for these cells. In addition, the Zn-transporter MT1A was

identified as a key gene which expression was affected by incubation with ZnO. MT plays a role in steering cytokine production in IPEC-J2 cells, especially, also because of the involvement of MT1A in the production of IL6, a cytokine that regulates HIF1-signalling (Mariani et al., 2008). Such indirect regulation by MT1A is probably more controlled and prevents overexpression of IL6. CSF2 was selected as immune modulator. CSF2 production by enterocytes may stimulate secretion of cytokines/chemokines by a small number resident macrophages/dendritic cells in the lamina propria in order to attract and activate other immune cells.

The previous (Jansman et al., 2016) and present in vivo study with piglets was carried out with animals in the post weaning period. Beside the multiple and complex effects of the weaning process on the piglets, no specific additional challenge was imposed to the piglets. Such an approach could be useful to evaluate the effects of dietary treatments on the responsiveness of the immune system, being influenced by the immune competence of the animals which is in part genetically determined and for another part developed during the previous period of life. Such
studies would involve evaluation of immunological response parameters specific for the imposed immune challenge. In the present study, IL-6, TNF-α and haptoglobin as acute phase protein in plasma were analysed on d 14 and 22/23. No effects of dietary ZnO administration on these immune molecules were observed. It is worthwhile to study these and other immune parameters as affected by dietary Zn treatment under immune challenge conditions.

It can be concluded from the present study that provision of a diet with a high concentration of zinc as zinc oxide (2730 mg/kg) in the immediate post weaning period (d 0-14 post-weaning) and/or during a day 14-23 post weaning (2850 mg/kg) induced some changes in intestinal microbiota composition, in particular in the ileum, and on the jejunal and ileal expression of genes. Effects were most pronounced in the immediate post weaning period (d 14 post-weaning), suggesting that this period is a more suitable window of opportunity to modulate in the intestinal microbiota with potential consequences for the functional development for the gut compared to later phases in life. Effects of the present and a previous study, as well as data presented in the literature suggest that high zinc diets in the post weaning period interfere with development of the piglet’s immune system and can be used as a model intervention for studying possibilities to modify development of immune competence. Further studies using piglets subjected to specific immune challenges can provide a more complete view on the biological and functional meaning of the observed changes induced by high zinc diets in the post-weaning period.

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6. Appendices

Appendix 1. Overview of taxa at family, genus and species level in jejunal and ileal digesta showing a difference in relative contribution at $P<0.05$ between dietary Zn treatment (group) x day (time/age), with the constraint that their overall contribution is at least 1% of the total microbiota.

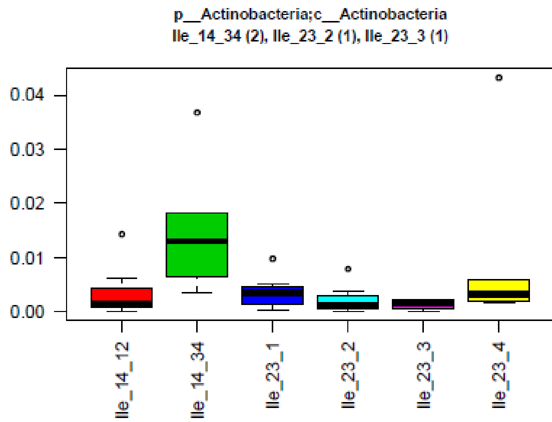
Jejunum

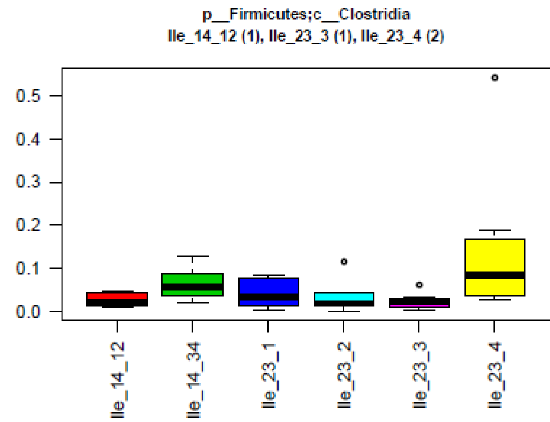
Level	Taxon (p_anova)	Significant comparisons (posthoc)
L2_Phylum	None	None
L3_Class	p__Firmicutes;c__Clostridia (p=0.0354) p__Firmicutes;c__Bacilli (p=0.0491)	None None
L4_Family	p__Proteobacteria;c__Gammaproteobacteria;f__Moraxellaceae (p=0.00639) p__Firmicutes;c__Clostridia;f__Veillonellaceae (p=0.0329) p__Firmicutes;c__Bacilli;f__Staphylococcaceae (p=0.0499)	None None None
L5_Genus	p__Firmicutes;c__Bacilli;f__Streptococcaceae;g__Lactococcus (p=0.00786) p__Proteobacteria;c__Gammaproteobacteria;f__Moraxellaceae;g__Moraxella (p=0.00976) p__Firmicutes;c__Clostridia;f__Veillonellaceae;g__Veillonella (p=0.0357) p__Firmicutes;c__Bacilli;f__Staphylococcaceae;g__Staphylococcus (p=0.0433)	14_34 vs 23_1, 23_3 and 23_4 None None None
L6_Species	p__Firmicutes;c__Bacilli;f__Streptococcaceae;g__Lactococcus;s__ (p=0.00797) p__Proteobacteria;c__Gammaproteobacteria;f__Moraxellaceae;g__Moraxella;s__ (p=0.00976) p__Firmicutes;c__Bacilli;f__Staphylococcaceae;g__Staphylococcus;s__ (p=0.0414)	14_34 vs 23_1, 23_3 and 23_4 None None

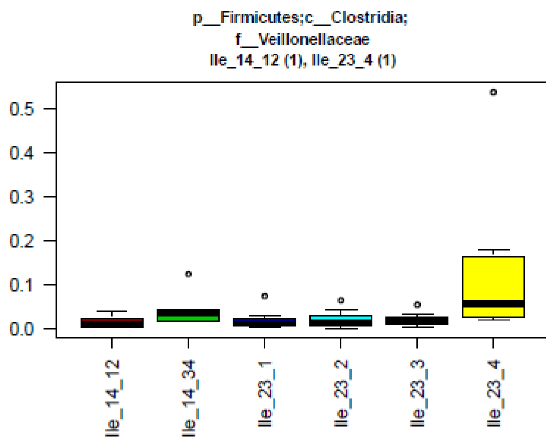
Ileum

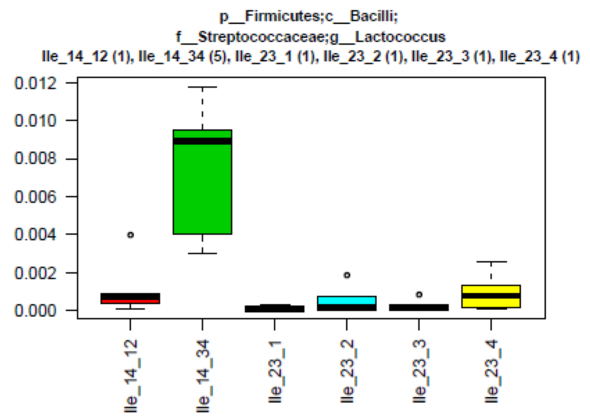
Level	Taxon (p_anova)	Significant comparisons (posthoc)
L2_Phylum	p__Actinobacteria (p=0.0175)	14_34 vs 23_2 and 23_3
L3_Class	p__Actinobacteria;c__Actinobacteria (p=0.0194) p__Firmicutes;c__Clostridia (p=0.0273)	14_34 vs 23_2 and 23_3 23_4 vs 14_12 and 23_3
L4_Family	p__Firmicutes;c__Clostridia;f__Veillonellaceae (p=0.0313) p__Actinobacteria;c__Actinobacteria;f__Actinomycetaceae (p=0.000113) p__Firmicutes;c__Bacilli;f__Streptococcaceae (p=0.016)	14_12 vs 23_4 None None
L5_Genus	p__Firmicutes;c__Bacilli;f__Streptococcaceae;g__Lactococcus (p=4.43e-12) p__Actinobacteria;c__Actinobacteria;f__Actinomycetaceae;g__Actinomyces (p=9.52e-05) p__Firmicutes;c__Bacilli;f__Streptococcaceae;g__Streptococcus (p=0.018) p__Firmicutes;c__Clostridia;f__Veillonellaceae;g__Veillonella (p=0.0287)	14_34 vs all of the rest None None None
L6_Species	p__Firmicutes;c__Bacilli;f__Streptococcaceae;g__Lactococcus; ;s__ (p=5e-12) p__Firmicutes;c__Clostridia;f__Veillonellaceae;g__Veillonella;s__dispar (p=0.00769) p__Bacteroidetes;c__Bacteroidia;f__Prevotellaceae;g__Prevotella; ;s__ (p=0.0287) p__Actinobacteria;c__Actinobacteria;f__Actinomycetaceae;g__Actinomyces ;s__ (p=9.52e-05) p__Firmicutes;c__Bacilli;f__Streptococcaceae;g__Streptococcus;s__ (p=0.0168)	14_34 vs all of the rest 14_34 vs 23_2 and 23_3 23_1 vs 14_12 and 23_3 None None

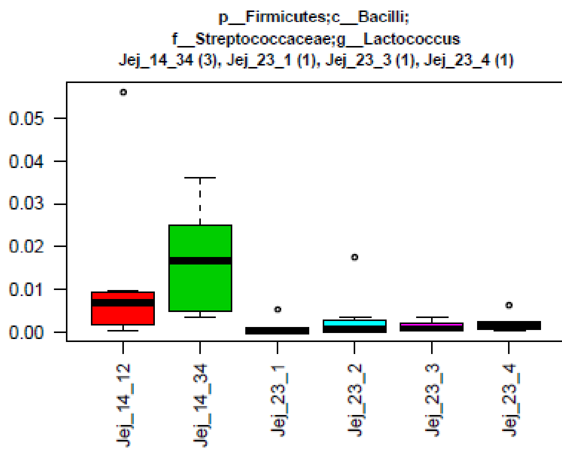
Appendix 2. Microbiota composition in jejunal and ileal digesta at different taxon levels differing significantly by age (time) and/or dietary treatment (proportion of total microbiota).











Appendix 3. Overview of microbiota composition at family level in jejunal digesta as affected by age (time) and dietary treatment (% of total microbiota).

Treatment	d 14		d 22/23			
Taxon	1/2	3/4	1	2	3	4
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Pasteurellaceae	6.9	22.5	5.0	19.3	5.3	18.9
k__Bacteria;p__Firmicutes;c__Bacilli;f__Turicibacteraceae	0.9	0.7	0.3	0.1	0.1	0.1
k__Bacteria;p__Firmicutes;c__Bacilli;f__Streptococcaceae	6.4	10.8	11.6	8.9	9.9	6.5
k__Bacteria;p__Firmicutes;c__Bacilli;f__Lactobacillaceae	66.4	42.1	65.5	47.7	64.3	35.9
k__Bacteria;p__Firmicutes;c__Clostridia;f__Veillonellaceae	7.0	9.3	7.1	13.8	10.1	27.2
k__Bacteria;p__Actinobacteria;c__Actinobacteria;f__Actinomycetaceae	0.7	1.1	1.3	0.7	0.8	0.6
k__Bacteria;p__Firmicutes;c__Bacilli;f__Staphylococcaceae	0.3	0.6	0.3	1.8	0.9	1.6
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Enterobacteriaceae	4.3	3.1	1.6	0.6	0.7	0.6
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;f__Erysipelotrichaceae	0.8	0.3	0.5	0.2	1.6	0.4
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Moraxellaceae	0.1	1.0	0.1	0.4	0.1	0.3
k__Bacteria;p__Firmicutes;c__Clostridia;f__Lachnospiraceae	0.1	0.0	0.1	0.0	0.2	0.1
k__Bacteria;p__Firmicutes;c__Clostridia;f__Clostridiaceae	0.1	0.0	0.3	0.0	0.2	0.1
k__Bacteria;p__Firmicutes;c__Bacilli;f__Leuconostocaceae	0.3	0.7	0.3	1.0	0.2	1.6
k__Bacteria;p__Cyanobacteria;c__Chloroplast;f__	0.3	1.7	1.8	0.5	0.7	0.9
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;f__Prevotellaceae	1.1	0.7	0.5	0.2	0.5	1.3
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;f__Neisseriaceae	0.1	0.8	0.3	0.3	0.2	0.4
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;f__Alcaligenaceae	0.0	0.1	0.1	0.1	0.1	0.1
k__Bacteria;p__Firmicutes;c__Clostridia;f__	0.0	0.0	0.1	0.1	0.1	0.0
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;f__Comamonadaceae	0.0	0.0	0.0	0.0	0.0	0.0
k__Bacteria;p__Firmicutes;c__Bacilli;f__Enterococcaceae	0.1	0.1	0.1	0.0	0.1	0.2
k__Bacteria;p__Firmicutes;c__Clostridia;f__Ruminococcaceae	0.1	0.1	0.1	0.0	0.3	0.1
k__Bacteria;p__Firmicutes;c__Bacilli;f__Gemellaceae	0.1	0.3	0.2	0.2	0.2	0.2
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Xanthomonadaceae	0.6	0.9	0.2	1.4	0.6	0.6
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;f__Helicobacteraceae	0.0	0.0	0.0	0.0	0.0	0.0
Unassigned;Other;Other;Other	0.6	0.9	0.9	0.5	0.6	0.7

Appendix 4. Overview of microbiota composition at genus level in jejunal digesta as affected by age (time) and dietary treatment (% of total microbiota).

	d 14		d 22/23			
Taxon	1/2	3/4	1	2	3	4
k__Bacteria;p__Firmicutes;c__Bacilli;f__Turicibacteraceae;g__Turicibacter	0.9	0.7	0.3	0.1	0.1	0.1
k__Bacteria;p__Firmicutes;c__Bacilli;f__Streptococcaceae;g__Streptococcus	5.2	9.1	11.5	8.5	9.8	6.3
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Pasteurellaceae;g__Actinobacillus	3.9	12.6	3.2	12.4	3.3	12.0
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Pasteurellaceae;Other	2.2	6.7	1.5	5.7	1.7	5.2
k__Bacteria;p__Firmicutes;c__Bacilli;f__Lactobacillaceae;g__Lactobacillus	66.4	42.0	65.5	47.7	64.3	35.9
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Pasteurellaceae;g__	0.7	3.1	0.3	1.1	0.3	1.6
k__Bacteria;p__Firmicutes;c__Clostridia;f__Veillonellaceae;g__Veillonella	4.9	8.5	6.7	13.4	9.4	25.7
k__Bacteria;p__Actinobacteria;c__Actinobacteria;f__Actinomycetaceae;g__Actinomyces	0.7	1.1	1.3	0.7	0.8	0.6
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Pasteurellaceae;g__Aggregatibacter	0.0	0.1	0.0	0.0	0.0	0.0
k__Bacteria;p__Firmicutes;c__Bacilli;f__Staphylococcaceae;g__Staphylococcus	0.2	0.6	0.3	1.8	0.9	1.6
k__Bacteria;p__Firmicutes;c__Bacilli;f__Streptococcaceae;g__Lactococcus	1.2	1.7	0.1	0.3	0.1	0.2
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Enterobacteriaceae;g__	4.2	2.5	1.4	0.3	0.5	0.4
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Moraxellaceae;g__Moraxella	0.0	0.9	0.1	0.3	0.1	0.2
k__Bacteria;p__Firmicutes;c__Clostridia;f__Clostridiaceae;g__	0.0	0.0	0.0	0.0	0.0	0.0
k__Bacteria;p__Firmicutes;c__Clostridia;f__Lachnospiraceae;g__	0.1	0.0	0.0	0.0	0.1	0.0
k__Bacteria;p__Cyanobacteria;c__Chloroplast;f__;g__	0.3	1.7	1.8	0.5	0.7	0.9
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;f__Prevotellaceae;g__Prevotella	1.1	0.7	0.5	0.2	0.5	1.3
k__Bacteria;p__Firmicutes;c__Bacilli;f__Leuconostocaceae;g__Weissella	0.1	0.2	0.1	0.9	0.2	1.5
k__Bacteria;p__Firmicutes;c__Clostridia;f__;g__	0.0	0.0	0.1	0.1	0.1	0.0
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;f__Alcaligenaceae;g__	0.0	0.1	0.1	0.1	0.1	0.1
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;f__Erysipelotrichaceae;g__	0.1	0.1	0.1	0.0	0.2	0.0
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;f__Comamonadaceae;g__Comamonas	0.0	0.0	0.0	0.0	0.0	0.0
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;f__Neisseriaceae;g__Neisseria	0.0	0.2	0.1	0.1	0.0	0.1
k__Bacteria;p__Firmicutes;c__Bacilli;f__Enterococcaceae;g__Enterococcus	0.1	0.1	0.1	0.0	0.1	0.2
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;f__Erysipelotrichaceae;g__[Eubacterium]	0.3	0.1	0.2	0.1	0.6	0.2

Appendix 5. Overview of microbiota composition at family level in ileal digesta as affected by age (time) and dietary treatment (% of total microbiota).

Treatment	d 14		d 22/23			
Taxon	1/2	3/4	1	2	3	4
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Pasteurellaceae	20.9	21.4	33.9	22.4	42.6	34.0
k__Bacteria;p__Firmicutes;c__Bacilli;f__Turicibacteraceae	13.2	23.8	8.0	12.8	9.2	6.7
k__Bacteria;p__Firmicutes;c__Bacilli;f__Streptococcaceae	3.9	21.2	12.8	21.6	4.3	16.9
k__Bacteria;p__Firmicutes;c__Bacilli;f__Lactobacillaceae	38.2	17.5	29.5	31.8	18.0	21.3
k__Bacteria;p__Firmicutes;c__Clostridia;f__Veillonellaceae	1.5	4.5	1.9	2.0	2.0	13.1
k__Bacteria;p__Actinobacteria;c__Actinobacteria;f__Actinomycetaceae	0.2	1.4	0.3	0.2	0.1	0.3
k__Bacteria;p__Firmicutes;c__Bacilli;f__Staphylococcaceae	0.1	0.4	0.1	0.2	0.3	0.3
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Enterobacteriaceae	18.8	4.1	5.9	5.5	21.2	4.0
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;f__Erysipelotrichaceae	0.2	0.4	0.3	0.1	0.1	0.1
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Moraxellaceae	0.1	0.1	0.1	0.1	0.2	0.0
k__Bacteria;p__Firmicutes;c__Clostridia;f__Lachnospiraceae	0.1	0.1	0.1	0.0	0.0	0.0
k__Bacteria;p__Firmicutes;c__Clostridia;f__Clostridiaceae	0.7	1.2	0.7	0.8	0.2	1.0
k__Bacteria;p__Firmicutes;c__Bacilli;f__Leuconostocaceae	0.1	0.4	0.0	0.2	0.0	0.1
k__Bacteria;p__Cyanobacteria;c__Chloroplast;f__	0.0	0.7	0.1	0.0	0.0	0.1
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;f__Prevotellaceae	0.3	0.4	2.6	0.5	0.2	0.6
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;f__Neisseriaceae	0.0	0.1	0.1	0.1	0.2	0.1
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;f__Alcaligenaceae	0.0	0.1	0.2	0.1	0.1	0.1
k__Bacteria;p__Firmicutes;c__Clostridia;f__	0.4	0.5	1.4	0.5	0.1	0.3
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;f__Comamonadaceae	0.0	0.0	0.0	0.0	0.0	0.0
k__Bacteria;p__Firmicutes;c__Bacilli;f__Enterococcaceae	0.1	0.1	0.1	0.0	0.0	0.1
k__Bacteria;p__Firmicutes;c__Clostridia;f__Ruminococcaceae	0.0	0.1	0.1	0.0	0.0	0.0
k__Bacteria;p__Firmicutes;c__Bacilli;f__Gemellaceae	0.0	0.2	0.1	0.1	0.0	0.1
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Xanthomonadaceae	0.0	0.1	0.1	0.0	0.0	0.0
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;f__Helicobacteraceae	0.0	0.0	0.0	0.0	0.0	0.0
Unassigned;Other;Other;Other	0.1	0.3	0.5	0.3	0.9	0.2

Appendix 6. Overview of microbiota composition at genus level in ileal digesta as affected by age (time) and dietary treatment (% of total microbiota).

	d 14		d 22/23			
Taxon	1/2	3/4	1	2	3	4
k__Bacteria;p__Firmicutes;c__Bacilli;f__Turicibacteraceae;g__Turicibacter	13.2	23.8	8.0	12.8	9.2	6.7
k__Bacteria;p__Firmicutes;c__Bacilli;f__Streptococcaceae;g__Streptococcus	3.8	20.4	12.8	21.5	4.3	16.8
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Pasteurellaceae;g__Actinobacillus	13.6	8.5	17.6	12.4	20.0	15.0
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Pasteurellaceae;Other	5.7	8.6	12.7	8.3	17.5	13.8
k__Bacteria;p__Firmicutes;c__Bacilli;f__Lactobacillaceae;g__Lactobacillus	38.1	17.4	29.5	31.8	18.0	21.2
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Pasteurellaceae;g__	1.4	4.0	3.4	1.5	4.5	4.6
k__Bacteria;p__Firmicutes;c__Clostridia;f__Veillonellaceae;g__Veillonella	1.3	4.4	1.6	1.9	1.9	12.4
k__Bacteria;p__Actinobacteria;c__Actinobacteria;f__Actinomycetaceae;g__Actinomyces	0.2	1.4	0.3	0.1	0.1	0.3
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Pasteurellaceae;g__Aggregatibacter	0.2	0.2	0.2	0.2	0.6	0.5
k__Bacteria;p__Firmicutes;c__Bacilli;f__Staphylococcaceae;g__Staphylococcus	0.0	0.4	0.1	0.2	0.3	0.3
k__Bacteria;p__Firmicutes;c__Bacilli;f__Streptococcaceae;g__Lactococcus	0.1	0.8	0.0	0.0	0.0	0.1
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Enterobacteriaceae;g__	18.8	3.7	5.4	4.8	21.1	3.8
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Moraxellaceae;g__Moraxella	0.1	0.1	0.1	0.1	0.2	0.0
k__Bacteria;p__Firmicutes;c__Clostridia;f__Clostridiaceae;g__	0.5	1.1	0.6	0.6	0.1	0.9
k__Bacteria;p__Firmicutes;c__Clostridia;f__Lachnospiraceae;g__	0.0	0.1	0.0	0.0	0.0	0.0
k__Bacteria;p__Cyanobacteria;c__Chloroplast;f__;g__	0.0	0.7	0.1	0.0	0.0	0.1
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;f__Prevotellaceae;g__Prevotella	0.3	0.4	2.6	0.5	0.2	0.6
k__Bacteria;p__Firmicutes;c__Bacilli;f__Leuconostocaceae;g__Weissella	0.0	0.1	0.0	0.2	0.0	0.1
k__Bacteria;p__Firmicutes;c__Clostridia;f__;g__	0.4	0.5	1.4	0.5	0.1	0.3
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;f__Alcaligenaceae;g__	0.0	0.0	0.2	0.1	0.1	0.1
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;f__Erysipelotrichaceae;g__	0.0	0.2	0.1	0.0	0.0	0.0
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;f__Comamonadaceae;g__Comamonas	0.0	0.0	0.0	0.0	0.0	0.0
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;f__Neisseriaceae;g__Neisseria	0.0	0.0	0.0	0.0	0.0	0.0
k__Bacteria;p__Firmicutes;c__Bacilli;f__Enterococcaceae;g__Enterococcus	0.1	0.1	0.1	0.0	0.0	0.1
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;f__Erysipelotrichaceae;g__[Eubacterium]	0.1	0.2	0.1	0.0	0.0	0.0

Appendix 7. Overview of differentially expressed genes in jejunal and ileal mucosa tissue as affected by experimental treatment and/or time of sampling (d 14, 22/23).

jejunum.14.H-jejunum.14.L (53)

ID	logFC	adj.P.Val
MT1F	5.31	1.1E-05
MT1A	5.01	1.1E-05
A_72_P339833	-1.01	1.5E-05
SLC39A4	-2.17	2.2E-05
A_72_P046286	3.29	5.9E-05
RGS7BP	4.22	2.3E-04
LOC516442	1.34	2.8E-04
MT-III	4.98	2.8E-04
A_72_P061851	-2.06	3.3E-04
RNF144A	1.01	6.2E-04
LOC396757	-1.97	1.1E-03
ANG1	-1.16	1.3E-03
ANG	-1.11	1.6E-03
A_72_P096656	1.15	1.6E-03
SERPINA6	1.60	1.6E-03
SFTPD	-3.04	1.6E-03
A_72_P328508	1.04	2.3E-03
BPIFB2	-2.88	5.8E-03
REG3G	-7.05	8.8E-03
WFDC3	1.41	1.1E-02
A_72_P145166	-1.25	1.5E-02
A_72_P332333	-1.66	1.5E-02
FAM177B	-1.01	1.5E-02
GDF15	-1.34	1.5E-02
RBMS2	-1.20	1.5E-02
EFNA4	1.40	1.7E-02

ID	logFC	adj.P.Val
A_72_P134456	-1.34	1.7E-02
LOC102161357	1.54	1.9E-02
LOC100523295	1.10	2.0E-02
LOC100517891	-1.53	2.2E-02
A_72_P125226	1.12	2.2E-02
A_72_P143791	-1.38	2.4E-02
DUOXA2	-1.42	2.4E-02
FABP3	-1.05	2.5E-02
GJB2	-1.07	2.6E-02
LOC101328813	-1.09	2.9E-02
GPX2	-1.45	3.2E-02
CLIC6	1.03	3.2E-02
CCL28	-1.16	3.4E-02
STEAP1	-1.06	3.4E-02
SULT2A1	-1.29	3.5E-02
CYP1A1	1.53	3.5E-02
A_72_P171746	1.08	3.5E-02
CCDC152	1.23	3.5E-02
A_72_P036541	1.26	3.5E-02
LOC102168197	-1.09	3.7E-02
A_72_P072971	-2.68	3.8E-02
A_72_P434529	1.09	3.8E-02
MUC20	-1.00	3.8E-02
SEC1P	-1.70	4.0E-02
TFR2	-1.86	4.1E-02
A_72_P109711	1.44	4.5E-02
SPAI-2	-1.05	4.7E-02

jejunum.23.HH-jejunum.23.LL (3)

ID	logFC	adj.P.Val
SLC39A4	-2.09	0.000
MT1F	3.90	0.008
MT1A	3.62	0.012

jejunum.23.LH-jejunum.23.HL (1)

ID	logFC	adj.P.Val
ARPP21	3.70	0.005

jejunum.23.HH-jejunum.23.HL (3)

ID	logFC	adj.P.Val
A_72_P12!	1.50	0.006
SLC39A4	-1.68	0.017
ARG2	-1.19	0.017

jejunum 23.HH-14.H (1)

ID	logFC	adj.P.Val
G6PC	-2.19	0.023

Appendix 7. continued.

jejunum 23.LL-14.L (110)

ID	logFC	adj.P.Val
LOC100737328	1.77	7.4E-09
INVS	1.06	2.1E-05
RAB31	1.07	1.0E-04
C2	-1.25	1.0E-04
TNNI3	1.02	2.0E-04
RBMS2	-1.61	2.4E-04
A_72_P025711	1.08	2.9E-04
LOC100157134	1.04	3.6E-04
LOC102161357	1.95	4.7E-04
SUCNR1	1.67	4.9E-04
SCD5	1.05	5.4E-04
PLA2G3	-1.00	5.6E-04
MAP6	1.45	6.7E-04
RNASE1	-1.20	6.7E-04
LOC100523295	1.32	9.9E-04
A_72_P405343	1.19	1.1E-03
NTS	1.38	1.1E-03
TMCO4	-1.17	1.2E-03
ABHD4	-1.54	1.2E-03
C9	-1.03	1.5E-03
DPEP1	2.44	1.6E-03
SEPP1	1.68	1.7E-03
PLK1	-1.02	1.7E-03
FN1	1.15	1.8E-03
A_72_P349933	1.77	2.0E-03
ANG1	-1.00	2.2E-03
FRZB	1.26	2.4E-03
KMO	-1.45	2.6E-03
LOC100129550	1.07	3.1E-03
LGALS9C	-1.20	3.3E-03
A_72_P217402	1.21	3.3E-03
A_72_P036816	-1.23	3.3E-03
A_72_P110401	1.62	3.4E-03
GJC2	1.11	3.6E-03
ACSL3	1.08	3.7E-03
BST2	-1.16	3.7E-03
CCDC152	1.35	4.0E-03
SULT2A1	-1.41	4.1E-03
FABP3	-1.09	4.3E-03
TMCO5B	-1.31	4.4E-03
GDA	1.49	4.4E-03
RNF152	1.20	4.6E-03
LOC100157505	-1.37	5.1E-03
ERH	-1.02	5.3E-03
CRYBB3	1.02	5.5E-03
AGXT2	-3.47	5.9E-03
KLHL3	1.42	6.0E-03
RGS22	-1.01	6.1E-03
A_72_P788198	6.67	6.2E-03
LGALS3BP	-1.25	6.4E-03
LOC100849881	2.31	6.7E-03
LGALS9	-1.12	7.1E-03
DUOXA2	-1.36	7.8E-03
GHRL	1.21	8.6E-03
CPO	1.58	8.9E-03

ID	logFC	adj.P.Val
LOC100622473	1.10	9.2E-03
A_72_P130061	1.37	1.0E-02
A_72_P344903	-1.00	1.1E-02
A_72_P022716	1.05	1.2E-02
LPL	-1.01	1.3E-02
FCN1	-1.09	1.3E-02
A_72_P333733	1.23	1.4E-02
EDN3	1.10	1.4E-02
LOC102164901	1.04	1.5E-02
A_72_P061851	-1.30	1.5E-02
GPX2	-1.31	1.5E-02
SPP1	-1.62	1.5E-02
TOR2A	-1.08	1.5E-02
SEMA5A	1.00	1.6E-02
CA12	1.28	1.7E-02
LOC100521594	1.61	1.7E-02
CLCA4	1.19	1.7E-02
CYP1A1	1.37	1.9E-02
A_72_P304429	1.58	2.2E-02
SFTPD	-1.99	2.3E-02
A_72_P172501	1.15	2.3E-02
A_72_P283964	1.40	2.4E-02
DPPA2	1.24	2.4E-02
BTN1A1	-1.48	2.4E-02
A_72_P706556	-1.30	2.5E-02
PTPRR	1.53	2.6E-02
CUBN	1.46	2.6E-02
A_72_P130731	-1.10	2.7E-02
LOC100296421	1.48	2.8E-02
A_72_P382663	1.15	2.8E-02
ABI3BP	1.23	2.8E-02
KCNIP4	1.71	3.0E-02
A_72_P390003	-2.22	3.1E-02
A_72_P092366	-1.05	3.2E-02
VIP	1.16	3.2E-02
A_72_P065056	-1.03	3.4E-02
C10ORF35	1.17	3.4E-02
A_72_P039546	-1.01	3.6E-02
A_72_P716933	-1.19	3.7E-02
LOC100517891	-1.14	3.7E-02
A_72_P239557	1.11	3.8E-02
MLKL	-1.21	3.8E-02
LOC100522330	1.10	3.8E-02
CLCA1	1.45	4.1E-02
CTRB1	1.78	4.2E-02
LOC101287912	-1.20	4.2E-02
MEG3	1.21	4.3E-02
CTLA4	-1.84	4.3E-02
A_72_P204712	-1.10	4.3E-02
A_72_P066171	4.61	4.4E-02
A_72_P417067	1.32	4.4E-02
A_72_P284589	1.03	4.5E-02
LOC100037924	-1.03	4.6E-02
FCGR1A	-1.41	4.9E-02
A_72_P105101	-1.09	4.9E-02

Appendix 7. continued.

ileum 23.LL-14.L (3)

ID	logFC	adj.P.Val
MFI2	-1.14	0.008
APOA4	-1.86	0.009
RBP2	-1.27	0.036

ileum.14.H-ileum.14.L (20)

ID	logFC	adj.P.Val
MT1F	5.18	2.5E-05
MT1A	4.79	4.2E-05
SLC39A4	-2.09	8.6E-05
RGS7BP	4.39	1.3E-04
SFTPD	-3.50	1.9E-04
C4BPB	-1.18	1.9E-04
PLSCR4	-1.31	8.3E-04
A_72_P145166	-1.58	8.8E-04
DMBT1	-3.57	1.0E-03
A_72_P046286	2.85	1.0E-03
SPP1	-2.42	1.4E-03
MUC20	-1.39	1.5E-03
MT3	4.50	1.7E-03
A_72_P206202	-1.51	4.0E-03
REG3G	-6.95	1.9E-02
ARL4A	-2.95	2.2E-02
A_72_P390003	-3.04	2.8E-02
LOC100517891	-1.62	2.8E-02
LOC100738775	-1.08	3.8E-02
PTGES	-1.27	5.0E-02

ileum.23.HH-ileum.23.LL (2)

ID	logFC	adj.P.Val
MT1F	3.97	0.030
MT1A	3.78	0.030

ileum.23.HH-ileum.23.HL (8)

ID	logFC	adj.P.Val
RGS7BP	4.22	0.001
A_72_P046286	2.89	0.004
A_72_P134456	-1.65	0.006
A_72_P125226	1.38	0.009
CXCL2	-1.52	0.009
LOC100517891	-1.75	0.026
A_72_P109711	1.81	0.028
A_72_P036541	1.49	0.035

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.

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