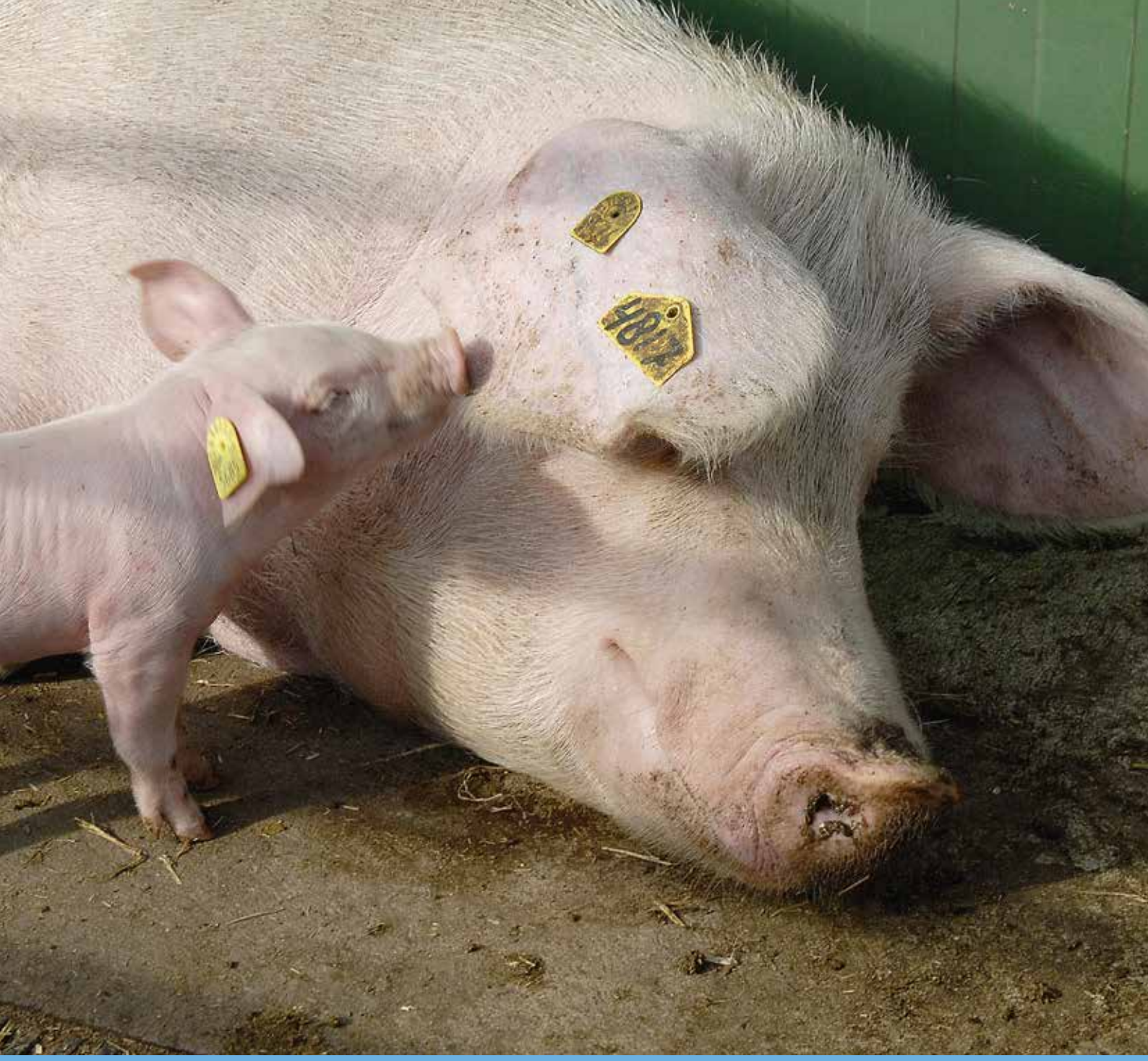

Effect of maternal antibiotic intervention in sows on gut development and microbiota in offspring

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LIVESTOCK RESEARCH
WAGENINGEN UR

Effect of maternal antibiotic intervention in sows on gut development and microbiota in offspring

Report of Feed4Foodure, VDI-2: 2013/2014

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LIVESTOCK RESEARCH
WAGENINGEN UR

Feed4 Foodure

Astrid de Greeff, Dirkjan Schokker, Petra Roubos, Peter Ramaekers, Jan Cornelissen, Carola van der Peet-Schwering, Paul Bikker, Stéphanie Vastenhouw, Freddy de Bree, Alex Bossers, Frank Harders, Mari Smits, and Johanna Rebel. 2015. *Transgenerational effect of maternal antibiotic intervention on gut development and microbiota in offspring; Report of Feed4Foodure, VDI-2.* Wageningen UR (University & Research centre) Livestock Research, Livestock Research Report 892 .

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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 / Central Veterinary Institute, part of Wageningen UR. 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 "Voeding, darmgezondheid, immuniteit", aims to contribute to a reduction in the use of antibiotics in livestock farming by increasing general (gut) health and disease resistance. The main goals are to develop innovative measurement techniques for (gut) health in animal husbandry.

The current report describes an experiment that was conducted within VDI workpackage-2 to investigate the effects of dietary inclusion of amoxicillin in the diet of sows from one week before farrowing until farrowing on gut health and performance parameters of their offspring. This experiment was performed at the Nutreco Swine Research Center in collaboration with scientists from Nutreco, Wageningen UR Livestock Research and Central Veterinary Institute, part of Wageningen UR. Experimental set-up and results were determined and discussed with representatives from the various private partners, including Agrifirm, ForFarmers-Hendrix, Nutreco, De Heus, Denkavit, van Drie groep, Sloten, MSD Animal Health and Darling Ingredients International. The authors thank the industrial partners of the project team for their input.

On behalf of Dr. Mari Smits, leader Feed4Foodure program line "Voeding, darmgezondheid, immuniteit",

Astrid de Greeff

Summary

A significant contribution to microbial colonization of piglets comes from the sow: via vertical transmission of vaginal flora during birth and transmission of mucosal immune memory and flora by feaces, colostrum and milk. In this study we determine the effect of an maternal nutritional intervention with an antibiotic on early microbial colonization of piglets. We used antibiotic treatment as a harsh intervention to investigate the hypothesis that the microbial composition in sows, may have an effect on the early microbial colonization of piglets. Two groups of gestating sows (n=15) were either treated with the antibiotic amoxicillin during the last week before farrowing, or left untreated. On day 21 pigs were weaned and transferred to pens in a nursery facility. immediately after birth of their first piglet a vaginal swabs was taken; fecal samples were collected on days, -28, -7, 0 (farrowing), 1, 7 and 28 (weaning). Fecal samples of piglets were collected after rectal stimulation on days 1, 7, 28, 32 and 49. On the same days, one pig per litter was sacrificed to harvest ingesta, intestinal scrapings and blood. The effect of treatment on microbial colonization of the vagina and faeces of sows and the jejunum of piglets was determined and correlated with each other. Furthermore, the effect of treatment on immune competence in the intestine of piglets was determined by studying gene expression profiles of jejunal scrapings of piglets to investigate the correlation between microbiota and intestinal immune development. The effects of oral amoxicillin during the last week of gestation were studied on piglet performance, microbiota composition and transcriptional responses. Although we showed that sow microbiota of both faecal and vaginal samples do not change considerably after amoxicillin treatment, changes in microbiota composition were induced and transmitted to piglets. Furthermore, morphological and transcriptional changes were induced in piglet jejunum suggesting that intestinal development of piglets from amoxicillin treated mothers is different from that of control controls. This differential intestinal development was not induced by changes in weight nor growth of piglets, since these parameters were not changed. Furthermore, differential intestinal development did not lead to different systemic inflammatory responses, since no difference in acute phase protein expression was found between the treatments. This implies that transgenerational effects can be induced after oral administration of feed ingredients / antibiotics and that these effects are mainly found in the process of intestinal development.

1 Background

1.1 'Voeding, darmgezondheid en immuniteit'

Within VDI the focus is to study how feed interventions can influence immune competence beneficially. To study this, model interventions are used under experimental conditions, both in animal models as well as in *in vitro* models. The ultimate goal is to define parameters that can be used to measure immune competence in livestock. Within VDI-2 the hypothesis is that maternal interventions can affect gut health in offspring. In this first study the main goal was to determine whether it is possible to affect microbiota composition and intestinal gene expression in offspring using a maternal feed intervention. Oral antibiotic administration (amoxicillin) was selected for this study as a model intervention to deliver proof of concept.

1.2 Introduction

In pig production, optimization of feed composition is important for production results and health of animals. The imbalance that is created due to selection on the number of ovulations on the one hand and functional uterine capacity to support optimal development of fetuses on the other hand, increases the number of low birth-weight piglets that may encounter major health problems due to malnutrition and poor immunity [1]. The early postnatal period is determinative for development of the gastrointestinal tract, since early-life conditions, specifically exposure to stress, affect gut microbial colonization and intestinal immune development, thereby making the early phase of life critical for intestinal immune development under regular production circumstances[2, 3]. The complex and dynamic interaction between the microbiota and its host shapes the repertoire of the microbiota and the immune system, such that the microbiota is normally restrained and well tolerated. At homeostasis, the microbiota benefits from the warm, nutrient-rich environment of its host, whereas the host benefits from adaptive digestive efficiency. [4, 5]. Shifts in the composition of the microbiota whether induced by dietary changes, antibiotic treatment or invasive pathogens, can disturb the balance of organisms leading to perturbation of immune regulatory networks. Especially at young age this perturbation can lead to permanent changes in immune networks that affect health. Colonization of the intestinal microbiota after birth plays an important role in development and programming of the neonatal gastrointestinal and immune systems. Key environmental factors that influence the colonization pattern are environment, hygiene status of the stable, delivery mode and nutrition[6]. The initial microbiota of vaginally born infants resembles that of their mother's vagina, while that of Caesarean section infants is dominated by skin microbes not related to those of the mother [7]. After this primary colonization, nutrition, environmental factors and condition of the sow have major impact on intestinal microbiota.

1.3 Objectives

The objective of this study was to demonstrate proof of concept for the hypothesis that by maternal feed interventions, immune competence and microbiota development of piglets can be manipulated and that parameters of immune competence can be identified. As a model intervention, amoxicillin treatment of sows is used. Antibiotic treatment is a harsh intervention to study gut health. However, antibiotics have been long used in low concentrations as feed additives to protect livestock from diseases and enhance growth. In the sow, antibiotics are expected to affect microbiota composition of the gut and perhaps also of the birth canal.

2 Material and Methods

2.1 Animal experiment

2.1.1 Experimental design, housing and diet

Thirty-one Hypor gilts and sows divided over three different batches were allotted to one of two treatments based on body weight and backfat thickness 4 weeks before farrowing (day 87 of gestation) and on parity. One treatment group (Control - C) received standard gestation feed and standard lactation feed (Table 1) within lactation (one week before expected day of farrowing until weaning). The second treatment group (antibiotic treatment – AB) received daily an oral antibiotic treatment with 15 mg / kg body weight of amoxicillin from one week before expected day of farrowing until farrowing as a topdress to the standard lactation feed. From farrowing till weaning they were fed the standard lactation diet. Sows were housed at Nutreco Swine Research Centre in gestation group housing and lactation departments. During lactation sows were housed individually. Sows had free access to water and were fed according to a standard feeding scheme. Feed intake was recorded using automated feeders. Farrowing was only induced, in sows that did not farrow on day 114 of gestation.

Table 1
Composition of gestation and lactation feed.

Ingredient	Unit	Gestation Diet	Lactation Diet
Wheat		10.00	10.00
Betain 96%		0.10	0.20
PHYZYME XP 5000 TPT		0.01	0.01
Sow premix STND/LAC		1.00	1.00
Maize		26.48	29.93
Barley		20.00	20.00
Soy bean meal 49, Cf		2.45	11.54
Sunflower seed meal		12.21	14.64
Beet pulp, Sug <10%		5.00	0.00
Soya hulls, CFiber 340		20.00	5.85
Limestone		0.48	1.32
Monocalcium phosphate		0.37	0.68
Na bicarbonate		0.33	0.43
Soya oil		1.24	3.86
L-Lysine HCl 98%		0.06	0.25
L-Threonine 98%		0.00	0.03
Choline Chloride 50%		0.08	0.08
Biotine-Mix		0.15	0.15
Vitamin E 50% adsorb		0.04	0.04
DM	g	883	887
CP	g	140	180
Ash	g	53	61
CF	g	113	65
EE	g	38	63
NDF	g	238	159
Starch am	g	329	350
ADL**	g	13	12
C18:0	g	1.0	1.9
C18:1	g	7.5	12.8
C18:2	g	18.0	30.9
C18:3	g	1.7	3.5
dEB	meq	210	220
EW		0.97	1.10

EWdracht		1.04	1.15
Total LYS	g	6.2	9.6
Total MET	g	2.5	3.1
Total MET+CYS	g	5.1	6.3
Total THR	g	5.0	6.7
Total TRP	g	1.5	2.0
SID ILEpig	g	4.4	6.1
SID LEUpig	g	8.5	11.6
SID LYSpig	g	4.8	8.4
SID M+Cpigs	g	4.2	5.4
SID METpig	g	2.2	2.8
SID THRpig	g	3.9	5.6
SID TRPpig	g	1.2	1.7
SID VALpig	g	5.3	7.2
Ca	g	6.5	9.5
Ca / P		1.5	1.6
P	g	4.5	5.9
IP	g	0.5	1.0
dP swine	g	2.4	3.3
I	mg	1.19	1.11
Cl	g	2.9	3.3
K	g	7.5	7.9
Na	g	2.3	2.5
Mg	g	2.2	2.3
Cu	mg	23	23
Mn	mg	50	49
Fe	mg	390	342
Se	mg	0.30	0.30
Zn	mg	136	135
Vitamin A	I.U.	10000	10000
Vitamin D	I.U.	2,000	2,000
Vitamin E	I.U.	60	60
Vitamin K	mg	2	2

The sows were weighed at the start of the trial (day 87 of gestation), at entrance to the lactation room (day 108 gestation), day 2 and 7 after farrowing and at weaning. A colostrum sample was collected after the first piglet was born and 7 days after farrowing. Sow faecal sampling was collected at the start of the trial (day 87 of gestation), 7 days before farrowing, day of farrowing and day 1 and 7 after farrowing and at weaning on 3 sows per batch per treatment in two batches (= 6 sows per treatment in total). A vaginal excretion sample (vaginal swab) was collected direct after the first piglet was born. The number of piglets born alive and still born piglets were recorded. Piglets were weighed immediately after birth, after 24 h (20 – 25 h), after 7 d, at weaning. (day 26) and 4 weeks after weaning (day 54). On day 1 and 7, and at weaning one male piglet was selected that was of average weight and in good health without prior veterinary treatments to be euthanized for collection of intestinal tissue. From the euthanized piglet digesta was collected from ileum and jejunum to analyse microbiota composition. Intestinal scrapings were collected from ileum and jejunum for gene expression analysis. Patches from jejunum and ileum were spread on cork and fixed in formalin for histological morphometric analysis. Blood was collected for systemic immunological parameters. To determine whether putative effects of maternal amoxicillin treatment in offspring were a direct effect of amoxicillin traces carried over via milk, or an indirect transgenerational effect of the treatment, amoxicillin levels in milk collected shortly after farrowing were determined by RIKILT.

2.1.2 Zootechnical data analysis (Statistics)

Statistical data analysis was done using Genstat v17.1.0 (VSN International Ltd., United Kingdom). Data were analysed using the model y = batch-effect + treatment-effect*time-effect + residual error in an ANOVA test with Bonferroni post hoc testing, unless otherwise indicated in materials and methods.

2.1.3 Acute phase proteins

Levels of acute phase proteins were determined in serum samples from piglets using commercial ELISAs according to instructions of the manufacturer. C-Reactive Protein (CRP) from , haptoglobin and Serum Amyloid A (SAA) ELISA kits were purchased at MT-diagnostics (Etten-Leur, The Netherlands). Data analysis was done using Graphpad software with a 2-way ANOVA following y = time-effect*treatment-effect + residual errors with Bonferroni post-hoc testing.

2.1.4

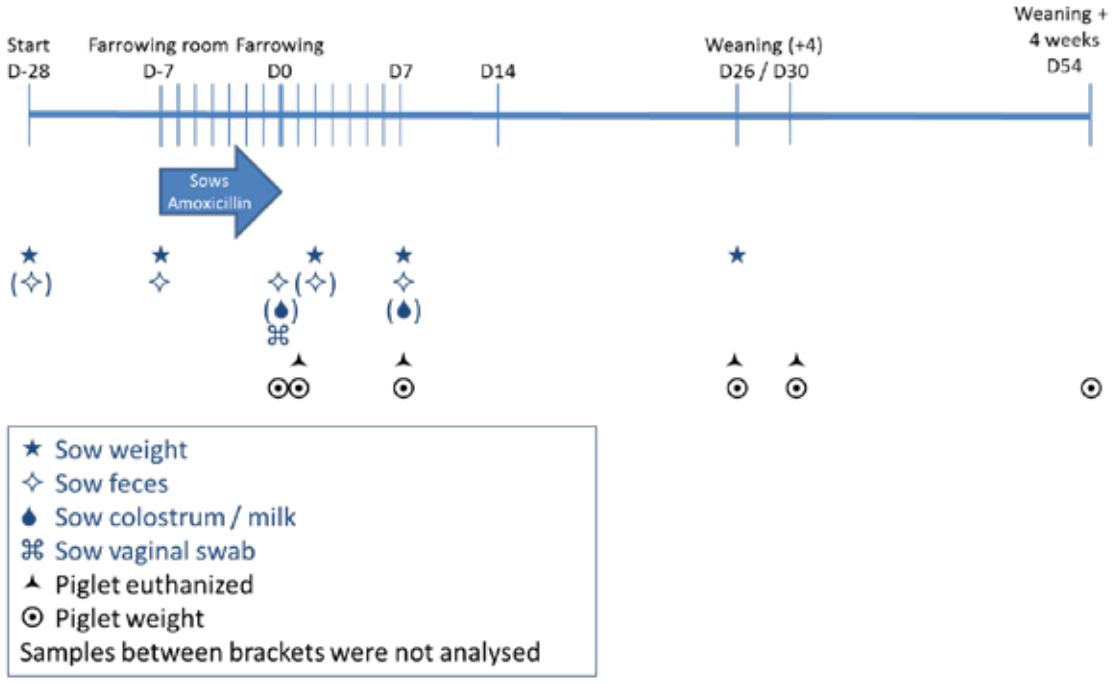

Figure 2.1 *Schematic representation of the experimental set-up.*
Sows entered the experiment 28 days before the expected farrowing date. The treatment started 7 days before expected farrowing date. The control treatment sows were fed regular lactation feed, the amoxicillin treatment sows received a top-coating of 15 mg / kg bodyweight amoxicillin until farrowing. Samples were taken according to the scheme.

2.2 Analysis of microbiota composition

2.2.1 DNA isolation

Microbiota composition was determined of sow faeces, sow vaginal swabs and piglet jejunal digesta. Samples were frozen on dry-ice after collection and stored at -80°C. To isolate DNA samples were mixed in a 1:1 ratio with phosphate buffered saline (PBS) and centrifuged for 5 min at 4°C at 300*xg*. Supernatant was collected and centrifuged for 10 m at 4°C at 9,000*xg*. DNA was extracted from the pellet using the "QIAamp DNA stool minikit" according to manufacturers' instructions. Quality and quantity of DNA was checked using the NANOdrop (Agilent Technologies).

2.2.2 Amplification and quantification 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.
To determine the amount of microbial content in populations a qPCR was performed on a 1:10000 dilution of isolated DNA using 16S primers as described above and Powr Sybr Green PCR Master mix (Applied Biosystems) with the following PCR conditions: 10 m at 95°C, 40 x (15s at 95°C, 30 s at 59°C, 36 s at 72°C), followed by a dissociation curve using the ABI7500 (Applied Biosystems). To correct for putative inhibition, 1 ng of an internal control was spiked to the samples and amplified in a separate qPCR using primers JR331 CCTGAAGTTCATCTGCACCA and JR328 CTTGTAGTTGCCGTCGTCCT with the following PCR conditions: 10 m at 95°C, 40 x (15s at 95°C, 30 s at 58°C, 36 s at 72°C), followed by a dissociation curve using the ABI7500 (Applied Biosystems). By dividing the amount of 16S by the amount of internal control, a relative abundance of microbial content is calculated. Data analysis was done using Graphpad software with a 2-way ANOVA following y = time-effect*treatment-effect + residual errors with Bonferroni post-hoc testing.

2.2.3 Sequence analysis and bioinformatics

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 [8]. Standard assembly based on amplicon was performed after removal of primer sequences. Data was filtered to yield high quality sequence data using the following settings: 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) [9, 10]. Furthermore chimeras were removed with Chimeraslayer [11].

2.2.4 Statistical analysis of microbiota data

The biodiversity of the jejunal microbiota 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 run on the family level microbiota data: y = Time + Treatment + Time* Treatment + error. Furthermore, statistical significance testing for over- and under-representation of the bacterial groups were 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.

2.3 Gene expression of intestinal tissue

2.3.1 RNA isolation

Total RNA was extracted from 50 to 100 mg jejunum tissue. Samples were homogenised using the TisuePrep Homogenizer Omni TP TH220P in 5 ml TRIzol reagent (Life Technologies). The homogenate was centrifuged for 5 m at 21,000 *xg*. 350 µl of supernatant was used to isolate RNA using the Direct-zol kit (Zymo Research) according to instructions of the manufacturer. Quality control was performed on the BioAnalyser (Agilent Technologies), quantity of RNA was determined using the Nanodrop (Agilent Technologies).

2.3.2 Microarrays: Labeling and hybridization procedure

Labelling of RNA was done as recommended by Agilent Technologies using the One-Color Microarray-Based Gene Expression Analysis Low input Quick Amp Labelling. 10 ng of total RNA was used as input,

600 ng of labelled cRNA was used to hybridise the porcine microarray (Agilent Technologies). Hybridisation was performed at 65°C for 17 h with head-over-head rotation. Microarrays were washed as recommended by the manufacturer. Microarrays were scanned using the Surescan high resolution scanner (Agilent Technologies) at a 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.

2.3.3 Microarray data analysis

The data were analysed by using R (v3.0.2) by executing different packages, including LIMMA and arrayQualityMetrics [12]. The data were read in and background corrected (method="normexp" and offset=1) with functions from the R package LIMMA from Bioconductor [13]. 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, 3) the gene/probe had to be expressed in each of the samples in the experimental condition. To test the differences between the experimental groups (Control versus amoxicillin treatment) the contrasts between control and amoxicillin treatment were studied for each time-point within the LIMMA package**.** DAVID was used to perform Functional Annotation Clustering (FAC) for the different contrasts. The up- and down-regulated genes were separately analysed.

2.4 Morphometric analysis of intestinal tissue

Formalinised samples were embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin for histologic examination. Villus length, crypt depth and mucosa length were determined using Imag- Pro plus software (Media Cybernetics, Rockville, USA). Proliferating cell nuclear antigen (PCNA) is a cell proliferation marker expressed by cells throughout the S to M phases of the cell cycle [14]. PCNA was detected by immunohistochemistry using a monoclonal antibody against recombinant rat PCNA (PC-10; DAKO). Briefly, paraffin-embedded tissue sections dewaxed in xylene, washed and incubated with the monoclonal antibody PC-10 at a 1:200 dilution overnight at 48°C. Endogenous peroxidase was inhibited by incubation in 1% H2O2 in 0.1 M Tris - HCl. The sections were then incubated with the biotinylated secondary antibody followed by peroxidase-conjugated streptavidin for 30 min each. Peroxidase activity was detected using 0.05% diaminobenzidine in 0.1 M Tris - HCl (pH 7.5) containing 0.01% H2O2. The slides were examined using light microscopy to detect the dark-brown nuclear staining of PCNA-positive cells. Data analysis was done using Graphpad software with a 2-way ANOVA following y = time-effect*treatment-effect + residual errors with Bonferroni post-hoc testing.

3 Results & conclusions

3.1 Zootechnical parameters

3.1.1 Sows

Sow weight and feed intake did not differ significantly between the control group and the amoxicillin group at any of the time-points (Figure 3.1 & 3.2).

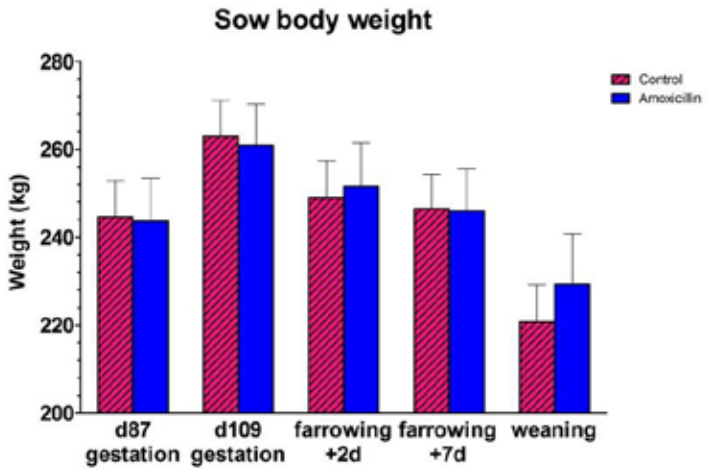

Figure 3.1 *Body weight of sows.*
Body weight of sows was determined at different time-points of sows of the control group (pink hatched bars) and sows of the amoxicillin treated group (blue bars). Error bars indicated Standard Error of the Mean (SEM).

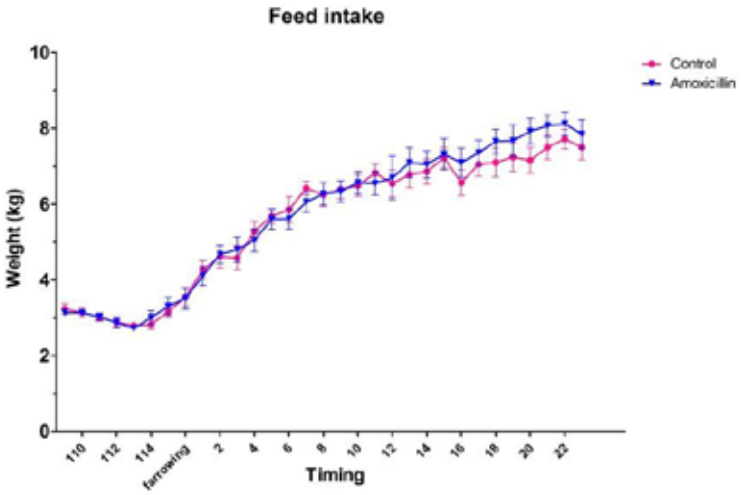

Figure 3.2 *Feed intake of sows.*
Feed intake of sows was determined daily of sows of the control group (pink circles) and sows of the amoxicillin treated group (blue triangles). Error bars indicated Standard Error of the Mean (SEM).

Sows in the amoxicillin group had significantly higher number of piglets born dead (Table 3.1). This increase in number of dead piglets could probably be explained by a significant difference in litter size where the amoxicillin treated group had larger litters. Since litter size was determined before treatment it is not likely this difference in reproduction was caused by the amoxicillin treatment. In conclusion, treatment of sows with amoxicillin in the last week before farrowing did not affect feed intake, weight development and reproduction parameters of sows in this study. Two samples of amoxicillin treated sows contained trace amounts of amoxicillin, all other sow milk amoxicillin levels

Table 3.1
Reproduction traits of sows.
The number of piglets born alive, born dead and weaned were registered for sows of the control group and the amoxicillin treated group.

Parameter	Mean ± SEM Control	Mean ± SEM Amoxicillin	p-value
Litter size	14.58 ± 0.15	15.06 ± 0.17	0.043
Piglets born alive	13.71 ± 0.13	13.19 ± 0.14	0.011
Piglets born dead	0.87 ± 0.067	1.87 ± 0.072	<0.001
Piglets weaned[1]	10.36 ± 0.075	10.23 ± 0.08	0.136

[1] Combined effect of piglets that were sacrificed for necropsy and piglets that died during the study.

(including control sows) were below detection level (data not shown). Based on these observations we concluded that all effects of maternal amoxicillin treatment could most likely be attributed to transgenerational effects of the treatment like maternal microbiota changes.

3.1.2 Piglets

Weight of piglets was determined as function of time (Figure 3.3). No significant differences in weight were found between piglets from sows of the control group and piglets from sows of the amoxicillin treated group. However, 4 weeks after weaning (day 54) piglets from sows of the control group are numerical on average 1 kg heavier than piglets from sows of the amoxicillin group. This difference in weight was not significant.

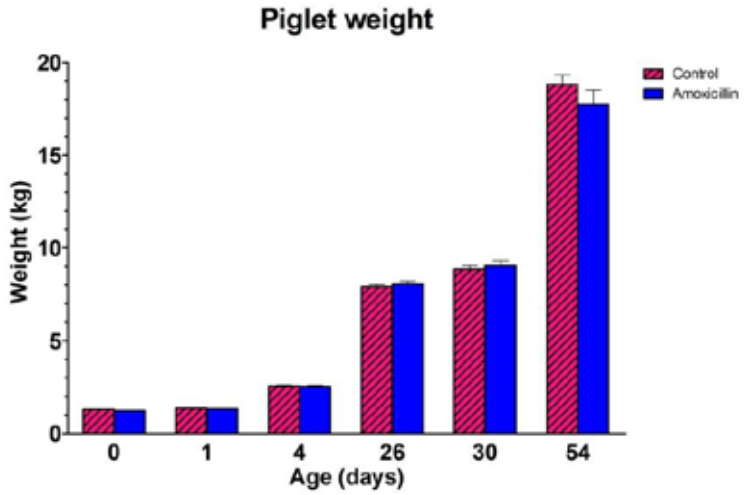

Figure 3.3 *Piglet weight as function of time.*
Weight of piglets from the control group (pink hatched bars) and the amoxicillin treated group (blue bars) was recorded as function of time. Error bars indicated Standard Error of the Mean (SEM).

3.2 Microbiota composition

3.2.1 General effect of amoxicillin treatment

Composition of the microbiota in faeces of sows was determined before treatment (7 days before farrowing), 1 day after farrowing and 1 week after farrowing. When data were visualized in a Principle Component Analysis (PCA), it could be seen that microbiota

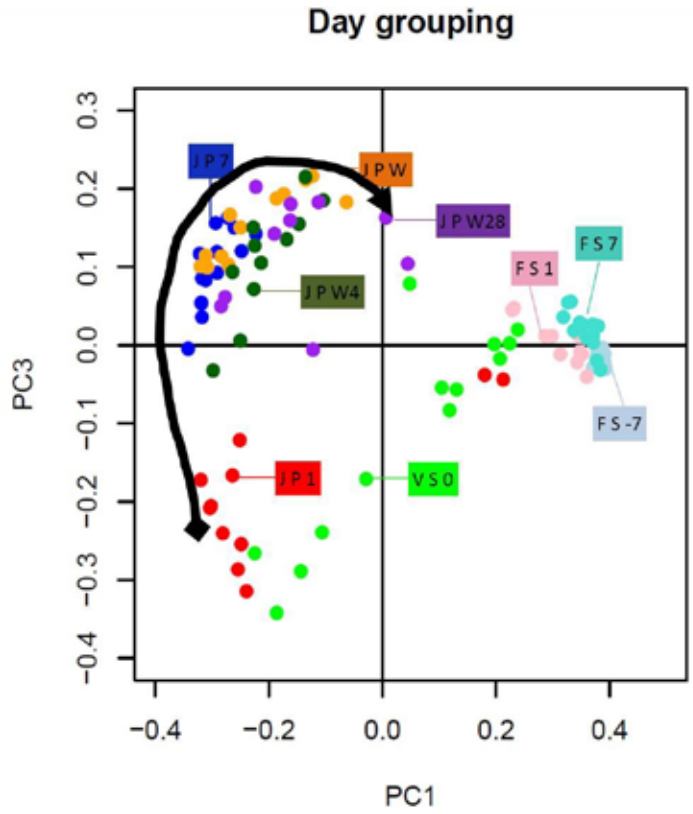

Figure 3.4 *Principle component analysis (PCA) of microbiota composition of different samples from sows and piglets.*
Microbiota composition of different samples was determined using 16S sequencing and data were visualized using a PCA plot. F S -7: faecal sample of sows 7 days before expected farrowing date (= pre-treatment); F S 1: faecal sample of sows on day 1 after farrowing; F S 7: faecal sample of sows 7 days after farrowing; V S 0: vaginal sample of sows taken after delivery of the first piglet; J P 1: jejunal digesta from piglet on day 1; J P 1: jejunal digesta from piglet on day 1; J P w: jejunal digesta from piglet on day of weaning; J P w4: jejunal digesta from piglet 4 days after weaning;J P w28 jejunal digesta from piglet 4 weeks after weaning. The black arrow indicates development of piglet jejunal digesta in time.

composition of sow faeces was comparable between sows (Figure 3.4). After a week of antibiotic treatment there was a small change in composition (the pink spots are slightly shifted to the left compared to the light blue spots), but one week after treatment this shift was returning to the original composition. Vaginal microbiota composition was different from the faecal microbiota and more variation between sows was observed for vaginal microbiota. Microbiota composition of jejunal digesta of piglets did not show large differences between the two treatment groups (data not shown). However, jejunal microbiota changed considerably in time, probably reflecting development of stable microbiota. In Figure 3.4 this process is visualized with a black arrow. A striking feature of this development is the resemblance on day 1 between microbiota composition of jejunal digesta of piglets on day 1 and the vaginal microbiota of sows during birth. This suggests that the first colonizers of the intestine are derived from the birth channel. In conclusion, data analysis showed that oral amoxicillin treatment of sows during 1 week before farrowing does not induce large changes in microbiota composition in both sow and piglets as detected by cluster analysis. Furthermore, we can conclude that vaginal microbiota of sows has influence on the first colonization of the piglet.
Amoxicillin not only affected the composition of microbial populations, but also the total amount of microbiota present in faeces (sow), vagina (sow) or digesta (piglet) (Figure 3.5). Although for sows the decrease of microbial content after amoxicillin treatment on day 0 is not significant, the trend is clearly present. For piglets the decrease of microbial content is numerical present on all days except for day 54. However, around weaning on day 26, the difference is statistical significant.

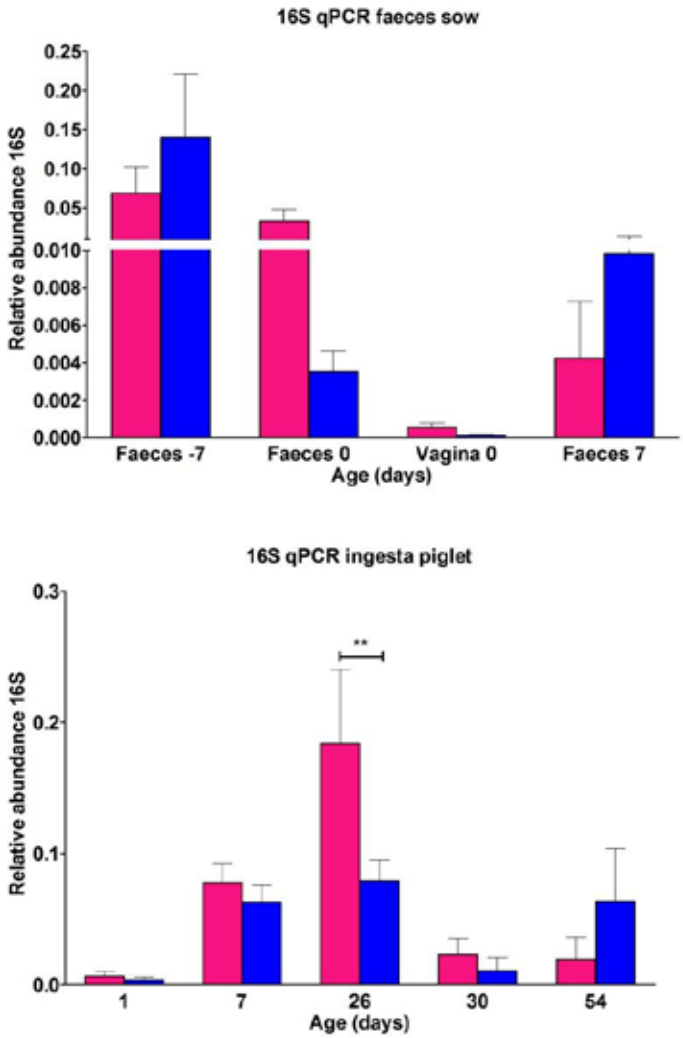

Figure 3.5. *Relative abundance of microbiota as determined by quantitative PCR. Using a quantitative PCR on 16S microbial rDNA the total amount of microbiota present in different population is determined in amoxicillin treated sows and their offspring (blue bars) and in control sows and their offspring (pink bars). Error bars indicate standard errors of the mean, ** p-value < 0.01 as determined with ANOVA*

The changes of microbiota in time were visualized in Figure 3.6, showing that numbers of *Lactobacilli* (orange bars) increase in time. Furthermore, it is clear that especially at day 1 piglets from amoxicillin treated sows have more streptococci (dark green), whereas later in time they have more clostridiaceae (light blue).

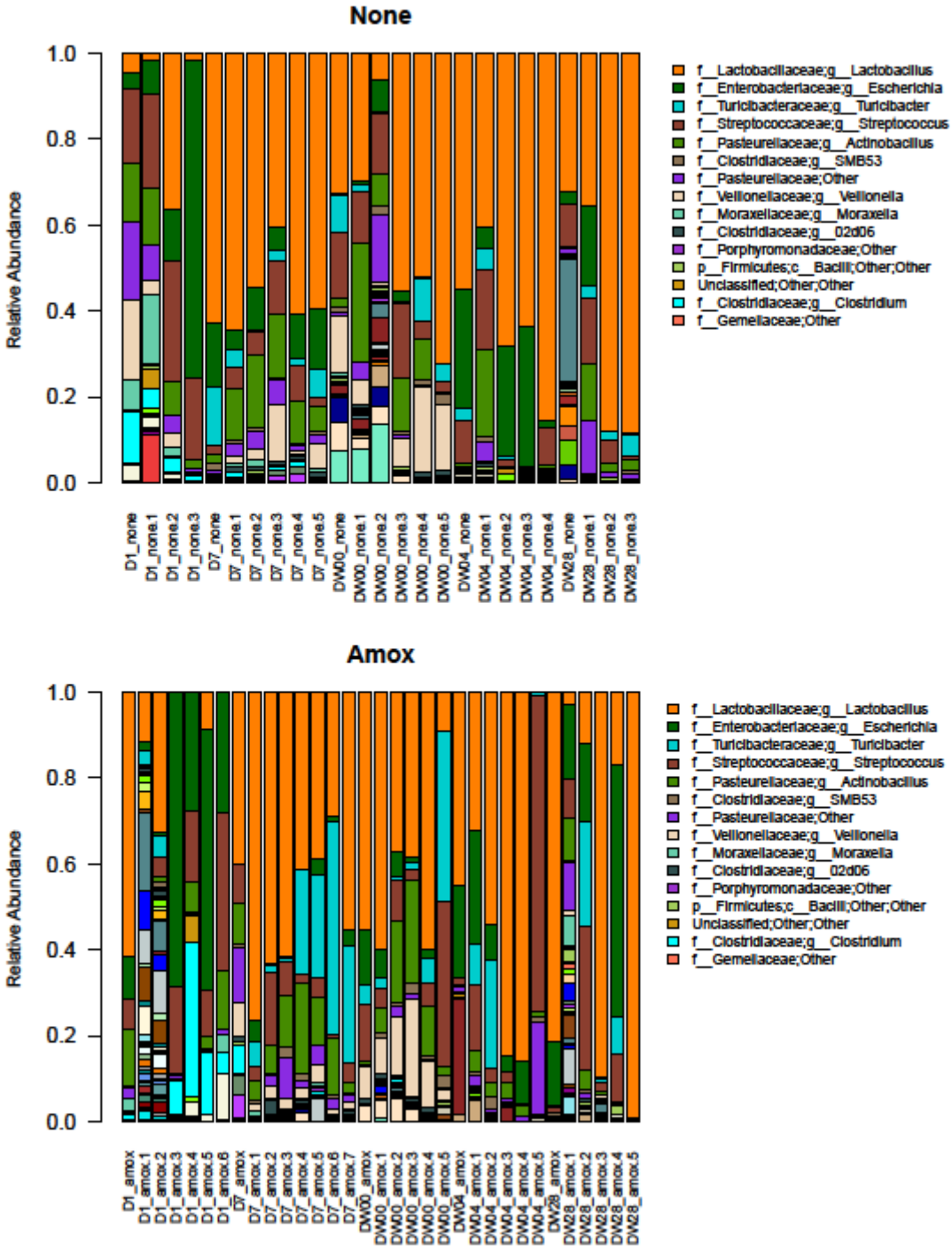

Figure 3.6. *Development of microbiota of piglets as function of time.*
Composition of microbiota of jejunal ingesta of piglets from control sows (upper panel) and amoxicillin fed piglets (lower panel)is depicted as function of time. Each bar represents one individual piglet. D1: 24 hrs after birth; D7: 7 days after birth, DW00: at weaning; DW4: 4 days after weaning; DW28: four weeks after weaning.

3.2.2 Diversity of microbiota

Diversity of microbiota (the number of different species in one sample) was used as a parameter to study effects on microbiota. Figure 3.7 shows that samples from different locations differ in their diversity, confirming observations in literature. Sow vaginal microbiota was less diverse than faecal

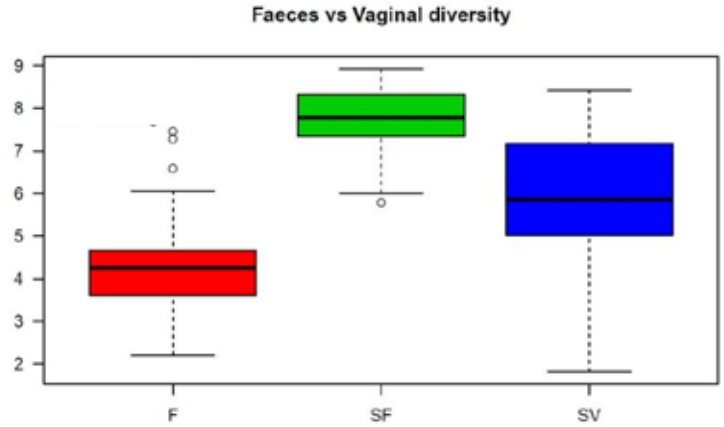

Figure 3.7. *Diversity of microbiota isolated from different locations.*
The Shannon index is a parameter that indicates how diverse a microbial population is. A higher Shannon index indicates more different microbial species in that samples. Shannon indices are compared between all microbiota samples derived from piglet digesta (F; red bar), sow faeces (SF; green bar), and sow vagina (SV; blue bar) in a box plot.

microbiota. Piglet microbiota of the jejunum was less diverse than sow faecal microbiota. When the data are studied in more detail it was clear that amoxicillin treatment of sows did not affect microbiota diversity in piglets (Figure 3.8) nor in sows (data not shown). The development of jejunal.

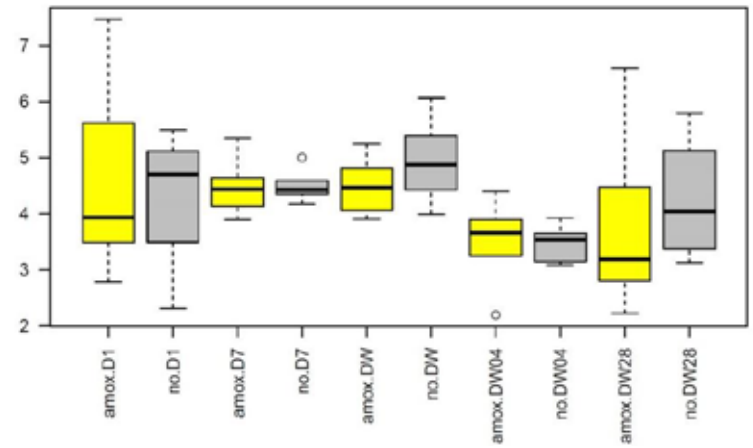

Figure 3.8. *Shannon index of jejunal digesta of piglets in time.*
Diversity of microbiota calculated as Shannon index for different moments in time as indicated. "Amox" indicates piglets from sows of the amoxicillin group, whereas "no" indicates piglets from sows of the control group.

microbiota was also reflected in the diversity. Immediately after birth there was large variation in diversity among piglets, reflecting the chaotic colonization of the naïve intestine. After 1 week, there was less variation, whereas diversity was still similar. Around weaning, the jejunal microbiota changed, variation between piglets increased whereas diversity decreased. This probably reflects the start of stable colonization, a process that is still ongoing 4 weeks after weaning. There was no difference in microbial diversity due to the amoxicillin treatment. Both for sows (data not shown) as well as for piglets microbial diversity was similar in both treatment groups.

3.2.3 Amoxicillin induced changes in microbiota composition

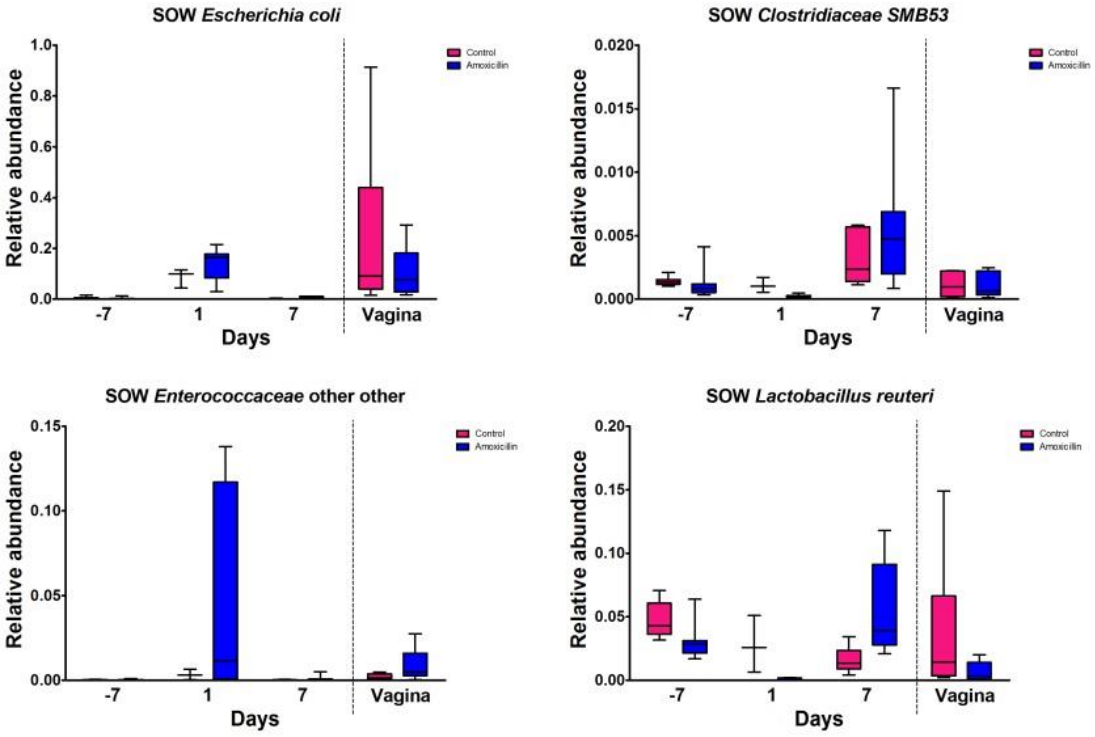

Figure 3.9 *Colonization of individual bacterial species in sow faeces or vagina. Bacterial species that were differentially present between treatment groups are depicted. Left of the dotted line are faecal samples of control sows (pink) or amoxicillin treated sows (blue).*

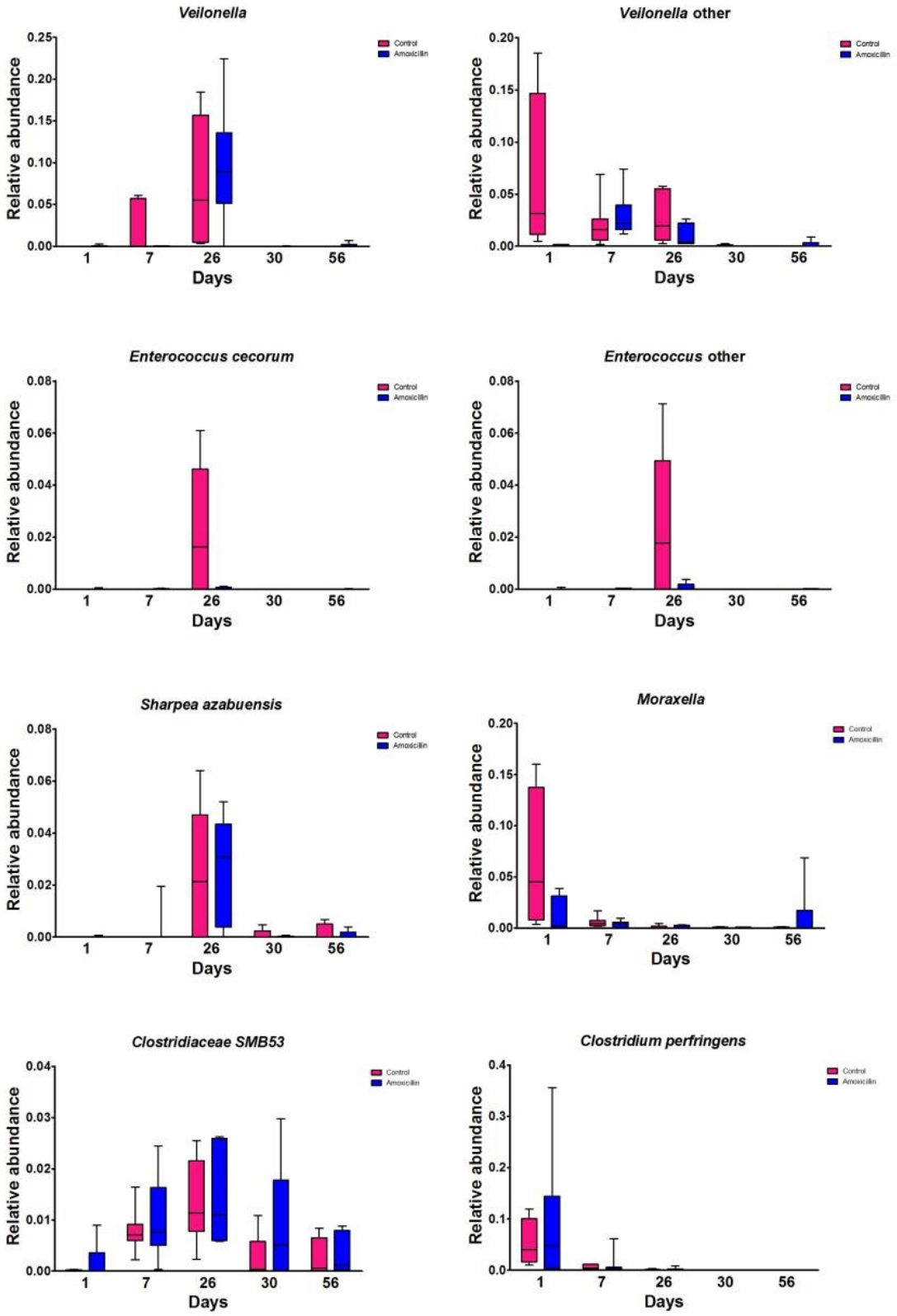

Figure 3.10. *Colonization of individual bacterial species in piglet jejunal digesta. Each panel represents one of the bacterial species that are differ in abundance between piglets from sows treated with amoxicillin (blue bars) and from control sows (pink bars).*

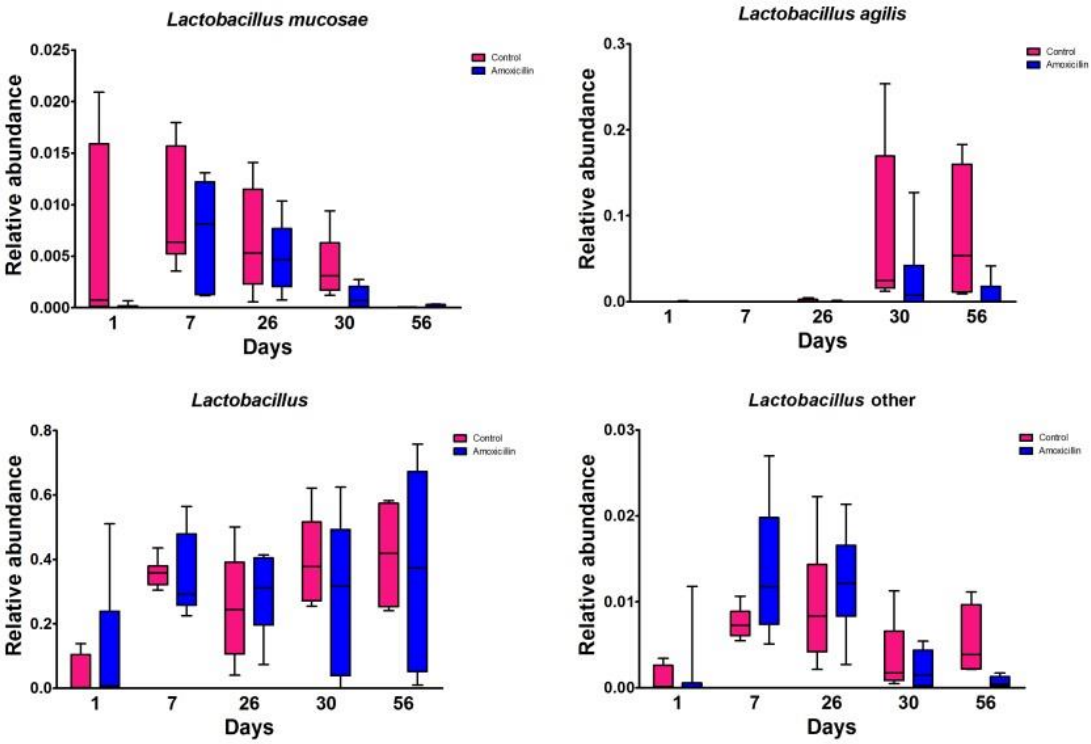

Figure 3.11. *Colonization of Lactobacilli in piglet jejunal digesta.*
Each panel represents Lactobacilli species that are different in abundance between piglets from sows treated with amoxicillin (blue bars) and from control sows (pink bars).

Although no large differences in microbiota diversity were induced by amoxicillin treatment of sows, there were significant differences in microbiota composition identified: Within sows 51 significant differences due to amoxicillin treatment were identified (whereas in piglets 16 significant differences were identified (see Appendix 1). In sows differences between control sows and amoxicillin treated sows were identified both in faecal microbiota as well as in vaginal microbiota. Bacterial species were either repressed by the amoxicillin (e.g. *Clostridiaceae* and *Lactobacillus reuteri*), or they were able to increase in numbers (e.g. *Enterococcaceae*) (Figure 3.9).

In piglets similar changes were identified (Figures 3.10 & 3.11). In total 16 bacterial species differed in relative abundance between treatment groups. Some species were more abundant in piglets from control sows, like *Enterococcus cecorum*, whereas others were more abundant in piglets from amoxicillin treated mothers, like *Lactobacillus* other. Interestingly, two species of *Clostridiaceae* were slightly more abundant in piglets from amoxicillin treated mothers. *Lactobacilli* are abundant in jejunal digesta of both groups, although they do differ in time and some species also differ between treatments. *Lactobacillus reuteri* was almost completely absent on day 1 in piglets from amoxicillin treated mothers, whereas it was abundant in piglets from control mothers. *Lactobacillus agilis* was more abundant in piglets from control mothers as well, but later in time, around weaning.

Based on the data, it is suggestive to speculate that piglets are indeed colonized by sow vaginal microbiota. There was similarity between significant changes in sow vaginal microbiota composition and colonisation of piglet jejunum on day 1, since changes in maternal microbiota are reflected in the piglet microbiota on day 1 (Figure 3.12). The best example for this phenomenom was colonization with *Veilonella*: in vaginal sow microbiota the amount of *Veilonella* was reduced due to amoxicillin treatment. This was reflected in the presence of *Veilonella* in piglet jejunal microbiota on day 1. After 1 week, this difference had disappeared. After weaning *Veilonella* was only present in piglet jejunal microbiota at very low numbers.

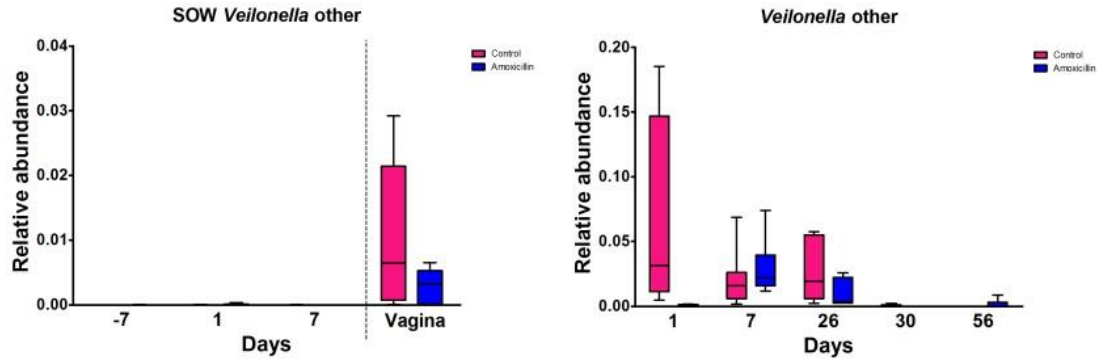

Figure 3.12. *Transmission of Veilonella from sow vagina to piglet jejunum. Relative abundance of Veilonella is depicted in sow vaginal microbiota (left panel) and piglet jejunal content (right panel) in animals from the control group (pink) or the amoxicillin treatment group (blue).*

3.2.4 Conclusions microbiota

In conclusion, amoxicillin induced small changes in microbiota composition in sow faecal and vaginal microbiota, as well in jejunal content of their offspring. There seems to be a correlation between vaginal microbiota of sows and early colonization of piglet intestinal microbiota. Furhtermore, amoxicillin treatmet of sows decreased microbial content numerically in sow faeces and vagina immediately after treatment. In piglets, the microbial content of piglets from amoxicillin treated mothers was significantly reduced around weaning.

3.3 Intestinal gene expression of piglets

3.3.1 General transcriptional effect of amoxicillin treatment

Gene expression was determined in jejunal scrapings of piglets either from control sows or from amoxicillin treated sows. To get insight in the treatment effect as well as in variability of data, data were visualized in a PCA plot (Figure 3.13). At all timepoints studied there was a clear distinction between piglets from control sows and piglets from amoxicillin treated sows, based on transcriptional profiling of intestinal gene expression. Furthermore, similar to the microbiota data, there was a clear time dependent development visable that was independent of treatment, as indicated by the arrow in Figure 3.13. These data suggest that amoxicillin treatment of sows induced changes in gene expression in intestinal tissue. More detailed analysis showed that biggest change in gene expression was induced from weaning onwards, when the highest number of genes is found to be regulated (Table 3.1). Strangely, at the age of 7 days no changes in gene expression could be identified. Table 3.1 illustrates that there is a large discrepancy between regulated probes found on the array, and regulated annotated genes. This is due to the fact that: 1) genes are represented by more than 1 probe on the array, and 2) annotation of function to genes is not complete for the porcine genome. About 30 – 50% of the porcine genome has been annotated.

Table 3.1
Number of regulated probes / genes in jejunal scraping from piglets from sows of the amoxicillin treated group compared to piglets from sows of the control group on days 1, 7, at weaning, weaning + 4 days and weaning + 4 weeks.

		Regulated Probes[1]		Regulated Annotated Genes[1]	
Comparison	Timepoint	Up	Down	Up	Down
Treatment vs Control	1	159	28	52	10
Treatment vs Control	7	0	1	0	0
Treatment vs Control	Weaning	194	301	95	148
Treatment vs Control	Weaning + 4d	11	153	1	84
Treatment vs Control	Weaning + 4w	154	272	67	156

[1] log (Fold Change) > |1.5| and adjusted p-value < 0.05

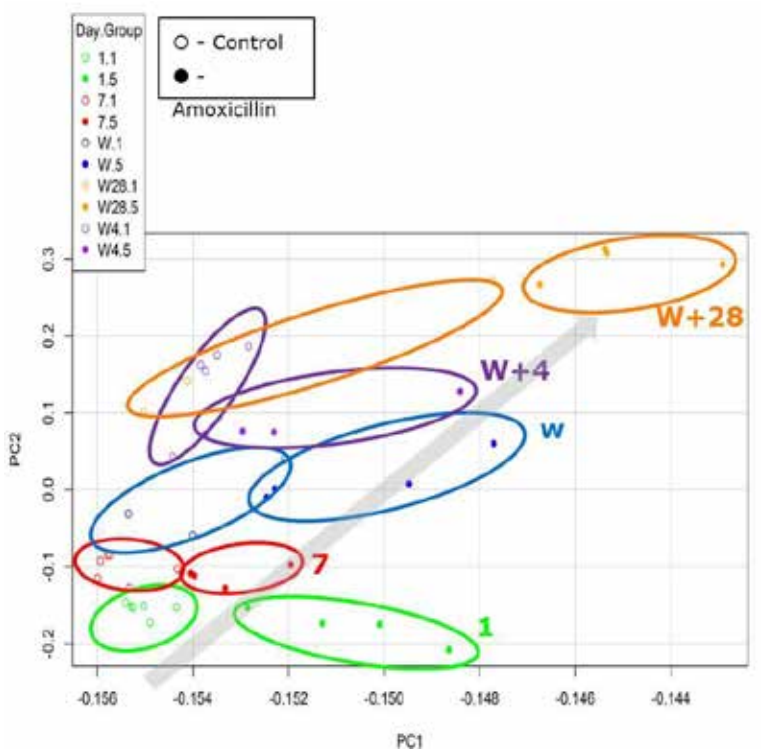

Figure 3.13. *PCA plot of gene expression data of jejunal scrapings of piglets. Gene expression in jejunal scrapings of piglets from control sows (open circles) or amoxicillin treated sows (closed circles) was determined using microarrays. Piglets were sampled at the age of 1 day (green), 7 days (red), at weaning (blue), 4 days after weaning (purple) and 4 weeks after weaning (orange). The grey arrow indicates gut development in time, independent of treatment group.*

3.3.2 Functional analysis of transcriptional changes

To understand more about the biological processes that were regulated in the jejunal scrapings of piglets, annotated genes were included in further functional and enrichment analyses to get a better understanding in which biological processes the regulated genes are involved. From these lists, both the significant up- and down-regulated genes were used as input for functional analyses. DAVID functional annotation clustering was performed resulting in multiple clusters with a significant Enrichment Score (ES). ES represents the geometric mean (in -log scale) of member's p-values in a corresponding annotation cluster. It is used to rank the biological significance of processes. An ES of 1 represents a p-value of ~0.1. Tables 3.2. and 3.3 demonstrate that more processes were down-regulated than upregulated. As expected based on the number of regulated genes, no processes were found regulated at the age of 1 week. On the other time-points several general processes were down-regulated in piglets from amoxicillin treated piglets. Although some processes were regulated at

Table 3.2
Functional Annotation Clustering of up-regulated genes in intestinal scrapings of piglets from sows from the amoxicillin group compared to the control group.

Condition	Day	Number of clusters	Generalised term[1]	Number of genes[2]	Enrichment score[3]
Treatment vs Control	1	2	Immunoglobulin domain	5	1.14
			Cell junction	4	1.001
Treatment vs Control	W	2	Transmembrane	56	1.65
			Regulation of cell activation	5	1.258
Treatment vs Control	W + 4d	0	-	0	-
Treatment vs Control	W + 4w	1	Appendage morphogenesis	3	1.357

[1] classification stringency was set at high
[2] adjusted p value < 0.05 and log Fold Change > |1.0|[3] clusters were indicated significant if above 1.00
3 clusters were indicated significant if above 1.00

Table 3.3

Functional Annotation Clustering of down-regulated genes in intestinal scrapings of piglets from sows from the amoxicillin group compared to the control group.

Condition	Day	Number of clusters	Generalised term[1]	Number of genes[2]	Enrichment score[3]
Treatment vs Control	1	4	Ion channel activity	5	1.64
			Ion transport	4	1.42
			Blood circulation	3	1.23
			Membrane fraction	6	1.2
Treatment vs Control	W	24	Regulation of protein metabolic process	9	2.65
			Apoptosis	17	2.17
			Negative regulation of macromolecule	20	2.12
			Organelle lumen	30	2.12
			Intracellular protein transport	13	2.12
			Macromolecule catabolic process	20	2.05
			Initiation factor	5	1.77
			GRAM	3	1.74
			Nucleotide-binding	33	1.45
			Protein catabolic process	5	1.45
			Regulation of apoptosis	17	1.41
			Embryonic limb morphogenesis	5	1.38
			Phosphate metabolic process	12	1.28
			Domain:Helicase C-terminal	5	1.25
			RNA catabolic process	4	1.17
			Bromodomain	3	1.17
			Vacuole	8	1.16
			Glycoprotein biosynthetic process	6	1.16
			Cytoplasmic vesicle	14	1.12
			Ubiquitin-protein ligase	5	1.09
			Macromolecular complex assembly	14	1.1
			Regulation of transcription	9	1.08
			Sequence motif:DEAD box	3	1.06
			GTPase activity	7	1.05
Treatment vs Control	W + 4d	4	Ribosome	5	2.63
			Cell cycle process	7	1.34
			Ubiquitin-dependent protein	3	1.09
			M phase	5	1.09
Treatment vs Control	W + 4w	14	Purine nucleoside binding	46	4.69
			Phosphorylation	25	2.73
			Serine/threonine-protein kinase	12	2.53
			Mitotic cell cycle	15	2.26
			Mitosis	9	1.67
			Repeat:RCC1 5	3	1.59
			Macromolecule catabolic process	20	1.47
			Zinc finger	3	1.46
			Cell fraction	21	1.42
			Endoplasmic reticulum part	10	1.4
			Helicase activity	6	1.27
			Organelle lumen	31	1.24
			Transcription	8	1.22
			Nucleotide kinase activity	3	1.13

[1] classification stringency was set at high
[2] adjusted p value < 0.05 and log Fold Change > |1.0|
[3] clusters were indicated significant if above 1.00

different time-points, most processes are specific for a certain time-point. This timing suggests that processes involved in intestinal development were also regulated due to the intervention. Processes that were regulated were very structural like regulation of metabolic processes, apoptosis, mitotic cell cycling and meiosis. This also suggests that intestinal development is organized differently in both treatment groups. No regulation of immunological processes was observed due to treatment

3.3.3 Network analysis of regulated processes

Network analysis of the data does not only take into account the regulated genes and processes as described above, but also includes additional information on protein-protein interactions described in literature. These interactions help to understand the biological response that is induced in the intestine of the piglets. To obtain a better understanding of the connections between all the regulated processes, functional association and data mining in the STITCH database was done. The STITCH database contains protein-protein interactions as well as protein-chemical interactions, allowing to link different processes via their interactions. This resulted in an interaction network (see Appendix 1 for all interaction networks). Based on the networks it could be concluded that cell cycle related processes were downregulated due to maternal amoxicillin treatment, especially around weaning. Several processes connected to the cell cycle, like protein synthesis and degradation were also downregulated. This could imply that maternal amoxicillin treatment leads to decrease of growth or differentiation in the gut.

3.4 Development of intestinal tissue

To determine whether maternal amoxicillin treatment affected intestinal development, villus height, crypt depth and mucosa length of jejunum and ileum were determined in piglets of amoxicillin treated and control sows. There were no significant difference in villus length nor in mucosa length, indicating

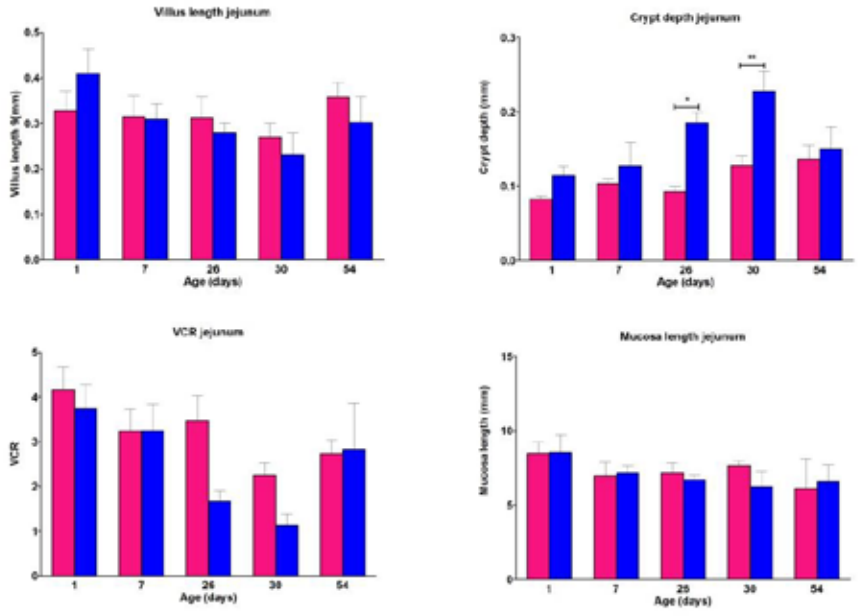

*Figure 3.12. Morphometric analysis of jejunum: villus length, crypt depth and mucosa length. Villus length, crypt depth, villus-crypt ratio (VCR) and mucosal length were determined in jejunum of piglets from sows treated with amoxicillin (blue bars) and of piglets of control sows (pink bars). Error bars indicate SEM. * p-value < 0.05; ** p-value < 0.01 as determined with ANOVA.*

that the absorptive capacity was comparable between treatments. There was a significant difference in crypt depth between the treatment groups, where piglets from amoxicillin treated sows showed deeper

crypts. Although these deeper crypts led to lower VCR, these differences were not significant. Since replication takes place in the crypts, these data suggest that offspring of amoxicillin treated mothers had potential different development compared to control piglets, although this did not lead to longer villi. In ileum mucosa similar processes took place (see Appendix 2 for results), suggesting that the changes were induced by general processes that were independent of the intestinal location in the small gut, but dependent on treatment.

To determine whether the observed differences in development are also reflected by differences in replication, PCNA staining was used. PCNA is generally used as a proliferation marker. In this study, no differences in replication capacity of intestinal cells were found between the treatment groups. Numbers of goblet cells present in mucosa were determined using a PASS staining. On day 1, significant differences existed in the number of goblet cells that were present in jejunum (Figure 3.13). Piglets that came from control sows, had much larger numbers of goblet cells. This suggests that maternal amoxicillin treatment decreased the number of goblet cells at birth, emphasizing that maternal amoxicillin treatment affected intestinal development of piglets. This difference in goblet cells was not found in the ileum (see Appendix 2). Since mucin, the secretion product of goblet cells, plays a role in microbial colonization, it cannot be excluded that this difference in goblet cells affects early colonization of piglets.

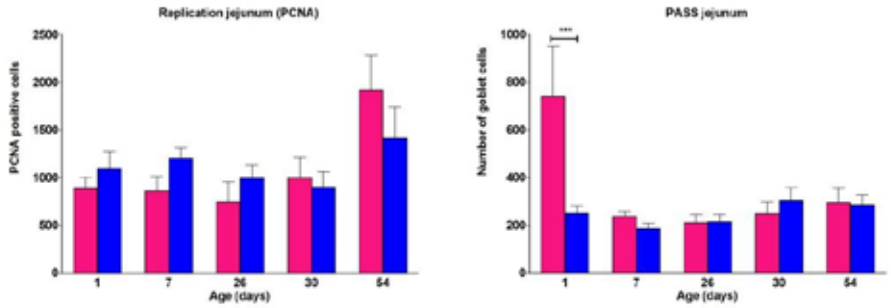

Figure 3.13. Morphometric analysis of jejunum: replication and goblet cells.
Replication was determined using a PCNA staining and number of goblet cells was determined with PASS staining in jejunum of piglets from sows treated with amoxicillin (blue bars) and of piglets of control sows (pink bars). Error bars indicate SEM.

3.5 Systemic responses

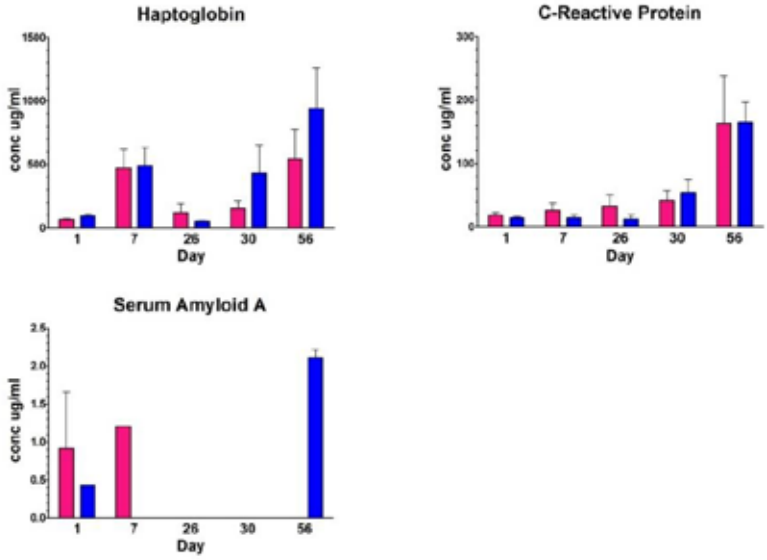

Figure 3.14. Expression of acute phase proteins in blood of piglets.
Protein expression levels were determined in blood of piglets from sows treated with amoxicillin (blue bars) and of piglets of control sows (pink bars). Error bars indicate SEM.

To determine if the observed changes in microbiota composition, gene expression and intestinal development also lead to systemic changes, expression of acute phase proteins in blood of piglets was determined. Acute-phase proteins are a class of proteins whose concentrations change rapidly in response to inflammation or injury. Levels of acute phase proteins are therefore indicative for the inflammatory state of the host. Three acute phase proteins were included for analysis: Haptoglobin, C-reactive protein (CRP) and serum amyloid A (Figure 3.14). The latter had very low expression levels in this experiment, many samples were below detection level. For CRP and haptogblobin the concentrations were higher, but no significant differences were found between the two treatment groups. This indicates that the observed intestinal changes do not induce systemic changes in inflammatory status of the piglets.

4 Discussion

4.1 General

It was shown that amoxicillin treatment of sows during the last week of gestation does not affect sow or piglet production parameters. Sow feed intake, weight and litter size were similar between treatment groups. Piglets from sows of different treatments did not differ in weight or weight gain. Although piglets from amoxicillin treated sows were 1 kg heavier at the age of 8 weeks, this was not significantly different from piglets from control sows. Maternal amoxicillin treatment did induce shifts in microbiota composition both in sows and in their offspring. Changes in microbiota composition could be detected in sow vaginal swabs, as well as in faecal sow samples. In piglets, changes in microbiota composition were observed in jejunal digesta. Furthermore, several networks of genes are differentially expressed between intestinal scrapings of piglets from amoxicillin treated sows and control sows. Among the differentially expressed pathways are processes that are involved in growth and development of cells, such as apoptosis, metabolic changes and meiosis. These changes suggest that intestinal development is differentially regulated between the treatment groups. As a result of this difference in intestinal development, amoxicillin treatment of sows induced changes in growth and differentiation of jejunal intestinal epithelia in time. So, we demonstrated that maternal (dietary) interventions (antibiotic treatments) affected gut and microbiota development in offspring.

4.2 Microbial colonization

4.2.1 Microbial colonization in sows

Amoxicillin treatment did not lead to large shifts in sow faecal microbiota nor in vaginal microbiota, although changes in relative amounts of individual bacterial species were observed. This is in accordance with a recent paper of Holman and Chénier, who concluded that there is considerable resilience to antibiotic perturbation of the gut microbiota in pigs [15]. They studied the effect of long-term treatment with antimicrobial growth promoters of piglets on microbiota composition, and concluded that mainly in the transition period of weaning small temporal changes in microbiota composition of piglets are induced. Similar to our study no changes in diversity of microbiota were found due to antibiotic treatment. Our study emphasizes this resilience of gut microbiota once it is settled. In this study, small shifts in microbiota composition were also found in vaginal microbiota. This suggests that orally applied amoxicillin can reach the vaginal mucosa and induce changes in microbiota. This is confirmed by the numerical decrease in microbial content that was found in vaginal microbiota of amoxicillin treated sows. Although not significant, the difference between treated and control sows is considerably. Alternatively, changes in vaginal microbiota composition could be induced via transmission of the faecal microbiota. This seems less likely, since different populations of microbiota are changed in vaginal microbiota compared to faecal microbiota.

4.2.2 Microbial colonization in piglets

In piglets, we focused our analysis on jejunal digesta. Since nutrient uptake occurs primarily in the small intestine, this is considered the region where bacterial activity could have the greatest influence on growth and performance of piglets [16]. Furthermore, it is expected that the small intestine is the main location for the cross-talk between microbiota and immune cells. This expectation is based on the observation that the number of immune cells is much higher in the mucosal tissues of the small intestine compared to the large intestine. Furthermore, it has been demonstrated that faeces alone is not informative of the ecology of specific intestinal habitats since each intestinal location has its own microbial targets that could be manipulated to influence gut health [17]. Therefore, we studied the effect of maternal amoxicillin treatment on intestinal development and microbiota composition in the jejunum.

Independent of the treatment groups, a clear microbiota development in piglets can be seen in time, both in diversity and in composition of microbiota. On the first day of life microbiota is very diverse, probably reflecting the early colonisers of the naïve intestine. Organisms that are present in the surroundings of the stable, or transmitted by the sow can colonise the piglets intestine. This is probably a random process. In time the microbiota develops in a more mature and dedicated microbiota. At the transition moment of weaning microbial diversity decreases. This is probably due to the change of diet, combined with the abrupt cessation of exposure to mother milk. After weaning the permanent microbiota starts to settle which is reflected in an increasing diversity. This pattern of diversity was also described by others [18].
Around weaning a significant decrease of microbial content was found in ingesta of piglets from amoxicillin treated piglets. This could either reflect a direct, post-poned effect of the amoxicillin treatment of sows. It could however, also be the consequence of the changed gene expression and deeper crypts. These changes could allow for differential microbial content within the groups, especially since all significant changes occur around weaning.
The bacterial species that were observed to differ in relative abundance in piglets between treatment groups are limited. They include several enterococcal and lactobacillus species, that are well known for their beneficial probiotic properties. These species are less abundant in piglets from amoxicillin treated mothers, especially around weaning. *Lactobacillus mucosae*'s relative abundance differs considerably between treatment groups on day 1. At this moment, there was a significant ($p<0.001$) difference in number of goblet cells in jejunum of piglets from different treatment groups. It is known that Lactobacilli in general and *Lactobacillus mucosae* in particular strongly adhere to mucus, that is produced by goblet cells [19-21]. This suggests that the differential numbers of goblet cells, might allow for differential colonization of the jejunum as was observed. Two species of clostridiaceae, among which *Clostridium perfringens*, seem to be more abundant in piglets from amoxicillin treated mothers. In literature, a possible relation was described between presence of Lactobacilli and absence of clostridaceae and the other way around, suggesting competitive exclusion between these organisms occurs [22]. This interaction might partly explain the observed increase in relative abundance of Clostridiaceae, especially on day 1. Furthermore, from human medicine it is well known that *Clostridium difficile* infections come up after antibiotic treatment [23]. The combination of maternal amoxicillin treatment combined with decreased relative numbers of Lactobacilli might therefore explain the relative abundance of Clostridiaceae.

4.2.3 Interaction of microbial colonization sows and piglets

Based on the overlap that was observed between sow vaginal microbiota, and piglets jejunal digesta on day 1, it could be speculated that during passage through the birth channel the first intestinal colonizers enter the gut. It is clearly visible that amoxicillin treatment reduces the number of *Veilonella* in the sow vagina as well as in the new-born piglets jejunal digesta. In a similar study with maternal antibiotic treatment, similar changes in relative abundance of groups within sow faecal microbiota and ileal microbiota of offspring was observed, also suggesting that piglets are colonized with maternal microbiota via direct transmission of bacteria [24]. This is also confirmed in the human field that already show that the initial microbiota of vaginally born infants resembles that of their mother's vagina, while that of C-section infants is dominated by skin microbes not related to those of the mother [7]. Therefore, we conclude that manipulation of vaginal microbiota of sows might be a first parameter that can be used to influence the first colonisers of piglet intestines. Although not much is known about factors that influence vaginal microbiota besides hormonal changes during gestation, it is a worthwhile direction to study in more detail.

4.3 Intestinal responses to amoxicillin: gene expression & morphology

In our study, in total 240 genes were differentially expressed between the treatment groups (over all time-points). Similar to microbiota development, a strong time dependent effect could be seen. Intestinal development is reflected in transcriptional changes in time. Since this was not part of the current study, these development changes that occurred in both treatment groups were not analysed in detail. In this study we focused on differences in gene expression between the two treatment groups.

Network analysis showed that most changes in expression occur around weaning. Some processes are regulated early in time. Immunoglobulin domain and cell junction are upregulated in the piglets from amoxicillin treated mothers, although these processes were only represented by few genes. These changes in expression suggest that intestinal development is different between treatment groups as was also reflected by significant differences in numbers of goblet cells as well as by the different composition of microbiota at that same time-point. It is however, difficult to exactly interpret these changes.

Around weaning, regulated genes are mainly involved in very general cellular processes, like 'apoptosis', 'ribosome', 'cell cycle' and 'ubiquitination'. This suggests that changes in replication or differentiation of cells are induced, e.g. the functioning of cells or the metabolic activity of the intestinal cells. These changes are most prominent around weaning. This is the moment piglets have to shift to solid feed, and the intestine has to adapt to these changes. The observed changes in gene expression probably reflect a change in development between the treatment groups. These changes coincide with changes in microbiota composition. One of the groups of bacteria that differ significantly, especially around weaning are enterococci and lactobacilli. Those are classes of bacteria that are known for their probiotic, beneficial effects to the host. Especially the enterococci, seem to be differentially present around weaning, coinciding with the strongest transcriptional changes. Therefore, it is very well feasible that abundant enterococcal presence affects gene expression patterns. In this case, relative low numbers of enterococci in piglets from amoxicillin treated mothers coincide with upregulation of processes involved in cell cycling.
Morphological data showed that around weaning piglets from amoxicillin treated sows have significantly deeper crypts in both jejunum and ileum, but no difference in villus height or replication was seen. Altered crypt depth indicates a modulation in the host in response to bacteria, probably mediated by the observed differences in microbiota composition in early life. [24]. Conventional pigs display deeper crypts than germfree or mono-colonized pigs [25]. Also in rats a conventional microbiota leads to deeper crypts than germfree rats [26]. Although we did not see major differences in diversity, changed microbiota could lead to deeper crypts and thus a difference in gut development. Taken together, the data indicate that although the crypts of piglets deepen around weaning as an effect of the amoxicillin treatment of sows without resulting in increased proliferation. In combination with the upregulation of cell cycle processes, ribosomal activity and protein degradation, this suggests that the intestinal development of these piglets is somehow different.

4.4 Conclusion and implication

In this study, proof of concept was delivered that by maternal interventions to sows, transgenerational effects can be sorted in piglets. Here the effects of oral amoxicillin during the last week of gestation were studied on piglet performance, microbiota composition and transcriptional responses. Although we showed that sow microbiota of both faecal and vaginal samples do not change considerably after amoxicillin treatment, they are very robust, changes in microbiota composition were induced and transmitted to piglets. Furthermore, morphological and transcriptional changes were induced in piglet jejunum suggesting that intestinal development of piglets from amoxicillin treated mothers is different from that of control controls. This differential intestinal development was not induced by changes in weight nor growth of piglets, since these parameters were not changed. Furthermore, differential intestinal development did not lead to different systemic inflammatory responses, since no difference in acute phase protein expression was found between the treatments. This implies that transgenerational

effects can be induced after oral administration of feed ingredients / antibiotics and that these effects are mainly found in the process of intestinal development.

In future experiments, more natural feed ingredients will be used to manipulate gut health of piglets. From the current study, we learned that vaginal microbiota should be included when looking at microbiota transmission. To be able to conclude whether microbiota composition and immune competence also affects health of animals, in future experiments a challenge should be included. It is very difficult to determine beneficial health effects of a dietary intervention in healthy piglets. Finally, assuming that the antibiotic treatment, from one week before farrowing to the day of farrowing, has a negative impact on the immune competence of piglets later in life, the data provided in this study may help to define relevant parameters for immune competence.

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Appendix 1 Bacterial species differentially present in microbiota

Table Appendix 1.1. Bacterial species that are differentially present in sows samples as determined by ANOVA. Indicated time-points are related to farrowing: -7 corresponds to 7 days before farrowing; 1 / 7 correspond to 1 or 7 days after farrowing; 0 indicates day of farrowing.

	Time-point	-7	-7	1	1	7	7	0	0
	Treatment	Control	Amoxicillin	Control	Amoxicillin	Control	Amoxicillin	Control	Amoxicillin
Bacterial Species	Sample	Faeces	Faeces	Faeces	Faeces	Faeces	Faeces	Vagina	Vagina
k__Bacteria;p__Firmicutes;c__Clostridia;f__Veillonellaceae;g__Phascolarctobacterium;s__		0.03889224	0.034788051	0.009192412	0.009449212	0.015456945	0.01524752	0.007690666	0.00945369
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;f__;g__;s__		0.086106803	0.07858383	0.05580663	0.031077641	0.129255452	0.078816588	0.014718861	0.019978206
k__Bacteria;p__Fibrobacteres;c__Fibrobacteria;f__Fibrobacteraceae;g__Fibrobacter;s__succinogenes		0.004543881	0.006209046	0.00092934	0.000554517	0.009032672	0.004489139	0.000370842	0.001229713
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;f__[Paraprevotellaceae];g__CF231;s__		0.01486086	0.016759238	0.000229644	0.000714833	0.010986577	0.016203479	0.00269672	0.002823396
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;f__Desulfovibrionaceae;g__Desulfovibrio;Other		0.006614047	0.004164737	0.015585878	0.019536985	0.005503521	0.005780389	0.0008588	0.003237852
k__Bacteria;p__Firmicutes;c__Clostridia;f__Dehalobacteriaceae;g__;s__		0.001946785	0.003538994	0.0059209	0.0050334	0.006037785	0.004052725	0.000283811	0.001209604
k__Bacteria;p__Bacteroidetes;Other;Other;Other;Other		0.01957643	0.018389891	0.004132416	0.003831808	0.013764825	0.017039707	0.002955354	0.004345138
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;f__Bacteroidaceae;g__Bacteroides;s__		0.000516042	0.000379033	0.05502592	0.030119549	0.001092347	0.002441511	0.000284819	0.00217603
k__Bacteria;p__Verrucomicrobia;c__Verruco-5;f__RFP12;g__;s__		0.017187411	0.016825189	0.001879444	0.001110088	0.0047665	0.004296791	0.003290097	0.006920685
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;Other;Other;Other		0.10861818	0.087451151	0.047006	0.031537539	0.071190117	0.057846309	0.017923028	0.030305449
k__Bacteria;p__Tenericutes;c__Mollicutes;f__;g__;s__		0.042637903	0.043030398	0.0797994	0.008164082	0.056220095	0.034907207	0.006352865	0.010366944
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;f__S24-7;g__;s__		0.057854173	0.079367701	0.028487527	0.02204676	0.070593825	0.108400764	0.007972189	0.010234934
k__Bacteria;p__Firmicutes;c__Clostridia;f__Veillonellaceae;g__Veillonella;Other		5.03863E-06	1.22365E-05	3.02878E-05	9.42064E-05	1.00527E-05	5.92373E-06	0.011483589	0.003003638
k__Bacteria;p__Firmicutes;c__Bacilli;f__Turicibacteraceae;g__Turicibacter;s__		0.016618648	0.013677936	0.043125678	0.00999373	0.020479931	0.023493953	0.004897901	0.010348322
k__Bacteria;p__Firmicutes;c__Clostridia;f__Veillonellaceae;g__Anaerovibrio;s__		0.004662561	0.007313522	0.000134217	0.000497592	0.000995808	0.003151138	0.002312934	0.000873247
k__Bacteria;Other;Other;Other;Other;Other		0.023693418	0.028132267	0.022923362	0.022538712	0.020659211	0.018868591	0.004747241	0.015670776
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;f__Erysipelotrichaceae;g__Bulleidia;s__p-1630-c5		0.001660672	0.002367046	0.002718963	0.001880257	0.005911009	0.00734269	0.001048185	0.00128399
k__Bacteria;p__Firmicutes;c__Bacilli;f__Streptococcaceae;g__Streptococcus;Other		0.000437181	0.000519402	0.003088408	0.001550025	0.000418478	0.000207782	0.023498522	0.01196386
k__Bacteria;p__Firmicutes;c__Bacilli;f__Staphylococcaceae;g__Staphylococcus;Other		7.54252E-06	3.5445E-06	2.43536E-06	1.96512E-05	2.1978E-06	7.33598E-07	0.009915321	0.00842903
k__Bacteria;p__Firmicutes;c__Clostridia;f__Clostridiaceae;g__SMB53;s__		0.00139508	0.001200211	0.001092046	0.000236789	0.003141548	0.005591213	0.001209701	0.001077932
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;f__Erysipelotrichaceae;g__[Eubacterium];s__biforme		0.001650965	0.004139447	0.015214467	0.013535259	0.005228259	0.005626656	0.00131012	0.001665094
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Moraxellaceae;g__Acinetobacter;Other		5.08411E-07	2.38897E-06	1.499E-06	1.48671E-06	5.85807E-07	1.79285E-06	0.012239366	0.043707572
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;f__Bacteroidaceae;g__Bacteroides;s__fragilis		2.46553E-05	3.76701E-05	0.006339061	0.08590837	3.15643E-05	7.73508E-06	0.000771758	0.000616912
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;f__Prevotellaceae;g__Prevotella;Other		0.024275893	0.037820589	0.033793497	0.021589763	0.02409441	0.018930679	0.007101358	0.015178633
k__Bacteria;p__Firmicutes;c__Bacilli;f__Enterococcaceae;Other;Other		0.000172548	0.000278704	0.003361267	0.04869932	0.000115536	0.000824424	0.002027598	0.008890102
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;f__Erysipelotrichaceae;Other;Other		0.003268566	0.00354369	0.005907304	0.001704388	0.004653463	0.006286886	0.002297125	0.001858197
k__Bacteria;p__Firmicutes;c__Bacilli;f__Lactobacillaceae;g__Lactobacillus;s__reuteri		0.047204255	0.03052428	0.027760941	0.00069111	0.016015379	0.056204645	0.043955164	0.006646851
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;f__Erysipelotrichaceae;g__Catenibacterium;s__		6.00093E-06	0.000656038	0.000930255	0.034417213	0.01247569	0.01390943	0.002134194	0.013048414
k__Bacteria;p__Firmicutes;c__Bacilli;f__Streptococcaceae;g__Streptococcus;s__		0.000129534	0.000188567	0.002055353	1.9842E-05	0.000325037	0.000133828	0.0299114	0.010964267
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Pasteurellaceae;Other;Other		5.71077E-06	1.94919E-05	0.000405596	9.06741E-05	2.79855E-05	1.27748E-05	0.066405852	0.025041
k__Bacteria;p__Firmicutes;c__Bacilli;f__Aerococcaceae;g__Aerococcus;s__		0	7.59361E-07	2.998E-06	3.32516E-06	0	2.94404E-06	0.010773957	0.02322711
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Moraxellaceae;g__Acinetobacter;s__lwoffii		0	2.31981E-06	0	2.46363E-06	0	3.26487E-06	0.013255871	0.034723828
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Enterobacteriaceae;g__Escherichia;s__coli		0.003768616	0.003163684	0.086443823	0.136136215	0.001193904	0.006583553	0.242424176	0.106897904
k__Bacteria;p__Firmicutes;c__Clostridia;f__Lachnospiraceae;Other;Other		0.005617423	0.006547145	0.005011887	0.013244763	0.009109626	0.014380945	0.002773076	0.004815828
k__Bacteria;p__Spirochaetes;c__Spirochaetes;f__Spirochaetaceae;g__Treponema;s__		0.029034273	0.040037009	0.044795697	0.037320019	0.035216693	0.038928695	0.00565671	0.007890528
k__Bacteria;p__Actinobacteria;c__Actinobacteria;f__Actinomycetaceae;g__Actinomyces;s__hyovaginalis		7.25018E-06	2.13893E-05	0.011132379	0.008603931	2.018E-05	5.81847E-06	0.000672501	0.000430131
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;f__Prevotellaceae;g__Prevotella;s__		0.088139333	0.089244563	0.03793212	0.064873166	0.077466253	0.068059786	0.02986661	0.037576614
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;f__Porphyromonadaceae;g__Parabacteroides;s__		0.005688124	0.003939747	0.011664244	0.018968481	0.00780791	0.009317439	0.000995436	0.001203015
k__Bacteria;p__Spirochaetes;c__Spirochaetes;f__Spirochaetaceae;Other;Other		0.001391677	0.002338889	0.010090562	0.008525428	0.000492065	0.000641231	0.000449182	0.000724327
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;f__Bacteroidaceae;g__Bacteroides;s__plebeius		1.52523E-06	1.21691E-06	0.032845283	0.02073875	7.26184E-06	4.62414E-05	6.68776E-07	6.59739E-06
k__Bacteria;p__Firmicutes;Other;Other;Other;Other		0.006669743	0.006988605	0.006934868	0.005873505	0.005416395	0.007566941	0.001816016	0.001706784
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;f__Erysipelotrichaceae;g__p-75-a5;s__		0.005105908	0.00474833	0.005066844	0.005232401	0.008204153	0.006884532	0.0018954	0.002559018
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;f__Bacteroidaceae;g__Bacteroides;s__coprophilus		3.4207E-06	1.14829E-06	0.010729413	0.002076159	4.96454E-07	3.46174E-05	1.20519E-06	2.91898E-06
k__Bacteria;p__Firmicutes;c__Bacilli;f__Streptococcaceae;g__Streptococcus;s__luteciae		0.018015628	0.036638402	0.0127672	0.006848943	0.002109145	0.005050223	0.051199086	0.002621252
k__Bacteria;p__Spirochaetes;c__Spirochaetes;f__Spirochaetaceae;g__Treponema;Other		0.006732037	0.006782204	0.004432292	0.002600843	0.00471122	0.004213982	0.002102251	0.001675355
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Pasteurellaceae;g__Actinobacillus;Other		1.0628E-05	2.34289E-05	0.000110583	0.00019247	2.07309E-05	1.72345E-05	0.095647632	0.094761929
k__Bacteria;p__Firmicutes;c__Bacilli;f__Lactobacillaceae;g__Lactobacillus;s__		0.124744098	0.073411144	0.082876657	0.042725031	0.147022614	0.118941329	0.042776528	0.039436058
k__Bacteria;p__Firmicutes;c__Clostridia;f__Ruminococcaceae;g__Oscillospira;s__		0.005941852	0.006408361	0.005449595	0.008918876	0.007517267	0.009609853	0.002695555	0.002558788

Table Appendix 1.2. Bacterial species that are differentially present in piglet samples as determined by ANOVA. Timepoints indicated represent age in days.

	Time-point	1	1	7	7	26	26	30	30	56	56
Bacterial Species	Treatment	Control	Amoxicillin	Control	Amoxicillin	Control	Amoxicillin	Control	Amoxicillin	Control	Amoxicillin
565 k__Bacteria;p__Firmicutes;c__Clostridia;f__Veillonellaceae;g__Veillonella;s__		1.3964E-06	0.000466669	0.016994015	0.000211893	0.07523289	0.096230635	4.2007E-05	0.000100853	8.46401E-06	0.001254352
384 k__Bacteria;p__Firmicutes;c__Bacilli;f__Lactobacillaceae;g__Lactobacillus;Other		0.000927652	0.001801034	0.007573056	0.013020236	0.009605418	0.012271487	0.003316558	0.002112942	0.005242505	0.000694655
607 k__Bacteria;p__Firmicutes;c__Erysipelotrichi;f__Erysipelotrichaceae;g__Sharpea;s__azabuensis		0	0.000123315	2.5914E-05	0.00280252	0.024687828	0.026598426	0.001003794	0.0001862	0.001685239	0.000864828
374 k__Bacteria;p__Firmicutes;c__Bacilli;f__Enterococcaceae;g__Enterococcus;s__cecorum		7.95996E-07	9.41543E-05	1.04476E-05	0.000114645	0.022460838	0.000288748	2.04635E-06	7.97213E-06	1.51507E-05	7.12528E-05
371 k__Bacteria;p__Firmicutes;c__Bacilli;f__Enterococcaceae;g__Enterococcus;Other		4.72145E-06	0.000107588	2.01423E-05	0.000165815	0.024790091	0.00085878	1.39604E-06	1.5351E-05	2.0503E-05	8.59385E-05
394 k__Bacteria;p__Firmicutes;c__Bacilli;f__Lactobacillaceae;g__Lactobacillus;s__mucosae		0.005596631	0.000142608	0.009894699	0.007070159	0.006462861	0.004954541	0.003823828	0.001006779	3.93201E-05	0.000134699
918 k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Moraxellaceae;g__Moraxella;s__		0.06338508	0.010756641	0.006077287	0.003891987	0.001009671	0.001227503	0.000424124	0.000511257	0.000382637	0.011465903
564 k__Bacteria;p__Firmicutes;c__Clostridia;f__Veillonellaceae;g__Veillonella;Other		0.063219373	0.000579332	0.021894879	0.029674536	0.026654771	0.00990468	0.000495156	2.92769E-05	4.89411E-05	0.001697396
387 k__Bacteria;p__Firmicutes;c__Bacilli;f__Lactobacillaceae;g__Lactobacillus;s__agilis		3.68871E-06	0.000171643	0	2.44484E-07	0.00106529	5.74432E-06	0.078989802	0.025908735	0.074816756	0.008718147
484 k__Bacteria;p__Firmicutes;c__Clostridia;f__Clostridiaceae;g__SMB53;s__		0.000113393	0.001851926	0.007825788	0.010071095	0.013392569	0.014283617	0.002451186	0.008995076	0.002409469	0.003196479
474 k__Bacteria;p__Firmicutes;c__Clostridia;f__Clostridiaceae;g__02d06;s__		0.000566132	0.002336288	0.012017938	0.005393656	0.007280476	0.009070414	0.00227352	0.002169354	0.000424026	0.002534147
482 k__Bacteria;p__Firmicutes;c__Clostridia;f__Clostridiaceae;g__Clostridium;s__perfringens		0.052233683	0.091561616	0.005670208	0.011528511	0.001227161	0.001490705	1.87358E-06	3.58584E-05	0	3.45643E-05
385 k__Bacteria;p__Firmicutes;c__Bacilli;f__Lactobacillaceae;g__Lactobacillus;s__		0.03547279	0.120119033	0.358730433	0.346113776	0.252073581	0.291493103	0.390395419	0.293025442	0.415659208	0.370579581
877 k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;f__Enterobacteriaceae;g__Escherichia;s__coli		0.24176701	0.276787594	0.083936974	0.019884572	0.018904833	0.047590763	0.18417956	0.115384253	0.053495984	0.178445244

Appendix 2 Interaction networks of regulated processes

Figure Appendix 2.1 Network of significant up-regulated genes on day 1 in jejunal scrapings of piglets from amoxicillin treated sows compared to piglets from control sows and their respective STITCH interaction partners. Highlighted are the 6 most frequent KEGG pathways.

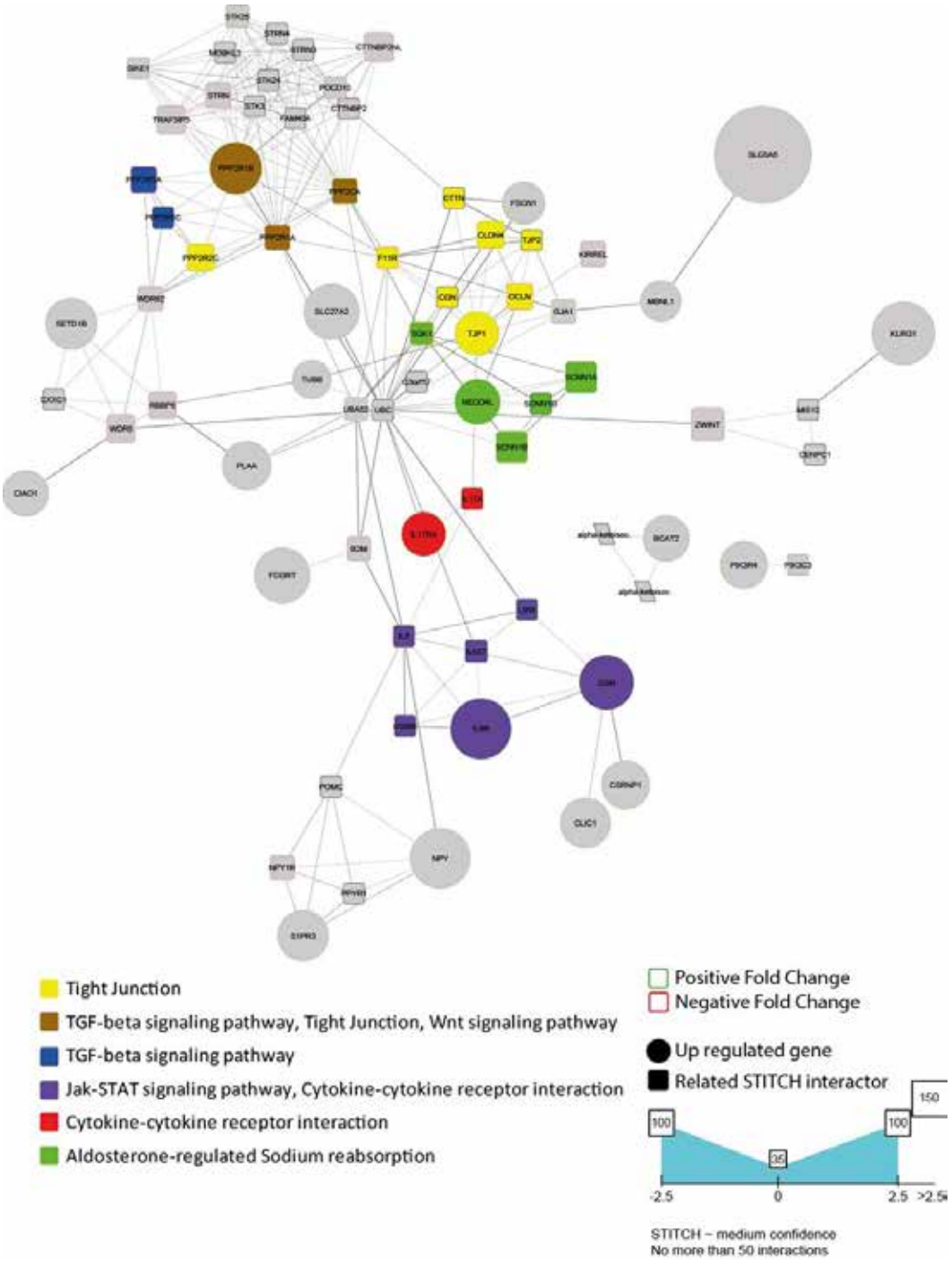


Figure Appendix 2.2 Network of significant down-regulated genes on day 1 in jejunal scrapings of piglets from amoxicillin treated sows compared to piglets from control sows and their respective STITCH interaction partners. Highlighted are the 6 most frequent KEGG pathways.

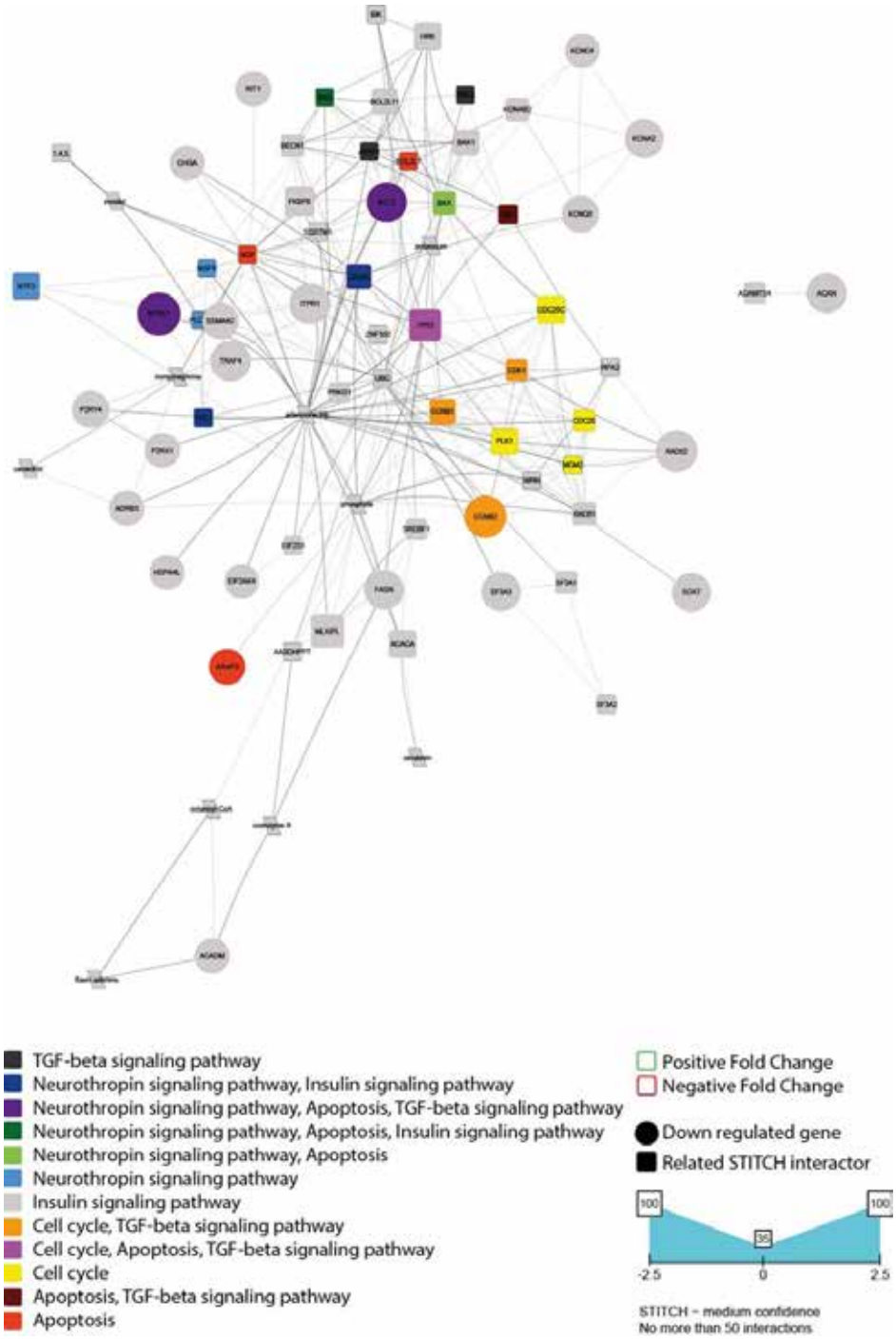


Figure Appendix 2.3 Network of significant up-regulated genes at weaning in jejunal scrapings of piglets from amoxicillin treated sows compared to piglets from control sows and their respective STITCH interaction partners. Highlighted are the 6 most frequent KEGG pathways.

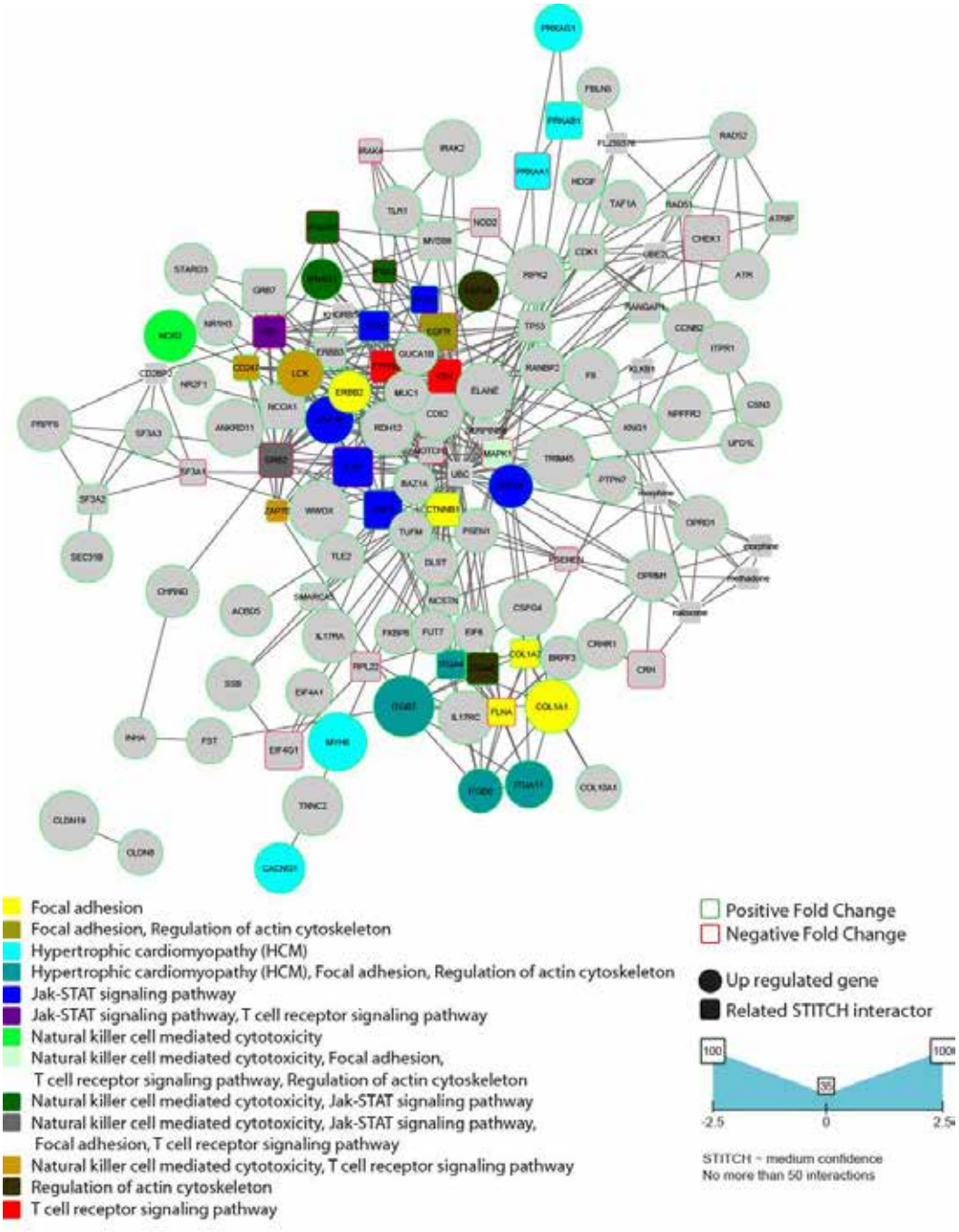


Figure Appendix 2.4 Network of significant down-regulated genes at weaning in jejunal scrapings of piglets from amoxicillin treated sows compared to piglets from control sows and their respective STITCH interaction partners. Highlighted are the 6 most frequent KEGG pathways.

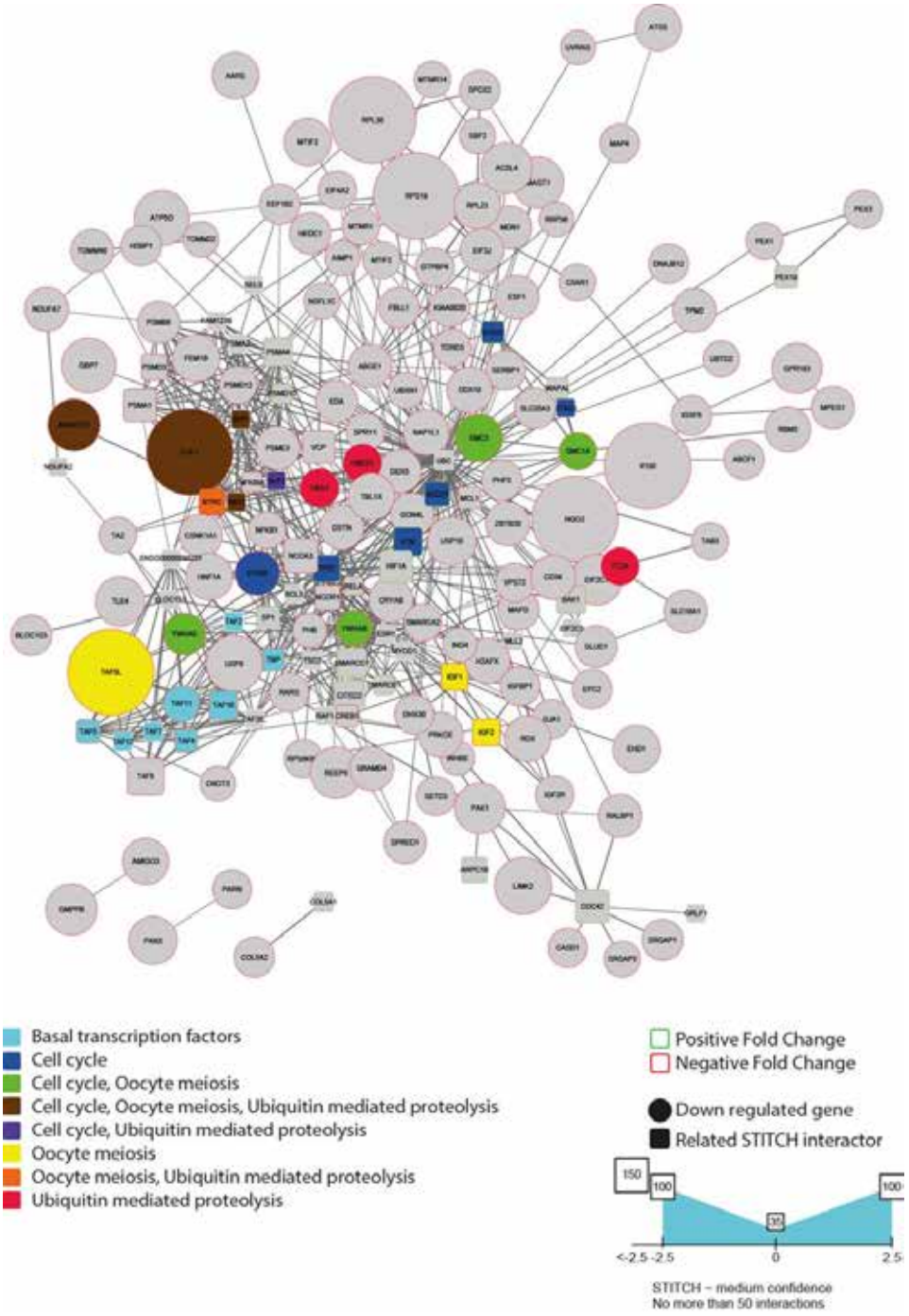


Figure Appendix 2.5 Network of significant up-regulated genes at weaning + 4 days in jejunal scrapings of piglets from amoxicillin treated sows compared to piglets from control sows and their respective STITCH interaction partners. Highlighted are the 6 most frequent KEGG pathways.

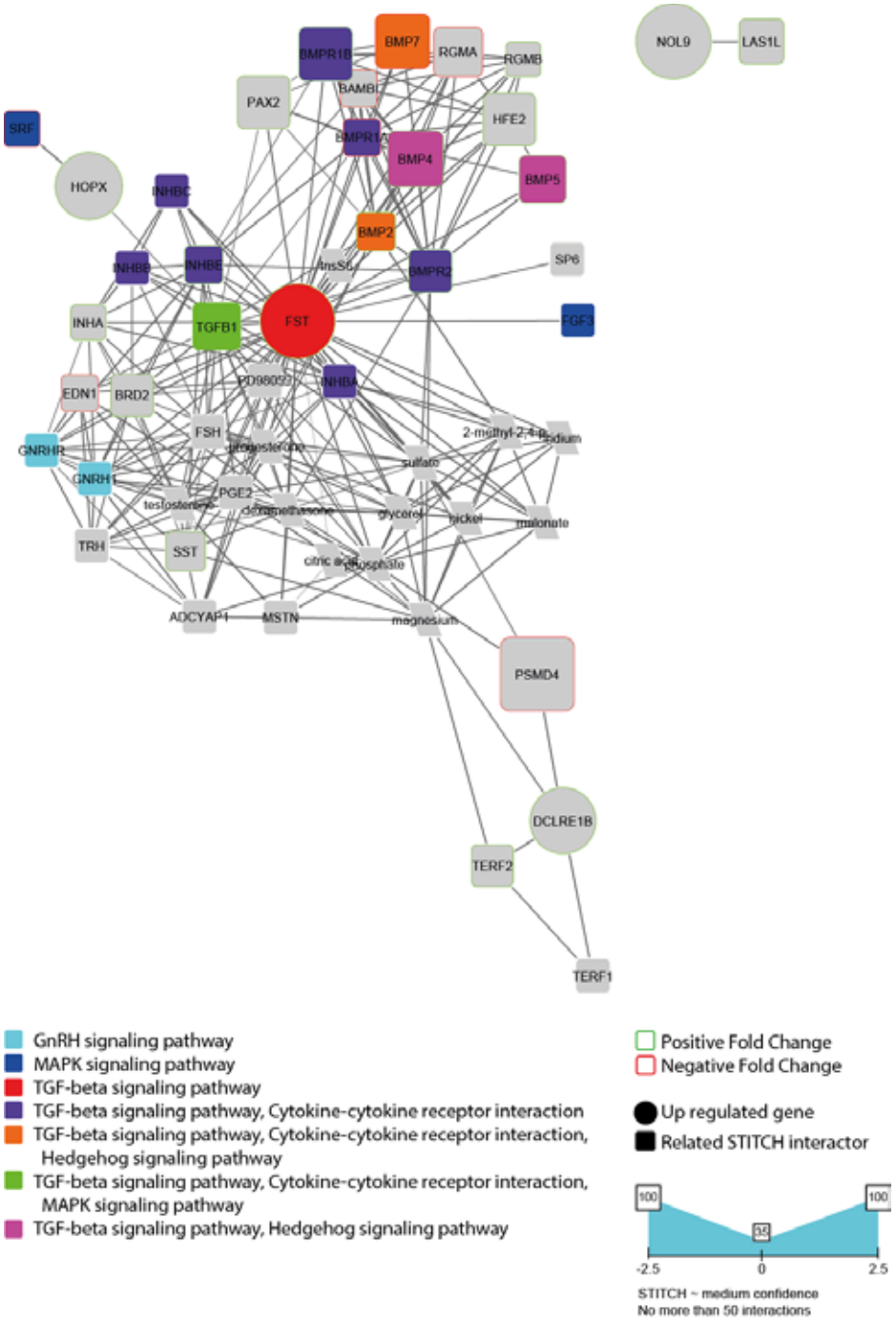


Figure Appendix 2.6 Network of significant down-regulated genes at weaning + 4 days in jejunal scrapings of piglets from amoxicillin treated sows compared to piglets from control sows and their respective STITCH interaction partners. Highlighted are the 6 most frequent KEGG pathways.

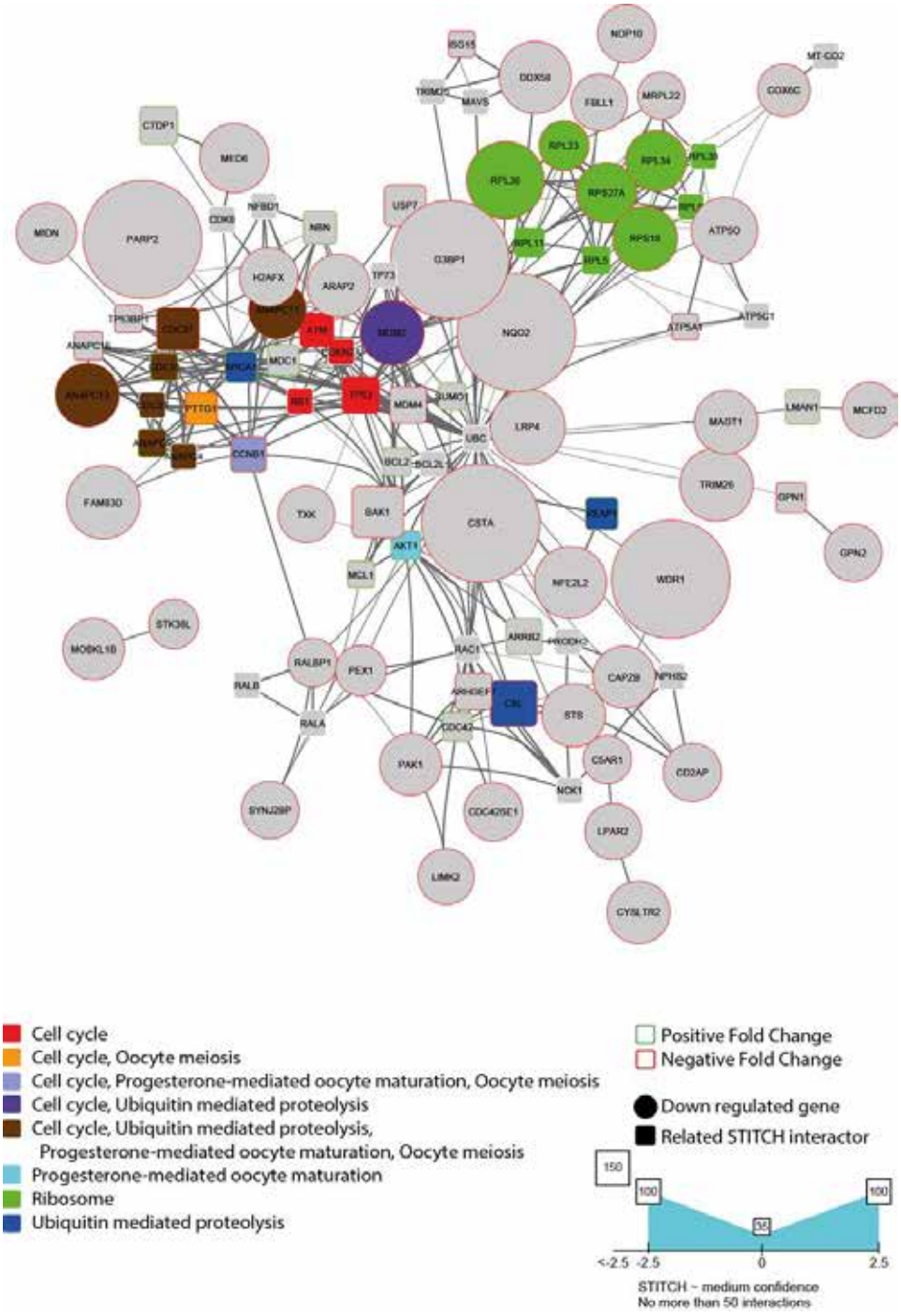
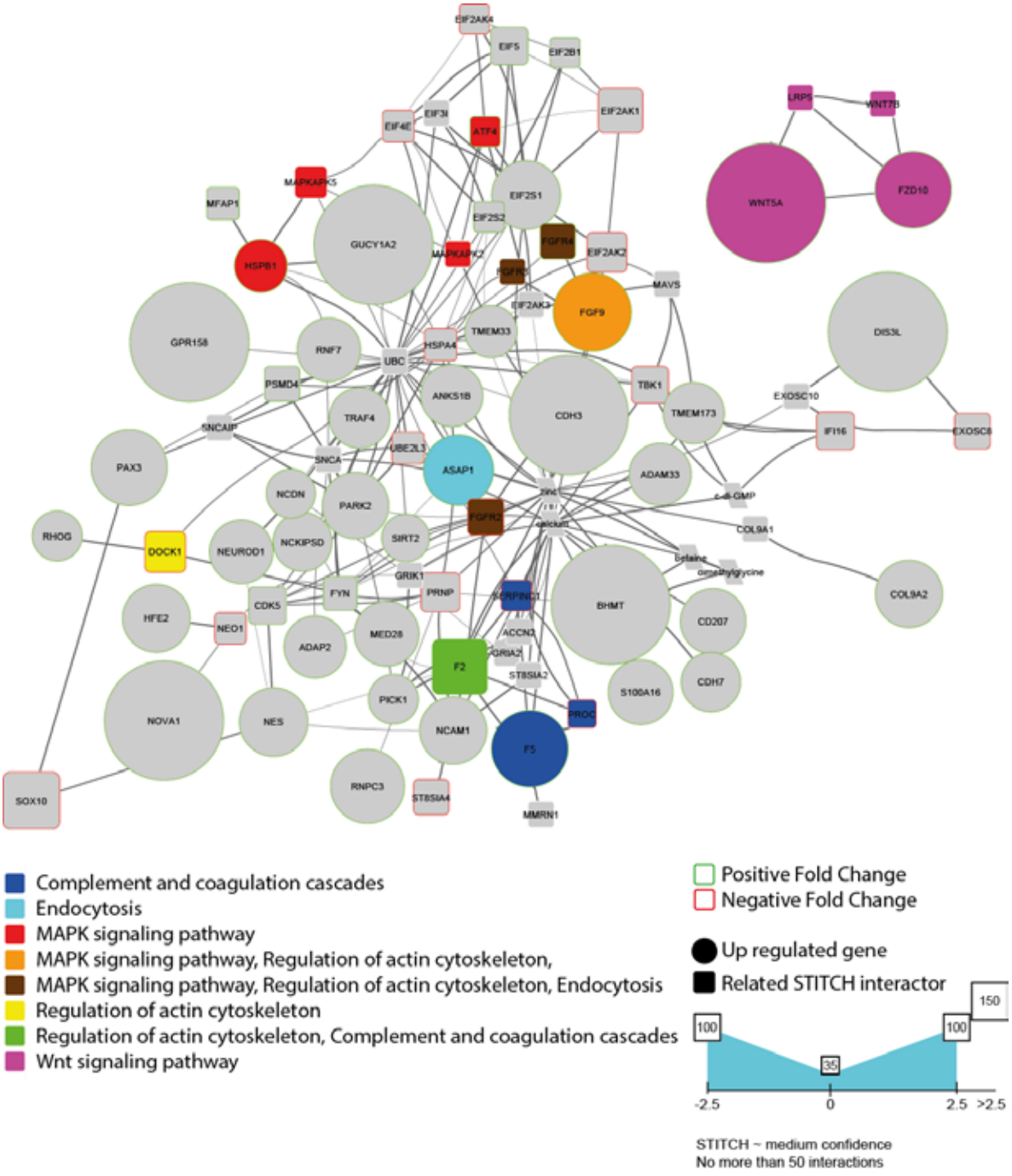


Figure Appendix 2.7 Network of significant up-regulated genes at weaning + 4 weeks in jejunal scrapings of piglets from amoxicillin treated sows compared to piglets from control sows and their respective STITCH interaction partners. Highlighted are the 6 most frequent KEGG pathways.



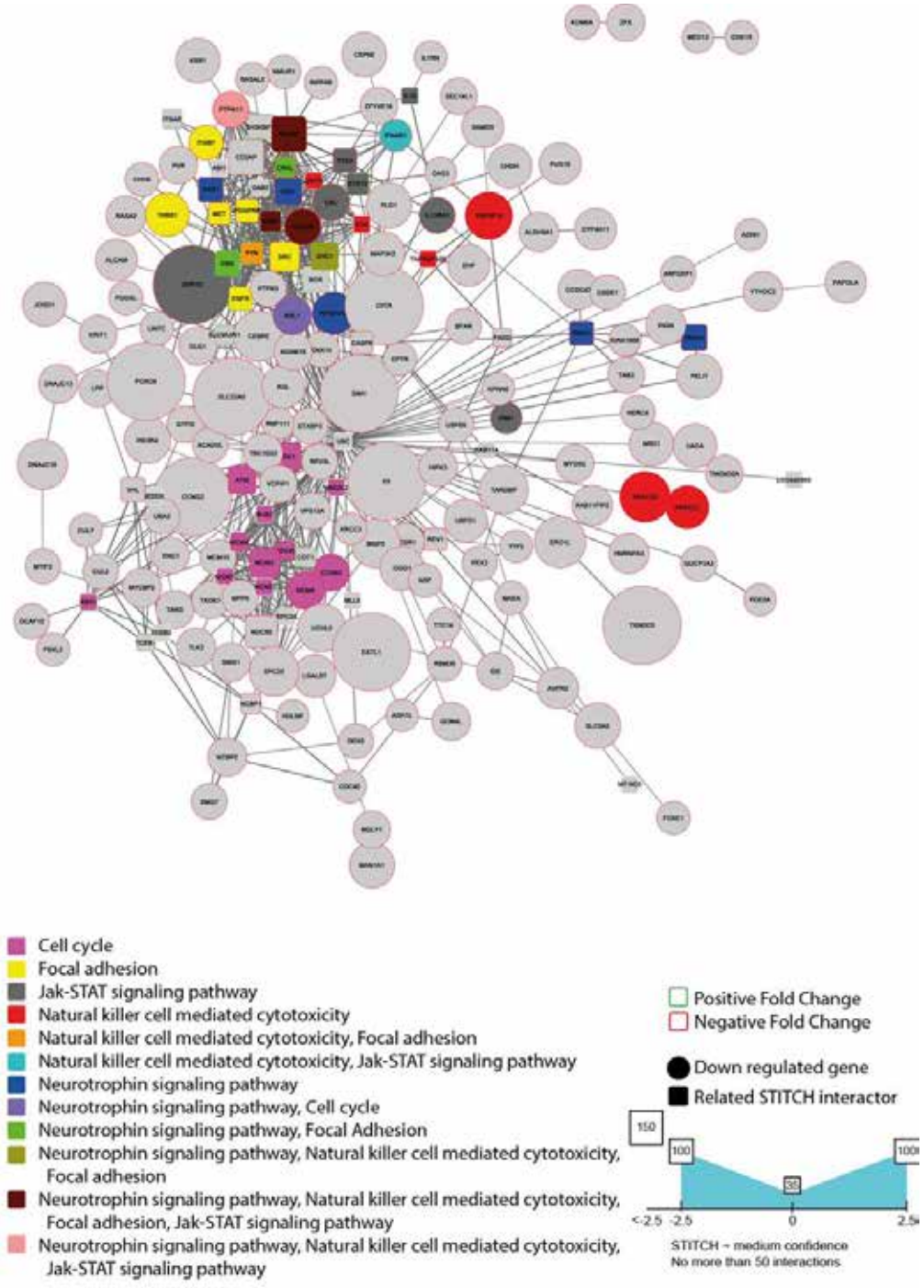
- Complement and coagulation cascades
- Endocytosis
- MAPK signaling pathway
- MAPK signaling pathway, Regulation of actin cytoskeleton,
- MAPK signaling pathway, Regulation of actin cytoskeleton, Endocytosis
- Regulation of actin cytoskeleton
- Regulation of actin cytoskeleton, Complement and coagulation cascades
- Wnt signaling pathway

- Positive Fold Change
- Negative Fold Change

- Up regulated gene
- Related STITCH interactor

STITCH ~ medium confidence
No more than 50 interactions

Figure Appendix 2.8 Network of significant down-regulated genes at weaning + 4 weeks in jejunal scrapings of piglets from amoxicillin treated sows compared to piglets from control sows and their respective STITCH interaction partners. Highlighted are the 6 most frequent KEGG pathways.



Appendix 3 Morphometric results ileum

*Figure appendix 2.1. Morphometric analysis of ileum: villus length, crypt depth and mucosa length. Villus length, crypt depth, villus-crypt ratio (VCR) and mucosal length were determined in ileum of piglets from sows treated with amoxicillin (blue bars) and of piglets of control sows (pink bars). Error bars indicate SEM. * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001 as determined with ANOVA.*

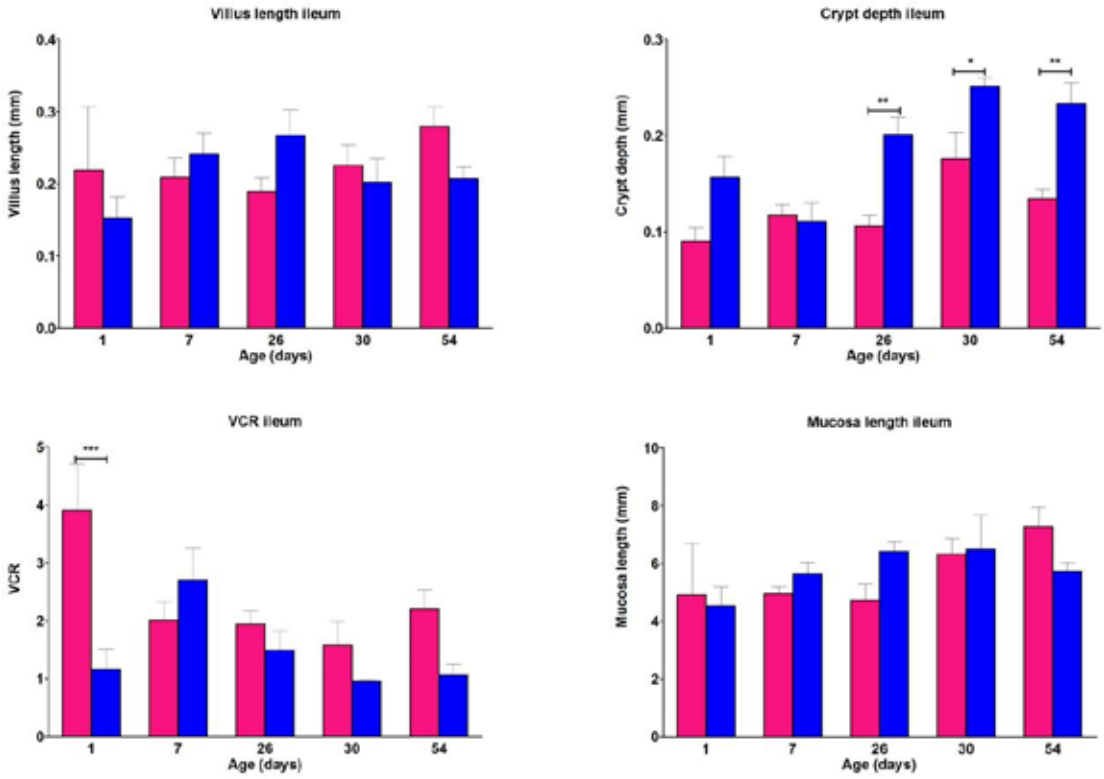
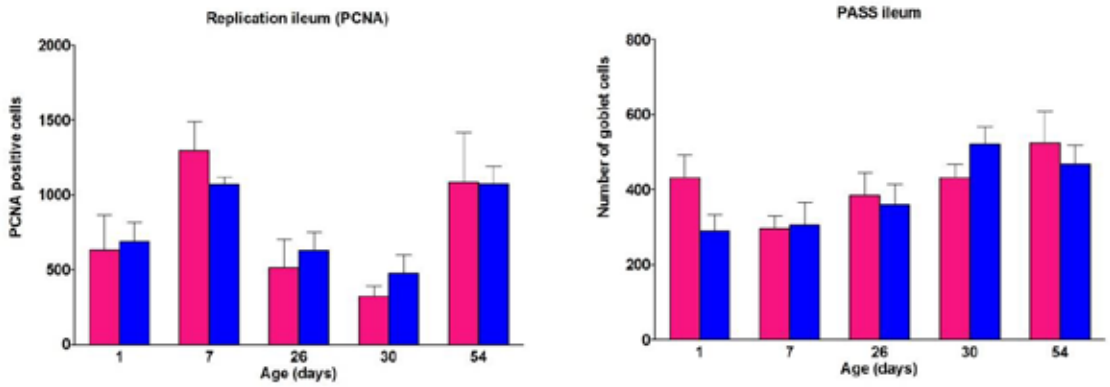


Figure appendix 2.2. Morphometric analysis of ileum: replication and goblet cells.
Replication was determined using a PCNA staining and number of goblet cells was determined with PASS staining in ileum of piglets from sows treated with amoxicillin (blue bars) and of piglets of control sows (pink bars). Error bars indicate SEM.



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