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LIVESTOCK RESEARCH
WAGENINGEN UR

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Gut microbial colonization and immune competence development are influenced by early-life environmental factors and interventions, such as oral treatment with antibiotics. Especially during this period the crosstalk between microbiota in the intestinal tract and host cells in the gut mucosal layer is important for immune development and immune programming. In humans, it has been shown that antibiotic treatment during early life can lead to a higher risk for developing immune system disorders such as allergy and asthma.

To investigate the effects on gut microbial colonization in early life, especially on the 'good' colonizers for the immune system development in broilers, we used a perturbation of the natural colonization by administering amoxicillin via the drinking water to 1-day-old chickens for a period of 24 hours. At three time-points after hatch (day 1 (prior to antibiotic administration), 5, and 14) birds were sacrificed to collect various intestinal samples in order to analyse the microbial composition and corresponding gene activity in the tissue.

We provide evidence that early temporary antibiotic treatment affects both the microbiota composition in the intestinal tract as well as the intestinal gene expression in broilers over a period of at least two weeks. Functional analysis of the changes in gene expression showed a significant effect on the expression of immune related processes. Primarily on day 5, most of these immune processes show lower activity of genes in antibiotic treated birds compared to the controls. The results of this study contribute to the identification of parameters for immune competence.

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Foreword

Feed4Foodure is a public-private partnership between the Dutch Ministry of Economic Affairs, a consortium of various organizations within the animal production chain and Wageningen UR Livestock Research. Feed4Foodure aims to contribute to sustainable and healthy livestock farming in the Netherlands, simultaneously strengthening its competitive position on the global market. The Feed4Foodure program line "Nutrition, Intestinal Health, and Immunity", aims to contribute to a reduction in the use of antibiotics in livestock farming by increasing general health and disease resistance. The main goals are to develop innovative measurement techniques for intestinal health and to test new health-promoting nutritional additives in the field of gut health and immunity.

The current report describes an experiment that was conducted to investigate the effect of a short-term antibiotic intervention in early life on microbial colonization and gut development in broilers. Such an intervention is expected to have negative effects on immune competence. This experiment was performed within the frame work of the Feed4Foodure program line "Nutrition, Intestinal Health, and Immunity" in combination with partners from Breed4Food.

For the current study, scientist of Wageningen UR Livestock Research, Wageningen UR CVI and the Dutch GD Animal Health worked together with representatives from the various private partners, including Agrifirm, ForFarmers, Nutreco, De Heus, Denkavit, and Darling Ingredients International. The authors thank the industry partners of the project team for their worthwhile input.

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

Summary

Gut microbial colonization and immune competence development are influenced by early-life environmental factors and interventions, such as oral treatment with antibiotics. The crosstalk between microbiota in the intestinal tract and host cells in the gut mucosal layer is important for immune development and programming. In humans, it has been shown that antibiotic treatment in early life can lead to a higher risk for developing immune system disorders such as allergy and asthma.

To investigate the effects on gut microbial colonization in early life we used a perturbation of the natural colonization by administering amoxicillin via the drinking water (67 mg amoxicillin/L) to 1-day-old chickens for a period of 24 hours. Such an intervention is expected to have negative effects on immune competence. A control group was included and not exposed to the antibiotic. Birds were housed in a floor pen system. At three time-points after hatch (day 1 (prior to antibiotic administration), 5, and 14) birds were sacrificed and jejunal digesta were collected to analyse the composition of resident microbiota and corresponding jejunal tissue samples were taken to perform genome-wide gene expression profiling to get insight into immune related processes in the mucosal layer. We provide evidence that early temporary antibiotic treatment affects both the microbiota composition in the intestinal tract as well as the intestinal gene expression in broilers over a period of at least two weeks. Functional analysis of the changes in gene expression showed a significant effect on the expression of immune related processes. Primarily on day 5, most of these immune processes show lower activity of genes in antibiotic treated birds compared to the controls.

Microbial colonization and intestinal immune development are affected by the antibiotic administration in early life. Microbiota composition and diversity changed between control and antibiotic treated birds. Expression of numerous genes changed and were related to changes in the activity of various biological processes in the small intestinal tissue, i.e. immune and cell cycle related processes. Validation of the functional changes was performed by staining immune cells in the small intestinal mucosa, i.e. changes in the number of monocytes/macrophages. In antibiotic treated birds the number of monocytes/macrophages in small intestinal tissue was lower compared to the controls. The generated data provides a valuable tool for identification of immune competence parameters.

1 Background

1.1 Overall aim of VDI3

Aim of VDI3 'Neonatal models' is the development and application of neonatal models with predictive value to study the impact and underlying mechanisms of nutritional- and microbial interventions and to identify indicators regarding the gut development in terms of immune development and functionality in order to have a maximum immune competence in later life. In the neonatal period a lasting influence of a (dietary) intervention can be exerted on the development and competence of the immune system. With such neonatal models, testing the functional effect of feed ingredients, i.e. raw materials, feed additives, and nutrient composition, in early life on animal health, immune competence and productivity can be examined.

1.2 Introduction

To influence the gut development at early age, we have chosen for an antibiotic treatment because it is known that antibiotics have a tremendous effect on the microbial population and a potential 'negative" effect on immune competence. The antibiotic used in this study was amoxicillin, which is used in broiler production systems in case respiratory or gastro-intestinal infections are detected. Amoxicillin is a moderate-spectrum β-lactam antibiotic and has proven effects on susceptible Gram-positive and Gram-negative bacteria.
Early life gut development encompasses morphological, functional, and immunological development. Here we are mainly interested in the immunological development, which after hatch develops rapidly in broiler chickens. in the first weeks of life, different categories of immunological processes can be identified [1-3]. Based on spatio-temporal gene expression profiles, the following sequential order in time was observed for immune related processes: 1) innate development and influx of immune cells to the gut from the lymphoid tissues; 2) immune differentiation and specialization; and 3) maturation and immune regulation [3].

For pigs, it has already been shown that perturbation of the intestinal microbial colonization during early life leads to altered microbiota composition and diversity for a certain period of time [4-6]. This perturbation also effected the expression of numerous immune related genes in the gut mucosal tissue for a longer period of time. This suggest an important role for the early microbial colonizers of the gut for the development and/or programming of the mucosal immune system. In addition, also studies in mice and humans show that modulating the microbial colonization in early life by antibiotics can lead to higher risk of developing immunity based disorders such as asthma and allergy [7-9]. Therefore, it is worthwhile to also investigate the importance of early life microbial colonization on immune status in broiler chickens, which is relevant for industry regarding the production of vital broiler chicks. In addition, detailed analysis of a variety of gut related parameters may help in the identification of parameters associated with immune competence.

In the present study, the antibiotic amoxicillin was administrated via the drinking water (67 mg amoxicillin/L) to 1-day-old chickens for a period of 24 hours, to perturb the 'natural' colonization of microbiota. In contrast the control group received no antibiotics. To find out whether the microbiota and host gene expression were altered by the treatment, we collected jejunal tissue and digesta at three time-points (day 1 (prior to antibiotic administration), 5, and 14). In depth sequencing of the resident microbiota was performed on the jejunal digesta samples and for the jejunal tissue genome wide gene expression studies were performed.

1.3 Objective

The objective of this study was to investigate the impact of a short-term perturbation during early life to the resident microbiota in jejunal digesta, i.e. microbiota composition and diversity, as well as the impact on the jejunal tissue gene expression and jejunal immune cells. Such measurements will contribute in identifying parameters associated with immune competence of broilers.

2 Material and Methods

2.1 Experimental design

1-day-old chickens (Cobb500) were housed in a floor pen system (16 pens in total) in which the chickens had *ad libitum* access to feed and water. We assumed that chickens will consume approximately 0.012 L water the first day. The antibiotic treatment consisted of 0.067 gram Octacillin® per litre drinking water, which corresponds to approximately 0.8 mg Octacillin® per bird per day. Octacillin® contains 687mg amoxicillin per gram Octacillin® corresponding to 800mg amoxicillin trihydrate. Birds were sacrificed for tissue sampling at day 1, before the antibiotic treatment. At this time-point 80 chickens were sacrificed (5 per pen). At days 5 and 14, 160 chickens were sacrificed, 80 control and 80 antibiotic treated chickens respectively. Subsequently these 80 samples of digesta and jejunal tissue were pooled for further analysis, samples of 10 chickens per pool and in total 8 pools (see Figure 1).

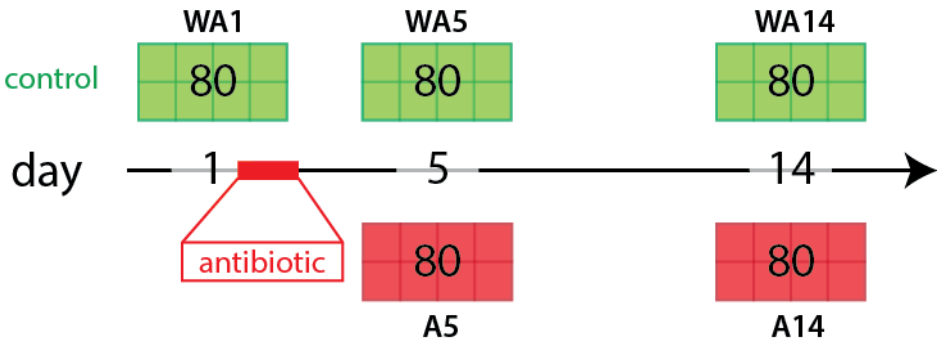


Figure 1. *Schematic representation of experimental design. Eighty birds were sacrificed at day 1, 5, 14 for control birds (without antibiotic), WA1, WA5, and WA14 respectively, and at day 5 and 14 for antibiotic treated birds, A5 and A14 respectively. The antibiotic (Octacillin® [amoxicillin]) was administrated for one day, starting at day 1 and lasting for 24 hours, via the drinking water. At all sampling days, jejunal digesta was taken for sequencing of the luminal microbiota. Jejunal tissue was taken for host gene expression and immunohistochemical staining of different immune cells.*

2.1.1 Housing and diet

The 1-day-old chicken were housed in a floor pen system and had ad libitum access to feed and water. The diet composition is depicted in Table 1.

Table 1

Ingredient and calculated nutrient composition of broiler starter diet.

Ingredient	% inclusion	Calculated nutrients	g/kg
Maize	35.03	Crude protein	201[1]
Soybean meal	30.00	Ether extract	61[1]
Wheat	27.60	Crude fibre	30[1]
Premix	2.00	Ash	52[1]
Soybean oil	1.92	Starch (Brunt)	376[1]
Palm oil	1.00	Ca	7.7[1]
Chalk	0.70	P	5.2[1]
Monocalcium phosphate	0.66	Cl	1.7
Sodium bicarbonate	0.23	Na	1.6
DL-methionine	0.26	Cu	0.01
Lysine HCl	0.22	K	8.6
L-threonine	0.05	Vitamin A (IU[2]/kg)	12500
Salt	0.19	Vitamin D3 (IU/kg)	3500
Enzyme (NSP degrading[3])	0.02	Vitamin E	0.1
Enzyme (4-Phytase[4])	0.01	6-phytase (PU[2]/kg)	500
		Digestible lysine	11.2
		Digestible methionine	5.4
		Digestible methionine + cysteine	8.3
		Digestible threonine	6.9
		Digestible tryptophan	2.2
		Digestible isoleucine	7.7
		Digestible valine	8.3
		Digestible arginine	12.5
		Digestible glycine + serine	16
		Absorbable phosphorus	4.2

[1] Analysed values

[2] International Units

[3] Hostazyme X®

[4] Phyzyme® XP 5000L

2.2 Microbiota data

2.2.1 DNA Extraction

Jejunal content was snap frozen in liquid nitrogen and stored at -80°C. For the microbial DNA extraction the following protocol was used. Jejunal content was mixed 1:1 with phosphate buffered saline (PBS) and vortexed, subsequently it is centrifuged for 5 minutes (300g) at 4°C. The supernatant was transferred to a new tube and spun for 10 minutes (9000g) at 4°C, thereafter supernatant was removed. DNA was extracted by using the QIAamp DNA Stool Mini Kit protocol as described by the manufacturer. The samples were eluted in 100 µl of the (provided) elute buffer and afterwards an optical density measurement to check the quality was performed on Nanodrop (Agilent Technologies).

2.2.2 Amplification of 16S rDNA (V3-PCR)

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

2.2.3 Sequence analysis and bioinformatics (QIIME)

Samples were sequenced by targeted-amplicon 16S sequencing using the MiSeq sequencer (Illumina) and analysed for taxonomy profile per sample with clustering by profile by using QIIME [10]. 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) [11, 12]. Furthermore chimeras were removed with Chimeraslayer [13].

2.2.4 Statistical analyses

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 ran on the family level microbiota data: $y = Time + Treatment + Time* Treatment + error$. RDA is a constrained linear ordination method, combining multiple regression with principal component analysis (PCA). Each canonical axis is the linear combination of all explanatory variables. RDA is often used in ecology and allows the user to specify the number of variables, i.e. by defining a model. With RDA it is possible to extract and summarize the variation of the dataset and therefor useful as explorative analysis and to identify trends in the data.

2.3 Transcriptomics data

2.3.1 RNA Extraction Tissue

Total RNA was extracted from 50 to 100 mg jejunum tissue. All samples were homogenised using the TisuPrep Homogenizer Omni TP TH220P) in TRizol reagent (Life Technologies) as recommended by the manufacturer with minor modifications. The homogenised tissue samples were dissolved in 5 ml of TRizol reagent. After centrifugation the supernatant was transferred to a fresh tube. Subsequently, Direct-zol™ RNA MiniPrep Kit by Zymo Research was used as described by the manufacturer. The RNA was quantified by absorbance measurements at 260 nm. Quality Control was performed by Agilent Bioanalyser.

2.3.2 Labelling, Hybridization, Scanning and Feature Extraction

Labelling of RNA was done as recommended by Agilent Technologies using the One-Color Microarray-Based Gene Expression Analysis Low input Quick Amp Labelling. The input was 10 ng of total RNA and 600 ng of labelled cRNA is used on the eight pack array. Hybridization was performed as described in the One-Color Microarray-Based Gene Expression Analysis Low input Quick Amp Labelling protocol from Agilent in the hybridization oven (G2545A hybridization Oven Agilent Technologies). The hybridization temperature is 65°C with rotation speed 10 rpm for 17 hours. After 17 hours the arrays are washed as described in the One-Color Microarray-Based Gene Expression Analysis Low input Quick Amp Labelling protocol from Agilent. The arrays where scanned using the DNA microarray scanner with Surescan high resolution Technology from Agilent Technologies. Agilent Scan Control with resolution of 5 µm, 16 bits and PMT of 100%. Feature extraction was performed using protocol 10.7.3.1 (v10.7) for 1 colour gene expression.

2.3.3 Data Analysis

The data were analysed by using R (v3.0.2) by executing different packages, including LIMMA [14] and arrayQualityMetrics [15]. The data were read in and background corrected (method="normexp" and offset=1) with functions from the R package LIMMA [14] from Bioconductor [16]. Quantile normalisation of the data was done between arrays. The duplicate probes mapping to the same gene were averaged (‘avereps’) and subsequently the lower percentile of probes were removed in a three-step procedure, 1) get the highest of the dark spots to get a base value, 2) multiply by 1.1, and 3) the gene/probe must be expressed in each of the samples in the experimental condition.

2.3.4 Statistical and Functional Genomics Analysis

To test the differences between the experimental groups (without antibiotics and antibiotics) on both day 5 and 14, the following contrasts, A5-WA5 and A14-WA14, were generated within the LIMMA package [14]**.** Database for Annotation, Visualization and Integrated Discovery (DAVID) was used to perform Functional Annotation Clustering (FAC) for the two different contrasts, i.e. A5-WA5 and A14-WA14. The up- and down-regulated genes were separately analysed. DAVID can be used to extract biological meaning from large gene lists. DAVID combines multiple databases, i.e. gene expression tissues, protein domains and interaction, pathways, sequence features, and Gene Ontology. FAC is a gene-term enrichment method that identifies the most over-represented biological terms associated to the gene list of interest. The results can help in gaining more (in-depth) understanding of the biological mechanisms.

2.4 Immunohistochemistry

Jejunal cryosections, 8 µm thick, were collected at day 1, 5, and 14 and stained with specific antibodies using an indirect immunoperoxidase staining method as described by Schokker *et al.* (2010) [17]. In brief, slides were treated for endogenous peroxidase activity, blocked with BSA, and incubated with monoclonal antibodies against $CD4^+$ cells, $CD8^+$ cells, or macrophage-like cells (CT-4, 1:200; CT-8, 1:200; and KUL01, 1:50, respectively; Southern Biotech, Birmingham, AL), followed by peroxidase-conjugated rabbit anti-mouse Ig (P0161, Dako, Denmark). Peroxidase activity was detected by 3,3-diaminobenzidine, and sections were counterstained with haematoxylin. Negative controls were performed by omitting of the primary antibody. One to three representative microscopic images were captured per sample and analysed using Olympus cellSens Dimension version 1.7.1 software. Positive-stained cells were counted, averaged per time point and group, and represented as positive-cells per tissue area (square mm).

The avian intestinal immune system consists of many immune cells, including macrophages and dendritic cells [18, 19] , plasma and (memory-) B cells [20], and T cells, e.g. helper T cells (T_H cells), cytotoxic T lymphocytes (CTLs), regulatory T cells, natural killer T cells and memory T cells [21].

In addition, intraepithelial lymphocytes (IELs) are abundantly dispersed in the mucosal layer. IELs monitor the gut lumen and eliminate distressed epithelial cells [22]. Other specific gut immune cells are lamina propria lymphocytes (LPLs), the phenotype of these LPLs is of activated memory T cells [23]. For the present study we have chosen three cell types which cover different parts of the immune system, i.e. these cells represent both innate and adaptive immunity in chickens.

3 Results

3.1 Performance

To investigate whether the antibiotic treatment affected the performance, body weight, feed intake and feed conversion ratio (FCR) were measured for multiple time-periods. Both body weight and FCR were statistically not significantly different between antibiotic-treated and control chickens. Body weight showed an increase in time, from approximately 130 g at day 5 to 2.3 kg at day 34 (Figure 2). FCR increased from approximately 0.8 at day 5 to about 2.3 at day 34 (Figure 3).

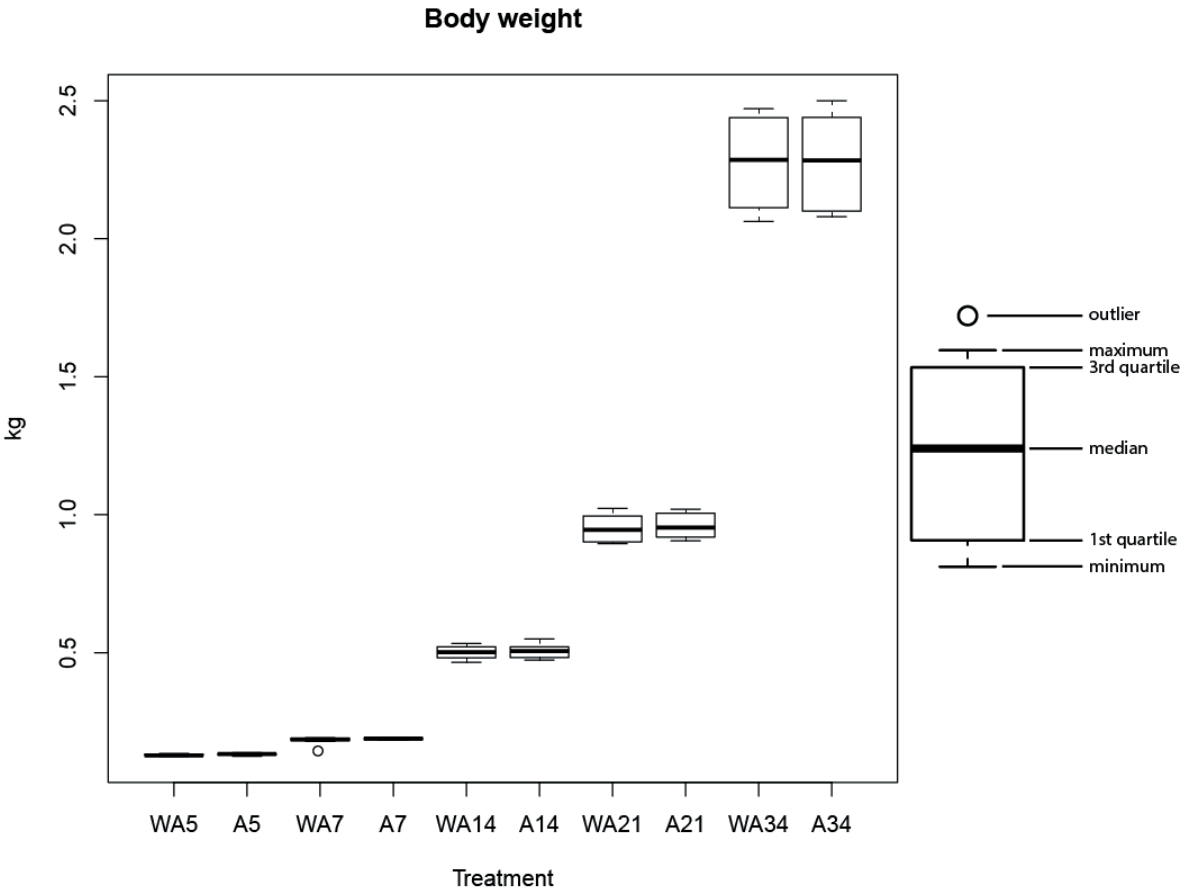


Figure 2. *Body weight of chickens per treatment in time.Chickens were weighed at multiple time-points. Furthermore, discrimination between the treatments was made. The x-axis depicts the treatment; without antibiotic (WA) or antibiotic treated (A), and time in days 5, 7, 14, 21, and 34. The y-axis depicts the body weight (alive) in kilograms. At each time point 8 pools of 10 chicken were measurement, i.e. the spread depicted here is the average between the pools.*

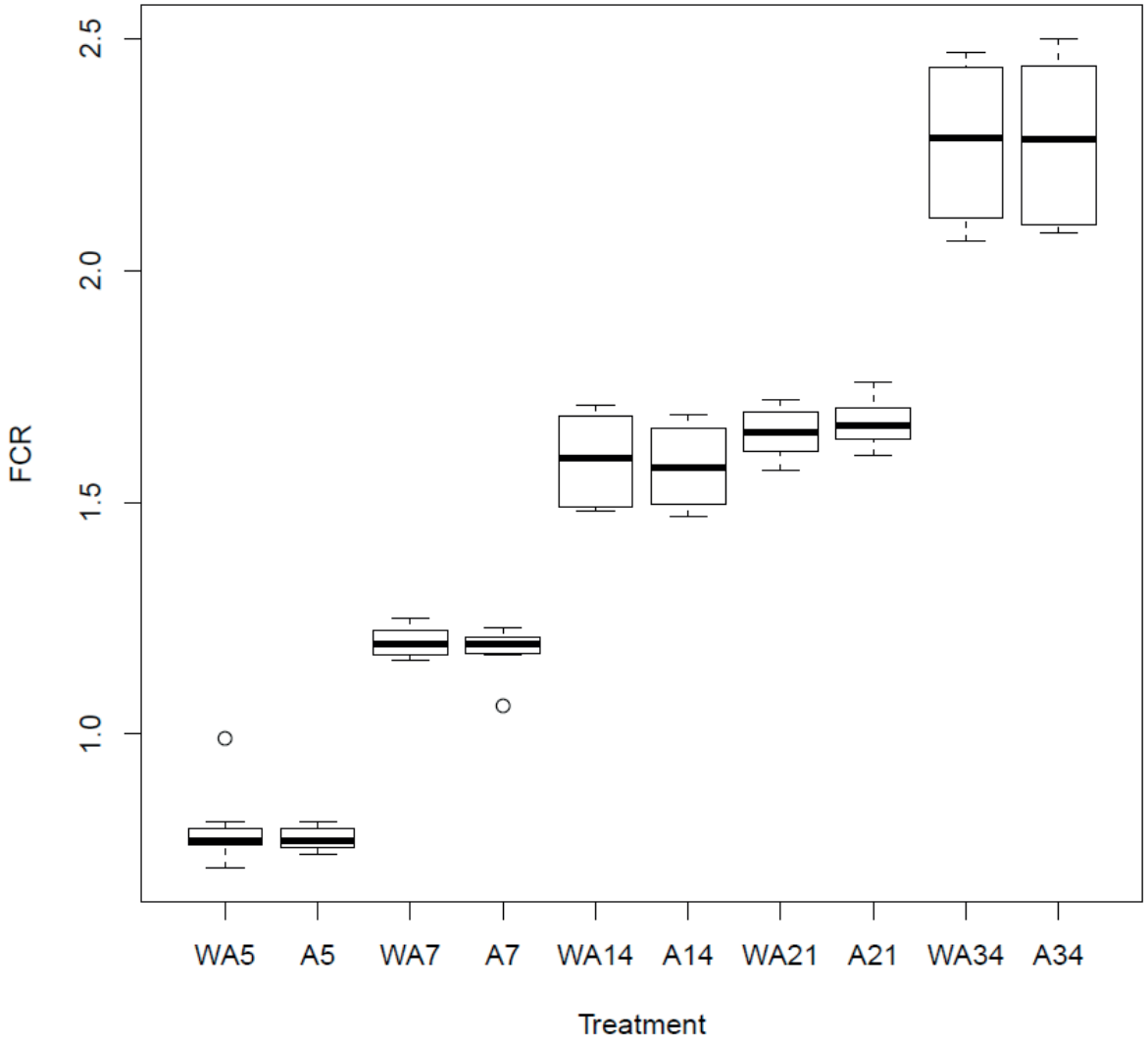


Figure 3. *Feed conversion ratio of chickens per treatment in time. Feed conversion ratio (FCR) was measured over different time periods and discrimination between the experimental treatments was made. The x-axis depicts the treatment; without antibiotic (WA) or antibiotic treated (A), and time in days 5, 7, 14, 21, and 34. The y-axis depicts the FCR. At each time point 8 pools of 10 chicken were measurement, i.e. the spread depicted here is the average between the pools.*

3.2 Microbiota analyses

To evaluate the impact of antibiotic treatment on the composition and diversity of intestinal microbiota, MiSeq data was used based on the 16S region of bacteria. To get generic insight into the microbiota and relative abundance of microbial groups in time, we selected the top 11 most abundant families over all three time-points (Table 2). This shows that the most dominant families, based on the relative contribution (RC), on day 1 were the Enterobacteriaceae (61.1%) and Enterococcaceae (25.9%). Because we were specifically interested in the temporal aspect, the following average relative contributions are averaged over control and antibiotic treated birds. However for day 5 the most dominant families were the Lactobacillaceae (76.1%) and Enterococcaceae (23.4%). At day 14, again a shift in dominant families is observed when compared to the previous recorded time-point, here Lactobacillaceae (76.3%) and Streptococcaceae (10.4%) are the most dominant families.

Table 2

Effect of antibiotic treatment on d 1 of age on intestinal microbiota composition on d 5 and 14

Phylum	Class	Family	WA1	WA5	A5	WA14	A14
Firmicutes	Bacilli	Enterococcaceae	**25.9**[1]	**21.7**	**25.2**	4.9	9.9
Firmicutes	Bacilli	Lactobacillaceae	0.5	**77.9**	**74.2**	**82.2**	**70.5**
Firmicutes	Bacilli	Leuconostocaceae	<0.01	0.04	0.08	0.1	0.2
Firmicutes	Bacilli	Streptococcaceae	0.4	0.2	0.3	**8.9**	**11.9**
Firmicutes	Clostridia	Clostridiaceae	6.0	<0.01	0.01	0.2	0.2
Firmicutes	Clostridia	Other	<0.01	<0.01	0.02	0.4	0.7
Firmicutes	Erysipelotrichi	Erysipelotrichaceae	<0.01	0.05	0.05	0.04	0.7
Tenericutes	Mollicutes		<0.01	<0.01	<0.01	0.02	1.1
Proteobacteria	Gammaproteobacteria	Enterobacteriaceae	**61.1**	0.05	0.04	3.0	3.2
Unclassified			3.5	<0.01	<0.01	<0.01	0.2
Bacteria_Other			1.9	<0.01	<0.01	<0.01	0.1
Total			*99.3*	*99.9*	*99.9*	*99.7*	*98.6*

In **bold** are the two most dominant families per time-point. Abbreviations: WA1; without antibiotic day 1, WA5; without antibiotic day 5, A5; with antibiotic day 5, WA14; without antibiotic day 14, A14; with antibiotic day 14.

[1] average relative contribution

To test the microbiota composition as a whole, multivariate redundancy analysis (RDA) of the approximate family-level was performed, which showed a clear separation of the time effect (days of age and time after antibiotic treatment) and high overlap is observed within days between the experimental treatments (Figure 4). To test whether specific microbial families (n=46) are significantly different between the treatment and control on a specific time-point, we employed the Wilcoxon signed-rank test. This resulted in eight significantly different family groups ($p<0.05$) for day 5, and three significantly different family groups ($p<0.05$) for day 14. When multiple testing correction is taken into account only three family groups remain for day 5, and for day 14 no family groups were left (Table 3). When zooming in on the microbiota diversity, calculated by the Shannon diversity index, based on the genus/species level data, no significant differences between the treatments were observed at day 5 or 14 (Figure 5).

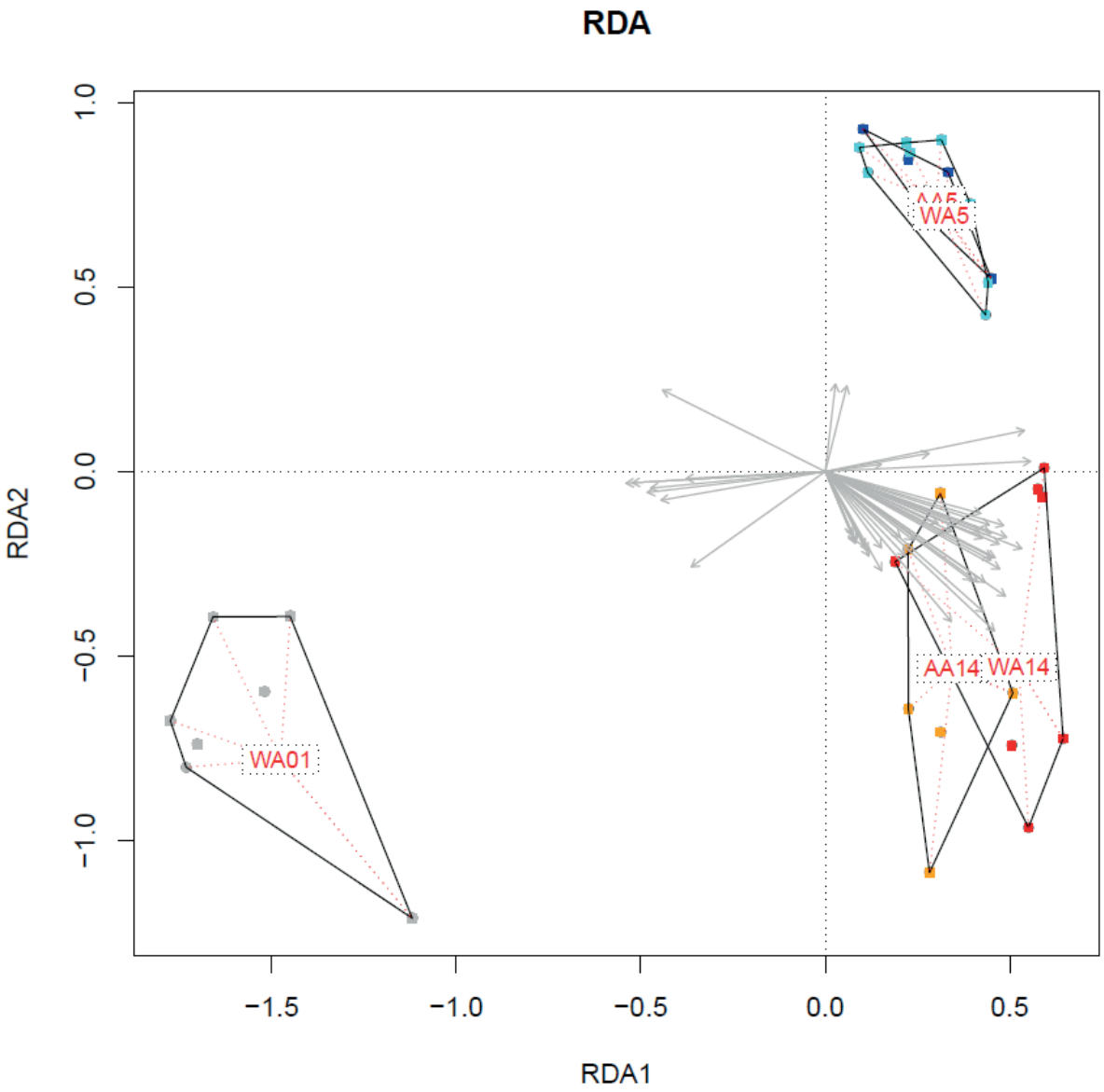


Figure 4. *Redundancy analysis (RDA) of family level microbial groups of jejunum digesta. The x-axis depicts explanatory axis 1 (RDA1) and y-axis depicts explanatory axis 2 (RDA2). Each condition is represented by a different colour (day 1, grey; day 5, where 'WA' is blue and 'A' is cyan; and day 14 where 'WA' is red and 'A' is orange). The grey arrows represent the environmental variables as constraining variables (i.e. the different microbial groups). The following model was used as input for the RDA: y = Time + Treatment + Time * Treatment + error.*

Abbreviations: WA1; without antibiotic day 1, WA5; without antibiotic day 5, A5; with antibiotic day 5, WA14; without antibiotic day 14, A14; with antibiotic day 14.

Table 3

Differences at family level microbial groups between control and by antibiotic treatment

Day	Phylum	Class	Family	p.value[1]	FDR[2]	WA5	A5	WA14	A14
5	Firmicutes	Bacilli	Bacillaceae	<0.01	0.04[3]	0.00	0.01	0.01	0.30
	Firmicutes	Bacilli	Carnobacteriaceae	<0.01	0.04	0.00	0.01	0.01	0.02
	Firmicutes	Bacilli	Leuconostocaceae	<0.01	0.04	0.04	0.08	0.12	0.16
	Actinobacteria	Actinobacteria	Nocardiopsaceae	0.02	0.11	<0.001	<0.001	<0.001	<0.001
	Firmicutes	Bacilli	Thermoactinomycetaceae	0.02	0.11	<0.001	<0.001	<0.001	<0.001
	Firmicutes	Clostridia	Ruminococcaceae	0.03	0.13	<0.001	<0.001	0.01	0.38
	Actinobacteria	Other	Other	0.04	0.15	<0.001	<0.001	<0.001	<0.001
	Unclassified	Other	Other	0.05	0.15	<0.001	<0.001	<0.001	0.15
14	Firmicutes	Bacilli	Enterococcaceae	0.01	0.24	21.7	25.2	4.91	9.89
	Firmicutes	Bacilli	Lactobacillaceae	0.03	0.24	77.9	74.2	82.2	70.5
	Firmicutes	Clostridia	Other	0.04	0.24	<0.001	0.02	0.37	0.73

[1] Wilcoxon signed-rank test; [2] FDR, False Discovery Rate; [3] Average relative contribution

Abbreviations: WA1; without antibiotic day 1, WA5; without antibiotic day 5, A5; with antibiotic day 5, WA14; without antibiotic day 14, A14; with antibiotic day 14.

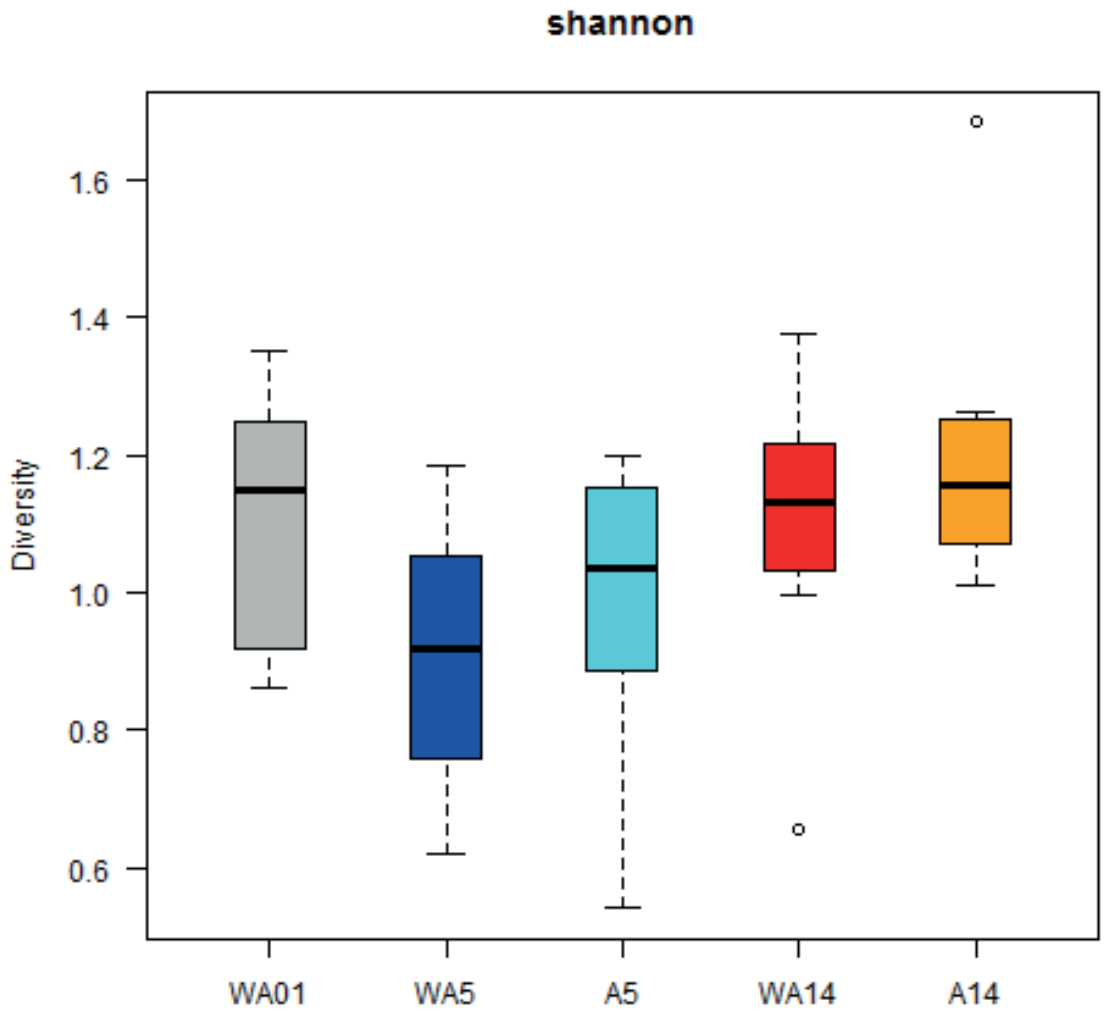


Figure 5. *Diversity of luminal microbiota in jejunum of broilers for different experimental conditions. The Shannon index (y-axis) was calculated for all five experimental conditions (WA1, WA5, A5, WA14, A14) (x-axis) based on the species level, which is a measure for the diversity of the sample. At each time point 8 pools of 10 chicken were measurement, i.e. the spread depicted here is the average between the pools.*
Abbreviations: WA1; without antibiotic day 1, WA5; without antibiotic day 5, A5; with antibiotic day 5, WA14; without antibiotic day 14, A14; with antibiotic day 14.

3.3 Transcriptomic analyses

Principal Component Analysis (PCA) was performed to get more insight into the variability in the jejunal transcriptomics data, taking into account the different treatment and time-points. Only the first and second principal component were taken into account for both analyses. Figure 6 shows that clustering occurs on days. Furthermore, within days the effect of treatment is not observed, because no clear distinction was found between samples of birds who had either or not received the oral antibiotic treatment on day 1. This analysis also shows that there was no clear sex difference (female vs. male), because within days and/or treatment they are scattered. To investigate the effect of the treatment in jejunum, an ANOVA analyses was performed. All probes/genes which were significant under $p_{adj} < 0.01$ were identified, as well as a cut-off for the statistical testing $p_{adj} < 0.05$, but including an absolute Fold Change > 1 (Table 4). The annotated genes from the $p_{adj} < 0.01$ list were taken for further functional and enrichment analyses. 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). The top 10 results from the DAVID functional annotation clustering are summarized in for the comparison between treatments on day 5 (Table 5) and on day 14 (Table 6). Especially at day 5 the dominant general terms in the down-regulated genes, i.e. lower expression in the antibiotic treated birds, are related to immune processes. For example, 'Immune response-regulating signal transduction', 'Positive regulation of immune system process', and 'Adaptive immune response'. Up-regulated genes, i.e. higher expression in the antibiotic treated birds, mainly processes related to cell development and function are observed. Including 'Extracellular matrix', 'Cell projection morphogenesis', 'Regulation of cell development', and 'EGF-like domain'. Contrasting results were seen at day 14, in which down-regulated genes are involved in the cell processes. The up-regulated genes do not show a coherent picture of similar dominant processes. Also at day 14 the enrichment scores are in general lower compared to day 5.

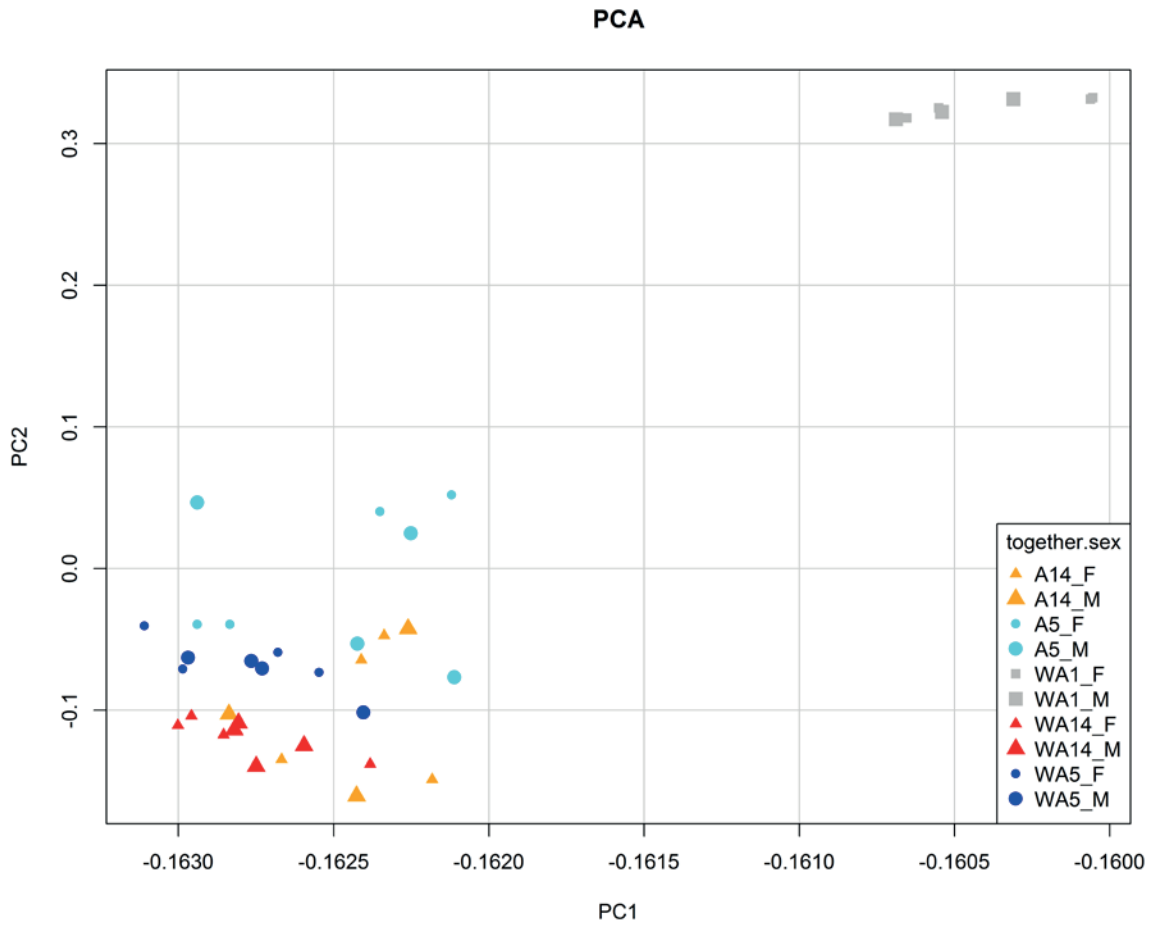


Figure 6 *Principal components analysis (PCA) of jejunal tissue transcriptomics data. The x-axis depicts principal component 1 (PC1) and y-axis depicts principal component 1 (PC2). Each day is represented by a different symbol (day 1, square; day 5, circle; and day 14, triangle). Each treatment within a particular day by a colour (day 1, grey; day 5, where 'WA' is blue and 'A' is cyan; and day 14 where 'WA' is red and 'A' is orange). The size of the symbols depicts sex, where small and large symbols represent female and male, respectively. Abbreviations: WA1; without antibiotic day 1, WA5; without antibiotic day 5, A5; with antibiotic day 5, WA14; without antibiotic day 14, A14; with antibiotic day 14.*

Table 4

Descriptive statistics of jejunal gene expression data comparing antibiotic versus control on days 5 and 14

Comparison	A5-WA5		A14-WA14	
Regulation	**Down**	**Up**	**Down**	**Up**
Number of probes				
p_{adj}[a] < 0.01	717	1156	457	354
p_{adj} < 0.05 and logFC[b] > |1|	26	65	17	21
Number of annotated genes				
p_{adj} < 0.01	489	556	182	234
p_{adj} < 0.05 and logFC > |1|	18	18	8	3

a, Adjusted p-value (False Discovery Rate); b, log Fold Change

Abbreviations: A5; with antibiotic day 5, WA5; without antibiotic day 5, A14; with antibiotic day 14, WA14; without antibiotic day 14.

Table 5

Functional annotation clustering (DAVID) of jejunum (ES>1.3) of the comparison antibiotic versus control on day 5 (p_{adj} < 0.01)

Down (lower in antibiotic treatment)		Up (higher in antibiotic treatment)	
ES	General Term	ES	General Term
4.83	intracellular organelle lumen	7.86	extracellular matrix
4.77	protein transport/localization	5.25	triple helix (hydroxyproline,hydroxylysine)
3.26	domain: BTB/POZ-like	5.16	Collagen triple helix repeat (hydroxyproline,hydroxylysine)
3.09	macromolecule/protein catabolic process	4.47	cell projection morphogenesis (neuron, differentiation)
2.65	immune response-regulating signal transduction	3.66	Fibrillar collagen
2.39	nuclear envelope-ER network	3.56	regulation of cell development (neuronal)
2.33	Pos. regulation of immune system process	3.08	positive regulation of transcription/macromolecule
2.27	cellular protein localization	3.07	EGF-like domain
2.19	adaptive immune response	2.57	response to steroid hormone stimulus (cortico/glucocortico)
2.08	Protease/peptidase activity	2.57	thrombospondin-type (Laminin G)

ES, enrichment score

Table 6

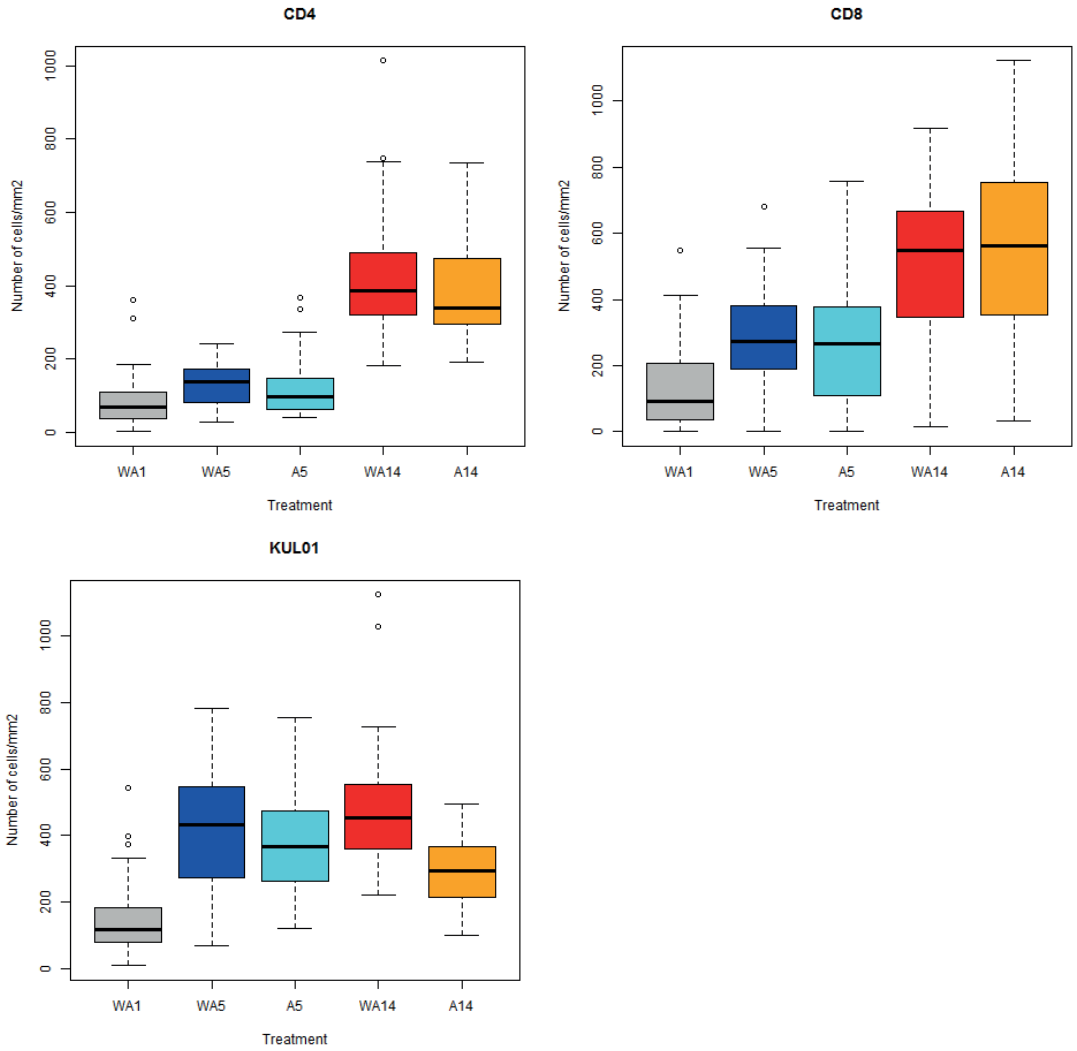
Functional annotation clustering (DAVID) of jejunum (ES>1.3) of the comparison antibiotic versus control on day 14 (p_{adj} < 0.01)

Down (lower in antibiotic treatment)		Up (higher in antibiotic treatment)	
ES	General Term	ES	General Term
2.49	positive regulation of biosynthetic process/transcription	4.5	organelle lumen (intracellular)
2.00	epithelium morphogenesis/development	2.38	transit peptide/Mitochondrion
1.60	macromolecule/protein catabolic process	1.84	sterol/steroid biosynthesis
1.48	intracellular organelle lumen	1.68	Heat shock protein (DnaJ)
1.47	blood vessel development	1.53	RNA recognition motif (RNP-1)
		1.51	translation initiation factor activity
		1.48	(negative) regulation of lipid storage
		1.43	Multiple Signalling Pathways (EPO/IGF1/IL6/TPO/IL2/PDGF/EGF)
		1.39	cellular protein localization/targeting
		1.32	zinc-binding (LIM domain)

ES, enrichment score

3.4 Immunohistochemistry

To investigate whether the gene expression differences observed in the jejunal tissue at day 5, were also translated into differences at the cellular level (i.e. immune cells), we have selected cellular immunity macrophage(-like) cells, $CD4^+$ and $CD8^+$ cells. Macrophage(-like) cells have a strong link to innate immunity, whereas $CD4^+$ and $CD8^+$ T cells have a strong link to adaptive immunity. All measurements were performed in gut tissue, jejunal mucosa were stained at 1, 5, and 14 days of life and quantified (Figure 7). The development of the adaptive immune system was shown by the increasing number of cells per consecutive time-points. However, no significant difference was observed on day 5 or 14 when testing for the effect of treatment on either $CD4^+$ or $CD8^+$ cells. However, for macrophages and monocytes ($KUL01^+$ cells) a trend was found at day 5 which showed lower number of macrophage and monocytes in the antibiotic treated birds compared to the control birds. At day 14 a significant difference was seen between the antibiotic treated birds compared to the control birds, where the antibiotic treated birds have a lower number of macrophage and monocytes.



Figures 7 *Immunohistochemistry (IHC) staining of immune cells for the different treatments in time. In all graphs, the horizontal axis depicts the experimental condition (treatment and time) and the vertical axis the number of cells per square millimetre of jejunal tissue. Left upper panel depicts CD4+ cells, right upper panel shows the CD8+ cells, and the lower left panel represents the KUL01+ cells (macrophage and monocytes). At each time point 8 pools of 10 chicken were measurement, i.e. the spread depicted here is the average between the pools.*

Abbreviations: WA1; without antibiotic day 1, WA5; without antibiotic day 5, A5; with antibiotic day 5, WA14; without antibiotic day 14, A14; with antibiotic day 14.

4 Discussion and Conclusions

In this report we provide evidence that a 1-day oral antibiotic treatment in early life (day 1) of broilers affects microbial composition as well as the intestinal gene expression on days 5 and 14. We show that multiple genes, involved in immune related processes, are lower expressed in the antibiotic treated broilers. These data suggest that due to the antibiotic treatment, the priorities of biological processes in the gut have shifted for these birds. The data suggest that antibiotic treated birds invest more in cellular processes, such as cell cycle, which could indicate that instead of investing in (adaptive) immunity, the barrier function is strengthened.
The immune system consists of both an innate part, the first line of defence against invading pathogens, and an adaptive part, which creates immunological memory after pathogen attacks. The gene expression differences in these immune processes apparently resulted in differences at the cellular level, i.e. the number of specific immune cells. We observed lower numbers of macrophages and monocytes in the mucosal layers of antibiotic treated broilers compared to the controls, which was most pronounced at day 14 of age.
It is evident that, similar to what has been found for other (mammalian) species, early colonization of the gut by microbiota is an important driver of immune development and/or programming. In humans it has also been shown that early life factors impact gut homeostasis, for example antibiotics can increase the risk of certain diseases like inflammatory bowel's disease [26]. However, besides gut related diseases, it has also been shown in humans that maternal and neonatal nutrition plays an important role in multiple developmental processes [27-29].
For industry, the observation in this study provides evidence that several immune competence parameters can be influenced during early life. This knowledge may be a driver for investigating which environmental conditions (i.e. climate conditions and pen design) and dietary interventions, could be beneficial for colonization in early life and consequently for immune development. Since the antibiotic treatment as has been used here, is regarded to have negative consequences for immune competence, the generated data provide a rich source of information for identifying parameters for broiler immune competence.

4.1 Microbial colonization and the effect of a short antibiotic treatment on health

Early life microbial colonization of the gut is important for the development and programming of the immune system. Less is known about the most important colonizers in the chicken gut. In the current study, we observed that at day 1 the families *Enterobacteriaceae* and *Enterococcaceae* were most abundant. Due to development of the gut and interaction with the environment the overall ecosystem shifted towards *Lactobacillaceae* being most dominantly present followed by *Enterococcaceae*. At day 14 *Lactobacillaceae* were still the most dominant and *Streptococcaceae* were second most abundant. This shows the generic succession of bacterial families in the chicken gut in time, regardless of the antibiotic treatment perturbation on day 1.

The impact of the antibiotic treatment is seen on bacterial family level, where minor changes in average relative contribution occurred at day 5 and major changes on day 14 (Table 2). *Lactobacillaceae* were more abundant at day 14 in controls compared to the antibiotic treated chickens, 82.2% and 70.5% respectively. This suggests that the early antibiotic treatment affects the microbial colonization and composition/diversity over a much longer period of time. This could be due to the antibiotic treatment as such or indicates that the ecosystem has shifted towards a different steady state (homeostasis). *Lactobacilli* are used as probiotics in chicken to improve intestinal health [30-32] and were reported to be involved in competitive exclusion of pathogens [33-35]. This suggests that, in general, high abundance of *Lactobacilli* is favourable for immune competence and intestinal health. This also implies that an perturbation with amoxicillin could have a negative effect on (gut) health. Under conditions of the present experiment the perturbation did not translate into differences in body weight gain and FCR. At day 14, a higher abundance of *Enterococcaceae* was observed in the antibiotic treated (9.9% ARC) compared to the control (4.9% ARC) chickens. Although *Enterococci* produce lactic acid [36, 37] and are commensals, these bacteria have intrinsic antibiotic

resistance mechanisms [38]. However, we lack data to confirm whether the *Enterococci* observed here have such intrinsic antibiotic resistance mechanisms.

To test whether significant differences occurred between the antibiotic treated and control birds, a Wilcoxon signed-rank test was performed. This was tested on all microbial groups at the family level, i.e. even low abundant families under 0.01%. This was intentionally done because pathobionts could be different between the treatment groups but are most often low abundant. At day 5 most of the differences between bacterial families were low abundant, whereas the differences at day 14 were in high abundant families. When zooming in on these differences no pathobionts could be detected, in contrary many of these families are lactic acid producing bacteria, such as the Lactobacillaceae, Enterococcaceae, Leuconostocaceae, and Carnobacteriaceae. Lactid acid producing bacteria are often classified as beneficial for the host.

Microbial diversity is a derivative read-out parameter for the resilience of the gut ecosystem; the more divers the microbial population, the more resilient the ecosystem is against (pathogenic) invaders. The diversity, as measured by the Shannon index, decreased from day 1 to day 5. This was observed in both control and antibiotic treated chickens, and could be due to the change in environment of the birds on day 1. At day 1, chickens were transported by truck from the hatchery to the experimental farm. Beforehand, we did not expect a large effect of transport by truck of chickens on the chicken gut microbiota, however, chickens could be still sensitive for environmental changes and other early settlers from different environments could be able to colonize one-day-old chickens during transport. Another factor, which may influence the outcome of the experiment, was the time of hatching. It is possible that a variation of up to 24 hours exists between hatching of the first and last chicken. To minimize the variation between these different hatching times, chickens were designated to different hatching groups related to the interval between hatching and transport. In total 8 pools of 10 chickens for tissue and digesta sampling after euthanasia were generated based upon this information. On day 1, two pools had 4 chickens that hatched 'early', 3 chickens 'middle', and 3 chickens 'late'. Four pools had 3 chickens that hatched 'early', 4 chickens 'middle', and 3 chickens 'late' and two pools had 3 chickens that hatched 'early', 3 chickens 'middle', and 4 chickens 'late'. On the later time-points (day 5 and 14) all pools consisted of 2 chickens that hatched 'early', 6 chickens 'middle', and 2 chickens 'late'.

The microbial diversity increased when comparing day 5 and 14. This was expected because the whole gut system develops towards a more stable system, already partly reflecting the 'adult' type microbiota.
When comparing the antibiotic treated chickens with their respective controls, we observed an numerically higher diversity at day 5 that was not notable at day 14. This increase in diversity was expected, as studies in pigs already showed that the microbial diversity slightly increases, becoming more chaotic, due to an early life antibiotic treatment, which corresponded to an altered microbial composition [5, 6, 39]. When comparing the microbiota composition of the antibiotic treated and control chickens, high overlap of microbial species was observed in both day 5 and 14. This shows that the gut ecosystem has a high resilience, because in two days after the termination of the 1-day antibiotic treatment the gut ecosystem has changed into a steady state.

Taken together, these data suggest that these (small) differences in microbiota in early life exert influence on the immune programming (see next paragraph). We still need more knowledge about this life phase to fully understand the gut (eco)system and its implication towards the immune system and health. Nevertheless, this is a first step to identifying key components, e.g. microbial families or species, which are important during colonization in early life or that are affected by antibiotic treatment.

4.2 Functional genomics approach identifies (dis)similarities between antibiotic treated and control chickens

The subtle differences in the early colonization of microbiota could have a direct impact on the expression of genes in small intestinal tissue. In this study, antibiotic treated chickens showed lower expression of genes involved in (generic) metabolic and immune related processes. Higher expression of genes, associated to cell structure/cell cycle and developmental processes, was observed in the antibiotic treated chickens, which may be related to the barrier function of the intestine. Similar observations have been shown in antibiotic treated pigs, where microbial changes lead to differentially expressed genes, that were partly involved in immune related processes [5, 6, 39]. These gene expression data suggest that a disturbed microbial colonization in the chicken gut leads to higher expression of genes linked to barrier function and lower expression of genes involved in immune related processes. The barrier function is the first line of defence against intruders, and epithelial cells are key players to maintain intestinal homeostasis [40]. Dysfunction of the intestinal barrier is linked to multiple gastrointestinal diseases [41-44]. A lower gene expression in the intestinal mucosa of the antibiotic group was observed for cell-mediated immunity. This is a remarkable observation, because the cell-mediated immunity develops after hatch and maturation in broilers occurs primarily in the first weeks [2, 3]. These data suggest that during the perturbation in early life intestinal cell-mediated immune development has lower priority compared to the intestinal barrier function. This strategy of coping with a perturbation in early life could be most cost effective for the birds, because strengthening the intestinal barrier results in less risk of a microorganism to invade the gut tissue and may be at the expense of the immune competence.

4.3 Differences in immune cell populations due to the antibiotic treatment

Development of the adaptive immune system occurs in the first weeks of life in broilers [1-3]. Both $CD4^+$ and $CD8^+$ cells increase in number in the small intestinal mucosa. This local expansion of immune cells is initiated by the microbiota and the diet. Immuno-histochemical staining was performed on jejunal tissue to investigate the impact of the antibiotic treatment of three major immune cell types; macrophages, $CD4^+$, and $CD8^+$ T cells. The (in)direct effect of amoxicillin on immune cells in healthy individuals is not well described. Different immune genes, however, are suggested to be influenced by the use of amoxicillin, including HLA-A, HLA-DQB1, HLA-DRB1, GSTM1, GSTT1, CYP11B2, CYP17A1, CYP19A1, CXCL8, and CRP [45-49]. These genes are involved in a range of processes including antigen processing and presentation, natural killer cell mediated cytotoxicity, and hematopoietic cell lineage. Macrolides, including amoxicillin, mainly influence the neutrophil (chicken equivalent are heterophils) function and immune response [50], and not the number of immune cells. In the current study, we observed that amoxicillin had a slight numerical impact on the $CD4^+$ cell population in the intestinal mucosa, which may be related to the lower microbiota diversity in digesta of the corresponding section of the gut. A less diverse microbial population may lead to fewer $CD4^+$ cells residing in the gut, because less immune surveillance is necessary for maintaining gut homeostasis. Contrary to $CD4^+$ cells, $CD8^+$ cells were not affected by the early antibiotic treatment. These data suggest that the adaptive immune system is not, or only partly, affected by the antibiotic treatment. The number of macrophages was significantly lower in antibiotic treated chickens on day 14 compared to control chickens, whereas only a decreasing trend in the number of macrophage-like was observed on day 5. The lower expression of genes involved in immune processes at day 5 in antibiotic treated chickens could have a direct effect on the number of macrophages. Another explanation could be that due to the augmented barrier function, macrophages are of less importance under such a condition. Although the reasoning behind the mechanism of fewer macrophages in the jejunum is not yet clear, the consequence could be that the antibiotic treated chickens have a lower immune capacity against invading pathogens.

4.4 Overall conclusion

Short term oral perturbation with amoxicillin in early life of chickens affects microbial colonization and the development of the immune system in the jejunum, that may result in a modified immune competence later in life. This was shown at the microbiota level (composition and diversity), but to a greater extent at the gene expression and related biological process level in the jejunal tissue, i.e. by a lower expression of genes involved in immune pathways. Validation by immunohistochemistry at the cellular level showed that amoxicillin treatment at early age decreases the number of macrophages and monocytes in jejunal tissue at days 5 and 14 compared to their controls.

4.5 Potential impact

The work described here shows that it is possible to modulate the intestinal immune development by affecting the intestinal microbiota at early age. Although, here we made use of an intervention that disturbed the colonization of the gut microbiota, i.e. many species were removed from the gut ecosystem, we did find an altered response from the broiler chickens with respect to development of the immune system (immune competence). The next step in follow-up research within VDI will be to identify functional ingredients and specific additives which modulate intestinal immune development in a beneficial way. Many studies on functional feed ingredients only consider their impact on the zootechnical performance, but do not provide details on their effects on the immune system. Future experiments should focus on dietary functional ingredients and specific additives that modulate the immune system in the gut, such as oligosaccharides, β-glucans, medium chain fatty acids, fish oil, and oat hulls. The functional consequences of dietary interventions influencing the immune system should be finally evaluated under conditions where the immune system is stimulated, e.g. by infectious agents that act in the digestive tract. To gain more knowledge and understand the mechanisms of modulating the development of the gut immune system, in the present study changes in community-scale gut microbiota and in expression of all genes of intestinal tissue were investigated. The explorative nature of this research has enabled us to define key components contributing to intestinal immune system development, such as genes involved in specific immune processes and the number of macrophages in intestinal tissue. Follow-up experiments are necessary which investigate dietary interventions in early life and their impact on intestinal immune system development and immune responses under infectious challenge conditions and the consequences for the zootechnical performance.

From this study we could identify potential indicators for improved immune competence. In the microbiota data, many lactic acid producing bacteria were different between antibiotic treated chickens and controls. For example, increased Lactobacillaceae in the controls may lead to higher levels of lactic acid, which is classified as beneficial for the host. Focusing on the transcriptomics data it is harder to identify a clear-cut potential indicator. A potential indicator could be genes and/or processes related to intestinal barrier function and cell-mediated immunity, these two aspects are important in early life for a proper functioning gut and the development of the intestinal immune system. To validate these gene expression differences, innate and adaptive immune cells were characterized in the mucosal tissue. We identified a decreased number of macrophages in antibiotic treated chickens, this may lead to a lower or delayed response to potential infectious agents in these chickens. Thus, the immune competence of these antibiotic treated chickens is lower and macrophages could be a potential indicator to address immune competence. In conclusion, several potential indicators for immune competence were identified at different biological levels.

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Wageningen UR Livestock Research develops knowledge for meticulous and profitable livestock farming. This is then translated into practical solutions and innovations, all the while, ensuring the dissemination of this knowledge. Together with our clients, we combine our scientific knowledge in the field of livestock farming systems and nutrition, genetics, health and environmental impact of livestock into workable livestock concepts for the 21st century.

The mission statement of Wageningen UR (University & Research centre) is 'To explore the potential of nature to improve the quality of life'. Within Wageningen UR, nine specialised research institutes of the DLO Foundation and Wageningen UR have joined forces in order to contribute to the solution of pressing questions in the field of healthy food and living environment. With around 30 branches, 6,000 employees and 9,000 students, Wageningen UR is one of the most prestigious knowledge institutes in its field worldwide. The integrated approach to problems and the collaboration between different disciplines, form the core of Wageningen's unique approach.

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

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