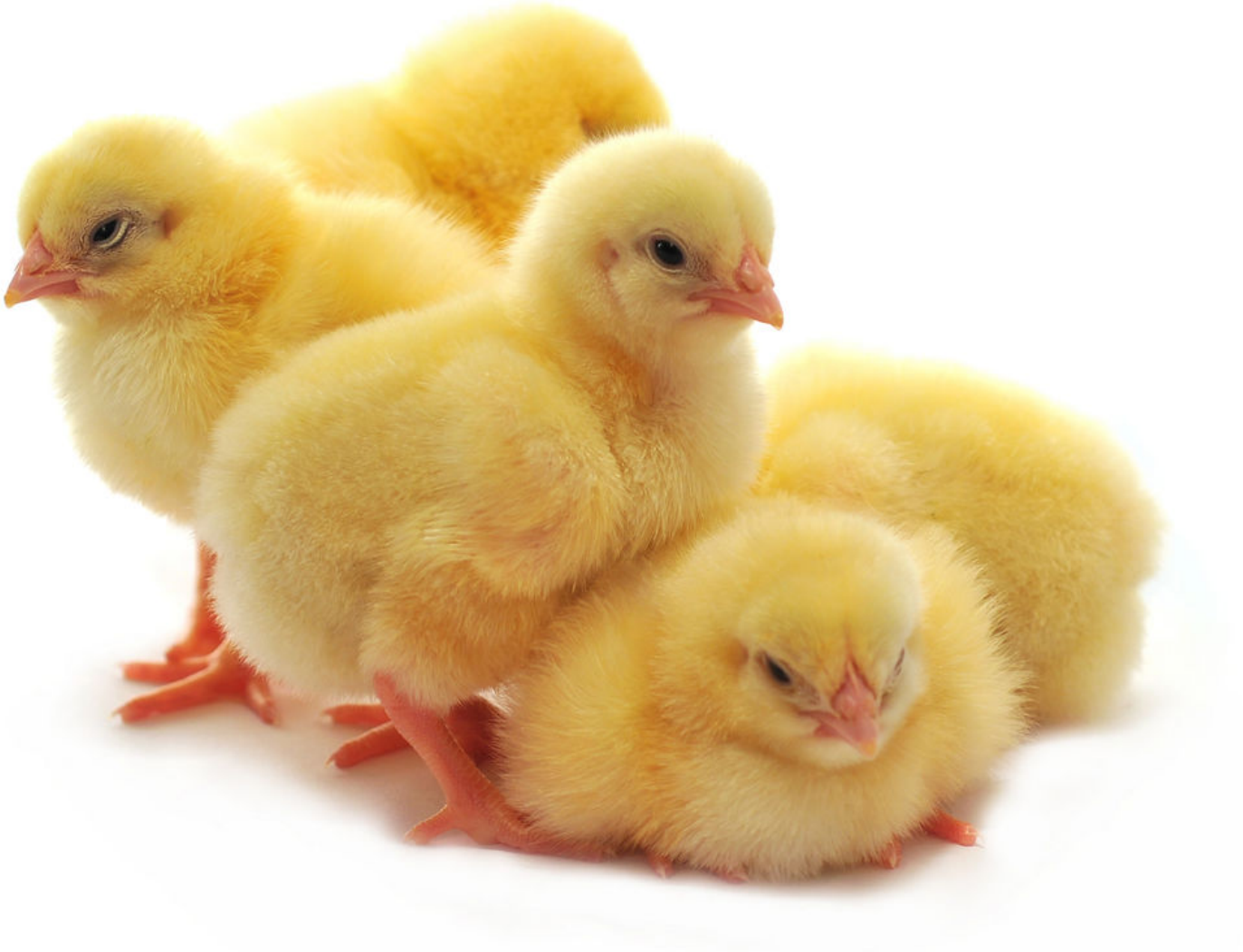

Nutritional interventions to modulate immune competence in broilers and correlation to quantitative disease phenotype after Necrotic enteritis challenge.

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Inhoud

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 / Wageningen Bioveterinary Research. Feed4Foodure aims to contribute to sustainable and healthy livestock farming in the Netherlands, simultaneously strengthening our competitive position on the global market. The Feed4Foodure program line "Nutrition, intestinal health, and immunity", aims to contribute to a reduction in the use of antibiotics in livestock farming by increasing general (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-11 as in kind contribution of Cargill.

For the current study, scientists of Wageningen UR Livestock Research and Wageningen Bioveterinary Research worked together with representatives from the various private partners of VDN (Vereniging Diervoederonderzoek Nederland). The authors thank the industry partners of the project team for their worthwhile input.

On behalf of Prof. Dr. Mari Smits, leader of the Feed4Foodure program line "Nutrition, intestinal health, and immunity".

Dr. Dirkjan Schokker

Short summary

This study investigated the effect of four different nutritional interventions, applied during the first week of life, on immune competence parameters of broiler chickens. The four (dietary) interventions were antibiotics in the drinking water, 25% inclusion of rye in the feed, beta-glucans from *Saccharomyces cerevisiae*, and coated butyrate. Broiler chickens (between 1 and 2 weeks of age) were subjected to a Necrotic Enteritis (NE) challenge. A positive and negative control treatment (challenged vs. non-challenged chickens, respectively) was included to estimate the effect of the NE challenge. It was hypothesized that applying a nutritional intervention in the first week of life would affect the microbiota colonization, immune system programming, and consequently the quantitative disease phenotype (lesions scores as a result of the NE challenge).
The NE challenge did affect the overall performance during the first 2 weeks post-challenge. The antibiotics group showed significant effects on different biological levels: (temporary) increased performance, deviating microbiota composition, increased gene expression in barrier function processes, whereas decreased gene expression in immune related processes, and a higher villi to crypt ratio compared to the (un)challenged control treatment. In the other treatments only significant differences in bacterial genera were observed.

In conclusion, this study has shown that it is probable to disturb the gut system development, whereby giving antibiotics (amoxicillin) gives an effect on multiple biological levels. In addition, the other dietary perturbations, i.e. change in feed composition (25% inclusion of rye), feed additives (beta-glucans or coated butyrate) only affected the microbiota composition to some extent.

1 Introduction

1.1 Importance of early life

According to EFSA/ECDC [1], microbial resistance to antibiotics is an alarming threat, putting human and animal health in danger. Agricultural use of antibiotics can be recognized as one of the major contributors to the development of resistant organisms [2]. Although in the last years the use of antibiotics in livestock production substantially reduced (56% over the period 2007-2012 in the Netherlands), to solve the current and future threats of multi-drug resistant organisms in (food)-animals to human health, a substantial further reduction should be warranted [3]. Appropriate nutrition may aid in minimizing the incidences of diseases in livestock, among others by enhancing or improving the immune response towards these diseases [4], consequently resulting in a further reduction of the use of antibiotics. Better understanding in the nuances of nutrition and immunity is important for optimizing bird health and productivity [4]. Several studies reviewed the impact of nutritional factors on immunity, e.g. Kidd [5].

Early life gut development encompasses morphological, functional, and immunological development. After hatch, the immune system develops rapidly in broiler chickens. In the first weeks of life, different categories of immunological processes can be identified [6-8]. 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. Studies in pigs and laying hens showed that perturbation of the intestinal microbial colonization during early life led to altered microbiota composition and diversity in later life [9-12]. This perturbation also affected the expression of numerous immune related genes in the gut mucosal tissue for a longer period of time. This suggests an important role of 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 [13-15]. These examples in different animal species show the importance to also investigate the impact of early life microbial colonization on the immune status in broiler chickens. Improving the immune competence of broiler chickens might contribute to their health status, and to a further reduction in the use of antibiotics.

The current study, however, focussed particularly on the impact of nutritional interventions in early life on immune competence, which is defined as the ability of the immune system to respond adequately to an antigenic stimulus, i.e. inducing an appropriate immune response with a balance between tolerance and inflammation [16]. In this study, immune competence related parameters were determined in broilers provided with nutritional interventions, e.g. amoxicillin, rye, beta-glucans, and coated butyrate, during the first week of life, followed by a Necrotic enteritis (NE) challenge in the second week of life. The reasons for selecting these interventions are explained below. NE is a multi-factorial disease process, in which a number of co-factors are usually required to precipitate an outbreak of the disease. Although, *C. perfringens* has been identified as the etiological agent of the disease, the predisposing factors that lead to over-proliferation of *C. perfringens* and the subsequent progression to disease are poorly understood [17]. NE has been the most significant threat for the poultry industry, which, in clinical form, causes high mortality and in subclinical forms, affects growth and feed conversion.

1.2 Feed interventions

1.2.1 Positive and negative control

A positive and negative control treatment (control diet, with and without NE challenge) allowed to distinguish the effect of the NE challenge as such (positive control) from the effect of an NE challenge combined with a nutritional intervention.

1.2.2 Antibiotics (amoxicillin – drinking water)

An antibiotic treatment at early age might impact the immune competence of broiler chickens. It was shown that young broiler chickens treated with enrofloxacine, florfenicol, or ceftiofur had reduced proportions of immune cells in the spleen, which might have an impact on the immune response to bacterial endotoxins in chicks [18]. Amoxicillin is used in broiler production systems to treat respiratory and gastro-intestinal infections. It is a moderate-spectrum β-lactam antibiotic and has proven effects on susceptible Gram-positive and Gram-negative bacteria [19]. In the current study, amoxicillin was used as antibiotic treatment, aiming to negatively influence the gut development at early age. The antibiotic was provided in the drinking water.

1.2.3 Rye (25%)

A high dietary inclusion level of rye was chosen due to the presence of soluble non-starch polysaccharides (NSP), particularly arabinoxylans, which are complex cell wall polysaccharides, being composed of two pentose sugars, arabinose, and xylose in a branched structure [20]. Arabinoxylans create a viscous environment within the intestinal lumen [20, 21]. Increased viscosity might impair digestibility and absorption of dietary nutrients, leading to a lowered growth rate and feed conversion ratio (FCR) [22, 23]. Dietary fibre may increase the secretion of mucus, which is produced by goblet cells [24]. Feeding rats various gelling agents increased the proliferation rate of the enterocytes of the jejunum and distal ileum, and decreased the activity of specific epithelial surface enzymes [25]. Smits and Annison (1996) hypothesized that an increased viscosity of the ileal digesta might change the morphology of the villi. Moreover, these authors emphasized that the microbiota may, at least partially, be indirectly responsible for the detrimental effects of viscous digesta. Based on observations in caecectomized broiler chickens, Choct et al. [26] concluded that the anti-nutritive effects of these pentosans in poultry are partially due to an increased activity of hindgut microbiota. Van der Klis and Van Voorst [27] reported that carboxymethyl cellulose increased the average retention time of digesta in the gastrointestinal tract, thereby giving the microbiota more time and more substrate to colonize the proximal small intestine. Feeding broiler chickens a wheat/rye-based diet, high in NSP, seriously decreased immunity-related parameters of the chickens. This has been indicated by induced villus fusion, reduced thickness of the tunica muscularis, induced T-lymphocyte infiltration, more and larger goblet cells, more apoptosis of epithelial cells in the mucosa, and a shift in microbiota [28]. Smits and Annison (1996) hypothesized that an increased viscosity of the ileal digesta, due to the presence of soluble NSP, might change the morphology of the villi. These NSP, when present at the surface of or within the mucus layer, may serve as substrate for microbial growth and may stimulate bacterial proliferation and attachment of bacteria to the mucins and glycocalix. Subsequently, some bacterial species, e.g. *Streptococcus faecium,* may cause atrophy of the villi [29, 30].
Based on the available literature, it was hypothesized that dietary inclusion of rye would increase viscosity of intestinal digesta, consequently resulting in an effect on nutrient absorption, gut wall morphology, composition of microbiota, and immune-related processes in the gut wall. Therefore, it was expected that rye in grower diets of broilers might be a helpful model ingredient to investigate the negative effects of nutrition on immune competence related parameters. In the current study, 25% rye was included in the diet (Note: that this was not a 1:1 exchange of wheat and rye). To maintain a high viscosity in jejunum and ileum, animal fat was used as the primary fat source instead of soya oil [31].

Saccharomyces cerevisiae, on activation of the transcription factors activator protein-1 and specificity protein-1 in normal human dermal fibroblasts. These authors showed that β-glucan stimulated fibroblast expression of neurotrophin 3, platelet derived growth factor A, platelet derived growth factor B, fibroblast growth factor acidic, fibroblast growth factor basic, transforming growth factor alpha, transforming growth factor beta, and vascular endothelial growth factor mRNA, all involved in wound healing. Wound healing is an immune mediated event [45], and agents which modulate the innate immune response might also contribute to the modulation of wound healing process [46-48]. In addition, Delatte et al. [49] reported that paediatric burns could effectively be treated with glucan–collagen mixtures. In another study, (1,3/1,6)-β-D-glucan affected lung immune development in the neonatal piglet because of a reduced mRNA expression of transforming growth factor (TGF) β2, and a trend to a reduced mRNA expression of TGF-β1 in lung tissue [50]. Thus, the effects of dietary β-glucans on immune responses can be mediated via regulating activities of growth factors. In the current study, the effect of dietary supplementation of 2 g/kg of β-glucans (85% pure product; Wellmune) to the starter diet on immune related parameters was investigated.

1.2.4 Butyrate

Dietary butyrate supplementation has been shown to improve growth performance and resilience of broiler chickens through distinct mechanisms operating on both eukaryotic and prokaryotic cells. Inconsistent effects of butyrate supplementation on performance have been reported, however, which seem to be partly explained by health status of the animal, diet composition, environmental conditions, coated versus uncoated butyrate and coating matrix, and the butyrate inclusion level [51]. Inconsistent effects of dietary butyrate supplementation on for example broiler gut integrity are reported, as reviewed by Moquet [52]. Firstly, butyrate influences endogenous avian cells in multiple ways: it functions as an agonist of free-fatty acid receptors and an inhibitor of pro-inflammatory pathways [53, 54]. Feeding 1 g/kg of unprotected butyrate to broiler chicken resulted in a significant increase in expression of genes encoding for antimicrobial peptides, which could subsequently induce chemotaxis of avian innate immune cells [55]. Butyrate can prevent inflammation and infiltration of immune cells in peripheral tissues by influencing cell adhesion and chemotaxis [56]. Broiler chickens that were provided 1 g/kg unprotected butyrate had reduced serum TNF-α and IL-6 levels at 21 d of age, while serum antioxidant indices were increased [57]. Moreover, butyrate influences the microbiota residing in the avian gastrointestinal tract (GIT) as a result of its bacteriostatic properties. In the current study, the effect of dietary supplementation of 0,417% Adimix Precision to realize a dosage of 0.1% pure butyrate to the starter diet on immune-related parameters was investigated.

1.3 Objective

The objective of this study was to determine the effect of nutritional interventions with amoxicillin, rye, beta-glucans, or coated butyrate, provided during the first week of life, before they were challenged by the Necrotic enteritis model, on performance and immune competence related parameters of broiler chickens. A positive and negative control treatment (challenged vs. unchallenged broilers) was included to estimate the effect of the NE challenge. In addition, we want to correlate these changes in immune competence related parameters to the quantitative disease phenotype of broilers during their second week of life.

2 Materials and Methods

2.1 Experimental design

The study consisted of 6 treatments (Table 1). One control treatment ("negative control") received no challenge, whereas the remaining five treatments received a NE challenge after the period where they received a (nutritional) intervention. Chickens received an experimental pre-starter diet during the first week of life. Afterwards, all pens received a common starter diet until 16 d of age and a common grower diet until 35 d of age. Treatments were randomly distributed over 12 blocks (divided over two rooms) into 72 pens, resulting in 12 replicates per treatment with 15 chickens each.

Table 1. *Experimental design*

Treatment	Description	NE Challenge	Inclusion level
Unchallenged control	Control	No	-
Challenged control	Control	Yes	-
ANT	Antibiotic (amoxicillin)	Yes	20mg / kg BW/ day
RYE	Rye	Yes	25%
BG	Beta-glucans	Yes	0.2%
BUTY	Butyrate	Yes	0.1%[1]

[1]Pure butyrate.

2.2 Animal experiment

2.2.1 Animal housing and management

One thousand and eighty Ross 308 male day-old chickens, derived from a 36-week old broiler breeder flock, were purchased from a commercial hatchery (Lunteren, the Netherlands) and randomly allocated across 72 pens. Each pen consisted of 15 chickens with an initial individual BW of 39.6 ± 0.8 (g). Pen sizes were in accordance with housing requirements as stated in Annex III of the 2010 / 63 / EU Directive and had a raised floor covered with a 2-cm layer of wood shavings. In total, there were 12 pens per treatment; 6 pens in each room. Each pen was equipped with nipple drinkers (adjustable in height) and a feeder that was positioned inside the pen for the first 14 d of age. From 14 d onwards, feed was supplied via a feeder trough in front of the pen.
Throughout the study, a strict hygiene protocol was maintained and access was restricted (shower-in, shower-out, and change of clothes) due to the applied NE challenge. Chickens were kept until 35 d of age and had *ad libitum* access to water and feed, except in advance of inoculation and lesion scoring (related to NE challenge). Immediately after inoculation, chickens were provided feed access again. The light schedule and climate settings used were in line with practice (Directive 2007 / 43 / EC). Chickens were spray-vaccinated against Newcastle Disease (Poulvac NDW-vaccine, Zoetis Inc., NJ) at 18 d of age.

2.2.2 NE challenge

To facilitate successful colonization of *Clostridium perfringens* in the small intestine, broiler chickens were first inoculated with a 1ml inoculant containing *Eimeria maxima (*~4,500 sporulated oocyst/ml) at 7 d of age. Next, broiler chickens were inoculated with a 1ml inoculant containing *C. perfringens* (~1 × 10^8 cfu / ml) at 13 d of age. In advance of inoculation at 7 and 13 d of age, chickens were feed deprived for about 5 hours. The *E. maxima* and *C. perfringens* were both obtained from the GD Animal Health (Deventer, the Netherlands) and were orally inoculated using a 1 cc syringe without needle. Broiler chickens from the control treatment that was not challenged, received a sterilized inoculum.

The purpose of the challenge was to influence growth performance without increasing mortality, resulting in a sub-clinical NE challenge.
At 14 and 15 d of age, 2 randomly selected broiler chickens per pen were selected for intestinal post-mortem lesion scores related to the *E. maxima* and *C. perfringens*. Birds were euthanized with an intermuscular administration of Zoletil (30 mg/kg; Virbac, Barneveld, the Netherlands) and subsequently bled. Lesion scores for *E. maxima*, characterized by haemorrhages, blood and orange mucus, were conducted in the second loop of the duodenum and jejunum until the Meckels diverticulum and ranged from 0-4, 0=no haemorrhages, 1= One or some haemorrhages, 2= Several haemorrhages, 3= Many haemorrhages, orange mucus in the lumen, 4= Many haemorrhages, mucosal damage (free blood in the lumen). Lesion scores for *C. perfringens*, characterized by white, brown, or grey spots, were conducted from the gizzard towards Meckels diverticulum and ranged from 0-4, 0=no lesions, 1= One to five lesion present in the intestine, 2= More than 5 single distinguished lesions, 3= Lesions merge or extend to a surface of more than 1 cm^2, 4= Pseudo membranes present in the intestine causing death. All chickens were scored by the same veterinarian (GD Animal Health, Deventer, the Netherlands) with solid expertise in distinguishing the different lesions types and lesion severities.

2.2.3 Diets and nutritional interventions

In advance of diet formulation, batches of corn, wheat, soybean meal, toasted soybeans, and rye were reserved and analysed. For each feeding phase (pre-starter, starter, grower) diets were formulated. Diet formulations were derived from a previous study focusing on the effects of rye inclusion on immunity related parameters and performance (Wageningen UR Livestock report 889; project BO31.03-005-001 Feed4Foodure).
For rye, no $ME_{broiler}$ value was defined in the CVB Feed Tables. Therefore, the $ME_{broiler}$ value for rye was calculated by deriving digestibility coefficients for crude protein (CP), crude fat (CFat), and nitrogen free extract (NFE) from the digestibility coefficients for rye in older birds, using similar analogy as for wheat. For wheat, the digestibility coefficients of CP, CFat, and NFE were 0.81, 0.60, and 0.90 for poultry, and 0.83, 0.53, and 0.84 for broiler chickens, respectively. As such, absolute differences in digestibility coefficients between poultry and broiler chickens were +0.02, -0.07, and -0.06, respectively. For rye, digestibility coefficients of crude protein, crude fat and NFE were 0.60, 0.31, and 0.82 for poultry, thus resulting in 0.62, 0.25, and 0.76 for broiler chickens. Using dCP, dCFat and NFE values of 93, 17 and 708 g/kg, respectively, the calculated $ME_{broiler}$ for rye was 2482 kcal/kg. Inclusion of rye was at the expense of wheat, while keeping the diets isocaloric. For the rye diet, animal fat was used as the primary fat source instead of soybean oil.
Butyrate was added at the expense of an inert filler (Diamol, Ligrana, Germany). A correction was made for the dietary sodium level by exchanging with sodium bicarbonate. Butyrate was dosed based on the intended pure butyrate level (0.1%). Adimix Precision (Nutriad, Belgium) contains 30% sodium butyrate, of which 6% is sodium. I.e. the actual butyrate level in Adimix Precision was 24%. Therefore, Adimix Precision was included at 0.417% to dose 0.1% pure butyrate.
Beta-glucans (85% pure product; Wellmune, United States) was added at the expense of an inert filler (Diamol, Ligrana, Germany) at 0.2%.
Pre-starter diets were manufactured using one basal diet. Diets were produced by Research Diet Services (RDS, the Netherlands) and pelleted at 2.5 mm (pre-starter), 2.5 mm (starter), and 3.0 mm (grower diet). Composition of the experimental diets is given in Table 2.
In addition to the control diet, broiler chickens of treatment 3 received an antibiotic (Octacillin; Dechra, the Netherlands) dissolved in water during the first week of life. The following assumptions were made for dosing calculations: 1) Intended dose of 20.0 mg amoxicillin per kg BW per day, 2) Octacillin (product name) contains 697 mg amoxicillin per gram of product, and 3) Octacillin dose: 28.7 mg per kg BW per day. Average bodyweight and average water intake were used to calculate the dosing of the Octacillin on a daily basis. Every morning a new solution (drinking water + Octacillin) was made for the pens receiving the antibiotic treatment. From day 3 until day 7, a calculation error resulted in double dosing of Octacillin from what was intended.

Table 2. Composition of the experimental diets

Diet code		1	4	5	6	7	8
Treatment		**1-3**	**4**	**5**	**6**	**1-6**	**1-6**
Phase		**0-7 d**	**0-7 d**	**0-7 d**	**0-7 d**	**7-16 d**	**16-35 d**
Ingredient composition, %							
Maize		29.7	26.8	29.7	29.7	40.6	42.8
Wheat		25.0	-	25.0	25.0	15.0	15.0
Rye		-	25.0	-	-	-	-
Soybean meal HP		33.6	33.8	33.6	33.6	35.0	32.8
Toasted soybeans		2.00	2.00	2.00	2.00	-	-
Animal fat		1.00	6.18	1.00	1.00	1.50	1.50
Soybean oil		3.51	1.00	3.51	3.51	3.67	4.21
Monocalcium phosphate		1.40	1.37	1.40	1.40	1.20	0.92
Limestone		1.36	1.38	1.36	1.36	1.32	1.06
Salt		0.27	0.25	0.27	0.27	0.30	0.30
Sodium bicarbonate		0.16	0.16	0.06	0.16	0.11	0.11
DL-Methionine		0.27	0.30	0.27	0.27	0.20	0.18
L-Lysine HCL		0.17	0.18	0.17	0.17	0.05	0.06
L-Threonine		0.07	0.09	0.07	0.07	-	-
L-Valine		0.04	0.07	0.04	0.04	-	-
DIAMOL		0.50	0.50	0.18	0.30	-	-
Adimix Precision		-	-	0.42	-	-	-
Beta glucans		-	-	-	0.20	-	-
Premix broilers starter[1]		1.00	1.00	1.00	1.00	1.00	-
Premix broilers grower[2]		-	-	-	-	-	1.00
Total		100.0	100.0	100.0	100.0	100.0	100.0
Crude protein	%	22.3	22.0	22.3	22.3	21.9	21.0
Crude fat	%	6.6	9.2	6.6	6.6	7.1	7.7
Crude fibre	%	2.7	2.6	2.7	2.7	2.6	2.6
Calcium	%	0.90	0.90	0.90	0.90	0.85	0.70
Phosphorous P	%	0.71	0.71	0.71	0.71	0.66	0.59
Sodium	%	0.16	0.16	0.16	0.16	0.16	0.16
Potassium	%	1.00	1.00	1.00	1.00	1.00	0.95
Chloride	%	0.23	0.23	0.23	0.23	0.23	0.23
Dig P	%	0.42	0.42	0.42	0.42	0.38	0.32
ME broiler	kcal	2800	2800	2800	2800	2875	2950
dig. Lysine	%	1.15	1.15	1.15	1.15	1.05	1.00
dig. Methionine	%	0.55	0.57	0.55	0.55	0.48	0.45
dig. Met.+Cys.	%	0.84	0.84	0.84	0.84	0.77	0.73
dig. Threonine	%	0.75	0.75	0.75	0.75	0.68	0.65
dig. Tryptophan	%	0.23	0.22	0.23	0.23	0.23	0.22
dig. Isoleucine	%	0.82	0.79	0.82	0.82	0.82	0.78
dig. Valine	%	0.92	0.92	0.92	0.92	0.88	0.85
dig. Arginine	%	1.33	1.29	1.33	1.33	1.32	1.26
C18:2 (Linoleic acid)	%	2.87	2.02	2.87	2.87	2.90	3.20

[1]Contributed per kilogram of diet: thiamine, 1.0 mg; riboflavin, 4.5 mg; niacinamide, 40 mg; D-pantothenic acid, 9 mg; pyridoxine-HCL, 2.7 mg; choline chloride, 500 mg; cyanocobalamin, 20 µg; vitamin E (DL-α-tocopherol), 33 IU; menadione, 2.3 mg; vitamin A (retinyl-acetate), 12,000 IU; cholecalciferol, 5,000 IU; biotin, 100 µg; folic acid, 0.5 mg; FeSO4·H2O, 150 mg; MnO2, 100 mg; CuSO4·5H2O, 40 mg; ZnSo4·H2O, 145 mg; Na2SeO3, 0.56 mg; KI, 2.0 mg; antioxidant (oxytrap PXN), 125 mg.
[2]Contributed per kilogram of diet: thiamine, 0.8 mg; riboflavin, 4.5 mg; niacinamide, 30 mg; D-pantothenic acid, 8 mg; pyridoxine-HCL, 1.9 mg; choline chloride, 400 mg; cyanocobalamin, 20 µg; vitamin E (DL-α-tocopherol), 22 IU; menadione, 2.3 mg; vitamin A (retinyl-acetate), 10,000 IU; cholecalciferol, 2,000 IU; biotin, 50 µg; folic acid, 0.5 mg; FeSO4·H2O, 150 mg; MnO2, 100 mg; CuSO4·5H2O, 40 mg; ZnSo4·H2O, 145 mg; Na2SeO3, 0.50 mg; KI, 1.9 mg; antioxidant (oxytrap PXN), 125 mg.

2.2.4 Performance and sampling

Group weights were recorded at start of the experiment (day 0), whereas individual body weights were recorded at 7, 13, 16, 21, 28 and 35 d of age. In addition, feed consumption for each pen was recorded on the same day the chickens were weighed. Based on body weight and feed consumption, the average daily gain (ADG; grams per day), average daily feed intake (ADFI; grams per day), and gain to feed ratio (G:F; kg of weight gain / kg of feed consumed) were calculated. Total feed consumption per pen was corrected for mortality, removal and outliers. On 7, 14, and 35 d of age, two chicks per pen were sacrificed in order to obtain tissue samples to investigate immunological and histological parameters.

2.3 Analysis of microbiota composition

2.3.1 DNA isolation

Microbiota composition was determined of broiler digesta from jejunum, ileum and colon. 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 min at 4°C at 9,000*xg*. DNA was extracted from the pellet using the "QIAamp FAST DNA stool minikit" according to manufacturers' instructions. Quality and quantity of DNA was checked using the NANOdrop (Agilent Technologies, CA, United States).

2.3.2 Sequence analyses of 16S rDNA

PCR was used to amplify the 16S rDNA V3/V4 fragment using forward primer V3_F (CCTACGGGAGGCAGCAG) and reverse primer V4_R (GGACTACHVGGGTWTCT). PCR conditions were as follows: 2 min 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. Samples were sequenced by targeted-amplicon 16S sequencing using the MiSeq sequencer (Illumina, CA, United States) and analysed for taxonomy profile per sample with clustering by profile by using QIIME [58]. Standard assembly based on amplicons 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) [59, 60]. Furthermore chimeras were removed with Chimeraslayer [61].

2.4 Gene expression of intestinal tissue

2.4.1 RNA isolation

Total RNA was extracted from 50 to 100 mg of mucosal mid-jejunum tissue. Samples were homogenised using the TissuePrep Homogenizer Omni TP TH220P in 5 ml TRIzol reagent (Life Technologies, CA, United States). The homogenate was centrifuged for 5 min at 21,000 *xg*. 350 µl of supernatant was used to isolate RNA using the Direct-zol kit (Zymo Research, CA, United States) according to instructions of the manufacturer. Quality control was performed on the BioAnalyser (Agilent Technologies, CA, United States), quantity of RNA was determined using the Tape station (Agilent 2200 tape station, Agilent technologies, CA, United States).

2.4.2 Microarrays: Labelling 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. 200 ng of total RNA was used as input, 600 ng of labelled cRNA was used to hybridise the porcine microarray (Agilent Technologies, CA, United States). 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, CA, United States) at a resolution of 3 µm, 20 bits and PMT of 100%. Feature extraction was performed using protocol 10.7.3.1 (v10.7) for 1 colour gene expression.

2.5 Histology

2.5.1 Morphometric measurements

Formalin fixed 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 Image-Pro Plus software v7.0.1 (Media Cybernetics, MD, USA). In **Appendix A**, an example

microscopic image is given accompanied by the measurements. Per animal ten crypts and their ten associated villi were measured.

2.5.2 Periodic acid-Schiff / alcian blue staining (goblet cells)

This is a combined method utilising the properties of both the periodic acid-Schiff (PAS) and alcian blue methods to demonstrate the full complement of tissue proteoglycans. The rationale of the technique is that by first staining all the acidic mucins with alcian blue, those remaining acidic mucins which are also PAS positive will be chemically blocked and will not react further during the technique. Those neutral mucins which are solely PAS positive will subsequently be demonstrated in a contrasting manner. Where mixtures occur, the resultant colour will depend upon the dominant moiety.

2.6 Statistical analyses

2.6.1 Performance data, lesion scores, mortality

For comparison of the different treatments, all data were subjected to mixed model analysis using the PROC Glimmix procedure in SAS (Version 9.3, 2011, SAS Institute Inc., NC, United States) according to the following statistical model:

$$Y_{ij} = \mu + \alpha_i + b_j + \varepsilon_{ij}$$

where:

Y_{ij} = a specific trait per experimental unit
μ = overall mean
α_i = fixed effect of dietary treatment fat level (i = 1-6)
b_j = random block effect (j = A - L)
ε_{ij} = error term

Lesion scores were analysed as ordinal data and mortality as binomial data, using the PROC GLIMMIX procedure in SAS and the same model parameters used as for performance data. For lesion scores, results are presented as the probability of a higher lesion score.
Data are expressed as least square (LS) means ± SEM. LSmeans were compared after being corrected with a Tukey test for multiple comparisons and effects were considered to be significant when $P \leq 0.05$.

2.6.2 Microbiota data

The biodiversity of the microbiota was calculated by the vegan package http://cran.r-project.org/web/packages/vegan/ within the R environment (R v3.0.2), by employing the Shannon diversity indices, as well as species and evenness. The Redundancy analysis (RDA) was also performed by using the vegan package. The following model was used on the family level microbiota data: Y = Time (day) + Treatment + error. Furthermore, statistical significance testing for over- and under-representation of the bacterial groups was made at the phylum / family / species level by performing the Wilcoxon signed-rank test, and P-values were also converted to false discovery rate (FDR) values to correct for multiple testing. Before day 7 of age 2 control groups exist, i.e. the unchallenged (n=24) and challenged controls (n=24). After quality control for jejunum samples, 19 unchallenged and 18 challenged control samples remained, 20 samples in the antibiotic group, 18 samples in the β-glucans group, 17 samples in the butyrate group, and 18 samples in the rye group. In colon, 23 unchallenged and 24 challenged control samples remained and 23 for the butyrate group.
For the semi-quantification of the microbiota, a polymerase chain reaction (PCR) was performed. With this PCR we focused on the 16S gene (representing the microbiota) and the Green Fluorescent Protein (GFP), a measure for how efficient the PCR has been performed. The threshold cycle value, so called Ct value, were calculated as well as a proxy for the quantity of each gene (i.e. 16S and GFP). Lastly the ratio between 16S Ct and GFP Ct, which could possibly be used to correct the average relative contributions. The latter we did not perform in this study.

2.6.3 Gene expression (microarray data)

The data were analysed by using R (v3.0.2) by executing different packages, including LIMMA and arrayQualityMetrics [62]. The data were read in and background corrected (method="normexp" and offset=1) with functions from the R package LIMMA from Bioconductor [63]. Quantile normalisation of the data was performed between arrays. The duplicate probes mapping to the same gene were averaged ('avereps') and subsequently the lower percentile of probes was removed in a three-step procedure: 1) get the highest of the dark spots to get a base value, 2) multiply by 1.1, 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, i.e. control versus treatment group), the contrasts between control and treatment group were studied for each time-point separately within the LIMMA package. Two control groups exist, i.e. the unchallenged (n=24) and challenged controls (n=22).

2.6.4 Histology

The data were analysed by using R (v3.0.2) by executing different packages, including 'aov' and 'TukeyHSD' to perform statistical testing between groups. The data were read in subsequently three separated ANOVAs were performed, for goblet cells, villi length, and crypt depth, when significant the Tukey post-hoc test was performed to identify which groups differed significantly.

3 Results

3.1 Effect of nutritional interventions on performance

Body weight

BW at 7 d of age was similar for both control treatments. From 7 d of age onwards (after inoculation with *E. Maxima* at 7 d of age), challenged broiler chickens fed the control diet has a lower BW compared to the unchallenged broiler chickens. The difference in BW between challenged and unchallenged broiler chickens persisted throughout the study, resulting in an absolute difference in BW of 150 g at 35 d of age (Table 3).
Provision of diets supplemented with rye, β-glucans or butyrate during the first week of life did not affect BW at 7 d of age compared to the (un)challenged control treatment. On the contrary, broiler chickens that received antibiotic treatment had a higher BW at 7 d of age compared to all other treatments. At 13 d of age, BW of broiler chickens receiving the antibiotic treatment remained numerically higher than the controls or broiler chickens receiving another treatment. At 16 and 21 d of age (after additional inoculation with *C. perfringens*), BW of broiler chickens receiving the antibiotic treatment was higher compared to all other challenged treatments (P<0.001; Table 3). No differences in BW at 35 d of age were found amongst challenged broiler chickens, regardless of treatment (Table 3).

Table 3. *Effect of (dietary) treatment and a necrotic enteritis (NE) challenge with inoculation of E. maxima at 7 d of age and C. perfringens at 13 d of age, on body weight of broiler chickens*

NE challenge	No	Yes	Yes	Yes	Yes	Yes		
Diet trt. (0-7d)	Control	Control	Antibiotic Octacillin	Rye 25%	β-glucans 0.2%	Butyrate 0.1%	SEM	P-value
0d	39.7 [a]	39.8 [a]	39.4 [a]	39.7 [a]	39.6 [a]	39.6 [a]	0.2	0.927
7d	195 [bc]	197 [b]	222 [a]	189 [c]	197 [b]	194 [bc]	2	<.001
13d	452 [a]	416 [bc]	443 [ab]	406 [c]	417 [bc]	414 [c]	8	<.001
16d	624 [a]	510 [c]	574 [b]	515 [c]	522 [c]	527 [c]	12	<.001
21d	1000 [a]	885 [b]	967 [a]	883 [b]	900 [b]	890 [b]	17	<.001
28d	1748 [a]	1599 [c]	1698 [ab]	1605 [c]	1623 [bc]	1609 [bc]	24	<.001
35d	2568 [a]	2418 [b]	2504 [ab]	2444 [b]	2426 [b]	2437 [b]	28	0.001

[a-c] LSmeans within a row lacking a common superscript differ (P≤0.05). Note that at day 0 and 7, the challenged birds were not yet challenged (highlighted in grey).

Performance 0 to 7 d of age: Provision of (dietary) treatments

From 0 to 7 d of age, provision of an antibiotic in the drinking water resulted in a higher ADG (25.8 g/d) and ADFI (23.4 g/d) compared to all other treatments (P<0.001; Table 4). Rye inclusion resulted in the lowest ADG. Provision of an antibiotic in the drinking water resulted in the highest G:F ratio (1.102), rye inclusion for the lowest (1.059; *P*=0.004; Table 4).

Performance 7 to 16 d of age: Necrotic enteritis challenge

From 7 to 13 d of age, after inoculation with *E. maxima*, the non-challenged control treatment had a higher ADG (43 g/d), ADFI (52 g/d) and G:F ratio (0.83) compared to the challenged control (ADG 37 g/d, ADFI 48 g/d, G:F ratio 0.757; P<0.05; Table 4).

From 13 to 16 d of age, after inoculation with *C. perfringens*, the non-challenged control treatment had higher ADG (58.8 g/d), ADFI (72.9 g/d), and G:F ratio (0.81) compared to the challenged control (ADG 34 g/d, ADFI 56 g/d, and G:F ratio 0.60; P<0.001; Table 4). Amongst the challenged treatments, ADG and ADFI of broiler chickens that received the antibiotic treatment were higher than the challenged control treatment (41.6 and 62.1 g/d vs. 29.3 and 53.5 g/d; P<0.001; Table 4). The other treatments showed intermediate results.
Throughout the challenge period (7 to 16 d of age), the non-challenged control treatment had a higher ADG, ADFI, and G:F ratio compared to the challenged control. No differences in performance were observed amongst dietary treatments (P<0.001; Table 4).

Table 4. *Effect of (dietary) treatment and a necrotic enteritis (NE) challenge with inoculation of E. maxima at 7 d of age and C. perfringens at 13 d of age on performance parameters (ADG, ADFI, and G:F)*

NE[1] challenge	No		Yes		Yes		Yes		Yes		Yes			
Treatment (0-7d)	Control		Control		Antibiotic Octacillin		Rye 25 %		β-glucan 0.2 %		Butyrate 0.1 %		SEM	P-value
ADG[2] 0-7d	22.2	bc	22.4	b	25.8	a	21.1	c	22.4	b	22.0	bc	0.3	<.001
ADFI[3] 0-7d	20.5	b	20.6	b	23.4	a	19.9	b	20.8	b	20.3	b	0.3	<.001
G:F[4] 0-7d	1.083	ab	1.086	ab	1.102	a	1.059	b	1.081	ab	1.084	ab	0.007	0.004
ADG 7-13d	43.1	a	36.3	b	36.6	b	36.3	b	36.8	b	36.6	b	1.1	<.001
ADFI 7-13d	51.8	a	48.0	b	49.1	ab	47.8	b	48.3	b	47.8	b	0.8	0.002
G:F 7-13d	0.831	a	0.757	b	0.744	b	0.758	b	0.760	b	0.765	b	0.013	<.001
ADG 13-16d	58.8	a	29.3	c	41.6	b	33.2	bc	32.0	bc	34.0	bc	2.7	<.001
ADFI 13-16d	72.9	a	53.5	c	62.1	b	56.9	bc	54.1	bc	54.4	bc	2.0	<.001
G:F 13-16d	0.807	a	0.544	b	0.654	b	0.579	b	0.589	b	0.620	b	0.030	<.001
ADG 7-16d	47.7	a	34.3	b	38.1	b	35.4	b	35.4	b	35.9	b	1.1	<.001
ADFI 7-16d	58.1	a	49.6	b	52.9	b	50.5	b	50.0	b	49.7	b	0.9	<.001
G:F 7-16d	0.822	a	0.691	b	0.715	b	0.702	b	0.708	b	0.721	b	0.012	<.001
ADG 16-21d	75.1	a	73.2	a	77.8	a	73.4	a	74.9	a	72.3	a	1.6	0.125
ADFI 16-21d	98.9	a	89.4	b	98.0	ab	91.7	ab	92.8	ab	92.0	ab	2.3	0.021
G:F 16-21d	0.759	b	0.820	a	0.797	ab	0.808	ab	0.809	ab	0.786	ab	0.013	0.015
ADG 21-28d	107.1	a	101.3	b	103.1	ab	103.1	ab	103.3	ab	101.9	ab	1.4	0.039
ADFI 21-28d	148.3	a	139.0	b	143.2	ab	140.7	ab	142.3	ab	140.2	b	2.0	0.015
G:F 21-28d	0.722	a	0.728	a	0.721	a	0.733	a	0.726	a	0.727	a	0.005	0.501
ADG 28-35d	115.9	a	118.2	a	114.9	a	119.2	a	115.4	a	116.9	a	2.2	0.677
ADFI 28-35d	177.4	a	176.2	a	175.3	a	176.1	a	176.0	a	175.5	a	1.8	0.971
G:F 28-35d	0.654	a	0.671	a	0.655	a	0.677	a	0.655	a	0.666	a	0.010	0.290
ADG 16-35d	101.8	a	99.8	a	100.5	a	101.2	a	100.2	a	99.4	a	1.1	0.646
ADFI 16-35d	145.7	a	139.1	a	142.4	a	140.8	a	141.5	a	140.0	a	1.7	0.076
G:F 16-35d	0.699	b	0.718	ab	0.706	ab	0.719	a	0.708	ab	0.710	ab	0.005	0.044
ADG 0-35d	64.4	a	58.5	b	60.4	b	59.2	b	59.4	b	58.6	b	0.9	<.001
ADFI 0-35d	86.1	a	79.0	b	81.4	b	80.0	b	80.8	b	79.3	b	1.1	<.001
G:F 0-35d	0.748	a	0.740	a	0.742	a	0.740	a	0.735	a	0.740	a	0.004	0.143

[1] Necrotic enteritis; [2] Average daily gain; [3] Average daily feed intake; [4] Gain to feed ratio;
[a-c] LSmeans within a row lacking a common superscript differ (P≤0.05).

Performance 16 to 35 d of age: Grower phase

From 16 to 35 d of age, ADG and ADFI were not affected by (dietary) treatment (P=0.646 and 0.076, respectively; Table 4). Non-challenged and challenged treatments had similar ADG, ADFI, and G:F from 28 d of age onwards (P>0.05; Table 4). For the overall grower phase (16 to 35 d of age), broiler chickens fed 25% rye inclusion had the highest G:F ratio whereas the non-challenged control treatment had the lowest G:F ratio (P=0.044; Table 4).

Performance 0 to 35 d of age: Overall study period

Overall, the non-challenged control treatment had the highest ADG (64.4 g/d) and ADFI (86.1 g/d) compared to all challenged treatments (av. ADG 59.2 g/d and ADFI 80.1 g/d; P<0.001; Table 4). On the contrary, G:F ratio was not different between challenged and non-challenged treatments (P=0.143; Table 4). Overall performance (ADG, ADFI and G:F ratio) did not differ significantly amongst any of the challenged treatments.

Mortality and culling

Results are reported as the probability for a higher mortality, so not as absolute mortality percentages per treatment. Culling is excluding broilers that were selected for dissection. Mortality and culling rates were not affected by (dietary) treatment (Table 5).

Table 5 *Effect of (dietary) treatment, and effect of necrotic enteritis (NE) challenge, on mortality and culling from 0 to 35 d of age. Percentages reported are the probabilities for a higher mortality*

NE challenge Dietary treatment (0-7d)	No Control	Yes Control	Yes Antibiotic Octacillin	Yes Rye 25%	Yes β-glucan 0.2%	Yes Butyrate 0.1%	SEM	P-value
Mortality, %	4.3	6.0	5.4	4.9	5.4	3.3	1.8	0.876
Mortality and culling, %	4.6	9.3	10.5	7.0	6.4	8.1	2.3	0.369

3.2 Effect of nutritional interventions on jejunal microbiota

Microbiota diversity

The microbiota diversity, Shannon index, was measured for all treatment groups with respect to jejunum. For the colon, only the control and butyrate groups were examined (Table 6).

Table 6. *Shannon diversity at 7 d of age for jejunum (all treatment groups) and colon (control and butyrate)*

7 d of age	Group[1]	Mean	SEM
Jejunum[2]	CON	0.46	0.07
	CON_NE[4]	0.53	0.05
	ANT	0.60	0.14
	RYE	0.48	0.12
	BG	0.65	0.08
	BUTY	0.4	0.08
Colon[3]	CON	1.50	0.08
	CON_NE[4]	1.48	0.08
	BUTY	1.37	0.08

[1]CON, unchallenged control (n=12); CON_NE, challenged control (n=12); ANT, antibiotics (n=12); RYE, rye (25%)(n=12); BG, beta-glucans (n=12); BUTY, coated butyrate(n=12), [2] ANOVA; p-value = 0.65, [3] ANOVA; p-value = 0.74,

[4]Note that these birds were not challenged yet.

Redundancy analysis

For both jejunum and colon, a redundancy analysis was performed for intestinal samples of broiler chickens at 7 d of age, in order to identify whether the intervention groups differed in overall microbiota composition. Intestinal microbiota composition of the broiler chickens that received an antibiotic in the drinking water deviated from the (un)challenged control groups, as a low overlap was observed (Figure 2). Other treatment groups shared the overall microbiota composition, but there were about 1 to 5 broiler chickens per treatment that showed more variation compared to the control treatment. In the colon no clear differences were observed between the butyrate group and the (un)challenged control groups (Figure 3).

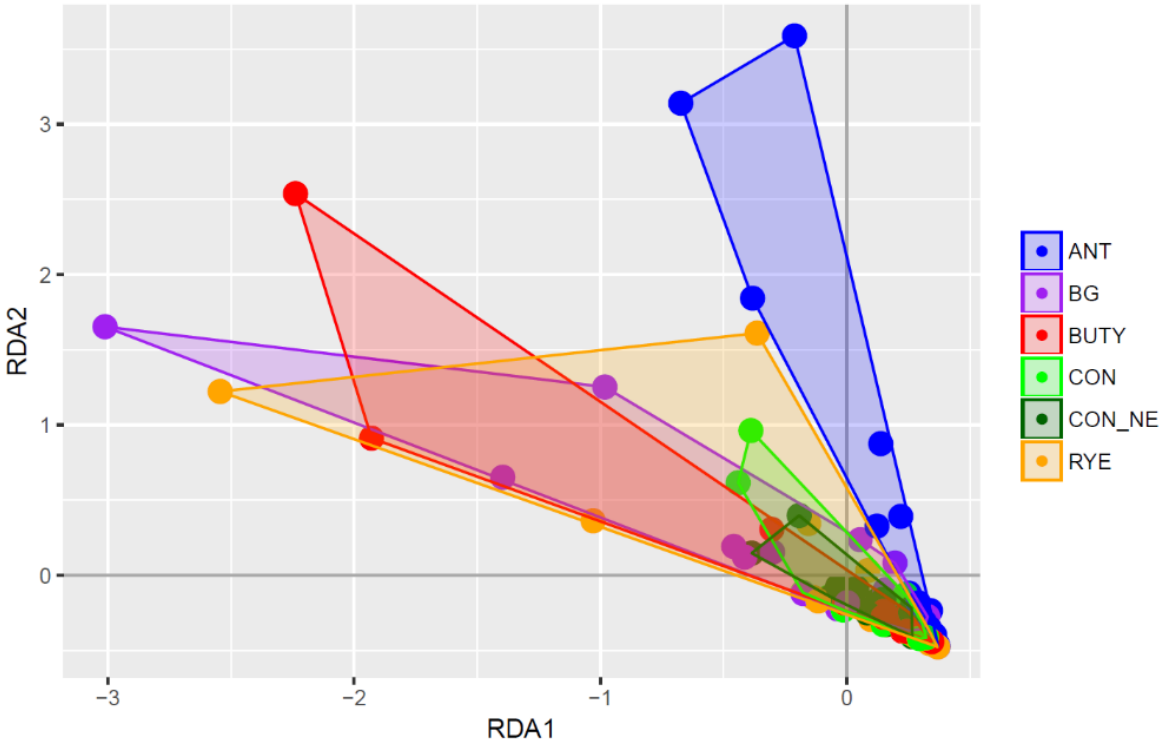

Figure 2. Redundancy analysis of jejunum microbiota (7 d of age)

The x-axis depicts RDA1 and the y-axis depicts RDA2. Each dot represents an individual broiler chicken, and each colour a (dietary) treatment: antibiotic in drinking water (ANT; Blue, n=20), β-glucans (BG; Purple, n=18), butyrate (BUTY; Red, n=17), unchallenged control (CON; Green; n=19), challenged control (CON_NE, Darkgreen, n=18) and rye (RYE; Orange, n=18). Note that the CON_NE birds were not challenged yet.

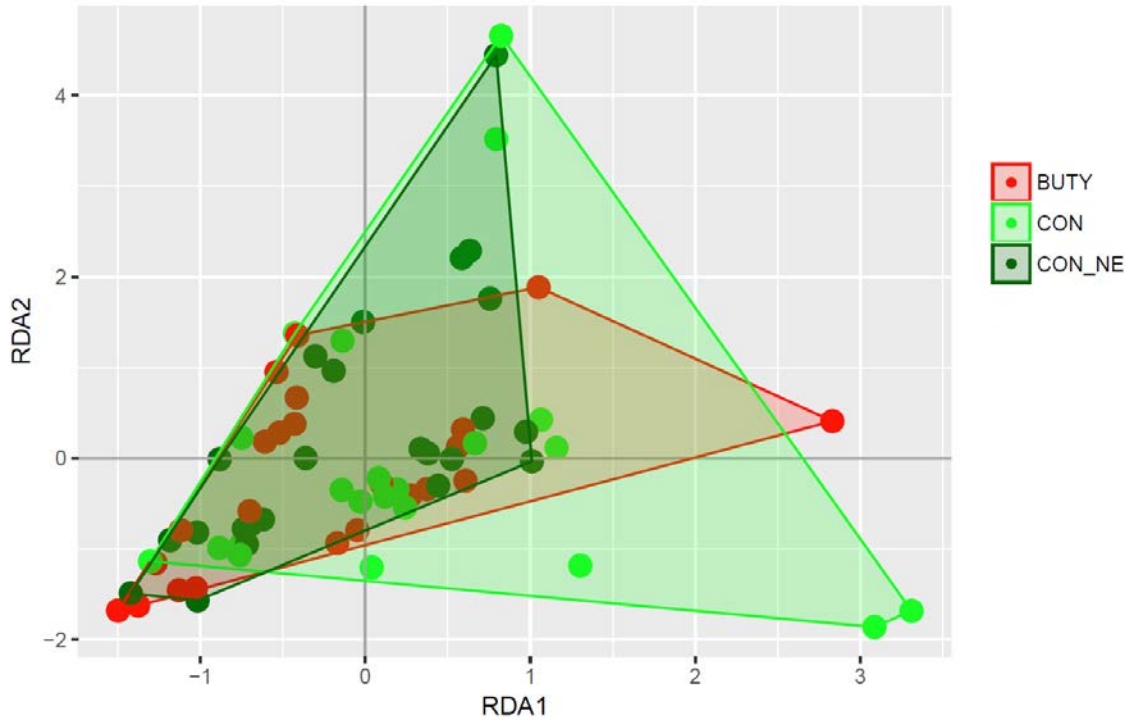

Figure 3. Redundancy analysis of colon microbiota (7 d of age).

The x-axis depicts RDA1 and the y-axis depicts PC1. Each dot represents an individual broiler chicken, and each colour a (dietary) treatment: unchallenged control (CON; Green, n=23), challenged control (CON_NE; Darkgreen, n=24) and butyrate (BUTY; Red, n=23). Note that the CON_NE birds were not challenged yet.

Microbiota composition on genus level

A test was performed at genus level in order to identify differences between the intervention groups and the (un)challenged controls. In the jejunum, the antibiotic treatment (ANT) showed 10 different bacterial genera and relatively large changes in Average Relative Contribution (ARC) were observed compared to the respective (un)challenged control (Table 7). For example, *Enterococcus* represented a 4.4% ARC in the unchallenged control group and 5.4% in the challenged control group versus 0.3% in the antibiotic group, whereas *Enterobacteriaceae* were higher in the antibiotic group (6.6% ARC) compared to the un- and challenged control groups (1.0 and 0.4% ARC respectively).
For the rye treatment, up to 13 different genera were significant different when comparing to the control group (Table 7). However, the ARC was generally low in both groups (all below 1%). Only two significant difference was observed, i.e. *Lactobacillus* and *Enterococcaceae*. The ARC of *Lactobacillus* was lower in the beta-glucan group (78.4%) compared to the (un)challenged control group, the ARC were 88.7% and 89.2% respectively. For the butyrate group, both jejunum and colon were investigated. In jejunum, four bacterial genera were significant and in colon as well four bacterial genera were significant, however not the same bacterial genera were significant in the two tissues. Moreover, in jejunum the ARC of all significant bacterial genera were low (under 1%). However, in colon the *Enterobacteriaceae* were decreased in the butyrate group (3.9% ARC vs. 4.7% ARC in the averaged control group).

Table 7. *Overview of statistical significant genera between (dietary) treatment groups versus (un)challenged controls in jejunum and colon of broiler chickens at 7 d of age*

TRT[1]	Phylum	Class	Family	Genus	CON[2]	CON_NE[2]	TRT[2]	TRT vs. CON		TRT vs. CON_NE	
								P-value	FDR[3]	P-value	FDR
ANT jejunum	*Actinobacteria*	*Actinobacteria*	*Dermabacteraceae*	*Brachybacterium*	<0.01	<0.01	0.039	<0.01	<0.01	<0.01	0.01
	Firmicutes	*Bacilli*			0.111	0.151	0.031	<0.01	<0.01	<0.01	<0.01
	Firmicutes	*Bacilli*	*Enterococcaceae*	*Enterococcus*	4.438	5.365	0.283	<0.01	<0.01	<0.01	<0.01
	Firmicutes	*Bacilli*	*Pediococcus*		<0.01	<0.01	0.857	<0.01	<0.01	<0.01	0.04
	Firmicutes	*Bacilli*	*Leuconostocaceae*		0.164	0.089	0.029	<0.01	0.15	<0.01	0.05
	Firmicutes	*Bacilli*	Other	Other	0.182	0.225	0.070	<0.01	<0.01	<0.01	<0.01
	Firmicutes	*Clostridia*	*Clostridiaceae*		<0.01	0.011	<0.01	>0.05	>0.05	0.02	0.38
	Firmicutes	*Clostridia*	*Clostridiaceae*	*Candidatus.Arthromitus*	0.629	0.521	<0.01	0.02	0.39	<0.01	0.02
	Firmicutes	*Clostridia*	*Peptostreptococcaceae*		0.048	0.040	<0.01	>0.05	>0.05	0.01	0.37
	Proteobacteria	*Gammaproteobacteria*	*Enterobacteriaceae*		1.046	0.356	6.643	0.02	0.39	<0.01	0.05
RYE jejunum	*Actinobacteria*	*Actinobacteria*	*Corynebacteriaceae*	*Corynebacterium*	0.043	0.064	<0.01	0.01	0.59	>0.05	>0.05
	Cyanobacteria	*Choloroplast*			0.241	0.212	0.105	0.01	0.59	0.02	0.56
	Firmicutes	*Bacilli*			0.111	0.151	0.050	>0.05	>0.05	0.00	0.42
	Firmicutes	*Bacilli*	*Leuconostocaceae*		0.164	0.089	0.010	0.02	0.59	0.01	0.44
	Firmicutes	*Bacilli*	Other	Other	0.182	0.225	0.095	>0.05	>0.05	0.01	0.44
	Firmicutes	*Clostridia*			0.297	0.496	0.790	>0.05	>0.05	0.04	0.58
	Firmicutes	*Clostridia*	*Clostridiaceae*	*Clostridium*	0.014	0.053	0.012	0.03	0.59	0.02	0.54
	Firmicutes	*Clostridia*	*Lachnospiraceae*	*Blautia*	0.081	0.139	0.104	>0.05	>0.05	0.02	0.54
	Firmicutes	*Clostridia*	*Lachnospiraceae*	*Coprococcus*	0.044	0.055	0.056	>0.05	>0.05	0.05	0.58
	Firmicutes	*Clostridia*	*Lachnospiraceae*	*Dorea*	0.026	0.035	0.040	>0.05	>0.05	0.04	0.58
	Firmicutes	*Clostridia*	*Ruminococcaceae*	*Ruminococcus*	0.066	0.095	0.211	>0.05	>0.05	0.05	0.58
	Proteobacteria	*Alphaproteobacteria*	*mitochondria*	Other	0.022	0.013	<0.01	>0.05	>0.05	0.01	0.44
	Other	Other	Other	Other	0.305	0.709	0.160	>0.05	>0.05	0.02	0.49
BG jejunum	*Firmicutes*	*Bacilli*	*Enterococcaceae*	Other	<0.01	<0.01	0.013	0.03	0.64	>0.05	>0.05
	Firmicutes	*Bacilli*	*Lactobacillaceae*	*Lactobacillus*	88.670	89.196	78.430	0.02	0.64	>0.05	>0.05
BUTY jejunum	Other	Other	Other	Other	0.12	0.08	0.21	>0.05	>0.05	0.01	0.62
	Firmicutes	*Clostridia*	*Clostridiaceae*	*Candidatus.Arthromitus*	0.63	0.52	<0.01	>0.05	>0.05	<0.01	0.38
	Firmicutes	*Clostridia*	*Lachnospiraceae*	*Blautia*	0.08	0.14	0.04	>0.05	>0.05	0.03	0.62
	Firmicutes	*Clostridia*	*Lachnospiraceae*	*Dorea*	0.03	0.03	0.01	>0.05	>0.05	0.04	0.62
BUTY colon	*Firmicutes*	*Bacilli*	*Enterococcaceae*	Other	0.013	0.039	0.035	0.03	0.63	>0.05	>0.05
	Firmicutes	*Clostridia*	*Clostridiaceae*	SMB53	<0.01	<0.01	0.015	0.02	0.63	>0.05	>0.05
	Firmicutes	*Clostridia*	*Veillonellaceae*	*Veillonella*	0.027	<0.01	<0.01	0.04	0.63	>0.05	>0.05
	Proteobacteria	*Gammaproteobacteria*	*Enterobacteriaceae*		6.551	2.964	3.894	0.05	0.63	>0.05	>0.05

Abbreviations used: TRT, treatment; CON, unchallenged control; CON_NE, challenged control; ANT, antibiotics; RYE, rye; BG, beta-glucans; BUTY, coated butyrate. [2] Values are average relative contribution, [3] False discovery rate

Semi-quantification of the microbiota

For semi-quantification of the microbiota, both the 16S and Green Fluorescent Protein (GFP) cycli were quantified by qPCR (Table 8). The first column shows the intervention groups. The second column depicts the Ct value (threshold cycle; is the PCR read-out) of the reference gene, here green fluorescent protein (GFP), for each intervention group. This gene is used to correct for the PCR-feasibility of the samples. This gene expression of the reference gene is a measure of the efficiency of the PCR. The third column contains the amount of GFP which is calculated based on the Ct value and the calibration line (data not shown). The fourth column contains the Ct value of our target genes (16S). The same gene (primer-pair) that was used in the microbiota sequence analysis. This gene is a measure of the amount of microbiota present in the intervention group. The fifth column contains the ratio of 16S Ct divided by GFP Ct.
The lowest quantity for the ratio of 16S and GFP was observed for control (CON) 0.06, whereas the highest was the rye (RYE) group (0.54). Both antibiotics (ANT) and beta-glucans (BG) where two times higher than the control group. The butyrate (BUTY) group was slightly higher than the controls. As shown in earlier work the use of antibiotics does not automatically result in a lower quantity, compared to the control the antibiotic group is even higher, although antibiotics suppress growth of certain bacterial groups it also enables all other bacterial species to flourish.

Table 8. *Results of semi-quantification of microbiota for different (dietary) treatment groups in jejunum of 7 days old-broilers*

Group	16S Ct	Quantity	GFP Ct	Quantity	ratio 16S/GFP Quantity
CON	19.7	75.8	26.4	1650.7	**0.06**
CON_NE[1]	19.1	119.8	25.8	1687.0	**0.07**
ANT	18.7	208.4	25.9	1888.2	**0.12**
RYE	19.9	78.2	26.8	1538.6	**0.54**
BG	19.7	97.6	26.9	1189.4	**0.12**
BUTY	18.6	155.9	25.8	2342.4	**0.07**

[1]Note that these birds were not challenged yet.

Abbreviations used: CON, unchallenged control; CON_NE, challenged control; ANT, antibiotics; RYE, rye (25%); BG, beta-glucans; BUTY, coated butyrate

3.3 Effect of feed interventions on transcriptomics in jejunum at day 7

The effect of the inclusion of amoxicillin, rye, β-glucans, or butyrate into the diet on gene expression in jejunum was studied. Here, we focused on the significant genes, followed by pathway analysis. This analysis was performed by transcriptomic profiling analysis.

3.3.1 Differential gene expression between feed interventions and control broilers

There were differences in jejunal gene expression between the different feed interventions and control birds at day 7 (Table 9). Only the antibiotic treatment (ANT) differed from the (un)challenged control groups. Antibiotic versus the unchallenged control showed 417 differentially expressed probes, mapping to 240 annotated genes, whereas the antibiotic versus the challenged control showed 470 differentially expressed probes, mapping to 266 annotated genes.

Table 9. *Differentially expressed genes[1] in jejunum at day 7*

Contrast	Probes	Annotated Genes
ANT vs. CON	417	240
ANT vs. CON_NE[2]	470	266
RYE vs. CON	0	0
RYE vs. CON_NE	0	0
BG vs. CON	0	0
BG vs. CON_NE	0	0
BUTY vs. CON	0	0
BUTY vs. CON_NE	0	0

[1] Adjusted p-value (False Discovery Rate) < 0.05 and FoldChange > |1.5|. [2] Note that these birds were not challenged yet.

Abbreviations used: CON, unchallenged control; CON_NE, challenged control; ANT, antibiotics; RYE, rye (25%); BG, beta-glucans; BUTY, coated butyrate

3.3.2 Pathway analysis

In order to investigate in which biological pathways these genes were involved, we performed a pathway analysis. An overview of the significant pathways is shown in Table 10, in total 28 pathways had an adjusted P-value below 0.05.

Table 10. *Significant pathways when comparing the antibiotic group vs. challenged and unchallenged controls*

Unchallenged control				
Pathway Name	**Score**	**Total Genes**	**Matched Genes**	**%**
NF-kappaB Signaling	**25.36**	313	16	5.11
Akt Signaling	**22.48**	681	22	3.23
TGF-Beta Pathway	**21.45**	652	21	3.22
PAK Pathway	**20.41**	682	21	3.08
Cytokine Signaling in Immune System	**19.82**	761	22	2.89
ERK Signaling	**19.29**	1177	28	2.38
PEDF Induced Signaling	**19.15**	721	21	2.91
Interleukin-4 and 13 Signaling	**16.76**	114	8	7.02
Cytokine Production By Th17 Cells in CF (Mouse Model)	**16.65**	54	6	11.11
Allograft Rejection	**16.02**	249	11	4.42
Th17 Cell Differentiation	**15.96**	162	9	5.56
IL-4 Signaling Pathways and Their Primary Biological Effects in Different Immune Cell Types	**15.79**	17	4	23.53
Type II Interferon Signaling (IFNG)	**15.49**	37	5	13.51
Innate Immune System	**14.60**	2132	37	1.74
NRF2 Pathway	**14.31**	145	8	5.52
NF-KappaB Family Pathway	**13.98**	241	10	4.15
Challenged control*				
Pathway Name	**Score**	**Total Genes**	**Matched Genes**	**%**
Collagen Chain Trimerization	**18.13**	184	11	5.98
NF-kappaB Signaling	**17.72**	313	14	4.47
Allograft Rejection	**16.49**	249	12	4.82
Akt Signaling	**15.41**	681	20	2.94
Cytokine Signaling in Immune System	**14.80**	761	21	2.76
Type II Interferon Signaling (IFNG)	**14.51**	37	5	13.51
PEDF Induced Signaling	**14.27**	721	20	2.77
ERK Signaling	**13.94**	1177	27	2.29
Development_Thrombopoetin Signaling Via JAK-STAT Pathway	**13.31**	23	4	17.39

*Note that these birds were not challenged yet.

Highlighted grey pathways are overlap between the antibiotic group and the unchallenged and challenged controls.

Subsequently, the most significant pathways were reviewed and general biological processes were extracted. This was followed by superimposing the gene expression data (fold change ANT vs. CON) on particular pathways of interest, i.e. the NF-kappa B signalling pathway (Figure 4), PI3K-Akt signalling pathway (Figure 5), Cytokine-cytokine receptor interaction (Figure 6), and Intestinal immune network for IgA production (Figure 7).

A highly significant pathway was the NF-kappa B pathway, which is important in regulation of cellular responses and can be induced by e.g. reactive oxygen species (ROS), TNFα, IL-1β, lipopolysaccharides (LPS). In this pathway the majority of genes are down-regulated in the antibiotics groups vs the (un)challenged controls, most of these are (in)directly involved in immune related processes. However, genes involved in cell survival are up-regulated. Another highly significant pathway was the PI3K-AKT

signalling pathway, where we mostly observed down-regulated genes, except for the mTOR signalling pathway part. mTOR signalling has a broad range of functions, including actin/cytoskeleton reorganization and cell survival. An interesting pathway related to immunity is the cytokine-cytokine receptor interaction pathway, where down-regulation of genes was observed for the following families chemokines, hematopoietins (mainly the IL2RG subset), interferon, IL-10, IL-1, and IL-17, and up-regulation for the families hematopoietins (mainly the IL6ST subset), PDGF, and TGFB. Most of these families are involved in immune related processes, like chemokines, hematopoietins, interferon, IL-10, IL-1, and IL-17. Whereas the PDGF and TGFB families are involved in developmental and wound healing functions, by stimulation of extracellular matrix production, influencing cell migration, cell adhesion, differentiation, and other cellular activities.

Because of the lower expression in immune related parts of the above mentioned pathways, we also investigated the intestinal immune network for IgA production pathway, in which we also observed that the majority of genes is down-regulated.

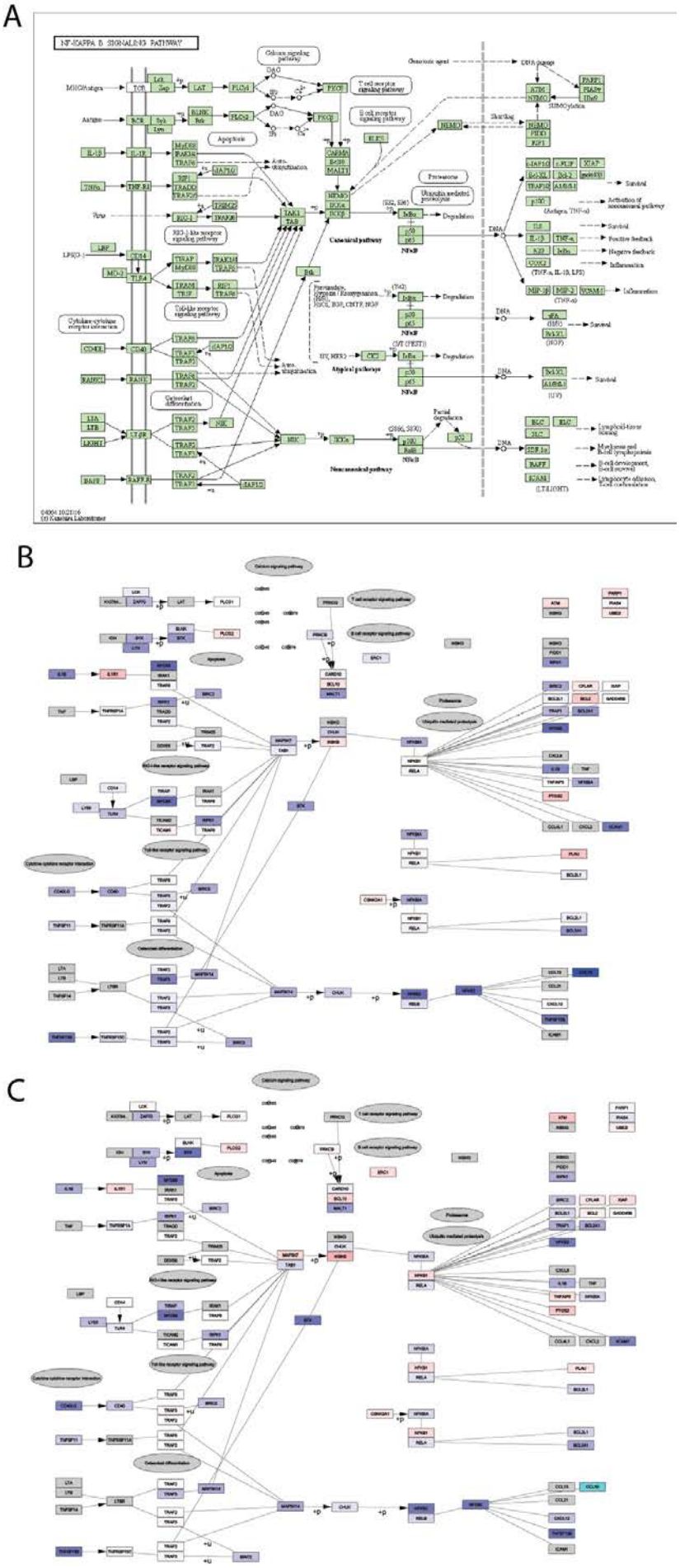

Figure 4. *NF-kappa B signalling pathway*

A) shows the NF-kappa B signalling pathway diagram as represented by Kyoto Encyclopedia of Genes and Genomes (KEGG). B and C show the same pathway as well, however the lay-out differs somewhat from the KEGG representation. In B genes are coloured by fold change (ANT vs. unchallenged control) and in C genes are coloured by fold change (ANT vs challenged control). Where blue denotes down-regulated genes and red denotes up-regulated genes, grey denotes not measured by our transcriptomic analysis. See Appendix B for more detailed explanation and pdf version for better resolution.

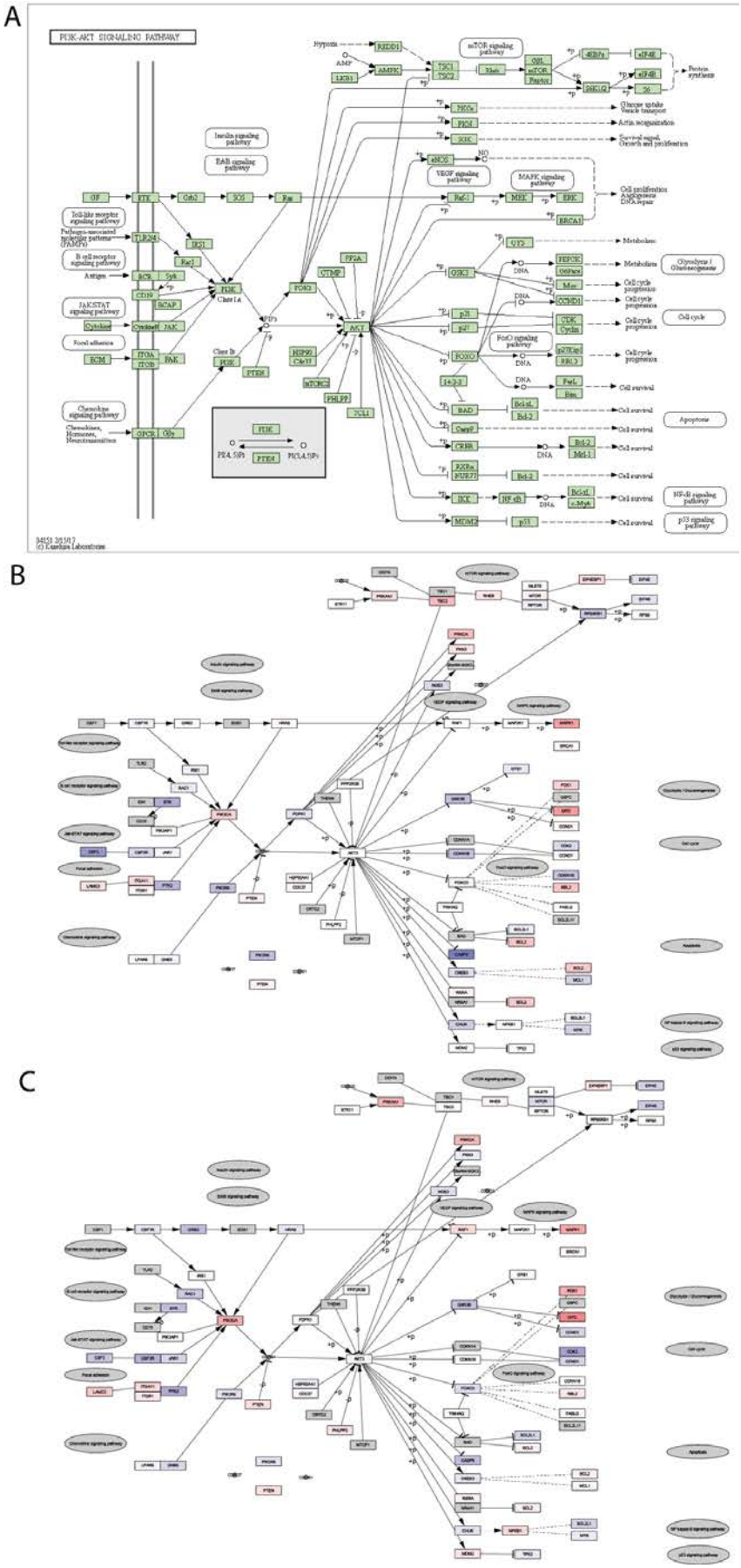

Figure 5. *PI3K-Akt signalling pathway*

A) shows the PI3K-Akt B signalling pathway diagram as represented by Kyoto Encyclopedia of Genes and Genomes (KEGG). B and C show the same pathway as well, however the lay-out differs somewhat from the KEGG representation. In B genes are coloured by fold change (ANT vs. unchallenged control) and in C genes are coloured by fold change (ANT vs challenged control). Where blue denotes down-regulated genes and red denotes up-regulated genes, grey denotes not measured by our transcriptomic analysis. See Appendix B for more detailed explanation and pdf version for better resolution.

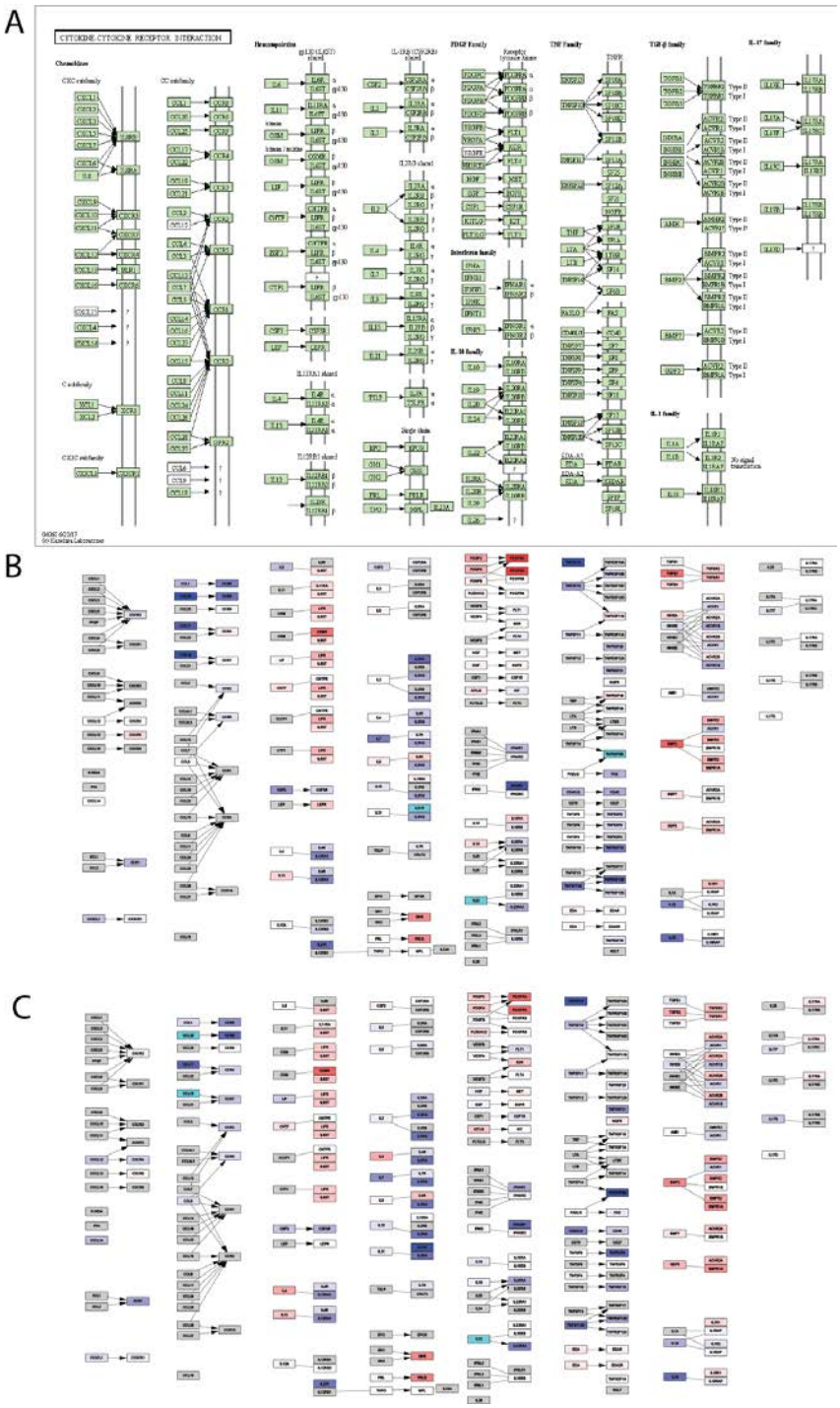

Figure 6. *Cytokine-cytokine receptor interaction*

A) shows the Cytokine-cytokine receptor interaction diagram as represented by Kyoto Encyclopedia of Genes and Genomes (KEGG). B and C show the same pathway as well, however the lay-out differs somewhat from the KEGG representation. In B genes are coloured by fold change (ANT vs. unchallenged control) and in C genes are coloured by fold change (ANT vs challenged control). Where blue denotes down-regulated genes and red denotes up-regulated genes, grey denotes not measured by our transcriptomic analysis. See Appendix B for more detailed explanation and pdf version for better resolution.

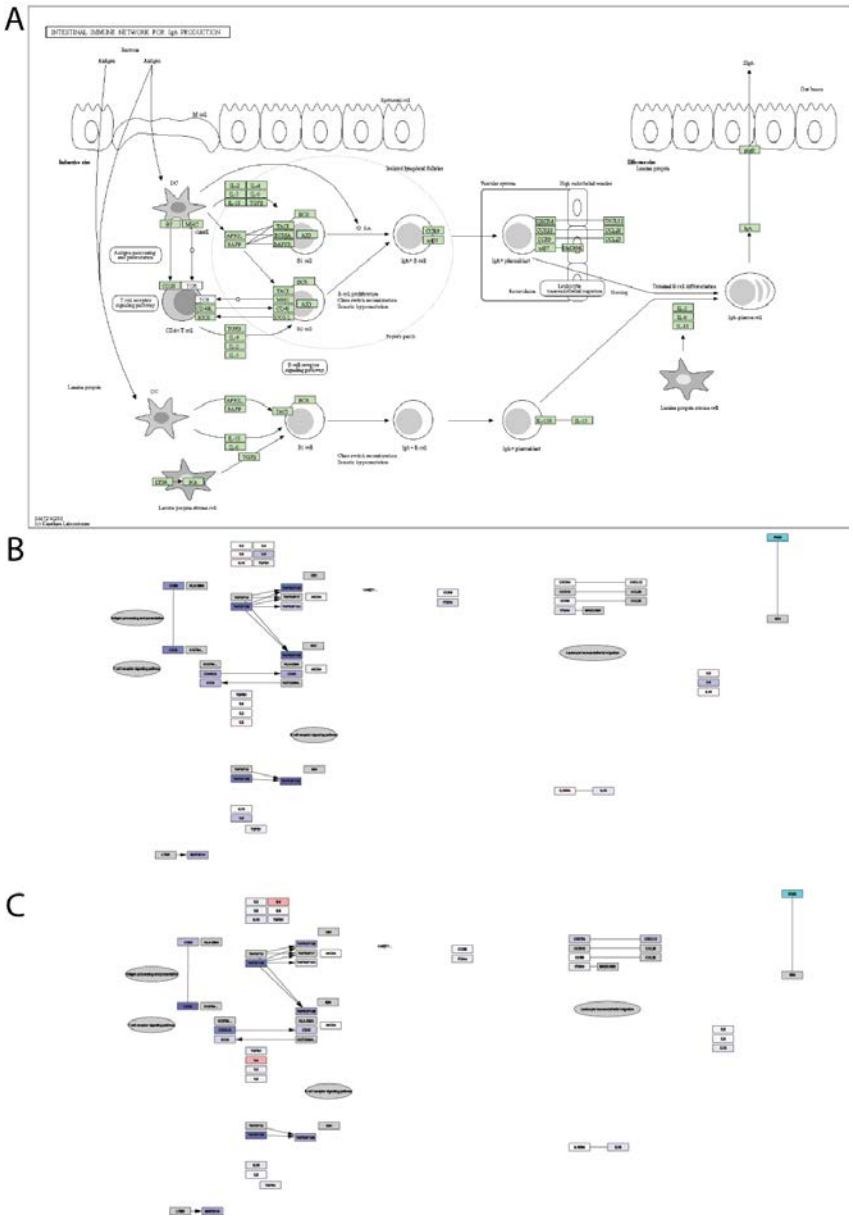

Figure 7. *Intestinal immune network for IgA production*

A) shows the intestinal immune network for IgA production pathway diagram as represented by Kyoto Encyclopedia of Genes and Genomes (KEGG). B and C show the same pathway as well, however the lay-out differs somewhat from the KEGG representation. In B genes are coloured by fold change (ANT vs. unchallenged control) and in C genes are coloured by fold change (ANT vs challenged control). Where blue denotes down-regulated genes and red denotes up-regulated genes, grey denotes not measured by our transcriptomic analysis. See Appendix B for more detailed explanation and pdf version for better resolution.

3.4 Effect of feed interventions on jejunal histology

Jejunal histology was measured on 7 and 14 d of age (Tables 11 and 12). At 7 d of age, broiler chickens that received the antibiotic treatment had a lower crypt depth and higher villi to crypt ratio (V:C) compared to the control treatment, whereas the number of goblet cells was not affected. A tendency was observed for lowered villi length comparing the antibiotic group versus the control group. Jejunal histology did not differ amongst any of the other (dietary) treatments.

Table 11. *Jejunum histology results for different (dietary) treatment groups of broiler chickens at 7 d of age*

	CON mean	CON sd[2]	CON_NE[1] mean	CON_NE sd	ANT mean	ANT sd	RYE mean	RYE sd	BG mean	BG sd	BUTY mean	BUTY sd
No. of goblet cells per mm2	1411	371.4	1528	432.8	1435	348.3	1542	354	1383.8	390.9	1654.5	502.1
Crypt depth (µm)	111.2	21.7	121.4	28.9	85.6	11.8	126.7	36.9	107.4	29.3	104.7	18.7
Villi length (µm)	501.2[a]	101.2	519.4[a]	133.5	437.6[b]	92.3	539.2[a]	112.4	465.6[a]	124.6	509.6[a]	105.9
V:C ratio	4.6[a]	0.7	4.4[(a)]	1.1	5.1[(b)]	0.9	4.4[a]	1	4.4[a]	0.8	4.9[a]	1

[1]Note that these birds were not challenged yet. [2] sd = standard deviation. Abbreviations used: CON, unchallenged control (n=12); CON_NE, challenged control (n=12); ANT, antibiotics (n=12); RYE, rye (n=12); BG, beta-glucans (n=12); BUTY, coated butyrate (n=12).
[a, b] Means in a row with different superscripts differ significantly (P<0.05) from (un)challenged controls.
[(a), (b)] Means in a row with different superscripts between brackets indicate a trend (0.05<P<0.10) from (un)challenged controls.

At 14 d of age, broiler chickens from the control treatment with NE challenge had deeper crypts and a lower villi to crypt ratio compared to the negative control, whereas villi length and the number of goblet cells was not affected. Jejunal histology did not differ amongst any of the other (dietary) treatments.

Table 12. *Jejunum histology results for different (dietary) treatment groups1 of broiler chickens at 14 d of age*

	CON mean	CON sd[1]	CON_NE mean	CON_NE sd	ANT mean	ANT sd	RYE mean	RYE sd	BG mean	BG sd	BUTY mean	BUTY sd
No. of goblet cells per mm2	1563.1	317.2	1360.9	398.8	1402	365.3	1345.9	591.4	1256.5	396.1	1461.2	386.8
Crypt depth (µm)	149.6[b]	36.7	245.6a[a]	50.3	203.9[(c)]	66.7	234.2[a]	58.3	239.5[a]	54.1	230.6[a]	51.4
Villi length (µm)	610.7	90.2	524	88.4	558	103.2	543.8	113.6	566.8	170.4	586.5	127
V:C ratio	4.3[b]	1	2.2[a]	0.7	3[a]	1.1	2.5[a]	0.8	2.5[a]	0.9	2.7[a]	0.8

[1]sd = standard deviation. [2]CON, unchallenged control (n=12); CON_NE, challenged control (n=12); ANT, antibiotics (n=12); RYE, rye (n=12); BG, beta-glucans (n=12); BUTY, coated butyrate (n=12).
[a,b] Means in a row with different superscripts differ significantly (P<0.05).
[(c)] Means in a row with different superscripts between brackets indicate a trend (0.05<P<0.10).

3.5 Effect of feed interventions on lesion scores

The probability for a higher lesion score for *E. Maxima* and *C. perfringens* at 14 and 15 d of age was not affected by (dietary) treatment (Table 13).

Table 13. *Lesion scores[1] for E. maxima and C. perfringens for different (dietary) treatment groups of broiler chickens at 14 and 15 d of age. Results are reported as the probability for a higher score*

NE challenge	No	Yes	Yes	Yes	Yes	Yes		
Diet treatment (0-7d)	Control	Control	Antibiotic	Rye	β-glucans	Butyrate	SEM	P-value
E. maxima, d14	0.31	0.28	0.16	0.46	0.35	0.47	0.13	0.201
C. perfringens, d14	0.32	0.3	0.22	0.36	0.22	0.4	0.11	0.601
E. maxima, d15	0.89	0.8	0.71	0.87	0.68	0.79	0.25	0.483
C. perfringens, d15	0.22	0.28	0.17	0.19	0.17	0.29	0.11	0.779

[1]*E. maxima* lesions scored on scale 0-4: 1= One or some haemorrhages, 2= Several haemorrhages, 3= Many haemorrhages, orange mucus in the lumen, 4= Many haemorrhages, mucosal damage (free blood in the lumen). *C. perfringens* lesions scored on scale 0-4: 1= One to five lesion present in the intestine, 2= More than 5 single distinguished lesions, 3= Lesions merge or extend to a surface of more than 1 cm^2, 4= Pseudo membranes present in the intestine causing death.

In addition to the probability scores of having higher lesion scores, we also generated a frequency table and calculated the percentage of positive birds (birds having lesions (Table 14). For the *Eimeria maxima* related lesions, the challenged control shows 75% birds positive for lesions, where the antibiotic group is lowest (63%) and the rye group highest (95%). For the *Clostridium perfringens* related lesions the challenged control shows 42% birds positive for lesions, where the antibiotic group is lowest (38%) and the butyrate group highest (63%).

Table 14. *Lesion scores[1] for E. maxima and C. perfringens for different (dietary) treatment groups of broiler chickens at 14 d of age. Results are reported as frequency or percentage positive of total.*

Score *Eimeria maxima* Treatment	ND	0	1	2	3	4	POS	TOTAL	%POS
CON	2	6	4	12	0	0	16	22	72.73
CON_NE	8	4	6	5	1	0	12	16	75.00
AMOX	5	7	7	5	0	0	12	19	63.16
RYE	5	1	7	10	1	0	18	19	94.74
BG	4	4	6	9	1	0	16	20	80.00
BUTY	3	3	4	13	1	0	18	21	85.71
Score *Clostridium perfringes* Treatment		**0**	**1**	**2**	**3**	**4**	**POS**	**TOTAL**	**%POS**
CON		8	4	9	3	0	16	24	66.67
CON_NE		14	1	0	3	6	10	24	41.67
AMOX		15	2	2	1	4	9	24	37.50
RYE		11	2	3	3	5	13	24	54.17
BG		14	4	1	2	3	10	24	41.67
BUTY		9	3	2	7	3	15	24	62.50

Abbreviations used: CON, unchallenged control; CON_NE, challenged control; ANT, antibiotics; RYE, rye; BG, beta-glucans; BUTY, coated butyrate.

4 Discussion

4.1 NE Challenge

Till date, necrotic enteritis is one of the most significant threats for the poultry industry. In clinical form it causes high mortality and in subclinical forms it affects growth and feed conversion. Necrotic enteritis is a multi-factorial disease process, in which a number of co-factors are usually required to precipitate an outbreak of the disease. Although *C. perfringens* has been identified as the etiological agent of the disease, the predisposing factors that lead to over-proliferation of *C. perfringens* and the subsequent progression to disease are poorly understood [17].

For the current study, the NE challenge model included an infection with *E. maxima* to predispose the intestinal tract for a subsequent *C. perfringens* infection. The aim of the challenge was to induce a subclinical infection, affecting growth performance but not mortality. Growth performance was indeed affected due to the NE challenge, as challenged broiler chickens fed the control diet had a reduced average daily gain and feed intake, and a worsened gain to feed ratio compared to unchallenged chickens fed the same diet. As intended, mortality was not affected by the NE challenge. The effect of the NE challenge on growth performance was significant from 7 to 16 d of age, where after birds gradually recovered from the challenge. The impaired BW development as a result of the NE challenge could not be fully compensated for during the experimental period, resulting in a BW difference of 150 g between the challenged and unchallenged birds at 35 d of age. These findings are in line with other studies using the NE model [64, 65].

For all treatments, including the unchallenged control treatment, intestinal lesions were scored at 14 and 15 d of age. Also in the unchallenged broiler group, chickens had lesions, and probably got infected. Although it may be hypothesized that unchallenged broiler chickens may have gotten infected through the (supposed to be sterilized) inoculum they received, this appears to be unlikely. Hygiene measures applied during the execution of the study were intended to prevent any contact between challenged and unchallenged birds (using pen dividers to prevent excretion from one into another pen), as well as transmission of spores from one pen to the other during measurements (bird weighing, etc.). Nevertheless, as challenged and unchallenged broiler chickens were housed in the same room, transmission may also have occurred through, e.g., dust particles in combination with ventilation.

4.2 Antibiotics

Broiler chickens that received antibiotics during their first week of life, had a higher body weight gain and feed intake compared to birds fed the control diet during this period, whereas gain to feed ratio was only numerically improved. Compared to the challenged control treatment, the numerically higher feed intake and body weight gain as a result of the antibiotic treatment remained until 16 d of age, where after both treatments performed similar unto the end of the experiment. The used antibiotic, amoxicillin, is a moderate-spectrum β-lactam antibiotic and has proven effects on susceptible Gram-positive and Gram-negative bacteria [19]. Because of this, it was hypothesized that amoxicillin supplementation could improve growth performance of the chicken, which indeed was the case during the period of supplementation. Many studies showed improved growth performance in antibiotic supplemented broilers [66, 67]. In the study of Salim et al. [66], in which broiler chickens were supplemented with virginiamycin up to 35 d of age, however, feed intake and feed conversion ratio were only improved during the first week of life, whereas body weight gain was improved up to 21 d of age. Broiler chickens fed the virginiamycin also had higher plasma immunoglobulin levels at 35 d of age compared to the control group, which might contribute to the improved growth performance.

In a study in which 3-week old turkeys were inoculated with several viruses and bacteria to induce respiratory diseases, a five-day treatment with amoxicillin, compared with the untreated group, did not cause a significant reduction in any of the measured immune related parameters [68]. The authors suggested that the bactericidal effect of amoxicillin is being diminished by its less efficient distribution throughout tissues and its relatively fast elimination, as compared to enrofloxacin and florfenicol, which were also included in that study.

In addition to the effect of antibiotics on performance, it has already been shown that a single therapeutic dose of amoxicillin for 24 hours in day old chicks has a negative effect on immune development [69]. In that study, numerically higher microbial diversity was observed in the antibiotic treated group at 5 d of age, as well as lower gene expression of immune related processes at 5 d of age, and subsequently a decreased number of macrophage-like cells in the mucosa at 14 d of age. In the present study, amoxicillin was administered from 0 to 7 d of age in the drinking water, and therefore a continuous pressure on the resident microbiota was established. Also a numerically higher diversity in microbiota (Shannon index) was observed in the current study, contrary to a previous study [69]. In the jejunum, *Enterococcus* was decreased to 0.3% ARC (4.9% ARC in CON). *Enterococci*, lactic acid producing bacteria, are important colonizers immediately after hatch, and up to 70-80% ARC is observed for different genetic lines (Cobb) [70]. In humans they are abundant and of commensal nature, however, they are also potentially pathogenic and have intrinsic (and acquired) mechanisms for antibiotic resistance and virulence factors [71]. Thus far, it is not known what the specific role of these bacteria is in the programming of the immune system.

Contrary to *Enterococci*, *Enterobacteriaceae* were increased in ANT 6.6% ARC compared to the unchallenged control 1.0% or challenged control 0.4% ARC. Members of the Enterobacteriaceae family are 'normal' residents of the gut. However, some of the species can produce endotoxins, which could be harmful to the host. Only the genus was identified in data analysis for the current study and therefore it is not possible to further characterize or interpret this without too much speculation.

Growth performance and microbiota composition in the jejunum at 7 d of age were significantly different between the antibiotic and control treatment, which was also reflected in the gene expression results. Multiple pathways were significantly enriched when comparing the two treatments and by superimposing the fold change data, striking differences in specific pathways of interest, i.e. PI3K-AKT, NFkB signalling pathway, cytokine-cytokine receptor interactions, and intestinal immune network for IgA production were observed. The PI3K-AKT pathway is involved in regulating cell cycle. The PI3K-AKT pathway is activated by many types of cellular stimuli or toxic insults and regulates fundamental cellular functions such as transcription, translation, proliferation, growth, and survival. These pathways can also regulate tight junctions in the gut by Toll-like receptor 2 (TLR2) [72] and play a key role in the modulation of immune responses [73, 74]. Thus, the relative higher expression for the antibiotic treatment could indicate that the barrier function is enhanced, or enhanced ECM regulation, or that the inflammatory response is modulated. To further investigate the latter, we also focused on the NFkB signalling pathway, cytokine-cytokine receptor interactions, and intestinal immune network for IgA production. In these pathways, a relatively lower expression of genes was observed for the antibiotic treatment compared to the (un)challenged controls. The NFkB signalling pathway regulates genes that are involved in immunity, inflammation, and cell survival. Thus, these data suggest that the antibiotic treatment has a decreased inflammation compared to control treatment. Similarly, less secretory IgA will be produced for the antibiotic treatment compared to the control treatment. Consequently, this may result in less entrapped dietary antigens and microorganisms in the mucus and neutralization of (endo)toxins [75, 76]. Whereas the cytokine-cytokine receptor interactions are involved in innate and adaptive inflammatory host response, cell growth, differentiation, cell death, angiogenesis, and wound healing. These cytokines and their receptors can be categorized by structure into different families. In our analysis we mainly observed lower expression regarding immune related cytokines in the antibiotic group compared to the (un)challenged controls, whereas higher expression was observed in families involved in ECM and/or wound healing related processes.

Taken together, the gene expression data suggest that broiler chickens from the control treatment showed higher gene expression in NFkB signalling and sIgA, and lower expression in PI3K-AKT/mTOR pathways. This could mean that there are different mechanisms for the host to maintain a certain homeostatic state during microbiota colonization and immune system programming in early life.

In the current study, broiler chickens from the antibiotic treatment had a higher villi to crypt ratio at 7 d of age compared to control chickens. Low V:C ratios, are associated with infection and are being considered as detrimental for the host [77]. Current differences in V:C ratio (5.1 vs. 4.5) may be significant, but the biological read-out is still in the range of a well-functioning gut.

In conclusion, administrating antibiotics in the first week boosts the short-term performance, changes the microbiota composition, modulates genes involved in barrier and immune function, and affects the intestinal morphology.

4.3 Rye

Rye-fed broiler chickens had a reduced daily gain during the first week of life compared to the unchallenged control treatment, whereas feed intake did not differ and the gain to feed ratio only tended to differ. The reduction in daily gain was in line with our expectations, because dietary inclusion of rye will increase viscosity of intestinal digesta, consequently impairing digestibility and absorption of dietary nutrients and leading to a depression in growth performance [21-23]. After the first week of age, none of the growth performance parameters of the rye treatment differed from the challenged birds fed the control diet. This indicates that feeding the rye-enriched starter diet had only temporary effects on growth performance. In literature, no experiments could be found that studied the temporarily supplementation of rye in broilers, meaning that it was not possible to compare current performance results with that of other findings.

Microbiota diversity and overall composition did not significantly differ between the rye treatment and the controls. However, the focused investigation on genera level, resulted in 13 significantly different bacterial genera, dominated by members of *Clostridia* and *Bacilli*. Quantification of the microbiota by qPCR resulted in 10-fold more microbiota in the rye treatment compared to the (un)challenged control treatment. It is hypothesized that this could be due to the fact that the intestinal content is more viscous, resulting in a slower passage rate and consequently change the microbiota composition [78]. However, this change in microbiota did not result in significant changes in either gene expression or histology in jejunum at 7 d of age, between the rye and control treatment.

In conclusion, inclusion of rye affected growth performance, but only during the age period it was provided and without carry over effects to later age. Certain bacterial genera were significantly affected, and the total amount of microbiota was increased compared to the control treatment.

4.4 Butyrate

In the current study, growth performance of the challenged broiler chickens fed the butyrate supplemented diet did not differ from the challenged broiler chickens fed the control diet. In contrast, another study showed that the addition of Na-butyrate, encapsulated in palm fat, to the feed of experimentally infected broilers with NE resulted in increased final body weight, at 35 d of age, whereas there was a tendency to reduced total feed consumption, improved feed conversion ratio, reduced cumulative mortality, and increased production number compared to the infected broilers fed the control diet [79]. In that study, however, broiler chickens received the butyrate supplemented diet during the whole experimental period (0 to 35 d of age), whereas in the current study the butyrate was fed only for the first 7 days. Apparently, there was no preventive effect of the short-term provision of butyrate on performance at later age, when challenged with necrotic enteritis. In a study of Jerzsele *et al.* [80], however, BW of NE infected broiler chickens fed coated butyrate during the entire experiment was not different compared to the BW of infected birds fed the control diet.

The microbiota diversity and overall composition at 7 d of age were not significantly affected by the butyrate treatment, in both jejunum and colon. When testing for individual bacterial genera, four genera in the jejunum and four genera in the colon were observed that were significantly different between the butyrate and control treatment. In the jejunum the following four were significanlty different: *Clostridiaceae candidatus.arthromitus*, *Lachnospiraceae blautia*, and *Lachnospiraceae dorea,* and a non-classified species. Whereas in colon, *Enterococcacea*e, *Clostridaceae* SBM53, *Veillonellaceae Veillonella* and *Enterobacteriacea*e were significantly different. All these observed bacterial groups encompass large families with diverse functions and are therefore not helpful in the biological interpretation, in other words there is still much functional annotation necessary to make these data more valuable. Gene expression and jejunum histology at 7 d of age did not show any significant effects between the butyrate and control treatment.

In conclusion, inclusion of butyrate did not affect growth performance. However, certain bacterial genera were significantly affected, but still lack the proper functional annotation.

4.5 Beta-glucans

In the current study, growth performance parameters of challenged broiler chickens fed the β-glucans supplemented diet did not differ from the challenged broiler chickens fed the control diet. Contrary to these findings in our study, in a study of Tian et al. [81], NE infected broiler chickens fed a β-glucan-supplemented diet had significantly increased body weight (13 to 21 and 0 to 42 d of age), improved feed efficiency (13 to 21 and 21 to 42 d of age), increased antibody levels against *C. perfringens,* and improved villi height and villi height to crypt depth ratio. In addition, infected broiler chickens that were supplemented with β-glucans had markedly reduced intestinal *C. perfringens* counts, and NE related lesion scores in the gut compared with infected broiler chickens fed the control diet. In that study [81], however, broiler chickens received the supplemented diet during the entire grow-out period (0 to 42 d of age), whereas broiler chickens in the current study were fed the β-glucans only for the first 7 d of age. Apparently, there was no effect of the short-term provision of β-glucans on performance at later age, when challenged with necrotic enteritis.
Microbiota diversity and overall composition did not significantly differ between the β-glucans and control treatment. However, when zooming into the bacterial genera level we did observe a significant difference in the genus *Lactobacillus*. The β-glucans treatment showed a decreased ARC (78.4%) compared to the unchallenged and challenged controls, respectively 88.7% and 89.2% ARC. We observed a significant increase in the ARC of *Enterococcaceae* in the antibiotic group compared to the unchallenged controls, however the ARC were relatively low around 0.01%. Like *Lactobacillus, Enterococcaceae* are also lactic acid producers, this suggests that maybe the decrease in *Lactobacillus* is compensated by an increase in *Enterococcaceae*. Gene expression and jejunum histology at 7 d of age did not show any significant effects between the β-glucans and control treatment.
In conclusion, inclusion of β-glucans did not affect growth performance. The observed difference in the genus *Lactobacillus* probably also does not functionally affect the gut system.

4.6 Quantitative disease phenotype

Despite the clear differences in the antibiotics group compared to the control group, in for example intestinal microbiota composition, host gene expression, and gut morphology, no significant differences were observed in the quantitative disease phenotype. However based on the birds positive for lesions, the antibiotic group scored lowest for lesions originating from both *Eimeria maxima* and *Clostridium perfringens,* respectively 12% and 4% lower than the challenged control. In addition, the antibiotic group also showed no birds or the least amount of birds having a severe score compared to all other groups including the challenged control.

It could be that the NE challenge model is too severe for the gut system and therefore no differences were observed when comparing challenged controls versus challenged birds that received antibiotics. A limitation to the correlation of the immune competence status to the quantitative disease phenotype is that the sacrificed birds on day 7 are of course different from the sacrificed birds for the quantitative disease phenotype on day 14.

4.7 Concluding remarks and future research

Except for the antibiotic treatment, providing diets enriched with rye, beta-glucans or coated butyrate during the first week of age, followed by an NE-challenge, did not show a programming effect on immune competence-related parameters.
The other objective of investigating the correlation between immune competence parameters before the NE challenge and the read-out of the NE challenge, so called quantitative disease phenotype, was not apparent. In retrospect, maybe the applied 'synchronized' challenge was too harsh on the gut system to measure the 'small' differences in immune competence by the different feed interventions.
Possible next steps could be to investigate the relation between the host gene expression and the gut microbiota, so-called correlation analysis. In addition, a data mining approach would also be worthwhile because of the plethora of data generated within this project. Furthermore, the current project investigated the immune competence development in great detail, it could also be interesting to see the different (immune) responses of the host on day 15 regarding the host gene expression and gut microbiota, in other words the 'molecular side' of the quantitative disease phenotype. In addition to the more local derived phenotypic aspects, mostly regarding (parts of the) gut, it is possible to generate data in blood. With these blood metabolite profiles one could investigate the systemic effects of the (dietary) intervention.

The results obtained in this study were used as input for the VDI-10 project in which a 'ruler' for immune competence has been designed and further tested. For example, the transcriptomics and microbiota data are fully integrated in the VDI-10 project, and also re-analysed to make these data comparable to the other datasets. The histology results are also used to fill the immune competence 'ruler'.

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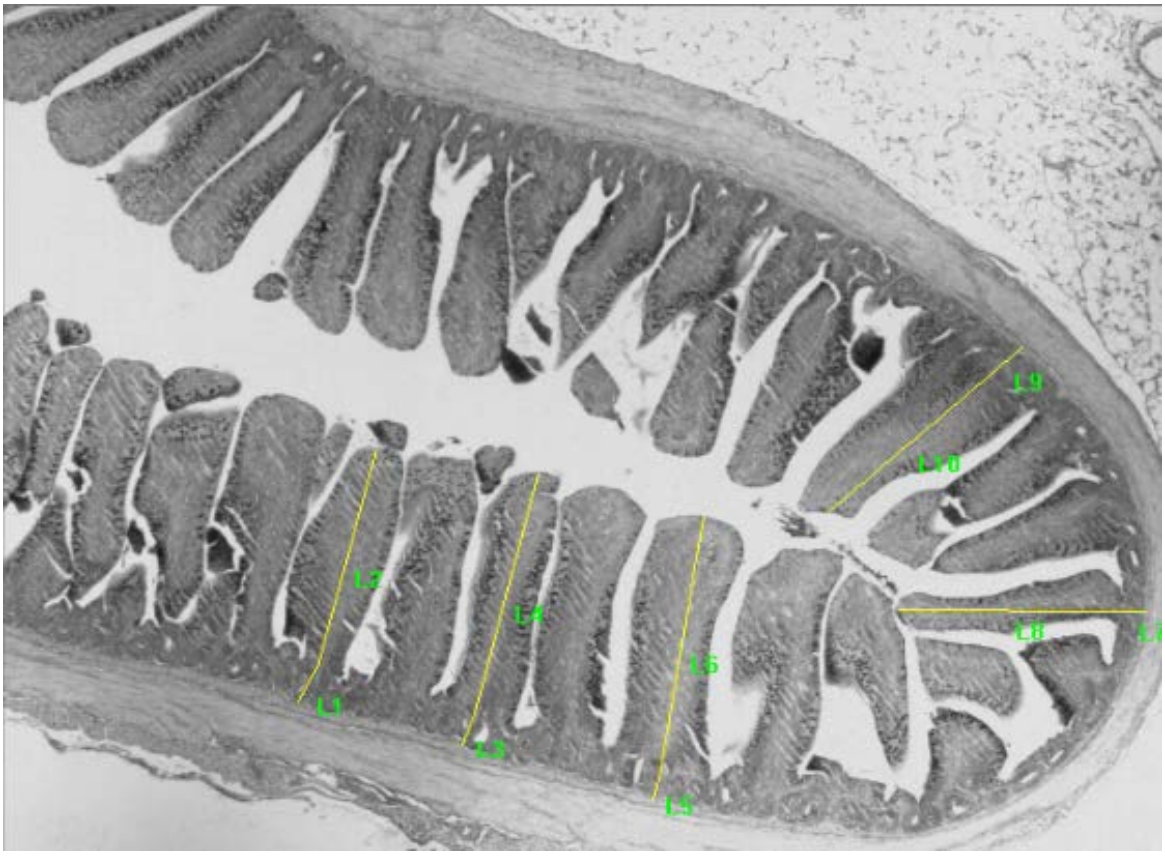
Appendix A

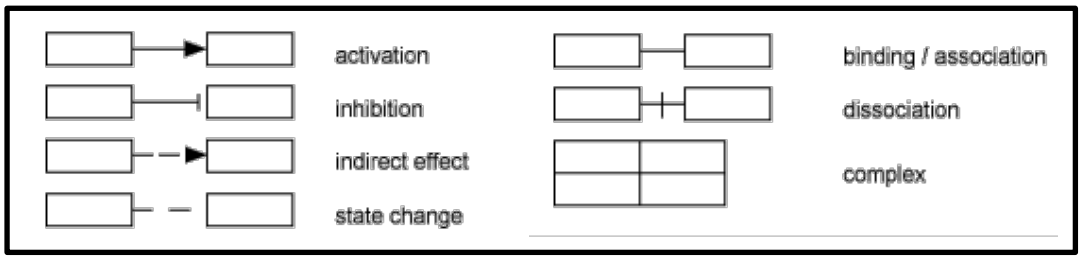
Figure legend: *Cross-section of jejunum of broiler (day 7)*



The yellow lines indicate either crypt depths (L1, L3, L5, L7, L9) or villi height (L2, L4, L6, L8, L10) in µm.

Appendix B

More explanation of Figures 4-7.
The top panel shows the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway of interest (indicated in the top left box), the green rectangles indicate genes or gene complexes, open circles indicate other molecules (mostly chemical compounds), and the white boxes with rounded edges depict links to other pathways. Solid arrows indicate molecular interactions, whereas dashed lines indicate indirect effects. Within the protein-protein interactions (between boxes) different activities are distinguished, +p is phosphorylation, -p dephosphorylation, +u is ubiquitination, +g is glycosylation, +m is methylation, see box below for the other activities.



In the bottom panel, a replica is generated based on the KEGG pathway, however small differences in lay-out can occur. The rectangles are now coloured based on their fold change between the antibiotic and control group. Blue denotes down-regulated genes and red denotes up-regulated genes, more intense colours depict a higher value i.e. down-regulation or up-regulation. Grey rectangles denote that these genes were not present on our platform, in other words they were not measured by our transcriptomic analysis. The bottom panel shows in one overview that different parts of a pathway can be up- or down-regulated and because different parts of a pathway can link to different biological functions. These representations help in further interpreting the underlying biological mechanisms.

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

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