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The purpose of this experiment was to evaluate the effects of five nutritional interventions, provided during d 14 – 28, including inclusion of a plant extract (quercetin); an insoluble fiber (oat hulls); a prebiotic (β-glucan); an anti-microbial protein (lysozyme), and ω-3 fatty acids from fish oil, on growth performance, composition of the intestinal microbiota, and morphology and gene expression of small intestine of broilers. Despite the different types of interventions, parameters related to immune competence were only marginally affected by the tested products. It seemed that in this study inclusion of oat hulls, and probably β-glucans, had perspective to improve immune competence. It is recommended to revaluate some of the tested interventions, especially dietary inclusion of oat hulls and β-glucans, in broilers starting from day-old onward.

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The ISO 9001 certification by DNV underscores our quality level. All our research commissions are in line with the Terms and Conditions of the Animal Sciences Group. These are filed with the District Court of Zwolle.

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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 the Animal Sciences Group of Wageningen UR. Feed4Foodure aims to contribute to sustainable and healthy livestock farming in the Netherlands, simultaneously strengthening our competitive position on the global market. The Feed4Foodure program line “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, and the use thereof in innovative products and diet compositions.

The livestock sector faces challenges to sharply reduce antibiotic consumption. The program Nutrition - Intestinal Health - Immunity (VDI) strives to reduce antibiotic treatment in animals through nutritional interventions, which should defense against infectious diseases. The purpose of this experiment was to evaluate the effect of five different nutritional interventions of broilers after the neonatal period (d 14-28) on the performance, composition of the intestinal microbiota, intestinal morphology and gene expression in small intestinal tissue. In the present experiment, the effect of several dietary interventions provided during d 14 – 28 of age on parameters related to immune competence of broiler chickens was examined. This experiment was performed within the framework of the Feed4Foodure program line “Nutrition, Intestinal Health, and Immunity”.

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, Cargill, DSM, Groep van Zes, DIVA, and Centrico. The authors thank the industry partners of the project team for their worthwhile input.

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

Summary

The livestock sector faces challenges to sharply reduce antibiotic consumption. The program Nutrition - Intestinal Health - Immunity (VDI) strives to reduce antibiotic treatment in animals through nutritional interventions, which should improve immune competence. This experiment was conducted to investigate the effects of five nutritional interventions between 14 and 28 days of age on broiler performance (d 14 – 35), and immune competence related parameters as composition of the intestinal microbiota (d 21 and 28) , and gene expression in gut tissue (d 21 and 28). A total of 1,008 one-day-old male Ross 308 chicks was randomly distributed over 36 floor pens (28 birds per pen). Birds were allocated to one of the six (five dietary interventions besides a control treatment) iso-caloric (ME_N 13 MJ/kg) experimental growing diets (d 15-28), with six replicate pens per treatment. Commercial starting and finishing diets were used from d1-d14 and d29-d35 of age, respectively. A high level (25%) of rapeseed meal, containing glucosinolates as antinutritional factor, was included to the diets to provide a nutritional challenge. Five various nutritional interventions, differing in mechanism of action, including a plant extract (quercetin, 400 mg/kg); an insoluble fiber (oat hulls, 50 g/kg); a prebiotic (β-glucan, 100 mg/kg); an anti-microbial protein (lysozyme, 40 mg/kg) and ω-3 fatty acids from fish oil (40 g/kg) were applied in the growing phase (d 15-28). Dissection was done on 6 birds/pen on d21 and d28 of age. Feed intake of broilers fed the diet including oat hulls and lysozyme was reduced during the first week of the growing period (d14-21) compared to other groups. Birds adapted to consume diets including oat hulls and lysozyme after the first week of consumption, and feed intake was not affected by treatment after that (d22-28, d14-28 and d29-33). During the first week of the growing period, a trend of decreased BWG of broilers fed the diet including lysozyme (compared to the control $P<0.05$), oat hulls and fish oil (compared to the control $P>0.05$) was observed, but over the second week of the growing period (d22-28), broilers fed the diet including lysozyme showed increased BWG compared to the control and birds fed diets including quercetin and β-glucan ($P<0.05$). Broilers fed the diets including fish oil and oat hulls also showed increased BWG during the second week of the growing period, but the differences with other groups were not significant. It seems that broilers fed diets including lysozyme, oat hulls and fish oil showed compensatory growth in the second week of the grower period. Considering the whole growing period, there was no significant difference in BWG between nutritional interventions. A trend of increased FCR in broilers fed the diets supplemented by lysozyme ($P<0.05$) and oat hulls ($P>0.05$) was observed during the first week of the growing period. Also an improved FCR in broilers fed the diets supplemented by lysozyme, oat hulls and fish oil was observed during the second week of growing period; nevertheless over the whole growing period, no significant effect of dietary treatment on FCR was observed. No carry-over effect of dietary treatment on BWG, feed intake and FCR was observed in the finishing period. Compared to the control diet, litter score improved by feeding oat hulls and fish oil (d28), and oat hulls and lysozyme (d33). On d21, hierarchical clustering of the group-averaged microbiota data showed no meaningful effect of dietary interventions based on the underlying taxonomic profiles. Alpha diversity by Shannon Index showed no differences between jejunum and ileum. Principal component analysis on microbiota showed that the oat hulls treatment was separated from data from the other experimental treatments, which were all centred around the origin, which is a general indication that the microbiota of the oat hulls fed birds differed from the other treatments. More specifically, a significant taxon-treatment association was found in the ileum within the genus Enterococcus, which was significantly higher in the oat hulls treatment compared to the control and fish oil fed birds, mainly at the expense of Lactobacilli. No effect of treatments on villus height of jejunum on d21 and of ileum (d21 and 28) was observed. Villus height of lysozyme fed birds in jejunum at d28 was decreased compared to the control. No effect of treatments on crypt depth of jejunum on d21 and of ileum was observed. Jejunum crypt depth of the birds fed lysozyme in d28 was decreased compared to the control. In jejunum (d21 and 28), the total surface area of villi occupied by goblet cells (μm^2) and average total villi surface area (μm^2) were higher in chickens fed diet included oat hulls compared with other dietary interventions. Only marginal effects of dietary interventions on the expression of genes were observed. Compared to the control birds, genes related to growth-factor-activity (directly involved in wound healing processes) were expressed more in the birds fed the β-glucan included diet, while the genes related to anion-transmembrane-transporter-activity in the broilers fed the quercetin and oat hulls included diet were expressed less. In conclusion, dietary inclusion of oat hulls and lysozyme resulted in temporary reduction in performance. Dietary oat hulls inclusion slightly affected overall microbiota composition within and between jejunal and ileal digesta.

Limited effects of nutritional interventions were observed on gene expression of the gut tissue, and the genes differently expressed between dietary treatments do not seem to be directly involved in immune related processes.
Despite the different types of interventions, parameters related to immune competence were only marginally affected by the tested products. It seemed that in this study inclusion of oat hulls, and probably β-glucans, had perspective to improve immune competence. It is recommended to revaluate some of the tested interventions, especially dietary inclusion of oat hulls and β-glucans, in broilers starting from day-old onward.

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1 Introduction

1.1 Immune competence and nutritional immunomodulation

Immune competence is defined as the ability of the immune system to respond adequately on an antigenic stimulus by an appropriate immune response with a balance between tolerance and inflammation. Since the immune system is highly complex and tightly regulated, a profound understanding of how the avian immune system works is necessary to take full advantage of immune competence. The current state of knowledge of avian immunity lags behind that of many mammalian species. This topic may become more important to the poultry industry, given the current interest in using non-drug interventions to support the health and rapid and efficient growth in poultry. Appropriate nutrition becomes even more critical as anti-bacterials, anti-parasitics and other additives that promote animal health are eliminated due to consumer demands. Nutrition may aid in minimising the incidence of diseases by enhancing immune competence. It can be questioned, however, whether the diets in use today for realizing maximal production efficiency are sufficient for optimal immunity. Klasing (2007) distinguished six different mechanisms by which diet might affect immunity: 1) feeding the cells of the immune system, 2) feeding pathogens, 3) modifying the responses of leukocytes, 4) protecting against immunopathology, 5) influencing the microbial ecology of the gut, and 6) stimulating the immune system. In general, the immune system of the bird can be influenced by nutrition in ways of anatomical development of lymphoid tissues, mucus production, synthesis of immunologically active substances, cellular proliferation, cellular activation and movement, intracellular killing of pathogens, modulation and regulation of the immune process (Gershwin et al., 1985; Klasing, K. C. 1997). Nutritional immunomodulation represents a rational goal to allow efficient production of healthy birds. One of the major factors driving interest in the development of nutritional immuno-modulation is the removal of sub-therapeutic dietary antibiotics from poultry diets in many parts of the world, and consumer and legislative pressures to reduce or eliminate antibiotic growth promoters use elsewhere. The use of antibiotic growth promoters in poultry diets may influence immune function in at least reducing the interaction of GI tract bacteria with the immune system of the bird suggesting that one of the mechanisms of action of it is to reduce the reliance of the bird on its own immune system to prevent clinical or subclinical disease (Korver, 2012). Consequently, reduced exposure of the bird to bacteria would reduce activation of the inflammatory response, and therefore the associated growth-suppressive effects. In an environment where growth-promoting antibiotics are not used, the bird would be more dependent upon its own immune system to avoid bacterial infections. The second manner is the environment under which many genetic selection programs for poultry take place. Commercial genetic selection under high sanitation may reduce the ability of poultry to respond to the diversity and intensity of field disease challenges. Thus, the need for a highly responsive inflammatory response may have been masked, and may have inadvertently been selected against.

1.2 Specific feed ingredients and omics techniques

The immuno modulatory effects of specific feed ingredients is assumed to be related to some functional components in these feed matrices. The mode of action of these components, however, is often unknown. Integration of “omics” techniques like transcriptomics, proteomics, and metabolomics, could fill in this knowledge gap. The data gained with these techniques would significantly contribute to the elucidation of the mechanisms how specific components and/or ingredients in feed positively influence functional processes in the GI tract, and with this, the overall performance of farm animals. Combined information about gene-chemical associations retrieved from on-line available databases can be used to predict the *in vivo* biological activity of natural occurring chemical compounds, i.e. predict their “defined mode of action”. Or contrariwise, this information can be used to identify compounds that have potential to influence the function of specific genes/proteins or biological processes. Such a data-mining approach has already accelerated the development of alternative or new dedicated supplements/additives for human foods. *In vivo* experiments are widely used to investigate the effects of nutritional interventions on immune related parameters in animals. In these studies animals are used as target animals, or as a model for humans. *In vivo* experiments, however, might increase the level of discomfort of the animals, are time and labour intensive, and costly. For some research questions, other types of experiments can be used as well, thereby lowering the level of disadvantages compared to *in vivo* experiments.

Based on the available literature, it was hypothesized that nutritional interventions resulting in better protection against potential pathogens, including improving intestinal integrity, improving the functioning of the local immune system in the intestine, gut wall morphology and composition of microbiota, with the result that there is less need for the immune system to combat against antigens via severe inflammatory reactions. Table 1, based on a report by Van Krimpen et al. (2014), shows the effects of some dietary interventions during the post-neonatal phase on immune competence related parameters.

Table 1 *Selection of nutritional interventions and their described effects (Van Krimpen et al., 2014) in the post-neonatal phase of monogastrics.*

Intervention	Gut Integrity	Local i.s.	Systemic i.s.	Microbiota	Performance
Glutamine	+		+		
Plants and herbs					
Chitosan			+		
Herb mixture		+	+		
Quercetin				+	+
Black cumin	+	+			+
Rice bran		+	+	+	
Alfalfa		+		+	
Probiotics					
Bacillus subtilis	+		+	+	
Lactobacillus bulgaricus		+	+		
Prebiotics/β Glucans (neonatal)	+	+			
Antimicrobial proteins					
Antimicrobial peptide-A3/P5[1]	+			+	+
Buforin II[2]	+			+	+
Lysozyme	+	+	+		+
Fish oil (maternal)		+			

[1] AMP-A3 and P5 have potential to improve performance, nutrient digestibility, intestinal morphology and to reduce pathogenic bacteria in weanling pigs

[2] Buforin II protects small intestinal mucosal membrane integrity of weaned pigs.

1.3 Nutritional interventions

In the present study, five different nutritional interventions, differing in mechanism of action, including a. a plant extract, quercetin (active substance of yellow onion), b. an insoluble fiber (oat hulls); c. a prebiotic (β-Glucan), d. an antimicrobial protein (Lysozyme), and e. Omega-3 fatty acids from fish oil were applied.

Quercetin is a flavonoid compound that belongs to the class of flavonols. It can be found in most edible fruits such as apples, mostly in peels (Manach et al., 2004), in vegetables such as red onions and capers (Bhagwat et al., 2013), and is potentially beneficial for human and animal health. It has been found that dietary quercetin can be absorbed and metabolized by rats (Crespy et al., 2001; Chang et al., 2004; de Boer et al., 2005; Gee et al., 2004), pigs (Cermak et al., 2003; de Boer et al., 2005), humans (Hong and Mitchell, 2004; Manach et al., 2004; Mullen et al., 2004; Spencer et al., 2003; Wang and Morris, 2005) and broilers (Rupasinghe et al, 2010). Rupasinghe *et al*. (2010) administered quercetin to a broiler diet as a pure substance and detected quercetin metabolites in the plasma and several tissues (liver, thigh, and breast muscle and duodenum). Many *in vitro* biological properties of quercetin and its glycosides, such as modulation of cell signalling pathways (Soundararajan et al., 2008), reduction of oxidative DNA damage (Wilms et al., 2005), lipid oxidation in rodents (Molina et al., 2003), and in vitro antioxidant activity (Arts et al., 2004) have been demonstrated. Studies in mice (Comalada et al., 2006; Hamalainen et al., 2007; Huang et al., 2010; Orsolić et al., 2004; Rotelli et al., 2003) and human (Boots et al., 2011; Sternberg et al., 2008) showed that quercetin has anti-inflammatory as well as anti-bacterial properties (Waage and Hedin, 1985).

Many feed ingredients, such as barley, oats, and soybean meal contain a considerable amount of insoluble fibre (Bach Knudsen, 1997). Dietary fibre has been considered to be a diluent in poultry diets, with negative effects on voluntary energy intake and nutrient digestibility. However, many

studies indicated that moderate amounts of fibre improve gizzard function (Hetland and Svihus, 2001; Rogel et al., 1987; Svihus, 2011), digestibility of non-fibre nutrients (Amerah et al., 2007; Amerah et al., 2009; Gonzalez-Alvarado et al., 2010; Jimenez-Moreno et al., 2009b; Mateos et al., 2012), gastrointestinal tract (GIT) health (Kalmendal et al., 2011; Mateos et al., 2012), and growth performance (Gonzalez-Alvarado et al., 2007; Jimenez-Moreno et al., 2013a) in broilers. Although it was expected that the beneficial effect of fiber is due to a slower digesta passage speed, better action of enzymes and / or killing microbes by pH reduction is also the case.

Prebiotics are antibiotic growth promotors (AGPs) alternatives having three criteria; non-digestible by host enzymes, fermentable in the gastrointestinal tract and selectivity in stimulation of intestinal microbiota and metabolic activity (Gibson *et al.*, 2004; Van Loo, 2004). Therefore, they improve the intestinal ecosystem, intestinal tissue, immunity and general host status (Gibson *et al.*, 2004). Prebiotics could alter the intestinal ecosystem via promoting competitive exclusion of pathogenic microbes and selective colonization by beneficial microbes, leading to improvement of poultry performance (Biggs *et al.*, 2007). Mannan-oligosaccharides (MOS) are mannose-rich carbohydrates, derived from yeast cell walls (Sentandreu and Northcote, 1968, 1969; Young et al., 1998), and can inhibit the growth of harmful bacteria and stimulate the non-specific immune system; activates the healthiness and growth performance of birds (Ferket, 2004), reduces serum cholesterol with no effect on the triglycerides (Yalçinkaya *et al.*, 2008). Currently, MOS products, particularly those derived from the cell wall of *Saccharomyces cerevisiae*, are extensively used as natural feed additives in livestock and poultry because of documented benefits in performance (Hooge, 2004; Rozeboom et al., 2005; Rosen, 2007) and gastrointestinal health (Spring et al., 2000; Sims et al., 2004; Baurhoo et al., 2007b; Yang et al., 2008; Baurhoo et al., 2009). Galacto-oligosaccharides is structurally similar to cell surface glycoconjugates that are used by pathogens for adherence in the gut and, in this way, protect against the colonization and growth of pathogens (Newburg, 2000; Searle et al., 2010; Shoaf et al., 2006). Prebiotics bind to dectin-1-Like receptor heterophils and monocytes, and as a result, heterophils are stimulated to increase production of cytokines. Studies have demonstrated that dietary immuno-modulators such as β-glucans show beneficial results in a wide variety of animal species (Chae et al., 2006; Guo et al., 2003). The immuno-stimulant and immuno-modulatory effects of β-glucan polysaccharides result in the regeneration of the host's ability to resist life-threatening opportunistic infections (Rieder et al., 2013). The immuno-modulatory process initiates when the β-glucan binds to cell surface receptors of macrophages, lymphocytes and neutrophils (Chen and Seviour, 2007; Guo et al., 2003). β-glucan activates B-lymphocytes and macrophages through dectin-1, CR3, lactosylceramide, scavenger receptors and Toll-like receptors (Le et al., 2011; Taylor et al., 2002), modulating the immune system and inducing the production of cytokines (Cheng et al., 2004). In the present experiment, a pure β- 1,3 glucan was added to diet that was produced by the unicellular *Euglena gracilis*. The product is widely used as a pure β-glucan in research on immune competence. The action of antimicrobial proteins is based on a direct inhibitory effect on proliferation of *Clostridium perfringens* and to the production of alpha-toxin.

Lysozyme (muramidase), one of the best known and best described animal proteins (Callewaert and Michiels, 2010), forms around 3.5% of total egg white protein (Burley and Vadehra, 1989) and has bactericidal properties (Fleming, 1922; Salton, 1957) due to its ability to hydrolyse β-linkages between N-acetylmuramic acid and N-acetyl glucosamine in the peptidoglycan cell walls of certain Gram-positive bacteria (Ohno and Morrison, 1989; Phillips, 1966). Following chemical and/or thermal modification, however, it has also been found to be bacteriostatic toward Gram-negative bacteria (Ibrahim et al., 1994; Johnson, 1994; Masschalck et al., 2001). One of the main roles of lysozyme is most probably to prevent invasion and proliferation of bacteria inside the egg during the pre-incubation and incubation periods (Board and Fuller, 1974; Wellman-Labadie et al., 2008b; D'Alba et al., 2010). Following the addition of substances like EDTA, butyplaraben or tripolyphosphate, the activity of lysozyme was enhanced enough to control growth of the Gram-negative *Escherichia coli* (*E. coli*) (Durance, 1994; Boland et al., 2003). In addition to its antimicrobial function, lysozyme has been shown to play an essential role as a general defence molecule in the innate immune system of many vertebrates due to its continuous presence in various body fluids, including mucus, ejaculate, and blood (Millet et al., 2007; Rowe et al., 2013). In most vertebrates, including birds, both plasma lysozyme concentration and activity has been observed to increase along with pathogen exposure or inflammation processes (Caruso et al., 2002; Maraghi et al., 2012; Millet et al., 2007) and is accompanied by an increased blood leukocyte profile (Figuerola et al., 1999; Maxwell and Robertson, 1998). Plasma lysozyme is not only lethal to lysozyme-susceptible pathogens but may also be involved in non-specific immune processes such as opsonisation, leading to phagocytosis of Gram-negative

bacterial invaders that are less responsive to lysozyme (Abergel et al., 2007; Callewaert et al., 2008). Evidence exists for both synergistic (Bugla-Ploskonska et al., 2008; Futoma-Koloch and Bugla-Ploskonska, 2009) interactions between lysozyme and plasma or serum complement activity. Complement represents just one of a number of bactericidal cascade mechanisms consisting of more than 30 individual plasma proteins that participate in pro-inflammatory, chemotactic and opsonic activities, thereby forming the intersection between non-specific humoral and cellular immune functions in vertebrates (Holland and Lambris, 2002).

Fish oil, derived from the tissues of oily fish, is a commonly and economic fishery sub-product, and can partially replace soybean oil in broiler diets. It contains n-3 polyunsaturated fatty (n-3 or omega-3 PUFA), such as eicosapentaenoic acid EPA (20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) (Hulan *et al.,* 1988). The n-6 PUFA arachidonic acid (ARA) is the precursor of prostaglandins, leukotrienes and related molecules, which have important roles in inflammation and regulation of immunity (Calder, 2006). Dietary supplementation with fish oil resulted in reduction of ARA in cell membranes, which was replaced with EPA, leading to decreased production of ARA-derived pro-inflammatory mediators (Calder, 2006; Cherian, 2011; Fritsche et al., 1991; He *et al.,* 2007; Kelley and Daudu, 1993; Korver and Klasing, 1997; Liu *et al.,* 2014; Parmentier et al., 1997; Yang *et al.,* 2008). In addition, it has been shown that EPA and DHA supplements in poultry diets are incorporated into egg yolks and are available to the developing embryo in the liver and residual yolk (Koppenol et al., 2014). The offspring of breeding chickens fed a diet supplemented with n-3 PUFAs showed changes in antibody and cell-mediated immune responses (Sijben et al., 2001; Wang et al., 2000, 2002). Imbalanced eicosanoid production can affect poultry health, promoting cardiac problems and sudden death (Ajuyah *et al.,* 2003; Bautista-Ortega *et al.,* 2009; Saki and Hemati Matin, 2011; Squires and Summers, 1993;). The n-3 PUFA also can be improved in poultry meat (Hulan *et al.,* 1989) and characteristics of processed products (Yang *et al.,* 2010). Fish oil should replace soybean oil in diets only at low levels in order to minimize its latent negative impact on the sensory attributes of meat broiler (Bou *et al.,* 2004; Lopez-Ferrer *et al.,* 2001). However, the sensory alterations were not evident at 2-4 % (Jeun-Horng *et al.,* 2002) fish oil supplementation in broiler diets. Based on their described impacts on health status of animals, it is hypothesized that dietary supplementation of quercetin, oat hulls, B-glucans, lysozyme or omega-3 PUFA affects immune competence of broilers.

This study aims, to evaluate the nutritional interventions that directly or indirectly (via the microbiota) engage the optimal development of the immune competence of broilers in the post-neonatal phase (after day 14 of age). Therefore, the effects of the 5 dietary interventions in the grower diets applied after the neonatal phase (d14-28) on growth performance of broilers, the composition of the microbiota (16S rRNA sequencing), and functional and immune status of small intestinal tissue by determining genome-wide gene expression profiles and gut morphology were determined.

2 Material and methods

The experimental protocol conformed to the standards for animal experiments and was approved by the Ethical Committee of Wageningen UR, the Netherlands (project nr. 44-00321/approval nr. 201309.b). Animal care guidelines were used as provided by the Euro guide recommendations for animal use for experimental and other scientific purposes (Forbes et al., 2007).

2.1 Start date and end date of the experiment

The experiment was performed from 26th of June (day 0) to 31th of July 2014 (day 35). Day-old chicks were purchased from the hatchery Van Hulst, Veldhoven. At the end of the experiment, the chickens were delivered to butchery Kapteijns, Diessen. The trial was performed using 1,080 day-old Ross 308 broiler cockerels.

2.2 Experimental facility and housing

The experiment was conducted at the practical research farm of feed producer De Heus (Eerde, The Netherlands).

Table 2 *Experimental design in summary.*

Item	Number
Treatments	6
Replicates (pens) per treatment	6
Total number of pens	36
Broilers per pen	28
Broilers per treatment	168
Total number of broilers	1,080 (1008 used for the study; 72 reserve birds)

2.2.1 Experimental animals

A total of 1,080 one-day-old male Ross 308 chicks were used and randomly allocated to six experimental treatments (Table 2). After arrival, the chicks were randomly picked from the chick boxes and distributed among the 36 pens: the first chick in pen 1, the second chick in pen 2, and so on until there are 28 chicks in each pen (36 pens with 28 chicks / pen) in a mechanically ventilated room provided with facilities to control temperature, ventilation and lighting. The chicks were then weighed as a group per pen. The distribution of treatments over the 36 pens was determined by drawing lots. The surface area of the pens was 1.5 m^2. Each pen contained a feeding trough and three drink cups belonging to a water line. Wood shavings were used as bedding material. The broiler unit was continuously illuminated during the first two days. The broilers were sexed at the hatchery. The birds were supplied by a commercial hatchery (Van Hulst, Veldhoven, The Netherlands). Chicks passed the adaptation period (0-14 days), followed by the experimental periods 14-21 days, 21-28 days, and the finishing period (28-35 days). During the first 14 days, all the animals received the same standard starter feed, where after the experimental diets were provided from 14-28 days. From day 29 onwards, a standard finisher diet was provided to all remaining birds. On day 21 and 28, randomly 6 chicks per pen were sacrificed for further analysis. The remaining 16 chicks per pen (depending on the base) were on day 35 discharged to a regular butcher. The experimental feeds were optimized by De Heus and produced by Research Diet Services in Wijk bij Duurstede (The Netherlands). All feeds were pelleted (3.2 mm diameter). After pelleting, starter feed was crumbled. Grower and finisher feeds were provided as whole pellet. The feed was delivered as bagged. Feed and water was unlimited provided to the animals.

2.2.2 Climate

Realized relative humidity and stable temperature was maintained by means of a climate computer. Temperature inside the room was increased to 36°C one day before arrival of the chicks. From d1, the

temperature was gradually decreased to 20°C according to Table 3. Relative humidity was gradually increased from 60 (d0) to 75% (d33) during the experiment.

Table 3 *Temperature schedule.*

Age (d)	Temperature (°C)
1	35
7	31
14	28
21	27
28	25
35	20

2.2.3 Light

On day 0 and 1, the animals were given continuous light (24L: 0D) with a light intensity of 20 lux. From day 2 onwards, a day-night light schedule of 18 hours light and 6 hours of darkness (18L: 6D) was used. The light intensity was set at 20 lux during the entire experimental period.

2.2.4 Health and Failure

All fallen animals were noted and weighed, whereas the failure cause was determined by section. The fallen animals were kept refrigerated and offered for daily section. During the test the animals were checked daily on abnormalities by the animal technicians. Animals with a deviation were removed from the trial after consultation with the researchers. At the time of removal, the animal weight was noted.

2.2.5 Vaccination

The chicks were vaccinated immediately after arrival at IB + NCD (vaccination coarse spray droplet).

2.3 Experimental measurements

2.3.1 Diets

Four iso-caloric and iso-nitrogenous experimental grower diets (ME= 11.8 MJ/Kg and crude protein= 195 g/kg) were formulated. Per kg of diet, 40 mg of Lysozyme (from chicken egg white, protein ≥90%, CAS Number 12650-88-3, Sigma-Aldrich, Zwijndrecht, The Netherlands), 100 mg β-glucans (β-1,3-Glucan from *Euglena gracillis* CAS Number 9051-97-2, Sigma-Aldrich, Zwijndrecht, The Netherlands) and 400 mg quercetin (purity >95%, CAS Number 117-39-5, Sigma-Aldrich, Zwijndrecht, The Netherlands) were supplemented on top of the control diet, while 40 g/kg fish oil was included in the control diet at the expense of animal fat. Oat hulls (50 g/kg) were inserted to the control diet at the expense of maize with increasing levels of soybean meal and soya oil (10 to 12.2 for soybean meal and 2 to 4.88 for soya oil). So the highest amount of crude fat was analysed in the oat hulls-included diet (12.6 vs 9.9% in other experimental diets). The composition of the grower diets is shown in appendix 1. The composition of the starter and finisher diets is shown in appendix 2. In the present experiment, rapeseed meal was added to nutritional interventions (25%) to cause a nutritional challenge. Compared to soybean meal, rapeseed meal contains relatively high amounts of non-starch polysaccharides, glucosinolates, sinapines, tannins, erucic acid and phytate, that might have antinutritional capacities and hamper among others absorption of nutrients by the gut cell wall. The chemical and nutritional composition of the used rapeseed extract comparing with canola meal (Khajali and Slominski, 2012) is presented in appendix 3. Diets were in duplo analysed for dry matter (International Organization for Standardization, 1998), crude protein (International Organization for Standardization, 1997), crude fat (International Organization for Standardization, 1999), crude fibre (International Organization for Standardization, 1988), ash (International Organization for Standardization, 2002), Starch content was analysed enzymatically as described by Brunt (1993). Sugars were extracted from the feed samples, using 40% ethanol, and determined as described by Suárez et al. (2006). Analysis of NDF was based on a modified method of Van Soest (1973), as described by Suárez et al. (2006).

2.3.2 Performance

Body weight of birds was determined per pen at 0, 14, 21, 28 and 33 days of age. Feed intake per pen was determined at 14, 21, 28 and 33 days of age (provided feed minus remaining feed at 14, 21, 28 and 33 days of age). Body weight (BW) gain and feed conversion ratio (FCR) of broilers in each pen was calculated at the end of each feeding period (at 14, 21, 28 and 33 days of age). Also BW gain and FCR for the entire growth period was calculated. Culling, mortality and general health were recorded daily (including probable causes of any culling, illness or deaths). D0 to d14 was defined as the starter period, d15 to d21 as the grower-1 period, d22 to d28 as the grower-2 period, and d29 – d33 as the finisher period.

2.3.3 Litter quality

Litter quality was visually scored on a 0 to 10 point scale on 28 and 33 days of age by one person. The scores and the description of each score are presented in Table 4.

Table 4 *Litter quality scores.*

Score	Description
10	Dry and friable litter
9	Dry and 10% caked litter
8	Almost dry and 20% caked litter
7	Almost dry and 30% caked litter
6	Almost dry and 40% caked litter
5	Moist and 50% caked litter
4	Moist and 60% caked litter
3	Moist and 70% caked litter
2	Wet and 80% caked litter
1	Wet and 90% caked litter
0	Wet and 100% caked litter

2.3.4 Dissection and sample collection

At 21, and 28 days of age, 6 birds per pen were euthanized by electrocution. Subsequently, the chest cavity and the abdomen were opened and the small intestine was ligated and removed from the bird. The digesta of the middle part of the jejunum, defined as the 10 cm before and after the middle of the jejunum, was collected from all birds. The digesta was collected by gently stripping the gut segment into a plastic container. The digesta of all animals per pen were pooled and immediately after collection frozen on dry ice and stored at -80°C until further analysis on microbiota composition. The digesta was collected in the order of pen numbering. Digesta samples were ground (0.5 mm) by CVI (The Netherlands).
From all birds three samples (2 cm per sample) of intestinal tissue of the jejunum were collected. One sample was put in a formalin filled tube and stored at -80°C until further analysis on intestinal morphology (villus height and crypt depth), and number and size of goblet cells. Sample two was directly placed into liquid nitrogen and stored at -80°C until further analysis on genome-wide gene expression profiling in jejunal tissue. Sample 3 was taken as reserve sample and also directly placed in liquid nitrogen, followed by storage at -80°C for future unforeseen analysis. Villus height, crypt depth (µm) and goblet cells were determined by GD Animal Health (Deventer, The Netherlands) per individual bird, whereas microbiota composition and genome-wide gene expression profiling were determined in pooled samples of birds per pen by CVI (The Netherlands).

2.3.5 Microbiota

Microbiota diversity index and microbiota composition of the jejunal and ileal digesta were measured in a pooled sample of digesta of six birds (content of 2 cm jejunum length per sample) per pen on days 21. Samples of day 28 were collected but not analysed, because it was hypothesised that). To isolate DNA samples of d21 were mixed in a 1:1 ratio with phosphate buffered saline (PBS) and centrifuged for 5 min at 4°C at 300xg. Supernatant was collected and centrifuged for 10 m at 4°C at 9,000xg. DNA was extracted from the most sensitivepellet using the “QIAamp DNA stool minikit” according to the dietary interventions.manufacturers’ instructions. Quality and quantity of DNA was

checked using the NANOdrop (Agilent Technologies). 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. Samples of jejunum were sequenced by targeted-amplicon 16S sequencing and analysed for taxonomy profile per sample, alpha diversity and beta diversity. This included possible taxonomy association with treatments. Pseudo reads were clustered into OTUs per sample at 97% similarity and OTU-representative sequences were aligned against the aligned Greengenes core set (13_8 release). Chimeras were removed with Chimeraslayer. Standard alpha diversity metrics ("Chao1", "observed species", "PD whole tree", "Shannon" ,based on the species level data) were calculated for the 97% similarity clustering with 94,038 sequences/sample.

2.3.6 Genome-wide gene expression profiling

RNA Extraction Tissue

Total RNA was extracted from 50 to 100 mg jejunum and ileal 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.

Labelling, Hybridization, Scanning and Feature Extraction

Labelling of RNA was done as recommended by Agilent Technologies, using the One-Color Microarray-Based Gene Expression Analysis Low input Quick Amp Labelling. The input was 10 ng of total RNA, and 600 ng of labelled cRNA was used on the eight pack array.

Hybridization was performed as described in the One-Color Microarray-Based Gene Expression Analysis Low input Quick Amp Labelling protocol from Agilent in the hybridization oven (G2545A hybridization Oven Agilent Technologies). The hybridization temperature was 65°C with rotation speed 10 rpm for 17 hours. After 17 hours the arrays were washed as described in the One-Color Microarray-Based Gene Expression Analysis Low input Quick Amp Labelling protocol from Agilent.

The arrays where scanned using the DNA microarray scanner with Sure scan high resolution Technology from Agilent Technologies. Agilent Scan Control with resolution of 5 µm, 16 bits and PMT of 100%. Feature extraction was performed using protocol 10.7.3.1 (v10.7) for one colour gene expression.

Data analysis

The data were analysed by using R (v3.0.2) by executing different packages, including LIMMA and arrayQualityMetrics. The data were read in and background corrected (method="normexp" and offset=1) with functions from the R package LIMMA (Gentleman et al., 2005) from Bioconductor (Gentleman et al., 2004). Quantile normalisation of the data was done between arrays. The duplicate probes mapping to the same gene were averaged (by using the avereps function in R/Bioconductor) 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.

To determine the effect of the experimental treatments on gene expression in intestinal tissue at each time point, six specific contrasts were defined within the R package LIMMA.

Functional association data mining

A Gene Set Enrichment Analysis (GSEA) was performed on all different contrasts compared to the respective control (Mootha et al., 2003, Subramanian et al., 2005). Default settings were used with the exception for the permutations which were performed on the gene set. Four gene set databases (v3.0) were loaded for analysis. Three Gene Ontology related gene sets ('biological processes', 'molecular function', and 'cellular component' and one pathway related database 'Kyoto Encyclopedia of Genes and Genomes (KEGG)' were used for all analyses. Furthermore, all annotated probes (i.e. genes) were used as input, after transforming them to human gene names.

Relative bursa and spleen weight
At 21 and 28 days of age bursa and spleen were dissected from six birds per pen for bursa and spleen weight determination. Bursa and spleen weights were expressed relative to the individual BW of the birds.

2.4 Statistical analysis

The experimental data (performance, litter quality, bursa/spleen content, and gut morphology data) were analysed using Genstat statistical software (Genstat 8 Committee, 2002). Statistical significance is declared at $P < 0.05$, with $0.05 < P < 0.10$ considered as a near-significant trend. The *P*-value of the treatment effect and the LSD (least significant difference ($P = 0.05$)) were provided per response parameter.

Response parameters were analysed using ANOVA (analyses of variance) according the following model:
$Y_{ij} = \mu + Treatment_i + Error_{ij}$

Where:

Y	Response parameter
μ	General mean
Treatment	Effect of diet (i=1…6)
Error	Error term

If an overall statistical treatment effect was found (*P* value < 0.05), a Fisher protected t-test was used for analysing pair-wise differences. Pairwise differences are marked with a letter in superscript. In running Shannon diversity index, for the comparisons, not one taxon came through the filter, α was <0.05 and relative-abundance limit was 0.005.

3 Results

3.1 General

No adverse events occurred during the course of the experiment. Average performance parameters of all broilers during the experiment are presented in Table 5. The average weight of the broilers at arrival was 40 gram. During the period that the experimental diets were provided, the performance level of the broilers in the present study was below the Ross 308 performance standards of male broilers (Ross, 2014), as shown by lower body weights at d21 and d28. Body weight at d33, however, met the Ross standard, indicating compensatory growth after the switch to commercial finisher diet. From d14 onwards (start of providing the experimental diets) mortality amounted 1.2%

Table 5 *Performance data of male broilers during the experiment between 0 and 33 days of age.*

Performance data	Experiment	Ross 308 standard performance data
Body weight day 0 (g)	40	42
Body weight day 14 (g)	485	488
Body weight day 21 (g)	912	959
Body weight day 28 (g)	1393	1576
Body weight day 33 (g)	2088	2075
Body weight gain day 28-33 (g/d)	139	100
Body weight gain day 0-33 (g/d)	60.2	59.8
Feed intake day 0-33 (g/d)	91.1	89.6
Feed conversion ratio day 0-33 (kg/kg)[1]	1.519	1.499

[1] Values on feed conversion ratio were based on feed intake and body weight gain, but were not corrected for mortality.

3.2 Diets

The results of the chemical analysis of the different diets are presented in Table 6, and the dietary ingredients and calculated nutrient concentrations of the experimental diets (grower diets) are presented in Appendix 1. Calculated composition of the starter and the finisher diets is presented in Appendix 2. Analysed crude protein, crude fat, crude fibre, starch and sugars contents were in agreement with the calculated concentrations.

Table 6 *Chemical analysis of the grower diets (g/kg).*

Days	Control	Quercetin	Oat hulls	Lysozyme	β-glucan	Fish oil
Moisture	107	106	97	104	105	103
Crude protein	198	196	201	200	196	199
Crude fat	105	105	130	104	104	104
Crude fibre	57	58	71	59	54	55
Ash	57	57	61	57	56	56
Starch	340	343	277	341	340	343
Sugars	45	46	42	46	46	46

3.3 Performance

The results of feed intake (g/d) of broilers per treatment for the growing (d14-28) and finishing periods (d29-33) are presented in Table 7. Feed intake of broilers fed the diet including oat hulls and lysozyme was reduced during the first week of growing period (d14-21) compared to other groups, but feed intake was not affected by treatment after that (d22-28, d14-28 and d29-33). Birds adapted to consume diets including oat hulls and lysozyme after the first week of consumption.

Table 7 *Feed intake (g/d) presented per treatment for the different experimental periods (d14-33).*

Days	Control	Quercetin	Oat hulls	Lysozyme	β-glucan	Fish oil	P-value	SE
14-21	117a	117a	112b	107c	117a	117a	< 0.001	2.3
22-28	167	178	177	185	171	177	0.135	6.6
14-28	142	147	145	146	144	147	0.494	3.1
29-33	235	235	235	237	234	235	0.995	5.5
14-33	176	182	181	184	177	181	0.436	4.5

The results of body weight gain (BWG, g/d) of broilers per treatment for the growing (d14-28) and finishing periods (d29-33) are presented in Table 8. During the first week of the growing period (d14-21), BWG of broilers fed the diet including lysozyme decreased compared to control ($P<0.05$), where a trend of decreased BWG was observed for the oat hulls and fish oil fed birds, compared to control ($P>0.05$) was observed. Birds adapted to the consumption of oat hull, fish oil and lysozyme diets in the first week. Over the second week of the growing period (d22-28), broilers fed the diet including lysozyme showed increased BWG compared to the control and birds fed the diets including quercetin and β-glucan ($P<0.05$). Broilers fed the diets including fish oil and oat hulls also showed numerically increased BWG during the second week of grower period (d22-28) but the differences with other groups were not significant. It seems that during the second week of the growing period (d22-28), broilers fed diets including lysozyme, oat hulls and fish oil tried to have compensatory growth. There was no significant difference in BWG between nutritional interventions considering the whole growing period.

Table 8 *Body weight gain (g/d) presented per treatment for the different experimental periods (d14-33).*

Days	Control	Quercetin	Oat hulls	Lysozyme	β-glucan	Fish oil	*P*-value	*SE*
14-21	64^{ab}	65^{a}	60^{b}	55^{c}	62^{ab}	61^{b}	0.002	2.4
22-28	62^{b}	65^{b}	74^{ab}	77^{a}	63^{b}	70^{ab}	0.004	4.1
14-28	63	65	67	66	63	66	0.109	1.6
29-33	138	141	137	140	141	137	0.818	4.0
14-33	83	85	85	85	83	85	0.666	1.8

The results of feed conversion ratio (FCR) of broilers per treatment for the growing (d14-28) and finishing periods (d29-33) are presented in Table 9. Compared to the control treatment, increased FCR in broilers fed the diets supplemented by lysozyme ($P<0.05$), and a trend to increased FCR in oat hulls ($P>0.05$), was seen during the first week of growing period (d14-21). Improved FCR in broilers fed the diets supplemented with lysozyme, oat hulls and fish oil compared to control was seen during the second week of the growing period (d22-28); nevertheless, over the whole growing period no significant effect of dieatry tratment on FCR was seen. No carry-over effect of dietary treatment on BWG, feed intake and FCR was seen in the finishing period.

Table 9 *Feed conversion ratio (FCR) presented per treatment for the different experimental periods (d14-33).*

Days	Control	Quercetin	Oat hulls	Lysozyme	β-glucan	Fish oil	P-value	SE
14-21	1.42^{b}	1.42^{b}	1.48^{ab}	1.54^{a}	1.45^{b}	1.45^{b}	0.014	0.034
22-28	1.94^{a}	1.87^{ab}	1.70^{c}	1.69^{c}	1.88^{ab}	1.77^{c}	0.001	0.049
14-28	1.64	1.62	1.59	1.64	1.64	1.60	0.200	0.025
29-33	1.70	1.67	1.71	1.70	1.67	1.71	0.716	0.036
14-33	1.66	1.64	1.63	1.64	1.65	1.63	0.601	0.021

3.4 Litter quality

The results of litter condition score per treatment for two different samplings (d28 and d33) are presented in Table 10.

Table 10 *Litter condition score1 presented per treatment for two different samplings (d28 and d33).*

Days	Control	Quercetin	Oat hulls	Lysozyme	β-glucan	Fish oil	*P*-value	*SE*
28	5.3^{c}	5.0^{c}	7.0^{a}	5.8^{bc}	5.3^{c}	6.2^{ab}	0.001	0.45
33	6.2^{b}	6.5^{b}	7.7^{a}	7.3^{a}	6.3^{b}	6.5^{b}	<0.001	0.30

[1]Litter score ranges from 10 (dry, friable) to 0 (wet/100% plaque).

At d 28, oat hulls and fish oil fed birds had improved litter score compared to the control diet. At d 33, oat hulls and lysozyme fed birds still had improved litter score compared to the control diet.

3.5 Microbiota

Microbiota composition and hierarchical clustering of jejunal and ileal digesta samples per pen at species level for d21 is presented in Figure 1. No large differences between pens were observed. For each sample, clustering of microbiota per treatment and per segment for d21 is presented in Figure 2. No (treatment) clustering in jejunum and ileum was seen. From the taxa-bars it was clear, that the ileum-jejunum samples were too much alike and that the microbiota composition/diversity was fairly similar for all treatments. This was the case for all taxonomic levels. Microbiota composition averaged per treatment per segment (d21) is presented in Figure 3. Small differences between treatments were observed. Dietary interventions including oat hulls and β-glucans tended to differ from the control, especially in ileum. From this it was clear, that by averaging and by clustering per tissue, there was no real clustering by treatment/group, indicating that nutritional interventions had no meaningful effect on the microbiota composition. Alpha diversity by Shannon index for ileum and jejunum is presented in Figure 4 and 5, respectively. No significant effects of treatment on diversity index in jejunum was seen, indicating that the degree of diversity in microbiota composition in these segments was not affected by dietary treatments. Principal component analysis on microbiota in ileum and jejunum are presented in Figures 6 and 7, respectively. Figure 7 showed that according to the principal component analysis the ileal microbiota composition at d21 of the oat hulls treatment (green triangle) differed from data from the other experimental treatments, which were all centred around the origin. This is a general indication that the microbiota of the oat hulls fed birds differed from the other treatments. More specifically, a significant taxon-treatment association was found in the ileum within the genus Enterococcus (Figure 8), which was significantly higher in the oat hulls treatment (8.1%) compared to the control (2.5%) and fish oil (3.5%) fed birds, mainly at the expense of Lactobacilli.

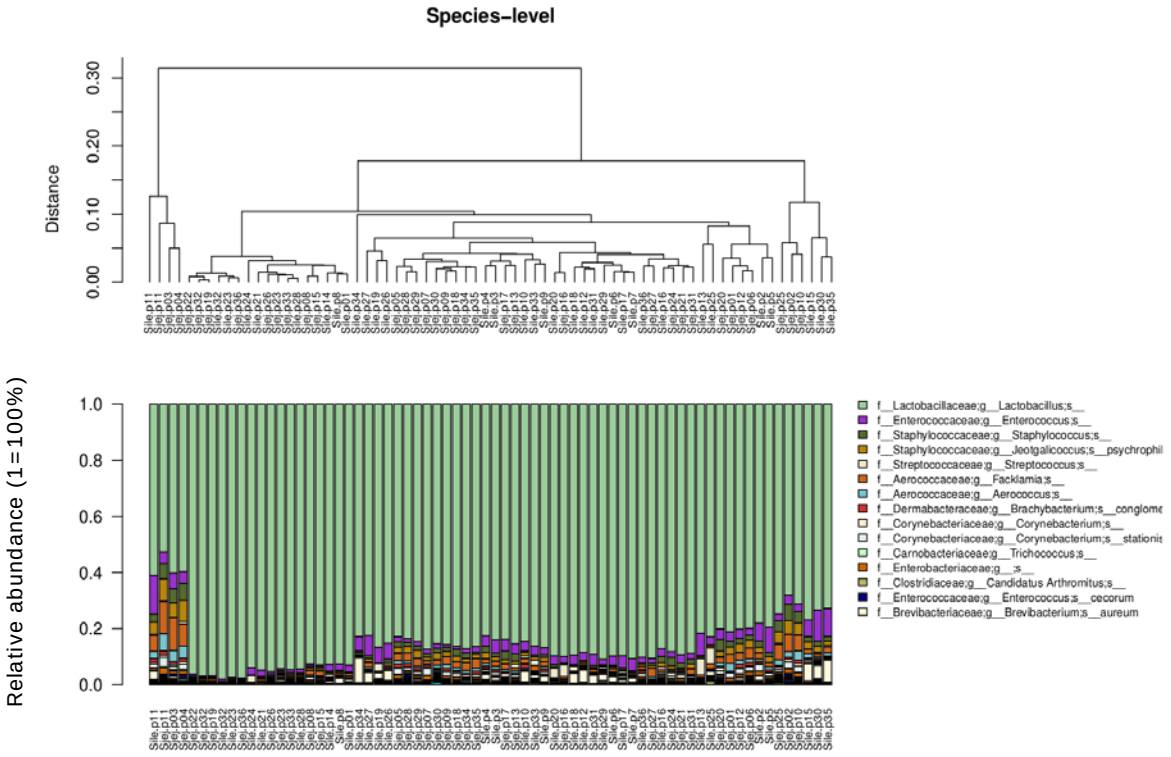

Figure 1 *Microbiota composition of jejunal and ileal digesta samples per pen (species level, d21). The upper figure shows the percentage distance in microbiota composition between pens, and the lower figure the relative abundance (1=100%) of the species per individual pen.*

Figure 2 *Hierarchical clustering of microbiota composition (percentage distance between pens) of jejunal and ileal digesta per treatment at pen level (d21).*

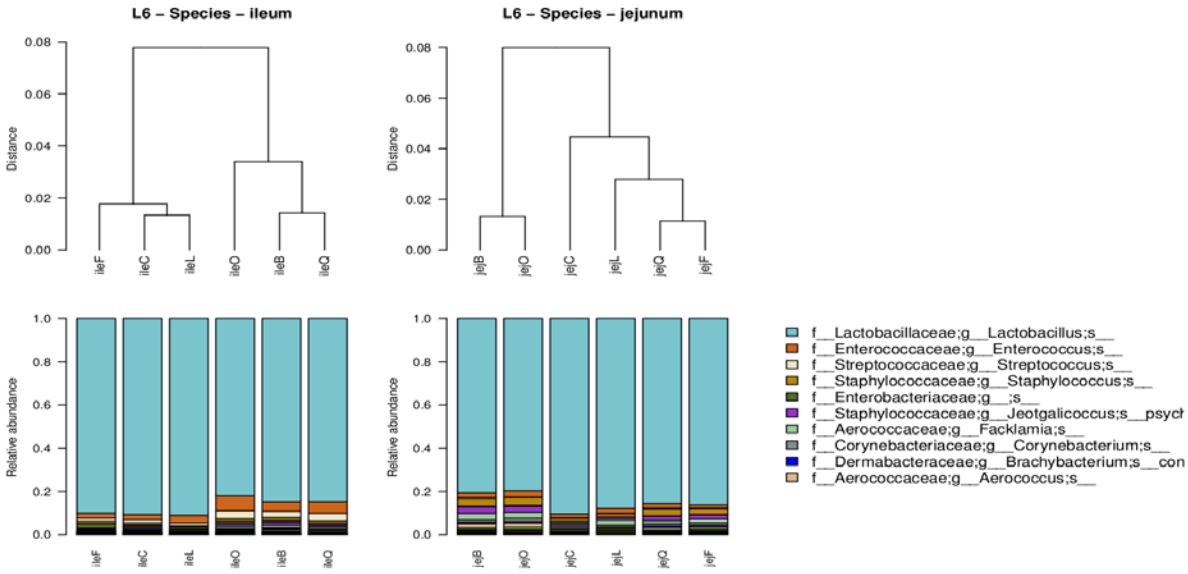

Figure 3 *Ileal (left panel) and jejunal (right panel) microbiota composition averaged per treatment (d21). The upper figure shows the distance (%) between treatments and the lower figure the relative abundance (1 = 100%) of microbiota species per treatment.*

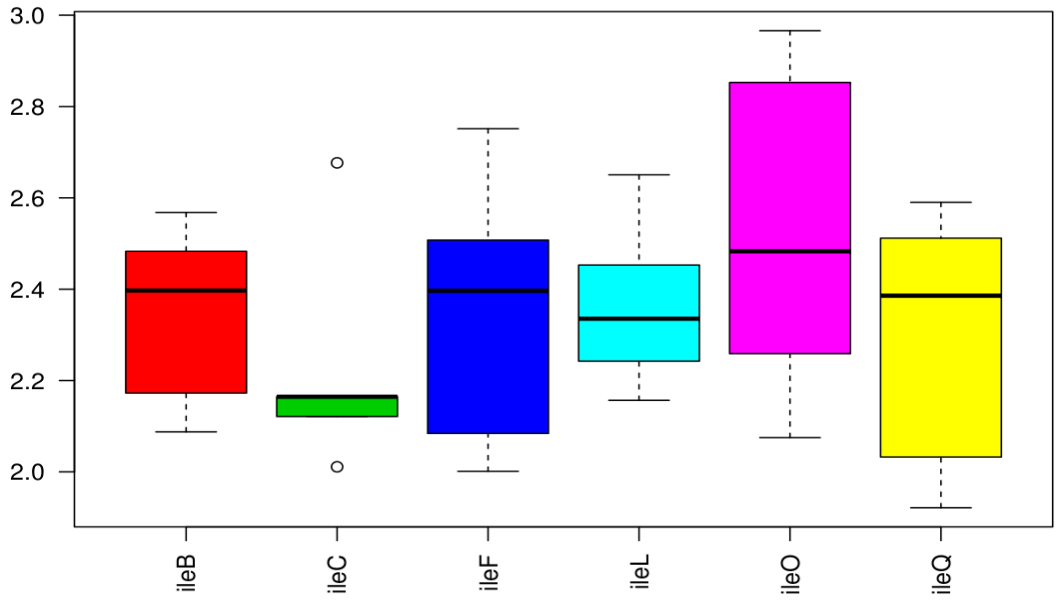

Figure 4 *Diversity in ileal microbiota composition (d21), expressed by the Shannon diversity index.*

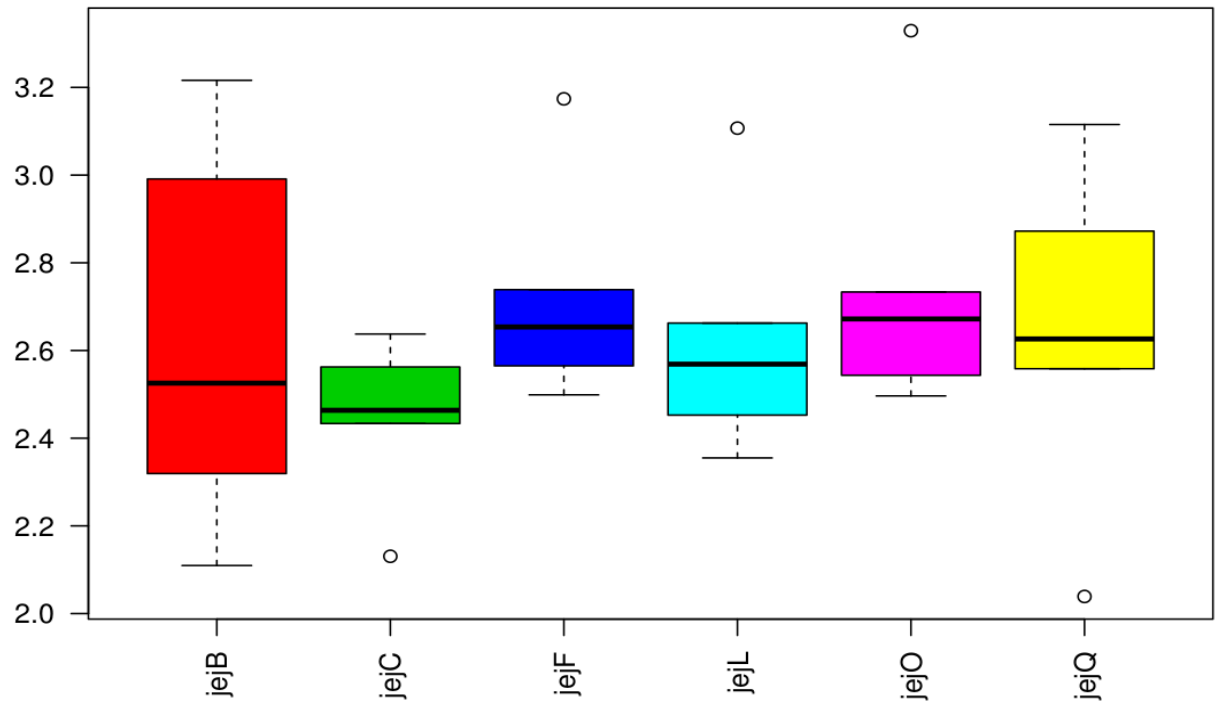

Figure 5 *Diversity in jejunal microbiota composition (d21), expressed by the Shannon diversity index.*

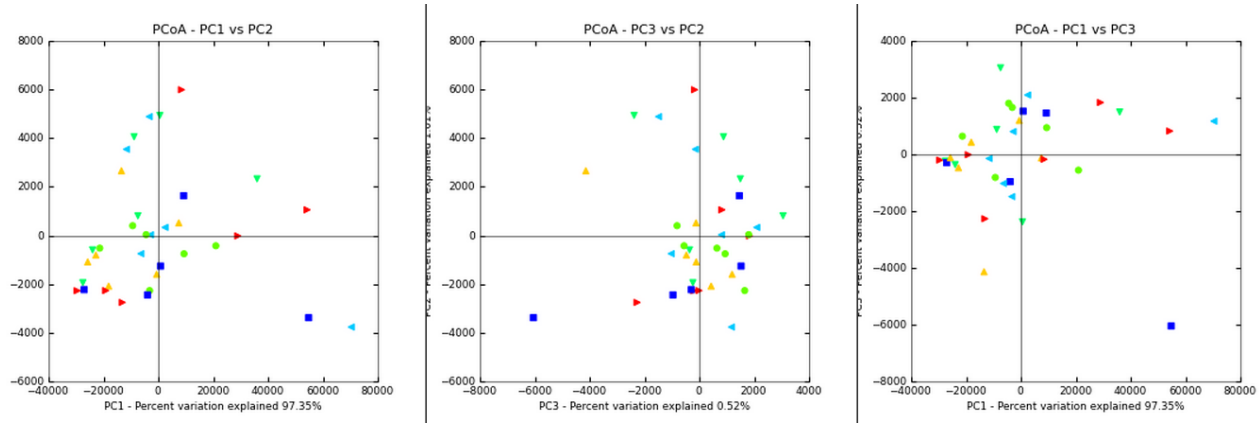

Figure 6 *Principal component analysis on microbiota in jejunum (d21).*

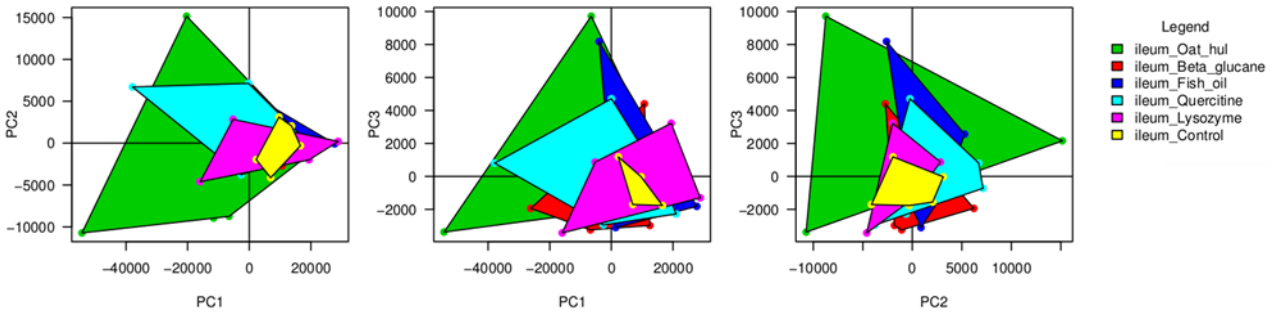

Figure 7 *Principal component analysis on microbiota in ileum (d21).*

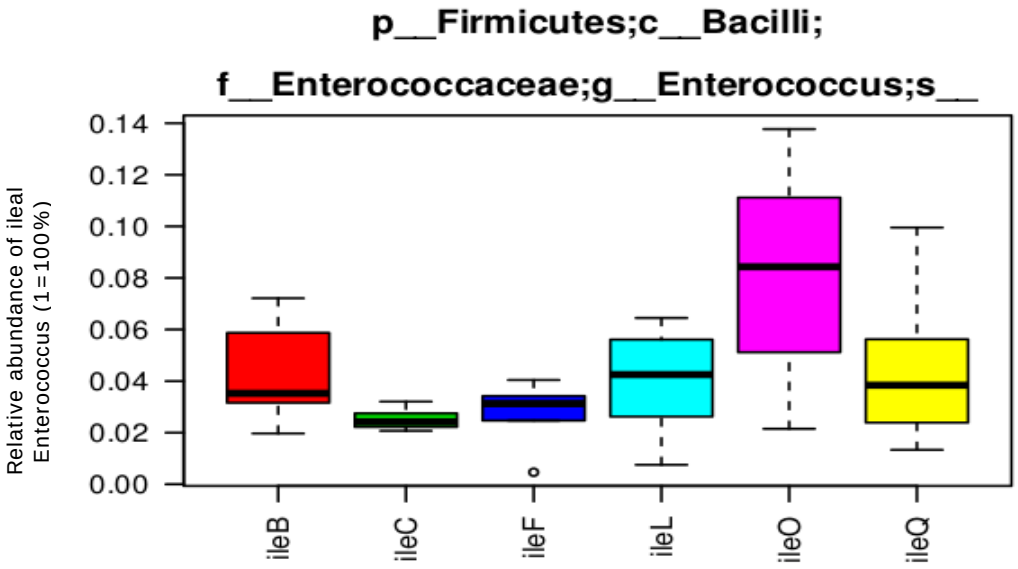

Figure 8 *Relative abundance of ileal Enterococcus (1=100%) per treatment (d21).*

3.6 Gut morphology

Results for villus height (µm) of jejunum and ileum presented per treatment for two different samplings (d21 and d28) are presented in Table 11. No effect of treatments on villus height of jejunum on d21 and of ileum (d21, d28) was observed. Villus height of lysozyme fed birds in jejunum at d28 was decreased compared to the control and oat hulls ($P < 0.005$).

Table 11 *Villus height (µm) of jejunum and ileum presented per treatment for two different samplings (d21 and d28).*

Days	Control	Quercetin	Oat hulls	Lysozyme	β-glucan	Fish oil	*P*-value	*SE*
Jej. 21	1388	1455	1477	1368	1422	1381	0.669	77.9
Jej. 28	1354^{ab}	1260^{abc}	1558^{a}	1076^{c}	1306^{abc}	1249^{bc}	0.005	120.4
IL. 21	482	542	479	434	579	427	0.875	150.3
IL. 28	810	759	796	838	796	743	0.687	65.1

Results for crypt depth (µm) of jejunum and ileum presented per treatment for two different samplings (d21 and d28) are presented in Table 12. No effect of treatments on crypt depth of jejunum on d21 and of ileum (d21, d28) was observed. Crypt depth of lysozyme fed birds in jejunum at d28 was decreased compared to the control and other dietary interventions ($P < 0.001$).

Table 12 *Crypt depth (µm) of jejunum and ileum presented per treatment for two different samplings (d21 and d28).*

Days	Control	Quercetin	Oat hulls	Lysozyme	β-glucan	Fish oil	*P*-value	*SE*
Jej. 21	323	274	335	301	291	305	0.800	45.3
Jej. 28	433^{ab}	451^{a}	421^{ab}	307^{c}	405^{b}	457^{a}	0.001	39.8
IL. 21	142	160	150	126	166	129	0.902	43.8
IL. 28	216	222	208	257	218	210	0.651	32.1

Results for Villus/crypt ratio of jejunum and ileum presented per treatment for two different samplings (d21 and d28) are presented in Table 13. No effect of treatments on villus/crypt ratio of jejunum on d21 and of ileum (d21, d28) was detected. Interventions deviated not significantly from the control in villus/crypt ratio in jejunum at d28, although villus/crypt ratio of the birds fed oat hulls tended to be higher as compared to the control.

Table 13 *Villus/crypt ratio of jejunum and ileum presented per treatment for two different sampling (d21 and d28).*

Days	Control	Quercetin	Oat hulls	Lysozyme	β-glucan	Fish oil	*P*-value	*SE*
Jej. 21	5.0	5.7	5.1	5.3	5.3	5.1	0.970	0.81
Jej. 28	3.3^{ab}	2.9^{bc}	4.0^{a}	3.8^{ab}	3.5^{ab}	2.9^{bc}	0.050	0.44
IL. 21	3.5	3.5	3.3	3.5	3.5	3.4	0.797	0.20
IL. 28	3.9	3.6	4.0	3.6	3.8	3.7	0.478	0.25

Effects of dietary interventions and sampling day on average number of goblet cells per villi, total surface area of villi occupied by goblet cells (μm^2), average goblet cell size (µm), average total villi surface area (μm^2), and goblet cell surface area relative to total villi surface area (%) of jejunum and ileum in two sampling days (days 21 and 28 of age) are presented in Tables 14 to 18, respectively. Among the measured characteristics of goblets cells, significant effect of dietary interventions were seen on total surface area of villi occupied by goblet cells (μm^2) as well as average total villi surface area (μm^2) of jejunum. In jejunum, the total surface area of villi occupied by goblet cells (μm^2) and average total villi surface area (μm^2) were higher in chickens fed diet included oat hulls compared with other dietary interventions.

Table 14 *Effects of dietary interventions on average number of goblet cells per villi of jejunum and ileum in two sampling days (days 21 and 28 of age).*

Section and age of sampling (d)	Control	Quercetin	Oat hulls	Lysozyme	β-glucan	Fish oil	SE	*P*-value Treatment	*P*-value Day	*P*-value Trt x Day
Jejunum										
Day 21	88.4	69.8	107.3	77.6	72.5	78.7	22.92	0.203	<0.001	0.997
Day 28	157.9	146.2	195.2	143.5	138.0	160.7	25.42			
Ileum										
Day 21	46.1	61.0	53.3	41.7	57.3	43.9	13.0	0.875	<0.001	0.977
Day 28	112.9	110.6	108.5	110.2	110.8	104.8	13.5			

Table 15 *Effects of dietary interventions on total surface area of villi occupied by goblet cells (µm2) of jejunum and ileum in two sampling days (days 21 and 28 of age).*

Section and age of sampling (d)	Control	Quercetin	Oat hulls	Lysozyme	β-glucan	Fish oil	SE	P-value Treatment	P-value Day	P-value Trt x Day
Jejunum										
Day 21	3116	2678	4176	2747	2787	3273	529.1	0.019	<0.001	0.935
Day 28	4675	3957	5884	4437	3619	4144	586.6			
Ileum										
Day 21	1253	1993	1713	1052	2022	951	476.0	0.343	<0.001	0.887
Day 28	3875	3491	3464	3276	3576	3175	491.8			

Table 16 *Effects of dietary interventions on average goblet cell size (µm) of jejunum and ileum in two sampling days (days 21 and 28 of age).*

Section and age of sampling (d)	Control	Quercetin	Oat hulls	Lysozyme	β-glucan	Fish oil	SE	P-value Treatment	P-value Day	P-value Trt x Day
Jejunum										
Day 21	44.9	48.7	47.8	44.7	48.0	51.1	5.95	0.990	<0.001	0.934
Day 28	29.7	26.6	30.3	31.5	26.7	26.3	6.59			
Ileum										
Day 21	24.0	30.6	28.8	22.3	36.2	15.1	6.68	0.540	0.224	0.826
Day 28	33.8	31.8	31.3	29. 6	32.4	30.4	6.92			

Table 17 *Effects of dietary interventions on average total villi surface area (µm2) of jejunum and ileum in two sampling days (days 21 and 28 of age).*

Section and age of sampling (d)	Control	Quercetin	Oat hulls	Lysozyme	β-glucan	Fish oil	SE	P-value Treatment	P-value Day	P-value Trt x Day
Jejunum										
Day 21	222358	212118	250921	225555	218541	226596	14928	0.014	0.659	0.279
Day 28	239986	219142	283921	199146	184542	238698	16535			
Ileum										
Day 21	58609	69226	69256	53767	79243	40505	16937	0.875	<0.001	0.823
Day 28	123879	122054	112071	124141	117799	118372	17547			

Table 18 *Effects of dietary interventions on goblet cell surface area relative to total villi surface area (%) of jejunum and ileum in two sampling days (days 21 and 28 of age).*

Section and age of sampling (d)	Control	Quercetin	Oat hulls	Lysozyme	β-glucan	Fish oil	SE	P-value Treatment	P-value Day	P-value Trt x Day
Jejunum										
Day 21	1.386	1.313	1.744	1.224	1.298	1.446	0.2292	0.618	<0.001	0.535
Day 28	1.955	1.807	2.083	2.453	1.989	1.772	0.2542			
Ileum										
Day 21	1.828	2.403	1.943	1.723	2.324	1.800	0.360	0.627	<0.001	0.947
Day 28	3.193	3.055	3.081	2.788	3.096	2.829	0.373			

3.7 Gene expression

Based on the principal component analysis (PCA), no significant effects of treatments on expression of genes was observed in jejunal and ileal tissue. Figure 9 shows the PCA 3D plot of gene expressions. One dot represents the overall expression data of a pooled tissue sample of six birds per pen. Clustering of gene expressions in the jejunum substantially differed from the ileum, indicating the functional differences between both segments. Within the gut sections, no clear differences in clustering of expressed genes occurred, showing that the dietary interventions in general had a limited effect on the biological processes in intestinal tissue. Nevertheless, dietary treatments affected a few specific biological processes in the ileum as presented in Table 19. Compared to the control birds, the genes related to growth-factor-activity pathway were higher expressed in chicken fed the diet including β-glucan (FDR q-value = 0.026), whereas the genes related to anion-transmembrane-transporter-activity pathway in the quercetin and oat hulls included diet were lower expressed (FDR q-value = 0.016). Individual genes in these pathways had low fold change values and high adjusted P-values, and therefore no indications were found that the individual genes in these pathways were related to immunity.

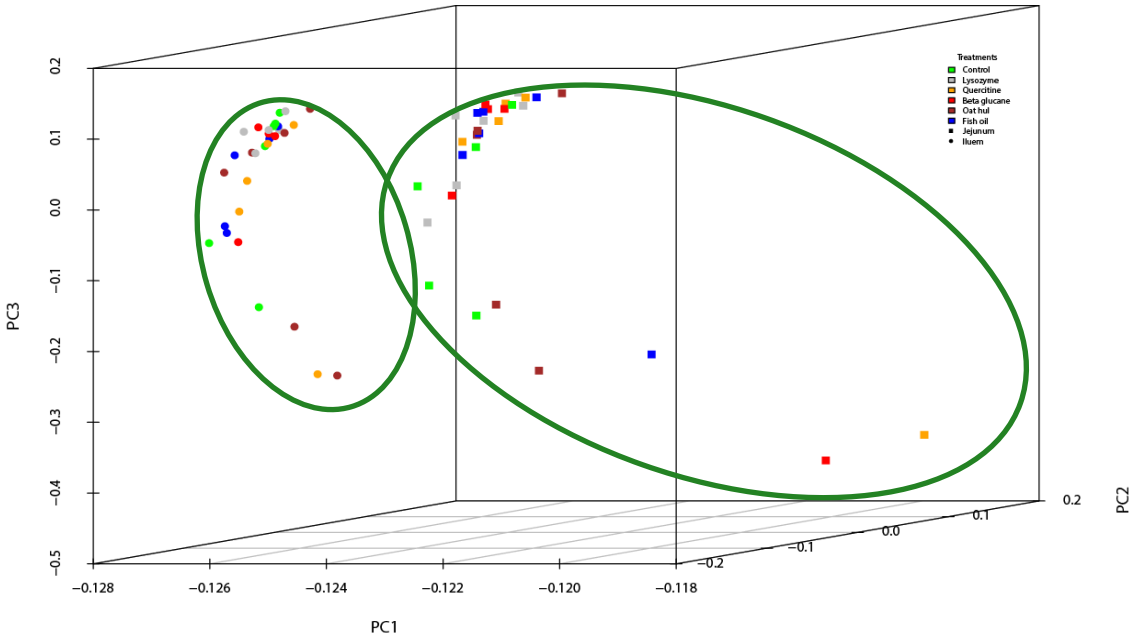

Figure 9 *Principal Component Analysis 3D plot gene expressions (PCA 3D plot) in jejunum and ileum (d21). Squares represent jejunal samples and circles ileal samples.*

Table 19 *Annotation of the different expressed genes per treatment as compared to the control treatment in ileal tissue to the related biological processes (d21).*

Compared to control	Annotation category	Size[1]	NES[2]	FDR q-value
β-glucans	GROWTH_FACTOR_ACTIVITY	27	1.98	**0.026**
β-glucans	HYDROLASE_ACTIVITY_HYDROLYZING_O_GLYCOSYL_COMPOUNDS	22	1.90	0.058
Lysozyme	CATION_HOMEOSTASIS	57	1.86	0.057
Lysozyme	ION_HOMEOSTASIS	67	1.79	0.064
Lysozyme	CELLULAR_CATION_HOMEOSTASIS	55	1.79	0.069
Lysozyme	CELLULAR_HOMEOSTASIS	76	1.77	0.070
Lysozyme	CHEMICAL_HOMEOSTASIS	82	1.82	0.076
Lysozyme	KEGG_GLYCINE_SERINE_AND_THREONINE_METABOLISM	21	1.79	0.079
Lysozyme	RESPONSE_TO_EXTRACELLULAR_STIMULUS	19	1.86	0.084
Lysozyme	RESPONSE_TO_NUTRIENT_LEVELS	18	1.90	0.088
Lysozyme	HOMEOSTATIC_PROCESS	113	1.79	0.093
Quercetin	KEGG_PRIMARY_IMMUNODEFICIENCY	27	1.99	0.055
Quercetin	ANION_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	24	-1.91	0.074
Oat hulls	ANION_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	24	-1.96	**0.016**
Oat hulls	EMBRYONIC_DEVELOPMENT	40	-1.84	0.096

[1] Size = number of different expressed genes involved in the specific annotation category.

[2] NES stands for Normalized Enrichment Score. It is a measure for over-representation of classes of genes in the tissues and may have an association with disease phenotypes

3.8 Organ weights

Results for the relative weight of bursa of Fabricius and spleen to body weight per treatment for two different samplings (d21 and d28) are presented in Table 20 and 21, respectively. The interaction between treatment and sampling day on relative weight of bursa was significant (P = 0.005). At d21, relative weight of bursa of birds fed fish oil was increased compared to the control group, whereas relative bursa weight of fish oil fed birds did not differ from the control at d28. There was no significant effect of treatment on relative weight of spleen, but the effect of sampling day was significant (P < 0.001). Relative weight of spleen was higher in d28 compared to d21.

Table 20 *Effects of nutritional interventions on relative weight of bursa of Fabricius to body weight in two sampling days (day 21 and 28 of age).*

Section and age of sampling (d)	Control	Quercetin	Oat hulls	Lysozyme	β-glucan	Fish oil	SE	*P*-value Treat-ment	*P*-value Day	*P*-value Trt x Day
Day 21	2.19^{abcd}	2.43^{de}	2.22^{bcd}	2.20^{abcd}	2.31^{cde}	2.54^{e}	0.530	0.764	<0.001	0.005
Day 28	2.14^{abc}	2.16^{abc}	2.33^{cde}	2.15^{abc}	2.04^{ab}	1.95^{a}				

Table 21 *Effects of nutritional interventions on relative weight of spleen to body weight in two sampling days (day 21 and 28 of age).*

Section and age of sampling (d)	Control	Quercetin	Oat hulls	Lysozyme	β-glucan	Fish oil	SE	*P*-value Treatment	*P*-value Day	*P*-value Trt x Day
Day 21	1.11^{b}	1.18^{b}	1.14^{b}	1.12^{b}	1.16^{b}	1.12^{b}	0.280	0.967	<0.001	0.316
Day 28	1.36^{a}	1.25^{a}	1.27^{a}	1.26^{a}	1.22^{a}	1.33^{a}				

Discussion

Nutritional challenge by dietary rapeseed inclusion

In the present study, rapeseed meal at a level of 250 g/kg was included in the experimental diets to cause nutritional challenge, because the in rapeseed meal present antinutritional factors hampers nutrient intake by the gut. The main components of rapeseed include protein, carbohydrates (i.e. simple sugars, sucrose, oligosaccharides, starch), fibre (i.e. NSP, lignin with associated polyphenols, glycoproteins), fat, and ash. As a consequence of the small size and high oil content in rapeseed, the resulting meal contains a relatively high proportion of fibre. The crude fibre (Kocher et al., 2000; Mawson et al., 1993), acid detergent fibre (ADF), neutral detergent fibre (NDF), and total dietary fibre values for rapeseed are higher than those of soybean meal due to a much higher content of lignin with associated polyphenols (tannins). The NSP, which include β–glucans, arabinoxylans and pectins, may decrease nutrient digestibility (Downey and Bell, 1990), mainly on crude fat and amino acids (Knudsen, 1997), as well as the performance of broiler chickens fed diets with high levels of rapeseed meal (Bedford, 2000; Koncicki et al. 1991; Haščik et al., 1994; Wetscherek et al., 1993). Rapeseed non-digested protein at ileal level may increase the amount of undigested amino acids reaching the hindgut that in turn may enhance proteolytic fermentation by resident microbiota (Libao-Mercado et al., 2009). As a side effect, hindgut protein fermentation can even further negatively affect performance of broilers due to the formation of toxic compounds such as amines, ammonia, skatole, or indoles (Gabriel et al., 2005). Rapeseed products have other nutritionally unfavourable substances such as glucosinolates, sinapine, tannin, erucic acid and phytate (Ciska and Kozowska, 1998; Korol et al., 1994; Mustapić and Pospišil, 1995).

During the period that the experimental grower diets were provided, the performance level of the broilers in the present study was below the Ross 308 performance standards of male broilers (Ross, 2012). The poor general performance of broilers was assumed to be mostly due to insertion of a high level of rapeseed meal to the diets, resulting in retardation of body weight gain and less efficient feed conversion. After the switch to the commercial finisher diets, compensatory growth occurred, resulting in body weights at d33 that met the Ross (2014) standard.

Performance and litter quality

Feed intake of broilers fed the diet including oat hulls and lysozyme was reduced during the first week of the growing period, and birds adapted to consume diets including oat hulls and lysozyme on the week after. During the first week of the growing period, temporary reduced feed intake of broilers fed the diet including oat hulls may partly due to the high dietary percentage of crude fat (10 vs. 13%), which can in turn reduce voluntary feed intake especially in young chicks (Fuller and Rendon, 1977). On the other hand, chicks fed the diet including oat might not able to compensate in feed intake because of physical limitation. During the first week of growing period, a trend of decreased BWG of broilers fed the diet including lysozyme, oat hulls and fish oil was observed. The decreased BWG of broilers fed the diet including oat hulls is mainly due to reduced feed intake which was mentioned before. There is no clear reason for the decreased BWG of broilers fed the diet including lysozyme, and fish oil. Over the second week of the growing period, broilers fed the diet including lysozyme showed increased BWG compared to the control and birds fed the diets including quercetin and β-glucan. Broilers fed the diets including fish oil and oat hulls also showed increased BWG during the second week of growing period. It seems that broilers fed the diets including lysozyme, oat hulls and fish oil had compensatory growth, and no significant difference in BWG between nutritional interventions considering the whole growing period were observed. Improved FCR in broilers fed the diets supplemented with lysozyme, oat hulls and fish oil was seen during the second week of the growing period. Improved FCR in broilers fed the oat hulls diet may be partly explained based on the studies by Qaisrani *et al* (2014 and 2015), where coarse diets improved gut integrity (villus height) and gizzard development.

The five different nutritional interventions applied in this study; quercetin, oat hulls, β-glucan, lysozyme, and fish oil; have also been used in previous investigations as immuno-modulatory agents. Based on the literature, the effectiveness and action mechanism of these dietary additives differ. In agreement with the results of the current study in terms of improved FCR in broilers fed the diets supplemented with oat hulls during the second week of the growing period, benefits of dietary inclusion of insoluble fibre on growth have been demonstrated with mash (Gonzalez-Alvarado *et al*., 2007; Jimenez-Moreno *et al*., 2011), whereas no effect has been observed with pellets (Amerah *et al*., 2009a; Hetland *et al*., 2003; van der Hoeven-Hangoor *et al*., 2014). In addition, Jimenez-Moreno *et al*

(2015) reported that the inclusion of oat hulls or sunflower hulls into low fibre diets was beneficial for improving broiler performance. Jimenez-Moreno *et al* (2009b) who arranged factorial three sources of fibres (none; 3% oat hulls, and 3% sugar beet pulp) and 2 fat sources (5% soybean oil and 5% yellow grease) reported improved performance and nutrient digestibility in young chicks, especially when saturated fats were used. In our study, the crude fat (soya oil which has mostly unsaturated lipids) percentage of diet including oat hulls was higher than the other diets, which in turn affected feed intake and FCR of broilers. On the other hand, improved FCR in broilers fed the oat hulls diet may be partly due to the effect of oat hulls in increasing the coarseness of the diet (Qaisrani *et al*., 2014 and 2015).

The effect of dietary fibre on gizzard development was found to depend on fibre source and its particle size (Amerah *et al*., 2009; Hetland *et al*., 2005; Mateos *et al*., 2012; Svihus, 2011) Sacranie *et al* (2012) showed that the addition of hulls (consisting of equal weights of hulls from oats and barley) increased gizzard weight and content and lowered gizzard pH. In the current experiment, gizzard weight and gizzard pH were not measured. FCR of the oat hulls fed birds during the second week of the growing period, however, significantly improved compared to the control group. This might be the result of increased gizzard weight and improved gizzard functioning, which adapted to the supplementation of the oat hulls diet. In agreement with our finding, improved feed conversion efficiency, corrected for insoluble fibre contents, was detected in broilers by inclusion of oat hulls in diets based on whole or ground wheat (Hetland *et al*., 2003).

Similar to the current study, Goliomytis *et al* (2014b) also added quercetin to feed and observed no effect on body weight and feed intake of broilers; but poorer FCR values and relative heart weight were obtained with increasing levels of dietary quercetin.

Oat hulls and fish oil fed birds had improved litter score compared to the control diet at d 28 and oat hulls and lysozyme fed birds still had improved litter score compared to the control diet at d 33. In agreement with our study, Van der Klis and de Lange (2013) reported that the inclusion of 5.0% oat hulls in iso-nutritive diets reduced water-to-feed ratio in broilers from 1 to 21 d of age. In addition, van der Hoeven-Hangoor *et al* (2014) reported that the inclusion of 2.5% coarse oat hulls in a wheat and soybean meal based diet with 2.4% crude fibre reduced excreta and litter moisture content in broilers.

It can be concluded that the dietary interventions had limited effect on performance levels of the birds. Litter score improved by feeding oat hulls and fish oil (d28), and oat hulls and lysozyme (d33), which might be in indication of better functioning of the GIT.

Gut microbiota

Gut microbiota have an important role in broiler health and performance (Gordon and Pesti 1971; Hammons *et al*., 2010; Klosterbuer *et al*., 2011, Torok *et al*., 2011b). Although in the present study, small differences between treatments were observed in terms of microbiota composition at day 21, both in jejunum and ileum, but in general the effects of the nutritional interventions were absent or small. This might be related to the timing of application of the interventions, being d14 - 28 of age. Supplementation of oat hulls tended to differ from the control, especially in ileum. It has been demonstrated that several factors can influence the host's gut microbiota, including age, genetics, housing environment and stress, although the greatest effecting factor by far is the host's diet (Lu *et al*., 2003; Burkholder *et al*., 2008; Torok *et al*., 2009, 2011b; Lumpkins *et al*., 2010). Nevertheless, changes in gut microbiota due to nutritional intervention does not always cause altered broiler performance (Gunal et al., 2006; Pedroso *et al*. 2006; Geier *et al*. 2009). The primarily focus in this study was to improve immune competence and not performance, among others by affecting microbiota composition. Multiple studies have been conducted to measure the effect of dietary manipulation on profile of the gut microbial community applying microbiological culturing techniques, culture-independent molecular techniques and indirect measurement of bacterial metabolic products (Bjerrum *et al*., 2006; Choct *et al*., 1999; Hubener *et al*., 2002; Zhu *et al*., 2002; Yin *et al*., 2010). The molecular techniques, including denaturing gradient gel electrophoresis, terminal restriction fragment length polymorphism, pyrotag sequencing and phylogenetic microarrays have been also used to investigate microbial community structure (Amend *et al*. 2010; Zhou *et al*., 2011; Fraher *et al*., 2012). Torok *et al* (2013) developed quantitative polymerase chain reaction (qPCR) assays to five potential performance-related bacteria (*Lactobacillus salivarius*, *L. crispatus*, *L. aviarius*, *Gallibacterium anatis* and *Escherichia coli*) and generic eubacteria. They detected that *L. salivarius*, *L. crispatus*, *L. aviarius*, *E. coli* and total eubacterial numbers were altered by diet, environment (litter), and/or sex of birds. In addition, Lactobacilli and total Eubacteria were decreased in birds that were more feed efficient, but *E. coli and G. anatis* was found not to be consistently linked with broiler performance.

In the current study, qPCR 16S sequencing technique was applied to determine all available gut microbiota at genus level. Alpha diversity by Shannon index showed a trend to a more diverse microbiota composition in the birds fed oat hulls. At genus-level, principal component analysis on

microbiota of ileum also showed that the diet included oat hulls was more separated from the cluster of the other samples, which were all centred around the origin. Higher abundance of *Enterococcaceae* was observed in ileum of birds fed the diet including oat hulls compared to the control and the fish oil included diets. The order Lactobacillae includes the family Enterococcaceae with four genera: Enterococcus, Melissococcus, Tetragenococcus, and Vagococcus. They can be found in diverse environments. In general, the Enterococcaceae are fastidious, and consequently, they thrive in environments where various nutritional needs are provided, usually by other living or dead organisms. In addition to the intestinal tracks of mammals, birds, fish, and insects, they can be recovered from decaying plant and animal material. Several Enterococcus species also produce lactic acid by homofermentative glucose metabolism. Enterococci are routinely cultivated on rich media with occasional pigment production and haemolysis (Palmer et al. 2012). Distinct strains of E. faecium and E. faecalis account for virtually all clinical infections caused by Enterococcaceae, particularly in nosocomial settings (Fisher and Phillips 2009). A number of Enterococcus species have been successfully used as probiotics for the treatment of diarrhea in humans and farm animals (Franz et al. 2011).

Lactobacillus is the major genus found in the ileum (70%), whereas the other genera include Clostridiaceae (11%), Streptococcus (6.5%) and Enterococcus (6.5%) (Lu *et al*., 2003). The microbiota is influenced by dietary ingredients such as medium chain fatty acids that decreased the growth of gram positive Firmicutes and some other species including Lactobacillus, Micrococcaceae and Enterococcaceae, whereas the growth of gram negative bacteria is increased (Van Der Hoeven-Hangoor *et al*., 2013). The latter authors related the change in microbiota population with higher dietary concentrations of medium chain fatty acids to the sensitivity of gram positive compared with gram negative bacteria. The higher level of crude fat (especially from soya oil) of the oat hulls included diet compared to other diets as well as higher amount of insoluble fibres might cause the higher abundance of *Enterococcaceae* observed in ileum of the birds. In line with our findings, Amerah *et al* (2009) showed that the microbial composition of birds fed diets supplemented with whole wheat or wood shavings differed from birds fed the control of cellulose supplemented diet.

In a study by Zhang *et al* (2010), a lysozyme-based antimicrobial blend was very effective in reducing negative health effects in broilers after necrotic enteritis challenged with *Eimeria maxima* and *Clostridium perfringens*. In the current study, Lysozyme addition did not affect the jejunal and ileal microbiota composition of healthy broilers.

Supplementation of β-glucans showed a slight trend to differ from the control, especially in ileum. In trying to explore the effect of oligosaccharides as nutritional intervention on gut microbiota, it has been detected that mannan-oligosaccharide linked with improved gut health, indicated by increased populations of beneficial bacteria such as lactobacilli and bifidobacteria in the guts of broilers and turkeys, while reducing the populations of *Salmonella* and *E. coli* (Sims *et al*., 2004; Baurhoo et al., 2007b; Brummer *et al*., 2010).

It can be concluded f jejunum and ileum had similar microbiota compositions. Nutritional interventions, however, only slightly affected the overall microbiota composition within and between jejunum and ileum. Only feeding oat hulls affected ileal microbiota composition, as shown by an increased amount of *Enterococcaceae* at the expense of *Lactobacillae*, indicating that oat hulls good have the capacity to affect immune competence.

Gut morphology

No effect of nutritional interventions on villus height of jejunum on d21 and of ileum (d21, d28) was observed. Villus height and crypt depth of lysozyme fed birds in jejunum at d28, however, were decreased compared to the control. Villi height reflects the absorptive capacity of the intestine (Teirlynck *et al*., 2009). Interventions deviated not significantly from the control in villus/crypt ratio in jejunum at d28, although villus/crypt ratio of the birds fed oat hulls tended to be higher as compared to the control, so it seems that the birds in oat hulls group adapted to use the diet with higher amount of fibre compared to the other dietary groups. Jiménez-Moreno *et al* (2013b) evaluated the effects of inclusion of oat hulls (OH) and sugar beet pulp (SBP) in the diet on the development of the gastrointestinal tract in broilers from 1 to 18 days of age and detected reduced villus height at d 12 with SBP inclusion, but no effects were detected with OH. In addition, the inclusion of up to 75 g OH or SBP/kg in low fibre diets increased the relative weight of the GIT and reduced digesta pH of the gizzard. Sadeghi *et al* (2015), reported a decreased effect of sugar beet pulp (SBP) on villus height in the duodenum and ileum compared with control, whereas no influence of rice hulls (RH), or combination of SBP and RH (SBP/RH) was detected. Supplementing soluble fibre has been reported to reduce villus height in broilers given 50 g/kg xanthan (Iji *et al*., 2001). No difference in jejunal villus height was observed using higher or equal ratios of insoluble to soluble fibres (Saki *et al*., 2011), indicating that insoluble fibres are not detrimental for jejunal villus height. Qaisrani *et al*. (2014 and

2015) demonstrated that broilers fed coarse rapeseed meal based diets had heavier gizzards and lower relative empty weights of the crop, duodenum, jejunum, and ileum compared with those fed the fine diets. In addition, dietary coarseness resulted in greater ileal protein digestibility, lower gizzard pH, greater villus height, lower crypt depth, reduced cecal branched chain fatty acids and lower biogenic amines in the cecal digesta compared with chickens fed the fine diets. In the study by Gao *et al*., (2008), supplementation with yeast culture (YC) increased villus height linearly, crypt depth in the duodenum, jejunum and ileum, and villus height to crypt depth ratio in duodenum, but decreased villus height to crypt depth ratio in jejunum and ileum. In the current experiment, however, dietary supplementation with β-glucans did not affect villus height in jejunum and ileum. Mannan-oligosaccharide and 1,3/1,6 *β*-glucan are components of the yeast cell wall (Morales-Lopez *et al*., 2009; Talbott, 2012) that modulate immunity (An *et al*., 2008; Cox *et al*., 2010; Shashidhara and Devegowda, 2003), promote growth of intestinal microflora (Spring *et al*., 2000; Stanley *et al*., 2000), and increase growth (Parks *et al*., 2001). Microbiota composition of the *β*-glucan fed birds, however, was not affected. Average total villi surface area (μm^2) was affected by dietary interventions in two sampling days (days 21 and 28 of age). Mannan-oligosaccharide has been linked with improved gut health, indicated by increased villi length and the number of goblet cells of broilers and turkeys (Sims et al., 2004; Baurhoo *et al*., 2007; Brummer *et al*., 2010). Shikha-Bhatia *et al* (2015) indicated that galacto-oligosaccharides may enhance mucosal barrier function through direct stimulation of intestinal goblet cells. In jejunum, the total surface area of villi occupied by goblet cells (μm^2) and average total villi surface area (μm^2) were higher detected in chickens fed diet included oat hulls compared with other dietary interventions. Rezaei *et al* (2011) showed that dietary supplementation with micronized insoluble fibre resulted in dose dependent increases in the ileal villus height:crypt depth ratio and number of goblet cells in male broilers. Increased intestinal villus height, villus thickness, villus height: crypt depth ratio and number of goblet cells was detected in quails (7 to 35 day of age) fed diet included processed rice hulls (Rezaei *et al*., 2014) which is in agreement with the current results. These findings show that feeding insoluble fibres to broilers might improve gut morphology, which is an indication of both good gut health and absorptive capacity, which could be indirectly beneficial for the immune competence of the birds. Further research is required to unravel the mechanisms behind, especially if the insoluble fibres are provided from day-old onwards.

Gut gene expression

The information presenting in this section about the gene activity/functionality are related to human data, because more and better gene annotation is available. In the present study, no significant effect of the nutritional interventions on expression of genes was observed in jejunum. Compared to the control birds, the genes related to growth factor activity were expressed more in the β-glucans included diet. Relation between β-glucans and growth factor activity and wound healing has been well-documented. Wei *et al* (2002) examined the effect of (1-3)-β-D-glucan phosphate, a highly purified water-soluble glucan isolated from *Saccharomyces cerevisiae,* on activation of the transcription factors activator protein-1 and specificity protein-1 in normal human dermal fibroblasts and 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. Wound healing is an immune mediated event (DiPietro, 1995) and agents which modulate the innate immune response are consequently accepted to modulate the wound healing process (Browder *et al*., 1988; Compton *et al*., 1996; Portera *et al*., 1997). Interestingly the observed effect of β-glucans as a nutritional intervention on up-regulating of the growth factor activity pathway is indirectly immune regulatory which was hypothesized beforehand. In addition, Delatte *et al* (2001) reported that paediatric burns can be effectively treated with glucan–collagen mixtures. In another study, the effects of (1,3/1,6)-β-D-glucan on lung immune development in the neonatal piglet was investigated and showed that β-glucan reduced the mRNA expression of transforming growth factor (TGF) β2 and tended to reduce the mRNA expression of TGF-β1 in lung tissue (Thorum *et al*., 2013). So the effects of dietary β-glucans on immune responses can be mediated via regulating activities of growth factors.

The genes related to anion transmembrane transporter activity in the quercetin and oat hulls included diet were expressed less. Effects of nutritional intervention on regulating gene expression of anion transmembrane transporter activity has been also reported (Lillycrop *et al*., 2010). Although the effectiveness of the nutritional interventions on regulating gene expression, tested in the current study, was very limited, Xiao *et al* (2012) indicated in 3-wk old broilers, that were supplemented with mannan-oligosaccharides (MOS) from day-old onwards, a total of 672 deferentially expressed genes (fold change >1.2) in the jejunum. The expressed genes were involved in diverse biological functions including energy production, cell death, and protein translation. In addition, these authors demonstrated that expression of 77 protein synthesis-related genes was deferentially regulated by MOS in the jejunum, whereas further pathway analysis indicated 15 genes related to oxidative phosphorylation were upregulated in the jejunum, and expression of genes important in cellular stress

response, such as peroxiredoxin 1, superoxide dismutase 1, and thioredoxin, were also increased by MOS. In addition, differential expression of genes associated with cellular immune processes, including lysozyme, lumican, β-2-microglobin, apolipoprotein A-1, and fibronectin 1, were observed in MOS-fed broilers. Royan *et al* (2011) also demonstrated fish oil as a more effective fat in up-regulating hepatic PPARα gene expression in broilers.

It can be concluded that changes in gene sets in the current study seem not to have an obvious relation with immune related processes, except for β-glucans which affected the gene expression of growth factor activity. The main reason for the observed discrepancy between our data and literature is not clear, but the age of the applied nutritional interventions could be the case. So it seems that nutritional modulation can be more effective when it is applied in post-hatched broilers chicks.

Other immune competence related parameters

Nutrients are the major decisive factors that determine the expression of genetic potential of birds in terms of growth and immunity (Katanbaf *et al*., 1988; Klasing and Barnes, 1988). As described below, information in literature is available regarding the modulating effects of the five nutritional interventions tested in the current study on immune responses related parameters

The bursa of Fabricius and thymus are the primary lymphoid organs in which the lymphocyte precursors develop into immune competent native lymphocytes. Secondary lymphoid tissues are the spleen, bone marrow, harderian gland, pineal gland and organised lymphoid tissues (Panda *et al*., 2015). The highest and lowest weight of bursa as a percentage of body weight was detected in fish oil on d21 and d28, respectively. Among treatments, relative weight of spleen was higher in d28 compared to d21.

The effects of the five nutritional interventions, used in the current study, on immunity related parameters of birds have been well documented in the literature.

Effect of interventions on bursa weight

In the current study, no effects of nutritional interventions on bursa and spleen weight were observed. In contrast, Wang *et al* (2000), who investigated the effects of fat source (sunflower oil, SO; animal oil, AO; linseed oil, LO; or menhaden fish oil, FO at 5%) on immune response of the offspring of the Single Comb White Leghorn laying hens, reported that growths of thymus, spleen, and bursa were significantly impacted by the amount of dietary PUFA, the ratio of n-6 to n-3 fatty acids, and n-3 PUFA components. Sridhar *et al* (2015), who studied dietary incorporation of n-3 PUFA rich vegetable oil sources (soybean oil, SO; mustard oil, MO; linseed oil, LO; and fish oil, FO) on immune response in Krishibro broilers, detected that FO improved the relative bursa weight compared to other nutritional interventions. Ao and Choct (2013) demonstrated that birds given mannan-oligosaccharide tended to have a heavier bursa and lower spleen/bursa weight ratio at 35 d of age. In the study of Wang et al (2000) however, effects were observed after supplementing omega-3 fatty acids via maternal nutrition or prenatally, whereas in the current study the fatty acids were supplemented in the adult phase.

Effect of interventions on antibody production and immune cells

Hager-Theodorides *et al* (2014) detected a linear dose-dependent manner of quercetin on IgY antibody production in response to SRBC immunization. Yang *et al* (2015), who evaluated the effects of EPA, DHA, and a combination of both, on the signalling pathways in lipopolysaccharide (LPS)-stimulated intestinal B lymphocytes from broiler chickens, observed that dietary n-3 PUFA suppressed the LPS stimulated proliferation of B lymphocytes by interfering with phosphatidylinositol signalling and the second messenger pathways. Dietary supplementation of MOS increased mucosal IgA secretions and humoral and cell-mediated immune responses of neonatal chicks (Gomez-Verduzco *et al*., 2009). Yitbarek *et al* (2015) who compared the effectiveness of a yeast-derived carbohydrates (YDC), and a blend of YDC and probiotics (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Streptococcus faecium*, *Bacillus subtilis)*, plus YDC as a sort of symbiotic, SNB) in pullets, showed that SNB compared to YDC had a more balanced T-helper (Th)-1/Th-2 response locally, and a more Th-2-dependent response systemically. It has been reported that dietary supplementation with yeast products derived from *Saccharomyces cerevisiae* can modulate both the innate and humoral immune system in broilers (Gao *et al*., 2008; Muthusamy *et al*., 2011; Yitbarek *et al*., 2013).

Although the composition of the yeast-derived components are variable, they essentially are rich sources of s1,3-1,6-glucan, mannan polysaccharides and nucleotides (Lipke and Ovalle, 1998). Yeast cell wall polysaccharides, including *β*-glucans and mannan, could act as microbe associated molecular patterns and modulated the immune system through pattern recognition receptors (PRR) (Ferket *et al*., 2002; Jawhara *et al*., 2012; Shashidhara and Devegowda, 2003). Modulation of PRR expressed by cells of the innate immune system including macrophages and dendritic cells would be followed by production of cytokines, some of which are involved in B cell development and antibody production (Reise-Sousa, 2004). Gomez-Verduzco *et al* (2009) demonstrated that supplementation of 0.05% of yeast cell wall in the diet increased humoral and cell mediated immune response in broiler chickens

following natural exposure to *Eimeria* spp. Lowery *et al* (2005) showed that a diet containing purified *β*-glucans induced an innate immune response against *Salmonella enterica* serovar *Enteritidis* in immature chickens. *In vivo* administration of β-glucans enhanced immune reactions and up-regulate the resistance of host against tumour cells (Kogan, 2000). Furthermore, administration of glucans to macrophages activated by LPS increased production of cytokines like interleukin-1 and TNF-α, which subsequently induced lymphocyte differentiation and proliferation to enhance immune responses (Adachi *et al*., 1994; Chihara, 1992). Alizadeh *et al* (2016) reported that dietary supplementation with 0.25% of yeast cell wall stimulated Th2 cell-mediated immune response indicating the immunomodulatory activities of these products following immunization with non-inflammatory antigens.

Zimmermann *et al* (2015) demonstrated in vitro dose-dependent cyto-protective and geno-protective effects of β-glucans on broiler chicken lymphocytes with damaged DNA by aflatoxin B1. A large number of *in vitro* and in vivo studies support an immuno-modulatory role for quercetin and other flavonoids in man and in rodents (García-Lafuente et al., 2009; González-Gallego et al., 2010; Serafini *et al*., 2010). Studies on human lymphocytes showed that flavonoids reduced immune cell proliferation and *in vitro* cytokine production in response to PHA stimulation (Pandey *et al*., 2005). Yang *et al* (2015) showed that EPA and DHA suppressed the LPS-stimulated proliferation of B lymphocytes by interfering with phosphatidylinositol signalling and the second messenger pathways.

In the current study, the production of antibodies and immune cells were not measured. Based on the information received from the expressed genes, however, there were no indications that immune related processed were affected by the applied interventions during the period of 15 to 28 days of age.

Effect of interventions on intestinal adhesion of pathogens

In broilers, β-glucan has been shown to be an excellent adjuvant for the avian influenza H5 subtype vaccine, enhancing the vaccine immunogenicity (Le *et al*., 2011). Mannan-oligosaccharide-bound pathogens were prevented from attaching to intestinal mannose residues (Spring *et al*., 2000), and mannose blocked the colonization of intestinal pathogens, such as *Salmonella* spp. and *Escherichia coli* (Kelly *et al*., 1994). MOS stimulated intestinal mucosal immunity mostly by acting as a non-pathogenic microbial antigen (Davis *et al*., 2004a,b).

In the current study, the adhesion of pathogens to intestinal tissue was not determined.

Conclusions

Based on results of the present investigation it can be concluded that:

- Supplementing oat hulls and lysozyme resulted in comparison with the control group in a temporary reduction in feed intake and body weight gain during the first week of supplementation. Birds compensated for it during the second week of supplementation, with overall no effects on performance.
- Compared to the control group, litter score improved by feeding oat hulls and fish oil (d28), and oat hulls and lysozyme (d33).
- No significant differences were observed in microbiota composition of jejunum and ileum.
- Nutritional interventions only slightly affected the microbiota composition in jejunum and ileum.
- Compared to the control group, feeding oat hulls resulted in an increased amount of *Enterococcaceae* in the ileum.
- Changes in gene sets seem not to have an obvious relation with immune related processes, except for β-glucans which affected the gene expression of growth factor activity.
- Adding lysozyme to feed decreased villus height in jejunum (d28) compared to the control and oat hulls fed birds.
- Feeding oat hulls resulted in an increased villus/crypt ratio, total surface area of villi occupied by goblet cells (μm^2) and total villi surface area (μm^2) in jejunum.

In the current study, five dietary interventions, largely varying in the expected mode of actions to affect immune competence of broilers, were tested in 14-28 d old broilers. Despite the different types of interventions, parameters related to immune competence were only marginally affected by the tested products. It seemed that in this study inclusion of oat hulls, and probably β-glucans had perspective to improve immune competence. The marginal effects could be related to the age of the birds (14-28 d of age), which might be a less sensitive age for affecting parameters related to immune competence. It is recommended to also evaluate some of the tested interventions, especially dietary inclusion of oat hulls and β-glucans, in broilers starting at early age. In a follow-up study, the effects of some of the investigated interventions on immune competence will be determined in young broilers under challenged conditions.

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Appendix 1 Calculated composition of the diets

Dietary ingredients	Control*	4 % Fish oil %	5 % Oat hulls
Maize	35.12	35.12	28.39
Wheat	10.0	10.0	10.0
Peas	8.77	8.77	5.46
Rapeseed extract	24.99	24.99	24.99
Limestone	1.18	1.18	1.14
Salt	0.21	0.21	0.22
L-Lysine HCL	0.23	0.23	0.21
DL-Methionine	0.19	0.19	0.19
L-Threonine	0.06	0.06	0.06
L-tryptophane	0.01	0.01	0.01
Premix	0.50	0.50	0.50
Sodium bicarbonate	0.16	0.16	0.16
MCP	0.99	1.02	1.02
Soya oil	2.0	2.0	4.88
Animal fat	5.59	1.59	5.59
Fish oil	0.0	4.0	0.0
Oat hulls	0.0	0.0	5.0
Soybean meal	10.00	10.00	12.18
Calculated amount			
ME (MJ)	11.77	11.77	11.79
Ca	0.87	0.87	0.87
Cl	0.22	0.22	0.22
K	0.79	0.79	0.80
Na	0.14	0.14	0.14
P	0.72	0.72	0.71
Available P	0.35	0.35	0.35
Dry matter	88.47	88.47	88.93
NSP	18.20	18.20	20.76
Crude fiber	4.83	4.83	5.99
Crude protein	19.49	19.49	19.51
Crude fat	9.93	9.93	12.63
Suger	4.36	4.36	4.44
Starch	30.73	30.73	25.66
Dig. Lys	1.00	1.00	1.00
Dig. Met	0.47	0.47	0.48
Dig. Ile	0.64	0.64	0.64
Dig. Thr	0.67	0.67	0.67
Dig. Trp	0.20	0.20	0.20
Dig. Val	0.77	0.77	0.77
C16:0	1.61	1.61	1.89
C18:2	2.42	2.42	3.80
C18:3	0.27	0.27	0.49

Appendix 2 Calculated composition of the starter and finisher diets

Dietary ingredients	Starter	Finisher
Maize	62.02	25.00
Soybean meal	32.23	19.64
Wheat	-	43.61
Rapeseed extract	0.24	5.00
Soya oil	1.50	1.20
Limestone	1.61	1.06
Monocalcuum phosphate	0.86	0.18
RDS Premix broiler 5%	0.50	0.50
L-Lysine HCL 79%	0.21	0.24
DL-Methionine 99%	0.29	0.20
L-Threonine 98%	0.07	0.07
Sodium bicarbonate	0.17	0.17
Salt-NaCl	0.23	0.19
Xylanase	-	0.01
Phytase	0.05	0.05
Salinocox 12%	0.06	-
Calculated amount		
Moisture	11.90	11.80
ME (MJ)	12.44	12.94
Ca	0.86	0.55
Cl	0.23	0.23
K	0.91	0.76
Na	0.14	0.15
P	0.55	0.40
Crude fiber	2.71	2.97
Crude protein	21.21	18.66
Crude fat	5.02	6.73
Ash	5.64	4.18
Starch-Am	39.19	41.08
Starch-Ew	41.75	43.46
dlys-P %	1.15	0.95
vMethP/vLys.-P	0.50	0.48
vCyst-P/vLys.-P	0.24	0.29
vM+CP/vLys.-P	0.74	0.76
vThreoP/vLys.-P	0.65	0.65
vTryP/vLys.-P	0.19	0.20
Synth.Lys/ vLys.-P %	14.50	19.90

Appendix 3 Chemical and nutritional composition of the rapeseed extract used in the present experiment comparing with canola meal

(Khajali and Slominski, 2012)

Component	Rapeseed extract	Canola meal
ME (Kcal/Kg)	1384	2000
Crude protein	33.5	36.5
Ether extract	2.6 or 3.8	3.6
Dry matter	87.3	90
Crude fiber	12.0	11.6
ADF	29.2	18.2
NDF	19.4	26.0
NSP	35.2	18.0
Sugars	9.0	8.6
Starch	6.1	2.4
Lignin and polyphenoles		10.4
Glycoproteins		3.3
Glucosinolates (µmol/g)		5.5
Ash	6.7	6.8
Ca	0.74	0.67
Available P	0.36	0.38
Arginine		2.04
Lysine	1.8	2.0
Threonine	1.47	1.57
Methionine	0.67	0.74
Cystine	0.84	0.85
Tryptophan	0.44	0.48

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

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