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Effect of nutritional interventions with quercetin, oat hulls, β-glucans, lysozyme and fish oil on performance and health status related parameters of broilers chickens

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ABSTRACT

1. An experiment was conducted to evaluate the effects of technical feed ingredients between 14 and 28 d of age on performance and health status of broilers (d 14–35) fed diets with a high inclusion rate of rapeseed meal as a nutritional challenge. It was hypothesized that the feed ingredients would improve health status related parameters.
2. A total of 1008 one-day-old male Ross 308 chicks were distributed over 36 floor pens and allocated to one of six iso-caloric (AME_N 13 MJ/kg) growing diets (d 15–28): a control and five test diets supplemented with quercetin (400 mg/kg), oat hulls (50 g/kg), β-glucan (100 mg/kg), lysozyme (40 mg/kg) or fish oil ω-3 fatty acids (40 g/kg), with six replicate pens per treatment.
3. Dietary inclusion of oat hulls and lysozyme resulted in a reduction in broiler performance during the first week after providing the experimental diets.
4. No effect of interventions on the microbiota diversity in the jejunum and ileum was observed. Ileal microbiota composition of birds fed oat hulls differed from the other groups, as shown by a higher abundance of the genus *Enterococcus*, mainly at the expense of the genus *Lactobacillus*.
5. In the jejunum, villus height and crypt depth of lysozyme-fed birds at d 28 were decreased compared to the control group. Higher total surface area of villi occupied by goblet cells and total villi surface area in jejunum (d 21 and 28) were observed in chickens fed oat hulls compared to other groups.
6. Genes related to the growth-factor-activity pathway were more highly expressed in birds fed β-glucan compared to the control group, while the genes related to anion-transmembrane-transporter-activity pathway in the quercetin- and oat hull-fed birds were less expressed. The genes differently expressed between dietary interventions did not seem to be directly involved in immune related processes.
7. It was concluded that the tested nutritional interventions in the current experiment only marginally effected health status related parameters.

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Broiler; gene expression; gut microbiota; nutritional interventions; performance; rapeseed meal

Introduction

Rapeseed meal (RSM) is a poorly digestible protein source with relatively low amino acid digestibility, and high dietary RSM inclusion level, particularly at inclusion levels of 25% or higher, can decrease broiler performance (Qaisrani et al. 2015a). Undigested protein at the ileal level may increase the amount of undigested amino acids reaching the hindgut, thereby enhancing proteolytic fermentation by resident microbiota (Libao-Mercado et al. 2009). This could result in the formation of toxic compounds, such as amines, ammonia, skatol or indoles that negatively influence the performance of broilers (Gabriel et al. 2006). In previous studies, the effects of some dietary manipulations to ameliorate the detrimental effects of feeding RSM on performance and gut health of broiler have been tested (Qaisrani et al. 2014, 2015b). Feeding coarse RSM-rich diets to broilers increased feed intake, body weight (BW) gain (BWG) and feed conversion ratio (FCR) compared with broilers fed fine RSM-rich diets (Qaisrani et al. 2014). Feeding RSM-rich diets to broilers could be partly counterbalanced by coarse grinding and dietary supplementation of butyric acid (Qaisrani et al. 2015b). There is a scarcity of published data regarding the effects of nutritional interventions via supplementation with technical feeding ingredients on the gut ecosystem in broilers challenged by feeding RSM-rich diets. To address this knowledge gap, in the present study, five different feed ingredients, differing in mode of action, were applied in RSM-rich diets with an RSM inclusion level of 25%. These were (i) the plant extract quercetin (active substance of yellow onion), (ii) an insoluble fibre (oat hulls), (iii) a prebiotic (β-glucan), (iv) an antimicrobial protein (lysozyme) and (v). omega-3 fatty acids from fish oil.

Quercetin, a flavonoid compound, can be absorbed and metabolized by broilers (Rupasinghe et al. 2010). Many *in vitro* and *in vivo* studies have demonstrated the 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), antioxidant activity (Arts *et al.*, 2004), anti-inflammatory properties in mice (Comalada et al. 2006; Huang et al. 2010) and humans (Sternberg et al. 2008; Boots et al. 2011) and anti-bacterial properties (Waage and Hedin 1985).

Many studies have shown that moderate amounts of fibre increased gizzard function (Rogel et al. 1987; Hetland and Svihus 2001; Svihus 2011), digestibility of non-fibre

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Supplemental data for this article can be accessed here.

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nutrients (Jimenez-Moreno et al. 2009; Gonzalez-Alvarado et al. 2010; Mateos et al. 2012), gastrointestinal tract (GIT) health (Kalmendal et al. 2011; Mateos et al. 2012) and performance characteristics (Gonzalez-Alvarado et al. 2007; Jimenez-Moreno et al. 2013a) in broilers.

Increased immunomodulatory effects of dietary β-glucans compared to the respective controls have been demonstrated in a wide variety of animal species (Guo et al. 2003; Chae et al. 2006; Chen and Seviour 2007). β-glucan activates the immune system, as shown by increased numbers of B-lymphocytes and macrophages (Taylor et al. 2002; Le *et al*., 2011), and higher levels of cytokines (Cheng et al. 2004). Results indicated that immune-enhancing effects in broiler chickens, and the alteration of immune-related gene expression profiles, favouring an enhanced T helper type-1 cell response during coccidiosis occurred at low dietary inclusion levels (Cox et al. 2010).

Lysozyme (muramidase), one of the best-described animal proteins (Callewaert and Michiels 2010), accounts for 3.5% of total egg white protein (Burley and Vadehra, 1989) and has bactericidal properties against Gram-positive bacteria (Phillips 1966; Ohno and Morrison 1989). It has bacteriostatic properties toward Gram-negative bacteria following chemical and thermal modification (Ibrahim et al. 1994; Johnson 1994; Masschalck et al. 2001). Plasma lysozyme may be involved in non-specific immune processes such as opsonisation, leading to phagocytosis of Gram-negative bacterial invaders that are less responsive to lysozyme (Callewaert et al. 2008). In addition, lysozyme plays 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 (Millet et al. 2007; Rowe et al. 2013). In most vertebrates, including birds, both plasma lysozyme concentration and activity increases along with pathogen exposure or inflammation processes (Millet et al. 2007; Maraghi et al. 2012), accompanied by increased blood leukocyte levels. (Maxwell and Robertson 1998).

It has been well-demonstrated that omega-3 polyunsaturated fatty acids (n-3 PUFA) from fish oil (Hulan *et al*., 1988), and in particular eicosapentaenoic acid EPA (20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), reduce arachidonic acid (ARA), a precursor of prostaglandins with important roles in inflammation and the regulation of immunity (Calder, 2006) in cell membranes. This leads to decreased production of ARA-derived pro-inflammatory mediators (Yang et al. 2008; Cherian 2011; Liu et al. 2014a, Liu et al. 2014b). EPA and DHA in poultry diets are incorporated into egg yolks, where they are available for the developing embryo via the liver and residual yolk (Koppenol et al. 2014). The offspring of broiler breeders fed a diet supplemented with n-3 PUFAs had increased antibody production and decreased cell-mediated immune responses (Wang et al. 2000, 2002).

The following study aimed to evaluate the various test feed ingredients, applied after the chick phase (d 14–28), e.g. quercetin, oat hulls, β-glucans, lysozyme and omega-3 PUFA, on the composition of the microbiota, gene expression in the small intestine, gut morphology and performance of broilers nutritionally challenged via a high inclusion rate of RSM in the diet. The RSM challenge was caused by the relatively high contents of non-starch polysaccharides, glucosinolates, sinapines, tannins, erucic acid and phytate in RSM, which might have antinutritional capacities and hamper absorption of nutrients by the gut mucosa.

Materials and methods

Animal ethics

The experiment was approved by the Ethical Committee of Wageningen UR, The Netherlands (project no. 44–00321/approval no. 201 309.b). Animal care guidelines were used as provided by the Directive 2010/63/EU (EU, 2010).

Housing, experimental animals and diets

A total of 1008 one-day-old male Ross 308 chicks, supplied by a commercial hatchery, were randomly distributed among 36 pens (1.5 m^2) and allocated to one of six experimental treatments (36 pens with 28 chicks/pen) in a mechanically ventilated room provided with facilities to control temperature, ventilation and lighting. Each pen contained a feeding trough, and three drinking bowls. Wood shavings were used as bedding material. Temperature inside the room was increased to 36°C one day before arrival of the chicks and from day one onwards, gradually decreased to 20°C. On day of placement and day one, the birds were given continuous light (24L: 0D) with a light intensity of 20 lx. From day two onwards, a day–night light schedule of 18 h light and 6 h of darkness (18L: 6D) was used. The light intensity was set at 20 lx during the entire experimental period. The chicks were vaccinated immediately after arrival against infectious bursitis and Newcastle's disease.

During the first 14 d, all the animals received the same standard starter feed, whereas the experimental (grower) diets were provided from 14–28 d, followed by a standard finisher diet from day 29 onwards. After pelleting, the starter feed was crumbled, whereas grower and finisher feeds were provided as pellets (3.2 mm). Feed and water were provided *ad libitum* to the animals. Six iso-caloric and iso-nitrogenous experimental grower diets (ME = 11.8 MJ/Kg and crude protein = 195 g/kg) were formulated. For each kg of diet, 40 mg of lysozyme (from chicken egg white, protein ≥ 90%, CAS Number 12 650–88-3, 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) or 400 mg quercetin (purity > 95%, CAS Number 117–39-5, Sigma-Aldrich, Zwijndrecht, The Netherlands) were supplemented on top of the control diet, while fish oil (40 g/kg) was supplemented to the control diet at the expense of animal fat and oat hulls (50 g/kg) were supplemented at the expense of maize with increasing levels of soybean meal and soya oil (10.0 to 12.2% for soybean meal and 2.0 to 4.9% for soya oil). The ingredient composition of the diets is shown in Table 1.

Qaisrani et al. (2014) showed that RSM inclusion levels of 25% or more had detrimental effects on bird performance. Therefore, a dietary RSM inclusion level of 25% was used to induce a nutritional challenge. Based on the results of Jimenez-Moreno et al. (2013b), who demonstrated that 5% inclusion of oat hulls resulted in an improved development of the GIT and structure of the jejunal mucosa, it was decided to include 5% oat hulls in the experimental diets. Based on results of Liu *et al.* (2014b), who tested quercetin in laying hens at inclusion levels from 0 to 600 mg/kg of diet, 400 mg/kg quercetin was included in

Table 1. Ingredients (%) and calculated nutrient composition (g/kg) of the experimental diets (as-fed basis).

	Starter	Grower			Finisher
		Control	4% Fish oil	5% Oat hulls	
Maize	62.02	35.12	35.12	28.39	25.00
Wheat	-	10.00	10.00	10.00	43.61
Peas	-	8.77	8.77	5.46	-
Soybean meal	32.23	10.00	10.00	12.18	19.64
Rapeseed meal	0.24	24.99	24.99	24.99	5.00
Limestone	1.61	1.18	1.18	1.14	1.06
Salt	0.23	0.21	0.21	0.22	0.19
L-Lysine HCL	0.21	0.23	0.23	0.21	0.24
DL-Methionine	0.29	0.19	0.19	0.19	0.20
L-Threonine	0.07	0.06	0.06	0.06	0.07
L-Tryptophan	-	0.01	0.01	0.01	-
Premix[1]	0.50	0.50	0.50	0.50	0.50
Sodium bicarbonate	0.17	0.16	0.16	0.16	0.17
Monocalciumphosphate	-	0.99	1.02	1.02	-
Soya oil	1.50	2.00	2.00	4.88	1.20
Animal fat	-	5.59	1.59	5.59	-
Fish oil	-	-	4.0	-	-
Oat hulls	-	-	-	5.0	-
Xylanase	-	-	-	-	0.01
Phytase	0.05	-	-	-	0.05
Salinocox 12%	0.06	-	-	-	-
Calculated amount					
ME (MJ)	12.44	11.77	11.77	11.79	12.94
Ca	8.6	8.7	8.7	8.7	5.5
Cl	2.3	2.2	2.2	2.2	2.3
K	9.1	7.9	7.9	8.0	7.6
Na	1.4	1.4	1.4	1.4	1.5
P	5.5	7.2	7.2	7.1	4.0
Retainable P	-	3.5	3.5	3.5	-
Dry matter	-	885	885	889	-
NSP[2]	-	182	182	208	-
Crude fiber	27.1	48.3	48.3	59.9	29.7
NDF	-	141.9	141.9	167.5	-
ADF	-	72.8	72.8	89.3	-
ADL	-	13.6	13.6	14.1	-
Crude protein	212.1	194.9	194.9	195.1	186.6
Crude fat	50.2	99.3	99.3	126.3	67.3
Sugar	-	43.6	43.6	44.4	-
Starch	-	307.3	307.3	256.6	-
Dig. Lys	-	10.0	10.0	10.0	-
Dig. Met	-	4.7	4.7	4.8	-
Dig. Ile	-	6.4	6.4	6.4	-
Dig. Thr	-	6.7	6.7	6.7	-
Dig. Trp	-	2.0	2.0	2.0	-
Dig. Val	-	7.7	7.7	7.7	-
C16:0	-	16.1	16.1	18.9	-
C18:2	-	24.2	24.2	38.0	-
C18:3	-	2.7	2.7	4.9	-

[1]Premix composition (per kg of diet): 12 000 IE retinol, 2400 IE cholecalciferol, 50 mg dl-a-tocopherol, 1.5 mg menadione, 2.0 mg thiamine, 7.5 mg riboflavin, 3.5 mg pyridoxine, 20 mcg cyanocobalamins, 35 mg niacin, 12 mg D-pantothenic acid, 460 mg choline chloride, 1.0 mg folic acid, 0.2 mg biotin, 80 mg iron, 12 mg copper, 85 mg manganese, 60 mg zinc, 0.40 mg cobalt, 0.8 mg iodine, 0.1 mg selenium, 125 mg anti-oxidant mixture.
[2]Calculated as: Dry matter – ash – crude protein – crude fat – starch – sugars

the experimental diet. Earlier findings suggested that 40 mg/kg of exogenous lysozyme supplementation could decrease *Clostridium perfringens* colonisation and improve intestinal barrier function and growth performance of chickens. Based on these findings, the same inclusion level was used in the current experiment (Liu et al. 2010). The addition of 100 mg/kg β-glucans was determined based on a broiler study (Cox et al. 2010). Finally, the dietary supplementation of 4% fish oil was based on a study conducted in maternal mice (Lauritzen et al. 2011).

Experimental grower diets were analysed in duplicate for dry matter (International Organisation for Standardisation, 1998), crude protein (International Organisation for Standardisation, 1997), crude fat (International Organisation for Standardisation, 1999), crude fibre (International Organisation for Standardisation, 1988) and ash (International Organisation for Standardisation, 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 Neutral Detergent Fibre (NDF) was based on a modified method of Van Soest (1973), as described by Suárez et al. (2006). The chemical analysis of the grower diets is shown in Table 2.

Table 2. Analysed contents (g/kg) of the grower diets, provided during d14 to d28.

	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

Performance and litter quality

BW of the birds was determined (average per pen) at placement and 14, 21, 28 and 33 d of age. Feed intake, BWG and FCR were determined per pen in the following periods: 14–21, 22–28, 29–33, 14–28 and 14–33 d of age. Culling, mortality and general health were recorded daily (including probable causes of illness or deaths). Litter quality was visually scored on a 1 (wet/100% plaque) to 10 (dry, friable) point scale on 28 and 33 d of age by one person.

Dissection and sample collection

At 21 and 28 d of age, six birds per pen were randomly selected and euthanised by electrocution. At 35 d of age, the remaining 16 chicks per pen (depending on the level of mortality) were sent of for commercial processing. After electrocution, the chest cavity and the abdomen of the euthanised birds were opened and the small intestine was ligated and removed. The digesta after the midpoint of the jejunum, defined as the 10 cm before and after the middle of the jejunum, was collected by flushing. The digesta of dissected animals were pooled per pen and immediately frozen on dry ice and stored at −80°C until further analysis for microbiota composition. The digesta was collected in the order of pen numbering. From all birds, three samples (2 cm per sample) of intestinal tissue of the jejunum were collected. A first sample was put in a formalin filled tube and stored at room temperature until further analysis for intestinal morphology (villus height and crypt depth), and number and size of goblet cells. The second sample was directly placed into liquid nitrogen and stored at −80°C until further analysis of genome-wide gene expression profiling in jejunal tissue.

Villus height, crypt depth and goblet cells were determined by GD Animal Health (Deventer, The Netherlands) per individual bird. Jejunal samples were fixed in 4% neutral buffered formalin, dehydrated and embedded in

paraffin. Tissue sections of 2 µm in size were stained with a combined Alcian Blue (pH 2.5)/PAS procedure. For each batch of slides stained, an Alcian Blue/PAS-positive control slide was included. Microscopic images of representative cross-sections of each tissue were captured by a microscope (Olympus BX41) connected to a digital camera (Olympus Dp26) and analysed using Olympus cellSens Dimension version 1.12 software. For each jejunal segment, 10 representative, and completely paired, villus-crypt units were measured. The villus:crypt ratio was determined as the length of the villi divided by the depth of the mucosal crypt region. The size and density of neutral (PAS positive) and acid mucin (Alcian Blue positive) producing goblet cells was determined and analysed in five representative, well oriented villi. The goblet cell size and density were reported as area per villus surface area and as percentages of the surface area. The average of each villus:crypt ratio, and the neutral and acid mucin producing goblet cell characteristics were calculated and reported per individual bird.

Microbiota composition and genome-wide gene expression profiling were determined from pooled samples from the birds per pen by CVI (The Netherlands). At 21 and 28 d 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.

Microbiota

Microbiota diversity index and composition in the jejunal and ileal digesta were measured in a pooled sample of digesta from six birds per pen (the content of 2 cm jejunum sections per sample) on day 21. To isolate DNA, samples were mixed in a 1:1 ratio with phosphate buffered saline and centrifuged for 5 min at 4°C at 300 × g. Supernatant was collected and centrifuged for 10 min at 4°C at 9000 × g. DNA was extracted from the most sensitive pellet using the QIAamp DNA stool minikit according to the manufacturers' instructions (Schokker et al. 2017). Quality and quantity of DNA was checked using the NANOdrop method (Agilent Technologies). PCR was used to amplify the 16S rDNA V3 fragment using the forward primer V3_F (CCTACGGGAG GCAGCAG) and reverse primer V3_R (ATTACCGCGG CTGCTGG). PCR conditions were as follows: 2 min at 98°C, 15 × (10 s at 98°C, 30 s at 55°C, 10 s at 72°C), 7 min at 72°C. PCR efficiency was checked using agarose gel by visual inspection. Jejunum digesta samples were sequenced by targeted-amplicon 16S sequencing and analysed for taxonomy profile per sample, alpha diversity and beta diversity. This included possible taxonomy associations 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/samples.

Genome-wide gene expression profiling

Tissue RNA extraction

Total RNA was extracted from 50 to 100 mg of each jejunum and ileal tissue. Each sample was 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, the Direct-zol™ RNA MiniPrep Kit from 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, hybridisation, scanning and feature extraction

Labelling RNA was performed 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. Hybridisation was performed as described in the One-Color Microarray-Based Gene Expression Analysis Low Input Quick Amp Labelling protocol from Agilent in a hybridisation oven (G2545A hybridisation Oven Agilent Technologies). The hybridisation temperature was 65°C with rotation speed 10 rpm for 17 h. Then the arrays were washed as described in the One-Color Microarray-Based Gene Expression Analysis Low Input Quick Amp Labelling protocol from Agilent. The arrays were scanned using the DNA microarray scanner with Surescan High Resolution Technology from Agilent Technologies. Agilent Scan Control with resolution of 5 µm, 16 bits and PMT of 100%. Feature extraction was performed using protocol 10.7.3.1 (v10.7) for one colour gene expression.

Data analysis

The data were analysed by using the software package R (version 3.0.2) by executing different packages, including LIMMA and array Quality Metrics. 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 was removed in a three-step procedure, (1) get the highest of the dark spots to obtain 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

Because far more human genes are annotated, and more information in databases is available for humans than for poultry, a human background was used for this functional analysis. A gene set enrichment analysis was performed on all different contrasts compared to the respective control (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 sets ('biological processes', 'molecular function' and 'cellular component') and one pathway related database Kyoto Encyclopedia of Genes and Genomes were used for all analyses. Furthermore, all annotated probes (i.e. genes) were used as input, after transforming them to human gene names.

Statistical analysis (except for gene expression data)

The experimental data (performance, litter quality, bursa/spleen content, microbiota genera and gut morphology data) were analysed using Genstat statistical software (Genstat 8 Committee 2002). Statistical significance was declared at $P < 0.05$, with $0.05 < P < 0.10$ considered as a nearly-significant trend. The *P*-value of the treatment effect and the least significant difference were provided for each response parameter. Response parameters were analysed using ANOVA according the following model:

$Y_{ij} = \mu + \text{Treatment}_i + \text{Error}_{ij}$,

where Y = Response parameter, μ = General mean, Treatment = effect of diet (i = 1…6) and Error = Error term.

Pairwise differences are marked with a letter in superscript. Effects of dietary treatments on microbiota composition were determined at taxonomical level, where the minimum relative-abundance limit of a taxon was set 0.005.

Results

Bird performance and litter quality

Feed intake during the first week of the growing period (d 14–21) was lower in broilers fed the diets including oat hulls (−5 g/d) or lysozyme (−10 g/d) compared to birds fed the control diet, but feed intake was not affected by treatment after the first week (d 22–28 and d 29–33) (Table 3). From d 14 to 21, BWG of broilers fed the lysozyme supplemented diet was lower compared to the control diet fed birds (55 *vs.* 64 g/d; $P < 0.05$), where BWG in this period tended to decrease in birds fed the oat hulls (60 g/d) or fish oil (61 g/d) supplemented diet.

During the second week of the growing period (d 22–28), BWG was higher in the birds fed the lysozyme supplemented diets compared to the control diet fed birds (77 *vs.* 62 g/d), where BWG of the broilers fed the oat hulls (74 g/d) and fish oil (70 g/d) supplemented diets was in between. From d 14 to 21, FCR was poorer in broilers fed the lysozyme supplemented diets compared to birds fed the control diet (1.54 *vs.* 1.42), where FCR of birds fed the diet containing the oat hulls was in between (1.48). During d 22 to 28, FCR was improved in broilers fed the diets supplemented with lysozyme (1.69), oat hulls (1.70) and fish oil (1.77) compared to control diet fed birds (1.94). No carry-over effects of dietary treatments on BWG, feed intake and FCR was observed in the finishing period (d 29–33). At d 28, birds fed diets containing oat hulls and fish oil had better litter scores compared to the control diet fed birds. At d 33, litter score was better in the birds fed diet including oat hulls or lysozyme compared to the control diet fed birds.

Microbiota

Microbiota composition, as shown by hierarchical clustering of jejunal and ileal digesta samples per pen at species level for d 21 (Figure 1) showed no large differences between pens, and consequently no significant treatment clustering in jejunum and ileum was seen. There was no real clustering by treatment, indicating that nutritional interventions did not have a meaningful effect on the microbiota composition.

Alpha diversity by Shannon index for ileum and jejunum showed no significant effects of treatment on diversity index (Figure 2), indicating that the degree of diversity in

Table 3. *Feed intake (g/d), body weight gain (g/d) and feed conversion ratio (FCR) presented per treatment for the different experimental periods, and litter conditionscore[1] presented per treatment for two different samplings (d 28 and d 33).*

Parameters	Control	Quercetin	Oat hulls	Lysozyme	β-glucan	Fish oil	*P*-value	*SE*
Feed intake (g/d)								
d 14–21	117[a]	117[a]	112[b]	107[c]	117[a]	117[a]	<0.001	2.3
d 22–28	167	178	177	185	171	177	0.135	6.6
d 14–28	142	147	145	146	144	147	0.494	3.1
d 29–33	235	235	235	237	234	235	0.995	5.5
d 14–33	176	182	181	184	177	181	0.436	4.5
Body weight (g)								
d 14	489.8	484.0	482.1	488.9	482.6	482.7	0.584	8.7
d 33	2120[ab]	2071[bc]	2138[a]	2079[abc]	2056[c]	2064[bc]	0.077	49.6
Body weight gain (g/d)								
d 14–21	64[ab]	65[a]	60[b]	55[c]	62[ab]	61[b]	0.002	2.4
d 22–28	62[b]	65[b]	74[ab]	77[a]	63[b]	70[ab]	0.004	4.1
d 14–28	63	65	67	66	63	66	0.109	1.6
d 29–33	138	141	137	140	141	137	0.818	4.0
d 14–33	83	85	85	85	83	85	0.666	1.8
Feed conversion ratio								
d 14–21	1.42[b]	1.42[b]	1.48[ab]	1.54[a]	1.45[b]	1.45[b]	0.014	0.034
d 22–28	1.94[a]	1.87[ab]	1.70[c]	1.69[c]	1.88[ab]	1.77[c]	0.001	0.049
d 14–28	1.64	1.62	1.59	1.64	1.64	1.60	0.200	0.025
d 29–33	1.70	1.67	1.71	1.70	1.67	1.71	0.716	0.036
d 14–33	1.66	1.64	1.63	1.64	1.65	1.63	0.601	0.021
Litter condition score[1]								
d 28	5.3[c]	5.0[c]	7.0[a]	5.8[bc]	5.3[c]	6.2[ab]	0.001	0.45
d 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).
[a]Means in a row with different superscripts differ significantly ($P < 0.05$).

microbiota composition in these segments was not affected by the dietary treatments.

The principal component analysis showed that the ileal microbiota composition at d 21 of the oat hulls treatment differed from those of the other experimental treatments, which were all centred on the origin (Figure 3). 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 observed in the ileum within the genus *Enterococcus* (Figure 4), which was higher ($P < 0.05$) 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 *Lactobacillus* spp.

Gut morphology

On d 21, no effects of dietary treatments on jejunal and ileal villus height, crypt depth and villus to crypt ratio were observed (data not shown). Villus height of the lysozyme fed birds (1076 μm) in the jejunum at d 28 was decreased compared to the control (1354 μm) and the oat hulls group (1558 μm) ($P = 0.005$). Crypt depth of lysozyme fed birds in the jejunum samples at d 28 (307 μm) was decreased compared to all other dietary interventions (on average 433 μm; $P = 0.001$). No effects of dietary treatments on ileal villus height, crypt depth and villus to crypt ratio at d 28 were observed.

Dietary treatments did not affect the jejunal and ileal average number of goblet cells per villi, average goblet cell size or goblet cell surface relative to the total villi surface area at either d 21 or d 28 (data not shown). Both at d 21 and 28, the jejunal total surface area of villi occupied by goblet cells was increased in the oat hulls fed birds (5030 μm^2 as average of d 21 and 28) compared to all other dietary treatments (on average 3543 μm^2; $P = 0.019$). Likewise, at d 21 and 28, the jejunal average total villi surface area was increased in the oat hull supplemented birds (267 420 μm^2) compared to all other dietary treatments (on average 218 668 μm^2; $P = 0.014$).

Figure 1. Hierarchical clustering of microbiota composition (percentage distance between pens) of jejunal and ileal digesta per treatment at pen level (d 21). Jej = jejunum, ile = ileum, C = control diet, L = lysozyme diet, Q = quercetin diet, B = Beta-glucan diet, O = oat hulls diet and F = fish oil diet.

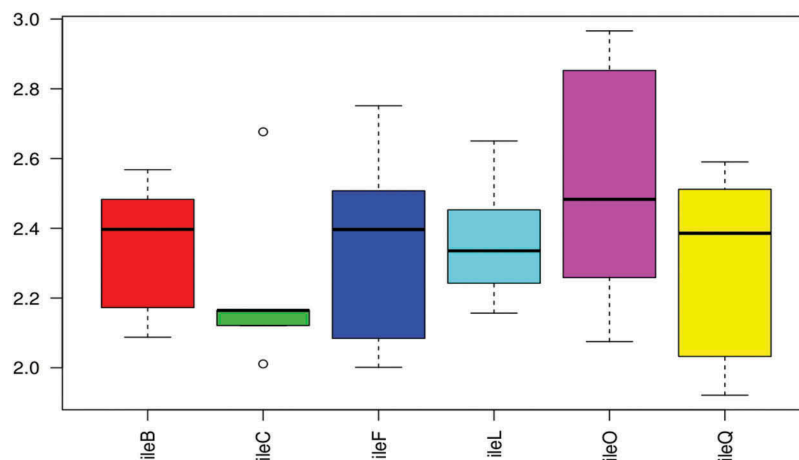

Figure 2. Diversity in ileal microbiota composition (d 21), expressed by the Shannon diversity index. C = control diet, L = lysozyme diet, Q = quercetin diet, B = Beta-glucan diet, O = oat hulls diet and F = fish oil diet.

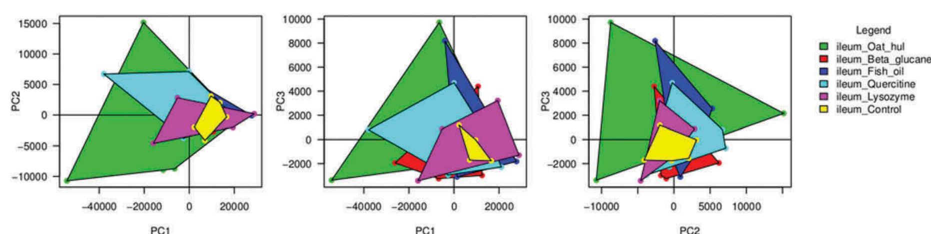

Figure 3. Principal component analysis on microbiota in ileum (d 21).

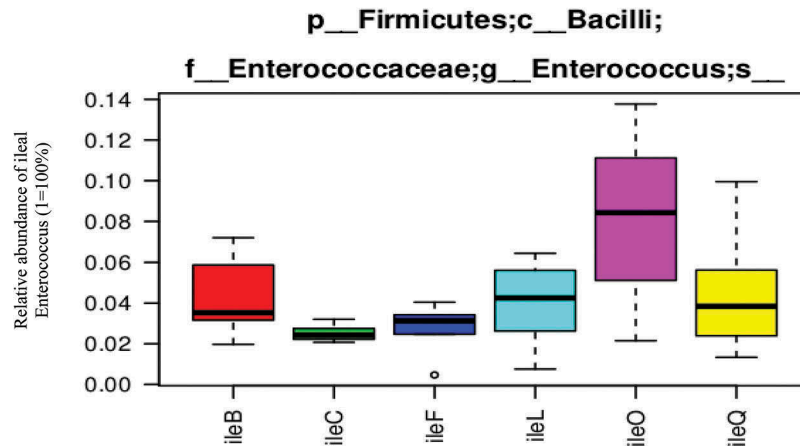

Figure 4. Relative abundance of ileal Enterococcus (1 = 100%) per treatment (d 21). C = control diet, L = lysozyme diet, Q = quercetin diet, B = Beta-glucan diet, O = oat hulls diet and F = fish oil diet.

Gene expression

Clustering of gene expression in the jejunum substantially differed in the ileum, thereby expressing the functional differences between both gut regions. Based on the principal component analysis, no significant effects of the feed treatments on expression of genes was observed in either jejunal or ileal tissue. Nevertheless, based on analysis of individual genes, the tested feed ingredients affected a few specific biological processes in the ileum (data not shown). Compared to the control birds, genes related to the growth-factor-activity pathway were more highly expressed in chickens fed the diet including β-glucan (FDR q-value = 0.026), whereas genes related to the anion-trans-membrane-transporter-activity pathway in the quercetin and oat hulls supplemented diet were less 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.

Organ weights

A significant interaction between treatment and sampling day for relative bursa weight was observed (P = 0.005; Table 4). At d 21, relative bursa weight of birds fed fish oil was increased compared to the control group, whereas relative bursa weight of these birds did not differ from the control at d 28. There was no significant effect of treatment on relative spleen weight, but the effect of sampling day was significant (P < 0.001). Relative spleen weight was higher at d 28 compared to d 21.

Table 4. Effects of nutritional interventions on weight of lymphoid tissues (bursa of Fabricius and spleen) relative to body weight (%) in two samplings (d 21 and d 28).

	Control	Quercetin	Oat hulls	Lysozyme	β-glucan	Fish oil
Bursa of Fabricius						
d 21	2.19^{abcd}	2.43^{de}	2.22^{bcd}	2.20^{abcd}	2.31^{cde}	2.54^{e}
d 28	2.14^{abc}	2.16^{abc}	2.33^{cde}	2.15^{abc}	2.04^{ab}	1.95^{a}
SE	0.530					
P-value Treatment			0.109			
P-value day			< 0.001			
P-value Trt x day			0.005			
Spleen						
d 21	1.11	1.18	1.14	1.12	1.16	1.12
d 28	1.36	1.25	1.27	1.26	1.22	1.33
SE			0.280			
P-value Treatment			0.313			
P-value day			< 0.001			
P-value Trt x day			0.316			

[a]Means in a row with different superscripts differ significantly (P < 0.05).

Discussion

Nutritional challenge by dietary RSM inclusion

During the grower period, the performance level of the broilers was below the Ross 308 performance standards for male broilers (Ross, 2012), which was assumed to be due to the high dietary RSM content. At d 28, average BW of the birds was 1393 g, whereas BW according to the ROSS (2014) standard was 1576 g, showing that the applied nutritionally challenge was effective. After the switch to the commercial finisher diets, it seemed that compensatory growth occurred, resulting in BWs at d 33 (2088 g) that met the ROSS (2014) standard (2075 g).

Performance and litter quality

Feed intake of broilers fed the diet supplemented with oat hulls or lysozyme was reduced during the first week of the growing period. This temporary change may have been partly the result of the high dietary percentage of crude fat (13 *vs.* 10%), which was needed to make it isocaloric to the other diets. It is known that high-fat diets reduce voluntary feed intake, especially in young chicks (Fuller and Rendon 1977). During the first week of the growing period, a trend (P > 0.05) for decreased BWG of broilers fed the diets supplemented with lysozyme, oat hulls or fish oil was observed, compared to the control group. The decreased BWG of the oat hulls fed broilers was likely due to reduced feed intake. Improved FCR in broilers fed diets supplemented with oat hulls during the second week of the growing period may be partly explained by the positive effects of the coarse oat hulls particles on gut integrity (villus height) and gizzard development (Qaisrani et al. 2014, 2015b). There was no clear explanation for the altered FCR in the lysozyme or fish oil fed broilers, which showed increased FCR during the first and

decreased FCR in the second week of the growing period, although the latter might be due to compensatory growth.

The effectiveness and suggested modes of action of the five feed ingredients applied in this study differ. No benefits of insoluble fibre on growth have been demonstrated in pelleted diets (Hetland et al. 2003; Van der Hoeven et al. 2014). In the current study, increased levels of soya oil, which contained mainly unsaturated lipids, were supplemented in the diet supplemented with oat hulls. The birds fed the diet containing oat hulls showed an increased feed intake and FCR, which might reflect the combined result of the increased coarseness of the diet (Qaisrani et al. 2014, 2015b), and higher crude fat level (Jimenez-Moreno et al. 2009, 2016).

In line with the findings of the current experiment, Gong et al. (2016) reported no effect of dietary supplemental lysozyme (100 ppm from d 0 to d 35) on broiler performance, both under clean and dirty conditions via providing used litter. Goliomytis et al. (2014) added quercetin to feed and observed no effect on BW and feed intake of broilers compared to the control group but reported a poorer FCR and higher relative heart weight with increasing supplementation levels of dietary quercetin.

Increased litter scores were observed in birds fed diets containing oat hulls and fish oil at d 28, and in those fed oat hulls and lysozyme at d 33. For diets with oat hulls this has already been shown (Van Der Klis and De Lange (2013), E et al. (2014)).

Gut microbiota

Gut microbiota have an important role in broiler health and performance (Klosterbuer et al. 2011). In the current study, small differences between treatments were observed in terms of microbiota composition at d 21, both in the jejunum and ileum. This may have been related to the timing of application of the interventions, i.e. d 14–28. The ileal microbiota composition of the oat hulls fed birds differed from the control, because of a higher share of *Enterocccecae* spp. It has been demonstrated that several factors can influence the host's gut microbiota, e.g. 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; Lumpkins et al. 2010). Nevertheless, changes in gut microbiota due to supplementation did not always cause altered broiler performance (Pedroso et al. 2006; Geier et al. 2009). The primary focus in this study was to increase health status and not performance related parameters, mainly by affecting microbiota composition. Gong et al. (2016) reported no effect of dietary supplemental lysozyme (100 ppm from d 0 to d 35) on gut microbiota of broilers under clean conditions. But when the experiment was repeated in dirty conditions with used litter, feeding lysozyme to birds from d 5 to d 14 and throughout the trial, reduced the number of *E. coli* in the ileum compared with feeding virginiamycin to birds.

A higher abundance of *Enterococcaceae* spp. was observed in the ileum of birds fed the diet including oat hulls compared to the control and the fish oil diets. Interestingly, it was observed that *Enterobacteriaceae* spp. were more prevalent in ileum of three-day-old broilers fed antimicrobial supplemented diets (zinc bacitracin), compared to broilers fed antimicrobial free diets (Torok et al. 2011), indicating that the effects of oat hulls on the broiler microbiota composition, to a certain extent, were comparable with that of the effect of antimicrobials. In a study of Lu et al. (2003), *Lactobacillus* spp. were the major genus observed in the ileum (70%), whereas the other genera included *Clostridiaceae* (11%), *Streptococcus* (6.5%) and *Enterococcus* spp. (6.5%). 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 or cellulose supplemented diet. It can be hypothesised that the higher amount of insoluble fibre, together with a higher level of unsaturated fatty acids (from soya oil), as present in the oat hull diet caused the higher abundance of *Enterococcaceae* spp. in the ileum of the birds.

Zhang et al. (2010) showed that a lysozyme-based antimicrobial blend, containing lysozyme and other natural antimicrobials, was effective in reducing negative health effects in broilers that suffered from necrotic enteritis, caused by a challenge with *Eimeria maxima* and *C. perfringens*. In the current study, lysozyme addition did not affect the jejunal and ileal microbiota composition of healthy broilers.

Overall, the current study showed that only the supplementation of oat hulls affected ileal microbiota composition, as shown by an increased amount of *Enterococcaceae* spp. at the expense of *Lactobacillae* spp.

Gut morphology

Villus height and crypt depth from the jejunum of the lysozyme fed birds at d 28 were decreased compared to the control. Villi height reflects the absorptive capacity of the intestine (Teirlynck et al. 2009). Shorter villi and deeper crypts are associated with the presence of pathogenic microorganisms (Cook and Bird, 1973; Gabriel et al. 2006). The supplemented treatments studied here did not deviate significantly from the control in villus/crypt ratio in the jejunum at d 28, although the villus/crypt ratio of the birds fed oat hulls tended ($P > 0.05$) to be higher compared to the control. Similarly, Jimenez-Moreno et al. (2013b) did not detect any effect of oat hulls inclusion on jejunal villus height of the GIT in broilers from d 1 to d 18. In the current experiment, dietary supplementation with β-glucans did not affect villus height in the jejunum and ileum.

Average total villi surface area was affected by dietary supplementation at the two sampling days, i.e. d 21 and d 28. The total surface area of jejunal villi occupied by goblet cells and the average total jejunal villi surface area was higher in the oat hull fed birds as compared with other treatments.

These findings show that feeding insoluble fibres to broilers may increase gut morphology, which is indicative of a high absorptive capacity and improved gut health. Further research is required to unravel the underlying mechanisms, especially if the insoluble fibres are provided to the birds from day-old onwards.

Gut gene expression

No significant effects of the treatments on expression of individual genes were observed in the jejunum. Birds fed the β-glucan diet showed, in ileal epithelial cells, more genes involved in the growth factor activity pathway compared to

the control and this pathway is related to wound healing. 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. These authors showed that β-glucan supplementation 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 (TGF) alpha, TGF beta and vascular endothelial growth factor mRNA. Wound healing is an immune-mediated event (Dipietro 1995), and agents which modulate the innate immune response may contribute to the modulation of wound healing process (Browder et al. 1988; Compton et al. 1996; Portera et al. 1997). Therefore, the observed effect of β-glucans on up-regulation of the growth factor activity pathway indirectly contributed to immune responses, which was hypothesised initially. In another study, (1,3/1,6)-β-D-glucan affected lung immune development in the neonatal piglet because of a reduced mRNA expression of TGF-β2 and a trend for reduced mRNA expression of TGF-β1 in lung tissue (Thorum et al. 2013). Thus, the effects of dietary β-glucans on immune responses can be mediated via regulating activities of growth factors.

The genes related to the anion transmembrane transporter activity pathway were less expressed in birds fed the quercetin and oat hulls supplemented diets compared to the control group. Effects of other treatments (e.g. restricted protein supply) in maternal rats regarding regulating gene expression of the anion transmembrane transporter activity pathway in the progeny have been reported (Lillycrop et al. 2010). Although the effectiveness of the different treatments in the current study on regulating gene expression was limited,

it can be concluded that changes in the gene sets from the current study seem not to have an obvious relation with immune related processes, except for β-glucans which affected the gene expression of the growth factor activity pathway.

Other health status related parameters

No effects from the dietary treatments were seen for bursa and spleen weights. In contrast, Wang et al. (2000), who investigated the effects of fat source (sunflower oil, animal oil, linseed oil or menhaden fish oil, at a 5% inclusion level) on immune response of the offspring of the Single Comb White Leghorn laying hens, reported that the development 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.

The studied nutritional challenge, because of the high dietary inclusion level of RSM, worked. The five supplementation factors applied, which were selected because of their different presumed mode of actions, only marginally affected parameters related to health status. It seemed that oat hulls, and probably β-glucans, were able to increase health status, where quercetin, lysozyme and fish oil were not. These marginal effects could be related to the age of the birds (14–28 d), which might be a less sensitive window for affecting parameters related to health status. It can be recommended that evaluation some of the treatments, especially oat hulls and β-glucans, needs to start at an early age in poultry.

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No potential conflict of interest was reported by the authors.

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