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LIVESTOCK RESEARCH
WAGENINGEN UR

Effects of rye inclusion in grower diets on immunity-related parameters and performance of broilers

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Feed4 Foodure

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An experiment was conducted to investigate the effects of dietary inclusion of three levels (0, 5, and 10%) of rye between 14 and 28 days of age on gut health and performance in broilers. A total of 960 one-day-old male Ross 308 chicks were allocated to 24 pens (40 birds per pen). The inclusion of 5 or 10% rye in the diet between day 14 and 28 in a broiler diet resulted in decreased performance and litter quality, but in increased villus height and crypt depth in the small intestine (jejunum) of the birds. Relative bursa and spleen weights were not affected by dietary treatments. In the jejunum, no effects on number and size of goblet cells, and only small effects on microbiota composition in the digesta were observed. Dietary rye inclusion affected expression of genes involved in cell cycle processes of the epithelial gut cells, thereby influencing cell growth, cell differentiation and cell survival. This observation is consistent with the observed differences in the morphology of the gut wall. Whether this also affected the barrier function of the epithelial layer, cannot be concluded. The complement and coagulation pathways, which are also affected by providing rye-rich diets, are parts of the innate immune system. These pathways are involved in eradicating invasive pathogens.
Overall, it is concluded that inclusion of 5% or 10% rye to the grower diet of broilers in the current study had limited effects on performance. Ileal gut morphology, microbiota composition of jejunal digesta, and gene expression profiles of jejunal tissue, however, were affected by dietary rye inclusion levels.

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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 current report describes an experiment that was conducted to investigate the effects of dietary inclusion of three levels (0, 5, and 10%) of rye between 15 and 28 days of age on gut health and zootechnical performance of broilers. Such an intervention is expected to have negative effects on immune competence and therefore will contribute to the identification of (negative) parameters for immune competence. 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, Denkavit, and Darling Ingredients International. 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

High dietary inclusion levels of rye grain are usually not recommended for growing chickens. The use of rye in broiler diets is restricted due to the presence of soluble non-starch polysaccharides (NSP), particularly arabinoxylans, which are expected to create a viscous environment within the intestinal lumen. Increased digesta viscosity is associated with impaired digestibility and absorption of dietary nutrients, finally leading to a depression in growth rate and worsened feed conversion ratio. It was hypothesized that an increased viscosity of intestinal digesta would negatively affect small intestinal morphology, composition of intestinal microbiota, and as a consequence immune related processes in the gut wall. Therefore, it was expected that the inclusion of rye in grower diets of broilers might be a model ingredient to investigate the relationships between diet composition, intestinal microbiota and gene expressions in the intestinal tissue at adult stage (15 to 21d of age) and would contribute to the identification of parameters associated with immune competence.

Therefore, within the Feed4Foodure program line "Nutrition, Intestinal Health, and Immunity", the Animal Sciences Group of Wageningen UR performed an experiment to evaluate the effect of dietary inclusion of rye in diets on immune competence and performance in adult broilers. A total of 960 one-day-old male Ross 308 chicks were randomly allocated to 24 pens. The experiment was carried out with three treatments (0% rye, 5% rye and 10% rye diet), and each treatment was replicated 8 times. The experimental unit was a pen with 40 male broilers at the start of the experiment (d0). The experimental diets were supplied from 15 to 28 d of age.

Based on results of the present experiment, the following conclusions can be drawn:

- Inclusion of 10% rye in the diet did not affect feed intake, but decreased body weight gain and increased feed conversion ratio.
- Litter quality was inversely related to the level of rye inclusion in the diet.
- Providing rye-rich diets resulted in increased jejunal villus height and crypt depth during the first week of provision, whereas the villus-crypt ratio was not affected. During the second week of the experiment, however, the level of rye inclusion had no effect on jejunal gut morphology.
- Inclusion of rye into the diet did not affect the number and size of jejunal goblet cells.
- Dietary inclusion of rye did not affect relative bursa and spleen weight.
- Dietary inclusion of rye did not affect the diversity of the jejunal microbiota, as determined by the Shannon index, although specific microbial strains were affected by rye inclusion.
- *Lactobacillus* species made about 75 - 80% of the jejunal microbiota; rye inclusion resulted in an exchange between the different *lactobacillus* species.
- At d28, the share of *Lactobacillus reuteri*, *Staphylococcus saporphyticus* and *Aerococcaceae* in the microbiota in jejunal digesta decreased with increasing dietary rye inclusion levels.
- Dietary inclusion of rye affected expression of genes in the small intestinal tissue involved in cell cycle processes of the epithelial gut cells, including proliferation, differentiation, motility, and survival, as well as in the complement and coagulation cascade. At 28 d of age, effects were more pronounced in birds fed the 10% rye diet, compared to birds fed the 5% rye diet.

Dietary rye inclusion affected expression of genes involved in cell cycle processes of the epithelial gut cells, thereby influencing cell growth, cell differentiation and cell survival. This observation is consistent with the observed differences in the morphology of the gut wall. Whether this also affected the barrier function of the epithelial layer, cannot be concluded. The complement and coagulation pathways, which are also affected by providing rye-rich diets, are parts of the innate immune system. These pathways are involved in eradicating invasive pathogens.
Overall, it is concluded that inclusion of 5% or 10% rye to the grower diet of broilers in the current study had limited effects on performance. Ileal gut morphology, microbiota composition of jejunal digesta, and gene expression profiles of jejunal tissue, however, were affected by dietary rye inclusion levels.

1 Introduction

High dietary inclusion levels of rye grain are usually not recommended for growing chickens. The use of rye in broiler diets may be limited due to the presence of soluble non-starch polysaccharides (NSP), particularly arabinoxylans. As shown in Figure 1, arabinoxylans are complex cell wall polysaccharides, being composed of two sugars, arabinose, and xylose in a branched structure (Smits and Annison, 1996).

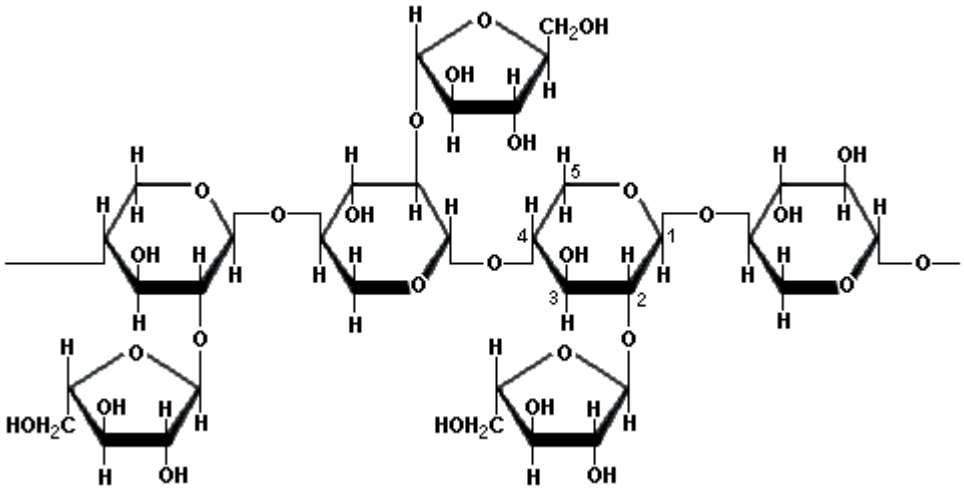

Figure 1 *Structure of arabinoxylans, the main soluble non-starch polysaccharide of rye*

Arabinoxylans create a viscous environment within the intestinal lumen (Smits and Annison, 1996; Jozefiak et al., 2007). Increased viscosity might impair digestibility and absorption of dietary nutrients, leading to a depression in growth rate and feed conversion ratio (FCR) (Antoniou and Marquardt, 1981; Bedford and Classen, 1993). The increased viscosity is due to the high levels of soluble carbohydrates in rye, which can hold water in the digesta producing a thick viscous solution and very wet excreta (Choct and Annison, 1990; Knudsen, 1997; Silva and Smithard, 2002). Dietary fibre may increase the secretion of mucus, which is produced by goblet cells (Satchithanandam et al., 1990). Feeding rats various gelling agents increased the proliferation rate of the enterocytes of the jejunum and distal ileum and decreased the activity of specific epithelial surface enzymes (Johnson and Gee, 1986). Smits and Annison (1996) hypothesized that an increased viscosity of the ileal digesta might change the morphology of the villi. Moreover, these authors emphasized that the microbiota may, at least partially, be indirectly responsible for the detrimental effects of viscous digesta. Choct et al. (1992) reported that the deleterious effect of wheat pentosans on the digestibility of long chain fatty acids was less pronounced in caecectomized broilers, and therefore the anti-nutritive effects of these pentosans in poultry are partially due to an increased activity of hindgut microbiota. In a study of Misir and Marquardt (1978), relative improvement of performance in broilers was higher if antibiotics were added to a rye-based diet compared to antibiotic supplementation to a control diet, indicating that the microbiota played a larger role in birds fed the rye-based diets. Smits and Annison (1996) reported that fibre viscosity in germ-free chicks had negligible effects on fat digestibility, indicating that viscous NSPs must modify bacterial activity in order to lower fat digestibility. Van der Klis and Van Voorst (1993) reported that carboxymethyl cellulose increased the average retention time of digesta in the gastrointestinal tract, thereby giving the microbiota more time and more substrate to colonize the proximal small intestine.
Feeding broilers a wheat/rye-based diet, high in NSP, compared with a maize-based diet, seriously decreased immunity-related parameters of the birds, as indicated by induced villus fusion, reduced thickness of the tunica muscularis, induced T-lymphocyte infiltration, more and larger goblet cells, more apoptosis of epithelial cells in the mucosa, and a shift in microbiota (Teirlynck et al., 2009). In

the study described above, however, it was not possible to identify the individual bacterial groups involved in the observed effects.

Based on the available literature, it was hypothesized that dietary inclusion of rye would increase viscosity of intestinal digesta, consequently resulting in an effect on nutrient absorption, gut wall morphology, composition of microbiota, and immunity-related processes in the gut wall. Therefore, it was expected that rye in grower diets of broilers might be a helpful model ingredient to investigate the negative effects of nutrition on the immunity-related parameters of these birds (15 to 28 d of age) and that it would help in the identification of parameters associated with (negative) immune competence parameters. It is expected that this study will help to better understand the impact of nutrition on gut health and immunity of broilers, which is the main focus of the Feed4Foodure program line 'Nutrition, Intestinal Health, and Immunity'.

2 Material and methods

2.1 Start date and end date of the experiment

Start date (day 0): 5 August 2013
End date (day 35): 9 September 2013

2.2 Experimental animals

A total of 960 one-day-old male Ross 308 chicks were used and randomly allocated to three experimental treatments. At the time of arrival at the experiment farm, day-old birds were weighed and randomly divided among the pens (8 pens per treatment). The broilers were sexed at the hatchery. All broilers were vaccinated at d 0 (at the experimental farm) against IB and NCD. The birds were supplied by a commercial hatchery (Van Hulst, Veldhoven, The Netherlands).

2.3 Experimental design

The experiment was carried out with three dietary treatments (0% rye, 5% rye and 10% rye diet). Each treatment was replicated 8 times. The experimental unit was a pen with 40 male broilers at the start (d0) of the experiment (Table 1).

Table 1
Experimental design in summary.

Item	Number
Treatments	3
Replicates (pens) per treatment	8
Total number of pens	24
Broilers per pen	40
Broilers per treatment	320
Total number of broilers	960

2.4 Rye

Rye (*Secale cereale*) is a grass grown extensively as a grain, a cover crop and as a forage crop. It is a member of the wheat tribe (*Triticeae*) and is closely related to barley (*Hordeum*) and wheat (*Triticum*). Compared to barley and wheat, rye has an intermediate total NSP content, but the highest soluble NSP content. Soluble NSPs are responsible for the increase in digesta viscosity after ingestion via the diet.

2.5 Experimental facility and housing

The experiment was conducted at the experimental facility of feed producer De Heus (Eerde, The Netherlands). Male broilers were housed in floor pens (40 birds per pen at d0) in a mechanically ventilated room provided with facilities to control temperature, ventilation and lighting. The surface area of the pens was 1.5 m^2. Each pen contained a feeding trough and three drink cups. Wood shavings were used as bedding material and bedding material was 'topped up' (ca. 1 kg/pen) at d 30 due to poor litter condition. The broiler unit was continuously illuminated during the first two days. From day three onwards the broiler house was illuminated according to the schedule 18L:6D with a light intensity of 20 lux at bird level. Temperature inside the experimental 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 2. Relative humidity was gradually increased from 60 (d0) to 75% (d35) during the experiment.

Table 2
Temperature schedule.

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

2.6 Experimental diets

The experimental diets were formulated according to commercial broiler diets and produced by Research Diet Services, Wijk bij Duurstede, The Netherlands. For each feeding period (starter, grower and finisher), diets were formulated to meet or exceed the nutrient requirements recommended by Ross 308 Guidelines for broiler chickens. The broilers received a standard starter and finisher broiler diet, whereas during the grower period (d 15 to 28) the three experimental grower diets were provided. Besides the 0% rye diet, two diets with different levels (5 and 10%) of rye inclusion were used. A standard Dutch broiler diet usually contains a high level of wheat (25-50%). Rye was chosen as test ingredient, because of its high insoluble NSP content. To prevent interaction with other insoluble NSP sources, maize (low in insoluble NSP) instead of wheat (intermediate in insoluble NSP) was included as the main cereal in the diets. To compensate for the lower energy value of rye compared to maize, the inclusion of lard was increased with increasing rye inclusion level.
Diets were formulated to be iso-nitrogenous and iso-caloric. Diets were not supplemented with NSP degrading enzymes. Starter diets were offered to all birds from day-old until 14 days of age, grower diets from 15 to 28 days and finisher diets from 29 to 35 days. The diets and water were provided ad libitum. All diets were pelleted (3.2 mm). After pelletizing, the starter diet was crumbled, whereas the grower and finisher diets were provided as pellets. The calculated compositions of the starter, grower and finisher diets are presented in Appendix 1.

2.7 Measurements

2.7.1 Diets

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). The relative viscosity of the feed samples was measured at 20ºC using a Brookfield viscometer in a model digestion system according to Bedford & Classen (1993).

2.7.2 Performance

Body weight of birds was determined per pen at 0, 14, 21, 28 and 35 days of age. Feed intake per pen was determined at 14, 21, 28 and 35 days of age (provided feed minus remaining feed at 14, 21, 28 and 35 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 35 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 – d35 as the finisher period.

2.7.3 Litter quality

Litter quality was visually scored on a 0 to 10 point scale on 21, 28 and 35 days of age by one person. The scores and the description of each score are presented in Table 3.

Table 3
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

2.7.4 Dissection and sample collection

At 14, 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 and stored at -80°C until further analysis on microbiota composition. The digesta was collected in the order of pen numbering. Digesta samples were freeze dried, and 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 to analyse intestinal integrity (villus height and crypt depth), number and size of goblet cells, and genome-wide gene expression profiling in jejunal tissue. 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).

2.7.5 Microbiota

Microbiota diversity index and microbiota composition of the jejunal digesta were measured in a pooled sample of digesta of six birds (content of 2 cm jejunum length per sample) per pen on day 14, 21, and 28. 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.

2.7.6 Genome-wide gene expression profiling

RNA Extraction Tissue

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

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 ('avereps') and subsequently the lower percentile of probes were removed in a three-step procedure , 1) get the highest of the dark spots to get a base value, 2) multiply by 1.1 and 3) the gene/probe must be expressed in each of the samples in the experimental condition.

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 (Table 4).

Table 4

Specific contrasts that were defined within the R package LIMMA.

Age	Condition 1	Condition 2
21	10% rye diet	0% rye diet
21	5% rye diet	0% rye diet
21	10% rye diet	5% rye diet
28	10% rye diet	0% rye diet
28	5% rye diet	0% rye diet
28	10% rye diet	5% rye diet

Functional association data mining

The Database for Annotation, Visualization and Integrated Discovery (DAVID version 6.7) website (Huang et al., 2009) and the "Set Distiller" module of GeneDecks (Stelzer et al., 2009) were used to assign genes to a specific pathway and processes. In comparison to poultry, the human genome is better annotated, and also more information within databases is available. Therefore, all analyses were performed using a human background. In DAVID, both KEGG and Biocarta pathways with a *P*-value of below 0.2 (EASE score) were retrieved.

Functional association between proteins encoded by differential expressed genes, ligands, and enzyme substrates/products linked to these proteins, were established using the (protein)-protein-chemical interaction web tool STITCH 3.1 (Kuhn et al., 2012). The confidence view, in which stronger associations are represented by thicker lines was used, and by using the 'add nodes' function once and subsequently using the 'remove nodes' function all output was similar (i.e. independent of ordering in gene list). When indicated the 'add nodes' function was used twice, to identify even more additional interaction partners. The output (.txt) from STITCH was imported into Cytoscape (Shannon et al., 2006) (3.1.0).
To investigate in which processes the 'network' genes were involved, these genes were loaded into DAVID again and a 'Functional Annotation Table' was generated using only the KEGG database.

2.7.7 Relative bursa and spleen weight

At 14, 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.

2.8 Statistical analysis

The experimental data (performance, litter quality and bursa/spleen content) 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…3)
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.

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. The performance level of the broilers in the present study was high and exceeded the Ross 308 performance standards of male broilers (Ross, 2012). For instance BW gain of the male broilers in the current study was 15% higher than the Ross 308 standard performance data. General bird health was good during the course of the study resulting in a low mortality rate (1.2%).

Table 5
Performance data of male broilers during the experiment between 0 and 35 days of age.

Performance data	Experiment	Ross 308 standard performance data
Body weight day 0 (g)	40	42
Body weight day 14 (g)	545	481
Body weight day 21 (g)	1112	945
Body weight day 28 (g)	1854	1553
Body weight day 35 (g)	2569	2250
Body weight gain day 0-35 (g/b/d)	2529	2208
Feed conversion ratio (kg/kg)[1]	1.428	1.566
Mortality (%)	1.2	n.a.[2]

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

3.2 Diets

The results of the analysis of the different diets are presented in Appendix 1. Analysed crude protein and crude fat contents were in agreement with the calculated concentrations. The calculated dietary NSP content was increased in the rye-containing grower diets, whereas the insoluble NSP content remained constant among the grower diets.

3.3 Performance

3.3.1 Starter period (d0-14)

Performance results during the starter period (between d 0 and 14) are presented in Table 6. Body weight of the birds at 0 days of age was, on average, 40 gram for all treatment groups. All birds received in this period the same standard starter diet. Therefore, as expected, no significant treatment effects on performance during the starter period between 0 and 14 days were observed.

Table 6
Body weight (g), body weight gain (g), feed intake (g), feed conversion ratio (g/g), and mortality (%) presented per treatment for the starter period (d0-14).

Diet	Body weight d14 (g)	BW gain d0-14 (g)	Feed intake d0-14 (g)	FCR d0-14 (g/g)	Mortality d0-14 (%)
0% rye	543	503	604	1.200	0.9
5% rye	551	511	612	1.199	0.9
10% rye	541	501	601	1.200	0.9
P-value	0.290	0.290	0.246	0.958	1.000
LSD	14	14	15	0.011	1.9

3.3.2 Grower-1 period (d15-21)

Performance results during the grower-1 period between 15 and 21 days of age are presented in Table 7. Broilers fed the 10% rye diet showed a decreased BW gain and BW between 15 and 21 days compared to the 5% rye diet whereas the 0% rye diet was intermediate. No effect on feed intake was found, however, FCR was higher for the broilers fed the 10% rye diet, whereas no effect was found between the 0% rye and 5% rye diet. No mortality occurred between 15 and 21 days of age.

Table 7

Body weight (g), body weight gain (g), feed intake (g), feed conversion ratio (g/g), and mortality (%) presented per treatment for the grower-1 period (d15-21).

Diet	Body weight d21 (g)	BW gain d15-21 (g)	Feed intake d15-21 (g)	FCR d15-21 (g/g)	Mortality d15-21 (%)
0% rye	1112^{ab}	569^{ab}	758	1.333^{b}	0.0
5% rye	1127^{a}	577^{a}	772	1.338^{b}	0.0
10% rye	1096^{b}	555^{b}	759	1.366^{a}	0.0
P-value	0.030	0.034	0.433	0.001	-
LSD	22	16	24	0.016	-

^{a,b} Means in a column with different superscripts differ significantly ($P < 0.05$).

3.3.3 Grower-2 period (d22-28)

Performance results during the grower period between 22 and 28 days are presented in Table 8. BW at d28 tended ($P = 0.06$) to decrease in birds that were fed the 10% rye diet. Because BWG was not significantly affected by dietary treatment in this period, the effect on BW was only slightly a direct diet effect in the period between 22 and 28 days. Differences in BW were mainly arising between 15 and 21 days (Table 6). FI and BWG were not affected by the inclusion rate of rye, although FCR was higher for the broilers fed the 10% rye diet compared to the 0% rye and 5% rye diet. No effect of the rye inclusion level on mortality was found between 22 and 28 days.

Table 8

Body weight (g), body weight gain (g), feed intake (g), feed conversion ratio (g/g), and mortality (%) presented per treatment for the grower-2 period (d22-28).

Diet	Body weight d28 (g)	BW gain d22-28 (g)	Feed intake d22-28 (g)	FCR d22-28 (g/g)	Mortality d22-28 (%)
0% rye	1868$^{(ab)}$	756	1128	1.493^{b}	0.0
5% rye	1877$^{(a)}$	749	1128	1.507^{b}	0.6
10% rye	1816$^{(b)}$	720	1124	1.562^{a}	0.3
P-value	0.057	0.214	0.985	0.003	0.234
LSD	53	44	67	0.037	0.7

^{a,b} Means in a column with different superscripts differ significantly ($P < 0.05$).
$^{(a,b)}$ Means in a column with different superscripts between brackets indicate a trend ($0.05 < P < 0.10$).

3.3.4 Finisher period (d29-35)

No effects on performance during the finisher period were observed (Table 9).

Table 9

Body weight (g), body weight gain (g), feed intake (g), feed conversion ratio (g/g), and mortality (%) presented per treatment for the finisher period (d29-35).

Diet	Body weight d35 (g)	BW gain d29-35 (g)	Feed intake d29-35 (g)	FCR d29-35 (g/g)	Mortality d29-35 (%)
0% rye	2578	710	1488	2.104	0.0
5% rye	2576	699	1485	2.128	0.0
10% rye	2552	736	1478	2.008	0.0
P-value	0.746	0.137	0.970	0.160	-
LSD	80	38	89	0.133	-

3.3.5 Total period (d0-35)

Performance results of the total growth period are presented in Table 10. Inclusion of rye did not affect BW, BW gain, Feed intake, FCR and mortality between 0 and 35 days of age. However, the 0% rye diet tended ($P = 0.08$) to a more favourable FCR compared to the 10% rye diet, while the 5% rye diet resulted in an intermediate FCR.

Table 10

Body weight (g), body weight gain (g), feed intake (g), feed conversion ratio (g/g), and mortality (%) presented per treatment for the total growth period (d0-35).

Diet	Body weight (g)	BW gain (g)	Feed intake (g)	FCR (g/g)	Mortality (%)
0% rye	2578	2538	3603	1.420$^{(b)}$	0.9
5% rye	2576	2536	3614	1.425$^{(ab)}$	1.6
10% rye	2552	2512	3612	1.438$^{(a)}$	1.2
P-value	0.746	0.746	0.976	0.075	0.764
LSD	80	80	115	0.016	1.8

$^{(a,b)}$ Means in a column with different superscripts between brackets indicate a trend ($0.05 < P < 0.10$).

3.4 Litter quality

The results of the visual scores of the litter quality are presented in Table 11. Litter quality was scored into 11 categories from 0 (dry and friable litter) to 10 (wet and 100% caked litter). Litter quality at day 21 and 28 was affected by rye inclusion level, whereas no treatment effect was found at day 35. Litter quality of the broilers fed the 10% rye diet at day 28 was extremely worse and therefore extra bedding material (ca. 1 kg per pen) was provided to all pens at day 30. From d30 to 35, no differences in litter quality were observed, resulting in a similar litter score for all treatments at d35.

Table 11

Litter quality at 21, 28 and 35 days of age.

Diet	21 d	28 d	35 d
0% rye	7.0^{a}	6.8^{a}	6.8
5% rye	6.5^{b}	6.4^{ab}	6.6
10% rye	5.6^{c}	3.0^{b}	6.6
P-value	<0.001	<0.001	0.887
LSD	0.45	0.53	0.61

^{a,b} Means in a column with different superscripts differ significantly ($P < 0.05$).

3.5 Gut morphology

Results of the villus height and crypt depth (µm) of the jejunum at day 14, 21 and 28 are presented in Table 12. Although all birds were fed the same standard starter diet during the starter period, villus height at d14 was highest in the broilers that in the grower period would be fed the 0% rye diet compared to the diets with rye inclusion (5 or 10%). From d14 to d21, increase in villus height of broilers fed the 5 and 10% rye diet was higher compared to broilers fed the 0% rye diet (Table 13), resulting in a numerical higher villus height for the broilers fed the 10% rye diet (P = 0.11). Crypt depth was not affected by level of rye inclusion at d28. At d21, crypt depth of broilers fed the 10% rye diet tended (P = 0.07) to increase compared to birds fed the 0% rye diet, while the birds fed the 5% rye diet showed an intermediate result.
From d14 to d21, the decrease in crypt depth showed a linear trend with increasing levels of rye inclusion in the diet (Table 13). Between 21 and 28d of age, crypt depth decreased more in broilers fed the 10% rye diet compared to crypt depth of birds fed the 0% rye and 5% rye diet.

Table 12

Villus height and crypt depth (µm) of the jejunum at 14, 21 and 28 days of age.

	Villus height (µm)			Crypt depth (µm)		
Diet	**14 d**	**21 d**	**28 d**	**14 d**	**21 d**	**28 d**
0% rye	1059[a]	1327	1463	244	189[(b)]	196
5% rye	981[b]	1408	1548	227	192[(ab)]	201
10% rye	999[b]	1447	1484	230	219[(a)]	192
P-value	0.017	0.108	0.214	0.459	0.074	0.351
LSD	53	115	102	30	29	14

[a,b] Means in a column with different superscripts differ significantly (P < 0.05).
[(a,b)] Means in a column with different superscripts between brackets indicate a trend (0.05 < P < 0.10).

Table 13

Change of villus height and crypt depth (µm) in the jejunum between 14 and 21 and 21 and 28 days of age.

	Villus height (µm)		Crypt depth (µm)	
Diet	**14 to 21 d**	**21 to 28 d**	**14 to 21 d**	**21 to 28 d**
0% rye	269[b]	136	-55[a]	7[a]
5% rye	428[a]	140	-35[ab]	10[a]
10% rye	449[a]	37	-11[b]	-27[b]
P-value	0.024	0.392	0.042	0.041
LSD	135	177	33	31

[a,b] Means in a column with different superscripts differ significantly (P < 0.05).
[(a,b)] Means in a column with different superscripts between brackets indicate a trend (0.05 < P < 0.10).

Results of the villus/crypt ratio and area of villi covered by goblet cells (%) of the jejunum at day 14, 21 and 28 are presented in Table 14. All birds were fed the same standard starter diet during the starter period until d14. No effects of dietary rye inclusion on villus-crypt ratio and area of villi covered by goblet cells (%) were found.

Table 14

Villus-crypt ratio and villus covered by goblet cells (%) of the jejunum at 14, 21 and 28 days of age.

	Villus-crypt ratio			Area of villi covered by goblet cells (%)		
Diet	**14 d**	**21 d**	**28 d**	**14 d**	**21 d**	**28 d**
0% rye	4.8	7.5	7.8	3.3	3.4	2.9
5% rye	4.7	7.7	8.0	3.3	2.9	3.0
10% rye	4.7	7.4	8.1	3.4	3.2	2.9
P-value	0.924	0.718	0.456	0.965	0.353	0.487
LSD	0.66	0.79	0.51	0.5	0.6	0.3

The number of goblet cells per villi (#) and the surface of the goblet cells (μm^2) of the jejunum at day 14, 21 and 28 are presented in Table 15. All birds were fed the same standard starter diet during the starter period until d14. The rye inclusion level did not affect the number of goblet cells per villi ratio and the surface of the goblet cells at d21. At d28, the 5% rye diet showed a tendency ($P = 0.09$) to an increased surface of goblet cells compared to the surface of goblet cells in broilers fed the 0% and 10% rye diets.

Table 15

Goblet cells per villi (#) and surface of goblet cells (μm^2) of the jejunum at 14, 21 and 28 days of age.

	Goblet cells per villi			Surface of goblet cells (μm^2)		
Diet	**14 d**	**21 d**	**28 d**	**14 d**	**21 d**	**28 d**
0% rye	261	366	332	6628	8661	8333[(b)]
5% rye	248	359	369	6045	8256	9517[(a)]
10% rye	248	378	336	6098	9026	8329[(b)]
P-value	0.714	0.854	0.193	0.926	0.710	0.093
LSD	38	74	45	1026	1971	1236

[(a,b)] Means in a column with different superscripts between brackets indicate a trend ($0.05 < P < 0.10$).

3.6 Relative weight of bursa and spleen

Relative bursa and spleen weight (% of BW) at day 14, 21 and 28 are presented in Table 16. All birds were fed the same standard starter diet during the starter period until d14. No effects of rye inclusion in broiler diets on relative bursa and spleen (% of BW) were found.

Table 16

Bursa and spleen (% of BW) at 14, 21 and 28 days of age.

	Bursa (% of BW)			Spleen (% of BW)		
Diet	**14 d**	**21 d**	**28 d**	**14 d**	**21 d**	**28 d**
0% rye	0.23	0.23	0.19	0.10	0.10	0.13
5% rye	0.25	0.25	0.19	0.10	0.11	0.13
10% rye	0.24	0.23	0.19	0.10	0.10	0.13
P-value	0.336	0.162	0.986	0.371	0.716	0.527
LSD	0.03	0.02	0.02	0.01	0.01	0.01

3.7 Microbiota

Values of the Shannon diversity index, indicating the diversity of the microbiota composition in jejunal digesta based on the genus/species level data, at different ages (d14, d21 and d28) and rye inclusion levels (no = 0%, moderate = 5%, high = 10%) are presented in Figure 2. No main effects of age and rye inclusion level in diets on diversity of microbiota were found.

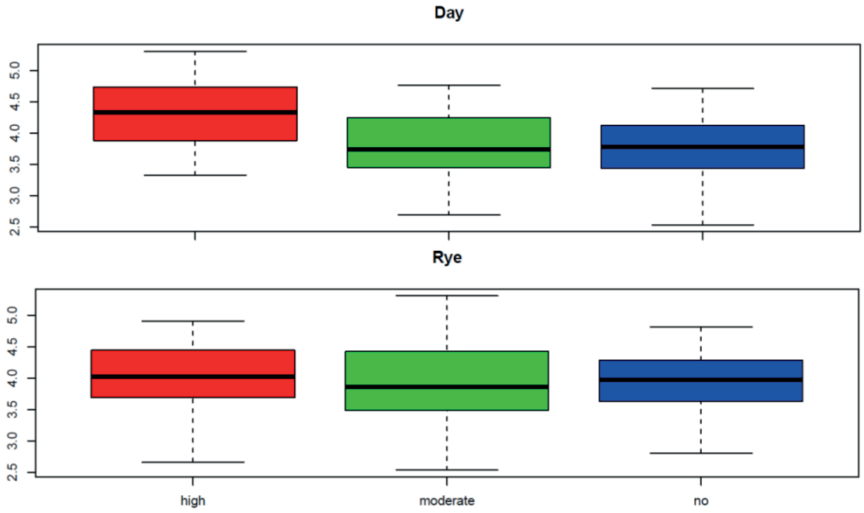

Figure 2 *Main effects of day of age and dietary rye content on the Shannon diversity index, showing the diversity of the jejunal microbiota of birds fed the diets containing 0% (no), 5% (moderate),or 10% (high) rye averaged over age, and the diversity of the jejunal microbiota of birds at d14, 21 and 28, averaged over dietary rye inclusion levels. All birds were fed the same standard starter diet during the starter period until d14.*

The interaction effects of dietary rye content and day of age on the Shannon diversity index, which indicates the diversity of the jejunal microbiota, are presented in Figure 3. No significant effect of rye inclusion was found on the Shannon microbiota diversity index at day 21 and 28.

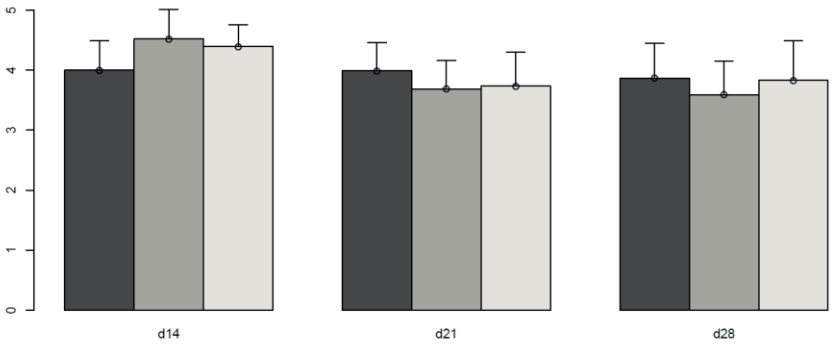

Figure 3 *Interaction effects of dietary rye content and day of age on the Shannon diversity index, showing the diversity of the jejunal microbiota of birds fed the diets containing 0% (left bar), 5% (middle bar), and 10% (right bar) rye at d14, 21 and 28. All birds were fed the same standard starter diet during the starter period until d14.*

Effects of rye inclusion level on the most abundant microbiota species (% of total) in jejunal digesta at d21 and 28 are presented in Table 17 and 18, respectively. Although the diversity index was not affected by dietary treatments, some changes in individual microbial species were observed. Microbiota composition at day 21 was affected by rye inclusion level. The share of *Lactobacillus reuteri* linearly increased from 28.0 to 39.0% with increasing rye inclusion level, mainly at the expense of other *Lactobacilli (undefined species*). The share of total *Lactobacilli* species was higher for the 5% rye diet (85.6%) compared to the 0% rye (78.1%) and 10% rye diets (78.8%). The other species showed more diffuse changes in dependence of dietary rye content.

Table 17

Microbiota composition (% of total) at 21 days of age.

Family	Genus	Species	0% rye	5% rye	10% rye	*P*-value
Lactobacillaceae	Lactobacillus	reuteri	27.98	34.96	38.99	< 0.001
Lactobacillaceae	Lactobacillus		33.23	38.92	17.82	< 0.001
Lactobacillaceae	Lactobacillus	salivarius	16.84	11.68	21.98	< 0.001
Planococcaceae	Staphylococcus	saprophyticus	4.50	1.73	5.11	0.002
Enterococcaceae	Other	Other	2.64	1.51	2.05	0.006
Corynebacteriaceae	Corynebacterium	stationis	2.73	1.79	3.06	0.261
Streptococcaceae	Streptococcus	luteciae	0.96	0.40	0.86	< 0.001
Aerococcaceae	Aerococcus		1.45	0.95	1.18	< 0.001
Staphylococcaceae	Jeotgalicoccus	psychrophilus	1.89	1.32	1.77	0.641
Aerococcaceae	Facklamia		1.64	1.72	1.53	0.110
Other	Other	Other	1.14	0.74	0.56	0.004
Total			95.02	95.72	94.92	

Microbiota composition at d28 was affected by rye inclusion level (Table 18). The share of *Lactobacillus reuteri*, *Staphylococcus saporphyticus* and *Aerococcaceae* decreased with increasing dietary rye inclusion levels. Contrary, the share of *Lactobacillus* (undefined species; from 16.2 to 22.3%) and *Lactobacillus salivarius* (from 11.8 to 13.5%) was found to increase with increasing rye inclusion levels. At d28, Lactobacilli species still were the most dominant ones, contributing for 73.8%, 80.1%, and 76.1% to the total microbiota when providing the 0%, 5% and 10% rye diets, respectively.

Table 18

Microbiota composition (% of total) at 28 days of age.

Family	Genus	Species	0% rye	5% rye	10% rye	*P*-value
Lactobacillaceae	Lactobacillus	reuteuri	45.81	40.97	40.33	< 0.001
Lactobacillaceae	Lactobacillus		16.19	19.71	22.33	< 0.001
Lactobacillaceae	Lactobacillus	salivarius	11.77	19.46	13.46	< 0.001
Planococcaceae	Staphylococcus	saprophyticus	6.07	3.75	4.01	0.002
Enterococcaceae	Other	Other	2.20	3.24	3.31	0.006
Corynebacteriaceae	Corynebacterium	stationis	2.99	2.21	3.33	0.261
Streptococcaceae	Streptococcus	luteciae	1.77	1.22	1.75	< 0.001
Aerococcaceae	Aerococcus		2.00	1.03	1.43	< 0.001
Staphylococcaceae	Jeotgalicoccus	psychrophilus	2.54	1.42	1.82	0.641
Aerococcaceae	Facklamia		1.73	0.97	1.69	0.110
Other	Other	Other	1.01	0.61	0.61	0.004
Total			94.10	94.59	94.08	

On average, at d28 the share of *Lactobacillus reuteri* as % of the total microbiota was 8.4% higher compared to d21, which was mainly compensated by a lower share of *Lactobacillus* (unspecified species; -10.6%) and *Lactobacillus salivarius* (- 1.9%).

The quantitative number of gut bacteria in the current study was not affected by dietary treatments at d21 and 28 (data not shown). Therefore, differences in proportions of species and absolute microbial load were not interfering.

3.8 Gene-expression

To explore the gene expression data in the jejunal tissue, first a two-dimensional Principal Component Analysis was performed on 63 samples (9 samples were considered as outlier after Quality Control). Figure 4 shows that, based on the genome-wide expression, the different experimental treatments were clearly separated in time (d 14, 21 and 28). Furthermore, it can be concluded from Figure 4 that the variation in gene-expression is very high for day 21. At d21, the expression in intestinal tissue of birds receiving a rye containing diet (filled symbols) is different from the 0% rye group (open circles). Also for d28, it seems that the gene expression in jejunal tissue is different in birds receiving a diet including rye compared to the 0% rye group, although the gene expression of treatments groups

showed more overlap compared to d21. Further statistical testing was performed to identify which genes differed between experimental treatments on the different time-points.

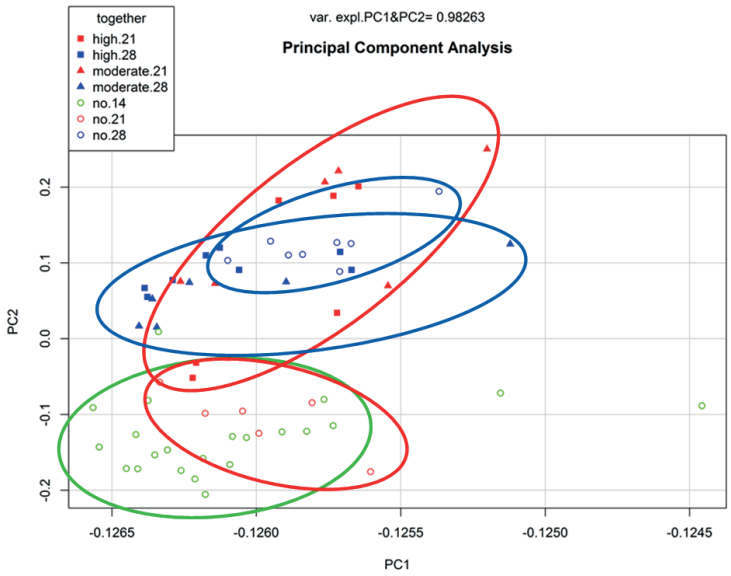

Figure 4 *Principal Component Analysis of gene expression data of jejunal tissue of chickens receiving a diet containing 0, 5 or 10% of rye.*
Each symbol represents all expressed genes (approximately 18k) of a particular sample. Three different treatments are depicted, no addition of rye (no; open circles), moderate (5% rye; filled triangles), and high (10% rye; filled squares). Colours represent the different time-points of sampling; day 14 (green), day 21 (red), and day 28 (blue).

An overview of the significant number of probes and annotated genes, and their respective regulation, up or down, is given in Table 19. This table clearly showed that the birds on the 0% rye diet had significantly more down-regulated genes compared to those on the 5% and 10% rye diets. At d28, more down-regulated genes were observed in birds fed the 5% and 10% rye diets compared to the 0% rye diet. The 10% rye diet had more up-regulated probes/genes compared to the 5% rye diet.

Table 19
Descriptive statistics of regulated probes/genes in jejunal tissue between dietary treatments at 21 and 28 days of the study.

	Probes[1]		Annotated genes[1]	
Contrast	Up	Down	Up	Down
Day 21				
5 vs. 0% rye diet	34	403	16	103
10 vs. 0% rye diet	14	313	3	103
10 vs. 5% rye diet	57	15	8	12
Day 28				
5 vs. 0% rye diet	4	22	4	6
10 vs. 0% rye diet	31	297	7	89
10 vs. 5% rye diet	5	282	2	94

[1] logFC>|1.3| and adjusted *P*-value < 0.05

To investigate in which processes these up- or down-regulated genes were involved, a functional analysis for all six contrasts was performed by the DAVID web tool at d21 (Table 20) and d28 (Table 21). Only significant effects (Enrichment Score above 1.00) are presented. Dietary inclusion of rye, however, affected expression of genes in the small intestinal tissue involved in cell cycle processes of the epithelial gut cells, including proliferation, differentiation, motility, and survival, as well as in the complement and coagulation cascade.

Table 20

Descriptive statistics of regulated functional annotation clusters and involved biological processes in jejunal tissue between dietary treatments at 21 days of the study.

Regulation	Clusters	Generalized term	# of genes	ES[1]
10 vs. 0% Up	0			
5 vs. 0% Up	2	Steroid, lipid, mitochondrial	3	2.07
		Extracellular region part,	3	1.04
10 vs. 5% Up	0			
10 vs. 0% Down	2	Blood coagulation,	3	10.8
		M phase, meiosis	4	1.08
5 vs. 0% Down	4	Meiosis, M phase	5	1.36
		Cell process, M phase	6	1.21
		Regulation of –ase activity	3	1.03
		Extracellular region	9	1.02
10 vs. 5% Down	0			

[1]ES, Enrichment score; clusters were indicated significant if above 1.00.

Table 21

Descriptive statistics of regulated functional annotation clusters and involved biological processes in jejunal tissue between dietary treatments at 28 days of the study.

Regulation	Clusters	Generalized term	# of genes	ES[1]
10% vs. 0% UP	0			
5% vs. 0% UP	0			
10% vs. 5% UP	2	Regulation of cell migration	5	2.24
		Neg. regulation of cell	2	1.56
10% vs. 0%	3	Regulation of cell migration	5	2.36
		Neg. regulation of cell	3	1.62
		Pos. Regulation of -ase	3	1.12
5% vs. 0%	0			
10% vs.	0			
10% vs. 0% UP	0			
5% vs. 0% UP	0			

[1]ES, Enrichment score; clusters were indicated significant if above 1.00.

Functional association and data mining

To zoom in on the genes in these significant functional annotation clusters, these genes were loaded into the STITCH database. This database contains protein-protein and protein-chemical interactions, which makes it possible to link different genes (nodes) by their interactions (edges), resulting in an interaction network. For each contrast that had one or more significant functional annotation clusters, a network based on the STITCH database was generated. In order to investigate the general trend in the up- or down-regulated genes, irrespective of treatment or time, and their corresponding processes, a merge of multiple networks was performed, resulting in two 'merged' networks, one for down-regulated genes (Figure 5) and one for up-regulated genes (Figure 6). Surprisingly, in both the up- and down-regulated network the 'ErbB signalling pathway' was found to be affected by dietary treatment. This pathway regulates various biological cell processes, including cell proliferation, cell differentiation, cell motility, and cell survival, as well as 'complement and coagulation cascades', which are involved in innate immunity and a nonspecific defence mechanism against pathogens. Because both up- and down-regulation of genes involved in this ErbB signalling pathway is observed, interpretation of the effect of these genes on the final regulation of such pathways is difficult.

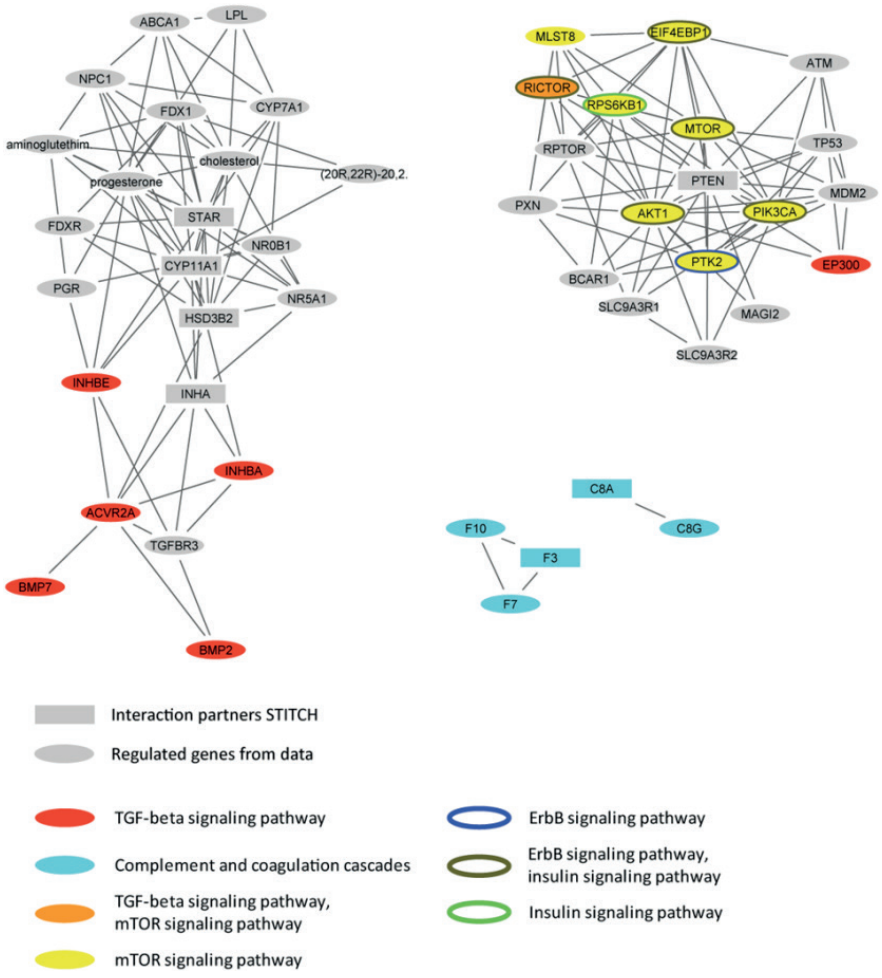

Figure 5 *Network of down-regulated genes and their respective STITCH interaction partners, highlighted are the five most dominant KEGG pathways (TGF-beta signalling pathway, complement and coagulation cascades, mTOR signalling pathway, ErbB signalling pathway, Insulin signalling pathway).*

A wide variety of cellular processes is regulated by TGF-beta family members, including proliferation, apoptosis, differentiation and migration. This was mainly shown in the network of up-regulated genes (and their interaction partners), strengthened by the observation that the 'mTOR signalling pathway' was affected as well. The latter is involved in protein synthesis, lipid synthesis, autophagy, energy metabolism, cell survival, cell metabolism, and cytoskeletal organization (REF; mTOR signalling in growth control and disease). These data together suggest that jejunal cell proliferation/differentiation is affected by the inclusion of rye in the diet. In contrast, the network of down-regulated genes affected by dietary treatment (and their interaction partners) were mainly related to 'cell cycle' processes. In conclusion, these data show that intestinal cell related processes are affected by diet composition i.c. the inclusion of rye in the diet.

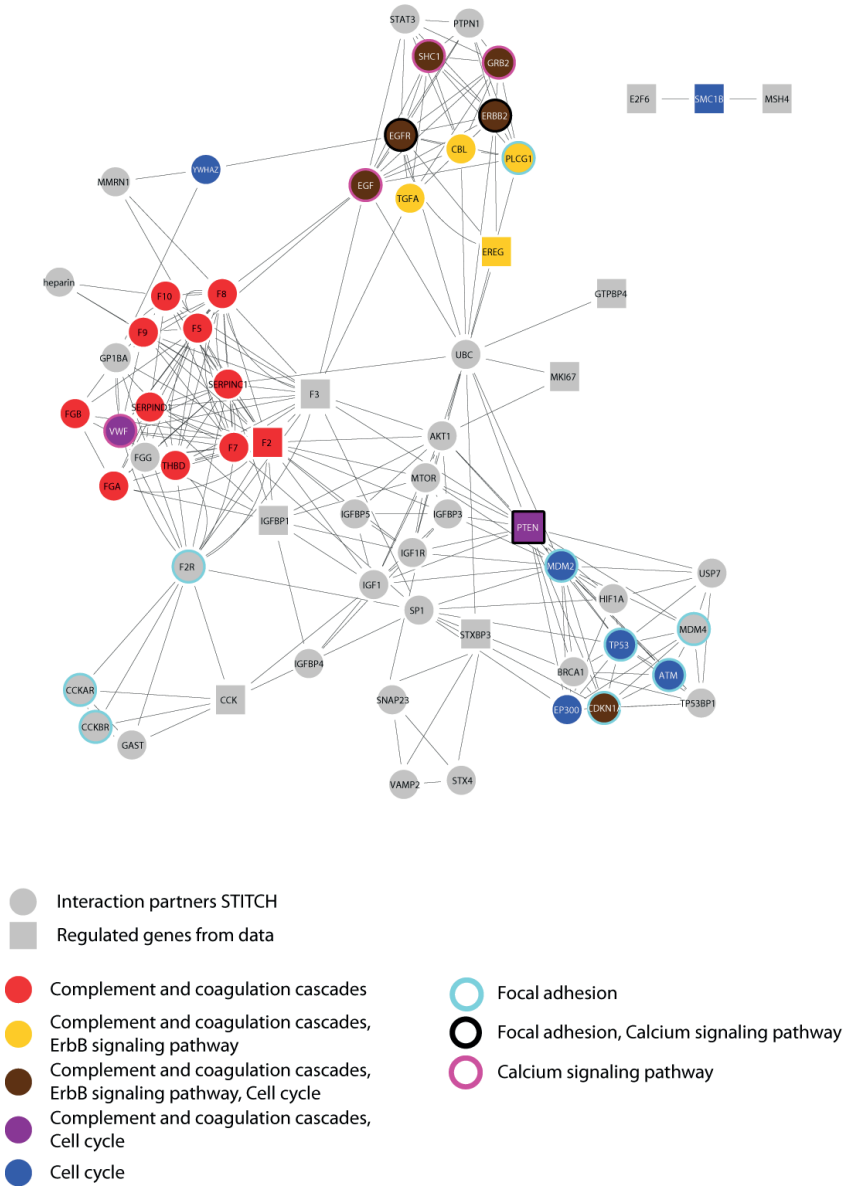

Figure 6 *Network of up-regulated genes and their respective STITCH interaction partners, highlighted are the 5 most dominant KEGG pathways (complement and coagulation cascades, ErbB signalling pathway, cell cycle, focal adhesion, calcium signalling pathway)*

4 Discussion

Effects of increased viscosity of digesta

Due to the presence of arabinoxylans, dietary inclusion of rye is known to create a viscous environment within the intestinal lumen (Smits and Annison, 1996; Lazaro et al., 2004; Jozefiak et al., 2007; Tellez et al., 2014). Although viscosity of intestinal digesta was not determined in the present experiment, the analysed viscosity of the diets doubled after inclusion of 5% rye and quadrupled after inclusion of 10% rye, which might reflect the contrasts in viscosity of the intestinal digesta *in vivo*. Based on these findings, it can be concluded that the 5% and 10% rye inclusion levels were sufficient for creating substantial contrasts in digesta viscosity.

In literature, increased viscosity was shown to impair digestibility and absorption of dietary nutrients, leading to a depression in growth rate and feed conversion ratio (FCR) (Antoniou and Marquardt, 1981; Bedford and Classen, 1993). Effects of rye on performance is dose, age but also dietary fat type dependent. Teirlynck et al. (2009) did not found an effect of 5% rye inclusion in the diet fed to broilers during the entire growth period (1 to 42 d), whereas Langhout (1998) after inclusion of 25% rye in the diet observed a 15% decrease in BWG and a 13% increase in FCR in broilers between 1 and 21 days of age. In line with these results, in the current experiment, BWG and FCR only worsened in broilers fed the 10% rye diets in the grower-1 period (d15-21), whereas in the grower-2 period (d22-28) only FCR worsened in the 10% rye treatment. After switching back to diets without rye from d29 onwards, birds that were fed the 10% rye diet seemed to compensate for the negative effects caused by the high viscous diet by utilising the nutrients more efficient, as shown by a higher body weight gain and more efficient FCR in the period of 29 to 35 d of age.

In the current experiment, saturated fat (lard) was the main fat source. In a study of Danicke et al (2000; 2003), the levels of jejunal and ileal digesta viscosity in broilers were significantly increased if the rye-rich (56% inclusion level) diets contained saturated tallow instead of unsaturated soybean oil as fat source. The increased digesta viscosity levels were associated with a lower feed intake and BWG.

Rye contains high levels of soluble carbohydrates, which can hold water in the digesta, consequently producing a thick viscous solution and very wet excreta (Choct and Annison, 1990; Knudsen, 1997; Silva and Smithard, 2002). In the rye-containing grower diets of the current experiment, the total NSP content was increased. The insoluble NSP content remained constant among the grower diets, and as a consequence, the soluble NSP content increased in the rye-containing diets, thereby increasing gut viscosity. Indeed, in the current study, litter score was drastically reduced in pens in which the 10% rye diets were supplemented, thereby reflecting the higher water content of the excreta.

Dietary fibre may increase the secretion of mucus, which is produced by goblet cells in the intestinal mucosa (Satchithanandam et al., 1990). Although not quantitatively analysed, Teirlynk et al. (2009) observed more and larger goblet cells, both on the villi and in the crypts at the age of 29 d in broilers given a wheat/rye diet than in those given a maize diet, especially in the ileum and caecum. In the current experiment, dietary rye level did not affect the number of goblet cells, their size, and their covered surface area on the villi. The goblet cell parameters in the current study, however, were determined in the jejunum, whereas the effects of Teirlynk et al. (2009) were observed in more distal gut segments.

Smits and Annison (1996) hypothesized that an increased viscosity of the ileal digesta, due to the presence of soluble NSPs, might change the morphology of the villi. These NSPs, when present at the surface of or within the mucus layer, may serve as substrate for microbial growth and may stimulate bacterial proliferation and attachment of bacteria to the mucins and glycocalix. Subsequently, some bacterial species, e.g. *streptococcus faecium,* may cause atrophy of the villi (Viveros et al., 1994; Smits et al., 2000). In line with this hypothesis, several authors observed similar or reduced villi length in broilers fed rye-based diets (Mathlouthi et al., 2002; Teirlynck et al., 2009). In the current experiment, however, villi length at d21 was increased in birds fed the 10% rye diet. In contrast to their working hypothesis, Smits et al. (2000) concluded that addition of non-fermentable carboxymethylcellulose to a broiler diet had a beneficial instead of a detrimental effect on the jejunal villus length. These authors attributed this effect to the fact that a non-fermentable NSP source was

used to increase gut viscosity, which was supported by data of Langhout et al. (1999) In a study of Iji et al. (2001) in which a diet supplemented with alginic acid (AL; a non-viscous NSP) was compared with gum arabic (GA, predominantly galacto-araban), ileal villus height was also increased in birds fed the viscous GA diet. Despite increased villus height, villus surface area of the GA fed birds was similar to the AL fed birds, indicating that a reduced diameter of the villi in the GA fed birds. Based on these findings, it can be hypothesized that in birds fed viscous diets villi length will increase in an attempt to maintain villus surface area. This hypothesis is supported by the findings in the current study.

Gut microbiota

The balance between beneficial and pathogenic bacteria is important to maintaining normal intestinal physiology, as this balance has direct effects on immune function and nutrient digestion and absorption. A healthy and diverse microbiota acts as a barrier to potentially pathogenic microorganisms (Klosterbuer et al., 2011). Smits and Annison (1996) emphasized that the change in microbiota composition or activity may, at least partially and indirectly be responsible for the detrimental effects of viscous digesta. Choct et al. (1992) reported that the deleterious effect of wheat pentosans on the digestibility of long chain fatty acids was less pronounced in caecectomized broilers, and therefore the anti-nutritive effects of these pentosans in poultry are partially due to an increased activity of hindgut microbiota. In a study of Misir and Marquardt (1978), relative improvement of performance in broilers was higher if antibiotics were supplemented to a rye-based diet compared to antibiotic supplementation to a control diet, indicating that the microbiota played a larger role in reducing performance level in birds fed the rye-based diets. Smits and Annison (1996) reported that fibre viscosity in germ-free chicks had negligible effects on fat digestibility, indicating that viscous NSPs must modify bacterial activity in order to lower fat digestibility. Van der Klis and Van Voorst (1993) reported that carboxymethylcellulose increased the average retention time of digesta in the gastrointestinal tract, thereby providing the microbiota more time and more substrate to colonize the proximal small intestine.

Langhout et al. (1999) showed that the inclusion of water-soluble pectins in diets of chicks changed the numbers of ileal microbes. The magnitude of these changes depended on the degree of methylation of the pectins. Numbers of *Enterococci*, *Bacteroidaceae*, *Clostridia*, and E. coli were increased with dietary addition of high-methylated citrus pectin, whereas inclusion of low-methylated citrus pectin in the diet only affected the number of Clostridia was increased. Teirlynck et al. (2009) also observed a shift in microbiota in broilers fed a wheat/rye-based instead of a maize-based diet. In this study, however, it was not possible to identify the individual bacterial groups involved in the observed effects. Young broilers, that were fed a diet containing 58% of rye had a significant increase in the number of total lactic acid bacteria, that were observed in the duodenum, in the ileum, and in the ceca when compared with broilers fed corn-rich diet. In these birds, a significant increase in the total number of coliforms was also observed in duodenum and ileum, whereas, an increase in total number of anaerobes was observed only in the duodenum(Tellez et al., 2014). In the current experiment, at 28 d of age the share of *Lactobacillus reuteri, Staphylococcus saporphyticus*, and *Aerococcaceae* decreased, whereas the share of *Lactobacillus* (undefined species) and *Lactobacillus salivarius* increased with increasing dietary rye inclusion levels. These changes, however, were not considered as detrimental for broiler gut health. Variations in proportions of species were related to the microbiota composition. *Lactobacillus reuteri* has been described as a probiotic and to be involved in the regulation of Tight Junction protein expression (Yang et al., 2015). Tight junctions are protein complexes that join two epithelial cells together to form a virtually impermeable barrier to fluid. Also *Lactobacillus salivarius* is regarded as a probiotic strain.

Gene expressions of gut cell walls

Based on the transcriptomics data of the current experiment, is was suggested that dietary rye addition affected cell proliferation and differentiation in intestinal jejunal tissue. These findings are in line with those of Pell et all. (1992), who observed that inclusion of guar gum, an soluble NSP source, to the diet exerted a positive effect on cell turnover in the jejunal and ileal crypts of rats. The increased expression of genes involved in cell cycle processes in the current experiment might be associated with the increased villi length and deeper crypts in birds fed the 10% rye diet. These findings suggest that inclusion of soluble NSP sources to the diet might exert positive trophic effects on the intestinal mucosa.

Within the framework of the current study, the used experimental diets were also tested in an *in vitro* bioassay using cultured Intestinal Porcine Epithelial Cells (IPEC-J2). In this assay, the effect of dietary rye supplementation on gene expressions of the IPEC-J2 cells were also determined, thereby evaluating the capability of this assay to predict enterocyte-specific physiological and immunological processes induced by nutrients/additives in the intestines of farm animals. Based on the gene expressions, it is concluded that rye influenced processes related to cell cycle progression in the IPEC-J2 cells, and showed only limited effect on genes related to immunological processes (Hulst et al., In progress). No correlation between viscosity of rye diets and biological processes was found.

Overall

Overall, it is concluded that inclusion of 5% or 10% rye to the grower diet of broilers in the current study had limited effects on performance. Ileal gut morphology, microbiota composition of jejunal digesta, and gene expression profiles of jejunal tissue, however, were affected by dietary rye inclusion levels. Our findings were less expressed compared to those of Teirlynck et al. (2009), who concluded that feeding broilers a wheat/rye-based diet, high in NSP, compared with a maize-based diet, decreased immunity-related parameters of the birds, as indicated by induced villus fusion, reduced thickness of the tunica muscularis, induced T-lymphocyte infiltration, more and larger goblet cells, and more apoptosis of epithelial cells in the mucosa. Although it is assumed that in both studies digesta viscosity is increased, differences between experiments might be associated with the age of providing rye-rich diets (d1 vs. d14) and differences in diet composition, e.g. the main dietary cereal (maize vs. rye).

Based on the available literature, it was hypothesized that dietary inclusion of rye in the current study would increase viscosity of intestinal digesta, consequently resulting in a negative effect on nutrient absorption, gut wall morphology, composition of microbiota, and immune related processes in the gut wall. Our working hypothesis, e.g. that rye could be a (negative) model ingredient that might contribute to a better understanding of the impact of nutrition on gut health and immunity of broilers was partly confirmed in the current experiment.

Conclusions

Based on results of the present experiment, the following conclusions can be drawn:

- Inclusion of 10% rye in the diet did not affect feed intake, but decreased body weight gain and increased feed conversion ratio.
- Litter quality was inversely related to the level of rye inclusion in the diet.
- Providing rye-rich diets resulted in increased jejunal villus height and crypt depth during the first week of provision, whereas the villus-crypt ratio was not affected. During the second week of the experiment, however, the level of rye inclusion had no effect on jejunal gut morphology.
- Inclusion of rye into the diet did not affect the number and size of jejunal goblet cells.
- Dietary inclusion of rye did not affect relative bursa and spleen weight.
- Dietary inclusion of rye did not affect the diversity of the jejunal microbiota, as determined by the Shannon index, although specific microbial strains were affected by rye inclusion.
- *Lactobacillus* species made about 75 - 80% of the jejunal microbiota; rye inclusion resulted in an exchange between the different *lactobacillus* species.
- At d28, the share of *Lactobacillus reuteri*, *Staphylococcus saporphyticus* and *Aerococcaceae* in the microbiota in jejunal digesta decreased with increasing dietary rye inclusion levels.
- Dietary inclusion of rye affected expression of genes in the small intestinal tissue involved in cell cycle processes of the epithelial gut cells, including proliferation, differentiation, motility, and survival, as well as in the complement and coagulation cascade. At 28 d of age, effects were more pronounced in birds fed the 10% rye diet, compared to birds fed the 5% rye diet.

Dietary rye inclusion affected expression of genes involved in cell cycle processes of the epithelial gut cells, thereby influencing cell growth, cell differentiation and cell survival. This observation is consistent with the observed differences in the morphology of the gut wall. Whether this also affected the barrier function of the epithelial layer, cannot be concluded. The complement and coagulation pathways, which are also affected by providing rye-rich diets, are parts of the innate immune system. These pathways are involved in eradicating invasive pathogens.

Overall, it is concluded that inclusion of 5% or 10% rye to the grower diet of broilers in the current study had limited effects on performance. Ileal gut morphology, microbiota composition of jejunal digesta, and gene expression profiles of jejunal tissue, however, were affected by dietary rye inclusion levels.

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Appendix 1 Calculated composition of the diets

	Starter	Grower			Finisher
		0% rye	5% rye	10% rye	
Ingredient (g/kg)					
Maize	572.1	599.4	539.8	480.3	646.5
Rye	0.0	0.0	50.0	100.0	0.0
Lard	25.0	30.6	37.9	45.2	36.5
Soybean oil	15.0	15.0	15.0	15.0	15.0
Soybean meal	291.4	263.3	265.6	268.0	213.4
Rapeseed meal	50.0	50.0	50.0	50.0	50.0
Monocalciumphosphate	13.5	12.3	12.2	12.0	11.3
Lime fine	17.0	15.1	15.1	15.1	13.2
Salt	2.2	2.3	2.3	2.3	2.3
Sodium bicarbonate	2.1	2.0	2.0	2.0	2.0
L-valine	0.1	0.0	0.0	0.0	0.0
Methionine 99%	2.9	2.6	2.6	2.7	2.3
L-lysine HCL 79%	2.3	1.9	1.9	1.8	1.9
Threonine 98%	0.8	0.6	0.6	0.6	0.6
Salinocox 12%	0.6	0.0	0.0	0.0	0.0
Premix	5.0	5.0	5.0	5.0	5.0
Calculated content (g/kg)					
Crude protein	212.4	199.8	200.9	201.9	178.4
Crude fat	67.8	73.7	79.5	85.2	80.3
ME_{br} (kcal/kg)	2875	2950	2950	2950	3050
dLysine	11.5	10.5	10.5	10.5	9.2
dMethionine	5.8	5.4	5.4	5.4	4.8
dMethionine+Cystine	8.5	8.0	8.0	8.0	7.2
dThreonine	7.5	6.9	6.9	6.9	6.2
dTryptophan	2.1	2.0	2.0	2.0	1.7
dIsoleucine	7.7	7.2	7.2	7.2	6.3
dArginine	12.4	11.6	11.6	11.6	10.1
dValine	8.6	8.0	8.0	8.0	7.1
Linoleic acid	25.0	25.8	25.6	25.4	26.8
totP	6.9	6.5	6.5	6.5	6.0
Chloride	2.3	2.3	2.3	2.3	2.3
Potassium	8.7	8.2	8.3	8.3	7.3
Sodium	1.6	1.6	1.6	1.6	1.6
Cupper (mg/kg)	18.5	18.1	18.2	18.3	17.4
tot Mg	2.0	1.9	1.9	1.9	1.7
tot Mn (mg/kg)	105.3	104.2	105.7	107.3	102.5
tot Zn (mg/kg)	93.6	92.5	92.8	93.0	90.9
Absorbable P	4.0	3.7	3.7	3.7	3.4
Analysed content (g/kg)					
Dry matter	893	891	893	897	887
Ash	63.8	57.2	57.0	57.2	50.6
Crude protein	211.7	195.6	197.0	196.9	175.2
Crude fat	- - -	82.0	85.5	90.1	- - -
Crude fibre	28.3	29.5	29.1	27.0	27.6
Starch	365.3	380.1	367.2	363.7	407.7
Sugars	47.7	44.2	43.2	49.6	40.4
NSP[1]	- - -	102.4	114.0	112.5	- - -
NDF	73.6	79.6	79.0	83.1	80.4
Soluble NSP[2]		22.8	35.0	29.7	
Viscosity (cPas)	1.5	2.0	4.4	8.3	2.2

[1] Calculated as: Dry matter – ash – crude protein – crude fat – starch- sugars

[2] Calculated as: NSP - NDF

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