Research article

Induction of gut leakage in young broiler chickens fed a diet with low rye inclusion

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ABSTRACT

The aim of the present study was to assess the absence of a non-starch polysaccharide (NSP) enzyme in a broiler diet containing a low level (10%) of rye inclusion. Two experimental groups with 40 Ross broilers each, were fed a diet containing 10% rye. One group was supplemented with a NSP enzyme, and the other was not supplemented with the enzyme to increase intestinal viscosity. The birds were fed the respective diets for 14 or 28 days. Intestinal sections were submitted to morphological, morphometric and mRNA-level gene expression analyses. To assess gut leakage, 150 min before euthanasia, broilers had no access to feed and received an oral gavage with fluorescein isothiocyanate-labelled dextran (FITC-d). Serum levels of FITC-d, D-lactate, tight-junction-associated protein 1 (TJAP1), citrulline and ovotransferrin were determined. A significant increase in FITC-d levels was observed in the 14-day-old birds fed the non-supplemented rye diet, and no other serum markers were affected. These birds presented a decreased villus height/crypt depth (VH:CD) ratio and an increased degree of damage in the jejunum. The ileum VH:CD increased, and the goblet cell number decreased in 28-day-old birds fed the non-supplemented rye diet. When broilers were fed the non-supplemented rye diet, the mRNA expression of the tight-junction zona occludens 1 (ZO1) was significantly decreased in the jejunum of 14-day-old broilers, whereas a significant decrease in jejunum mRNA expression of ZO2 and mucin-2 (MUC2) was observed in the jejunum of 28-day-old broilers. In contrast, a significant increase in the mRNA expression of ZO2 was observed in the ileum from 28-day-old broilers fed the non-supplemented rye diet. In conclusion, a 10% rye diet causes intestinal stress in young broiler chickens when the feed is not supplemented with a NSP enzyme. This study may be applied as experimental model of mild gut leakage of broiler chickens.

1. Introduction

Optimal performance in broiler chickens depends on the capacity of their intestine to selectively absorb dietary nutrients and avoid the invasion of toxins or pathogenic micro-organisms. One of the protective mechanisms of the intestine is its ability to control the passage of solutes, which is well controlled by tight junctions. These tight junctions can discriminate solutes that can traverse the intestinal barrier from undesirable substances. Functional disturbances in tight junctions can be caused by different stress factors such as a high mycotoxin level in the diet (Omealeere et al., 2013), heat stress (Santos et al., 2015, 2019), feed restriction for 24 h (Baxter et al., 2017) and the inclusion of high levels of rye in the diet of broiler chickens (37%; Tellez et al., 2014; 58% Vicuna et al., 2015). All these models can induce intestinal inflammation and mimic situations of environmental and dietary stress regardless of the age of the bird. However, increased intestinal permeability may also occur in broiler chickens that are not experiencing stress conditions with visible clinical symptoms, especially the youngest ones.

Young broiler chickens produce limited amounts of pancreatic and intestinal mucosal enzymes (Almirall et al., 1995; Uni et al., 1999; Sklan and Noy, 2000), which are positively correlated with their body weight (Shakouri et al., 2008). Moreover, their immature gut does not support the transit of a viscous digesta with the same efficiency as that of older birds (Smulikowska, 1998) because of decreased intestinal contractile

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activity (Smulkowska et al., 2002). In newly hatched broilers, the intestine will undergo morphological changes before the chicken reaches maturity. For instance, the number of intestinal crypts will reach a plateau 2–3 days post-hatching (dph), whereas the villus width and length of the jejunum will reach their plateau only 7 and 10 dph, respectively (Sklan, 2001; Geyra et al., 2001). Although rye appears as an alternative feedstuff for poultry, its high levels of non-starch polysaccharides (NSPs) lead to increased digesta viscosity, and the subsequent decrease in the feed passage rate will induce overgrowth of bacteria, as well as inflammation and gut permeability (Vicuna et al., 2015). It has already been demonstrated that dietary rye at inclusion levels above 30% (37%; Tellez et al., 2014; 58% Vicuna et al., 2015) is able to induce leaky gut. In practice, rye inclusion in poultry diets does not exceed 10%. However, based on their immature gut, we hypothesise that a low rye inclusion level (10%) in the diet is sufficient to induce leaky gut in young broiler chickens. Therefore, these young birds could be used as models without experiencing extreme stress.

Several methods are used to evaluate gut integrity. The first is the detection of large molecules that pass through the intestine and reach the blood circulation. To evaluate this, broiler chickens are submitted to an oral inoculation of fluorescein isothiocyanate-labelled dextran (FITC-d), a large molecule that should not easily cross the epithelial barrier (Vicuna et al., 2015). Other serum markers for intestinal integrity include tight-junction-associated proteins (TJAPs) that will reach blood circulation when disrupted, and D-lactate as an indicator of leaky gut accompanied by bacterial proliferation (Ducatelle et al., 2018).

Rye may also lead to intestinal inflammation; serum markers for such stress conditions include citrulline and ovotransferrin (Baxter et al., 2019). Furthermore, intestinal stress may result in morphological and molecular changes, which include changes in VH and CD (Santos et al., 2019). Furthermore, intestinal stress may result in morphological and molecular changes, which include changes in VH and CD (Santos et al., 2019). Other serum markers for intestinal integrity include tight-junction-associated proteins (TJAPs) that will reach blood circulation when disrupted, and D-lactate as an indicator of leaky gut accompanied by bacterial proliferation (Ducatelle et al., 2018).

### Table 1. Composition of the experimental diets.

| Ingredients (%) | Starter (D0-14) | Grower (D14-28) |
|-----------------|----------------|----------------|
| Corn            | 24.66          | 25.00          |
| Corn gluten meal| 8.14           | 6.86           |
| Soybean meal    | 27.56          | 24.43          |
| Wheat           | 20.00          | 19.96          |
| Rye             | 10.00          | 10.00          |
| Soybean full fat| 0.00           | 4.25           |
| Palm oil        | 5.083          | 6.00           |
| Salt            | 0.110          | 0.231          |
| Limestone       | 1.701          | 1.354          |
| Monocalcium Phosphate | 1.265   | 0.866         |
| Sodium Bicarbonate | 0.309   | 0.100         |
| Lysolecithin    | 0.376          | 0.263          |
| DL-Methionine   | 0.209          | 0.175          |
| Threonine       | 0.037          | 0.008          |
| Tryptophan      | 0.001          | 0.000          |
| Arginine        | 0.050          | 0.000          |
| Vitamin & Mineral premix | 0.500   | 0.500         |
| **Total**       | **100.00**     | **100.00**     |
| E-Brillers kcal/kg | 2.900       | 3.000          |
| DM g/kg         | 885            | 885            |
| Ash g/kg        | 58.7           | 51.8           |
| Crude protein g/kg | 240           | 230            |
| Crude fat Atl g/kg | 78.4       | 94.6           |
| Crude fibre g/kg | 21.2          | 22.3           |
| Ca g/kg         | 9.5            | 7.5            |
| P g/kg          | 6.4            | 5.4            |
| Mg g/kg         | 1.5            | 1.5            |
| K g/kg          | 8.4            | 8.5            |
| Na g/kg         | 1.4            | 1.3            |
| Cl g/kg         | 2.0            | 2.5            |
| retPint g/kg    | 3.8            | 3.0            |
| avCap g/kg      | 9.5            | 7.5            |
| dEBL meq        | 220            | 202            |
| US ratio        | 1.7            | 1.8            |
| dLYS g/kg       | 12.0           | 11.0           |
| dMET g/kg       | 5.5            | 5.0            |
| dCVS g/kg       | 3.1            | 3.0            |
| dM + C g/kg     | 8.7            | 8.0            |
| dTHR g/kg       | 7.5            | 6.9            |
| dTRP g/kg       | 2.2            | 2.1            |

The challenge diet was either or not supplemented with the NSP enzymes xylanase and glucanase (50 g/ton diet).

2. Materials and methods

#### 2.1. Ethics statement

This study was conducted according to the guidelines of the Animal and Human Welfare Codes/Laboratory practice codes in the Netherlands. The protocol was approved by the Ethics Review Committee: Body of Animal Welfare at SFR (AVD246002016450).

#### 2.2. Animals and experimental design

One-day-old male Ross broilers, purchased from a local commercial hatchery, were used in this study, with two dietary treatments of 40 chicks each. The birds were housed in cages (2 birds per cage) of 2 m² with wood shavings as bedding material. The two experimental groups consisted of a 10% rye diet supplemented or not with a commercial NSP enzyme blend (50 g/ton diet, xylanase and glucanase). Diet (Table 1) was offered as mash and birds had free access to the feed and water. The experiment had a starter phase from D0-14 and a grower phase from D14-28, and birds were euthanized at two time points, i.e. 20 per treatment at D14 and 20 per treatment at D28, for sampling. To assess gut leakage, 150 min before euthanasia, broilers received an oral gavage with FITC-d (MW 3,000–5,000; Sigma Aldrich Co., St. Louis, MO) diluted in Milli-Q water, (2.2 mg/ml; 1 ml/bird) (Vicuna et al., 2015). The birds were not fed between gavage and euthanasia to avoid feed consumption interference. After euthanasia, samples of mid-jejunum and distal ileum of all birds (40 birds per treatment; 20 per feeding period) were collected for histologic and blood sampling. Blood was collected for the measurement of FITC-d levels, as well as for the analysis of other markers for gut integrity and inflammatory response. Samples of mid-jejunum and distal ileum were collected from 20 birds per treatment (10 per feeding period) and submitted to mRNA expression analyses.

#### 2.3. Serum analysis

Serum was derived from blood (~10 ml collected per bird at slaughter), harvested by centrifugation (15 min at 1500 × g), protected from light and stored at −20 °C. For detection of FITC-d levels in serum, a protocol previously described (Baxter et al., 2017) was applied and fluorescence levels were measured at an excitation wavelength of 485 nm and emission wavelength of 528 nm (plate reader Infinite® 200 Pro, Tecan, Männedorf, Switzerland).

Levels of D-Lactate (μmol/ml), citrulline (ng/ml), ovotransferrin (ng/ml), and tight junction associated protein-1 (ng/ml) were measured using...
assay kits from MyBiosource Inc. (San Diego, CA, USA), coded as MBS2604186, MBS4191177, MBS2610621, and MBS9915242, respectively. Absorbance was measured using a spectrophotometer (plate reader Infinite® 200 Pro, Tecan, Tecan Group Ltd., Männedorf, Switzerland) at a wavelength of 450 nm.

### 2.4. Histological analysis of jejunum and ileum

Jejunum and ileum samples from each bird were submitted to morphometric and morphological analyses as previously described (Santos et al., 2015). In brief, histological slides (periodic acid–Schiff (PAS)-hematoxylin staining) from the intestinal sections were prepared and evaluated with the help of the viewer software (NDP.view2; Hamamatsu), and analysed using the analysis software (NDP.analyze; Hamamatsu). Villus height (VH), crypt depth (CD), and villus area (μm²) from each intestinal sample were measured (five intact villi per intestinal segment), and the VH:CD ratio was calculated. Furthermore, the number of goblet cells was quantified and the goblet cells density per villus was calculated. For this, intestinal sections were stained with Alcian Blue. To evaluate the degree of mucosal damage, the Chiu/Park scale was applied. For this, intestinal sections were stained with Alcian Blue. To evaluate the degree of mucosal damage, the Chiu/Park scale was applied (Santos et al., 2015). In brief, a section of the complete intestinal segment was used for mucosa analysis, which was classified from normal to demonstrating severe damage. The scale comprises four degrees: (degree 0) no damage, (degree 1) very slight damage (e.g. more than five times the amount of rye that we added to the present diet). Differently from pigs, chicken enterocytes cannot synthesize L-citrulline from glutamine (Wu et al., 1995). In avian species, arginine generates a limited amount of citrulline via nitric oxide synthase, resulting in very negligible plasma levels (He et al., 2021). An increase in the levels of ovotransferrin in the serum of chickens is related to pathogen infection. D-lactate is another biomarker of increased intestinal permeability, but its presence in serum requires higher intestinal stress than does that of FITC-d (Gilani et al., 2016). Increased serum levels of FITC-d alone indicated that the present challenge was not severe enough to prevent gut integrity. From an animal welfare perspective, such a protocol allows the evaluation of gut integrity in young chicks without submitting them to highly stressful conditions.

### 2.5. mRNA expression in jejunum and ileum

RNA from samples of jejunum and ileum was isolated using the SV Total RNA Isolation System (Promega, Madison, WI, USA). For each intestinal segment within each treatment, 20 samples were collected (10 per feeding period). Subsequently, 1 μg of extracted total RNA was reverse transcribed with the iScriptTM cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). The cDNA was diluted to a final concentration of 30 ng/μl qPCR was performed using the MyIQ single-color real-time PCR detection system (Bio-Rad) and MyIQ System Software Version 1.0.410 (Bio Rad Laboratories Inc., USA). Primers, as presented in Table 2, were commercially produced (Eurogentec, Maastricht, the Netherlands) and their cycling conditions and melting curves were assessed before running the analysis of the samples. Data were analysed using the efficiency-corrected DeltaDelta-Cq method (Pfaffl, 2001). The fold-change values of two housekeeping genes: hypoxanthine-guanine phosphoribosyl transferase (HPRT) and β-actin (ACTB). The mRNA expression level of markers involved in gut integrity, i.e. claudin-3 (CLDN3), CLDN5, zona occludens-1 (ZO1), and ZO-2 were evaluated in the jejunum and ileum. Furthermore, mRNA expression of a marker involved in mucus production (mucin-2; MUC2) was also measured. Each sample was evaluated in triplicate.

#### 2.6. Statistical analysis

Statistical analysis was carried out with GenStat® for Windows (20th edition; VSN International, Hemel Hempstead, UK). All parameters were analysed with one-way ANOVA with Fisher's least significant difference (LSD) post-hoc test to compare the treatment means within each period. Values with P ≤ 0.05 were considered statistically significant.

### 3. Results and discussion

The average body weight (BW) of the birds fed the non-supplemented and fed the enzyme-supplemented one were 416 vs 427 g at D14, and 1281 vs 1249 g at D28, respectively. Other authors demonstrated that dietary supplementation with 10% rye results in a significant decrease in body weight gain in broiler chickens (Van Krimpen et al., 2017). However, no significant differences were observed in the present study.

The serum FITC-d levels were significantly increased in 14-day-old birds fed the non-supplemented rye diet. None of the other serum parameters, i.e. citrulline, ovotransferrin and D-lactate were affected by the dietary treatment, regardless of the age of the birds (Figure 1). Young chickens may experience gut leakage in the first days of life without presenting symptoms of a disease, as confirmed by the fact that markers of inflammation such as citrulline or ovotransferrin were not affected by the diet. Baxter et al. (2019) observed a significant increase in citrulline levels in the plasma of broiler chickens fed a diet containing 58% rye. This is more than five times the amount of rye that we added to the present diet. Differently from pigs, chicken enterocytes cannot synthesize L-citrulline from glutamine (Wu et al., 1995). In avian species, arginine generates a limited amount of citrulline via nitric oxide synthase, resulting in very negligible plasma levels (He et al., 2021). An increase in the levels of ovotransferrin in the serum of chickens is related to pathogen infection. D-lactate is another biomarker of increased intestinal permeability, but its presence in serum requires higher intestinal stress than does that of FITC-d (Gilani et al., 2016). Increased serum levels of FITC-d alone indicated that the present challenge was not severe but sufficient to impair gut integrity. From an animal welfare perspective, such a protocol allows the evaluation of gut integrity in young chicks without submitting them to highly stressful conditions.

Figure 2 shows that at D14, a significant decrease in the VH/CD ratio was observed in the jejunum of broiler chickens fed the non-supplemented rye diet. Damage in the jejunum from the chickens fed the non-supplemented rye diet was higher than degree 2. This means that, instead of no damage (degree 0) or damage restricted to the villus

### Table 2. Primers used for the quantification of genes of interest (GOI) and housekeeping genes (HKG) expression.

| Genes | Accession no | Primer sequence | Annealing T° | Pairs of bases | Reference          |
|-------|--------------|-----------------|--------------|---------------|--------------------|
| HKG   |              |                 |              |               |                    |
| HPRT  | NM_204848.1  | F: CGGTGGCTGCTTACTAATGCAAG R: GATACTCCACATTCGAGGAG | 65           | 90            | Santos et al. (2019) |
| ACTB  | NM_205518.1  | F: ATGGGATCGACGAGGAGGTA R: TTATGGCGATTATATGGGTTTGT | 65           | 127           | Varasteh et al. (2015) |
| GOI   |              |                 |              |               |                    |
| CLDN3 | NM_204202    | F: AGCCCTCCTCTCAGCAG R: TTCTCCGCGAGCCTTC | 56           | 185           | Ozden et al. (2010) |
| CLDN5 | NM_204201    | F: CATCCTCTCTGCTGAGCAG R: GCACAGAGATCTGCAGAGTGC | 58           | 111           | Osselare et al. (2013) |
| ZO1   | XM_413773    | F: CTTCAGCTGTTTCTGCTCCTTC R: CTGGTTGTCTCATGGTGGTGC | 59           | 131           | Osselare et al. (2013) |
| ZO2   | NM_204918    | F: GCCAGGCTCTAGCGACACTC R: CACAGGCGCAGGCTACAG | 64           | 87            | Osselare et al. (2013) |
| MUC2  | BX930545     | F: ATGCCAGTTAACACAGGACTC R: GTGGACGCAGACAGTTC | 61           | 110           | Forder et al. (2012) |
tip (degree 1), these birds also presented extension of the sub-epithelial space (degree 2) and denudation of the villus (degree 3). Images of the jejunal and ileal sections are given in Figure 3. At D28, the CD of the ileum of chickens fed the non-supplemented rye diet was significantly decreased accompanied by a significant increase in the ileal VH/CD ratio when compared with them fed with NSP enzyme. When exposure to a certain source of stress remains for a longer time, the ileum will also react and present morphometric changes (Santos et al., 2019), as observed in the present study. The ileum was affected only after 28 days of exposure to the challenge diet, showing a significant decrease in CD but maintaining a similar VH to that of the supplemented rye diet. Therefore, this intestinal section was more efficient in combating stress when compared with the jejunum, maintaining a similar intestinal absorption area to that of birds fed the NSP enzyme-supplemented rye diet. It was also remarkable that the ileum of 28-day-old birds fed the non-supplemented rye diet presented a significant decrease in the number of goblet cells per villus. This result differs from those of other studies that actually showed an increase in the number of goblet cells in the ileum of broiler chickens fed rye diets as a result of an excessive stimulation of the immune system (Teirlynck et al., 2009). According Maiorka et al. (2003), a decrease in the number of goblet cells is one of the signs of suboptimal intestinal development. In the present study, acute inflammation was not stimulated, neither was the expression of mucin-2 (MUC2) increased. As shown in Figure 4, gut leakage was also confirmed by a decrease of tight-junction mRNA expression in the jejunum. This effect was first observed in the jejunum at D14, when zona occludens 1 (ZO1) expression decreased followed by a decrease in ZO2 expression in the jejunum and increased ZO2 expression in the ileum at D28. At D28 ileal ZO1 expression was similar between dietary treatments but was accompanied by downregulation of ZO2. Both ZO1 and ZO2 are peripheral membrane proteins crucial to tight-junction assembly and maintenance. These proteins interact with others, such as claudins, occludins and actin (Turner, 2009). Downregulation of ZO1 expression does not completely hamper the function of tight junctions but delays their activity, which is compensated by ZO2 upregulation (Roehlen et al., 2020). At D28, some recovery of ZO1 expression was observed in the ileum, suggesting an intestinal adaptation at D28 related to the increased FITC-d levels only at D14 and not at D28 in challenged birds. The significant decrease in the number of goblet cells in the ileum of 28-day-old challenged birds was not accompanied by a significant change in the expression of MUC2. On the other hand, the number of goblet cells per jejunal villus was not affected, but MUC2 expression was significantly decreased in 28-day-old broiler chickens fed the non-supplemented rye diet. This shows that, although the number of goblet cells in the jejunum was the same regardless of diet, they were not functioning at a similar capacity. The present model was able to induce gut leakage in young (14-day-old) broiler chickens, and it was not acute enough to affect older (28-day-old) chickens. Such a compensatory mechanism was previously demonstrated in broiler chickens (Maiorka et al., 2003; Santos et al., 2021) and might be an adaptive gut response after exposure to diverse sources of stress or dietary challenges (Lamot 2017). It is important to bear in mind that mRNA expression will not necessarily reflect the actual protein expression in tissue. Therefore, the analysis of protein expression remains needed to support these findings.
supplementation would differ between the two diets. Wheat starch is more rapidly digested than corn starch (Giuberti et al., 2012), and the proximal ileal digestibility coefficients of starch are negatively correlated with the level of 12 amino acids (Moss et al., 2018). Moreover, Crystal et al. (2021) recently demonstrated that it is not possible to compare a NSP-rich grain like wheat with corn because wheat in the diet will result in inadequate detoxification of ammonia together with competition for intestinal uptake between amino acids and starch. Importantly, NSP not only increases the digesta viscosity but also decreases the digestibility of protein, fat and starch (Bedford et al., 1991; Meng et al., 2005).

In conclusion, it was possible to induce and measure gut leakage in young broiler chickens fed a diet containing 10% rye. Although most negative effects were measured in young (14-day-old) broilers, intestinal changes remained in 28-day-old birds. The jejunum was more sensitive

Figure 3. Representative images of periodic acid–Schiff (PAS)-hematoxylin stained sections showing: jejunum sections from 14- (A, C) and 28-days-old (F, H) broilers fed the rye diets with (A, F) or without enzyme (C, H); ileum sections from 14- (B, D) and 28-days-old (G, I) broilers fed the rye diets with (B, G) or without enzyme (D, I). Magnification of 200×. The black arrows indicate denudation of villi tips; the red arrow indicates extension of the sub-epithelial space, and the yellow arrow indicates denudation of the villus. Scale bars; 100 μm.
than the ileum in the present study, probably because jejunal development, which is accomplished a few days post-hatching, was affected by the rye diet.

**Declarations**

**Author contribution statement**

Regiane R. Santos: Conceived and designed the experiments; performed the experiments; analyzed and interpreted the data; wrote the paper.

Marjolein A.M. Oosterveer-van der Doelen: Performed the experiments; analyzed and interpreted the data; wrote the paper.

Monique H.G. Tersteeg-Zijderveld: Analyzed and interpreted the data; wrote the paper.

Francesc Molist: Conceived and designed the experiments; contributed reagents, materials, analysis tools or data; wrote the paper.

Ronette Gehring: Contributed reagents, materials, analysis tools or data; wrote the paper.

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**Data availability statement**

Data included in article/supplementary material/referenced in article.

**Declaration of interests statement**

The authors declare no conflict of interest.

**Additional information**

No additional information is available for this paper.

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