Final Report

Project code: 018-422

Prepared by: Dr Reza Barekatain

Date: 26-11-2019

Potential non-invasive biomarkers of intestinal inflammation and permeability in broiler chickens

Milestone 1.4

Potential non-invasive biomarkers of intestinal inflammation and permeability in broiler chickens

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In submitting this report, the researcher has agreed to Poultry Hub Australia publishing this material in an edited form.

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Project Summary

Project Title	Potential non-invasive biomarkers of intestinal inflammation and permeability in broiler chickens
Project No.	018-422
Date	Start:01-12-2018 End: 30-11-2019
Project Leader(s)	Dr Reza Barekatain, Prof Gordon S. Howarth, Dr David Cadogan and Dr Stuart Wilkinson
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Project Aim	- To investigate potential biomarkers of gut barrier function in excreta to facilitate non-invasive assessment of gut integrity without a need to euthanize or bleed the birds. - To compare two different gut leakage (also called barrier dysfunction) models for potential biomarkers. - To investigate the relevance of specific biomarkers in relation to a selected feed additive (Bacillus based probiotics)
Background	Managing gut health through barrier function is regarded as a new frontier for disease prevention across different species. In poultry, few objective measures have been identified that could relate to the functionality of the intestinal barrier and further, detection of inflammation with currently methods are mainly invasive, complex and time-consuming. Little research has been conducted on identification of biomarkers related to gut permeability and inflammation that could be sampled non-invasively, are simple and field-relevant.
Research Outcome	Fibronectin, intestinal alkaline phosphatase and lipocalin 2 in excreta were found to be responsive to the gut leaky model, in particular synthetic glucocorticoid, dexamethasone. Subject to further validations, these three proteins hold great promise to be used as novel biomarkers of intestinal integrity.
Impacts and Outcomes	Relatively rapid assessment of gut health will pay large dividends to the industry by minimising costs of poor enteric health. A fully set up and validated ELISA assay can be completed in less than one day allowing an accelerated decision making process to address on farm gut health issues.
Publications	Barekatain R., Howarth G.S., Willson N.L., Cadogan D. and Wilkinson S. 2020. Selected excreta biomarkers of intestinal barrier function in broiler chickens subjected to two gut leakage models with or without probiotic supplementation. World's Poultry Congress 2020, Paris, France. (Submitted for approval – a copy in Appendix) Barekatain R., Howarth G.S., Willson N.L., Cadogan D. and Wilkinson S. 2020. Quantification of selected excreta biomarkers in broiler chickens in response to two different gut barrier dysfunction models with or without in feed probiotic supplementation. Animal Science and Biotechnology. (To be submitted after approval sought)

Executive Summary

Increased intestinal permeability (IP) and inflammation are both linked with the functionality of the intestinal barrier and in particular enterocytes. When the intestinal barrier function is disturbed, toxins, harmful bacteria and unwanted materials are passed through the intestine and activate the immune response which in turn cause intestinal inflammation and ultimately compromise performance. Currently, almost all assessment methods of the intestinal barrier function are invasive. These methods include differentially sized sugar tests, tissue sampling, or blood collection. Such methods are often complex, time-consuming and less field relevant. The present project aimed to quantify selected proteins as biomarkers in excreta to facilitate non-invasive assessment of gut barrier function using enzyme-linked immunosorbent assays (ELISA). It was also hypothesised that probiotics as feed additives may counteract the gut barrier dysfunction. A 3 × 2 factorial arrangement of treatments was used with the main factors being gut barrier dysfunction models (control, rye-based diet, and dexamethasone – DEX) with and without probiotic supplementation (a three-strain *Bacillus*). 72 male Ross 308 day-old chickens were given a commercial starter diet with or without probiotic supplementation. From days 13 to 21 of age, birds were transferred into individual cages and allowed access to experimental treatments. Each of the 6 experimental treatments was replicated 12 times. Birds in the DEX group were injected with DEX on days 14, 16, 18 and 20 (0.5 mg/kg BW). On d 21 of age, fluorescein isothiocyanate dextran (FITC-d) uptake into serum was examined to test IP. Fresh excreta samples were collected on d 20. A total of 9 biomarkers were assessed. The biomarkers included Alpha 1 Antitrypsin (A1AT), Intestinal Fatty Acid Binding Protein (IFABP-2), Lipocalin 2 (LCN2), Fibronectin (FN), Intestinal Alkaline Phosphatase (IAP), Lactoferrin (LTF), ovotransferrin (OVT) and superoxide dismutase [Cu-Zn] (SOD1). Calprotectin was also attempted however, none was detected in excreta samples which indicates that this protein may not be expressed in chickens. Only DEX increased FITC-d passage to the blood, indicating a greater IP. The excreta concentrations of A1AT and IFABP-2 were unaltered by the experimental treatments. DEX increased ($P<0.05$) FN concentration in excreta compared with control birds. Conversely, inclusion of rye in the diet reduced ($P<0.05$) FN. Independently, DEX decreased IAP ($P<0.05$) in excreta compared with control and rye-fed birds. The concentration of LCN2 also tended to increase in birds under stress, stimulated by DEX. Lactoferrin was below the detectable range of the ELISA assay. There was no demonstrable effect of probiotic addition on any of the studied parameters. The results of this project showed that among the tested biomarkers FN, IAP and LCN2 revealed promise as biomarkers of intestinal barrier function (intestinal permeability and inflammation) quantified by ELISA kits. Further studies are required to validate these results and compare litter samples as well as fresh digesta; as the homogeneity of samples at farm level may be a determining factor in obtaining accurate results. Further biomarkers will need to be identified through both proteomic and direct measurement approaches, as multiple biomarkers are more representative of gut integrity compared to a single biomarker. Should the research into excreta biomarkers be successfully adopted, a significant benefit for the poultry industry could be achieved through minimising the cost of poor enteric health, stress and digestive disorders.

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Introduction

Maintaining and improving gut health is fundamentally important as the gut supports optimal digestion and therefore performance and profitability of production. Managing gut health through barrier function is regarded as a new frontier for disease prevention across different species (Vancamelbeke and Vermeire, 2017). In poultry, considerable research has been done on improving animal performance and gut health through various nutritional approaches. However, few objective measures have been identified that could relate to the functionality of the intestinal barrier and detection of inflammation. The complex structure of the epithelium consisting of a mucus layer covering a single layer of epithelial cells, plays a crucial role in controlling the permeability and selective absorption of nutrients (Ulluwishewa et al., 2011). Disruption of tight junction proteins, alteration in the mucus layer or changes in proliferation of epithelial cells could compromise gut integrity, increase bacterial translocation and eventually cause inflammation. Inflammation often leads to impaired performance that is a significant loss to the poultry industry. Little research has been conducted on identification of biomarkers related to gut permeability and inflammation that could be non-invasive, simple and field-relevant. Currently very few biomarkers of gut barrier failure and inflammation have been identified for poultry and the assessment is limited to often invasive methods of intestinal tissue sampling or analysis of differentially sized sugars via methods that require birds to be bled and/or euthanized (Gilani et al., 2016). There are very recent efforts to identify biomarkers of gut barrier function based on pathogenic and necrotising agents (De Meyer et al., 2019). However, ideal biomarkers should not only be reflective of one particular model and it is important to associate potential biomarkers to other available physiological (i.e. stress) and nutritional models. Besides, still less data is available for excreta biomarkers; and intestinal contents, including colon, have been used as a proxy to excreta (De Meyer et al., 2019).

It is clear that not a single universal biomarker exists for assessing gut health (Ducatelle et al., 2018), therefore there is a need for a set of objective biomarkers that could be detectable in biological fluids as well as excreta as a primary step to facilitate non-invasive, farm-relevant evaluation of intestinal inflammation and barrier function. Despite the lack of data for poultry, research in humans (Kosek et al., 2013) has identified some faecal biomarkers related to gut inflammation and permeability such as alpha1-antitrypsin inhibitor (AAT, biomarker of gut permeability). There are also some recent reviews listing potential biomarkers that could be detected in excreta (Celi et al., 2019). In most cases, it is unknown how useful excreta biomarkers can be in poultry and even the reference values are lacking. So far little attempt has been made to link these potential biomarkers with inducing agents of gut inflammation and permeability such as stress, bacterial, hormonal and feed-related factors.

Dietary manipulation and feed additives are among strategies to combat gut barrier dysfunction and enteric disorders. Probiotics, in particular, are shown to help maintain intestinal barrier function through modulating microbiota composition, maintaining permeability, enhancement of immune responses and physical characteristics of the mucous layer (Wang et al., 2018). Gadde et al. (2017) found that dietary supplementation of *Bacillus subtilis*-based probiotics positively influenced gut barrier integrity by increasing tight junction gene expression. However, little is known in poultry whether any of the excreta biomarkers can be affected by probiotics. If excreta biomarkers are found respondent to a relevant dietary probiotic, this could facilitate rapid and non-invasive assessment of dietary interventions directly linked with intestinal functions.

This project proposes a suite of potential biomarkers detectable in excreta of broiler chickens and aims to test them via the use of poultry models of intestinal barrier dysfunction (gut

leakage models) and through the application of feed-related factors. It also aimed to simultaneously investigate possible counteracting effects of probiotic supplementation on the studied biomarker. Chicken specific reagents of these biomarkers have only recently been made available. Therefore the potential of ELISA was investigated since the method is relatively rapid and can directly quantify proteins.

Objectives

It was hypothesized that excreta samples collected from broiler chickens would contain biomarkers that can be analysed by relatively simple ELISA assays, thereby facilitating potentially non-invasive field-relevant assessment of gut inflammation and permeability. This is an area of research that is largely untapped in poultry.

Briefly, specific objectives/aims of this project were to:

1. Evaluate the usefulness of sampling excreta to assess gut inflammation
2. Identify a suite of novel gut inflammation and permeability biomarkers that could be potentially detected in the excreta.
3. Compare different inflammation and permeability models for these potential biomarkers
4. Assess the relevance of these biomarkers in relation to a selected feed additive (probiotics) known to affect intestinal integrity

Strategies incorporated into this proposal by which the above objectives may be achieved:

1. Inclusion of feed or stress related factors as different potential models that could potentially cause intestinal inflammation and permeability
2. Selecting a probiotic product as a potential anti-inflammatory feed additive for leaky gut models
3. Assessing ELISA biomarker kits for chicken-specificity and commercial viability

All the main objectives of the project were met and the results are presented and discussed in the relevant sections.

Methodology

All the experimental procedures were approved by the Animal Ethics Committees of The Primary Industries and Regions South Australia and the University of Adelaide.

The experiment comprised a 2 × 3 factorial arrangement of treatments. The main factors were gut barrier dysfunction models (control, rye-based diet and dexamethasone – DEX) without and with probiotic supplementation of the diets. The probiotic used in the study was a three-strain *Bacillus* product (Enviva Pro 202 BA, Dansico, Dupont) with minimum activity of 2.5 x 10^9 colony forming unit/g. The probiotic was supplemented at 60 ppm to the diets. The probiotic was provided by the Feedworks company and their recommendation was followed for the inclusion rate. A total of 72 male Ross 308 day-old chickens were obtained from Aviagen hatchery (Goulburn, NSW) and were brought to the poultry research facility at the Roseworthy Campus of the University of Adelaide. Upon arrival, birds were kept in two groups

in raised pens and were given starter diets with or without probiotic supplementation. On day 13 of age, birds were transferred to a total of 72 individual metabolism cages for experimental procedures. Half of the birds received the diets supplemented with probiotic while the remaining half were fed unsupplemented diets. Each of the 6 experimental treatments was replicated 12 times. Body weight of each bird was recorded at the beginning and the end of 8 days in metabolism cages. Feed consumption was also individually recorded and feed conversion ratio was subsequently calculated. All birds had unrestricted access to feed and water throughout the experiment. Birds were maintained on 16 hours of light and 8 hours of darkness except for the first 3 days when they were exposed to 23 hr light. The room temperature was kept at 34 ºC during the first 3 d followed by a gradual decrease to 23 ºC by the end of study at d 21 of age.

Experimental diets were formulated to be iso-nitrogenous and iso-energetic (Table 1). Main ingredients were analysed for nutrient composition using near-infrared spectroscopy (Evonik Industries). Digestible amino acids for rye were the average values obtained from Zuber et al. (2016). A rye-based diet was used as a model of gut barrier dysfunction accordingly to the literature (Latorre et al., 2015). Dexamethasone was used as another model based on previous experiments (Barekatain et al., 2019; Wideman Jr and Pevzner, 2012). Birds were given experimental diets from d 13 to 21. Birds in DEX group were injected with DEX at 0.5 mg/kg BW on d 14, 16, 18 and 20 of age. The DEX preparation of solution for each injection followed the procedure previously described by Wideman Jr and Pevzner (2012).

FITC-d test

On d 21, each bird was given an oral gavage of FITC-d (2.2 mg/bird) similar to previous studies (Barekatain et al., 2019; Gilani et al., 2018). After 150 min, blood collection was carried out from the live bird via the jugular vein. Blood samples were kept at room temperature for at least 3 hours to allow clotting. Subsequently, serum samples were separated after centrifuging blood tubes at 1000 g for 15 min. The concentration of FITC-d was determined using a Synergy MX plate reader (Biotek Instruments, Bedfordshire, UK) with excitation and emission wavelengths set at 485 and 530 nm, respectively. Standards and samples were analysed in triplicates. On d 21, all the birds were then euthanized for recording the weights of bursa, spleen and liver.

Excreta collection and processing

On the evening of day 20 of age, following the last DEX injection, all the excreta trays were cleaned and fresh excreta samples were collected for each of the 72 birds within 6 hours. Excreta samples were then stored at -80 C until analysis. The frozen excreta samples were thawed and subsequently diluted (1:10) with PBS. Samples were then thoroughly mixed and then centrifuged at 1500 g and 4° C for 20 min. Aliquots of the supernatants for each sample were then obtained and kept in -80° C until used for assays.

ELISA assays

Commercial ELISA kits for chicken alpha 1 antitrypsin (A1AT) (MBS028567), intestinal fatty acid binding protein (IFABP-2) (MBS741864), chicken Lipocalin 2 (LCN2) (MBS005459), Chicken Fibronectin (FN) (MBS778116), Chicken Lactoferrin (LTF) (MBS268795), Chicken Intestinal Alkaline Phosphatase (IAP) (MBS734160), Chicken ovotransferrin (OVT) (MBS944289) were sourced from MyBioSource (San Diego, CA). The kit for chicken superoxide dismutase [Cu-Zn] (SOD1) was sourced from Wuhan Fine Biotech Co., Ltd. (Hubei, China). All the assays were precisely carried out according to the manufacturer's instructions. Each blank and standard solutions were replicated 3 times on each plate and samples were assayed in duplicates. The optical density for all the assays were determined using a microplate reader (Bio-Rad Benchmarch Plus^{TM}, CA, USA).

Statistical analysis

All the data were subjected to statistical analysis using two-way ANOVA (SAS Statistical package 9.4). The main effects of gut barrier dysfunction and probiotic as well as their interaction were assessed. When significant difference was detected, means were separated and compared using Least Square Differences test. Data were checked for normal distribution. For ELISA assays, occasional outliers were removed from the data if there were ± 3 times of the standard deviation. Each individually housed bird and its respective sample was considered an experimental unit. The level of significance was considered $P < 0.05$ and tendency was considered for $0.05 \leq P \leq 0.10$.

Results

Performance of birds and intestinal permeability

Feed consumption, body weight gain and FCR of individually birds housed birds are shown in Table 2. There was no interaction between the gut leakage models and supplementation of probiotic for any of the performance parameters. No effect of probiotic was also observed for any of studied parameters. Both rye-based diet and DEX decreased feed intake ($P<0.0001$) compared with control group of birds. Body weight gain ($P<0.0001$) and FCR ($P<0.0001$) were also compromised most by DEX followed by the rye-based diet compared with control birds.

As shown in Table 3, DEX injection severely reduced the weight of spleen ($P<0.0001$) and bursa ($P<0.0001$) and enlarged the liver ($P<0.0001$) compared with rye fed or control groups of birds. Feeding rye reduced the relative weight of liver and increased bursa ($P<0.0001$) only compared with control birds.

The concentration of FITC-d in blood is demonstrated in Figure 1. While DEX increased ($P<0.001$) the passage of FITC-d from the intestine into the blood, there was no significant effect of rye inclusion or probiotic.

Excreta biomarkers

None of the biomarkers were affected by dietary supplementation of probiotic. The concentration of AAT assayed by ELISA is shown in Figure 2. With an average concentration of 55.8 µmol/ml in the excreta supernatant, the AAT was not affected by any of the experimental factors. As shown in Figure 3, DEX increased ($P<0.05$) FN concentration by 28% (15.7 vs 20.2 ng/ml) compared with control birds. Conversely, inclusion of rye in the diet reduced ($P<0.05$) FN by 25.7%. Calprotectin was also assayed for the excreta but none was detected suggesting that this protein may not be expressed in chickens.

The concentration of IFABP-2 in excreta remained similar for the experimental treatments showing an average of 44.1 pg/ml (Figure 4). As illustrated in Figure 5, LPN2 tended ($P=0.086$) to increase in excreta of birds injected with DEX compared with control and rye diet. Compared to control birds, a marked 34% elevation ($P<0.001$; 0.406 vs 0.304 µg/ml) in excreta OVT was observed in birds fed rye-based diet (Figure 6). There was no significant difference between DEX and control for OVT. Illustrated in Figure 7, injection of birds with DEX significantly decreased IAP ($P<0.05$) in excreta by compared with both control and rye-fed birds. The superoxide dismutase in excreta was not affected by the experimental factors showing a high variability (SD=0.67) with an average of 0.73 ng/ml (Figure 8).

Table 1. Ingredient and nutrient composition of normal and rye-based experimental diets

	Rye-based diet	Normal
Rye	52.547	0.000
Wheat	0.000	65.215
Soybean meal	34.638	26.284
Canola oil	8.681	4.260
Limestone	1.221	1.150
Di-calcium phosphate	1.424	1.558
Sodium chloride	0.101	0.128
Sodium bicarbonate	0.498	0.404
Vitamin and mineral premix[1]	0.160	0.160
Choline Cl 70%	0.053	0.054
L-lysine HCl 78.4	0.169	0.342
DL-methionine	0.352	0.270
L-threonine	0.158	0.177
Dry Matter	90.42	89.94
ME Poultry (kcal/kg)	3000	3000
Crude Protein	21.5	21.621
Crude fat	10.098	5.818
Crude Fiber	3.809	2.422
Dig Arg	1.37	1.23
Dig Lys	1.15	1.15
Dig Met	0.61	0.55
Dig M+C	0.87	0.87
Dig Trp	0.26	0.27
Dig Leu	1.24	1.28
Dig Ile	0.82	0.82
Dig Thr	0.77	0.77
Dig Val	0.90	0.90
Calcium	0.87	0.87
Available Phosphorus	0.43	0.43
Sodium	0.20	0.20
Chloride	0.20	0.20

[1]Vitamin and mineral concentrate supplied per kilogram of diet: retinol, 12,000 IU; cholecalciferol, 5,000 IU; tocopheryl acetate, 75 mg, menadione, 3 mg; thiamine, 3 mg; riboflavin, 8 mg; niacin, 55 mg; pantothenate, 13 mg; pyridoxine, 5 mg; folate, 2 mg; cyanocobalamin, 16 µg; biotin, 200 µg; cereal-based carrier, 149 mg; mineral oil, 2.5 mg; Cu (sulfate), 16 mg; Fe (sulfate), 40 mg; I (iodide), 1.25 mg; Se (selenate), 0.3 mg; Mn (sulfate and oxide), 120 mg; Zn (sulfate and oxide), 100 mg; cereal-based carrier, 128 mg; mineral oil, 3.75 mg.

Table 2. Growth performance broilers subjected to two leaky gut models with and without probiotic [1]

	Feed intake (g/bird)	Body weight gain (g/bird)	FCR (g feed per g gain)
Main effects			
Leaky gut model			
Control (n=24)	599[a]	452[a]	1.33[c]
Rye-based diet (n=24)	526[b]	356[b]	1.48[b]
DEX (n=24)	510b	156[c]	3.34[a]
Probiotic			
No (n=36)	543	322	2.042
Yes (n=36)	547	321	2.056
SEM	5.59	4.63	0.030
Source of variation			
Model	<.0001	<.0001	<.0001
Probiotic	0.77	0.84	0.81
Model × Probiotic	0.08	0.41	0.99

[1] Means within a column not sharing a superscript differ significantly at the P level shown for the main effects.
[2] Values in parenthesis represent the number of replicates/birds
[3] Pooled standard error of mean (n=72)

Table 3. Relative wright (g/100g BW) of spleen, bursa and liver of broilers subjected to two leaky gut models with and without probiotic [1]

	Spleen	Bursa	Liver
Main effects			
Leaky gut model			
Control (n=24)	0.071[a]	0.24[b]	2.68[b]
Rye-based diet (n=24)	0.072[a]	0.27[a]	2.37[c]
DEX (n=24)	0.034[b]	0.06[c]	4.17[a]
Probiotic			
No (n=36)	0.059	0.19	3.14
Yes (n=36)	0.059	0.19	3.01
SEM	0.0014	0.005	0.036
Source of variation			
Model	<0.0001	<0.0001	<0.0001
Probiotic	0.976	0.083	0.723
Model × Probiotic	0.693	0.222	0.068

[1] Means within a column not sharing a superscript differ significantly at the P level shown for the main effects.
[2] Values in parenthesis represent the number of replicates/birds
[3] Pooled standard error of mean (n=72)

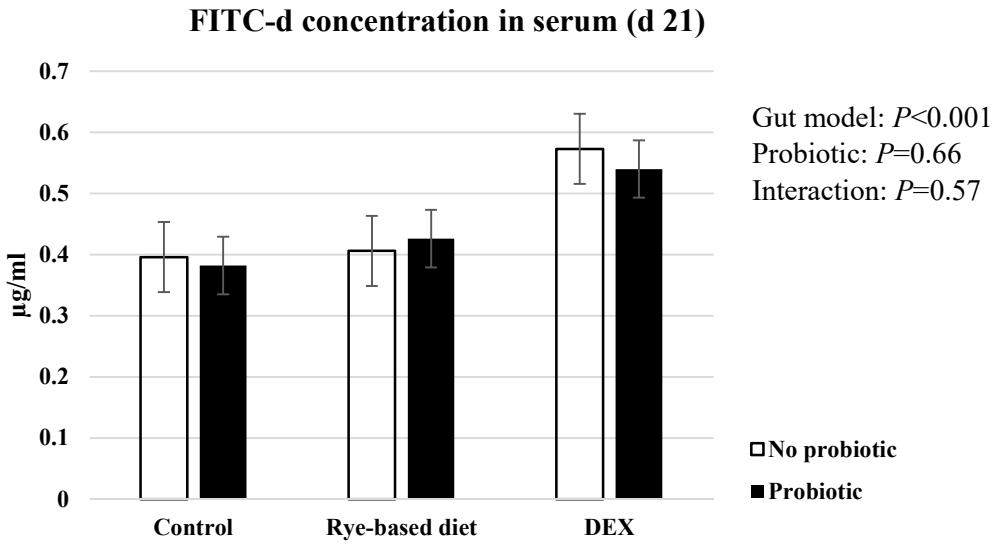

Figure 1. Serum FITC-d concentration of broilers subjected to two leaky gut models with and without probiotic. The error bars represent standard error of the mean.

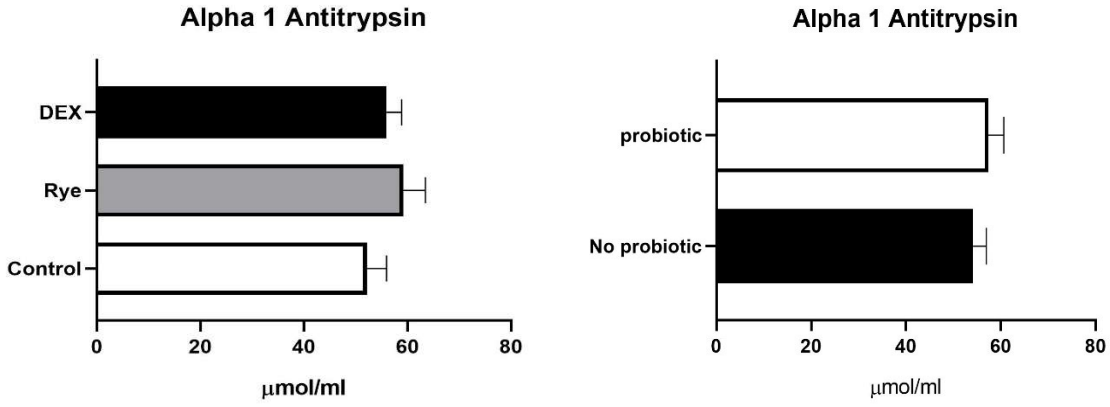

Figure 2. The main effects of gut barrier dysfunction models ($P>0.05$) and probiotic supplementation ($P>0.05$) on concentration of Alpha 1 antitrypsin in excreta samples (n=72). The error bars represent standard error of the mean (SEM).

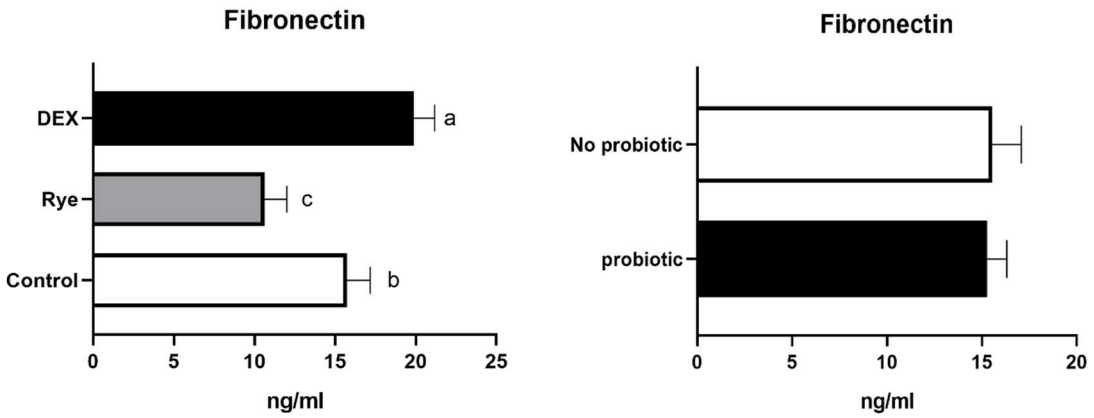

Figure 3. The main effects of gut barrier dysfunction models (P<0.05) and probiotic supplementation (P>0.05) on concentration of Fibronectin in excreta samples (n=72). The error bars represent standard error of the mean (SEM). Bars marked with a different letter are statistically different (P<0.05).

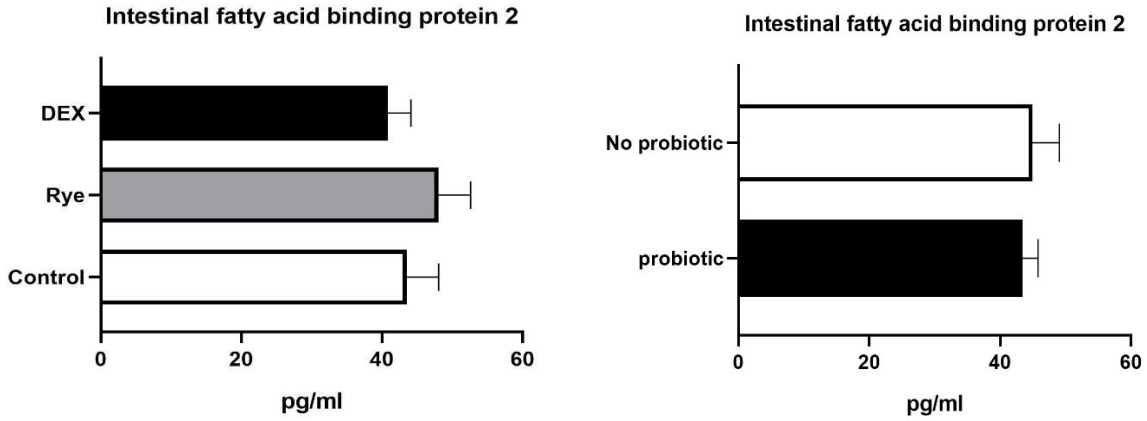

Figure 4. The main effects of gut barrier dysfunction models (P>0.05) and probiotic supplementation (P>0.05) on concentration of intestinal fatty acid binding protein 2 in excreta samples (n=72). The error bars represent standard error of the mean (SEM).

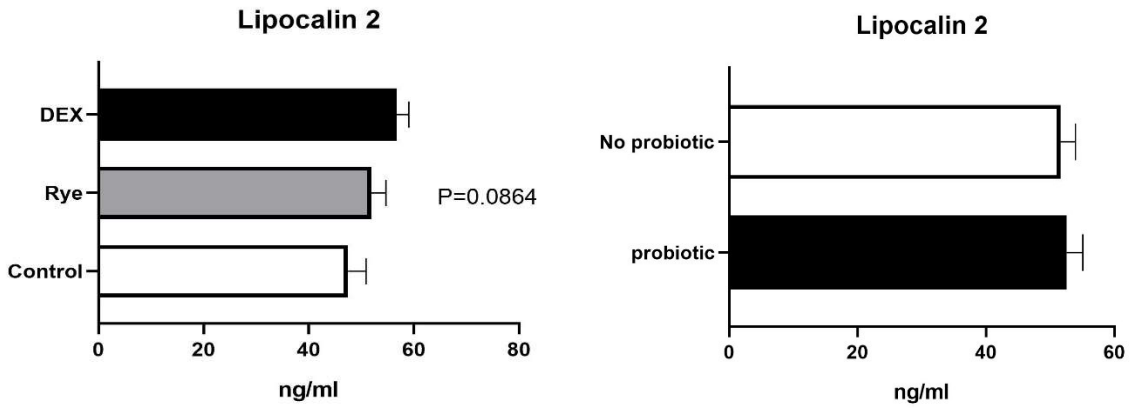

Figure 5. The main effects of gut barrier dysfunction models and probiotic supplementation on concentration of Lipocalin 2 in excreta samples (n=72). The error bars represent standard error of the mean (SEM).

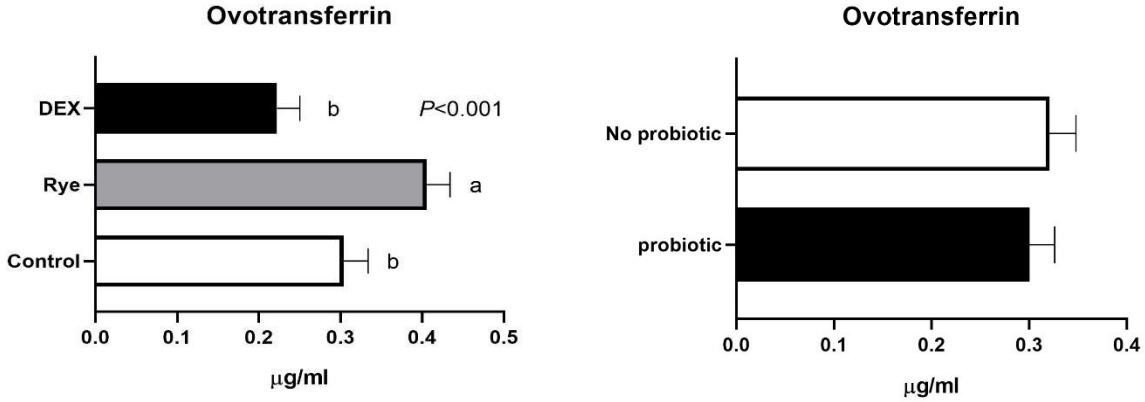

Figure 6. The main effects of gut barrier dysfunction models and probiotic supplementation on concentration of ovotransferrin in excreta samples (n=72). The error bars represent standard error of the mean (SEM). Bars marked with a different letter are statistically different (P<0.05).

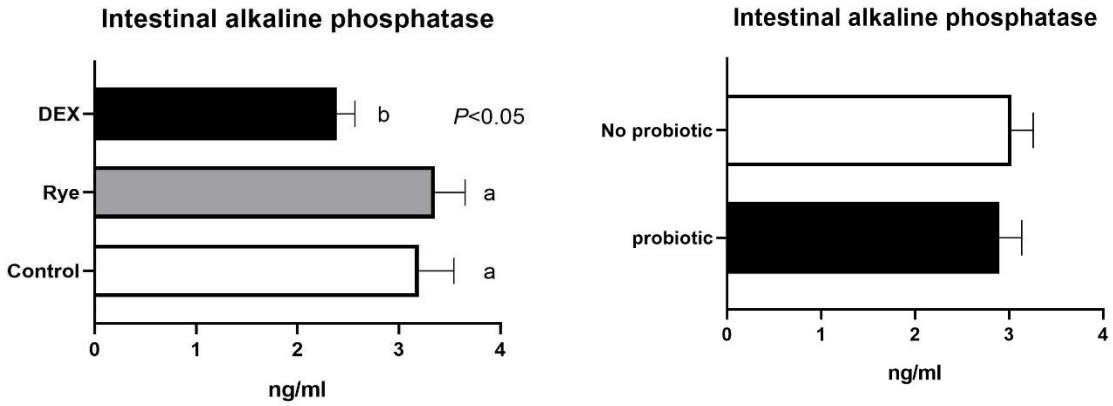

Figure 7. The main effects of gut barrier dysfunction models and probiotic supplementation on concentration of intestinal alkaline phosphatase in excreta samples (n=72). The error bars represent standard error of the mean (SEM). Bars marked with a different letter are statistically different (P<0.05)..

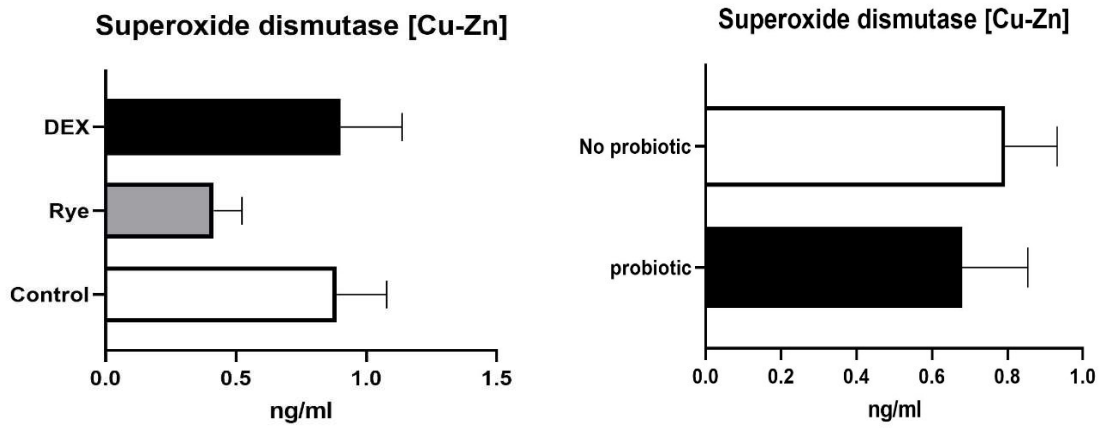

Figure 8. The main effects of gut barrier dysfunction models (P>0.05) and probiotic supplementation (P>0.05) on concentration of superoxide dismutase [Cu-Zn] in excreta samples (n=36). The error bars represent standard error of the mean (SEM).

Discussion of Results

Two different models were used to induce gut leakage in the birds and consequently study a suit of potential biomarkers in excreta and a possible counteracting effect of a probiotic product with anti-inflammatory properties. Dexamethasone successfully caused the gut leakage demonstrated by an increase in the passage of FITC-d into blood in agreement with recent observations (Barekatain et al., 2019; Duff et al., 2019). Such increased permeability along with distinct retardation in growth and atrophy of immune organs unequivocally supports that glucocorticoids have profound effects on gut barrier function. The mechanisms by which DEX can stimulate stress and impact gut integrity are mainly through GC type 1 receptors, mobilising glucose, and immunosuppression (Ünsal and Balkaya, 2012). The rye-based diet however failed to increase FITC-d concentration in serum contrary to other studies (Kuttappan et al., 2015; Tellez et al., 2014). Notably, in the current study a wheat-based control diet was used which may have diminished the effect of rye as a less digestible and rich source of non-starch polysaccharide. In hindsight a maize-based control diet known to have less soluble NSP content would have possibly provided a better opportunity to detect the effects for this particular model.

In the literature, both positive (Hernandez-Patlan et al., 2019) and lack of responses (Song et al., 2014) to probiotics for intestinal barrier function have been documented. The positive effect of the probiotic is mainly associated with changes in microbiota. The absence of a probiotic effect in this study may be explained by the relatively short period of study, housing conditions (i.e cage vs floor) and the basal diet composition and possibly viable organisms of the tested probiotic. Besides, the effects of probiotics can be strain dependent and non-ubiquitous nature for intestinal barrier function (Wang et al., 2018) that may be another factor to explain the lack of probiotic effect in the present study. Given the absence of response to probiotics for all the parameters tested in this study, the discussion is mainly focused on the tested biomarkers in response to the gut barrier dysfunction models.

Fatty acid binding proteins (FABP) are molecules that coordinate lipid responses in cells and are known to be involved in various metabolic and inflammatory pathways (Furuhashi and Hotamisligil, 2008). There are several FABP in different tissues including liver, intestine, heart, adipocyte and brain. However, intestinal FABP (IFABP) is expressed in the intestine and is also known as FABP-2 making it a potential candidate for intestinal barrier function. As for the expression of IFABP, it is expressed in every section of the intestine, but most abundantly in the distal region of the small intestine (Furuhashi and Hotamisligil, 2008). With mucosal damage and subsequent "leaky gut", IFABP can leak into the circulation from the epithelium leading to an increased concentration in plasma and subsequently voided in urine (Adriaanse et al., 2013). Very little research on the leaky gut and IFABP has been conducted in poultry limiting it to gene expression in the intestine or plasma concentration (Chen et al., 2015). In the current study, it was hypothesised that IFABP-2 could be detected in the excreta of poultry and could be used as a non-invasive tool to detect intestinal inflammation and permeability. Despite successful quantification of IFABP-2 in excreta samples, there was no significant differences between any of the experimental treatments for IFABP. It may be possible that IFABP-2 can be a faecal biomarker under more severe intestinal damage conditions (Reisinger et al., 2012) compared to the models tested in the current study. The relatively large molecular size of IFABP-2 being around 15000 Da (Ni et al., 2015) may have prevented the passage through tight junction proteins or transcellular pathways at a high rate, as opposed to FITC-d with a much smaller molecular size (4000 Da). This may be a possible explanation as to why no changes in FABP-2 was observed even in birds injected with DEX.

AAT is produced by the liver and is present in serum. This protein is resistant to proteolysis in the intestine and reflects the loss of proteins to the intestinal lumen (Schwiertz et al., 2018). AAT concentration increases in the gut in cases of higher permeability or when the mucosal barrier is disrupted. This is through extravasation from serum into the gut and ultimately in faecal material, making it viable as a candidate for intestinal permeability (Wang et al., 2015). We could not substantiate any differences between the treatments in relation to the concentration of AAT in excreta of chickens. In accordance with this result, Gilani et al. (2017) failed to find an association between concentration of AAT with two gut leakage models caused by fasting and dextran sodium sulfate in chickens. It therefore appears that AAT is not responsive to different models available in poultry and that the suitability of this protein for a non-invasive assessment of gut barrier dysfunction can be questioned.

Lipocalin 2 (LCN2) is a glycoprotein proven to be a sensitive biomarker of various metabolic and inflammatory diseases as well as intestinal inflammation in rats and humans. LCN2 is a protein that limits bacterial growth by sequestering iron in the gut environment (Flo et al., 2004). The concentration of LCN2 is typically low in biological fluids but elevated under inflammatory conditions (Abella et al., 2015). The concentration of LCN2 is elevated in faecal material of mice subjected to dextran sulfate sodium induced colitis (Chassaing et al., 2012) or when fed high-fat and salt diets (Agus et al., 2016). LCN2 is expressed in neutrophils and in high permeability situations, it can leak into the intestinal lumen from activated neutrophils making it a suitable faecal biomarker for non-invasively assessing inflammation and permeability (Wells et al., 2016). LCN2 has not been studied in poultry as an excreta biomarker of barrier dysfunction. The observed tendency for elevated LCN2 in excreta of birds under DEX injections can simply indicate the extensive effect of GC on intestinal barrier function as well as potential for this glycoprotein to be used as a biomarker of intestinal inflammation in poultry. DEX is shown to upregulate the expression of LCN2 in murine chondrocytes (Owen et al., 2008). Nevertheless, further verifications are warranted in future experiments under different models.

Ovotransferrin (OVT) is an acute phase protein and its elevated levels can be used as a biomarker of inflammation in poultry in response to various inflammation induced by chemical, bacterial or viral factors (Rath et al., 2009). It is believed that the loss of plasma proteins into the gastro intestinal tract is linked with disturbance of the intestinal barrier. In the current study the excreta concentration of OVT was elevated in birds fed rye-based diet compared with other treatments. This result suggests a possible loss of intestinal integrity as a result of feeding high levels of rye and consequences on systemic inflammation. Elevated OVT in faecal material has recently been shown in response to necrotic enteritis (Goossens et al., 2018) making it a worthwhile candidate for future validation studies.

Superoxide dismutase (SOD), an antioxidant enzyme, was assessed as a potential biomarker in excreta as this enzyme is responsive to oxidative stress and subsequent damage to intestinal barrier integrity. DEX is known to induce oxidative stress (Feng and Tang, 2014), and therefore it would be prudent to expect a change in SOD level. However, the lack of differences in SOD in the present study may be explained by considerably high variation in data obtained from individual birds for this particular assay. Assessed in serum samples, Baxter et al. (2019) also found no change in SOD in response to a rye-based diet using a leaky gut model.

Fibronectin (FN) is a ubiquitous extracellular matrix (ECM) glycoprotein involved in tissue integrity through cell adhesion, proliferation and migration and is produced by multiple cell types (Dhanani et al., 2017) including intestinal epithelial cells (Kolachala et al., 2007). It has been documented that the FN levels are altered in patients suffering from ulcerative colitis or Crohn's disease with major involvement in wound healing processes (Kolachala et al., 2007). FN in a soluble form can be found in body fluids and in its insoluble form in the basement membrane and ECM of intestinal wall (De Meyer et al., 2019). Host mucosal damage can expose ECM and protein such as FN can be released into the intestinal content and eventually excreta. In case of inflammation or chronic injury FN is expected to increase and therefore can be a potential biomarker for intestinal inflammation (Kolachala et al., 2007). In the present study, the elevated concentration of FN in excreta of birds received repeated DEX injections is in agreement with increased permeability and intestinal barrier failure of these birds likely resulting from intestinal damage. Indeed, it has been shown that DEX can stimulate expression of FN (McKeown-Longo and Etzler, 1987). Consistent with our results, recently De Meyer et al. (2019) found a higher level of FN in colonic contents, as a proxy to excreta content, of birds challenged with a gut leakage model caused by necrotic enteritis. The present study is the first report of the elevated FN on actual excreta samples of broiler chickens in response to both nutritional and physiologically induced gut leakage models. Indeed FN is shown to be a stress responsive protein (Dhanani et al., 2017) and the stress stimulated by DEX in the present study further supports the idea that this protein may be a suitable biomarker of intestinal barrier failure and inflammation under stress conditions. The lower FN content of excreta in birds ingesting a rye-based diet compared with control birds cannot simply be explained by the data of the current study. However, it is probable that the nutrient composition in particular carbohydrates of wheat vs rye, or a possible negative effect on energy utilisation (Kono et al., 1988) may have contributed to the observed differences in FN. Despite the lack of any demonstrable effect of probiotic in the present study, ECM binding ability through its proteins, particularly FN, is worthy of future consideration for efficacy of selected probiotic strains, in particular, *Lactobacillus* sp. (Sánchez et al., 2009). Probiotic bacterial strains can compete with pathogenic bacteria for binding receptors such as FN (Štyriak et al., 2003) and therefore under unfavourable increase of FN presence of probiotic may be beneficial. Nevertheless, further studies are required to establish a range of FN concentrations in poultry; with any abnormal

concentration presenting an opportunity for diagnosis and progression of particular intestinal disorders.

Enterocytes secrete intestinal alkaline phosphatase (IAP) both apically and basolaterally. IAP plays pivotal roles in regulation of bicarbonate secretion, absorption of long chain fatty acids and mitigation of intestinal inflammation as well as influencing both composition and translocation of gut microbiota (Lallès, 2014). Thomas and Henton (1985) were the first to propose IAP as a faecal biomarker of intestinal damage in rats. In poultry, data for IAP as a faecal biomarker are indeed scarce. Nevertheless, when intestinal damage occurs digestive enzyme secretion may reduce therefore making them a viable biomarker (Ducatelle et al., 2018). In the present study, DEX significantly reduced IAP in the excreta compared to control and rye fed birds. There may be a few possible explanations for alteration in IAP. Firstly, possible damage to enterocyte caused by DEX-stress stimulated may have negatively affected secretion of IAP. Secondly, repeated GC exposure may increase intestinal permeability which eventually can make the birds susceptible to mucosal inflammation (Duff et al., 2019). Thirdly, the lower IAP may also at least in part explain the impaired intestinal barrier function in DEX injected birds with recent evidence of IAP impacting key tight junction proteins (Lallès, 2019). However it should be noted that depending on the type of AP isoforms, both upregulation or down regulation of IAP is possible in response to inflammation or a metabolic disorder. The inflammation may lower the IAP but not tissue non-specific AP isoforms (Lallès, 2014). Therefore, it is prudent to assume that IAP measured in this study has originated from the small intestine. A caveat in using IAP as a biomarker may be the confounding factors such as differences in feed intake or dietary composition that can change intestinal production and release of IAP (Lallès, 2015). The potential of IAP as a biomarker and a potent controlling agent for intestinal inflammation and barrier function warrants further research in poultry.

Implications

Detection of a gut health problem at an early stage benefits the industry through reducing cost of poor enteric health and associated compromised performance. This will allow rapid intervention to address the issue through management strategies, feed additives or seeking veterinary advice. Subject to further validation studies, the Australian poultry industry can adapt the results of this study by adapting a rapid test that allows sample screening at the farm level or through developing devices that can detect the intensity of a particular biomarker on a real-time basis. It appears that ELISA with careful set up and modification can be a useful test particularly when reliable commercial kits are made available. An ELISA assay, in most cases, can be completed in less than one day if that assay is fully validated.

In quest of suitable biomarkers of intestinal barrier function across species, there is a consensus that a suite of multiple biomarkers is superior to any single one to represent the status of a gut integrity related issue (Ducatelle et al., 2018; McCormick et al., 2017). Similarly in this study, it appeared that the response of biomarkers to different gut leakage models may differ which further emphases the use of multiple biomarkers. The present study identified candidate biomarkers that could be detected in the excreta of broiler chickens as a non-invasive method to assess gut barrier function and inflammation. Among tested biomarkers, FN and IAP were found to be responsive to stress induced by DEX and were consistent with results obtained for permeability as assessed by FITC-d concentration in blood samples. A promising similar trend

was also identified for LCN2 for the first time in this study. Future validation studies at flock level are required in order to facilitate the use of biomarkers in the poultry industry.

Recommendations

Given the novelty of the current research and significance of potential benefit to the industry the following recommendations can be made:

- Validation studies need to be conducted for the tested biomarkers as well as more potential biomarkers specifically in the excreta of chickens.
- In addition to ELISA assays, a proteomics analysis of excreta samples need to be performed to capture all possible proteins that may alter in response to various gut barrier dysfunction models.
- Application of feed additives requires a separate project testing different strains of probiotics with anti-inflammatory effects.

Acknowledgments

The financial support from Poultry Hub is gratefully acknowledged. Technical help and assistance from SARDI staff Mr Derek Schultz and Kylee Swanson was integral in successful completion of this project. Dr Nicky-Lee Willson from the University of Adelaide generously developed her time to assist with setting up and running ELISA assays for which the principal investigator is grateful. Thank you also to Dr Kapil Chousalkar for bleeding birds.

Media and Publications

The results of this project will be presented in form of at least two conference abstract/papers and a full peer-reviewed manuscript planned for Journal of Animal Science and Biotechnology. The first abstract has been prepared and submitted to the Poultry for approval which will be submitted to the World's Poultry Congress 2020 in Paris, France.

Barekatain R., Howarth G.S., Willson N.L., Cadogan D. and Wilkinson S. 2020. Selected excreta biomarkers of intestinal barrier function in broiler chickens subjected to two gut leakage models with or without probiotic supplementation. World's Poultry Congress 2020, Paris, France. (Submitted – a copy in Appendix)

Barekatain R., Howarth G.S., Willson N.L., Cadogan D. and Wilkinson S. 2020. Quantification of selected excreta biomarkers in broiler chickens in response to two different gut barrier dysfunction models with or without in feed probiotic supplementation. Animal Science and Biotechnology. (To be submitted after approval sought)

Intellectual Property Arising

While the information obtained in this study are not patentable, most of the biomarkers tested in this project have been quantified in excreta of broiler chickens for the first time. Therefore, the project presents novel and innovative data that have a high scientific and industry value. We plan to publish the results in a scientific journal as soon as practical. We

therefore request that, where possible, this report is not made available online until the full publication of the results are made in 2020.

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Appendix

Poultry Hub Project: 018-422

Abstract for World's Poultry Congress 2020 – Paris, France

Selected excreta biomarkers of intestinal barrier function in broiler chickens subjected to two gut leakage models with or without probiotic supplementation

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Perturbation of the intestinal barrier can lead to increased intestinal permeability (IP), elevated inflammation, high risk of enteric diseases and compromised performance in poultry. Common assessments of intestinal barrier function have been mainly through invasive methods involving differentially sized sugar tests, tissue sampling, or blood collection. Such methods are often complex, time-consuming and less field relevant. The present study aimed to identify selected biomarkers in excreta of broilers to facilitate the non-invasive assessment of gut barrier function. A 3 × 2 factorial arrangement of treatments was used with the main factors being gut barrier dysfunction models (control, rye-based diet, and dexamethasone – DEX) with and without probiotic supplementation (a three-strain *Bacillus*). Seventy-two male Ross 308 day-old chickens were kept in two groups given the same diets with or without probiotic supplementation. From days 13 to 21 of age, birds were individually housed and subjected to experimental treatments. Each of the 6 experimental treatments was replicated 12 times. On d 14, 16, 18 and 20, birds in the DEX group (n=24) were injected with DEX (0.5 mg/kg BW). Fluorescein isothiocyanate dextran (FITC-d) uptake into serum was used to test IP on d 21. Fresh excreta samples were collected on d 20. The excreta concentrations of Alpha 1 Antitrypsin (A1AT), Intestinal Fatty acid Binding Protein (IFABP-2), Fibronectin (FN) and Intestinal Alkaline Phosphatase (IAP) were measured using chicken specific ELISA assays. Data were subjected to two-way ANOVA to assess main effects and interaction. Treatment means were separated by Fisher's LSD test (P<0.05). DEX and rye-based diet depressed feed intake, weight gain and increased feed conversion ratio compared with control birds. Only DEX increased FITC-d passage to the blood, indicating a greater IP. The excreta concentration of A1AT and IFABP-2 were unaltered by the experimental treatments. DEX increased (P<0.05) FN concentration in excreta compared with control birds. Conversely, inclusion of rye in the diet reduced (P<0.05) FN. Independently, DEX decreased IAP (P<0.05) in excreta compared with control and rye-fed birds. There was no demonstrable effect of probiotic addition on any of the studied parameters. Subject to further validation studies the results reveal that FN and IAP, determined by ELISA, show promise as excreta biomarkers for rapid assessment of gut barrier function in poultry.

Key words: Intestinal permeability, ELISA, dexamethasone, rye, inflammation