# THE ROLE OF DIETARY INTERVENTIONS IN BROILER INTESTINAL HEALTH AND IMMUNITY

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**By**

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# DECLARATION

This thesis has not been previously submitted to this or any other university for examination for a higher degree. The work presented here is entirely my own except where otherwise acknowledged. This thesis may be made available for consultation within the university library. It may be copied or lent to other libraries for purposes of consultation.

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Conor McCaffrey

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# Acknowledgements

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# Abstract

The innate immune system represents the host's initial defence mechanism which responds to a diverse range of antigenic stimuli. This response is characterised by the activation of pathogen recognition receptors upon recognition of microbial patterns. This action results in the activation of the transcription factor NF-κB, MAPK signalling pathways and the induction of pro-inflammatory cytokines. The gastrointestinal mucus layer serves as an essential innate barrier to pathogenic adhesion and subsequent infection. Dysregulation in the integrity or function of the mucosal layer has been implicated in malabsorption, leading to reduced animal productivity. The objective of the work described in this thesis was to evaluate the effect of dietary supplementation of mannan-rich natural formulations on broiler tissue morphology, mucin barrier integrity, digestive enzyme capacity, apoptotic and necroptotic cell death levels and the expression of inflammatory cytokines. Supplementation of diet with each mannan-rich fraction (MRF) increased villus height and villus surface area. In addition, crypt depth was increased upon dietary inclusion of naturally-derived additives. Dietary inclusion of mannan-rich fraction (MRF) supplements (supplements 1 to 3) enhanced goblet cell density and mucus layer thickness. Incorporation of mannan-rich fraction 1 (MRF-1) and mannan-rich fraction 2 (MRF-2) enhanced the activities of digestive enzymes maltase, sucrase and alkaline phosphatase. The expression of apoptotic cell death markers (TUNEL staining and caspase-3 expression) was not altered statistically upon incorporation of dietary additives. Receptor-interacting serine/threonine kinase 3 (RIPK3) expression was significantly decreased upon MRF-1 supplementation in the duodenum, jejunum and caecum. Supplementation of mannan-rich fraction 3 (MRF-3) significantly enhanced protein expression levels of p-Akt. MRF-2 and MRF-3 dietary incorporation led to a significant decrease in RIPK3 protein expression levels. Dietary additive inclusion had little effect on serum and tissue lysate expression of

inflammatory cytokines. The work in this thesis demonstrates a role for mannan-rich fractions in improving broiler tissue morphology, barrier integrity and digestive enzyme capacity, thus potentially enhancing overall broiler performance.

# Abbreviations

| | |
|---|---|
| AGP | Antimicrobial growth promoters |
| AI | Apoptotic index |
| AIM | A*bsent in melanoma* |
| *Akt* | *Protein kinase B* |
| ALP | Alkaline phosphatase |
| APC | Antigen presenting cell |
| APS | Ammonium persulfate |
| BAD | Bcl-2-associated death promoter |
| BAK | Bcl-2 homologous antagonist/killer |
| BAX | Bcl-2-associated X protein |
| BSA | Bovine serum albumin |
| C | Carboxy; cysteine |
| CadF | Campylobacter adhesion to Fibronectin |
| CARD | Caspase activation and recruitment *domains* |
| CD | Cluster of differentiation |
| CD | Crypt depth |
| *C. difficile* | *Clostridium difficile* |
| *C. jejuni* | *Campylobacter jejuni* |
| Cia | Campylobacter invasion antigens |
| CpG | 2′-deoxyribo cytidine-phosphate-guanosine |
| CWP | Cell wall proteins |
| CXCL | Chemokine (CXC motif) ligand |
| DAB | 3, 3 -diaminobenzidine |
| DAMP | Danger-associated molecular patterns |

| | |
|---|---|
| DAPI | 4`, 6-Diamidino-2-Phenylindole |
| DC | Dendritic cell |
| DFM | Direct fed-microbials |
| $dH_2O$ | Distilled water |
| DNA | Deoxyribonucleic acid |
| DPX | DePeX |
| *E. coli* | *Escherichia coli* |
| ELISA | Enzyme-linked immunosorbent assay |
| ERK | Extracellular signal–regulated kinases |
| EtOH | Ethanol |
| FCR | Feed conversion ratio |
| FOS | Fructooligosaccharides |
| g | Gravity |
| GC | Goblet cell |
| GlcN | Glucosamine |
| GI | Gastrointestinal |
| GPI | Glycosylphosphatidylinositol |
| GBS | Guillain-Barré Syndrome |
| $H_2O_2$ | Hydrogen peroxide |
| HCl | Hydrochloric acid |
| HRP | Horseradish peroxidase |
| IAP | Inhibitor of apoptosis protein |
| IB | Immunoblot |
| IFN | Interferon |
| IL | Interleukin |
| iNOS | Inducible nitric oxide synthase |
| IRAK | Interleukin-1 receptor-associated kinase |

| | |
|---|---|
| IRF | Interferon regulatory factor |
| IκB | Inhibitor of κB |
| JNK | c-Jun n-terminal kinase |
| L | Litre |
| LPS | Lipopolysaccharide |
| LRR | Leucine-rich repeat |
| MAPK | Mitogen-activated protein kinase |
| MASP | MBL-associated serine proteases |
| MBL | Mannose-binding lectin |
| mg | Milligram |
| MHCII | Major histocompatibility complex II |
| mL | Millilitre |
| MLT | Mucus layer thickness |
| MOS | Mannanoligosaccharides |
| MRF | Mannan-rich fraction |
| MyD88 | Myeloid differentiation protein 88 |
| NaOH | Sodium hydroxide |
| NBF | Neutral buffered formalin |
| NF-κB | Nuclear factor kappa B |
| OCT | Optimum cutting temperature |
| OD | Optical density |
| NLR | Nod-like receptor |
| PAGE | Polyacrylamide gel electrophoresis |
| PAMP | Pathogen associated molecular patterns |
| PAS | Periodic-acid Schiff |
| PBS | Phosphate buffered saline |
| PBST | PBS-TWEEN |

| | |
|---|---|
| Pir | Protein with internal repeats |
| pNP | p-Nitrophenol |
| pNPP | p-Nitrophenol phosphate |
| PRR | Pathogen recognition receptor |
| RIPK3 | Receptor-interacting serine/threonine-protein kinase |
| RNA | Ribonucleic acid |
| RT | Room temperature |
| *S. cerevisiae* | *Saccharomyces cerevisiae* |
| SMAC | Second mitochondria-derived activator of caspase |
| SOD1 | Superoxide dismutase |
| T | Tonne |
| T3SS | Type III Secretion System |
| TBS | Tris-buffered saline |
| TBST | Tris-buffered saline containing Tween-20 |
| TEMED | N N N' N' – Tetramethylethylene-diamine |
| TGO | Tris-glucose oxidase |
| TLR | Toll-like receptor |
| TMB | 3 3' 5' 5'-Tetramethylbenzidine |
| TNF | Tumour necrosis factor |
| TRIF | TIR-domain-containing adapter-inducing interferon-β |
| TUNEL | Terminal deoxynucleotidyl transferase dUTP nick end labelling |
| TXN | Thioredoxin |
| V | Volts |
| v | Volume |
| VH | Villus height |
| VSA | Villus surface area |
| w | Weight |

| | |
|---|---|
| YCW | Yeast cell wall |
| µg | Microgram |
| µL | Microlitre |
| µM | Micromolar |

# Chapter 1: Introduction

## 1.1 Role of Dietary Formulation additives in the poultry industry

### 1.1.1 Overview of poultry industry

Poultry production has increased exponentially within the past half-century, to meet the demands of an ever-growing population. In 2014, poultry meat production totalled over 13 million tonnes in the European Union, representing almost a 10% increase from 2007 (Eurostat). Within the past 50 years, significant improvements have been made in poultry production technologies. This has resulted in drastically reduced mortality rates, enhanced weight gain - with birds almost five times larger compared to broilers bred in 1957- and improvements in feed conversion ratios (the ratio at which feed input results in overall weight gain) from 3.26 in 1957, to 2.68 in 2001 (Havenstein *et al.*, 2003). A 'broiler' is the term attributed to any chicken that has been bred specifically for the purpose of poultry meat production (Le Bihan-Duval *et al*., 1999) Progressions in broiler chicken diet strategies have also led to improvements in body weight and growth performance. Antibiotic use in broiler production commenced in the mid-20$^{th}$ century (Castanon, 2007). The inclusion of antimicrobial growth promoters (AGP) in broiler feed had led to significant improvements in animal growth and performance (Castanon, 2007). The supplementation of animal feed with antibiotics at sub-therapeutic levels greatly alleviated pathogen invasion and broiler mortality rates (Miles *et al*., 2006). Several mechanisms have been postulated for how antibiotic supplementation could improve broiler intestinal health and growth including: reducing the colonisation levels of pathogenic bacteria such as *E. coli* and *S. aureus* and the enhancement of animal production through increased nutrient absorption, alterations of the composition of intestinal microflora and intestinal physiology (Niewold, 2007; Schlundt and Aarestrup, 2017).

### 1.1.2 Decline of antibiotics

The addition of antibiotics at a sub-therapeutic level in animal feed has resulted in the formation of a favourable environment for the onset of microbial antibiotic resistance (Roto *et al*., 2015). Resistant bacteria have the potential to be transferred from animal to human at several phases of animal processing, such as during the slaughtering or processing stages (Verraes *et al*., 2013). Mounting public concern regarding the risk posed by antimicrobial resistance led to the outright ban of the use of antibiotics in poultry production in the European Union (EPC, 2005). There has been a plethora of conflicting evidence regarding the effects of the ban on AGPs. The sharpest effect of the ban was felt in the pork industry, most notably in piglets (Casewell *et al.*, 2003; Hao *et al*., 2014; Schlundt and Aarestrup, 2017). Incidences of disease rapidly increased shortly after the ban and animal mortality increased. Animal diseases such as necrotic enteritis again gained prominence following the ban on antibiotic growth promoters (Tsiouris *et al*., 2016). Necrotic enteritis is a primary concern in the poultry industry due to the high mortality rates and decreased production associated with the disease (Timbermont *et al*., 2011). The sub-clinical form of this disease is considered to be of higher risk as symptoms are not apparent and the disease can progress undetected, resulting in greater economic losses (Dahiya *et al.*, 2006). Broilers infected with necrotic enteritis have a reduced ability to digest and absorb available nutrients, resulting in lower feed conversion ratios and reduced weight gain (Paiva and McElroy, 2014). The negative effects of the ban of AGPs have led to renewed focus on the development of naturally-derived dietary formulations for incorporation into animal feed.

### 1.1.3 Alternatives for Antibiotics

Several approaches to enhance broiler health and growth involve the use of biologics incorporated into animal feed either alone or in combination. The most well-defined alternatives include: in-feed enzymes, prebiotics, direct-fed microbials, β-glucans and mannanoligosaccharides.

#### 1.1.3.1 In-feed Enzymes

In-feed enzymes have long been used in animal nutrition. There are several different types of in-feed enzymes utilised in the animal feed industry. One such type of enzymes are microbial phytases, which break down phytate to release residual phosphorous (Ravindran, 2013). Phosphorous has been shown to be essential for broiler growth and maintenance in addition to tissue repair (Adedokun & Adeola, 2013). The majority of absorption of phosphorous occurs in the duodenum and jejunum (Liu *et al*., 2016). Another popular type of feed enzyme are glucanases. Glucanases have been demonstrated to improve boiler performance and the absorption of fat, starch and protein (Annison, 1992; Marquardt, Boros, Guenter, & Crow, 1994). The main goal for the use of in-feed enzymes is to enhance nutrient digestibility, however the role of intestinal microbiota in influencing diet is only recently coming to light. Evidence has shown that unassimilated nutrients from a high-energy can contribute to intestinal microbiota overgrowth and subsequent induction of inflammation, an energy expensive process (Pan & Yu, 2014; Wu, Stanley, Rodgers, Swick, & Moore, 2014). The investigation of the interplay between intestinal microbiota and influences on diet is an ever-expanding field.

### 1.1.3.2 Probiotics

Probiotics have been defined as the use of feed products that may be derived from live or naturally occurring microorganisms, differing from prebiotics in that prebiotics may also contain enzymes or crude extracts (Brashears *et al.*, 2003; McAllister *et al*., 2011). Probiotics have been postulated to convey beneficial effects on poultry through modulation of the immune system, the promotion of commensal microbiota growth and the inhibition of pathogenic bacteria proliferation in addition to increasing digestive enzyme activity (Dahiya *et al*., 2006; McAllister *et al*., 2011). A reported role of probiotics is to maintain a healthy balance between 'beneficial' and 'non-beneficial' microbiota (Pan & Yu, 2014). Under conditions of stress, 'non-beneficial' bacteria may exponentially proliferate and lead to reduced feed-intake and gastroenteritis (Jadhav, Sharma, Katoch, Sharma, & Mane, 2015). The incorporation of probiotics into broiler feed has led to reduced incidences of avian coccidiosis, an important finding in the poultry industry due to the effects of increased mortality and reduced feed efficiency upon infection of broilers (Brashears *et al*., 2003). The inclusion of *Lactobacillus*- and *Bacillus*- based probiotics into animal feed led to two reported benefits: the reduction of colonisation levels of enteric pathogens in the gastrointestinal layer and a reported increase in the levels of beneficial bacteria in the microbiome (Gebert *et al*., 2007). Evidence has also emerged of probiotic supplementation increasing antibody response levels during an oral pathogenic challenge (Brisbin *et al.*, 2008).

### 1.1.3.3 Prebiotics and Mannanoligosaccharides

#### 1.1.3.3.1 Prebiotics

Prebiotics have been defined as substances that are indigestible to the host but can alter the activity and composition of gut microflora to confer a beneficial effect on the host (Gaggia *et al*., 2010; Roberfroid, 2007). Recent evidence has demonstrated the possibility of yeast cell wall derivatives regulating innate signalling pathways, including the mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling cascades through interactions with essential signalling proteins (Tian *et al*., 2015). Prebiotics have been demonstrated to alter the composition of intestinal microbiota, increasing the presence of potentially beneficial microbiota such as bifidobacterial and lactobacilli (Gaggia *et al.*, 2010). These bacteria have been shown to modulate the immune response, adhere to the gastrointestinal layer and confer a role in the regulation of gut motility (Bernardeau *et al*., 2006; Hamilton-Miller, 2004; Picard *et al*., 2005). Commonly incorporated types of prebiotics in the poultry industry include inulin and fructooligosaccharides (FOS). FOS are composed of short chains of fructose while inulin can consist of between 20 and several thousand fructose molecules (Hughes *et al.*, 2017) These prebiotics have been demonstrated to select for microorganisms hypothesised to promote nutrient assimilation and also to prevent the adhesion and colonisation of potentially pathogenic bacteria (Barko, McMichael, Swanson, & Williams, 2017; Ricke, 2015; Roberfroid, 2007).

Mannan-rich fraction 1 (MRF-1), mannan-rich fraction 2 (MRF-2), and mannan-rich fraction 3 (MRF-3) (Alltech Inc., Lexington, Kentucky, USA) are naturally-derived dietary supplements designed to take account of the growing use of natural alternatives to antibiotic growth promoters. These supplements are derived from the cell wall of *Saccharomyces*

*cerevisiae* and can potentially modulate the immune response, improve intestinal capacity and function, as well as enhancing animal performance and growth.

#### 1.1.3.3.2 Mannanoligosaccharides

Mannanoligosaccharides (MOS) derived from the outer yeast cell wall of *S. cerevisiae* have been hypothesised to improve gastrointestinal health, growth, mortality rates and performance of broiler chickens (Xiao *et al*., 2012). The incorporation of MOS into feed has been reported to potentially increase villus length (therefore potentially increasing the surface area available for nutrient absorption), increase goblet cell presence (thereby enhancing mucous lining protection), alter crypt depth and also cause a decrease in the levels of *E. coli* and *Salmonella* colonisation (Baurhoo, Letellier, Zhao, & Ruiz-Feria, 2007; Griggs & Jacob, 2005; Sims, Dawson, Newman, Spring, & Hoogell, 2004). Several different mechanisms have been suggested for the action of MOS in animal health and productivity. Reduced bacterial presence due to MOS is hypothesised to be a result of the interaction of MOS with mannose-binding lectins on bacterial cell surfaces (D. Kelly, Begbie, & King, 1994). Bacteria containing type-1 fimbriae, such as *E. coli* and *Salmonella*, attach to the intestinal layer though mannose residues present in intestinal mucins (Baurhoo *et al.*, 2007). Upon incorporation of MOS into broiler diets, enteric pathogens will instead attach to mannose residues present in MOS, resulting in their excretion from the animal (Line *et al.*, 1998). This mode of pathogen exclusion greatly protects the intestinal layer from pathogenic bacteria and invading toxins while also permitting host energy to be utilised for growth, and not the repair and regeneration of the intestinal layer (Benites *et al*., 2008). Immunomodulating effects were observed in broilers fed with MOS such as the increased expression of cytokines and genes associated with antioxidant activity such as *SOD1* and *TXN* (Xiao *et al*., 2012). Additionally, it has been reported that MOS-incorporation

led to reduced coccidiosis infection in broilers due to improved gut morphology (Elmusharaf, Peek, Nollet, & Beynen, 2007).

## 1.2 Characteristics and composition of the yeast cell wall

### 1.2.1 Yeast cell wall overview

It is clear that yeast cell wall extracts can regulate intestinal health and disease. The yeast cell wall (Fig. 1.1) provides mechanical strength, structural integrity and osmotic protection for yeast cells (Levin, 2011). It can account for almost 20% of total cell mass (Aguilar-Uscanga & François, 2003). This rigid layer can vary in thickness between 100 and 200nm (Lipke & Ovalle, 1998). The yeast cell wall is typically comprised of glucans and mannans, which together form an intertwined layer, providing strength and solidity to the cell wall (Erwig and Gow, 2016; Kollar *et al*., 1997). Glucans such as (1→3)-β glucans play an essential role in the maintenance of cell wall shape whereas (1→6)-β-D-glucans function as a point of attachment for the majority of polysaccharides present on the cell wall (Kwiatkowski *et al*., 2009; Lowman *et al*., 2011). Mannans present on the outer portion of the yeast cell wall can convey a protective effect, prevent the adhesion and infiltration of foreign toxins and pathogens (Erwig and Gow, 2016). The cell wall is composed of an inner layer (consisting primarily of β1,3- glucan molecules) that serves as a scaffold for other proteins and which also protects an outer layer of glycoproteins involved in the permeability of the cell wall and cell-cell adhesion events (Levin, 2011; Luo *et al*., 2015).

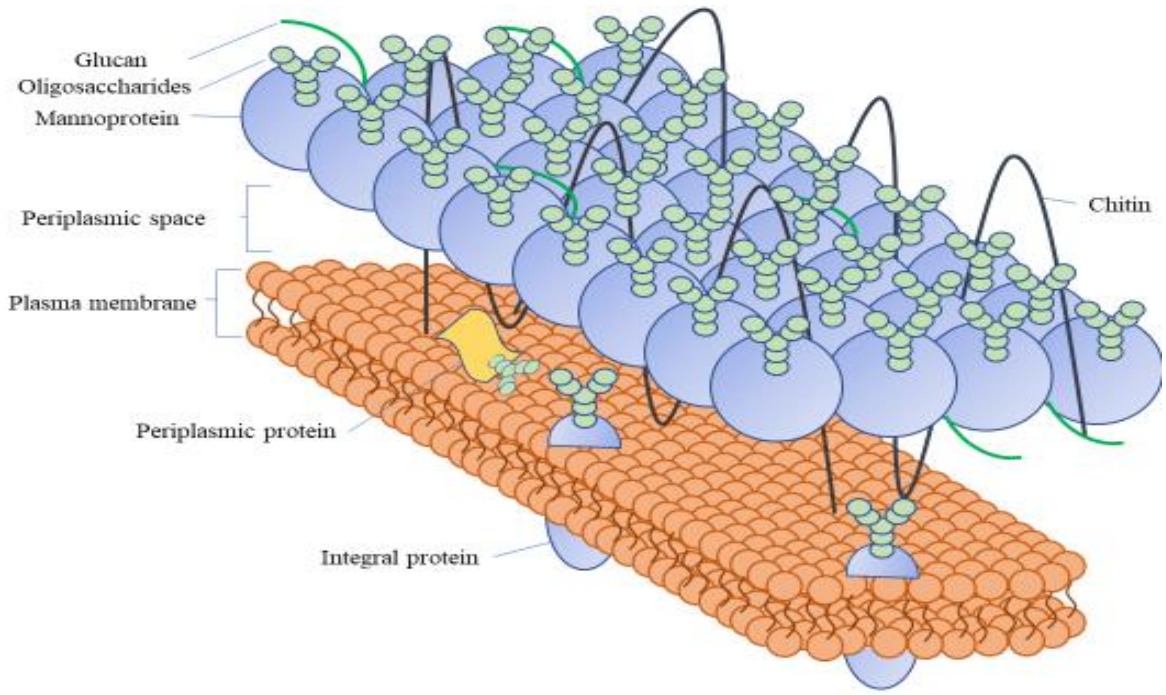



**Figure 1.1 Overview of the yeast cell wall**

## 1.2.2 Functions of the yeast cell wall

The yeast cell wall (YCW) serves several essential functions: the determination of cellular shape, the regulation of cell-cell recognition events, communication with the external environment and also the maintenance of internal osmotic homeostasis, which prevents the cell bursting in response to alterations in environmental or physiological conditions (Kogan and Kocher, 2007; Kollar *et al*., 1997). The composition of the cell wall provides protection against mechanical or physical stress, due to the high elasticity and mechanical strength of the wall (Aguilar-Uscanga & François, 2003; Martinez de Marañon, Marechal, & Gervais, 1996). The elasticity of the wall is generally attributed to the presence of 1,3-β-glucan molecules, which can adopt a helical conformation and exist in varying states of extension (Smits, C Kapteyn, van den Ende, & Mklis, 1999). The inner layer also serves as an attachment site for proteins that are structural components of the outer layer of the YCW (Klis, Mol, Hellingwerf, & Brul,

2002). The cell wall may also function as a scaffold for proteins (Levin, 2011). β1,3- glucan molecules are the major constituents of these scaffolds that serve as a site of attachment for heavily-glycosylated external proteins that have been implicated in determining the permeability of the cell wall (Klis *et al*., 2002; Levin, 2011; Lipke & Ovalle, 1998). The glycoproteins associated with the outer layer of the cell wall thereby serve to protect the cell from potentially pathogenic proteins and cell wall-degrading enzymes such as cellulose and hemicellulose enzymes (Levin, 2011; Murashima, Kosugi, & Doi, 2003).

### 1.2.3 YCW glycoproteins

Two general classes of outer layer glycoproteins exist: glycosylphosphatidylinositol (GPI)-modified cell wall proteins (CWPs), which are indirectly linked to β1,3- glucans through β1-6, glucan chains and protein with internal repeats (PIR-) CWPs that are directly linked to β1,3-glucans (Klis *et al*., 2002; Yin, de Groot, de Koster, & Klis, 2008). External glycoproteins serve numerous essential functions including the maintenance of cell wall integrity by crosslinking polysaccharides, facilitating mating and flocculation between cells, iron uptake, retention of water, association with epithelial cells and also resistance to oxidative stress (Klis, Boorsma, & De Groot, 2006; Levin, 2005, 2011; Yin et al., 2008). GPI-CWPs can be linked to the non-reducing end of a β1,6-glucan through the reducing end of a mannose residue (after cleavage of the GPI between its GlcN and Man residue) or to a glucose residue on a β1,6-glucan chain (Fujii, Shimoi, & Iimura, 1999; Kollár *et al*., 1997). PIR-CWPs are ester-linked directly to β1,3-glucan chains through amino acid side chains present in the repeat portion of the protein (Ecker, Deutzmann, Lehle, Mrsa, & Tanner, 2006; Orlean, 2012).

## 1.3 Role of the intestine in maintenance of health in broilers

### 1.3.1 Gastrointestinal tract overview

The gastrointestinal (GI) (Fig. 1.2) tract absorbs and digests available nutrients and also acts as a selective barrier that prevents the entry of potentially pathogenic bacteria with wide-ranging implications for the overall health and performance of commercial poultry (Yegani & Korver, 2008). The components of the avian GI tract include the oesophagus, crop, proventriculus, gizzard, small intestine (duodenum, jejunum and ileum) and the large intestine (caecum, colon and cloaca) (Yegani & Korver, 2008). The majority of food digestion and absorption occurs in the small intestine (Taghipoor, Barles, Georgelin, Licois, & Lescoat, 2014). The GI tract is composed of four layers: the mucosa, the submucosa, the muscular layer and the adventitial layer (Cheng *et al*., 2010). The mucosal layer of the small intestine can be broadly divided into two fundamental structures: the villi and the crypts (Clevers, 2013) (see Figure 1.3). Villi extend from the epithelial layer of the mucosa to allow a greater surface area available for nutrient absorption. Each villus contains microvilli that together form a brush-border to further increase the surface area present for digestive enzyme activity (Crawley, Mooseker, & Tyska, 2014). Digestive enzymes (e.g. maltase, sucrase) play an important role in nutrient digestibility, intestinal homeostasis and overall bird performance through the availability of a higher volume of nutrients (Pinheiro *et al*., 2004). The crypts of Lieberkühn are essential for the maintenance of immune homeostasis and the self-renewal of cells involved in host defence (Guezguez, Paré, Benoit, Basora, & Beaulieu, 2014; Scoville, Sato, He, & Li, 2008). The epithelial sublayer of the mucosa has been the subject of extensive research regarding bacterial-host interactions with implications in immune function (Abreu, 2010; Ashida, Ogawa, Kim, Mimuro, & Sasakawa, 2011; Canny, Swidsinski, & McCormick, 2006). The GI tract serves as a hospitable environment for many bacterial species but is also a viable

target for many enteric pathogenic species, including *C. jejuni*, *E. coli* and *Clostridium difficile* (Guerrant, Steiner, Lima, & Bobak, 1999).

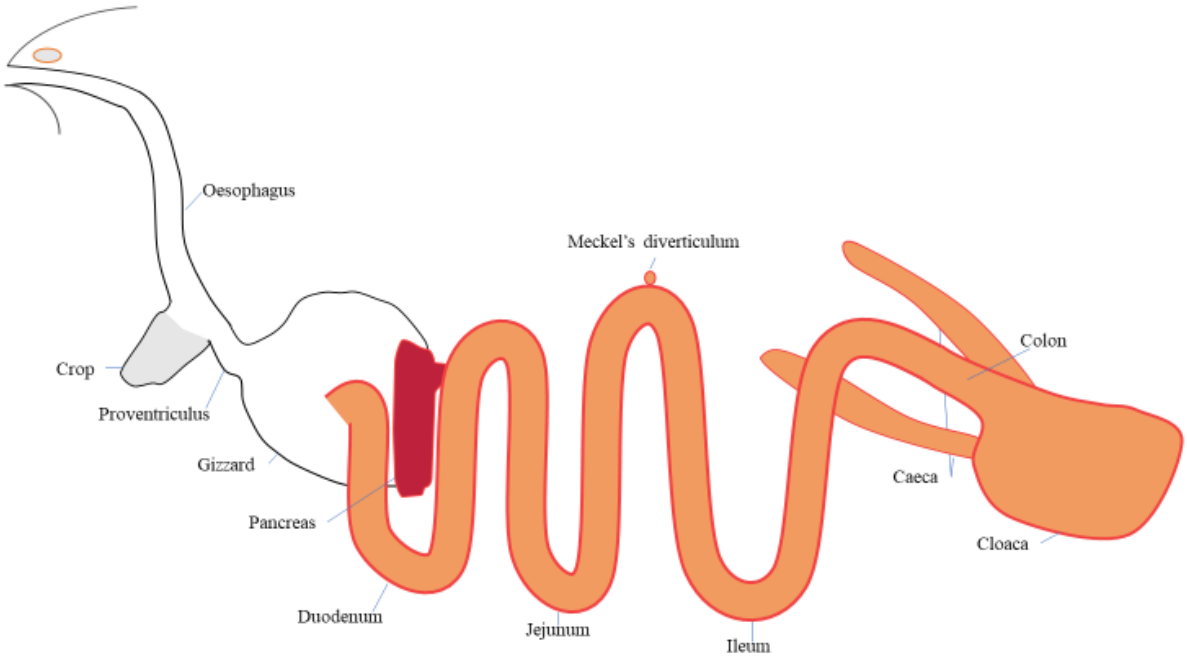



**Figure 1.2 Overview of broiler digestive tract**

## 1.3.2 Cell types present in intestinal tract

Many different cell types are present in the intestinal tract (van der Flier & Clevers, 2009). Enterocytes function primarily to assist in nutrient absorption (Snoeck *et al*., 2005). Goblet cells along the epithelial lining secrete mucins that protect and lubricate the mucosa (Johansson, Sjövall, & Hansson, 2013). The mucosal layer formed by mucins secreted by goblet cells acts as a first line of defence from pathogenic bacteria (Petersson *et al*., 2011). Recent evidence has pointed towards a role of mucins in interacting with dendritic cells and T regulatory cells to maintain gut homeostasis in addition to acting as a barrier for the diffusion of nutrients (De Santis *et al*., 2015; Petersson *et al*., 2011). The efficient uptake of available nutrients is essential for bird weight gain and subsequently bird performance. Paneth cells are

found in the crypts and are essential in maintaining intestinal barrier integrity (therefore maintaining the resistance of broilers to pathogenic bacterium invasion) through the action of controlled release of defensin lysozymes and tumour necrosis factor (TNF) (Clevers, 2013) (see Figure 1.3).

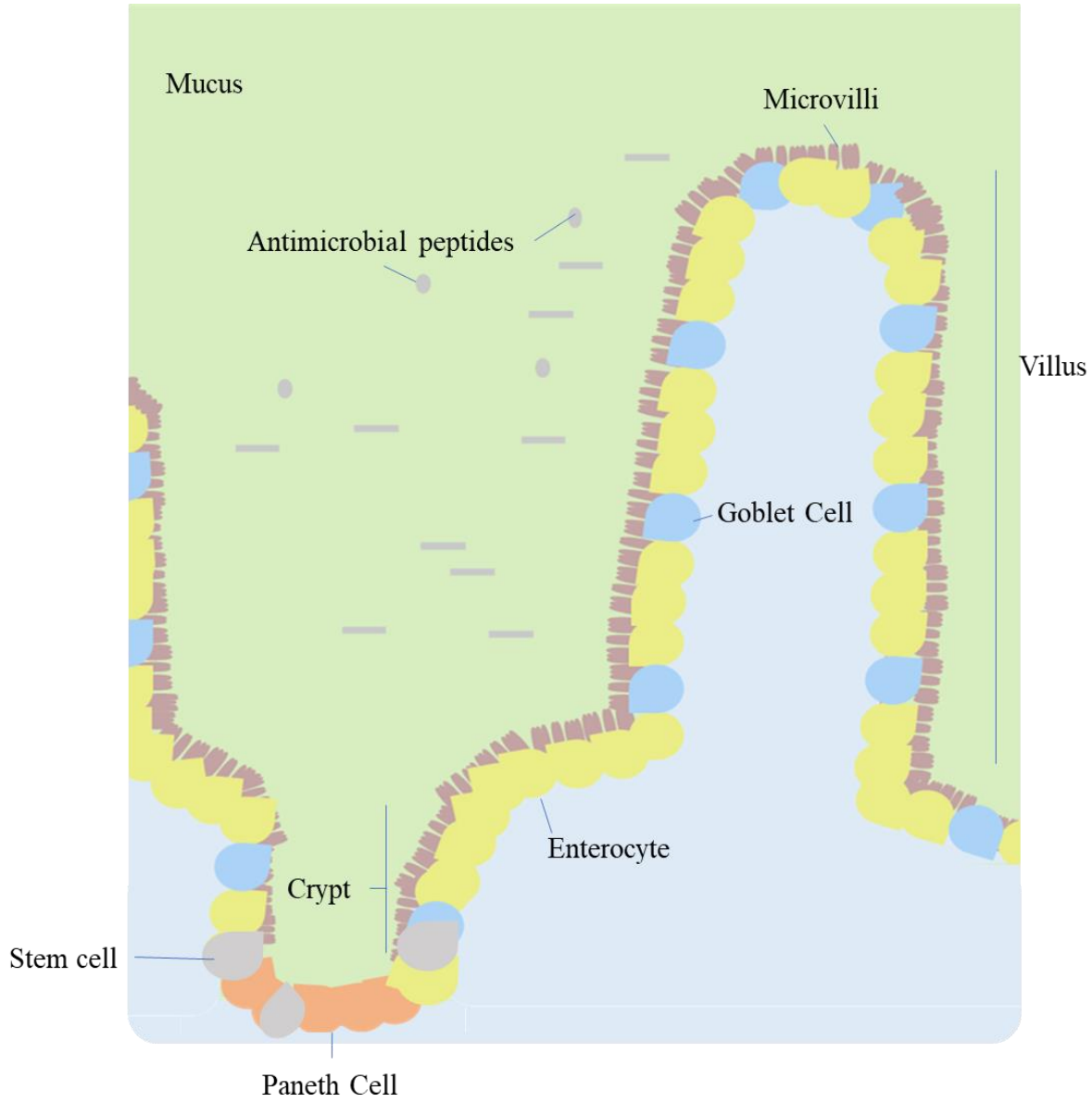



**Figure 1.3 Overview of the brush border membrane and protective mucus**

Goblet cells secrete mucin glycoproteins that form a mucus layer. Antimicrobial peptides are unable to adhere to the brush border membrane due to the action of the protective mucus layer. The function of the mucus layer ensures that energy available from nutrients is utilised for productive purposes such as enhancing broiler performance as opposed to combating pathogenic infection.

### 1.3.2 Role of cell death in maintenance of intestinal homeostasis

Intestinal epithelial cells play an essential role not only in nutrient absorption, but in the maintenance of intestinal homeostasis. The protective layer formed by single epithelial cells act as a barrier from potentially pathogenic microorganisms which may potentially impact broiler performance (Crawley *et al*., 2014). The efficient regulation of cell death in the epithelial layer is essential for immune defence (Negroni, Cucchiara, & Stronati, 2015). Dysregulation of intestinal cell death has been associated with the onset of chronic inflammation, intestinal tumour development and inflammatory bowel disease (Delgado, Grabinger, & Brunner, 2016; Negroni *et al*., 2015). Excessive epithelial cell death has also been linked to breakdowns in gut barrier function, leading to complications such as infectious enteritis (Halpern & Denning, 2015). Several different cellular death processes in the intestinal epithelium have been described, namely apoptosis, necroptosis and pyroptosis (Delgado *et al*., 2016; Halpern & Denning, 2015; Negroni *et al*., 2015).

Apoptosis is a caspase-dependant form of cell death, characterised by cell shrinking and a condensed nuclear structure, a phenomenon known as pyknosis (Davidovich, Kearney, & Martin, 2014). Two major apoptotic pathways have been described in the literature: the intrinsic pathway and the extrinsic pathway (Davidovich *et al*., 2014). The intrinsic pathway is characterised by mitochondrial stress as a result of factors such as heat shock or DNA damage. These factors trigger the binding of pro-apoptotic proteins BAX and BAD to the outer

membrane of the mitochondria in addition to the recruitment of BAK, a pro-apoptotic protein residing internally in the mitochondria (Negroni *et al.*, 2015). This action results in the release of cytochrome c, which binds to apoptotic protease activating factor -1 and adenosine tri-phosphate (ATP) (Delgado *et al*., 2016). This formation binds to pro-caspase-9, forming a complex known as an apoptosome which cleaves pro-caspase-9, thereby activating it and in turn activating caspase-3 (Dejean, Martinez-Caballero, & Kinnally, 2006). Second mitochondria-derived caspases (SMACs) are present as a result of increased mitochondrial permeability. SMACs function to bind to inhibitors of apoptosis proteins (IAPs), thereby deactivating them and allowing the intrinsic apoptotic pathway to continue (Elmore, 2007). Caspase-3 acts as an effector protein to initiate the process of cellular degradation (Davidovich *et al*., 2014).

The extrinsic pathway of apoptosis is characterised by the interaction between transmembrane receptor and extrinsic signals (Elmore, 2007). The binding of Fas or tumour-necrosis factor-α (TNF-α) to their respective receptors initiate a cascade of events resulting in the recruitment of adaptor proteins FADD (upon binding of Fas ligand to Fas receptor) or TRADD with the recruitment of FADD (upon binding of TNF-α ligand to TNF-α receptor) (Elmore, 2007). The recruitment of FADD leads to the association of pro-caspase-8, forming a death-inducing signalling complex (DISC) and the activation of caspase-8 (Martin & Henry, 2013). These actions result in the activation of effector protein caspase-3 and the degradation process resulting in the death of the cell. Apoptosis is essential for the maintenance of internal homeostasis (Negroni *et al.*, 2015). The apoptotic process is considered an anti-inflammatory process, meaning that the process of apoptosis is immunologically silent, in contrast to another form of cell death known as necroptosis.

Necroptosis is a caspase-independent form of cell death involving the receptor interacting protein kinase (RIP) family of proteins (Delgado et al., 2016). The necroptotic pathway is

similar to the extrinsic apoptotic pathway as the binding of TNF-α and Fas to their respective cell surface receptors initiates a series of events resulting in the phosphorylation of RIP1 and RIP3, to form a RIP1-RIP3 heterodimer (Pasparakis & Vandenabeele, 2015). This action leads to the auto-phosphorylation of RIP3 and facilitates the recruitment of mixed-lineage kinase domain-like protein (MLKL) in order to form a necrosome (Zhou & Yuan, 2014). This results in the formation of MLKL homo-trimers, leading to the subsequent release of intracellular contents (Davidovich *et al*., 2014). Necroptosis is considered an inflammatory form of cell death which can severely impact broiler homeostasis, as a result of the release of danger-associated molecular patterns (DAMPs) upon plasma membrane permeabilisation (Pasparakis & Vandenabeele, 2015). This is similar to another form of cell death known as pyroptosis.

Pyroptosis is a necrotic, caspase-1 dependant form of cell death (Man, Karki, & Kanneganti, 2017). The pyroptotic process is characterised by cellular swelling, chromatin condensing and the loss of mitochondrial membrane potential (Shimada *et al*., 2012). In this pathway, pathogen-associated molecular patterns (PAMPs) or DAMPs activate their respective stimuli which in turn leads to the recruitment of the inflammasome adapter apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) and cysteine-protease caspase-1 (Shimada *et al*., 2012). This complex is known as an pyroptosome and results in the activation of caspase-1 (Fernandes-Alnemri *et al*., 2007). Cellular death through pyroptosis results in the release of DAMP molecules such as ATP, providing a potent signal for the induction of a pro-inflammatory response (Fernandes-Alnemri *et al*., 2007). Pyroptosis leads to the subsequent release of IL-1β and IL-18.

The regulation and characterisation of cell death in the intestinal epithelium is essential due to the role the epithelial layer plays in nutrient absorption and host defence. Breakdowns in the function or integrity of the epithelial layer though excessive intestinal cell death can result in

the onset of inflammatory disorders that impact the vital functions of the epithelium and by extension overall broiler gut health.

## 1.4 Innate immune system overview

### 1.4.1 Innate immunity and role of pathogen recognition receptors (PRRs)

Innate immunity is the first line of defence against invading pathogens. The innate immune system is comprised of several distinct components including physical and biochemical barriers such as skin, lactic and fatty acids as well as immune cells such as dendritic cells, natural killer cells, monocytes and macrophages (Thaiss *et al*., 2016). The innate immune response has the ability to in turn activate the adaptive immune response. The adaptive immune response can recognise specific pathogenic organisms as a result of several evolutionary mechanisms, such as clonal selection and hypervariability (Maxwell *et al*., 2006). This can occur in several ways, primarily through the activation of previously immature dendritic cells as a result of pathogenic infection (Clark and Kupper, 2005). The recognition of foreign pathogen associated molecular patterns (PAMPs) leads to the presentation of microbial peptides by major histocompatibility complex II (MHCII) in DCs whilst also inducing expression of co-stimulatory molecules like CD80 and CD80 that are essential for activation of naïve T-cells (den Haan *et al.*, 2014). Dendritic cells are the only cell subtype that can present antigen to both $CD4^+$ and $CD8^+$ T cells (Clark and Kupper, 2005). These antigen-presenting cells (APC) migrate to a network of lymph vessels and interact with B cells and T cells in order to determine the specific role and effect the adaptive immune response will play in responding to a pathogen. Several features of the adaptive immune response can in turn affect the innate response. T-regulatory cells (Tregs) have been demonstrated to supress the inflammatory

response of the innate immune system (Thaiss *et al.*, 2016). IL-10 has been hypothesised to play an essential role in this action (Thaiss *et al*., 2016). Type-1 helper cells (Th1) can activate the innate immune system through several mechanisms, including cell-cell interaction and secretion of IFN-γ (Shanker, 2010). The introduction of YCW material and consequent recognition of foreign particles by the host leads to an innate immune reaction, characterised by the detection of PAMPs by pathogen recognition receptors (PRRs) (Pinto, Barreto-Bergter, & Taborda, 2008). Extensively-studied classes of PRRs include Toll-like receptors (TLRs), nucleotide oligomerisation domain protein-1 and 2 (NOD-1 and NOD-2) and Dectin proteins -1 and -2 (Kumagai & Akira, 2010). NOD-like receptors are cytoplasmic PRRs that recognise bacterial fragments, with NOD-1 and NOD-2 among the best defined in literature (Girardin *et al*., 2003). NOD-like receptors are composed of three structural domains: a nucleotide-binding domain, an N-terminal caspase activation and recruitment domain (CARD) and a C-terminal leucine-rich repeat domain (LRR) (Sukhithasri, Nisha, Biswas, Anil Kumar, & Biswas, 2013). Binding of ligand results in a conformational change leading to the downstream translocation of NF-κB into the nucleus from the cytosol and transcriptional activation of inflammatory genes (Martinon & Tschopp, 2005). TLRs are transmembrane proteins that recognise a wide range of non-self components. For example, TLR2 can identify various pathogenic species such as lipopolysaccharides (LPS), flagella and viral dsRNA (Calich *et al*., 2008); (Akira & Takeda, 2004). TLR activation induces a conformational change resulting in an immune response via the NF-κB pathway or the production of type-1 interferons due to the activation of the TRIF-IRF pathway in the case of TLR3 (Akira & Takeda, 2004). TLR4 activation can result in the activation of both pathways in addition to the MAPK pathway (Akira & Takeda, 2004). The adaptor protein MyD88 is critical for all TLRs, with the exception of TLR3 (West, Koblansky, & Ghosh, 2006). Ten different chicken TLR members have been identified at present (Ruan *et al*., 2015). TLRs in chickens recognise similar ligands to their mammalian

counterparts (Abdul-Cader *et al*., 2017). Chicken TLR15 is unique to the avian species and recognises extracellular proteases (de Zoete *et al.*, 2011). Chicken TLR15 has been found to be expressed primarily in the bone marrow and moderately in the small intestine and liver (de Zoete *et al*., 2011). Chicken TLR21 is considered a functional homologue of mammalian TLR9 (Abdul-Cader *et al*., 2017). The stimulation of TLR21 in response to 2′-deoxyribo cytidine-phosphate-guanosine (CpG) results in the activation of NF-κB and the upregulation of pro-inflammatory genes (Keestra *et al.*, 2010) Dectin proteins recognise yeast cell wall components such as β-glucans and induce phagocytosis (Levitz, 2010). This process also results in the activation of NF-κB and cytokine induction (Levitz, 2010).

Components of the YCW have been reported to initiate a feature of the innate response mechanism that conveys quite precise immunological protection (Shashidhara and Devegowda, 2003). The complement system is composed of serum and membrane-bound proteins that act together as a cascade in order to opsonise pathogens and to induce various inflammatory responses that result in the maintenance of tissue homeostasis (Merle *et al*., 2015). Mannan and β-glucans have been hypothesised to result in the activation of the alternative and lectin-mediated complement pathway (Shashidhara and Devegowda, 2003). Pathogen-recognition molecules of the lectin pathway include mannose-binding lectin (MBL) and collectins which may have binding potential to mannans and β-glucans, respectively. MBL pathogen binding results in a conformational change that leads to the recruitment of MBL-associated serine proteases (MASPs) (Kjaer *et al*., 2013). This action facilitates the subsequent cleavage of complement proteins C4 and C2 and formation of the C3 convertase (C4b2a) (Merle *et al*., 2015). The end results of this cascade is the establishment of a membrane attack complex which dysregulates pathogen cellular integrity, leading to cellular apoptosis (Kjaer *et al.*, 2013). Complement molecule C3b can also be deposited on the microbial cell wall in order

to enhance recognition and eventual phagocytosis of the pathogen by the cells of the innate immune system (Merle *et al*., 2015).

## 1.5 Potential markers for innate immune response to yeast cell wall introduction

### 1.5.1 Markers from innate signalling pathways

A wide range of markers exist that can be used to analyse a host's immune response upon recognition of foreign particles. As described above, TLRs play a crucial role in the recognition of foreign PAMPs, which makes them good candidates as markers for the activity of the immune response. MyD88 is an adaptor protein essential for the action of all TLRs, with the exception of TLR3 (Phongsisay, 2015). Activation of MyD88 leads to the recruitment of interleukin-1 receptor-associated kinases (IRAK) and the stimulation of a signalling cascade resulting in the upregulation of proinflammatory genes through the canonical NF-κB pathway (Phongsisay, 2015; Lawrence, 2009). Other potent activators and therefore potential immune markers, include the proinflammatory cytokines IL-1β and TNF-α (Lawrence, 2009). Il-1β is initially present in an inactive form (pro-IL1β) that is processed by caspase-1 into its mature bioactive form (Kesavardhana and Kanneganti, 2017). Activation of IL-1β or TNF-α leads to the stimulation of the canonical NF-κB pathway and the upregulation of pro-inflammatory cytokines such as IL-1β, IFNβ, IL-6 and IL-12 in a positive feedback loop mechanism (Newton & Dixit, 2012). The TRIF-IRF pathway can be activated upon ligand binding to TLR3/4 with the resulting signalling cascade leading to the stimulation of IRF3 and chemokines such as CXCL10 (Honda & Taniguchi, 2006). Activation of the MAPK cascade results in the upregulation of IL-10, TNF, IL-1β and the downregulation of iNOS and IFN-β (Arthur & Ley, 2013). It has been demonstrated that IL-18 plays a role in regulating both the innate and

acquired immune response through IFN-γ and the activation of the CD134 response pathway upon initial stimulation by MyD88 and IRAK-4 (Maxwell *et al*., 2006). The role of IL-18, also known as IFN-gamma activating factor, in intestinal inflammation has been attributed to regulating the action of IL-1β in addition to playing a role in the Th1 response, in synergy with IL-12 (Dinarello *et al*., 2013; Sivakumar *et al*., 2002). The proteolytic processing of both pro-IL-18 and pro- IL-1β is dependent on the action of caspase-1 in humans (Kesavardhana and Kanneganti, 2017).

Caspase-1 is converted into its active form as a result of NLR assembly into a multi-protein complex known as an inflammasome (Dubois *et al*., 2016). The activation of the inflammasome leads to the processing of pro- caspase-1 which in turn results in the upregulation of both IL-18 and IL-1β in mammals (Man and Kanneganti, 2016). This action leads to the execution of a form of inflammation-associated cell death known as pyroptosis (Man and Kanneganti, 2016). Pyroptosis is a caspase-1 dependent, rapid form of cell death that shares features with both apoptosis and necrosis (Fink and Cookson, 2005). Pyroptosis is characterised by cellular swelling, dysregulation of the plasma membrane and the release of danger-associated molecular patterns (DAMPs) (Kesavardhana and Kanneganti, 2017). Apoptosis can be characterised by the presence of cleaved-caspase-3, a critical executioner of the apoptotic cycle (Brauchle *et al*., 2014). Necroptosis, a type of cell death initiated upon the induction of an inflammatory response, can be characterised upon the cleavage of receptor-interacting serine/threonine-protein kinase 3 (RIPK3) (Kim and Pasparakis, 2014). Five PRRs have at present been identified to initiate inflammasome assembly upon recognition of their respective stimuli: NLRP1, NLRP3, NLRC4, AIM2 and pyrin (Sharma and Kanneganti, 2016). These distinct inflammasomes are termed ‘canonical inflammasomes’ as their mode of action implicates the incorporation of pro-caspase 1 in mammals (Kesavardhana and Kanneganti, 2017). The role of inflammasomes in avian species is poorly defined (Ye *et al*., 2015). In

chickens, pro- IL-1β does not contain a cleavage site for caspase -1, suggesting an alternative mechanism for IL-1β processing (Bird *et al*., 2002). Sequence analyses of the NLPR3 gene in yellow chicken revealed only 52% homology with mammalian NLRP3 (Bird *et al.*, 2002; Ye *et al*., 2015). The presence and expression of the NLRP3 inflammasome has been recently demonstrated in the literature (Ye *et al*., 2015). The downstream effects of NLRP3 inflammasome activation are conserved in mammal and avian species (Ye *et al*., 2015). Elevated expression levels of chIL-1β have been reported in response to pathogenic infection (Berndt *et al*., 2007). Chicken IL-18 has been reported to exhibit similar biological activity to mammalian IL-18 (Gibson *et al.*, 2014). Caspase-1 has been predicted to cleave the inactive form of chIL-18, with a mechanism of action similar to that observed in mammalian species (Gibson *et al*., 2014).

## 1.6 Pathogenic infection in broilers

### 1.6.1 Colonisation in birds

A wide range of enteric pathogens can potentially invade and infect broilers, increasing mortality rates and decreasing weight gain and performance (Awad *et al.*, 2017). Examples of the most common enteric pathogens include *Salmonella, E. coli*, and *C. jejuni*. Enteric pathogens preferentially colonise the intestinal tract of birds due to favourable environmental conditions (Dasti, Tareen, Lugert, Zautner, & Gross, 2010). The highest levels of colonisation occurs in the crypts of the caeca and the small intestine (Beery, Hugdahl, & Doyle, 1988; Conlan, Coward, Grant, Maskell, & Gog, 2007). The pathogenesis of *Salmonella* involves the breakdown of the mucosal barrier in the small intestine. The interaction between *Salmonella* and the epithelial layer results in the recruitment of macrophages to the site of infection (Henderson *et al*., 1999). *Salmonella* bacterium have demonstrated the ability to replicate through use of the host's macrophages (Henderson *et al.*, 1999). In broiler chickens, *C. jejuni*

colonisation generally occurs after 14 days, which corresponds to diminished maternal antibody protection (Sahin *et al*., 2001). *C. jejuni* O-linked glycosylated flagella are critical in successful host colonisation and the release of invasive antigens (Cia) (Dasti *et al*., 2010; Logan *et al*., 2009). Proteins such as CadF have been implicated in adhesion to host cell and stimulation of signalling cascades resulting in internalisation of the bacterium (Dasti *et al*., 2010; Young, Davis, & Dirita, 2007).

Evidence has also emerged of the ability of enteric pathogens to stimulate signalling cascades involved in the innate immune response, potentially resulting in the onset of autoimmunity (MacCallum, Haddock, & Everest, 2005; Phongsisay, Hara, & Fujimoto, 2015; Samuelson *et al*., 2013). Campylobacter invasive antigens (Cia) are introduced into host cell via a Type III Secretion System (T3SS) originating from the flagellum (Neal-McKinney & Konkel, 2012). An effector protein, CiaD, has been experimentally shown to be a potent activator of MAPK signalling pathway components ERK1/2 and p38 (Neal-McKinney & Konkel, 2012). Glycoconjugates present on *C. jejuni* have been shown to induce the NF-κB pathway (Al-Sayeqh, Loughlin, Dillon, Mellits, & Connerton, 2010; Phongsisay *et al*., 2015). *C. jejuni* can initiate the activation of the NF-κB signalling cascade through TLR4 and chicken TLR21 in intestinal epithelial cells leading to the secretion of IL-8 (Al-Sayeqh *et al*., 2010). Li *et al*. (2011) demonstrated that *C. jejuni* can stimulate the production of proinflammatory IL-8 and anti-inflammatory IL-10 in epithelial cells.

## 1.7 Context of this study

The perceived ability of mannan-containing feed additives to modulate the innate immune response of the host and to potentially reduce the colonisation levels of pathogenic agents such as *C. jejuni, E. coli, Salmonella* and *Clostridium difficile* warrants further investigation. This knowledge will be extremely beneficial in understanding the mechanism of action of yeast cell

wall derivatives and also possibly formulating additional alternatives to antibiotic-growth promoters. The data available for the effect of natural-feed additives is limited, and this research will help to fully elucidate the effect of dietary additives on a wide range of parameters, with the overall aim to find a better alternative to antibiotic-growth promoters. With respect to this, the objectives of this study were the following:

- To determine if MRF supplementation could lead to alterations in intestinal enzyme capacity
- To explore the role of MRF supplementation on intestinal tissue morphological features and integrity
- To investigate if MRF incorporation leads to alterations in inflammatory cytokine expression
- To fully delineate the effect of MRF supplementation on gut barrier function through analysis of cellular death levels

# Chapter 2: Materials and Methods

## 2.1 Materials

### 2.1.1 Reagents

| Reagents | Supplier |
|---|---|
| Acetic Acid glacial, ≥99.85% | Sigma-Aldrich |
| Acetic Acid | Fisher-Scientific |
| Alcian Blue | Abcam |
| APS | Sigma-Aldrich |
| Bradford Reagent Dye | Bio-Rad |
| BSA | Sigma-Aldrich |
| Coverslips | Fisher Scientific |
| BSA | Sigma-Aldrich |
| DAPI (4`, 6-Diamidino-2-Phenylindole) for nucleic acid staining | Sigma-Aldrich |
| DPX Mounting Medium | Sigma-Aldrich |
| DNA ladder & Loading dye | Promega |
| Eosin Y | Sigma-Aldrich |
| Ethanol | Sigma-Aldrich |
| Glucose | Sigma-Aldrich |
| Glucose Oxidase from *Aspergillus Niger* | Sigma-Aldrich |
| Haematoxylin | Sigma-Aldrich |
| Hydrochloric Acid (HCL) | Sigma-Aldrich |
| $H_2O_2$ | Sigma-Aldrich |
| Imidazole | Sigma-Aldrich |

| | |
|---|---|
| ImmPACT DAB Peroxidase (HRP) Substrate | Vector Laboratories |
| Isopropanol | Sigma-Aldrich |
| Maleic Acid | Sigma-Aldrich |
| Maltose | Sigma-Aldrich |
| Microscope Slides | VWR |
| o-dianisidine dihydrochloride | Sigma-Aldrich |
| NaOH | Sigma-Aldrich |
| Periodic Acid | Sigma-Aldrich |
| Phosphatase Buffered Saline | Oxoid |
| Ponceau S | Sigma-Aldrich |
| Potassium Dichromate 99% | Sigma-Aldrich |
| Pre-stained molecular weight marker | Invitrogen |
| Protogel | National Diagnostics |
| Schiff Reagent | Sigma-Aldrich |
| SDS | Sigma-Aldrich |
| Milk Powder | Sigma-Aldrich |
| Sodium Citrate | Sigma-Aldrich |
| Sucrose | Sigma-Aldrich |
| TEMED | Sigma-Aldrich |
| TMB | Sigma-Aldrich |
| Thermo Scientific™ Shandon™ Peel-A-Way Disposable Embedding Molds | Fisher Scientific |
| Tissue-Tek® Cryomold® Mold | VWR |
| Tissue-Tek® OCT Compound | VWR |

| | |
|---|---|
| Tris-base | Sigma-Aldrich |
| Tris-HCl | Sigma-Aldrich |
| Triton-X-100 | Sigma-Aldrich |
| Trizma | Sigma-Aldrich |
| Tween-20® | Sigma-Aldrich |
| VectaMount Permanent Mounting Medium | Vector Laboratories |
| VECTASTAIN® ELITE® ABC HRP Kit (Peroxidase Rabbit IgG) | Vector Laboratories |
| Xylene, reagent grade | Sigma-Aldrich |
| 0.1% Peroxidase Solution | Sigma-Aldrich |
| 4% Paraformaldehyde | Sigma-Aldrich |
| 10% Neutral Buffered Formalin | Sigma-Aldrich |
| 96-well NUNC™ "Maxisorb" plates | Costar |

### 2.1.2 Kits

| Kit | Supplier |
|---|---|
| In Situ Cell Death Detection Kit, Fluorescein | Sigma-Aldrich |
| Chicken IL-6 (Interleukin 6) ELISA Kit 96T | Elabscience |
| Chicken IL-10 (Interleukin 10) ELISA Kit 96T | Elabscience |
| Chicken IL-1β (Interleukin 1 Beta) ELISA Kit 96T | Elabscience |
| Chicken IL-18 (Interleukin 18) ELISA Kit 96T | Abbexa |
| Chicken IL-12 (Interleukin 12) ELISA Kit 96T | Abbexa |

## 2.1.3 Antibodies

### 2.1.3.1 Antibodies for Immunoblotting

| Primary Antibody | Supplier | Dilution | Diluent |
|---|---|---|---|
| Akt | Cell Signalling | 1:1000 | 5% BSA TBST |
| Phospho-Akt | Cell Signalling | 1:1000 | 5% BSA TBST |
| RIPK3 | Biorbyt | 1:150 | 5% BSA TBST |

| Secondary Antibody | Supplier | Dilution | Diluent |
|---|---|---|---|
| Anti-mouse HRP | Cell Signalling | 1:3000 | 5% Milk TBST |
| Anti-rabbit HRP | Cell Signalling | 1:3000 | 5% Milk TBST |
| IRDye® 680RD Goat anti-Mouse | Li-cor | 1:5000 | 5% Milk TBST |
| IRDye® 800CW Goat anti-Rabbit | Li-cor | 1:5000 | 5% Milk TBST |

### 2.1.3.2 Antibodies for Immunohistochemistry

| Antibody | Supplier | Dilution | Diluent |
|---|---|---|---|
| Caspase-3 | Abcam | 1/75 | PBST |
| RIPK3 | Biorbyt | 1/200 | PBST |

## 2.1.4 Buffers

| Buffer Name | Composition |
|---|---|
| Block buffer (Immunoblotting) | TBS, 0.1% (v/v) Tween-20 with 5% (w/v) non-fat dry milk |
| Maleate Buffer | 50mM Maleic Acid, 1M NaOH, pH 6, 0.056M Maltose or Sucrose. |
| Tris-HCl | 0.5M Trizma base, HCl, pH 7. |
| Detergent | 1part Triton X-100 with 4 parts 95% Ethanol |
| Laemmli sample buffer | 62.5 mM Tris-HCl, pH 6.8, 10% (w/v) glycerol, 2% (w/v) SDS, 0.7 M β-mercaptoethanol and 0.001% (w/v) bromophenol blue |
| 0.1% Peroxidase Solution | 10mg 0.1% Peroxidase Solution to deionised water |
| o-dianisidine dihydrochloride | 50mg to 5mL deionised water |
| Tris Glucose Oxidase (TGO) Solution (100mL) | Tris-HCl, 1 % Detergent, 0.1% Peroxidase Solution, o-Dianisidine Dihydrochloride, 0.1g Glucose Oxidase from *Aspergillus Niger* |
| 0.5% Periodic Acid Solution | 0.5% Periodic Acid, Distilled Water |
| 1% Acid Alcohol | 2-Propanol (Isopropanol), $dH_2O$, HCl |
| Eosin Y | 1g Eosin, 1.6g Potassium Dichromate, $dH_2O$ |
| Permeabilisation Solution (TUNEL) | 0.1% Triton X-100, 0.1% Sodium Citrate, $dH_2O$ |
| Fixation Solution (TUNEL) | 4% Paraformaldehyde, PBS, pH 7.4 |
| TUNEL Positive Control Solution | 50mM Tris-HCl, 30U/mL DNase I Recombinant, BSA, pH 7.5 |
| Phosphate Buffered Saline (PBS) | 2.7mM KCl, 1.5mM $KH_2PO_4$, 137mM NaCl, 8mM $NaHPO_4$, pH 7.4 |

| | |
|---|---|
| Phosphate Buffered Saline Tween 20 (PBST) | 2.7mM KCl, 1.5mM $KH_2PO_4$, 137mM NaCl, 8mM $NaHPO_4$, 0.05% Tween®, pH 7.4 |
| Ponceau stain | 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid |
| SDS running buffer | 25 mM Tris, 192 mM glycine, 0.1% SDS |
| Tris buffered saline (TBS) | 25 mM Tris, pH7.4, containing 0.14M NaCl |
| 0.3% $H_2O_2$ (Endogenous peroxidase activity) | 3mL 30% $H_2O_2$ in 300mL $H_2O$ |

## 2.2 Methods

### 2.2.1 Trial Outline

A 35-day trial involving 492 male broiler chickens was carried out to determine the possible effect, compared to a control (birds fed a normal corn and soybean diet), of three naturally-derived dietary supplements on broiler intestinal health and immunity. The boiler chickens were housed on-site at Agri-Food Biosciences Institute (AFBI, Belfast, UK). The experiment was set up in a randomised complete block design with each bird allocated at random to each of 12 pens at day of hatch. Three pens were allocated for each treatment (41,41 and 43 birds respectively). The supplements were mannan-rich fractions (MRF) extracted from the cell wall of *Saccharomyces cerevisiae*. Basal diets consisted primarily of a standard wheat/soybean diet which met full dietary nutritional requirements. Feed and water were provided *ad libitum*. Broiler diets consisted of three distinct phases: starter (day 0 - day 10), grower (day 11 – day 25) and finisher (day 26 – day 35). Broiler dietary supplements were tailored at each stage with supplements included at the manufacturer's recommended inclusion levels. MRF-1 was incorporated at 1.3 kg/T, 1 kg/T and 0.6 kg/T in starter, grower and finisher feed, respectively. MRF-2 was supplemented into broiler diets at 1 kg/T for each stage. MRF-3 was incorporated into broiler diets at 1 kg/T for each phase. At day 0, 7, 21 and 35, all birds were weighed and

the resulting values averaged per pen. In order to assess possible differences in weight gain and feed-conversion ratios, all feed intake was carefully monitored. All procedures were subject to the approval of the local Animal Welfare Ethics Review Board and subsequent approval by a Home Office Inspector. All procedures were carried out under the strict guidelines of the Animal (Scientific Procedures) Act 1986.

### 2.2.2 Sample Collection and Preparation

#### 2.2.2.1 Sample Collection and Preparation

Birds were sacrificed at day 35 by cervical dislocation followed by exsanguination. Biological tissue samples were retrieved from four intestinal sections per bird: the duodenum, jejunum, ileum and the caecum. At day 35, serum samples were also collected. Histological tissue samples were suspended in 1x phosphate buffered saline (PBS) and stored at 4°C overnight. The samples were then immersed in OCT compound, and placed in a cryomold and stored at -80°C until further processing. Tissue samples for digestive enzyme analyses were flash frozen, kept on dry ice and finally stored at -80°C.

#### 2.2.2.2 Sample preparation- Digestive Enzyme Assays

All steps were carried out on ice unless otherwise stated, and all equipment and labware were pre-chilled in advance of sample preparation. Tissue samples were removed from -80°C storage and kept on ice prior to homogenisation to prevent possible enzyme degradation. Segments weighing 10mg were removed from each intestinal tissue sample using a scalpel. Tissue segments were placed, using forceps, into a flat-bottomed plastic cylindrical tube containing 500µL of distilled water. Homogenisation was carried out using an T10 basic ULTRA-TURRAX® homogenizer at medium speed. Samples were homogenised for a total of

2 minutes. Homogenised samples were aliquoted into 100µL Eppendorf tubes, briefly centrifuged and the supernatant stored at -20°C for further analyses.

#### 2.2.2.3 Sample Preparation- Histological Analyses

Tissue samples embedded in OCT compound were removed from -80°C storage. Tissue sections were prepared using a Thermo Shandon Cryotome™ M165. The temperature of the chamber was set at -18°C. Tissue blocks were removed from Cryomold® mold and fitted inside the pre-chilled cryotome chamber and allowed to equilibrate to chamber temperature. Tissue sections were trimmed at 15µm until the full intestinal tissue was exposed. 10µm sections were then cut in continuous fashion and transferred onto VWR® microscopic slides. Three sections were placed on each slide. Slides were labelled and stored at -80°C prior to staining. Unused tissue blocks were re-embedded in OCT compound and returned to -80°C storage.

### 2.2.3 Bradford Assay

Total protein content of homogenised tissue was quantified using the Bradford method (Bradford, 1976). The Bradford assay was carried out using the microplate method. Pre-determined concentrations of BSA standards and samples were diluted in 18µL of distilled water and mixed with 180µL of Bradford assay reagent. Protein concentration was determined by the resulting formation of colourimetric product. The OD value of each well was recorded at 595nm using a TECAN Infinite® M200 PRO with TECAN i-control™ software. Protein concentrations of homogenised sample were extrapolated from a BSA standard curve of known concentrations ranging from 5mg/mL to 0mg/mL. Samples were analysed in triplicate and standard concentrations in duplicate. Data analysis was carried out using GraphPad® Prism 5 software (GraphPad® Software).

### 2.2.4 Maltase Enzyme Capacity Analysis

Maltase enzyme capacity was assayed using a tris-glucose oxidase (TGO) reaction according to the method of Dahlqvist (1968), with slight modification. The assay was carried out in two steps: first the intestinal homogenate was incubated in the presence of maltose substrate solution and then glucose product was recorded using a spectrophotometer after addition of TGO solution. Sample dilutions were carried out using ice-cold deionised water. 10µL of appropriately diluted intestinal homogenate was added to each well in triplicate of a NUNC™ Maxisorb 96-well plate in the presence of 10µL of substrate solution (50mM maleate buffer, 1M NaOH, pH 6, 0.056M maltose). The plate was covered to prevent evaporation and incubated at 37°C for 15 minutes. A glucose standard curve was constructed in order to accurately record the concentration of glucose emitted which is proportional to digestive enzyme capacity. Glucose standards were prepared ranging from 200µg/mL to 0µg/mL with distilled water. Following the first incubation, 20µL of each standard was added to the plate in duplicate. 200µL of TGO solution was added to each well on the microplate and incubated for an additional 15 minutes. The absorption of standards and samples were recorded at 420nm using a TECAN Infinite® M200 PRO with TECAN i-control™ software. The concentration of glucose released from intestinal homogenate was determined by interpolating the sample absorbance from the known concentrations of the glucose standard curve. Sample concentrations were converted to µmol of glucose released per minute and results expressed as µmol glucose released per minute per µg protein content. Data analysis was carried out using GraphPad Prism 5 software (GraphPad® Software).

### 2.2.5 Sucrase Enzyme Capacity Analysis

Sucrase enzyme capacity was assayed using a TGO reaction according to the method of Dahlqvist (1968), with slight modification. The assay was carried out in two steps: first the intestinal homogenate was incubated in the presence of sucrose substrate solution and then glucose product was recorded using a spectrophotometer after addition of TGO solution. Sample dilutions were carried out using ice-cold deionised water. 10µL of appropriately diluted intestinal homogenate was added to each well in triplicate of a NUNC™ Maxisorb 96-well plate in the presence of 10µL of substrate solution (50mM maleate buffer, 1M NaOH, pH 6, 0.056M sucrose). The plate was covered to prevent evaporation and incubated at 37°C for 15 minutes. A glucose standard curve was constructed in order to accurately record the concentration of glucose emitted which is proportional to digestive enzyme capacity. Glucose standards were prepared ranging from 200µg/mL to 0µg/mL with distilled water. Following the first incubation, 20µL of each standard was added to the plate in duplicate. 200µL of TGO solution was added to each well on the microplate and incubated for an additional 15 minutes. The absorption of standards and samples were recorded at 420nm using a TECAN Infinite® M200 PRO with TECAN i-control™ software. The concentration of glucose released from intestinal homogenate was determined by interpolating the sample absorbance from the known concentrations of the glucose standard curve. Sample concentrations were converted to µmol of glucose released per minute and results expressed as µmol glucose released per minute per µg protein content. Data analysis was carried out using GraphPad Prism 5 software (GraphPad® Software).

## 2.2.6 Alkaline Phosphatase Enzyme Capacity Analysis

Tissue homogenate was prepared and stored at -20°C prior to analysis. The procedure was carried out as per manufacturer's instructions (Abcam). For the preparation of 5mM p-nitrophenol phosphate (pNPP) solution, 2 pNPP tablets (sufficient for 100 assays) were re-constituted in 5.4mL Assay Buffer. Concentrated ALP enzyme was diluted with 1mL Assay Buffer and kept on ice during the assay. Working solution of ALP enzyme was aliquoted and unused solution stored at 4°C. A pNPP standard curve was prepared ranging from 20nmole/well to 0nmol/well. Optimal sample dilutions were calculated prior to analysis to ensure results fell within range of standard curve. Sample dilutions were carried out using Assay Buffer. Appropriately diluted sample was added to each well in triplicate and volume topped up to 80µL using Assay Buffer. 120µL of each standard was added to the plate in duplicate. Distilled water was used as a negative control. 20µL Stop Solution was immediately added to control well. 50µL of 5mM pNPP Solution was added to each sample and control well. 10µL of ALP enzyme solution was added to each pNPP standard well. The plate was covered and then incubated for 60 minutes at 25°C. The enzyme will convert pNPP substrate to an equal amount of coloured p-Nitrophenol (pNP). 20µL of Stop Solution was then added to each sample and standard well. The plate was placed on an ELISA shaker for 30 seconds and absorbance read at 405nm using a TECAN Infinite® M200 PRO with TECAN i-control™ software. pNP activity was determined by interpolating the sample absorbance from the known concentrations of the pNPP standard curve. Sample concentrations were converted to µmol of pNP released per minute and results expressed as µmol pNP released per minute per µg protein content. Data analysis was carried out using GraphPad Prism 5 software (GraphPad® Software).

## 2.2.7 Histological Staining

### 2.2.7.1 Haematoxylin & Eosin Y Staining (H&E Staining)

Sections were fixed onto glass slides using 10% neutral buffered formalin (NBF) for 30 minutes. The slides were then washed in 1x PBS for 2 minutes, twice. Sections were placed in xylene for 20 minutes. Samples were then dehydrated in 100% EtOH for 10 minutes. The sections were incubated in 95% EtOH and 80% EtOH for 2 minutes and rinsed in $dH_2O$ for 2 minutes. The slides were then incubated in haematoxylin for 3 minutes and rinsed in running tap water until the excess haematoxylin was removed. The samples were decolourised in 1% acid alcohol for 20 seconds and rinsed in running tap water for 5 minutes. The sections were then immersed in eosin Y for 3 minutes and rinsed in running tap water for 5 minutes. The slides were then dehydrated in 80% EtOH for 5 minutes. This was followed by incubation in 95% EtOH and 100% EtOH for 5 minutes each time. DPX mounting gel was added to each slide and a coverslip carefully lowered to avoid any air bubbles. Nail varnish was blotted onto the coverslip edge to anchor the coverslip. Slides were left to dry overnight.

### 2.2.7.2 Periodic Acid-Schiff Staining

Sections were fixed onto glass slides using 10% NBF for 30 minutes. The slides were then washed in 1x PBS for 2 minutes, twice. Sections were placed in xylene for 20 minutes. Samples were then rehydrated in 100% EtOH for 10 minutes. The sections were then incubated in 95% EtOH and 80% EtOH for 2 minutes and rinsed in $dH_2O$ for 2 minutes. The slides were oxidised in 0.5% periodic acid solution for 5 minutes and then rinsed in distilled water. Sections were placed in Schiff reagent for 15 minutes to allow tissue samples to develop a light pink colour.

The slides were washed in lukewarm tap water for 5 minutes to allow sections to develop a dark pink colour. The tissue samples were counterstained in Mayer's haematoxylin for 1 minute and washed in tap water for 5 minutes. The slides were then dehydrated in 80% EtOH for 5 minutes. This was followed by incubation in 95% EtOH and 100% EtOH for 5 minutes. DPX mounting gel was added to each slide and a coverslip carefully lowered to avoid any air bubbles. Nail varnish was blotted onto the coverslip edge to anchor the coverslip. Slides were left to dry overnight.

### 2.2.8 TUNEL Staining

TUNEL staining was carried out using the *in situ* Cell Death Detection Kit, Fluorescein (Roche), according to manufacturer's instructions, with slight modifications. Intestinal tissue sections were fixed with 4% paraformaldehyde in PBS, pH 7.4 for 20 minutes at room temperature (RT). Slides were then washed in PBS for 30 minutes. Sections were incubated in permeabilisation solution for 2 minutes at 4°C. TUNEL reaction mixture was prepared by adding 50µL enzyme solution to 450µL label solution. 50µL of TUNEL positive control solution was added to the positive control slide and incubated at RT for 10 minutes. Slides were rinsed twice in PBS and the area around the samples dried. 50µL of the TUNEL reaction mixture was added to each slide and 50µL of label solution added to the negative control slide. All samples were incubated in a humidified chamber for 1 hour at 37°C in the dark. Tissue samples were rinsed 3 times with PBS for 3 minutes and two separate drops of Gel Mount-DAPI was added to each slide. A coverslip was carefully lowered onto sample to avoid any air bubbles. Tissue intestinal samples were analysed using fluorescence microscopy (Olympus IX81) and images were obtained using 20x magnification. The apoptotic index (AI) was used

as a quantitative measure of apoptosis in tissue samples. This index is defined as the percentage of apoptotic cells per total number of analysed cells. The apoptotic index can be described by the following formula:

$$\text{Apoptotic Index (AI)} = \frac{\text{Number of TUNEL positive cells} * 100}{\text{Total number of nuclei analysed}}$$

## 2.2.9 Histological Analyses

### 2.2.9.1 Villus Height, Villus Surface Area and Crypt Depth

Villus height (VH), villus surface area (VSA) and crypt depth (CD) were analysed using H&E staining. Morphometric investigations for VH was calculated as the average of 15 vertically-orientated villi from a total of 9 birds. Data was expressed in µm. VSA was calculated as the average of 15 vertically-orientated villi per bird from a total of 9 birds using the following equation as adapted from Iji *et al*. (2001):

$$VSA = ((Villus\ height\ at \frac{1}{3} height\ of\ villus + villus\ height\ at\ \frac{2}{3}\ height\ of\ villus) * 2^{-1} * villus\ height)$$

Crypt depth was calculated as the average of 20 well-defined crypts from a total of 9 birds. Data was expressed in µm. Two sections of each tissue were analysed per bird for each morphological measurement.

### 2.2.9.2 Muscularis Mucosae Thickness, Mucus Layer Thickness and Goblet Cell Density

Muscularis mucosae layer thickness was examined using Periodic Acid Schiff (PAS) staining and expressed in µm. Five points along the muscularis layer from each bird was measured for an average value from a total of 9 birds. Goblet cell density was calculated by counting the number of goblet cells present on the midpoint of the villus (165µm) on a total of 5 vertically-

orientated villi per bird to find an average value from a total number of 9 birds. Mucus layer thickness was expressed using the PAS stain and counterstaining with haematoxylin. Data was expressed from an average of 5 measurements per bird from a total of 9 birds. Two sections were analysed per bird for each morphological measurement.

### 2.2.10 Enzyme-Linked Immunosorbent Assay (ELISA)

#### 2.2.10.1 IL-6, IL-10 and IL-1β ELISA

Samples (intestinal tissue homogenate (see section 2.2.2.2) and serum) were collected and stored at -20°C prior to ELISA analysis which was carried out using the chicken IL-6, IL-10 and IL-1β ELISA kits from Elabscience. Micro ELISA plate strips were pre-coated with antibody against IL-6, IL-10 and IL-1β. 100µL of standards ranging from 15pg/mL to 1000pg/mL for IL-6 and IL-10 and concentrations ranging from 6.25pg/mL to 400pg/mL for IL-1β were added to pre-determined wells. Standard dilutions were carried out using reagent diluent. 100µL of reagent diluent was added to designated wells to act as a blank (0pg/mL). 100µL of serum or appropriately diluted intestinal tissue homogenate sample was added to each sample well and gently mixed. Intestinal tissue homogenate samples were diluted in reagent diluent. Plates were sealed with an adhesive film to prevent possible evaporation and incubated in a humidified chamber at 37°C for 90 minutes. After incubation, the contents of the plates were decanted and 100µL of biotinylated detection antibody was added to each well. The adhesive film was re-applied to the plates and gently tapped to ensure thorough mixing and incubated at 37°C for 1 hour. Plates were then washed three times with wash buffer (320µL) provided and dried against thick clean absorbent paper to ensure complete removal of residual wash buffer. The plates were placed on an orbital shaker during wash steps. 100µL of horseradish peroxidase (HRP) conjugate was then added to each well and plates were sealed with new adhesive film and incubated for 30 minutes at 37°C. After incubation, plates were

again washed five times using wash buffer provided and 90µL of Substrate Solution was added to each well. The micro ELISA plates were then incubated at 37°C in the dark for 15 minutes. The reaction was stopped by the addition of 50µL of stop solution. The optical density (OD) was determined for each well at 450nm and 590nm using a ELx800TM microplate reader with Gen5 Data Analysis software (Biotek™). The concentrations of IL-6, IL-10 and IL-1β in each experimental sample were extrapolated from a standard curve of known concentrations. Standard samples were added in duplicate to ensure accurate readings. Data analysis was carried out using GraphPad Prism 5 software (GraphPad® Software).

### 2.2.10.2 IL-12 and IL-18 ELISA

Samples (intestinal tissue homogenate and serum) were collected and stored at -20°C prior to ELISA analysis which was carried out using the chicken IL-12 and IL-18 ELISA kit from Abbexa. Micro ELISA plate strips were pre-coated with antibody against IL-12 and IL-18. 100µL of standards ranging from 31pg/mL to 2000pg/mL for (IL-12) and ranging from 7pg/mL to 500pg/mL (for IL-18) were added to designated wells. Standard dilutions were carried out using sample diluent. 100µL of sample diluent was added to designated wells to act as a blank (0pg/mL). 100µL of serum or appropriately diluted intestinal tissue homogenate sample was added to each sample well and gently mixed. Intestinal tissue homogenate samples were diluted in sample diluent. Plates were sealed with an adhesive film to prevent possible evaporation and incubated in a humidified chamber at 37°C for 2 hours. After incubation, the contents of the plates were decanted and 100µL of detection reagent A was added to each well. The adhesive film was re-applied to the plates and gently tapped to ensure thorough mixing and incubated at 37°C for 1 hour. Plates were then washed three times with wash buffer (320µL) provided and dried against thick clean absorbent paper to ensure complete removal of residual wash buffer. The plates were placed on an orbital shaker during wash steps. 100µL of detection reagent B

was then added to each well and plates were sealed with new adhesive film and incubated for 1 hour at 37°C. After incubation, plates were washed five times using wash buffer provided and 90µL of TMB Substrate Solution was added to each well. The micro ELISA plates were then incubated at 37°C in the dark for 25 minutes. The reaction was stopped by the addition of 50µL of stop solution. The optical density (OD) was determined for each well at 450nm and 590nm using a ELx800TM microplate reader with Gen5 Data Analysis software (Biotek™). The concentrations of IL-12 and IL-18 in each experimental sample were extrapolated from a standard curve of known concentrations. Standard samples were added in duplicate to ensure accurate readings. Data analysis was carried out using GraphPad Prism 5 software (GraphPad® Software).

### 2.2.11 Immunohistochemical Analyses

#### 2.2.11.1 Caspase-3 and receptor-interacting protein kinase 3 (RIPK3)

Tissue sections were stored at -80°C prior to immunohistochemical analyses. Slides were removed from cold storage and allowed to come to room temperature prior to fixation. Slides were fixed using 4% paraformaldehyde (v/v) for 25 minutes in fumehood. Samples were then rinsed 2x times in 300mL of 10mM PBS, pH 7.4 for 5 minutes. Endogenous peroxidase activity was blocked using 0.3% (v/v) $H_2O_2$ in water for 30 minutes. Sections were washed 2x in PBS-Tween®-20 (PBST) for 5 minutes. Tissue sections were then incubated for 1 hour with 2.5% normal goat serum blocking solution (Vector Labs). After incubation, excess serum was blotted from sections and slides were washed in PBS. Sections were then incubated with primary antibody overnight diluted in PBST at 4°C as per dilutions outlined in Section 2.1.3.1. Following primary antibody incubation, slides were washed 2x with PBST for 5 minutes. Tissue samples were then incubated for 45 minutes with VECTASTAIN ® Elite ® goat anti-rabbit biotinylated secondary antibody (Vector Labs). VECTASTAIN ® ABC reagent was

prepared 30 minutes after biotinylated secondary antibody incubation. Following secondary antibody incubation, slides were washed 3x times in PBST, for 5 minutes. Sections were then incubated in VECTASTAIN ® ABC reagent mixture for 40 minutes. Samples were washed 3x times in PBST. Tissue sections were incubated in DAB peroxidase (HRP) substrate kit until the desired stain intensity developed and then rinsed with tap water. Following DAB substrate incubation, sections were rinsed in distilled water for 1 minute. The slides were then incubated in haematoxylin for 20 seconds and rinsed several times with water. The samples were dehydrated in 85% EtOH for 5 minutes. This was followed by incubation in 95% EtOH and 100% EtOH for 5 minutes. DPX mounting gel was added to each slide and a coverslip carefully lowered to avoid any air bubbles. Nail varnish was blotted onto the coverslip edge to anchor the coverslip. Tissue intestinal samples were analysed using light microscopy (Nikon® Eclipse E600) and images were obtained using 40x magnification.

### 2.2.12 Protein Immunoblotting

#### 2.2.12.1 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was conducted according to the method of Laemmli (Laemmli, 1970) as modified by Studier (Studier, 1973). Samples were prepared by performing protein equalisation and denaturing in SDS-PAGE by boiling for 5 minutes at 95˚C. Sample and pre-stained protein markers (Promega, 10-250kDa) were loaded into separate wells. Electrophoresis was performed initially at 70V through a 5% SDS polyacrylamide stacking gel and then through an 8-15% polyacrylamide resolving gel at 100V for 1.5-2 hours. The percentage gel chosen was dependant on the predicted size of the protein of interest with lower percentage gels prepared for the identification of higher molecular weight proteins.

### 2.2.12.2 Immunoblotting

Following the separation of proteins by electrophoresis, the protein gel was transferred electrophoretically to nitrocellulose membranes in a Hoefer TE 70 Semiphor semi-dry transfer unit at 200mA for 120 minutes. Pre-cut Whatmann filter paper and nitrocellulose were equilibrated with cold 1X Transfer buffer (25mM Tris Base, 0.2M glycine and 20% (v/v) methanol) 5 minutes prior to addition to transfer unit. Three layers of Whatmann paper were placed on the bottom surface on the transfer unit followed by one layer of nitrocellulose. The resolving gel was removed from the electrophorator, washed briefly in transfer buffer and then placed on the nitrocellulose. Finally, three more layers of Whatmann filter paper were added to the sandwich and the unit was closed. Following the transfer, the membrane was stained with Ponceau S to ensure correct transfer of proteins and rinsed in TBST. The membrane was then blocked with TBST containing 5% (w/v) skimmed milk powder on an orbital shaker for 15 minutes at room temperature to prevent non-specific antibody binding to membrane. The membranes were incubated in the presence of primary antibody diluted in either TBST containing 5% (w/v) skimmed milk powder or TBST containing 5% (w/v) BSA overnight at 4°C as per dilutions outlined in Section 2.1.3.1. Following incubations, the membranes were washed in TBST 3x times for 5 minutes on the shaker. Membranes were then incubated in a secondary antibody specific to the primary antibody diluted in TBST containing 5% (w/v) skimmed milk powder (anti-rabbit, anti-mouse or anti-goat) at RT for 1 hour. The nitrocellulose membranes were then washed 3x times in TBST under constant agitation. The immunoreactive bands were then visualised using the Odyssey infrared imaging system from Licor Biosciences or using enhanced chemiluminescence development (ECL) for sensitive proteins.

# Chapter 3: Results

## 3.1 Prologue

The innate immune system represents the initial line of defence from invading microorganisms (Netea *et al.,* 2016). This system is characterised by the presence of immune cells such as macrophages, natural killer cells, neutrophils and dendritic cells in addition of the action of PRRs upon recognition of their respective stimuli (Thaiss *et al*., 2016). The activation of PRRs such as TLRs in response to their antigenic stimuli results in the upregulation of several inflammatory signalling components such as NF-κB and MAPK and the onset of adaptive immunity, a more specific response to the threat posed by antimicrobial peptides. (Thaiss *et al*., 2016).

The intestinal tract is exposed to a diverse range of foreign particles and microbial species. The epithelial layer of the intestinal tract acts as a selective barrier, obstructing the passage of these potentially pathogenic organisms into systemic circulation and possibly initiating an inflammatory response (Snoeck *et al*., 2005). The mucus layer is composed of mucin glycoproteins which provide an extremely efficient system for preventing the adherence of bacteria to the epithelial layer (Cornick *et al*., 2015). Breakdowns in the function and integrity of this mucosal barrier can lead to alterations in intestinal homeostasis, and the onset of various inflammatory disorders, such as necrotic enteritis and ulcerative colitis (Cornick *et al*., 2015; O'Reilly *et al*., 2017).

It has been suggested the MRF dietary inclusion into animal diets can lead to an increased bird weight gain and an enhanced immune response (Corrigan *et al*., 2017; Wang *et al*., 2017). The enhanced immune defence (as a result of increased mucin production or improved integrity of the gastrointestinal barrier) in broilers may result in lower incidences of disease occurrence through a reduction in pathogen colonisation levels, thereby enhancing the partitioning of available nutrients towards productive processes such as bird weight gain and feed conversion

ratios. The capacity of digestive enzymes to break down and absorb available nutrients plays an essential role in enhanced broiler weight gain. Despite the research into the role naturally-derived dietary supplements can play on bird weight gain and performance, the impact of dietary supplements on the expression of pro- and anti-inflammatory cytokines remains to be fully delineated. The effect of dietary supplements on intestinal tissue morphology and the expression of markers of cellular death remains a significant topic in research, as they can be informative towards the overall health of the host. Efficient and regulated levels of cell death are essential for the maintenance of optimal gut barrier function (Delgado *et al*., 2016). Apoptosis is generally considered an anti-inflammatory process, due to the diminished activity of DAMPs (Szondy, Sarang, Kiss, Garabuczi, & Köröskényi, 2017). Necrotic cell death, conversely, has been shown to convey pro-inflammatory properties, potentially resulting in excessive inflammation and epithelial barrier dysfunction (Davidovich *et al.*, 2014).

The work presented in this thesis aims to investigate the effect of natural-derived dietary supplements on the levels of digestive enzymes maltase, sucrase and alkaline phosphatase. The potential effect of MRF incorporation on intestinal tissue histological parameters will also be examined to determine if MRF supplementation can improve tissue morphology. Analyses of the effect of MRF inclusion on inflammatory cytokine expression levels in serum and gut homogenate will also be carried out in addition to examinations of cellular death levels as a measure of gastrointestinal barrier integrity and function, with overall implications for broiler intestinal health and immunity.

## 3.2 Results

### 3.2.1 Mannan-rich fraction supplementation results in alterations to gut tissue morphology

| | Control | MRF-1 | MRF-2 | MRF-3 |
|---|---|---|---|---|
| **Villus Height (µm)** | | | | |
| Duodenum | 1157.71±41.82 | 1623.83 ±56.33*** | 1256.01±59.58 | 1506.29±51.61*** |
| Jejunum | 901.49 ±20.75 | 1187.70 ±47.47*** | 1132.53±50.28*** | 1156.68±63.57** |
| Ileum | 529.73 ±18.83 | 731.97 ±22.47*** | 736.14±29.29*** | 676.21±38.48** |
| Caecum | 231.39 ±9.66 | 315.70 ±5.26*** | 327.30±9.52*** | 317.28±17.96** |
| **Villus Surface Area (VSA) ($mm^2$)** | | | | |
| Duodenum | 0.16±0.02 | 0.24±0.01*** | 0.20 ±0.01 | 0.21 ±0.01* |
| Jejunum | 0.10 ±0.01 | 0.15±0.01*** | 0.13 ±0.01** | 0.12 ±0.01* |
| Ileum | 0.05±0.01 | 0.09 ±0.01*** | 0.09±0.01** | 0.07±0.01* |
| Caecum | 0.01±0.001 | 0.02±0.001*** | 0.02±0.001*** | 0.02±0.001** |
| **Crypt Depth (µm)** | | | | |
| Duodenum | 160.63±13.74 | 235.47±6.94*** | 254.77±20.36** | 209.63±7.80** |
| Jejunum | 120.88±6.29 | 172.09±8.10*** | 228.37±11.90*** | 143.20±6.15* |
| Ileum | 82.61±3.43 | 139.39±4.70*** | 178.29±7.13*** | 111.64±2.66*** |
| Caecum | 111.71±5.38 | 104.00±2.88 | 121.82±8.45 | 144.03±8.16** |
| **Muscularis Mucosae Thickness (µm)** | | | | |
| Duodenum | 41.33±1.84 | 29.62±1.65*** | 28.07±0.58*** | 29.13±1.25*** |
| Jejunum | 47.96±1.14 | 37.11±1.41*** | 37.47±1.61*** | 31.91±2.31*** |
| Ileum | 51.16±2.04 | 45.76±1.40* | 41.62±1.45** | 43.44±1.77* |
| Caecum | 32.91±1.88 | 26.07±1.10** | 26.51±1.56* | 26.76±0.70** |

**Table 3.1 Effect of mannan-rich fraction supplementation on intestinal mucosal morphometry of broiler chickens at day 35.**

Statistical significance between control and each treatment was determined using two-tailed Student's t-test (*P<0.05; **P<0.01; ***P<0.001).

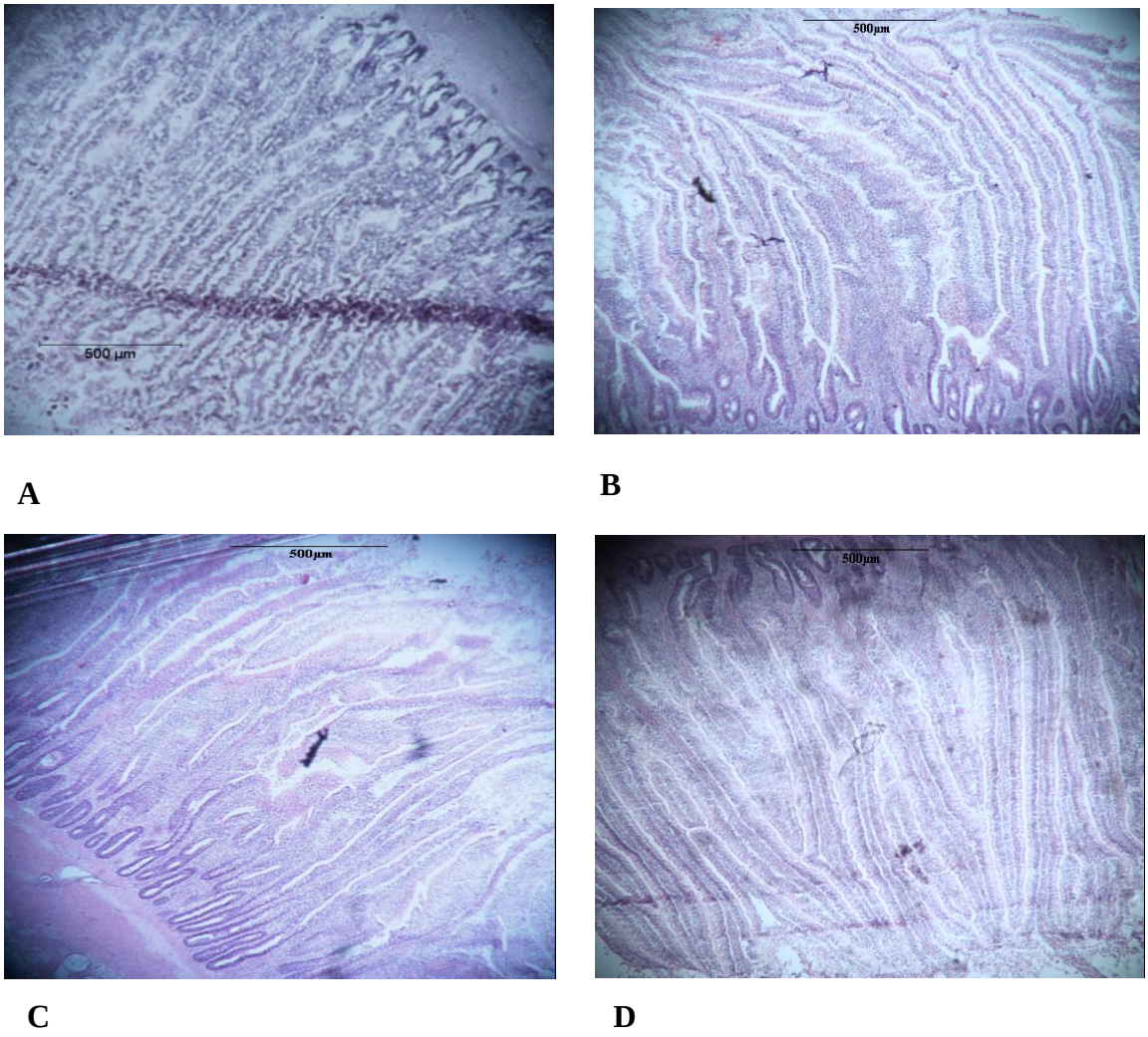
[1] Each value represents the mean of 9 birds per treatment.

| | Control | MRF-1 | MRF-2 | MRF-3 |
|---|---|---|---|---|
| **Mucus Layer Thickness (µm)** | | | | |
| Duodenum | 18.69±0.80 | 21.71±1.09* | 21.13±0.82* | 22.22±0.98* |
| Jejunum | 21.58±0.66 | 24.29±0.47** | 24.47±0.80* | 23.78±0.43* |
| Ileum | 24.29±0.51 | 28.51±0.90*** | 26.29±0.75* | 25.56±0.44 |
| Caecum | 18.13±0.50 | 22.24±0.52*** | 22.07±0.34*** | 20.78±0.84* |
| **Goblet Cell Density** | | | | |
| Duodenum | 16.20±0.50 | 18.24±0.82* | 19.87±0.47*** | 19.53±0.81** |
| Jejunum | 19.10±0.85 | 23.20±0.99** | 23.30±0.62** | 23.17±0.80** |
| Ileum | 23.57±0.43 | 26.59±0.61** | 26.57±0.87** | 25.84±0.94* |
| Caecum | 9.00±0.22 | 9.83±0.33 | 10.34±0.26** | 10.43±0.21*** |

**Table 3.2 Effect of mannan-rich fraction supplementation on intestinal mucosal morphometry of broiler chickens at day 35.**

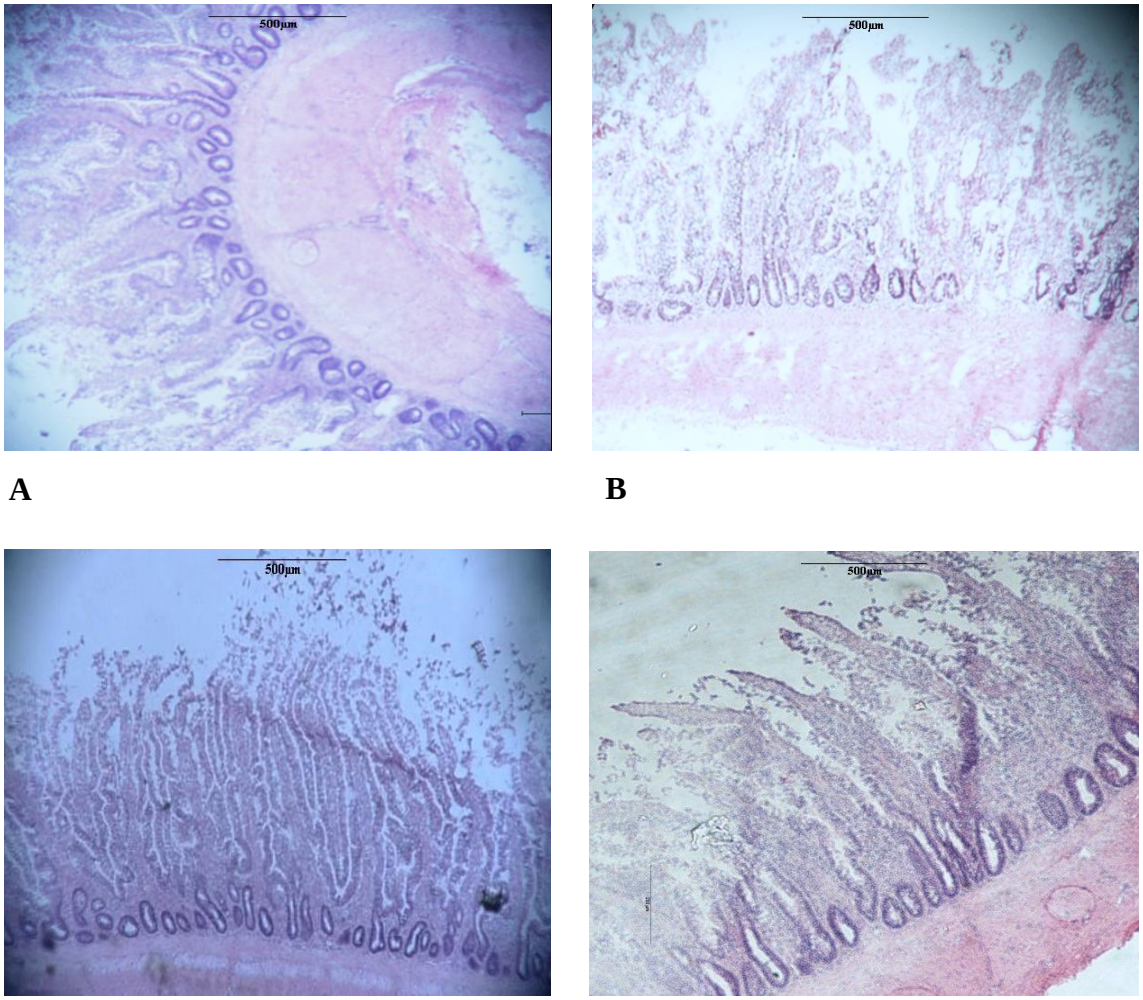
Statistical significance between control and each treatment was determined using two-tailed Student's t-test (*P<0.05; **P<0.01; ***P<0.001).

[1] Each value represents the mean of 9 birds per treatment.



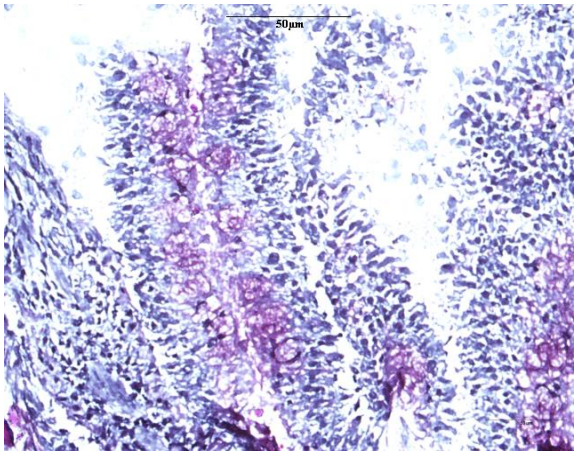

**Figures 3.1 Representative samples demonstrating the effect of mannan-rich fraction supplementation on *in situ* villus height in broiler chickens at day 35.**

Representative samples of effect of (A) control diet, (B) MRF-1 supplementation, (C) MRF-2 supplementation and (D) MRF-3 supplementation on villus height in duodenum from chicken 35 days after diet supplementation. Samples were stained with H&E. Morphometric investigations for VH was calculated as the average of 15 vertically-orientated villi from a total of 9 birds under 10x magnification.

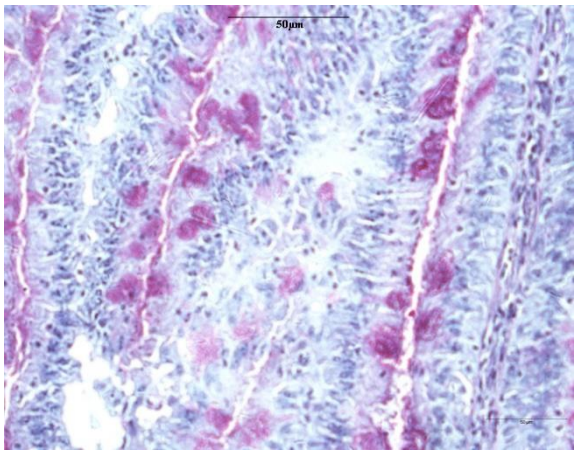



**Figures 3.2 Representative samples demonstrating the effect of mannan-rich fraction supplementation on *in situ* crypt depth in broiler chickens at day 35.**

Representative samples of effect of (A) control diet, (B) MRF-1 supplementation, (C) MRF-2 supplementation and (D) MRF-3 supplementation on crypt depth in jejunum from chicken 35 days after diet supplementation. Samples were stained with H&E. Morphometric investigations for crypt depth was calculated as the average of 20 well-defined crypts from a total of 9 birds under 10x magnification.




**A**

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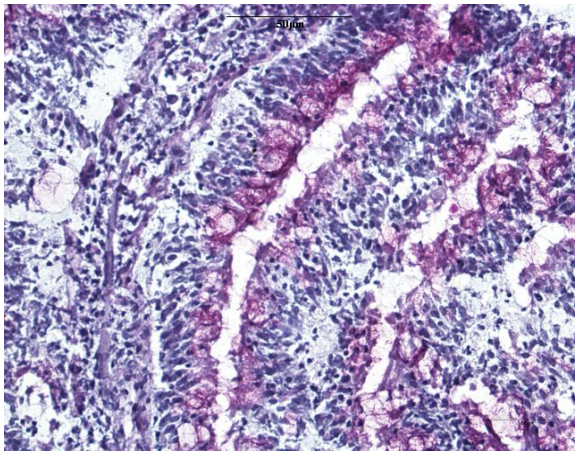


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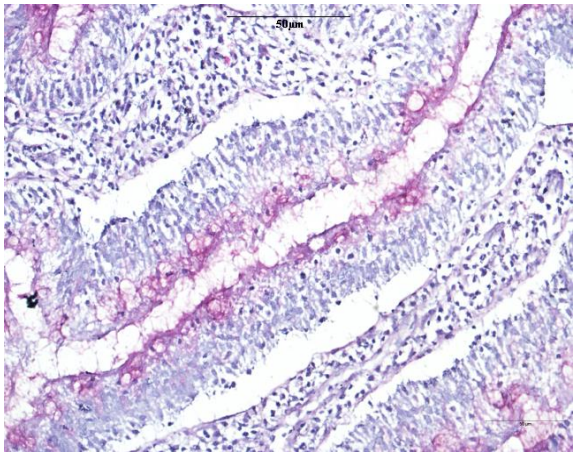



**D**

**Figures 3.3 Representative samples demonstrating the effect of mannan-rich fraction supplementation on *in situ* goblet cell density in broiler chickens at day 35.**

Representative samples of effect of (A) control diet, (B) MRF-1 supplementation, (C) MRF-2 supplementation and (D) MRF-3 supplementation on goblet cell density in ileum from chicken 35 days after diet supplementation. Samples were stained with Periodic Acid Schiff (PAS) staining. Data was expressed from an average of 5 measurements per bird from a total of 9 birds under 40x magnification. Two sections were analysed per bird for each morphological measurement

Given that mannan-rich fractions (MRF) have been hypothesised to potentially alter tissue integrity in the intestine, it was important to identify if MRF supplementation could result in improvements in intestinal tissue morphology. To this end, intestinal tissue sections were prepared and stained using H&E staining. The villus height (VH), villus surface area (VSA) and crypt depth (CD) from a total of 9 birds per treatment was measured using light microscopy and ImageJ software. Incorporation of MRF-1 (Figure 3.1B), MRF-2 (Figure 3.1C) and MRF-3 (Figure 3.1D) into broiler diet resulted in a significant increase in VH in each intestinal section with the exception of MRF-2 incorporation in the duodenum (Table 3.1 & Figure 3.1 A-D). The effect of MRF supplementation on VSA was similar to the results observed for VH (Table 3.1). Incorporation of MRF-1, MRF-2 and MRF-3 into broiler diet resulted in a significant increase in VSA in each intestinal section. The increase in VH and VSA upon mannan-rich fraction dietary supplementation compared to the control may increase the surface area available for nutrient absorption (Baurhoo *et al.,* 2007). Mannan-rich fraction dietary inclusion also resulted in a significant increase in crypt depth in small intestinal tissue for each treatment (Table 3.1 & Figure 3.2 A-D). MRF-1 (Figure 3.2B) dietary inclusion into broiler diet resulted in a slight decrease in crypt depth in caecal tissue compared to the control (Figure 3.2A), whereas MRF-3 (Figure 3.2D) supplementation led to a significant increase in crypt depth in the caecum. The incorporation of MRF-2 (Figure 3.2C) into broiler diet resulted in a slight increase in crypt depth, suggesting an overall differing effect of MRF supplementation on small intestinal and caecal crypt depth. The crypt is involved in the formation of new intestinal cells (Benites *et al*., 2008). The base of these crypts house intestinal stem cells, which are involved in the formation of each epithelial cell type present in the crypt and the villi (Benites *et al*., 2008). The increase in crypt depth observed in small intestinal tissue (Table 3.1) as a result of mannan-rich fraction incorporation may potentially result in a more developed villus.

Since it was demonstrated that MRF addition into broiler diet may result in alterations to intestinal tissue morphology that could potentially enhance nutrient absorption in broiler chickens, the next step involved examining if MRF incorporation could convey an increased protection from pathogenic invasion and adhesion, thereby permitting the greater volume of available nutrients to be utilised for enhancing bird performance and growth. To achieve this, intestinal tissue sections from each treatment were stained using PAS staining. Muscularis mucosae thickness, goblet cell density and mucus layer thickness were examined from a total of 9 birds per treatment. Dietary incorporation of each fraction led to a significant decrease in muscularis mucosae thickness in each intestinal section compared to the control (Table 3.1). Goblet cell density was significantly increased as a result of MRF-1 (Figure 3.3B) dietary inclusion in intestinal tissue while MRF-2 (Figure 3.3C) incorporation into broiler diet significantly enhanced goblet cell density in each intestinal tissue (Table 3.2 and Figure 3.3 A-D). MRF-3 (Figure 3.3D) supplementation resulted in a significant increase in goblet cell density in the duodenum, jejunum, ileum and the caecum (Table 3.2). The thickness of the mucus layer was increased significantly in each intestinal tissue upon MRF dietary inclusion with the exception of MRF-3 in the ileum (Table 3.2). The muscularis mucosae acts as a boundary between the mucosal and submucosal layer of the GIT (Cheng *et al*., 2010). A decrease in the thickness of this layer may in theory assist in the translocation of nutrients into systemic circulation (Cheng *et al*., 2010). The increase in goblet cell density and corresponding increase in mucus layer thickness as a result of MRF supplementation suggest towards an increased protective effect compared to the control unsupplemented broiler chickens. Goblet cells secrete mucin glycoproteins which from a protective mucus layer that protects the intestinal epithelium from bacterial adhesion (Johansson *et al*., 2013).

These results suggest that MRF incorporation into broiler diet can lead to an increase in surface area available for nutrient absorption and an enhanced protection from pathogenic invasion.

### 3.2.2 MRF-1 and MRF-2 dietary supplementation leads to enhanced digestive enzyme maltase capacity

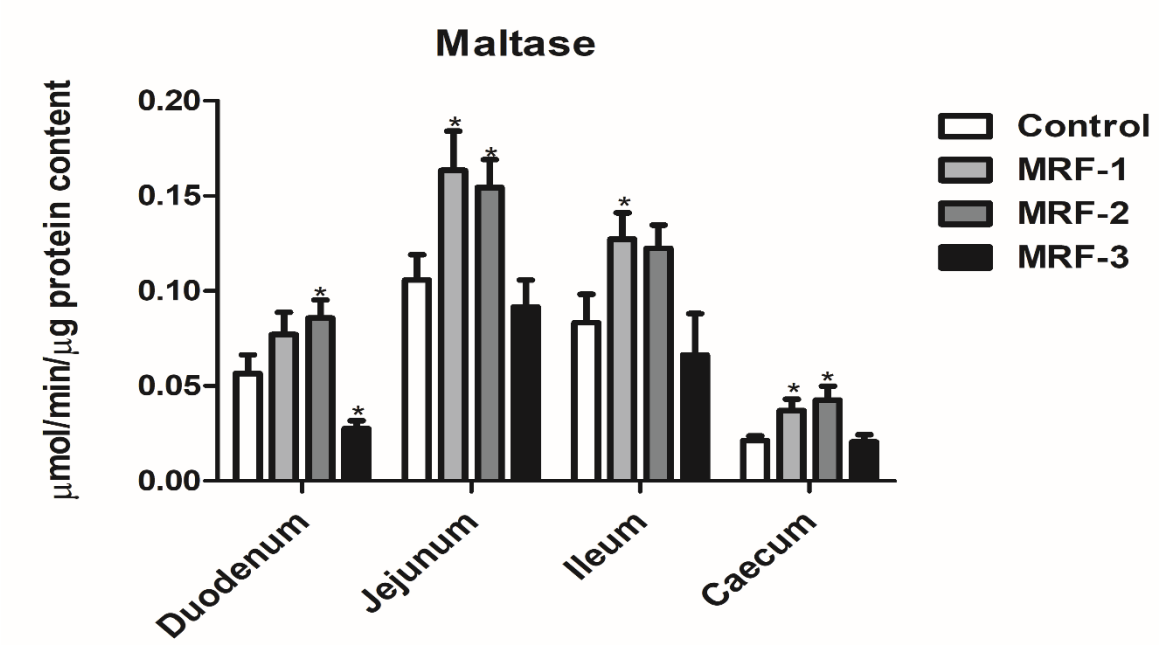



**Figure 3.4 Effect of mannan-rich fraction supplementation on maltase digestive enzyme capacity**

Digestive enzyme capacity analysis of maltase enzyme per µg of intestinal tissue lysate in duodenum, jejunum, ileum and caecum on day 35. The four bars represent, from left to right, control unsupplemented, MRF-1 supplement, MRF-2 supplement and MRF-3 supplement. Data are presented as the mean +/- SEM of maltase activity normalised per µg of homogenate proteins from 10 birds. Statistical significance between control and each treatment was determined using two-tailed Student's t-test (*= $P<0.05$).

Given that mannan-rich fractions have been hypothesised to potentially alter digestive enzyme capacity in the GIT, the initial analyses to assess the efficacy of mannan-rich dietary interventions focused on its potential to affect digestive enzyme maltase capacity. Intestinal tissue from broiler chickens (n=10) was homogenised and assayed for maltase enzyme capacity by means of maltose substrate solution and TGO solution. The digestive enzyme maltase was chosen for analysis as it plays an essential role in providing energy for maintenance and for growth (Horvatovic *et al*., 2015). Enzyme capacity was assessed through enzymatic assays. Incorporation of MRF-1 into broiler diet led to a significant increase in maltase enzyme capacity in the jejunum, ileum and caecum (Fig. 3.4). Supplementation of broiler diet with MRF-2 resulted in significantly enhanced enzyme capacity compared to the control in the duodenum, jejunum and caecum (Fig. 3.4) whereas dietary inclusion of MRF-3 led to a significant decrease in enzymatic capacity in the duodenum and a decrease (not reaching statistical significance) in maltase capacity in the jejunum, ileum and caecum (Fig. 3.4). These results indicate that MRF-1 and MRF-2 incorporation into broiler diet may result in enhanced digestive enzyme maltase capacity, thereby potentially increasing nutrient absorption.

### 3.2.3 MRF-1 and MRF-2 dietary supplementation leads to enhanced digestive enzyme sucrase capacity

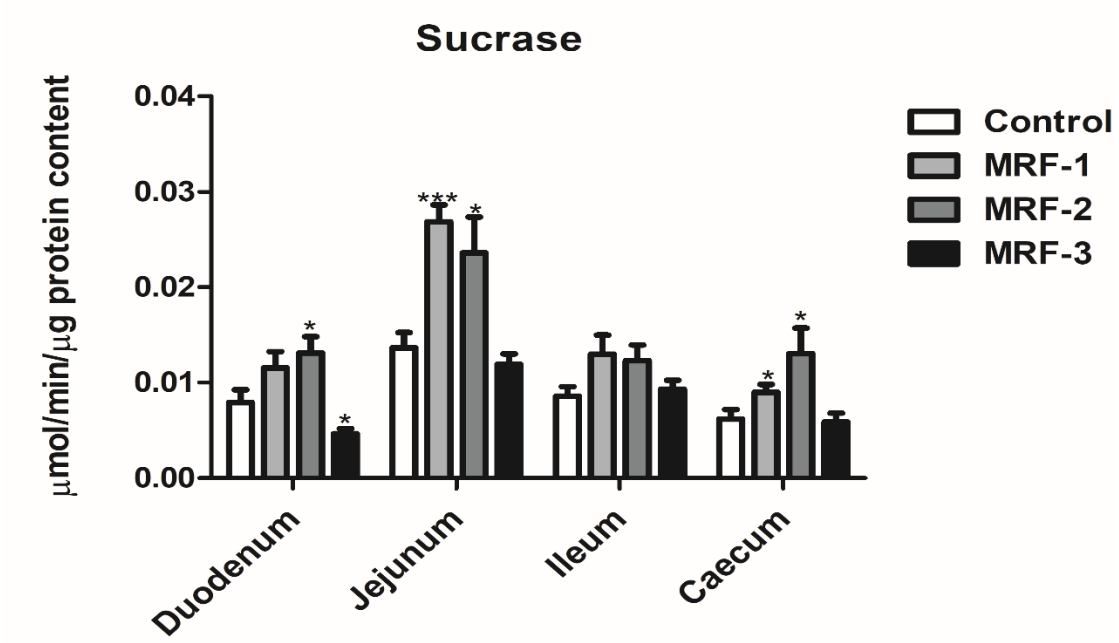



**Figure 3.5 Effect of mannan-rich fraction supplementation on sucrase digestive enzyme capacity**

Digestive enzyme capacity analysis of sucrase enzyme per µg of intestinal tissue lysate in duodenum, jejunum, ileum and caecum on day 35. The four bars represent, from left to right, control unsupplemented, MRF-1 supplement, MRF-2 supplement and MRF-3 supplement. Data are presented as the mean +/- SEM of sucrase activity normalised per µg of homogenate proteins from 10 birds. Statistical significance between control and each treatment was determined using two-tailed Student's t-test (*= P<0.05, ***=P<0.001).

Since the data above suggests that MRF-1 and MRF-2 dietary inclusion can result in enhanced digestive enzyme maltase, it was important to ascertain whether MRF supplementation can alter the enzymatic capacity of the sucrose, an enzyme that catalyse hydrolysis of the disaccharide sucrose. To achieve this, tissue lysates from broiler chickens (n=10) were assayed for sucrase enzyme capacity in the presence of sucrose substrate solution and TGO solution. Digestive enzyme sucrase was chosen for analysis as it hydrolyses sucrose to fructose and glucose, playing a significant role in carbohydrate digestion (Horvatovic *et al.*, 2015). Enzyme capacity was assessed through enzymatic assays. Supplementation of MRF-1 led to a significant increase in sucrase enzyme capacity in the jejunum and the caecum (Fig. 3.5). Incorporation of broiler diet with MRF-2 resulted in a significant increase in enzyme capacity in the duodenum, jejunum and caecum (Fig. 3.5) while integration of MRF-3 into broiler diet led to a significant decrease in the duodenum and a reduction (not reaching statistical significance) in sucrase enzyme capacity in the jejunum and the caecum, compared to the control (Fig. 3.5). These results indicate that MRF-1 and MRF-2 incorporation into broiler diet may result in enhanced digestive enzyme sucrase capacity, permitting the absorption of a greater volume of nutrients. This result was consistent with the findings that MRF-1 and MRF-2 dietary inclusion enhanced the capacity of the disaccharide maltase (Section 3.2.2).

### 3.2.4 MRF-1 and MRF-2 dietary supplementation leads to enhanced alkaline phosphatase enzyme capacity

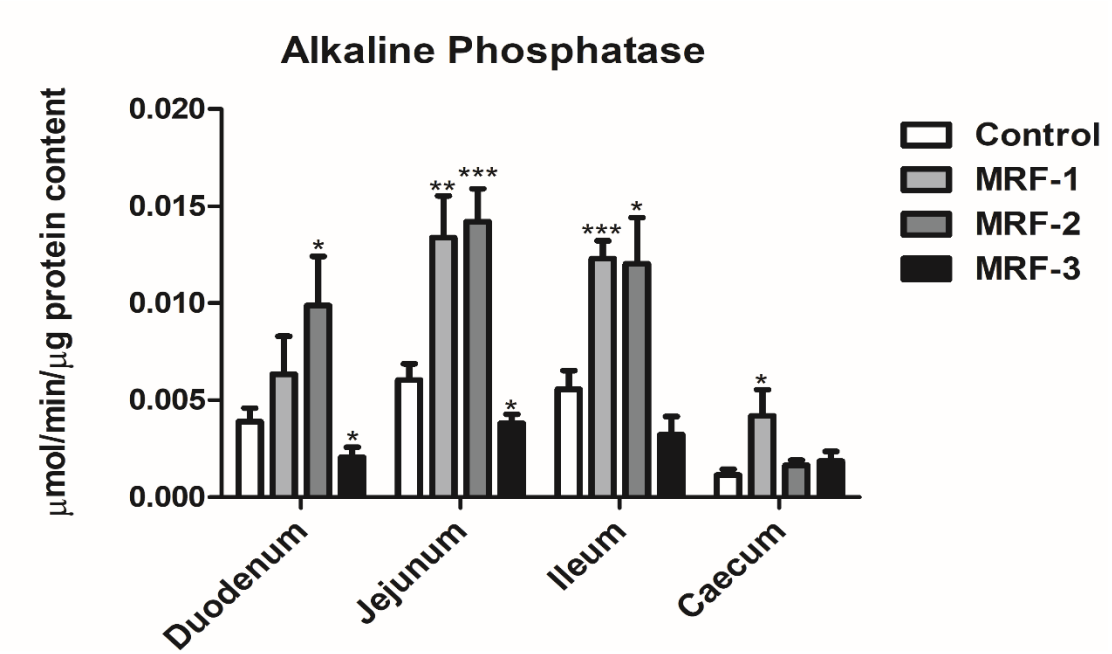


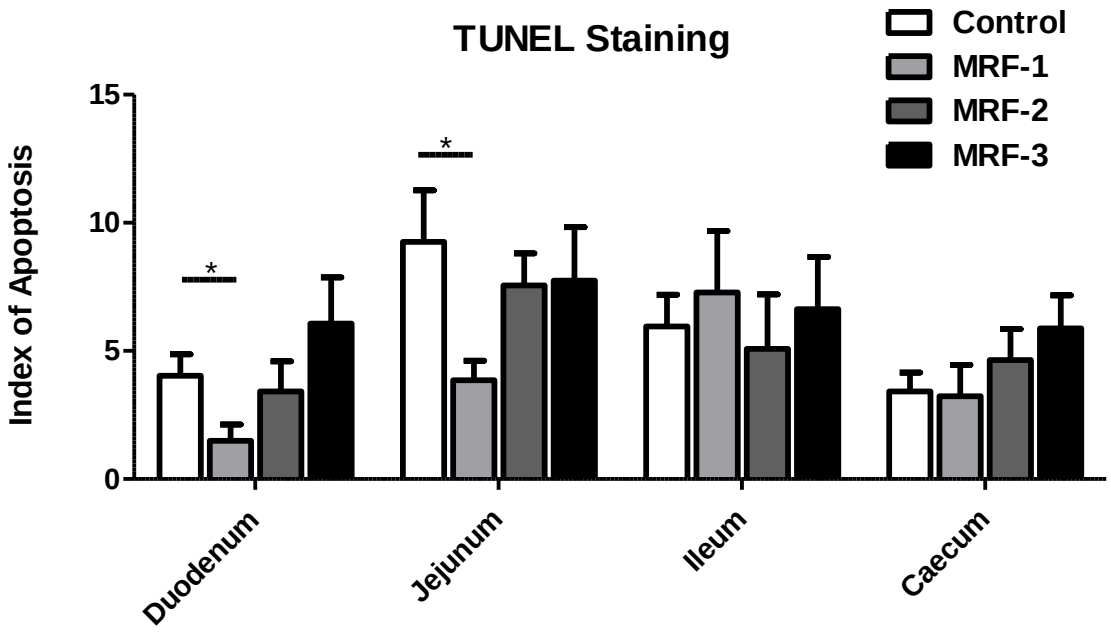

**Figure 3.6 Effect of mannan-rich fraction supplementation on alkaline phosphatase digestive enzyme capacity**

Digestive enzyme capacity analysis of alkaline phosphatase per µg of intestinal tissue lysate in duodenum, jejunum, ileum and caecum on day 35. The four bars represent, from left to right, control unsupplemented, MRF-1 supplement, MRF-2 supplement and MRF-3 supplement. Data are presented as the mean +/- SEM of alkaline phosphatase activity normalised per µg of homogenate proteins from 10 birds. Statistical significance between control and each treatment was determined using two-tailed Student's t-test (*= P<0.05, **=P<0.01, ***=P<0.001).

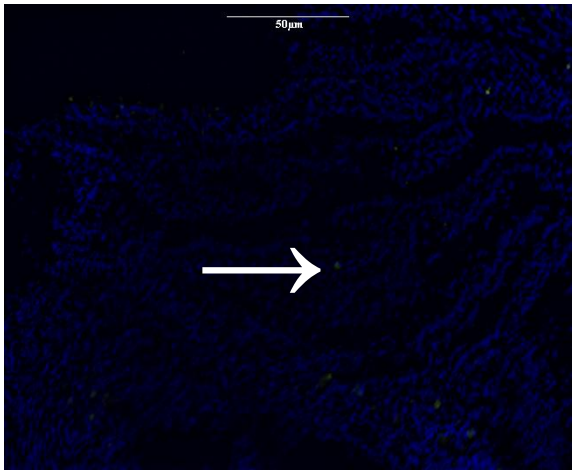
Given that MRF-1 and MRF-2 appear to enhance the enzymatic capacity of the disaccharides maltase and sucrase, potentially increasing the amount of carbohydrates available to the host, the role of MRF incorporation on alkaline phosphatase enzyme capacity was next elucidated. Tissue lysates from broiler chickens (n=10) were assayed for alkaline phosphatase using the ALP assay kit (colourimetric) from Abcam, as per the manufacturer's instructions. Alkaline phosphatase was selected for analysis as it regulates the secretion of bicarbonate, which protects the cells lining the GIT from potential damage due to bacterial invasion (Bilski *et al*., 2017). The enzymatic capacity of ALP was measured through a colourimetric reaction. Incorporation of MRF-1 led to a significant increase in maltase enzyme capacity in the jejunum, ileum and the caecum compared to the control (Fig. 3.6). Supplementation of broiler diet with MRF-2 resulted in significantly enhanced enzyme capacity in the jejunum and the ileum (Fig. 3.6), whereas addition of MRF-3 led to a significant decrease in alkaline phosphatase enzyme capacity in the duodenum and jejunum with a slight increase in the caecum (Fig. 3.6). These results suggest that MRF-1 and MRF-2 integration into broiler diet results in enhanced alkaline phosphatase enzyme capacity, thereby potentially providing increased protection from pathogenic invasion and adhesion.

The overall results from the digestive enzyme capacity analyses indicated that MRF-1 and MRF-2 incorporation into broiler diet resulted in enhanced digestive enzyme capacity, which is consistent with the observed differences in VH and VSA outlined in Section 3.2.1.

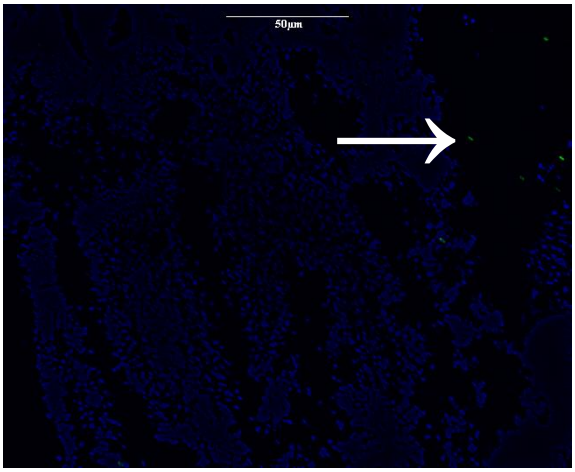
### 3.2.5 Mannan-rich fraction dietary supplementation leads to alteration of *in situ* apoptotic levels in intestinal tissue



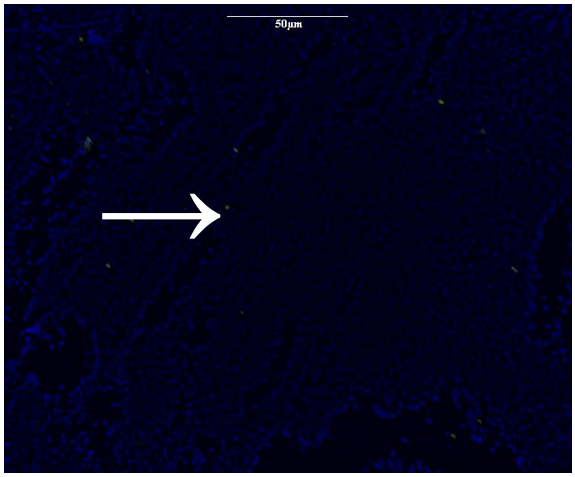

**A**



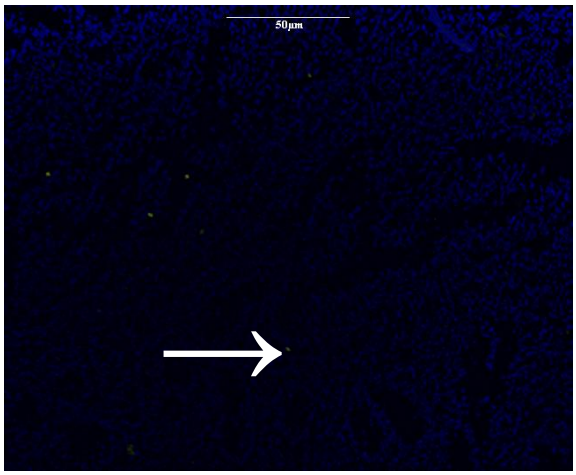

**B**




**C**



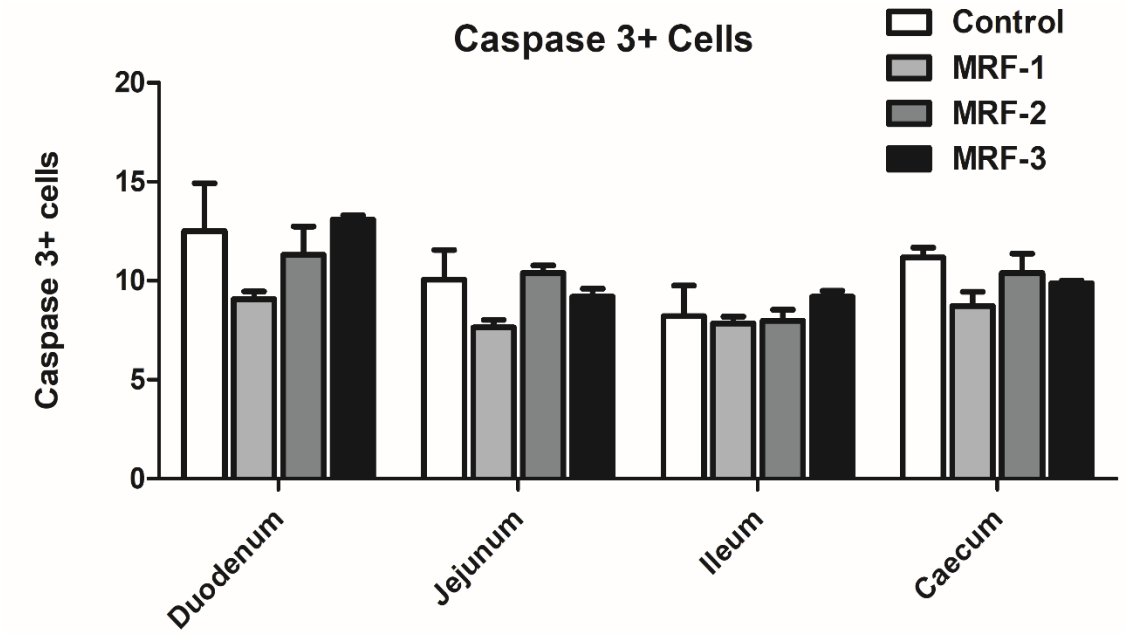

**D**



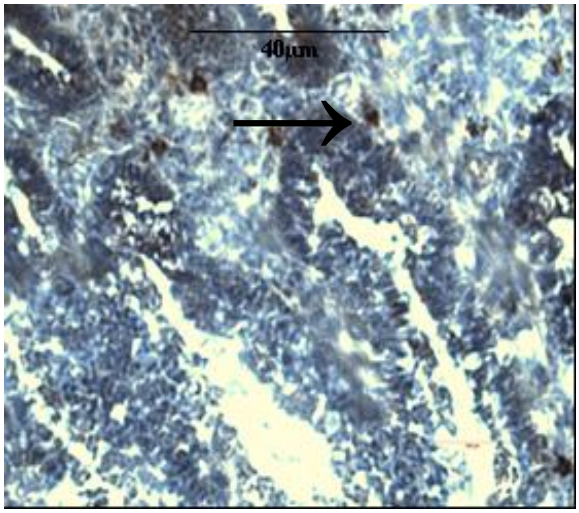

**E**

**Figure 3.7 Effect of mannan-rich fraction supplementation on intestinal apoptosis, as measured by TUNEL staining in broiler chickens at day 35**

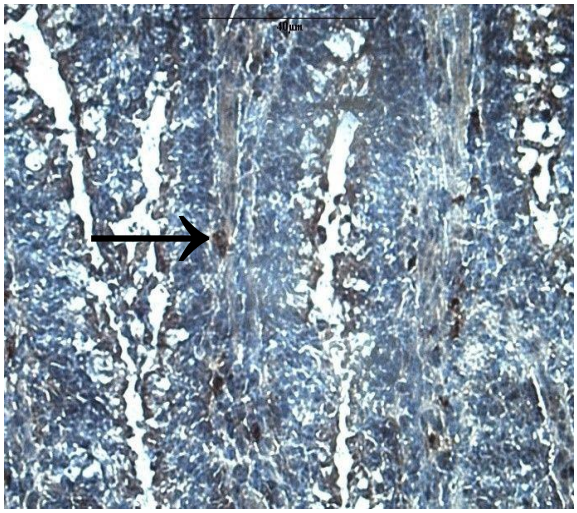
Broiler chickens were supplemented with control chow diets or supplemented with MRF-1, MRF-2, MRF-3. (A) Intestinal tissue was processed for TUNEL staining with index of apoptosis calculated as % of cells that show positive TUNEL staining. Representative images of TUNEL staining of ileal intestinal tissue from chickens fed on (B) Control diet, (C) MRF-1, (D) MRF-2 and (E) MRF-3 are also shown. Data are presented as the mean +/- SEM from 9 birds for TUNEL staining with four sections analysed per bird under 20x magnification. Statistical significance between control and each treatment was determined using two-tailed Student's t-test (*= $P<0.05$).


Caspase 3+ Cells
Control
MRF-1
MRF-2
MRF-3
Caspase 3+ cells
20
15
10
5
0
Duodenum
Jejunum
Ileum
Caecum

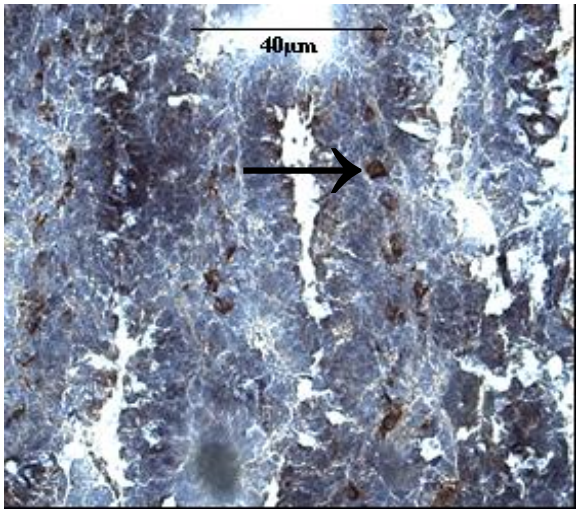
**A**



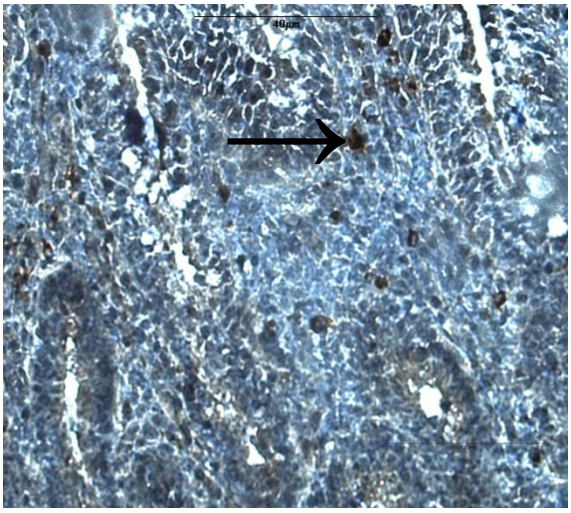

**B**



**C**




**D**



**E**

**Figures 3.8 Effect of mannan-rich fraction supplementation on intestinal apoptosis, as measured by Caspase-3 immunostaining in broiler chickens at day 35 post supplementation**

Broiler chickens were supplemented with control chow diets or supplemented with MRF-1, MRF-2, MRF-3. (A) Intestinal tissue was processed for caspase-3 staining with caspase-3 positive cells indicated as % of cells that show positive caspase-3 staining. Representative images of caspase-3 staining of duodenal intestinal tissue from chickens fed on (B) Control diet, (C) MRF-1, (D) MRF-2 and (E) MRF-3 are also shown. Data are presented as the mean +/- SEM from 5 birds for caspase staining with four sections analysed per bird under 40x magnification. Statistical significance between control and each treatment was determined using two-tailed Student's t-test (*= $P<0.05$

The above data (Section 3.2.2- 3.2.4) suggests that MRF supplementation into broiler chicken diet results in enhanced activity or levels of digestive enzymes that will feed carbohydrate metabolism whilst histological data suggest that supplementation can also change tissue architecture to a form that would suggest increased protective barrier against infection. Given such morphological and structural changes, studies were then conducted to assess if MRF supplementation could regulate cell death in intestinal tissue. Tissue samples were first examined for levels of apoptosis. Apoptosis is an immunologically silent form of cell death, essential for the maintenance of cellular homeostasis (Szondy *et al*., 2017). In order to assess the levels of apoptosis, TUNEL staining and caspase-3 immunohistochemical analysis was carried out on each intestinal segment. DNA fragmentation in intestinal tissue sections from broiler chickens was identified using the *in situ* cell death detection kit (fluorescein) from Roche, as per the manufacturer's instructions. TUNEL-positive cells (cells undergoing extensive DNA damage as a consequence of the process of apoptosis) were quantified in each intestinal section from a total of 9 birds per treatment. Supplementation of MRF-1 (Fig. 3.7C) into broiler diet resulted in a significant decrease in TUNEL positive cells in the duodenum and jejunum (Fig. 3.7A). The incorporation of MRF-2 (Fig. 3.7D) and MRF-3 (Fig. 3.7E) to the diet had no statistically significant effects on the number of TUNEL positive cells (Fig. 3.7A). The effect of MRF-1 (Fig. 3.7C) incorporation into broiler diet on cellular apoptosis seemed to be less pronounced towards the ileum and the caecum. Since TUNEL is a measure of DNA fragmentation, these data were also supported by analysis of processed levels of caspase-3, one of the executioner caspases in the apoptotic pathway. Immunohistochemical analysis of caspase-3 positive cells (Fig. 3.8A) revealed a similar trend to the results observed for TUNEL staining (Fig. 3.7A). Dietary inclusion of MRF-1 (Fig. 3.8C) resulted in no change in caspase-3 positive cells in the duodenum, jejunum and the caecum (Fig. 3.8A). Whilst integration of MRF-2 (Fig. 3.8D) into broiler diet led to a decrease in caspase-3 positive cells

in the duodenum and caecum, this did not reach statistical significance (Fig. 3.8A). Supplementation of MRF-3 (Fig. 3.8E) into broiler diet had little effect on the presence of caspase-3 positive cells, compared to the control (Fig 3.8B).

These results suggest that dietary inclusion of MRF-1 into broiler diet may reduce the levels of apoptosis in intestinal tissue.

### 3.2.6 Mannan-rich fraction supplementation resulted in a decrease in RIPK3 expression levels

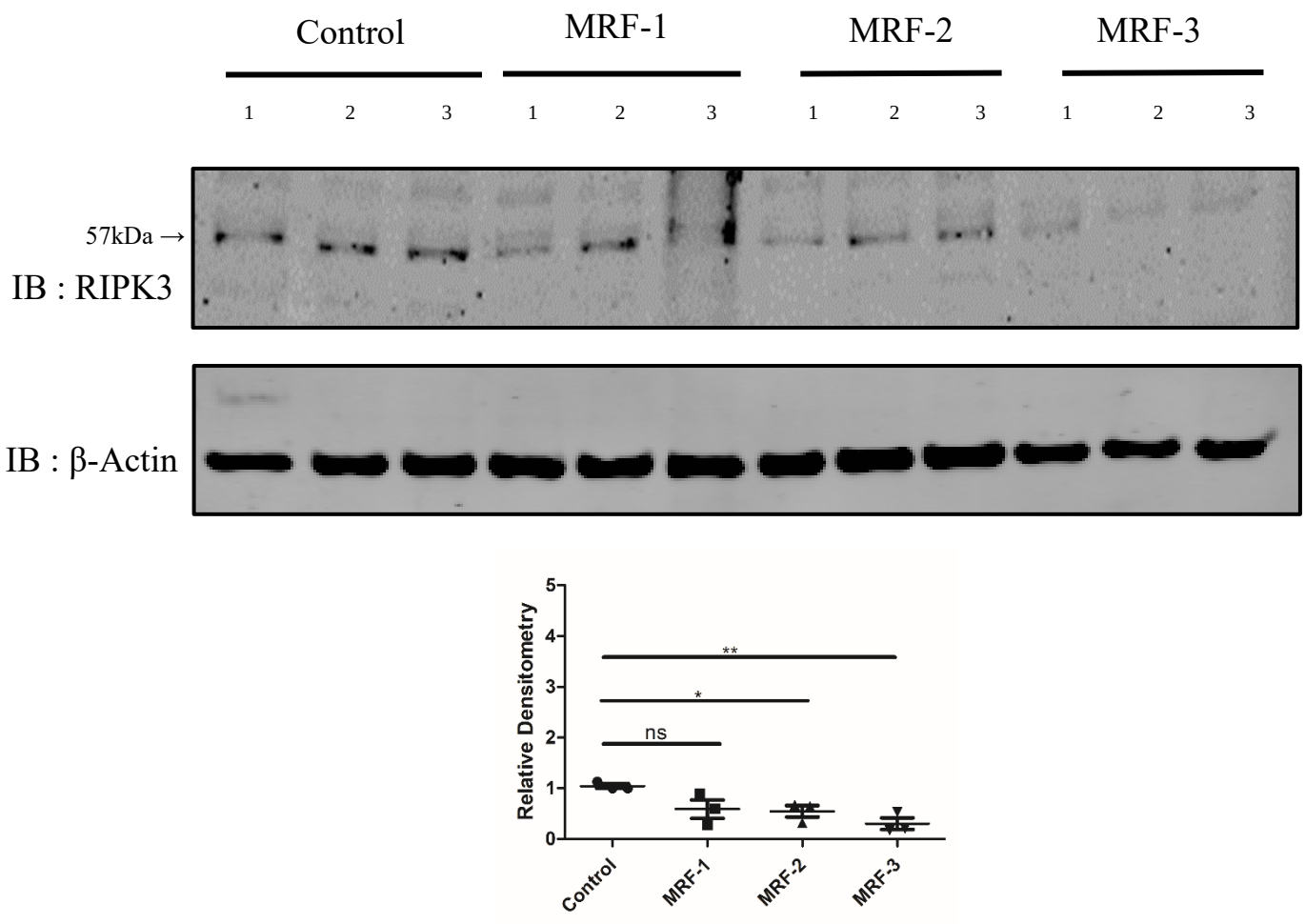
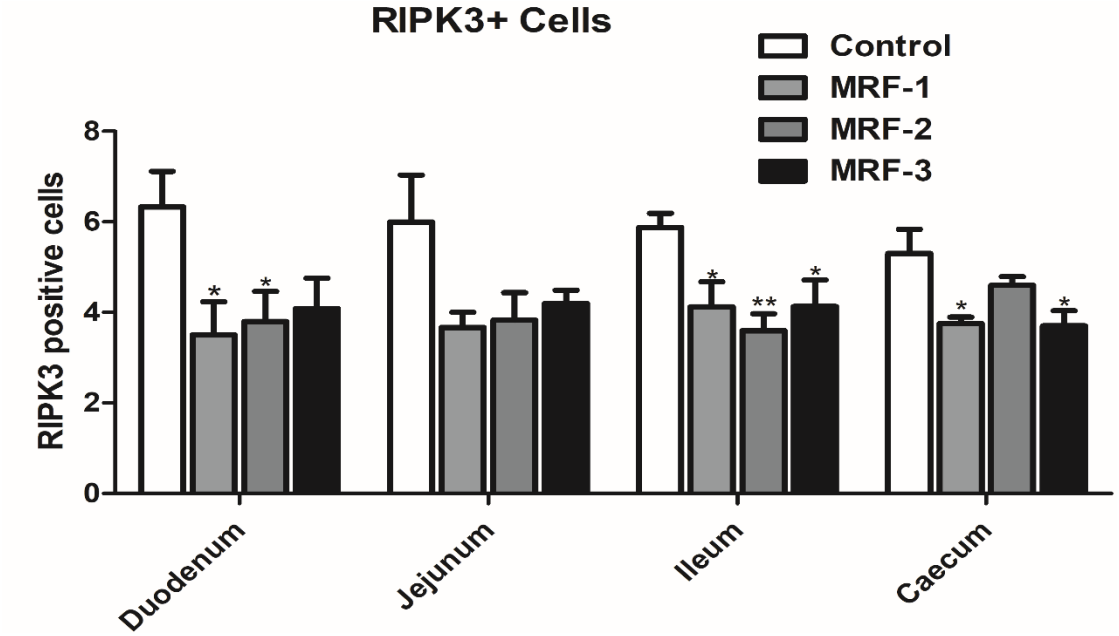
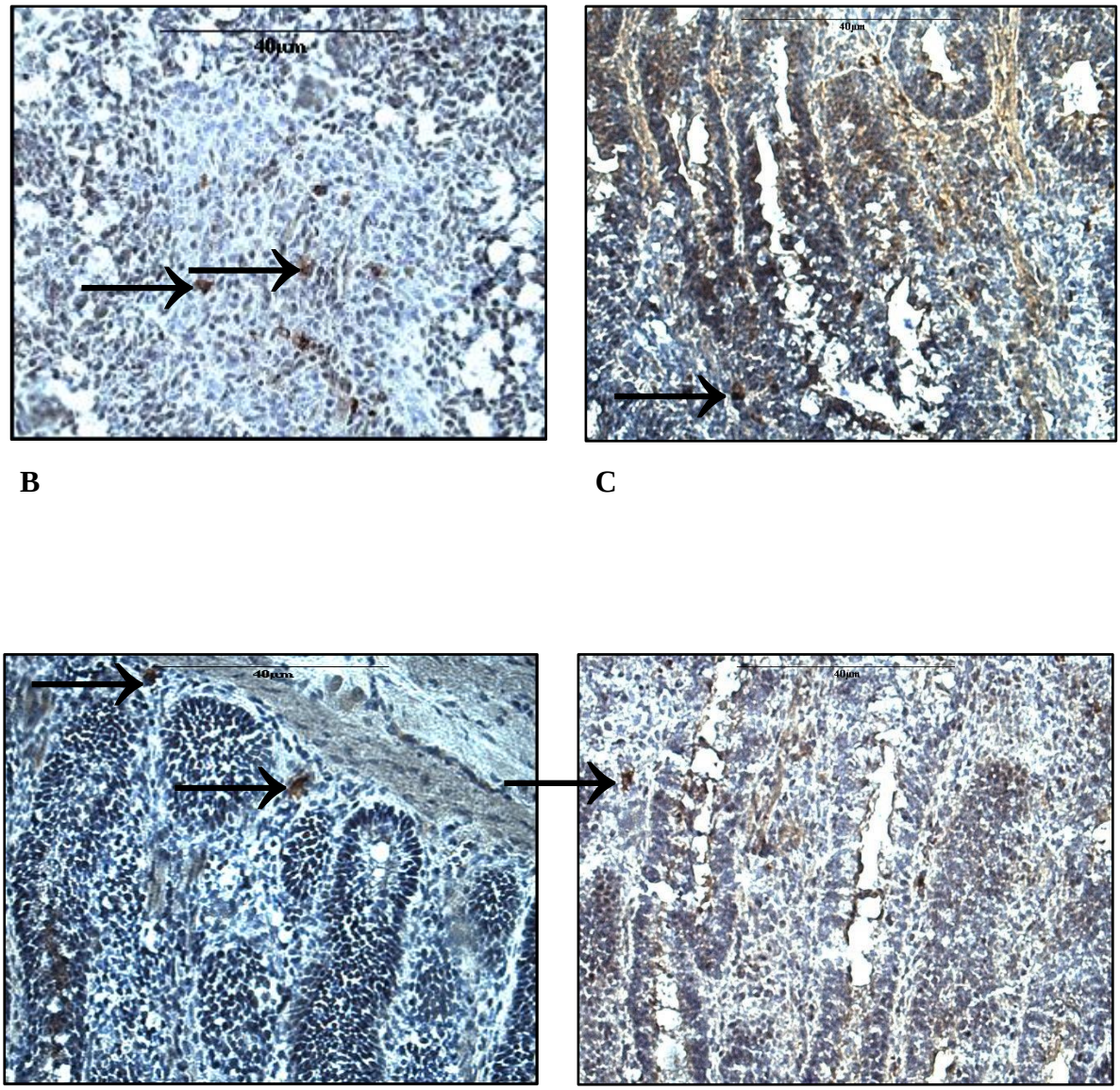



**Figure 3.9 Effect of mannan-rich fraction supplementation on RIPK3 protein expression levels**

Immunoblot analysis of RIPK3 in tissue lysates from control unsupplemented and mannan-rich fraction supplemented broiler chickens on day 35. β-actin was used as a loading control. Each lane represents a separate biological replicate (n=3). Data for relative densitometry was relative to sample C2. (*, $P < 0.05$, **, $P < 0.01$). Error bars, SEM.


RIPK3+ Cells
Control
MRF-1
MRF-2
MRF-3
RIPK3 positive cells
8
6
4
2
0
*
*
*
**
*
*
*
Duodenum
Jejunum
Ileum
Caecum

**A**




**Figures 3.10 Effect of mannan-rich fraction supplementation on *in situ* necroptotic levels in broiler chickens at day 35.**

Broiler chickens were supplemented with control chow diets or supplemented with MRF-1, MRF-2, MRF-3. (A) Intestinal tissue was processed for RIPK3 staining with RIPK3positive cells indicated as % of cells that show positive RIPK3 staining. Representative images of RIPK3 staining of duodenal intestinal tissue from chickens fed on (B) Control diet, (C) MRF-1, (D) MRF-2 and (E) MRF-3 are also shown. Data are presented as the mean +/- SEM from 5 birds for caspase staining with four sections analysed per bird under 40x magnification. Brown staining (labelled) indicates RIPK3 staining. Statistical significance between control and each treatment was determined using two-tailed Student's t-test (*= $P<0.05$, **=$P<0.01$).

Given that mannan-rich fraction supplementation in broiler diets has been shown to potentially decrease apoptotic levels in intestinal tissue, it's effect on RIPK3 (a marker of necroptosis) expression levels was next explored. Necroptosis is generally induced in response to a bacterial or viral infection (Pasparakis & Vandenabeele, 2015). Necroptotic cell death, a type of programmed necrotic cell death, is characterised by the activation of MLKL into phospho-MLKL by RIPK3 (Kim and Pasparakis, 2014). The necroptotic marker RIPK3 was chosen for analysis due to the unavailability of phospho-MLKL antibodies reactive to chickens. RIPK3 (in addition to MLKL) is present in both the cytoplasm and nucleus of the cell, playing a key role in the formation of the necrosome (Nogusa *et al*., 2016). Tissue lysates from each intestinal section were analysed for protein expression through western blot from a total of 3 birds per treatment. The incorporation of each dietary supplement led to a decrease in RIPK3 expression levels, and a significant decrease upon MRF-2 (Fig. 3.9) and MRF-3 (Fig. 3.9) dietary incorporation.

To further characterise the effect of MRF supplementation on RIPK3 expression levels, it was necessary to assess its effect though immunohistochemical analysis. Immunohistochemical analysis was carried out on each intestinal segment on a total of 5 birds per treatment. Incorporation of MRF-1 (Fig. 3.10C) resulted in a significant decrease in RIPK3 levels in the duodenum, ileum and the caecum compared to the control (Fig. 3.10B). Dietary supplementation of MRF-2 (Fig. 3.10D) into broiler diet led to a significant decrease in RIPK3 cells in the duodenum and the ileum. The inclusion of MRF-3 (Fig. 3.10E) into broiler diet resulted in a significant decrease in RIPK3 levels in the ileum and the caecum, with a slight decrease present in the duodenum and the jejunum.

These results indicate that MRF incorporation into broiler diet may result in a decrease in RIPK3 expression levels in intestinal tissue. These results are consistent with the decrease in apoptotic levels observed in Section 3.2.5

### 3.2.7 Mannan-rich fraction supplementation resulted in alterations in p-Akt protein expression

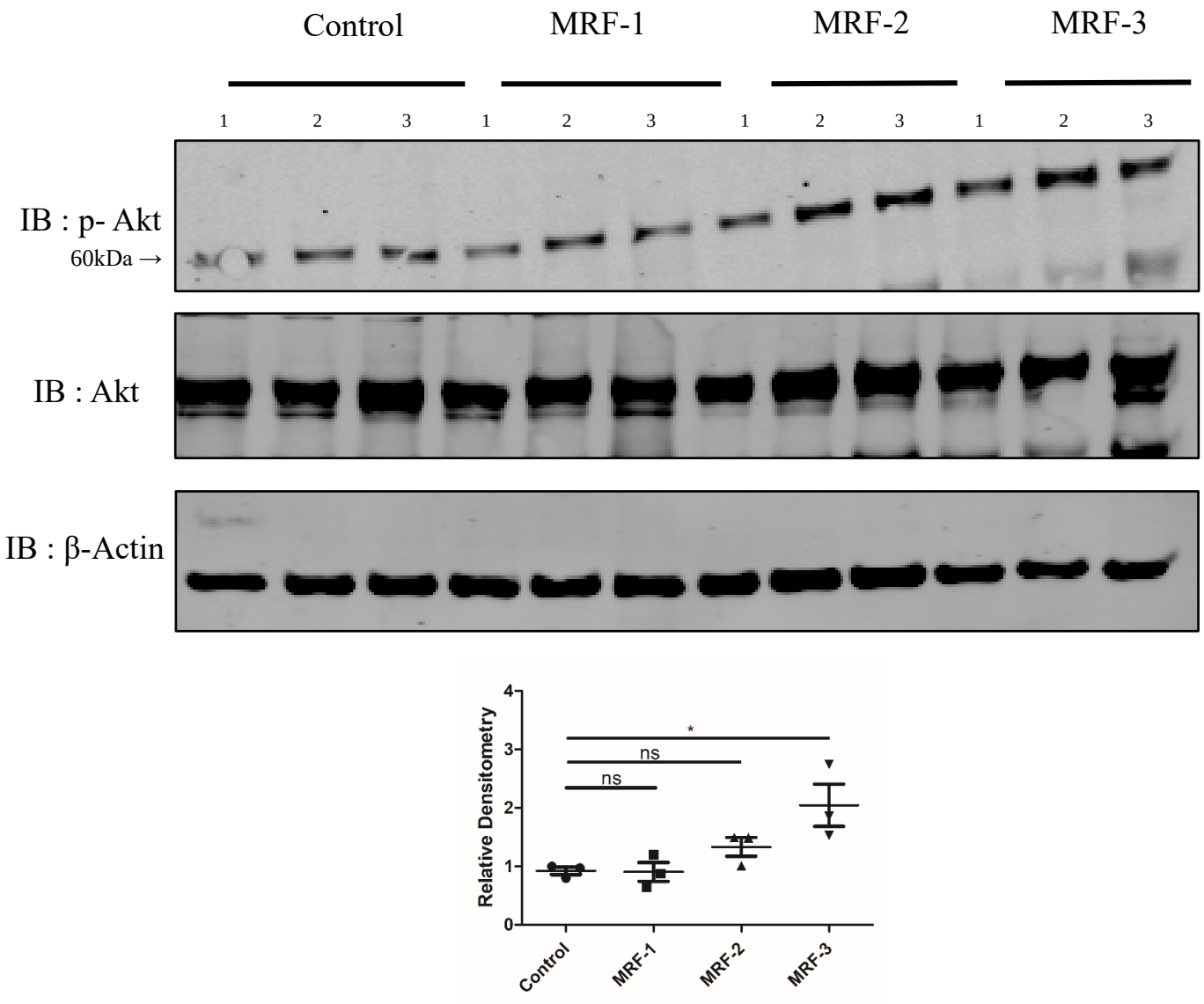



**Figure 3.11 Effect of mannan-rich fraction supplementation on p-Akt protein expression levels**

Immunoblot analysis of p-Akt in tissue lysates from control unsupplemented and mannan-rich fraction supplemented broiler chickens on day 35. β-actin was used as a loading control. Each lane represents a separate biological replicate (n=3). Data for relative densitometry was relative to sample C2. (*, $P < 0.05$, **, $P < 0.01$). Error bars, SEM.

As a result of the observed decrease in RIPK3 expression levels upon MRF incorporation, the effect of MRF supplementation on p-Akt expression levels was next explored. Phospho-Akt is intrinsically linked to several signalling pathways with implications for cell survival, growth and inhibition of apoptosis through the inactivation of apoptosis-associated molecules such as Bad and c-Raf (McCubrey *et al.*, 2007). Tissue lysates from each intestinal section were analysed for protein expression through western blot from a total of 3 birds per treatment. The addition of MRF-1 into broiler diet resulted in a negligible effect on p-Akt expression (Fig. 3.11). Supplementation of MRF-2 led to a slight increase compared to the control while dietary inclusion of MRF-3 resulted in a significant increase in p-Akt expression (Fig. 3.11).

## 3.2.8 Mannan-rich fraction supplementation had no effect on expression of inflammatory cytokines in serum

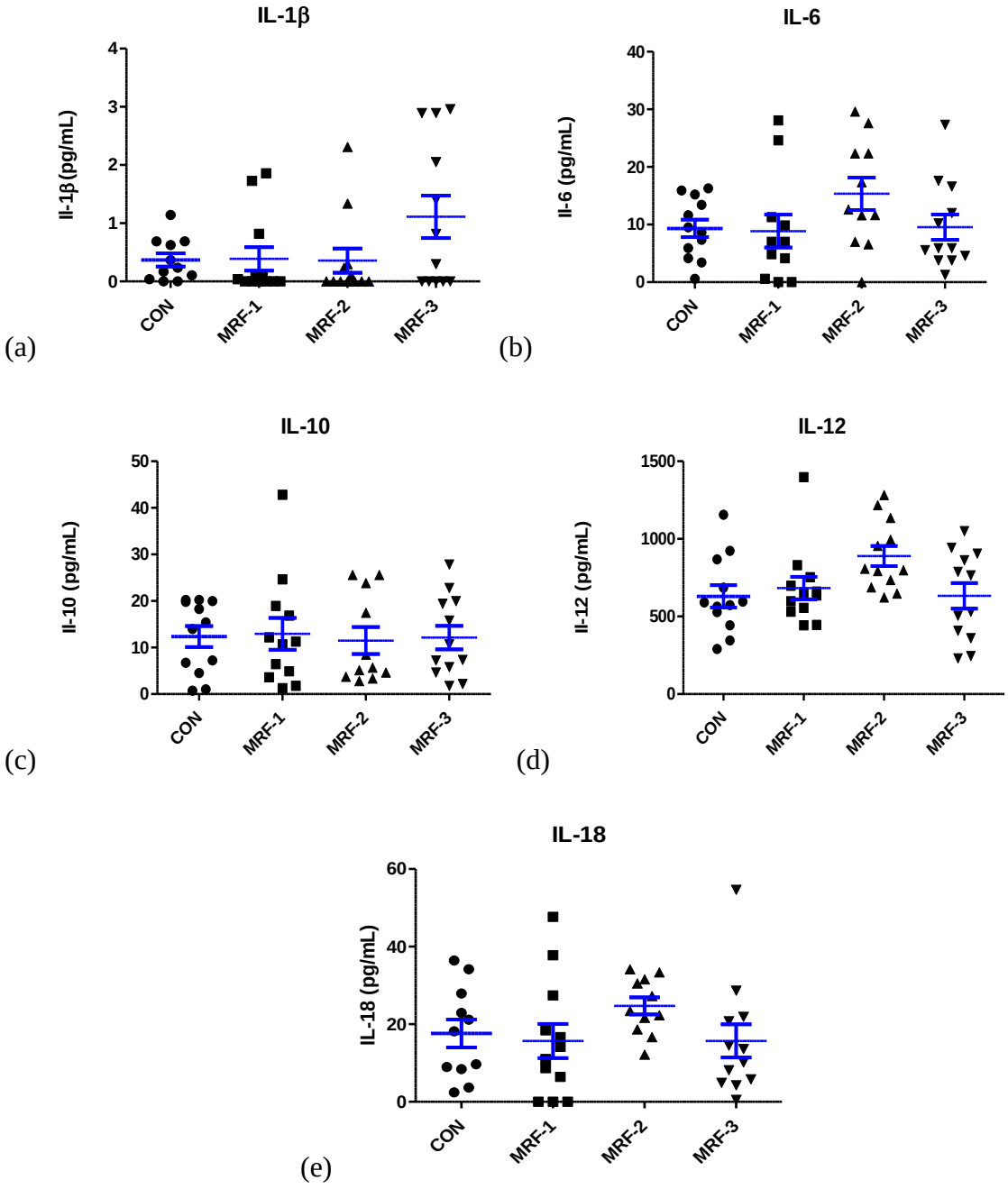



**Figure 3.12 Effect of mannan-rich fraction supplementation on serum levels of pro- and anti-inflammatory cytokines**

Serum cytokine analysis of control and mannan-rich fraction supplemented broiler chickens (n=12). Broiler chickens were sacrificed and serum analysed by ELISA for (a) IL-1β (b) IL-6 (c) IL-10 (d) IL-12 and (e) IL-18. Data are presented as the mean +/- SEM from 12 birds. Statistical significance was determined using two-tailed Student's t-test (*, $p < 0.05$).

Given that the tissue morphological analyses suggested that MRF supplementation may improve tissue integrity and therefore enhance the ability of the GIT to prevent bacterial adhesion, the next step involved examining if MRF incorporation altered the expression levels of inflammatory cytokines in serum. Serum was collected from broiler chickens on day 35 and the expression levels of IL-1β (Fig. 3.12a), IL-6 (Fig. 3.12b), IL-10 (Fig. 3.12c), IL-12 (Fig. 3.12d), and IL-18 (Fig. 3.12e) were assessed by ELISA. Analysis of IL-12 and IL-18 was carried out using a chicken IL-12 and chicken IL-18 ELISA kit (Abbexa) and was performed as per manufacturer's instructions. Analysis of IL-1β, IL-6 and IL-10 was carried out using specialised kits from Elabscience and was performed as per manufacturer's instructions. None of the supplements affected the serum levels of any pro-inflammatory cytokines.

### 3.2.9 Mannan-rich fraction supplementation alters inflammatory cytokine expression levels in intestinal tissue

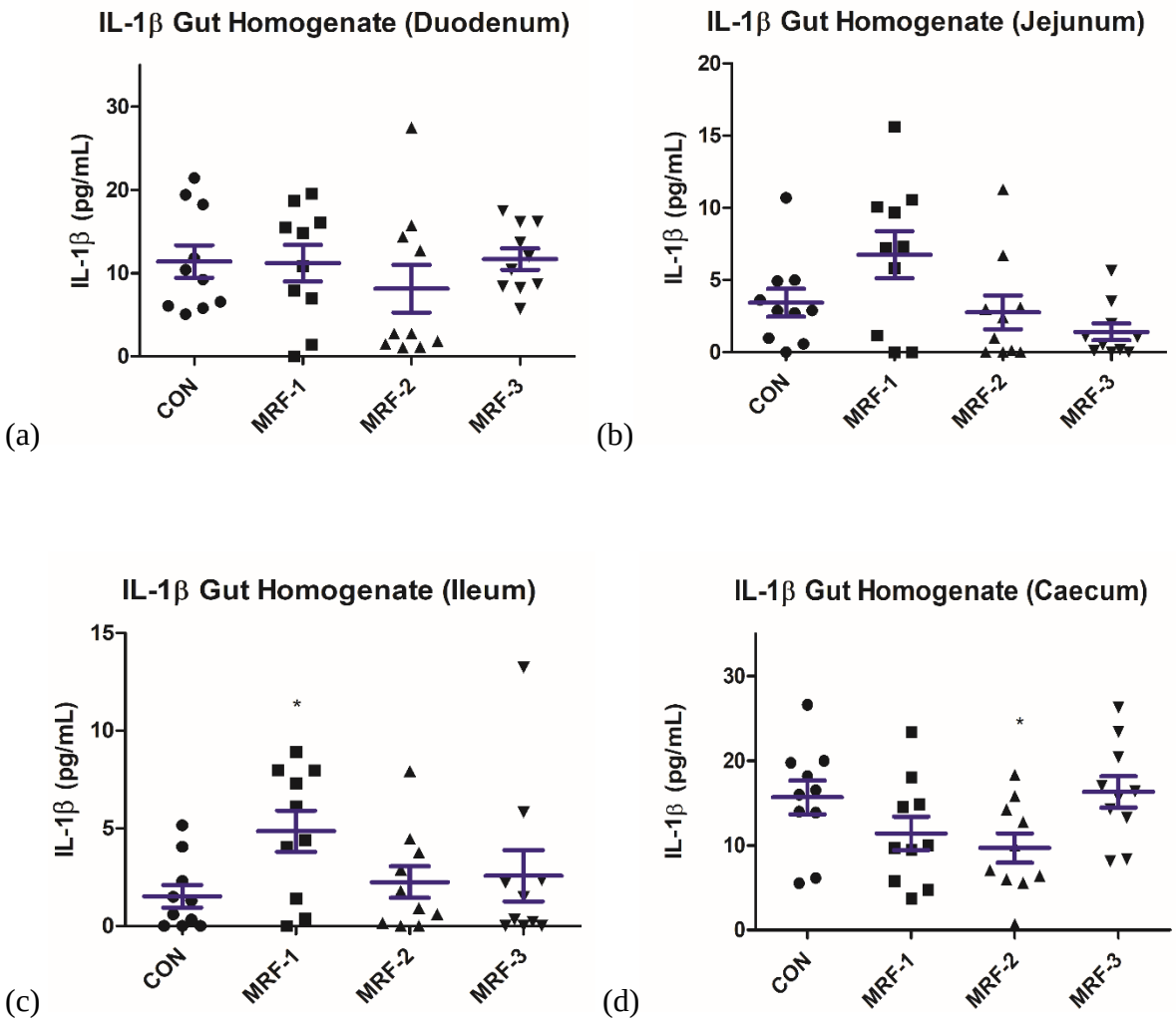



**Figure 3.13 Effect of mannan-rich fraction supplementation on intestinal expression of IL-1β**

Tissue lysate cytokine analysis of control and mannan-rich fraction supplemented broiler chickens in the (a) duodenum, (b) jejunum, (c) ileum and (d) caecum (n=10). Broiler chickens were sacrificed and tissue lysate analysed by ELISA for IL-1β expression. Data are presented as the mean +/- SEM from 10 birds. Statistical significance was determined using two-tailed Student's t-test (*, $p < 0.05$).

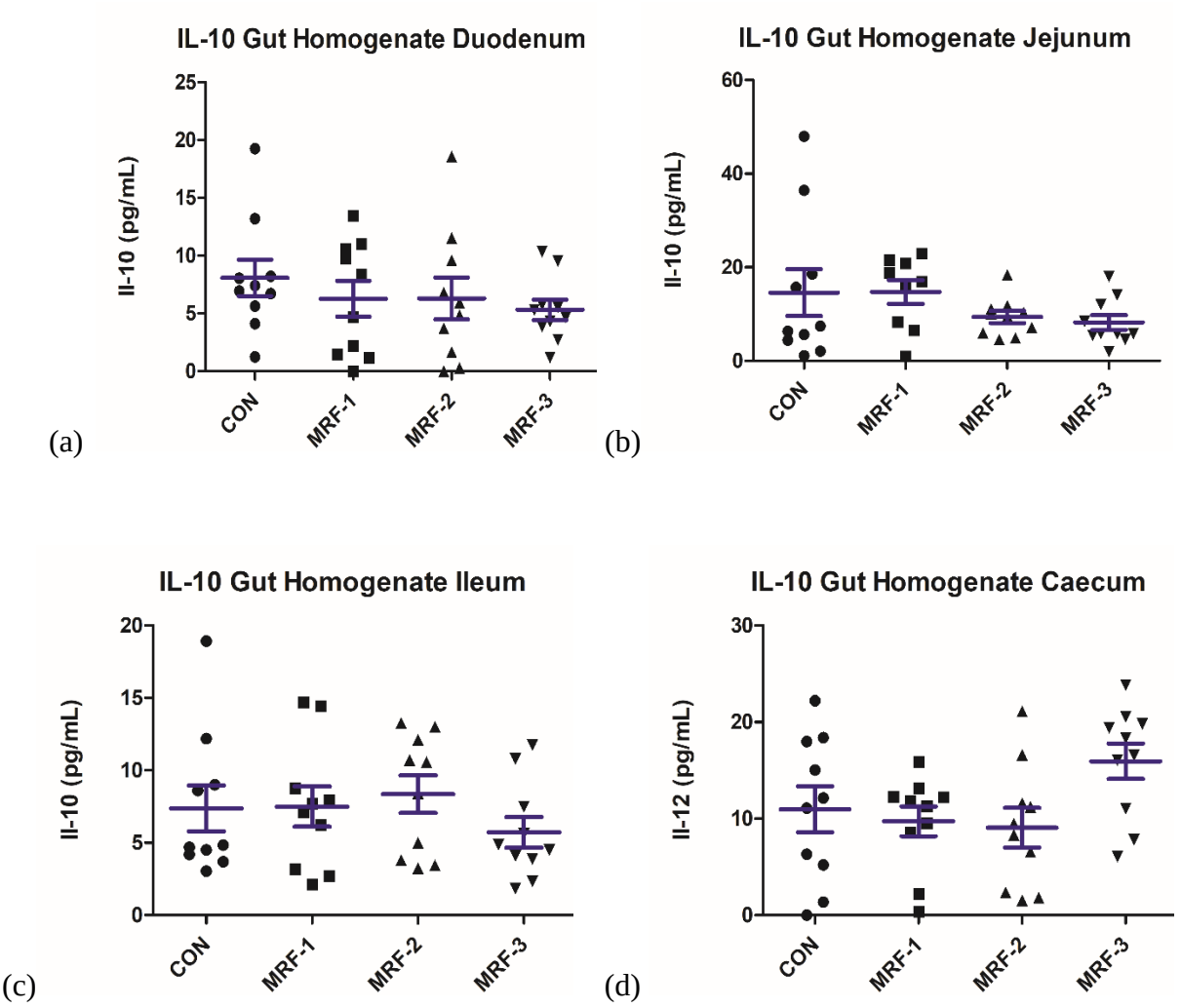



**Figure 3.14 Effect of mannan-rich fraction supplementation on intestinal expression of IL-10**

Tissue lysate cytokine analysis of control and mannan-rich fraction supplemented broiler chickens in the (a) duodenum, (b) jejunum, (c) ileum and (d) caecum (n=10). Broiler chickens were sacrificed and tissue lysate analysed by ELISA for IL-10 expression. Data are presented as the mean +/- SEM from 10 birds. Statistical significance was determined using two-tailed Student's t-test (*, $p < 0.05$).

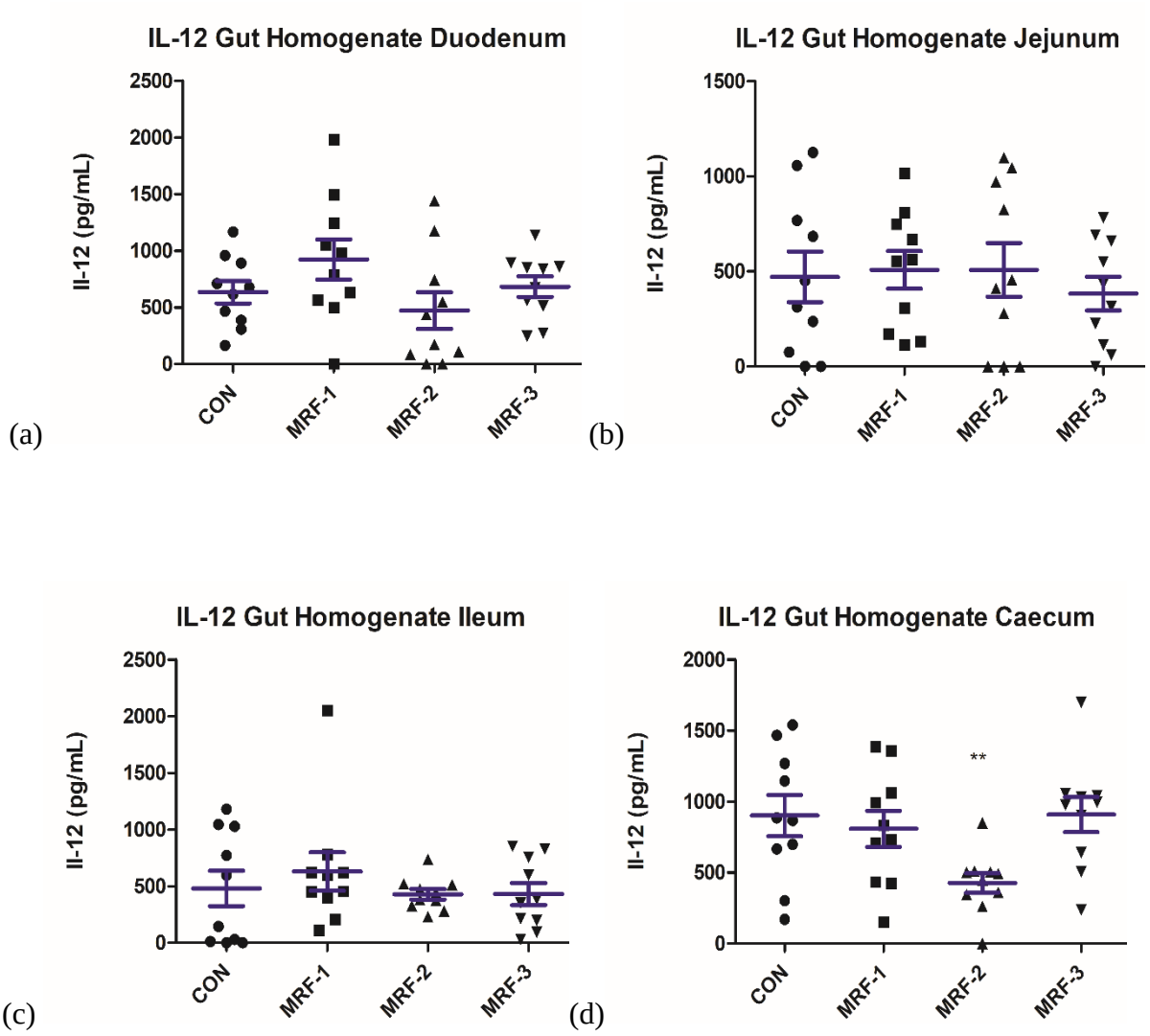



**Figure 3.15 Effect of mannan-rich fraction supplementation on intestinal expression of IL-12**

Tissue lysate cytokine analysis of control and mannan-rich fraction supplemented broiler chickens in the (a) duodenum, (b) jejunum, (c) ileum and (d) caecum (n=10). Broiler chickens were sacrificed and tissue lysate analysed by ELISA for IL-12 expression. Data are presented as the mean +/- SEM from 10 birds. Statistical significance was determined using two-tailed Student's t-test (*= $p < 0.05$, **= $p<0.01$).

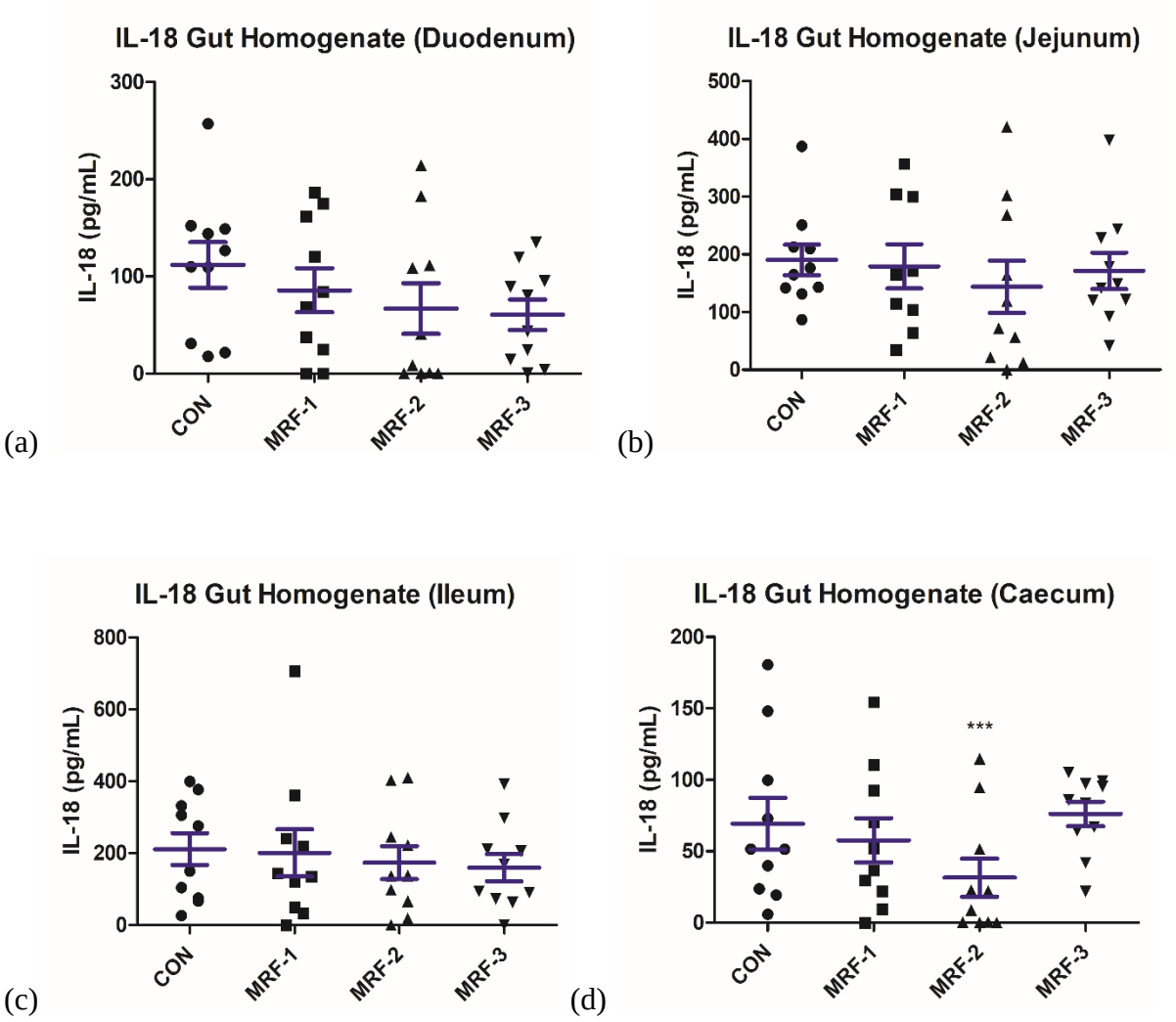



**Figure 3.16 Effect of mannan-rich fraction supplementation on intestinal expression of IL-18**

Tissue lysate cytokine analysis of control and mannan-rich fraction supplemented broiler chickens in the (a) duodenum, (b) jejunum, (c) ileum and (d) caecum (n=10). Broiler chickens were sacrificed and tissue lysate analysed by ELISA for IL-18 expression. Data are presented as the mean +/- SEM from 10 birds. Statistical significance was determined using two-tailed Student's t-test (*= $p < 0.05$, ***= $p<0.001$).

While the work in the previous section indicated that MRF dietary inclusion had little effect on serum inflammatory cytokine expression levels, the more localised role of MRF supplementation in controlling intestinal expression of inflammatory cytokines was next explored. Tissue lysates from broiler chickens (n=10) were assayed for the expression level of inflammatory cytokines IL-1β (Fig. 3.13), IL-10 (Fig. 3.14), IL-12 (Fig. 3.15) and IL-18 (Fig. 3.16) in each intestinal section. The analysis for IL-1β and IL-10 was carried out using chicken ELISA kits from Elabscience as per manufacturer's instructions. The analysis for IL-12 and IL-18 was carried out using chicken ELISA kits from Abbexa and as per manufacturer's instructions.

Incorporation of MRF-1 led to a slight increase in IL-1β expression in the jejunum (Fig. 3.13B) and a significant increase in expression in the ileum (Fig. 3.13C) compared to the control (Fig.3.13C). There was no change in IL-1β expression in the caecum (Fig. 3.13D). Dietary addition of MRF-2 resulted in a little change in IL-1β expression in duodenal (Fig. 3.13A) and jejunal (Fig. 3.13B) tissue, with a significant decrease observed in the caecum (Fig 3.13D). A slight increase in IL-1β expression was observed in the ileum (Fig. 3.13C). The expression levels of IL-1β upon MRF-3 dietary inclusion was negligible in each intestinal tissue (Fig. 3.13A-D).

Incorporation of MRF-1 led to a reduction in IL-10 (Fig. 3.14) expression in each intestinal tissue analysed with the exception of the ileum (Fig. 3.14C), however this did not reach statistical significance. The inclusion of MRF-2 into broiler diet resulted in a slight decrease in IL-10 expression in each intestinal tissue, with the exception of the ileum (Fig 3.14C). The expression levels of IL-10 upon MRF-3 dietary inclusion was decreased in the duodenum (Fig. 3.14A), jejunum (Fig. 3.14B) and the ileum (Fig. 3.14C), however this did not reach statistical significance. The expression level of IL-10 was unchanged in the caecum (Fig. 3.14D).

Incorporation of MRF-1 led to a slight increase in IL-12 expression in each intestinal tissue analysed (Fig. 3.15A-D) with the exception of the caecum (Fig. 3.15D), where a slight reduction was observed. Supplementation of broiler diet with MRF-2 resulted in a decrease in IL-12 expression in the duodenum (Fig. 3.15A) and ileum (Fig. 3.15C) compared to the control (Fig. 3.15C), however this did not reach statistical significance. There was a significant decrease in IL-12 expression levels in the caecum (Fig. 3.15D). The change in expression levels of IL-12 upon MRF-3 dietary inclusion was negligible in the duodenum (Fig. 3.15A), ileum (Fig. 3.15C) and the caecum (Fig. 3.15D). The expression level of IL-12 was unchanged in the jejunum (Fig. 3.15B), upon MRF-3 incorporation.

Dietary incorporation of broiler diet with MRF-1 led to a consistent decrease in IL-18 expression in each intestinal tissue analysed (Fig. 3.16A-D). The inclusion of MRF-2 resulted in a decrease in IL-18 expression, with a significant decrease in expression levels in the caecum (Fig 3.18D). The alteration of IL-18 expression upon MRF-3 dietary incorporation was negligible in the jejunum (Fig. 3.18B). The expression level of IL-18 was decreased in the duodenum (Fig. 3.18A) and ileum (Fig. 3.18C). MRF-3 addition into broiler diet led to an increase in IL-18 expression in the caecum (Fig. 3.18D).

These results suggest that MRF incorporation into broiler diets may alter the expression levels of inflammatory cytokines in tissue lysates. MRF inclusion has been shown to generally increase IL-1β expression levels in this study, which is consistent with previous findings incorporating alternative mannan-rich fractions (Lamkanfi *et al.*, 2009; Saegusa *et al.*, 2009). IL-1β and IL-18 are closely linked to the NLRP3 inflammasome in mammals (Kesavardhana and Kanneganti, 2017). This association is not yet well defined in avian immunology. In mammals, the activation of caspase-1 leads to the upregulation of both IL-1β and IL-18 however in avians, pro- IL-1β does not contain a cleavage site for caspase-1 (Ye *et al.*, 2015). This may suggest an alternative activation mechanism for pro- IL1β activation and perhaps an

alternative mode of action. Further research is necessary to fully elucidate the mechanism of pro- IL-1β processing. The role of IL-18 in immunology remains heavily debated. Increased IL-18 expression had initially been associated with enhanced epithelial layer integrity and cellular proliferation (Dupaul-Chicoine *et al*., 2010; Zaki *et al*., 2010). Recent evidence has suggested a link between increased IL-18 expression levels and disease progression in addition to dysregulation of the mucosal barrier (Nowarski *et al*., 2015). Nowarski *et al.* (2015), have also reported that increased IL-18 expression may lead to the inhibition of goblet cell formation, therefore resulting in a decreased mucus layer thickness. The results observed for MRF incorporation on IL-18 expression are consistent with this finding, as are the results for mucus layer thickness and goblet cell density in Section 3.2.1.

The above results suggest an effect of MRF supplementation on inflammatory cytokine expression levels, however the consequences of these alterations remain somewhat unclear.

# Chapter 4: Discussion

The poultry industry is one of the most rapidly expanding sectors worldwide (GLEAM 2). Almost 100 million tonnes of poultry meat is produced globally each year to meet the demands of a continually-growing population (Mottet and Tempio, 2017). With the ever-increasing expansion of the poultry industry and poultry meat production, the development of effective dietary supplements is of critical importance. Advances in the formulation and development of naturally-derived dietary supplements has been brought into sharp focus as a result of the ban on antibiotic growth promoters (AGPs) (Verraes *et al*., 2013). The ban on AGP dietary incorporation into animal feed resulted initially in an increase in disease incidence and animal mortality rates (Schlundt and Aarestrup, 2017). The increased disease incidence and mortality rates led to much-improved efforts to develop effective natural feed additives. Investigations into the efficacy of natural feed additives initially focussed primarily on observed differences on bird weight gain and feed-conversion ratios (Corrigan *et al*., 2017; Demir *et al*., 2005; Taylor *et al*., 2003; Wood *et al*., 1996). Furthermore, these studies demonstrated that an increased weight gain and an improved broiler performance could be obtained upon natural feed dietary supplementation. Recently, studies determining the effect of natural feed additives in animal diets have progressed to examine the effects of supplementation on animal health through analysis of various markers of intestinal health and function (Kiczorowska *et al*., 2017; Lillehoj and Lee, 2012). The majority of markers employed for intestinal health and function analysis consist of components of the innate immune system and mucosal integrity (Kiczorowska *et al*., 2017; Niewold, 2007). These components may include cell death markers, inflammatory cytokine expression levels in addition to the enumeration of goblet cell presence. The structure and integrity of the mucosal layer is critically important for optimal nutrient absorption in addition to host defence from pathogenic invasion and adhesion (Petersson *et al*., 2011). Dysregulation of the mucus layer has been associated with impaired nutrient transport and increased pathogenic adhesion (Cornick *et al*., 2015). MRF-containing supplements

derived from the cell wall of *Saccharomyces cerevisiae* may confer the host with an enhanced innate response and improved intestinal function. For this reason, investigations of the effects of mannan-rich fraction dietary inclusion into broiler diet are of vital importance for elucidating the potential benefits of MRF incorporation on broiler intestinal health and immunity.

While the inclusion of mannan-rich fractions has been postulated to affect broiler intestinal health (Corrigan *et al*., 2017), the work presented in this thesis demonstrates the potential role mannan-rich fractions may have on broiler digestive enzyme capacity, tissue morphology, serum and tissue lysate inflammatory cytokine expression, cell death and the protein expression levels of signalling components. In order to achieve this, a trial involving 492 male broiler chickens was carried out. Broilers were split randomly into 4 groups and fed either a basal diet or a basal diet including a mannan-rich fraction supplement. On day 35, serum and tissue samples were obtained, processed, and stored for future analyses.

The majority of nutrient absorption occurs in the small intestine, primarily in the villi (Clevers, 2013). The height and surface area of villi was demonstrated to increase upon mannan-rich fraction incorporation into broiler diet. The increase in VH and VSA may in theory increase the surface area available for nutrient absorption, thereby improving villus development and general global gut health. This observation is in agreement with the increased digestive enzyme capacity observed upon mannan-rich fraction supplementation. Previous studies into the effect of MRFs on broiler health have shown an increase in bird weight gain on day 35, compared to a control (Corrigan *et al*., 2017). Crypt depth was also shown to increase upon MRF incorporation, whereas previous reports (analysing different products) on the effect of dietary additives have suggested otherwise (Luo *et al*., 2015; Xiao *et al*., 2012). The observed increase in crypt depth in this trial may be a result of increased enterocyte migration to the villus to account for the enhanced villus surface area and corresponding increase in digestive enzyme

capacity. In addition, the decrease in the thickness of the muscularis mucosae may potentially aid in the passage of nutrients into systemic circulation (Cheng *et al*., 2010). These findings provide strong evidence that the increases in VH, VSA and the decrease in muscularis mucosae thickness may point towards a role of MRF incorporation in potentially improving nutrient availability and absorption. Such findings are consistent with previous reports detailing the effects of naturally-derived dietary additives into cattle and piglets (Jiang *et al*., 2015; Zitnan *et al*., 2008). Enhanced nutrient availability and absorption would in theory result in an enhanced bird weight gain and improved gastrointestinal integrity.

Another essential function of the gastrointestinal system is protection from invading pathogens and bacterium (Cheng *et al*., 2010). This role is served by the presence of mucins, which form a mucus layer that prevents the invasion and adhesion of foreign pathogens (Cornick *et al*., 2015). The observed increase in alkaline phosphatase enzyme capacity indicates towards an increased protection upon MRF incorporation. Goblet cell density and mucus layer thickness were examined in order to further delineate the potential role of mannan-rich fraction supplementation in broiler intestinal immunity. The evidence provided here proposes that MRF incorporation may result in an increased goblet cell density and mucus layer thickness. Goblet cells secrete mucins, which are the constituent component of the mucus layer. This mucus layer provides a protective barrier between the external environment and epithelial cell surface (Johansson *et al*., 2013). Goblet cells also interact with resident gut microbiota to maintain barrier function (J. R. Kelly *et al.*, 2015). The composition of gut microbiota in the intestine has been demonstrated to adjust in response to stress or infection, possibly leading to increased inflammation (Jang *et al*., 2017). This is a very interesting concept in light of the fact that breakdowns in tissue barrier integrity has been previously reported to result in malabsorption of available nutrients (Awad *et al*., 2017). An increased mucus layer thickness potentially

provides enhanced protection from invading pathogens and would, crucially, therefore permit the increased volume of nutrients to be utilised for growth rather than for the repair of damaged tissues as a result of pathogenic invasion. Further investigations into the interplay between the protection offered from the mucus layer from pathogen adhesion and the utilisation of available nutrients towards productive processes may deepen our understanding of the benefits of improved tissue morphology.

The results of a trial that formed the basis for this study (Corrigan *et al*., 2017) demonstrated that MRF incorporation into broiler diet led to an increased weight gain, when compared to a control. The work presented in this study establishes that the incorporation of MRF-1 and MRF-2 into broiler diet results in enhanced digestive enzyme capacity. Increases in the capacity of digestive enzymes maltase and sucrase may in theory increase the ability of the host to intake a greater volume of nutrients. This is intriguing as an increased rate of nutrient absorption may lead to correlated improvements in tissue integrity and bird weight gain. This result may in theory explain the demonstrated increase in weight gain and feed conversion ratios observed in the Corrigan *et al*., trial. Furthermore, the capacity of enzyme alkaline phosphatase was also determined to be augmented upon MRF-1 and MRF-2 incorporation. Alkaline phosphatase serves as an essential defence mechanism of the gastrointestinal tract, with roles including the prevention of pathogen translocation into systemic circulation (Bilski *et al*., 2017). It could be speculated that the increases in disaccharide enzyme capacity and alkaline phosphatase capacity indicate that the supplementation of mannan-rich fractions may result in an increased ability to intake a greater volume of nutrients in addition to increased protection from pathogenic invasion.

Cellular death is a hallmark of inflammation (Yang *et al*., 2015). Several types of cell death exist, such as apoptosis (considered to be immunologically silent), necroptosis (an

inflammatory form of cell death) and pyroptosis (a highly inflammatory form of cell death) (Sharma and Kanneganti, 2016). Enhanced cellular death levels may be the result of the onset of inflammation (Yang *et al*., 2015). Several markers exist for identifying the type of cell death occurring. TUNEL staining and caspase-3 are considered markers for apoptotic cell death while RIPK3 has been implicated as an essential component of necroptotic cell death (Zhu *et al*., 2016). Incorporation of MRF-1 led to a significant decrease in TUNEL-positive staining in the duodenum and jejunum and little effect in the ileum and caecum. However, the effect of MRF-2 and MRF-3 incorporation was less pronounced. There was generally a slight decrease in caspase-3 positive cells upon mannan-rich fraction incorporation. It was intriguing that the effects of mannan-rich fraction dietary inclusion appeared to lessen somewhat towards the large intestine when compared to the effects observed in the duodenum and jejunum. Interestingly, there was a notable decrease in RIPK3 cells upon dietary supplementation. This effect was most pronounced upon MRF-1 dietary inclusion. It could be speculated that the increases in mucus layer thickness (as a direct result of the enhanced goblet cell density) and alkaline phosphatase enzyme capacity are playing a critical part in the observed increased broiler intestinal health.

The data presented in this thesis suggests a role of MRF incorporation resulting in decreased cellular death levels in broilers. This potential role was further explored through analysis of p-Akt and RIPK3 protein expression levels in duodenal tissue. Phospho-Akt is implicated in several cellular pathways involving apoptosis, cellular proliferation and cellular differentiation (Hart and Vogt, 2011). Western blot analysis of p-Akt expression revealed a significant increase in expression upon MRF-3 incorporation into broiler diet. Interestingly, incorporation of MRF-2 and MRF-3 also led to a significant reduction in RIPK3 protein expression levels. This finding was consistent with the immunohistochemical analysis of RIPK3 in Section 3.2.6.

RIPK3 is a necroptotic cell death marker, a highly inflammatory form of cell death (Nogusa *et al*., 2016). Interestingly, knockdown of RIPK3 in mice has been demonstrated to induce a switch from TNF-mediated necroptosis to a kinase-dependant apoptotic form of cell death (Remijsen *et al*., 2014). As apoptosis is an immunologically silent process, the effect of RIPK3 knockdown on intestinal inflammation would be an interesting avenue to explore. Increased p-Akt expression has been linked to the inhibition of apoptosis (Granado-Serrano *et al*., 2006; Hart and Vogt, 2011). The findings illustrated here can be linked to the decreased expression of apoptotic cell markers in Section 3.2.5. Similarly, the decrease in expression of apoptotic cell markers can potentially be attributed to the enhanced mucus layer thickness and increased alkaline phosphatase enzyme capacity. The ratio of cellular death through processes such as apoptosis and the formation of new intestinal cells are essential towards preserving intestinal homeostasis. The maintenance of homeostasis is a critical factor in barrier function in the intestine (Williams *et al*., 2015). Recent evidence has suggested that regulated levels of apoptosis convey no effect on the function of the epithelial barrier, however increased levels of apoptosis- as a result of intestinal infection or inflammation- can contribute to intestinal barrier dysfunction (through the loss of epithelial tight junction proteins) and increased permeability (Demehri *et al*., 2013). The results from this trial are in agreement with the study by Demehri *et al*., as MRF incorporation has been demonstrated to result in a reduction in apoptotic markers and an improvement in intestinal morphometric features.

The onset of inflammation can be characterised by alterations in the expression of a range of inflammatory cytokines, primarily IL-18, IL-12 and IL-1β (Netea *et al*., 2016). However, MRF incorporation into broiler diet had no effect on the expression levels of inflammatory cytokines in serum. The expression of IL-10 was not significantly altered in tissue lysates upon mannan-rich fraction incorporation. There was a significant decrease in IL-12 expression upon MRF-2

incorporation in the caecum. Furthermore, IL-1β expression was significantly increased in the ileum upon MRF-1 incorporation and significantly decreased in the caecum upon MRF-2 supplementation. Interestingly, IL-18 expression was almost consistently decreased upon dietary incorporation of each mannan-rich fraction. This divergent finding between IL-1β and IL-18 warrants further investigation. IL-1β and IL-18 are considered to be closely linked due to their dual activation upon inflammasome assembly (Kesavardhana and Kanneganti, 2017). The slightly diverging results presented in this thesis may be as a result of alternative activation mechanisms or mode of action in avians as compared to mammals. This deficit in our understanding of IL-1β/IL-18 in inflammasome activation in avian species requires further attention to elucidate their potential role in inflammatory diseases. The exact implication of increased IL-18 expression is subject to renewed discussion (Dupaul-Chicoine *et al*., 2010; Nowarski *et al*., 2015; Siegmund, 2010). Recent evidence has pointed towards increased IL-18 expression driving disease progression (Nowarski *et al*., 2015; Vanden Berghe *et al*., 2014). Nowarski *et al*., (2015) demonstrated that deletion of *IL-18* and its receptor conferred host protection in a colitis mouse model. It was also demonstrated that increased IL-18 expression resulted in a reduction of goblet cell maturation. The data presented in this thesis illustrate that dietary incorporation of mannan-rich fractions results in a consistent decrease in IL-18 expression. This finding may be explained somewhat by the increased mucus layer thickness and improved tissue integrity, conferring greater protection to the host as a result of dietary supplementation.

In conclusion, this study has described the effect of MRF incorporation into broiler diet on intestinal health and immunity. The role of MRF inclusion in enhancing digestive enzyme capacity was elucidated, with overall implications for bird weight gain. Histological analyses of intestinal tissue upon MRF supplementation demonstrated an improved tissue architecture,

compared with the control. Increases in VH and VSA may lead to an enhanced surface area available for nutrient absorption and assimilation whilst a higher density of mucin-secreting goblet cells may confer a greater protection to the host from pathogenic invasion and subsequent induction of inflammation through enhanced barrier function. The role of MRF incorporation in reducing the expression of RIPK3, a marker of the necroptotic cell death process, was also highlighted. The incorporation of mannan-rich fractions into broiler diet has been demonstrated to potentially increase bird weight gain through enhanced digestive enzyme capacity and confer greater protection from invading microorganisms as a result of improved barrier function and tissue morphology. Future work will be required to further elucidate the role of IL-1β/IL-18 activation in inflammasome assembly and ultimately inflammatory disease progression. Further understanding of the avian immune system and the interplay of *Campylobacter*-specific TLRs such as TLR4 and TLR21 (the avian equivalent of mammalian TLR9) will tie in with this work to provide a clearer portrait of the underlying mechanisms of avian intestinal health and disease.

# Chapter 5: Bibliography

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