Effects of orally administered ovine serum immunoglobulin in the normal and *Salmonella enteritidis* – challenged growing rat.

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I am dedicating my thesis to my guru, his holiness arulthiru BANGARU ADIGALAR

Abstract

Immunoglobulins (Ig) are the primary anti-infective component of plasma, colostrum and breast milk. They are the specialized glycoproteins that protect the body from harmful bacteria, viruses and other environmental pathogens by either binding to them or by forming an encapsulating barrier. The development of antimicrobial and immunomodulatory products from natural sources for dietary supplementation in both animals and humans is an active area of research. Purified Ig from sheep plasma (ovine serum Ig) is one such candidate product.

Based on the results of the numerous background growth studies of others, the objectives of this study were to determine whether orally administered ovine serum Ig affected growth performance, digestive organ weights, gut morphology, immunity, the gut microbiota, goblet cell numbers, mucin gene expression and digesta mucin protein contents in the growing rat. The study also sought to understand whether orally administered ovine serum Ig prevented or lessened the negative effects of *Salmonella enteritidis* ATCC 13076 (a pathogen) in the *S. enteritidis*−challenged growing rat. The presence of ingested intact Ig in different parts of the digestive tract was also determined. Investigations were undertaken in normal and *S. enteritidis*−challenged Sprague-Dawley male growing rats. Diets were iso-caloric and had similar protein and amino acid contents. The diets were fed for 21 days (for non-challenged rats) and for 18 days (for the challenged rats).

An ovine Ig fraction improved food conversion efficiency, the weights of several digestive organs and gut histology. Compared with spray-drying, a freeze-drying procedure preserved a higher degree of immunological activity.

In immunity studies, an ovine Ig fraction selectively enhanced ($P < 0.05$) various indices of immune function such as phagocytic acitivity, lymphocyte proliferation and gut and plasma antibodies. In microbiolgical studies, the number of lactobacilli in the gut were increased ($P < 0.05$) by feeding the ovine Ig. Ovine Ig also influenced the transcription and translation of gut mucin protein as evidenced by increased ($P < 0.05$) mucin gene expression and digesta mucin protein concentrations as well as an increased goblet cell count.

After gavaging with *S. enteritidis*, the rats fed the IOI (inactivated ovine Ig) and BD (basal diet) diets grew considerably more slowly (growth declined)

than the challenged rats fed the FDOI (freeze-dried ovine Ig) diet and the latter rats showed no sign of infection. The villus length, crypt depth, villus:crypt ratio and villus surface area (VSA) of the duodenum and jejunum were generally greater ($P < 0.05$) in rats challenged with *S. enteritidis* and receiving the FDOI diet compared to either the unchallenged rats fed the BD diet (except duodenal and jejunal VSA) or the challenged rats fed the BD or IOI diets. Several measures of immune modulation were affected as was the bacterial composition of the gut microflora. The ileal and colonic digesta for the FDOI-fed rats had higher ($P < 0.05$) numbers of goblet cells and higher ($P < 0.05$) digestive luminal mucin protein concentrations than the challenged rats fed either the BD- or IOI-supplemented diets.

Intact ovine Ig were detected in the luminal contents from the stomach through to the colon in the growing rat fed orally with ovine Ig fraction. The amounts (percentages of digesta dry matter) of intact ovine Ig for rats fed the FDOI diet were 2.17%, 3.12%, 5.31%, 2.03% and 5.76% for stomach chyme, duodenal, jejunal, ileal and colonic digesta respectively. Overall, the accumulated amount was 18.4%, which indicates the presence of a high level of active material throughout the digestive tract.

In conclusion, purified ovine Ig improves growth of healthy rats and protects against enteric infection by immunomodulation, mucin protein and/or modification of commensal microbial composition. The results contribute to knowledge of how orally administered ovine Ig can modulate and enhance key indicators of gut function and overall growth performance in the growing rat.

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AA – Amino acid

ADCC – Antibody dependent cell-mediated cytotoxicity

ADFI – Average daily feed intake

ADG – Average daily gain

AMI – Antibody-mediated immunity

AP – Animal plasma

BD – Basal diet

BSA – Bovine serum albumin

C3b – complement component 3

cAMP – Cyclic adenosine monophosphate

cGMP – Cyclic guanosine monophosphate

CD – Crohn's disease

CD4 – cluster of differentiation 4

CD8 – cluster of differentiation 8

cDNA – complementary DNA

CDR – complementarity determining regions

CFU – Colony forming unit

C_H – Constant heavy chain

C_L – Constant light chain

CLR – C-type lectin receptor

Con A – Concanvallin A

CPM – Counts per minute

CT – cholera toxin

CXC R1 – chemokine receptor 1

d – day

DC – Dendritic cells

DGGE – Denaturing Gradient Gel Electrophoresis

DNA – Deoxyribonucleic acid

dsRNA – double-stranded RNA viruses

EAggEC – enteroaggregative E. coli

EDTA –Ethylenediaminetetraacetic acid

EHEC– enterohemorrhagic *E. coli*

ELISA – Enzyme Linked Immunosorbent Assay

ELLA – Enzyme-Linked Lectin Assay

EPEC – enteropathogenic *E. coli*

ETEC – enterotoxigenic *E. coli*

Fab – “Fragment, antigen-binding”

FACS – Fluorescence-activated cell sorter

FAE – Follicle-Associated Epithelium

Fc – “Fragment, crystallisable”

FcR – Fc receptor

FCγbp – Fc-gamma binding protein

FDOI – Freeze dried ovine immunoglobulin

FOS – fructo-oligosaccharides

GALT – Gut associated lymphoid tissue

G:F – Gain:feed ratio

GIT – Gastrointestinal tract

GOS – galacto-oligosaccharides

H_2O_2 – hydrogen peroxide

HD – human defensins

HIP / PAP – hepatocarcinoma-intestine-pancreas / pancreatic-associated protein

HRP – Horseradish peroxidase

HSP – Heat shock protein

HVR – Hyper variable region

IBD – Inflammatory bowel disease

IEC – intestinal epithelial cells

IFNγ – Interferon gamma

Ig – Immunoglobulin/ Immunoglobulins

IgA – Immunoglobulin A

IgD – Immunoglobulin D

IgE – Immunoglobulin E

IgG – Immunoglobulin G

IgM – Immunoglobulin M

IgY – Egg yolk antibodies

IL-1– Interleukin -1

IL-1R – Interleukin -1 receptor

IMO – isomalto-oligosaccharide

iNOS – inducible nitric oxide synthase

IOI – Inactivated ovine immunolglobulin

IPS-1 – Interferon promoter stimulator-1

JAM – junctional adhesion protein

kDa – kiloDalton

LEAPs – liver-expressed antimicrobial peptides

LPS – lipopolysaccharide

LT – Heat Labile Toxin

LTB4 – leucotrien B 4

M-SAA3 – mammary-associated serum amyloid A isoform 3

MALT – mucosal-associated lymphoid tissue

M-cell – Microfold cells

MDA5 – Melanoma differentiation associated gene 5

MHC – Major histocompatibility complex

mRNA – messenger ribonucleic acid

MUC – Mucin genes (human)

Muc – Mucin genes (rat)

NA – Natural antibodies

NF-κB – Nuclear factor kappa B

NLR – Nucleotide-binding domain leucine-rich repeat

NK cells – Natural killer cells

NO – Nitric oxide

NOD – Nucleotide-binding domain

NOS – nitric oxide synthase

$O^{\cdot}{}_2^-$ – Superoxide anion

OIC – Ovine serum immunoglobulin concentrate

OPD – o-phenylenediamine dihydrochloride

PAMPs – Pathogen-associated molecular patterns

PBL – Peripheral blood leukocytes

PBS-T – Phosphate buffer saline–tween 20

PCR – Polymerase chain reaction

PHA – Phytphaemagglutinin

pIgR – Polymeric immunolglobulin receptor

PLA_2 – Phospholipase A_2

PP – Peyer's patch

PRR – pattern recognition receptors

qRT-PCR – Quantitative Real Time –Polymerase Chain Reaction

RELMβ – resistin-like molecule beta

RLR – Rretinoic-acid-inducible protein 1–like receptors

RNA – Ribonucleic acid

s – Seconds (time)

SAP130 – Sin3-associated polypeptide p130

SDAP / SDBP – Spray dried animal plasma / Spray dried bovine plasma

SDOI – Spray dried ovine immunoglobulin

SDP – Spray dried plasma

SDPP – Spray dried porcine plasma

SDS-PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEB – *Staphyloccocus aureus* enterotoxin B

SED – subepithelial dome

SEM – Standard error of mean

SIgA – Secretory immunoglobulin A

SIgM – Secretory immunoglobulin G

ST – Stable enterotoxins

TER – transepithelial electric resistance

TFP – trefoil factor peptides

TGFβ – Transforming growth factor

T_H1 – T helper cells 1

T_H2 – T helper cells 2

T_H17 – T helper cells 17

TLR – Toll like receptor

TNFα – Tumour necrosis factor alpha

UC – Ulcerative colitis

VR – Variable region

VSR – Villous surface area

WGA – Wheat germ agglutinin

Wk – Week

ZO – *zonula occluden*

Zot – *Zonula occludens* toxin

LIST OF PUBLICATIONS

Peer-reviewed articles published or in progress

1. **P Balan**, KS Han, K Rutherfurd-Markwick, H Singh and PJ Moughan. (2011). Immunomodulatory effects of ovine serum immunoglobulin in growing rats gavaged with *Salmonella enteritidis* J Nutr. 141, 950-956.
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5. **P Balan**, KS Han, K Rutherfurd-Markwick, H Singh and PJ Moughan. (2010) Immunomodulatory effects of ovine serum immunoglobulin in the growing rat. Animal. 4, 1702-1708.
6. **P Balan**, KS Han, SM Rutherfurd, H Singh and PJ Moughan. (2009) Orally administered ovine serum immunoglobulins influence growth performance, organ weights, and gut morphology in growing rats. J Nutr.139, 244-9.

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8. SM Rutherfurd, TK Chung**,** DV Thomas, ML Zou, KS Han, **P Balan**, E Maier and PJ Moughan (2011). Effect of microbial phytase on broiler performance, AME, toe ash, bone mineral density, ileal and colonic microbial population, gut mucin production and the digestibility of phytase P, minerals and amino acids.

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Manuscripts in preparation

11. **P Balan** and PJ Moughan. Immunoglobulins – Review
12. **P Balan** and PJ Moughan. Ovine serum immunoglobulin supplements prevent the release of mucosal proinflammatory mediators in the growing rat challenged with *Salmonella enteritidis*.
13. **P Balan** and PJ Moughan. Stimulatory effect of ovine serum Ig on multiplication of lactic acid bacteria under *in vitro* condition.
14. **P Balan**, SM Rutherfurd and PJ Moughan. Effects of ovine serum immunoglobulin on dental health and immunomodulation in the cat.
15. **P Balan**, G Mal, S Das and PJ Moughan. Synergistic antimicrobial activity of curcumin, manuka honey and whey protein isolate.
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17. KS Han, **P Balan**, A Purba and PJ Moughan. Effect of Korean traditional foods-derived polysaccharides on lymphocyte proliferation of Peyer's patch, cytokine and immunoglobulin production in the ileum of growing rats.
18. SM Rutherfurd, TK Chung, **P Balan**, KS Han, E Maier and PJ Moughan. Effect of three microbial phytases on mucin output and gut microbiota in broilers fed low-phosphorus corn-soybean diets.
19. SJ Henare, SM Rutherfurd, M Zou, **P Balan**, KS Han, N Strobinger, MJ Boland and PJ Moughan. Gastrointestinal interactions of kiwifruit: Effects on mucin production in piglets.
20. SM Rutherfurd, SJ Henare, RK Richardson, ML Zou, **P Balan**, C Sawatdeenaruenat and PJ Moughan. The effect of dietary protein content on endogenous ileal tryptophan flow in the growing rat.

Patent application

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27. **P Balan**, KS Han, H Singh and PJ Moughan. Immunomodulation of ovine serum immunolgobulins in the growing rat. Poster presentation at the Functional food conference, Riddet Institute, Palmerston North, New Zealand, February 2009.
28. KS Han, R Sengupta, **P Balan**, A Deglaire, H Singh and PJ Moughan. Effect of bioactive protein on mucin gene expression in rat small intestine. Poster presentation at the 17th Queenstown Molecular Biology Meeting. Queenstwon, New Zealand. 2007.

Chapter 1

Review of Literature

1.1. The gut: its structure and function

The general structure of the alimentary tract comprises a tubular structure, running from the mouth to the anus. In an adult human, the gastro-intestinal tract (GIT) measures up to 5 m long in a live subject, or up to 9 m without the effect of muscle tone and it consists of the upper and lower GI tracts. The GIT may be further divided into stomach, small (0.25 m duodenum, 2.5 m jejunum and 3.4 m ileum) and large (caecum, colon and rectum) intestine (*Guyton, 2006*).

Throughout its length, the alimentary tract is structured similarly (similar general histology), in the form of four concentric layers: the mucous membrane or mucosa; the submucosa; the muscle layer (muscularis) and the serosa or adventitia. The above structures can be considered as basic components which are fundamentally similar throughout the entire tract, but they vary somewhat in nature and thickness from region to region based on functional requirements. The mucosa is the innermost layer of the GIT, which surrounds the lumen within the tract. This layer comes in direct contact with food (or the bolus) and it is responsible for absorption and secretion, important processes in digestion. The mucosa can be divided into the epithelium, lamina propria and muscularis mucosae. The submucosa is comprised of a dense irregular layer of connective tissue (with large blood vessels) and lymphatics and nerves, branching into the mucosa and muscularis externa. The muscularis externa (muscle layer) is comprised of an inner circular layer and an outer longitudinal muscular layer. The circular muscle layer prevents food from travelling backwards and the longitudinal layer shortens the tract. The co-ordinated contractions of these layers are called peristalsis and they propel the bolus through the GIT. The serosa is the outermost layer (*Magee & Dalley, 1986*).

The intestinal surface in an adult human covers an area of approximately 100 m^2 and it is lined by a single layer of columnar intestinal epithelial cells (IECs), which form a barrier between the intestinal lumen and the host's connective tissue (*Artis, 2008*). The gut's epithelial cells are frequently replaced, by a method which involves the proliferation of stem cells and this rapid and constant epithelial cell turnover is important for its normal physiological function. The newly produced cells begin at the junction of the villus and the crypt and then migrate, either to the top of the villus, or to the

base of the crypt. These cells differentiate into either enterocytes or goblet cells (this is explained elsewhere in this literature) or Paneth cells, which secrete antimicrobial peptides that protect the contents of the intestine, with specific functions for each group of cells. The final digestion and absorption of nutrients occurs in the villi which line the inner surface of the small intestine. The turnover of villus enterocytes is very rapid, since the cells which are removed, due to apoptosis (cell death) and exfoliation (occurring at the tip of the villi), are replaced every two to three days. The re-population of crypts and villi is continuous, as a result of the replication rate of the stem cells (*Artis, 2008*).

Intestinal epithelial cells have microvilli on their apical surface, with a filamentous brush border glycocalyx at their tips (*Maury et al., 1995*). While this structure helps to avoid penetration by foreign antigens, these cells also express major histocompatibility complex class II receptors (MHC II), to aid antigen presentation to immune cells as required. Highly conserved microbial structures can also be recognised by epithelial cells, through surface toll-like receptors (TLRs) and they can initiate cellular signalling responses on their own (*Mowat, 2003*). Gut epithelial cells are continually exposed to numerous macro-molecules and pathogens, including chemical irritants, digested foods, toxins, resident bacteria and intestinal pathogens and their products (*Moncada & Chadee, 2002*).

1.1.1. Tight junctions (TJ)

Intestinal epithelial cells are held together by TJ and adherent junctions (in the form of adhesion belts between epithelial cells in the small intestine). TJ correspond to an important barrier within the paracellular pathway between gut epithelial cells. Disruption of TJ results in intestinal hyperpermeability (termed as “leaky gut”) and it is implicated in the pathogenesis of various acute and chronic diseases (*Liu et al., 2005*).

1.1.1.1. TJ structure and composition

Gut epithelial cells stick to each other through junctional complexes, which are located at the lateral membranes. The interepithelial junction consists of three major components with occlusive properties: (a) TJ; (b) adherens junctions (AJs); and (c) desmosomes. The TJ is occasionally also called the *Zonula occludens*, since it represents the most important barrier within the

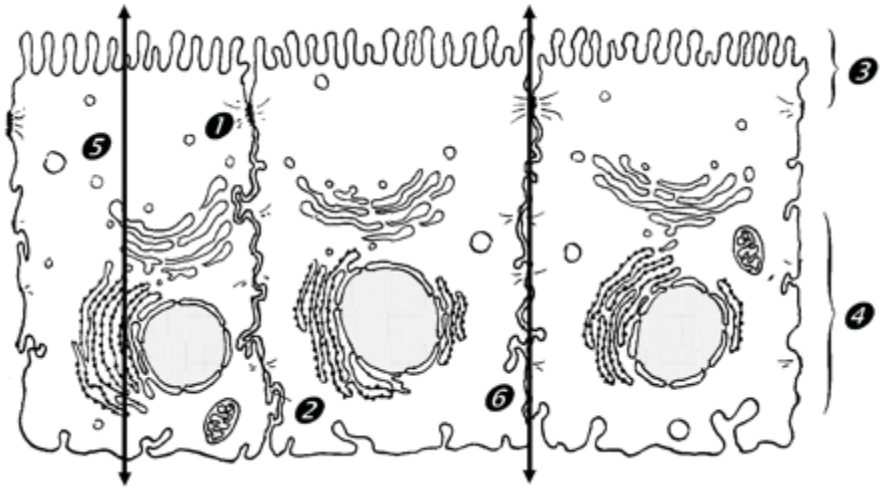
paracellular pathway and between intestinal epithelial cells (*Gonzalez-Maris cal et al., 2003*). These structures are comprised of complex proteins, of which more than 40 have been studied in detail. The most important proteins, which build up the TJ, have been reported as: occludin, tricellulin (*Ikenouchi et al. 2005*), claudin, (*Tsukita & Furuse, 1999, 2000*) and junctional adhesion protein (JAM) (*Gonzalez-Mariscal et al., 2003*). Occludin was once thought to be the most important protein impacting on TJ function. Studies on occludin "knockout" mice, however, found that the mice did not lose their intercellular structural morphology and that the barrier function of the gut was not altered when studied electrophysiologically, despite growth failure and other phenotypic abnormalities (*Saitou et al., 2000*). In the literature, reports have suggested that claudin-1, the intestine-associated family member (*Furuse et al., 1999; Kinugasa et al., 2000*), can be directly associated with occludin (laterally) in the membrane (within the same cell) but not intercellularly and that the grouping of these two proteins (functioning together) perform the vital "gatekeeper" or barrier function of the TJ (*Gonzalez-Mariscal et al., 2003*). These sealing proteins, which are both trans-membrane proteins, interact with cytoplasmic plaques, which consist of different types of cytosolic proteins (including the *zonula occluden* proteins ZO-1, ZO-2 and ZO-3) that work as adaptors between the TJ proteins and actin and myosin contractile elements within the cell. Working together, they open and close the paracellular junctions (*Liu et al., 2005; Anderson & Van Itallie, 2009*).

1.1.1.2. Permeability through TJ

The control of intestinal transport function is based, to a large extent, on the junctional complex connecting enterocytes together (Figure 1.1). This regulates the extent to which solutes and water are absorbed or secreted. As a barrier between the luminal and basolateral compartments, TJ exclusively control the passive diffusion of ions and other small solutes, through the paracellular pathway and thereby influence any gradient created by the activity of pathways associated with the transcellular route. The presence of paracellular pathways with high permeability, such as those in the small intestine, allows rapid transepithelial diffusion and prevents the presence of a large transepithelial electrical potential and concentration gradient across the gut epithelium. Alternatively, these routes facilitate the absorption of some solutes attached to fluid absorption, via convection (solvent drag) and without

additional expenditure of energy (*Pácha, 2000*). Pappenheimer & Reiss (1987) reported that the effective pore size of an adult intestinal epithelium was able to allow passage of solutes of 5,000 Da at the tight junction. Therefore, even small macromolecules (not only ions and nutrients) and water would possibly pass through this route under normal physiological conditions (*Atisook & Madara, 1991*). In addition, TJ are dynamic structures that readily adjust to a range of physiological conditions and they may modify the size of the effective pore (*Ballard et al., 1995; Pácha, 2000*).

Figure 1.1 Schematic diagram of intestinal epithelium*.



*Reproduced with permission (The American Physiological Society) from Pácha, (2000). Schematic diagram of intestinal epithelium. The tight junctions (1) define the boundary between the apical (3) and basolateral (4) membranes and prevent free movement of water and solutes through the lateral intercellular space. The permeability of tight junctions significantly influences transepithelial transport (2, lateral intercellular space; 5, transcellular transport; 6, paracellular transport).

1.1.1.3. Factors affecting TJ

Various factors, which have been found to affect the TJ, include food, enteric microbes and certain disease states.

1.1.1.3.1. Food

Glutamine (a conditionally essential dietary amino acid) plays a key role in numerous metabolic processes, and supplementation with glutamine has been found to improve intestinal barrier function in highly stressed patients. Critically ill patients are frequently deprived of glutamine, and the breakdown of barrier function can result in pathology, by allowing greater toxins and/ or

antigenic penetration across the intestinal barrier (*Liu et al., 2005*). Celiac disease (gluten-induced enteropathy) is one of the major disease entities, wherein a breakdown of barrier function plays a key role in pathogenesis. An important food protein, involved in the pathogenesis of gluten-induced enteropathy, is gliadin. This molecule and related proteins have been identified as possible triggers for various auto-immune diseases, including celiac disease and type 1 diabetes. Gliadin and structurally similar agents have been reported as damaging intercellular junctions (*Liu et al., 2005; Clemente et al., 2003*). Certain lipids also have an effect on the TJ. Sucrose monoester fatty acid, a food-grade surfactant, for example, causes significant decreases in transepithelial electric resistance (TER), actin break-up, structural disruption of TJ in Caco-2 cells and an increase in the paracellular uptake of food antigen (*Mine & Zhang, 2003*). Sodium caprate (C10), a medium-chain lipid, results in intestinal hyperpermeability and dilatation of tight junctions. Both the omega-3 fatty acid, eicosapentaenoic acid (EPA, C20) and gamma linolenic acid (C18) have been found to up-regulate TJ function, by increasing occludin in a human vascular endothelial cell line (*Liu et al., 2005*).

1.1.1.3.2. Microbes

A vast number of microbes, which target the TJ, have been reported in the literature (*Hecht, 2001*). There is “cross talk” and interaction between the enteric pathogens (through various virulence factors) and intestinal epithelial cells. Enteropathogenic *Escherichia coli* (*E. coli* / EPEC) damage the tight junctions, in part by changing occludin distribution from the TJ into the cytosol (*Simonovic et al., 2000*). Nusrat *et al*. (2001) have shown that *Clostridium difficile* toxins A and B cause disruption of apical and basal F actin and dissociation of occludin ZO-1 and ZO-2 from the lateral TJ membrane. Various studies have reported that rotavirus infection of polarised epithelial cells leads to a paracellular leak and F-actin modification (*Tafazoli et al., 2001*). An endogenous protein known as “zonulin”, which is functionally and immunologically related to *zonula occluden* toxin, from *Vibrio cholerae* (*V. cholerae*), has been found to disrupt intercellular TJ by interacting with cell membrane receptors (*Wang et al., 2000*).

1.1.1.3.3. Diseases

Maaser & Kagnoff (2002) have shown that occludin is down-regulated in patients suffering from inflammatory bowel disease, which could explain the enhancement of paracellular permeability and neutrophil transmigration.

1.1.2. M-cells

The mucosal-associated lymphoid tissue (MALT), consisting of immunoreactive cells and organised lymphoid tissues, is present in close contact with all mucosa throughout the body. In the gut, it is termed gut-associated lymphoid tissue (GALT), which consists of both isolated and aggregated lymphoid follicles (*Neutra et al., 2001*). In these sites, antigen recognition and mucosal immune responses are initiated. Aggregated lymphoid follicles are found in the Peyer's patches (PP) of the small intestine; appendix vermiformis and caecum; colon; and rectum patches. GALT is one of the largest lymphoid organs in the body. Typical GALT structures can be seen in PP and aggregated lymphoid follicles in the small intestinal mucosa (*Neutra et al., 2001; Corr et al., 2008*). The follicle-associated epithelium (FAE) is a one-cell-thick layer comprised of enterocytes and specialised epithelial cells, termed M-cells (*Owen & Jones, 1974*). The FAE overlies the PP and forms the interface between the intestinal lymphoid system and the intestinal luminal environment. M-cells (the presence of 'microfolds' on the apical surface of these epithelial cells) are specialised epithelial cells found in the FAE of PP, the isolated lymphoid follicles, the appendix and MALT sites outside the GIT. M-cells differ morphologically and enzymatically from adjacent enterocytes. M-cells (Figure 1.2) function as gatekeepers to the mucosal immune system, constantly sampling the lumen of the small intestine and transporting antigen to the underlying mucosal lymphoid tissue for processing and initiation of immune responses (*Kraehenbuhl & Neutra, 1992; Neutra et al., 1996a, b; Corr et al., 2008*). Pathogens, such as *Salmonella typhimurium*, exploit this M-cell sampling process, as a means for translocating to underlying tissue (*Jensen et al., 1998; Corr et al., 2008*).

1.1.3. Paneth cells

Paneth cells, one of the four major epithelial cell lineages in the small intestine, are found at the base of the crypts and they have apically orientated secretory granules. These granules are comprised of high amounts of

antimicrobial peptides, such as α-defensins, lysozyme and phospholipase A_2 (*Ouellette, 1997; Porter et al., 1997; Ayabe et al., 2004; Ouellette, 2010*). In contrast to villus enterocytes, which have a lifetime of two to three days, Paneth cells live for more than 20 days and they are replaced at a slower rate. Paneth cells release α-defensins and other granule constituents in response to carbamyl choline and bacteria (or their antigens), such as lipopolysaccharide (LPS) and muramyl dipeptide. Although Paneth cells might not selectively discriminate between commensal or virulent bacteria, they do recognise bacterial factors (*Ayabe et al., 2000*). Paneth cells are responsible for maintaining the homeostasis of the villus–crypt micro-environment by controlling the entry of microbes (*Ayabe et al., 2004*).

1.1.4. Peyer's patches

PP are groups of lymphoid nodules, identified by Peyer in 1677, which are present in the small intestine (usually the ileum). They occur massed together on the intestinal wall, opposite the line of attachment of the mesentery. PP consist of a subepithelial dome area, B-cell follicles and interfollicular T-cell areas (Figure 1.2) (*Artis, 2008*).

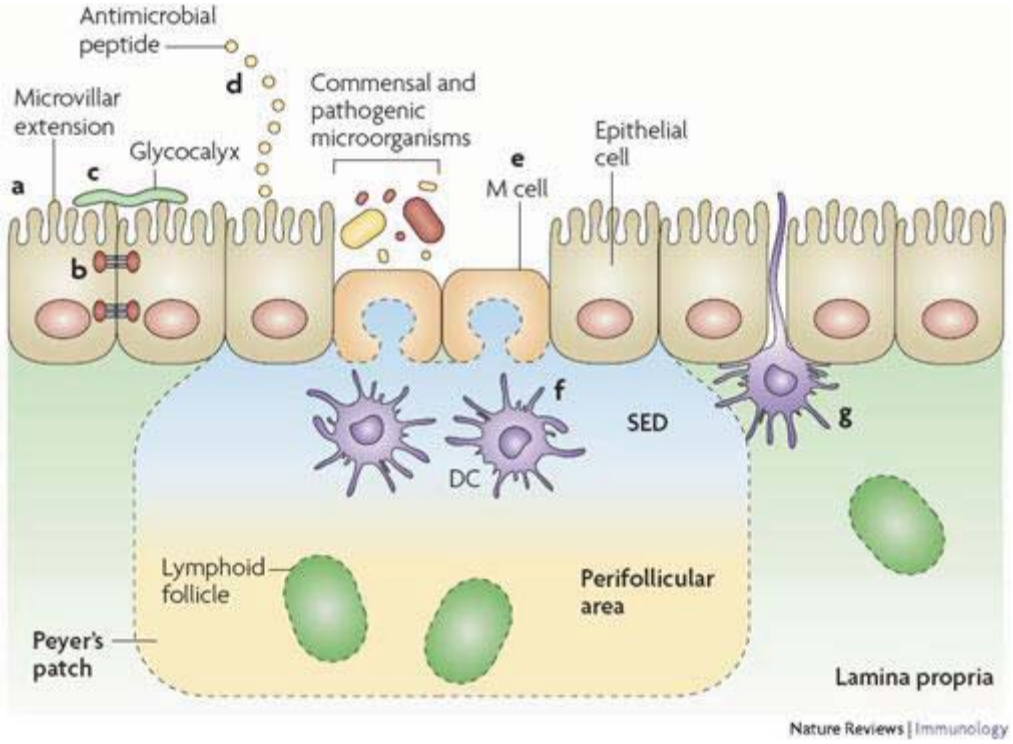
1.1.5. Lamina propria

The lamina propria is a connective tissue, directly found under the mucosal epithelial-cell surface of the GIT (Figure 1.2). It is covered by blood and lymphoid vessels and physically supports epithelial cells through the basal membrane. It is enriched with innate and adaptive immune cells (*Artis, 2008*).

1.1.6. Dendritic cells (DC)

Dendritic cells are specialised intestinal cells found in the lamina propria of the small intestine (Figure 1.2). Rescigno *et al*. (2001) reported that DC present in the intestine could extend their dendrites into the gut lumen and directly contact (engulfing) the luminal bacteria, a function that depends on CX_3C-chemokine receptor 1 (CX_3CR1) (*Neiss et al., 2005*). This function is regulated by the composition of commensal bacteria (*Chieppa et al., 2006*). Niess *et al*. (2005) reported that mice, lacking in CX_3CR1, resulted in DC failing to sample luminal antigens and this led to an impaired resistance to *S. typhimurium* infection, thus suggesting a vital role for luminal sampling in the correct development of protective immune responses in the GIT.

Figure 1.2 The intestinal epithelial cell lining*.



*Reproduced with permission (Nature Publishing Group) from Artis, (2008). Simple columnar epithelial cells demonstrate physical and biochemical adaptations to preserve barrier integrity including actin-rich microvillar extensions (a), epithelial-cell tight junctions (b), apically attached and secreted mucins that form a glycocalyx (c) and the production of various antimicrobial peptides (d). Specialized intestinal epithelial cells called M (microfold) cells overlie PP and lymphoid follicles to help in luminal sampling. M-cells show reduced mucin secretion and consist of modified apical and basolateral surfaces (e) to assist uptake and transport of intestinal contents to professional antigen-presenting cells that inhabit the subepithelial dome (SED) of the PP and lymphoid follicles (f). Specialized dendritic cell (DC) subsets can also extend dendrites between the tight junctions of intestinal epithelial cells to sample luminal contents (g).

1.1.7. Gut enzymes and secretions

In the mouth, the salivary gland secretes salivary amylase, which converts starch to maltose. The wall of the stomach is lined with millions of gastric glands, which together secrete 400–800 ml of gastric juice, at each meal. Several types of cells are present in the gastric glands, such as parietal cells, chief cells, mucus secreting cells and hormone secreting cells. When peptides are detected in the stomach the hormone gastrin is released into the bloodstream and this hormone causes the gastric glands, which are present in the lining of the stomach wall, to produce a gastric juice (*Isenman et al.,*

1999; *Guyton, 2006*). Gastric juice is an acid juice (pH 1-3). Its main components are HCl (hydrochloric acid); rennin; pepsinogen; gelatinase; gastric amylase; and gastric lipase. The HCl keeps the stomach pH acidic. Since HCl is a strong acid, it destroys most of the pathogens present in the food and it also helps to convert pepsinogen into pepsin (*Guyton, 2006*). Rennin (present in infants but not in adults) is an enzyme which assists in the digestion of milk. Pepsin digests other proteins, which are present in food, to smaller peptide fragments. Gelatinase helps in digesting type I and type V gelatin and type IV and V collagen, which are proteoglycans present in meat. Gastric amylase assists in the digestion of the starch, which was not digested in the mouth. Gastric lipase helps to digest fat into fatty acids and glycerol. These digestion processes take place in the stomach (*Guyton, 2006*). The remaining food (now called chyme) is passed through the abdomen into the duodenum, through the pyloric sphincter, which is present at the lower end of the abdomen. The GIT releases hormones (gastrin, secretin, cholecystokinin and grehlin) to help regulate the digestion process (*Isenman et al., 1999*; *Guyton, 2006*). Brunner's glands are duodenal glands, which are present throughout the duodenum. The chief function of these glands is to produce an alkaline solution (containing bicarbonate ion), to help the duodenum to provide an alkaline condition for the intestinal enzymes to be active, thus enabling absorption to take place and also the lubrication of the intestinal walls. There are also three juices secreted in the duodenum: (1) bile juice, (2) pancreatic juice and (3) intestinal juice. Bile juice is secreted by the liver and is stored mainly in the gall bladder. Bile juice contains bile salts, which help to emulsify fats (it breaks it down into small globules, which can be easily digested by the enzyme lipase present in the pancreatic juice) (*Guyton, 2006*). Pancreatic juice contains trypsin (enterokinase is present in the intestinal juice and it converts the trypsinogen inactive form to active trypsin). It also breaks down protein to smaller peptide fragments (similar to pepsin). Chymotrypsin has the same function as that of trypsin and pancreatic lipase acts on fats and converts them into fatty acids and glycerol. Pancreatic amylase assists in the digestion of starch. Intestinal juice is secreted by the intestinal walls and it contains the following enzymes: enterokinase (which activates trypsinogen to tryspsin), erepsins (which help in the conversion of polypeptides to amino acids), maltase, sucrase, and lactase, which help in the digestion of maltose, sucrose and lactose, to glucose (*Isenman et al., 1999*; *Guyton, 2006*). The main function of the ileum is to absorb vitamin B12, bile

salts and digested contents. The villi contain large numbers of capillaries which carry the amino acids (AA) and glucose (produced by digestion) to the liver through the hepatic portal vein. Lacteals are small lymph vessels, found in villi, and they absorb fatty acids and glycerol. The large intestine is mainly responsible for holding the waste and for water re-absorption in addition to maintaining water balance and absorbing some vitamins, such as vitamin K. (*Isenman et al., 1999*; *Guyton, 2006*).

1.2. The gut's role in host health

The gut's digestive process and immunity are complex and integrated biological processes. The major function of the small intestine is to extract nutrients from food and transfer them into the body, in a usable form. Another important function, however, is to protect against potentially harmful microbes and pro-inflammatory luminal antigens. The intestinal mucosal immune system must distinguish between pathogens, which invade from the external environment, and the resident microbiota that colonise the intestine. The gut is the main site of digestion and absorption and it is also one of the primary organs directly exposed to the luminal environment. Luminal macro-molecules function as substrates for digestion, through their corresponding enzymes. Both micro-(minerals and vitamins) and macro-nutrients (AA, glucose and fatty acids) have been identified as regulating signals in the modification of gene expression both at the transcriptional and translational level (*Fafournoux et al., 2000; Paoloni-Giacobino et al., 2003*).

Gut function can be compromised by numerous factors, such as diet, bacterial antigens, toxins, immune status, age, diseases (infectious and non-infectious organisms, inflammatory bowel disease, ulcers etc.) and drugs (including non-steroidal anti-inflammatory and antibiotics). It has been well established that infection, inflammation and immunolgical stress can inhibit growth of the gut. In the gut, contact with antigens, such as pathogenic and non-pathogenic organisms can stimulate the production of pro-inflammatory cytokines such as interleukin-1, interleukin-6 and tumour necrosis factor-α and acute phase proteins (*Johnson, 1997*). The consequent demand for AA reduces the efficiency of dietary protein use for organ protein synthesis, resulting in overall growth inhibition. Various nutritional strategies (discussed below) are considered to lower or minimise gut disturbance and over-

stimulation of the immune system by preventing microbial growth or colonisation in the gut, especially in the small intestine, or by indirectly assisting mucosal integrity (*Touchette et al., 2002*). Food supplementation, such as with animal plasma, may improve small intestinal morphology (independent of feed intake) and this could result in a more functional and intact gut structure (especially villi), through which nutrients could be more efficiently absorbed (*Touchette et al., 1997*). In addition, a lower activation of the gut immune system could lead to a higher availability of nutrients and energy for growth, resulting in increased growth (*Demas et al., 1997; Torrallardona et al., 2003*).

Humoral and cell-mediated immunity work together and function as a key role in protecting the gut. However, a primary line of protection is provided by non-immune structural and dynamic processes. Pathogens attached to the mucosa are removed (or washed) by the luminal loss of epithelial cells, together with exudation of fluid from the crypt. The above mechanism facilitates protection of the epithelial surface from pathogenic bacterial invasion (*Eckmann, 2006*).

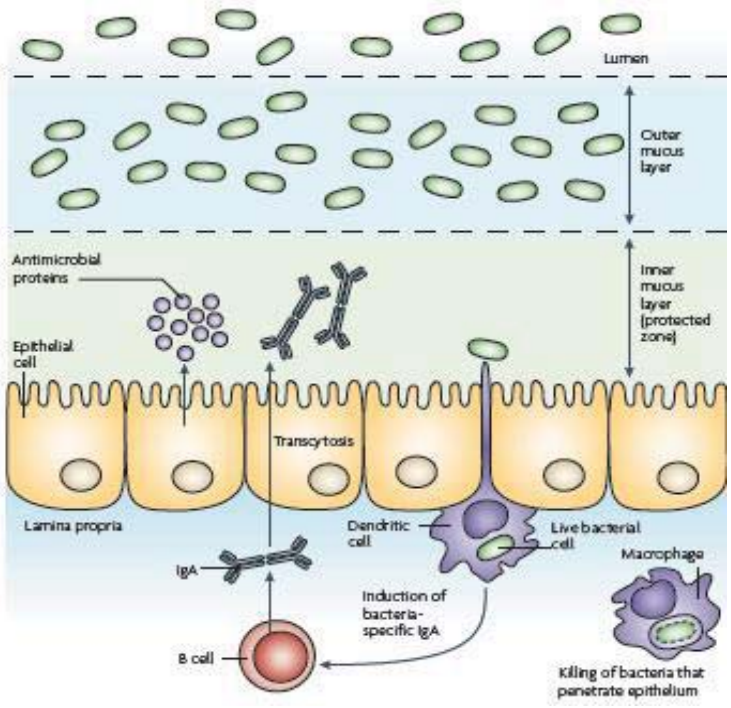
1.2.1. The gut barrier

The gut barrier is a complex structure, which separates the host from the external environment. Physical barriers include cellular and stromal structures from the vascular endothelium to the epithelial cell lining. The mucosal epithelium of the gut represents a vital barrier to a broad spectrum of noxious and immunogenic substances within the gut lumen. The destruction of the integrity of the mucosal epithelial barrier is seen in the course of diverse intestinal disorders, such as inflammatory bowel diseases (IBD), celiac disease, intestinal infections, and various other diseases. Additionally, even under normal physiological conditions, short-term damage of the epithelial surface mucosa may be induced by proteases, residential flora, dietary compounds, or other factors (Dignass, 2001).

The gut's barrier is comprised of four layers of defence (Figure 1.3). The first barrier is formed by a thick mucus layer, which covers the entire intestinal epithelium. Here, the commensal bacteria are distributed all over the mucus layer, thereby making the inner layer more resistant to (microbiological barrier within the gut) infiltration by pathogenic bacteria (*Johansson et al., 2008*). The second barrier is formed by the secretion of antimicrobial proteins

by gut epithelial cells which help to neutralise bacteria that may penetrate the mucus layer. The third barrier is formed by a physical barrier composed of a layer of epithelial cells along the gut and finally, the fourth layer is formed by an

Figure 1.3 Gut barrier*.



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immunological barrier mainly consisting of macrophages, dendritic cells and gut or intestinal immunoglobulin A (IgA). Intestinal IgA decreases the number of bacteria which adhere to the epithelial layer (*Suzuki et al., 2004b*) and restricts bacterial translocation across the epithelium (*Macpherson et al., 2000; Macpherson & Uhr, 2004*). Dendritic cells contribute to IgA induction, by sampling bacteria at the apical epithelial surface and transporting small numbers of live bacteria to inductive sites (*Macpherson & Uhr, 2004*). Secreted IgA can also be directed against the bacterial populations which are present in the outer mucus layer, or in the gut lumen, thus influencing the microbial community's composition (*Peterson et al., 2007*).

1.3. Gut microbiota

The human gut coexists in mutualism with a huge population of bacteria,

which contribute significantly to the normal digestive function. The human gastrointestinal microbiota is a complex ecosystem of approximately 300 to 500 bacterial species, comprised of nearly two million genes (the "microbiome"). Before birth, the human gut is sparsely colonised with a microbial population but, during delivery, it becomes more colonised, as a result of contact with the vaginal and faecal flora and colonisation also occurs with the first feed (*Palmer et al., 2007; Quigley, 2010*). The entire gut is colonised by microbes. However, numbers and species composition may vary significantly, according to location in the gut. The oral cavity contains a particularly complex microbiota, including members such as *Prevotella, Porphyromonas, Peptostreptococcus, Bacteroides, Fusobacterium, Eubacterium* and *Desulfovibrio* (*Willis et al., 1999*). The stomach is not greatly colonised with microbiota, due to its low pH and typically has ~10^3 CFU/g. These include mainly lactobacilli, streptococci and yeasts (*Bernhardt & Knoke, 1997; Holzapfel et al., 1998*).

Similar to the stomach, the duodenum also has a lower microbial population, due to rapid food movement and also because of the secretion of pancreatic and biliary fluids, which result in a non-favourable environment for microbes. However, there is a progressive increase in both numbers and type of species along the jejunum (10^4/g of contents) and ileum (10^7/g of contents) (*Salminen et al., 1998*). Gram-negative facultative organisms and obligate anaerobes start to appear from the ileo-caecal junction (*Holzapfel et al., 1998*). Finally, the colon is the most densely populated area of the gut, with 10^{12}/g of contents (*Gibson & Roberfroid, 1995*), comprised mainly of anaerobes, such as *Bacteroides, Porphyromonas, Bifidobacterium*, *Lactobacillus* and *Clostridium*, with anaerobic bacteria outnumbering aerobic bacteria, by a factor of 100-1000:1 (*Neish, 2009*). The colonic environment is most favourable for microbial growth, since transit time is slow and there is a greater availability of nutrients and a high pH. Several hundred species are present and a considerable number cannot be cultured by conventional methods (*Suau et al., 1999*).

The microbial population plays a major role in gastro-intestinal functions, such as digestion and resistance to pathogens and immunity. The bacterial activities of the GIT can influence nutrient utilisation in humans. The large numbers of microbes, present in the GIT, require and consume energy. This energy normally comes from the diet and thus these microbes use some of the energy which would otherwise be available for the host. The vital role of

the gastro-intestinal microbiota in health and disease is being increasingly documented, which makes it interesting for nutritionists and microbiologists to explore and understand the interplay between microbes and host, in order to develop strategies on how to protect the host from enteric diseases (*Cebra, 1999*). Bacterial populations as high as 10^{10} to 10^{11} bacteria / g of gut content (belonging to as many as 350 to 400 different species) are present in the hindgut of mammalian species, including humans. Bacterial cells, mainly anaerobes, have a great influence on the immunological, nutritional and physiological processes in the host (*Savage, 1977*).

The nature of the GIT microbiota in health and disease has been widely studied in human subjects and animals. The GIT microbiota is an extremely diverse ecosystem present from birth. The complexity of the microbiota is dependent upon many factors, such as the host's genotype and physiology, the accessibility of nutrients from the diet, and competitive mechanisms amongst the bacteria themselves (*Savage, 1977*).

The host's health is influenced by the activities and composition of the microbiota and this may result in positive or negative effects, (*Gibson & Roberfroid, 1995; Salminen et al., 1998*). The chief positive effect of an optimal microbiota includes a resistance to colonisation by pathogens (such as through adhesion mechanisms on the GIT mucosa, competition for nutrients, the production of short chain fatty acids as end products of the fermentation of carbohydrates, proteins in the gut, and the production of antimicrobial compounds (bacteriocins). Moreover, the GI microbiota is important for mucosal integrity (*Delzenne & Williams, 2002*) and the development and regulation of intestinal immune responses, including oral tolerance towards food-borne antigen structures (*Guarner & Malagelada, 2003; Mazmanian et al., 2005*).

The influence of the microbiota on chronic mucosal inflammation is not fully understood, but defensive and anti-cancerous effects by probiotics and prebiotics, via modulation of mucosal cyclooxygenase (COX) expression *in vitro,* have been reported (*Mäkivuokko et al., 2005; Nurmi et al., 2005*). Negative activities of the microbiota include the production of potentially toxic and carcinogenic compounds, derived from proteolytic metabolic processes (for example, ammonia, phenols), the translocation of opportunistic pathogens across the mucosal barrier to the mesenteric lymph nodes and other extra-intestinal sites (*Cummings & Macfarlane, 1991; Gibson &*

Roberfroid, 1995), and GIT infections caused by ingested pathogens, resulting in diarrhoea. Therefore, the modulation of the GIT microbiota, through diet, may be advantageous for the host.

1.3.1. Commensal bacteria

Commensal bacteria have co-evolved with their host and they are vital for the development of a normal healthy gut (*Ley et al., 2006*). Commensal bacteria are acquired shortly after birth and they have been found to reach a density of 10^{12} per ml of intestinal contents in the adult human colon (*Savage, 1977*). Therefore, the numbers of commensal bacteria are 10 times greater than the combined number of somatic and stem cells within the human body (*Hooper & Gordon, 2001*).

Commensal bacteria positively influence various processes such as the digestion and absorption and storage of nutrients, in addition to providing a defence against pathogen colonisation through competition for nutrients and the secretion of antimicrobial substances and micro-niche exclusion (*Artis, 2008*). Furthermore, commensal bacteria assist in angiogenesis and the development of the intestinal epithelium and they have been found to be essential for the normal development and functioning of the immune system (*Artis, 2008*).

1.3.2. Probiotics

The word ‘probiotic’ is derived from the Greek word meaning “for life”, and probiotics are defined as live organisms that, when ingested in sufficient amounts, facilitate a health benefit for the host (*Schrezenmeir & de Vrese, 2001*). There are numerous commercially available supplements containing viable micro-organisms with probiotic properties. The most commonly used probiotics are lactobacilli, bifidobacteria and nonpathogenic yeasts. Although probiotics have been proposed for use in gastrointestinal, inflammatory, infectious, neoplastic and allergic disorders, the ideal probiotic strain (for use in any of these indications) has yet to be identified. The interpretation of available data on probiotics is further confused by variability in strain selection, dose, delivery vehicle and evaluation of viability and efficacy (*Shanahan, 2003*). In 1908, Metchnikoff described probiotics, related to longevity of individuals and a regular intake of a fermented milk product. Over the years, since that time, numerous products have emerged in health food

stores and supermarket shelves throughout the world, which include the term 'probiotic' on their label (*Quigley, 2010*).

1.3.3. Prebiotics

Prebiotics are defined as non-digestible food ingredients, which beneficially modulate the host, by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, which have the potential to up-regulate health (*Gibson & Roberfroid, 1995; Quigley, 2010*).

Compared with probiotics, which introduce exogenous bacteria into the human colon, prebiotics stimulate the growth of a limited number of health-promoting species, which are already present in the colon and especially (but not exclusively) lactobacilli and bifidobacteria. For example, the oligosaccharides in human breast milk (also referred to as human milk oligosaccharides) are considered a prebiotic, since they support the preferential growth of bifidobacteria and lactobacilli in the colon. This may well account for some of the immunological and other benefits that have accrued to breast-fed infants (*Depeint et al., 2008; Quigley, 2010*). Many of the prebiotic ingredients are oligosaccharides, such as galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS), inulin and isomalto-oligosaccharide (IMO) (*Crittenden & Playne, 1996; Van Loo et al., 1999; Cummings et al., 2004*)

Due to their chemical structure, prebiotics are not digested and absorbed in the small intestine, but instead they are fermented in the colon, by endogenous bacteria, resulting in lactic and short-chain carboxylic acids (*Quigley, 2010*). Recent studies have shown that prebiotics produce quite selective changes in the composition of the microbiota (*Vos et al., 2007*) and this may have benefits for the alleviation of irritable bowel syndrome (*Silk et al., 2009; Quigley, 2010*).

1.3.4. Synbiotics

Synbiotics, defined as a combination of probiotics and prebiotics, aim to increase the survival and activity of proven probiotics, *in vivo*, in addition to stimulating indigenous bifidobacteria and lactobacilli (*de Vrese & Schrezenmeir, 2008*).

1.4. Gut mucosal layer

The mucus layer, which coats the GIT, is the front line of innate host defence, largely due to the secretory products of intestinal goblet cells. Goblet cells synthesise secretory mucin glycoproteins (MUC2) and bioactive molecules, such as epithelial membrane-bound mucins (MUC1, MUC3 and MUC17); trefoil factor peptides (TFP); resistin-like molecule beta (RELMβ); and Fc-gamma binding protein (Fcγbp) (*Kim & Ho, 2010*). TFP are a family of small proteins, which are secreted onto the apical gut mucosal surface where they are present in high concentrations. These peptides appear to protect the epithelium and support healing after injury (*Ogata & Podolsky, 1997*). RELMβ is a cysteine-rich cytokine expressed in the GIT and involved in insulin resistance and gastrointestinal nematode immunity. Hogan *et al*. (2006) reported RELMβ as an important molecule in homeostatic gastrointestinal function and colonic inflammation. Fcγbp is immunologically distinct from known Fcγ receptors and its structure contains repeated cysteine rich unit sequences, which resemble those present in mucins. Kobayashi *et al*. (2002), using an immunohistochemical study, reported that mucin secreting cells in the colon, small intestine, gall bladder, cystic duct, choledochus, bronchus, submandibular gland and cervix uteri, contained Fcγbp. With the use of immunodotblot and immunoprecipitation analysis and a coating of IgG and monoclonal antibodies, they found that the fluids secreted by these cells were capable of binding to IgG. The surface mucus cells in the stomach were variably positive for Fcγbp. Due to proteolytic degradation, Fcγbp in the gut's lavage fluid, did not possess IgG binding activity, but this activity was present in the mucus covering the colon.

The mucus forms a continuous gel which covers the epithelial surface (*Kindon et al., 1995; Matsuo et al., 1997*). The enhanced presence of goblet cells would assure an improved synthesis of mucus, which has important immune and non-immune functions that would help to protect the epithelial surface. Reports have been published that show an increase in goblet cells by dietary intervention, such as probiotic bacteria (*Dock-Nascimento et al., 2007*; *Vinderola et al., 2007*) and keratinocyte growth factor (*Fernandez-Estivariz et al., 2003*). Other investigators have shown that chronic protein depletion, or protein-energy under-nutrition, decreased goblet cell number or

mucin synthesis in rodent and pig proximal small intestines, respectively (*Neutra et al., 1974; Sherman et al., 1985*). Recently, King *et al*. (2008) reported that feeding pigs with either colostrum or plasma resulted in an increase in villus and crypt goblet cells.

In response to stimulation, intestinal goblet cells may increase their discharge of mucin through two processes. In many mucus secreting cells, compound exocytosis is induced which results in deep cavitation of the apical membrane surface of the mucus cells (*Specian & Oliver, 1991; Forstner, 1995*). Some intestinal goblet cells may also react to stimulation by a second process which decreases the intracellular store of mucus granules, but without cavitation (*Specian & Oliver, 1991; Forstner, 1995*). The latter mechanism results in a total loss of mucus granules. The goblet cells are no longer stained by alcian blue and they appear identical to other epithelial cells. The mucus layer provides a physical barrier between the gut lumen and epithelium and it functions as an important structure for host-bacteria and bacteria-bacteria interactions. The powerful hydrophobic surface of the mucus layer averts the influx of water-soluble toxins into the epithelium (*Lugea et al., 2000*). A layer of surfactant lipids found along the top of the mucus gel, which are secreted by epithelial cells, provides the surface hydrophobicity (*Lichtenberger, 1995*).

In many mammalian species (including humans), surface hydrophobicity is very high on top of the gastric and colonic mucosa, whereas it is much lower throughout the small intestine which constitutes the major absorptive surface. Mucus layer thickness may rise to 450 μm in the stomach, but the small intestine is covered with a much thinner mucus layer and it is discontinuous above the PP. Hydrophobicity and the mucus' layer thickness increases after the ileo-caecal valve and gradually increase from the ascending colon. The mucus layer thickness reaches up to 285 μm in the rectum (*Matsuo et al., 1997; Lugea et al., 2000*). Modification of the properties of this protective layer of mucus may affect the intestinal absorption of both dietary nutrients and endogenous macromolecules and ions (*Dryden et al., 1985; Quarterman, 1987*). The mucus coat separates mucosal cells from the exterior environment and provides a defence from harsh substances, allowing lubrication of the cell surface and regulation of ion fluxes (*Forstner & Forstner, 1994*). Mucus provides a vital role for the intestinal surface integrity in health, and the malfunction of mucus secretion (and/or mucin expression) might result in several pathologies, such as inflammatory intestinal diseases and

cancer (*Ho et al., 1993*). Microbes, such as bacteria, viruses and protozoa, become trapped in the mucus layer and are expelled from the GIT by peristalsis. Other important components of mucus are comprised of various proteolytic enzymes, which serve not only to help in digestion of polypeptides, but also to alter and/or diminish the immunogenic properties of these peptides, because peptides of less than 8 to 10 AA are poor immunogens (*Forstner & Forstner, 1994*).

Mucins are the high molecular weight glycoproteins secreted by goblet cells, which are found in the columnar epithelium. They consist of a core protein, which is comprised of tandem repeats of AA sequences, rich in threonine, proline and/or serine. The hydroxyl residues are heavily substituted with *O*-linked oligosaccharides. *N*-acetylglucosamine, *N*-acetylgalactosamine, fucose and galactose are the four primary mucin oligosaccharides. Mucin oligosaccharide chains are often terminated with sialic acid or sulphate groups (*Forstner et al., 1995; Herrmann et al., 1999; Deplancke & Gaskins, 2001*). Mucins are classified into neutral and acidic subtypes and the acidic mucins are further divided by the presence of sulphated (sulphomucins), or non-sulphated (sialomucins) groups. Neutral mucins appear to be the major subtype expressed in gastric mucosa and in the small intestine: However, acidic mucin dominates in the large intestine (*Meslin et al., 1999; Deplancke & Gaskins, 2001; Kleessen et al., 2003*). Fontaine *et al*. (1996) reported that acidic mucins protected against bacterial translocation, due to the presence of sulphated mucins, which appear to be resistant to bacterial glycosidases and host proteases (*Deplancke & Gaskins, 2001*). Mucin acts as the main structural component of the mucus layer, giving rise to its polymeric, viscoelastic and protective properties. These are located at the edge of the epithelial cells. The mesh-like structure of the mucin gel prevents the diffusion of unwanted macromolecules and it also serves numerous other functions, such as lubrication for the passage of particulate matter; preservation of a hydrated layer over the epithelium; providing a barrier to noxious substances; and forming a permeable gel layer, for the exchange of gases and nutrients, with the underlying epithelium (*Laboisse et al., 1996*). Preventing the entry of enteric pathogens is one of its well established functions. Since mucins play an important role in protecting the underlying epithelium, any quantitative alteration in mucus secretion may change this defensive barrier and therefore this may have important physiological implications.

Mucins can be divided into three distinct subfamilies: cell-surface

mucins, secreted gel-forming mucins, and secreted non-gel-forming mucins. Secreted gel-forming mucins and not the secreted non-gel-forming mucins, have the capacity to form oligomers and the capability to build up the dense, visco-elastic mucus gel that covers many epithelia. Structural and functional properties of secreted non-gel-forming mucins are not well described (*Dekker et al., 2002; Linden et al., 2008*). Secreted mucins facilitate the formation of the mucus gel, but the function of membrane bound mucins is not well characterised, despite the fact that they are located on the surface of epithelial cells throughout the body. Gastrointestinal diseases and stress modify the mucus layer, both quantitatively and qualitatively. Crohn's disease (CD), colitis and gastric carcinogenesis, in which *Helicobacter pylori* (*H. pylori*) is implicated, alter the mucin secretion and reduce the number of goblet cells, which result in less mucin and leads to the degradation of the mucus layer (*Corfield et al., 2000; Babu et al., 2006*).

Until recently, 21 different mucin genes have been identified, cloned and partially sequenced, in humans (*Dekker et al., 2002*) and the majority of their homologues have been identified in mice and rats (*Perez-Vilar & Hill, 1999*). $Muc2^{-/-}$ *mice* developed spontaneous inflammation, presumably due to the absence of the major component of intestinal mucus, leading to increased exposure to normal intestinal microbial flora (*Velcich et al., 2002; Van der Sluis et al., 2006*). Up-regulation of MUC2 and MUC3 expression in colonic cells has been correlated with a decreased binding of enteropathogenic *E. coli* (*Mack et al., 1999, 2003*). Larson *et al*. (2003) found that colostrum-associated M-SAA3 (mammary-associated serum amyloid A isoform 3) peptide enhanced innate protection, by stimulating human intestinal epithelial cells to express MUC3. In the colon, the mucus layer acts as a physical barrier to protect and maintain epithelium integrity (*Tai et al., 2007*). It has been found that mice deficient in mucin production spontaneously developed a colitis-like phenotype (*Van der Sluis et al., 2006*).

1.4.1. Diet effects on mucin

Growing rats fed with dietary fibres with increasing viscosities had an increase in ileal endogenous nitrogen flow and mucoprotein (sialic acid) secretion (*Larsen et al., 1994*). Also, Barcelo *et al*. (2000) reported that dietary fibre (alginate, ulvan), uronic acids (glucuronic acid, galacturonic acid) and short chain fatty acids stimulate secretory activity of intestinal goblet cells.

Caballero-Franco *et al*. (2007) studied three bacterial groups (lactobacilli, bifidobacteria and streptococci) contained in a probiotic formula and reported that the *Lactobacillus* species was the strongest potentiator of mucin secretion *in vitro*. Claustre *et al*. (2002), using isolated vascularly perfused rat jejunum and ELISA, found that peptides, from a casein hydrolysate, clearly stimulated the expression of mucin genes in the rat jejunum. Trompette *et al*. (2003) reported that luminal administration of β-casomorphin-7, a fragment of β-casein, caused mucin release in the rat jejunum. Zoghbi *et al*. (2006), using rat and human intestinal mucin-producing cells (DHE and HT29-MTX) and quantitative RT-PCR and ELISA, found that β-casomorphin-7 may contribute significantly to mucin production, via a direct effect on intestinal goblet cells and the activation of mu-opioid receptors. Recently, Han *et al*. (2008), using qPCR, found that feeding rats with hydrolysed casein (HC) influenced mucin gene expression in the rat intestine, *in vivo*. The higher ileal endogenous nitrogen loss, identified in that study, may have resulted from a higher secretion of mucins into the intestine, with diet HC. Also, they reported that HC promoted a significant increase of Muc3 mRNA in the small intestinal tissue and Muc4 mRNA in the colon.

1.4.2. Mucus synthesis and secretion: the effect of microbial derived factors

Mucus provides various ecological advantages for the intestinal microbes. Mucin oligosaccharides serve as a direct source of carbohydrates and peptides and exogenous nutrients for bacteria, including a source of vitamins and minerals which are probably concentrated within the mucus matrix. Microbes, which are able to colonise mucus, can evade rapid expulsion, via the hydrokinetic properties of the GIT. Microbial colonisation of mucus would thus impart a growth advantage for the bacteria. Both commensal and pathogenic microbes could derive considerable benefit from an ability to chemically regulate mucus synthesis (or secretion) from host goblet cells (*Deplancke & Gaskins, 2001; Derrien et al., 2004*).

A well-defined mucin secretagogue (secretory mucins are stored in secretory granules and released at the apical surface in response to mucin secretagogues) is the cholera toxin (CT) of *V. cholerae*, which triggers massive mucin release, via a cAMP-dependent mechanism (*Lencer et al., 1990*) and it can also increase the mucin secretion directly (*Epple et al.,*

1997). The *Listeria monocytogenes* (*L. monocytogenes*) toxin (listeriolysin O) and entamoeba secretagogue have also been found to induce mucus exocytosis and depletion (*Coconnier et al., 1998; Belley et al., 1999*). LPS from *H. pylori* caused a rapid mucin discharge from a human gastric mucosa biopsy segment and persistent incubation led to a concentration dependent decrease in mucin synthesis and secretion (*Slomiany et al., 1992a*). Furthermore, the addition of *H. pylori* not only suppressed total mucin synthesis, but it also inhibited MUC1 and MUC5AC gene expression, in a human gastric cell line, which substantiates the ability of microbes to directly influence MUC gene expression (*Byrd et al., 2000*).

1.4.3. Microbial mucolysis

Previous studies have established that mucins are degraded in the intestine (*Lindstedt et al., 1965; Hoskins & Zamcheck, 1968; Vercellotti et al., 1977*). Both host digestive enzymes and bacterial enzymes are known to degrade soluble mucins (*Stanley et al., 1986*). Mucus degradation by microbial (both commensals and pathogenic) enzymes appears to be a common feature amongst bacteria (*Corfield et al., 1992; Deplancke & Gaskins, 2001*).

Enzymatic digestion of the mucus coat allows access to readily available sources of carbon and energy and that helps bacteria to gain access to the epithelial surface. Mucin degradation is a multistep process, which starts with proteolysis of the non-glycosylated regions of the mucin glycoproteins by the host and microbial proteases (*Quigley & Kelly, 1995*).

The first step markedly reduces mucin gelation and viscosity and this results in the accumulation of highly glycosylated sub-units (> 500-kDa), which are resistant to additional proteolytic attack. Microbial enzymes then degrade the mucin glycopeptides, based on the complexity of the oligosaccharide chains, which also differ in size, degree of branching, type of linkage and the presence of terminal sialic acid or sulphate groups. Terminal sulphate, sialic acid residues and oligosaccharide side chains, are then cleaved by bacterial sialidases, glycosulphatases and specific glycosidases (*Corfield et al., 1992*). *E. histolytica* binds specifically to *N*-acetylglucosamine and harbours the corresponding glycosidase (*Belley et al., 1999*). *S. typhimurium* attaches specifically to a glycoprotein containing sialic acid (*Vimal et al., 2000*) and it has a sialidase (*Hoyer et al., 1992*). *H. pylori* bind to sulphated glycoconjugates (*Bravo & Correa, 1999*) and harbour a

glycosulphatase (*Slomiany et al., 1992b*). Furthermore, *H. pylori* also has the enzymatic capability to damage the oligomeric structure of mucin, allowing the pathogen to move freely within the mucus layer, supported by its highly active flagellum and its ability to down-regulate mucin synthesis (*Dohrman et al., 1998; Windle et al., 2000*). In one study, mucin degradation was studied with one *Clostridium* (RS42) and two *Bacteroides* (RS2 and RS13) strains, which had been isolated from a pig colon mucosa. The results showed that the three strains of colon bacteria were able to hydrolyse mucin carbohydrate (*Stanley et al., 1986*).

However, the interactions of the gut microbiota, especially probiotic bacteria with intestinal mucus, are poorly understood. Ruseler-van Embden *et al*. (1995), using both *in vitro* and *in vivo* studies, found that *L. casei*, *L. acidophilus* and *B. bifidum* strains all colonised a rat's intestinal mucus, but they were not found in the deep crypts suggesting that there was no degradation of mucus glycoproteins, either *in vitro* or *in vivo*. Zhou *et al*. (2001), using a SDS-polyacrylamide gel electrophoresis (SDS-PAGE) technique, demonstrated that probiotic lactic acid bacteria *L. rhamnosus* (HN001), *L. acidophilus* (HN017) and *B. lactis* (HN019) were unable to degrade gastrointestinal mucin, *in vitro*.

1.5. Gut Immune System

The immune system is a system of protective biological structures and processes, which ensures the host's protection against disease, by detecting and destroying pathogens, such as bacteria, parasites and viruses, or those diseases from within, such as cancerous cells (tumour cells), or those that make auto-antibodies (*Bower, 1990*). The GIT of mammals is heavily colonised with a vast number of symbiotic microbial communities and the tract is also a major portal of entry for pathogenic bacteria. To manage these complex microbial challenges, intestinal epithelial cells generate a diverse repertoire of proteins (or peptides), from multiple distinct protein families. These proteins are secreted apically into the luminal environment of the intestine, where they play a pivotal role in protecting against enteric infections and they may also function to limit opportunistic invasion by pathogenic bacteria (*Wilson et al., 1999; Salzman et al., 2003b*). Different organs and tissues (with various functions) are involved in the efficient development of the

immune response. These are mainly classified into two types based on their functions: primary lymphoid organs and secondary lymphoid organs. In most mammals, bone marrow and thymus are the primary lymphoid organs, the sites at which the maturation of lymphocytes takes place. The lymph nodes, spleen and MALT, including GALT, are the secondary (or peripheral) lymphoid organs, which trap antigen, resulting in sites for mature lymphocytes to encounter trapped antigen. Based on molecular and functional characteristics, the host defences (or immune response), is commonly divided into two components: the innate immune system and the adaptive (or specific) immune system (*Eckmann, 2006*).

Innate immunity is characterised by the natural ability to detect microbes and generate antimicrobial defences, before encountering specific microbes. The host molecules, involved in these processes, are coded for in the germ line of the host and they are usually generated in a functional form by the host cells, which provide rapid and fast immune (innate) responses against microbial pathogens or infections. Immunological memory does not form within the innate immune system. In contrast, adaptive immunity, also known as the specific or acquired immune system, has the ability to develop precise specificity against a large range of microbes and antigens and it is able to develop immunological memory. This is due to the generation of new sensor and effector molecules that can recognize and protect against microbial pathogens and which are not encoded in the genome of the germ line. This is possibly due to a process of somatic DNA recombination and the reshuffling and editing of the segments of particular groups of immune genes, which results in new genes being encoded with unique new proteins, in the form of specific B-cell receptors (Ig, either as a membrane bound or secreted form) and T-cell receptors, within individual immune cells. After the first encounter with an antigen, immune responses (within an adaptive immunity) develop but with a delay or sometimes after several days. However, with subsequent exposure to the same antigen, this results in a rapid and long sustained immune response (*Eckmann, 2006*).

1.5.1. Innate immune system

The innate immunity is comprised of anatomic barriers including skin and mucous membranes and a cellular component; a physiological barrier (including temperature, low pH and chemical mediators); phagocytic/

endocytic barriers; and finally inflammatory barriers, which are the first line of defence. Together, these barriers work as a primary layer of natural defence against invading micro-organisms, with the general goal of preventing their entry into the body. An immune system, capable of protecting the host against microbial overgrowth and attack, requires specific sensor molecules for microbe detection (and differentiating from self) and effector molecules, for the subsequent measures needed to control and destroy them. The innate arm of the immune system uses many (a few hundred) germ line encoded gene products, which work as sensors for the microbial signature structures and as effectors with antimicrobial activity. In contrast, adaptive immunity uses products coded by millions of somatically rearranged immunoglobulin and T-cell receptor genes, such as sensors and effectors (*Eckmann, 2006*).

1.5.1.1. Sensor Molecules

The gut is frequently in contact with vast numbers of commensal bacteria and it is repeatedly exposed to foodborne and waterborne microbes both harmless and potentially pathogenic. Under normal conditions, this causes an immense challenge for the intestinal immune system, while it attempts to restrain and control the enteric bacteria and also prevent their entry into the remainder of the body. Effective control of intestinal microbes can happen without specific recognition (e.g. killing by stomach acid) (*Eckmann, 2006*). However, this may compromise the host's health (a problem associated with acid hypersecretion). Detection of enteric bacteria is mediated by various mechanisms, which depend on host specific receptors for conserved bacterial structures, which are not normally found in the host. These are called molecular patterns and when they are associated with pathogens, they are called pathogen-associated molecular patterns (PAMPs). Examples of PAMPs are LPS, peptidoglycans, lipoteichoic acid, bacterial flagellin and unmethylated bacterial DNA. The receptors that recognise PAMPs are called pattern recognition receptors (PRR), which (on interaction) activate cellular signal transduction pathways, which then activate innate immunity. The detection of PAMPs, by PRRs, results in antigen-presenting cell activation and this promotes adaptive immunity. Other endogenous molecules, called danger-associated molecular patterns, can also induce immune responses. This important group of PRRs are Toll-like receptors (TLR); RIG-I-like receptors; NOD-like receptors (NLR); and C-type lectin receptors (*Eckmann,*

2006; Kumagai & Akira, 2010).

1.5.1.1.1. Toll-like receptors

A mammalian homologue of the drosophila Toll was identified, after the discovery of the function of the drosophila (*Drosophila melanogaster*) Toll in the host's defence against fungal infection (*Medzhitov et al., 1997*). Later, a family of proteins, structurally related to drosophila Toll, was identified and together they were referred to as the Toll-like receptors (TLRs). The intracellular signalling cascades, triggered by these PRRs, led to a transcriptional expression of inflammatory mediators, which synchronise the elimination of pathogens and infected cells. The signalling pathway of drosophila Toll shows a remarkable similarity to the mammalian IL-1 pathway, which leads to the activation of nuclear factor-*κ*B (NF-*κ*B) a transcription factor responsible for many aspects of inflammatory and immune responses (*Takeuchi & Akira, 2010*).

The TLR family comprises 10 members (TLR1-TLR10) (*Medzhitov et al., 1997; Rock et al., 1998; Takeuchi et al., 1999; Chuang & Ulevitch, 2000; Du et al., 2000; Chuang & Ulevitch, 2001*). TLRs are comprised of a sub-family of five members (TLR2, TLR3, TLR4, TLR5 and TLR9 sub-families), within the larger super-family of interleukin-1 (IL-1R) receptors, based on structural similarity within their cytoplasmic regions (consisting of 'Toll/IL-1R', named as TIR motif). In contrast, the extracellular regions are quite different. The extracellular region of the IL-1Rs possesses Ig-like domains and TLRs comprised of multiple leucine-rich repeats. The TLR2 sub-family is composed of TLR1, TLR2, TLR6 and TLR10. The TLR3 sub-family composed of TLR3; TLR4 sub-family composed of TLR4; TLR5 sub-family composed of TLR5; and TLR9 sub-family composed of TLR7, TLR8 and TLR9 (*Eckmann, 2006*). Various ligands (or activators) are found to be recognised by TLRs: tri-acyl lipopetides and di-acyl lipopeptides are known to activate TLR1 and TLR6; peptidoglycans and lipopeptides activate TLR2; LPS, anthrolysin O activates TLR4; ssRNA activates either TLR7 or 8; CpG (deoxy-cytidylate-phosphate-deoxy-guanylate) DNA activates TLR9; dsRNA activates TLR3; and flagellin activates TLR5 (*Eckmann, 2006*).

TLR1, TLR2 and TLR6 are constitutively expressed in moncoytes/ macrophages, dendritic cells and B cells, in most organs. Stomach, colon, lung and skin epithelial cells have an expression of TLR5. The bacterial

flagella are mainly recognised by TLR5. TLR3 is expressed in dendritic cells, normal human small intestine and in the upper crypts of the colon, but the expression is very low or absent in monocytes, neutrophils, T and B lymphocytes. TLR7 is produced constitutively in the normal human small intestine and the colon. However, TLR8 is expressed only in the colon and not in the small intestine. TLR10 is expressed in B cells, but weakly in dendritic cells and in the colon and small intestine, under normal conditions (*Eckmann, 2006*).

TLR4 is the best studied mammalian receptor. TLR is predominantly expressed by macrophages, dendritic cells and B cells, which are present throughout the body. In normal conditions, human intestinal epithelium expresses very low levels of TLR4, but expression dramatically increases under inflammatory conditions (*Cario & Podolsky, 2000*). LPS (a chief constituent of the outer membrane of gram-negative bacteria) is primarily recognised by TLR4. However, TLR4 by itself does not function as an LPS receptor; it requires a helper molecule, called MD-2. Cells that lack either TLR4 or MD-2 (or both) do not respond to LPS (*Shimazu et al., 1999*). Together with TLR4 and MD-2, two other proteins, namely LPS-binding protein (LBP) and CD 14 (cluster of differentiation 14), are involved in precise LPS detection (*Eckmann, 2006*). In the lamina propria, only a small percentage of CD14 expressing macrophages are present and that is the reason why there is a general lack of LPS responsiveness within these cell populations. In contrast, in acute colonic inflammation there is an increase in the number and proportions of CD14 expressing macrophages, due to an influx of $CD14^+$ cells from the blood circulation (*Grimm et al., 1995; Rogler et al., 1997*) and this is accompanied by an increased LPS response from those cells (*Rugtveit et al., 1997*). These findings suggest that, under normal conditions, intestinal epithelial cells and macrophages are hypo-responsive to LPS and therefore, LPS from commensal bacteria does not trigger mucosal inflammation. In contrast, under inflammatory conditions, expression of the crucial components of the LPS receptor complex and subsequent LPS responsiveness can be induced, thus demonstrating that a mucosal response to LPS can be controlled by the mucosal micro-environment (*Eckmann, 2006*). The work described above shows that the TLR4 has been recognised as an essential component in the recognition of LPS. However, other reports have shown that LPS can also be recognised independently of TLR4. A study with affinity chromatography, peptide mass fingerprinting and fluorescence

resonance energy transfer, has found four molecules on the cell surface, which can attach to LPS. These are the heat shock protein 70 (Hsp70), Hsp90, chemokine receptor 4 (CXCR4), and growth differentiation factor 5 (*Triantafilou et al., 2001*).

1.5.1.1.1.1. RIG-I–like receptors

TLRs can detect viruses that are present in the endosomal compartment (ie, 'outside' the cell) and promote antiviral responses, such as the production of type I interferon. However, viruses usually replicate inside the cell and therefore, an intracellular receptor (or receptors) is necessary for a well-organised antiviral immunity. RIG-I and the homologues melanoma differentiation associated gene 5 (MDA5) and LGP2 (RIG-I–like receptors [RLRs]) are the intracellular receptors for RNA viruses (*Akira et al., 2006; Kato et al., 2008; Satoh et al., 2010*). These receptors transmit their signal through a common adaptor protein, interferon promoter stimulator-1 (IPS-1), in order to induce type I interferon production and antiviral responses (*Kawai & Akira, 2006*).

1.5.1.1.1.2. Nod-like receptors (NLR)

Another class of PRRs, NLRs, have been identified as crucial sensors of bacterial infection. Nod1 and Nod2 are NLRs that detect PAMPs, which are derived from the bacterial cell wall. Unlike TLRs, Nod1 and Nod2 are located in the cytoplasm and they can perform TLR-independent antibacterial responses (*Akira et al., 2006*). Nod1 is produced, constitutively, in the epithelial cells of the stomach, the small intestine and the colon (*Inohara et al., 1999; Kim et al., 2004*). Nod1 works as a microbial sensor and it mediates detection of a specific diaminopimelic acid-containing dipeptide (or tripeptide motif) which is found in the cell wall of gram-negative bacteria and gram-positive bacteria and which is not present in the eukaryotes (*Girardin et al., 2003*). Nod2 is expressed predominantly in macrophages, dendritic cells and neutrophils (*Ogura et al., 2001*), as well as in paneth cells in the small intestine (*Ogura et al., 2003*). Reports have shown that Nod2 is involved in defence against the parasite *Toxoplasma gondii* (*Shaw et al., 2009*). Cytokines, such as TNFα and IFNγ, can induce the expression of Nod2 in cultured intestinal epithelial cells, suggesting that under normal conditions, the expression may be very low, but it can be increased under inflamed

conditions (*Ogura et al 2003; Rosenstiel et al., 2003*). Other NLRs, such as NALP1, NALP3, Ipaf and Naip, are components of a molecular complex called the inflammasome (*Fritz et al., 2006*).

1.5.1.1.1.3. C-type lectin receptor (CLR)

CLRs are also receptors for endogenous ligands. CLRs, such as Mincle (*Yamasaki et al., 2008*) and Clec9a/DNGR-1 (*Sancho et al., 2009*), detect damaged but not apoptotic cells. Mincle recognises Sin3-associated polypeptide p130 (SAP130), a component of small nuclear ribonucleoprotein, which is released from damaged cells (*Kumagai & Akira, 2010*).

1.5.1.2. Effector Molecules

The host's immune defence against commensal and pathogenic microbes requires not only a capacity to detect their presence but also to control and kill them. Under normal conditions only a few (or no) enteric bacteria can reach systemic locations. Mammals have a varied set of antimicrobial molecules, which include inorganic disinfectants (such as hydrogen peroxide and nitric oxide) and specialised proteins (including defensins, hepcidins and cathelicidins) and proteins (such as lysozyme, secreted phospholipase A_2 and angiogenins etc). Based on structural characteristics, these antimicrobial peptides may be divided into two major groups, peptides with β-sheet structure (eg, defensins and hepcidins) and peptides with α-helical conformation (cathelicidins) (*Eckmann, 2006*).

1.5.1.2.1. Hydrogen peroxide and nitric oxide

The innate immune system is comprised of circulating and fixed phagocytes, such as neutrophils, eosinophils, monocytes/macrophages, natural killer cells and dendritic cells. Initially, neutrophils attach to pathogenic micro-organisms and they phagocytose and kill them. Phagocytosis is assisted by opsonisation. Opsonins activate neutrophils, resulting in an oxidative burst that produces free radicals, such as hydrogen peroxide (H_2O_2) and superoxide anion ($O^{\cdot}{}_2^-$). These substances eradicate the bacteria and the neutrophil, with a release of toxic waste products. Although this response is beneficial, to a certain extent, a continuous and extensive inflammatory phase can be harmful to the host. Monocytes (in blood) and macrophages (in tissues) are also the machinery of innate immunity. They engulf and digest

antigens and then process them through an oxidative burst reaction. In addition, they present antigen particles to T cells (T lymphocyte), via MHC I, or II receptors for immune recognition (*Abbas et al., 1991*).

Nitric oxide has a very powerful antimicrobial activity. In combination with $O^{\cdot}_2{}^{-}$, it results in more powerful and potent antimicrobial substances. Reports have shown that the antimicrobial activity of macrophages against bacteria, fungi, parasitic worms and protozoa is due to nitric oxide and other substances derived from it (*Goldsby et al., 2003b*).

1.5.1.2.2. Defensins

Defensins are 2-6-kDa cationic peptides with antimicrobial properties. Based on their structure and the spacing of disulphide bonds, they are divided into two major groups: α and β defensins. They cause microbial 'death by lysis' by means of a disruption to the integrity of the microbial cell membranes, which vary from the host cell membranes due to the absence of cholesterol and high phospholipids content (*Eckmann, 2006*).

1.5.1.2.2.1. α-defensins

Based on comparative genome analysis, humans express 11 α-defensin genes, of which 6 (*DEFA1-6*) are expressed and the remaining 5 (*DEFA7P-11P*) are likely to be pseudo-genes (*Patil et al., 2004*). In humans, neutrophils and Paneth cells present in the crypts of the small intestine express α-defensins. Four α-defensins (*DEFA1-4,* coding for human neutrophil peptides, HNP 1-4) are found in neutrophils and *DEF5* (coding for human defensins, HD5) and *DEF6 (*coding for HD6) are expressed primarily in Paneth cells (*Cunliffe, 2003*). HD5 can also be detected in small intestinal cells, which are villus epithelial cells with a similarity to goblet cells (*Cunliffe et al., 2001*) and α-defensins (such as HD5) are synthesised as precursor proteins, which need to be processed to become biologically active, usually by the enzyme trypsin, which is co-localised with HD5 in human Paneth cells (*Ghosh et al., 2002*). Moreover, during post-translation processing defensin production can also be controlled at the expression level. Oral infection of mice with virulent *S. typhimurium* resulted in down-regulation of cryptdin-1 (expressed only in mice but not in humans; similar to α-defensin genes) in the small intestine within 24 h (*Salzman et al., 2003a*), suggesting that enteric pathogens may have created strategies to ease the chemical barrier formed by α-defensins in the

small intestine (*Eckmann, 2006*).

1.5.1.2.2.2. β-defensins

In humans, at least 31 β-defensin genes (*DEFB*) have been reported, located in five separate gene clusters (*Maxwell et al., 2003*). β-defensins are largely expressed in the epithelial surface of the intestine, lungs, skin, kidneys and testis, showing that they defend the host from exposure to environmental pathogens and/or microbes. In the human intestinal tract, *DEFB1* (coding for human β-defensin, hBD-1) is constitutively expressed in the small intestinal and colonic epithelial cells, but not up-regulated by inflammatory stimuli or infection caused by invasive microbes (*O'Neil et al., 1999*). Protozoan parasites, such as *C. parvum* infection (both *in vivo* and *in vitro*) cause suppression and down-regulation of epithelial β-defensin expression in the intestinal epithelial cells (*Zaalouk et al., 2004*). Moreover, reports have shown that hBD-1 can destroy *C. parvum* sporozites, *in vitro* (*Zaalouk et al., 2004*). Together these data clearly suggest that suppression of epithelial β-defensin expression by pathogens is a virulence strategy to evade attack by the host's defence. The expression of hBD-2 differs from hBD-1, since they are not constitutively present in the normal small intestine, but they are strongly expressed in the presence of enteric pathogens or the presence of inflammatory mediators such as IL-1 (*Zaalouk et al., 2004*). Wehkamp *et al*. (2003) reported that in a normal human colon, hBD-3 is rarely expressed, but it is strongly induced in inflamed areas of patients suffering from CD and ulcerative colitis (UC). In contrast, Fahlgren *et al*. (2004) found that hBD-3 and hBD-4 are both constitutively expressed in both normal small and large intestinal epithelium. Expression of both defensins was markedly increased in patients suffering UC, but not in patients with CD. Discrepancies in the results of the two studies may be due to the difference in the starting materials and assay sensitivity (*Eckmann, 2006*). In summary, β-defensins can be classified into two categories based on intestinal regulation. The defensin hBD-1 is a highly expressed constitutive epithelial β-defensin with a low capacity for additional up-regulation, whereas hBD-2, hBD-3 and hBD-4 show varying degrees of constitutive epithelial expression and a strong inducibility to pathogenic and inflammatory stimuli (*Eckmann, 2006*).

1.5.1.2.3. Hepcidins

Hepcidins, also called liver-expressed antimicrobial peptides (LEAPs), consist of 20 to 25 AA containing four pairs of cysteines, which form intramolecular disulphide bridges (*Eckmann, 2006*). Hepcidins/LEAP-1 (*Krause et al., 2000; Park et al., 2001*) and LEAP-2 (*Krause et al., 2003*), the two peptides found in humans, are expressed constitutively at high levels by hepatocytes in the liver (*Park et al., 2001; Krause et al., 2003*) and they are present at different levels in serum and urine (*Park et al., 2001; Kemna et al., 2005*). Both hepcidins/LEAP-1 and LEAP-2 have strong *in vitro* antimicrobial activity against gram-positive bacteria such as streptococci and staphylococci and fungi, which include *Candida albicans* (*C. albicans), Aspergillus* and *Saccharomyces cerevisiae*. However, their activity against gram-negative bacteria is confined to certain species, such as *E. coli* (*Krause et al., 2000; Park et al., 2001*).

1.5.1.2.4. Cathelicidins

Cathelicidins are cationic antimicrobial peptides, in which a highly conserved N-terminal structural domain (cathelin) is linked to a C-terminal peptide with antimicrobial activity (*Hase et al., 2002; Lehrer & Ganz, 2002; Zanetti, 2004*). Approximately 30 different cathelicidins have been identified in mammals. However, only one has been identified in humans (human cationic protein 18-kDa, hCAP18/LL-37) (*Bulet et al., 2004*). The cathelicidin hCAP18/LL-37 is expressed in neutrophils, mast cells and epithelial cells. In the GIT, cathelicidin protein is formed, constitutively, in differentiated surface and upper crypt epithelial cells in the colon and in Brunner's glands in the duodenum, but not in the deeper regions of the colon crypts or epithelial cells of the small intestine (*Hase et al., 2002; Eckmann, 2006*). Commensal bacteria are not required for constitutive LL-37 production in the intestinal epithelial cells (*Hase et al., 2002*) and its expression is not up-regulated by inflammatory stimuli or in inflamed conditions *in vivo* (*Hase et al., 2002*). LL-37 is also formed on the surface epithelial and parietal cells of the stomach (*Hase et al 2002*), implying that it could be active as it passes to the small intestine. Apart from epithelial cells, LL-37 is also found in neutrophils in inflamed areas of colonic mucosa.

1.5.1.2.5. Lysozyme

Lysozyme, (N-acetyl-muramylhydrolase or muramidase) is a 14-kDa cationic protein which promotes antibacterial activity by hydrolysing peptidoglycans in the bacterial cell wall. A single lysozyme gene is found in humans, but two closely related (lysozyme M and P) genes are found in mice (*Cortopassi & Wilson, 1990; Chung et al., 1998; Cross et al., 1988*). Lysozyme is formed in human neutrophils, macrophages, Paneth cells in the small intestine and in alveolar type 2 epithelial cells in the lungs. Lysozyme expression is induced in either (or both) colonic or small intestinal goblet cells of patients suffering from UC, CD or celiac disease (*Fahlgren et al., 2003; Forsberg et al., 2004*). Oral infection of mice with a virulent strain of *S. typhimurium* can down-regulate small intestinal lysozyme expression within 24 hours, thus suggesting (as discussed in section-1.5.1.2.2.1) that enteric pathogens may contain strategies to prevent a lysozyme-dependent host immune defence. Small antimicrobial peptides derived from lysozyme are formed by partial proteolytic degradation (*Pellegrini et al., 1997*) and these small peptides exert antimicrobial activity in the intestinal lumen. Lysozyme can also work synergistically with other antimicrobial molecules (eg. lactoferrin), to destroy many enteric bacteria, such as *V. cholerae, Salmonella* and *E. coli* (*Ellison & Giehl, 1991*).

1.5.1.2.6. Secreted Phospholipase A_2 (PLA_2)

PLA_2 is an enzyme which catalyses the hydrolysis of glycerophospholipids, at the *sn*-2 (stereochemical number) position, to discharge free fatty acids and lysophospholipids (*Chakraborti, 2003*). On the basis of structural characteristics, calcium requirements and cellular localisation, PLA_2 can be divided into three major groups: (1) calcium-dependent, low-molecular weight (13-17-kDa), secreted enzymes ($sPLA_2$); (2) calcium-dependent, high-molecular weight (50-100-kDa) cytosolic enzymes ($cPLA_2$); and (3) calcium-independent intracellular enzymes ($iPLA_2$). One member of $sPLA_2$ (called group 2a $sPLA_2$) is a 14-kDa cationic protein, which has antibacterial activity due to its ability to destroy phospholipids in the bacterial cell membrane (*Buckland & Wilton, 2000*). It has a strong preference for phosphatidylglycerol, which is present only in the bacterial cell membrane, but not in eukaryotic membranes. Similar to lysozyme, group 2a $sPLA_2$ is very effective against gram-positive bacteria, such as *L. monocytogenes* and

Staphylococcus aureus (*S. aureus*), where it can gain entry into the cell membrane but not in gram-negative bacteria (*Buckland & Wilton, 2000; Koduri et al., 2002; Dubouix et al., 2003*). However, these enzymes work in synergy with other host-derived molecules (possessing bactericidal and membrane permeability properties), to also gain entry into the outer cell membrane of gram-negative bacteria (*Weiss et al., 1994*). Group 2a $sPLA_2$ is expressed primarily in Paneth cells in the normal small intestine and it can be released into the lumen by bacterial LPS stimulation (*Qu et al., 1996*). This gene is also expressed in the small intestinal and colonic enterocytes during intestinal inflammation (*Haapamäki et al., 1997*, *1999*).

1.5.1.2.7. Lectins

Antimicrobial lectins belong to a newly described family of inducible antimicrobial molecules with potential activity against gram-positive bacteria. They have been identified in humans as a hepatocarcinoma-intestine-pancreas/pancreatic-associated protein (HIP/PAP) and also in mice (with the name RegIIIγ). These small secreted C-type lectins, consist of a carbohydrate recognition domain (16-kDa) linked to an N-terminal signal peptide (*Zhang et al., 2003*). The expression of these proteins is constitutive throughout the intestinal epithelium, principally by Paneth cells in the small intestine and endocrine cells in the colon (*Christa et al., 1996; Narushima et al., 1997*). Both RegIIIγ and HIP/PAP are directly bactericidal, at low (micro-molar) concentrations for gram-positive bacteria (*Cash et al., 2006*). The antibacterial activity of RegIIIγ and HIP/PAP rely upon binding to the bacterial membrane, through interactions with peptidoglycan (*Cash et al., 2006*). Since peptidoglycan is present on the surface of gram-positive bacteria (but it is concealed in the periplasmic space of gram-negative bacteria) this binding activity provides a molecular explanation for the gram-positive specific bactericidal effects of these lectins.

1.5.1.2.8. Other proteins

Apart from the specialised antimicrobial peptides, such as defensins and lysozyme, other proteins which can prevent or kill bacteria, have been identified in the gut. Reports have shown that the normal human colonic mucosa contains numerous peptides, which possess antimicrobial activities, such as ubiquicidin (which has antimicrobial activity against pathogens like *L.*

monocytogenes and *S. typhimurium*), ribosomal proteins L30, L39 and S19; histones H1.5 and H2B; and eosinophil cationic protein (*Howell et al., 2003; Tollin et al., 2003; Eckmann, 2006*). Histones can neutralise endotoxins (*Augusto et al., 2003*) and they are present in normal human villus epithelial cells, where they are released during apoptosis in order to kill bacteria thus showing a link between cell death and antimicrobial defence (*Rose et al., 1998*). In summary, the innate immune system possesses various genome encoded effector molecules that show antimicrobial activity. These molecules are active against many microbial targets, making them highly effective for the control of infections, with not only commonly encountered microbes, but also those not previously encountered by the host. Altogether, the cooperation between the sensor and effector molecules (of the innate immunity) facilitates a primary host defence, which is vital for rapidly controlling pathogen attacks until adaptive immunity can develop powerful immune defences against particular microbial targets (*Eckmann, 2006*)

1.5.2. Adaptive immune system

Adaptive immunity is divided into humoral and cell-mediated immunity, composed of B lymphocytes and T lymphocytes respectively. The adaptive immune system is able to recognise and eliminate specific foreign molecules (i.e. antigens), pathogens and micro-organisms which cause diseases. Adaptive immunity shows four key characteristics: (a) antigen specificity (antibodies can distinguish subtle differences among the antigens); (b) diversity (the immune system is capable of producing tremendous diversity in its recognition molecules, allowing it to recognise billions of unique structures on foreign antigens); (c) immunologic memory (once the immune system has recognised and facilitated its action on an antigen, it acquires immunologic memory, (i.e. a second encounter with the same antigen leads to a very high state of immune reactivity); and (d) self/non-self recognition (the immune system normally only responds to foreign antigens, showing that they can recognise self from non-self molecules. A failure in the latter system may lead to autoimmune disease, which can be fatal to the host (*Goldsby et al., 2003a*).

B lymphocytes mature in bone marrow and respond to stimulation by certain antigens and then differentiate into plasma cells, which synthesise and secrete antibodies, commonly termed Ig. Details about Ig are discussed in

later sections. T lymphocytes arise from bone marrow but, unlike B cells, T cells migrate to the thymus and mature there. Antigen-presenting cells (APC), such as macrophages, are responsible for triggering a specific immune response. Interaction between an antigen and macrophage leads to the production of IL-1, which causes T cells to produce IL-2 and other lymphokines. Production of IL-2 helps to stimulate T and B cells to form clones, which bear receptors specific to the sensitising antigen. These clones form the long-lived memory cells, which proliferate and release lymphokines upon re-exposure to the same antigen. These clones, together with macrophages, can eliminate the antigen. Faults in cell-mediated immunity are linked to bacterial, fungal, mycobacterial, parasitic and viral infections (*Bower, 1990; Abbas et al., 1991*). Apart from mediating delayed hypersensitivity, organ graft rejection, elimination of pathogenic micro-organisms and the destruction of malignant cells, T cells also regulate the responses of other immune cells. There are two sub-populations of T cells: T helper (T_H) and T cytotoxic (T_C) cells. T_H and T_C cells can be differentiated by the presence of either CD4 or CD8 membrane glycoproteins on their surface (*Goldsby et al., 2003a*). The sub-sets of T cells are comprised of helper-inducer T cells $CD4^+$ (T_H), which assist plasma cells to produce antibodies and release lymphokines, which alter the interaction between lymphocytes and other cells. Cytotoxic-suppressor T cells, $CD8^+$ (T_C) may destroy target cells and inhibit antibody responses or inhibit inflammatory responses (*Bower, 1990; Abbas et al., 1991*).

Naive T cells recognise an antigen by using the MHC molecules via the T-cell receptor complex, which can be developed into four major subsets: T_H1, T_H2, Treg/T_H3 and T_H17, based on the pattern of cytokines they secrete. The key Th1 cytokines are IFNγ, IL-2, IL-3, IL-12, IL-17 and TNFα, which are predominantly involved in cell mediated immune response and they confer immunity against harmful intracellular agents, such as bacteria, viruses and tumour cells and also down-regulate intestinal IgA concentrations. They also activate and differentiate T and B lymphocytes, in addition to macrophages, whereas cytokines, such as IL-4, IL-5 and IL-6 (which are secreted by T_H2 cells) confer humoral responses, activate B lymphocytes, up-regulate mucosal immunity and mediate responses against extracellular parasites, in addition to up-regulating IgA concentration. Treg/T_H3 cells secrete IL-10 and TGFβ (transforming growth factor β) cytokines. A balance between T_H1 and T_H2 cytokines may be necessary to maintain a normal IgA immune response

(*Mosmann & Coffman, 1989; Kramer et al., 1995; Reiner & Seder, 1995; DiPiro, 1997*).

Mucosal immunity can be seen as a first line of defence that minimises the requirement for systemic immunity, which is chiefly pro-inflammatory and therefore a 'two-edged sword'. Many genes are involved in controlling innate and adaptive immunity with various modifications incorporated over millions of years. The mucosal immune system has developed two non-inflammatory layers of defence: immune exclusion, performed by secretory antibodies (mostly by sIgA, sIgG and sIgM), to prevent surface colonisation of micro-organisms and diminish penetration of potentially dangerous soluble substances; and immunosuppressive mechanisms, to evade local and peripheral hypersensitivity to antigens that are normally innocuous. The latter mechanism is known as 'oral tolerance' when induced via the gut (*Brandtzaeg, 1996*), and this probably explains why an obvious and continual allergy to food proteins is relatively rare (*Bischoff et al., 2000*). A similar down-regulatory mechanism of the immune system is normally created against antigenic materials of commensal microbial flora (*Duchmann et al., 1997; Helgeland & Brandtzaeg, 2000; Moreau & Gaboriau-Routhiau, 2000*).

Mucosally induced tolerance is a strong adaptive immune function. Significant amounts of food and drink pass through the gut of an adult every year, which results in an extensive uptake of intact antigens, usually without causing any harm. The gut mucosa is comprised of approximately 80% of the body's activated B cells, which are terminally differentiated to Ig-producing plasmablast and plasma cells. Dimeric IgA and pentameric IgM, produced by gut lymphocytes, are actively transported through secretory epithelial cells with the help of a polymeric Ig receptor (pIgR) — otherwise called a membrane secretory component or SC. This binds to the small peptide referred to as the 'joining' (J) chain, which is incorporated selectively into dimeric IgA and pentameric IgM (*Brandtzaeg et al., 1999*). Immune exclusion is then carried out by the generated sIgA and sIgM antibodies, in cooperation with innate non-specific defence mechanisms. Moreover, some serum derived (or locally produced) IgG antibodies may be passively transferred to the gut lumen, by paracellular transcytosis. However, as IgG is a complementing activating antibody (a cascade of proteins in the blood that form part of innate immunity) its role in surface defence may result in a pro-inflammatory reaction, which could expose the epithelial barrier function to

potential microbes (*Brandtzaeg & Tolo, 1977*).

Foreign material, which has penetrated through the epithelial barrier, is removed by a coordinated mechanism called 'immune elimination'. It symbolises a 'second line' of mucosal defence that relies partly on systemic immune components (serum-derived Ig), in addition to locally produced antibodies, possibly often working together with CD 8^+ T cells and NK cells. Non-specific biological amplification systems of innate immunity may enhance immune elimination. This situation may lead to obvious immunopathology or inflammatory reaction, if removal of the antigen is not achieved quickly, thereby giving rise to an inflammatory disease. Such 'frustrated' immunological elimination mechanisms are obvious in the pathogenesis of many gut disorders, such as food allergy, coeliac disease, Crohn's disease and inflammatory bowel disease (*Brandtzaeg et al., 1987; Brandtzaeg, 1997*; *Brandtzaeg et al., 1999*).

1.6. Host–pathogen interactions

The host immune system has been exploited by numerous gut pathogens. Some pathogens (bacterial, parasitic and viral agents) have developed numerous ways to promote their attachment, colonisation and ability to translocate to various organs. Examples of pathogens (and how they have developed their own exclusive approach) are discussed in the following section.

E. coli is the most abundant facultative anaerobe in the normal microbiota of the mammalian colon (*Kaper et al., 2004*). Different types of *E. coli* have been correlated with intestinal diseases, including enterohemorrhagic *E. coli* (EHEC); EPEC; enterotoxigenic *E. coli* (ETEC); and enteroaggregative *E. coli* (EAggEC).

Intestinal infections in infants are very common where hygiene is poor, and these result in diarrhoeal diseases. In their first year of birth, infants in underdeveloped countries suffer (on average) from at least ten episodes of diarrhoea (*Black et al., 1989*). In some countries, diarrhoea is the main reason for the death of young children (*Wadstrom, 1979*). In a normal biological state, close contact is mutually favourable to both bacteria and host. However, certain strains of pathogenic *E. coli,* such as EPEC contribute to the development of acute gastroenteritis. Whilst some of these types of *E.*

coli have very precise virulence factors, such as Shiga toxins produced by EHEC and heat-stable and labile toxins produced by ETEC, several other virulence factors are noticeably play a role in pathogenesis. One potential pathogenic mechanism, which is attracting attention, is the effect of *E. coli* bacterial products on intestinal lymphocytes. Recognised products (lysates) produced by the EPEC chromosomal genes were able to abolish cytokine production, by the use of human peripheral blood and gut lamina propria lymphocytes (*Klapproth et al., 1996*). The IL-2 and IL-4, which were produced due to mitogen-stimulated lymphoctes, were directly inhibited by lysates obtained from Shiga toxin producing *E. coli*, *E. coli* RDEC-1; *Citrobacter rodentium*; and an EPEC espB insertion mutant (*Malstrom & James, 1998*). In addition, as reported by Klapproth *et al*. (2000), lymphocyte proliferation and IL-2, IL-4 and IFNγ production (in response to numerous stimuli) have also been found to be inhibited by lymphostatin, which is a toxin secreted by an EPEC strain. Overall, these data suggest that enteric bacterial products may modify immune homeostasis in the GIT through the inhibition of regulatory cytokine production which could then add to bacterial pathogenesis.

Salmonella sp. invade epithelial M-cells and induce apoptosis of phagocytic cells (for their survival), by using a type III secretion system (*Sansonetti, 2002*). However, in contrast to *Shigella*, they often remain intracellular and they have evolved elaborate mechanisms to bypass immune defences and cause enterocolitis or systemic disease (*Martinoli et al., 2007*). *S. typhimurium* use their long fimbriae to adhere to murine M-cells. This results in the ruffling of the cell membrane and macropinocytosis (*Sansonetti, 2002*). *Salmonella* serotypes also secrete effectors, such as SopE and SopE2, which interact with the actin cytoskeleton and allow entire engulfment of the bacterium (*Hornef et al., 2002*). Some strains have transformed PAMPs, such as hepta-acylated lipid A, which results in low stimulatory LPS and/or a deficiency of flagellin, which makes these organisms less reactive. PAMPs are highly conserved microbial structures, comprised of LPS, peptidogylcans and bacterial DNA, which can start cellular signalling responses through interaction with innate host receptors such as Toll-like receptors (TLRs) (*section-1.5.1.1; Hornef et al., 2002*). Once inside the cells, *Salmonella* is able to exist or resist immune attack through various mechanisms.

1.6.1. Breakdown of gut barrier function

The gut barrier helps to preserve a close mutual benefit between gut microbes and the host animal. In addition, the functionality of this barrier is maintained by a complex network of physical, physiological and immune factors, which are comprised of dietary influences, the host's environment (which can be modified by age and external factors such as antibiotics and immune competency) and the indigenous microbial flora of the gut (*Rolfe, 1997*). Changes in these factors may result in non-efficient removal or degradation of harmful ingested antigens and/or disturbance of regulatory cell function, which results in mucosal damage, increased gut permeability and overgrowth and translocation of harmful pathogens. Carcinogens or toxins, which may be directly toxic to the gut mucosa are introduced by diet.

Antibiotics are beneficial for the treatment of diseases caused by certain enteric pathogens. However, they may also add to disease by eliminating certain microbes from the indigenous microbiota which inhibit or suppress the growth of pathogenic microbes in the GIT. In addition, extensive use of antibiotics may support colonisation by antibiotic resistant strains of bacteria, which can cause serious consequences if these strains become pathogenic. An excellent example of the suppressive phenomenon of antibiotics is infection with *C. difficile*, which leads to pseudomembranous colitis (*Rolfe, 1997*). The indigenous microbiota provides protection against infection from certain pathogens. "Colonisation resistance" or "bacterial antagonism" (*Rolfe, 1997*) demonstrates the capability of the host to obstruct the colonisation of the GIT by exogenous pathogens. It is necessary to understand that interplay between microbes, within the gut lumen, results in both direct and indirect modes of protection from pathogens. Modification of this interplay may not only affect the integrity of the mucosal barrier, but it may also facilitate certain pathogens to overcome or avoid mucosal immune responses.

1.6.2. Translocation of pathogens

Failure or breakdown of the mucosal barrier network favours the entry of pathogenic organisms through host cells. The process of migration of viable microbes, from the lumen of the GIT to extra-intestinal locations such as the mesenteric lymph nodes, liver, spleen and bloodstream, is called

"translocation" or "bacterial translocation" (*Berg, 1999*). Factors which favour translocation include bacterial overgrowth, immune deficiencies and mucosal injury, with loss of barrier integrity. Indication of an increased translocation of pathogens has been found in much pathology (*Berg, 1999*) and this has been found to be linked with cases of post-operative sepsis (*O'Boyle et al., 1998*) and also with spontaneous bacterial peritonitis in cirrhotic rats (*Guarner et al., 1997*). Pathogens are able to survive and spread to other organs, since they significantly translocate through cells and they can also prevent detection by the host immune system. However, non-pathogenic organisms are also found to translocate under normal conditions and the experimental introduction of pathogenic organisms (past the GIT mucosal barrier) is not always associated with disease (*Drevets, 1999*). Some researchers have disputed the necessity of this mechanism, for inducing disease or pathogenesis (*Alverdy et al., 2003)*.

A variety of factors, as described above, can influence bacterial overgrowth and result in an increased translocation. For some organisms, such as *E. coli*, the prospective for translocation is directly associated with the level of organisms. However, microbes, such as *Pseudomonas* sp. and gram-negative enterobacteria, are more capable of translocating across cells, than other microbes in the gut, such as obligate anaerobes (e.g. *Bacteroides* sp.). This may describe why opportunistic infections and sepsis within immuno-compromised hosts are caused by these former mentioned organisms.

This capability for translocation is independent of the ability to attach to epithelial cells, or of host genetic factors (*Berg, 1999*), but it may be increased by defective host immune responses and mucosal injury. Translocation regularly occurs across epithelial cell walls. However, ingested agents (castor oil) and pathological conditions (shock) can cause direct damage to tight junctions between epithelial cells and this may then allow microbes to exploit other portals of entry into the mucosa (*Berg, 1999*).

1.6.3. Microbial toxins

Interaction between enteric pathogens and the intestinal host may be activated by either microbial invasion (as discussed above) or by toxins (*Fasano, 2002*). Intestinal cells function through well-established intracellular signal transduction pathways, to control water and electrolyte fluxes across the intestinal mucosa: (1) cyclic adenosine monophosphate (cAMP), (2) cyclic

guanosine monophosphate (cGMP), (3) calcium dependent pathways and (4) nitric oxide (NO).

1.6.3.1. cAMP

Being a vastly heterogeneous group of micro-organisms, *E. coli* cover almost all features of possible interaction between intestinal microbiota and the host, ranging from the role of being merely a harmless presence, to that of a highly pathogenic micro-organism. Indeed, the *E. coli* species consists of many strains that differ widely from each other in terms of biological features and virulence properties (*Levine, 1987; Fasano, 2002*). Some ETEC strains produce the heat-labile toxin (LT), which is structurally and functionally similar to the CT, which is produced by *V. cholerae* and both activate the adenylate cyclase/cAMP pathway (*Fasano, 1999*). LT induces mild diarrhoea, commonly referred as 'travellers' diarrhoea but, in contrast, CT cause a severe, sometimes life-threatening clinical condition, which is typical of cholera infection. CT and LT share a common structure each comprised of an A (active) sub-unit of ~25-kDa and a ring of 5 B (binding) sub-units of ~11-kDa (AB5). Rodighiero *et al*. (1999) have shown that the difference in toxicity of CT and LT is due to a 10 AA segment, within the A2 fragment of CT, which is responsible for a higher stability to the CT holotoxin during uptake and transportation into intestinal epithelia (*Fasano, 2002*).

1.6.3.2. cGMP

Apart from LT, ETEC also secretes heat-stable enterotoxins (STs). ST_a (heat-stable toxin A) is a small peptide, which induces guanylate cyclase, resulting in an increased intracellular concentration of cGMP which stimulates chloride secretion and causes diarrhoea (*Fasano, 1999*). TolC, an ETEC outer membrane protein, appears to be important for the translocation of the toxin across the outer membrane (*Yamanaka et al., 1998*). In addition to LT and ST exotoxins, ETEC also possesses a LPS endotoxin, which greatly increases the expression of the inducible nitric oxide synthase II (NOS II) and its effector enzyme soluble guanylate cyclase in mice colonic cells. This results in increased levels of cGMP and leads to hypersecretion and diarrhoea (*Closs et al., 1998; Fasano, 2002*).

1.6.3.3. Calcium signalling

Toxins, including *Cryptosporidium* toxin (*Guarino et al., 1994a*) and the *H. pylori* vacuolating toxin (*Guarino et al., 1998; Boncristiano et al., 2003*), appear to function through calcium signalling (*Fasano*, 2002).

1.6.3.4. Nitric oxide

Nitric oxide (NO) in gut fluid and electrolytes, balances changes according to the pathophysiological conditions that stimulate this pathway. Under physiological circumstances, there is a pro-absorptive effect of NO due to the involvement of the enteric nervous system (*Izzo et al., 1998*). In contrast, high NO production (working as a secretagogue) causes diarrhoea in humans (*Dykhuizen et al., 1996; Fasano, 2002*).

1.6.3.5. Pore forming toxins

Some bacterial toxins are cytolytics, since they induce the formation of large pores in host cell membranes, while other toxins, which form smaller pores, display lesser cytotoxic effects and some of them have been involved in various aspects of bacterial virulence. *C. perfringens* enterotoxin (CPE) is released during bacterial lysis and binds to a brush border receptor of the host enterocyte. Following this binding, CPE associates with a 70-kDa membrane protein and results in the formation of pores, through which water, ions, nucleotides and AA leak. *S. aureus* generates a toxin which also forms pores, but its mechanism of action involves the formation of oligomers, which contain only toxin molecules (*Popoff, 1998; Fasano, 2002*).

1.6.3.6. Toxins blocking and inducing protein synthesis

Shiga toxin (released by *S. dysenteriae*) and SLTs (Shiga-like toxins) share a structure, typical of CT and LT, but they operate through a different mechanism of action. The A1 sub-unit of Shiga toxin and SLTs attaches and inactivates the 60S sub-unit of the host cell ribosome and this action results in the complete disruption of cell protein synthesis (*Popoff, 1998*). In contrast, up-regulation of protein synthesis, particularly pro-inflammatory mediators, is one of the other explained mechanisms by which bacterial toxins induce diarrhoea. Nielsen *et al*. (1998) have reported that staphylococcal enterotoxin A stimulates tyrosine phosphorylation of numerous host intracellular proteins; down regulation of the T cell receptor; and production of IFNγ, which is a key

cytokine in the pathogenesis of intestinal inflammatory and secretory processes (*Nielsen et al., 1998; Fasano, 2002*).

1.6.3.7. Toxins influencing the enterocyte actin cytoskeleton

Zonula occludens toxin (Zot) is a toxin produced by *V. cholerae*. Zot up-regulates intestinal permeability by interacting with a mammalian cell receptor and subsequently activates an intracellular signalling which results in the disassembly of the intercellular tight junctions (*Fasano et al., 1991; Fasano et al., 1995; Fasano, 2002*).

1.7. Immunoglobulins

Ig are glycoproteins produced by plasma cells, in response to antigens or immunogens. Ig are a group of specialised bioactive proteins, present in plasma, serum and other tissue fluids, including the milk of all mammals (*Späth, 1999*).

1.7.1. Functions of Ig

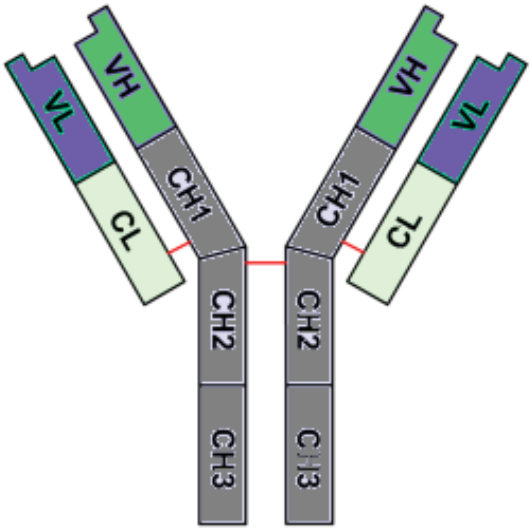
The function of these molecules is to bind to invading pathogens and to activate specific functions, which help to prevent infection and destroy disease causing agents. They function in cell killing, inflammation and prevention of pathogen attachment (such as bacteria and viruses) and they have an integral role in the immune defence system. The body's immune system produces these specialised molecules. These are released in response to the host being exposed to an immunogenic or foreign substance (antigen), such as an infectious pathogen or microbe. Their action is to remove and/or neutralise potential disease-causing agents. A specialised feature of antibodies is that they are directed specifically to the antigen that induced their formation (*Späth, 1999*).

1.7.2. Structure of Ig

The basic structure of Ig consists of two identical heavy and two identical light chains, linked together by intra-and inter-disulphide bonds. Each heavy chain can be divided into a minimum of four regions (only two in a light chain and each is variable and constant), one variable (V) and at least three constant regions, based on the type of Ig isotype (Figure 1.4). The first 110 or more AA

of the amino terminal regions (of heavy or light chains) are responsible for determining antigen specificity, since this directly interacts with the antigen, and thus differs greatly amongst antibodies of different specificity. The remainder of the three to four segments in a heavy chain (only one in a light chain) do not differ a great deal in their AA sequence, which is called the constant (C) region. In the variable region, there are two regions called hypervariable (HVR) or complementarity determining regions (CDR) and a framework region. HVR or CDR is comprised of AA sequences (of the variable regions of Ig) and most of their variability resides in the two regions called the hypervariable region or the complementarity determining region.

Figure 1.4 General structure of immunoglobulin*.



*Adapted and modified from Goldsby *et al*., (2003c). Light chains have one variable (VL) and one constant (CL) unit; heavy chains have one variable unit (VH) and three or four constant (CH) units.

The framework region is present between the HVR and CDR, in the variable region. The constant region can be expressed in two light chain classes or isotypes and five major heavy chain classes. The heavy chain constant region (as opposed to the smaller light chain) determines the functions and properties of the antibody molecule, with the specific heavy chain isotype presenting a unique effector function. In general, heavy and light chains can be divided into two regions, based on their variability in the AA sequences. These are: (1) Light Chain, comprised of V_L (110 AA) and C_L (110 AA) and (2) Heavy Chain, comprised of V_H (110 AA) and C_H (330-440 AA /or C_H, C_H, C_H each 110 AA). The heavy and light chain genes are divided

into multiple gene segments, which are serially arranged along the chromosome. These genes encode for a 50-kDa heavy chain and a 25-kDa light chain glycoprotein. These multiple gene segments consist of different versions of the V (variable), D (diversity, only in the heavy chain) and J (joining) gene segments (*Steiner, 1996*; *Späth, 1999*; *Goldsby et al., 2003c*).

IgM is the first antibody produced during the first interaction (primary immune response) with an antigen. Although IgM tends to interact with antigens with low affinity, its pentameric structure with ten antigen-binding sites allows for high-avidity binding to an antigen, which has several representations of the same epitope. However, IgM function is restricted due to its size and short half-life. As a result, after the primary immune response, class switch recombination occurs through a second DNA recombination event which will result in the expression of antibodies of the IgG, IgA or IgE isotypes. Although the later antibodies have a lower antigen valency compared to IgM, the affinity of isotypes (IgG, IgA or IgE) is typically high enough to allow for high avidity binding of pathogens or antigens (*Späth, 1999*; *Goldsby et al., 2003c; Sulentic, 2008*).

1.7.2.1. Classes and types of Ig

1.7.2.1.1. Ig classes

Based on differences in the AA sequences, in the constant region of the heavy chains, the Ig can be divided into five different classes: (1) IgG, Gamma (γ); (2) IgM, Mu (μ); (3) IgA, Alpha (α); (4) IgD, Delta (δ); (5) IgE, Epsilon (ε). All Ig, within a given class, will have very similar heavy chain constant regions. These differences can be identified by sequence studies, or more commonly by serological methods (i.e. by the use of antibodies directed to these differences) (*Späth, 1999*).

IgG coats micro-organisms for uptake by other cells (opsonisation). IgG also crosses the placenta of the pregnant woman, to provide passive immunity to the foetus and to increase the complement function, primarily located in serum. IgG is the most prevalent class of Ig in all species. IgA primarily located in body fluids, safeguards mucous membranes by preventing bacteria from attaching to the mucosal surface. IgM primarily located in blood, firstly reacts to antigens, via agglutination and bacteriolysis. IgD primarily located in serum and in plasma membrane of B lymphocytes is involved in the differentiation of B lymphocytes. IgE functions in

hypersensitivity reactions and allergic responses, phagocytosis and other Ig activity. IgE is primarily located in plasma and tissue and on surface membranes of basophils and mast cells (*Späth, 1999*).

1.7.2.1.2. Ig Subclasses

The classes of Ig can be further subdivided into sub-classes, based on small differences in the AA sequences, in the constant region of the heavy chains. All Ig, within a sub-class, will have similar heavy chain constant region AA sequences. These differences are most commonly identified by serological methods. IgG Subclasses comprised of IgG1, γ^1 heavy chains, IgG2, γ^2 heavy chains, IgG3, γ^3 heavy chains and IgG4, γ^4 heavy chains. IgA Subclasses comprised of IgA1, α 1 heavy chains and IgA2, α 2 heavy chains (*Späth, 1999*).

1.7.2.1.3. Ig Types

Ig can also be classified by the type of light chain that they possess. Light chain types are based on differences in the AA sequence in the constant region of the light chain, such as Kappa (κ) light chains and Lambda (λ) light chains. The light chains can be further divided into sub-types on the basis of differences in the AA sequences in the constant region of the light chain: κ subtypes κ1 and κ2; λ subtypes – λ 1, λ 2, λ 3 and λ 4 (*Goldsby et al., 2003c*).

1.7.3. Ig: Mechanism of action

1.7.3.1. Opsonisation

One of the important antibacterial defences is the promotion of phagocytosis of antigens by macrophages and neutrophils. F_C receptors (F_CR) are present on the surface of phagocytes such as macrophages and neutrophils. Binding of antibodies, coupled with antigens or bacterial cell to the F_CR, produces an interaction that leads to the binding of the antigen to the phagocyte membrane. This cross linking (i.e. phagocyte with F_C receptor-antibody-antigen complex) leads to the initiation of signal transduction pathways, which results in the phagocytosis of the antigen-antibody complex. Once inside the phagocyte, the antigens are attacked with numerous destructive processes, such as enzymatic digestion, oxidative damage and the membrane damaging

effects of antibacterial peptides (*Späth, 1999*; *Goldsby et al., 2003b*).

1.7.3.2. Activation of complement

Complement is serum glycoproteins which can perforate the cell membrane. IgM and the majority of the IgG subclasses can activate a complement system. Complement component 3 (C3b) is a protein fragment, which attaches non-specifically to the cell and antigen-antibody complexes near the site at which the complement was activated. Numerous cell types, such as erythrocytes and macrophages, express receptors for C3b and so they bind to cells or complexes (antigen-antibodies) to which C3b is attached. Binding of C3b by macrophages results in phagocytosis of the cell or complexes attached to the C3b. The interaction between the antibody and complement system is crucial for the inactivation and removal of antigens and the killing of pathogens (*Späth, 1999*; *Goldsby et al., 2003c*).

1.7.3.3. Antibody Dependent Cell-mediated Cytotoxicity (ADCC)

Numerous types of cells (such as macrophages, natural killer cells, monocytes, neutrophils and eosinophils) that have cytotoxic potential, express membrane receptors for the F_C region of the antibody molecule. Antibody dependent cell-mediated cytotoxicity, by causing cell lysis (of the target cells), is initiated when any of the above mentioned cells (with F_CR) bind to the F_C region of the antibody which is specifically bound to the target cells (such as virus infected cells of the host). Although cytotoxic cells are non-specific for antigen, the specificity of the antibody directs them to the specific target cells (*Späth, 1999*; *Goldsby et al., 2003c*).

1.7.3.4. Intestinal Ig – Immune exclusion and inclusion

A total of 2.5 g/day of sIgA (secretory or intestinal IgA) is secreted into the lumen of the GIT, making it the most heavily produced protein in the body, by weight (*Conley & Delacroix, 1987*). The sIgA can bind to microbes and prevent them from attaching to (or penetrating) the epithelial lining, thereby preventing microbial translocation, which can result in sepsis and the death of the host (*Brandtzaeg et al., 1985*). The sIgA exerts its function by aggregating bacteria, resulting in a clearance of the gut bacteria (*Williams & Gibbons, 1972*) and preventing invasion inside the host tissue (*Brandtzaeg et al., 1985;*

Brandtzaeg, 1998). This model of sIgA activity is called 'immune exclusion', a term coined for the non-inflammatory antibody shielding of internal body surfaces (*Brandtzaeg, 2009*). In addition to the immune exclusion function displayed by sIgA, it has two other potential modes and sites of action, in mucous membranes (*Kaetzel et al., 1991; Mazanec et al., 1992*). Firstly, during transportation through the epithelial cell lining, after polymeric immunoglobulin receptor (pIgR)-mediated endocytosis, IgA may be able to interact with intracellular pathogens, such as viruses, blocking replication, assembly, and/or budding. Such intra-epithelial cell neutralisation has been shown by IgA monoclonal antibodies (MAbs) against sendai virus, influenza virus and rotavirus (*Mazanec et al., 1992; Mazanec et al., 1995; Burns et al., 1996*). Secondly, IgA in the lamina propria, beneath the mucosal epithelium, may form a complex with antigens and then transport them, via the pIgR, across the epithelial cells and into the secretions (*Kaetzel et al., 1991; Robinson et al., 2001; Yan et al., 2002*).

A report has shown that sIgA may actually interact with the commensal bacteria in an agonistic (pro-microbial) way, inside the intestinal lumen (*Mestecky et al., 1999*). The currently accepted hypothesis is that sIgA-mediated aggregation of bacteria decreases the adherence of those bacteria to epithelial surfaces (*Williams & Gibbons, 1972*). Williams & Gibbons, (1972), reported that bacteria agglutinated by sIgA attached to epithelial surfaces *in vitro,* less well than the unagglutinated bacteria. Binding of *Streptococcus sanguis* (*S. sanguis*), *S. mitis* and two strains of *S. salivarius* to cultured human epithelial cells was reduced in the presence of sIgA to around 65 to 70%. Therefore, the report by Williams & Gibbons (1972), presents a relatively simple and negative relationship between aggregation and adhesion, using a particular set of conditions. Thus, aggregation of commensal gut bacteria by sIgA (*Bollinger et al., 2003*) and perhaps by other components of the natural immune system, will not only avoid the translocation of those bacteria through the gut barrier, but it may also be agonistic by selectively facilitating the adherent growth of the commensals under the flowing conditions present in the gut. A revised model, involving both inclusion (maintaining bacteria inside the gut) and exclusion (preventing bacteria from crossing the epithelial barrier), may thus be possible (*Everetta et al., 2004*).

If indeed sIgA exerts pro-microbial activity towards the normal flora, how then can the same molecule be antagonistic toward pathogens?

Possibly, the pro-microbial activity of sIgA towards the commensal gut flora is, in fact, the mechanism by which sIgA is antagonistic toward pathogens. This may be possible, given that the normal flora is protective against pathogens *(Harp et al., 1992; MacDonald & Pettersson, 2000*). The idea is certainly attractive in terms of its simplicity and utility. Thus, this new model of immune exclusion/inclusion accounts for the evidence pointing towards a pro-microbial activity of sIgA and (at the same time) a protective effect contributed by the same molecule (*Everetta et al., 2004*).

1.7.4. Antibody-mediated immunity

Antibody-mediated immunity (AMI) refers to the positive effects mediated by Ig. Historically, the efficiency of AMI against a pathogen was recognised by the following benchmarks: (1) Experiment showing that transfer of immune sera to a naive host reduces susceptibility to infection and/or disease; (2) A display of a correlation between the presence of a specific antibody in a host and resistance to an infection and/or disease; (3) Demonstration of an association between deficiency in AMI and vulnerability to infection and/or disease.

The first benchmark, discovered in 1890s, was that naive animals might be protected against toxins with immune sera (*Behring & Kitasato, 1890*). The above discovery gave rise to the second benchmark, which was illustrated by the recognition that the acquirement of specific antibodies conferred immunity to certain pathogens. The third benchmark was based on the observation that certain antibody deficiencies conferred susceptibility to certain classes of pathogens (*Ochs & Smith, 1996*). Natural antibodies (NA) are a first line of defence against infections. Innate immunity is essentially important as a first line of defence against pathogens, in avoiding microbial invasion and in alerting other components of the body's immune system. In the absence of internal or external antigenic stimulation, NA as part of the innate immune system, are present in the serum and they are also present in new-born and germ-free animals. NA are mostly of the IgM isotype. However, a number of IgG and IgA NA have also been described. NA are encoded by germline variable (V) genes, without extensive somatic mutations and they have a wide range of binding avidities, ranging from 5×10^{-3} to 5×10^{-11} M. Infections of the vital organs are prevented by NA, through the targeting of antigen to the spleen. Targeting antigen to the splenic marginal zone, by NA,

is vital for the induction of early neutralising antibody responses in the absence of T_H cells. NA also enhances specific B and T cell responses by activating the complement cascade (*Matter & Ochsenbein, 2008*).

In the past, effective antimicrobial therapy was carried out by using passive antibodies. *Behring and Kitasato,* in 1890, discovered the successful protection of specific antibodies against bacterial toxins (*Casadeval et al., 2004*) and this discovery has led to the treatment of infectious diseases with antibodies (*Casadevall & Scharff, 1994, 1995*). Serum from immune human donors, or immunised animals is a source of antibody preparation. Therapy, which uses this form of preparation, is called 'serum therapy'. Sulphonamides and other antimicrobial agents, introduced in 1935, led to the rapid decline of serum antibody therapy. In the 1940s, serum therapy was almost abandoned as an anti-microbial medication, except in a few treatments for toxins, venoms and some viral infections. Recently, antibiotic side effects and the adverse effects and high cost of new anti-microbial medications have led researchers to investigate the use of antibodies for antimicrobial and other treatments (*Casadeval et al., 2004*). Further, the treatment and control of infectious diseases has become increasingly complicated because of the recent incidence of opportunistic infection and the emergence of resistant pathogenic micro-organisms to antibiotics (*Weiner et al., 1999*).

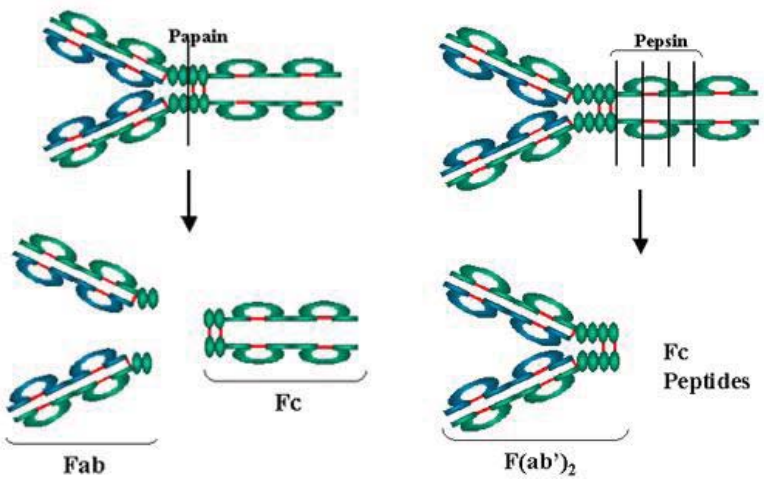
1.7.5. Digestion of Ig by digestive enzymes

Proteolytic digestion of Ig fragments is very useful when determining structure and function relationships in Ig. Papain digestion breaks the Ig molecule in the hinge region, before the H-H inter-chain disulphide bond (Figure 1.5). This results in two identical fragments, which contain the light chain and the V_H and C_{H1} domains of the heavy chain. These fragments are called the Fab fragments, as they contain the antigen binding sites of the antibody. Each Fab fragment is monovalent whereas the original molecule is divalent. Papain digestion also produces a fragment that contains the remainder of the two heavy chains, each containing a C_{H2} and C_{H3} domain, called 'Fc fragment' since it is easily crystallisable (*Goldsby et al., 2003c*).

Pepsin treatment of Ig results in cleavage of the heavy chain, after the H-H inter-chain disulphide bonds, resulting in a fragment that has both antigen binding sites, called $F(ab')_2$, because it is divalent (Figure 1.5). The Fc region of the molecule is digested into small peptides by pepsin. The $F(ab')_2$

binds to antigen, but it does not mediate the effector functions of antibodies which are less allergenic (*Lefranc-Millot et al., 1996*) than the whole or parent molecule.

Figure 1.5 Immunoglobulin digestion* by enzymes.



*Reproduced from Mayer, (2009).

Several studies have shown that orally administered Ig retains its activity within the GIT and is immunologically effective post-digestion. Blum *et al*. (1981) found undigested and partially digested Ig with intact opsonic activity in faecal samples of low birth weight infants who had been given Ig orally. Rodriguez *et al*. (2007) reported that porcine Ig partially resisted the digestion process in the GIT of adult dogs and cats fed diets containing Ig derived from pig blood. The levels of intact Ig in the faeces of dogs and cats consuming diets containing Ig were similar to those found in humans (*Blum et al., 1981; Kelly et al., 1997*) and in pigs (*Morel et al., 1995*). They also reported that Fab fragments were found after digestion, which is consistent with the *in vitro* findings of Nezlin (*Nezlin, 1998*), who reported that Fab is more resistant to the digestion process than the remainder of the Ig molecule. The $F(ab')_2$ fragment, which is released after pepsin digestion (*Gorevic et al., 1985*) retains the capacity to bind bacteria by neutralisation and opsonisation since it contains the antigen binding sites. Several reports have demonstrated that human serum and bovine Ig resist digestion in the upper GIT of humans and can retain toxin binding and neutralizing activity (*Losonsky et al., 1985; Kraehenbuhl & Neutra, 1992; Warny et al., 1999*). In suckling and possibly

mature animals, Ig may be phagocytosed by the enterocyte and escape partial digestion by intracellular proteases and therefore reach the circulation by exocytosis on the basolateral surface of the cell (*Hemmings & Williams, 1978; Sanderson & Walker, 1993*). Also, Ig prevents pathogens, such as bacteria and viruses, from damaging the gut wall thereby resulting in a more intact and absorptive intestinal wall.

1.7.6. Ig recovery

The average recovery of intact Ig, following *in vivo* digestion or *in vitro* simulated digestion (based on 15 published reports) was ~24% (Table-1.1). None of these studies have reported total loss of IgG. Roos *et al*. (1995) demonstrated that the recovery of immunologically active nitrogen from digested IgG and IgM, was approximately 19%, which was considerably higher than that from other milk proteins (5-6% active) which have ileal digestibilities of > 90%. As explained previously, as a result of digestion of IgG, IgG $F(ab)_2$, or F(ab) and Fc fractions are formed, out of which both IgG $F(ab)_2$ and/or F(ab) retain biological activity (*Lefranc-Millot et al., 1996*).

1.7.7. Sources of Ig

1.7.7.1. Monoclonal antibodies

Monoclonal antibodies were first described by Kohler and Milstein (*1975*). It was immediately recognised that they had great potential, as powerful and specific therapeutic agents. During attempts to make use of that potential, large numbers of clinically-relevant monoclonal antibodies have been derived and many of these have been tested *in vivo* (*Thistlethwaite et al., 1984; Goldstein, 1986*).

Morelli *et al*. (1996) demonstrated that chemotherapy, which induced gastrointestinal toxicity (mucositis) in mice, was prevented by oral administration of anti-doxorubicin monoclonal antibody (MAD11). Gastrointestinal mucositis is a common and painful condition which affects a section of cancer patients receiving chemotherapy. The experiment revealed that orally administered MAD11 antibodies, (a) were not absorbed into the blood (b), reduced the degree of doxorubicin-induced apoptosis in murine intestinal crypts and (c), and reduced body weight loss in mice treated with 12 mg/kg body weight of doxorubicin. Finally, Morelli *et al*. (1996) concluded that this type of treatment may be useful in preventing anthracycline induced

gastrointestinal mucositis in cancer patients.

1.7.7.2. Egg yolk antibodies (IgY)

Numerous articles have reported the use of antibodies developed through egg yolk. Shimamoto *et al*. (2002) studied a highly specific chicken egg yolk-derived anti-*H. pylori* antibody and they examined its efficacy to induce passive immunity and a bacteriostatic effect on *H. pylori*. These authors concluded that the antibody had a bacteriostatic effect on *H. pylori*, it inhibited *H. pylori* urease activity and it inhibited *H. pylori* infection in Mongolian gerbils and humans. An IgY sample was also tested against *C. parvum* infection in SCID (severe combined immunodeficiency) mice and it was found to be effective against the infection (*Kobayashi et al., 2004*). Suzuki *et al*. (2004a) reported the effect of IgY prepared from egg yolks of hens immunised with *H. pylori* infection. They showed that IgY mitigates *H. pylori* associated gastritis and attenuated gastric urease activity. Recently, specific and nonspecific IgY have been used against *S. enteritidis* and *S. typhimurium* outer membrane proteins (OMP) against two *Salmonella* sp. Effects of IgY were investigated *in vitro* and results showed that both specific and non-specific IgY reduced the decrease in TER of the infected Caco-2 cell monolayers and blocked the adhesion of *Salmonella* sp. in a concentration-dependent manner (*Chalghoumi et al., 2009*). A study was also conducted to measure IgY inhibition of fluconazole-sensitive and resistant strains of *C. albicans* to assess their potential use in the prevention and treatment of oral candidiasis. Results showed that the IgY extract inhibited the growth (by 82.98%) of *C. albicans* during 24-48 hour time period (*Wang et al., 2008*).

1.7.7.3. Colostrum and milk Ig

Various components in colostrum and milk exhibit specific biological activity, apart from their well-established nutritional qualities. During the past two decades, there has been a growing interest in these beneficial physiological effects. Colostrum is a complex fluid rich in nutrients and it is also characterised by its elevated levels of bioactive components, such as Ig, growth factors (insulin-like growth factors), TGFβ2, growth hormone, lactoferrin, lysozyme and lactoperoxidase (*Elfstrand et al., 2002*). The number of IL-12^{+}CD11b^{+} cells in the spleen, the formation of superoxide by peritoneal macrophages, the number of natural killer cells in PP and splenocytes, and

the cytotoxic activity of spleen cells towards an erythroleukemia cell line (K562) were higher in mice which had been given an IgG (from cow's milk)-supplemented diet, when compared to the control diet. However, the number of $IFN\gamma^+CD4^+$ and $IL\text{-}4^+CD4^+$ cells in PP or spleen and the level of total or ovalbumin-specific intestinal IgA and serum IgG were significantly lower in mice which had been fed the IgG-supplemented diet. These results suggest that cow's milk IgG, given orally, may stimulate some innate cellular immune systems whilst suppressing humoral adaptive immune response in the mouse (*Ohnuki et al., 2006*). Tacket *et al*. (1988) reported that bovine milk Ig concentrate protects against ETEC (oral challenge) in human volunteers.

Recently, Berge *et al*. (2009) reported that supplementing calves with oral colostrum resulted in higher grain consumption and weight gain over the first 28 days of life. Colostrum supplementation during the first two weeks of life lessened diarrhoeal disease in the pre-weaned calves and thereby reduced the amount of antimicrobial treatments needed. Tzipori *et al*. (1986) demonstrated that Ig derived from bovine colostrum has been shown to be an effective treatment for diarrhoea caused by *Cryptosporidium*. Immunodeficient individuals, particularly those with acquired immune deficiency syndrome (AIDS), are especially susceptible to Cryptosporidiosis. He *et al*. (2001) found that bovine colostrum, fed to eighteen healthy volunteers with an attenuated *S. typhi* Ty21a oral vaccine, resulted in an enhanced human immune response, such as an increase in IgA. Crooks *et al*. (2006) demonstrated that colostrum supplementation (vs. placebo) of 35 distance runners (aged 35 to 58 years) resulted in increased sIgA.

1.7.7.4. Animal plasma

Spray-dried plasma (SDP) is an abattoir by-product obtained from animal blood, after exclusion of cells, concentration and spray drying. Three types of SDP products are generally available and these have been prepared from bovine plasma (SDBP), porcine plasma (SDPP) and plasma of unknown or mixed animal species (SDAP) (*Lallès et al., 2009*). Several studies have reported benefits from including SDP in the post-weaning diet of production animals, with improvements observed in feed intake, growth rate and intestinal growth (*Gatnau et al., 1989; Hansen et al., 1993; Kats et al., 1994; Quigley et al., 2002; Campbell et al., 2003; Torrallardona et al., 2003; Campbell et al., 2004, 2006; Pierce et al., 2005, Campbell et al., 2010; Gao et*

al., 2011). The beneficial effects of SDAP on growth have also been reported in dogs (*Quigley et al., 2004*), mice (*Thomson et al., 1994, 1995*) and rats (*Garriga et al., 2005; Pérez-Bosque et al., 2006, 2008, 2010a, 2010b*).

Table 1.1 Reports of Ig recovery after oral administration.

Donor	Recipient	Formulation	Source	% intact [1]	Reference	Year
Porcine	cat	Coated over kibbles	IgG serum	14	Rodriguez *et al*.	2007
Porcine	dog	Coated over kibbles	IgG serum	9.2	Rodriguez *et al*.	2007
Bovine	human	Liquid	IgG colostrum	>50	Pacyna *et al*.	2001
Bovine	adult	Powder	IgG colostrum	49	Warny *et a*l.	1999
Human	rat	Liquid	IgG serum	25.7	Gmoshinskiĭ *et al*.	1998
Bovine	human	Powder	IgG colostrum	25	Kelly *et al*.	1997
Bovine	-[4]	Powder	IgG colostrum	25	Kelly *et al*.	1997
Bovine	piglet	Powder	IgG	45-50[2]	Morel *et al*.	1995
Bovine	human	Powder	IgG colostrum	19[3]	Roos *et al*.	1995
Bovine	-[4]	Powder	IgG colostrum	20-42	Petschow & Talbott	1994
Bovine	human	Powder	IgG colostrum	10-20	McClead *et al*.	1988
Bovine	infant	liquid	IgG milk	10	Hilpert *et al*.	1987
Human	infant	liquid	IgG serum	25	Losonsky *et al*.	1985
Bovine	-[4]	Powder	IgG colostrum	50	McClead & Gregory	1984
Human	infant	Liquid	IgG serum	25	Losonsky *et al.*	1985
Human	infant	Liquid	IgG serum	4-12	Blum *et al*.	1981

[1] % of original dose recovered in faeces or an in vitro sample, [2] % recovered in the proximal gut, [3] % recovered in the terminal ileum, [4] results from *in vitro* studies

Earlier studies have reported that the effect of SDP is due to an increase in feed intake which is due to the improved palatability of the diets. This theory was supported by a two-choice feed preference study in which piglets preferred a diet containing SDP over a diet containing dried skimmed milk (*Ermer et al*., *1994*). However, the higher feed intake could also possibly have been due to the better health of the piglets. A study with piglets fed with or without plasma protein, revealed that its effects are independent of intake thus proving a specific biological effect (*Jiang et al*., *2000a*). Protein utilisation was improved by SDP in piglets and this could have been due to lower protein catabolism by the gut microbiota (*Jiang et al*., *2000b*). SDP was found to influence the intestinal mass and the cellularity of the lamina propria, indicating there was antimicrobial activity, but data directly focusing on the effects of SDPP on gut bacteria or microbiota are limited. In one study, SDAP was found to increase the number of lactobacilli in the ileal and caecal contents of piglets (*Torrallardona et al*., *2003*), but a later study could not confirm this finding (*Torrallardona et al*., *2007*). The reason for these discrepancies is not clear, but it might be due to a difference in the diet used (concentration of the SDP) and the duration (days) of the studies. Discrepancies have also been reported in regards to the change in the villus architecture. SDP may (*Owusu-Asiedu et al*., *2003a, 2003b*) or may not (*Jiang et al*., *2000b*; *Owusu-Asiedu et al*., *2002*; *Torrallardona et al*., *2003*; *Nofrarias et al*., *2006*) increase villus height, in the jejunum of pigs (*Lallès et al., 2009*).

The biological effects of animal plasma appear to be more pronounced in piglets during a pathogen challenge (*Coffey & Cromwell, 1995*). Lallès *et al.* (2009) reported that the beneficial effects of plasma were mediated by its Ig fraction and its inhibitory activities against pathogenic microbes and enterotoxins. Therefore, differences in IgG content (both quantitative and qualitative) might influence the efficacy of various SDP sources. SDP, with guaranteed elevated levels of Ig, was shown to be superior to conventional plasma (*Bosi et al*., *2001*). Furthermore, Bosi *et al*. (2004) reported that piglets challenged with *E. coli* K88 (K88 fimbrial appendages present on *E. coli* mediates adhesion of the bacterial cell to the brush borders of the epithelial cells lining the small intestine) had a lower concentration of specific IgA anti-K88 in plasma and saliva when they were fed a plasma source rich in IgG, thus suggesting a protective effect against the

adhesion of *E. coli* K88 to the enterocytes. The observed effects of dietary SDAP and its Ig fraction on growth in animals may be due to a greater availability of nutrients and energy for growth, consequent upon a lower degree of immune cell activation (*Demas et al., 1997*) and an altered integrity and structure of the intestinal mucosa (*Fiocchi, 1997*). It can be summarised that SDP is a useful protein source for production animal diets and, apart from its beneficial effects on growth, feed intake and feed efficiency, there is clear evidence that SDP (due to mainly its Ig fraction) prevents the binding of pathogens to the gut wall and reduces the incidence of post-weaning diarrhoea (*Lallès et al., 2009*).

1.7.7.5. Plasma Ig

Recently, Han *et al.* (2009) in an *in vitro* experiment demonstrated the anti-pathogenic effect of ovine Ig concentrate (OIC) extracted from lamb's blood. They reported that OIC was able to bind to the cell walls of 13 strains of bacteria (both gram-positive and gram-negative). They also found that OIC was able to bind to the LPS obtained from *E. coli* O111:B4 and *S. typhimurium*. They concluded that OIC might be a potential supplement for protection against pathogenic bacteria.

Various articles have demonstrated the benefits of porcine plasma Ig concentrate (IgC), in the treatment of mild intestinal inflammation (induced by administration of staphylococcal enterotoxin B [SEB]) rat model. Dietary supplementation, with IgC, prevented the SEB-induced activation of CD3, CD4 (T helper lymphocytes), CD8 (T suppressor/or cytotoxic lymphocytes), CD25 (activated T lymphocytes), Tγδ lymphocytes and Natural Killer (NK) cells in lamina propria (*Pérez-Bosque et al., 2008*), expression of IFNγ, TNFα, IL-6 and LTB_4 in PP and mucosa (*Pérez-Bosque et al., 2010a, 2010b*), and water content of faeces (*Pérez-Bosque et al., 2004*), dextran and horseradish peroxidase flux, across the intestinal wall (*Pérez-Bosque et al., 2006*). Dietary plasma protein, containing Ig, has been found to ameliorate SEB effects on mucosal inducible nitric oxide synthase (iNOS), cryptdin 4 and β-defensin 1 expression (*Pérez-Bosque et al., 2010a*).

Nollet *et al*. (1999) evaluated the protection of non-immune plasma powder in weaned pigs, by challenging with $F18^+$ *E. coli* strain expressing F18 fimbriae and producing SLTIIv and LT-toxins. They reported that both morbidity

and mortality, caused by an enterotoxaemic *E. coli* could be reduced by the oral administration of dried non-immune plasma powder to weaned pigs.

1.7.8. Ig as a dietary supplement

Ig is the primary anti-infective component of blood serum, colostrum and breast milk. These specialised proteins protect the body from harmful bacteria, viruses and other environmental pathogens by either binding to them or by the formation of an encapsulating barrier (*Kraehenbuhl & Neutra, 1992*). Ig safeguards the body from pathogens, such as harmful bacteria, viruses, and other environmental pathogens, either by forming an encapsulating barrier or binding to them. Animal studies and reports have demonstrated that oral supplementation of Ig restores health in animals, which are experiencing stress on their immune systems. Oral supplementation with Ig also promotes the integrity of the digestive tract barrier, which is a constant combat zone between the immune system, pathogenic organisms and environmental toxins. Ig supplementation may be beneficial for people undergoing stress and various other health problems when antibody synthesis and secretion are reduced (*Greenberg & Cello, 1996*).

Eibl *et al*. (1988) reported that children (born prematurely), with necrotising enterocolitis, were prophylactically treated with an oral administration of purified human Ig. Ig have also been used, therapeutically, against *C. jejuni* (*Hammarström et al., 1993*) and *C. difficile* (*Tjellstrom et al., 1993*) which are responsible for diarrhoea in immuno-compromised patients, in addition to chronic diarrhoea of unknown aetiology in normal infants (*Casswall et al., 1996*). The successful intervention of *H. pylori*, rotavirus and cryptosporidial infection, using bovine Ig, has also been reported (*Bogstedt et al., 1996*).

Guarino *et al*. (1996) have reported the effect of human serum Ig against rotavirus infection in Caco-2 cells. Rotavirus infection resulted in a progressive decrease in TER and a parallel reduction in cell viability. These effects were reduced in the Ig treated cells. Infections of cells were prevented by pre-incubating with Ig. The addition of Ig partially prevented the decrease in TER and later, positive values showed the restoration of the monolayer's integrity. The above finding clearly shows that Ig is effective in decreasing cell damage and preventing infection by direct anti-rotavirus action. Guarino *et al*. (1994b) conducted a double-blind, placebo-controlled study with oral Ig, for the treatment

of acute rotavirus gastroenteritis in ninety-eight children. Ig treated children exhibited significantly faster clinical improvement, both in clinical condition and stool pattern, such as mean total duration of rota viral diarrhoea, viral excretion and length of hospital stay when compared to the control children. Losonsky *et al*. (1985) examined the pharmacokinetics and immunologic activity of human serum Ig (HSG) possessing anti-rotavirus activity, in immuno-deficient patients with viral gastroenteritis. They reported that orally administered HSG could survive in the GIT in an immunologically active form (> 50% was in macro-molecular form in the stool) and it has potential for the prevention of gastrointestinal infections (generation of rotavirus-specific immune complexes in GIT). Passive antibody therapies have also been reported in piglets (*Bridger & Brown, 1981; Lecce et al., 1991*), cows (*Snodgrass et al., 1980; Castrucci et al., 1988*) and lambs (*Snodgrass & Wells, 1976*), with variable outcomes.

Kuhls *et al*. (1995) reported that human serum Ig preparation was shown to reduce the intensity of *C. parvum* infection, in SCID (severe combined immune deficiency) mice, when administered prophylactically, but not therapeutically. A 10-year old boy suffering from acute pre-B cell leukaemia showed a prompt resolution of symptoms and the disappearance of *C. parvum* infection, using human serum Ig administered orally (*Borowltz & Saulsbury, 1991*). Tzipori *et al*. (1994) reported that bovine colostrum-Ig treatment reduced the weight of *C. parvum* infection, moderately, in SCID mice and only slightly in piglets. They also reported that the moderate effect of Ig was probably due to its rapid transit rate through the gut and inactivation in the stomach.

Schneider *et al*. (2006) found that oral Ig had the potential to be used safely as a therapeutic aid, in the treatment of chronic gastrointestinal disturbances associated with autism disorder and it might be related to pervasive developmental disorders. Earnest *et al*. (2005) reported that consuming bovine Ig appeared to reduce total cholesterol and low-density lipoprotein cholesterol in the milder hypercholesterolaemic patients. They suggested this might be due to the antibodies, which bind directly to the cholesterol and hinder their absorption. In a double blind cross-over study of hypercholesterolaemic patients, the consumption of 90g of immune milk appeared to reduce the plasma cholesterol and blood pressure (*Sharpe et al., 1994*). Golay *et al*. (1990) also reported that milk from immunised cows could result in a significant reduction of elevated

blood cholesterol concentrations. They also reported that a specific increase in Ig content (in the skimmed milk) appeared to lower the serum cholesterol.

Hepatitis A is a common contagious viral disease present in low-income countries. Hepatitis A is transmitted primarily by faecal-oral spread from person to person. Liu *et al*. (2009) concluded that Ig appeared to be effective for the prevention (pre-exposure and post-exposure prophylaxis) of hepatitis A, in both children and adults.

Pontes *et al*. (2005) reported the role of intravenous IgG and IgM antibodies, in the protection of mice challenged with *E. coli* serotype O6:K2ac. Their results showed that serum from animals pre-treated with purified IgG (and not IgM) did not show any detectable pro-inflammatory cytokine, such as TNFα and IL-6.

1.8. Conclusion

The GIT of the host is heavily colonised with 10-100 trillion microbes representing thousands of species and it is also a major portal of entry for pathogenic bacteria. To control these complex microbial challenges, intestinal epithelial cells generate a diverse repertoire of protective proteins (or peptides). These proteins are secreted (or membrane bound, such as mucins, Ig etc) apically into the luminal environment of the intestine where they play a crucial role in protecting against enteric infections. From the above discussed literature, it is obvious that pathogens have developed virulence strategies to evade this protective mechanism. Therefore, exogenous administration of therapeutic formulations or supplements is vital to overcome these potential problems.

The identification and understanding of the importance of Ig has been known for decades. However, an antibiotic-resistant, side effect, adverse effects and the high cost of new anti-microbial agents, has made researchers re-evaluate and re-use Ig, for anti-microbial and other treatments. Currently, the treatment and control of infectious diseases has become increasingly difficult due to the incidences of opportunistic infection and the emergence of antibiotics-resistant pathogenic micro-organisms.

Furthermore, the benefits of including Ig (from colostrum or animal plasma) have been reported and they have been found to be effective in the suppression of pathogens, its toxins and inflammatory mediators and cytokines (due to pathogenic infection) and also in up-regulating anti-inflammatory cytokines, TGFβ. Ig, extracted from animal plasma, appears to be effective in passive immunisation since it contains a range of antibodies against various pathogens. Mammals born with (or that have developed) an inability to produce Ig suffer from chronic infections and other diseases which are closely related to a compromised immune system. In addition, Ig plays a key role in the regulation of immune response. Many researchers believe that a weak immune system leads to illness, but even over-stimulation can lead to a reduced appetite and tiredness and fever, all of which are associated with infection.

From the above literature, we can assume that the gut's immune system, together with its microbiota and mucin, are closely interlinked with each other. Modification or changes in any one system may have an impact on another and

ultimately affect the growth of the host.

Whilst some of the orally-supplemented Ig remains in the digestive tract and it is not absorbed into the bloodstream, it nevertheless benefits the body's overall immunity. Complex interactions between cells and cytokines directly connect the GIT to the respiratory tract and other mucosal surfaces. As a result of a reduction in the demand for synthesis of Ig, to inhibit the growth of opportunistic organisms or reduce absorption of toxins in the gut, supplemental Ig resources are preserved or redirected, to wherever they may be needed. Ig are vital proteins for immunity and they also preserve the gut's barrier function, in addition to reducing the burden on the immune system, so that nutrients gained from food are used by the body, for productive functions and not to fuel an immune response.

In New Zealand, approximately 40 million sheep are processed annually, from which 60 million litres of blood (a by-product) are produced. Currently, this blood is used as a low-value product, or discarded as waste. It would be more beneficial if the high-value components of ovine blood were separated from this by-product. Recently, in our laboratory, Han *et al*. (2009) demonstrated an anti-pathogenic (*in* v*itro*) effect of ovine Ig concentrate (OIC) extracted from lamb's blood. They reported that OIC was able to bind to the cell walls of 13 strains of bacteria (both gram-positive and gram-negative). They also found that OIC was able to bind to the LPS obtained from *E. coli* O111:B4 and *S. typhimurium*. However, to our knowledge, there has not been any work reported in any animal models (*in vivo*), regarding the effects of ovine serum Ig.

On the basis of the above discussed literature, the following studies were designed to test the hypothesis that oral administration of purified, polyspecific, pooled ovine serum Ig may be effective in improving the growth performance, strengthen mammalian immune function, and modify the mucin protein presumably via Ig-mediated binding of potential pathogenic bacteria (preventing attachment) (*Han et al., 2009*) and/or by support the development of beneficial bacteria in the gut. This thesis also sought to present evidence that oral administration of pooled ovine serum Ig is a safe and potentially effective approach in preventing and treating enteric infections by pathogenic bacteria such as *Salmonella*. Therefore, the major objectives of the experimental work described in this thesis were as follows:

- To ascertain whether orally administered ovine serum Ig can improve growth performance, digestive organ weight and gut morphology, in the normal growing rat; and whether the method of manufacture of ovine serum Ig affects its bioactivity.
- To investigate whether orally administered ovine serum Ig modulates immunity, in the normal growing rat.
- To explore potential changes in the gut microbiota by feeding the rat with ovine serum Ig.
- To assess whether orally administered ovine serum Ig can influence the number of goblet cells, mucin gene expression and digesta mucin protein content in the GIT of the normal growing rat.
- To understand whether orally administered ovine serum Ig prevents or lessens the effects of *S. enteritidis* (a pathogen) on growth performance, gut organ weight, gut morphology, mucin production and immunity and the associated gut microbiota, in the *S. enteritidis*-challenged growing rat.
- To determine whether ovine serum Ig can be detected in its intact form in the rat's digestive tract (stomach through to colon).

If successful, ovine Ig can be used in the following areas of potential application such as: replacement for antibiotic therapy, controlling and preventing diarrhoeal diseases caused by microbial pathogens, gastric and duodenal ulcer caused by *H. pylori* infection, protecting and reducing mucositis caused by chemo and radiotherapy, lowering cholesterol in humans and optimal immune support for athletes. In porcine, bovine and poultry industry, Ig can be used as the supplement for promoting growth, immunity and reduction in pathogenic bacterial load.

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Chapter 2

Orally administered ovine serum immunoglobulins influence growth performance, organ weights and gut morphology in growing rats

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Literature reported in Chapter 1 shows that animal plasma is effective in supporting growth in the growing pig model and plasma Ig seems to be the most important factor. There are no reports regarding the effects of sheep Ig in mammalian models. Therefore, the objective of this work and that reported in accompanying Chapters was to ascertain the biological activity of ovine Ig given orally to growing rats. In this Chapter, we sought to determine whether orally administered ovine serum Ig affected growth performance, gut morphology and histology in the growing rat.

2.1. Abstract

In this study, our aim was to determine whether orally administered ovine serum Ig improved growth performance, organ weights, and gut morphology in growing rats and whether the method of manufacture of ovine serum Ig affected its bioactivity. Ninety Sprague-Dawley male rats were used in a 21-d growth study and were fed a basal control diet (BD; no Ig) and 5 test diets: spray-dried porcine plasma (SDPP), freeze-dried ovine Ig (FDOI), 2 concentrations of spray-dried ovine Ig ($SDOI_{100}$ and $SDOI_{150}$), and inactivated ovine Ig (IOI). Diets were isocaloric and contained the same amount of the first limiting amino acids, methionine plus cysteine. The body weight gain:feed ratio was higher ($P < 0.05$) for the FDOI-fed rats than for the BD- and IOI-fed groups. FDOI rats had higher jejunum ($P < 0.05$) and colon weights ($P < 0.05$) at the end of the study than rats in the BD group. Compared with the $SDOI_{100}$-fed group, the FDOI group supported higher ($P < 0.05$) duodenum and colon weights. For gut morphology, the FDOI and the BD and IOI groups differed ($P < 0.05$). The FDOI-fed rats had longer ($P < 0.05$) villi and greater villi surface areas in the duodenum, jejunum, and ileum than the rats fed $SDOI_{100}$. An ovine Ig fraction selectively improved growth performance, organ weight, and gut morphology in growing rats. Compared with spray-drying, a freeze-drying procedure appears to preserve a higher degree of immunological activity.

2.2. Introduction

Several studies have demonstrated the benefits of including spray dried animal plasma (SDAP) in the postweaning diet of production animals, with improvements observed for feed intake, growth rate and intestinal growth (*Gatnau et al., 1989; Hansen et al., 1993; Kats et al., 1994; Quigley et al., 2002; Campbell et al., 2003; Torrallardona et al., 2003; Campbell et al., 2004; Pierce et al., 2005*). Beneficial effects of SDAP on growth have also been reported in dogs (*Quigley et al., 2004*), mice (*Thomson et al., 1994*), and rats (*Garriga et al., 2005; Pérez-Bosque et al., 2006, 2008*). The effects of animal plasma appear to be more pronounced during higher pathogen challenge (*Coffey & Cromwell, 1995*). Although the mechanism of action of SDAP has not been fully elucidated, a reduction in the degree of adhesion to pathogenic bacteria appears to be involved. Bosi *et al*. (2004) demonstrated that Ig present in SDAP may bind to antigens in the lumen of the gastrointestinal tract (GIT) and prevent their attachment to the mucosa. Inflammatory cytokine expressions also seems to be reduced in tissues exposed to lipopolysaccharide (*Touchette et al., 2002*) or *Escherichia coli K88* (*Bosi et al., 2004*) in the presence of SDAP. The effects of dietary SDAP and its Ig fraction on growth in animals may be due to a greater availability of nutrients and energy for growth, consequent upon a lower degree of immune cell activation (*Demas et al., 1997*), and an altered integrity and structure of the intestinal mucosa (*Fiocchi, 1997*).

The treatment and control of infectious diseases has become increasingly complicated due to the recent emergence of pathogenic microorganisms resistant to antibiotics and thus incidence of opportunistic infection (*Weiner et al., 1999*). Eibl *et al*. (1988) reported that children (born prematurely) with necrotizing enterocolitis were prophylactically treated with oral administration of purified human Ig. Ig have also been used therapeutically against *Campylobacter jejuni* (*Hammarstrom et al., 1993*) and *Clostridium difficile* (*Tjellstrom et al., 1993*) that are responsible for diarrhea in immunocompromised patients as well as chronic diarrhea of unknown etiology in normal infants (*Casswall et al., 1996*). The successful intervention of *Helicobacter pylori*, rotavirus and cryptosporidial infection using bovine Ig has also been reported (*Bogstedt et al., 1996*).

Several studies (*Blum et al., 1981; Eibl et al., 1988; Hammarstrom et al.,*

1993; Tjellstrom et al., 1993; Bogstedt et al., 1996; Casswall et al., 1996) have shown that orally administered Ig retains its activity within the GIT and is effective post-digestion. Blum *et al*. (1981) found undigested and partially digested Ig with intact opsonic activity in fecal samples of low birth weight infants who had been given Ig orally.

The objective of this work was to ascertain whether orally administered ovine serum Ig affected growth performance, gut morphology, and histology in the growing rat. We compared a freeze-dried ovine Ig (FDOI)-containing diet, a casein-based control diet (BD; no Ig) and a negative control [inactivated ovine Ig-containing diet (IOI)]. The study also included evaluation of a commercially available spray-dried porcine plasma preparation (SDPP) and spray-dried ovine Ig (SDOI) at 2 dietary concentrations. A secondary objective was to ascertain whether spray-drying influenced the activity of the ovine Ig and whether SDPP and SDOI had similar effects.

2.3. Materials and methods

2.3.1. Preparation and ELISA of ovine serum Ig and SDPP

An ovine Ig fraction was prepared according to a procedure previously developed in our laboratory (*Han et al., 2008*). Briefly, ammonium sulfate was added to ovine serum to obtain a 1.7 M ammonium sulfate solution. The precipitated protein was centrifuged at 11,000 x *g* for 20 min. The precipitate was dissolved in distilled water and then ultrafiltered through a 12-kDa membrane using an ultra-filtration unit (Model x702, XTravert, PDL Electronics). The final product was either freeze-dried or spray-dried (internal temperature, 180°C; exit temperature, 80°C). A portion of FDOI was inactivated (IOI) by dry heating (121°C, 15 min). Inactivation of Ig in IOI was confirmed using a circular dichroism spectrometer (Chirascan, Applied Photophysics) based on the method of Vermeer and Norde (2000). Commercial SDPP (AP920) was obtained from American Protein Corporation.

The content of ovine IgG was analyzed by direct ELISA. Sheep IgG from Auspep was used as a reference antigen for construction of the standard curve. Flat-bottomed Immunopure Polysorp 96-well plates (Nunc) were coated with

reference antigens or freeze-dried or spray-dried ovine Ig fraction in PBS buffer (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4). The plates were incubated overnight at 4°C, washed 5 times with PBST (PBS containing 0.05% Tween-20) and then the remaining binding sites were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h at 37°C. The plates were then washed 5 times with PBST. The 96-well plates were incubated with horseradish peroxidase-conjugated rabbit anti-sheep IgG (AbD Serotec) at a 1:20,000 dilution in PBST for 2 h at 37°C. The plates were washed five times and developed using *o*-phenylene diamine (Sigma) for 20 min. The reaction was stopped by the addition of 2 M H_2SO_4 and the absorbance was read at 450 nm using an ELISA plate reader (Perkin Elmer).

The content of IgG in SDPP was analyzed by sandwich ELISA using a pig IgG ELISA Quantitation kit (Bethyl Lab.) according to the manufacturer's instruction. The 96-well plates (Nunc) were coated with goat anti-pig IgG-affinity purified. The plate was incubated for 1 h at room temperature, washed 3 times with wash buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0), and the remaining binding sites blocked with 1% BSA (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0) for 30 min at 37°C. The plates were then washed 3 times. Samples of SDPP and pig serum as a reference were added to the plates and incubated for 1 h at room temperature before being washed 3 times. The 96-well plates were incubated with goat anti-pig IgG-horseradish peroxidase conjugate at a 1:50,000 dilution in wash buffer for 1 h at 37°C and then absorbance was measured as described earlier. The ELISA was performed four times for each sample and the data were averaged.

2.3.2. Animal study

This work was approved by the Massey University Animal Ethics Committee (MUAEC 06/132) and procedures complied with the New Zealand Code of Recommendations and Minimum Standards for the Care and Use of Animals for Scientific purposes (New Zealand Animal Welfare Advisory Committee, 1995).

Ninety Sprague-Dawley male rats (140–160 g body weight) were housed singly in stainless steel cages with free access to water in a room maintained at 22 ± 2°C with a 12 h light/dark cycle.

The rats were given 1 wk to acclimatize during which time they consumed

the basal diet *ad libitum*. After acclimatization, the rats were randomly allocated to 1 of 6 diets (Table 2.1, *n*=15 per diet) to undergo the 3-wk growth study.

The diets were formulated with methionine plus cysteine as the first limiting amino acids (AA) to meet or exceed National Research Council (*NRC, 1995*) recommendations for growing rats for the major nutrients (Table 2.1). The diets included a BD and 5 test diets containing SDPP, FDOI, and SDOI at 2 dietary concentrations ($SDOI_{100}$ and $SDOI_{150}$) and a negative control diet IOI. The freeze- and spray-dried ovine Ig fractions were included in the diet at 3.07% (FDOI), 3.28% ($SDOI_{100}$, IgG contributed in this diet is similar to IgG in the SDPP diet), and 3.40% (IOI) such that the amount of IgG in each diet was equal to the amount of IgG contributed by the SDPP diet. For the $SDOI_{150}$ diet, SDOI was included at 4.92% to provide 1.5 times the amount of IgG present in the SDPP diet (Table 2.1 and 2.2). All food was consumed in powdered form *ad libitum*.

2.3.3. Chemical analysis

Dry matter, ash, crude protein, total fat and gross energy were determined according to methods described by AOAC (2003). Carbohydrate (nitrogen-free extractive) was determined by calculation using the formula [100 – (moisture + fat + ash + crude protein) = carbohydrate]. AA were determined in duplicate diet samples after acid hydrolysis (6 M HCl for 24 h at 110°C) using ion exchange HPLC with ninhydrin post column derivatization. Methionine and cysteine were oxidized to methionine sulphone and cysteic acid, respectively, using perfomic acid, followed by hydrolysis and then determined using ion exchange HPLC with *o*-phthaldialdehyde post column derivatization.

2.3.4. Growth performance

Body weights were measured at d 1, 7, 14, and 21 and food intake was recorded daily from d 1 to 21. Daily gain and daily feed intake were calculated. The gain:feed (G:F) ratio was calculated by dividing the daily gain by the daily feed intake.

2.3.5. Post-mortem procedure and organ weights

At d 21 of the experiment, rats were anesthetized by intraperitoneal injection (0.1 mL/100 g live weight) of a mixture containing 2 parts acepromazine maleate BP (ACP, 2 g/L), 5 parts ketamine (100 g/L), 1 part xylazine (10%), and 2 parts sterile water. Rats were then killed by exsanguination.

The stomach was removed and its contents emptied. The tissues were then dried with absorbent paper and weighed. The entire small intestine from the pylorus to the ileocecal valve was removed and contents were flushed out using PBS. The tissues were then blotted dry and weighed. The same procedure was followed for the cecum and colon. The liver was also removed and weighed. The remaining portion of the rat was weighed as empty body weight. Relative weights of the organs (weight of organ per unit body weight) were calculated.

2.3.6. Gut morphology

The small intestine was dissected free of mesenteric attachments and was divided approximately into duodenum, jejunum, and ileum. Lengths of tissue (2 cm) were collected from the duodenum (from pylorus end), jejunum (middle of intestine), and ileum (anterior to ileocaecal valve) and stored immediately in 10% buffered formalin for 24 h. After fixation, each tissue was excised, dehydrated, and embedded in paraffin wax. From each tissue, transverse sections were cut, stained with hemotoxylin, eosin, and alcian blue, and examined under a light microscope. The parameters measured were as follows: villus height (h) measured from the tip of the villus to the villus-crypt junction, crypt depth (d) measured from the crypt-villus junction to the base of the crypt, villus width at mid-villus height (mh), and villus surface area (VSA) [calculated as $VSA = [(\pi \times mh \times h) + \pi \times (mh/2)^2$ and where $\pi = 3.14$]. The villus:crypt ratio was calculated by dividing the villus height by crypt depth. The 5 longest and straightest villi and their associated crypts from each transverse tissue section of 10 rats in each group were measured. Colon tissue was prepared as described for the small intestine and crypt depths were measured for 5 elongated and straight crypts from each transverse tissue section of 10 rats in each group.

Table 2.1 Ingredient compositions and determined nutrient and energy contents of the control and test diets.

	Diet[1]					
	BD	**SDPP**	**FDOI**	**$SDOI_{100}$**	**$SDOI_{150}$**	**IOI**
	g/kg dry weight					
Ingredient						
Wheat starch	492	480	493	492	493	490
Sucrose	100	100	100	100	100	100
Cellulose (Avicel)	50	50	50	50	50	50
Soyabean oil	70	70	70	70	70	70
Casein	186	98	154	153	136	153
Vitamin premix[2]	50	50	50	50	50	50
Mineral premix[3]	50	50	50	50	50	50
L-Methionine		1.7	0.6	0.6	0.9	0.6
L-Cysteine	2.4		1.8	1.8	1.1	1.7
SDPP		100				
Ovine IgG fraction			30.7	32.8	49.2	34.0
Nutrient[4]						
Gross energy, *kJ/g*	17.5	17.5	17.6	17.6	17.5	17.6
Dry matter, %	92.2	92.5	92.4	92.4	92.0	92.3
Crude protein, %	16.9	17.0	16.8	16.8	16.7	17.0
Carbohydrate, %	64.4	63.6	64.6	64.8	64.3	64.3
Fat, %	7.15	7.42	7.15	7.15	7.08	7.29
Ash, %	3.74	4.52	3.75	3.75	3.84	3.77
Methionine+cysteine, *g/kg*	8.22	8.21	8.19	8.17	8.21	8.07
Lysine, *g/kg*	13.67	14.01	13.31	13.47	13.26	13.45

[1]All diets were formulated to meet or exceed the nutrient requirements of growing rats
[2]The mixture supplied (mg/kg diet): retinol acetate, 5.0; DL-α-tocopheryl acetate, 200; menadione, 3.0; thiamin hydrochloride, 5.0; riboflavin, 7.0; pyridoxine hydrochloride, 8.0; D-pantothenic acid, 20; folic acid, 2.0; nicotinic acid, 20; D-biotin, 1.0; myoinositol , 200; choline chloride, 1500; (μg/kg diet) ergocalciferol, 25; cyanocobalamin, 50.
[3]The mixture supplied (g/kg diet): Calcium hydrogen phosphate, 6.29; Magnesium oxide, 1.06; Potassium chloride, 5.24; Sodium chloride, 1.97; (mg/kg diet) Chromic potassium sulfate, 1.97; Cupric carbonate, 10.7; Ferric citrate, 424; Manganese sulfate, 78.0; Zinc oxide, 48.2; (μg/kg diet) Cobalt chloride, 29.0; Potassium iodate, 151; Ammonium molybdate, 152; Sodium selenite. 151.
[4]values for nutrients were from chemical analysis except for carbohydrate, which was calculated by difference, methionine, cysteine and lysine were calculated values.

2.3.1. Statistical analysis

Results were given as means ± SEM. Data were analyzed using a 1-way ANOVA using the General Linear Models procedure of SAS/PROC GLM and means were compared using Tukey's test. Weekly data for daily gain, daily feed intake, and G:F ratio were analyzed over 3 wk and subjected to a repeated-measures analysis using the General Linear Models procedure of SAS/PROC GLM (SAS, version 9.1, SAS Institute). Statistical significance was accepted at $P < 0.05$.

Table 2.2 Protein and Ig concentrations of the Ig fractions and estimated Ig concentration in the test diets.

Fraction	Protein	Ig concentration[1]	Estimated Ig concentration in each diet
		%	
FDOI	94	73.0	2.24
SDOI	90	68.4	2.24, 3.37[2]
IOI	85	-[3]	2.29
SDPP	78	22.4	2.24

[1]The values were determined by ELISA. [2]$SDOI_{100}$ and $SDOI_{150}$, respectively. [3]It was not possible to determine the amount of IOI due to difficulty with solubilizing the material. A concentration of 66% was estimated based on the protein and Ig concentrations of the FDOI.

2.4. Results

2.4.1. Biological activity of freeze-dried ovine Ig

2.4.1.1. Growth performance

At the end of wk 3, overall daily gain and daily feed intake were not affected by diet (BD, FDOI, and IOI), but the G:F ratio was higher ($P < 0.05$) for rats

consuming the FDOI diet than for those fed the BD diet or the IOI diet (Table 2.3). Similarly, in a repeated-measures analysis (data not shown), daily gain and daily feed intake over the 3 wk were not affected by diet, but the G:F ratio was greater (P = 0.004) for rats receiving the FDOI diet compared with those consuming the BD or IOI diets. We considered the G:F ratio as the most sensitive measure of growth performance.

Table 2.3 Growth performance of rats fed diets containing ovine Ig for 21 d[1].

	Diet				
	BD	**FDOI**	**IOI**	**SEM**	***P* Value**
Feed intake, *g/d*	22.25	22.32	21.92	0.64	0.710
Weight gain, *g/d*	8.46	9.22	8.83	0.40	0.740
G:F ratio, *g/g*	0.38[b]	0.42[a]	0.39[b]	0.01	0.015

[1]Values are means ± SEM, n=15. Means in a row with superscripts without a common letter differ, P<0.05.

2.4.1.2. Organ weights

The relative weights of stomach, ileum, cecum, and liver and the empty body weight (Table 2.4) were not affected by diet, whereas the relative weights of the duodenum, jejunum, and colon (Table 2.4) were generally higher for rats receiving the FDOI diet compared with those fed the BD or IOI diets (P = 0.001–0.069).

2.4.1.1. Gut morphology

The villus length, crypt depth, villus:crypt ratio, and villus surface area of duodenum, jejunum, and ileum generally were greater (P < 0.01) in rats receiving the FDOI diet than for those fed either the BD or IOI diet (P < 0.05) (Table 2.5). The colon crypt depth was also higher (P < 0.001) in rats receiving the FDOI diet than in rats fed the BD or IOI diets.

Table 2.4 Relative organ weights and empty body weight of rats fed diets containing ovine Ig for 21 d[1].

	Diet			SEM	*P* value
	BD	**FDOI**	**IOI**		
	g/100g body weight				
Stomach	0.50	0.47	0.48	0.01	0.481
Duodenum	0.38^{ab}	0.43^{a}	0.37^{b}	0.01	0.019
Jejunum	1.03^{b}	1.17^{a}	1.10^{ab}	0.03	0.026
Ileum	0.94	0.97	0.93	0.16	0.713
Caecum	0.45	0.45	0.49	0.03	0.122
Colon	0.48^{b}	0.55^{a}	0.49^{b}	0.02	0.024
Liver	4.59	4.64	4.86	0.13	0.192
Empty body	94.73	95.23	94.90	1.69	0.144

[1] Values are means ± SEM, *n*=15. Means in a row with superscripts without a common letter differ, *P*<0.05.

2.4.2. Comparison between freeze- and spray-dried ovine Ig

2.4.3. Growth performance

Daily gain, daily feed intake, and the G:F ratio did not differ among the rats fed the FDOI, SDPP, $SDOI_{100}$, and $SDOI_{150}$ diets ($P > 0.05$) (data not shown).

2.4.3.1. Organ weights

The relative weight of the duodenum was greater in rats fed the FDOI diet than in those fed the SDPP, $SDOI_{100}$, and $SDOI_{150}$ diets ($P < 0.05$) (Table 2.6). Rats given the FDOI diet also had heavier ($P < 0.05$) colons than rats receiving the SDPP and $SDOI_{100}$ diets, whereas the relative weights of stomach, jejunum, ileum, and cecum and empty body weight did not differ among the 4 diet groups. Rats fed the SDPP diet had a higher ($P < 0.01$) relative liver weight than those fed the FDOI, $SDOI_{100}$, or $SDOI_{150}$ diets (Table 2.6).

2.4.3.2. Gut morphology

When comparing the effect of the FDOI, SDPP, $SDOI_{100}$, and $SDOI_{150}$ diets on gut morphology, rats fed the SDPP and FDOI diets had higher ($P < 0.05$) villus lengths in the duodenum and jejunum than in rats fed the $SDOI_{100}$ diet. In the ileum, rats receiving the FDOI and $SDOI_{150}$ diets had greater ($P < 0.001$) villus lengths and cyrypt depths than rats fed the SDPP and/or $SDOI_{100}$ diets. Rats receiving the SDPP diet had a higher ($P < 0.05$) villus:crypt ratio in the duodenum than rats fed the $SDOI_{100}$ diet. Rats given the FDOI diet had greater ($P < 0.01$) villus surface areas in the duodenum, jejunum, and ileum than rats fed the SDPP, $SDOI_{100}$, and $SDOI_{150}$ diets. Rats fed the $SDOI_{150}$ diet had greater jejunal villus surface areas ($P < 0.001$) and ileum villus surface areas ($P < 0.001$) than rats fed the SDPP diet (Table 2.7).

2.5. Discussion

Plasma proteins have been used in the diets of several species of animal (*Gatnau et al., 1989; Hansen et al., 1993; Kats et al., 1994; Quigley et al., 2002; Campbell et al., 2003; Torrallardona et al., 2003; Campbell et al., 2004; Pierce et al., 2005; Pérez-Bosque et al., 2006*) as a supply of AA and also as a source of Ig, which are the primary antiinfective component of blood serum, colostrum, and breast milk. These specialized proteins protect the body from harmful bacteria, viruses, and other environmental pathogens by either binding to them or by the formation of an encapsulating barrier (*Kraehenbuhl & Neutra, 1992*).

In the gut, exposure to antigens such as pathogenic and nonpathogenic organisms induces the production of proinflammatory cytokines such as IL-1, IL-6, and tumor necrosis factor-α and acute phase proteins (*Johnson, 1997*). The consequent demand for AA reduces the efficiency of dietary protein utilization, resulting in overall growth inhibition. Plasma Ig are considered to lower stimulation of the immune system by preventing microbial growth or colonization in the gut, especially in the small intestine, and/or by indirectly assisting mucosal integrity (*Touchette et al., 2002*). Lowered activation of the immune system may thus lead to a higher availability of nutrients and energy for growth (*Demas et al., 1997; Torrallardona et al., 2003; Garriga et al., 2005*).

Table 2.5 Intestinal morphology of rats fed diets containing ovine Ig for 21 d[1].

	Diet			SEM	*P* value
	BD	**FDOI**	**IOI**		
Duodenum					
Villus length, μm	564[b]	706 [a]	573[b]	13.4	<0.001
Crypt depth, μm	205[ab]	223[a]	186[b]	6.97	<0.001
Villus:Crypt ratio	2.79[b]	3.21[a]	3.15[ab]	0.114	<0.01
Villus surface area, mm^2	0.02[b]	0.04[a]	0.02[b]	0.0013	<0.001
Jejunum					
Villus length, μm	389[c]	570[a]	445[b]	11.3	<0.001
Crypt depth, μm	131[ab]	151[a]	117[b]	6.31	<0.001
Villus:Crypt ratio	3.03[b]	3.92[a]	3.96[a]	0.213	<0.001
Villus surface area, mm^2	0.01[c]	0.04[a]	0.05[b]	0.0009	<0.001
Ileum					
Villus length, μm	187[c]	327[a]	235[b]	11.2	<0.001
Crypt depth, μm	106[b]	137[a]	104[b]	5.49	<0.001
Villus:Crypt ratio	1.78[b]	2.43[a]	2.48[a]	0.142	0.008
Villus surface area, mm^2	0.004[b]	0.018[a]	0.006[b]	0.0005	<0.001
Colon					
Crypt depth, μm	202[b]	311[a]	195[b]	11.69	<0.001

[1] Values are means ± SEM, *n*=10. Means in a row with superscripts without a common letter differ, *P*<0.05.
[2] Formula for villus Surface Area = (3.14 × *mh* × h) + 3.14 × $(mh/2)^2$, where *mh* = villus width at midvillus height, h = villus height measured from the tip of the villus to the villus-crypt junction and crypt depth is measured from villus-crypt junction to the base of the crypt.

Ig are at least partially resistant to digestion in the upper gastrointestinal tract. Rodriguez *et al*. (2007) recently reported that porcine Ig partially resisted the digestion process in the GIT of adult dogs and cats fed diets containing SDAP or concentrated Ig derived from pig blood. The concentrations of intact Ig in the feces of dogs and cats consuming diets containing Ig were similar to those found in humans (*Blum et al., 1981; Kelly et al., 1997*) and in pigs (*Morel et al., 1995*).

Table 2.6 Relative organ weights of rats fed diets containing ovine Ig and SDPP for 21 d[1].

	Diet				SEM	*P* value
	SDPP	**FDOI**	**$SDOI_{100}$**	**$SDOI_{150}$**		
			g/100g body weight			
Duodenum	0.39[b]	0.43[a]	0.38[b]	0.38[b]	0.01	0.010
Colon	0.45[b]	0.55[a]	0.48[b]	0.55[a]	0.02	0.024
Liver	5.21[a]	4.63[b]	4.74[b]	4.65[b]	0.13	0.007

[1]Values are means ± SEM, *n*=15. Means in a row with superscripts without a common letter differ, *P*<0.05.

Rodriguez *et al*. (2007) also reported that Fab fragments were found after digestion which is consistent with the *in vitro* findings of Nezlin (1998), who reported that Fab is more resistant to the digestion process than the rest of the Ig molecule. The Fab fragment, which is released after pepsin digestion (*Gorevic et al., 1985*), retains the capacity to bind bacteria by neutralization and opsonization, as it contains the antigen binding sites. Indeed, several reports have demonstrated that human serum and bovine Ig resist digestion in the upper GIT of humans and retain toxin binding and neutralizing activity (*Losonsky et al., 1985; Kraehenbuhl & Neutra, 1992; Warny et al., 1999*). In suckling and possibly mature animals, Ig may be phagocytosed by the enterocyte, escape partial digestion by intracellular proteases and reach the circulation by exocytosis on the basolateral surface of the cell (*Hemmings & Williams 1978; Sanderson & Walker, 1993*). Also, Ig prevents pathogens like bacteria and viruses from damaging the gut wall, thereby resulting in a more functional (intact and absorptive) intestinal wall. Several studies have shown that animals fed with plasma proteins have an improved gut morphology as evidenced by longer villus height and greater villus:crypt ratio (*Spencer et al., 1997; Touchette et al., 1997; Owusu-Asiedu et al., 2003*) and increased villus surface area (*Gatnau et al., 1995*). This may be due to Ig preventing damage to the gut wall.

Table 2.7 Intestinal morphology of rats fed diets containing ovine Ig and SDPP for 21 d[1].

	Diet					
	SDPP	**FDOI**	**$SDOI_{100}$**	**$SDOI_{150}$**	**SEM**	***P* value**
Duodenum						
Villus length, μm	719[a]	706[a]	663[b]	698[ab]	13.40	0.020
Villus:Crypt ratio	3.42[a]	3.21[ab]	2.88[b]	3.16[ab]	0.11	0.017
Villus surface area, mm^2	0.034[b]	0.040[a]	0.032[b]	0.035[b]	0.0013	0.002
Jejunum						
Villus length, μm	562[a]	570[a]	526[b]	546[ab]	11.30	0.021
Villus surface area, mm^2	0.022[c]	0.033[a]	0.024[bc]	0.026[b]	0.0009	0.002
Ileum						
Villus length, μm	256[b]	327[a]	271[b]	315[a]	11.18	0.001
Crypt depth, μm	116[b]	137[a]	136[a]	141[a]	5.49	0.001
Villus surface area, mm^2	0.008[c]	0.018[a]	0.011[b]	0.011[b]	0.0005	0.002

[1] Values are means ± SEM, n=15. Means in a row with superscripts without a common letter differ, P<0.05.
[2] Formula for Villus Surface Area= (3.14 × *mh* × h) + 3.14 × $(mh/2)^2$, where *mh* = villus width at midvillus height, h=villus height measured from the tip of the villus to the villus-crypt junction and crypt depth is measured from villus-crypt junction to the base of the crypt.

In this study, the daily gain across all rats and diets was 9 g/d, which is much higher than a typical growth rate of 6 g/d reported by the NRC (1995) for similar-aged rats. This is probably due to the highly digestible, high protein, and high energy diets used in our study. It may also have been due to a lower incidence of subclinical disease infection. This study may have thus been less sensitive than some for assessing growth performance. Daily gain and daily feed

intake did not differ among the diets, but the G:F ratio, which is a more sensitive measure of growth performance, for the rats fed FDOI was significantly higher than that for rats fed the BD (control) and IOI-supplemented diets. Moreover, at the end of wk 1, 2, and 3, rats fed the FDOI had a significantly higher G:F ratio than rats fed the BD or the IOI diet.

The FDOI-fed rats responded favorably with a better G:F ratio, which is consistent with results reported from other studies (*Coffey & Cromwell, 1995; Gatnau et al., 1995; Owen et al., 1995; Weaver et al., 1995; Jiang et al., 2000; Pierce et al., 2005*) and support the earlier suggestion by Pierce *et al*. (2005) that higher growth performance with Ig may be due to a nutrient-sparing effect. A FDOI diet was utilized for growth to a greater extent when compared with a control diet or to a diet containing IOI. Given that the Ig in the IOI treatment was inactivated as confirmed by circular dichroism spectral analysis (data not shown), it would suggest that the active Ig was responsible for the greater growth efficiency, possibly via a sparing of nutrients that would otherwise have been used for immune response activation. It should be noted that our testing regimen was conservative, because some Ig may not have been digested, rendering the test diets more limiting in methionine than anticipated based on formulation.

Because our test proteins did not affect the daily feed intake, the study was suitable for determining whether there were any trophic effects of the Ig fraction. Rats receiving the FDOI had significantly heavier duodenums, jejunums, and colons compared with those fed the BD or IOI diet.

Important differences in intestinal morphology were also observed. Touchette *et al*. (1997) reported that animal plasma improved small intestinal morphology independent of feed intake. The rats fed the FDOI diet had significantly longer villi, greater crypt depths, and greater villi surface areas in all small intestinal sections. These results suggest that consumption of freeze-dried (undenatured) Ig leads to improved gut morphology.

Growth performance did not differ among rats fed the FDOI, $SDOI_{100}$, $SDOI_{150}$, or SDPP diets. Spray drying may, however, affect the activity of Ig, because rats fed the $SDOI_{100}$, $SDOI_{150}$, and SDPP had lower ($P < 0.05$) duodenal weights than those fed the FDOI diet. A similar trend was observed for colon weights, where the FDOI-fed rats had significantly heavier colons than the $SDOI_{100}$-fed rats.

For most of the organs examined, organ weight was not adversely affected by spray drying of the Ig. Rats receiving the SDPP diet had significantly higher relative liver weights than those fed the other 3 diets. This result is similar to the findings of Owusu-Asiedu *et al*. (2003), who also found that SDPP affects liver weight by unknown mechanism. Gut morphology was influenced by spray drying of the material and spray drying may result in partial denaturation of ovine Ig.

In conclusion, FDOI improved growth performance and the weights of some digestive organs and improved gut morphology in growing rats. Spray drying the ovine Ig fractions may result in a partial loss of Ig activity.

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Chapter 3

Immunomodulatory effects of ovine serum immunoglobulin in the growing rat

Animal (2010) 4, 1702-1708

The results reported in Chapter-2 show that in growing rats, active Ig was responsible for the greater growth efficiency, and that the FDOI diet improved the weights of some digestive organs and positively supported gut morphology; and that spray drying the ovine Ig fractions may result in a partial loss of Ig activity. Aspects of immunity, however, were not examined. Therefore, the objective of this study was to ascertain whether orally administered ovine serum Ig selectively modulates immune function in the growing rat.

3.1. Abstract

This study aimed to determine whether orally administered ovine serum Immunoglobulin (Ig) modulates aspects of immunity such as phagocytosis, lymphocyte proliferation, cytokine production, intestinal and plasma Ig concentrations in growing rats. Forty-five male Sprague-Dawley rats (*n*=15/group) were used in the 21 day study, and fed a basal control diet (BD; no Ig) or two test diets: freeze-dried ovine Ig (FDOI) and inactivated ovine Ig (IOI). Phagocytic activity of peripheral blood leukocytes and lymphocyte proliferation in the presence of the mitogen concanavalin A (ConA) was greater ($P < 0.05$) for the FDOI-fed rats than for the BD- and IOI-fed groups. ConA-stimulated and unstimulated spleen cell culture produced higher ($P < 0.05$) interferon-γ and interleukin-4, respectively, from rats fed the FDOI than rats fed the BD diet. In the jejunum, ileum and plasma, rats fed FDOI produced higher ($P < 0.05$) concentrations of secretory IgA (sIgA) than rats fed IOI or BD. Rats fed the FDOI-supplemented diet had greater jejunal ($P = 0.037$) and lower plasma ($P = 0.025$) rat IgG concentrations than rats fed either the BD or IOI-supplemented diet. In conclusion, an ovine Ig fraction selectively modulated various indices of immune function.

3.2. Implications

There has been no evidence suggesting the effects of ovine serum Ig on animal immunomodulation. The results of this study clearly demonstrate an effect of ovine serum Ig on immunomodulation in the growing rat. The proliferation of lymphocytes, phagocytic activity and rat digesta antibodies were all significantly enhanced. The results now need to be confirmed in production animals, but ovine Ig may offer a novel means of enhancing immunity.

3.3. Introduction

The development of immunomodulators from natural sources for dietary supplementation in both animals and humans is an active area of research. Recently, immunonutrition has been applied to improve the clinical course of recovery for severely sick or surgical patients who often need an exogenous supply of nutrients delivered through the parental or enteral routes (*Calder, 2007*).

Ig are the primary anti-infective component of plasma, colostrum and breast milk. These specialized proteins protect the body from harmful bacteria, viruses and other environmental pathogens by either binding to them or by forming an encapsulating barrier (*Kraehenbuhl & Neutra, 1992*). Human Ig has been used as a prophylactic treatment for children born prematurely with necrotizing enterocolitis (*Eibl et al., 1988*). To date, Ig has been used therapeutically for intervention in *Campylobacter jejuni* (*Hammarstrom et al., 1993*), *Clostridium difficile* (*Tjellstrom et al., 1993*), Helicobacter *pylori*, rotavirus and cryptosporidial infection (*Bogstedt et al., 1996*).

Several studies carried out in production animals at weaning (*Kats et al., 1994; Torrallardona et al., 2003; Pierce et al., 2005*) have demonstrated beneficial effects from the ingestion of spray-dried animal plasma (SDAP) containing Ig, such as increased intestinal tissue growth rate, increased feed intake and higher body weight (BW) gain. Furthermore, the beneficial effects of feeding SDAP are more pronounced with higher pathogen challenge, as when pigs were housed in an on-farm conventional nursery, which was exposed to a

greater load of subclinical pathogenic organisms, the animals exhibited an enhanced growth rate and feed intake compared to those reared in the off-site experimental (cleaner) settings (*Coffey & Cromwell, 1995*). A study by Bosi *et al*. (2004) suggests that the Ig present in SDAP appears to elicit its effect by binding to the pathogenic bacteria, thereby preventing their attachment to the mucosa in the lumen of the gastrointestinal tract (GIT).

An important consideration with orally administered Ig is whether the proteins become denatured during their passage through the GIT, thus losing activity. Several reports have shown that orally administered Ig retains activity in the GIT (*Tjellstrom et al., 1993; Bogstedt et al., 1996; Casswall et al., 1996*), and partially digested or undigested Ig with opsonic activity has been found in the faeces of low BW infants who had been treated orally with Ig (*Blum et al., 1981*).

Recently, I have reported that freeze-dried ovine serum Ig (FDOI) selectively improves growth performance, organ growth and gut morphology in rats when compared with a control diet and with inactivated ovine Ig (IOI; *Chapter-2*). There have been no reports, however, regarding the effects of ovine Ig on immunomodulation in animals.

The objective of this study was to ascertain whether orally administered ovine serum Ig selectively modulates immune function in the growing rat. A number of indices of immune function were studied including phagocytic activity, lymphocyte proliferation of spleen cells, cytokine production and IgA and IgG concentrations in intestinal digesta and plasma. Comparison was made among a diet containing FDOI, a casein-based control diet (BD, no Ig) and a negative control diet (IOI-containing diet).

3.4. Material and methods

3.4.1. Preparation and quantitation of ovine serum Ig

An ovine Ig fraction was prepared by ammonium sulphate precipitation of fresh lamb's blood as described previously (*Chapter-2, Section-2.3.1*). The final product was freeze-dried, a portion of which was inactivated by dry heating (121°C, 15 min) to produce the IOI.

The IgG content in the ovine Ig fraction was analysed by direct ELISA using a rabbit anti-sheep IgG (AbD Serotec, Oxford, UK) according to the method as reported in Chapter-2, Section-2.3.1. The ELISA was performed in quadruplicate for each sample and the data were averaged.

3.4.2. Animal study

The study was approved by the Massey University Animal Ethics Committee (06/132) and procedures complied with the New Zealand Code of Recommendations and Minimum Standards for the Care and Use of Animals for Scientific purposes (New Zealand Animal Welfare Advisory Committee, 1995). Forty-five male Sprague–Dawley rats (140 to 160 g BW, animals reported in *Chapter-2*) were housed singly in stainless steel cages with free access to water in a room maintained at 22 ± 2°C with a 12 h light/dark cycle.

The rats were given 1 week to acclimatize, during which they consumed the basal diet *ad libitum*. After acclimatization, the rats were randomly allocated to one of three diets (Table 3.1, *n*=15 per diet) for the 3-week study.

The diets were supplemented with synthetic methionine and cysteine to meet or exceed the National Research Council (*NRC, 1995*) recommendations for the growing rat, as methionine plus cysteine was the first limiting amino acid (Table 3.1). The diets included a BD, a test diet containing FDOI and a negative control diet, IOI. The ovine Ig fractions that consist of IgG at a concentration of 73% were included in the diets (FDOI and IOI) at a concentration approximately equal to the amount of SDPP IgG used in previous studies (*Pierce et al., 2005; Balan et al., 2009*) (Table 3.1). Food was provided *ad libitum* in powdered form.

3.4.3. Chemical analysis

Dietary dry matter, ash, crude protein, total fat and gross energy were determined as described previously (*Chapter-2, Section-2.3.3*).

Table 3.1 Ingredient composition and determined nutrient and energy content of the control and test diets.

	Diet[1]		
	BD	**FDOI**	**IOI**
	g/kg air dry weight		
Ingredient			
Wheat starch	492	493	490
Sucrose	100	100	100
Cellulose (Avicel)	50	50	50
Soyabean oil	70	70	70
Casein	186	154	153
Vitamin premix[2]	50	50	50
Mineral premix[3]	50	50	50
L-Methionine		0.6	0.6
L-Cysteine	2.4	1.8	1.7
Ovine IgG fraction		30.7	34
Nutrient[4]			
Gross energy, kJ/g	17.5	17.6	17.6
Dry matter, %	92.2	92.4	92.3
Crude protein, %	16.9	16.8	17
Carbohydrate, %	64.4	64.6	64.3
Fat, %	7.2	7.2	7.3
Ash, %	3.74	3.75	3.77
Immunoglobulins[5], %		2.24	2.29
Methionine+cysteine, g/kg	8.22	8.19	8.07
L-Methionine, g/kg	5.02	5	4.95
L-Cysteine, g/kg	3.2	3.2	3.12
Aspartic acid, g/kg	13.99	13.13	13.43
Threonine, g/kg	7.45	7.64	7.74
Serine, g/kg	8.12	8.67	8.54
Glutamic acid, g/kg	31.03	32.28	31.93
Glycine, g/kg	4.19	3.95	3.91
Alanine, g/kg	6.72	5.93	5.6
Valine, g/kg	10.7	11.93	11.67
Histidine, g/kg	5.23	5.02	5.11
Lysine, g/kg	13.67	13.31	13.45
Arginine, g/kg	7.64	7.02	6.74

BD=basal diet; FDOI=freeze-dried ovine immunoglobulin; IOI=inactivated ovine immunoglobulin. [1]All diets were formulated to meet or exceed the nutrient requirements of growing rats. [2]The mixture supplied (mg/kg diet): retinol acetate 5.0, DL-tocopheryl acetate 200, menadione 3.0, thiamin hydrochloride 5.0, riboflavin 7.0, pyridoxine hydrochloride 8.0, D-pantothenic acid 20, folic acid 2.0, nicotinic acid 20, D-biotin 1.0, myo-inositol 200, choline chloride 1500; (µg/kg diet) ergocalciferol 25, cyanocobalamin 50. [3]The mixture supplied: (g/kg diet) Calcium hydrogen phosphate 6.29, Magnesium oxide 1.06, Potassium chloride 5.24, Sodium chloride 1.97; (mg/kg diet) Chromic

potassium sulphate 1.97, Cupric carbonate 10.7, Ferric citrate 424, Manganese sulphate 78.0, Zinc oxide 48.2; (µg/kg diet) Cobalt chloride 29.0, Potassium iodate 151, Ammonium molybdate152, Sodium selenite 151. [4]values for nutrients were from chemical analysis except for carbohydrate which was calculated by difference, methionine and cysteine were calculated values. [5]Estimated Ig concentration in each diet.

3.4.4. Post-mortem procedure

At day 21 of the experiment, rats were anaesthetized by the intra-peritoneal injection (0.1 ml/100 g live weight) of a mixture containing two parts acepromazine maleate BP (0.2%, w/v, ACP), five parts ketamine (10%, w/v), one part xylazine (10%, w/v) and two parts sterile water. A blood sample was collected by cardiac puncture and a portion was immediately transferred to a heparinized vacutainer tube (Becton Dickinson, NJ, USA) to isolate plasma for measurement of plasma IgA and IgG. The remaining heparinized blood was used for assessing the phagocytic activity of peripheral blood leukocytes (PBLs). The spleen was removed aseptically for the lymphocyte proliferation assay. The jejunum (middle of the intestine) and ileum (anterior to ileocaecal valve) were collected and washed intra-luminally with 5 ml of phosphate buffer saline (PBS) containing 1% protease inhibitor (Sigma, Auckland, New Zealand). The suspensions were then centrifuged (HERAEUS, Fresco 17, Thermo Electron Corporation, MA, USA) at 3000 *g* for 10 min to remove debris and the clear supernatant was stored at −20°C until the measurement of intestinal IgA and IgG.

3.4.5. Assessment of phagocytosis

Assessment of the phagocytic activity of PBLs by flow cytometry was based on the method of Wan *et al*. (1993) with some modifications. Briefly, 5 µl fluorescein isothiocyanate-labelled *Escherichia coli* (FITC-labelled *E. coli*) K-12 (1×10^9/ml, Molecular Probes Incorporated, OR, USA) and 100 µl blood were mixed in glass tubes on ice and incubated at for 30 min at 37°C in a 5% humidified CO_2-air atmosphere. The PBLs were fixed for 1 min with 100 µl paraformaldehyde (8%, v/v) and erythrocytes lysed by the addition of 1 ml ice-cold water. Following centrifugation, the resulting pellets were resuspended in 0.5 ml of PBS, thoroughly mixed and transferred to an FACS tube containing 50 µl trypan blue (0.4% in PBS, w/v) to quench extraneous fluorescence. The level of phagocytic

activity was determined using an FACS (Fluorescence-activated cell sorter)-Calibur flow cytometer (Becton Dickinson, USA). Results were expressed as the proportion PBLs population which contained FITC-labelled *E. coli* based on minimum of 10,000 gated events (lymphocytes, platelets and erythrocytes excluded).

3.4.6. Preparation of splenocytes

Single-cell suspensions were prepared from spleen in complete RPMI-1640 medium (RPMI-1640 medium supplemented with 10% foetal calf serum, 10 mM HEPES, 2 mM-L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin sulphate and 50 mM 2-mercaptoethanol; all reagents from Gibco, USA). The spleen tissue was mechanically disrupted by cutting into small pieces using scissors and sucking up and down with a sterile 1 ml syringe. The resulting spleen cell suspension was transferred to a 15-ml tube containing 5 ml RPMI-1640 and centrifuged at 1000 x *g* for 10 min. Supernatants were then discarded. Erythrocytes were lysed using 5 ml ACK lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA) with occasional mixing during a 5-min incubation period. The remaining leukocyte suspensions were washed twice in complete RPMI-1640 medium and then resuspended in 3 ml RPMI-1640 medium, from which 100 µl of suspended cells were transferred to a flow cytometer tube containing 400 µl of PBS and 2 µl of propidium iodide. Viable lymphocytes were identified and counted using Becton Dickinson Immunocytometry Systems 'Cell Quest' software on an FACS Calibur flow cytometer (Becton Dickinson) by gating around the mixed lymphocyte cell population. Spleen lymphocyte cell numbers were adjusted to a final concentration of 2×10^6 lymphocyte-like cells/ml in complete RPMI-1640 immediately before use.

3.4.6.1. Lymphocyte proliferation assay

Aliquots of 10^5 spleen lymphocyte cells in 50 µl of complete RMPI-1640 medium were added in triplicate to the wells of a 96-well, flat-bottomed tissue culture plate (Nunc, MO, USA) and cultured in the presence of either 2.5 µg/ml concanavalin A (Con A; Sigma), 5 µg/ml lipopolysaccharide (LPS; Sigma), diluted (1:49) phytohaemagglutinin (PHA; Gibco) or complete RPMI-1640 in place of the mitogen (control wells). The cells were then cultured for 48 h at 37°C in a 5%

humidified CO_2-air atmosphere, before being pulsed for 18 h with 0.5 mCi methyl-^{3}H-thymidine (Amersham Biosciences, USA) per well. Each plate was then harvested onto a 96-well glass fibre mat using a Tomtek cell harvester 96 (Hamden, USA) and counted using a Wallac MicroBeta Trilux 1450 liquid scintillation and luminescence counter (PerkinElmer Life Sciences, USA). Stimulation index was calculated as counts per minute (cpm) in wells with mitogen divided by cpm in wells without mitogen.

3.4.7. Analysis of cytokines

In all, 2 ml of the spleen lymphocyte cell suspensions (4×10^6 cells) were added to each well of a 24-well plate (Costar, USA) and cultured in the presence and absence of ConA (1 mg/ml; Sigma, NZ) for 48 h at 37°C. Cell-free supernatant fractions were harvested and stored at −20°C until assayed. The presence of interferon-γ (IFNγ) and interleukin-4 (IL-4) in the culture supernatants was determined by sandwich ELISA using IFNγ and IL-4 cytokine DuoSet ELISA kits (R&D systems, DuoSet®, UK) according to the manufacturer's instruction.

3.4.8. Quantitative analysis of IgA and IgG

A sandwich rat IgA and IgG ELISA was used to quantify the IgA and IgG concentrations in plasma and intestinal digesta (jejunum and ileum); all antibodies were obtained from AbD Serotec, Oxford, UK. Briefly, for IgA quantitation, an ELISA plate was coated with mouse anti-rat IgA antibody (PRP01), and incubated for 1 h at room temperature. After washing, purified rat IgA kappa standards (MCA191) or suitably diluted samples were added to designated wells. Following further washings, horseradish peroxidase-conjugated mouse anti-rat IgA antibody (STAR111P) was added to each well as a detection antibody. After an incubation period, TMB enzyme substrate (3,3',5,5'-Tetramethyl-Benzidine) was added to each well. After 20 min, the reaction was stopped with 2 M H_2SO_4 and the absorbance was determined at 450 nm (OD_{450nm}). The ELISA procedure for IgG was similar to that described for IgA quantitation, using purified rat IgG standards (PRP12), but the antibodies used were goat anti-rat IgG (STAR71) and horseradish peroxidase-conjugated sheep anti-rat IgG (AARP10P).

3.4.9. Statistical analysis

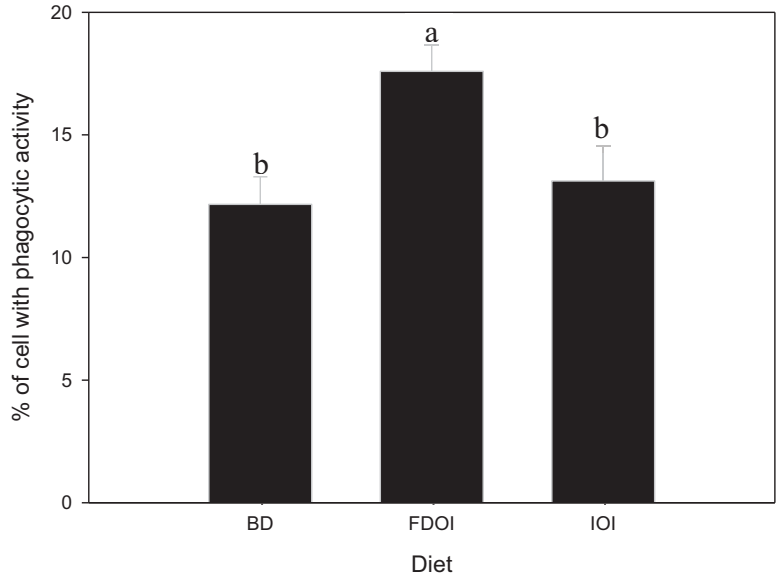
Results are expressed as mean ± SEM. Data were analysed using a one-way ANOVA using the GLM procedure of SAS/PROC GLM (SAS, version 9.1, SAS Institute Inc., Cary, NC, USA) and means were compared using Tukey's test. Statistical significance was accepted at $P < 0.05$.

3.5. Results

3.5.1. PBL phagocytosis

The phagocytic activities of PBLs from rats fed BD, FDOI and IOI diets for 3 weeks are shown in Figure 3.1. Levels of phagocytic activity were greater ($P = 0.002$) in rats receiving the FDOI diet than in rats fed either the IOI or the BD diet. There was no difference ($P > 0.05$) between rats fed the BD and IOI-supplemented diets.

Figure 3.1 Phagocytic activity of peripheral blood leukocytes in rats fed a diet containing ovine Ig for 21 days[1].



BD=basal diet; FDOI=freeze-dried ovine immunoglobulin; IOI=inactivated ovine immunoglobulin. [1]Values are means ± S.E. (n=15); [a,b]Means superscripts without a common alphabet differ, $P < 0.05$.

3.5.2. Lymphocyte proliferation

ConA-induced lymphocyte proliferative responses (stimulation index) of spleen cells in rats fed the FDOI diet were greater (P = 0.024) when compared to rats fed either the BD or the IOI diet (Table 3.2). Feeding the rats with different diets (BD, FDOI and IOI) did not result in any difference (P > 0.05) in spleen lymphocyte proliferative responses to LPS or PHA.

Table 3.2 Spleen lymphocyte proliferative responses (stimulation index) to ConA, LPS and PHA for rats fed a diet containing ovine Ig for 21 days[1].

	Diet				
	BD	**FDOI**	**IOI**	**SEM**	***P* Value**
ConA	6.12[b]	41.23[a]	12.80[b]	4.79	0.024
LPS	1.84	10.50	3.41	3.11	0.114
PHA	2.02	9.09	3.63	0.35	0.215

ConA=concanavalin A; LPS=lipopolysaccharide; PHA=phytohemagglutin. Ig=immunoglobulin; BD=basal diet; FDOI=freeze-dried ovine immunoglobulin; IOI=inactivated ovine immunoglobulin. [1]Values are means ± SEM. (n=15), means in a row with superscripts without a common alphabet differ, P < 0.05.

3.5.3. Cytokine analysis

IFNγ and IL-4 productions in ConA stimulated and unstimulated (*in vitro*) spleen cell cultures are shown in Table 3.3. In stimulated spleen cells, IFNγ was higher (P = 0.008) in rats fed the FDOI diet than in rats fed the BD diet. The level of IL-4 production was not affected by the diets (BD, FDOI and IOI). In unstimulated spleen cells, IFNγ production was not affected (P > 0.05) by the dietary intake of BD, FDOI and IOI, but the IL-4 concentrations were found to be higher (P = 0.044) in rats fed the FDOI diet than in rats receiving the BD diet.

3.5.1. Rat IgA and IgG concentrations in intestinal digesta and plasma

The IgA concentrations in the jejunal digesta were higher (P = 0.037) in rats receiving the FDOI diet than in rats fed the IOI diet. In the ileal digesta and plasma, IgA concentrations were higher (P = 0.05 and P = 0.041, respectively) in rats fed the FDOI diet than in rats receiving the BD diet (Table 3.4).

The IgG concentrations in the jejunal digesta were higher (P = 0.037) in rats receiving the FDOI diet than in rats fed either the BD or the IOI diet. Ileal IgG concentrations were not affected (P > 0.05) by the dietary consumption of BD, FDOI and IOI. Plasma IgG levels were higher (P = 0.025) in rats receiving either the BD or the IOI diet than in rats fed the FDOI diet (Table 3.4).

Table 3.3 IFNγ and IL-4 production (pg/ml) by spleen lymphocyte cells from rats fed a diet containing ovine Ig for 21 days[1] with and without ConA stimulation.

	Diet			SEM	*P* Value
	BD	**FDOI**	**IOI**		
	ConA stimulated				
IFNγ	1022.51[b]	1460.60[a]	1179.32[ab]	18.84	0.008
IL-4	4.43	4.48	5.03	0.22	0.977
	ConA unstimulated				
IFNγ	23.90	97.10	75.90	13.71	0.251
IL-4	2.18[b]	4.42[a]	3.09[ab]	0.18	0.044

IFNγ=interferon-γ; IL interleukin; Ig=immunoglobulin; ConA=concanavalin A; BD=Basal diet; FDOI=Freeze-dried ovine immunoglobulin; IOI=Inactivated ovine immunoglobulin. [1]Values are means ± SEM. (n=15), means in a row with superscripts without a common alphabet differ, P < 0.05.

3.6. Discussion

Phagocytic cells (polymorphonuclear leukocytes, monocytes and macrophages) and natural killer cells are the major effectors of innate immunity. These are considered as the first line of defence against foreign pathogens *(Watzl et al., 2005*). Oral administration of bovine colostrum is known to stimulate host non-specific immunity by increasing the systemic immune response or by modulating immunocompetent cell function (*Sugisawa et al., 2001*). In this study, the phagocytic activity of PBLs was enhanced in rats fed the FDOI-supplemented diet compared to rats fed either the BD or the IOI diet. Therefore, I can suggest that feeding the rats with FDOI may improve their innate immunity via enhancement of phagocytic activity of PBLs.

Table 3.4 IgA and IgG concentrations of intestinal digesta and plasma for rats fed a diet containing ovine Ig for 21 days[1].

	Diet			SEM	*P* Value
	BD	**FDOI**	**IOI**		
	Jejunum (μg/ml)				
IgA	1278.28^{ab}	2047.33^{a}	999.16^{b}	29.30	0.037
IgG	177.50^{b}	254.51^{a}	193.02^{b}	7.78	0.037
	Ileum (μg/ml)				
IgA	3156.32^{b}	4063.20^{a}	3576.99^{ab}	27.64	0.050
IgG	149.51	178.34	166.12	6.49	0.416
	Plasma (mg/ml)				
IgA	1.14^{b}	1.58^{a}	1.40^{ab}	0.04	0.041
IgG	2.17^{a}	1.01^{b}	2.15^{a}	0.01	0.025

BD=basal diet; FDOI=freeze-dried ovine immunoglobulin; IOI=inactivated ovine immunoglobulin. [1]Values are means ± SEM. (*n*=15), means in a row with superscripts without a common alphabet differ, $P < 0.05$.

Further study is required to determine a definitive mechanism of action. However, it is possible that it may be similar to that proposed by Rutherfurd-Markwick *et al.* (2005) who reported that mice fed whey protein concentrate (WPC) exhibited significant increases in both phagocytosis by blood leukocytes and ConA-induced proliferation of spleen lymphocytes, compared to those from mice receiving a milk protein-free diet. They suggested that constituent cysteine residues of WPCs may provide a substrate for glutathione synthesis and that this serves to potentiate antioxidant activity and membrane function of immune system cells sufficient to promote increased cellular activity (*Bounous et al., 1989*).

Lymphocyte proliferative responses to mitogens (i.e. stimulation index) are widely used to ascertain T (ConA is a T-cell mitogen) and B (LPS is a B-cell mitogen)-cell function (*Gill et al., 1992*). In this study, rats fed the FDOI diet had a higher stimulation index in ConA-stimulated spleen lymphocytes compared to rats receiving the BD and IOI diets. This result may indicate that the FDOI diet enhanced T-cell function.

Naive T cells recognize an antigen using the major histocompatibility

complex molecules via the T-cell receptor complex, and can be developed into three major subsets: Th1, Th2 and Treg/Th3 based on the pattern of cytokines they secrete. The key Th1 cytokines such as IFNγ, IL-2, IL-3, IL-12 and TNFα are involved predominantly in cell-mediated immune response and confer immunity against harmful intracellular agents such as bacteria, viruses and tumour cells, and also downregulate intestinal IgA concentrations. They also activate and differentiate T and B lymphocytes as well as macrophages, whereas cytokines such as IL-4, IL-5 and IL-6, which are secreted by Th2 cells, confer humoral responses, activate B lymphocytes, upregulate mucosal immunity and mediate responses against extracellular parasites, as well as upregulating IgA concentration. Treg/Th3 cells secrete IL-10 and TGFβ cytokines. A balance between Th1 and Th2 cytokines may be necessary to maintain a normal IgA immune response (*Kramer et al., 1995; Reiner & Seder, 1995; DiPiro, 1997*).

In this study, since the spleen lymphocytes from the rats fed the FDOI diet showed a greater stimulation index in the presence of ConA, we analysed cell culture supernatants for their cytokine pattern (IFNγ and IL-4) to ascertain T-cell polarity. In FDOI-fed rats, the pattern of cytokine expression indicates that there might have been a selective activation of Th1 cells in the presence of ConA and selective activation of Th2 cells (to a lesser extent than Th1 cells) in the absence of the mitogens. A balance between Th1 and Th2 cytokines is necessary for normal immune function because if the diet influences persistent Th1 polarity, it may lead to cell-mediated immunity and cytotoxic T-lymphocyte induction, resulting in inflammatory responses, whereas persistent Th2 polarity leads to more IgE production, resulting in allergy and hypersensitivity reactions (*Thyphronitis et al., 1989*). In this study, I found no significant differences in the plasma IgE levels of rats fed BD, FDOI and IOI (data not shown), suggesting that the rats fed the FDOI diet had not developed any allergic type reaction. Our findings are similar to that of Boudry *et al.* (2007) who reported an immunomodulatory effect of bovine colostrum on the gut associated lymphoid tissue of weaned piglets, which responded by producing both Th1 cytokines and Th2 cytokines at different levels. They concluded that Th1/Th2 bipolar response protects the piglets from both allergic (food) and infectious (pathogens) diseases.

When Ig present in the FDOI diet is delivered orally, the gut-associated lymphoid tissue may be its primary target, resulting in stimulation of IgA secretion in the intestine. The immunological defence against bacteria and various potential pathogens in the gut is carried out by intestinal IgA in a process known as immune exclusion where intestinal IgA binds to the bacteria, pathogen or its antigen, thereby preventing their translocation (*Amin et al., 2007*). Rats consuming the FDOI diet in this study had greater amounts of secretory IgA (sIgA) in both jejunal and ileal digesta that might play a major role in protecting the intestine from invading pathogens (*Mayer, 1997*) and suppressing inflammatory processes (*Parlesak et al., 2002*). Rats fed the FDOI diet also had greater amounts of jejunal IgG. Taken together, the elevated amounts of intestinal digesta IgA and IgG of rats fed the FDOI diet indicate that the animals receiving this diet may be better able to preserve a functional (intact and absorptive) gut compared to rats fed the other diets, by preventing the adverse effects of possible pathogens in the GIT. This is in line with our previous observations that villus length, crypt depth, villus/crypt ratio and villus surface area of the small intestine were greater in rats receiving the FDOI diet than in rats fed either the BD or the IOI diet (*Chapter-2, Section-2.4.1.3*). Because of the intact and absorptive gut (without subclinical infection), the FDOI-fed rats responded favourably with a better G:F ratio (*Chapter-2, Section-2.4.1.1*), which is consistent with results reported from other studies (*Coffey & Cromwell, 1995; Pierce et al., 2005*) and support the earlier suggestion by Pierce *et al*. (2005) that higher growth performance with SDAP is due to its Ig content.

Plasma IgA plays an important role in inhibiting allergen absorption (*Benyacoub et al., 2003*). In this study, plasma IgA concentrations were also greater in rats fed the FDOI diet than in rats fed the BD diet. This is similar to the results from a study (*Puri et al., 1996*) in which diets supplemented with *Enterococcus faecium* (SF68) stimulated immune function in dogs by increasing the concentrations of intestinal and plasma IgA. Intestinal IgA, produced by B lymphocytes in the intestinal cells, may cross the mucosal barrier and enter the bloodstream influencing the plasma IgA concentration (*Puri et al., 1996*).

In this study, rats fed the FDOI diet had lower plasma IgG levels when compared to rats fed the BD and IOI diets. A possible explanation for this could be that the ovine Ig present in the FDOI may remove pathogens or

microorganisms in the GIT, resulting in less pathogenic antigens being transported across the gut to blood vessels resulting in a lower production of rat plasma IgG. Torrallardona, (2010) showed that serum contains antibodies against pathogenic bacteria. Therefore serum Ig may provide passive antimicrobial protection (and also from subclinical infection) by exclusion of opportunistic pathogens and also help in preventing mucosal damage (*Chapter-2, Section-2.4.1.3*). The gut is a battleground for beneficial and harmful microbes and toxins. If pathogens are suppressed by Ig there may be a possibility for the growth of beneficial microbes. In our previous study, we found that *Lactobacillu*s species were up regulated in ileal and colonic digesta in the rats fed the FDOI diet when compared to the rats fed the BD diet (*Chapter-4, Section-4.4*). Livingston *et al.* (2009) reported that the percentage of Foxp3+ T cells (Forkhead box P3 regulatory T cells) from mice colonized by *Lactobacillus* species was found to be significantly higher than that from *Lactobacillus*-free mice in both mesenteric lymph nodes and spleen cell populations, clearly showing that mice colonized with *Lactobacillus* species developed more tolerance to commensal (where the body does not mount an immune response to gut commensal bacteria) of the immune system in gut-associated lymphoid tissue than mice without *Lactobacillus*. This information suggests that rats fed the FDOI diet may develop tolerance by upregulation of the *Lactobacillus* species in the gut.

Ig are at least partially resistant to digestion in the upper GIT. Rodriguez *et al*. (*2007*) recently reported that porcine Ig partially resisted the digestion process in the GIT of adult dogs and cats fed diets containing SDAP, or concentrated Ig derived from pig blood. Indeed, reports have shown that human serum Ig and bovine Ig resist digestion in the upper GIT of humans and retain toxin binding and neutralizing activity (*Losonsky et al., 1985; Warny et al., 1999*). Given that circular dichroism analysis (data not shown) confirmed that the Ig in the IOI treatment was inactivated, our results suggest that active (undenatured) Ig in the FDOI diet was the active ingredient responsible for the observed immune responses.

In conclusion, orally administered FDOI selectively modulated immune function in the growing rat through increasing phagocytic activity of PBLs and lymphocyte proliferation, and by influencing the balance between Th1 and Th2 cytokine productions, and the sIgA and sIgG in the intestinal contents and

plasma. Altogether, these effects may result in the animal having increased resistance to infection. To our knowledge, this is the first report of the immunomodulatory effects of dietary ovine serum Ig in an animal species.

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Chapter 4

Dietary supplementation with ovine serum immunoglobulins results in enrichment of *Lactobacillus johnsonii* in the growing rat

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The results in Chapter-2 showed that in growing rats, orally fed FDOI supported growth. In Chapter-3, selective modulation of the immune system (increasing phagocytic activity of PBLs and lymphocyte proliferation, secretory IgA and IgG in the intestinal contents and plasma) was shown. Recently, our own group have demonstrated the anti pathogenic activity (*in vitro*) of ovine Ig concentrate, but there are no reports regarding the effects of plasma Ig on the mammalian microbiota. Therefore, the aim of this Chapter was to ascertain whether oral administration of ovine serum Ig modulates the gut microbial population in the growing rat.

4.1. Abstract

This study aimed to investigate whether orally administered ovine serum immunoglobulins (Ig) modulate the gut microbiota in the growing rat. Thirty Sprague-Dawley male rats were used in a 21 day study and were fed either a basal control diet (BD; no Ig) or a diet containing freeze-dried ovine Ig (FDOI). Ileal and colonic digesta samples were collected for isolation of bacterial DNA. DGGE profiles and DNA sequence analysis suggested that the FDOI-fed rats selectively enriched for *Lactobacillus* sp. and the increment of *L. johnsonii* was confirmed by qPCR. The finding suggests that *Lactobacillus* sp. such as *L. johnsonii* in the gut can be up-regulated by feeding ovine Ig.

4.2. Introduction

Orally administered Ig present in spray dried animal plasma (SDAP) have been shown to confer multiple benefits in terms of health and growth in farm animals (*Gatnau et al., 1986; Pierce et al., 2005*) and these effects appear to be greater under conditions of pathogen challenge (*Coffey & Cromwell, 1995*). A reduction in the degree of adhesion of pathogenic bacteria to the gut mucosa may be involved (*Bosi et al., 2004*).

There are several reports in the literature whereby Ig have been shown to target specific pathogenic bacteria. For instance, children with necrotizing enterocolitis were treated prophylactically with human Ig (*Eibl et al., 1993*). Ig have also been used therapeutically for intervention of *Campylobacter jejuni* (*Hammarstrom et al., 1993*), *Clostridium difficile* (*Tjellstrom et al., 1993*), *Helicobacter pylori*, rotavirus and cryptosporidial infection (*Bogstedt et al., 1996*). Ig concentrate reduced the negative effects of *Staphylococcus aureus* enterotoxin B (SEB) in rats (*Pérez-Bosque et al., 2008*) and increased the number of lactobacilli in the ileal and caecal contents of piglets fed a diet containing SDAP (*Torrallardona et al., 2003*). Recently, our own group in an *in vitro* study reported that ovine serum Ig exerted inhibitory activity and binding activity to gram-positive and gram-negative pathogens as well as their LPS and enterotoxin (*Han et al., 2009*).

Given the reported effects of Ig on specific pathogenic bacteria in the gut, it is possible that the feeding of Ig may also influence the commensal bacteria in the digestive tract. There are, however, no reports on the effects of ovine serum Ig on the gut microbiota. We used PCR-DGGE and qPCR to ascertain whether short term (21 days) oral administration of ovine serum Ig modulates the gut microbial population in the growing rat.

4.3. Materials and Methods

4.3.1. Preparation and quantitation of ovine serum Ig

An ovine serum Ig fraction was prepared according to the procedures described in an earlier study, using an ammonium sulphate precipitation method (*Chapter-2*). The fraction was freeze-dried and the IgG content in the ovine Ig fraction was

analyzed by direct ELISA using a rabbit anti sheep IgG (AbD Serotec, UK) according to the earlier reported method (*Chapter-2, Section-2.3.1*). The ELISA was performed in quadruplicate for each sample and the data were averaged. The respective protein and Ig concentrations of the FDOI were 94% and 73%.

4.3.2. Animal study

This study was approved by the Massey University Animal Ethics Committee (MUAEC 06/132) and procedures complied with the New Zealand Code of Recommendations and Minimum Standards for the Care and Use of Animals for Scientific purposes (New Zealand Animal Welfare Advisory Committee, 1995). Thirty Sprague-Dawley male rats (animals reported in Chapter-2, 5 weeks-old, 140 to 160 g body weight) were housed singly in stainless steel cages with free access to water in a room maintained at 22±2°C with a 12 h light/dark cycle.

The rats were given 1 week to acclimatize to their surroundings during which time they consumed a basal diet *ad libitum*. After acclimatization, the rats were randomly allocated to two diets (*refer Chapter-3, Table-3.1*, *n*=15 per diet): a basal diet (BD) and a test diet containing FDOI, and underwent a 3-wk study. The diets were formulated, with methionine plus cysteine as the first limiting amino acid, to meet or exceed National Research Council (*NRC, 1995*) recommendations for growing rats for the major nutrients (*refer Chapter-3, Table-3.1*). The FDOI fraction was included in the FDOI diet at 3.07%. The amount of ovine Ig included in the FDOI diet was equal to the amount of porcine IgG contributed by spray dried porcine plasma diets used in other studies (*Pierce et al., 2005; Chapter-2, Section-2.3.2*). Food was given in a powdered form *ad libitum*.

4.3.3. Chemical analysis

Dietary dry matter, ash, crude protein, total fat and gross energy were determined as described previously (*Chapter-2, Section-2.3.3*).

4.3.4. Post-mortem procedure

At day 21 of the experiment, the rats were anaesthetized by an intraperitoneal injection. Rats were then killed by exsanguination. The abdomen was opened

and the entire digestive tract was dissected, and the ileum and colon were isolated. The ileum (anterior to the ileocaecal valve) was washed intraluminally with 5 ml of phosphate buffer saline (PBS) and digesta were collected. A similar procedure was followed for collecting digesta from the colon. The collected digesta were stored immediately at -80°C until bacterial DNA isolation.

4.3.5. Isolation of bacterial DNA from digesta and PCR-DGGE

The ileal and colonic digesta samples were thawed at 4°C and centrifuged at 10000 *g* for 30 min at 4°C. From the above samples, 0.4 g of pellet was used to extract the bacterial DNA using a QIA amp® DNA stool mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The PCR reactions were completed using HotStarTaq master mix (Qiagen, Germany). Individual samples and pooled samples (*n*=15) of each group were amplified using an iCycler® Thermal Cycler (Bio-rad, USA) with primers (10 pM each) specific for conserved sequences flanking the variable V7&8 region of 16S rDNA, as described previously (Muyzer *et al*., *1993*) (forward: U968-GC, 5' CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC 3', reverse: L1401-R, 5' CGG TGT GTA CAA GAC CC 3'). The underlined sequence in U968-GC corresponds to the GC clamp. The PCR reaction condition was: initial activation step at 95°C for 2 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 45 sec, extension at 72°C for 1min, and a final extension at 72°C for 10 min followed by cooling to 4°C. PCR products (430 to 470 bp) were stored at -20°C.

DGGE analysis of PCR products was performed using the DCode™ system (Bio-Rad) at 130 V for 6 h in a 1.25 X TAE buffer at a constant temperature of 60°C on 8% polyacrylamide gels. Optimal separation was achieved by a gradient of 22 to 55% denaturant (100% denaturant was defined as 7 M urea and 40% [v/v] formamide). The DNA bands were visualized by ethidium bromide staining (50 µg/ml) and then photographed under UV transillumination with the GelDoc™ XR 2000 (Bio-Rad, USA).

4.3.5.1. DGGE profile analysis

Diversity data base version 2003 (Phoretix software, Nonlinear dynamics Ltd,

Newcastle Upon Tyne, UK) was used to analyze the intensity and migration patterns of the DGGE gel profiles within each lane of a gel. The above information was used to measure and analyze the Shannon's diversity index.

4.3.5.2. Sequencing of DGGE gel bands

Bands of specific interest indicating different intensities between the two diets were excised from DGGE gels using a sterile scalpel, placed in 100 µl sterile water, and incubated at 4°C overnight for diffusion of DNA into water. The bands were reamplified and again run on the DGGE system to make sure the correct mobility of reamplified bands within the gels (Bourne *et al*., 2008). Five µl of diffused sample was amplified as a template DNA using HotStarTaq master mix (Qiagen, Germany) with primers U968/L1401-R without the GC clamp. PCR reaction was initially activated at 95°C for 15 min, followed by amplification as described above. A portion of the PCR product was run on a 1 % agarose gel to confirm product recovery. PCR product was then purified using Wizard® SV gel and PCR Clean-up system (Promega, USA), and cloned into the pGEM-T-easy vector (Promega, USA) according to manufacture's instructions.

The cloned (2 to 3 clones per sample) products (600ng of DNA template) were sequenced using T7-SP6 primers (6.4pmol), the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer Corp., Foster City, CA) and ABI310 Sequencer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. DNA sequencing was carried out by the Alan Wilson Centre Genome Service (Massey University, New Zealand). Analysis of nucleotide sequence data was completed using the GenBank database with the BLASTN search program (http://www.ncbi.nlm.nih.Gov/sut-ils/genom_table.cgi). Nucleotide sequence data of excised DGGE bands reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB544006 to AB544012 and in appendix A.

4.3.5.3. *L. johnsonii* preparation and DNA extraction

L. johnsonii ATCC 33200 was used for the standard curve preparation. Prior to use, *L. johnsonii* were subcultured three times in MRS broth at 37°C for 18 hours

and cells were concentrated by centrifugation at 4500 × *g* for 5 min. DNeasy tissue kit (QIAGEN, Hilden, Germany) was used for DNA extraction according to manufacturer's instruction. Briefly, bacterial cells (approximately 1 x 10^9 cells) were harvested in a microcentrifuge tube by centrifuging for 10 min at 5000 x *g*. Supernatant was then discarded. Bacterial pellet were then resuspended in 180 µl enzymatic lysis buffer and incubated for at least 30 min at 37°C. Then, 25 µl proteinase K and 200 µl Buffer AL (without ethanol) were added and mixed by vortexing and incubated at 56°C for 30 min. To this 200 µl ethanol (96–100%) was added, and mixed thoroughly by vortexing. This mixture was pipetted into the DNeasy Mini spin column placed in a 2 ml collection tube. The tubes (with the column) were then centrifuged at > 6000 x *g* for 1 min. Flow-through and collection tube were discarded. The DNeasy Mini spin column was placed in a new 2 ml collection tube, 500 µl Buffer AW1 was added, and centrifuged for 1 min at > 6000 x *g*. Same procedure was followed as above but instead of using Buffer AW1, 500 µl Buffer AW2 was added and centrifuged for 3 min at 20,000 x g to dry the DNeasy membrane. Finally, for eluting the DNA (after discarding flow-through and collection tube), the DNeasy Mini spin column were placed in a clean 2 ml microcentrifuge tube, and 100 µl Buffer AE was added directly onto the DNeasy membrane and incubated at room temperature for 1 min, and then centrifuged for 1 min at > 6000 x *g*. DNA concentrations were measured by absorbance using a NanoDrop® ND-1000 Spectrophotometer.

L. johnsonii ATCC 33200 has one 16S rRNA copy number and its genome size is 1772891 bp. This was converted into a mass per genome, using Avogadro's number Na (6.02214 × 10^{23} bp/mol) and an average molecular weight of a base pair M (609.5 g/mol): Mass of the DNA in ng = 10^9 × (1772891× M)/Na. The DNA ng/mg of digesta estimates were converted to estimate *L. johnsonii* cells per mg of digesta by dividing the estimated DNA mass by this genome mass.

4.3.5.4. Quantitative Real-Time PCR

Quantitative real-time PCR was performed using the LightCycler 480 system with the SYBR Green I master kit (Roche, USA). The primers for *L. johnsonii* were adapted from published specific primers: 5'- CAC TAG ACG CAT GTC TAG AG and 5'- AGT CTC TCA ACT CGG CTA TG (Furet *et al.*, 2004). The specificity of primers was then evaluated using the Probe Match tool on the Ribosomal Database Project website (http://rdp.cme.msu.edu). The reaction mixture consisted of 10 µl SYBR Green I master, 0.5 µM each primer and 2 µl DNA sample in a final volume of 20 µl. An initial DNA denaturation step at 95°C for 5 min was followed by 45 amplification cycles (95°C for 10 sec, 60°C for 25 sec and 72°C for 15 sec) and cooling at 4°C. The standard curve (appendix B) was constructed by plotting the *Cp* values against serial dilutions of DNA solutions isolated from *L. johnsonii* ATCC 33200 using a DNeasy Blood and Tissue kit (Qiagen, Germany).

4.3.6. Statistical analysis

Results were given as means ± SE. Data were analyzed using the SAS/ PROC TTEST (SAS, version 9.1, SAS Institute Inc, Cary, NC). Statistical significance was accepted at $P < 0.05$.

4.4. Results

4.4.1. Bacterial communities in ileal digesta

Shannon's diversity index was found to be significantly higher ($P = 0.005$) for the rats fed the FDOI diet than for rats fed the BD diet for ileal digesta. Two prominent DGGE bands (IL-1 and IL-2) obtained from a pooled (n=15) ileal digesta sample of rats fed the FDOI diet were isolated and sequenced (Table 4.1 and Figure 4.1A). Band IL-1 had a DNA sequence with 99% similarity to the 16S rDNA V3 region of *L. johnsonii* strain NCC and band IL-2 had similarity of 99% to *L. johnsonii* MH21. To confirm the abundance of *L. johnsonii* in ileal digesta, the cell numbers of this strain were determined by qPCR and were found to be significantly higher ($P < 0.05$) in the FDOI group when compared to the BD group

(Figure 4.2).

Table 4.1 Identified bacterial species from DNA sequencing of the PCR-DGGE bands shown in Figure 4.1.

Sample ID	Diet	Nearest Neighbour	Similarity with nearest neighbour	Accession no. of nearest neighbour
IL-1	FDOI	*L. johnsonii* strain NCC	99%	HQ828141.1
IL-2	FDOI	*L. johnsonii* strain MH21	99%	FJ542292.1
CBD1	BD	*Parasutterella excrementihominis*	98%	AB370250.1
CFD2	FDOI	*Lactobacillus* sp. BL304	99%	FJ557009.1
CFD3	FDOI	*L. johnsonii* strain NCC	99%	HQ828141.1
CFD4	FDOI	*L. johnsonii* DPC 6026	99%	CP002464.1
CFD5	FDOI	Uncultured bacterium clone RL199_aaj42f04	99%	DQ793351.1

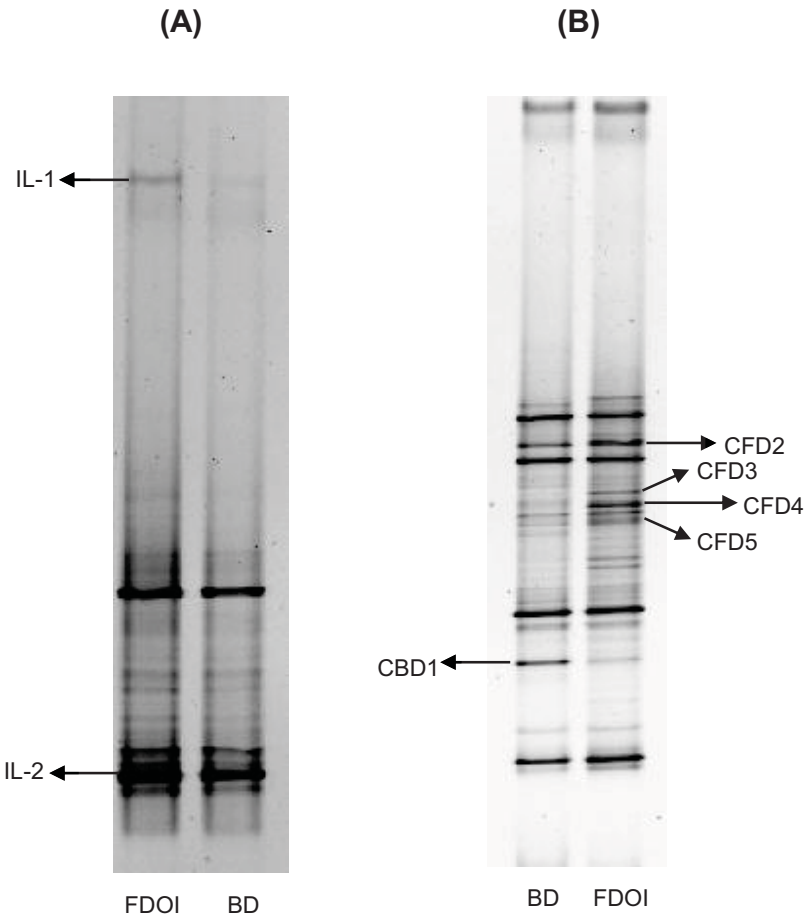
BD, Basal diet; FDOI, Freeze-dried ovine immunoglobulin.

4.4.2. Bacterial communities in colonic digesta

There was no significant ($P > 0.05$) difference for Shannon's diversity index between the rats fed the FDOI or BD diets. One prominent band (CBD1 from the BD diet group) and four bands (CFD2, CFD3, CFD4 and CFD5) from the FDOI diet group were obtained from a pooled (n=15) colonic digesta sample, isolated and sequenced (Table 4.1 and Figure 4.1B). Band CBD1 had a DNA sequence with 99% similarity to *Parasutterella excrementihominis*; CFD2, CFD3 and CFD4 had similarities up to 99% to *Lactobacillus* sp. BL304, *L. johnsonii* NCC and *L. johnsonii* DPC 6026, respectively. CFD5 had a similarity of 99% to uncultured bacterium clone RL199_aaj42f04 (next close relative to *Clostridiaceae* bacterium of 94% sequence similarity from cultured bacteria). The numbers of *L. johnsonii*

species determined by qPCR in colonic digesta were significantly higher ($P < 0.05$) in the FDOI group when compared to the BD group (Figure 4.2).

Figure 4.1 Lanes showing DGGE bands from pooled DNA samples (*n*=15) of ileal (A) and colonic (B) digesta used for sequencing for the identification of bacteria in rats fed a basal diet or a diet containing ovine Ig for 21 days[1].



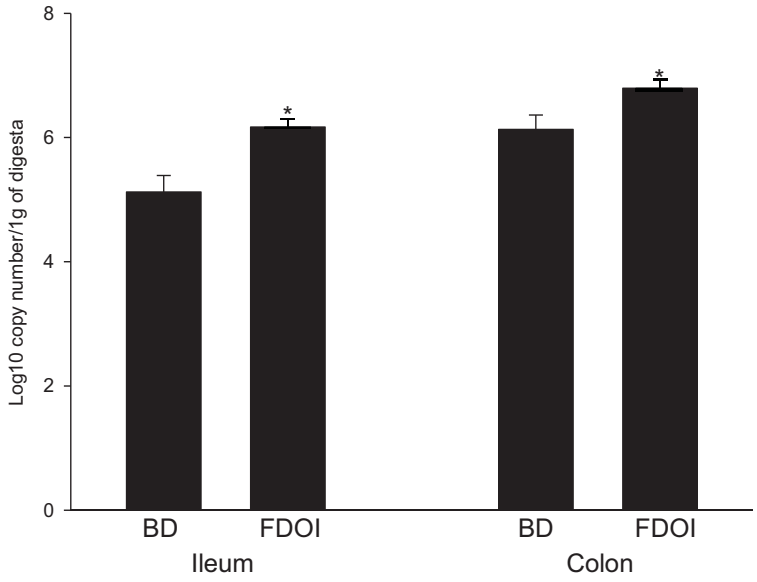
[1]BD, Basal diet; FDOI, Freeze-dried ovine immunoglobulin. Bands were selected for sequencing only when they were showing an obvious difference between the diets

4.5. Discussion

A diverse microbiota resides in the mammalian gut and influences the physiology, biochemistry and immunology of the host (*Hooper et al., 2001*). The vital role of the GIT microbiota in both the health and disease of the host has

been increasingly documented. It is important to develop a better understanding of the microbiota and factors that affect its composition. The limitations of traditional methods for studying the composition of the intestinal microbiota can be overcome by applying various molecular techniques such as PCR-DGGE, 16s rDNA sequencing and qPCR (*Muyzer et al., 1993*).

Figure 4.2 Quantification of *L. johnsonii* strains using quantitative real-time PCR in ileal and colonic digesta of each diet group[1].



*, $P < 0.05$. BD, Basal diet; FDOI, Freeze-dried ovine immunoglobulin. [1]Data presented are mean values (± SE, n=15) of duplicated real-time PCR analyses of individual DNA extracts obtained from each rat.

Gut biological diversity can be analyzed in different ways. The two main factors to take into consideration are evenness and richness. Evenness reflects the individual distribution of each bacterial species in the ecosystem and richness reflects the number of different kinds of organisms present in a sample. The diversity of species affects a number of processes in ecological communities, including productivity, stability, and susceptibility to invasive species. As the evenness and richness of species increase, so the diversity increases and this might stabilize the ecosystem (*Hooper et al., 2005*). A greater degree of species richness in the gut microbiota is related to a decreased ability of pathogens to

colonize the gut (*Dillon et al., 2005*) because bacteria in the GIT compete with each other for nutrients and colonization sites.

In the present work, the inclusion of an FDOI fraction in the diet led to a statistically significant increase in Shannon's diversity index (0.27 for BD group and 0.41 for FDOI group) on the DGGE gel in ileal digesta, reflecting an increase in microbial diversity. However, a comparable effect of diet at the colonic level was not observed. The apparent effect of the FDOI was more evident in the less complex ileal microbial population.

Ig is at least partially resistant to digestion in the upper GIT. Rodriguez *et al*. (2007) recently reported that porcine Ig partially resisted digestion in the GIT of adult dogs and cats fed diets containing spray dried animal plasma or concentrated Ig derived from pig blood. The Fab fragment, which is released after pepsin digestion (*Gorevic et al., 1985*), retains the capacity to bind bacteria by neutralization and opsonisation as it contains the antigen binding sites.

The DNA sequencing of DGGE bands and quantification using real-time PCR from the ileal or colonic digesta of rats fed the FDOI diet demonstrated a relative increase of bacteria such as *L. johnsonii* part of the probiotic lactic acid bacteria group (*Liévin-Le Moal et al., 2006*; *Makras et al., 2006*; *Inoue et al., 2007*; *Anwar et al., 2008*; *Denou et al., 2008*). *L. johnsonii*, was previously classified in the *L. acidophilus* group (*Fujisawa et al., 1992*). *L. johnsonii* has been extensively studied for its clinical and nutritional health benefits (*Haller et al., 2000*; *Kaburagi et al., 2006; Liévin-Le Moal et al. 2006*). Mice fed *L. johnsonii* have been shown to have increased levels of intestinal IgA, serum IgA and splenocyte proliferation (*Brassart & Schiffrin, 1997*). This is in line with other work from our group (*Chapter-3*) which showed that orally administered FDOI selectively modulated immune function in the growing rat by increasing phagocytic activity of PBL and spleen lymphocyte proliferation, and by increasing cytokine production and the secretory IgA and IgG in the intestinal contents and plasma. It is possible that an increase in the number of bacteria such as *L. johnsonii* consequent upon administration of ovine serum Ig may positively modulate the diverse indices of immunity in animals resulting in increased resistance to infection.

In conclusion, feeding rats the FDOI for 21 days had an effect on the microbial composition of ileal digesta. This observation is of specific interest

since orally administered ovine Ig might help to establish an intestinal environment that improves beneficial bacteria overgrowth. To our knowledge, this is the first time a direct effect of ovine serum Ig on the composition of gut microbiota has been reported.

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Chapter 5

Dietary supplementation with ovine serum immunoglobulins is associated with increased gut mucin secretion in the growing rat

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The findings of Chapter-2 and -3 clearly demonstrate that ovine Ig improved growth and immunity in the growing rat. The results in Chapter-4 show that ingestion of an ovine Ig fraction selectively enriched *Lactobacillus* sp. such as *L. johnsonii* in the gut of the growing rat. If there is a change in the immunity and gut microbial population, there may also be a change in gut mucin as these are interlinked. The following study, therefore, investigated whether orally administered ovine serum Ig affects gut mucin in the growing rat.

5.1. Abstract

The aim was to determine whether orally administered ovine serum immunoglobulins (Ig) influence goblet cell count, mucin gene expression and digesta mucin protein content in the gastrointestinal tract of the growing rat. Thirty Sprague-Dawley male rats were used in a 21 day study and were fed a control diet (BD; no Ig) and a test diet containing freeze-dried ovine Ig (FDOI). When compared to the BD diet, rats consuming the FDOI diet had significantly ($P < 0.05$) more intact and cavitated goblet cells in villi throughout the small intestine. A similar result was found for crypt goblet cells in the small intestine and colon. Muc5Ac mRNA expression in the stomach was significantly ($P < 0.05$) higher for rats fed the FDOI diet when compared to rats fed the BD diet. Muc2, Muc3 and Muc4 mRNA expression in the ileum of the FDOI diet group were significantly higher ($P < 0.05$) than for the BD diet group. Mucin protein content was found to be significantly ($P < 0.05$) higher in the stomach, ileal and colonic digesta of rats fed the FDOI diet when compared to rats fed the BD diet. In conclusion, orally administered FDOI influenced the transcription and translation of gut mucin protein in the growing rat as evidenced by increased mucin gene expression and digesta mucin protein concentrations as well as an increased goblet cell count.

5.2. Introduction

The mucosal surface of the gastrointestinal tract (GIT) is made up of a complex organization of epithelium, immune cells and resident microflora (*McCracken & Lorenz, 2001*). Gut epithelial cells are continually exposed to numerous macromolecules including chemical irritants, digested foods, toxins, resident bacteria, intestinal pathogens and their products (*Moncada & Chadee, 2002*). The mucus coat separates and defends mucosal cells from the exterior environment, allows lubrication of the cell surface, and regulates ion fluxes (*Forstner & Forstner, 1994*). Mucus plays a vital role in intestinal surface integrity, and malfunction of mucus secretion and/or mucin expression may result in several pathologies such as inflammatory intestinal diseases and cancer (*Ho et al., 1993*). Mucin producing goblet cells along with antimicrobial peptides, luminal Ig, lysozymes and resident microflora collectively contribute to innate immunity and provide the front line of defence against pathogens.

Mucins are the high molecular weight glycoproteins secreted by goblet cells which are found in the columnar epithelium (*Herrmann et al., 1999*). They act as the main structural component of the mucus layer, giving rise to its polymeric, viscoelastic and protective properties (*Laboisse et al., 1996*). A well established function of mucins is their role in preventing entry of enteric pathogens via the gut. As mucins play an important role in protecting the underlying epithelium, any quantitative alteration in mucus secretion may change this defensive barrier and have important physiological implications.

Mucins can be divided into three distinct subfamilies: secreted gel-forming mucins, cell-surface mucins and secreted non-gel-forming mucins (*Linden et al., 2008*). Secreted mucins facilitate the formation of the mucus gel but the function of membrane-bound mucins is not well characterized, even though they are located on the surface of epithelial cells throughout the body.

Gastrointestinal diseases and stress modify the mucus layer both quantitatively and qualitatively. Crohn's disease, colitis and gastric carcinogenesis associated with *Helicobacter pylori* alter mucin secretion and reduce the number of goblet cells which leads to degradation of the mucus layer (*Corfield et al., 2000; Babu et al., 2006*). To date, 21 different mucin genes have been identified, cloned and partially sequenced in humans (*Dekker et al., 2002*),

and most of their homologues have been identified in mice and rats (*Perez-Vilar et al., 1999*).

A number of reports have been published regarding the use of dried animal plasma in the post-weaning diet of animals with enhanced growth performance and reduced levels of gastrointestinal infection being reported (*Gatnau et al., 1989; Torrallardona et al., 2003; Pierce et al., 2005*). Animal plasma seems to be effective during pathogen challenge (*Coffey & Cromwell, 1995*). Ig present in plasma has been found to bind to pathogenic bacteria or to antigens in the gut lumen thereby preventing attachment to the mucosa (*Bosi et al., 2004*). Human Ig has also been used as a prophylactic treatment for children born prematurely with necrotizing enterocolitis (*Eibl et al., 1988*).

Recently, we have reported that feeding a freeze-dried ovine serum Ig preparation selectively improved growth performance, the weight of some digestive organs and gut morphology in the growing rat (*Chapter-2*), preserved a higher degree of immunological activity (*Chapter-3*) and selectively altered the composition of the gut microbiota in growing rats (*Chapter-4*). However, there are no reports in the literature regarding the effects of ovine Ig on gut mucin secretion in animals. The objective, therefore, was to investigate whether orally administered ovine serum Ig affects gut mucin in the growing rat. Comparison of goblet cell count, mucin gene expression and digesta mucin concentrations was made between a casein-based control diet (BD) and a similar test diet but supplemented with freeze-dried ovine Ig (FDOI).

5.3. Materials and methods

5.3.1. Preparation and quantitation of ovine serum Ig

An ovine serum Ig fraction was prepared according to the procedures described in an earlier study, using an ammonium sulphate precipitation method (*Han et al., 2009*). The IgG content in the ovine Ig fraction was determined in quadruplicate by direct ELISA using a rabbit anti sheep IgG (AbD Serotec) as described in Chapter-2 (*Section-2.3.1*). The respective protein and Ig concentrations of the FDOI were 94% and 73%.

5.3.2. Animal study

This study was approved by the Massey University Animal Ethics Committee (MUAEC 06/132) and procedures complied with the New Zealand Code of Recommendations and Minimum Standards for the Care and Use of Animals for Scientific purposes (New Zealand Animal Welfare Advisory Committee, 1995). Fourteen Sprague-Dawley male rats male rats (animals reported in Chapter-2, 140 to 160 g body weight) were housed singly in wire-bottomed stainless steel cages and had free access to water in a room maintained at 22±2°C with a 12 h light/dark cycle.

The rats were given 1 wk to acclimatize to their surroundings during which time they consumed a basal diet *ad libitum*. After acclimatization, the rats were randomly allocated to two diets (*refer Chapter-3, Table-3.1*, *n*=7 per diet) including a control diet (BD) and a test diet (FDOI) and underwent a 3-wk study.

The diets were formulated with methionine plus cysteine as the first limiting amino acid to meet or exceed National Research Council (*NRC, 1995*) recommendations for growing rats for the major nutrients (*refer Chapter-3, Table-3.1*). The freeze-dried ovine Ig fraction was included in the FDOI diet at 3.07%. The amount of ovine Ig included in the FDOI diet was equal to the amount of porcine IgG contributed by spray dried porcine plasma diets used in other studies (*Pierce et al., 2005; Balan et al., 2009*). Food was given in a powdered form *ad libitum*.

5.3.3. Chemical analysis

Dietary dry matter, ash, crude protein, total fat and gross energy were determined as described previously (*Chapter-2, Section-2.3.3*).

5.3.4. Post-mortem procedure

At day 21 of the experiment, rats were anaesthetised by intra-peritoneal injection (0.1mL/100g live weight) of a mixture containing Acepromazine Maleate BP (ACP, 2 mg/mL) 2 parts, Ketamine (100 mg/mL) 5 parts, Xylazine (10%) 1 part and Sterile Water 2 parts. Rats were then killed by exsanguination.

The stomach of each rat was removed and its contents collected and

stored at -20°C. The tissues were then dried with absorbent paper and weighed. The entire small intestine from the pylorus to the ileocecal valve was removed, dissected free of mesenteric attachments and divided approximately into duodenum (upper 11 cm from the pylorus end), and the rest was divided into two parts, i.e. jejunum (central of the small intestine) and ileum (anterior to the ileocaecal valve). The contents of each part were flushed using phosphate buffer saline and stored at -20°C. The tissues were immediately frozen on dry ice and then stored at -80°C for subsequent analysis of mucin gene expression. For goblet cell count, lengths of tissues (2 cm) were collected from the middle of the duodenum (from pylorus end), jejunum (middle of intestine), ileum (anterior to ileocaecal valve) and colon, and stored immediately in 10% buffered formalin for 24 h.

5.3.5. Intact and cavitated goblet cell count

Each tissue fixed with formalin was excised, dehydrated and embedded in paraffin wax. From each tissue, transverse sections were cut, stained with haematoxylin, eosin and Alcian blue, and the goblet cells were examined under a light microscope. Goblet cells were measured in intact villi (from the tip of the villus to villus-crypt junction) and crypts (from villus-crypt junction to the base of the crypt). Positive staining with Alcian blue indicated goblet cells containing acid mucins. For duodenum, jejunum and ileum, the 5 longest and straightest villi, and their associated crypts from each transverse tissue section of rats in each group were used to enumerate intact and cavitated goblet cell numbers. For colon tissue, goblet cells in crypt were measured from 5 elongated and straight crypts from rats in each group. All goblet cells were also examined for the presence of cavitation of their apical membrane. A goblet cell with clear apical indentation into the intracellular store of mucus granules was taken as showing signs of the cavitation that accompanies recent compound exocytotic activity.

5.3.6. RNA extraction and quantitative real-time polymerase chain reaction

The procedure followed the method described by Han *et al*. (2008). Six rats were randomly selected from each group to determine the expression of mucin genes.

Total RNA was extracted from stomach, ileum and colon tissue with Trizol reagent (Invitrogen Co, NZ) according to the manufacturer's guidelines. Tissue samples (100mg) were homogenized in 1 ml of TRIZOL reagent using homogenizer (Polytron, Tekmar's TISSUEMIZER). To this 0.2 ml of chloroform were added. Samples were then vortex vigorously for 15 seconds and incubated at room temperature for 3 min. Samples were then centrifuged at 12000 x *g* for 15 min at 4°C. Following centrifugation step, upper aqueous phase was carefully transferred without disturbing the interphase into fresh tube. RNA was then precipitated from the aqueous phase by mixing with 0.5 ml isopropyl alcohol and then incubated at 24°C for 10 min and then centrifuged at 12000 x *g* for 10 min at 4°C. After removing the supernatant, the RNA pellet was washed with 1 ml of 75% ethanol and the samples were mixed by vortexing and centrifuged at 7500 x *g* for 5 minutes at 4°C. Remaining ethanol was removed after repeating the washing procedure. RNA pellet was air-dried for 10 min. The RNA was dissolved in DEPC-treated water by passing solution a few times through a pipette tip. Finally RNA concentration and purity was determined using a NanoDrop® ND-1000 Spectrophotometer at 260 nm and 280 nm.

Total RNA was digested (see below) by RNAfree DNase I (Promega Co, USA) to ensure that there was no contamination by genomic DNA. The DNase digestion reaction was set up using following mixture: 1–8µl of RNA in water, 1µl of RQ1 RNase-Free DNase 10X Reaction Buffer, 1u/µg RNA RQ1 RNase-Free DNase and Nuclease-free water to a final volume of 10µl and incubated at 37°C for 30 min. Samples were then incubated at 65°C for 10 min to inactivate the DNase. Several random samples were then run in Real-Time PCR to confirm the absence of DNA in the samples.

Briefly, the first strand of cDNA was synthesized utilizing the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, USA). DNase treated RNA (1 µg), anchored-oligo(dT) 18 primer (2.5 µM), transcriptor reverse transcriptase (10 U), deoxynucleotide mix (1 mM each), RNase inhibitor (20 U) and reaction buffer (8 mM $MgCl_2$) were mixed to yield a final volume of 20 µL, and incubated at 50°C for 60 min and then at 85°C for 10 min. The real-time PCR measures were performed in duplicate using the LightCycler 480 system with the LightCycler 480 Probes Master Kit (Roche Diagnostics, USA). Primers and probes for mucin genes and an internal reference β-actin gene were designed

with the assistance of Roche Universal Probe Library Assay Design Center (https://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp) (Table 5.1, Appen-ix-D, Han *et al*., 2008). The reaction mixture contained 2 µL of diluted cDNA (1:5), forward and reverse primers (0.4 µM each), probe (0.1 µM), and 10 µL of LightCycler 480 probes master in a final volume of 20 *µ*L. The cycling conditions of PCR were as follows: preincubation at 95°C for 10 min, followed by 50 amplification cycles (95°C, 10 s, and 55°C, 45 s), and cooling (40°C, 10 s). Real-time monitoring was achieved by measuring the fluorescence at the end of the elongation phase.

Table 5.1 Primers and probes for quantitative Real Time PCR[1].

Gene	Primers	Probes	Genbank accession no.
Muc2	5′-TGAGGTAGACAGAGCGACCA 5′-GGAGTCCAAGCAGGGAGAG	5′-GCCTGAAG	U68172
Muc3	5′-CTTGAGGAGGTGTGCAAGAAA 5′-CCCCAGGGTGACATACTTTG	5′-GGAGAGGA	U76551
Muc4	5′-GCTTGGACATTTGGTGATCC 5′-GCCCGTTGAAGGTGTATTTG	5′-CATCACCA	AH003319
Muc5Ac	5′-ACTTGACGGCCACTGTTACT 5′-TAACCCCTTTGACCACCTGA	5′-ATGGCTGC	U83139
β-actin	5′-CCCGCGAGTACAACCTTCT 5′-CGTCATCCATGGCGAACT	5′-CAGCTCCT	NM 031144

[1]Muc, Mucin

5.3.7. Enzyme-linked lectin assay for mucin

Stomach chyme, Ileal and colonic digesta of six rats from each group were freeze-dried and assayed for glycosylated mucin by an enzyme-linked lectin assay (ELLA) using wheat germ agglutinin (WGA) as the lectin, as described by Trompette *et al*. (2004) with some modification. Porcine gastric mucin (Sigma, NZ) was used as the standard. Briefly, various dilutions of standards and samples were prepared in carbonate buffer (0.5 M, pH 9.6) and coated on 96-well microtiter plates (NUNC microplates, USA). After incubating overnight at

4°C, the plates were washed three times with PBS Tween (PBS-T, pH 7.2). Microplates were then blocked with 300 µl of a PBS-T solution containing bovine serum albumin (BSA, 5 g/L) (PBS-T-BSA) and incubated for 1 h at 37°C. Plates were washed again, and 100 µl of biotinylated WGA (Sigma, NZ) in PBS-T-BSA was added and the plates were incubated for 1 h at 37°C. Plates were washed and incubated with 100 µl of avidin-peroxidase conjugate (Sigma, NZ) for 1 h at room temperature. After washing, 100 µl of OPD solution (Sigma, NZ) was added to each well and the plate was incubated in the dark at room temperature for 10 min. The reaction was stopped by adding 50 µl of sulphuric acid (1 M). Absorbance was read at 492 nm using an ELISA reader (Anthos 2010, Austria). Mucin concentration in samples was calculated with reference to a porcine gastric mucin standard curve. Data were expressed as µg or ng mucin per g freeze-dried digesta matter.

5.3.8. Statistical analysis

This study was part of large growth trial. Unless otherwise indicated, all values, n=15. Results were given as means ± SEM. Intact and cavitated goblet cell numbers were analyzed using the Mann-Whitney t test. Mucin gene expression and mucin protein were analyzed using the SAS/ PROC TTEST (SAS, version 9.1, SAS Institute Inc, Cary, NC). The PROC TTEST procedure shows two t statistics: one calculated under the equal variance assumption and the other for unequal variance. We applied the equal variance test (F test) first. If the null hypothesis was not rejected, we directly read the t statistic and its p-value for the pooled analysis. If the null hypothesis was rejected, we read the t statistic and its p-value for a Satterthwaite or Cochran/Cox approximation. Statistical significance was accepted at $P < 0.05$.

5.4. Results

5.4.1. Food intake and growth rate

Food intake and growth rate over the 21-day trial were not affected by the dietary treatments (BD and FDOI). Mean (± SE) food intake was 22g/d (± 0.65) and

mean growth rate was 9g/d (± 0.36) across the diets. On the day of slaughter, mean (± SE) food intake was 21g/d (± 0.80) across the diets (*Chapter-2*).

5.4.2. Intact and cavitated goblet cell count

When compared to the BD diet, rats consuming the FDOI diet had a higher ($P < 0.05$) number of goblet cells in the villi of duodenum, jejunum and ileum. Crypt goblet cells were also higher in number ($P < 0.05$) for the FDOI diet in the duodenum, jejunum, ileum and colon (Table 5.2, Figure 5.1).

In ileal segments, histological analysis indicated that rats fed the FDOI diet had significantly ($P < 0.05$) more cavitated goblet cells when compared to the rats fed the BD diet. A similar result was observed in the duodenum, jejunum and colon (only crypt) for the rats fed the FDOI diet (Table 5.3, Figure 5.1).

5.4.3. Mucin gene expression

Muc5Ac mRNA expression in the stomach was significantly ($P = 0.021$) higher for rats fed the FDOI diet compared to rats fed the BD diet (Figure 5.2). Further, Muc2, Muc3, and Muc4 mRNA expression in the ileum was higher ($P < 0.05$) for rats given the FDOI diet (Figure 5.2). There was no significant ($P > 0.05$) difference between the two groups for mucin gene expression in the colon.

5.4.4. Quantification of mucin protein

Mucin protein (ELLA test) was significantly ($P < 0.05$) higher in stomach chyme, and ileum and colon digesta for rats fed the FDOI diet compared to rats fed the BD diet (Table 5.4).

Table 5.2 Goblet cell counts in the small intestine and colon for rats fed a diet containing ovine Ig for 21 d[1].

Organ	Diet		SEM	*P* value
	BD	FDOI		
	Villus goblet cells[2]			
Duodenum	109.4	208.8	8.85	<0.001
Jejunum	78.4	134.2	5.02	<0.001
Ileum	84.7	126	8.24	0.015
	Crypt goblet cells[2]			
Duodenum	98.8	167.8	7.07	<0.001
Jejunum	52.2	109.4	7.98	<0.001
Ileum	58.3	97.8	6.42	0.011
Colon	88.4	127.9	7.90	0.01
	Villus goblet cells[3]			
Duodenum	0.19	0.36	0.02	<0.001
Jejunum	0.20	0.33	0.01	<0.001
Ileum	0.47	0.65	0.04	0.05
	Crypt goblet cells[3]			
Duodenum	0.47	0.78	0.03	<0.001
Jejunum	0.38	0.75	0.01	<0.001
Ileum	0.54	0.86	0.06	0.016
Colon	0.43	0.60	0.04	0.038

[1]Values are means ± SEM, *n*=7. [2] Number of goblet cells per five complete villi and their associated crypts. [3] Number of goblet cells per µm for five complete villi and their associated crypts. BD, Basal diet; FDOI, Freeze-dried ovine immunoglobulin diet.

Table 5.3 Cavitated goblet cell counts in the small intestine and colon for rats fed a diet containing ovine Ig for 21 d[1].

	Diet			
Organ	**BD**	**FDOI**	**SEM**	***P* value**
	Villi cavitated goblet cells[2]			
Duodenum	9.3	15.5	1	0.021
Jejunum	10	13.2	1.1	0.019
Ileum	10.6	17.8	2.8	0.034
	Crypt cavitated goblet cells[2]			
Colon	7.2	15.8	3.7	0.032
	Villi cavitated goblet cells[3]			
Duodenum	0.017	0.026	0.001	0.001
Jejunum	0.025	0.032	0.002	0.027
Ileum	0.059	0.091	0.010	0.038
	Crypt cavitated goblet cells[3]			
Colon	0.035	0.075	0.011	0.019

[1]Values are means ± SEM, *n*=7. [2] Number of goblet cells per five complete villi and their associated crypts. [3] Number of goblet cells per µm for five complete villi and crypts. BD, Basal diet; FDOI, Freeze-dried ovine immunoglobulin diet.

Table 5.4 Mucin protein in stomach chyme and ileal and colonic luminal digesta for rats fed a diet containing ovine Ig for 21 d[1].

	Diet			
organ	**BD**	**FDOI**	**SEM**	***P* value**
Stomach (ng/g)	1231.4	3319.2	310.39	0.013
Ileum (µg/g)	1612.4	3363.0	399.21	0.029
Colon (ng/g)	2796.9	5560.3	346.91	0.018

[1]Values are means ± SEM, *n*=6, BD, Basal diet; FDOI, Freeze-dried ovine immunoglobulin diet.

5.5. Discussion

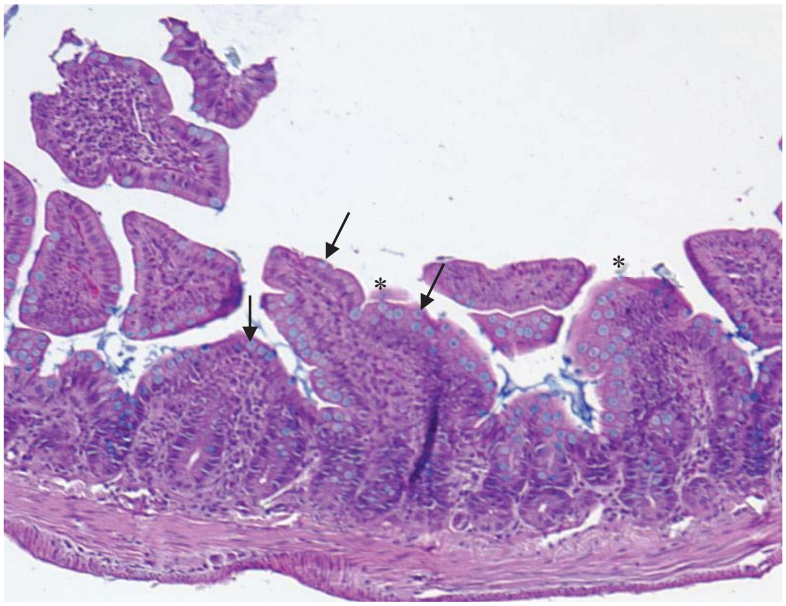
Ig are the primary anti-infective components of body fluids. These specialized proteins protect the body from harmful pathogens by either binding to them or by formation of an encapsulating barrier (*Kraehenbuhl & Neutra, 1992*). It is known that orally administered Ig is able to retain its activity in the GIT (*Eibl et al., 1988; Hammarstrom et al., 1993; Tjellstrom et al., 1993; Bogstedt et al., 1996*). Rodriguez *et al*. (2007) showed that porcine Ig partially resisted the digestion process in the GIT of adult dogs and cats fed diets containing spray-dried animal plasma or concentrated Ig derived from pig blood. Human serum and bovine Ig resist digestion in the upper GIT of humans and retain toxin binding and neutralizing activity (*Losonsky et al., 1985; Warny et al., 1999*).

One of the important functions of the gut is to prevent pathogenic bacteria and toxins from reaching the systemic circulation, organs and internal tissues (*Allori et al., 2000*). The gut mucins afford the first line of defence against bacteria and are one of the main constituents of the gut barrier (*Allori et al., 2000*).

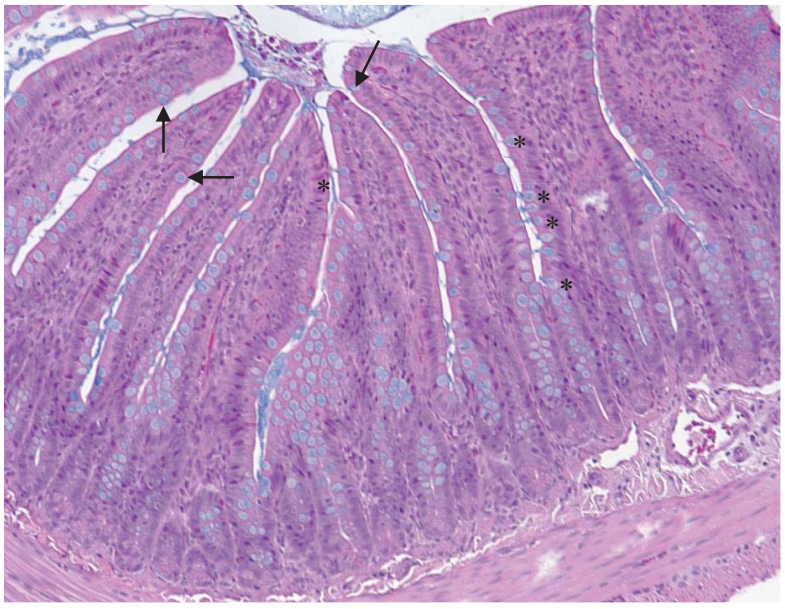
Mucins are produced in the gut by specialized goblet cells and an enhanced production of goblet cells may lead to an improved synthesis of mucus that may help to protect the epithelial surface. The number of goblet cells in gut tissue may be increased by dietary interventions such as probiotic bacteria (*Dock-Nascimento et al., 2007; Vinderola et al., 2007*) and keratinocyte growth factor (*Fernandez-Estivariz et al., 2003*). Other investigators (*Neutra et al., 1974; Sherman et al., 1985*) have shown that chronic protein depletion or protein-energy under-nutrition decrease goblet cell number and mucin synthesis in the proximal small intestine of rodents and piglets. Recently, King *et al*. (2008) reported that feeding pigs with either colostrum or dried plasma resulted in an increase of villus and crypt goblet cells. In their study, the lack of signs of intestinal inflammation may signify an augmentation of intestinal barrier function. In the present study, the rats fed the FDOI diet had a significantly higher number of villi and crypt goblet cells in the duodenal, jejunal, ileal and colonic segments when compared to the BD diet. Rats fed the FDOI diet also appeared to have, based on visual examination of a light micrograph (Figure 5.1), more sizeable and more intact ileal villi and crypts.

Figure 5.1 Transverse sections of rat ileum.

(A)

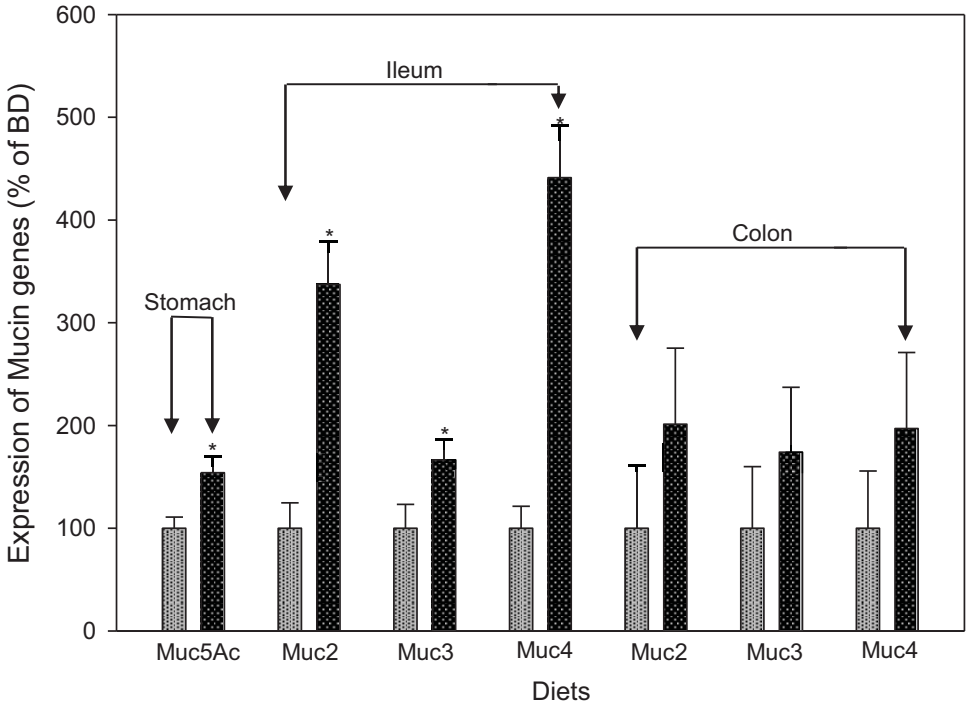


(B)



Section was stained with haemotoxylin, eosin and alcian blue. Goblet cells (alcian blue stained) are filled with mucus. Arrows indicate examples of stained mucus cells. Stained mucus cells exhibit deep apical membrane cavitations from which streams of stained mucus (asterisk) emerged to join the mucus blanket in the ileal lumen. (A) Section from a rat fed with BD diet (shows crypt damage, villus fusion, tip denudation, and atrophy, and low alcian blue stained goblet cells) and (B) FDOI diet.

Figure 5.2 Expression of mRNA levels of mucin genes (Muc5Ac, Muc2, Muc3 and Muc4) in the stomach, ileum and colon of rats fed a diet containing ovine Ig for 21 d[1].



[1]Values are means ± SE (*n*=6). * denotes that expression of mRNA levels of mucin genes of the indicated diet group is significantly ($P < 0.05$) differ from other group. BD, ▒; FDOI ▓.

In response to stimulation, intestinal goblet cells may increase their discharge of mucin by two processes. In many mucus cells, a phenomenon of compound exocytosis is induced, resulting in deep cavitation of the apical membrane surface of mucus cells (*Specian & Oliver, 1991; Forstner, 1995*). Some intestinal goblet cells may also react to stimulation by way of a second process that decreases the intracellular store of mucus granules but without cavitation (*Specian & Oliver, 1991; Forstner, 1995*). The latter mechanism results in a total loss of mucus granules and goblet cells are no longer stained by alcian blue and appear identical to other epithelial cells. Higher luminal mucin protein and more intact and cavitated goblet cells suggest that the FDOI diet fed to the

rats induced more mucus discharge than for the rats fed the BD diet (Figure 5.1).

Muc2$^{-/-}$ mice develop spontaneous inflammation, presumably due to the absence of the major component of intestinal mucus, leading to increased exposure to the normal intestinal microbial flora (*Velcich et al., 2002; Van der Sluis et al., 2006*). Up-regulation of MUC3 expression in colonic cells has been correlated with decreased binding of enteropathogenic *E. coli* (*Mack et al., 1999; Mack et al., 2003*). Larson *et al*. (2003) demonstrated that colostrum-associated M-SAA3 (mammary-associated serum amyloid A isoform 3) peptide enhanced innate protection by stimulating human intestinal epithelial cells to express Muc3.

In the present study, stomach Muc5Ac, ileal Muc2, Muc3 and Muc4 expression increased by 154%, 337%, 166% and 441%, respectively in the rats fed the FDOI diet when compared to rats fed the BD diet. The mRNA levels in the stomach tissue were highly correlated with chyme mucin protein concentrations (r=0.83; $P < 0.005$). Similarly in the ileum, mRNA levels of Muc2, Muc3 and Muc4 were significantly correlated (r=0.97; $P < 0.001$, r=0.95; $P < 0.001$ and r=0.96; $P < 0.001$) with digesta mucin protein contents. Conversely, in the colon, mRNA levels of Muc2, Muc3 and Muc4 were not significantly correlated (r=0.62; $P > 0.05$, r=0.61; $P > 0.05$ and r=0.63; $P > 0.05$) with mucin protein. The observed up-regulation of mucin genes (mainly stomach Muc5AC and ileal Muc2) may have resulted in the observed increase in luminal mucus in the stomach and intestine, though this has not been confirmed in the present work. A lack of correlation between mucin gene expression and colonic luminal mucin content may reflect the microbial degradation of mucins in the colon (Barcelo *et al*., 2000). Further work is warranted to demonstrate the causal links between mucin gene expression and digesta mucin protein content to explain regional variations in the expression of these genes along the gut.

Other studies have reported an effect of specific dietary components on gut mucin secretion. Claustre *et al*. (2002) found that milk protein hydrolysates (casein and lactalbumin hydrolysates) and β-casomorphin-7 induced mucin release in rat jejunum. Growing rats fed with dietary fibers with increasing viscosities were associated with an increase in ileal endogenous nitrogen flow and mucoprotein (sialic acid) secretion (*Larsen et al., 1994*). Also, Barcelo *et al*. (2000) reported that dietary fibres (alginate, ulvan), uronic acids (glucuronic acid, galacturonic acid), and short chain fatty acids may stimulate secretory activity of

intestinal goblet cells. Caballero-Franco *et al*. (2007) studied the three bacterial groups (lactobacilli, bifidobacteria, and streptococci) contained in a probiotic formula and reported that the *Lactobacillus* species were the strongest potentiator of mucin secretion *in vitro*. In the present study, mucin protein was found to be significantly higher in the luminal contents of the stomach, ileum and colon for the FDOI fed rats when compared to the BD fed rats. Previously, we have observed that feeding rats an FDOI containing diet results in an increase in the *Lactobacillus* species in both ileal and colonic digesta (*Chapter-4, Section-4.4*). Thus feeding the FDOI diet may have led to an increase in *Lactobacillus* species which might have potentiated the mucin secretion *in vivo*. As discussed in previous Chapters (*Chapters-1, 2, 3 & 4*), serum contains antibodies against numerous pathogenic bacteria (*Torrallardona, 2010*). Therefore serum Ig may provide passive antimicrobial protection (and also from subclinical infection) by exclusion of opportunistic pathogens and also help in preventing mucosal damage. The intestinal tract is a battleground for beneficial and pathogenic microbes and toxins. If pathogens are suppressed by Ig there may be a possibility for the growth of beneficial bacteria, which was evident in the previous Chapter (*Chapter-4, Section-4.4*). The increased gut mucin for FDOI-supplemented rats reported in this Chapter is contradictory to the observation reported in the earlier Chapter (*Chapter-2*, *Section-2.4.1.1*), where we reported that G:F ratio was greater ($P < 0.05$) for rats receiving the FDOI diet compared with those consuming the BD or IOI diets. But this may be explained due to lack of subclinical infection (passive prevention of pathogenic bacteria by Ig) for those rats fed with FDOI which resulted in better growth performance.

Overall, it appears that FDOI leads to an enhanced gut mucin secretion in the rat. This may be associated with an enhanced mucus layer overlying the epithelial lining of the gut which may in turn enhance GIT protection (*Linden et al., 2008*).

In conclusion, orally administered FDOI selectively altered mucin gene expression and gut digesta mucin protein contents as well as the number of tissue goblet cells in growing rats. To our knowledge, this is the first report regarding the effects of ovine serum Ig on mammalian mucin secretion.

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Chapter 6

Dietary supplementation with ovine serum immunoglobulin influences growth performance, organ weight, gut morphology and intestinal mucin production in growing rats challenged with *Salmonella enteritidis*

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Results of the previous four Chapters (*Chapters 2, 3, 4, & 5*) clearly demonstrate that orally fed ovine Ig modulates the growth performance, immunity, microbiota and digesta mucin in the normal growing rat. Reports are found in the literature regarding the counteracting effects of animal plasma in challenged (pathogenic bacteria) animal models. The aim of this study was to determine whether dietary supplementation with ovine serum Ig modulates growth and intestinal mucin production in growing rats challenged with *Salmonella enteritidis*.

6.1. Abstract

The aim was to determine whether orally administered ovine serum Ig prevented the effects of *Salmonella enteritidis* on growth performance, organ weight, gut morphology and mucin production in the *S. enteritidis*−challenged growing rat. The four treatment groups consisted of non-challenged rats fed a casein-based control diet (BD) and three groups of rats challenged with $1X10^7$ CFU *S. enteritidis* and fed either a casein-based diet, a diet containing freeze-dried ovine Ig (FDOI) or a diet containing inactivated ovine Ig (IOI). The rats were randomly allocated to one of the four treatments (n=15) and received their respective diets for an 18d experimental study. Challenging took place on Day 15. Average daily gain and body Gain:Feed ratio (post gavage) were significantly ($P < 0.05$) higher for the rats fed the FDOI diet compared to those fed the BD and IOI diets. At the end of the study, the small intestine and colon were significantly ($P < 0.05$) heavier for the challenged rats fed the FDOI diet compared to the challenged rats fed either the BD or IOI diets. Moreover, the relative weights of the caecum, liver and spleen of the challenged rats fed the BD or IOI diet were significantly ($P < 0.05$) heavier compared to the challenged rats fed the FDOI diet. Generally, the challenged rats fed the FDOI diet had significantly ($P < 0.05$) more sizeable and intact gut morphological structures, higher goblet cell count and luminal mucin protein than the challenged rats fed either the BD or IOI diets. Overall, a FDOI fraction clearly prevented the deleterious effects of *S. enteritidis*.

6.2. Introduction

Animal Plasma (AP) is a potent source of immunoglobulins (Ig) and when included in the diets of healthy weaned production animals has been reported to improve voluntary feed intake, growth rate and intestinal growth (*Hansen et al., 1993; Kats et al., 1994*). This effect may be due to a greater availability of nutrients and energy for growth, consequent upon a lower degree of immune cell activation (*Demas et al., 1997*). For animals that have undergone an immune challenge such as infection with *E. coli* the effects of AP on growth were even more pronounced (*Bosi et al., 2004*). It has been also suggested that Ig present in AP may bind to bacteria in the lumen of the gastrointestinal tract (GIT) and prevent their attachment to the mucosa (*Bosi et al., 2004*) thereby reducing the degree of adhesion of pathogenic bacteria to the gut wall (*Coffey & Cromwell, 1995*).

Orally administered human Ig has been shown to reduce the subclinical symptoms of necrotizing enterocolitis (*Eibl et al., 1988*) while Ig have also been used therapeutically against *Campylobacter jejuni* (*Hammarstrom et al., 1993*) and *Clostridium difficile* (*Tjellstrom et al., 1993*) a major cause of diarrhoea in immunocompromised patients as well as chronic diarrhoea of unknown aetiology in normal infants (*Casswall et al., 1996*). The successful treatment of *Helicobacter pylori*, rotavirus and cryptosporidial infection using bovine Ig has also been reported (*Bogstedt et al., 1996*). Moreover, reports have been published demonstrating positive effects of Ig concentrate in rats challenged with *Staphylococcus aureus* enterotoxin (*Pérez-Bosque et al., 2008, 2010*). Orally administered Ig appears to be at least partially resistant to digestion in the GIT and intact active Ig has been reported in the stools of low birth weight infants given Ig orally (*Blum et al., 1981*). Roos *et al*. (1995) demonstrated that as much as 19% of ingested IgG and IgM remains immunologically active in the gut of adult humans.

Recently, our own group has reported that feeding freeze-dried ovine serum Ig (Ig extracted from lamb's blood) to normal growing rats improved growth performance, increased the weight of some organs and improved the gut architecture (*Chapter-2*). It also supported a higher degree of immunological

activity (*Chapter-3*), selectively altered the composition of gut microbiota and modulated the transcription and translation levels of mucin in the GIT (*Chapter-4 & 5*). In this study, the aim was to extend our recent work to the animal challenged with *S. enteritidis*, subsequently the present study examined the effect of dietary ovine Ig on growth performance, organ weights, gut morphology and intestinal mucin production in rats following challenging with *S. enteritidis*.

6.3. Materials and methods

6.3.1. Preparation and quantitation of ovine serum Ig

Freeze-dried ovine Ig (FDOI) and inactivated ovine Ig (IOI) was prepared as described in Chapter-2 (S*ection-2.3.1*).

The IgG content in the ovine Ig fraction was determined as described in Chapter-2 (S*ection-2.3.1*). The respective protein and Ig concentrations of the FDOI were 96.5% and 73.6%, while in the IOI they were 94.3% and 71.9%, respectively.

6.3.2. Experimental diets

Three semi-synthetic diets were formulated, 1) a basal casein-based diet containing no Ig, 2) the basal casein-based diet supplemented with FDOI and 3) the basal casein-based diet supplemented with IOI. The ingredient composition and determined nutrient composition of the diets are shown in Table 6.1. All diets exceeded the recommended nutrient requirement levels for the growing rat for all nutrients (*National Research Council*, *NRC, 1995*). The FDOI and IOI fractions were included in the respective diets at a concentration approximately equal to the amount of spray dried porcine plasma (SDPP) IgG used in previous studies (*Pierce et al., 2005; Chapter-2*)

Table 6.1 Ingredient composition and determined nutrient and energy content of the control and test diets[1].

	Diet[1]		
	BD	**FDOI**	**IOI**
	g/kg dry weight		
Ingredient			
Wheat starch	489.5	492.2	491.7
Sucrose	100	100	100
Cellulose (Avicel)	50	50	50
Soyabean oil	70	70	70
Casein	186	152.8	152.8
Vitamin premix[2]	50	50	50
Mineral premix[3]	50	50	50
Methionine	1	1.6	1.6
Cysteine	3.4	2.8	2.7
Ovine IgG fraction		30.5	31.2
Nutrient[4]			
Gross Energy, kj/g	17.7	17.7	17.8
Dry matter, %	92.2	92.5	92.7
Crude Protein, %	16.9	16.9	17.2
Carbohydrate, %	64.4	64.6	64.2
Ash, %	3.9	3.9	4.1
Immunoglobulins[5] (%)		2.24	2.24

[1]All diets were formulated to meet or exceed the nutrient requirements of growing rats
[2]The mixture supplied (mg/kg diet): retinol acetate 5.0, DL-tocopheryl acetate 200, menadione 3.0, thiamin hydrochloride 5.0, riboflavin 7.0, pyridoxine hydrochloride 8.0, D-pantothenic acid 20, folic acid 2.0, nicotinic acid 20, D-biotin 1.0, myo-inositol 200, choline chloride 1500; (*μ*g/kg diet) ergocalciferol 25, cyanocobalamin 50. [3]The mixture supplied: (g/kg diet) Calcium hydrogen phosphate 6.29, Magnesium oxide 1.06, Potassium chloride 5.24, Sodium chloride 1.97; (mg/kg diet) Chromic potassium sulphate 1.97, Cupric carbonate 10.7, Ferric citrate 424, Manganese sulphate 78.0, Zinc oxide 48.2; (*μ*g/kg diet) Cobalt chloride 29.0, Potassium iodate 151, Ammonium molybdate152, Sodium selenite 151. [4]values for nutrients were from chemical analysis except for carbohydrate which was calculated by difference. [5]Estimated Ig concentration in each diet. BD, Basal diet; FDOI, Freeze-dried ovine immunoglobulin; IOI, Inactivated ovine immunoglobulin.

6.3.3. Animal study

This work was approved by the Massey University Animal Ethics Committee (MUAEC 09/29) and procedures complied with the New Zealand Code of Recommendations and Minimum Standards for the Care and Use of Animals for Scientific purposes (New Zealand Animal Welfare Advisory Committee, 1995). Sixty Sprague-Dawley male rats (8 wk of age) were housed (single room under PC2 conditions with HEPA filtered air), individually in stainless steel cages in a room maintained at 22 ± 2°C with a 12 h light/dark cycle (in Small Animal production Unit, SAPU, Massey University, Palmerston North). The animals had unrestricted access to water at all times. During the first week the rats were acclimatized to the cages and received the basal diet. The diet was provided *ad libitum* as a dry powder. At the end of the acclimatization period the rats were randomly allocated to the four experimental treatments such that there were 15 rats per treatment. The experimental treatments were as follows: 1) non-challenged rats fed the basal diet (BD) 2) rats challenged with *S. enteritidis* and fed BD, 3) rats challenged with *S. enteritidis* and fed the FDOI-containing diet and 4) rats challenged with *S. enteritidis* and fed the IOI-containing diet. The rats received their respective diets ad *libitum* for 18 days (experimental period). On day 15, the rats in treatment groups 2, 3 and 4 were challenged with a single dose of *S. enteritidis* ATCC 13076 (1 ml of saline solution containing 3% sodium bicarbonate with $1X10^7$ viable *S. enteritidis*). *S. enteritidis* (strain 1891, originally from England) was purchased from New Zealand Reference Culture Collection - Environmental Science and Research (NZ). Prior to use, *S. enteritidis* was subcultured three times in LB (luria-bertani) broth (Difco, USA). The exact viable count of *S. enteritidis* inoculum was determined by plating on brilliant green agar (Oxoid, England). Rats in the group 1 (unchallenged BD) challenged with 1 mL of saline solution without *S. enteritidis*. The body weights of the rats were measured at days 1, 7, 14,15,16,17 and 18 of the experimental period, and the food intake was recorded daily. Average daily gain (ADG) and average daily feed intake (ADFI) were determined. Gain:Feed ratio (G:F) was calculated by dividing the daily gain by the daily feed intake. As many of the challenged rats showed symptoms of *S. enteritidis* challenge including nasal discharge, and red crusts around eyes and nostrils after 3 d of gavage and for ethical reasons the study was culminated after 3 d of challenge. However, the effects of *S. enteritidis* were

expected to be acute and marked and a 3 d challenge period was considered adequate to test the effects of Ig.

On the final day (day 18) of the experimental period, the rats were anaesthetized by intra-peritoneal injection (0.1 mL/100 g live weight) of a mixture containing acepromazine maleate BP (ACP, 2 mg/mL) 2 parts, ketamine (100 mg/mL) 5 parts, xylazine (10%) 1 part and sterile water 2 parts. Rats were then killed by exsanguination. The stomach of each rat was removed and its contents emptied. The tissues were then dried with absorbent paper and weighed. The entire small intestine from the pylorus to the ileocecal valve was removed, dissected free of mesenteric attachments and divided approximately into duodenum (upper 11 cm from the pylorus end), and the rest was divided into two parts, i.e. jejunum (central of the small intestine) and ileum (anterior to the ileocaecal valve). The contents were flushed out using phosphate buffer saline. The tissues were then blotted dry and weighed as described above. The same procedure was followed for the caecum and colon. The liver and spleen were also removed and weighed. The remaining portion of the animal was weighed as the empty body weight. Relative weights of the organs were calculated with respect to empty body weight.

6.3.4. Chemical analysis

Dietary dry matter, ash, crude protein, total fat and gross energy were determined as described previously (*Chapter-2, Section-2.3.3*).

6.3.5. Gut morphology

Tissue sections, 2 cm in length, were collected from the middle of the duodenum, jejunum and ileum, and immediately placed (and subsequently stored) in 10% buffered formalin for 24 h. After fixation, each tissue was excised, dehydrated and embedded in paraffin wax. From each tissue, transverse sections were cut, stained with haematoxylin, eosin and Alcian blue, and examined under a light microscope. The 5 longest and straightest villi, and their associated crypts from each transverse tissue section were randomly selected from 10 rats for each experimental treatment and the villus height, crypt depth and villus surface area for each section measured as described previously (*Chapter-2, Section-2.3.6*).

Colon tissue samples were prepared as described for the small intestine samples and the crypt depths were measured for 5 elongated and straight crypts from each transverse tissue section selected from 10 rats for each experimental treatment.

6.3.6. Intact goblet cell count

Goblet cells were measured in intact villi (from the tip of the villus to villus-crypt junction) and crypts (from villus-crypt junction to the base of the crypt). Positive staining with Alcian blue indicated goblet cells containing acid mucins. For duodenum, jejunum and ileum, the 5 longest and straightest villi, and their associated crypts from each transverse tissue section of 10 randomly selected rats in each group were quantified. For colon tissue, goblet cells in crypt were measured from 5 elongated and straight crypts from each transverse tissue section of 10 randomly selected rats in each group.

6.3.7. Enzyme-linked lectin assay for mucin

Ileal and colonic digesta of ten randomly selected rats from each group were freeze-dried and assayed for glycosylated mucin by enzyme-linked lectin assay (ELLA) using wheat germ agglutinin (WGA) as the lectin, as described in Chapter-5, Section-5.3.7. Mucin concentration in samples was calculated with reference to a porcine gastric mucin standard curve. Data were expressed as µg or ng mucin per mL wet digesta.

6.3.8. Statistical analysis

Results are presented as means ± SEM. Data were analysed using a one-way ANOVA (GLM procedures of SAS, version 9.1, SAS Institute, Cary, NC) and means were compared using Tukey's method. ADG, ADFI and G:F were also analyzed before and after gavage with *S. enteritidis* using a repeated measures analysis (SAS, version 9.1, SAS Institute Inc, Cary, North Carolina). For the goblet cell numbers, the Mann-Whitney U test was used. For the comparison of the results obtained from the four groups, the Kruskal-Wallis test was applied; $P < 0.05$ was considered to be statistically significant.

6.4. Results

6.4.1. Growth performance

ADFI, ADG and G:F were determined for the period prior to challenging with *S. enteritidis* (day 1 to 14) and after challenging with *S. enteritidis* (day 15 to 18) (Table 6.2). Prior to challenging, there was no significant ($P > 0.05$) effect of diet on ADFI. For ADG there was no significant ($P < 0.05$) difference across the rats fed the BD and FDOI diets, however, ADG was significantly ($P = 0.013$) lower for the rats receiving the IOI diet compared to the rats fed the FDOI diet. G:F ratio was higher ($P = 0.003$) for the rats consuming the FDOI diet than for those fed the BD or IOI diets. After challenging with *S. enteritidis*, ADFI was not significantly ($P > 0.05$) different between treatments but ADG and G:F were significantly ($P = 0.001$ and $P = 0.018$ respectively) lower for the challenged rats fed the BD diet when compared to the unchallenged rats receiving the BD diet and there was no significant ($P > 0.05$) difference between the challenged rats fed either BD or IOI. In contrast, the ADG and G:F were significantly ($P < 0.05$) higher for the challenged rats fed the FDOI in comparison with the challenged BD- and IOI-diet fed rats. When comparing the pre- and post-challenging periods, challenging with *S. enteritidis* resulted in a significant ($P < 0.05$) lowering of both ADG and GF for the rats fed either BD or IOI, but this effect was not observed ($P > 0.05$) in rats fed the FDOI diet.

6.4.2. Organ weights

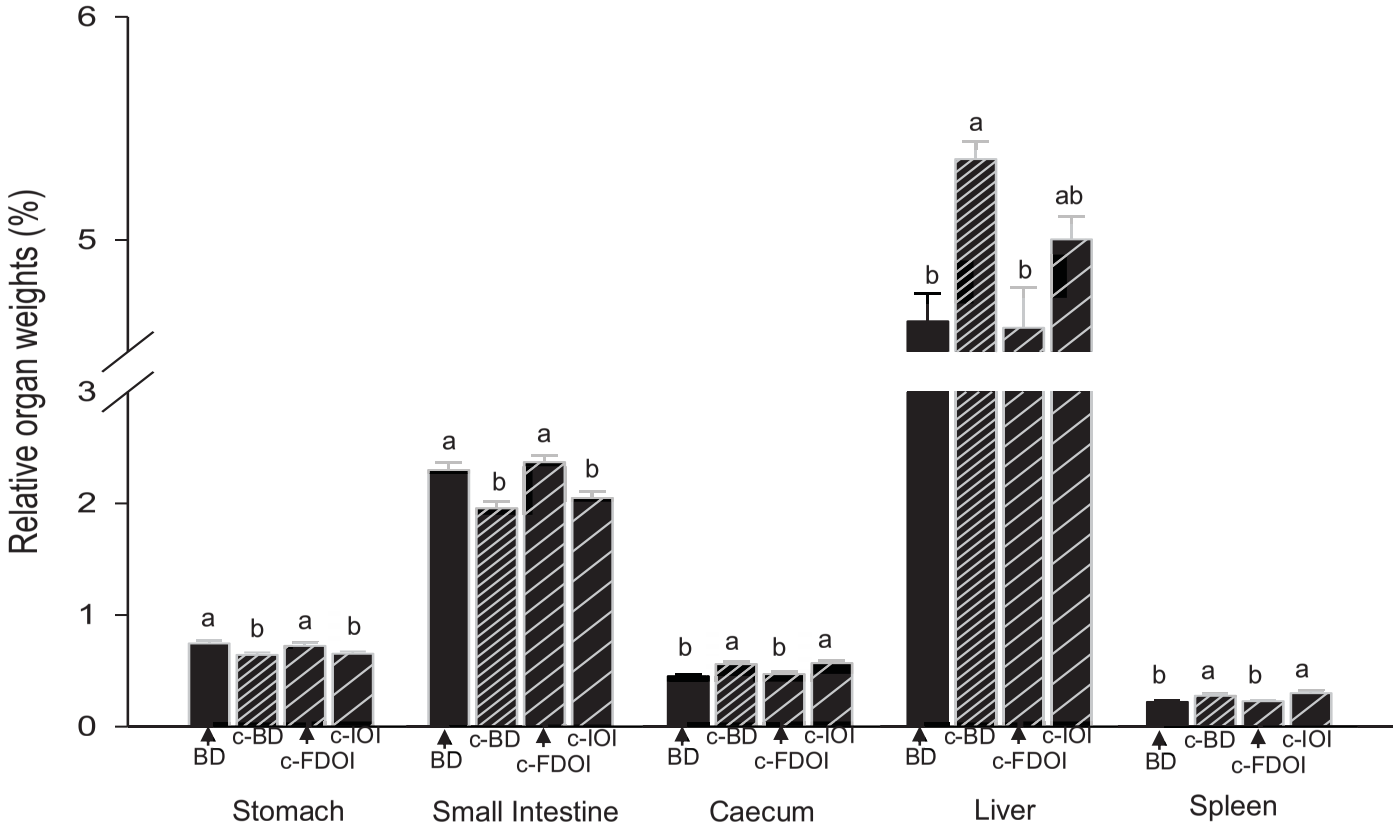
Mean organ weights relative to empty body weight of the rats on the final day of the study are shown in Figure 6.1. Challenging with *S. enteritidis* resulted in significantly ($P < 0.05$) lower stomach and small intestine and higher caecum, liver and spleen weights for the rats fed the BD diets. Furthermore, these organ weights in the challenged rats fed the IOI diet were not significantly ($P > 0.05$) different from the challenged rats fed the BD diet. In contrast, the weights of the organs in the challenged FDOI fed rats were not significantly ($P > 0.05$) different from the unchallenged BD diet fed rats but were significantly ($P < 0.05$) different from the challenged BD- and IOI-diet fed rats.

Table 6.2 Growth performance of rats before and after challenging with *S. enteritidis*[1].

Diet:	BD	BD	FDOI	IOI	SEM	*P* Value
		Before challenge				
ADFI, g/day	25.02	25.69	25.33	24.17	1.32	0.866
ADG, g/day	5.00^{ab}	5.00^{abx}	6.26^{a}	3.84^{bx}	0.33	0.013
G:F, g/g	0.19^{b}	0.20^{bx}	0.26^{a}	0.16^{bx}	0.01	0.003
		After challenge				
ADFI, g/day	27.14	22.44	23.76	24.55	0.70	0.156
ADG, g/day	5.04^{a}	1.81^{by}	5.68^{a}	2.61^{by}	0.35	0.001
G:F, g/g	0.21^{a}	0.09^{by}	0.24^{a}	0.10^{by}	0.02	0.018

[1]BD=Basal diet; FDOI=Freeze-dried ovine immunoglobulin; IOI=Inactivated ovine immunoglobulin; ADFI, Averaged daily feed intake; ADG, Averaged daily gain; G:F, Gain:Feed ratio. ^{a,b} Means in a row without a common superscript differ and $^{x, y}$ Mean in a column (pre and post challenging) for same variable without a common superscript differ significantly ($P < 0.05$).

Figure 6.1 Mean (*n*=15) relative organ weights (%, empty body weight) for the growing rat fed diet BD and the growing rat with *S. enteritidis* and fed either diet BD, FDOI or IOI-supplemented diets.



BD=basal diet; FDOI=freeze-dried ovine immunoglobulin; IOI=inactivated ovine immunoglobulin; c, rats challenged with *S. enteritidis*.
[a, b] Means in an organ without a common superscript differ significantly, $P < 0.05$.

6.4.3. Gut morphology

Gut morphology data are shown in Table 6.3 and stained transverse sections of gut tissue in Figure 6.2, 6.3, 6.4 & 6.5. The villus length, crypt depth, villus:crypt ratio and villus surface area (VSA) of the duodenum and jejunum were generally significantly ($P < 0.01$) greater in rats challenged with *S. enteritidis* and receiving the FDOI diet compared to either the unchallenged rats fed the BD diet (except duodenal and jejunal VSA) or the challenged rats fed the BD or IOI diets. In the ileum, challenging with *S. enteritidis* resulted in significantly ($P < 0.001$) lower villus length, villus:crypt ratio and VSA for the challenged rats receiving either the BD, FDOI or IOI diets when compared to the unchallenged rats fed the BD diet. In contrast challenged rats fed the FDOI diet had greater crypt depth than the rats in the other treatment groups. The colon crypt depth was also significantly greater ($P < 0.001$) in the unchallenged rats receiving the BD diet and the FDOI diet-fed challenged rats when compared to the challenged rats fed the BD or IOI diets. In the stained transverse sections, severe mucosal histological damage (villus shortening and fusion, crypt loss, inflammatory infiltration) was found for the challenged rats fed the BD diet when compared to the unchallenged rats receiving the BD diet and a similar trend was observed for the challenged IOI fed rats. In contrast, these effects were completely negated for the challenged rats fed the FDOI diet.

6.4.4. Goblet cell count

Goblet cell count data are shown in Table 6.4. The number of goblet cells in the villi and crypts of duodenal, jejunal and ileal segments were significantly ($P < 0.05$) lower for the challenged rats receiving diet BD compared to their unchallenged counterparts. This lowering of goblet cell numbers was not evident for the challenged rats fed the FDOI diet. Moreover, the latter rats had a significantly ($P < 0.05$) higher number of goblet cells compared to unchallenged rats fed the BD diet in villi and crypts for some of the intestinal segments.

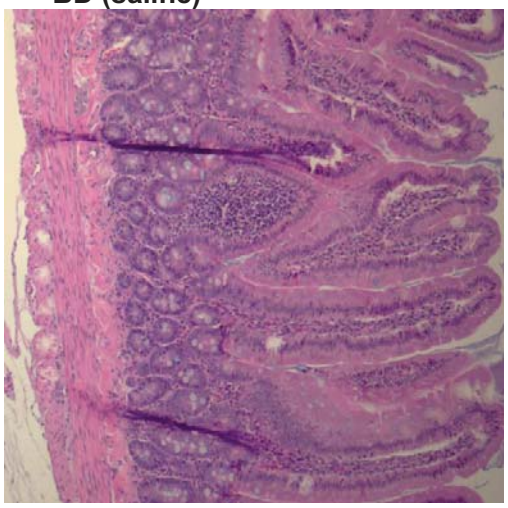
Table 6.3 Mean (*n*=10) intestinal morphology characteristics in the growing rat after challenging with *S. enteritidis*[1].

	Unchallenged	Challenged with *S. enteritidis*				
Diet:	**BD**	**BD**	**FDOI**	**IOI**	**SEM**	***P* value**
Duodenum						
Villus length, µm	516.60[b]	300.06[d]	657.59[a]	365.26[c]	10.75	<0.0001
Crypt depth, µm	187.26[b]	141.08[c]	248.89[a]	138.11[c]	4.66	<0.0001
Villus:Crypt ratio	2.79[b]	2.18[c]	3.53[a]	2.69[b]	0.17	<0.0001
Villus surface area[2], mm^2	0.059[a]	0.027[b]	0.063[a]	0.027[b]	0.004	<0.0001
Jejunum						
Villus length, µm	428.12[b]	278.41[d]	538.73[a]	326.90[c]	9.68	<0.0001
Crypt depth, µm	133.39[b]	114.42[c]	180.36[a]	110.23[c]	2.87	<0.0001
Villus:Crypt ratio	3.28[b]	2.46[b]	4.25[a]	3.08[b]	0.31	0.001
Villus surface area, mm^2	0.055[a]	0.028[b]	0.054[a]	0.031[b]	0.004	<0.0001
Ileum						
Villus length, µm	301.94[a]	165.84[c]	256.78[b]	179.27[c]	6.84	<0.0001
Crypt depth, µm	172.92[b]	102.78[c]	195.50[a]	102.18[c]	5.12	<0.0001
Villus:Crypt ratio	1.76[a]	1.64[a]	1.41[b]	1.79[a]	0.07	0.001
Villus surface area, mm^2	0.052[a]	0.024[c]	0.038[b]	0.029[bc]	0.005	0.001
Colon						
Crypt depth, µm	243.62[a]	166.90[b]	272.51[a]	147.11[b]	12.36	<.00001

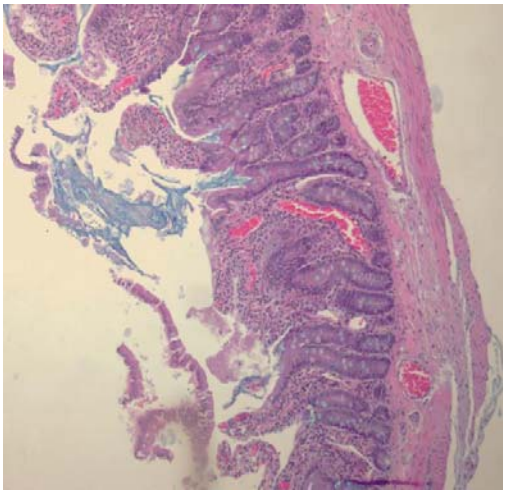
[1]BD=Basal diet; FDOI=Freeze-dried ovine immunoglobulin; IOI=Inactivated ovine immunoglobulin. [2]Villus Surface Area (VSA in mm^2) = (3.14×*mh*×h)+3.14×(*mh*/2)^2. where *mh(µm)* = villus width at midvillus height, h*(µm)*=villus height measured from the tip of the villus to the villus-crypt junction and crypt depth is measured from villus-crypt junction to the base of the crypt. [a,b,c] Means in a row without a common superscript differ, $P < 0.05$.

Figure 6.2 Transverse sections of rat duodenum. Section was stained with haemotoxylin, eosin and alcian blue.

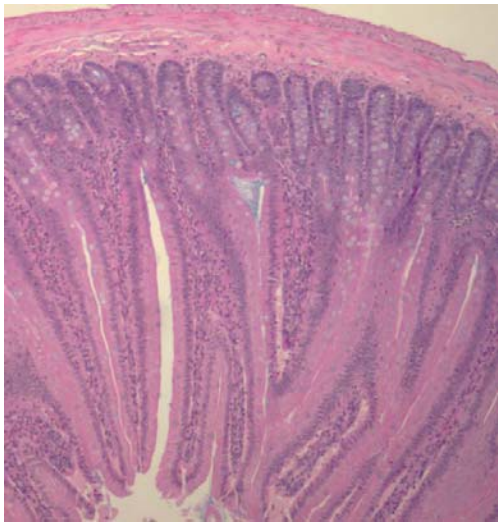
BD (saline)



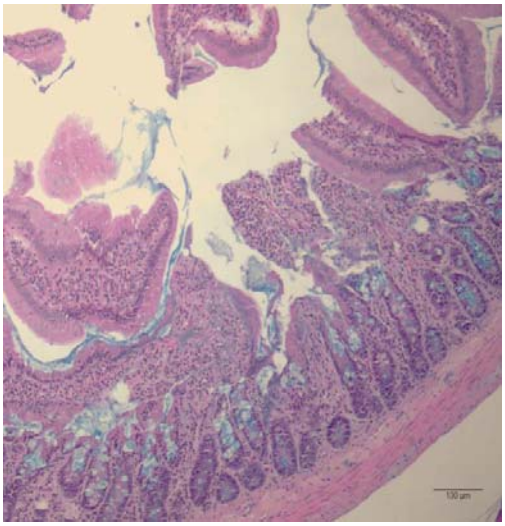
BD (challenged with *S. enteritidis*)



FDOI (challenged with *S. enteritidis*)



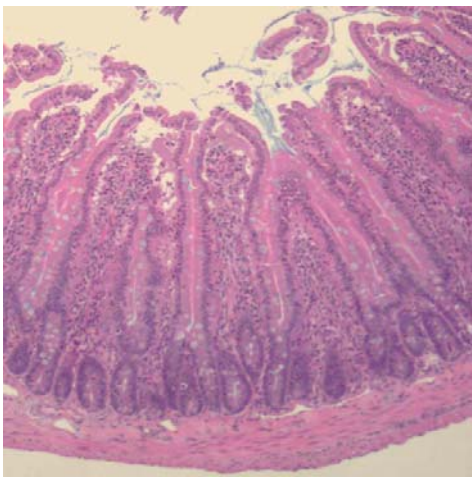
IOI (challenged with *S. enteritidis*)



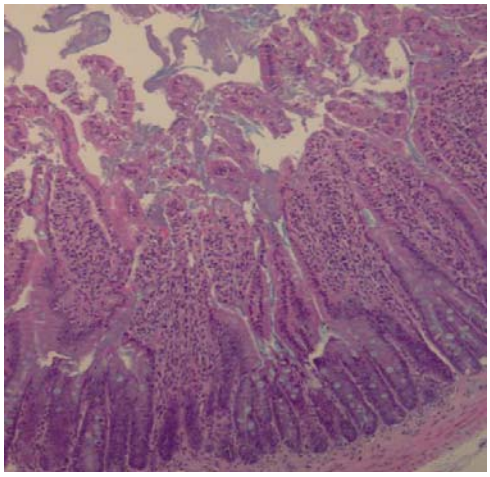
The intestinal (duodenum) damage resulting due to *S. enteritidis* administration to rats (BD and/or IOI, challenged group) is clearly evident by crypt loss, villus fusion, tip denudation, and atrophy, and low alcian blue stained goblet cells. But this is not evident for rats fed the FDOI and challenged with *S. enteritidis*.

Figure 6.3 Transverse sections of rat jejunum. Section was stained with haemotoxylin, eosin and alcian blue.

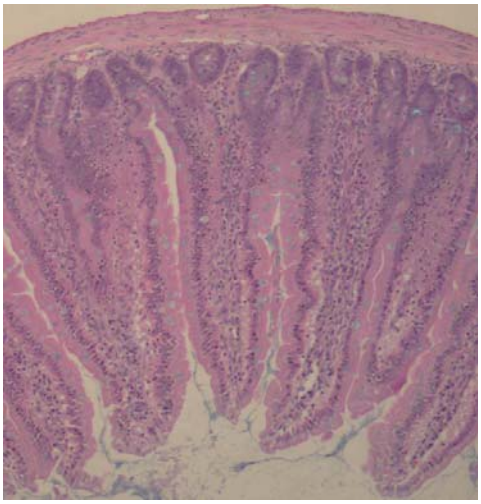
BD (saline)



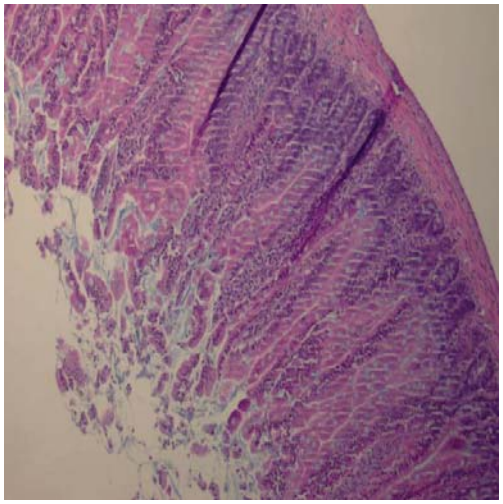
BD (challenged with *S. enteritidis*)



FDOI (challenged with *S. enteritidis*)



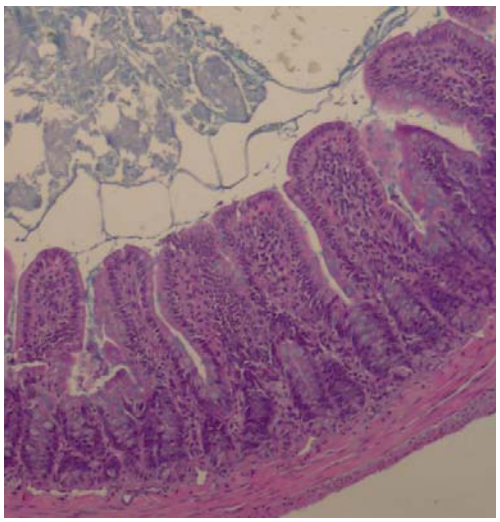
IOI (challenged with *S. enteritidis*)



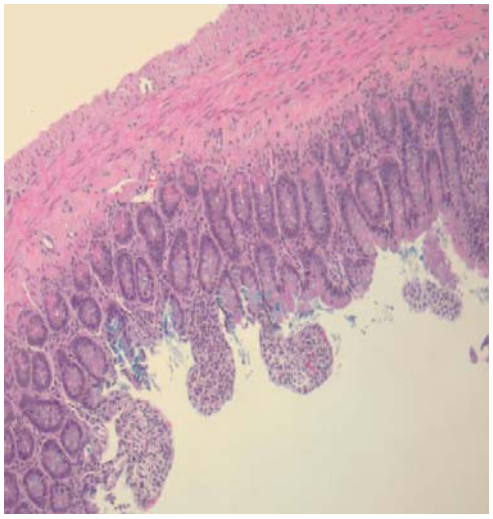
The intestinal (jejunum) damage resulting due to *S. enteritidis* administration to rats (BD and/or IOI, challenged group) is clearly evident by villus fusion, tip denudation and atrophy, crypt loss and loss of intact crypts, and low alcian blue stained goblet cells. But this is absent for rats (normal histological appearance) fed the FDOI and challenged with *S. enteritidis*.

Figure 6.4 Transverse sections of rat ileum. Section was stained with haemotoxylin, eosin and alcian blue.

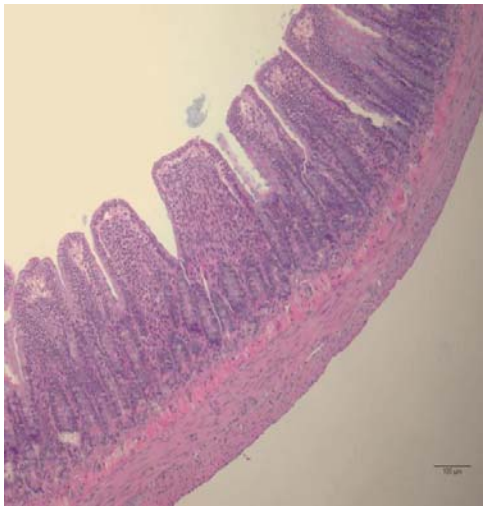
BD (saline)



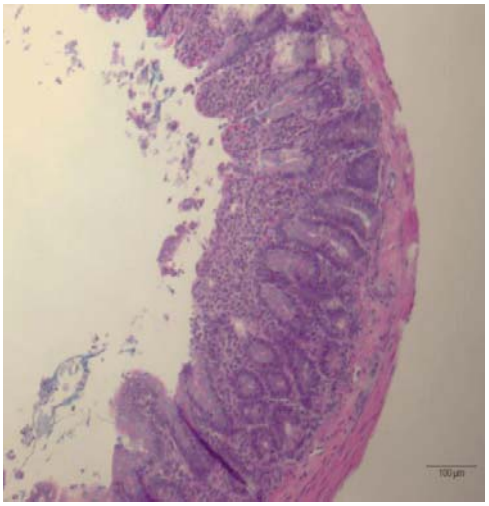
BD (challenged with *S. enteritidis*)



FDOI (challenged with *S. enteritidis*)



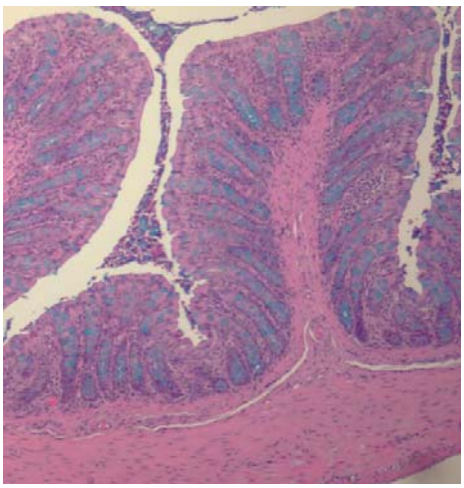
IOI (challenged with *S. enteritidis*)



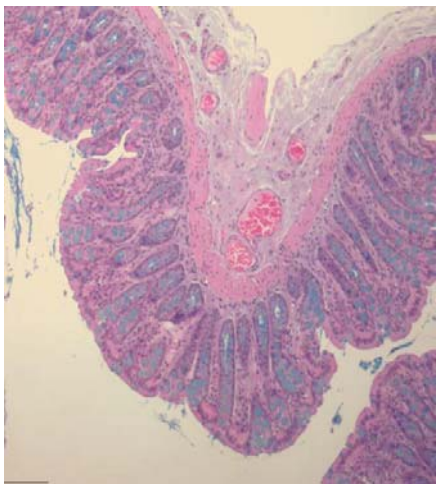
The intestinal (ileum) damage resulting due to *S. enteritidis* administration to rats (BD and/or IOI, challenged group) is clearly evident by villus fusion, tip denudation and atrophy, crypt loss and loss of intact crypts (pathalogical appearance), and low alcian blue stained goblet cells. But this is not evident for rats fed the FDOI and challenged with *S. enteritidis*.

Figure 6.5 Transverse sections of rat colon. Section was stained with haemotoxylin, eosin and alcian blue.

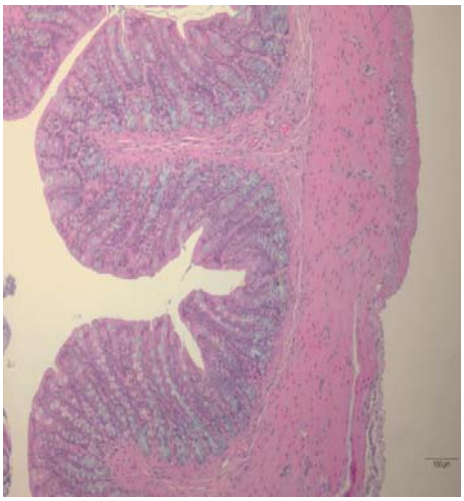
BD (saline)



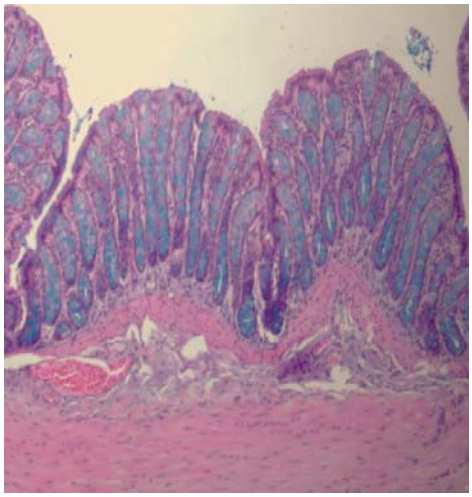
BD (challenged with *S. enteritidis*)



FDOI (challenged with *S. enteritidis*)



IOI (challenged with *S. enteritidis*)



S. enteritidis administration to rats had low histological damage in colonic segements for rats fed either BD or IOI. Bu there is evidence of intact crypts loss and low alcian blue stained goblet cells. But this is not seen for rats fed the FDOI and challenged with *S. enteritidis*.

Table 6.4 Mean (*n*=10) goblet cell counts in the intestine of growing rats after challenging with *S. enteritidis*[1].

	Unchallenged	Challenged with *S. enteritidis*				
Diet:	**BD**	**BD**	**FDOI**	**IOI**	**SEM**	***P* value**
	Villus goblet cells[2]					
Duodenum	104.57[b]	25.71[d]	137.86[a]	51.86[c]	7.96	<0.0001
Jejunum	99.14[a]	18.71[c]	107.14[a]	37.14[b]	6.16	<0.0001
Ileum	83.43[b]	29.57[c]	100.00[a]	38.43[c]	4.73	<0.0001
	Crypt goblet cells[2]					
Duodenum	64.33[b]	33.33[c]	76.00[a]	29.67[c]	3.1	0.001
Jejunum	46.57[b]	22.00[c]	54.29[a]	21.71[c]	2.45	<0.0001
Ileum	34.00[a]	18.86[b]	39.00[a]	18.57[b]	1.87	0.001
Colon	212.43[a]	138.00[b]	224.00[a]	138.14[b]	8.27	0.001
	Villus goblet cells[3]					
Duodenum	0.19[b]	0.04[d]	0.24[a]	0.09[c]	0.0038	<0.0001
Jejunum	0.25[a]	0.05[c]	0.26[a]	0.09[b]	0.0043	<0.0001
Ileum	0.46[b]	0.16[c]	0.51[a]	0.20[c]	0.0104	<0.0001
	Crypt goblet cells[3]					
Duodenum	0.27[b]	0.14[c]	0.33[a]	0.12[c]	0.0050	<0.0001
Jejunum	0.33[b]	0.16[c]	0.38[a]	0.15[c]	0.0117	<0.0001
Ileum	0.3[a]	0.17[b]	0.33[a]	0.16[b]	0.0116	0.001
Colon	1.39[a]	0.91[b]	1.46[a]	0.91[b]	0.0152	0.001

[1]BD=Basal diet; FDOI=Freeze-dried ovine immunoglobulin; IOI=Inactivated ovine immunoglobulin. [2] Number of goblet cells per five complete villi and their associated crypts. [3] Number of goblet cells per µm for five complete villi and their associated crypts.

6.4.5. Quantification of luminal mucin protein

Data for the mucin contents of intestinal digesta are shown in Table 6.5. In the ileum and colon, mucin protein content was significantly ($P < 0.05$) lower for the rats challenged and fed either the BD or IOI diets than for the unchallenged rats fed the BD diet. In contrast, in both segments, for the challenged rats fed the FDOI diet, mucin protein content was significantly ($P <$

0.05) higher than for the challenged rats fed either the BD or IOI diets, but was not different ($P > 0.05$) from the unchallenged rats fed the BD diet.

Table 6.5 Mean (n=10) concentration of mucin protein in the ileal and colonic luminal digesta for the growing rat following challenging with *S. enteritidis*[1].

	Unchallenged	Challenged with *S. enteritidis*				
Diet:	**BD**	**BD**	**FDOI**	**IOI**	**SEM**	***P* value**
Ileum (µg/mL)	10.69[a]	3.03[b]	9.95[a]	3.58[b]	1.85	0.007
Colon (ng/mL)	46.39[a]	5.95[b]	50.22[a]	9.51[b]	11.56	0.015

[1]BD=Basal diet; FDOI=Freeze-dried ovine immunoglobulin; IOI=Inactivated ovine immunoglobulin.

6.5. Discussion

Prior to *S. enteritidis* administration, rats fed the FDOI diet had a greater G:F ratio than the rats fed either the BD or IOI diets. This finding is consistent with Chapter-2 (*Section-2.4.1*) where rats fed a FDOI-based diet had a higher G:F ratio than rats fed with a basal diet containing no Ig or a diet containing IOI. In contrast, Owusu-Asiedu *et al*. (2002) found no difference in G:F ratio in pigs fed diets containing either an unautoclaved or autoclaved (or denatured) spray dried porcine plasma.

The experimental period during which the rats were challenged with *S. enteritidis* was restricted to 3 days for ethical reasons. After challenging with *S. enteritidis* over three days, the decrease in ADG and efficiency were drastic and highly statistically significant. In contrast, feed intake and water intake (data not shown) did not vary ($P > 0.05$) across the diets. Although ADG declined, we have no information on the composition of the body weight loss. This relatively short challenging period was deemed to be sufficient since the effects of *S. enteritidis* were expected to be acute. The dramatic effects of challenging on the BD-fed rats compared to the unchallenged counterparts observed for most of the parameters measured in this study clearly support this assumption. After challenging with *S. enteritidis*, the rats fed the IOI-supplemented and BD grew considerably more slowly than either the challenged rats fed the FDOI diet or the unchallenged rats fed the BD

diet. Furthermore the challenged FDOI-fed rats and the unchallenged BD-fed rats grew at the same rate suggesting that dietary Ig may be able to prevent colonization in the first place or reduce the *S. enteritidis* load to that of a normal unchallenged animal. When comparing the pre- and post-gavage periods, the ADG of the challenged rats fed either BD or IOI diets decreased by 64% and 32%, respectively. Similarly, the G:F ratio of the same rats decreased by 55 % and 27%, respectively. In contrast, for the unchallenged rats fed the BD diet and the challenged rats fed the FDOI diet, there was no significant reduction in growth performance between the pre- and post-gavage periods. These results clearly show that the dietary FDOI firstly enhanced the growth performance of the normal rat and secondly, completely negated any effects of *S. enteritidis* administration on growth.

Similar results have been reported in other studies using healthy animals and given diets containing FDOI (*Chapter-2, Section-2.4.1*), spray dried animal plasma (*Weaver et al., 1995*) and spray dried porcine plasma (SDPP) (*Gatnau et al., 1995; Owen et al., 1995*). Pierce *et al*. (2005) conducted a comprehensive study examining the effects of dietary SDPP, spray dried bovine plasma, porcine Ig and bovine Ig in healthy early weaned piglets and showed that all Ig preparations resulted in higher growth rates. They concluded that the improved growth performance was due to a nutrient-sparing effect as a result of the action of the consumed Ig. Johnson (1997), reported that oral administration of antigens such as either pathogenic or non-pathogenic organisms induced production of the pro-inflammatory cytokines IL-1, IL-6 and TNF-α and acute phase proteins, thereby increasing the demand for amino acids for non-growth functions and reducing overall animal growth. Consequently, a lowered activation of the immune system may lead to a greater amount of dietary nutrients and energy being available for growth (*Coffey & Cromwell, 1995*).

The small intestine and colon of the rats challenged with *S. enteritidis* and receiving the FDOI-containing diet were heavier (possibly due to the presence of more, and larger villi) when compared to similarly challenged rats fed the control diet but were the same weight as those for the unchallenged rats fed BD diet. In addition, in this study weights of the caecum, liver and spleen were also higher in the challenged rats fed either the BD or IOI diets compared to challenged rats fed the FDOI diet or unchallenged rats fed the BD diet. Some of our results are similar to the recent findings of Lu *et al*. (2010) where they found that mice challenged with *Salmonella tyhpimurium*

had a significant increase in both liver and spleen weights after infection. Similarly, feeding FDOI diet prophylactically may have reduced the *S. enteritidis* attachment to the gut wall. This clearly suggests that the anti-*Salmonella* Ig present in the FDOI diet may have prevented translocation of *S. enteritidis* (*Han et al., 2009*).

In the present study, oral dosing with *S. enteritidis* to rats and fed the FDOI diet had longer villi, greater crypt depths and greater villi surface area in most of the small intestinal sections, which may have resulted in a greater absorption of available nutrients from the gut leading to the higher growth rate observed for the FDOI-fed and *S. enteritidis*-challenged rats. In contrast, challenging with *S. enteritidis* appeared to cause villus atrophy for those rats fed either BD or IOI based supplanted diets. This was similar to the finding of Gatnau *et al*. (1995) who reported that feeding either spray dried porcine plasma or its corresponding Ig fraction to healthy weaned pigs resulted in an increased villus surface area, while Touchette *et al*. (1997) reported that healthy weaned piglets fed a spray dried plasma had longer villi with a greater villus:crypt ratio compared to animals not receiving the sprayed dried plasma. Chapter-2 (*Section-2.4.1.3*) showed that in normal rats fed the FDOI-based diet there was an increase in villus length, crypt depth, villus:crypt ratio and villus surface area compared to similar rats fed BD (containing no Ig) or a diet containing inactivated ovine Ig.

Mucins are the high molecular weight glycoproteins secreted by goblet cells present in the columnar epithelium (*Moncada & Chadee, 2002*) and are one of the main constituents of the gut barrier that protects the gut from bacterial infection (*Moncada & Chadee, 2002*). In the present study, oral dosing with *S. enteritidis* dramatically reduced the amount of mucin in the gut and this was likely caused, at least in part, by the observed reduction in the number of villi and crypt goblet cells in the small and large intestine. These results are consistent with the findings of other workers (*Lambert et al., 1979*). In contrast, dietary freeze-dried ovine Ig, but not inactivated ovine Ig, appeared to completely negate the negative effects of *S. enteritidis* on mucin secretion and goblet cell numbers. FDOI-based diets have also been shown to increase villi and crypt goblet cell numbers throughout the gut in healthy rats (*Chapter-5*), while dietary colostrum or plasma resulted in an increase of villus and crypt goblet cells in healthy pigs (*King et al., 2008*). Depletion of the goblet cells after infection has also been reported in a murine model infected with *Citrobacter rodentium* (*Bergstrom et al., 2008; Lindé et al., 2008*).

Moreover, pathogens such as *H. pylori* have been shown to specifically inhibit mucin synthesis in the gut (*Slomiany & Slomiany, 2006*).

In conclusion, it appears that feeding freeze-dried ovine immunoglobulin to rats negated the negative impacts of orally administered *S. enteritidis* to such a degree that the performance of the *S. enteritidis* challenged rats fed FDOI was the same or superior to normal unchallenged rats.

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Chapter 7

Immunomodulatory effects of ovine serum immunoglobulin in growing rats challenged with *Salmonella enteritidis*

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It appears from the previous Chapter (Chapter 6) that feeding ovine Ig to rats negated the negative effects of *Salmonella enteritidis* to such a degree that the performance of the *S. enteritidis* challenged rats fed FDOI-based diets was the same or superior to normal unchallenged rats. Moreover, in Chapters 3 & 4 we demonstrated that feeding ovine Ig to normal growing rats resulted in up-regulation of various indices of immune function and positively modulated the gut microbial population, especially *Lactobacillus sp*. Therefore, the aim of the work described in this Chapter was to determine the effect of dietary ovine Ig on aspects of immunity and the associated gut microflora in *S. enteritidis*-challenged rats.

7.1. Abstract

This study aimed to determine whether orally administered ovine serum immunoglobulins modulate aspects of immunity and associated gut microflora in growing rats challenged with *Salmonella enteritidis*. The four groups consisted of rats fed a casein-based control diet (BD, unchallenged) and three groups of rats challenged with $1X10^7$ viable *S. enteritidis* and fed either a BD diet, a BD diet containing freeze-dried ovine Ig (FDOI) and a BD diet containing inactivated ovine Ig (IOI). The rats were randomly allocated to one of the four diets (n=15) and received their respective diets for an 18 day experimental period. Oral challenging took place on day 15. Phagocytic activity of peripheral blood leukocytes (PBL) and lymphocyte proliferation in the presence of the concanavalin A (Con A) was greater ($P < 0.05$) for the unchallenged BD- and the challenged FDOI-fed rats than the challenged rats fed either the BD or IOI diets. ConA stimulated Peyer's patch cells and splenocytes from the challenged rats fed the FDOI diet produced higher ($P < 0.05$) IFNγ, IgA, IgG than the challenged rats fed either the BD or IOI diets. The challenged FDOI-fed rats had higher ($P < 0.05$) anti-*Salmonella* sIgA (secretory IgA) and sIgG (secretory IgG) in the ileal and colonic digesta and plasma. Analysis of a DGGE profile revealed that six bands out of ten had sequence similarity to *Leuconostoc citreum, Weissella cibaria*, and *Lactobacillus johnsonii* strains of bacteria in the ileum and colon of the challenged FDOI-fed rats. In conclusion, an ovine Ig fraction positively modulated various indices of immune function and associated gut microflora in growing rats challenged with *S. enteritidis*.

7.2. Introduction

Several studies carried out in production animals at weaning have established beneficial effects from the ingestion of spray dried animal plasma (SDAP) containing Ig, such as increased intestinal tissue growth rate, increased feed intake and higher body weight gain (*Kats et al., 1994; Torrallardona et al., 2003; Pierce et al., 2005*). Bosi *et al*. (2004) reported that the Ig present in SDAP appear to exhibit beneficial effects by binding to pathogenic bacteria, thereby preventing their attachment to the mucosa of the gastrointestinal tract (GIT).

Ig are a primary anti-infective component of plasma, colostrum and breast milk. These specialized proteins protect the body from harmful bacteria, viruses and other environmental pathogens by either binding to them or by forming an encapsulating barrier (*Kraehenbuhl & Neutra, 1992*). Orally administered human Ig have been used as a prophylactic treatment for children born prematurely with necrotizing enterocolitis (*Eibl et al., 1988*). To date, Ig have been used therapeutically for intervention of *Campylobacter jejuni* (*Hammarstrom et al., 1993*), *Clostridium difficile* (*Tjellstrom et al., 1993*), *Helicobacter pylori*, rotavirus and cryptosporidial infection. Reports have shown that orally administered Ig retain activity even after digestion in the GIT (*Tjellstrom et al., 1993; Bogstedt et al., 1996*). Roos *et al*. (1995) demonstrated in an *in vivo* study that the recovery of immunologically active nitrogen from digested IgG and IgM was around 19% in the terminal ileum of healthy human adults.

Han *et al*. (2009) using an *in vitro* approach reported an inhibitory activity of ovine Ig concentrate against 13 strains of pathogenic bacteria including *Salmonella* sp. Previously, our group has reported that feeding freeze-dried ovine serum Ig (FDOI; and Ig extracted from lamb's blood) to normal growing rats improved growth performance, increased the weight of some organs and improved gut architecture, such as increased villus height, surface area and crypt depth (*Chapter-2*). This has also been shown to be the case in *S. enteritidis*-challenged growing rats (*Chapter-6*). We have also found that feeding FDOI to growing rats led to a higher degree of immunological activity (phagocytic activity, lymphocyte proliferation, cytokine production) and intestinal and plasma Ig levels (*Chapter-3*). This may have been associated with an observed selective up-regulation of beneficial

bacteria such as *Lactobacillus* sp. in the gut microbiota (*Chapter-4, Section-4.4*). On the basis of these findings we hypothesize that administered ovine Ig may strengthen mammalian immune function and may support the development of beneficial bacteria in the gut, and may thus be helpful in protecting against various enteric infections by pathogenic bacteria such as *S. enteritidis*.

This study aimed to extend previous work which had been conducted with normal rats to animals challenged with *S. enteritidis*. The effect of dietary ovine Ig on aspects of immunity and the associated gut microflora was investigated. Comparison was made between rats fed a casein based diet containing no Ig (BD, unchallenged) and *S. enteritidis*−challenged rats fed either the BD or a casein-based diet supplemented with either FDOI or inactivated freeze-dried ovine Ig (IOI).

7.3. Materials and methods

7.3.1. Preparation and quantitation of ovine serum Ig

An ovine Ig fraction was prepared by ammonium sulphate precipitation of fresh lamb's blood as described previously (*Chapter-1*). The final product was freeze-dried and a portion was inactivated by autoclaving (121°C, 15 min) to produce the IOI. The IgG content in the ovine Ig fraction was determined by direct ELISA using a rabbit anti sheep IgG (AbD Serotec, Oxford, UK) as described previously (*Chapter-2*).

7.3.2. Experimental diets

Three semi-synthetic diets were formulated, 1) a basal casein-based diet containing no Ig, 2) the basal casein-based diet supplemented with FDOI and 3) the basal casein-based diet supplemented with IOI. The ingredient composition and determined nutrient composition of the diets are shown in Table 6.1 (*Chapter-6, Section-6.3.2*). All diets were formulated to exceed the recommended nutrient requirements for the growing rat (*National Research Council, NRC, 1995*) for all nutrients. The FDOI and IOI fractions were included in the respective diets at a concentration approximately equal to the amount of SDPP IgG used in previous studies (*Pierce et al., 2005; Chapter-2*).

7.3.3. Animal study

This work was approved by the Massey University Animal Ethics Committee (MUAEC 09/29) and procedures complied with the New Zealand Code of Recommendations and Minimum Standards for the Care and Use of Animals for Scientific purposes (New Zealand Animal Welfare Advisory Committee, 1995). Animal trial was carried out as described in Chapter-6 (*Section-6.3.3*).

7.3.4. Chemical analysis

Dietary dry matter, ash, crude protein, total fat and gross energy were determined as described previously (*Chapter-2, Section-2.3.3*).

7.3.5. Post-mortem procedure

On the final day (d 18) of the experimental period, the rats were anaesthetized by intraperitoneal injection (0.1 mL/100g live weight) of a mixture containing 2 parts acepromazine maleate BP (ACP, 2g/L), 5 parts ketamine (100 g/L), 1 part xylazine (100 g/L) and 2 parts sterile water. Blood was collected by cardiac puncture and a portion was immediately transferred to either an EDTA Vacutainer® blood collection tube (BD, NZ) to isolate plasma for the measurement of plasma IFNγ TNFα, IL-4, IL-10, IgA, IgG and IgE or to a heparinVacutainer® tube (BD, NZ) for assessing the phagocytic activity of PBL. The PP and spleen were removed aseptically for preparation of cell suspensions for use in the lymphocyte proliferative assay. The ileum (anterior to ileocaecal valve) and entire colon were collected and washed intraluminally with 5mL of phosphate buffer saline containing 1% protease inhibitor (Sigma, NZ). The suspensions were then centrifuged at 3000 x *g* for 10 min to remove debris and the clear supernatant was stored at -20°C until the measurement of intestinal IgA and IgG.

7.3.6. Assessment of phagocytosis

Assessment of the phagocytic activity of PBL by flow cytometry was performed as previously described in Chapter-3, Section-3.4.5. The level of phagocytic activity was expressed as a proportion of phagocytically active PBL.

7.3.7. Lymphocyte proliferation assay

Lymphocytes were isolated from the spleens of unchallenged (BD fed) and challenged (BD-, FDOI-, IOI-fed) rats as described previously (*Chapter-3, Section-3.4.6*), with the exception that the viable lymphocytes were identified (by trypan blue exclusion) and manually counted on a haemocytometer. A similar procedure was followed to isolate leukocytes from PP. The assay was performed based on a colorimetric MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay. Aliquots of 10^5 spleen lymphocyte cells in 100 µL of complete RPMI 1640 medium were added in triplicate to the wells of a 96-well, flat-bottomed tissue culture plate (Nunc, MO, USA) and cultured in the presence of either 20 mg/L Concanavalin A (Con A) (Sigma, NZ), or 20 mg/L Lipopolysaccharide (LPS) (Sigma, NZ), or complete RPMI-1640 in place of the mitogen (control wells). After 72 h incubation at 37°C in the 5% CO_2 incubator, 10 µL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 5 g/L, Sigma, NZ) was added into each well and the material incubated for another 4 h at 37°C in the 5% CO_2 incubator and then 100 µL of acid-isopropanol (0.04 N HCl in isopropanol) was added into each well and mixed for 5 min to completely dissolve the precipitate. Absorbance was measured at 570 nm using an ELISA plate reader (Anthos Microplate reader, UK). A similar procedure was followed for the PP cell proliferative assay (*Lee et al., 1993*). Proliferative response was calculated as absorbance in wells with mitogen divided by absorbance in wells without mitogen.

7.3.8. Analysis of cytokines

Spleen cell suspensions (4 X 10^6 cells/mL) were added to each well of a 24-well plate (Costar) and cultured in the presence/absence of ConA (20 mg/L; Sigma, NZ) for 48 h at 37°C. A similar procedure was followed for the PP cells. Cell-free supernatant fractions were harvested and stored at -20°C until assayed. The presence of IFNγ and IL-4 in the culture supernatants was determined by sandwich ELISA using IFNγ and IL-4 cytokine DuoSet ELISA kits (R&D systems, UK). Cytokine concentrations were calculated by subtracting the amount of cytokine detected from the mitogen stimulated cell culture with those from unstimulated cell cultures. The presence of the cytokines IFNγ, IL-4, TNFα and IL-10 was also measured in plasma. The TNFα and IL-10 in the plasma were determined by using Bender

MedSystems (Rat IL-10 and TNFα MODULE SET, Austria) according to the manufacturer's instructions.

7.3.9. Quantitative analysis of IgA, IgG and IgE

A sandwich rat IgA and IgG ELISA was used to quantify the IgA and IgG concentrations in plasma, in intestinal digesta (ileum and colon), and in ConA stimulated (stimulated minus unstimulated) culture supernatants of both spleen and PP cells, as described previously (*Chapter-3, Section-3.4.8*). All antibodies were from AbD Serotec (UK). Similarly, for IgE quantitation, an ELISA procedure was followed using purified rat IgE kappa standard (PRP07A), goat anti rat IgE (MCA193) and horseradish peroxidase-conjugated mouse anti rat kappa lambda (MCA1296P, 1:2000).

7.3.10. Quantitative analysis of salmonella specific IgA and IgG

Salmonella specific IgA and IgG were measured as described previously (*Biedrzycka et al., 2003*) with some modification. Microplates were coated with 100 µL of an autoclaved suspension of *S. enteritidis* ATCC 13076 (10^8 cfu/mL in carbonate buffer, pH 9.6) by incubating for 20 h at 37°C. Plates were then fixed with 70% methanol (300 µL), and washed (3 times) with phosphate buffered saline with 0.05% Tween-20 (PBS-T), pH 7.4. The unbound sites on the microplates were blocked with 3% bovine serum albumin (Sigma, NZ), incubated at 37°C for 1h and then washed (3 times) with PBS-T. Rat ileal and colonic digesta samples (1/100 diluted with PBS-T for IgA and undiluted for IgG) and plasma samples (diluted 1/100 and 1/10 for IgG and IgA respectively) were added in duplicate to microplates, which were incubated at 37°C for 1h and then washed (3 times). Peroxidase-conjugated (100 µL/well) mouse anti-rat antibody IgA and sheep anti rat IgG (STAR111P and AARP10P, AbD Serotec) were added, incubated at 37°C for 1 h and washed 3 times. TMB enzyme substrate (3,3',5,5'-Tetramethyl-benzidine) was added (100 µL/well) to the plates and incubated at 37° C for 20 minutes and then the reaction was stopped by addition of 50 µL of 1 M H_2SO_4 to each well. Absorbance was determined at 450 nm on an Anthos ELISA plate reader (Austria) and antibody concentration in each sample was expressed as optical density (OD_{450nm}).

7.3.11. Isolation of bacterial DNA from digesta and PCR-DGGE

Isolation of bacterial DNA from ileal and colonic digesta, PCR-DGGE and sequencing of DGGE gel bands was performed as previously described in Chapter-4, Section-4.3.5 with some modification. The PCR reaction was initially activated at 95°C for 15 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 45 sec, extension at 72°C for 1 min, and a final extension at 72°C for 10 min and cooling to 4°C.

DGGE analysis of PCR products (430 to 470 bp) was performed using the DCode™ system (Bio-Rad, USA) at 130 V for 6 h in a 1.25 x TAE buffer at a constant temperature of 60°C on 8% polyacrylamide gels. Optimal separation was achieved by a gradient of 35 to 55% denaturant. The DNA bands were visualized by ethidium bromide staining (50 mg/L) and then photographed under UV transillumination using the GelDoc™ XR 2000 system (Bio-Rad, USA).

Nucleotide sequence data of excised DGGE bands reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB576354 to AB576363 and in appendix E.

7.3.12. Statistical analysis

This study was part of a larger growth trial. For most variables there were 15 rats per treatment, but in some cases a lower sample size was used. Results are expressed as mean ± SEM. Data were analyzed using a one-way analysis of variance using the General Linear Models procedure of SAS/PROC GLM (SAS, version 9.1, SAS Institute Inc, Cary, NC) and means were compared using Tukey's test. Statistical significance was accepted at $P < 0.05$.

7.4. Results

The effect of administration of *S. enteritidis* to the rats was acute. Most of the rats from the BD- and IOI-supplemented diet groups showed symptoms of *S. enteritidis* infection (nasal discharge and red crusts around nostrils) after 3 d of challenging, the study culminated for ethical reasons. After challenge with *S. enteritidis* over three days, there was a marked decrease in body wt gain/d

and food conversion efficiency ($P < 0.05$). In contrast, feed intake and water intake did not vary ($P > 0.05$) across the groups fed different diets. Although body wt gain/d declined, we have no information on the composition of the body weight loss. These results were discussed fully elsewhere (*Chapter-6, Section-6.4.1*).

7.4.1. Hematology

There was no significant ($P > 0.05$) difference in RBC (red blood corpuscles), hemoglobin, lymphocytes and monocytes across all treatment groups. Dietary intervention and *S. enteritidis* did not affect the hematology profiles ($P > 0.05$) except in rats challenged with *S. enteritidis* and fed either the BD- or IOI-supplemented diet had significantly higher ($P < 0.05$) neutrophil, WBC (white blood corpuscles) count when compared to the challenged rats fed the FDOI diet or unchallenged rats receiving the BD diet (Table 7.1).

Table 7.1 Mean haematological parameters (n=15) in the growing rat after challenging with *S. enteritidis*[1].

	Unchallenged	**Challenged with *S. enteritidis***				
Diet :	**BD**	**BD**	**FDOI**	**IOI**	**SEM**	***P* value**
		Hematology				
RBC	7.97	8.18	8.14	8.32	0.22	0.754
WBC	6.79[b]	13.20[a]	7.66[b]	10.98[a]	1.37	0.043
Hemoglobin	122.3	154.2	155.0	156.4	3.19	0.365
Neutrophils	0.80[b]	4.42[a]	0.91[b]	3.50[a]	0.89	0.048
Lymphocytes	5.67	7.05	5.54	6.20	0.92	0.409
Monocytes	0.18	1.42	0.19	1.27	0.43	0.061

[1]BD=Basal diet; FDOI=Freeze-dried ovine immunoglobulin; IOI=Inactivated ovine immunoglobulin. RBC (10^{12}/L); WBC (10^9/L); Hemoglobin (g/L); Neutrophils (10^{12}/L); Lymphocytes (10^9/L); Monocytes (10^9/L). [a,b]Means in a row without a common superscript differ, $P < 0.05$

7.4.2. PBL phagocytosis

Levels of phagocytic activity were greater ($P < 0.05$) in the unchallenged BD-fed rats than the challenged rats fed either the BD or IOI diets. But, phagocytic activity was significantly ($P < 0.05$) greater in the challenged FDOI-fed rats than the challenged rats fed either BD or IOI diets. There was no difference ($P > 0.05$) between the unchallenged BD-fed rats and the challenged FDOI-fed rats, or between the challenged rats fed either BD or IOI

diets (Table 7.2).

7.4.3. Lymphocyte proliferation

Peyer's patch cells (PP) stimulated with the mitogens ConA or LPS resulted in higher (P < 0.05) lymphocyte proliferative responses in the challenged FDOI-fed rats when compared to the challenged rats fed either the BD or IOI diets (Table 7.2).

ConA-induced lymphocyte proliferative responses of spleen cells in the challenged FDOI-fed rats were significantly greater (P < 0.05) when compared to the other three groups (Table 7.2). However, consumption of the different diets (BD, FDOI and IOI) did not result in any difference (P > 0.05) in splenocyte lymphocyte proliferative responses to LPS.

Table 7.2 Phagocytic activity of peripheral blood leucocytes and proliferative responses of Peyer's patch cells and spleen cells to ConA and LPS in the unchallenged rats fed the BD diet and rats challenged with *S. enteritidis** and fed either the BD, FDOI or IOI diets[1].

	Unchallenged	**Challenged with *S. enteritidis***				
Diet :	**BD**	**BD**	**FDOI**	**IOI**	**SEM**	***P* Value**
	Phagocytic activity of PBL[1]					
	9.82^{a}	6.78^{b}	8.94^{a}	6.63^{b}	0.064	0.01
	Lymphocyte proliferative response					
	Peyer's patch cells[2]					
ConA	1.13^{ab}	1.06^{bc}	1.19^{a}	1.03^{c}	0.032	0.01
LPS	1.06^{ab}	0.99^{b}	1.09^{a}	0.99^{b}	0.026	0.03
	Spleen cells[2]					
ConA	1.16^{b}	1.14^{b}	1.53^{a}	1.26^{b}	0.07	0.02
LPS	1.09	1.07	1.17	1.11	0.02	0.91

[1]Values are means ± SE, n=8. [2]Values (OD at 570nm) are means ± SEM, n=8, mitogen stimulated minus unstimulated, * 3 d of challenge. Means in a row with superscripts without a common letter differ, P < 0.05. BD=Basal diet; FDOI=Freeze dried ovine immunoglobulin; IOI=Inactivated ovine immunoglobulin.

7.4.4. Cytokine and Ig analysis in PP and spleen cell culture supernatants (ConA stimulated minus ConA unstimulated)

In PP cell cultures, IFNγ production was higher (P = 0.002) in the challenged FDOI-fed rats than the other three groups. IL-4 levels were not affected (P > 0.05) by dietary treatment. IgA and IgG levels were higher (P < 0.05) for the challenged FDOI-fed rats compared to either the challenged rats receiving BD or IOI diets. However, there was no difference (P > 0.05) between the unchallenged rats fed the BD and challenged rats fed FDOI diet. In splenocyte cultures, IFNγ, IgA and IgG levels were the highest (P < 0.05) in the challenged FDOI-fed rats when compared to the other groups (Table 7.3). IL-4 levels were not affected (P > 0.05) by dietary treatment (Table 7.3).

Table 7.3 IFNγ, IL-4, IgA and IgG production by ConA stimulated Peyer's patch cells and spleen cells in unchallenged rats fed the BD diet and rats challenged with *S. enteritidis** and fed either the BD, FDOI or IOI diets[1].

	Unchallenged	Challenged with *S. enteritidis*				
Diet :	**BD**	**BD**	**FDOI**	**IOI**	**SEM**	***P* Value**
	Peyer Patches cells					
IFNγ, ng/L	3540.5^{b}	3198.5^{b}	4856.2^{a}	3372.8^{b}	298.28	0.002
IL-4, ng/L	202.8	201.6	207.0	201.1	5.76	0.941
IgA, mg/L	4348^{ab}	1582^{c}	6390^{a}	2776^{bc}	653.34	0.001
IgG, mg/L	876.5^{a}	353.7^{b}	1082^{a}	84.8^{b}	144.19	0.002
	Spleen cells					
IFNγ, ng/L	2568.8^{b}	2144.3^{b}	5650.0^{a}	2154.5^{b}	936.74	0.032
IL-4, ng/L	230.2	240.1	234.2	238.5	7.39	0.841
IgA, mg/L	6694^{b}	2586^{d}	8384^{a}	3860^{c}	653.17	0.002
IgG, mg/L	199.2^{b}	133^{b}	302.7^{a}	160^{b}	26.24	0.004

[1]Values (ConA stimulated minus ConA unstimulated) are means ± SEM, n=8, *3 d of challenge, Means in a row with superscripts without a common letter differ, P < 0.05. BD=Basal diet; FDOI=Freeze-dried ovine Ig; IOI=Inactivated ovine Ig.

7.4.5. Cytokine and Ig analysis in plasma

Dosing with *S. enteritidis* resulted in significantly ($P < 0.05$) higher IFNγ, TNFα, IL-10 and IgA concentrations for the challenged BD-fed rats compared to the unchallenged BD-fed rats. This effect was completely negated in the challenged rats fed the FDOI-based diet. Challenged rats receiving the IOI diet were found to have similar ($P > 0.05$) cytokine (except TNFα) and IgA concentrations when compared to the challenged rats fed the BD diet. Plasma IgE concentrations were higher ($P = 0.01$) in the challenged rats receiving the IOI diet than for the other groups (Table 7.4).

Table 7.4 IFNγ, IL-4, IL-10, TNFα, IgA, IgG, and IgE concentrations in plasma for the unchallenged rats fed the BD diet and rats challenged with *S. enteritidis** and fed either the BD, FDOI or IOI diets[1].

	Unchallenged	**Challenged with *S. enteritidis***				
Diet :	**BD**	**BD**	**FDOI**	**IOI**	**SEM**	***P* Value**
IFNγ, ng/L	526^{b}	14017.1^{a}	760.3^{b}	7531.7^{ab}	1931.04	0.05
IL-4, ng/L	42.1	44.2	40.0	47.4	7.40	0.96
TNFα, ng/L	323.8^{b}	439.1^{a}	343.8^{b}	365.7^{b}	10.57	0.01
IL-10, ng/L	109.3^{b}	141.2^{a}	114.6^{b}	147.8^{a}	5.88	0.01
IgA, mg/L	464.8^{b}	1153.5^{a}	593.5^{b}	1025.4^{a}	135.95	0.01
IgG, mg/L	23.5	19.8	21.0	16.7	1.18	0.12
IgE, mg/L	1 2405^{b}	14716^{b}	14758^{b}	27169^{a}	1710.30	0.01

[1]Values are means ± SEM, n=8, *3 d of challenge, Means in a row with superscripts without a common letter differ, $P < 0.05$. BD=Basal diet; FDOI=Freeze-dried ovine immunoglobulin; IOI=Inactivated ovine immunoglobulin.

7.4.6. Ig analysis in intestinal digesta

In the ileal digesta, challenging with *S. enteritidis* led to significantly ($P = 0.03$) higher IgA concentrations for the challenged rats fed the BD or IOI diets compared with the unchallenged rats fed the BD diet. This effect was not found in the challenged FDOI-fed rats which had similar ileal digesta IgA

concentrations (P > 0.05) to the unchallenged BD-fed rats. However, IgG levels were not affected (P > 0.05) by dietary treatment.

In the colonic digesta, the challenged rats fed the BD diet had significantly (P < 0.05) higher IgA and IgG concentrations than to the unchallenged rats fed the BD diet. This effect was not evident (P > 0.05) for the challenged rats fed the FDOI diet (Table 7.5).

Table 7.5 Ig concentrations in intestinal digesta for unchallenged rats fed the BD diet and rats challenged with *S. enteritidis** and fed either the BD, FDOI or IOI diets[1].

	Unchallenged	**Challenged with *S. enteritidis***				
	BD	**BD**	**FDOI**	**IOI**	**SEM**	***P* Value**
		Ileal digesta				
IgA, mg/L	10751^{b}	20522^{a}	11194^{b}	20750^{a}	2490.44	0.03
IgG, mg/L	777.3	1266.8	787	1136.4	176.42	0.16
		Colonic digesta				
IgA, mg/L	1300.9^{b}	3485.9^{a}	1412.0^{b}	2840.6^{ab}	462.51	0.02
IgG, mg/L	401.7^{b}	819.5^{a}	427.5^{b}	713.7^{ab}	92.59	0.05

[1]Values are means ± SEM, n=8, *3 d of challenge, Means in a row with superscripts without a common letter differ, P < 0.05. BD=Basal diet; FDOI=Freeze-dried ovine immunoglobulin; IOI=Inactivated ovine immunoglobulin.

7.4.7. Anti-S. enteritidis (ASE) Ig analysis in intestinal digesta

Feeding rats (challenged with *S. enteritidis*) the FDOI diet significantly (P < 0.05) enhanced ASE-sIgA in the ileal digesta, colonic digesta and plasma when compared to the other groups. The levels of ASE-IgG were also found to be significantly (P < 0.05) enhanced in the colonic digesta and plasma but not in the ileal digesta of the challenged rats fed the FDOI diet compared to the other groups (Table 7.6).

Table 7.6 Concentrations of specific anti-*S. enteritidis* IgA and IgG in intestinal digesta and plasma for unchallenged rats fed the BD diet and rats challenged with *S. enteritidis** and fed either the BD, FDOI or IOI diets[1].

	Unchallenged	Challenged with *S. enteritidis*				
	BD	**BD**	**FDOI**	**IOI**	**SEM**	***P* Value**
			Anti-Salmonella IgA[2]			
Ileal digesta	0.36^{c}	0.55^{b}	0.86^{a}	0.57^{b}	0.03	0.01
Colonic digesta	0.31^{c}	0.45^{b}	0.93^{a}	0.47^{b}	0.04	0.01
Plasma	0.20^{b}	0.27^{b}	0.45^{a}	0.29^{b}	0.05	0.01
			Anti-Salmonella IgG[2]			
Ileal digesta	0.37	0.50	0.50	0.50	0.02	0.88
Colonic digesta	0.32^{b}	0.48^{b}	0.81^{a}	0.57^{b}	0.08	0.01
Plasma	0.34^{b}	0.39^{b}	0.83^{a}	0.42^{b}	0.09	0.01

[1]Values are means ± SEM, *n*=8, *3 d of challenge, Means in a row with superscripts without a common letter differ, $P < 0.05$. [2]Data are shown in absorbance (450 nm). BD=Basal diet; FDOI=Freeze-dried ovine immunoglobulin; IOI=Inactivated ovine immunoglobulin.

7.4.8. Bacterial communities in ileal and colonic digesta

Ten prominent DGGE bands obtained from pooled (*n*=15) ileal and pooled colonic digesta samples for the challenged rats fed the FDOI diet were isolated and sequenced (Fig 7.1 and Table 7.7). Bands ILFS1, ILFS2, ILFS3, ILFS4 and ILFS5 from the ileal digesta had sequence similarities between 95% and 99% to *Leuconostoc citreum, Weissella cibaria* (ILFS2 & ILFS4), and *L. johnsonii* strain (ILFS3 & ILFS5). Bands CFS1, CFS2, CFS3, CFS4 and CFS5 from the colonic digesta had sequence similarities of 99% or 100% to *L. johnsonii* (CFS1 & CFS5), *L. citreum, Akkermansia muciniphila* and an uncultured bacterium clone (next close relative to *Lactobacillus johnsonii* strain of 93% sequence similarity from cultured bacteria).

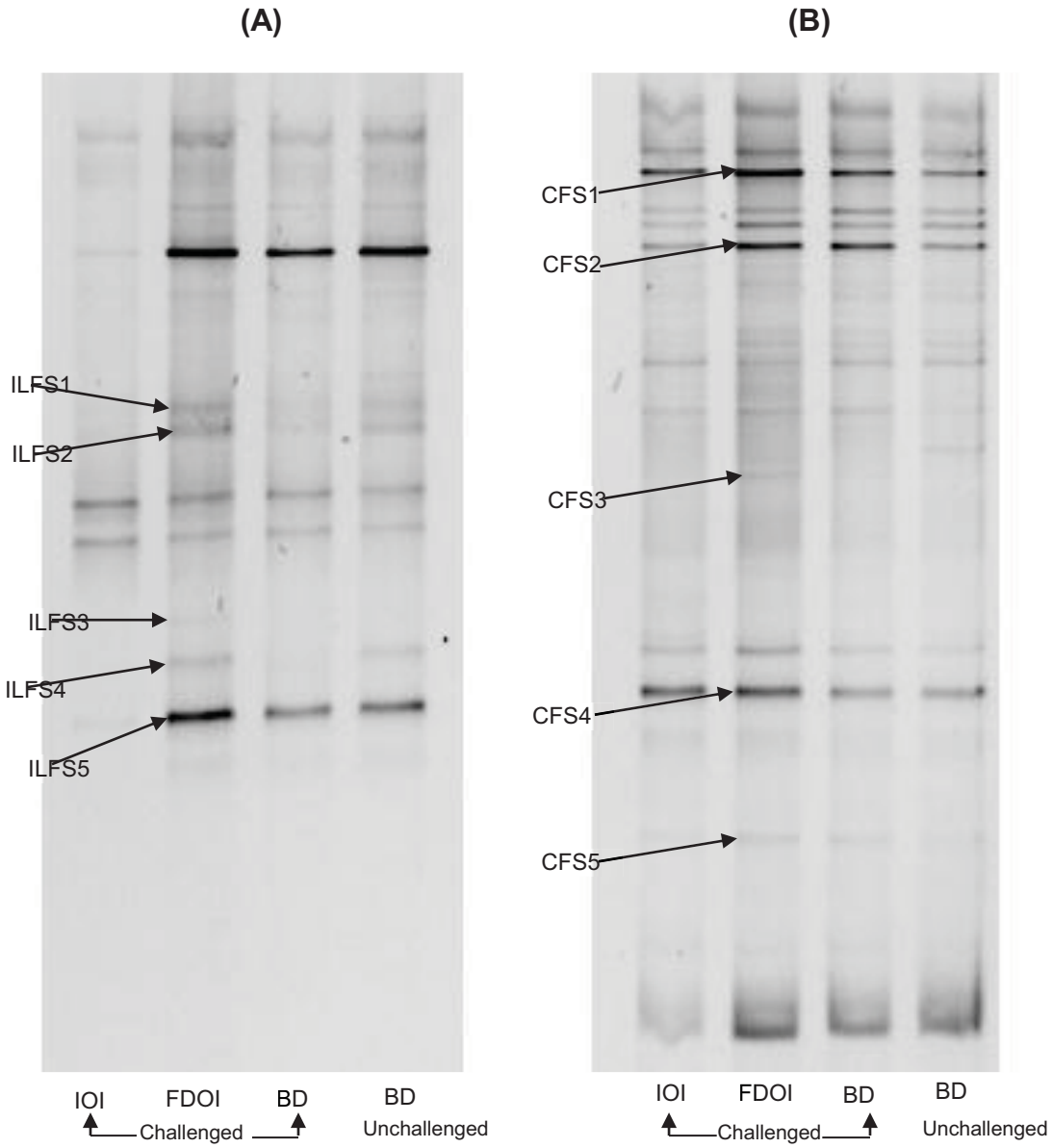
Table 7.7 Identified bacterial species from DNA sequencing of the PCR-DGGE bands for the challenged FDOI-fed rats (refer Figure 7.1).

Sample ID	Nearest Neighbour	Similarity with nearest neighbour	Accession no. of nearest neighbour
ILFS1	*L. citreum*	95%	AB362721.1
ILFS2	*W. cibaria* strain	99%	GU372716.1
ILFS3	*L. johnsonii strain* MCM	99%	HQ828141.1
ILFS4	*W. cibaria* strain M544M	99%	GU372715.1
ILFS5	*L. johnsonii strain* DPC	99%	CP002464.1
CFS1	*L. johnsonii* strain MCM	99%	HQ828141.1
CFS2	*L. citreum*	100%	AB572028.1
CFS3	*A. muciniphila* ATCC	99%	CP001071.1
CFS4	Uncultured bacterium clone	100%	EU451589.1
CFS5	*L. johnsonii* strain DPC	99%	CP002464.1

7.5. Discussion

The GIT acts as a protective barrier between the gut lumen, which contains large numbers of pathogens and antigens derived from food, and the internal millieu. Penetration of luminal antigens is controlled by the intestinal mucosa and the production of immunological responses in the gut. Changes in the regulation of these processes can lead to intestinal inflammation. Even in the absence of inflammatory stimuli, the healthy gut is in a proinflammatory condition; that is characterized by the production of proinflammatory cytokines such as IFNγ and TNFα (*O'Farrelly, 1998*).

Figure 7.1 Lanes showing DGGE bands from pooled DNA samples (*n*=15) of ileal (A) and colonic (B) digesta used for sequencing for the identification of bacteria in unchallenged rats fed the BD diet and rats challenged with *S. enteritidis* and fed either the BD, FDOI or IOI diets. Bands were selected for sequencing only when they were showing an obvious difference between the diets.



BD, Basal diet; FDOI, Freeze-dried ovine immunoglobulin; IOI, Inactivated ovine immunoglobulin.

The initial phase of *S. enteritidis* infection is characterized by the increased phagocytic activation and secretion of inflammatory cytokines in response to *S. enteritidis*, followed by specific immune responses against *S. enteritidis* (*Stoycheva et al., 2005*).

In this study, the phagocytic capacity of PBL was investigated as a

parameter of innate immunity and showed a marked increase for the challenged rats fed the FDOI diet when compared to the challenged rats fed either the BD or IOI diets, indicating a highly activated status (Table 7.2). Neutrophils are key cellular components in inflammatory response and play a vital role in host defense against bacterial infections and diseases through oxygen-dependent and -independent microbicidal mechanisms (*Elsbach et al., 1992*). A high white blood cell count can also indicate an infection. In this study, neutrophils were more than 5 times lower (P = 0.048) in the unchallenged rats fed the BD diet when compared with their challenged counterparts (Table 7.1). However, the neutrophil and white blood cell count in the challenged rats receiving the FDOI diet was similar (P > 0.05) to that in the unchallenged rats fed the BD diet, suggesting that FDOI counteracted the effects of infection. During (3 d of challenge) rapid production of neutrophils in the bone marrow, they may have decreased maturation period, and more immature neutrophils may have released into the blood to provide less active neutrophils which may explain the less phagocytic capacity for the challenged rats fed either BD or IOI diets (*Marsh et al., 1967*; *Terashima et al., 1996*).

Lymphocyte proliferative responses to mitogens are widely used to ascertain T (ConA is a T cell mitogen) and B (LPS is a B cell mitogen) cell function (*Gill et al., 1992*). Our findings demonstrated that the oral feeding of FDOI to challenged rats enhanced (P < 0.05) the capacity of PP cells and splenocytes to proliferate in response to mitogens ConA (both in PP and spleen) and LPS (in PP only) when compared to the challenged rats fed either the BD or IOI diets. This demonstrates that feeding of active ovine Ig significantly increased immune responsiveness against *S. enteritidis* in terms of cell proliferation at both the gut associated lymphoid tissue (in PP) and at the systemic level (in splenocytes). This is similar to our previous findings in unchallenged rats, where ConA induced lymphocyte proiferative responses of spleen cells from rats fed the FDOI diet were significantly higher than those from rats fed either the BD or the IOI diets (*Chapter-3*). The increased lymphocyte proliferative activity in rats fed the FDOI diet might be due to the presence of higher concentrations of intact bioactive Ig in the gut (*Silva & Malcata, 2005*).

In this study, since the PP cells and splenocytes from the challenged rats fed the FDOI diet showed a greater lymphocyte proliferative response in the presence of ConA, we analyzed the cytokine and Ig profiles from these cell culture supernatants in the presence and absence of ConA. The

challenged rats fed the FDOI diet showed significantly enhanced (130 to 300%) production of IFNγ, IgA and IgG in ConA stimulated supernatant of PP and splenocyte cultures compared to the challenged rats fed the BD or the IOI diets. These results are also consistent with our previous findings (*Chapter-3*) where we reported that consumption of FDOI stimulated IFNγ production in mitogen stimulated splenocyte cultures, suggesting that FDOI consumption may provide protection against *S. enteritidis*.

IFNγ and TNFα have been shown to suppress the invasiveness of *Salmonella* into the host cells (*Degré et al., 1989*). The primary antibacterial effect of IFNγ seems to be by suppression of bacterial growth by induction of nitric oxide (*Makela & Hormaeche, 1996*). In the present study, plasma IFNγ, TNFα, IL-10 and IgA concentrations were significantly lower for the challenged rats fed the FDOI diet when compared to the challenged rats fed the BD diet, clearly suggesting that there was a higher translocation of *S. enteritidis* into the blood for the challenged rats fed the BD than for the challenged rats fed the FDOI diet. In line with this result, Stoycheva & Murdjeva (2005) reported that serum levels of cytokines such as IFNγ, TNFα, IL-12 and IL-10 were significantly increased in patients with gastroenteric *Salmonella* infection in comparison with healthy controls.

Data from our previous study (*Chapter-6, Section-6.4.2*), showed that spleen size was significantly smaller in challenged rats fed the FDOI diet compared to challenged rats fed either the BD or IOI diets. Taken together these results suggest that the Ig (anti-*Salmonella*) present in the FDOI diet may have prevented (by binding) *S. enteritidis* from spreading from the gut to the systemic organs thus resulting in lesser infection (*Han et al., 2009*).

IgA works in a process known as immune exclusion, where intestinal IgA binds to the bacteria, pathogen or its antigen thereby preventing translocation (*Amin et al., 2007*). In this study, feeding FDOI significantly ($P < 0.05$) decreased the sIgA in both the ileal and colonic digesta when compared to the challenged rats fed either the BD or IOI diets. This indicates that the infection burden was greatly reduced in the lumen of rats fed the FDOI. Similar results were observed with the rat digesta IgG levels. In contrast to the above results, we found an increase in *Salmonella* specific IgA and IgG in both the gut (ileal and colonic) and plasma for challenged rats fed the FDOI diet, showing that feeding the FDOI diet stimulated specific humoral immune responses against *S. enteritidis*, perhaps by increasing either B cell proliferation or differentiation of B cells to secrete *Salmonella*-specific sIgA

and sIgG antibodies (*Jain et al., 2008*, *2009*). Rodenburg *et al*. (2007) using microarray technique demonstrated that immunity genes in ileal mucosa, Peyer pathces and colonic segments are up-regulated (compared to challenged and unchallenged rats) from 1d of challenge. Moreover ovine Ig was fed prophylactically (for two weeks before challenge) may have influenced increase in *Salmonella* specific Ig.

In ileal and colonic digesta, DGGE band number, Shannon's diversity index and peak intensity (only in colon) were found to be significantly ($P < 0.05$) higher for rats challenged with *S. enteritidis* and fed the FDOI diet when compared to the rats in the other treatment group (data not shown). DNA sequencing of the DGGE bands from the ileal and colonic digesta of the challenged rats fed the FDOI diet demonstrated a relative increase in bacteria such as *L. citreum*, *L. johnsonii* and *W. cibaria* (only ileum). *Leuconostoc* sp, *Lactobacillus* sp. and *W. cibaria* are members of the probiotic lactic acid bacteria group (*Makras et al., 2006*; Denou *et al., 2008; Kang et al., 2009*). *L. johnsonii*, was previously classified in the *L. acidophilus* group (*Fujisawa et al., 1992*). *A. muciniphila* which was isolated only from the colonic digesta, is known for its gut mucin modifying ability (*Collado et al., 2007*). In the present study, feeding the FDOI diet prophylactically resulted in the enrichment of lactic acid bacteria. This was similar to our previous finding (*Chapter-4, Section-4.4*) where we found that normal rats fed the FDOI diet showed an increase in abundance of *L. johnsonii* in both ileal and colonic contents. Moreover, this up-regulation of beneficial bacteria may have assisted to prevent the *S. enteritidis* attachment to the gut in the challenged rats fed the FDOI diet. The increased colonization of *Lactobacillus* sp. may benefit the gut in reducing *S. enteritidis* infections by competitive exclusion and adhesion to the intestinal epithelial cells (*Makras et al., 2006*; *Jankowska et al., 2008*). Bovee-Oudenhoven *et al*. (1997) reported that feeding rats with dietary calcium phosphate increased the endogenous lactobacilli which in turn led to an increase in resistance to *S. enteritidis* infection. Also, Ten-Bruggencate *et al*. (2006) reported that by feeding dietary fructooligosaccharides to healthy men increased bifidobacteria, lactobacilli, and lactic acid content in the gut. Reports have shown that *Lactobacillus* sp. fed to mice may modulate levels of intestinal IgA, serum IgA and splenocyte proliferation, which is in line with this study (*Kaburagi et al., 2007*).

Overall, our results are consistent with the earlier reports by Jain *et al*. (*2008*, *2009*) where they reported that feeding a probiotic formulation to non-

challenged and *S. enteritidis*-challenged mice resulted in increased phagocytic activity, higher spleen lymphocyte proliferative response, increased production of IFNγ in cell cultures stimulated with mitogen and higher anti-*Salmonella* IgA levels. They also concluded that pre-feeding the probiotic for 7 days prior to *S. enteritidis* challenge was more effective than pre-feeding for 2 days. In our study, the FDOI diet was pre-fed for 14 days prior to *S. enteritidis* challenge and was continually fed until the end of the study, and this may have resulted in a higher colonization of the beneficial bacteria.

Reports have demonstrated that bovine Ig resist digestion in the upper GIT of humans and retain toxin binding and neutralizing activity (*Warny et al., 1999*). Recently, Han *et al.* (2009) in an *in vitro* experiment demonstrated the anti-pathogenic effect of ovine Ig concentrate (OIC). They reported that OIC was able to bind to the cell walls of 13 strains of bacteria (both gram-positive and gram-negative). They concluded that OIC might be a potential supplement for protection against pathogenic bacteria. Given that circular dichroism analysis confirmed that the Ig in the IOI treatment was inactivated, our results suggest that active (undenatured) Ig in the FDOI diet was responsible for the observed immune responses.

Finally, FDOI selectively modulated the immune function through enhanced innate and specific immune responses, such as phagocytic activity of PBL, and enhancing lymphocyte proliferation, production of cytokines and *Salmonella*-specific sIgA and sIgG in the intestinal contents and plasma. In conclusion, orally administered FDOI significantly reduced *S. enteritidis* challenge in the growing rat by passive prevention of infection challenge and / or preventing translocation of *S. enteritidis*. Taken together, these effects resulted in the rat having an increased resistance to infection.

7.6. Literature cited

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Chapter 8

Intact ovine serum immunoglobulin in the luminal digesta of the growing rat

Submitted – Animal

There is ample evidence that orally fed ovine Ig affect growth performance, the gut microbiota and gut mucin synthesis and modulate immunity in the normal and *Salmonella enteritidis*-challenged growing rat. This infers that Ig must at least partially resist digestion in the alimentary canal. Various studies have reported that Ig extracted from different sources are able to resist digestion in the gut. The aim of this study was to determine whether intact ovine Ig is present in the chyme and digesta of growing rats administered ovine Ig.

8.1. Abstract

The aim was to recover intact ovine serum immunoglobulin from the luminal contents of the growing rat. Sprague-Dawley rats (*n*=5) were given a diet containing ovine Ig (FDOI). Samples of stomach chyme and digesta from the duodenum, jejunum, ileum and colon were collected and subjected to ELISA and Western blot analysis. Concentrations of intact ovine Ig were found to be 13.87, 20.02, 34.05, 13.0 and 36.91 µg wet digesta for stomach chyme, duodenal, jejunal, ileal and colonic digesta, respectively. Qualitative detection by Western blot revealed the presence of intact ovine IgG with a ~150 kDa MW. This was detected in all of the gut segments (stomach chyme, duodenal, jejunal, ileal and colonic digesta) for growing rats fed the FDOI diet. No Ig was detected in rats fed the BD or the IOI diets. In conclusion, intact ovine Ig were present in apparently high amounts throughout the digestive tract of the growing rat given the FDOI diet.

8.2. Introduction

Immunoglobulins are glycoproteins that are found in the blood and other bodily fluids of vertebrates, and function as antibodies to detect, neutralize and destroy foreign objects, such as bacteria and viruses. Ig are mainly produced in the lymph tissues. Ig consist of two identical heavy and two identical light chains linked together by intra- and inter-disulphide bonds (*Goldsby et al., 2003*). When Ig are ingested by an animal, they are subjected to the proteolytic enzymes pepsin, trypsin, chymotrypsin and the peptidases. Given that orally administered Ig (eg spray-dried plasma, colostrum) have been put forward as anti-bacterial, immune-enhancing and potentially growth-promoting agents in animals (*Bosi et al*., *2004; Pierce et al., 2005; Lallès et al., 2009; Campbell et al., 2010; Gao et al., 2011*), it is important to consider whether they exhibit resistance to these proteolytic enzymes, and can remain biologically active at different sites in the digestive tract.

In vitro digestion studies have shown that pepsin can cleave the heavy chain of the Ig molecule after inter-chain disulphide bonds, resulting in a fragment that has antigen binding sites. The fragment is called $F(ab')_2$ because it is divalent. The Fc region of the molecule is digested into small peptides. The $F(ab')_2$ does bind to antigens and can therefore be effective, but it does not mediate the effector functions of antibodies (*Lefranc-Millot et al., 1996*). Several studies have shown that orally administered Ig retains its biological activity within the gastrointestinal tract post-digestion. Undigested and partially digested Ig with intact activity have been found in the digestive tracts of adult and infant humans, dogs, cats and pigs (*Blum et al., 1981; Morel et al., 1995; Kelly et al., 1997; Rodriguez et al*., *2007*). The average recovery of intact Ig following *in vivo* digestion or *in vitro* simulated digestion based on 15 published reports was around 24% (C*hapter-1, Section-1.7.6*). No study has reported the complete loss of Ig during digestion. It would seem that Ig given to animals orally, are at least partially resistant to digestion and at least in the upper digestive tract. The objective of this study was to determine the resistance to digestion in the rat of an Ig preparation from ovine blood. The study sought to detect amounts of intact Ig in the chyme and digesta of rats fed orally with ovine serum immunoglobulins.

8.3. Materials and methods

8.3.1. Animal trial

This study was part of a large growth study reported in Chapter-2. The study was approved by the Massey University Animal Ethics Committee (MUAEC 06/132) and procedures complied with the New Zealand Code of Recommendations and Minimum Standards for the Care and Use of Animals for Scientific Purposes (New Zealand Animal Welfare Advisory Committee, 1995). Forty five Sprague-Dawley male rats (animals reported in Chapter-2, 140 to 160 g body weight) were housed singly in wire-bottomed stainless steel cages and had free access to water in a room maintained at 22±2°C with a 12 hour light/dark cycle. Animal trial was carried out as described in Chapter-2 (*Section-2.3.2*). The freeze-dried ovine Ig fractions were included in the diet at 3.07% (FDOI) and 3.40% (IOI) such that the amount of IgG in each diet was equal to the amount of IgG contributed by a commercially relevant spray dried porcine plasma diet (*Chapter-2, Table 2.1*). The animals were given the diets *ad libitum* and water was freely available.

8.3.2. Post-mortem procedure

At the end of the 3-week feeding period each rat was anaesthetized by intra-peritoneal injection (0.1mL/100g live weight) of a mixture containing acepromazine maleate BP (ACP, 2 mg/mL) 2 parts, ketamine (100 mg/mL) 5 parts, xylazine (10%) 1 part and sterile water 2 parts. Rats were then killed by exsanguination. The stomach was removed immediately and its contents collected and stored at -20°C. The entire small intestine and colon were removed. The small intestine was divided approximately into duodenum, jejunum and ileum. The contents of each part and the colonic contents were flushed using phosphate buffer saline and stored at -20°C. For analysis of intact ovine Ig, the chyme and intestinal digesta contents were spun at 2000 *g* for 15 min at 4°C and the supernatant was transferred into a tube and used for sandwich ELISA (*n*=5, randomly selected rats from each group) and Western blot (five randomly selected samples from each group were pooled). The remaining sample was stored at -80°C.

8.3.3. Quantitative analysis of intact ovine IgG

The content of intact ovine IgG in the gut digesta was analyzed by sandwich

ELISA. Sheep IgG from Auspep was used as a standard for construction of the standard curve. Flat-bottomed Immunopure Polysorp 96-well plates (Nunc) were coated with rabbit anti-sheep IgG (Heavy & Light chains) at a 1:20,000 dilution in carbonate buffer. After incubating overnight at 4°C, the plates were washed three times with PBS Tween (PBS-T, pH 7.2). Microplates were then blocked with 300 µl of a PBS-T solution containing rabbit serum (5 g/L) and incubated for 1 h at 37°C. Plates were washed again and purified sheep IgG or suitably diluted samples (stomach chyme, duodenal, jejunal, ileal and colonic digesta) in PBS buffer (pH 7.4) were added to designated wells and incubated for 1 h at 37°C. The plates were then washed three times with PBS-T. The plates were then incubated with horseradish peroxidase-conjugated rabbit anti-sheep IgG (AbD Serotec) at a 1:30,000 dilution in PBS-T for 2 h at 37°C. The plates were washed three times and developed using TMB enzyme substrate (3,3',5,5'-Tetramethyl-Benzidine). The reaction was stopped by adding 50 µl of sulphuric acid (2 M) and the absorbance was read at 450 nm using an ELISA plate reader. The recovery of intact ovine Ig in the luminal digesta was calculated based on the known ovine IgG intake**.** Only IgG was measured in this study, as it was the major constituent among the Ig (IgG, IgA, IgM, IgD and IgE) and the concentration of IgG was 73% of the total protein (94%) in the ovine Ig fraction (*Chapter-2, Table 2.1 and 2.2*).

8.3.4. Qualitative analysis of intact and digested sheep IgG

The qualitative analysis of intact and digested (Fab, Fc fractions) sheep IgG in the gut digesta was determined using native gels followed by Western blot. Native PAGE was performed at room temperature using Tris/glycine (15%) polyacrylamide gels and a Tris/glycine buffering system (pH 8.5). All samples were prepared by mixing an equal volume with 6 x Tris/glycerol sample buffer. Gels were typically loaded with 5 µL of NativeMark^{TM} unstained native marker, two standards, (sheep serum and freeze-dried ovine Ig each 3 µL) and 15 µL of luminal samples per lane and electrophoresed at a constant voltage of 150 V. After separation by native PAGE (Poly Acrylamide Gel Electrophoresis), the protein samples present on the gel were transferred onto polyvinylidene fluoride (PVDF) membranes (Invitrogen, California, USA). Transfer of proteins was performed at 15 V for approx 50 min in 0.025 M Tris, 0.19 M glycine and 20% v/v methanol (pH 9.2). After transfer, non-specific

sites on the membranes were blocked with 5% non-fat milk in 0.05 M Tris, 0.5 M NaCl and 0.05% Tween-20 (TTBS) for 90 min at room temperature. Blots were incubated with either horseradish peroxidase-conjugated rabbit anti-sheep IgG (H&L) (AbD Serotec, Oxford, UK) or Fab specific or Fc specific antibodies respectively for 90 min at room temperature. Anti-Fab (Jackson Immunoresearch Labs Inc, Pennsylvania, USA) recognizes Fab, while anti-Fc (Jackson Immunoresearch Labs Inc, Pennsylvania, USA) recognizes the Fc portion. Blots were washed 3 times with TTBS. The membranes were exposed to Metal Enhanced DAB Substrate kit (Thermo scientific) for 5 min. Blots were dried and protein bands were visualized by exposing blots to the light source.

8.4. Results

8.4.1. Food intake and growth rate

Food intakes and growth rates over the 21-day trial were not affected by the dietary treatments (BD, FDOI, and IOI). Mean (± SE) food intake was 22g/day (± 0.65) across the diets. On the day of slaughter, mean (± SE) food intake was 21g/day (± 0.80) (BD, FDOI, IOI) and thus Ig intakes for the rats fed either the FDOI diet or the IOI diet were similar.

8.4.2. Quantitative analysis of intact ovine IgG

Absolute concentrations and the amount given as a percentage of total daily Ig intakes of intact ovine IgG present in stomach chyme and duodenal, jejunal, ileal and colonic digesta are shown in Table 8.1. Intact ovine Ig was not detected in rats fed either BD diet (no ovine Ig) or the IOI diet, but intact IgG was detected in rats fed the FDOI diet. The overall accumulated amount (from stomach to colon) was 18.4% of the daily intake of Ig at a given time after feeding with ovine Ig.

8.4.3. Qualitative detection of intact and digested ovine IgG

Western blot was used to detect intact and digested ovine Ig. Intact ovine IgG with a molecular weight of approximate 150 kDa was detected in all the gut segments (stomach, duodenum, jejunum, ileum and colon) for rats fed the FDOI diet (Figure 8.1), but no Ig was evident in the rats fed the BD or IOI diets (data not shown). Digested fragments $F(ab)_2$, F(ab) or Fc were not

detected.

Table 8.1 Amounts of intact ovine IgG in stomach chyme and intestinal digesta from growing rats fed with a diet containing ovine Ig[1].

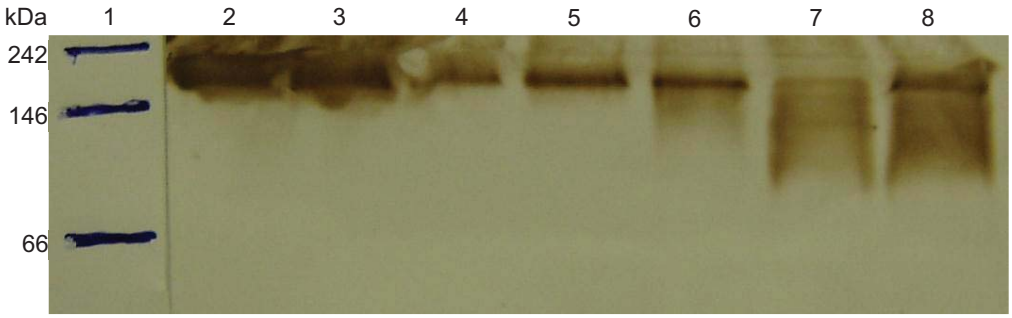
	Diet		
	BD	FDOI	IOI
	Whole Ig (μg)		
Stomach	0	13.87 ± 0.03	0
Duodenum	0	20.02 ± 0.61	0
Jejunum	0	34.05 ± 6.54	0
Ileum	0	13.00 ± 1.13	0
Colon	0	36.91 ± 1.60	0
	Relative to Ig intake (%)		
Stomach	0	2.17 ± 0.00	0
Duodenum	0	3.12 ± 0.03	0
Jejunum	0	5.31 ± 1.02	0
Ileum	0	2.03 ± 0.18	0
Colon	0	5.76 ± 0.25	0

[1]Values are means ± SE (*n*=5), BD=Basal diet; FDOI=Freeze-dried ovine immunoglobulin; IOI=Inactivated ovine immunoglobulin. Relative amount = (amount of IgG in digesta contents/total IgG ingested daily) X 100. The IgG ingested daily was around 620 μg.

8.5. Discussion

Studies have demonstrated that oral supplementation with Ig supports enhanced growth in animals (*Gatnau et al., 1989; Pierce et al., 2005*). Ig may prevent pathogens (bacteria and viruses) from damaging the gut wall, thereby resulting in a more intact and absorptive intestinal wall. Reduction in even sub-clinical infection may spare amino acids from the synthesis of immune proteins and thus increase amino acid supply for body protein synthesis (*Demas et al., 1997; Johnson, 1997; Torrallardona et al., 2003; Garriga et al., 2005*).

Figure 8.1 Detection of intact ovine IgG in chyme and intestinal digesta for rats fed a diet containing ovine IgG for 21 d[1].



[1] Characterization of ovine Ig fractions. Lane 1, NativeMarker 2; Lane 2, Sheep IgG; Lane 3, Freeze-dried ovine Ig; Lane 4, pooled chyme sample; Lane 5, pooled duodenal digesta; Lane 6, pooled jejunal digesta; Lane 7, pooled ileal digesta; Lane 8, pooled colonic digesta. All samples are from rats fed with the freeze-dried ovine immunoglobulin (FDOI) diet. Gels were subjected to Native PAGE separation and blotted and investigated with a rabbit anti-sheep IgG (H&L).

Several reports have shown that human serum and bovine Ig may resist digestion in the upper gastrointestinal tract of humans and retain toxin binding and neutralizing activity in the digestive lumen (*Losonsky et al., 1985; Kraehenbuhl & Neutra, 1992; Warny et al., 1999*). Furthermore, in suckling and possibly mature animals, Ig may be phagocytosed by the enterocyte, escape partial digestion by intracellular proteases and reach the circulation by exocytosis on the basolateral surface of the cell (*Hemmings & Williams, 1978; Sanderson & Walker, 1993*).

In the present study, we determined the concentration of intact ovine Ig in growing rats fed a diet containing freeze-dried ovine Ig using sandwich ELISA, and confirmed the results qualitatively using Western blot. The overall accumulated amount of intact ovine Ig (from stomach to colon) was found to be 16.5 ± 2.33 % of the amount of Ig ingested daily, at one point in time of sample collection. Given that the animals were fed the diet *ad libitum* over a 24 hour period, this suggests a high level of retention of active Ig in the digestive tract. Surprisingly, we found intact ovine Ig to be present in the colon, suggesting that orally administered ovine Ig can partially escape digestion by proteolytic and bacterial enzymes. This may be due to the *ad libitum* feeding of the diets to the rats in this study, in contrast to other studies which used restricted or single dose feeding. Contrary to what some other workers (Gmoshinskiĭ *et al., 1998; Rodriguez et al*., *2007*) have reported, we did not detect any digested Ig fragments such as $F(ab')_2$, F(ab) or Fc using Western blot. This may have been due to concealed epitopes. Also, most

reported studies have been carried out using bovine, human or porcine but not with ovine Ig. Gmoshinskiĭ *et al*. (1998) used a rat model, all other studies used other animal models or were *in vitro* studies. Rabbit anti-sheep IgG (heavy & light chains), which was used here, can detect ovine $F(ab')_2$ or Fab (both made of heavy and light chains), but no fragments were detected. ELISA, however, can give false negatives. The ELISA result, was confirmed using Western blot. Intact ovine Ig (in ELISA & Western blot) or digested Ig (Western blot) were not detected for rats fed the IOI diet, suggesting that heat inactivation may cause conformational changes to the Ig that might prevent contact to certain epitopes for binding with rabbit anti sheep IgG, or Fab or Fc.

One of the important characters of the dietary ingredient or component to be efficient prebiotic is to resist the digestion by the digestive enzyme and reach safely to the large intestine where it can be utilized by the resident microbiota (*Chapter 1, Section 1.3.3*). Since higher amount of intact ovine Ig was present in the colonic digesta, this could explain, Ig has the ability to support and/or enhance (prebiotic acitivty) the growth of lactobacilli and other beneficial bacteria in the gut as reported in earlier Chapters (*Chapters-4 & 7, Sections-4.4, & 7.4.8*). This clearly demonstrates ovine Ig can escape partial digestion by proteolytic enzymes and may work as a prebiotics in the gut. Further work is warranted to demonstrate the exact mechanism of prebiotic activity of ovine Ig.

In conclusion, intact ovine Ig are present in the luminal contents of the rat from the stomach right through to the colon when freeze-dried ovine Ig is given orally. The results provide a basis to explain the reported anti-bacterial, immune-enhancing and growth-enhancing properties of orally administered Ig.

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Chapter 9

Overall Discussion and Conclusions

9.1. Discussion and conclusions

Spray-dried animal plasma is a by-product of the meat processing industry, and there is a wealth of published scientific evidence showing that animal plasma products offered orally may lead to improvements in the growth rate of young animals. The ingestion of animal plasma may lead to increased food intake, higher growth rate, and improvement in intestinal morphology, an augmented immune system and beneficial changes in the composition of the gut microflora. In many of these studies it has been shown that the Ig fraction of the plasma was largely responsible for the observed biological effects (*Gatnau et al., 1989; Hansen et al., 1993; Kats et al., 1994; Thomson et al., 1994; Quigley et al., 2002; Campbell et al., 2003; Torrallardona et al., 2003; Campbell et al., 2004; Quigley et al., 2004; Garriga et al., 2005;* Pérez-Bosque *et al., 2004, 2006, 2008; Pierce et al., 2005*).

In this thesis findings are presented on various effects of an orally administered purified immunoglobulin sourced from ovine serum. An ovine serum Ig fraction was prepared from fresh lamb's blood according to a procedure previously developed in our laboratory using an ammonium sulfate precipitation method (*Han et al., 2009*). The ovine serum Ig is a dry powder, stable at room temperature. The animal model used here was the normal and *Salmonella enteritidis*−challenged rat. In the first study (*Chapter*-2) the aim was to ascertain whether the orally administered ovine serum Ig improved growth performance, increased gut organ weights and altered gut morphology in the normal growing rat, and whether the method of manufacture of the ovine serum Ig affected its bioactivity. The research objective was then directed towards studying immune modulation. The objective of the second study (*Chapter-3*), therefore, was to determine whether orally administered ovine serum Ig modulated immune parameters such as phagocytosis, lymphocyte proliferation, cytokine production and intestinal and plasma Ig concentrations in the growing rat. A further study, (*Chapter-4*) was undertaken to explore potential changes in the gut microbiota consequent upon feeding rats the ovine Ig. In Chapter-5, a study is described which sought to determine whether orally administered ovine serum Ig influenced goblet cell count, mucin gene expression and digesta mucin protein content in the gastrointestinal tract of the normal growing rat. Encouraged by the results of the various studies using normal healthy growing rats, a set of studies was undertaken (C*hapters-6 and 7*) using a challenged rat model (*S.*

enteritidis−challenged growing rat). Specifically, the studies investigated whether orally administered ovine serum Ig prevented negative effects of *S. enteritidis* on growth performance, digestive organ weights, gut morphology and gut mucin production, measures of immunity, and associated changes in the gut microflora. It became clear during the work that ovine serum Ig administered orally in the food had multiple effects upon gut structure and function in the growing rat. It would thus appear likely that the Ig material retained activity in its transit through the digestive tract. A final study (C*hapter-8*) was undertaken to confirm this premise and specifically to determine the amounts of intact ovine Ig in different regions of the gut.

Ig are the major anti-infective constituent of blood serum, colostrum and breast milk. These specialized proteins defend the body from pathogenic bacteria, viruses and other environmental pathogens by either binding to them or by the formation of an encapsulating barrier (*Kraehenbuhl & Neutra, 1992*). In the gut, exposure to antigens such as pathogenic and non-pathogenic organisms induces the production of pro inflammatory cytokines such as IL-1, IL-6 and TNFα and acute phase proteins (*Johnson, 1997*). The consequent increased demand for amino acids from a stimulated immune system reduces the efficiency of dietary protein utilization and may result in an overall lowering of growth. Plasma Ig are considered to lower stimulation of the immune system by preventing microbial growth or colonization in the gut, especially in the small intestine, and/or by indirectly assisting mucosal integrity (*Touchette et al., 2002*). Lowered activation of the immune system may lead to a lower utilization of nutrients and energy by the immune system itself and thus a greater supply of nutrients (especially amino acids) and energy for growth (*Demas et al., 1997*). This mechanism may even be important in apparently non-diseased animals where a sub-clinical microbial burden may still be operative.

The studies presented in this thesis were based upon hypotheses that oral administration of purified ovine serum Ig may be effective in improving the growth performance, strengthen immune function and modify mucin protein, presumably via intact Ig binding (preventing attachment to the host) of potential pathogenic bacteria and/or by supporting the development of beneficial bacteria in the gut of normal and challenged rat model. To accomplish this, two animal trials (normal and challenged model) were conducted. In the work described in Chapter-2 comparison was made among an iso-caloric freeze-dried ovine Ig (FDOI) containing diet, a casein based

control diet (no Ig, BD) and a negative control (inactivated freeze-dried ovine Ig containing diet, IOI). Growing rats were fed the FDOI diet for 21 days and at the end of this period, there was a higher ($P < 0.05$) G:F ratio and a heavier duodenum, jejunum, and colon for rats consuming the FDOI diet compared to those animals fed the BD or IOI diets. This overall result concurred with results reported by other workers investigating the effects of animal serum Ig or animal plasma (*Coffey & Cromwell, 1995; Gatnau et al., 1995; Owen et al., 1995; Weaver et al., 1995; Jiang et al., 2000; Pierce et al., 2005*). In the present work, villus length, crypt depth, villus:crypt ratio, and villus surface area of the duodenum, jejunum, and ileum were generally greater ($P < 0.01$) for rats receiving the FDOI diet compared to the controls. The colon crypt depth was also higher ($P < 0.001$) in rats receiving the FDOI diet. Touchette *et al*. (1997) also reported that animal plasma given orally improved small intestinal morphology independent of the level of food intake. The rats fed the FDOI diet in the presently reported study had significantly longer villi, greater crypt depths, and greater villi surface areas in all small intestinal sections. Feeding rats with freeze-dried (undenatured) Ig leads to improved gut morphology, and could potentially support an increased uptake of dietary nutrients from the gut. This hypothesis is worthy of investigation in future work.

A secondary objective of the first study was to ascertain whether the spray-drying process influenced the activity of the ovine Ig and whether SDPP and SDOI (at two levels of dietary inclusion, 100 and 150% of ovine Ig) had similar effects. Growth performance did not differ among rats fed the FDOI, $SDOI_{100}$, $SDOI_{150}$, or SDPP diets. Spray drying may, however, affect the activity of Ig, as rats fed the $SDOI_{100}$, $SDOI_{150}$, and SDPP had significantly ($P < 0.05$) lower duodenal weights than those fed the FDOI diet. A similar trend was found for colon weights, where the FDOI-fed rats had significantly heavier colons than the $SDOI_{100}$-fed rats. When comparing the effect of the FDOI, SDPP, $SDOI_{100}$, and $SDOI_{150}$ diets on gut morphology, generally, FDOI fed rats had superior gut morphology when compared to the rats fed the other diets, suggesting that gut morphology was also influenced to some extent by spray drying of the material. Spray drying may result in partial denaturation of the ovine Ig.

Chapter-3 presents results of the effect of ovine Ig on indices of immune function including phagocytic activity, lymphocyte proliferation of spleen cells, cytokine production, and IgA and IgG concentrations in intestinal

digesta and plasma. After 21 days of receiving ovine Ig, levels of phagocytic activity of PBLs, and ConA-induced lymphocyte proliferative responses were higher ($P < 0.05$) for rats receiving the FDOI diet than in rats fed either the IOI or the BD diets. Sugisawa *et al.* (2001) showed that oral administration of bovine colostrum stimulated non-specific immunity of the host by increasing the systemic immune response or by modulating immunocompetent cell function. Therefore, in this study, the feeding of rats with FDOI may have improved their innate immunity via enhancement of phagocytic activity of PBLs. Lymphocyte proliferative responses to mitogens are widely used to ascertain T-(ConA is a T cell mitogen) and B-(LPS is a B cell mitogen) cell function (*Gill et al., 1992*). Our findings suggest that the FDOI diet may have enhanced T-cell function. In stimulated spleen cells, IFNγ was higher ($P = 0.008$) in rats fed the FDOI diet compared to that in rats fed the BD diet.

Intestinal IgA functions as an immunologic defence against bacteria and various potential pathogens in the gut (*Amin et al., 2007*). Rats fed the FDOI diet had greater amounts of secretory IgA in both jejunal and ileal digesta which could potentially play a role in protecting the intestine from invading pathogens and suppressing inflammatory processes (*Parlesak et al., 2002*). The rats fed the FDOI diet also had greater amounts of jejunal rat IgG. The higher intestinal digesta IgA and IgG oncentrations of rats fed the FDOI diet indicate that these animals may be better equipped to handle the adverse effects of potential pathogens in the GIT. This is in line with our earlier observation (*Chapter*-2) that villus length, crypt depth, villus:crypt ratio and villus surface area of the small intestine were greater in rats receiving the FDOI diet. This study clearly demonstrated an effect of ovine serum Ig on immunomodulation in the growing rat through an increased proliferation of lymphocytes, increased phagocytic activity and an increased concentration of rat antibodies in the gut digesta.

The control and treatment of antibiotic-resistant pathogens in animals and humans are difficult (*Weiner et al., 1999*), and plasma Ig may be an effective alternative to antibiotics for controlling such pathogens. Effects of Ig against pathogens are well documented. For instance, human Ig was used prophylactically to treat children suffering from necrotizing enterocolitis (*Eibl et al., 1988*). Ig have also been used therapeutically against *Campylobacter jejuni* (Hammarström *et al.,* 1993), *Clostridium difficile* (Tjellstrom *et al.,* 1993), *Helicobacter pylori*, rotavirus and cryptosporidial infection (*Bogstedt et al., 1996*). An Ig concentrate was found to reduce the negative effects of

Staphylococcus aureus enterotoxin B (SEB) in rats (*Pérez-Bosque et al., 2008*). As Ig affect the pathogens they may also affect the gut commensal bacteria, and this thinking led us to investigate the influence of ovine Ig on the composition of the gut microflora. We used PCR-DGGE and qPCR to ascertain whether short term (21 days) oral administration of ovine serum Ig modulates the gut microbial population in the growing rat (*Chapter*-4). In ileal digesta, we found two prominent probiotic bacterial bands in rats fed the FDOI diet; these bands had DNA sequences with 99% similarity to the 16S rDNA V3 region of *Lactobacillus johnsonii* strain NCC and with 99% similarity to *L. johnsonii* MH21 (*Inoue et al., 2007*; *Anwar et al., 2008*). In the colon, three prominent probiotic bacterial bands in rats fed the FDOI diet had respective similarities up to 99% to *Lactobacillus* sp. BL304, *L. johnsonii* NCC and *L. johnsonii* DPC (*Inoue et al., 2007*; *Anwar et al., 2008*). The numbers of *L. johnsonii* determined by qPCR in both ileal and colonic digesta were significantly higher in the FDOI group when compared to the BD group. The feeding of FDOI to the rat may increase the number of beneficial probiotic bacterial species such as *Lactobacillus* sp. (*Makras et al., 2006*; *Inoue et al., 2007*; *Anwar et al., 2008*; *Denou et al., 2008*). Brassart & Schiffrin (1997) showed that mice given *L. johnsonii* orally had increased levels of intestinal IgA, serum IgA and splenocyte proliferation. This is in line with the results discussed here (*Chapter*-3) in which we found that orally administered FDOI selectively modulated immune function in the growing rat by increasing phagocytic activity of PBLs and spleen lymphocyte proliferation, and by increasing cytokine production and the secretory IgA and IgG in the intestinal contents and plasma. There may be a connection between the increase in the number of bacteria such as *L. johnsonii* consequent upon administration of ovine serum Ig and the modulation of the various indices of immunity in animals. This warrants further study.

The mucus coat separates and defends mucosal cells from the exterior environment (*Forstner & Forstner, 1994*). Mucus (a major part is mucin) plays a pivotal role in intestinal surface integrity, and failure of mucin secretion and/or mucin expression may result in several pathologies such as inflammatory intestinal diseases and cancer (*Ho et al., 1993*). Mucin-producing goblet cells along with antimicrobial peptides, luminal Ig, lysozymes and resident microflora collectively contribute to innate immunity and provide the front line of defence against pathogens. A well documented function of mucins is their role in preventing entry of enteric pathogens via the gut. As

mucins play a crucial role in protecting the underlying epithelial lining, any quantitative alteration in mucin secretion may change this defensive barrier and thus have important physiological implications. Reports have shown that gastrointestinal diseases and stress modify the mucus layer both quantitatively and qualitatively. Crohn's disease, colitis and gastric carcinogenesis associated with *Helicobacter pylori* alter mucin secretion and reduce the number of goblet cells which leads to degradation of the mucus layer (*Corfield et al., 2000; Babu et al., 2006*). The aim of the work described in Chapter-5 was to determine whether orally administered ovine serum Ig affected gut mucin in the growing rat and could assist to explain the observed immune modulation and changes in gut bacteria. A comparison of the number of goblet cells, mucin gene expression and digesta mucin concentrations was made between rats given a casein-based control diet (BD) and rats receiving a similar test diet but containing freeze-dried ovine Ig (FDOI). The rats consuming the FDOI diet (21 days) had a higher ($P < 0.05$) number of goblet cells in the villi and the crypt of the duodenum, jejunum and ileum. Muc5Ac (only in stomach) and Muc2, Muc3, and Muc4 mRNA expressions in the ileum were higher ($P < 0.05$) for rats given the FDOI diet. Finally, the concentration of mucin protein was found to be significantly ($P < 0.05$) higher in stomach chyme, and ileal and colonic digesta for rats fed the FDOI diet. King *et al*. (2008) found that feeding pigs with either colostrum or dried plasma resulted in an increase in villus and crypt goblet cells. The increased intestinal mucin found by feeding the ovine Ig could potentially lead to an enhanced gut barrier in the rats receiving the FDOI diet.

Caballero-Franco *et al*. (2007) studied three bacterial groups (lactobacilli, bifidobacteria, and streptococci) contained in a probiotic formula and found that the *Lactobacillus* sp. was the strongest potentiator of gut mucin secretion *in vitro*. Thus feeding the FDOI diet which led to an increase in *Lactobacillus* sp. in the gut may have potentiated the mucin secretion *in vivo*.

In summary, it is concluded that the FDOI positively influenced growth rate, gut microarchitecture, immunity, gut microbiota and gut mucin content when it was fed to normal growing rats. These observations are consistent with those for production animals at weaning following the ingestion of spray dried animal plasma (SDAP) containing Ig.

It is well known that the beneficial effects of feeding SDAP are more pronounced with higher levels of pathogen challenge (*Coffey & Cromwell*, 1995). This observation was the basis for the studies reported in Chapters-6

and 7, which investigated whether orally administered ovine serum Ig prevented the negative effects of *S. enteritidis* in the *S. enteritidis*-challenged growing rat. The four treatment groups were normal rats fed a casein-based control diet (BD) and three groups of rats each challenged with *S. enteritidis* ($1X10^7$ viable bacteria per rat) and fed either a casein-based diet, a diet containing freeze-dried ovine Ig (FDOI) or a diet containing inactivated ovine Ig (IOI). The rats received their respective diets for 18 days, with challenging taking place on day 15.

As anticipated, prior to challenging, there was no significant ($P > 0.05$) effect of diet on ADFI. For ADG there was no significant ($P < 0.05$) difference across the rats fed the BD and FDOI diets, but G:F ratio was higher ($P = 0.003$) for the rats consuming the FDOI diet. After challenging with *S. enteritidis*, however, both ADG and G:F were significantly ($P < 0.05$) higher for the challenged rats fed the FDOI diet in comparison with the challenged rats fed the BD- and IOI-diets. The challenged rats fed the IOI and BD diets grew considerably more slowly than either the challenged rats fed the FDOI diet or the unchallenged rats fed the BD diet. Furthermore, the challenged FDOI diet-fed rats and the unchallenged BD diet-fed rats grew at similar rates clearly demonstrating a prophylactic effect of the FDOI. The dietary Ig may have been able to prevent colonization by the *S. enteritidis* and/or may have reduced the pathogen load.

The small intestine and colon of the rats challenged with *S. enteritidis* and receiving the FDOI diet were heavier when compared to similarly challenged rats fed the control diet, but were the same weight as those for the unchallenged rats fed BD diet. The caecum, liver and spleen weights were higher in the challenged rats fed either the BD or IOI diets compared to the challenged rats fed the FDOI diet or unchallenged rats fed the BD diet. Some of the results reported here are similar to the recent findings of Lu *et al*. (2010) who found that mice challenged with *S. tyhpimurium* had a significant ($P < 0.05$) increase in both liver and spleen weights after infection. The challenged rats fed the FDOI diet had more intact gut morphology in most of the small intestinal sections studied. In contrast, challenging with *S. enteritidis* led to some degree of villus atrophy for those rats fed either BD or IOI. Gatnau *et al*. (1995) reported that feeding either spray dried porcine plasma or its corresponding Ig fraction to healthy weaned pigs resulted in an increased villus surface area, while Touchette *et al*. (1997) reported that healthy weaned piglets fed a spray dried plasma had longer villi with a greater

villus:crypt ratio compared to animals not receiving the sprayed dried plasma. This result was also consistent with our previous findings (*Chapter*-2) that in normal unchallenged rats fed the FDOI-based diet there was an increase in villus length, crypt depth, villus:crypt ratio and villus surface area compared to similar rats fed BD diet (containing no Ig) or a diet containing inactivated ovine Ig.

Challenging with *S. enteritidis* dramatically reduced the amount of mucin in the gut of rats fed either the IOI- or the BD-diets and this may have been caused, at least in part, by the observed reduction in the number of villi and crypt goblet cells in the small and large intestines. In contrast, dietary freeze dried ovine Ig, but not inactivated ovine Ig, appeared to completely negate the effect of *S. enteritidis* on mucin secretion and goblet cell numbers. FDOI-based diets have also been shown to increase villi and crypt goblet cell numbers throughout the gut in healthy rats (*Chapter-5*), while King *et al*. (2008) reported that feeding dietary colostrum or plasma to healthy pigs results in an increase of villus and crypt goblet cells. Infection may cause a depletion of goblet cells as has been reported in a murine model *Citrobacter rodentium* infection (*Lindén et al., 2008*). Moreover, certain pathogens such as *H. pylori* have been shown to specifically inhibit mucin synthesis (*Slomiany & Slomiany, 2006*).

An ovine Ig fraction selectively modulated various indices of immune function and associated gut microflora in growing rats inoculated with *S. enteritidis* (*Chapter*-7). Phagocytic activity of PBLs and lymphocyte proliferation in the presence of the mitogen ConA was greater for the unchallenged BD and the challenged FDOI-fed rats than for the challenged rats fed either the BD or IOI diets. ConA stimulated Peyer's patches and splenocyte cultures from the challenged rats fed the FDOI diet produced higher cytokine such as: IFNγ, and IgA and IgG than for the challenged rats fed either the BD or IOI diets. *Salmonella* LPS stimulates IFNγ, TNFα, IL-1, IL-6, and IL-8 production (*Galdiero et al., 1993; Henderson & Wislon, 1996; Trebichavský, 1999*) during infection. Of these, IFNγ and TNFα have been shown to suppress the invasiveness of *Salmonella* into the host cells (*Degré et al., 1989*). Dosing with *S. enteritidis* resulted in significantly higher IFNγ, TNFα, IL-10 and IgA concentrations for the challenged BD-fed rats compared to the unchallenged BD-fed rats. This effect was completely negated in the challenged rats fed the FDOI diet. Arnold *et al*. (1993) reported that during the *Salmonella* infection, TNFα are produced to control the infection, but the

presence of TNFα results in reduction in the goblet cell numbers, change in mucin composition, shortened and blunted villi with large lesions, oedema, and inflammatory cells. But these effects were completely abolished when animals were pre-treated with antibody to TNFα. This clearly demonstrates that TNFα may play important role during the pathogenesis of *Salmonella* infection. However, in this study, challenged rats fed the FDOI diet had significantly lower levels of TNFα in the serum and significantly higher numbers of goblet cells, higher mucin content and intact villus architecture (longer) in ileal and colonic segments (*Chapter-6, Sections-6.4.3, 6.4.4 & 6.4.5*), clearly suggesting that ovine Ig was able to abolish *Salmonella* challenge by reducing TNFα production and its associated pathology. Conversely, this was not evident in challenged rats fed diet BD which had higher levels of TNFα and higher numbers of pathological lesions. The *S. enteritidis*-challenged FDOI-fed rats had significantly higher amounts of anti-*Salmonella* sIgA and sIgG in the ileal and colonic digesta and plasma. The challenged FDOI-fed rats had selectively enriched bacteria such as *L. citreum* and *L. johnsonii* in both the ileum and colon when compared to the other groups. These results are generally consistent with the earlier findings (*Chapters-3 and 4*).

This up-regulation of certain lactic acid bacteria (*Inoue et al., 2007*; *Anwar et al., 2008*) may have inhibited *Salmonella* attachment to the gut in the challenged rats fed the FDOI diet. Bovee-Oudenhoven *et al*. (1997) found that feeding rats with dietary calcium phosphate increased the endogenous lactobacilli which in turn led to an increase in resistance to *S. enteritidis* infection. *Lactobacillus* sp. fed to mice may alter levels of intestinal IgA, serum IgA and splenocyte proliferation, which is in line with the results reported here (*Kaburagi et al., 2007*). Also, the present results are consistent with the earlier findings of Jain *et al*. (2008, 2009), who reported that feeding a probiotic formulation to non-challenged and *S. enteritidis*-challenged mice resulted in increased phagocytic activity, a higher spleen lymphocyte proliferative response, increased production of IFNγ in cell cultures stimulated with mitogen and higher anti-*Salmonella* IgA levels. The latter researchers also concluded that pre-feeding the probiotic for 7 days prior to *S. enteritidis* challenge was more effective than pre-feeding for only 2 days. In our study, the FDOI diet was pre-fed for 14 days prior to *S. enteritidis* challenge and was continuously fed until the end of the study. This may have resulted in a elevated numbers of commensal bacteria.

The final experimental Chapter of this thesis (*Chapter-8*) reports the recovery of intact ovine Ig from various parts of the digestive tract of the growing rat, and provides a basis for the various physiological and other effects observed throughout this study, consequent upon feeding ovine Ig to the rat. Intact Ig was detected (quantitatively by ELISA and qualitatively by Western blot) in stomach chyme and duodenal, jejunal, ileal and colonic digesta of rats fed the FDOI diet. Intact ovine Ig was not detected in rats fed the IOI diet, reinforcing that heat inactivation may cause conformational changes in the Ig preventing access to certain epitopes essential for binding with rabbit anti sheep IgG, or Fab or Fc. In the case of rats fed the BD diet intact Ig was not detected in the gut. Contrary to previous studies (*Gorevic et al., 1985; Morel et al., 1995; Nezlin, 1998*), pepsin digested fragments ($F(ab)_2$ or Fab and Fc) were not detected (Western blot) in rats fed the different diets (FDOI and IOI). These results provide clear evidence that intact Ig can be detected in the gut post-digestion, and may be active (not the digested fragments) throughout the gut of the growing rat fed the FDOI diet.

With the above discussed findings (*Chapters-2* to *7*), we can accept the hypothesis (mentioned in *Chapter-1, Section-1.8*) that oral administration of purified, intact ovine serum Ig (and not the digested fragments, such as: $F(ab)_2$ or Fab and Fc) are effective in improving the growth performance, strengthening mammalian immune function, and up-regulate the mucin protein and support the development of beneficial bacteria in the gut. An understanding of the detailed mechanistic basis of the effects of ovine serum Ig on the growth, immunity, microbiota and mucin awaits future research. However, the results reported in this thesis suggest several mechanisms by which ovine serum Ig may influence the positive effect. Passive inhibition of the adhesion of the pathogenic bacteria to the host intestinal wall may be mediated by antibodies present in FDOI (*Han et al., 2009*). Sheep are exposed to a much harsher environment than the average human. As a result, it is plausible that animal's immune system must be stronger and better adjusted to counterattack the full spectrum of pathogens by producing antibodies against them. It is well known that animal serum contains antibodies that are effective against a variety of pathogens like *E. coli*, *Salmonella*, *Listeria*, *C. difficile*, rotavirus, and many more (*Eibl et al., 1988; Weiner et al., 1989; Gatnau et al., 1995; Casswall et al., 1996; Pierce et al., 2005; Han et al., 2009*). Therefore serum Ig may provide passive antimicrobial protection (and also from subclinical infection) by exclusion of

opportunistic pathogens. Another possible mechanism of enhanced intestinal function may be through supporting elevated numbers of lactic acid bacteria (*Lactobacillus* sp*, L. johnsonii*, *Leuconostoc citreum*, *Weissella cibaria*) which influence the immunity and gut mucin content (*Chapters-3, 4, 5, 6 & 7*). *L. johnsonii*, was previously classified in the *L. acidophilus* group (*Fujisawa et al., 1992*). The increased colonization of lactobacilli into the gut may prevent *S. enteritidis* pathogenesis in four ways: firstly, by reducing the intestinal pH by producing various organic acids, such as lactic acid, citric acid, etc., which may inhibit the growth of *S. enteritidis* by bacteriocidal action (*Bovee-Oudenhoven et al., 1997; Makras et al., 2006*), secondly, by producing antimicrobial substances such as bateriocins (*Muriana & Klaenhammer, 1991a; 1991b*), thirdly, by competitive exclusion of *S. enteritidis* from GIT epithelial cell contact sites by lactobacilli and other beneficial bacteria colonization of the gut mucosa (*Jankowska et al., 2008*) and fourthly, by augmentation of the immune system by lactobacilli immunostimulation, such that this might have conferred enhanced immune-mediated protection against *S. enteritidis* (*Gill et al., 2003; Jain et al., 2009*).

The following conclusions can be made:

(1) FDOI improved feed conversion efficiency, the weights and relative weights of some digestive organs, and gut morphology in the normal growing rat. Spray-drying the ovine Ig fractions may result in a partial loss of activity.

(2) FDOI selectively modulated immune function in the normal growing rat by increasing phagocytic activity of PBLs and lymphocyte proliferation, and by influencing the balance between Th1 and Th2 cytokine production, and the secretory IgA and IgG in the intestinal contents and plasma.

(3) Feeding normal rats FDOI for 21 days had an effect on the microbial composition of digesta. This observation is of specific interest since orally administered ovine Ig may help to establish an intestinal environment that improves beneficial bacterial overgrowth.

(4) Orally administered FDOI selectively altered mucin gene expression, digesta mucin protein content as well as the number of goblet cells in the gastrointestinal tract of normal growing rats.

(5) Feeding FDOI to rats negated the impact of orally administered *S. enteritidis* (a pathogen) to such a degree that the performance of the *S. enteritidis* − treated rats fed FDOI was similar to normal uninfected rats.

(6) FDOI significantly reduced the effects of *S. enteritidis* challenge in the growing rat by selectively modulating the immune function through enhanced innate and specific immune responses, such as phagocytic activity of PBLs, and enhanced lymphocyte proliferation, production of cytokines and *Salmonella*−specific secretory IgA and IgG in the intestinal contents and plasma. Taken together, these effects appeared to result in the animal having an increased resistance to infection.

(7) Intact ovine Ig were present and can be measured in the lumenal contents (stomach to colon) of rats fed the FDOI diet

9.2. Future directions

1. Effects of FDOI on growth performance and gut morphology were successfully shown in the growing rat. However, these outcomes have to be investigated further in other animal models such as the pig and ultimately in humans.
2. Effects of FDOI on immunmodulation were reported in the growing rat. Further immunophenotyping such as: anti CD45 (for B lymphocytes), anti CD3 (for T Lymphocytes), anti CD4 (for T helper lymphocytes), anti CD8 (for T suppressor/ cytotoxic cells), anti CD25 (for activated T lymphocytes) and anti NKR-P1A (for detecting Natural killer cells) should to be carried out to investigate which immune cells are targeted by FDOI.
3. Universal primer was used to detect and identify microbial changes in the rat digesta and there was up-regulation of beneficial bacteria such as *Lactobacillus* sp. High-throughput sequencing (HTS) technologies could be applied to identify more subtle microbial changes due to the incorporation of FDOI in the diet.
4. The mucin study demonstrated that the FDOI led to an increase in the number of goblet cells, mucin gene expression in the stomach (Muc5Ac) and ileal digesta (Muc2, Muc3 and Muc4) and mucin protein concentrations in ileal and colonic digesta. More candidate mucin genes should be studied in all segments of the gut.
5. The various biological effects of FDOI in *S. enteritidis* challenged rats were investigated. More experiments should be conducted using a wider range of pathogens and their cell wall components such as LPS.
6. Micro-array technologies and proteomics can be employed to identify and investigate the expression of key genes and proteins in the gut and serum that are modulated by ovine Ig.

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Appendix

Appendix A - Nucleotide sequence data of excised DGGE bands (*Chapter-4*)

A 1 Sample ID – IL-1 (AB544006)

GAACGCGAAGAACCTTACCAGGTCTTGACATCCAGTGCAAACCTAAGAG
ATTAGGTGTTCCCTTCGGGGACGCTGAGACAGGTGGTGCATGGCTGTC
GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTGTCATTAGTTGCCATCATTAAGTTGGGCACTCTAATGAGACTGCC
GGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCC
TTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAACGAGAAGC
GAACCTGCGAAGGCAAGCGGATCTCTTAAAGCCGTTCTCAGTTCGGACT
GTAGGCTGCAACTCGCCTACACGAAGCTGGAATCGCTAGTAATCGCGGA
TCAGCACGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGA

A 2 Sample ID – IL-2 (AB544007)

TGAACGCGAAGAACCTTACCACGGGGGTCTTGTACACACCGAACGCGA
AGAACCTTACCAGGTCTTGACATCCAGTGCAAACCTACGAGATTAGGTG
TTCCCTTCGGGGACGCTGAGACAGGTGGTGCATGGCTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCA
TTAGTTGCCATCATTAAGTTGGGCACTCTAATGAGACTGCCGGTGACAAA
CCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGACCT
GGGCTACACACGTGCTACAATGGACGGTACAACGAGAAGCGAACCTGC
GAAGGCAAGCGGATCTCTTAAAGCCGTTCTCAGTTCGGACTGTGGGCTG
CAACTCGCCTACACGAAGCTGGAATCGCTAGTAATCGCGGATCAGCACG
CCGCGGTGAATACGTTCCCGGGTCTTGTACACACCG

A 3 Sample ID – CBD1 (AB544008)

GAACGCGAAGAACCTTACCTACCCTTGACATGTCAGGAAGCTCTTGTAA
TGAGAGTGTGCCCGCAAGGGAGCCTGAACACAGGTGCTGCATGGCTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTGTCACTAGTTGTTACGAAAGGGCACTCTAGTGAGACTGCCGGT
GACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTA
TGGGTAGGGCTTCACACGTCATACAATGGTCGGAACAGAGGGCAGCGA
AGTCGTGAGACGGAGCCAATCCCAGAAAACCGATCGTAGTCCGGATTG
CAGTCTGCAACTCGACTGCATGAAGTCGGAATCGCTAGTAATCGCGGAT
CAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGA

A 4 Sample ID – CFD2 (AB544009)

GAACGCGAAGAACCTTACCAGGTCTTGACATCCAGTGCAAACCTAAGAG
ATTAGGTGTTCCCTTCGGGGACGCTGAGACAGGTGGTGCATGGCTGTC
GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTGTCATTAGTTGCCATCATTAAGTTGGGCACTCTAATGAGACTGCC
GGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCC
TTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAACGAGAAGC
GAACCTGCGAAGGCAAGCGGATCTCTTAAAGCCGTTCTCAGTTCGGACT
GTAGGCTGCAACTCGCCTACACGAAGCTGGAATCGCTAGTAATCGCGGA
TCAGCACGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGA

A 5 Sample ID – CFD3 (AB544010)

TGAACGCGAAGAACCTTACCAGGTCTTGACATCCAGTGCAAACCTAAGA
GATCAGGTGTTCCCTTCGGGGACGCTGAGACAGGTGGTGCATGGCTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTGTCATTAGTTGCCATCATTAAGTTGGGCACTCTAATGAGACTGC
CGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCC
CTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAACGAGAAG
CGAACCTGCGAAGGCAAGCGGATCTCTTAAAGCCGTTCTCAGTTCGGAC
TGTAGGCTGCAACTCGCCTACACGAAGCTGGAATCGCTAGTAATCGCGG
ATCAGCACGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCG

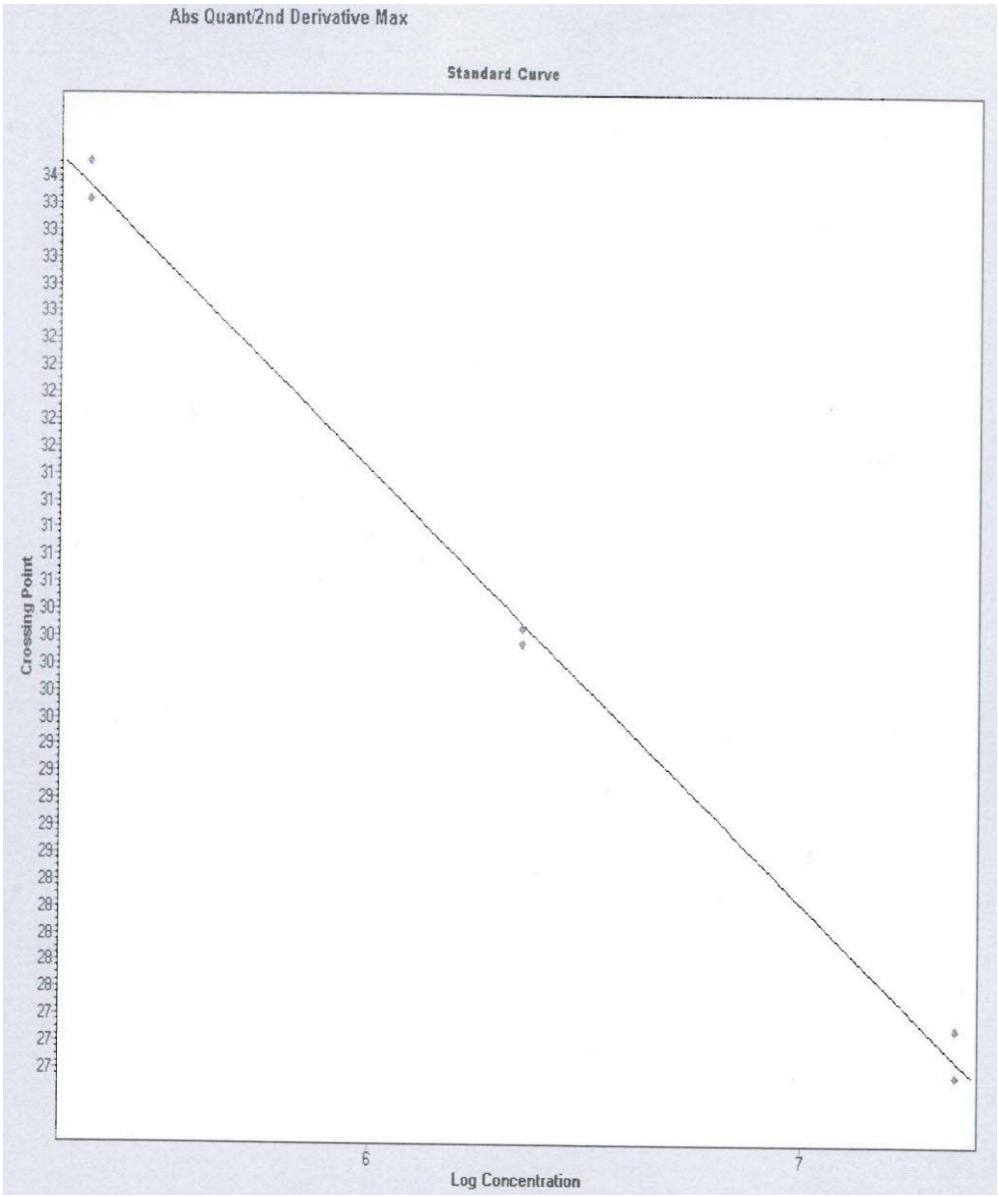
A 6 Sample ID – CFD4 (AB544011)

GAACGCGAAGAACCTTACCAGGTCTTGACATCCAGTGCAAACCTAAGAG
ATTAGGTGTTCCCTTCGGGGACGCTGAGACAGGTGGTGCATGGCTGTC
GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTGTCATTAGTTGCCATCATTAAGTTGGGCACTCTAATGAGACTGCC
GGTGACAAGCCGGAGGAAGGTGGGGGTGACGTCAAGTCATCATGCCCC
TTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAACGAGAAGC
GAACCTGCGAAGGCAAGCGGATCTCTTAAAGCCGTTCTCAGTTCGGACT
GTAGGCTGCAACTCGCCTACACGAAGCTGGAATCGCTAGTAATCGCGGA
TCAGCACGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGA

A 7 Sample ID – CFD5 (AB544012)

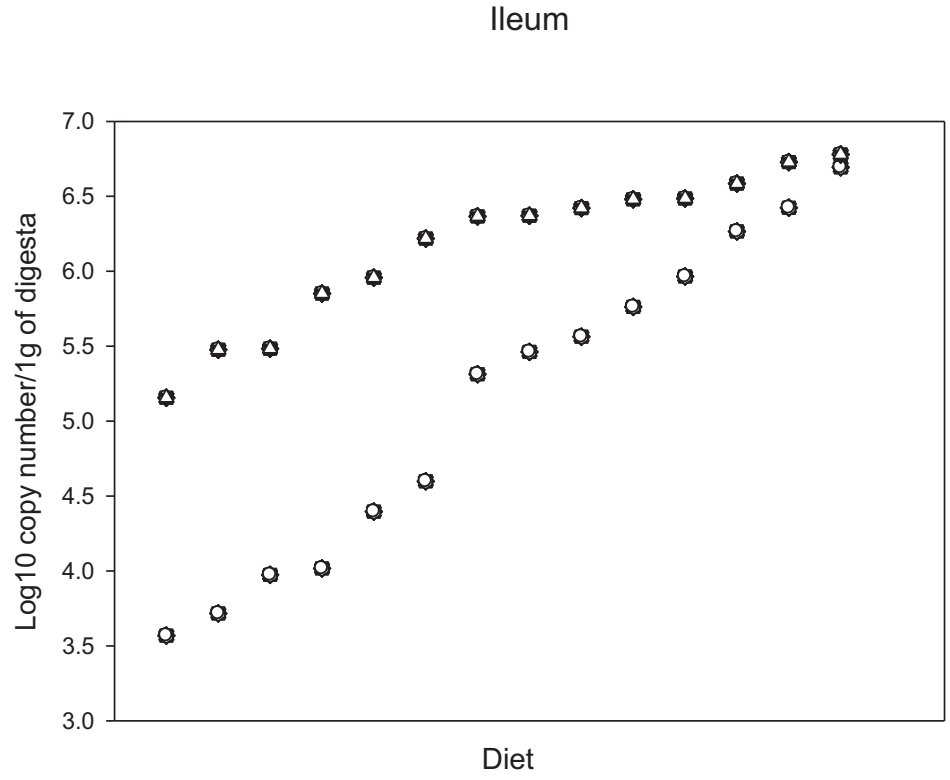
GAACGCGAAGAACCTTACGGGTCTTGTACACACCGATGCCCGCTCCGTA
ATGGGAGTTTTTCTTCGGAACATCGGTGACAGGTGGTGCATGGTTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CCTATCTTTAGTAGCCAGCATTTAAGGTGGGCACTCTAGAGAGACTGCC
AGGGATAACCTGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCC
TTATGACCAGGGCTACACACGTGCTACAATGGCGTAAACAAAGGGAAGC
AAAGCTGTGAGGCCGAGCAAATCCCAAAAATAACGTCTCAGTTCGGATT
GTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGCGAA
TCAGAATGTCGCGGTGAATACKTTCCCGGGTCTTGTACACACCGA

Appendix B - Standard curve for *L. Johnsonii* ATCC 33200

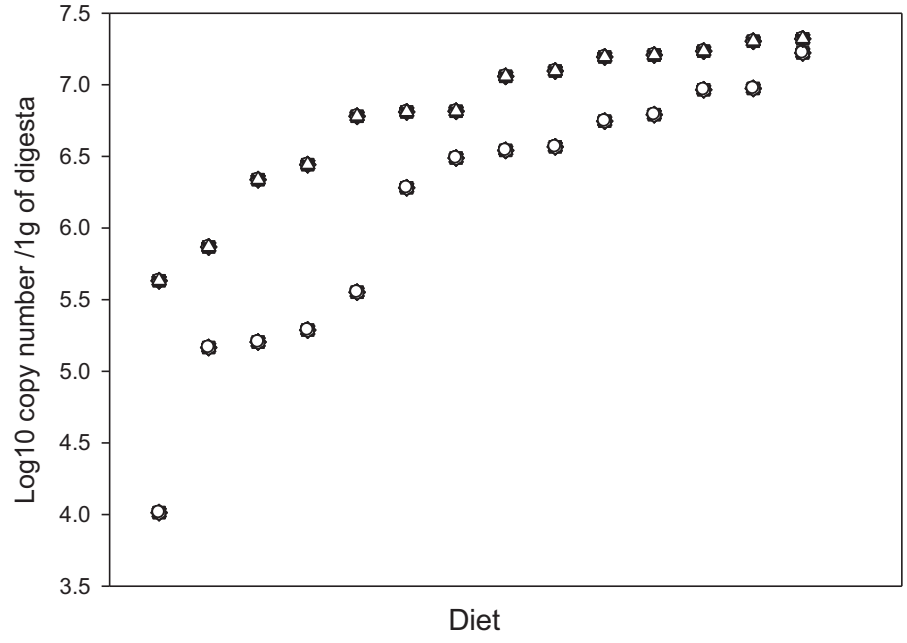


Appendix C - Scatter plot of the 15 individual qPCR data (A), ileal digesta and (B), colonic digesta

(A)



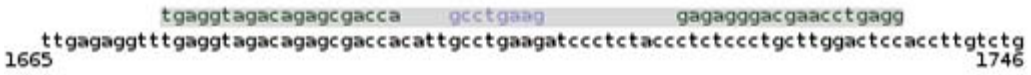
(B)



Appendix D - Full length of each amplicon of mucin genes and the internal probes

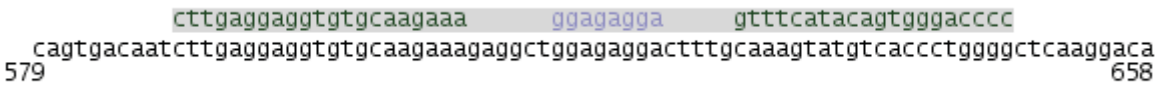
D 1 Muc2

Amplicon (62 nt)
tgaggtagacagagcgaccacattgcctgaagatccctctaccctctccctgcttggact cc



D 2 Muc3

Amplicon (60 nt)
cttgaggaggtgtgcaagaaagaggctggagaggactttgcaaagtatgtcaccctgggg



D 3 Muc4

Amplicon (64 nt)
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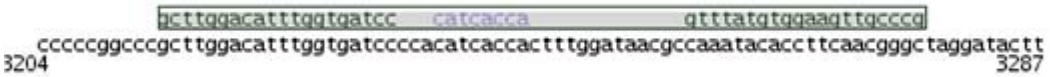

D 4 Muc 5AC

Amplicon (62 nt)
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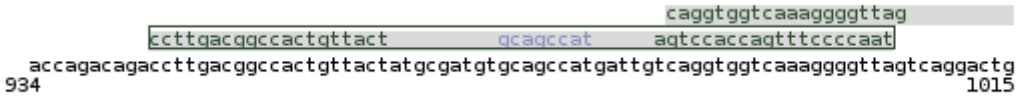
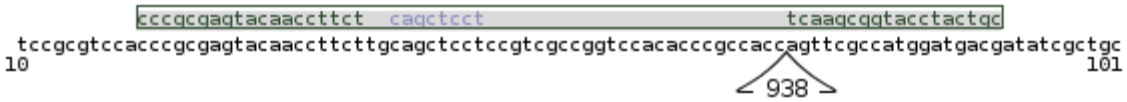


D 5 Beta-actin

Amplicon (72 nt)
cccgcgagtacaaccttcttgcagctcctccgtcgccggtccacacccgccaccagttcgccatggatgacg



Appendix E - Nucleotide sequence data of excised DGGE bands (*Chapter-7*)

E 1 Sample ID – ILFS1 (AB576354)

GAACGCGAAGAACCTTACCTAAGCTTGACWTCCTTTTGACCGATGCCTA
ATCGCATCTTTCCCTTCGGGGACAGAAGTGACAGGTGGTGCATGGTTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTATTGTTAGTTGCCAGCATTCAGTTGGGCACTCTAGCGAGACTG
CCGGTGACAAACCGGAGGAAGGCGGGGACGACGTCAGATCATCATGCC
CCTTATGACCTGGGCTACACACGTGCTACAATGGCGTATACAACGAGTT
GCCAACCTGCGAAGGTGAGCTAATCTCTTAAAGTACGTCTCAGTTCGGA
CTGCAGTCTGCAACTCGCCTACACGAAGCTGGAATCGCTAGTAATCGCG
GATCAGCACGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGA

E 2 Sample ID – ILFS2 (AB576355)

GAACGCGAAGAACCTTACCAGGTCTTGACATCCCTTGACAACTCCAGAG
ATGGAGCGTTCCCTTCGGGGACAAGGTGACAGGTGGTGCATGGTTGTC
GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTATTACTGGTTGCCAGCATTTAGTTGGGCACTCTAGTGAGACTGC
CGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCC
CTTATGACCTGGGCTACACACGTGCTACAATGGCGTATACAACGAGTTG
CCAACCCGCGAGGGTGAGCTAATCTCTTAAAGTACGTCTCAGTTCGGAT
TGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGG
ATCAGCACGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGA

E 3 Sample ID – ILFS3 (AB576356)

TGAACGCGAAGAACCTTACCAGGTCTTGACATCCAGTGCAAACCTAAGA
GATTAGGTGTTCCCTTCGGGGACGCTGAGACAGGTGGTGCATGGCTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTGTCATTAGTTGCCATCATTAAGTTGGGCACTCTAATGAGACTGC
CGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCC
CTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAACGAGAAG
CGAACCTGCGAAGGCAAGCGGATCTCTTAAAGCCGTTCTCAGTTCGGAC
TGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGG
ATCAGCACGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCG

E 4 Sample ID – ILFS4 (AB576357)

GAACGCGAAGAACCTTACCAGGYCTTGACATCCCTTGACAACTCCAGAG
ATGGAGCGTTCCCTTCGGGGACAAGGTGACAGGTGGTGCATGGTTGTC
GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTATTACTAGTTGCCAGCATTTAGTTGGGCACTCTAGTGAGACTGCC
GGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCC
TTATGACCTGGGCTACACACGTGCTACAATGGCGTATACAACGAGTTGC
CAACCCGCGAGGGTGAGCTAATCTCTTAAAGTACGTCTCAGTTCGGATT
GTAGGCTGCAACTCGCCTACATGAAGTCGGAACCGCTAGTAATCGCGGA
TCAGCACGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGA

E 5 Sample ID – ILFS5 (AB576358)

GAACGCGAAGAACCTTACCAGGTCTTGACATCCAGTGCAAACCTAAGAG
ATTAGGTGTTCCCTTCGGGKACGCTGAGACAGGTGGTGCATGGCTGTC
GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACAAGCGCAAC
CCTTGTCATTAGTTGCCATCATTAAGTTGGGCACTCTAATGAGACTGCCG
GTGACAAACCGGAGGAAGGCGGGGATTCCTCCAAGTTATCCCTGGCCT
TCTCTCTAGAGTGCCCAACTTAATGCTGGGAACTAAAGGCAAGGGTTGC
GCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACTA
CCATGCACCACCTGTCACTTCTGTCCCCGAAGGGAAAGATGCGATTAGG
CATCGGTCAAAAGGATGTCAAGCTTAGGTAAGGTTCTTCGCGTTCA

E 6 Sample ID – CFS1 (AB576359)

GAACGCGAAGAACCTTACCAGGTCTTGACATCCAGTGCAAACCTAAGAG
ATTAGGTGTTCCCTTCGGGGACGCTGAGACAGGTGGTGCATGGCTGTC
GTCAACTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
CCTTGTCATTAGTTGCCATCATTAAGTTGGGCACTCTAATGAGACTGCCG
GTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCT
TATGACCTGGGCTACACACGTGCTACAATGGACGGTACAACGAGAAGCG
AACCTGCGAAGGCAAGCGGATCTCTTAAAGCCGTTCTCAGTTCGGACTG
TAGGCTGCAACTCGCCTACACGAAGCTGGAATCGCTAGTAATCGCGGAT
CAGCACGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGA

E 7 Sample ID – CFS2 (AB576360)

TGAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGAAGCTTTTAGAG
ATAGAAGTGTTCTCTTCGGAGACAAAGTGACAGGTGGTGCATGGTCGTC

GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTATTGTTAGTTGCCAGCATTCAGTTGGGCACTCTAGCGAGACTGC
CGGTGACAAACCGGAGGAAGGCGGGGACGACGTCAGATCATCATGCCC
CTTATGACCTGGGCTACACACGTGCTACAATGGCGTATACAACGAGTTG
CCAACCTGCGAAGGTGAGCTAATCTCTTAAAGTACGTCTCAGTTCGGAC
TGCAGTCTGCAACTCGACTGCACGAAGTCGGAATCGCTAGTAATCGCGG
ATCAGCACGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCG

E 8 Sample ID – CFS3 (AB576361)

GAACGCGAAGAACCTTACCTGGGCTCGACATGTAATGAACAACATGTGA
AAGCATGCGACTCTTCGGAGGCGTTACACAGGTGCTGCATGGCCGTCG
TCAGCTCGTGTCGTGAGATGTTTGGTTAAGTCCAGCAACGAGCGCAACC
CCTGTTGCCAGTTACCAGCACGTGAAGGTGGGGACTCTGGCGAGACTG
CCCAGATCAACTGGGAGGAAGGTGGGGACGACGTCAGGTCAGTATGGC
CCTTATGCCCAGGGCTGCACACGTACTACAATGCCCAGTACAGAGGGG
GCCGAAGCCGCGAGGCGGAGGAAATCCTAAAAACTGGGCCCAGTTCGG
ACTGTAGGCTGCAACCCGCCTACACGAAGCCGGAATCGCTAGTAATGG
CGCATCAGCTACGGCGCCGTGAATACGTTCCCGGGTCTTGTACACACC
GA

E 9 Sample ID – CFS4 (AB576362)

TGAACGCGAAGAACCTTACCAGGCCTTGACATCCCCCTGGATATCCGGT
AATGCGGATAGGCCTTCGGGACAGGGGAGACAGGTGGTGCATGGTTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTGTCATTRGTTSCCWTCATTAAGTTGGGCACTCTAATGAGACTGC
CGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCC
CTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAACGAGAAG
CGAACCTGCGAAGGCAAGCGGATCTCTTAAAGCCGTTCTCAGTTCGGAC
TGTAGGCTGCAACTCGCCTACACGAAGCTGGAATCGCTAGTAATCGCGG
ATCAGCACGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCG

E 10 Sample ID – CFS5 (AB576363)

TGAACGCGAAGAACCTTACCAGGTCTTGACATCCAGTGCAAACCTAAGA
GATTAGGTGTTCCCTTCGGGGACGCTGAGACAGGTGGTGCATGGCTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTGTCATTAGTTGCCATCATTAAGTTGGGCACTCTAATGAGACTGC
CGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCC
CTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAACGAGAAG
CGAACCTGCGAAGGCAAGCGGATCTCTTAAAGCCGTTCTCAGTTCGGAC
TGTAGGCTGCAACTCGCCTACACGAAGCTGGAATCGCTAGTAATCGCGG
ATCAGCACGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCG