

**An *in vivo* model to study mucin-bacterial interactions during
early post-hatch development of broiler chickens**

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Abstract

Mucins, synthesised and secreted by goblet cells, possess potential binding sites for both commensal and pathogenic organisms, and may perform a defensive role during establishment of the intestinal barrier in newly hatched chickens. Increasing interest has been directed toward bacterial interactions within the mucus layer, and the mechanisms by which bacterial colonisation can influence mucus composition during early development. This is important, firstly, as a means to understand initiation of infection and secondly, to optimise the gut microflora for enhanced animal production. Currently, information on mucosal-bacterial interactions in poultry is limited. In order to observe the effects of bacterial exposure on intestinal goblet cell mucin production during early development, differences in the small intestine of conventionally-raised (CV) and low bacterial load (LBL) broiler chicks were examined during the first 7 days post-hatch.

The initial aim of the study was to construct a small-scale, economical isolator system to hatch and raise chicks in a bacterial-free environment as a means to observe bacterial interactions with the intestinal mucosa in chickens exposed to normal environmental conditions. The design and construction of flexible plastic isolators for incubation and brooding are described, along with methodologies for preparation of eggs for entry into the isolators, incubation and hatching. Two trials were conducted, the first in August 2005 and the second in March 2006. It was found that the isolator system was successful in producing low bacterial load chicks for comparative studies with conventionally raised chicks, without compromising body weight.

A histological study was then conducted whereby ileal and jejunal goblet cells were stained with either periodic acid-Schiff or high iron diamine/alcian blue pH 2.5 to

discriminate between neutral, sulphated and sialyated acidic mucins. Total goblet cell numbers and goblet cell and villous/crypt morphology were also examined. Bacterial colonisation of CV animals induced an increase in sialic acid moieties in both ileal and jejunal goblet cell such that initiation of these changes occurred at day 3-4 post-hatch. Differences in intestinal morphology were also consistent with other germ-free animal studies.

In order to further understand the extent to which bacteria affected mucin composition, purified, isolated oligosaccharide fractions from ileal mucin at d 4 and 7 post-hatch were collected and analysed using mass spectrometry techniques to determine any structural differences in chain length or chain number between LBL and CV animals. No differences in chain length or number were observed between CV and LBL animals at either d 4 or 7 post-hatch with both groups equally displaying chain lengths of both low and high molecular weights.

Although structural differences in mucin oligosaccharides were not observed between LBL and CV animals, bacterial binding assays utilising whole ileal sections were employed to determine whether or not the differences in mucin composition between LBL and CV animals during early development may have deterred or enhanced binding of certain bacterial species. *Escherichia coli* and *Lactobacillus salivarius* were selected for the experiment. Binding of *L. salivarius* to ileal sections was very low whereas *E. coli* binding was greater, and more pronounced in LBL animals, especially at d 7 post-hatch. No statistically significant differences were observed in binding of *E. coli* to purified ileal mucin from LBL and CV animals at either d 4 or d 7 post-hatch. Correlations between *E. coli* and *L. salivarius* adherence to ileal tissue and mucin samples, and goblet cell parameters, were not statistically significant when fitted as co-variates. It was concluded

that the changes in mucin composition played a minor role in bacterial adhesion of *L. salivarius* and this *E. coli* serotype.

In summary, this thesis explores the physiological changes in goblet cell mucin production in response to bacterial exposure post-hatch. The thesis outlines the complexity of mucosal-bacterial interactions which would benefit from the employment of specialised techniques such as nuclear magnetic resonance spectroscopy and microarray technologies to examine a greater range of mucin structures and gene expression. This thesis provides support for future investigations into the influence of intestinal microflora on mucosal and mucin dynamics of poultry and the potential development of prebiotics for use in animal production.

Declaration

This thesis contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution and to the best of my knowledge and belief contains no material previously published or written by another person except where due reference has been made in the text.

I give consent to this copy of my thesis being made available in the University of Adelaide Library.

Date

Rebecca Forder

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Abbreviations

°C	-	Degrees Celsius
AB	-	Alcian blue
ANOVA	-	Analysis of variance
BSA	-	Bovine serum albumin
CV	-	Conventionally reared
d	-	Day
DMSO	-	Dimethyl sulfoxide
DTT	-	Dithiotreitol
FITC	-	Fluorescein isothiocyanate
GF	-	Germ free
h	-	Hours
HCl	-	Hydrochloric acid
HID	-	High iron diamine
kDa	-	Kilodaltons
LBL	-	Low bacterial load
LPS	-	Lipopolysaccharide
min	-	Minutes
mL	-	Millilitres
MS	-	Mass spectrometry
MUC	-	Mucin core peptide gene
NFW	-	Nuclease free water
NMR	-	Nuclear magnetic resonance
OCT	-	Optimal cutting temperature
PAS	-	Periodic acid-schiff

PBS	-	Phosphate-buffered saline
PC2	-	Physical containment level 2
PLB	-	Protein loading buffer
PVC	-	Polyvinyl chloride
SDS-PAGE	-	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHQ	-	Super high quality
SPF	-	Specific pathogen free
TGS	-	Tris-glycine-saline
TRITC	-	Tetramethylrhodamine B-isothiocyanate
WGA	-	Wheat germ agglutinin

Publications

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This thesis is dedicated to Dan Thompson, an amazing man who touched the hearts of so many. I will miss you.

1979-2007

Chapter 1 Literature Review

1.1. Introduction

The gastrointestinal tract of poultry is densely populated with micro-organisms (both commensal and pathogenic) capable of intense metabolic activities (Macfarlane & Macfarlane, 2006; Smith & Bryant, 1979). Enteric infections with pathogenic bacteria can cause mortality in both humans and animals, and are responsible for reducing growth rates and consequent economic losses in animal production.

The first line of defence that bacteria encounter when trying to traverse the intestinal mucosa, is the overlying mucus-gel layer. The formation of the mucus-gel is through goblet cell secretion of polymeric mucin glycoproteins (Forstner & Forstner, 1994; Klinken *et al.*, 1995). These glycoproteins prevent noxious agents coming in contact with the underlying epithelial cells by competing with bacteria for adherence via heterogenous oligosaccharide chains, whilst simultaneously providing a desirable environment for specific microflora proliferation. Thus, the type and quantity of mucus are essential components in the establishment of the intestinal barrier.

Recently, there has been increasing interest toward the interactions of both commensal and pathogenic bacteria within the mucus layer and how colonisation can influence mucus composition during early development. The banning of antibiotics in the United Kingdom and European Union, has caused increased interest in the development of new products, which are advertised as potential antibiotic replacements (such as prebiotics or probiotics; Roberfroid, 2001). Antibiotic replacements have also been shown to improve performance compared to negative controls but the ability to measure this in the field is limited (Garland, 2004).

In order to maximise the efficacies of pre- and pro-biotics it is necessary to understand how bacteria colonise and their consequent effects on mucosal dynamics, especially during early development. Rodent based experiments investigating this area are quite extensive and have been used as models to determine bacterial effects on the causality of intestinal mucosal disorders associated with human health. Information on mucosal-bacterial interactions in poultry is limited, thus it would be beneficial to develop an understanding of these interactions in chickens as there are vast developmental differences between mammalian and avian species. This review provides insight into the mechanisms associated with production of the mucus layer and how interactions with intestinal microflora can exert changes in mucin composition and mucosal cytoarchitecture.

1.2. Goblet cells

Goblet cells are specialised epithelial cells responsible for the secretion and distribution of high molecular weight mucin glycoprotein (Perez-Vilar *et al.*, 2006). The cells consist of a narrow basal region, which includes the nucleus lying adjacent to the basal membrane, and a swollen apical region containing vast amounts of secretory granules, which are separated from other cytoplasmic constituents (Hodges, 1974). Comprising 16% of the total crypt cell population in mammals, goblet cells tend to reside in higher numbers in the crypts but are scattered amongst the enterocytes lining the villi in the small and large intestine (Hodges, 1974). Histologically, they are found in different stages of activity indicated by either greatly swollen or thin darkly stained appearances; the latter believed to be regenerating goblet cells (Hodges, 1974).

Mature goblet cells arise by differentiation of pluripotent stem cells at the base of intestinal crypts forming pre-goblet cells or oligomucus cells (Uni *et al.*, 2003a). These are poorly differentiated cells and have fewer mucosal granules. The pre-goblet cells are confined to

crypt bases or mid crypts in both the small and large intestine with some regional variation (Katz *et al.*, 2002). Goblet cells are similar in appearance when newly formed, however when migration occurs they undergo maturation acquiring more numerous mucus granules in addition to a highly organised array of microtubules and intermediate filaments termed theca. The mucus granules of the goblet cells and the striated border of the columnar cells undergo histochemical changes during migration and maturation (Suprasert *et al.*, 1987). The theca swells, separating the granules from the cytoplasm giving the distinctive goblet shaped appearance (Katz *et al.*, 2002; Uni *et al.*, 2003a). The goblet cells then migrate upwards toward the villus tip and are eventually sloughed into the lumen. In poultry, migration of goblet cells from crypt to villus tip takes 2 to 3 days (Uni *et al.*, 2003a).

Differentiation, proliferation and death of goblet cells is under tight genetic control. Growth factors such as keratinocyte growth factor (KGF) have been shown to induce proliferation and differentiation of epithelial cells throughout the gastrointestinal tract, including mucosal cells and has been specifically reported to promote goblet cell differentiation *in vitro* and *in vivo* (Fernandez-Estivariz *et al.*, 2003; Iwakiri & Podolsky, 2001). Goblet cell differentiation by KGF is reported to occur through the up regulation of trefoil peptides. Trefoil peptides are major constituents of the mucus layer and have been speculated to stimulate gut epithelial cell migration and mucosal repair by promoting maintenance of mucosal integrity (Mashimo *et al.*, 1996). It has been observed that trefoil peptides are selectively expressed in intestinal goblet cells and expression is correlated with goblet cell differentiation (Iwakiri & Podolsky, 2001).

Zinc-finger transcription factor (Klf4), is another example of a reported goblet cell specific differentiation factor. Expressed in the epithelia of the skin, lungs and gastrointestinal tract, Klf4 is involved in cell proliferation and differentiation as well as the maturation of

goblet cells from pre-goblet cells to mature goblet cells (Katz *et al.*, 2002). However the regulation of complex signalling involved in goblet cell differentiation is not yet fully understood and the identification of different factors controlling regulation of the goblet cell lineage *in vivo* is still elusive (Fernandez-Estivariz *et al.*, 2003).

1.3. The mucus layer

The mucus gel layer is an integral structural component that overlies the surface of the intestinal mucosa. It is a complex mixture of glycoproteins, water, cellular macromolecules, electrolytes, micro-organisms and sloughed cells (Faure *et al.*, 2003; Turck *et al.*, 1993). Throughout the gut, the mucus layer varies in its morphology and function. For example, the stomach contains a continuous layer of mucus up to 450 microns thick, where as the mucus layer in the small intestine is thin and discontinuous. The mucus layer in the colon is the thickest, increasing gradually from anterior to posterior.(Deplancke & Gaskins, 2001).

The properties of the mucus gel layer as a protective barrier for the gastrointestinal mucosa are numerous (Claustre *et al.*, 2002; Deplancke & Gaskins, 2001). It maintains the integrity of the underlying epithelium by protecting against vigorous digestive processes both chemical and mechanical by (a) creating an unstirred layer for lubrication (Claustre *et al.*, 2002; Deplancke & Gaskins, 2001; Lien *et al.*, 2001) and (b) acting as a diffusion barrier and by preventing large molecular weight compounds such as proteolytic enzymes from degrading the underlying mucosa (Lien *et al.*, 2001). It also has the ability to trap toxins and bacteria preventing infection by limiting their diffusion across the epithelium. Binding sites on mucus glycoproteins compete with receptors on the underlying epithelial cells thereby retarding access of micro-organisms to the mucosal surface reducing colonisation and favouring their removal (Mack *et al.*, 2003). By trapping pathogens, the

mucus layer can indirectly participate in the immune response due to interactions with secretory immunoglobulin A (Faure *et al.*, 2002).

The amount and composition of mucus is a balance between the degradation of luminal mucus and its components by chemical and physical forces and their renewal by goblet cell secretions from the intestinal crypts (Lien *et al.*, 2001). The effectiveness of the mucus barrier can also be greatly affected by drug-induced changes in response to illness. For example, adherence of mucin to the epithelial layer via mucin binding protein (MBP) can be weakened in response to particular drugs (Slomiany *et al.*, 2001). Other stressors such as starvation, decreases mucin quantity and secretion in both the rat (Sherman *et al.*, 1985) and the chicken (Smirnov *et al.*, 2004), which can affect digestive function and defence. In order to understand how the mucus layer may interact with external factors it is necessary to first understand its structure and biosynthesis.

1.3.1. Mucin structure

The mucus layer contains visco-elastic components termed mucins or mucin-type glycoproteins; proteins with carbohydrates covalently bound through glycosidic bonds (Beeley, 1985). Synthesised and secreted by goblet cells (Forstner & Forstner, 1994; Sharma *et al.*, 1997), mucins can be discharged in response to a wide variety of stimuli with the potential for changes in the type and quality of mucin secreted. The complexity of particular changes is of significance when describing the mechanisms involved in its role in first line defence of the mucosa.

Mucins are heterogenous, highly *O*-glycosylated glycoproteins with high molecular weights (Claustre *et al.*, 2002; Freitas *et al.*, 2002). They are described as having core peptides (~1,500 to >4,500 amino acids in length) with two regions: a major domain,

densely glycosylated *O*-linked oligosaccharide branches, rich in serine, threonine and proline and constitute 20-55% of the amino acid composition (Forstner & Forstner, 1994; Klinken *et al.*, 1995), and a minor portion; poorly or non-glycosylated, rich in cysteine located at the C- and N-terminal regions of the core protein (Claustre *et al.*, 2002). There is an alternation between the glycosylated and non-glycosylated domains, with *O*-linked glycosylated regions comprising 70-80% of the polymer. The non-glycosylated ends are more susceptible to degradation by proteases (Deplancke & Gaskins, 2001).

N-glycosylation, although low in abundance, does exist particularly in the cysteine rich N- and C-terminal regions. N-linked oligosaccharides bind to the amine end of asparagine and cysteine. They are of high mannose configuration, and may play a role in mucin polymerisation by maintaining a slow folding rate for mucin monomers allowing for maturation and stability of mucin peptides (Bell *et al.*, 2003). The continual removal and deletion of glucose of N-linked oligosaccharides are also reported to be important in maintaining optimal protein interactions during biosynthesis (Bell *et al.*, 2003; Table 1.2)

Mucin core peptide genes (MUC genes) are responsible for expression of the mucin peptide backbone (Forstner & Forstner, 1994). These genes are characterised by the possession of tandem repeats (PTS domains), which account for the high proline, threonine and serine content as well as the production of unique mucin core proteins (Forstner & Forstner, 1994; Klinken *et al.*, 1995). There have been nine MUC genes identified in the human (Deplancke & Gaskins, 2001) with 11 epithelial mucin genes reported in the gastrointestinal tract of rats (Faure *et al.*, 2003). Epithelial mucins that have been characterised in humans (MUC1-8) and are subdivided into two groups, the membrane anchored mucins and the secretory mucins (Smirnova *et al.*, 2003). In the chicken genome three transmembrane mucins (MUC4, MUC13 and MUC16) and four secretory mucins

(MUC6, MUC2, MUC5AC and MUC5B) have been identified (Lang *et al.* 2006). These genes share similar homology to human MUC genes, however the chicken has an additional gene (Between MUC2 and MUC5AC) not found in mammals, that codes for a mucin protein similar to human MUC2 but lacks a PTS domain (Lang *et al.*, 2006).

Expression of these genes is regulated in a tissue-specific and cell-regulated manner. For example, MUC2 is observed to be widely expressed in goblet cells of the small intestine and colon, whereas MUC5AC is weakly expressed in the intestine and colon but widely expressed in the stomach. Regional expression of MUC genes is further outlined in Table 1.1. The differences in expression in regions of the intestinal tract suggest that each mucin has its own specific function in maintaining mucosal integrity. For example, mice deficient in MUC2 were observed to have aberrant intestinal crypt morphology with altered cell maturation and migration (Claustre *et al.*, 2002).

Table 1.1. Expression of MUC genes in different regions of the gut using Northern Blot analysis (Klinken *et al.*, 1995).

	MUC1	MUC2	MUC3	MUC4	MUC5AC	MUC5B	MUC6	MUC7	MUC8
Stomach	+	-	+	+	++	+	++	-	ND
Small Intestine	+	++	++	+	±	+	±	ND	-
Colon	+	++	+	+	±	+	±	ND	ND

(++, strongly positive; +, positive; ±, trace positive; -, negative; ND, not detected)

Structurally, mucins consist of four subunits, linked by disulphide bonds and arranged into a 3-dimensional polymeric structure. They have been noted to exist as “windmill” structures in which the protein backbones of all four mucin subunits are linked in a common non-glycosylated region by disulphide bridges (Allen *et al.*, 1982; Figure 1.1). The ability to form disulphide bonds enables the non-glycosylated cysteine regions to form

large polymeric complexes, necessary for gel formation and viscosity (Allen *et al.*, 1982). These interactions are strong enough to resist osmotic pressure and solubilisation, but not strong enough to resist gel-spreading or mechanical disruption (Lien *et al.*, 2001).

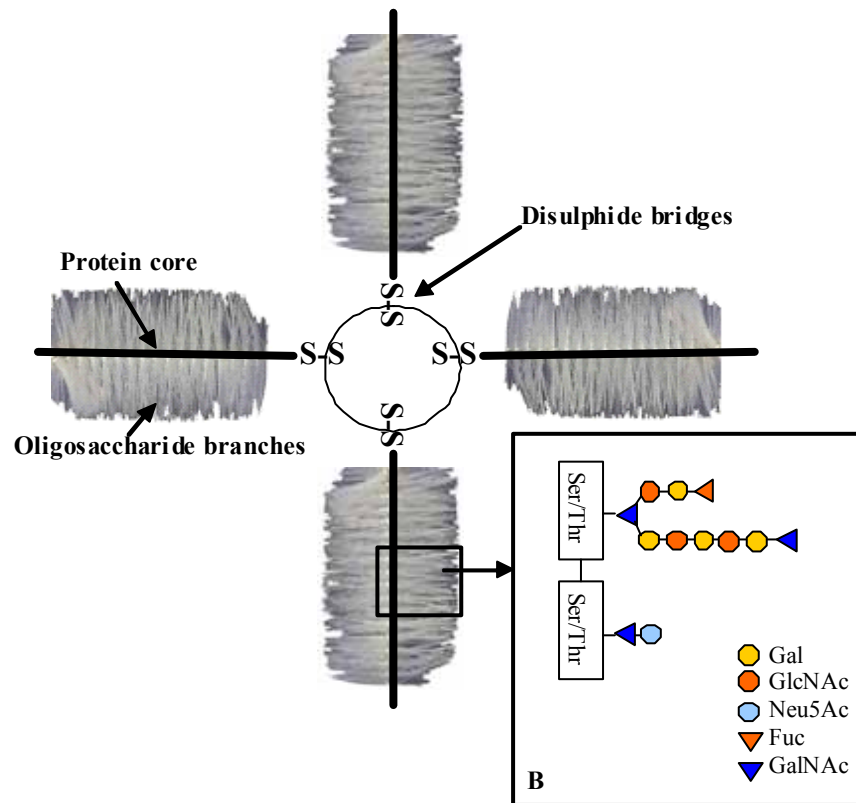


Figure 1.1. Diagrammatic representation of mucin glycoprotein structure, adapted from Allen *et al.*, 1982, and inset, examples of *O*-linked oligosaccharides; elongation occurs through the attachment of GalNAc to the hydroxyl group of serine and/or threonine residues, adapted from Nelson & Cox, 2000.

Mucin oligosaccharide chains that attach to the protein core comprise of the primary sugars, N-acetylglucosamine (GlcNAc), galactose, N-acetylgalactosamine (GalNAc), fucose (Fuc), as well as sialic acid (NeuAc), mannose and glucose (Forstner *et al.*, 1995; Kiernan, 1990). The sugars are linked via an alpha-glycosidic bond between N-acetylgalactosamine and either serine or threonine (which attaches to the oxygen atom of the lateral chain; Freitas *et al.*, 2002; Satchithanandam *et al.*, 1990; Figure 1.1). Oligosaccharide chains often terminate with sialic acid or sulphate groups, accounting for the polyanionic nature of mucins at a neutral pH (Deplancke & Gaskins, 2001). Sulphate moieties are usually attached by ester linkages to GlcNAc, galactose and sometimes to GalNAc residues. The amount and type of carbohydrates present denotes the charge of the *O*-glycan chain. For example, both sialic acid and sulphate moieties have strong negative charges. This property enables separation of oligosaccharides on ion exchange columns, or histochemical studies into neutral or acidic species, and to further separation of sulphated or carboxylated acidic subtypes (Deplancke & Gaskins, 2001). The classification of neutral mucins is not strictly accurate in the chemical sense, but is used to describe sugar residues that do not have sulphate-esters, carboxylic acids or nitrogen containing functional groups. Glucose, galactose, mannose and fucose are the principle “neutral sugars” (Kiernan, 1990).

Membrane-bound glycosyltransferases transfer monosaccharide units from nucleotide sugar donors in the Golgi apparatus to form mucin oligosaccharide chains. Sulphate in particular is transferred to peripheral or backbone oligosaccharide chains from 3'-phosphoadenosine-5' phosphate (PAPS) by golgi sulphotranferases (Deplancke & Gaskins, 2001). The many different combinations of potential oligosaccharides, including the diversity in the length, composition, branching and the degree of sulphation and acetylation (Corfield *et al.*, 1992; Uni *et al.*, 2003a) explain the inherent heterogeneity of mucin and also determine their specific functions (Klinken *et al.*, 1995). These variations

can also be dependent on the species and intestinal region examined. For example, 20 sugar residues have been reported in pig gastric mucus (Allen *et al.*, 1982) whereas in the rat, colonic mucins range in length from 2 to 12 sugars (Slomiany *et al.*, 1980). It has been reported that glycan structure is genetically determined for each individual, with specific genes coding for specific enzymes involved in glycosylation processes (Freitas *et al.*, 2002).

1.3.2. Biosynthesis and packaging

The processes involved in the biosynthesis and packaging of mucin require the involvement of many post- and co-translational modifications in both the rough endoplasmic reticulum and the Golgi complex within goblet cells. Tables 1.2 and 1.3 provides a brief outline of the various intracellular steps of mucin synthesis and packaging (Forstner & Forstner, 1994; Matthews & Van Holde, 1996).

Table 1.2. Synthesis of mucins

Procedure	Site	Initiation
Ribosomal translation forming nascent peptides.	Membrane-bound ribosomes	
<i>N</i> -glycosylation to C- and N- terminal ends. Assists in the efficient transfer of nascent mucins to the RER.	Cistern of the rough endoplasmic reticulum (RER)	Transfer of high-mannose oligosaccharides from dolichol pyrophosphate (Dol-P-P) to asparagine during translation.
Post-translational modifications (folding and oligomerisation by disulphide bridging).	RER	Process is energy dependant. Controlled by molecular chaperones. Trimming of terminal glucose from N-linked oligosaccharides. Influences binding of the chaperones calnexin and calreticulin (Bell <i>et al.</i> , 2003).
N-linked high mannose oligosaccharide removal by glycosidases. Further processing removal of terminal $\alpha(1-2)$ -linked mannose residues to form Man oligosaccharides.	Cis-cisternae of the golgi complex	Starting point for complex oligosaccharide synthesis (O-glycosylation).
<u>O-glycosylation</u> GalNAc addition to hydroxylated amino acid of the mucin core Cis-golgi.	Cis golgi	
Linear expansion by oligomerisation of mucin peptide/oligosaccharide elongation. Addition of terminal branches of complex N-linked oligosaccharide. O-linked glycosylation completion. Addition of terminal sialic acid, fucose, and sulphate residues.	Trans-golgi	Oligosaccharide elongation specific membrane-bound glycosyltransferases. Transfer monosaccharides from nucleotide sugar donors.

Table 1.3. Packaging of mucins in goblet cells

Procedure	Site	Initiation
Mucin collected at nodular dilations. Dilation bud forms. Golgi tubules form immature condensing granules which are membrane bound.	Tubular components of trans region of golgi stack	
Condensing granules mature	Most numerous near trans golgi	Signal for storage is carried within 1° structure of secretory proteins.
Condensation; Increased electron density. Granule membrane Ca^{2+} -ATPase or channel to concentrate the cation. Release of Ca^{2+} unshields mucins – mutual repulsion of polymer chains causing expansion of granules detaching from the cell (Paz <i>et al.</i> , 2003).	Moves to apex of goblet cell by microtubules	Increase in intragranular Ca^{2+} . Charge shielding, to keep negatively charged mucins condensed and tightly packed (Paz <i>et al.</i> , 2003). Stabilises by increasing polymer-polymer affinity, which is required for packaging of mucins in goblet cells (Paz <i>et al.</i> , 2003).

1.3.3. Granule discharge and intracellular transport pathways

Secretion of mucus from goblet cells is through exocytosis. Fusion of plasma membrane and mucin granules occurs at the site of a fusion pore and is probably mediated by specific attachment and pore proteins (Forstner & Forstner, 1994). Mucin secretion involves transformation of the apical cytoplasm into a low-opacity amorphous material, presumably derived from the digestion of pre-existing organelles in the area (Hafez, 1977).

There are two distinct processes involved in mucus secretion, baseline secretion and compound exocytosis. Baseline secretion, or simple exocytosis, constitutively release newly synthesised mucin granules that preferentially move along the periphery of the apical granule mass (Forstner & Forstner, 1994; Deplanke & Gaskins, 2002). On exposure to mucin secretalogues, goblet cells undergo compound exocytosis, an accelerated secretory event resulting in the acute release of centrally stored mucin granules (Deplanke & Gaskins, 2002). A wide range of receptor-mediated bioactive factors can release mucin onto the mucosal surface. Hormones, neuropeptides and other chemicals from both host and bacterial origin are examples that can elicit these changes (Forstner & Forster, 1994; Deplanke & Gaskins, 2002). Although the cytoarchitectural organisation of goblet cells and mucin is well known, information on how these factors contribute to heterogeneity of mucin glycoprotein or the differentiation of stem cells toward the goblet cell lineage is yet to be determined.

1.3.4. Region specificity of mucin

There are distinct differences in mucin glycosylation patterns through the gastrointestinal tract. It has been speculated that particular regions of the gut may require different mucin compositions to ensure the protection of the underlying epithelium (Robbe *et al.*, 2003). For example, neutral mucins are the predominant sub-type expressed in gastric mucosa,

and acidic mucins are expressed throughout the intestinal epithelium, dominating in the large intestine (Sheahan & Jervis, 1976). It was found that mucin isolated from the colon of pigs comprised of negatively charged sialic acid residues on the colonic glycoprotein, similarly the small intestinal mucin also contained sialic acid but also GalNAc and ester sulphate residues (Allen *et al.*, 1982). A high sialic acid content has also been reported in the colon of adult Leghorn chickens and mice (Kandori *et al.*, 1996; Suprasert *et al.*, 1987).

The use of lectin binding to peripheral sugars of mucin oligosaccharide chains in germ-free mice indicated regional differences in mucin carbohydrate moieties with *Sambucus nigra* agglutinin (SNA), having a high affinity for sialic acid, was more predominant in the colon, where as *Griffonia simplicifolia* (GSI) and Jacalin, having high affinities for galactose was concentrated to the brush border of the ileum (Freitas *et al.*, 2002). It was speculated that sialo-transferases and alpha-galactotransferases follow a tissue-specific and carbohydrate species-species regulation and that expression of glycans depends on the proximal gradient (small to large intestine) and on the cell lineage (absorptive, goblet, crypt and Paneth cells) (Freitas *et al.*, 2002).

Evidence of regional specific glycosylation, using techniques such as mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) has been reported in humans. It was found that sialic acid residues increased from ileum to colon, whereas fucose decreased. A gradient of increasing sialylation has been reported along the gastrointestinal tract with greater number of acid glycans (Robbe *et al.*, 2003). This was also observed by Slomiany *et al.*, 1980, with isolated colonic mucin oligosaccharides terminating with sialic acid.

In the colonic epithelium, a change in the mucin composition of goblet cell mucus granules during cell migration implies regional differences along the crypt/villus axis (Suprasert *et al.*, 1987). It was observed that the crypts had less acidic mucins when compared to villous epithelium with lower concentrations of alpha-D-mannose, alpha-D-glucose residues and sialic acid-galactose dimers in mucin granules. Changes in mucin composition due to cellular migration towards the villous tip may be a consequence of increased exposure to the luminal environment, and acts as a protective mechanism against potential harmful substances (Suprasert *et al.*, 1987).

The high degree of diversity in glycan expression in different regions of the intestine may be an explanation for the regional specific colonisation of bacteria along the digestive tract (Robbe *et al.*, 2004; Suprasert *et al.*, 1987). A large variety of bacterial strains have specific mechanisms to maximise adhesion to epithelial cells via manipulation of mucins glycoproteins. This will be discussed in subsequent sections.

1.4. Development – changes in mucin composition with age

Goblet cells first arise in the embryonic gut at day 14 of incubation but are few in number (Black & Smith, 1989). Goblet cells are first detected along the villi three days prior to hatch (Romanoff, 1960; Uni *et al.*, 2003b). In embryonic duodenum, at 18d incubation, 13% of epithelial cells were goblet cells, which were maintained throughout the first week post-hatch. This was also similar for jejunum and ileum (19%). On the day of hatch, goblet cells comprised 23% of epithelial cells in jejunum and 26% in the ileum, remaining the same until day 7 post-hatch (Uni *et al.*, 2003a).

The production of mucus in poultry, occurs during late embryonic development and immediately post-hatch (Uni *et al.*, 2003b). Differences in fluorescein isothiocyanate

(FITC)-conjugated lectin binding were observed in embryonic and day old chicks with variation of intestinal mucin among the examined intestinal segments. During days 18-21 of incubation, and day one post-hatch, the duodenum possessed goblet cell mucin containing GalNAc, galactose and α -L-fucose at day 18 indicated by soybean agglutinin (SBA), peanut agglutinin (PNA) and *Lotus tetragonolobus* (LTA) lectin reactivity. At day 20, reactions with wheat germ agglutinin (WGA), indicated an appearance of GlcNAc with possible sialic acid binding (Bryk *et al.*, 1999). In the ileum, SBA lectin reactivity was observed from day 17 to 19 with only WGA staining detected at hatch. WGA was detected in the colon at day one, interestingly *Ulex europaeus-I* (UEA-I) and LTA was detected, indicating two different types on linkage of α -L-fucose. Lectins specific for sialic acid were not used in this study and thus the profile of sialic acid throughout the intestine of poultry is yet to be determined.

Shub *et al.*, 1983, demonstrated age-related changes in composition and structure of mucin in newborn and adult rats showing differences in chemical and physical characteristics of mucin glycoprotein. Mucin from newborn rat intestine had a higher content of threonine, alanine and valine and a lower content of serine, proline, tyrosine and lysine. Newborns also contained a smaller carbohydrate content compared to adult mucin samples due to lower amounts of fucose and N-acetyl-galactosamine. Both adults and newborns had sialic acid and ester sulphate groups, however, newborn rats had a higher sulphate content (Shub *et al.*, 1983). These results were consistent with results in pigs, with mucins from 21-day-old sows having higher fucose and glucosamine and lower sialic acid and sulphate content compared to newborns (Turck *et al.*, 1993). In poultry, goblet cells were also observed to contain mostly acid mucins with less than 1% being periodic acid-Schiff (PAS) positive (indicating neutral mucins) during late incubation and that the ratio remained similar after hatch (Uni *et al.*, 2003a). The measurement of glycosyltransferase activity in the intestine

of mice further confirmed these results, with high sialyltransferase activity and low fucosyltransferase activity detected in suckling mice. However, the activity of these enzymes were reversed as the animals aged (Dai *et al.*, 2002; Nanthakumar *et al.*, 2003).

The rise in acid mucins has been speculated to coincide with maturation of barrier function. Their presence may be of particular importance as an innate barrier, as the acquired immune system is not fully functional in the neonatal intestine and thus more susceptible to infection. These observations may highlight the potential protective properties of mucin during early development.

1.5. Germ-free versus conventionally raised animals

Numerous rodent studies comparing Germ-free (GF) and Conventionally (CV) raised animals show distinct changes in mucosal morphology and mucus composition associated with the presence of intestinal microflora. Compared to CV rodents, GF rodents exhibited a decrease in colonic goblet cell size and number (Kandori *et al.*, 1996), with a consequent reduction in mucus layer thickness, indicating a reduction in mucus production (Abrams *et al.*, 1963b; Szentkuti *et al.*, 1990). Under germ-free conditions, staining densities of neutral and sialo-mucin containing goblet cells were lower, however, greater amounts of sulpho-mucin were observed in the small intestine, without any significant difference in numbers of mucin containing cells (Sharma & Schumacher, 2001). In the colon, the acidic to neutral mucin ratios were greater, with sialo-mucin containing cells in higher abundance in CV animals. Additionally, Meslin *et al.*, observed that numbers of goblets cells containing sialomucin was decreased in the intestinal mucosa but increased in the caecum. Sulpho-mucin in CV animals increased in the intestinal mucosa but was decreased in the colon (Meslin *et al.*, 1999a). However, mucus content (determined by mucin fractions) revealed there was an increase in sialomucin in all three sites. Using GF rodents, mucin

histochemistry of neonatal colonic crypts was examined. Both CV and GF animals had a similar mucin composition of predominantly sulphated mucin. Over time, sulphated mucins were still preserved in colonic crypts of GF animals, however in colonised animals, goblet cells containing sulphated mucins were only found in hyperplastic crypts, with sialomucins occurring more apically (Hill *et al.*, 1990).

Using FITC-conjugated lectins, researchers have been able to obtain a map of the peripheral sugars present in different intestinal regions of GF and CV rodents. Both GF and CV rats and mice exhibited similar staining with most lectins used (Freitas *et al.*, 2002; Kandori *et al.*, 1996; Sharma & Schumacher, 1995). However, UEA-I (L-fucose) stained only in goblet cells of conventional mice in both the ileum and the colon (Freitas *et al.*, 2002). This was not observed in the colon of rats with GF animals exhibiting a similar weak to nil staining intensity to CV animals (Sharma & Schumacher, 1995; Szentkuti & Enss, 1998). By contrast, GF rodents exhibited no binding of PNA (specific for Gal β 1,3-GalNAc), *Datura stramonium* agglutinin (DSA; specific for Gal β 1,4-GlcNAc) and WGA (specific for GlcNAc β 1,4n and sialic acid) however, binding was observed in CV colonic goblet cells. Concanavalin A (ConA; specific for mannose), was bound to goblet cells in GF but not CV animals. Although lectins for sialic acid such as *Limax flavus* agglutinin (LFA) were detected in both CV and GF animals, staining intensity for sialic acid residues was greater in CV animals (Szentkuti & Enss, 1998). In the small intestine, minor changes in the localisation and intensity of goblet cell staining was observed with *Helix pomatia* agglutinin (HPA; specific for GalNAc β (1,4)Gal), becoming associated with immature mucus vesicles, compared to labelling of mature mucus vesicles in GF mice (Freitas *et al.*, 2002). In the caecal mucosa, Kandori *et al.*, 1996, found that the binding of most lectins including PNA and WGA, was similar in both CV and GF animals. *Dolichos biflorus* agglutinin (DBA) and UAE-1, however, exhibited strong binding to goblet cells in CV

mice but not in GF animals (Kandori *et al.*, 1996) indicating a higher concentration of N-acetyl D-galactosamine and fucose.

1.6. Bacterial adhesion

Host-bacterial interactions are complex as they involve fixation of bacteria to a specific site along the gastrointestinal tract (Robbe *et al.*, 2003). As mentioned previously, mucin glycoprotein can prevent bacterial binding to epithelial cells by competing with bacteria for adherence via their extremely heterogeneous oligosaccharide chains. Mucin oligosaccharides also provide binding sites for microbial secretory products, such as large biomolecules including microbial toxins (cytotoxins, cytolytins, or invasins), surface proteins, hormones and antibodies (Freitas *et al.*, 2002). However, the high carbohydrate content of mucin provides a nutritional substrate for specific microflora, which may enhance colonisation, and possible infection (Deplancke & Gaskins, 2001; Robbe *et al.*, 2003). The diversity of the filamentous carbohydrate structures of mucus provides a vast library of potential target recognition sites for glycosidases, or lectin-like adhesins of both commensal and pathogenic organisms (Forstner & Forstner, 1994; Klinken *et al.*, 1995).

The intestinal microflora has the ability to colonise the gut by specific attachment properties. Adhesion can occur utilising one or more of the following mechanisms; passive Van der Waals' attractive forces, electrostatic interaction, hydrophobic steric forces and lipoteichoic acids. Frequently, active adhesion can also occur through the production of structural external appendages such as lectins, pilli or fimbriae (Gusils *et al.*, 2002) and/or extracellular polymers. Lipopolysaccharides (LPS), are major outer surface membrane endotoxins and are present in almost all gram-negative bacteria, stimulating innate immunity and inflammation (Gusils *et al.*, 2002; Smirnova *et al.*, 2003). Utilising these

properties, bacteria can bind to glycoconjugate receptors on the surfaces of mucus and/or brush border membranes of the gastrointestinal tract (Sharma *et al.*, 1997).

Different bacterial species have specific binding affinities to different sugars. For example, *L. animalis* has binding sites for glucose and/or mannose, whereas binding to intestinal mucus by *L. fermentum* was reduced with the addition of sialic acid or mannose (Gusils *et al.*, 2003). Different fimbriae also have specific affinities for different sugar moieties (Table 1.4). *E. coli* for instance, can bind to mannose via P or type 1-fimbria (Firon *et al.*, 1984) and sialic acid by way of S-fimbriae (Herias *et al.*, 1995).

Adherence to epithelial cells can also differ with age. It was observed that diarrhoea causing *E. coli* adhered to enterocytes more readily in neonatal pigs than in older pigs for both K99⁺ (Nagy *et al.*, 1992; Runnels *et al.*, 1980), K88⁺ (Conway *et al.*, 1990) and 987P⁻ serotypes (Dean-Nystrom & Samuel, 1994). These observations were also demonstrated in the mouse and calf (Runnels *et al.*, 1980). Different serotypes of *E. coli* adhered in different numbers. For example, K99⁺ serotype adhered more significantly than 123, with K88⁺ having the greatest adherence. It was suggested that these strains had more avid adhesive forces because of greater numbers of pilli present on the bacterial surface, or alternatively more host cell receptors on epithelial cells (Runnels *et al.*, 1980). One explanation for reduced adherence in older animals could be a protective function of mucin hindering attachment to the underlying cells, therefore reducing infection. The increased mannose content of mucin in older animals (Shub *et al.*, 1983) does coincide with binding of type-1 fimbriae of *E. coli* (Firon *et al.*, 1984).

Table 1.4. Possible mucin sugar receptors for species specific bacterial adhesion (Forstner & Forstner, 1994; Gusils *et al.*, 2003; Vimal *et al.*, 2000)

Receptor/sugar	Bacteria
Oligomannosides of mucin N-glycans	Type 1 pilli of specific isolates of verotoxin-producing <i>E. coli</i> O157:H7
Mannose containing glycoprotein fragment from mouse colonic mucus	LPS adhesion of human faecal <i>E. coli</i>
D-galactosamine containing receptors	K88 pillus-associated antigens of <i>E. coli</i> K12
Saccharides	K99, F41 and Fy pilli of enterotoxigenic <i>E. coli</i>
Putative fuctose-containing receptors	<i>Salmonella typhimurium</i> ; <i>Campylobacter jejuni</i> LPS adhesions
Gal-GalNAc receptors	<i>Entamoeba histolytica</i> trophozoites
Gal β 1,3 GlcNAc or Gal β 1,4 GlcNAc	<i>Pseudomonas aeruginosa</i>
GlcNAc-containing core mucins structures	Strains of <i>Pseudomonas cepacia</i>
Sialic acid-containing mucin structures	S-fimbriae of <i>E. coli</i>
250kDa neutral mucin specifically mannose	<i>Salmonella typhimurium</i> , pilli adhesion
Glucose and or mannose	<i>L. animalis</i> CRL1014

Different strains of bacteria exhibit a high tendency to compete for specific binding sites. For example, desired bacteria, such as bifido-bacteria, can bind to either mucus or epithelial cells and prevent pathogenic colonisation. This can be achieved by either lowering pH, due to production of fermentation products such as acetate and lactate, or by the production of inhibitory substances (bacteriocins; Kleessen *et al.*, 2003). Other reports have suggested that bacterial strains such as *L. fermentum* spp *cellobiosus* and *L. casei* can adhere to receptors on both mucins and epithelial cells, by way of possessing high cellular hydrophobicity and lectin like proteoic structures inhibiting pathogenic bacterial adhesion (Gusils *et al.*, 2002; Styriak *et al.*, 2003). A decrease in *E. coli* adherence to epithelial cells, with the addition of Lactobacillus species in culture, has been reported (Gusils *et al.*, 2003; Otte & Podolsky, 2004) However, similar experiments using salmonella found no differences in adhesion of salmonella species to mucus or intestinal epithelial due to the addition of lactic acid bacteria (Gusils *et al.*, 2003). Suggesting different adhesion properties between particular bacterial species can reduce or eliminate competition for colonisation.

1.7. Microbial modulation of mucus production

Bacteria can affect the mucus layer by influencing the production or quantity of mucus secreted at different stages of synthesis, by either affecting goblet and other underlying cell proliferation and morphology (Meslin *et al.*, 1999b), or the mucin carbohydrate structure itself (Smirnova *et al.*, 2003). However, the mechanisms by which bacteria cause these changes are still largely undefined.

Intestinal microbes may directly affect goblet cell functions through the local release of their bioactive factors or in response to host-derived bioactive factors generated by activated epithelial or underlying lamina propria cells after their contact with intestinal

bacteria (Deplancke & Gaskins, 2001). Mucus expression by goblet cells can be altered to increase mucus production and consequent protective properties through the formation of viscous polymeric structures. *In vitro* experiments using cultured HT29 colonic goblet cells showed that certain strains of lactobacilli induced upregulation of MUC3 expression, correlating with MUC3 mucin secretion, with a means to also inhibit enteropathogenic *E. coli* (Mack *et al.*, 2003). Similarly, when isolated rat colonic loops were exposed to the probiotic formula VSL#3, mucin secretion and MUC2 gene expression was significantly increased (Caballero-Franco *et al.*, 2007). From the bacterial groups present in the VSL#3 mixture, *Lactobacillus* species was the strongest stimulator of mucin secretion *in vitro*.

Short chain fatty acids (SCFAs) produced by bacteria have been shown to be involved in increased MUC2 expression through the stimulation of prostaglandin 2 (PGE₂) by the host. PGE₂ has been shown to regulate mucus secretion of epithelial cells through association with myofibroblasts in the underlying lamina propria (Willemsen *et al.*, 2003). Additionally, the SCFA butyrate, produced by bacteria fermentation, was found to up-regulate MUC3 and MUC5B gene expression in HT29-C1.16E colonic goblet cells. Expression was greatly enhanced, including that of MUC2 and MUC5AC, when butyrate was the primary energy source (Gaudier *et al.*, 2004).

Further investigation is needed to determine how products of carbohydrate fermentation exert feedback control on goblet cell functions. Changes to goblet cell and mucin dynamics could either be a result of bacteria colonisation alone or in conjunction with changes in luminal environment, such as decreased pH due to bacterial fermentation processes.

1.7.1. Indirect response through host-derived factors

Lipopolysaccharides (LPS) have been found to induce morphological changes in goblet cells and goblet cell proliferation in different types of mucosa, stimulating mucin secretion in numerous tissues including the intestine, through upregulating gene transcription in epithelial cells (Smirnova *et al.*, 2003). Using human colonic cell line HT29-MTX (a well-characterised mucin-secreting cell sub population), it was found that LPS stimulated goblet cell mucin expression and secretion of MUC5AC (predominant) and MUC5B. LPS was also found to stimulate IL-8 (pro-inflammatory cytokine) secretion and expression. It was speculated that LPS-induced mucin secretion occurred because of IL-8 mediation (Smirnova *et al.*, 2003). Several interleukins such as IL-1, IL-4 and IL-6 have been reported to increase expression of mucin genes. For example, the pro-inflammatory cytokine IL-6 was found to stimulate LS180 cells and increase the expression of MUC2, MUC5AC, MUC5B and MUC6 (Enss *et al.*, 2000). Expression of cytokines, IL-1 and IL-6 have been shown to increase in the presence of intestinal microflora (Shirkey *et al.*, 2006).

Studies have been conducted focussing on mechanisms, such as receptors and pathways, involved in the hypersecretion of mucus, but at present still require further investigation. Expression of mucin genes is controlled by transcription factors, which have been found to be upregulated by LPS (Zen *et al.*, 2002). Studies have reported that increased expression of MUC2 and MUC5AC by LPS from *E. coli* (Oss:B5) involves a pathway initiated through binding to CD14 receptors on biliary epithelial cells (BEC). This event causes an upregulation of the transcription factor, tumour necrosis factor (TNF- α). Upon release from the cell, TNF- α binds to its corresponding TNF receptor which then increases protein kinase C (PKC) activity, and intern, stimulates mucin transcription (Zen *et al.*, 2002).

Through a nuclear factor kappaB (NF- κ B) transcription factor, LPS has also been found to affect mucin expression in colonic and airway epithelial cell lines. Using *P. aeruginosa*, LPS was found to bind to LPS-binding protein (LBP), activating a protein kinase pathway (c-Src dependant Ras-Mek1/2-MAPK-pp90rsk pathway), which then stimulates NF- κ B. This then binds to a κ B site in the 5' flanking region of the MUC2 gene, causing an increase in expression (Li *et al.*, 1998).

Virulent factors of *Helicobacter pylori* such as *CagA* and *CagE* have been reported to activate the NF- κ B transcription factor, which stimulated the transcription of cytokines such IL-6 (Pritts *et al.*, 2002) and IL-8 (Maeda *et al.*, 2001) leading to possible MUC gene expression. Toll-like receptors (TLR4) activated by LPS, are thought to be involved in the stimulation of NF- κ B and thus cytokine expression. Su *et al.*, found that *H. pylori* activated TLR4 mRNA expression, but this did not result in increased cytokine expression in all three gastric epithelial cell lines (AGS, MKN45 and T84; Su *et al.*, 2003).

1.7.2. Microbial modulation of glycosylation

It has been found that cytokines can upregulate specific glycosyltransferase genes involved in the elongation of mucin oligosaccharide chains. In a human airway epithelial cell line (H292) IL-4 and IL-13 were found to upregulate C2GnT-M (core 2 β 1,6 *N*-acetylglucosaminyltransferase; Beum *et al.*, 2005). There is also evidence that butyrate, a product of carbohydrate fermentation, not only upregulates glycosyltransferase enzymes, but also genes involved in inflammation and cell adhesion, primarily Galectin-1. The addition of butyrate to a colonic cell line (HT29.Cl.16E) resulted in the upregulation of 3 sialotransferases and one sulphotransferase involved in mucin glycosylation (Gaudier *et al.*, 2004).

Comparison studies using GF and CV rodents, found that the induction of α 1,2-fucosyltransferase activity was induced with the addition of intestinal microbes to germ free mice (Nanthakumar *et al.*, 2003; Umesaki *et al.*, 1982). Similarly, in conventionally reared mice, β 1,4-galactosyltransferase (β GT) expression increased rapidly to adult levels by 4 week post-natal, however GF mice displayed low levels of enzyme activity but was rapidly induced with the reintroduction of intestinal microbes from adult rats (Nanthakumar *et al.*, 2005). Enzyme activity was also found to be parallel to mRNA expression, suggesting that intestinal microbes have an effect on transcriptional regulation during development.

At present, the mechanisms behind intestinal microflora and the control of glycosylation is still illusive. The connection between upregulation of cytokines, MUC and glycosyltransferase gene expression suggests that goblet cells and mucus production contribute to mucosal immunity (Smirnova *et al.*, 2003) and warrants further investigation in order to understand these pathways in response to pathogens and overall bacterial colonisation.

1.7.3. Microbial mucolysis

Mucolysis involves the proteolysis of the non-glycosylated ends of mucin. The mucin chain is broken into smaller subunits, reducing mucus viscosity and gelation (Deplancke & Gaskins, 2001). The ability to enzymatically degrade mucus can facilitate colonisation on the epithelial surface and is common among pathogenic and commensal bacteria (Corfield *et al.*, 1992; Deplancke & Gaskins, 2001). Enzymes such as O-acetylsterases, arylesterases and glycosulphatases can break down mucins and thus can find specific fixation sites on epithelial cells (Robbe *et al.*, 2003).

A correlation may exist between the type of glycoconjugate to which microbes preferably bind and the glycosidase they possess (Deplancke & Gaskins, 2001). For example, *H. pylori* binds to sulphate glycoconjugates (Bravo & Correa, 1999) and also secretes glycosulphatases, which can cleave sulphate from its linkage to mucin sugars (Robertson & Wright, 1997).

The hydroxyl groups of sialic acids are highly substituted by acetyl esters, which serve as added protection, as they block against further glycosidic degradation by sialidases. Two or more acetyl groups have been reported to inhibit enteric bacterial sialidases (Belley *et al.*, 1999; Corfield *et al.*, 1992). Both sialic acid and sulphate groups have been shown to possess protective properties (Corfield *et al.*, 1992; Robertson & Wright, 1997). For example, the addition of sulphated glycoconjugates *in vitro* was found to inhibit *Clostridium perfringens* neuraminidase activity, reducing mucin degradation (Mian *et al.*, 1979).

1.8. Conclusion

The heterogenous and polymeric nature of mucins secreted by goblet cells allows for further investigations into mucosal-bacterial interactions. Although there are many studies describing the impact of bacteria on mucus composition in the gut, the mechanisms behind these changes are poorly understood. Possible changes in mucin composition through the upregulation of MUC and glycosylation gene expression raise questions as to how bacteria may influence intestinal cell signalling. There are essentially two outcomes to induced changes to mucin composition; firstly, to facilitate the breakdown of the mucus layer and thus promote colonisation, or secondly, to illicit an immune response, beneficial to the host, thereby preventing colonisation and infection of pathogens.

The establishment of a normal intestinal microflora during early post-hatch development is imperative for the health and productivity of the animal. Understanding the nature of the mucus layer and its interactions with the existing microflora during early life will provide a greater knowledge base for combating infection, increasing growth, as well as assisting in the production of new therapies and interventions, reducing reliance on antibiotics.

It was therefore hypothesised that when compared to broiler chicks reared under conventional brooding conditions, chicks reared with a minimal exposure to microflora during early post-hatch development would exhibit differences in goblet cell and mucin production. Furthermore, any changes in mucin composition would be of benefit to the host as a defensive strategy during this period when the immune system is still immature. The following chapters describe the development of a low bacterial load isolation system for hatching and rearing chickens and the use of this system for studying histological changes in the small intestine of chickens and changes to the composition and structure of mucins secreted by goblet cells.

Chapter 2 Animal Trials

A small-scale, low-cost isolation system for the incubation and rearing of low bacterial load chicks as a model to study microbial-intestinal interactions

2.1. Introduction

Flexible film isolators are widely employed for housing animals under germ-free, gnotobiotic and specific pathogen-free (SPF) conditions (Dennet & Bagust, 1979; Phillips *et al.*, 1962). Such housing is useful as it allows the study of mechanisms underlying the pathology caused by bacterial infection. Isolation systems are usually a component of larger scale SPF or Physical Containment Level 2 (PC2) animal housing facilities, which are costly both in terms of construction and management (Cooper, 1970). Since facilities of this nature were not available on site, the aim was to establish a convenient, cost-effective, clean room environment to produce and house chicks with a minimal load of intestinal microflora to facilitate the study of mucosal-microbial interactions.

Many isolator systems for rearing chicks have placed eggs in the isolator during the hatching period or even at one day of age (Dennet & Bagust, 1979; Drew *et al.*, 2003; Phillips *et al.*, 1962). The feasibility of incubation and hatching chicken eggs within an isolator system was examined in an attempt to minimise exposure to the outside environment. This section describes the details of the flexible-plastic isolators used for the incubation, hatching and rearing of low bacterial load (LBL) broiler chicks, along with microbiological results, and growth rate comparisons with conventionally reared chicks.

2.2. Materials and methods

2.2.1. Isolator construction

A small, non-SPF animal housing facility (~49.48m³) situated at the University of Adelaide's Roseworthy Campus was used for the experiment. Two isolators were prepared for the incubation, hatching and rearing of low bacterial load chicks (University of New England, Armidale, New South Wales) (Appendix 1). One isolator was designated for incubation and hatching (Isolator type B, 500 x 1000 x 1300 mm) (Figure 2.1) and the other for brooding (Isolator type A, 500 x 500 x 1300 mm) (Figure 2.2). The isolators consisted of a metal frame with two solid metal ends, which accommodated the air inlet (Figure 2.2.b) and outlet (Figure 2.1.d), the access box (Figure 2.2.a), temperature probe inlet (Figure 2.2.f) and drinker line (Figure 2.2.f). The pressed metal floor was elevated above the outer plastic of the isolator in order to allow passage of faeces (Figure 2.1). The isolator frame was covered in disposable PVC flexible plastic (0.2mL Dry Slip, 1.36 x 100m, Halifax Vogel Group, Turrella, New South Wales), which was replaced after each trial (Figure 2.2.c).

The access box was used as a means of transporting items into and out of the isolator. It consisted of two sections (one with a glued extension) of 150mm i.d. PVC pipe and two sealed lids. Access to the isolator was achieved by inserting glove ports into the plastic walls. Gauntlet rings constructed from 200mm i.d. PVC pipe (Virginia Irrigation Service Pty Ltd., Virginia, South Australia), were used to attach a pair of 81.3cm long, 20.3cm port neoprene dry box gloves (Mohawk Industry and Nuclear Supply, Inc. CT, USA). Two glove ports were attached on opposite sides of the type B isolator (Figure 2.1.b) and one on the type A isolator (Figure 2.2.d).

A portable air filter (Model LR2, 0.2 μ m, 5m² high efficiency filter media area, Ozone Pollution Technology, Silverwater New South Wales) was used to supply microbe-free air at the correct positive air pressure and flow to both isolators via a 50mm diameter dual spigot (Type A isolator, 6.5m³ Air Req/hr, minimum backpressure 250Pa; Type B isolator, 13m³ Air Req/hr, minimum backpressure 250Pa; Figure 2.3.d). A supplied in-line silencer (Figure 2.3.b) was attached to minimise noise. The air filter was connected to the isolator's air inlet connection via a 50mm diameter duty hose and air valve (Figure 2.3.d). The presence of the air valve provided an effective means of blocking the passage of air during fumigation.

The air relief line (Figure 2.1.d) consisted of a long section of 25mm diameter PVC pipe that hung parallel to the isolator end to exhaust waste air from the isolator. At the bottom of the pipe there was a fitting to accommodate a cylindrical mesh filter to prevent insects from entering. To avoid dust, insects and other contaminants from the isolator becoming airborne in the room, the mesh filter was submerged in a dilute insecticide solution (Coopex[®], 1g/100ml, Intensive Farming Supplies, Cavan, South Australia).



Figure 2.1. Incubation and hatching isolator (B). (a) access box, (b) glove port, (c) *Maino* 50-egg incubator, (d) air relief line with mesh filter submersed in bucket of insecticide solution.

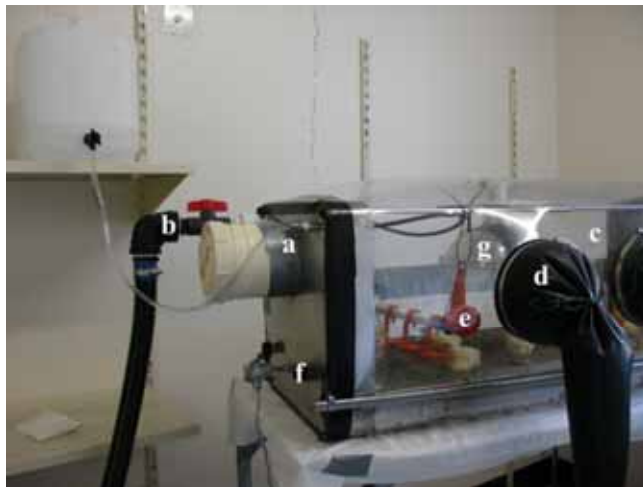


Figure 2.2. Brooding isolator (A), (a) access box, (b) air inlet; (c) flexible plastic walls; (d) gauntlet rings with neoprene gloves; (e) drinker and drinker line; (f) temperature probe; (g) brooding lamp.



Figure 2.3. The Ozone Air Filter, (a) on/off switch and variable speed motor (control of airflow); (b) air entry into filter; (c) silencer; (d) dual spigot/hose connection.

2.2.2. Testing for leaks

All external air access to the isolator was sealed. The air filter was turned on and the air valve was opened to distend the isolator. Air leaks were detected by applying a detergent solution to areas for detection of air bubbles. Any leaks were repaired, residual pressure released and all airlines resealed.

2.2.3. Sterilisation of isolators

All equipment used for the construction and set-up of the isolator was first thoroughly cleansed with a warm 6% v/v sodium hypochlorite solution. Once assembled, the air inlet and outlet were closed, and the isolators fumigated with formaldehyde gas for 24h (Isolator A, 9ml formaldehyde solution (37-40%) added to 5g potassium permanganate (Sigma, Sydney); Isolator B, 18ml formaldehyde solution (37-40%) added to 11g potassium permanganate) and then ventilated for another 24h. After ventilation, the isolators were fogged with Virkon S[®] (10g/L) disinfectant as a secondary treatment. Insecticide treatment (Coopex[®] 1g/100ml) was also applied to possible entry points for insects.

2.2.4. Isolator type B: incubation and hatching

Two Maino 50-egg incubators (Model ME3, type A with automatic egg turning, Intensive Farming Supplies, Cavan, South Australia) were used for incubating and hatching under isolation (Figure 2.1.c). The incubators were lightweight and portable, consisting of a plastic tub and lid with a metal egg tray that held 50 chicken eggs. Although the incubators had automatic temperature and egg-turning control the humidity needed to be manually maintained using the two small rectangular basins provided with the incubators and a large shallow basin that sat underneath the egg tray. Humidity was measured using a small digital humidity metre, which could be attached to the inside of each incubator (Incubators and More, Adelaide, South Australia). The isolator was stocked with sterile MilliQ water for use in the basins. All equipment necessary for incubation, candling and hatching was placed in the isolator before securing the flexible plastic.

Before use within the isolator a preliminary experiment was conducted to determine the hatching rate of broiler chicks using these portable incubators and also to optimise the equipment to achieve the necessary humidity and temperature for successful incubation and hatching.

2.2.5. Isolator type A: brooding

A drinker was constructed based on the Iberline Poultry Nipple System (Intensive Farming Supplies, Cavan, South Australia), using square pipe and fittings, 3 nipples, 3 orange water collection cups (Figure 2.2.e) and a sight tube to flush out water. Drinking water was gravity-fed from an autoclaved plastic 10L water drum via a flexible water line that passed into the drinker through an inlet located on the metal isolator wall (Figure 2.2). The height of the drinker could be adjusted using cable ties as the chicks grew. Brooding temperature was achieved by the attachment of a brooding lamp and a 150W ceramic heating bulb

(Intensive Farming Supplies, Cavan, South Australia). This was secured to the top of the isolator frame via a metal plate (Figure 2.2.g). After the plastic film had been fitted, the lamp power cord was pulled through the plastic film and sealed. Brooding lamp temperature was controlled by way of a universal temperature monitor (Model PM4-RT 240) and an industrial temperature probe (Model Pt100 RTD, P-RTQ12SA-1, Amalgamated Instruments Co. Pty. Ltd., Hornsby, New South Wales). The brooding lamp power cord was plugged into the monitor, with temperature set at $32 \pm 1^\circ\text{C}$. The probe was connected to the isolator via a designated access port (Figure 2.2.f).

2.2.6. Sterilisation of feed and drinking water

Chicks received high-energy broiler starter crumble (Ridley brand, Gawler Stock Feeds, Gawler, South Australia), which was separated into 500g portions and vacuum-sealed. The bags were sterilised by gamma irradiation at 25 kilograys (Steritech, Dandenong, Victoria). Feed was administered by transferring a bag of feed through the access box via a soak in Virkon S[®]. Once in the isolator, the bag was opened and feed dispensed into a standard plastic feeder (Intensive Farming Supplies, Cavan, South Australia). The bag was removed through the access box and discarded. MilliQ water was autoclaved in glass 1L bottles at 121°C for 20mins and transferred into the sterile water drum. After each trial, excess water was removed, and the water drum sterilised.

2.2.7. Procedure for low bacterial load chicks

Eggs (n=100), of similar weight (~50g) were collected from a commercial Cobb 500 fast-feathering flock from HiChick Hatchery, Bethel SA. Two trials were conducted, one in August 2005, and the second in March 2006. The eggs collected were from different flocks. Since eggs were sourced from a commercial flock and not from a SPF or germ-free source, eggs were selected from hens that had commenced egg production 3 weeks prior to

collection. This was to reduce bacterial transfer from the oviduct and or cloaca to the egg, which is greatly increased in older hens (Dennet & Bagust, 1979). All eggs were laid within a two-hour time period before collection (0900-1100h). Eggs were dipped in Ambicide[®] (Independent Veterinary Supplies, Melrose Park, South Australia) at 42.2°C and air-dried (42°C). Once dry, they were placed in sterile plastic boxes fitted with HEPA venting filters (Whatman HEPA-Cap 75, Singapore). They were then transported to Roseworthy and pre-incubated in the clean room at 17°C for 3 d and 26°C for 12 h.

After pre-incubation, the eggs were washed prior to entry into the isolator, using modified methods (Drew *et al.*, 2003; Wang & Slavik, 1998). All equipment was cleaned and sterilised either by autoclave or by soaking overnight in Virkon S[®] (10g/ml). 10-15 eggs were removed from the sterile boxes and placed into a large aluminium foil tray containing 1% sodium hypochlorite (44°C). Eggs were gently scrubbed using a hard bristled toothbrush to remove any organic matter attached to the shell. Once all were washed, they were placed into a plastic egg tray and then immediately into a drying oven for 2 min at 42°C.

The inside of the access box was saturated using Virkon S[®] spray. Ensuring that the inner lid was securely attached, the egg tray was placed in the box. Once inside the isolator, each egg was weighed, assigned a number and placed in the incubator. The process was repeated for the remaining eggs until all were washed and set in the incubators. The incubators were set at 37.7°C with humidity of 50-55% from day 0 to day 18. Incubators were checked morning and evening, the water basins were refilled with sterile MilliQ water and the egg turning device monitored to ensure even turning was being accomplished. Eggs were candled on day 5 of incubation to observe development. Non-

developing eggs were removed and yolks swabbed for detection of bacterial contamination.

On day 18 of incubation, the turning tray containing the eggs and the large shallow basin was removed and the eggs placed on the incubator floor. Four more small basins were placed against the edge of the incubator. Mesh screens were constructed to guard the basins and prevent the newly hatched chicks from drinking and/or spilling the water. Incubators were set at 36.7°C with a humidity of 60-65%.

At hatch, 21 chicks were transferred into the brooding isolator via an autoclaved cylindrical polypropylene container that was further soaked in Virkon S®. After each transfer the container was sprayed with Virkon S® and wiped using a disposable sterile cloth. Another 21 chicks were removed from the isolator and placed into conventional brooding pens (2 x 1.5m) at the PPPI poultry research unit. Chicks were reared on woodshavings, and maintained at 32 °C via a heat lamp. Freshwater was supplied using the Iberline Poultry Nipple System (Intensive Farming Supplies, Cavan, South Australia), using square pipe and fittings, nipples and orange water collection cups. Both groups were allowed free access to irradiated feed and water.

2.2.8. Cultures

Rectal swabs were collected daily from 3 chicks per group for 7 days to observe bacterial growth. Copan mini tip Amies agar gel transport swabs were used for rectal sampling (Interpath Services Pty, Ltd. West Heidelberg, Victoria). The swabs were thin aluminum wire with a narrow swab shaft, this enabled efficient sample collection. Surface swabs (Standard Copan Amies agar gel swabs) were also collected to monitor the sterility of the equipment. Gribbles Veterinary Pathology, Glenside SA, performed all swab analysis as

well as the assessment of bacterial status of the irradiated feed. Swabs were set up on the following plates: Blood Agar (to grow both Gram negative (GN) and Gram positive (GP) organisms), Macconkey Agar (to grow GN organisms), CAN agar (to grow GP organisms), Selenite broth (pre-enrichment for *Salmonella*) and XLD agar (selective for *Salmonella sp.*). Plates were incubated at 37°C and read at 24 and 48 h. A smear was made using the swab and Gram stained to detect GN and GP organisms.

2.2.9. Animal welfare

A preliminary trial, consisting of only 50 eggs was conducted to determine the efficacy of incubating, hatching and brooding chicks under the designed isolation conditions. The initial trial proved to be successful with the newly hatched chicks responding well to the bacterial-free conditions.

In all trials, animals were monitored daily for loss of condition. From the 3 chicks that were swabbed, body weights were also recorded to observe growth in each environment over the 7 d period. All experimental work was approved by the Animal Ethics Committees of the University of Adelaide and the Department of Primary Industries and Resources of South Australia.

2.2.10. Statistical analysis

Statistical analyses were performed using the SPSS software package V11.5 (SPSS Inc., Chicago, IL) Student t-tests were used to compare body weights between CV and LBL animals. Significance was determined as $P < 0.05$.

2.3. Results

2.3.1. Hatchability

Hatchability within the isolators was 60%, i.e. 60 viable chicks from 100 eggs. Chicks with poor condition, characterised by the presence of mottled feathers and/or irregular leg development, usually observed in chicks that hatch late, were excluded from the trial and humanely euthanased via cervical dislocation. The trial required a total of 42 chicks, hence the chicks used in the trial were those obtained on the first day of hatch. There were no losses of those 42 chicks during either trials.

2.3.2. Microbial analysis

2.3.2.1. Trial 1

Swab analysis of yolk sacs from removed candled eggs (n=10) showed all were negative for contamination except one in which the yolk sac had a heavy growth of coagulase negative staphylococci. Surface swabs showed that both isolators and incubators were bacteria-free before the addition of eggs and chicks and remained so until d 2 post-hatch when a light growth of coagulase negative staphylococci and *Streptococcus* species was detected in isolator A. Chicks remained bacteria-free until 48 h post-hatch when a light growth of *Bacillus* species and coagulase negative staphylococci was detected in rectal swab samples. One chick from this group had a high growth of *Streptococcus* species (Table 2.1). Conventionally reared chicks possessed the same bacterial species as LBL chicks with the addition of *E. coli* and *Proteus mirabilis*. Growth of these species was heavier compared to LBL chicks.

Table 2.1. Trial 1, August 2005. Microbial swab analysis of low bacterial load (LBL) and conventionally reared (CV) chicks.

Age, d	LBL		CV	
	Species	Growth	Species	Growth
1		0		0
	<i>Bacillus</i> sp.		<i>E. coli</i>	
2	Coagulase negative staphylococci	+	<i>Proteus mirabilis</i>	+++
	<i>Streptococcus</i> sp. (1 chick only)	++++	<i>Streptococcus</i> sp.	
3	<i>Bacillus species</i>		<i>Proteus mirabilis</i>	
	<i>Enterococcus faecalis</i>		<i>E. coli</i>	+++
	Mixed anaerobes	+	<i>Enterococcus faecalis</i>	
			Mixed anaerobes	
4	Coagulase negative staphylococci		<i>Proteus mirabilis</i>	
	Alpha haemolytic streptococcus		<i>E. coli</i>	+++
	Mixed anaerobes	+	Alpha haemolytic streptococcus	
			<i>Mixed anaerobes</i>	
5	Coagulase negative staphylococci		<i>Proteus mirabilis</i>	
	Alpha haemolytic streptococcus	++	<i>E. coli</i>	+++
	Mixed anaerobes		Alpha haemolytic streptococcus	
6	Coagulase negative staphylococci		<i>Proteus mirabilis</i>	
	Alpha haemolytic streptococcus	++	<i>E. coli</i>	+++
	Mixed anaerobes		Alpha haemolytic streptococcus	
7	Alpha haemolytic streptococcus		<i>Proteus mirabilis</i>	
	Mixed anaerobes	+++	<i>E. coli</i>	+++
			Alpha haemolytic streptococcus	

0: no growth; +: none –light; ++: light-moderate; +++: moderate-heavy; ++++: heavy
(Light growth, 10-50 colonies; Moderate growth 50-100 colonies; Heavy growth >100 colonies)

2.3.2.2. Trial 2

Similar to Trial 1, culture and microscopy of swabs collected from non-developing eggs removed at candling detected no bacterial growth in the yolk (n=10). Surface swabs of

isolators and incubators showed all were bacteria-free before the addition of eggs and chicks and remained so until d 2 post-hatch when light growth of *Bacillus cereus* was observed. Rectal swabs of chicks in both groups also remained bacterial free until 48 h post-hatch, as observed in Trial 1, but the presence of bacterial species in LBL chicks was remarkably different with growth of only *Bacillus cereus*. The conventionally raised group, however, showed no growth of this species, but a heavy growth of *E. coli* and *Enterococcus faecalis* from d 2-7 post-hatch (Table 2.2). Analysis of irradiated feed and drinking water samples showed no growth of bacteria after 48 h incubation in culture.

Table 2.2. Trial 2 March 2006. Microbial swab analysis of low bacterial load (LBL) and conventionally reared (CV) chicks.

Age, d	LBL		CV	
	Species	Growth	Species	Growth
1		0		0
2	<i>B. cereus</i>	+	<i>Enterococcus faecalis</i> <i>Pseudomonas</i> species	++++
3	<i>B. cereus</i>	++++	<i>Enterococcus faecalis</i> Non-haemolytic <i>E. coli</i>	++++
4	<i>B. cereus</i>	++	<i>Enterococcus faecalis</i> Non-haemolytic <i>E. coli</i>	++++
5	<i>B. cereus</i>	+++	<i>Enterococcus faecalis</i> Non-haemolytic <i>E. coli</i>	++++
6	<i>B. cereus</i>	++	<i>Enterococcus faecalis</i> Non-haemolytic <i>E. coli</i>	++++
7	<i>B. cereus</i>	+++	<i>Enterococcus faecalis</i> Non-haemolytic <i>E. coli</i>	++++

0: no growth; +: none –light; ++: light-moderate; +++: moderate-heavy; ++++: heavy (Light growth, 10-50 colonies; Moderate growth 50-100 colonies; Heavy growth >100 colonies)

2.3.3. Body weights

There were no significant differences in body weight between the two groups in either trial over the 7 d time period ($P > 0.05$; Figure 2.4).

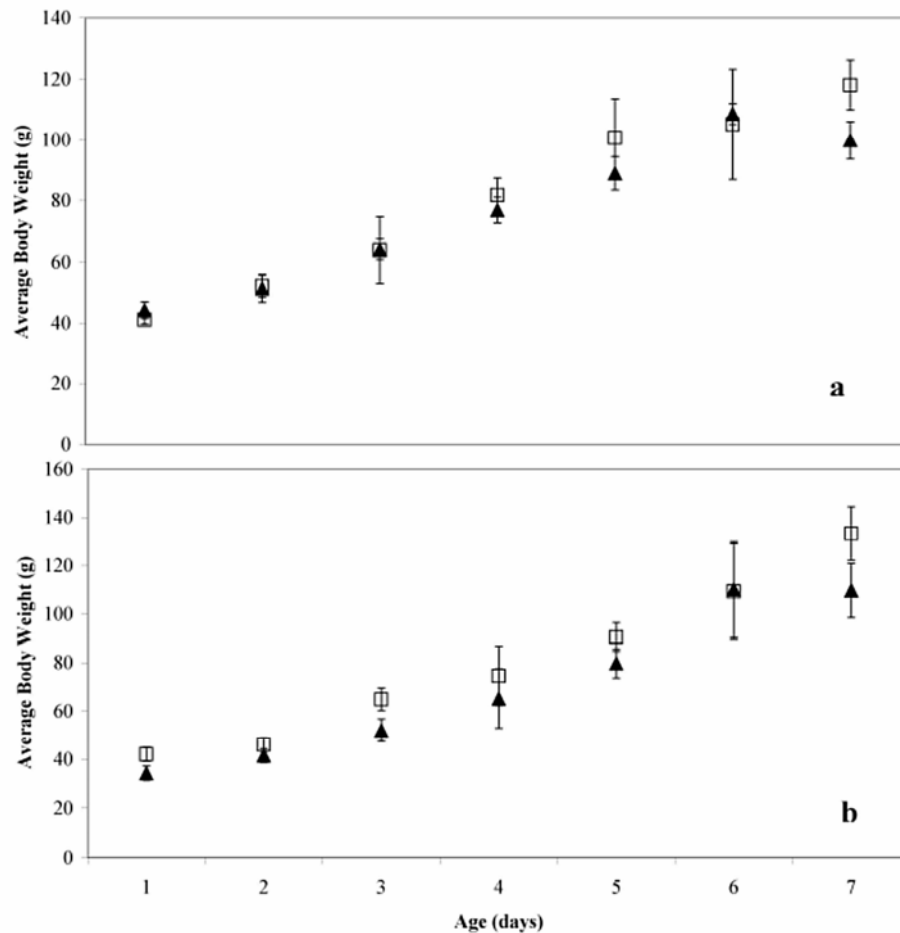


Figure 2.4. Comparative average growth rates of low bacterial load (LBL) (□) and conventionally reared (CV) (▲) Cobb broiler chicks over 7 d given irradiated broiler starter crumble for Trials 1 (a) and 2 (b). Each point represents the average weights and standard deviations of the three chicks sacrificed on day of measurement.

2.4. Discussion

Flexible film isolators were designed and assembled as a means for studying microbial-mucosal interaction in early post-hatch chicks. The isolator system with all parts provided was established in less than one week including cleaning and fumigation with minimal labour, for under \$6000 (AUS). Ideally this set up would benefit its location in a HEPA filtered barriered animal housing facility, in which traffic of people and materials could be controlled more effectively. Although meticulous in maintaining cleanliness and avoiding possible contamination, appropriate wash in/wash out facilities would have been preferred.

The presence of bacteria in CV and LBL groups was first observed at d 2 post-hatch indicating that it may have taken at least 48 h for bacteria to establish themselves in the gastrointestinal tract, either through environmental exposures or as a result of digestion of the yolk (Coloe *et al.*, 1984; Deeming, 2005; van der Wielen *et al.*, 2002). The presence of bacterial species in LBL chicks from rectal swabs during both trials indicated possible contamination.

Feed and drinking water tested negative for bacterial growth. All equipment showed no bacterial growth before chicks were transferred into isolator A and remained so until day 2 post-hatch. The majority of eggs removed at candling were negative for bacterial growth; however the detection of staphylococci in the yolk sac of a removed egg, suggested a possible bacterial contamination within certain eggs before entry into the isolator. Subsequent chick development, would allow the spread of bacteria within the isolator during hatching. In the current study, late-hatch chicks which were of poor condition were removed from isolator B and excluded from the trial. Whether these chicks contained pathogens warrants further investigation for future studies to determine the exact source of contamination. The differences in bacterial profiles observed between trials suggested that

the species of bacteria contaminating the eggs may have been defined by the microbial population of the source flock, indicating the importance of flock and egg selection for isolation experiments.

Coagulase negative staphylococcus, alpha haemolytic streptococcus and *Enterococcus* species have been found to exist naturally in the gut of poultry (Devriese *et al.*, 1991; Salanitro *et al.*, 1978). *Bacillus cereus* has also been found in broiler faecal samples (Barbosa *et al.*, 2005) and thus suggests all are resident commensals of the chicken intestinal microflora. The observed normal growth rates and no loss of condition in LBL chicks indicated no pathogenic effect of these species. A study on *B. cereus* and its ability to persist in the intestine of rats showed no detection of endotoxin, suggesting that the rat gut physiology may not allow the bacterial spores to germinate and produce endotoxins (Wilcks *et al.*, 2006). This may also be true for the gut of chickens.

It is interesting to note that in Trial 2 only the LBL group had *B. cereus* present. Competition between species, whereby this bacillus species cannot successfully colonise in the presence of certain bacteria, is a possible explanation. Since the LBL environment appeared to be free of other bacterial species, the *Bacillus* sp. had the potential to thrive, which would explain the increase in growth over the 7-day period.

Previous studies using germ-free chickens have used a series of culture methods to detect contamination (Drew *et al.*, 2003; Phillips *et al.*, 1962; Wagner, 1959), and have reported negative bacterial growth in these animals and claimed them to be germ-free. Consistent with this study, the eggs used in the trials of these investigators were obtained from commercial layer flocks, where all hens would have been exposed to a variety of bacterial species. In the current study, it was very difficult to avoid contamination in both trials. The

cleaning methods for entry into the isolator may have been more successful in other studies than the methods presented here, or the use of a smaller number of eggs may have reduced the chance of contamination.

Despite the presence of bacterial species in both trials, the isolator design was able to produce chicks with a lower bacterial load than conventionally raised chicks without compromising body weight. The isolator design was also economical in terms of costs and labour, providing a suitable means to study mucosal-microbial interactions in the gut of poultry.

Chapter 3 Histological Study

Bacterial modulation of small intestinal goblet cells and mucin composition
during early post-hatch development of poultry

3.1. Introduction

The intestinal mucosa is densely-populated with micro-organisms (both commensal and pathogenic) capable of intense metabolic activities, such as the fermentation of complex carbohydrates (Macfarlane & Macfarlane, 2006). Enteric infections with pathogenic bacteria play an important role in animal health with the initiation and perpetuation of diseases such as diarrhoeal disease caused by enterotoxigenic *E. coli* in neonatal pigs, calves and lambs (Runnels *et al.*, 1980) and Necrotic Enteritis, which in poultry, is responsible for reducing growth rates and consequent economic losses in animal production (Wages & Opengart, 2003).

The overlying mucus-gel layer is the first line of defence that foreign bacteria and other pathogens encounter when attempting to traverse the intestinal mucosa. Formation of the mucus-gel is through goblet cell secretion of polymeric mucin glycoprotein (Forstner & Forstner, 1994; Klinken *et al.*, 1995). These glycoproteins compete with bacteria for adherence via heterogenous oligosaccharide chains (Belley *et al.*, 1999), thereby preventing noxious agents from coming into contact with the underlying epithelial cells. However, simultaneously, the high carbohydrate content of mucin provides a nutritional source for specific microflora which may benefit their proliferation (Deplancke & Gaskins, 2001). Thus, the chemical composition of mucus is essential for establishment of the intestinal barrier.

Histologically, mucins can be separated into two broad categories; neutral and acidic, with the latter further sub-divided into sulpho- and sialo-mucin types (Forstner & Forstner, 1994; Kiernan, 1990). These terms are derived from the chemical nature of the oligosaccharide sugar moieties, and histological techniques have been applied to detect

whether particular mucins derive their acidity or neutrality from the presence of these sugar groups (Kiernan, 1990).

Numerous rodent studies have compared germ-free (GF) and conventionally (CV) raised animals showing distinct changes in mucosal morphology and mucus composition associated with the presence of intestinal microflora. Compared to CV rodents, GF rodents exhibited a decrease in goblet cell size and number (Kandori *et al.*, 1996), with a consequent reduction in mucus layer thickness, indicating a reduction in mucus production (Abrams *et al.*, 1963b; Szentkuti *et al.*, 1990). Compared to CV rodents, GF animals displayed less neutral mucin and sulpho-mucin but greater amounts of sialo-mucin in small intestinal mucin fractions (Meslin *et al.*, 1999b; Sharma & Schumacher, 2001).

High levels of sulpho- and sialo-mucins reportedly coincide with maturation of intestinal barrier function (Fontaine *et al.*, 1996) in newborn rats (Shub *et al.*, 1983) and pigs (Turck *et al.*, 1993). Their presence during early development may be of particular importance as an innate barrier, since the acquired immune system is not fully functional in the neonatal intestine, rendering it more susceptible to infection (Cebra, 1999; Deplancke & Gaskins, 2001).

Currently, little information is available describing the effects of bacterial colonisation on the secretory pattern of small intestinal mucins during early development of chicks. Reference to similar numbers of goblet cells containing acidic mucins compared to neutral mucins in conventionally reared poultry has been reported, however ratios of acidic subtypes have not been described (Uni *et al.*, 2003a). Thus the aim of this study was to investigate the effects of bacterial exposure on mucin production in ileal and jejunal goblet

cells during early post-hatch development of chickens reared under isolation and conventional conditions.

3.2. Materials and methods

3.2.1. Intestinal sample collection

Chicks were hatched and reared by the methods described in Chapter 2. Three chicks were removed from both the brooding pen and the isolator and sacrificed by cervical dislocation from 1-7 d post-hatch. Segments (1 cm) of jejunum (adjacent to Meckel's diverticulum) and ileum (adjacent to caecal tonsils) were dissected, flushed with cold sterile saline solution, opened longitudinally and placed, mucosa side up, onto small pieces of blotting paper. The intestinal specimen was then fixed in 10% buffered formalin. This process was performed for each chick using autoclaved sterile instruments for each dissection. Fixed samples were dehydrated, cleared and embedded in paraffin wax for subsequent histological analysis. Consecutive longitudinal sections (7 μm) were placed individually onto poly-L-lysine coated slides. Sections were then deparaffinised in Histolene (Fronine Laboratory Supplies Pty. Ltd., Riverstone, New South Wales) and rehydrated in preparation for staining.

3.2.2. Neutral and acidic mucin staining

Since individual goblet cells can potentially produce all three types of mucin concurrently, the determination of mucin type required a series of alternative staining techniques (Kiernan, 1990) (Appendix 2). For neutral mucins, sections were subjected to mild acid hydrolysis to eliminate the contribution of sialic acid residues, before periodic acid-Schiff staining (PAS). After rinsing with both tap and distilled water, sections were immersed in periodic acid solution (Sigma, St-Louis, MO, USA) for 20 min, washed and immersed in Schiff's Reagent (Sigma, St-Louis, MO, USA) for a further 20 min. Sections were rinsed in tap water for 10 min, dehydrated and mounted in Entellan® (ProSciTech, Kirwan, Queensland). Staining of acidic mucins required a technique that enabled distinct differentiation between sialo- and sulpho-mucins. For this purpose, high iron diamine

(HID)/Alcian blue (AB) pH 2.5 staining was used. Sections were treated in HID solution for 16 h at room temperature, rinsed and immersed in AB pH2.5 for 5 min. Sections were then rinsed, dehydrated and mounted in Entellan® (ProSciTech, Kirwan, Queensland) and examined by light microscopy (Olympus BX60 microscope, Tokyo, Japan) using a 20x objective and digital colour images (SHQ [3072 x 2304 pixels]) captured using an Olympus Camedia C-7070, wide zoom (5.7-22.9mm; 1:2.8-4.8), 7.1MP, 4x optical zoom camera (Tokyo, Japan) (Appendix 2).

3.2.3. Morphometry

Ileal and jejunal sections from each animal were stained with PAS and HID/AB pH 2.5. Image analysis programs ImageJ® 1.33o (Rasband, 1997-2004) and VideoPro® (Version 6.210, Leading Edge Pty Ltd., Australia) were used in conjunction to measure a variety of parameters for each of the stained sections in which 10 villi/section were measured. Image J® was used to calculate the number of goblet cells per unit of epithelial area (mm^2) and individual goblet cell areas (μm^2) (Appendix 2). For HID/AB pH 2.5 individual counts were obtained for goblet cells that stained either blue or brown. Goblet cells staining both brown and blue were counted separately and termed “intermediate”. The summed values provided a total count of goblet cells in HID/AB pH2.5 sections. VideoPro® was used to compute measurements of total villus area (μm^2); epithelial area (μm^2), which was the lamina propria area subtracted from the total villus area; villus length and breadth (μm); crypt depth (μm) and total goblet cell area expressed as a proportion of epithelial area (μm^2).

3.2.4. Statistical analysis

Statistical analyses were performed using the SPSS software package V11.5 (SPSS Inc., Chicago, IL). Group (LBL vs CV) x age effects were analysed using a 2-way ANOVA

fitted with a Bonferroni adjustment. A multivariate linear mixed model was used to determine differences in mucin composition of goblet cells between groups. Student t-tests were used to compare acidic and neutral goblet cell numbers. Significance was determined as $P < 0.05$.

3.3. Results

3.3.1. Cell numbers

Numbers of goblet cells containing total acidic mucins in both the ileum and jejunum did not differ significantly between CV and LBL animals at any time point (Table 3.1). In the CV group there was a significant decrease in ileal goblet cells from day 1 to 4 post-hatch ($P < 0.05$) but at day 7, the number had almost returned to day-old values. Compared to ileal goblet cells, jejunal goblet cells exhibited a marked decrease in acidic mucins at day 4 ($P < 0.05$) which remained similar at day 7. Overall, there were greater numbers of goblet cells containing acidic mucins in the ileum compared to the jejunum in both the LBL and CV groups ($P < 0.05$) (Figure 3.1).

At day 1 post-hatch, all ileal goblets cells were stained brown (HID positive brown stain) indicating the presence of sulphated mucin. However at day 4, goblet cells containing sialo-mucin (AB positive blue stain) appeared and were more abundant in CV animals, compared to LBL chicks, in which goblet cells containing sulphated mucins were still predominant (Table 3.1). Although not statistically significant at day 7, the CV group tended to have fewer goblet cells containing sulpho-mucins than the LBL group ($P = 0.108$), and a greater number of sialo-mucins with an increase in cell number from day 4 (Table 3.1, Figure 3.2). Intermediate cells containing both sulphated and sialo-mucin increased in number from day 1 to day 4 in LBL and CV animals which was maintained at day 7. By day 7, goblet cell numbers were greater in CV animals (Table 3.1). The jejunum exhibited a similar trend to the ileum in terms of mucin composition, however, the density of all goblet cells containing each mucin type in this region was lower than that observed in the ileum ($P < 0.05$).

Table 3.1. Differences in acidic goblet cell mucin composition in low bacterial load (LBL) and conventionally reared (CV) chicks during the first 7 days post-hatch in the jejunum and ileum.

Age, d	Mucin Type							
	Total acidic ¹		Sulphated		Sialyated		Intermediate	
	CV	LBL	CV	LBL	CV	LBL	CV	LBL
Jejunum								
1	2.4 ± 0.3 ^a	2.7 ± 0.4 ^a	2.4 ± 0.3 ^a	2.7 ± 0.4 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a
4	1.3 ± 0.2 ^b	1.6 ± 0.2 ^b	0.5 ± 0.2 ^b	1.1 ± 0.3 ^b	0.4 ± 0.1 ^b	0.1 ± 0.03 ^{**}	0.5 ± 0.2 ^b	0.4 ± 0.1 ^{ab}
7	2.2 ± 0.3 ^a	2.6 ± 0.1 ^a	0.9 ± 0.2 ^b	1.9 ± 0.4 ^{ab*}	0.4 ± 0.1 ^b	0.2 ± 0.1 [*]	0.9 ± 0.2 ^b	0.6 ± 0.2 ^b
Ileum								
1	2.9 ± 0.2 ^a	2.8 ± 0.4	2.9 ± 0.2 ^a	2.8 ± 0.4	0.0 ± 0.0 ^a	0.0 ± 0.0	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a
4	1.8 ± 0.2 ^b	2.6 ± 0.1	0.7 ± 0.4 ^b	2.0 ± 0.3 [*]	0.5 ± 0.1 ^b	0.04 ± 0.02 [*]	0.7 ± 0.1 ^b	0.6 ± 0.2 ^b
7	3.6 ± 0.5 ^a	3.2 ± 0.3	1.4 ± 0.3 ^b	2.3 ± 0.3	1.0 ± 0.2 ^c	0.3 ± 0.2 ^{**}	1.2 ± 0.2 ^b	0.7 ± 0.2 ^{b*}

¹Values are number of goblet cells ($\times 10^3$)/mm² expressed as means ± SE

*Significantly different from CV chicks ($P < 0.05$); ** $P < 0.01$

^{a,b,c}Means with different superscripts within the same column and tissue type differ significantly ($P < 0.05$)

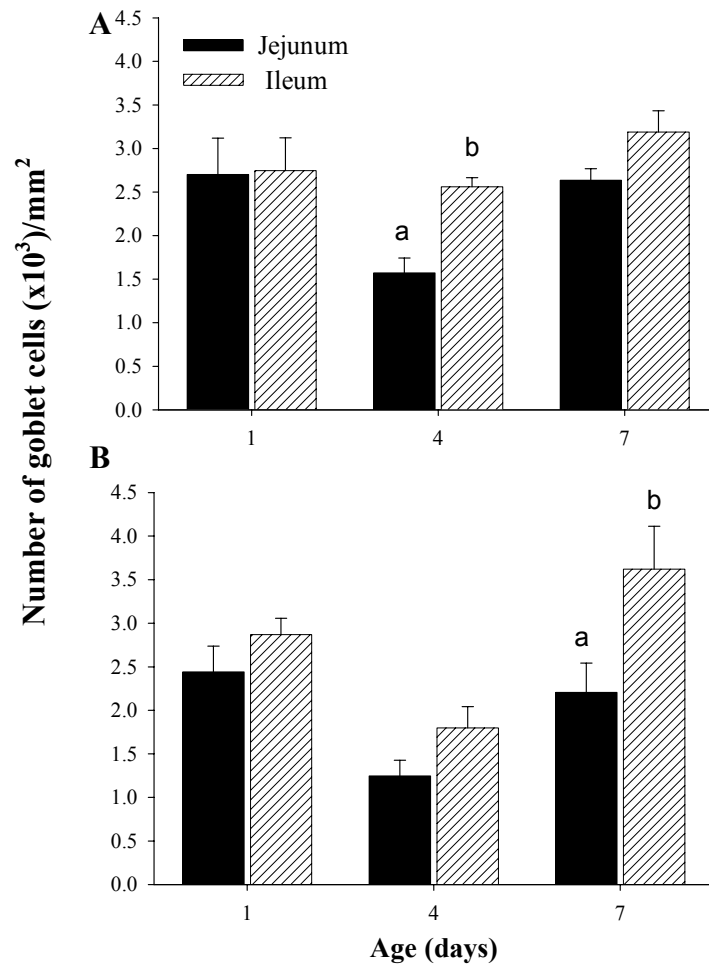


Figure 3.1. Numbers of goblet cells containing acidic mucins in the ileum and jejunum, at d 1, 4 and 7 post-hatch. Both low bacterial load (A) and conventionally raised (B) groups are shown. (Values are means \pm SE). Within the same day, bars with different letters (a, b) differ significantly ($P < 0.05$).



Figure 3.2. High iron diamide-alcian blue (HID/AB) pH 2.5 stained ileal sections from (A) conventionally raised and (B) low bacterial load chicks at d 7 post-hatch (magnification: 200X). Note the prominent alcian blue staining of villus goblet cells in the conventionally raised chick, compared to the low bacterial load chick. All goblet cells of crypts in both animals demonstrated a strong affinity of HID (brown stain).

The number of goblet cells containing neutral mucins did not differ significantly between CV and LBL animals at any time point for either the jejunum or ileum. However, for CV chicks, there was a significant decrease in ileal goblet cell number from day 1 to day 4 ($P < 0.05$) followed by an increase similar to day 1 values by day 7 ($P < 0.001$) (Figure 3.3). There were no significant changes in jejunal goblet cell numbers over time for either CV or LBL chicks (Figure 3.3). When the number of goblet cell containing neutral mucins (PAS stained sections) was compared to numbers of cells containing acidic mucins (HID/AB pH 2.5 stained sections) from both LBL and CV animals, there were no significant differences observed (Table 3.2).

3.3.2. Goblet cell area

Goblet cell areas containing total acidic mucins and areas of individual brown and blue stained cells in HID/AB stained sections were closely correlated with the changes in cell numbers (Figure 3.4), with the increase in cell number contributing to the increase in total goblet cell area. There was no difference in total jejunal PAS stained goblet cell area between CV and LBL chicks, with only a decrease in jejunal goblet cell area in CV chicks from day 1 to day 4, which was maintained at day 7 ($P < 0.05$). There were no changes in ileal PAS stained goblet cell area over the 7 day period in either LBL or CV animals. However, when compared to the LBL chicks, CV animals had a significant decrease in goblet cell area at day 4 (Figure 3.5). Further analysis of average individual goblet cell area (μm^2) at day 4 revealed CV chicks possessed smaller goblet cells compared to LBL chicks (LBL, $157 \pm 3 \mu\text{m}^2$; CV, $109 \pm 5 \mu\text{m}^2$, $P < 0.01$) and that this was not dependent on villus area ($r = 0.009$).

3.3.3. Villus breadth

In both the jejunum and ileum, a temporal change in villus breadth occurred only in CV chicks with an increase from day 1 to day 4, which remained similar at day 7 (Table 3.3). There were no differences in villus breadth between CV and LBL animals in the ileum. However, in the jejunum, villus breadth was greater in CV chicks at day 4 (Table 3.3).

Table 3.2. Comparisons between mean ileal and jejunal goblet cell numbers containing acidic and neutral mucin. There were no significant differences between mucin types in either low bacterial load (LBL) or conventionally raised (CV) groups ($P > 0.05$).

Age, d	LBL		CV	
	Acidic ¹	Neutral	Acidic	Neutral
Jejunum				
1	2.7 ± 0.4	2.7 ± 0.5	2.4 ± 0.3	2.7 ± 0.2
4	1.6 ± 0.2	2.0 ± 0.2	1.3 ± 0.2	1.7 ± 0.2
7	2.6 ± 0.1	2.8 ± 0.2	2.2 ± 0.3	2.2 ± 0.3
Ileum				
1	2.8 ± 0.4	2.7 ± 0.4	2.9 ± 0.2	2.8 ± 0.2
4	2.6 ± 0.1	2.5 ± 0.1	1.8 ± 0.2	1.9 ± 0.1
7	3.1 ± 0.3	3.1 ± 0.3	3.6 ± 0.5	3.2 ± 0.1

¹Values are number of goblet cells ($\times 10^3$)/mm² expressed as means ± SE

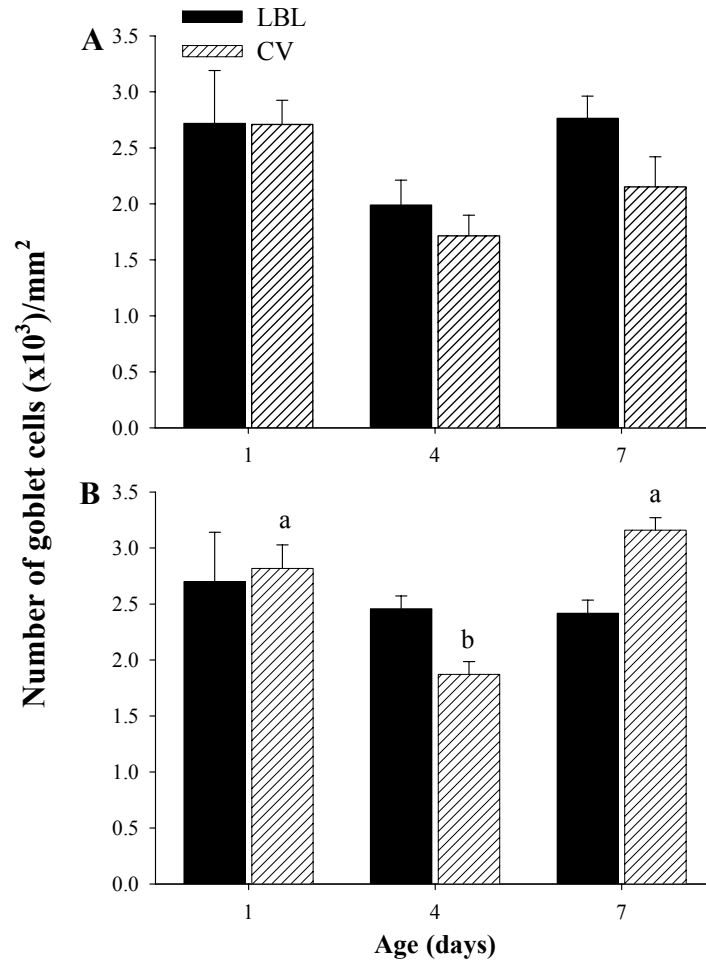


Figure 3.3. Differences in goblet cell numbers containing neutral mucin, between low bacterial load (LBL) and conventionally raised (CV) animals in the jejunum (A) and ileum (B) at days 1, 4 and 7 post-hatch (Values are means \pm SE). Bars with different letters, differ significantly from each other ($P < 0.05$).

3.3.4. Villus length

Ileal villus length increased from day 1 to day 4 in CV chicks, remaining similar at day 7 compared to LBL chicks in which there were no changes in villus length. In the jejunum, there was a greater increase in villus length, with both CV and LBL animals displaying a marked increase from day 1 (Table 3.3). Statistically-significant differences between CV and LBL chicks were observed only in the jejunum, with a greater villus length in CV animals as opposed to LBL at both day 4 and day 7 (Table 3.3).

3.3.5. Villus area

Total villus area and epithelial area in the ileum and jejunum were increased in CV and LBL chicks from day 1 to day 4, with no further increase observed at day 7 (Table 3.3). Differences between CV and LBL were observed at day 4, with the CV chicks exhibiting a greater villus area, which was more prominent in the jejunum (Table 3.3). The jejunum villus and epithelial areas of CV animals tended to remain greater at day 7 compared to LBL villi, although statistical significance was not quite attained (epithelial area, $P = 0.058$; total villus area $P = 0.067$).

3.3.6. Crypt depth

The villus crypt axis of poultry is not developed until day 5 post-hatch (Uni *et al.*, 2000); hence crypt depth measurements were only conducted in day 7 animals. It was found that ileal crypt depth in LBL animals was significantly lower than in CV animals (LBL, $99 \pm 4 \mu\text{m}$; CV, $149 \pm 7 \mu\text{m}$, $P < 0.05$). Crypt depth in the jejunum did not differ significantly between LBL and CV animals (LBL, $122 \pm 27 \mu\text{m}$; CV, $161 \pm 27 \mu\text{m}$, $P = 0.18$).

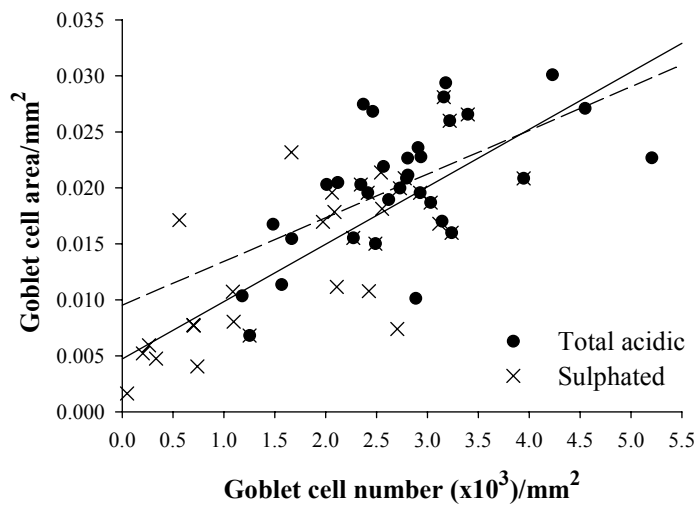


Figure 3.4. Correlations between ileal goblet cell number and goblet cell area for total acidic mucins (---, $r = 0.67$) and sulphated mucins only (—, $r = 0.79$) ($P < 0.05$), (low bacterial load and conventional animals combined).

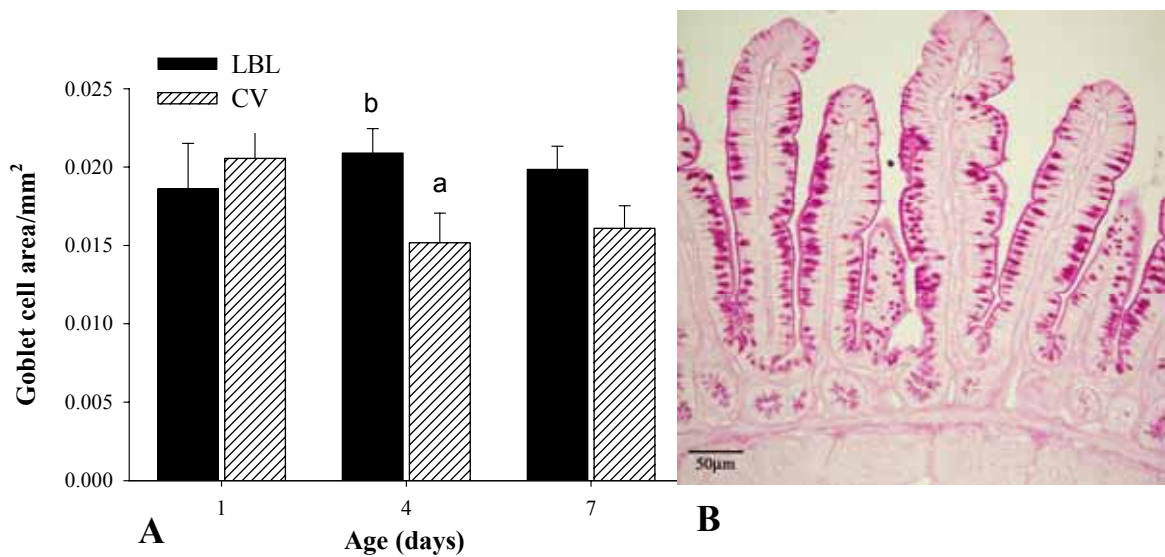


Figure 3.5. (A) Low bacterial load (LBL) versus conventionally raised (CV) total goblet cell area of ileal cells stained with periodic acid-Schiff (PAS) at d 1, 4 and 7 (Values are means \pm SE). Within the same day, bars with different letters (a, b) differ significantly ($P < 0.05$). (B) Typical section of ileal goblet cells stained with PAS.

Table 3.3. Differences in villus morphology in the ileum and jejunum of conventionally reared (CV) and low bacterial load (LBL) chicks during the first 7 days post-hatch

Age, d	1		4		7	
Jejunum	CV	LBL	CV	LBL	CV	LBL
Area ($\mu\text{m}^2 \times 10^3$) ¹	18.8 ± 3.5 ^a	18.2 ± 2.3 ^c	60.6 ± 7.5 ^b	39.6 ± 4.9 ^{d*}	59.0 ± 8.4 ^b	42.5 ± 5.5 ^d
Epithelial area ($\mu\text{m}^2 \times 10^3$)	12.2 ± 2.3 ^a	11.6 ± 1.3 ^c	46.7 ± 5.6 ^b	31.1 ± 3.9 ^{d*}	45.4 ± 6.3 ^b	32.8 ± 3.4 ^d
Length (μm)	286 ± 31 ^a	232 ± 15 ^c	494 ± 38 ^b	392 ± 28 ^d	570 ± 68 ^b	438 ± 30 ^{d*}
Breadth (μm)	105 ± 9 ^a	114 ± 10	190 ± 16 ^b	148 ± 12 [*]	166 ± 14 ^b	145 ± 12
Ileum						
Area ($\mu\text{m}^2 \times 10^3$)	16.1 ± 1.7 ^a	20.9 ± 5.1 ^c	50.6 ± 5.8 ^b	31.1 ± 3.8 ^{d*}	39.5 ± 5.5 ^b	33.0 ± 1.7 ^c
Epithelial area ($\mu\text{m}^2 \times$	11.1 ± 0.8 ^a	13.7 ± 3.8	35.5 ± 2.8 ^b	22.6 ± 2.1 [*]	29.5 ± 3.1 ^b	25.6 ± 1.5
Length (μm)	219 ± 35 ^a	266 ± 32	429 ± 18 ^a	352 ± 9	404 ± 36 ^b	357 ± 25
Breadth (μm)	78 ± 14 ^a	111 ± 16	196 ± 8 ^b	162 ± 15	150 ± 18 ^b	134 ± 3

¹Values are number of goblet cells($\times 10^3$)/mm² expressed as means ± SE

*Significantly different from CV chicks ($P < 0.05$)

^{a,b}Means for CV animals with different superscripts within the same row and tissue type differ significantly ($P < 0.05$)

^{c,d}Means for LBL animals with different superscripts within the same row and tissue type differ significantly ($P < 0.05$)

3.4. Discussion

Mucins are high-molecular weight, highly glycosylated glycoproteins produced by goblet cells (Forstner & Forstner, 1994). The chemical nature of these glycoproteins provide potential binding sites for microflora, which may prevent attachment to the mucosal surface (Corfield *et al.*, 1992; Forstner & Forstner, 1994). Utilising histological methodologies, it was demonstrated that microflora could affect small intestinal goblet cell mucus composition, and that these changes occurred between days 3 and 4 post-hatch. Although the total number of goblet cells containing acidic mucins was not influenced by limiting bacterial exposure, mucin composition was altered, with a decrease in sulpho-mucin and an increase in sialo-mucin content. The distinct differentiation between goblet cell staining in HID/AB sections in CV animals was interesting as HID possesses a very strong affinity for sulpho-mucin (Kiernan, 1990). The presence of blue stained goblet cells implied that those goblet cells were producing mucins with primarily sialic acid residues, with no sulphate groups.

The preservation of sulpho-mucins, evident throughout the first week of development in low bacterial load chickens, was consistent with other developmental studies (Hill *et al.*, 1990). A high degree of sulphation is characteristic of immature goblet cells (Turck *et al.*, 1993), since the level of intestinal microflora was low, the retention of sulpho-mucin during post-hatch development may be indicative of an immature gut, outlining the influence of bacteria on mucin production and overall gut maturity. The reasons for this are still unclear. However, some bacteria possess mucin-specific glycosidases and proteases, which are able to degrade mucus and facilitate colonisation of the epithelial surface (Corfield *et al.*, 1992; Deplancke & Gaskins, 2001). Bacteria such as *Helicobacter pylori* secrete glycosulphatases, which can cleave sulphate from its linkage to mucin sugars (Robertson & Wright, 1997). The switch from predominately sulpho-mucins to

acetylated sialo-mucin in neonatal animals, could represent a defence strategy. The hydroxyl groups of sialic acids are highly substituted by acetyl esters, which serve as added protection as they block against further glycosidic degradation, with reports that two or more acetyl groups inhibit enteric bacterial sialidases (Belley *et al.*, 1999; Corfield *et al.*, 1992). Both sulphate and sialic acid groups have protective properties (Corfield *et al.*, 1992; Robertson & Wright, 1997), as colonisation becomes greater the need for greater protection against mucus degradation is increased, which would explain the observed increase in sialo-mucin production. Moreover, in the current study, the greater number of goblet cells containing acidic mucin in the ileum compared to the jejunum would suggest the distal ileum may be a preferred region for bacterial colonisation. This is consistent with other findings using chicks, which have demonstrated a distal increase in the density of goblet cells along the duodenal-ileal axis (Uni *et al.*, 2003a).

In the current study, intestinal crypts showed a predominant HID positive staining for sulpho-mucins in both CV and LBL animals, with the sialylated stained goblet cells tending to be located from mid to villus tip. Migration rate of goblet cells from crypt to villus tip has been reported to take approximately 2-3 days in poultry (Imondi & Bird, 1966; Uni *et al.*, 2000), with conventionally raised animals having a greater rate of migration than germ free animals (Cook & Bird, 1973). Considering these differences in migration rate and the absence of sialo-mucin in intestinal crypts, the change in mucin composition in conventionally raised animals along the villus may have been due to differences in the luminal environment and not to the rate of migration of goblet cells during post-hatch development.

The presence of neutral mucins in ileal and jejunal goblet cells of day old chicks, was consistent with previous studies in poultry (Uni *et al.*, 2003a), but differed from

mammalian models. It has been reported that little to no neutral mucin was detected in the lower small intestine and colon of neonatal rodents and pigs, but neutral mucin content increase with age (Deplancke & Gaskins, 2001; Hill *et al.*, 1990; Turck *et al.*, 1993). Germ-free studies have used rodent and pig models, which during the first few weeks after birth are dependent on maternal milk resources. Chicks, however, must have the capacity to digest complex carbohydrates immediately after hatch (Sklan, 2001). Thus the gut of a day-old chick requires advanced intestinal development compared to a day old rodent or pig, in which the intestine is comparable to chicks at day 18 of incubation (Uni *et al.*, 2003a). In the current study, whether the presence of neutral mucins in the ileum and jejunum was due to bacterial colonisation or dietary components, or both, is yet to be determined. Bacterial species, mainly Type-1 fimbriated, have been demonstrated to possess receptors for mannose residues *in vitro* (Firon *et al.*, 1984; Marc *et al.*, 1998; Vimal *et al.*, 2000). As the animals age, there is increased bacterial adhesion to mannose and, consequently, reduced susceptibility to infection (Nagy *et al.*, 1992). The production of neutral mucins could therefore serve as a protective mechanism against invasion by pathogenic bacteria (Dean-Nystrom & Samuel, 1994; Runnels *et al.*, 1980). In this study, since neutral mucins in both the LBL and CV chicks displayed similar patterns, their presence at this time point could be the result of increased intestinal maturity to facilitate the breakdown of a complex carbohydrate diet, rather than a means of preventing bacterial attachment. Future studies could measure neutral mucin content, particularly that of mannose residues, at a later stage of development, in order to determine the extent to which bacteria may influence the production of this mucin type.

The differences in villus length, breadth and area between the ileum and jejunum were consistent with previous findings conducted in poultry, with the jejunum displaying an increase in all three parameters compared to the ileum (Iji *et al.*, 2001), thus supporting its

importance as the preferred site for nutrient digestion (Iji *et al.*, 2001). It has been well documented that villus length and crypt depth increase with age (Iji *et al.*, 2001; Uni *et al.*, 1995) which was observed in both CV and LBL animals. The increased mucosal development observed in CV animals compared to LBL animals may have been due to bacterial-diet interactions and the need for greater absorptive area to accommodate the by-products associated with microbial fermentation. The increased villus length and crypt depth evident in conventionally raised animals was consistent with previous studies (Abrams *et al.*, 1963a; Cook & Bird, 1973; Kleessen *et al.*, 2003). However this was not true of other reports in which crypt depth was found to be greater in CV animals, but villus length was significantly decreased (Shirkey *et al.*, 2006). These latter studies were conducted using pigs, thus species differences in overall intestinal morphology and also contributing factors such as microbial colonisation and digestive enzyme function, may have contributed to the contrasting results.

In the current study, marked changes in mucin composition in conventionally raised animals at day 4 post-hatch may have coincided with an increase in immune system development. With the depletion of the yolk sac, and subsequent reduction in maternal antibody resources, the stimulation of goblet cells to alter mucin glycosylation may have functioned to defend against pathogenic infection at this stage in development. At day 4 post-hatch, it has been reported that an up-regulation of mRNA expression of proteins involved in immune function such as antimicrobial peptides and pro-inflammatory cytokines, was greatly increased in the gut associated lymphoid tissue (GALT) (Bar-Shira *et al.*, 2003). Cytokines have been reported to increase the extent of mucin production and goblet cell proliferation (Blanchard *et al.*, 2004), and also to produce changes in the glycosylation of mucins (Beum *et al.*, 2005). Bacterial endotoxins, such as LPS, are the major outer surface membrane component of gram-negative bacteria, and have been found

to up-regulate mRNA expression and secretion of cytokine IL-8 and mucin genes MUC5AC and MUC5B (Smirnova *et al.*, 2003). Relative expression of pro-inflammatory cytokines IL-1 β and IL-6 has been reported to be highest in pigs inoculated with adult porcine faeces and non-pathogenic *E. coli* compared to GF animals (Shirkey *et al.*, 2006). both of which have been reported to trigger mucin release and up-regulate MUC gene expression (Deplancke & Gaskins, 2001; Enss *et al.*, 2000).

Conclusion

Findings from the current study provide insight into the influence of intestinal microflora on goblet cell and mucosal cytoarchitecture during early post-hatch development. The histological change in acidic mucin profile between LBL and CV animals was dramatic, thus it was further hypothesised that the chemical structure of mucin oligosaccharides were altered in response to bacterial exposure. The extent to which mucin oligosaccharides differed between LBL and CV animals is explored in Chapter 4.

Chapter 4 Mass Spectroscopy Analysis

Effects of bacterial exposure to mucin oligosaccharide structure during post-hatch development

4.1. Introduction

Mucins are heterogenous, large molecular weight, highly *O*-glycosylated glycoproteins (Claustre *et al.*, 2002; Freitas *et al.*, 2002). *N*-acetyl-galactosamine (GalNAc), *N*-acetyl-glucosamine (GlcNAc), galactose, fucose (Fuc), mannose, glucose and sialic acid (NeuAc) are the principle sugars of *O*-linked mucin oligosaccharide chains (Deplancke & Gaskins, 2001; Freitas *et al.*, 2002; Uni *et al.*, 2003b). The sugars are linked via an alpha-glycosidic bond between GalNAc and either serine or threonine of the protein core (Freitas *et al.*, 2002; Satchithanandam *et al.*, 1990). Oligosaccharides are added to mucins by specific membrane-bound glycosyltransferases that transfer monosaccharide units from nucleotide sugar donors in the golgi apparatus of the goblet cell (Deplancke & Gaskins, 2001). There are hundreds of glycosyltransferase genes, which account for the many different possible combinations of oligosaccharides, including the diversity in chain length, composition, branching and the degree of terminal sulphation and acetylation (Beyer *et al.*, 1981; Uni *et al.*, 2003b).

The high degree of heterogeneity allows for many potential binding sites for bacterial attachment (Klinken *et al.*, 1995). Bacterial species have the ability to adhere to the epithelial surface using external appendages such as fimbriae or extracellular polymers like LPS (Gusils *et al.*, 2002; Smirnova *et al.*, 2003). These can then bind to glycoconjugate receptors of mucins, preventing adhesion to the underlying enterocytes (Sharma *et al.*, 1997). Thus, changes to mucin oligosaccharide structure may provide a means to prevent mucosal infection by inhibiting adhesion of certain bacterial species.

Structural studies using mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) have been performed on rat colonic mucin (Slomiany *et al.*, 1980) and also in the small and large intestine of humans (Robbe *et al.*, 2004; Robbe *et al.*,

2003). Using this technology, a gradient from fucose to sialic acid terminating oligosaccharides from ileal to colonic regions in humans, and similarly exhibited in rats, has been detected. The increase in sialic acid moieties was proposed to be linked to the presence of microflora in these regions (Robbe *et al.*, 2004). In addition, a decrease in sulphate and sialic acid content with an increase in age may coincide with the maturation of barrier function. A higher acidic mucin content observed in neonates and hatchlings could serve as a protective mechanism against pathogenic infection during early development (Bryk *et al.*, 1999; Shub *et al.*, 1983; Turck *et al.*, 1993). Histological studies (described in the previous chapter) point to the same protective mechanism in the ileum at approximately d 4, emphasising its importance as a potential site for microbial colonisation.

In the current study, glycoprotein purification and MS techniques were applied to test the hypothesis that exposure to intestinal microflora impacted on the overall mass and structure of ileal mucin oligosaccharides of purified mucin samples from LBL and CV chicks at d 4 and 7 post-hatch.

4.2. Materials and methods

4.2.1. Intestinal sample collection

Chicks were hatched and reared by the methods described in Chapter 2. Three chicks were removed from both the brooding pen and the isolator and sacrificed by cervical dislocation from 1-7 d post-hatch. The ileum was dissected from low bacterial load (LBL) and conventionally reared (CV) chicks, and flushed with cold sterile saline solution. Ileal segments (~ 3-5 cm, adjacent to the caecal tonsils) were collected. The ileal mucosa from these segments was recovered by scraping with the edge of a glass slide. The contents were wrapped in aluminium foil and immediately frozen in liquid nitrogen. The samples were stored at -80°C until analysis.

4.2.2. Purification of intestinal mucin samples

Mucin purification was adapted from a previous protocol (Faure *et al.*, 2002; Appendix 3). Briefly, 300 mg of frozen sample was homogenised in cold 2 mL of 0.05M Tris/HCl buffer pH 7.5 and kept on ice. When all samples were homogenised they were partially digested with 100 µL Flavourzyme® (protease from *Aspergillus oryzae*, 83.8 µL/mL) (Sigma, St-Louis, MO, USA) and incubated at 37°C for 80 min under agitation (120 RPM). Samples were added to tubes containing guanidium hydrochloride (Sigma, St-Louis, MO, USA) to a concentration of 4M and further reduced by adding 350 µL of 100mM dithiotreitol (DTT) solution and incubated at room temperature for 2 h under agitation (160-180 RPM). 500mM iodoacetamide solution was prepared in which 200 µL was added to each sample to undergo alkylation. Samples were further incubated at room temperature for 2 h under agitation. After incubation the samples were frozen at -20°C for chromatography.

Mucins were purified by passing 1mL sample through PD-10 columns (Sigma, St-Louis, MO, USA) filled with Sepharose CL-4B (Sigma, St-Louis, MO, USA) equilibrated and

eluted with 2M guanidium hydrochloride in 0.05M Tris/HCl buffer pH 7.5. Mucin containing fractions were collected and dialysed (The dialysis tubing retained proteins of molecular weight greater than 12000-14000 Da) (Sigma, St-Louis, MO, USA) against deionised water for 48 h at 4°C. Samples were freeze-dried and stored at -20°C.

4.2.3. Determination of purity

The purity of all mucin samples was confirmed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to confirm the absence of contaminating proteins (polyacrylamide gel gradient with the Mini-PROTEAN® 3 cell system, Bio-Rad Laboratories Pty. Ltd., Reagents Park, NSW) (Appendix 3). Samples were applied to SDS-page after heating for 5 min at 95 °C in sample buffer containing 5% (v/v) 2-mercaptoethanol and 3% (v/v) SDS. Mucins were electrophorised either through a pre-cast Tris-HCl 5% (100-250kD) gel or a Tris-HCl 7.5% (40-200kD) gel (Bio-Rad Laboratories Pty. Ltd., Reagents Park, NSW) for 25 min at 200V. Bovine serum albumin (Sigma, St-Louis, MO, USA) and porcine gastric mucin (Sigma, St-Louis, MO, USA) were used as positive controls. Purified samples from intestinal ileal mucosa of LBL and CV chicks were loaded into the other wells. Size of protein bands were established by use of a high range SeeBlue® Plus2 pre-stained protein standard (Invitrogen Life Technologies, VIC). Gels were stained with either Coomassie brilliant blue R250 (Sigma, St-Louis, MO, USA) or with periodic acid-Schiff (Konat *et al.*, 1984).

4.2.4. Alkali/borohydride treatment of mucin glycoproteins.

Alkali/borohydride treatment was used to release oligosaccharides from the protein backbone (Piller & Piller, 1993) (Appendix 3). Briefly, samples were dissolved in distilled water to a total concentration of 25 mg/mL. An equal volume of freshly prepared 2M NaBH₄ (Sigma, St-Louis, MO, USA) in 0.1M NaOH was added to the mucin sample,

vortexed and incubated at 45°C for 16 h. After incubation, the samples were cooled to room temperature and excess NaBH₄ was neutralised, drop by drop, using 5 volumes of 0.25M acetic acid in methanol. The samples were then dried under a stream of nitrogen and the method repeated using acidified methanol, and twice again with methanol alone.

The released oligosaccharides were purified from proteins and amino acids by gel filtration through a column containing Sephadex G-50 superfine media (Amersham Biosciences, Rydalmere, NSW), (1.5 x 100 cm Eco-Glass column, Bio-Rad Laboratories Pty. Ltd., Reagents Park, NSW) equilibrated and developed in 100mM NaCl (flow rate, 6.18 mL/hr), whereby 5mL fractions were collected (Pharmacia P-1 peristaltic pump and FRAC-100 fraction collector) (Amersham Biosciences, Rydalmere, NSW; Appendix 3). Carbohydrate containing fractions were detected using the phenol-sulphuric acid assay (Piller & Piller, 1993) and absorbance read at 485 nm. Proteins were detected using UV absorption at 280nm. The recovered oligosaccharides were freeze-dried and desalted over a small column (Bio-Rad Eco-glass column 1 x 20 cm) of Bio-Gel P-2 fine media (Bio-Rad Laboratories Pty. Ltd., Reagents Park, NSW) equilibrated and eluted with water under gravity. Oligosaccharides were confirmed by further detection at 485nm. Fractions were freeze-dried, weighed and stored at -20°C until further analysis.

4.2.5. Analysis of oligosaccharide fractions

4.2.5.1. Mass spectrometry: time-of-flight (TOF)

Spectra were run on a Micromass Q-Tof2 Hybrid Quadrupole time-of-flight (TOF) mass spectrometer using MassLynx 4.1 software. Oligosaccharide samples were run in a 50/50 acetonitrile/H₂O solvent. All analysis was conducted in the positive ion mode.

4.2.5.2. Tandem mass spectrometry (MS/MS)

Using the same equipment, MS/MS was carried out on the peaks of interest obtained during the first MS experiment. A parent ion (indicated on the spectrum) was isolated and further fragmented, creating daughter ions with the potential to provide structural information on oligosaccharide sugars.

4.3. Results

4.3.1. SDS-PAGE

Purified mucin samples were detected by SDS-PAGE. The mucin sample appeared as high-molecular weight band just entering the running gel (Figure 4.1A, arrow). There was a slight contamination of smaller proteins in some mucin samples but was reduced by increasing dialysis time (Figure 4.1B, arrow).

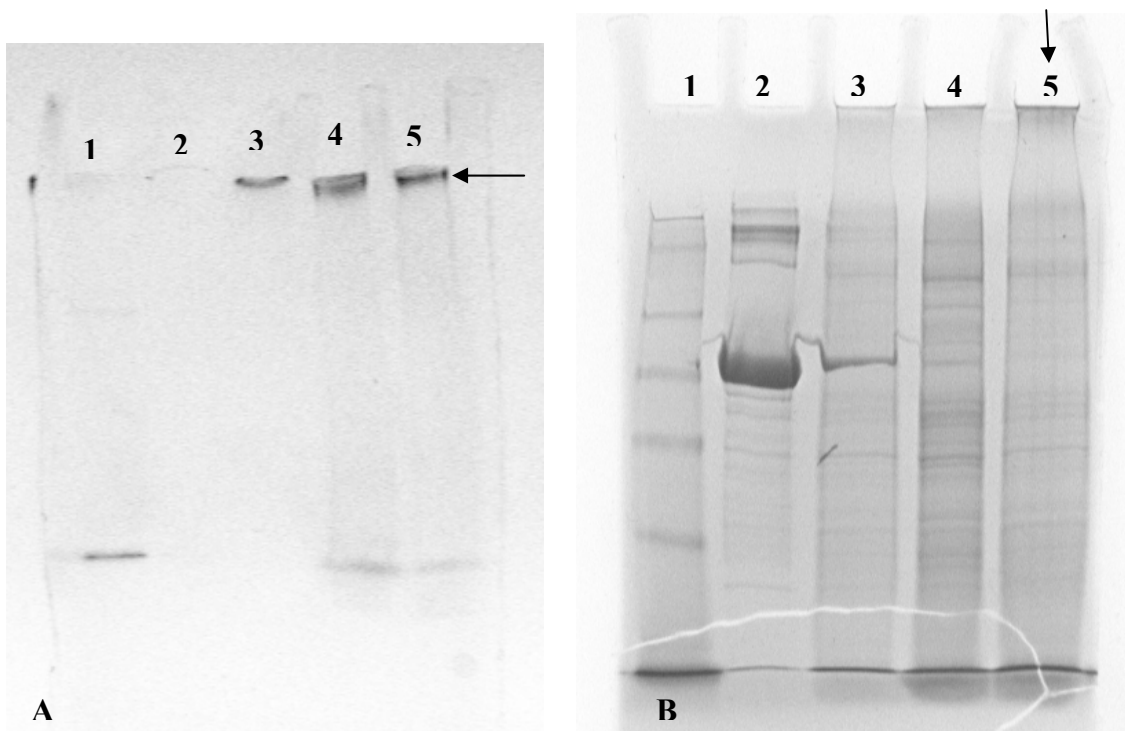


Figure 4.1. SDS-PAGE electrophoresis of purified mucin samples. The gels were stained with (A) periodic acid/Schiff's reagent and (B) Coomassie blue. Lane 1-marker; 2-control, BSA; 3-control, porcine gastric mucin; 4-ileal mucin sample; 5-ileal mucin sample with increased dialysis time.

4.3.2. Fraction assays

A separation between protein and oligosaccharides was obtained for each mucin sample run through the gel column. There were very sharp inclines and declines in concentrations

of both protein and oligosaccharides in different fractions (Figure 4.2). Fractions were collected that contained oligosaccharides devoid of protein (Figure 4.2).

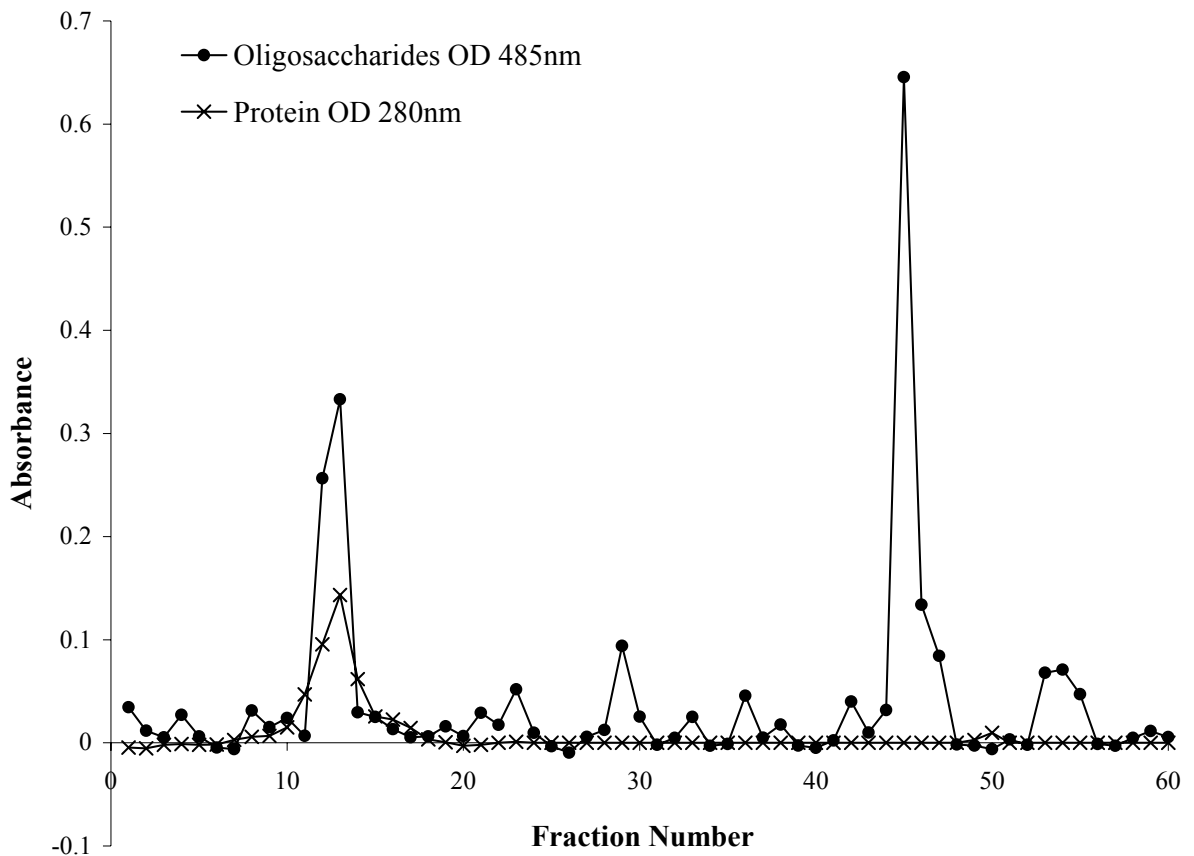


Figure 4.2. Gel filtration on Sephadex G-50 superfine of alkaline borohydride-treated chick ileal mucin. The sample was applied to a column (1.5 x 100 cm) equilibrated with 100mM NaCl. Fractions of 5mL were collected and monitored for protein (absorbance at 280 nm OD -×-×-) and oligosaccharides (phenol-sulphuric acid method, absorbance at 485 nm -●-●-) Tubes 28-31, 42-48 and 52-55 were collected for further desalting.

4.3.3. Oligosaccharide weights

Weighed quantities of mucin oligosaccharide from LBL and CV animals, all contained approximately 2 mg of sample prior to spectrometric analysis.

4.3.4. Mass spectrometry: time-of-flight (TOF)

Desalted oligosaccharides were directly analysed by MS-TOF after alkaline borohydride-treatment. Based on the mass of the compounds obtained by MS analysis, peaks of both low molecular weight and high molecular weights were common to both CV and LBL animals (353 and 381; 782 and 784) (Figures 4.3 and 4.4). There were no differences between the mass of the oligosaccharide compounds between LBL and CV animals at either time point. Neither was there a difference from d 4 or 7 post-hatch. The number of oligosaccharide peaks was also found not to differ between groups or days (Table 4.1).

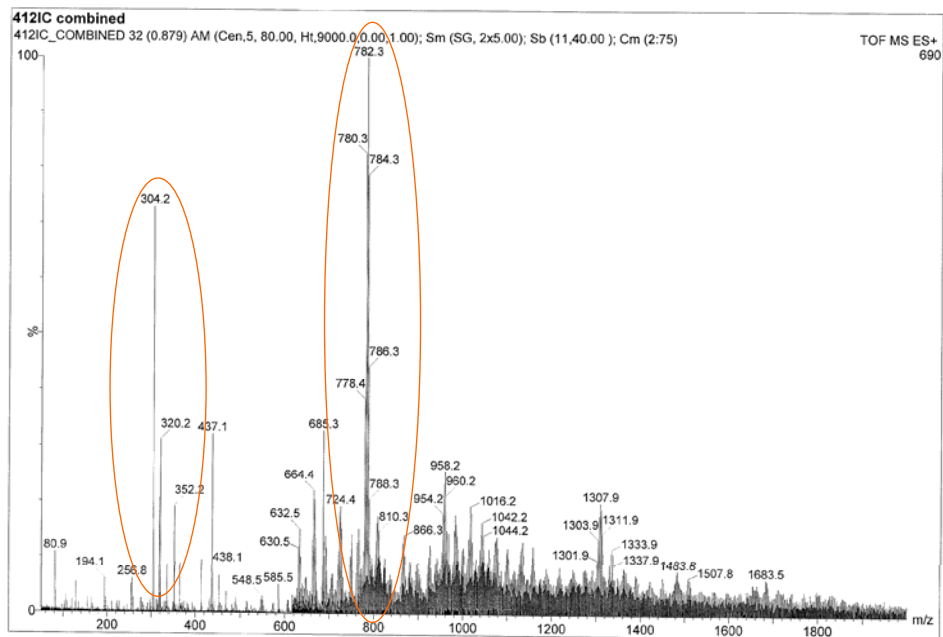
4.3.5. Tandem MS/MS

Results from MS/MS were inconclusive; the oligosaccharides could not be broken down successfully. Although oligosaccharides were detected when measured by spectrophotometry, it appeared that some samples may have contained salt contaminants, which had not been removed successfully during the desalting process.

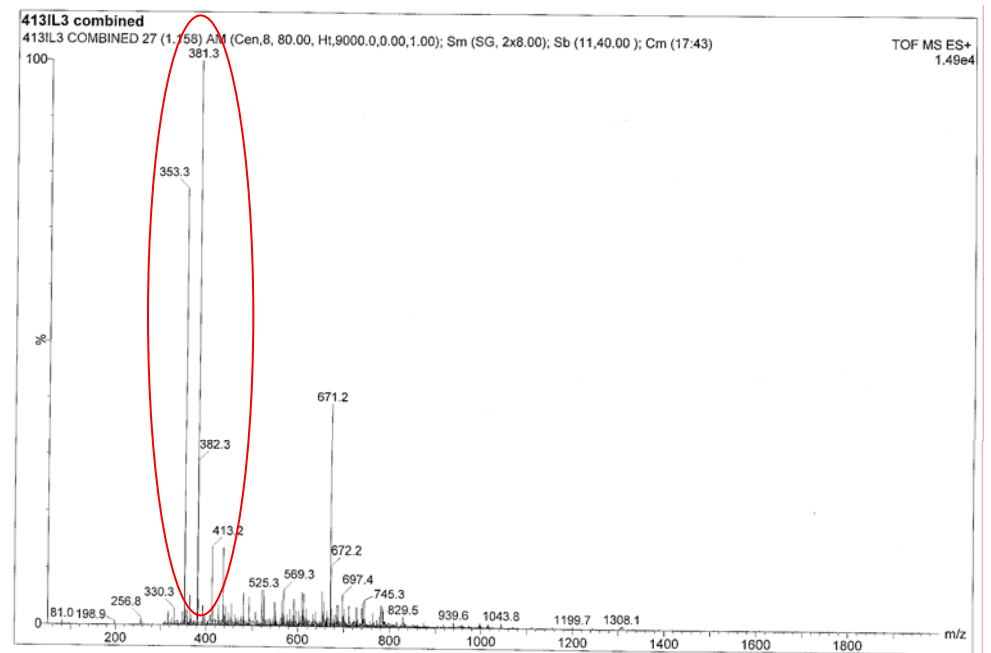
Table 4.1. Number of low and high molecular weight (MW) peaks detected using MS-TOF. Peaks represented mucin oligosaccharides of ileal mucin of low bacterial load (LBL) and conventionally reared (CV) chicks at d 4 and 7 post-hatch.

Age, d	4		7	
	LBL	CV	LBL	CV
Low MW (~ 350m/z¹) (total number of peaks)	4	5	4	5
High MW (~780m/z) (total number of peaks)	4	5	4	3

¹ Mass-to-charge ratio

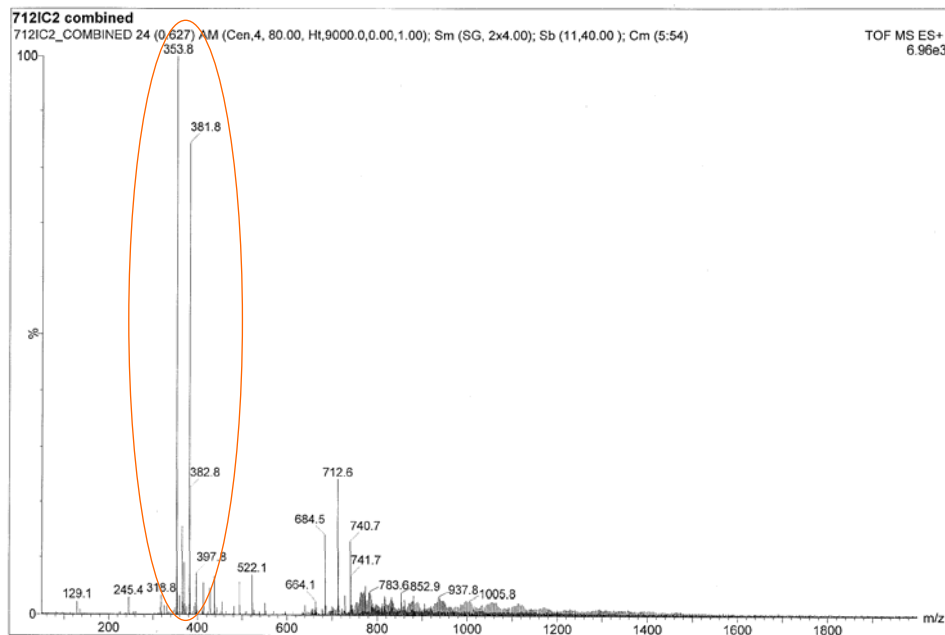


A

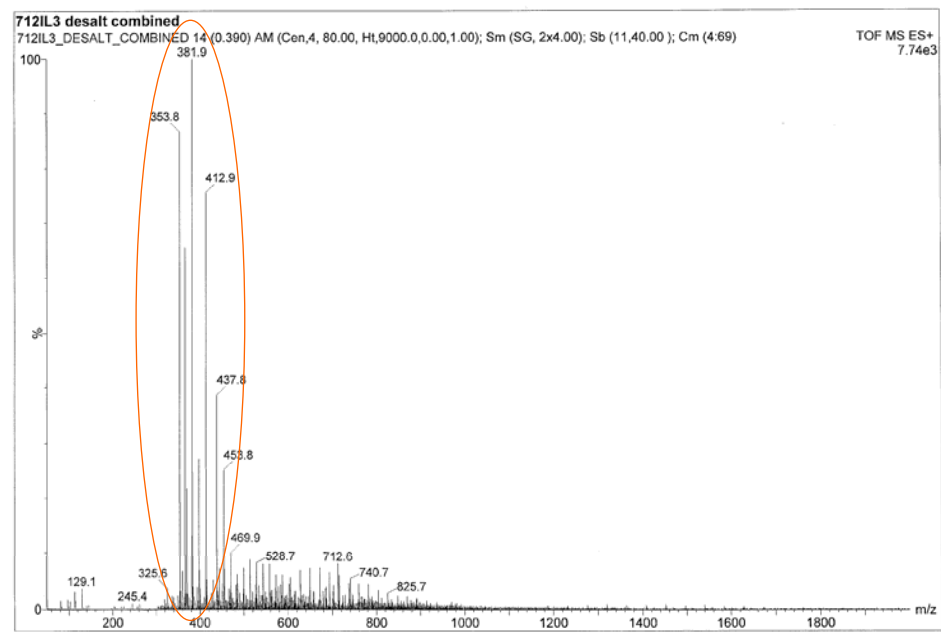


B

Figure 4.3. Examples of MS-TOF spectra of the total oligosaccharides, acquired in the positive ion mode, from ileum of d 4 chicks post hatch. (A) Conventionally reared chick and (B) low bacterial load chick. Red circles indicate the main peaks common to both groups.



A



B

Figure 4.4. Examples of MS-TOF spectra of the total oligosaccharides, acquired in the positive ion mode, from ileum of d 7 chicks post-hatch. (A) Conventionally reared chick and (B) low bacterial load chick. Red circles indicate the main peaks common to both groups.

4.4. Discussion

The intestinal mucus layer contains visco-elastic components termed mucins or mucin-type glycoproteins (Beeley, 1985). Synthesised and secreted by goblet cells (Forstner & Forstner, 1994; Sharma *et al.*, 1997), mucins can be discharged by a wide variety of stimuli, including the colonisation of bacteria, with the potential for changes in the type of mucin secreted. Structural changes to mucin oligosaccharide chains may be of significance when describing the mechanisms involved in its protective role preventing pathogen attachment and reducing potential intestinal infection.

With the exception of lectin histochemistry studies, no structural studies have been conducted investigating the glycosylation of mucin oligosaccharides in the intestine of poultry, nor whether exposure to intestinal micro-organisms can influence mucin oligosaccharide chain structures. Preliminary structural analysis by mass spectrometry was conducted on intestinal samples from both LBL and CV animals at d 4 and 7 post-hatch, to examine differences in mucin oligosaccharides following exposure to bacteria.

In the current study, it was found that limiting the exposure of chickens to bacteria did not change the length or number of oligosaccharide chains of ileal mucin samples at either d 4 or 7 post-hatch. It has been reported that the initiation of glycosylation, i.e., the addition of *N*-acetylgalactosamine (GalNAc) to the mucin protein core chain, may influence the subsequent glycosylation events in terms of lengths and numbers of *O*-linked chains (Tetaert *et al.*, 2001). Indeed cooperation between the enzymes that control GalNAc addition, *N*-acetylgalactosaminyltransferases (GaNTases), and their binding domains could potentially increase sugar/peptide affinity and would participate in greater glycosylation of mucins independent of the peptide core sequence (Tetaert *et al.*, 2001).

The maturity of the gastrointestinal tract may be an important factor when examining changes to mucin glycosylation. The carbohydrate content of mucin glycoprotein was previously measured in newborn and adult rats. In newborn rats the GalNAc content was much lower compared to adults (Shub *et al.*, 1983). These results were further confirmed when the enzyme activity of a *N*-acetylgalactosaminyltransferase (β 1,4GalNAc-transferase) was measured during post-natal development of rats. Enzyme activity was reported to be very low in suckling rats, increasing with age (Dall'Olio *et al.*, 1990). A histochemical study reporting the binding affinities of lectins in pre-hatch and day-old chicks, detected a strong binding affinity for GalNAc specific conjugated-soybean agglutinin (SBA) (Byrk *et al.*, 1999). Lectin studies have the ability to detect only terminal and sub-terminal sugar residues. The study failed to give a defined quantitative measurement of GalNAc detection thus the concentration of this sugar in the small intestine during post-hatch development is yet to be determined. It could be proposed that the efficiency of GaNTases, and thus the initiation of glycosylation through GalNAc attachment, may be limited during post-hatch development, hence limiting the ability to produce large numbers of mucin oligosaccharide chains with higher molecular weights.

The regulation of GaNTases by intestinal microflora has yet to be determined. The influence of bacteria on other glycosyltransferase activity was demonstrated when β 1,4-galactosyltransferase (β GT) was increased in germ-free mice with the addition of microbes from adult animals. However, activity was not altered when inoculated with intestinal microbes from suckling mice (Nanthakumar *et al.*, 2005). Thus the intestinal microflora present during early development may not be sufficient to cause a major effect on glycosyltransferase activity, and subsequently mucin oligosaccharide production. Moreover, in a previous study, larger weight oligosaccharides were observed in the ileum of adult human patients (Robbe *et al.*, 2003). Since oligosaccharides of smaller molecular

weight, observed in the current study, may have been related to the age of the animal, it would be interesting to compare the mucin oligosaccharide profile of older birds to post-hatch chicks. This would determine whether age affects oligosaccharide length and structure, distinguishing a possible correlation with the increasing complexity of the intestinal microflora as the animal matures.

The inability to successfully analyse individual sugar structures by MS/MS meant that structural information of these oligosaccharides could not be obtained. All analysis was conducted in the positive ion mode. A similar analysis could be conducted in the negative ion mode (determines structures with a negative charge). For this study, analysis in the negative ion mode was not possible, but may provide useful information for future studies.

A potential progression of this work would be to conduct an advanced study of the diversity of mucin sugars using 2D-NMR to determine whether there are subtle differences in isomeric structure. However, for this type of analysis, larger quantities of sample (5-25 mg) are required. Samples used in the current study, after purification, only rendered small amounts of oligosaccharides, which were not sufficient to conduct NMR analysis. Therefore, a greater amount of mucin sample would be required for future experiments.

Conclusion

This study describes the processes involved in preparing pure mucin samples for oligosaccharide isolation, to enable the analysis of intestinal mucin glycosylation of poultry. The present analysis did not support or refute the hypothesis tested that exposure to microflora influences the structure of oligosaccharide chains in mucins secreted differentially by intestinal goblet cells of early post-hatch chickens. It should be noted that the MS techniques used were not adaptable to replication in sufficient number to permit

statistical analysis. Future studies would benefit a systematic study of mucin oligosaccharide structures of CV and LBL chicks.

The understanding of intestinal mucin oligosaccharide structures has great potential for determining attachment sites for specific bacterial species in the gastrointestinal tract of the chicken. Furthermore, it may provide a means to ascertain functional changes in oligosaccharide profile during early development. Although structural differences of mucin oligosaccharides were not observed between LBL and CV animals, results obtained from histological studies (described in Chapter 3) indicate further experiments are warranted to establish whether mucin composition does indeed influence the binding of certain bacterial species. Differences in bacterial adhesion to ileal tissue of LBL and CV chickens and the relationship with changes to mucin composition are discussed in Chapter 5.

Chapter 5 Bacterial Binding Assays

Bacterial adhesion to ileal tissue and mucin samples of conventionally reared
and low bacterial load chicks

5.1. Introduction

Adhesion is an important prerequisite for bacterial colonisation. Bacteria possess specific attachment properties which enable them to bind to epithelial cells. External appendages such as fimbriae and extracellular polymers (LPS) can adhere to receptors on epithelial cells (Lu & Walker, 2001) and the mucus layer (Forstner *et al.*, 1995). Mucin glycoproteins compete with bacteria for receptors to reduce adherence. However, adherence to mucin glycoprotein may also facilitate bacterial colonisation, as mucins provide temporary attachment, allowing the secretion of secondary virulence factors such as cytotoxins, cytolysins or invasins (Forstner & Forstner, 1994).

Different bacterial species have specific binding affinities to mucin oligosaccharide sugar sub-units. For example, *L. animalis* possess binding sites for glucose and/or mannose, whereas *L. fermentum* possess receptors for sialic acid, fucose and mannose (Gusils *et al.*, 2003). *E. coli* serotypes, bind to mannose via P or type 1-fimbria (Firon *et al.*, 1984) and sialic acid via S-fimbriae (Herias *et al.*, 1995). The ability to bind to mucin glycoproteins is strain specific, with different species and serotypes displaying varying degrees of binding activity (Jin & Zhao, 2000; Jonsson *et al.*, 2001).

Adherence of enterotoxigenic *E. coli* to epithelial cells has been shown to decrease with age with older animals being less susceptible to infection. Younger animals, specifically neonates, are more vulnerable to pathogenic invasion. It has been speculated that the protective function of mucin in younger animals hinders bacterial attachment to the underlying intestinal epithelium (Conway *et al.*, 1990; Dean-Nystrom & Samuel, 1994).

Assays to measure bacterial binding to intestinal tissue, as well as intestinal mucin, have been conducted in a variety of animal models, including poultry (Gusils *et al.*, 2003; La

Ragione *et al.*, 2005), however, little is known of the influence of mucin composition on bacterial adhesion. The aim of this study was to determine whether the differences in ileal mucin composition between low bacterial load (LBL) and conventionally reared (CV) chicks during early development (described in previous chapters), may have deterred or enhanced binding of certain bacterial species.

E. coli was selected on the basis that it is commonly found in poultry, and because it is a resilient bacterium for use in *in vitro* experiments. *E. coli* has been reported to cause extensive enteric infections in young neonates (Mouricout *et al.*, 1990), especially during early development (Amit-Romach *et al.*, 2004). Although *E. coli* is not widely reported to cause enteric infections in poultry, it is known to infect the yolk sac (Giovanardi *et al.*, 2005), respiratory system (La Ragione *et al.*, 2000) as well as being a food-borne pathogen associated with poultry products (La Ragione *et al.*, 2005). *Lactobacillus salivarius* was also selected for the study as it is also a commensal species. Moreover, Lactobacilli are considered by some to be potential probiotics that may be of benefit in maintaining intestinal microbial balance (Styriak *et al.*, 2003).

5.2. Materials and methods

5.2.1. Bacteria

Bacterial cultures of *E. coli* and *L. salivarius* were provided from a collection held by Dr V. Torok, South Australian Research and Development Institute. *E. coli* had originally been isolated from the gut of poultry, while *L. salivarius* was a commercially supplied pure culture. Both bacterial strains were obtained from single colony isolates. Identity of each strain had previously been confirmed by genome sequence information from the 16S ribosomal gene. Glycerol stocks of *E. coli* and *L. salivarius* were streaked onto Bacto™ Tryptic Soy agar (Becton, Dickinson and Company, USA) and M.R.S. agar (Oxoid, Australia) plates, respectively. Plates were incubated at 37°C overnight under anaerobic conditions. Single colonies of *E. coli* and *L. salivarius* were isolated from plates and grown in 10 mL Bacto™ Tryptic Soy broth and M.R.S broth, respectively, for 16 h at 37°C under anaerobic conditions. Cell density was measured by spectrophotometry and calculated using the following formula (Dr Valeria Torok, *pers. com.*, 2007):

$$\#cells = 0.01 \times (OD600 \times 22.93 \times 10^8) \text{ cells/mL}$$

OD600 = optical density measured at 600nm

5.2.2. Preparation of fluorescein isothiocyanate-conjugated bacteria

A 10 mL volume of 6×10^{10} cells/mL was centrifuged at 2000g for 20 min at 4°C and the excess media removed. The bacterial cells were conjugated with fluorescein isothiocyanate (FITC) (Sigma, St. Louis, Mo.) as described previously (Edelman *et al.*, 2003). Briefly, bacteria were incubated for 30 min at room temperature with occasional mixing in 2mL 0.1M Na₂CO₃ pH 9.0 containing 0.9% (wt/vol) NaCl and 150µg/mL of

FITC (FITC dissolved in dimethyl sulfoxide (DSMO) 1mg/mL before adding to the buffer).

The suspension was diluted to 20 mL with phosphate-buffered saline (PBS), pH 7.1; containing 0.05% (v/v) Tween-20 (Sigma, St. Louis, Mo.). Bacteria were collected by centrifugation (20 min, 3150 rpm, 4°C) and further washed with 20 mL PBS-Tween. After a final centrifugation, the cells were then suspended in 1 mL PBS and stored in 100 µL aliquots at -20°C.

5.2.3. Intestinal samples

Frozen sections of ileal tissue from LBL and CV chicks, previously embedded in optimal cutting temperature (O.C.T.) compound (ProSciTech, Queensland, Australia), were cut using a cryostat. Sections (5µm), were mounted on glass slides (Starfrost, ProSciTech, Queensland, Australia), and stored at -80°C until needed. Before bacterial adhesion, sections were fixed for 15 min at room temperature with cold 3.5% (w/v) paraformaldehyde in PBS pH 7.1 and washed three times with 50mL of PBS.

5.2.4. Bacterial adhesion

Adhesion of *E. coli* was detected using fluorescence as described previously (Edelman *et al.*, 2003; Nowicki *et al.*, 1986), (Appendix 4). Briefly, FITC-labelled bacteria were thawed and diluted in PBS containing 0.01% (w/v) Tween and 1% (w/v) bovine serum albumin (BSA) at a concentration of 1×10^9 cells/mL. The bacterial suspension (50µL) was pipetted onto each section and incubated at room temperature for 1 h in a humidity chamber. Sectioned were rinsed three times in 50mL of PBS for 5 min each under gentle agitation.

For identification of intestinal morphology, tissue sections were subsequently counter-stained with tetramethylrhodamine B-isothiocyanate (TRITC)-conjugated wheat germ agglutinin (WGA) from *Triticum vulgare* (Sigma, St. Louis, Mo.). Sections were incubated with TRITC-WGA (50 μ L of 20 μ L/mL in Dulbecco's PBS solution (Sigma, St. Louis, Mo.) for 30 min at room temperature. Sections were rinsed individually to avoid residual staining, and then all slides were rinsed in PBS as described above.

5.2.4.1. Microscopy

An anti-fade mounting medium (Dr Graham Webb, *pers. com.*, 2007; Appendix 4) was used to coverslip the slides. A microscope equipped with an epi-illuminator and filter system for FITC and TRITC fluorescence was used for microscopy (Olympus BX60 microscope, Tokyo, Japan). Images were analysed using Video Pro[®] (version 6.210, Leading Edge Pty. Ltd., Australia). The image background was adjusted to produce a defined, clearer image to use for analysis (Appendix 4). The image was then sharpened, and a "luminance" function was selected to produce a grey-scale image. A threshold was then set to highlight only illuminated bacteria. Five images per section were analysed. Bacterial adherence was expressed as the area of bacterial fluorescence/total area of ileal tissue (μm^2).

5.2.5. Preparation of *E. coli* for mucin binding assays

A 10mL volume of 6×10^{10} cells/mL was centrifuged at 2000g for 20 min at 4°C and the excess media removed. Cells were diluted to 20mL with PBS, pH 7.1; containing 0.05% (v/v) Tween-20 (Sigma, St. Louis, Mo.) and the bacteria were collected by centrifugation (20 min, 2000g, 4°C) and washed once with 20mL PBS-Tween. The cells were then suspended in 1mL PBS and stored in 100 μ L aliquots at -20°C.

5.2.6. Binding to ileal mucin

Mucin samples were obtained as per the method described in Chapter 4. Mucin (300 μ L, 0.5 mg/ml) was pipetted onto 3-well diagnostic slides (ProSciTech, Queensland, Australia), with PBS pH 7.1 as a negative control and porcine gastric mucin as a positive control overnight at room temperature. The slides were then washed three times in PBS pH 7.1 for 5 min before and after blocking with 1% BSA in PBS for 1 h. *E. coli* cell suspension (BSA-PBS, 200 μ L of 1×10^9 cells) was pipetted into each well and incubated at room temperature for 1h. Unbound *E. coli* was removed by washing three times in PBS pH 7.1 for 5 min. Bacteria were stained with methylene blue for 1 min, rinsed in distilled water and observed using light microscopy (Olympus BX60 microscope, Tokyo, Japan).

5.2.6.1. Microscopy

Images were captured at 1000x magnification under oil immersion. Image analysis (Video Pro[®] version 6.210, Leading Edge Pty. Ltd., Australia) was used to calculate the number of cells/ mm^2 of the image (super high quality (SHQ), 3072 x 2304 pixels). Twenty images per sample were analysed. Images were calibrated using a 1mm calibration slide.

5.2.7. Statistical analysis

Data were analysed using GenStat 8th edition (VSN International Ltd., UK). Group x age affect was tested using a two way-analysis of variance (ANOVA). Simple regression analysis was used to detect relationships between bacterial adherence and goblet cell parameters measured in Chapter 3. The relationships were further tested by fitting individual goblet cell mucin composition values as covariates to the group x age ANOVA. Significance was determined as $P < 0.05$.

5.3. Results

5.3.1. *E. coli* binding to ileal sections

There were no significant differences in binding of *E. coli* between CV and LBL animals at 24 h or 96 h post-hatch, with both groups displaying similar binding affinities to the ileal sections tending to be concentrated in the vicinity of the villus tips (Figure 5.1). Although no statistically significant differences were observed between groups at these time points, there was a dramatic decrease in binding from day 1 to day 4, in both LBL and CV animals ($P < 0.05$) (Figure 5.2). By day 7 post-hatch, binding had significantly increased with greater adhesion observed in LBL animals ($P < 0.01$; Figure 5.2). In LBL and CV sections, bacterial adhesion tended to concentrate along the edges of the intestinal villi (Figure 5.3).

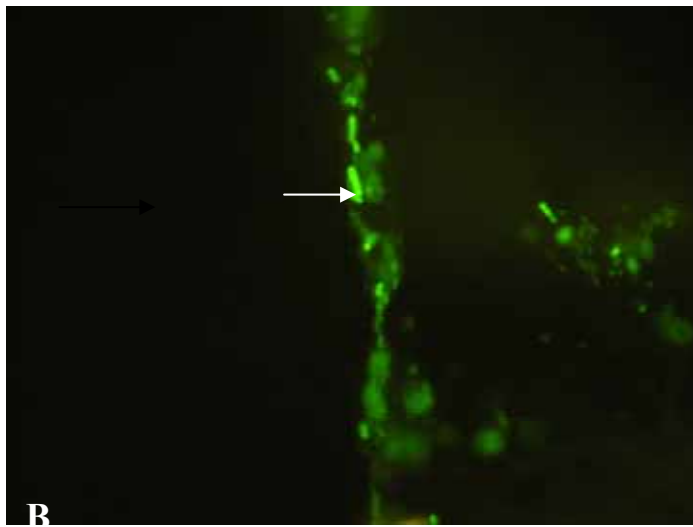
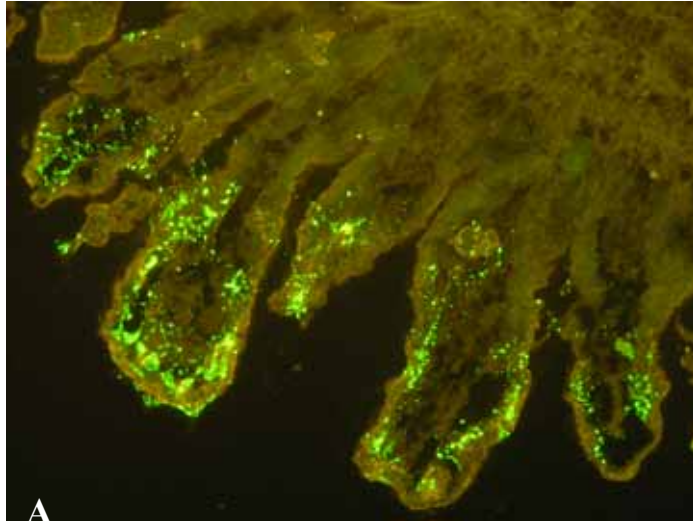


Figure 5.1. (A) Typical binding of FITC-conjugated *E. coli* to ileal tissue. Note the concentration at the villus tips. Magnification 200x (B) 1000x oil magnification of a single bound *E. coli* (arrow). Photomicrographs are from day old chicks.

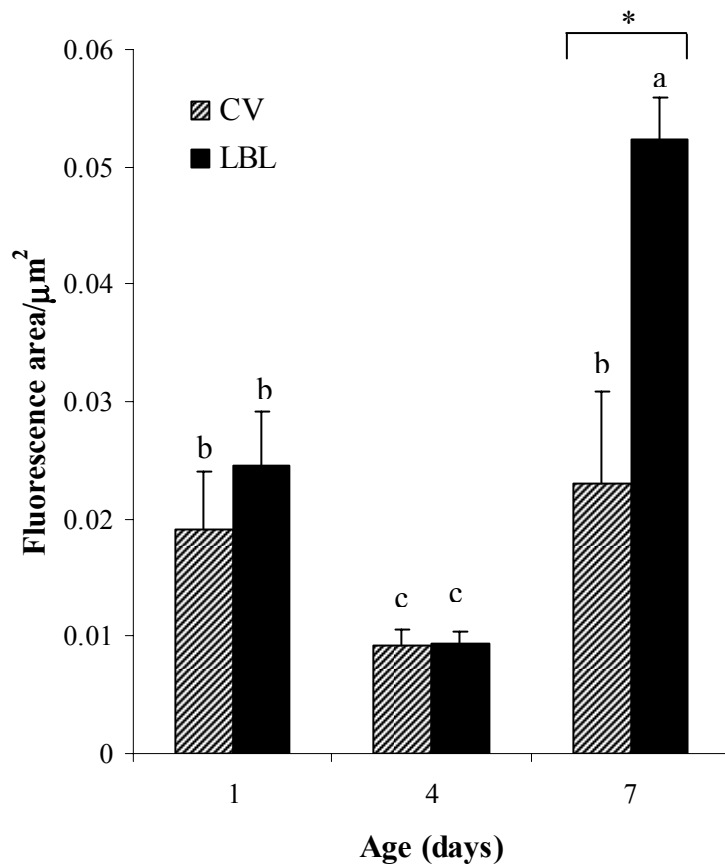


Figure 5.2. Bacterial fluorescence area of *E. coli* bound to ileal tissue in conventionally reared (CV) and low bacterial load (LBL) chicks at d 1, 4 and 7 post-hatch. Values are means \pm SE. Within the same day, bars with and * differ significantly ($P < 0.01$). Bars with different letters, differ significantly from each other ($P < 0.05$).

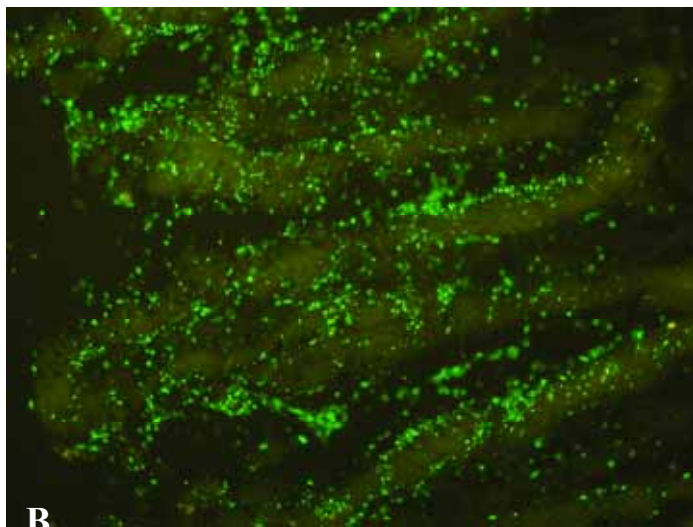
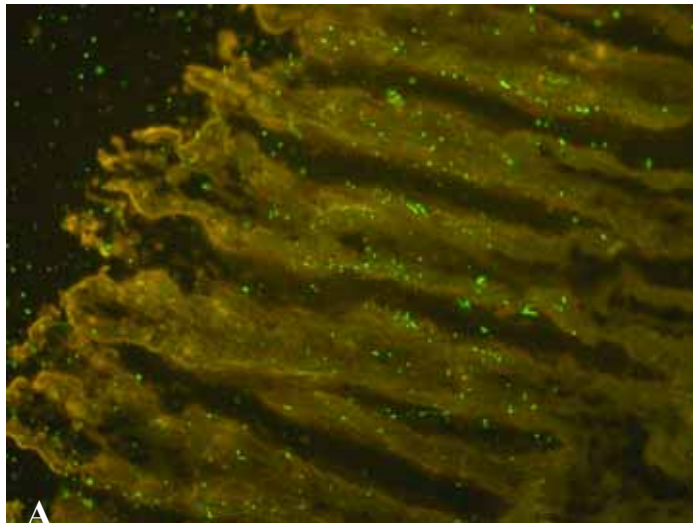


Figure 5.3. Differences in binding of FITC-conjugated *E. coli* to ileal tissue in (A) a conventionally reared (CV) chick and (B) a low bacterial load (LBL) chick at d 7 post-hatch. Magnification 200x.

5.3.2. *L. salivarius* binding to ileal sections

There were no significant differences in the binding of *L. salivarius* between CV and LBL animals at any time point (Figure 5.4). However, over time, LBL and CV animals displayed increasing binding activities. For LBL animals there was a gradual increase in binding from d 1 though to d 7 post-hatch. In CV animals there was a slight increase in adhesion at d 4, which returned to values similar to d 1 at d 7 post-hatch. It was also observed that the *Lactobacilli* did not adhere to ileal sections in as greater abundance as *E. coli*, although adherence was similarly localised to the villus epithelial surface (Figure 5.5).

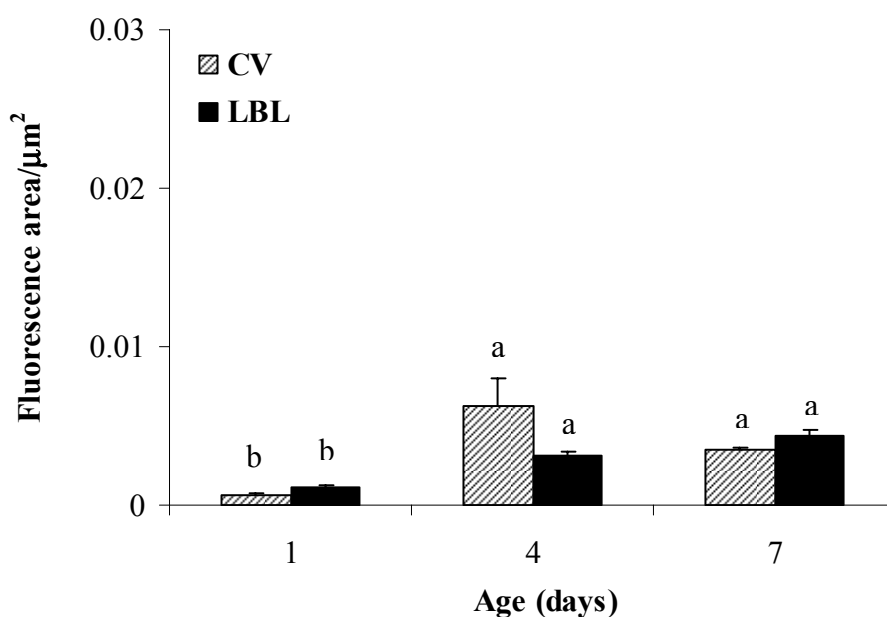


Figure 5.4. Bacterial fluorescence area of *L. salivarius* bound to ileal tissue in conventionally reared (CV) and low bacterial load (LBL) chicks at d 1, 4 and 7 post-hatch. Values are means \pm SE. Bars with different letters, differ significantly from each other ($P < 0.05$).

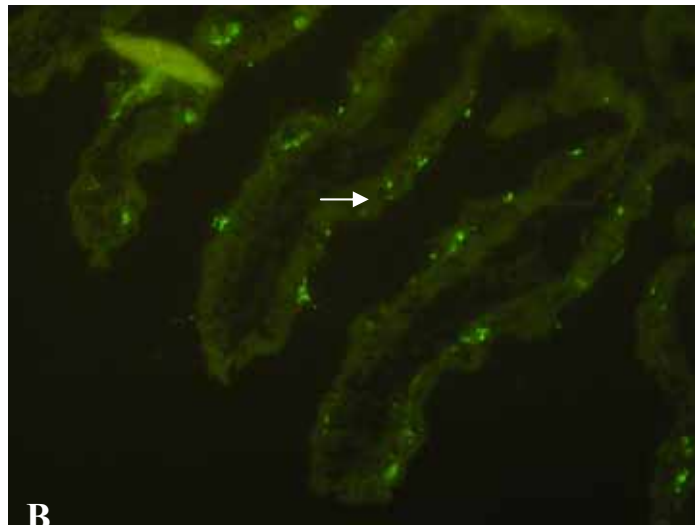
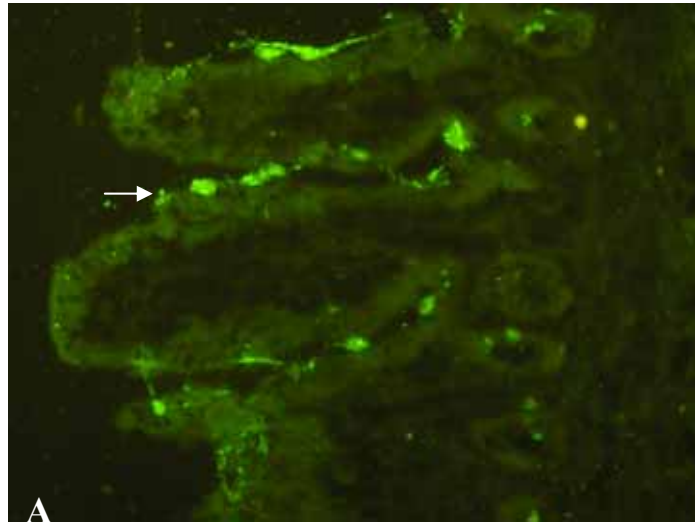


Figure 5.5. (A) Typical binding of FITC-conjugated *L. salvarius* to ileal tissue at d 4 post-hatch, and (B) at d 7 post-hatch. Note the adhesion to the surface of the villus epithelium (white arrows). Magnification 200x.

5.3.3. *E. coli* binding to ileal mucin

There were no significant differences in binding of *E. coli* to purified ileal mucin of LBL and CV animals at either d 4 or d 7 post-hatch ($P > 0.05$; Figure 5.6). The number of bacteria bound to ileal mucin was not correlated to values obtained from binding to whole tissue samples ($P > 0.05$). Binding to ileal mucin tended to be highly variable among individual animals resulting in large standard errors.

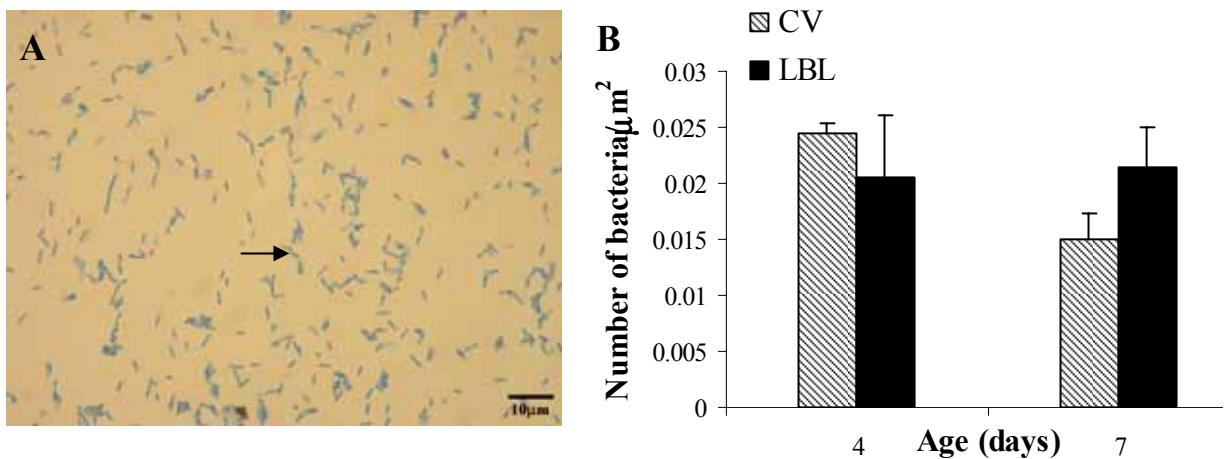


Figure 5.6. (A) Typical *E. coli* adhesion to intestinal mucin. Bacteria are stained with methylene blue (black arrow). Magnification 1000x under oil. (B) Binding activity of *E. coli* at d 4 and 7 post-hatch of conventionally reared (CV) and low bacterial load (LBL) chicks. Values are means \pm SE.

5.3.4. Correlations with goblet cell mucin composition

There was no relationship between increased numbers of goblet cells containing sialo-mucin observed in CV animals (described in Chapter 3), and binding of *E. coli* in either CV or LBL animals ($P > 0.05$). A moderate correlation between the decrease in goblet cell numbers containing total acidic mucins observed in CV animals and bacterial binding was detected ($r = 0.58$, $P < 0.05$). However, when the number of goblet cells containing acidic mucin was fitted as a covariate, statistical significance was no longer evident ($P > 0.05$). This was also observed for *L. salivarius*, with no significant correlations between goblet cell numbers ($P > 0.05$). However, the increased number of goblet cells containing sialo-mucin was moderately correlated with the increase in lactobacillus binding in CV animals ($P < 0.05$). However, once sialo-mucin goblet cell values were fitted as a covariate, the relationship was not statistically significant ($P > 0.05$).

5.4. Discussion

The mucus layer has been described as a site of attachment for bacteria as a defensive mechanism to prevent pathogen attachment to the epithelial surface. In poultry, the ileum has been reported to be the preferred site for bacterial colonisation (La Ragione *et al.*, 2005). In the present study, a decrease in bacterial binding in ileal sections at d 4, in both LBL and CV animals was detected. It was hypothesised that a linear increase in bacterial adhesion would be observed over time. However, there was a dramatic decrease in bacterial binding from d 1 to day 4, followed by a 2-fold increase at day 7. Day-old chicks have been reported to be highly susceptible to colonisation by O157 *E. coli*, possibly due to the presence of an immature immune system and the possession of little to no competitive gastrointestinal microflora (La Ragione *et al.*, 2005). This may have been indicative of the observations encountered for day-old chicks in the current experiment and also an explanation for the increased binding observed at d 7 in LBL animals, as the gut remains relatively immature under isolation conditions.

The moderate correlation between the decrease in acid goblet cell number and bacterial binding to ileal tissue suggested that the decrease in total acidic mucin may have been affecting adhesion of this bacterium, and thus the decrease in binding observed at d 4. However, this hypothesis was disproved by fitting acidic mucin values as a covariate. Thus, other factors, not necessarily changes in goblet cell mucin production, may have been influencing the inhibition of binding at this time point. The utilisation of molecular techniques in a past study revealed there were no detectable *E. coli* in the small intestine at d 4 post-hatch, with *Lactobacillus* in high abundance (Amit-Romach *et al.*, 2004). Although not statistically significant, in the current study, there was a slight elevation in *L. salivarius* binding in CV animals at d 4 post-hatch. The addition of *Lactobacillus* species *in vitro* is known to inhibit *E. coli* adherence (Larsen *et al.*, 2007; Otte & Podolsky, 2004).

Lactobacilli have also been involved in the upregulation of mucin gene expression (MUC2 and MUC3) which has been reported to inhibit *E. coli* adherence (Mack *et al.*, 1999). Thus it would be beneficial to observe the effects of lactobacilli on competitively excluding the adhesion of this *E. coli* serotype. In addition, determining whether these “beneficial” bacteria could mediate a decrease in *E. coli* adhesion at this time point through the regulation of mucin gene expression could also be examined. Using different *Lactobacillus* strains with greater binding capacity may be useful in investigating this hypothesis. Different *Lactobacillus* species exhibit different adhesion properties (Gusils *et al.*, 2002). The low binding values of *L. salivarius* in the current study indicated that the species may have had a lower affinity for intestinal tissue or mucin. *In vitro* mucin binding studies could be informative to detect specific interactions with mucin glycoproteins and this species.

The *E. coli* species used in the current study bound to purified ileal mucus equally well in LBL and CV animals. This observation contrasted with the inhibition of *E. coli* adherence to the epithelial surface of CV ileal tissue sections at d 7 post-hatch. Whether this was due to changes in mucin composition can not be determined. However, it can be hypothesised that the increase in sialo-mucin in CV may have been a means of increasing binding of mucin glycoproteins to the epithelial surface thus preventing binding of this *E. coli* serotype. It was demonstrated in the gnotobiotic rat that bacterial Sfim⁺ wildtype and Sfim⁻ mutants (which bind to sialic acid residues), both adhered to epithelial cells and intestinal mucus with both wildtype and mutant bacteria colonising well in the gut during early development (Herias *et al.*, 2001). The absence of sialic acid residues in LBL animals may have contributed to the high binding activity observed in this study.

The K99⁺ *E. coli* serotype, possesses receptors for sialic acid. In neonatal mice, piglets and calves, adhesion of this serotype to epithelial cells was greatest compared to mature animals. This was not found to be the case with K88 *E. coli* which remained at high levels into adulthood (Runnels, 1980). However, K88 colonisation to epithelial cells was reduced in the presence of ileal mucus isolated from older animals (Conway *et al.*, 1990). This suggests that during early development, the change in sialo-mucin composition may inhibit binding of particular bacterial species by competing with epithelial surface receptors. It would be interesting to conduct *in vitro* experiments in which sialic acid sugars could be added to an intestinal epithelial cell line culture in order to observe whether there are changes in bacterial adherence.

Assay analysis was restricted as identification of the specific serotype of pathogenic *E. coli* used in the present study was not conducted. Thus it was difficult to determine the structural adhesive properties of this bacterium and its binding to intestinal tissue and mucin. In future studies, different bacterial species could be utilised for these assays, especially those with a greater association with enteric infections of poultry.

In vivo models could provide an improved indication of bacterial colonisation in response to changes in mucin profile. Inoculation of LBL animals with different species of bacteria, pathogenic and commensal, may be a more efficient method to observe relationships between changes in intestinal mucin profile and colonisation. It has been observed that different species of *Lactobacillus* adhere differently when used in *in vivo* and in *in vitro* models (Gusils *et al.*, 1999; Larsen *et al.*, 2007). In addition, fimbriae are not strictly essential for the colonisation of bacterial species to the intestinal mucosa (Marc *et al.*, 1998). In poultry, flagella are also involved in colonisation as these assist in penetration of the mucus layer (Best *et al.*, 2005; La Ragione *et al.*, 2000). Moreover, the bacteria used in

the present assays were immobile, as a result, the *in vitro* studies may not have been a true indication of the adherence of bacteria in response to changes in the intestinal mucin profile.

The colonisation of bacteria to the intestinal mucosa biofilm is a complex process, with many factors contributing to successful adhesion. From the current study, it can be concluded that the changes in mucin composition played a minor role in bacterial adhesion of *L. salvarius* and this *E. coli* serotype. In this instance, changes in mucin composition exhibited in CV animals may have represented a mechanism to compete for epithelial attachment. The complex nature of bacterial adhesion to the intestinal mucosa warrants further investigation, especially the adhesion of bacterial species, such as *Clostridium perfringens*, which are specifically involved in the intestinal disease necrotic enteritis, an economically significant problem in poultry production.

Chapter 6 General Discussion

The overlying mucus-gel layer (biofilm) is the first line of defence that bacteria encounter when attempting to traverse the intestinal mucosa. The formation of the mucus-gel occurs through goblet cell secretion of polymeric mucin glycoproteins (Forstner & Forstner, 1994; Klinken *et al.*, 1995). These glycoproteins prevent noxious agents from coming into contact with the underlying epithelial cells by competing with bacteria for adherence via heterogenous oligosaccharide chains, whilst simultaneously providing a desirable environment for proliferation of specific bacterial species. Thus, the type and quantity of mucus are essential components in establishment of the intestinal barrier. Currently, there is little information available describing the effects of bacterial colonisation on the secretory pattern of small intestinal mucins during post-hatch development.

The primary aim of this research was to investigate the effects of bacterial colonisation on mucin production in intestinal goblet cells during early post-hatch development of chicks, and secondly, to develop a new *in vivo* model system to study bacterial-intestinal interactions. The first objective was to design and establish a means of effectively hatching and rearing low bacterial load chickens to study microbial-intestinal interactions in comparison with conventionally reared animals. Chicks were incubated, hatched and reared under low bacterial load conditions. This resulted in the successful production of chickens with a minimal bacterial load with distinct differences in microbial profile. Body weight was not compromised and was comparable to conventionally reared chickens.

The second objective was to utilise histological techniques to determine whether limiting bacterial exposure influenced goblet cell dynamics and mucin production. Using different staining techniques to distinguish between mucin types, it was found that total goblet cell numbers and morphology of goblet cells containing neutral and acidic mucins did not differ significantly between CV and LBL animals. However, significant differences in

acidic mucin composition, from primarily sulphated to an increase in sialyated sugars at d 3-4 post-hatch, were observed in CV chicks, with greater numbers of jejunal and ileal goblet cells displaying this mucin type.

On the basis of histological results obtained from this study, it was hypothesised that bacteria were influencing the chemical structure of the mucin oligosaccharide chains, as these are the primary target sites for bacterial attachment. Purification techniques, such as gel filtration chromatography, were employed to isolate mucin oligosaccharide fractions. Using mass spectrometry, a profile of the length and number of oligosaccharide chains was obtained. There were no differences observed for either chain length or number between LBL and CV animals, with no differences observed between d 4 and 7 post-hatch.

Although structural differences in mucin oligosaccharides were not observed between LBL and CV animals, the changes to mucin production in goblet cells, observed in histological studies suggested the need for further experiments to determine whether the mucin composition indeed influenced the binding of certain bacterial species. For these studies, *E. coli* and a *Lactobacillus* species were selected. Although not significant between LBL and CV animals at d 1 or d 4, there was a significant decrease in binding from day 1 to day 4, with the most dramatic reduction occurring in LBL animals. At d 7 post-hatch, binding had significantly increased, with greater adhesion observed in LBL animals. There were no significant differences in the binding of *L. salivarius* between CV and LBL animals at any time point. However, over time, LBL and CV animals displayed different binding patterns. No statistically significant differences were observed in binding of *E. coli* to purified ileal mucin from LBL and CV animals at either d 4 or d 7 post-hatch. Correlations between *E. coli* and *L. salivarius* adherence to ileal tissue and mucin samples, and goblet cell parameters, were not statistically significant. It was concluded that the changes in

mucin composition played a minor role in bacterial adhesion of *L. salivarius* and this *E. coli* serotype.

The switch from predominantly sulfo-mucins to acetylated sialo-mucin in goblet cells in histological sections of conventionally-reared chicks presumably represented a defensive mechanism by the host during early post-hatch development, as originally hypothesised. Moreover, based on the current histological studies it was further hypothesised that the sialylation of mucins inhibited the binding of certain bacterial species onto the epithelial surface, thus preventing potential invasion.

Previous studies have indicated that the hydroxyl groups of sialic acids are highly substituted by acetyl esters. These serve as added protection as they block against further glycosidic degradation, with reports that two or more acetyl groups may inhibit enteric bacterial sialidases (Belley *et al.*, 1999; Corfield *et al.*, 1992). Both sulphate and sialic acid groups exhibit protective properties against bacterial degradation (Corfield *et al.*, 1992; Robertson & Wright, 1997). As exposure to bacteria becomes greater, the need for protection against mucus degradation is increased, which would explain the observed increase in sialo-mucin production in the current study. Bacterial binding assays from previous studies demonstrated that the addition of mucin glycoprotein *in vitro* inhibited *E. coli* adhesion to epithelial cells (Conway *et al.*, 1990). In Chapter 5, it was proposed that *in vitro* experiments could be conducted whereby sialic acid sugars could be added to an intestinal epithelial cell line culture in order to observe whether these sugars could cause changes to bacterial adherence.

In addition, sialic acid has been reported to promote Ca^{2+} binding which is involved in the differentiation of embryonic goblet cells (Black & Smith, 1989) and the binding of

immunoglobins such as IgG to intestinal brush border membranes (Gill *et al.*, 1999). Whether sialylation of intestinal mucins plays a role in these processes is yet to be determined. Nevertheless, this provides insight into the role the mucus layer plays in pathogenic defence and its importance during this developmental stage.

The extent by which mucin composition is regulated is not completely defined. Further emphasis should be placed on measuring the expression of genes involved in mucin production, such as mucin core peptide (MUC) and glycosyltransferase genes. More importantly, the upregulation of these genes by bacteria and the relationship with inflammatory stimuli during post-hatch development should also be investigated. There have been numerous references to the upregulation of mucin gene expression through either direct bacterial attachment or by an indirect immune response to pathogens. For example, the upregulation of MUC gene expression has been shown to be stimulated through binding of bacterial LPS, and also the upregulation of cytokine production. During this developmental period, it has been reported that an upregulation of mRNA expression of proteins involved in immune function, such as antimicrobial peptides and pro-inflammatory cytokines, was greatly increased in the gut associated lymphoid tissue (GALT) (Bar-Shira *et al.*, 2003). Overall, this defines the potential importance of goblet cells and mucus production during post-hatch development while the immune system is still developing. From this, it was hypothesised that the intestinal immune development stimulated the production and composition of mucin as a defensive mechanism due to bacterial colonisation during the first few days post-hatch. A study of cytokine gene expression and their relationship with the upregulation of mucin genes could be applied using microarray technology to examine an extensive range of these genes and any patterns of expression.

The addition of non-digestible carbohydrates termed “prebiotics” to the diet, has been reported to decrease bacterial numbers, particularly intestinal pathogenic species, as well as promoting colonisation of beneficial bacteria (Kleessen & Blaut, 2005; Ten Bruggencate *et al.*, 2004). Studies outlining the benefits of these prebiotics have obtained bacterial counts predominantly from digesta samples (Baurhoo *et al.*, 2007; Spring *et al.*, 2000; Ten Bruggencate *et al.*, 2004) and not on adhesion to epithelial cells or competition with other bacterial species. Since many bacterial species, both beneficial and pathogenic, share similar adhesion receptors, the addition of particular oligosaccharides may disrupt the balance of beneficial commensals such as *Lactobacilli*, by preventing adhesion to epithelial cells or the mucus layer, thus facilitating pathogenic attachment. Understanding the structural nature of intestinal mucin oligosaccharides through the employment of mass spectrometry or nuclear magnetic resonance technologies, in conjunction with the application of *in vitro* bacterial binding assays, will enable the screening of a greater range of oligosaccharides with potential prebiotic properties. This will assist in the identification of specific oligosaccharide structures which could inhibit the binding of pathogens without compromising intestinal commensals, or alternatively, to promote binding of beneficial bacteria. Preliminary *in vitro* experiments could be conducted to determine adhesion properties, which could then be tested *in vivo* to determine their efficacy in the gastrointestinal tract of poultry. The developed low bacterial load isolation system, would be an effective model system to assist in the development of these potential dietary supplements to promote the growth of poultry through manipulation of the gut microflora.

It has been demonstrated that diet composition impacts on mucin dynamics. The composition of the diet in terms of fibre and carbohydrate content, not only influences mucin composition but also goblet cell number (Satchithanandam *et al.*, 1990; Sharma *et al.*, 1997) and mucus secretion (Lundin *et al.*, 1993; Ouwehand *et al.*, 2000). Differences

in mucin composition have been observed with changes in diet texture, i.e. a finely powdered diet versus a coarser commercial diet. Greater numbers of goblet cells containing acidic mucins with binding specificities for predominantly sialic acid as well as N-acetyl-glucosamine were detected in intestinal samples of conventionally reared animals fed a coarser diet (Brunsgaard, 1998; Sharma & Schumacher, 1995; Sharma & Schumacher, 2001). Administering a carbohydrate mixture consisting of maltose, sucrose and dextrin to the amniotic fluid of d 17 chick embryos was found to increase the number of goblet cells containing acidic mucin by 50% when compared to controls (Smirnov *et al.*, 2006). Mucin mRNA expression was also increased from d 17 of incubation to 3 d post-hatch. These findings emphasise the importance of diet on mucin dynamics and intestinal maturity during early development.

It would be interesting to administer a simplified diet to chicks under isolation conditions and observe the effect of diet on mucin-bacterial interactions. A purified diet was proposed in the initial research proposal but was found not to be feasible in terms of costs, as pre-formulated purified diets are expensive and difficult to prepare. Understanding the impact of diet on goblet cell and mucus ontogeny could assist in developing a convenient and effective way of manipulating intestinal microflora as well as the administration of pre-biotic compounds.

It was surprising that no differences in mucin oligosaccharide profile were observed between LBL and CV animals given that histology results were so distinctive. Changes in mucin oligosaccharide profile would have provided a better insight into the diversity of the oligosaccharide chains during this developmental period. It would have also been beneficial to analyse samples using NMR to obtain specific chemical structures. Work of this nature is indeed possible (Robbe *et al.*, 2004). The minute amount of mucin sample

able to be obtained from very young chickens prevented this approach in the current study and may have contributed to the overall outcomes of Chapter 4. Future collaborations with laboratories equipped for such analysis would be greatly effective to successfully isolate these mucin sugars.

A greater number of animals used for the isolation experiments would have also been beneficial. Due to the size of the brooding isolator the number of chicks was restricted. Replication of the trial enabled a total of 6 birds for each time point. An increase in bird number may have given a better defined profile for analysis of oligosaccharide structure. Correlations between changes in mucin composition and bacterial adhesion could also have been strengthened. The amount of mucus obtained from each intestinal region in day old chicks was minute, and when purified only yielded micrograms of mucin sample. As a result, for example, day old chick samples had to be pooled for each time point.

During the first 7 d of post-hatch development, the acidic mucin profile of the small intestine appeared to be of most importance in the chicken. This concurs with other developmental studies conducted in other animals in which the intestinal acidic mucin profile was greater in younger animals compared to adults (Fontaine *et al.*, 1996; Shub *et al.*, 1983; Turck *et al.*, 1993). It would be interesting to investigate the mucin profile of older birds, both LBL and CV, to observe whether this response changed over time.

Conclusion

The interactions between bacteria and the intestinal mucosa of poultry are highly complex. This thesis sought to provide a better understanding of the physiological responses associated with bacterial colonisation and mucin dynamics during early post-hatch development. Throughout the study, many factors have been speculated to contribute to

changes in mucin and goblet cell dynamics in response to microbial colonisation. The development of gut immunity and its interactions with mucin dynamics and bacterial colonisation warrants further investigation, more specifically, to determine their involvement in the pathogenesis of intestinal diseases. The application of the low bacterial load model system will facilitate development of dietary interventions to manipulate the bacterial profile and assist in preventing intestinal infections during post-hatch development, thus improving overall broiler performance, health and welfare.

Appendices

Appendix 1: Construction of isolators

Instructions for the construction of the isolators were outlined in a booklet prepared by Dr Gordon Firth (*pers com*, 8th Nov 2004). All tools, fittings and consumables, such as cloth ducting tape and plumbers tape were purchased from a local hardware store.

Cleaning

Isolators and all parts were initially cleaned and/or soaked with Domestos[®] house cleaner. Prior to assembly, the isolators were transported into the animal housing facility where they were steam-cleaned and wiped with an antimicrobial microfibre cloth.

Fitting the access box

The access box, a device constructed from 150mm PVC pipe, was used as a means of transporting items in and out of the isolator. It consisted of two sections (one with a glued extension) of PVC pipe and two lids. The section with the glued extension was fitted from the inside of the isolator such that it butted-up against the inner wall. Holding it in place, the remaining end was slipped onto the extension from the outside, aligning the two small holes on the top of the pipe. A small cable tie was threaded through the two holes and was used to hold the box together. To secure the assembly of the access box, cloth duct tape was used to seal both the outside and inside join between the access box and the isolator, and the join between the two access box sections. The external lid was securely attached to one end, and the internal lid was left on the floor of the isolator. Any unused designated attachment points to the isolator were either sealed with tape or with plastic screw-on caps.

Setting up for incubation (Type B isolator only)

The two 50-egg incubators along with 5 water containers, a power board, two candling lamps, a 250ml glass beaker and a small aluminium tray for fumigation were added to isolator B. The power board mains connection was disconnected and the remaining cord was run through a designated hole in the isolator wall. The gap between the cord and the hole was sealed with silicon and the mains connection reattached.

Setting up for brooding (Type A isolator only)

Drinker

The original design for the drinker required a 415mm length of 20mm diameter PVC pipe with 6 equally spaced nipple drinkers attached. However in initial trials, it was found that chicks were not successfully drinking from the drinker. The design was therefore reviewed. A new drinker was constructed based on the Iberline Poultry Nipple System¹, using a square pipe and fittings, 3 nipples, 3 orange water collection cups and a sight tube to flush out water.

Water was gravity-fed from a plastic 10L water drum into the drinker via an inlet on the end wall of the isolator and a flexible water line. Large cable ties from the isolator frame suspended the drinker. The cable ties were adjusted, so that the drinker was maintained at an appropriate height for day-old chicks. The cable ties could then be further adjusted to more suitable heights as the chicks grew in size.

¹ Intensive Farming Supplies, Cavan, South Australia

Testing for leaks

Air was bled from the nipples and the lines were allowed to fill with water to test for leaks, which were sealed using marine silicon. Afterwards, the water supply was turned off.

Attaching the brooding lamp

As there was no access to hot water, hot water brooding was not possible. Therefore, a brooding lamp was fitted to ensure the chicks were maintained at the correct temperature. The hot water brooder coil connections were subsequently sealed by attaching plastic caps to the bulkhead threads protruding through the isolator.

A metal attachment was designed and constructed to attach a brooding lamp within the isolator. The attachment was fixed to the top of the isolator by way of the top two metal rods of the isolator frame and lay directly in the centre of the isolator (Figure 7.1a). The brooding lamp was attached via a ceramic light bulb fitting 150W². The fitting could be unscrewed into two components, the top component containing the power cord, sat on top of the metal plate (Figure 7.1b). The lamp was attached underneath the metal plate and was secured to the top fitting by re-screwing the lower fitting into to the top fitting (Figure 7.1c). The globe was attached by screwing into the lower fitting. The power cord was pulled through the top of the plastic isolator wall and sealed using silicon and cloth tape.

² Intensive Farming Supplies, Cavan, South Australia

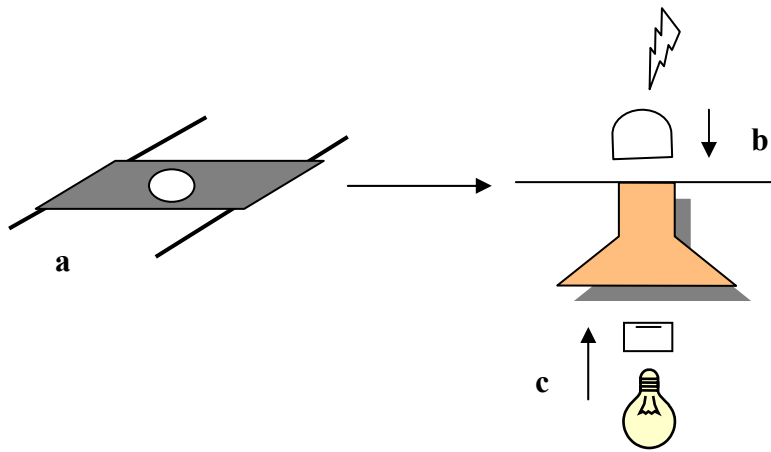


Figure 7.1. Attachment of the brooding lamp. Metal brooding lamp attachment connected to isolator frame (a), power attachment sits on top of the metal plate (b), lamp is connected and secured beneath the metal plate (c).

Brooding lamp temperature was controlled by a universal temperature monitor (Model PM4-RT 240) and an industrial temperature probe positioned close to the isolator floor (Pt 100 RTD, P-RTQ12SA-1)³. The brooding lamp power cord was plugged into the monitor, with temperature set at $32 \pm 1^\circ\text{C}$. The probe was connected to the isolator via a designated access port in one of the metal ends and a compression fitting that was attached to the probe.

Feeder

A standard plastic chicken feeder, which can be purchased from most poultry equipment suppliers, was used to deliver food to the chicks. A piece of plastic sheeting (500x 300mm) was taped centrally on the floor of the isolator and the feeder was placed so that it was half on the sheet. This allowed feed to be placed onto the plastic to teach the chicks how to use the feeder.

³ Amalgamated Instrument Co. Pty Ltd, Hornsby, NSW

Attaching flexible plastic walls

Before the plastic was attached, the join between the floor and each isolator end was taped to form a secure seal. The sides, top and bottom of the isolator were covered in plastic film (PVC soft formulation stack 0.2ml thickness 1.36m x 100m)⁴. Plastic was cut 1.5x the length of the isolator for type A brooding isolator and 3x the length for type B isolator. The plastic was firstly placed on top of the frame and temporarily taped to the back shoulder of the metal isolator ends. It was passed under and around the isolator leaving a 50mm overlap at its starting point. The plastic was pulled tight and even, before taping to the frame. The tape should securely seal the joins around the isolator ends and the plastic overlap.

After a night of running air into the isolator, it was found that the tape along the isolator frame had lifted, letting air escape from the isolator. When taping the edges, it is suggested that the tape is placed over the edge of the metal sides thus preventing the tape from lifting. External bars were inserted on the sides of the isolator to hold the plastic against the edge of the floor. This was to ensure that the chicks did not fall between the gaps created by the positive pressure.

Inserting glove ports and gloves

The gauntlet rings consisted of 200mm i.d. PVC pipe⁵. These were used to attach a pair of 32" long, 8" port Neoprene dry box gloves⁶. Using a marker pen the inside of two gauntlet

⁴ Cat # 003166, Halifax Vogel Group 08 83486800

⁵ This pipe was actually PVC pressure pipe, although slightly larger than the supplied gauntlet rings, they still were able to attach gloves. Virginia Irrigation Service Pty Ltd, Old Port Wakefield Rd, Virginia.

⁶ Mohawk Industry and Nuclear Supply, Inc., CT USA. Contact Jim Francoline at jfrancoline@mohawksafety.com.

rings was traced onto the plastic approximately a shoulder width apart. Keeping within 25-30mm within each outline a small circle was cut-out from the plastic. The plastic around the edge of the holes was softened using a hot air gun and whilst pliable the gauntlet ring was forced through the hole into the isolator and then pulled half way back, allowing it to settle in the plastic. The rings were then taped securely to the plastic. The gloves were attached inside out, thumbs innermost, palms down. The glove is slipped over the gauntlet ring and secured using a clamping ring.

Attaching the air relief outlet

The air relief line consisted of a 1m length of 25mm PVC pipe with an elbow connection; which hung parallel to the isolator end to allow waste air expulsion from the isolator. At the lower end of the pipe there was a fitting to accommodate a cylindrical mesh filter to prevent insects from entering the isolator. To avoid dust and other contaminants from the isolator becoming air borne in the room, the mesh filter was submerged in Coopex[®] insecticide solution. When the isolators were not in use, the mesh filter was removed and the outlet was closed securely with tape.

Attaching the airline

The air filter was connected to the isolator's air inlet connection via a 50mm diameter regular duty hose and appropriate fittings (Figure 7.2). The attached air valve was to control the amount of air entering the isolator and to provide an effective means of blocking the passage of air during fumigation. However since the air filter had a variable speed motor this was used primarily to control airflow.



Figure 7.2. Fittings required for air inlet connection from 50mm to 25mm, including air valve (centre)

Testing for leaks

To test for leaks, all external air accesses to the isolator were closed and/or sealed. The air filter was turned on and the air valve was opened to distend the isolator with air. Leaks were detected by the presence of hissing or feeling air currents on the back of the hand. The air supply was then turned off after 24h and the isolator was monitored to see how quickly the air subsided. All leaks were repaired, residual pressure released and all air-lines resealed.

Fumigation

Formaldehyde fumigation was conducted to produce a sterile environment within isolators. Before fumigation all air inlets and outlets were closed, and a respirator fitted with appropriate formaldehyde cartridges. Safety goggles were worn to prevent any eye contact with escaped gas. Formaldehyde solution (30-40%) was added to potassium permanganate in an aluminium tray which created a gas within the isolator. Fumigation continued overnight and was performed during a time when there would no contact with personnel.

A-type isolator: 9mL Formaldehyde solution (37-40%)

	5g	Potassium Permanganate
B-type isolator:	18mL	Formaldehyde solution (37-40%)
	11g	Potassium Permanganate

To expel the waste gas, a valve was attached to the air outlet line along with a 20m piece of hose (25mm). The hose was run out through the window of the building into an area where gas could be expelled safely. The air filter was turned on overnight to expel gas. When completed, the hoses were disconnected and the outlet pipe was sealed with tape.

After fumigation, the isolators were fogged with VirkonS[®] (20g/L) disinfectant using a spray bottle as a secondary treatment to ensure that the isolators were contaminant-free. Insecticide treatment (Coopex[®] 1g/100ml) was also administered in a location where insects could gain access into the isolator. All solutions were prepared using sterile MilliQ water. There was very little airflow within the isolator without positive pressure. Thus, after spraying, the isolator was not completely dry, therefore the air supply was turned on to ensure that the isolator dried out completely.

Maintaining cleanliness of room

Preparation before entering

Before entering the room, all researchers were required to wear coveralls/lab coats, hats, shoe covers and masks if coming into contact with eggs or chicks. There was an antibacterial “germ stopper” mat (Southern Cross Science Pty. Ltd., Panorama, SA) outside the room to remove contaminants on the bottom of the shoe covers. Once in the room, it was required that hands be washed before wearing and removing gloves.

Waste disposal

Upon leaving the room, gloves, wipes and other waste, were removed and placed in an autoclave bag. All waste was removed from the room daily and disposed of in the appropriate biohazard bins. Animal tissue waste was placed in autoclave bags and stored at -20°C for later waste collection.

Appendix 2: Staining protocols for goblet cell mucin glycoprotein

Periodic acid-Schiff reaction (PAS) (Kiernan, 1990)

Periodic acid solution and Schiff's Reagent were purchased pre-prepared for staining (Sigma, St-Louis, CT). All solutions were brought to room temperature before staining.

Histolene	10 min	}	Hydration step (removal of paraffin)
100% EtHOH	2 min		
80% EtHOH	2 min		
30% EtOH	2 min		
RO H ₂ O	2 min		
Periodic acid	20 min		(protocol recommended 10-30 min oxidation)
Running tap H ₂ O	3 min		
Schiff's Reagent	20 min		
Running Tap H ₂ O	10 min	}	Dehydration steps
70% EtHOH	1 min		
80% EtHOH	1 min		
100% EtHOH	2 min		
Histolene	5 min		

Sections stained a bright pink colour, indicated the presence of hexose-containing and sialic acid-containing mucosubstances. Sialic acid residues can be removed using mild acid hydrolysis before staining with PAS.

Mild acid hydrolysis

Take sections to water

Immerse in 0.05M Sulphuric acid for 60min at 80°C in water bath

Rinse well in running tap water, approximately 5 min

Proceed with PAS staining

High iron diamine (HID)/Alcian blue pH 2.5

All reagents purchased from Sigma Chemicals, except for 60% ferric chloride (Jomar Biosciences Pty. Ltd., SA)

HID solution

120mg	N,N-dimethyl-meta-phenylenediamine-dihydrochloride
20mg	N,N-dimethyl-para-phenylenediamine-dihydrochloride
50ml	Distilled water
1.4ml	60% ferric chloride

Dissolve the two salts in distilled water and then add the ferric chloride. Make solution fresh into Coplin jars for staining.

Alcian blue (pH 2.5)

5g	Alcian blue-tetrakis (methyl-pyridinium) chloride
500ml	3% aq. glacial acetic acid

Dissolve powder in glacial acetic acid, may need to use a magnetic stirrer. Test pH; adjust with concentrated acetic acid.

Staining protocol

Take sections to water using same solutions as for PAS staining.

HID solution 16 h room temperature

Running tap H₂O 3 min

Alcian Blue pH 2.5 5 min

Running Tap H₂O 3 min

Dehydrate and mount using same solutions as for PAS staining.

Sialyated mucins are stained blue; sulphated mucins are stained brown.

Image analysis

Image analysis was conducted using the programs Image J[®] 1.33 (Rasband, 1997-2004) and VideoPro[®] (version 6.210, Leading Edge Pty. Ltd., Australia). Image J was used to count goblet cell numbers. Video pro was used to measure epithelial area (mm²), total villus area (mm²), villus length (µm), villus breadth (µm), crypt depth (µm) and total goblet cell area (µm²). All images were calibrated using a 1mm calibration slide and a programme window size of 760 x 570 mm.

Particle counting

The “*Plugins/Particle counting/Cell Counter*” plugin was used to calculate total goblet cell numbers by automatically tallying the number of goblet cells using a marking tool. PAS, and HIDAB pH 2.5 stained sections were analysed by an identical method. In HID sections, different coloured markers were assigned to identify the goblet cells that stained only brown, only blue, and also if the cell had both brown and blue staining (intermediate).

Cells were counted by the presence of the apical part of the cell (goblet shaped). Cell counts per villus were expressed as the number of cells per epithelial area (mm^2).

Total goblet cell area

VideoPro[®] allowed for the selection of individual villi such that areas of goblet cells could be calculated as total area of goblet cells per total villi or epithelial area. Using the “*Edit/Draw/line*” tool, each villi was highlighted. “*Binary/Store/Load*” enabled the selection to be stored while goblet cells were selected. “*Colour/Process*” tool enabled the separation of goblet cells from the remainder of the image. (If HID sections, settings were R 0, G 0, B 255; if PAS sections, settings were R 0, G 255, B 0) (Figure 7.3b). By clicking “*Transmit*” the image was processed. “*Filter /Process/Average*” smoothed the image to achieve a better differentiation between goblet cells.

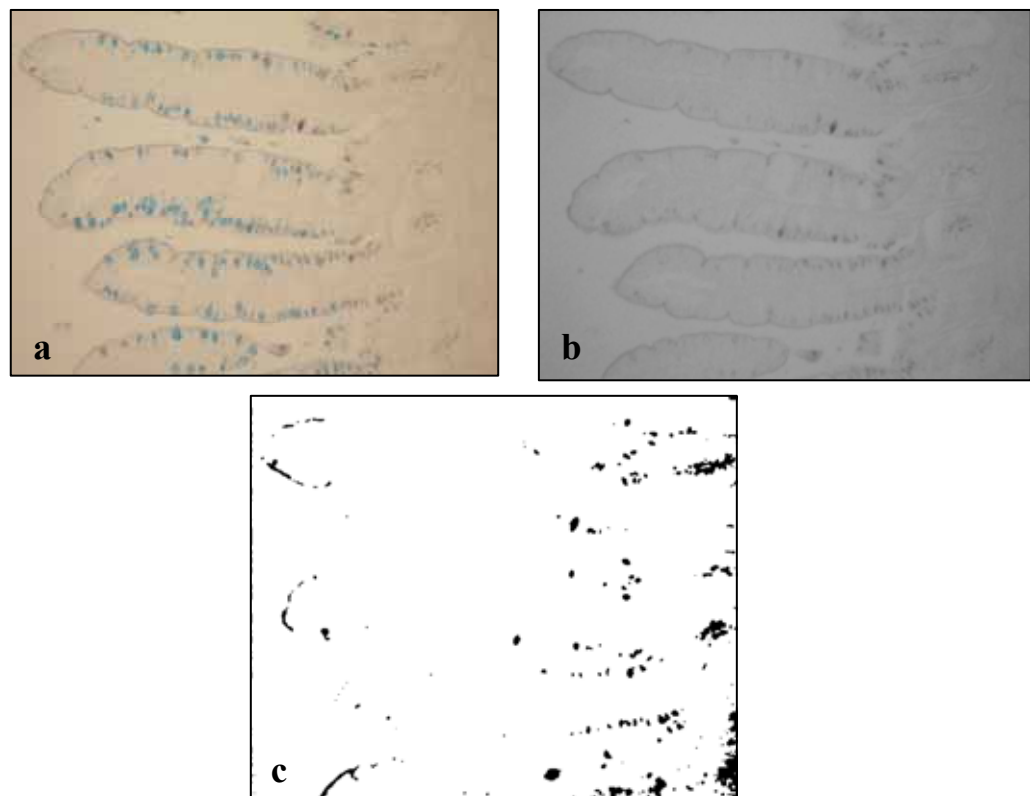


Figure 7.3. Stages of image analysis to highlight brown-stained goblet cells in HID/AB pH 2.5 ileal sections. Original Image (a), adjusted colour parameters to highlight goblet

cells of interest (b), converted binary image with goblet cells highlighted, ready for measurement (c).

“Colour/Histogram” converts to a binary image, boxes to detect black and immediate should be selected in the Histogram window. By clicking on the *“Auto”* button, the goblet cells were highlighted (Figure 7.3c), in some cases the threshold required adjustment depending on the image quality. In some sections, highlighting the goblet cells was difficult, and the specific threshold required tended to fill the entire image. Using the function *“Adapt”* allowed for a more concise representation of the goblet cells by removing unwanted “noise”. *“Binary/Store/And”* isolated the villus area measured previously, so that only goblet cells in that villus were measured. *“Measure/Field”* then calculates the area. By using *“Binary/Store/Initial”* the original villus area could be reviewed and morphometry parameters measured.

Appendix 3: Purification of mucin glycoprotein from gastrointestinal mucus

Solutions (Faure et al., 2002)

0.05 M Tris/HCl buffer, pH 7.5

6.06 g Tris powder, complete to 1 L with deionised water, adjust the pH with HCl 6M

100 mg/mL Flavourzyme solution

100 mg Flavourzyme, complete to 1 mL with 0.05M Tris/HCl buffer, pH 7.5

0.05 M Tris/HCl buffer containing 2M guanidinium, pH 7.5

191.06 g/L Guanidinium hydrochloride. Complete to 1 liter with 0.05M Tris/HCl buffer pH 7.5. The final pH does not need to be readjusted

100 mM Dithiothreitol (DTT) solution

154 mg DTT /10 mL Tris/HCl buffer 0.05M, pH 7.5

500 mM Iodoacetamide solution

925 mg/10 mL Tris/HCl buffer 0.05M, pH 7.5

Purification Procedure

Samples were kept on dry ice and a plate of glass placed on top. Samples were processed one sample at a time. Approximately 300mg of sample (still frozen) was cut with a scalpel on the glass plate. The tissue was directly weighted in a plastic tube and placed immediately in ice/water for homogenization. 2mL of 0.05M Tris/HCl buffer, pH 7.5 (cold buffer) was immediately added to the sample and homogenised at a low setting (6000 rpm), at 4°C, for 30 sec. The homogenate was kept on ice/water until the end of the homogenization procedure.

Partial digestion with Flavourzyme®

When all the samples were homogenised, Flavourzyme (Sigma, St-Louis, MO) was added to each sample (ratio: 10mg Flavourzyme per 300mg tissue, i.e.100µL of 100 mg/mL Flavourzyme solution per tube). The quantity of Flavourzyme must be adapted to the tissue weight.

After shaking briefly (vortex), the tubes were incubated in a water bath, at 37°C for 80 min, under agitation (120/min). During the incubation, another set of plastic tubes were prepared containing 0.8 g of guanidinium hydrochloride (final concentration of guanidinium hydrochloride = 4M). *The quantity of guanidinium hydrochloride must be adapted to the final volume of the reaction.*

The tubes were refreshed on ice/water for about 5 min to stop the enzymatic activity of Flavourzyme. The digested samples were emptied into the plastic tubes containing guanidinium hydrochloride and shook briefly (vortex).

Reduction with DTT

Homogenates were further solubilised under reducing conditions. A 100mM DTT solution was prepared (just before the end of incubation with Flavourzyme). After incubation, 350 µL of 100mM DTT was added per tube (final concentration of DTT = 10mM) and agitated briefly (vortex). The samples were incubated at room temperature for 2 h under agitation (160-180/min).

Alkylation with iodoacetamide

A 500mM iodoacetamide solution was prepared (just before the end of the reduction with DTT). 200µL of 500mM Iodoacetamide was added per tube (final concentration of Iodoacetamide = 25 mM) and shook briefly (vortex). Samples were further incubated at

room temperature for 2 h under agitation (160-180/ min). Samples were frozen at -20°C overnight or until use for further purification (maximum time tested = 4 days).

Sepharose CL-4B chromatography

Preparation before the day of the chromatography:

The supplied frit was placed into each of the columns (PD10 plastic columns, Sigma, St-Louis, MO) (Figure 7.4). A stopcock was attached to the bottom of the column and closed. A plastic 10mL reservoir was attached to the top of each column (Amersham Biosciences, Rydalmere, NSW). Sepharose CL-4B resin was washed as per instruction by Sigma protocol, and then washed 4 times in de-gassed 0.05M Tris/HCl buffer pH 7.5. The columns were packed with 10mL of resin under gravity by opening the valve and allowing buffer to flow through the column. When packed the valve was closed, allowing a cm of buffer to sit on top of the gel bed. For one sample, 2 columns were commonly required. Deionised water for dialysis (at 4°C) should be refreshed (4.5L cold deionised water for 64mL of sample).

The PD10 columns were washed with at least 25 mL of 0.05M Tris/HCl buffer containing 2M guanidinium. Samples were defrosted at room temperature. When the columns were washed, buffer was run into bed and flow stopped by closing the valve. Gently, 1 mL of sample per column was added, allowing the sample to run into the column. Without disturbing the gel bed, the sample was eluted by adding 0.05M Tris/HCl buffer pH 7.5 containing 2M guanidinium to the columns. Fractions (1 mL) were collected; with fractions 3 to 6 were stored on ice (mucin-containing fractions). Once the fractions were collected, the columns were washed with 0.05M Tris/HCl containing 2M guanidinium buffer until the resin recovered its initial white colour.



Figure 7.4. Equipment for sepharose CL-4B chromatography, (a) reservoir, (b) PD-10 column, (c) frit, (d) stopcock.

Dialysis (4°C)

Before dialysis, dialysis tubes were cut (wearing gloves) and washed in deionised water overnight (12000-14000 molecular weight cut-off, diameter 16mm). Fractions 3 to 6 of one sample were poured into a dialysis tube (2 chromatographies per sample, thus 4 mL to dialyse per sample). The samples were dialysed against deionised water for 48 h at 4°C (4.5 L cold deionised water for approximately 64 mL of sample). The water baths were changed twice each day, in the morning and in the evening. Samples were recovered in glass tubes and freeze-dried. Purified mucins were stored dried at -20°C until analysis.

SDS-Page: determination of mucin purity

The purity of all mucin samples were confirmed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-page), (Mini-PROTEAN® 3 cell system, Bio-Rad Laboratories Pty. Ltd., Reagents Park, NSW) to confirm the absence of contaminating proteins. Mucins were electrophoresed either through a pre-cast Tris-HCL 5% (100-250kD) gel or a Tris-HCL 7.5% (40-200kD) gel (Bio-Rad Laboratories Pty. Ltd., Reagents Park, NSW).

Freeze-dried mucin samples were resuspended in 0.2 mL of nuclease free water (NFW). 20 µL of 2x protein loading buffer (PLB) was added to 20 µL of sample and denatured at 95°C for 5 min. Electrophoresis was carried out in tris-glycine-saline (TGS) running buffer for 25 min at 200V.

Solutions

All solutions were prepared in accordance with Bio-Rad Laboratories recommendations.

TGS Running Buffer 5x

25 mM Tris Base	7.5g
192 mM Glycine	36g
0.1% SDS	25mL (10% w/v SDS)

Make up to 500mL with MilliQ H₂O

Stock solution of protein loading buffer (PLB)

0.5M Tris-HCL, pH 6.8

10% (w/v) SDS

80% (v/v) Glycerol

PLB/ Sample Buffer (2x)

62.5 mM Tris-HCL Buffer pH 6.8

25% Glycerol

2% SDS

5% 2-Mercaptoethanol

Make up to desired volume with MilliQ H₂O

Make up sample buffer without 2-Mercaptoethanol. Add last before adding to sample.

Sample loading sequence, wells 1-10

1. 15 μ L (10 μ L SeeBlue® Plus2 Pre-stained Protein Standard + 20 μ L PLB)
 2. 15 μ L Protein control, Bovine Serum Albumin (BSA) (Sigma, St-Louis, MO)
(Concentration 5 μ g/ μ L, dilution 2 μ L in 8 μ l NFW+ 20 μ l PLB)
 3. 15 μ L Mucin control porcine stomach mucin (Sigma, St-Louis, MO)
(Concentration 10 μ g/ μ L, dilution 1 μ L in 9 μ L NFW + 20 μ L PLB)
 4. 15 μ L mucin prep 1 + 2x PLB (10 +20 μ L in tube)
 5. 15 μ L mucin prep 2 + 2x PLB (10 +20 μ L in tube)
-
6. 15 μ L (10 μ L SeeBlue® Plus2 Pre-stained Protein Standard + 20 μ L PLB)
 7. 15 μ L Protein control Bovine Serum Albumin (BSA)
(Concentration 5 μ g/ μ L, dilution 2 μ l in 8 μ l NFW+ 20 μ L PLB)
 8. 15 μ L Mucin control porcine stomach mucin
Concentration 10 μ g/ μ l, dilution 1 μ L in 9 μ L NFW + 20 μ L PLB
 9. 15 μ L mucin prep 1 + 2x PLB (10 +20 μ L in tube)
 10. 15 μ L mucin prep 2 + 2x PLB (10 +20 μ L in tube)

After electrophoresis, the gel was cut in half and one side stained with Periodic acid-Schiff (mucin) and the other with Commassie Blue (protein)

Commassie blue stain for detection of protein (500mL)

- | | |
|-------|--|
| 0.5g | Commassie Brilliant Blue (Sigma, St-Louis, MO) |
| 250mL | Methanol |
| 50mL | Glacial acetic acid |
| 200mL | MilliQ water |

Stain for 1h at room temperature and then destain in a container of boiling MilliQ water, with an equal size piece of woollen fabric to absorb excess Commassie stain, in a microwave, until gel is clear.

PAS Staining for polyacrylamide gels (Konat *et al.*, 1984)

Fixative solution for PAS staining

40%	Ethanol
5%	Glacial acetic acid
55%	Distilled H ₂ O

0.2% Sodium Metabisulfite

0.4mg	Sodium Metabisulphite
200mL	5% acetic acid

0.2% Potassium Metabisulfite

0.4g	Potassium Metabisulfite
200mL	40% ethanol-5% acetic acid solution (same as fixative solution)

Fix gel overnight. Stain with periodic acid solution (Sigma, St-Louis, MO) for 2-3 h. Treat with 0.2% sodium metabisulfite for 2-3 h, with one solution change after 30 min. Incubate in Schiff's reagent (Sigma, St-Louis, MO) for 18 h at room temperature. Further incubate in 0.2% Potassium metabisulfite solution for 90 min at 55°C. De-stain gel in 40% ethanol-5% acetic acid solution. Gels photographed using Gel Doc.

Alkali/borohydride treatment of glycoprotein (Piller & Piller, 1993)

Solutions

0.1M NaOH solution

4g NaOH

1L Distilled water

To make up 10mL of 2M NaBH₄

0.76g NaBH₄

10mL 0.1M NaOH

0.25M acetic acid in Methanol

To make up 500mL

7.15mL Glacial acetic acid

492.85mL Methanol

Procedure

Dissolve the sample in distilled water to a concentration of 25 mg/mL. Add equal volume of freshly prepared 2M NaBH₄ in 0.1M NaOH, mix the solution rapidly and incubate 16 h at 45°C. Leave the tube caps loose. Cool the vial to room temperature and destroy the excess NaBH₄ in fume hood, with addition of 5 volumes of 0.25M acetic acid in methanol, do this drop by drop as the reaction can be violent. Remove the formed boric acid by evaporation of solution under a stream of nitrogen and repeat the procedure twice with the same amount of acidified methanol and twice with methanol alone.

Purification of mucin oligosaccharides⁷

The released oligosaccharides were purified from proteins by gel filtration on a Sephadex G-50 superfine packed column (1.5 x 100 cm) and desalted on a Bio-gel P2 fine gel packed column (1 x 20 cm) (Figure 7.5a). The amount of gel needed for preparation is determined by hydrated bed volume specific for each gel, this should be given by the manufacturer. For Sephadex G-50, it is 9-11 mL/g, for Bio-Gel P-2 it is 3 mL/g. 1.5 x the bed volume is required for a correctly packed column. The bed volume can be calculated using the following equation:

$$\text{Volume of the column} = \pi^2 \times h$$

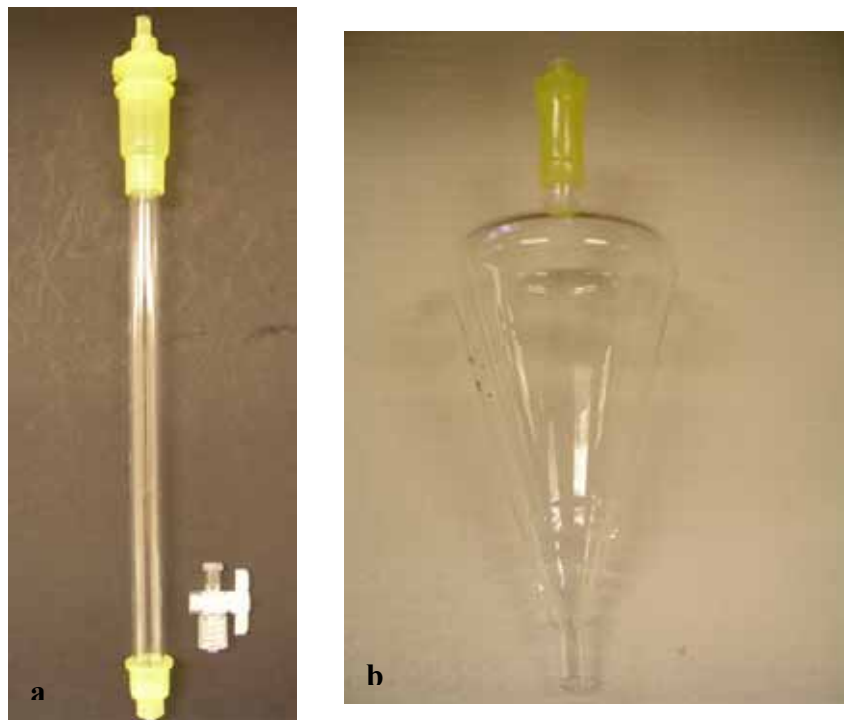


Figure 7.5. Bio-Rad Glass Eco-column with stopcock (a) and glass 500mL reservoir (b).

⁷ Determining the volume of the column and flow rate was taken from “Gel Filtration - principles and methods” Amersham Biosciences

Preparing the gel (as per Bio-Rad manual protocol⁸)

Gradually add the dry gel media to the buffer solution designated for the chromatography. Use twice as much buffer as the expected packed cell volume. Allow the gel to hydrate for at least 4 h at room temperature or 1 h in a 100°C water bath. After hydration, decant off half of the buffer, transfer to a filter flask and degas under vacuum for 5-10 min. Swirl the flask occasionally. Add two bed volumes of degassed buffer and swirl gently. Allow the gel to settle and remove supernatant by suction to remove the fine particles. Repeat 4 times to remove 90% of the fines.

Packing the column (as per Bio-Rad manual protocol)

Clean the column firstly with 20% ethanol and then distilled water. Attach a stop-cock to the bottom of the column and ensure it is closed. Add degassed buffer so that 20% of the column is filled. The slurry volume is usually greater than the volume of the column, so a 500 mL glass packing reservoir is attached to the top of the column (Bio-Rad Laboratories Pty. Ltd., Reagents Park, NSW; Figure 7.5b). Pour the slurry into the column in a single motion, to ensure even packing and avoid trapping air bubbles. When a 2-5 cm bed is formed, gently fill the reservoir with de-gassed buffer until full. Run degassed buffer through the pump at the designated flow rate to remove any air bubbles. Attach the pump connections to the reservoir meniscus to meniscus. Open the stop-cock valve and allow column to flow until packed. When the column is packed, close the valve and remove the reservoir and any excess gel. Reconnect to the pump (meniscus to meniscus) and pass 2 bed volumes of buffer through the column.

⁸ Manuals are supplied with purchase of gel media

The flow rate is dependant on the type of gel used and the column size. Recommended flow rate for Sephadex is 2-5 cm/h and Bio-Gel P2 is 5-10 cm/h.⁹ cm/h can be converted to mL/h using the flowing equation:

$$Y/60 \times (\pi \times d^2)/4 = \text{flow rate (mL/h)}$$

Where

Y= linear flow (cm/h)

d = column internal diameter in cm

Load sample without disturbing the bed. Fill the top of the column with buffer and reconnect to the pump ensuring there are no air bubbles. Collect fractions.

⁹ Bio-Gel P2 packed columns can be packed and run under gravity as long as the water volume is monitored to ensure the gel bed does not dry out. A plastic funnel can be used instead of the glass reservoir for packing as less buffer volume is required.

Appendix 4: Solutions for bacterial binding assays

Stock solution for flurochrome

1mg fluorescein isothiocyanate (FITC)

1mL dimethyl sulfoxide (DSMO)

Dissolve FITC in DSMO

0.1M Sodium carbonate buffer pH 9.0

To make 1M solution

10.8g Na_2CO_3

80mL distilled water (dH_2O)

Dissolve Na_2CO_3 in dH_2O , use magnetic stirrer. Adjust pH with concentrated HCl

Bring volume up to 100 mL with dH_2O . To make a 0.1M solution, dilute 1 in 10.

0.1M Na_2CO_3 buffer containing 0.9% w/v NaCl

To make up 100 mL

0.9g NaCl

100mL 0.1M Na_2CO_3 buffer

Dissolve NaCl in buffer. Before use, flurochrome solution is added to buffer at a concentration of 75 $\mu\text{g}/\text{mL}$. Therefore for 5 mL solution add 365 μL of FITC stock solution

0.1M PBS pH 7.1¹⁰

0.2M Monobasic stock

13.9g sodium phosphate monobasic

500mL dH₂O

0.2M Dibasic stock

28.4g sodium phosphate dibasic (anhydrous form)

1L dH₂O

0.1M phosphate buffer solution

To make 600 mL

99mL 0.2M monobasic stock

201mL 0.2M dibasic stock

300mL milliQ H₂O

Check pH, adjust with 1N NaOH

0.1M PBS pH 7.1

To make 500 mL

4g NaCl

500mL 0.1M phosphate buffer solution

Check pH and adjust with 1N NaOH

¹⁰ PBS and distilled water used for solutions were filtered before use, using bell filters (Millipore)

3.5% w/v paraformaldehyde in PBS

To make 100 mL

3.5g paraformaldehyde

100mL 1x PBS pH 7.1

Dissolve paraformaldehyde in ~80 mL PBS using a heat plate and magnetic stirrer, check pH and adjust with 1N NaOH. Bring to 100 mL with 1 x PBS pH 7.2

1x PBS pH 7.1 with 0.05% v/v Tween 20

To make 100 mL

50 µL Tween 20 (Sigma, St. Louis, Mo.)

99.5 mL 1x PBS pH 7.1

Measure out 100 mL PBS, remove 50 µL, then add Tween 20

1x PBS pH 7.1 with 0.01% v/v Tween 20 and 1% w/v BSA

To make 100mL

1g BSA

10µL Tween 20

99.9mL 1x PBS pH 7.1

Measure 100 mL of PBS remove 10 uL, dissolve BSA in PBS and then add Tween

Preparation of stock solution of WGA

To make 1mL

50µg WGA

1mL 1 x Dulbecco's PBS solution (Sigma, St. Louis, Mo.)

Dissolve WGA in PBS solution

Anti-fade mountant pH 7.0

200mg	p-phenylenediamine.2HCl
20mL	PBS pH 7.1
180mL	Glycerol

Dissolve p-phenylenediamine.2HCl in PBS. Adjust pH to 7.0 using saturated NaOH Add to glycerol, mix and check the pH. Store at -20°C in the dark.

Methylene blue staining

0.3g	Methylene blue
100mL	Distilled water

Dissolve the dye in water, filter using 540 Whatman filter paper.

Air-dry slides, and fix bacteria by passing through flame 3-4 times. Stain for one minute by coating slides in solution. Wash with water, let dry and examine under oil immersion.

Microscopy

Using VideoPro[®] (Version 6.210, Leading Edge Pty Ltd., Australia), fluorescent bacteria were able to be highlighted and total area measured (total area of bound bacterial/mm² of ileal tissue). Steps are provided;

“Image/Background /Options /Radius (100)/Tile (4)”; dark background selected

“Background/Create”

“Background/Correct”

“Filter/ Process/Sharpen”

“Colour/Process/Luminance”

“Colour/Histogram/Detect”

“Measure/Field”

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