The interaction of probiotic bacteria and an oligosaccharide-enriched fraction from goat whey on *in vitro* intestinal barrier function and mucin production

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

Massey University

Manawatu, New Zealand

Alicia Barnett

2014

Abstract

Multiple interactions occur in the human large intestine between the host, the intestinal microbiota and fermentable carbohydrates which transit relatively intact through the small intestine. A major site at which many of these interactions occur is the intestinal epithelium, which is formed from a single layer of epithelial cells. The cellular composition of the epithelial layer in the human small and large intestine varies in respect to the numbers of absorptive enterocytes and mucus-secreting goblet cells. For the human intestine the proportion of goblet cells among epithelial cell types is thought to increase from the duodenum (4%) to the distal colon (16-24%).

Epithelial cell co-culture models were developed containing absorptive enterocytes (Caco-2 cells) and mucus-secreting goblet cells (HT29-MTX cells) that more closely simulate the cell proportions found in the small (90:10) and large intestine (75:25). Trans-epithelial electrical resistance (TEER) of the co-cultures was more similar to reported values of *ex vivo* intestinal tissue of human small and large intestine than either of the two mono-cultures. Additionally, the mucus layer thickness present at the apical surface of 75:25 co-cultures (cellular composition representative of the large intestine) was similar to the reported thickness of the inner mucus layer of human large intestine. Introduction of an oligosaccharide-enriched fraction (OEF) from goat whey to the epithelial co-culture models was shown to modulate barrier integrity as measured by TEER, in a dose-dependent manner. Oligosaccharides (1 mg/mL) increased TEER and mucin gene/protein expression of epithelial co-cultures. Finally, the interaction between probiotic bacteria and the OEF and their individual or combined effects on intestinal epithelial barrier integrity and mucin gene/protein expression was investigated.

The OEF supported the growth of selected probiotic strains, and enhanced the adhesion of defined strains to the epithelial co-cultures. When in combination with the OEF, *Lactobacillus plantarum* 299v enhanced TEER and mucin gene/protein expression, the increase of which was

greater than that for either component alone. This suggests that an interaction between *Lactobacillus plantarum* 299v and the OEF exists which enhances barrier integrity through increased TEER and mucin gene/protein expression, all of which are essential components of the intestinal barrier.

The research presented in this dissertation has indicated that *in vitro* epithelial co-cultures can be used as a model to improve our understanding of the mechanisms through which probiotic bacteria/food components and intestinal epithelial cells interact, and these key findings will assist in the development of strategies to improve intestinal barrier function using novel dietary components.

Acknowledgements

There are many people with whom I owe my gratitude for their guidance, support and encouragement. I would like to begin by thanking my supervisors: Prof Warren McNabb, Dr Nicole Roy and Dr Adrian Cookson for always being available to answer my questions and for your unwavering support. I also acknowledge AgResearch and the Riddet Institute, a Centre of Research Excellence, for funding this research and providing me with excellent research facilities.

A big thanks goes out to Dr Mark McCann and Kelly Armstrong. Although I thank you both for your technical assistance relating to specific aspects such as cell culture and qPCR, I offer you greater thanks for your unfailing support, guidance and friendship.

I would also like to thank my many colleagues in AgResearch, especially the PhD students I shared an office with during the course of this project, for their friendship, support and help over the past few years. Thank you to Dr Amy Van Wey Lovatt for listening and encouraging me through the triumphs and troubles, especially in the writing stage of this study, and providing valuable and entertaining discussions on matter related (or not) to my research. Thank you also to Denise Martin, whose help has been invaluable in carrying out many of the tasks and activities along the road to completing this thesis.

To Louise Searle and Malcolm Powell, even though you are no longer with us, I dedicate my undying thanks. You believed in my abilities before I had even considered them. You were always in my thoughts and you gave me the courage to keep on going, because you always believed that that eureka moment was just around the corner.

Most of all, I would like to thank my husband, Alan Barnett. For you there is the most “mahusive” thanks for your constant encouragement, understanding and love. I promise from now on I will try even harder to make the time to beat you at golf.

Table of contents

List of figures

List of tables

List of abbreviations

Abs	**Absorbance**
ADAM10	**A disintegrin and metalloproteinase 10**
A-GM1	**Asialo-GM1**
AI-2	**Autoinducer-2**
ANOVA	**Analysis of variance**
ATCC	**American Type Culture Collection**
ATP	**Adenosine triphosphate**
BSA	**Bovine serum albumin**
cDNA	**Complimentary DNA**
CFU (cfu)	**Colony forming unit**
CK	**Cysteine knot**
CL	**Cell lysate**
DMEM	**Dulbecco's modified eagle's medium**
DMSO	**Dimethylsulfoxide**
DNA	**Deoxyribonucleic acid**
DP	**Degree of polymerisation**
DSM	**Deutsche Sammlung von Mikrooganismen (German Collection of Microorganisms and Cell Cultures)**
DSS	**Dextran-sodium-sulphate**
EDTA	**Ethylenediamine tetra-acetic acid**
EGF	**Epidermal Growth Factor**
EGFR	**Epidermal Growth Factor Receptor**
EPEC	**Enteropathogenic *Escherichia coli***
ERK	**Extracellular regulated kinase**
FBS	**Foetal bovine serum**

FOS	**Fructo-oligosaccharide**
G	**G protein**
GlcNAc	***N*-acetylglucosamine**
GOS	**Galacto-oligosaccharide**
GPCR	**G-protein-coupled-receptor**
HBEFG	**Heparin-binding epidermal growth factor**
HMO	**Human milk oligosaccharide**
IBD	**Inflammatory bowel disease**
Ig	**Immunoglobulin**
IL	**Interleukin**
LPS	**Lipopolysaccharides**
LTA	**Lipoteichoic acids**
M199	**Medium 199**
MAD	**Median absolute deviation**
MAPK	**Mitogen activated protein kinases**
MAPKK	**MAPK kinase**
MAPKK K	**MAPK kinase kinase**
MEK1	**MAPK ERK Kinase**
mL	**Millilitre**
MTX	**Methotrexate**
***MUC*/MUC**	**Mucin (gene/protein)**
mRNA	**Messenger RNA**
MRS	**de Man, Rogosa and Sharpe**
Neu5Ac	***N*-acetyl-neuraminic acid**
Neu5Gc	***N*-glycolylneuraminic acid**
NF-κB	**Nuclear factor kappa B**

OD	**Optical density**
OEF	**Oligosaccharide-enriched fraction**
OF	**Oligo-fructose**
PAS	**Periodic acid Schiff**
PBS	**Phosphate buffered saline**
PCR	**Polymerase chain reaction**
PET	**Polyester (Transwell cell culture inserts)**
PKC	**Protein kinase C**
PLC	**Phospholipase C**
qPCR	**quantitative real time PCR**
RNA	**Ribonucleic acid**
ROS	**Reactive oxygen species**
SCFAs	**Short chain fatty acids**
SEA	**Sea urchin sperm protein, Enterokinase, and Agrin**
SEM	**Standard error of the mean**
SM	**Spent media**
STP	**Serine, threonine and proline**
TEER	**Transepithelial electrical resistance**
TGF-α	**Transforming Growth Factor-α**
TLR4	**Toll-like receptor 4**
TMB	**Tetramethylbenzidine solution**
TNF	**Tumour necrosis factor**
TNBS	**Trinitrobenzenesulphonic acid**
µm	**Micrometre (micron)**
VNTR	**Variable number tandem repeat**
vWF	**von Willebrand Factor**

Introduction

The various surfaces of the adult human body are home to an estimated 100 trillion microorganisms, which are thought to outnumber the cells of their host by 10-fold [1]. The largest body surface in contact with the external environment (200 to 300 m^2) is that of the gastrointestinal tract, which includes the oesophagus, stomach, small intestine and large intestine [2]. The gastrointestinal tract acts as a barrier between the external environment and the internal milieu. The intestinal barrier function consists of multiple defence mechanisms which can basically be subdivided into a physical and an immunological barrier [3]. The physical barrier is composed of a lining of epithelial cells, connected by tight junctions which seal the paracellular pathway, preventing exposure of the internal milieu to potentially harmful intraluminal microbiota and microbial products. The physical barrier is further reinforced by the presence of a mucus layer produced and secreted by goblet cells.

Mucins, high molecular weight glycoproteins, are a major structural component of mucus, and when secreted from goblet cells, form a viscous gel-like structure covering the epithelial surface. The mucus layer provides protection, both chemically and physically, and contributes to the elimination of the intestinal contents [4]. The mucus layer is also the first point of contact between the host and the intestinal bacteria whether they are commensal, transient or pathogenic [5] and provides adhesion sites for bacterial colonisation.

Bacterial colonisation of the gastrointestinal tract begins immediately after birth and is influenced by extrinsic factors such as the mode of delivery of the new-born, any environmental contamination, and the sanitary conditions during the birthing process [6], but can also vary as a consequence of whether the infant is breast-fed or formula-fed [7]. Studies of the intestinal microbiota of breast-fed and formula-fed infants have reported the microbial diversity to be different within the first few weeks of life, even though total bacterial numbers were similar for each group [8]. In breast-fed infants up to 90% of the intestinal microbiota were represented by bifidobacteria and lactobacilli species, whereas in formula-fed infants

these two genera represented only 40 to 60% [6]. Colonisation occurs through specific attachment of bacterial surface proteins (lectins) to complementary oligosaccharides of the mucosa or mucus layer and to the surface of food components, or non-specifically through low affinity hydrophilic and/or hydrophobic interactions with mucins [9-14].

Bacterial adherence to the mucus layer/mucosal surface and their secreted products, such as *N*-(3-oxododecanoyl) homoserine lactone [15], alter mucin gene and protein expression through activation of different signalling cascades and secretory elements resulting in changes to the mucus layer [16-19]. These changes may affect not only the thickness of the mucus layer but also the composition of the mucins themselves through increased acidification [20, 21].

Generally a symbiotic relationship exists between bacteria and host, which can be viewed as mutualistic-commensalism [22]. There is an increasing gradient of endogenous microbiota throughout the intestine which increases from stomach; approximately 100 bacterial cells per mL of lumen contents; to the colon, 10^{11} to 10^{12} bacterial cells per mL of luminal contents [23, 24]. Although the isolation and function of many bacteria from the intestine has yet to be determined, it is known that some species of bacteria colonise the large intestine in preference to other gastrointestinal tract compartments, such as the small intestine. Of these, bifidobacteria and lactobacilli are normally present in the large intestine of healthy humans in numbers ranging from 10^8 to 10^{10} cfu/mL and 10^6 to 10^8 cfu/mL, respectively [25, 26]. These bacterial species survive in the large intestine, through their ability to degrade and utilise mono and disaccharides derived from a diverse range of carbohydrates, using various exo- and endoglycosidases [27].

The introduction of readily fermentable components (starch, non-starch polysaccharides, and/or non-digestible oligosaccharides) in the diet is known to selectively stimulate the growth of specific strains of bacteria in the intestine [28], and as a consequence alter the end products of bacterial fermentation. The production of SCFAs, such as butyrate are known to modulate mucin gene expression, [29, 30] and also intestinal epithelial barrier permeability [31].

However, the relationship between dietary components, the intestinal bacterial community and host function remains to be fully understood. Efforts to further understand the interactions of bacteria, oligosaccharides, epithelial barrier integrity and mucus production in the small and large intestine will have a major impact on the potential health promoting effects oligosaccharides exert on host function and interactions with bacteria.

Chapter 1: Review of literature†

† Selected material from this section was published as a critical review in the Journal Food and Function: Barnett AM, Roy NC, McNabb WC & Cookson AL. The interactions between endogenous bacteria, dietary components and the mucus layer of the large bowel. Food & Function; 2012, 3 (7): p. 690 – 699.

1.1 The human intestinal epithelium

The human intestine is divided anatomically into two major subdivisions, the small and the large intestine. The small intestine, about 2 to 4 m in length in (living) humans, is further subdivided into three regions: (1) the proximal duodenum; (2) the middle jejunum; and (3) the distal ileum. The mucosa of the small intestine has large circular folds called plicae circulares and smaller, finger-like projections, called villi, which together provide an increased surface area for absorption. The main functions of the small intestine are digestion, absorption of nutrients and production of gastrointestinal hormones [32].

The large intestine, about 2 m long, is approximately twice the diameter of the small intestine and includes four anatomically defined regions: the caecum, appendix, colon, and rectum. The colon is regionally divided into ascending, transverse, descending, and sigmoid areas. The large intestine has crypts which invaginate deep into the submucosa, but unlike the small intestine there are no villi present (Figure 1-1). Functionally, the large intestine completes the absorption process, and retrieves water and sodium from the luminal contents which become faecal residue. Large amounts of mucus, and some hormones, are secreted but no digestive enzymes [32].

The intestinal epithelium consists of a single cell layer separating the external luminal environment, which harbours bacteria some of which are pathogenic, from the sterile internal milieu. The epithelial cells originate from multi-potent stem cells present in the base of the many crypts that undergo proliferation and differentiation as they migrate to the intestinal surface [33, 34] (Figure 1-2). Four main differentiated epithelial cell types; Paneth cells, enteroendocrine, absorptive enterocytes and goblet cells; make up the epithelium [4, 34]. Epithelial cells are normally replaced every 2 to 5 days with the exception of Paneth cells which have a life-span of approximately 20 days [34]. Paneth cells reside at the bottom-most positions of the crypts of the small intestine and ascending colon and secrete antimicrobial

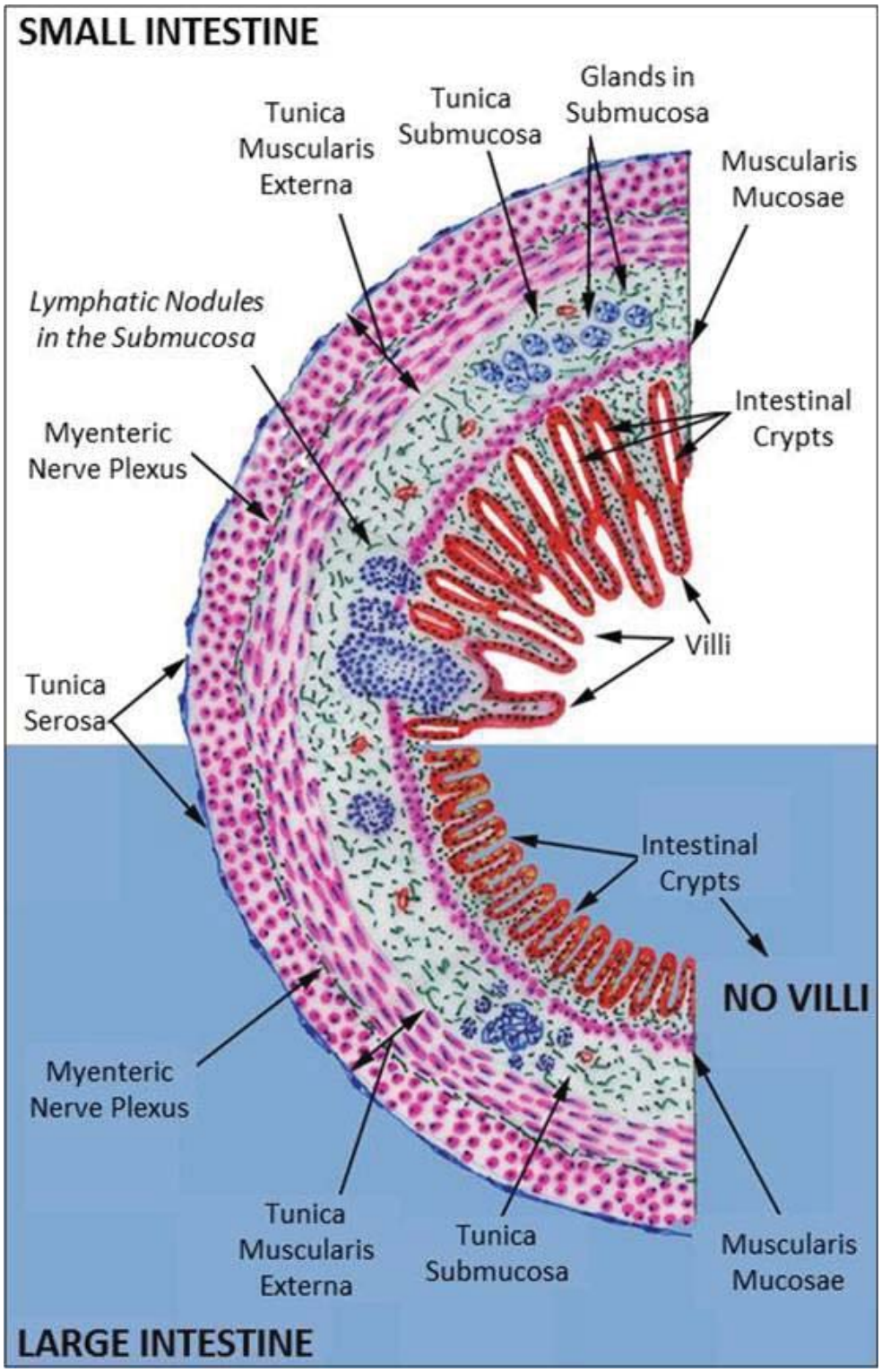


Figure 1-1 The two major subdivisions of the intestine highlighting their similarities and their differences [35]

The most visible and significant difference is the presence of villi in the small intestine, and their absence in the large intestine. The villi are a means to enhance the absorptive surface in the small intestine, but would be a hindrance to movement of the semi-solid faecal mass in the large intestine. The deep intestinal crypts are where new epithelial cells are generated by mitosis.

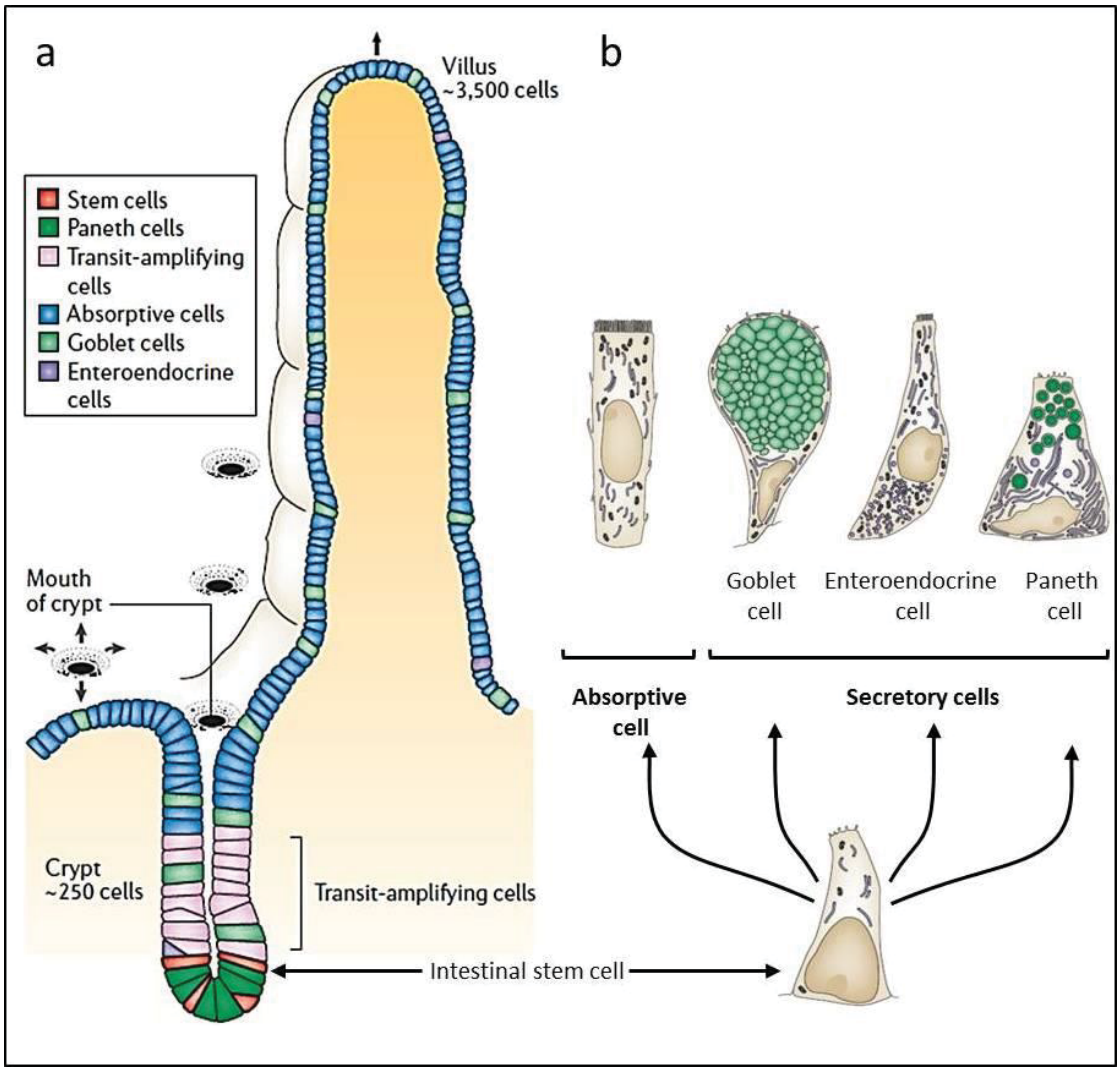


Figure 1-2 The distribution of epithelial cell types in the mammalian intestine [36]

a) A villus of the small intestine with one of the crypts that contribute to renewal of its epithelium. Stem cells lie near the crypt base, above which are transit-amplifying cells, and above these in the neck of the crypt and on the villus, lie post-mitotic differentiated cells (absorptive cells, goblet cells, and enteroendocrine cells). In the colon there are no villi, but the organisation is otherwise similar; cells are discarded into the intestinal lumen after they emerge onto the exposed flat surfaces around the mouths of the crypts.

b) Differentiated epithelial cell types of the intestinal epithelium. There are four classes of terminally differentiated cells. Absorptive cells have a brush border (a dense array of microvilli) on their apical surface. The other three classes are all secretory: goblet cells secrete mucus, and their apical cytoplasm is generally distended with mucus-filled secretory granules; enteroendocrine cells (of which there are many subtypes) are smaller and secrete various hormones; and Paneth cells secrete antibacterial proteins (lysozyme and defensins). Paneth cells differ from the other differentiated cell types in that they lie at the bottoms of the crypts and seem to be absent in the mammalian large intestine.

agents (defensins) and lysozyme, which play an essential role in the control of the microbial environment of the small intestine [32, 33, 37-39]. Enteroendocrine cells, represent less than 1% of epithelial cells, and regulate the activity of the digestive system by producing a variety of hormones including serotonin, substance P, and secretin [32]. Absorptive cells are the most abundant epithelial cell type in the intestinal epithelium. These are polarised cells that have an apical brush border [40], and regulate nutrient and fluid uptake and express selective enzymes and transporter proteins [41]. Goblet cells are specialised epithelial cells [33], the basal regions of which are narrow and include a large vesicular nucleus lying adjacent to the basal membrane. The apical region contains many membrane-bound secretory vesicles separate from other cytoplasmic constituents [20, 42]. Immature goblet cells formed at the base of the crypts, are poorly differentiated cells with few mucus vesicles.

Maturation of cells, during migration from their basal origins, results in increased numbers of mucus vesicles being acquired. The theca (intermediate filaments) and other cytoskeletal components become highly organised, to allow separation of the mucus granules from the cytoplasm below the apical membrane and give rise to their characteristic goblet-like shape. Mature cells which have undergone apoptosis are sloughed off into the lumen, the whole migration process taking just 2-5 days in humans, mice and rats [4, 34, 43-45]. Goblet cells secrete mucins into the lumen of the intestine to trap and expel micro-organisms and to lubricate the luminal surface [33]. The proportion of goblet cells among epithelial cell types increases from the duodenum (4%) to the distal colon (16-24%) [4, 33, 34, 46].

1.2 The intestinal epithelial barrier

The physical component of the intestinal barrier is the epithelium and an important component of the epithelium is the intercellular junctional complex, consisting of tight junctions, adherens junctions, desmosomes and gap junctions [47]. Tight junctions are anchored in the cell via the filamentous actin cytoskeleton [48]. Zonula occludens proteins are important intracellular tight junction proteins linking the cell cytoskeleton to the

transmembrane tight junction proteins: claudins, occluden and junctional adhesion molecules (JAM). Whereas occluden and JAM have a regulatory role, claudins are transmembrane proteins mainly responsible for the intestinal barrier function [3]. Tight junctions regulate the entry of luminal nutrients, ions and water while restricting pathogen entry and thus regulate the barrier function of the epithelium [49] . The physical barrier is reinforced by the presence of a mucus layer.

1.3 Mucins and the mucus layer(s)

1.3.1 Types of mucins

Mucus, which is secreted by goblet cells, is a thick gelatinous fluid that protects most surfaces of the body constituted by living epithelial cells (e.g. nose, lungs, intestines, digestive system). Mucus provides a dynamic semipermeable barrier that enables the exchange of nutrients, water, and gases while remaining impermeable to most bacteria [50]. Mucins are high-molecular weight glycoproteins that are major structural components of mucus, along with other secretory products including trefoil peptides, resistin-like molecule β and immunoglobulins [51-53]. Currently, 18 human mucin-type glycoproteins have been assigned to the *MUC* gene family which code for the protein cores of mucins as identified by the Human Genome Organisation Gene Nomenclature Committee (Table 1-1) [54, 55]. Although mucins are classified into two types, membrane-bound and secreted (secreted mucins are further sub-divided into gel forming and non-gel forming), a structural feature common to both mucin types is the variable number tandem repeat (VNTR) domains [4, 56, 57]. These domains are rich in serine, threonine and proline (STP) residues that are linked to a variety of *O*-linked oligosaccharide side chains, that make up 70% of the weight of the molecule [4]. The specific sequence and number of tandem repeats is highly variable between different mucins, but is unique for each mucin and their repetitive nature provides numerous sites for *O*-glycosylation with mucins typically containing 5 to 100 potential glycosylation sites per repeat [56-58] .

Table 1-1 Known human mucin genes, their functions and tissue expression [55].

Mucin gene	Type	Function	Tissue expression
MUC1	Membrane	Cellular signal transduction, barrier activity	Lung, cornea, salivary glands, oesophagus, stomach, pancreas, caecum, colon, breast, prostate, ovary, kidney, uterus, cervix
MUC3A	Membrane	Epithelial cell protection, adhesion modulation and cell signalling	Thymus, small intestine, colon, kidney
MUC3B	Membrane	Possibly cellular signal transduction	Small intestine, colon
MUC4	Membrane	Involved in intestinal epithelial cell differentiation, renewal and lubrication	Lung, cornea, salivary glands, oesophagus, small intestine, colon, kidney
MUC12	Membrane	Involved in epithelial cell regulation	Lung, middle ear, thymus, small intestine, pancreas, colon, liver, kidney, uterus, prostate
MUC13	Membrane	Epithelial barrier function	Lung, conjunctiva, stomach, small intestine, colon, kidney
MUC15	Membrane	Epithelial barrier function	Conjunctiva, tonsils, thymus, lymph node, breast, small intestine, colon, liver, spleen, prostate, ovary, leukocytes, bone marrow
MUC16	Membrane	Role in ovarian cancer	Conjunctiva, ovary
MUC17	Membrane	Extracellular matrix constituent	Intestinal cells, conjunctival epithelium
MUC20	Membrane	Cellular signal transduction	Lung, liver, kidney, colon, placenta, prostate
MUC21	Membrane	Mediates cell adhesion	Lung, large intestine, thymus, testis, colon
MUC2	Secreted	Primary extracellular matrix constituent in colon, lubricant activity	Lung, conjunctiva, ear, stomach, small intestine, colon, nasopharynx, prostate
MUC5AC	Secreted	Involved in intestinal epithelial renewal, primary component of airway mucus	Lung, conjunctiva, middle ear, stomach, gall bladder, nasopharynx
MUC5B	Secreted	Lubrication	Lung, middle ear, sublingual gland, larynx, submucosal glands, oesophageal glands, stomach, duodenum, gall bladder, nasopharynx
MUC6	Secreted	Involved in renal morphogenesis processes	Stomach, duodenum, gall bladder, pancreas, kidney
MUC7	Secreted	Facilitating clearance of oral bacteria	Lung, lachrymal glands, salivary glands, nose
MUC8	Secreted	Unknown	Oviduct
MUC19	Secreted	Major gel forming mucin of middle ear	Lung, salivary gland, kidney, liver, colon, placenta, prostate

O-linked side chains of STP residues within the VNTR domains have three structural regions (i) core, (ii) backbone and (iii) peripheral regions (Figure 1-3). The core region is created by the addition of alpha-*N*-acetylgalactosamine (α-GalNAc) to STP residues. The backbone regions consist of alternating D-galactose and N-acetyl-D-glucosamine residues in β-1-3 (type 1) or β-1-4 (type 2) linkages in either branched or linear forms [59]. Peripheral regions further characterise the mucin by conferring specific charge (negative) and antigenic (blood group and Lewis antigens) properties with the addition of sialic acid or sulfate esters. Linkage of sialic acid residues is via α-2-3 and α-2-6 linkages in most oligosaccharides, while di-, tri- or polysialyl linkages occur via α-2-8 bonds [59].

The presence of poly-*O*-acetylated sialic acid increases hydrophobicity and resistance to degradation by bacterial enzymes in the colon, and accounts for the poly-anionic nature of mucins at neutral pH [5, 59]. Although acidic mucin oligosaccharide chains are categorised as either sialomucin or sulfomucin, it should be noted that sialic acid and sulfate acid groups may be present in both types, but it is the overall predominance of one of these acid groups that determines the final categorisation [60]. Mucins which have neither sulfate nor sialic acid sugar residues, but instead have glucose, galactose, mannose or fucose, are termed as neutral mucins. The biological importance of the different oligosaccharide side chains of mucins can be related to their role in: i) the transportation of water and electrolytes; ii) barrier function; iii) maintaining a balance between the host/pathogenic and host/symbiotic bacterial interactions by providing attachment sites for different types of bacteria; and iv) maintaining the viscoelastic properties of the mucus layer [52, 61]. In the colon, sulfated mucins predominate creating a strongly negative surface with increased rigidity [62]. Any changes in mucin glycosylation as a result of diseases such as Inflammatory Bowel Disease or cancer can alter barrier function by decreasing sulfated residues and increasing sialic acid residues, leading to decreased viscosity [62, 63].

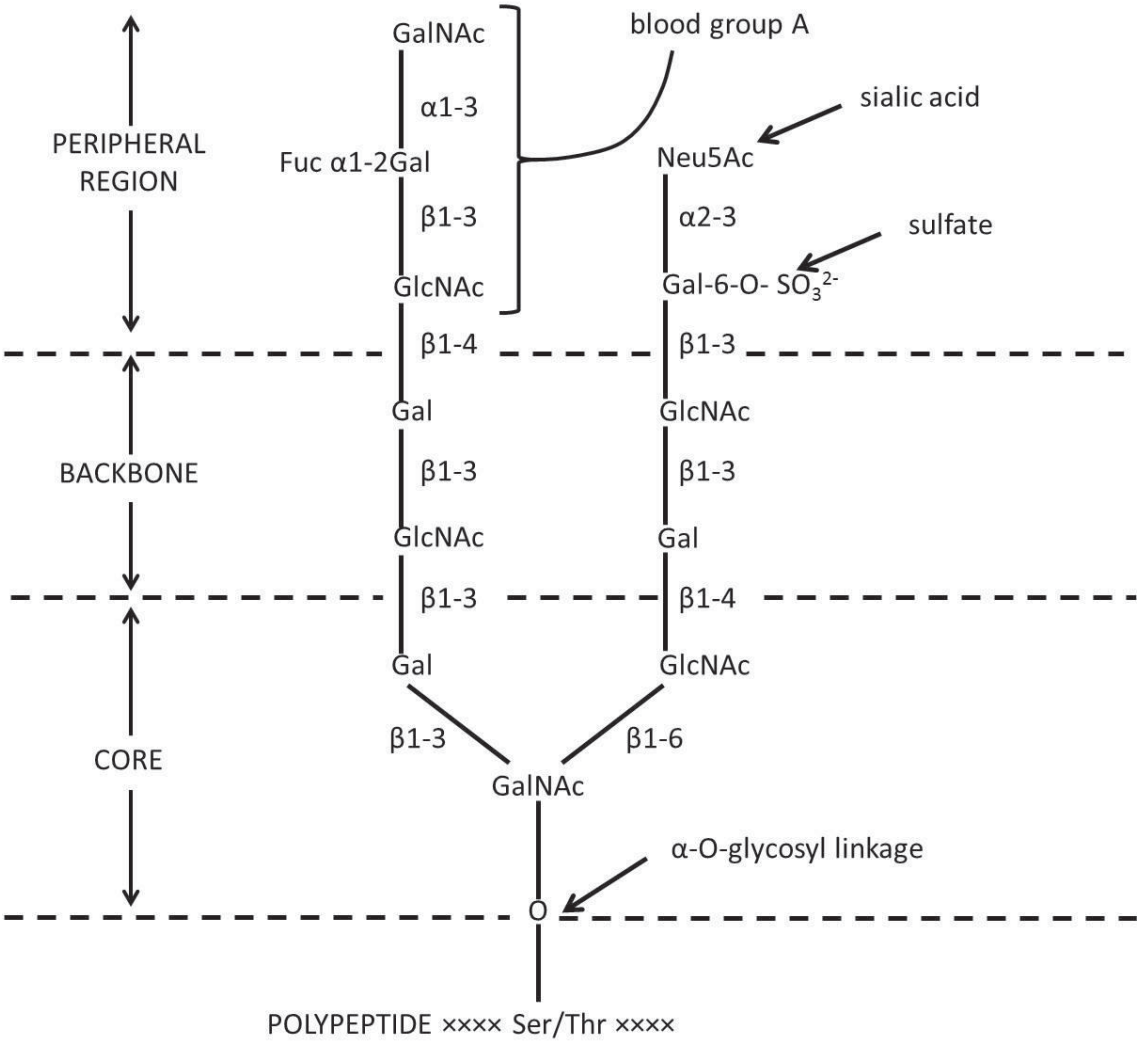


Figure 1-3 *O*-glycosylation of mucin

A theoretical oligosaccharide structure showing the core, backbone and peripheral regions [64, 65]. The *O*-glycosidic linkage to the polypeptide serine (Ser) and threonine (Thr) residues is through *N*-acetylgalactosamine (GalNAc). Backbone repeats of galactose (Gal) and *N*-acetylglucosamine (GlcNAc) are shown. Sialic acid (Neu5Ac), sulfate (SO_3^{2-}) and fucose (Fuc) residue at peripheral termini.

Glycosylation of mucins usually occurs with *O*-linked side chains, although N-linked chains do occur, but these usually arise outside of the VNTR domain [57, 66, 67]. N-glycosylation, which occurs less frequently than *O*-glycosylation, is thought to play a role in the early stages of mucin biosynthesis [59, 68] and is crucial for the function, stability, folding, transport, and secretion of glycoproteins. Of particular relevance is the role of N-glycosylation in the sorting and apical expression of glycoproteins in polarised cells [68].

Other regions/domains encoded by *MUC* genes are mucin type specific. For example, membrane-bound mucins are structurally different from secreted mucins (Figure 1-4). All membrane-bound mucins share common features. In addition to the VNTR sequences membrane-bound mucins possess transmembrane domains and carboxy-terminal cytoplasmic "tail" domains. The cytoplasmic tail, thought to play a role in signal transduction, has sites for serine phosphorylation and tyrosine sulphation [58, 59, 66, 67, 69-72]. Most membrane-bound mucins (exceptions include *MUC4, MUC15* and *MUC20*) contain Sea urchin sperm protein, Enterokinase, and Agrin (SEA) modules in the extracellular domain that are heavily *O*-glycosylated and may mediate protein binding to other carbohydrate moieties or function as proteolytic sites [58, 59, 69, 73, 74]. These domains may disassociate in response to mechanical stress, allowing the shedding of mucin without disruption to the epithelial cell membrane [50]. Epidermal Growth Factor (EGF)–like domains exist in *MUC3A, MUC3B, MUC4, MUC12, MUC13,* and *MUC17*. These domains show homology to EGF and, although their function is unclear, are thought to be involved in epithelial growth modulation and to mediate interaction between mucin subunits [58, 59, 69, 71-74]. Although these are classed as membrane-bound mucins, soluble forms arise as a consequence of cleavage of gene-coded peptides and alternate splicing events. Splice variants have been identified for most of the membrane-bound mucins [70, 72, 75-83].

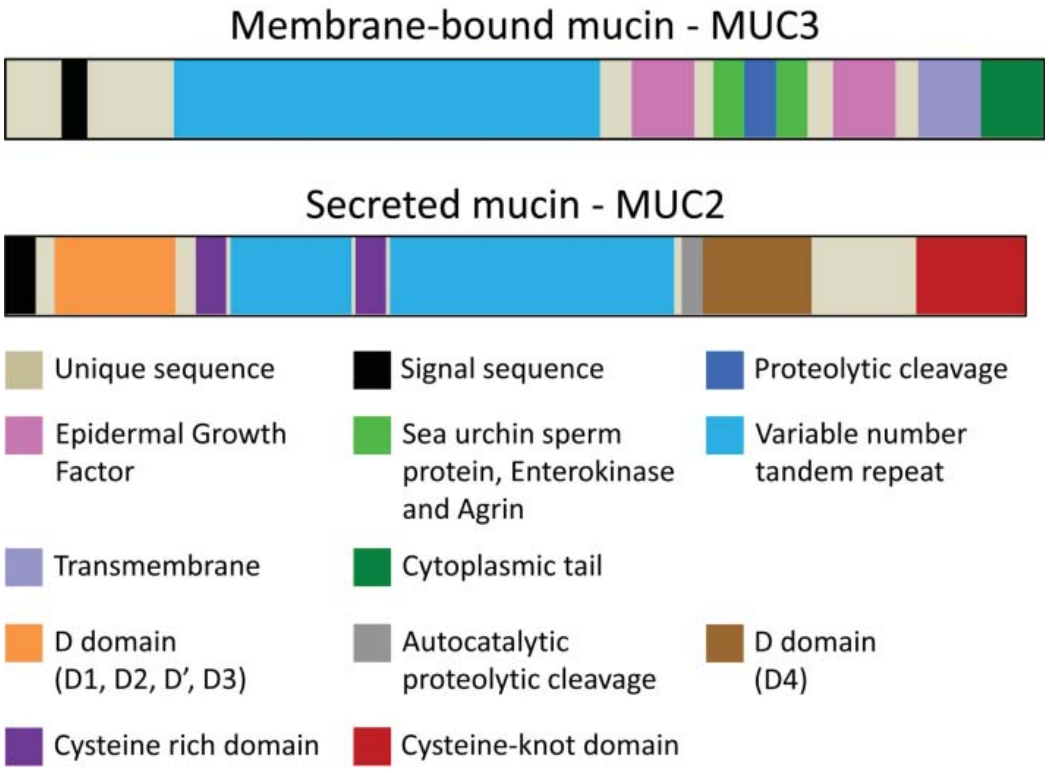


Figure 1-4 Domain structures of membrane-bound and secreted mucins [58, 64]

Autocatalytic proteolytic cleavage site in some mucins (demonstrated for MUC2) that is cleaved between glycine, aspartic acid and proline, histidine (GD and PH) residues. *Cysteine rich domain* is rich in cysteine residues and is not heavily glycosylated. *Cysteine-knot domain* mediates dimerisation of mucin molecules. *Cytoplasmic tail* is found on the cytoplasmic side of the cell surface membrane. It often contains sites of phosphorylation that interact with mediators of signal transduction and might mediate association with cytoskeletal elements. *D domains (D1, D2, D', D3)* mediate trimerisation of secreted mucin core proteins such as MUC2. *D domain (D4)* includes the GDPH autocatalytic site near the amino terminus of MUC2. *Epidermal growth factor*-like domains mediate interactions between mucin subunits. *Proteolytic cleavage site* facilitates the creation of mucin subunits that remain associated. *Sea urchin sperm protein, Enterokinase and Agrin (SEA)* is widely distributed among cell surface associated proteins that are heavily *O*-glycosylated and postulated to function in protein binding to carbohydrate moieties. *Signal sequence* directs insertion into the endoplasmic reticulum for secretion or cell surface delivery. *Transmembrane domain* spans the membrane and creates an integral membrane protein, which is found in membrane-bound mucins. *Variable number tandem repeat* domain is rich in serine, threonine and proline residues. They are heavily *O*-glycosylated and are characteristic of mucin core proteins. They are highly polymorphic for length and sequence variability.

Unlike membrane-bound mucins, secreted mucins are typically characterised by the presence of cysteine-rich regions (cysteine knot – CK) and D domains. The D domains have extensive similarity to the von Willebrand factor (vWF) and mediate oligomerisation. The D domains located at the carboxy-terminal (D1, D2, D', and D3) form disulphide-linked dimers, whilst the amino terminal D4 domain forms disulphide-linked trimers (Figure 1-4) [58, 84]. The CK domain is involved in the initial dimerisation of the apomucin within the rough endoplasmic reticulum [57, 58, 69].

1.3.2 Mucins of the gastrointestinal tract

Throughout the healthy human gastrointestinal tract, different mucins are secreted. For example, in the healthy human intestine, MUC2 is the main secreted mucin making up the mucus layer, whereas in the stomach MUC5AC and MUC6 are produced [85]. Similarly, the thickness of the mucus layer varies, throughout the gastrointestinal tract (Figure 1-5 A) and relates directly to the physiological demands at that location. For example, the stomach mucus layer is notably thicker than in most other regions (120 µm; ± 14 µm) to prevent luminal pepsin from diffusing to the epithelial surface [86-88]. The colon is also covered by a thick mucus layer (93 µm; ± 62 µm) [86, 89, 90] which protects the epithelium from invasion of colonic bacteria and damage by bacterial toxins and enzymes [50, 91], and functions as a lubricant to facilitate an easy passage of increasingly solid material through the last part of the gastrointestinal tract [92]. It is generally believed that a thicker mucus layer in these regions, provides better protection to the underlying epithelium [93]. In contrast to the thick mucus layers of the stomach and colon, a thinner and patchy layer [4] is present in the small intestine, which is thought to allow the exchange of nutrients and gases between the epithelium and the luminal surface [94, 95].

1.3.3 The mucus layer(s)

Mucus occurs in two distinct layers; an inner gel-like layer which is firmly attached to the epithelium and an outer "loose" layer [96]. Membrane-bound mucins form the glycocalyx

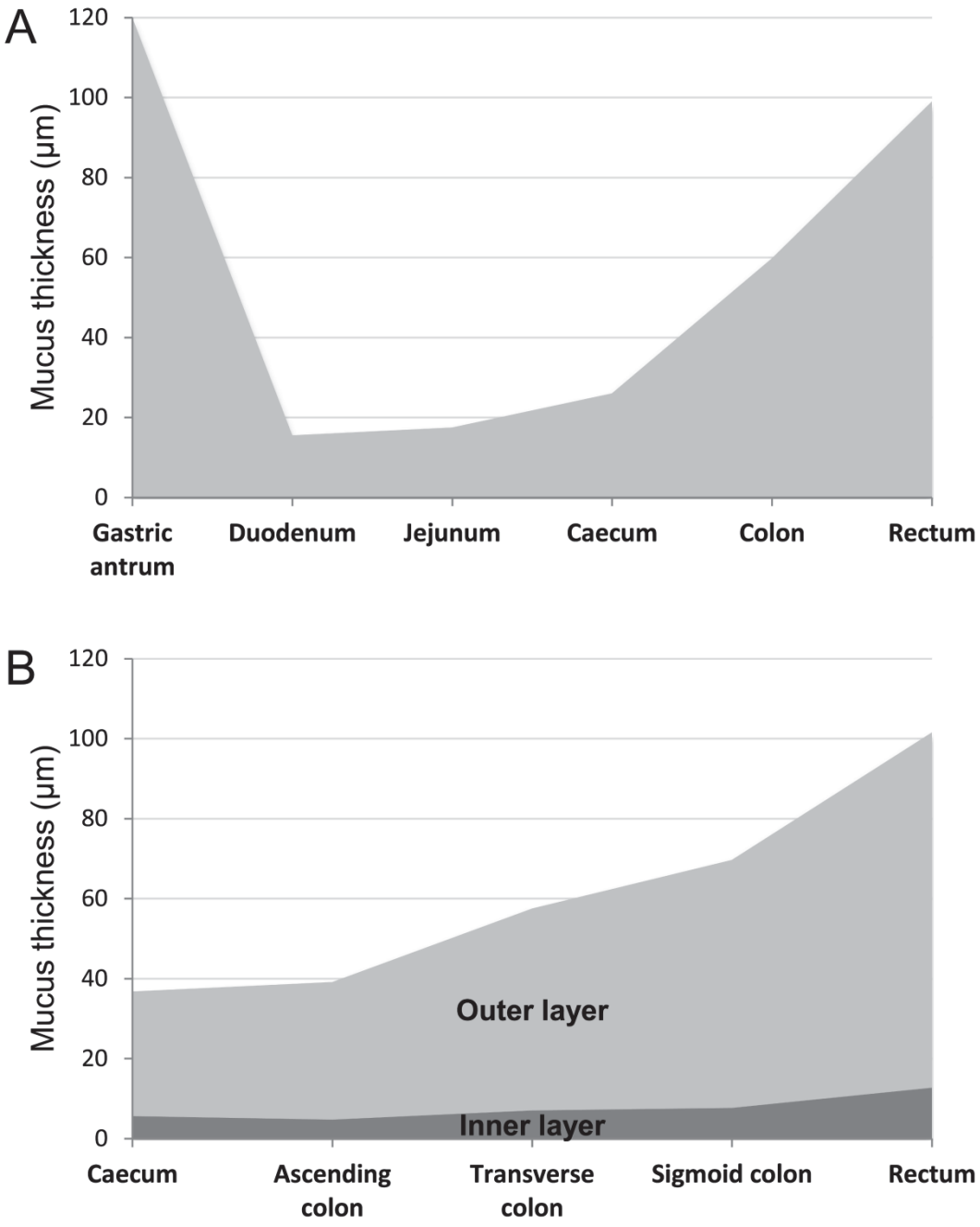


Figure 1-5 Schematic showing the thickness of the mucus layers as determined from published *in vivo* human studies

A) The overall mucus layer thickness of representative regions of the gastrointestinal tract and B) the thicknesses of the two mucus layers of the large intestine [64].

present at the apical surface of most epithelial cells and it is thought that an interaction exists between these and the firm gel-like layer formed by secreted mucins [93, 97, 98]. The inner layer has a stratified, mesh-like appearance [96, 97], created by entangled mucin fibres that are filled by a low viscosity fluid [62]. This layer is commonly referred to as a "viscoelastic gel" because it has variable properties that are between those of a viscous liquid and an elastic solid. These biophysical rheological properties are influenced by the fluid filled microscopic domains between the mucin fibres and the composition/charge of the mucin fibres themselves [62]. The loose outer mucus layer does not have a well-defined outer limit and has a disorganised appearance. This is due in part through degradation by the commensal bacteria, and the mucus being transported distally with the intestinal content [93].

Early estimates placed the outer mucus layer at twice the thickness of the inner layer, but it has subsequently been shown to be far greater than this (Figure 1-5 B), although this is variable between individuals and species [21, 97, 99]. The looseness of the outer mucus layer makes it a suitable habitat for bacterial colonisation and provides adhesion sites for bacterial attachment [97]. In contrast, the structure of the inner layer results in increased protection for the underlying epithelium, both from the physical environment of the colon and from bacterial invasion [96, 97].

1.4 Commensal intestinal microbiota

In its simplest form, a microbial ecosystem is the complex of microbes in a specified environment in combination with the surroundings with which the organisms are associated. Habitats and niches within these ecosystems are normally occupied by communities of indigenous microbes [1], known as the indigenous microbiota. Generally, a symbiotic relationship exists between some bacteria and host and can be viewed of in terms of mutualistic-commensalism. Symbiosis is a relationship between two different species where at least one partner benefits without harming the other, whilst commensalism refers to partners that co-exist without detriment but without obvious benefit [22].

The opposite situation is termed dysbiosis [100] and is a state in which the microbiota produces harmful effects via: 1) qualitative and quantitative changes in the intestinal microbiota itself; 2) changes in their metabolic activities; and 3) changes in their local distribution [101]. These factors result in an overgrowth of potentially pathogenic micro-organisms and the release of potentially toxic products that play a role in many chronic diseases such as inflammatory bowel disease [101-103].

Due to the complexity of the microbial community, and a limitation in culture based techniques, the identity of all intestinal bacterial species is not known. However, there are believed to be 1,000 to 1,150 prevalent bacterial species which colonise the adult human intestine [104], with 30 to 40 species comprising up to 99% of the total population [105]. Of these, bacteria from the genera *Bacteroides, Bifidobacterium, Eubacterium, Streptococcus, Clostridium* and *Lactobacillus* predominate [34].

1.4.1 Establishment of the indigenous microbiota

It is generally believed pristine habitats, such as the gastrointestinal tract of a new born baby, are colonised by microbes in characteristic succession and start immediately after birth [1]. However, recent studies have raised the possibility that a developing foetus *in utero* may be exposed to the maternal commensal microbiota during pregnancy. For example, DNA from intestinal bacteria was found in human placental samples suggesting that horizontal transfer of bacterial DNA from mother to foetus may occur via the placenta [106]. Bacteria from the genera *Enterococcus, Streptococcus, Staphylococcus,* and *Propionibacterium* were isolated from umbilical cord blood of healthy neonates born by elective caesarean section [107]. In another study, bacteria were isolated from the meconium obtained from healthy neonates, suggesting this material was not sterile [108]. In addition, bacteria found in amniotic fluid may have originated from the maternal digestive tract [108], and since amniotic fluid surrounds and is continuously swallowed by a foetus, this may provide an opportunity for these bacterial species to colonise the foetal intestine. However, to what extent, bacterial colonisation of the

intestinal tract occurs before birth has yet to be determined. Therefore, it is generally believed colonisation of the intestinal tract occurs during the birthing process and shortly thereafter [109]. The types of microbes that colonise during this time are influenced by forces exerted by the environment and by changes in the environment [1]. For example, the environmental conditions under which infants are born and nurtured may affect their exposure to microbes and may influence the composition of the commensal microbiota [109]. Diet also plays an important role in intestinal bacterial colonisation. The intestinal microbiota of full-term breast-fed infants and formula-fed infants differs with bifidobacteria and *Lactobacillus* predominating in breast-fed infants, and coliforms, enterococci and *Bacteroides* predominating in formula-fed infants. The differences in the intestinal microbiota of breast-fed and formula-fed infants have been related to differences in some biochemical parameters; for example, acetic acid is found at higher concentrations in breast-fed infants and degradation of mucin begins later in breast-fed infants than in formula-fed infants [109]. Although many factors are known to influence the composition of the intestinal microbiota (diet, host genotype and microbial interactions) it is generally considered that once it is established post-weaning, the microbiota remains stable throughout adult life but slowly changes with increased age with higher levels of Proteobacteria occurring [110] (Table 1-2).

1.4.2 Distribution of microbiota in the gastrointestinal tract

Bacterial numbers and composition varies along the length of the human gastrointestinal tract (Figure 1-6), with the stomach having the lowest microbial density of 10^1 to 10^3 cfu/mL [24] as a consequence of the gastric acidity killing many ingested microbes and a rapid transit time (30 minutes) of digesta through the stomach [111, 112]. Lactic acid producing bacteria are the main organisms recovered from the stomach due to retarded rates of multiplication and representative species include lactobacilli together with streptococci and bifidobacteria [25]. The transit of digesta through the small intestine is between 15 minutes and 5 hours.

Table 1-2 Main bacterial phyla compositions vary in age in the human intestine (data adapted from Xu *et al*. [113])

Phyla composition of faecal samples (%)	Physiological status		
	Children (1 to 6 y)	Adults (18 to 50 y)	Elderly (65 to 104 y)
Firmicutes	27.3 – 63.7	51.0 – 76.0	40.0 – 79.0
Bacteroidetes	22.4 – 57.5	19.0 – 41.0	16.0 – 57.0
Actinobacteria	6.7 – 10.1	0.2 – 3.5	0.4 – 4.0
Proteobacteria	0.8 – 6.7	1.2 – 5.0	1.0 – 2.5

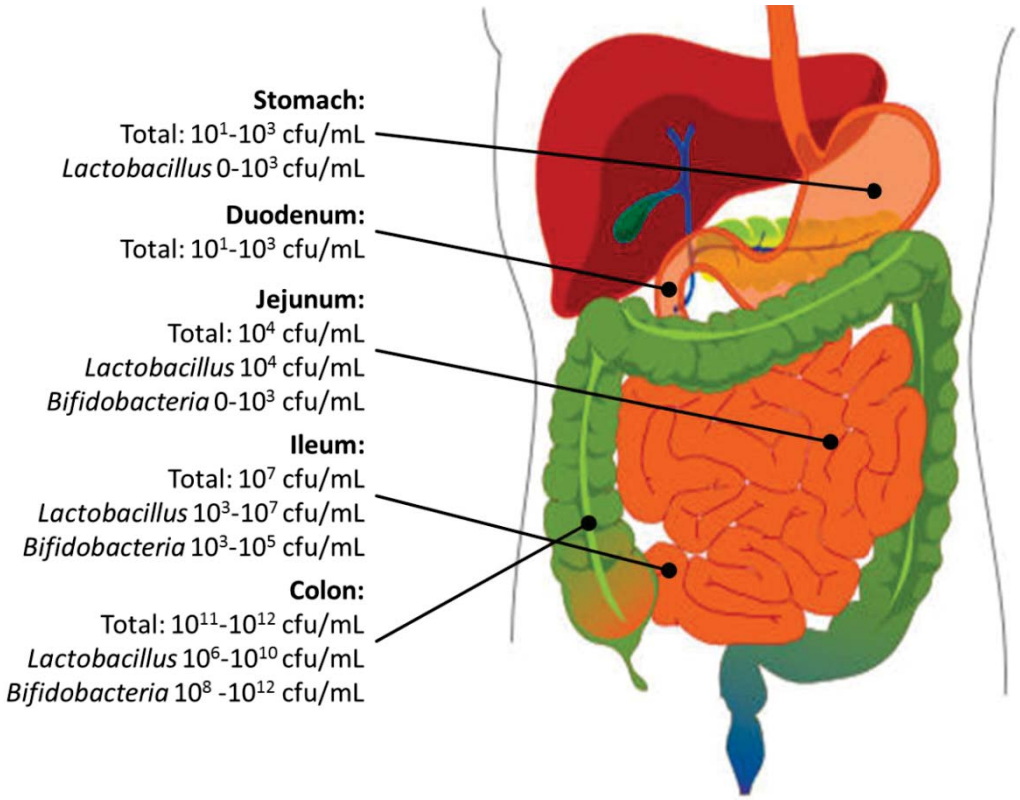


Figure 1-6 Variation in microbial numbers and composition across the length of the human intestinal tract

The density of the commensal intestinal microbiota and the prevalence of representative bacteria (*Lactobacillus* and bifidobacteria) increases from the proximal to the distal segments of the intestinal tract. Data taken from O'Hara and Shanahan (2006) [24] and Goldin (2008) [114]. Figure adapted from Wikimedia Commons [115].

Cfu/mL = Colony forming units per millilitre from liquid intestinal contents.

Consequently, this rapid flow of material prevents permanent microbial colonisation [116],thus, microbial densities in the duodenum and jejunum are relatively low (10^1 and 10^3 cfu/mL respectively) but increase dramatically in the ileum (10^7 cfu/mL) [103]. Transit time within the large intestine, 1 to 3 days [117], provides an ideal environment for bacterial growth, and in the colon microbial density increases to 10^{11} to 10^{12} cfu/mL [103].

Some bacteria, such as bifidobacteria and lactobacilli colonise the intestine preferentially. In healthy humans lactobacilli are normally present in the stomach in numbers of 0 to 10^3 cfu/mL; in the distal ileum lactobacilli are recovered in numbers of 10^3 to 10^7 cfu/mL [25], and in the colon at 10^6 to 10^{10} cfu/mL [26]. The presence of such bacteria in these environments are thought to have beneficial effects on host health through increasing resistance to gastroenteritis, improving blood lipid levels, decreasing the risk of cancer related disease, enhancing gastrointestinal immunity and improving lactose tolerance [118].

Bifidobacteria and lactobacilli are able to colonise and survive in the colon, an environment poor in free mono and disaccharides (these sugars are preferentially consumed by the host and microbes of the upper intestinal tract), through their ability to utilise diverse carbohydrates [27]. These bacteria produce lactic acid as an end product of fermentation but may also yield acetate, butyrate, ethanol, carbon dioxide and formic acid [13, 119].

1.5 Probiotics

Although bifidobacteria and lactobacilli naturally occur within the colon, dietary supplementation can occur with beneficial bacteria in the form of probiotics. Probiotics have been defined as “live microbes that, when administered in adequate amounts, confer a health benefit to the host” [19]. In relation to food, probiotics are considered as “viable preparations in foods or dietary supplements to improve the health of humans and animals” [120]. In general probiotics are members of the *Lactobacillus* and *Bifidobacterium* genera [121]. However, probiotic properties have been demonstrated by other micro-organisms (Table 1-3).

Table 1-3 Microorganisms considered as probiotics (data from Holzapfel *et al.* (2001) [120])

Lactobacillus species	*Bifidobacterium* species	Other lactic acid bacteria	Non-lactic acid bacteria
L. acidophilus	*B. adolescentis*	*Enterococcus faecalis*	*Bacillus cereus var.* toyoi
L. amylovorus	*B. animalis*	*Enterococcus faecium*	*Escherichia coli* strain nissle
L. casei	*B. bifidum*	*Lactococcus lactis*	*Propionibacterium freudenreichii*
L. crispatus	*B. breve*	*Leuconstoc mesenteroides*	
L. dulbrueckii subsp.*bulgaricus*	*B. infantis*	*Pediococcus acidilactici*	
L. gallinarum	*B. lactis*	*Sporollactobacillus insulinus*	
L. gasseri	*B. longum*	*Streoptococcus thermophilus*	
L. johnsonii			
L. paracasei			
L. plantarum			
L. reuteri			
L. rhamnosus			

The mode of action of probiotic health benefits include: i) reduced luminal pH through stimulation of the lactic acid-producing microbiota [122, 123]; ii) direct antagonistic effects on pathogens [122]; iii) competing for pathogen binding and receptor sites [124, 125]; and iv) improving immune function and stimulating immunomodulatory cells [118].

Probiotics are used as either freeze-dried preparations or in two major kinds of food products; suspended in milk or as a fermented dairy product [121]. Delivery in these formats provides protection for the probiotic and enhances their survival. However, delivery through milk products, especially those containing fat, has been shown to reduce the adhesion capabilities of some probiotics [121]. Thus, it can be noted that adhesion is not only species and strain specific but also related to how the bacteria have been grown and ultimately delivered to the host.

It is believed that ingested lactobacilli and bifidobacteria do not pose an increased risk of infection compared to that already associated with commensal strains; however, infection does occur rarely. Infection by *Lactobacillus rhamnosus* developed in a 67-year old man after removal of carious teeth. This man consumed probiotic capsules that contained a mixture of *L. rhamnosus, Lactobacillus acidophilus* and *Enterococcus faecalis.* As the capsules were too large to swallow, the contents were removed, chewed and then swallowed with milk. A few days after the dental extraction was performed, the patient developed endocarditis, and *L. rhamnosus* was isolated from several blood cultures. Further analysis showed that one of the organisms cultured from the probiotic capsule was indistinguishable from that isolated from the blood culture [26].

Certain activities have proven useful in defining microbes as probiotics: i) acid and bile tolerance; ii) survival through the intestinal tract; iii) ability to adhere to intestinal surfaces; iv) temporary colonisation within the colon; and v) antagonistic activity against pathogens [121]. These activities do not deal directly with health promoting aspects except for activity against pathogens. Probiotics inhibit the growth of pathogens and have been used in the treatment of

a variety of intestinal disorders including diarrhoea, malabsorption and *Clostridium difficile* colitis [126]. Some probiotic strains inhibit the growth and virulence of enteric bacterial pathogens through the direct production of bacteriocins (anti-microbial peptides) [122]; whilst others may influence gene expression of pathogens through quorum sensing mechanisms and reduce their virulence potential. For example, *L. acidophilus* (strain La-5) reduces the secretion of autoinducer-2 (AI-2) molecules by the enterohaemorrhagic *Escherichia coli* serotype O157:H7, resulting in reduced expression of genes that are critical for mediating intimate bacterial binding to host cell surfaces [122]. Another antagonistic mechanism probiotics employ against pathogens is enhanced secretion of mucins as occurs with *Lactobacillus plantarum* (strain 299v). As a consequence of upregulated mucin secretion, the mucus layer thickness is increased and acts as an antibacterial shield that prevents the binding of enteric pathogens and increases pathogen clearance from the lumen [122, 127].

1.6 Bacterial adherence

Bacterial adherence to the intestinal surface may occur through non-specific physicochemical interactions, such as low affinity hydrophilic/hydrophobic interactions [13]. Although low affinity bonds have short half-lives, if numerous low affinity bonds are formed the bacterium is able to remain linked to mucin fibres of the mucus layer [50]. Exopolysaccharides, which are extracellular polysaccharides that are attached to the bacterial cell wall or secreted into the surrounding environment, are known to contribute to the bacterial cell surface physiochemical properties and can directly affect their adhesion properties by shielding other cell surface adhesins [13]. For *Lactobacillus* species, specific protein-mediated adhesion mechanisms have been reported [9]. For example, an extracellular mucus-binding protein was identified in *Lactobacillus reuteri* 1063, [10, 11] and a fibronectin-binding protein and a surface layer protein were identified in *L. acidophilus* [12], all of which were required for adhesion. The binding affinity of different bacteria can also be mediated by the presence of different sugars in mucin fibres. For example, *Bifidobacterium animalis* binds specifically to glucose and/or

mannose sugars, whilst adhesion of *Lactobacillus fermentum* is reduced by the addition of sialic acid or mannose [128]. Adherence of bacteria to the epithelial surface or mucus layer has been noted to be strain specific rather than species specific. For example *L. rhamnosus* strain GG and *B. animalis* subsp *lactis* Bb-12 have increased adherence to human intestinal mucus than other strains of the same species [129].

Bacteria which are able to colonise the mucus layer have the potential to use the carbohydrate mucin as carbon, nitrogen and energy sources which may ultimately enhance colonisation [130-132]. A variety of hydrolytic enzymes are required to degrade mucin before it can be assimilated by the bacteria, but not all bacteria possess the different enzymes. Therefore, mucin degradation is usually a co-operative activity involving a number of different bacterial species [131]. *Bifidobacterium bifidum* and *Bifidobacterium longum* produce unique enzymes, 1,2-α-$_L$-fucosidase and endo-α-*N*-acetylgalactosaminidase, which are able to release terminal α-linked $_L$-fucose from oligosaccharides and hydrolyse the *O*-linked α-linkages between galactosyl β-1-3-*N*-acetylgalactosamine (respectively) [132]. Due to the uniqueness of 1,2-α-$_L$-fucosidase and endo-α-*N*-acetylgalactosaminidase enzymes it has been proposed that their activity may benefit these bacterial species in colonisation and promote their growth in these restricted environments [27]. In this way, certain colonic bacteria may modulate mucus layer thickness, and if numbers of these bacteria increase there is the possibility that mucin degradation would out-weigh mucin synthesis. However, there is evidence that mucin degradation by bacterial enzymes is rate-limiting [60, 131, 133].

Not only do bacteria adhere to the colonic mucus layer and the mucosa, they are also known to adhere in large aggregates/numbers to the surface of digesta where they form micro-colonies or biofilms [116]. In the strict sense a biofilm is “a polysaccharide matrix-enclosed bacterial population adherent to each other and/or to surfaces or interfaces” [134]. It has been hypothesised biofilm formation upon the surfaces of food residues occurs through an orderly process involving initial adhesion of planktonic (free-floating) cells, where they take on a

sessile lifestyle and form micro-colonies [135]. As micro-colonies increase in size a biofilm is formed, which is followed by secondary colonisation by other bacterial species [22]. Because of their surface-adherent nature and multicellular composition biofilms behave differently to their planktonic counterparts [134]. Biofilm communities are more efficient at digesting polysaccharides and produce acetate as their main fermentation product, while non-adherent communities more readily ferment oligosaccharides and produce high levels of butyrate [136]. Bifidobacteria predominate in biofilm communities on food particles in the colon and it is thought this provides them an advantage over non-adherent bacteria (free floating planktonic bacteria) in competing for nutrients [116]. *L. rhamnosus* GG, has also been shown to form biofilms *in vitro* on a polystyrene surface [137] and is able to adhere to, and colonise the human intestinal mucosa [138], but, its ability to form biofilms on food particles has yet to be established.

1.7 Bacterial modulation of mucin expression

Bacteria can influence the production and secretion of mucus by altering the morphology and proliferation of goblet cells and modifying the carbohydrate structure of mucin molecules with increased acidification being observed [20, 21]. This may occur as a direct result of bacterial adhesion to the mucus layer or mucosal surface, or as a consequence of secreted bacterial products, such as cholera toxin from the bacterium *Vibrio cholerae*, that can activate different signalling cascades and secretory elements, or sometimes as a result of the end products of bacterial carbohydrate fermentation [16-19].

Secretion of membrane-bound and secreted mucins occurs via constitutive and inducible pathways [139]. Non-regulated constitutive secretion is accomplished by the periodic exocytosis of single mucin granules which are synthesised in the Golgi and transported to the apical plasma membrane at a slow, variable rate. Unstimulated baseline secretion of mucin involves non-receptor mediated transport of granules from Golgi vesicles to the cell surface [16], resulting in a turnover of intracellular stores every 24 hours [44, 140, 141].

The regulated, inducible secretion pathway involves mucins that are concentrated and stored in granules where they form a highly polyanionic matrix. Initially this pathway is independent of gene expression [142] and for this reason mucin protein levels do not always correspond to transcript levels [143]. Stimulated or accelerated mucin secretion can be induced upon secretagogue stimulation through cholinergic, purinergic and neurotensin receptors by compounds such as carbochol, phorbol 12-myristate 13-acetate (regulated by calcium and protein kinase C signalling pathways) [59, 140, 141, 144] or by a wide variety of molecules including hormones (neuropeptides), growth factors and cytokines [16, 98]. Just as secretagogues are known to stimulate mucin secretion, bacteria (both pathogenic and commensal species) are also known to alter mucin gene and protein expression levels [19, 118, 145-147].

Certain *Bifidobacterium* and *Lactobacillus* species alter *MUC3* transcript levels in colonic tissue and cells [18, 148]. Furthermore, oral administration of 5 x 10^6 CFU per day of *B. bifidum* to neonatal rats has decreased the expression level of *Muc2* [148]. Using the intestinal epithelial cell line HT29, expression levels of *MUC2* and *MUC3* mRNA and their corresponding proteins were increased when cultured with *L. plantarum* strain 299v and *L. rhamnosus* strain GG [18]. The thickness of the mucus layer, which ultimately acts as an antibacterial shield that prevents the binding of enteric pathogens and increases pathogen clearance from the lumen, was increased by *L. plantarum* strain 299v *in vitro* [122, 127].

Bacterial products from Gram negative bacteria such as lipopolysaccharides (LPS) and flagellin A, and lipoteichoic acids (LTA) from Gram positive bacteria are able to modulate mucin production (Figure 1-7). LPS stimulates both the innate and adaptive immune system, through binding to LPS binding protein (LBP) and triggering the Rat sarcoma/Mitogen-activated protein kinase, extracellular regulated kinase kinase/Extracellular regulated kinase (Ras/MEK1/ERK1) pathway using Toll-like receptor 4 as a co-receptor [16, 98]. Flagellin binds to Asialo-GM1, a cell surface glycolipid receptor, which leads to the release of adenosine triphosphate (ATP) and

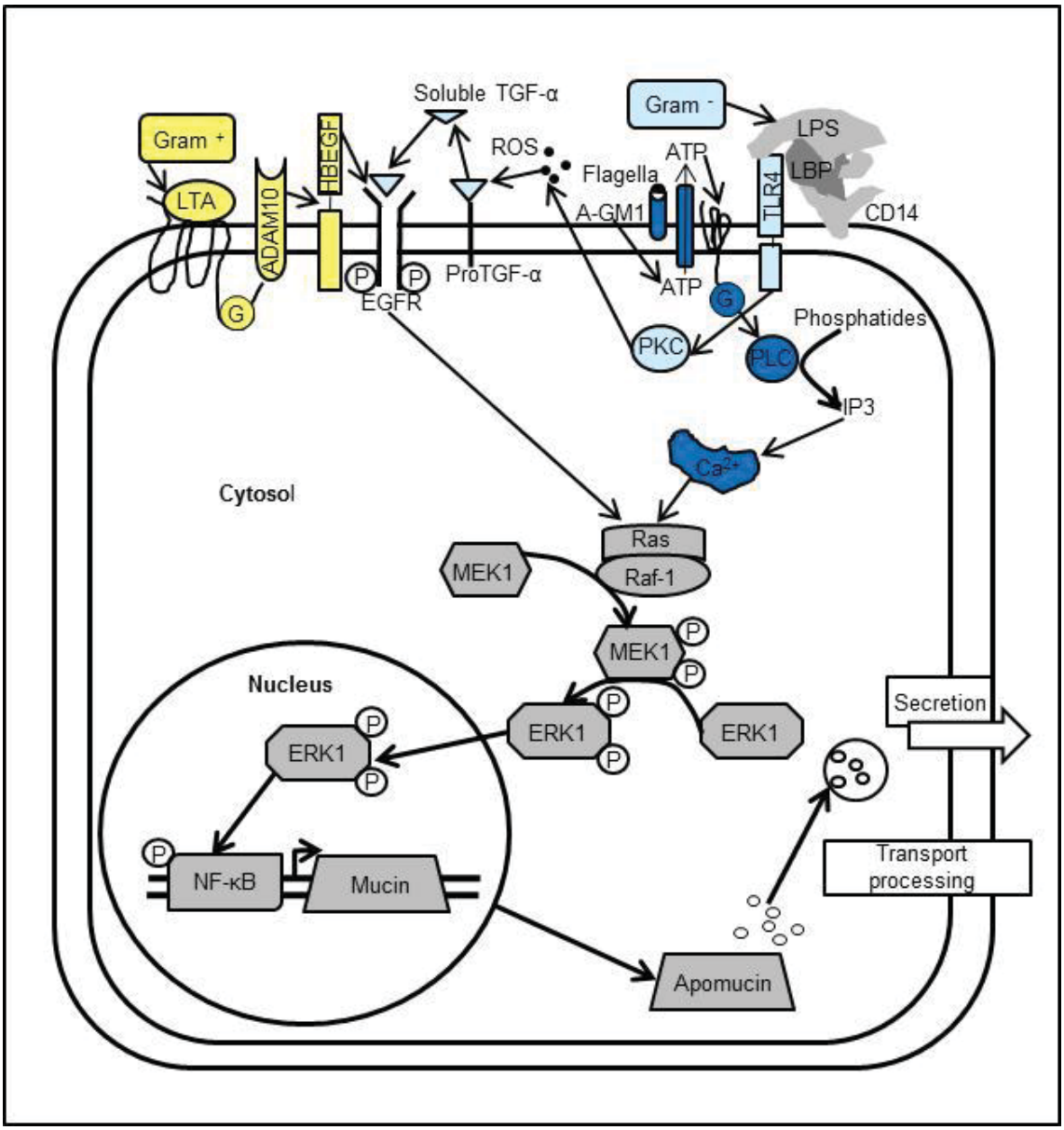

Soluble TGF-α
Gram +
Gram -
LPS
ROS
ATP
LBP
Flagella
TLR4
CD14
LTA
ADAM10
HBEGF
A-GM1
ATP
G
EGFR
ProTGF-α
Phosphatides
PKC
PLC
IP3
Ca2+
Cytosol
Ras
Raf-1
MEK1
MEK1
Nucleus
ERK1
ERK1
ERK1
Secretion
NF-κB
Mucin
Transport
processing
Apomucin

Figure 1-7 Modulation of mucin expression by components of Gram positive and Gram negative bacteria (adapted from Dharmani et al. (2009) [16])

LTA from Gram positive bacteria binds to the platelet-activating factor receptor (a G-protein-coupled receptor), and causes ADAM10 to cleave HBEGF and activate EGFR. This activates Ras which binds and activates Raf-1. Raf-1 phosphorylates MEK1 on two serine residues, activating it. MEK1 phosphorylates ERK1 on a threonine and tyrosine residue, allowing ERK1 to move into the nucleus and phosphorylate NF-κB (transcriptional factor) stimulating the transcription of mucin genes.

LPS from Gram negative bacteria predominantly, binds to LBP and Toll-like receptor 4 (LBP also binds to CD14). Binding activates PKC and the production of reactive oxygen species (ROS). ROS activates protease TNF-α- converting enzyme and cleaves pro-TGF-α resulting in the release of soluble TGF-α. TGF-α binds to and activates EGFR and the subsequent Ras pathway.

Flagellin binds to the surface glycolipid receptor, Asialo-GM1 causing the release of ATP and its subsequent binding to the G protein-coupled receptor (GP-CR). This activates phospholipase C (PLC) causing an increase in intracellular calcium levels. This increase also ultimately results in the activation of the Ras pathway and mucin transcription.

LTA = Lipoteichoic acids, LPS = lipopolysaccharide, LBP = lipopolysaccharide binding protein, ADAM10 = A disintegrin and metalloproteinase disintegrin and metalloproteinase, A-GM1 = Asialo-GM1, ATP = adenosine tri-phosphate, EGFR = epidermal growth factor receptor, ERK1 = extracellular regulated kinase, HBEGF = heparin-binding epidermal growth factor, G = G protein, MEK1 = MAPK ERK kinase (MAPK = mitogen activated protein kinase), NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells, PKC = protein kinase C, PLC = phospholipase C, Ras = Rat sarcoma, Raf-1 = rapidly accelerated fibrosarcoma, ROS = reactive oxygen species, TGF-α = transforming growth factor- α, TLR4 = Toll-like receptor4.

subsequent binding to the cell surface G-protein-coupled receptor (GPCR), and an increase in intracellular calcium levels through phospholipases C activation. Increased intracellular calcium levels leads to the downstream activation of the Ras pathway and activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mucin transcription [16, 98]. LTA, from Gram-positive bacteria, binds and activates another GPCR platelet-activating factor receptor, leading to the activation of A disintegrin and metalloproteinase (ADAM10). ADAM10 cleaves the transmembrane heparin-binding epidermal growth factor and activates epidermal growth factor receptor (EGFR) and the engagement of the Ras/Raf/MEK1/ERK1 pathway. Ultimately NF-κB acts as a transcription factor inducing mucin transcription [16, 98, 149-151]. EGFR is a member of the tyrosine kinase receptor family, and when bound to its ligand results in phosphorylation of the tyrosine residue and activation of enzyme dual oxidase and the production of reactive oxygen species (ROS). ROS induces the protease tumour necrosis factor-α (TNF-α) converting enzyme to cleave pro-transforming growth factor-α (pro-TGF-α) and the release of TNF-α, which binds to EGFR and initiation of the mitogen-activated protein kinase (MAPK; mitogens are signals that act from outside cells to induce mitosis and cell division) signalling cascade and mucin production [16, 98, 152].

Extracellular regulated kinase (ERK) is a member of the MAPK family whilst Raf-1 is a member of the MAPK kinase kinase (MAPKKK) family. Kinases of both of these MAPK families are specific for serine or threonine residues, whilst MAPK ERK kinase (MEK), a member of the MAPK kinase (MAPKK) family, is known to phosphorylate a serine and tyrosine residue in their substrate, a MAPK i.e. ERK [152].

Activation of these pathways is known to have a potent secretagogue effect on goblet cells, which leads to enhanced mucin secretion but can also lead to a depletion of mucus granular stores. *Helicobacter pylori* is known to inhibit mucin biosynthesis, which leads to a depletion in the gastric mucus layer [153]. Thus, bacteria are able to deplete the mucus layer by inducing hypersecretion of stored mucin pools or decreasing mucin biosynthesis [153], or increase the

mucin layer for their own benefit and to the detriment of other bacterial species. Therefore, bacterial modulation of mucin expression could be viewed as an adaptive mechanism for their growth and survival in the colonic environment.

1.8 Dietary modulation of mucin production and the bacterial community

Modulation of the intestinal microbiota can occur as a consequence of diet. For example a diet high in starch, non-starch polysaccharides or non-digestible oligosaccharides can cause significant changes in the growth of colonic bacteria, which in turn, can alter the population sizes of specific bacterial species [136]. Other non-digestible oligosaccharide dietary components that have been shown to modulate the colonic microbiota include fructo-oligosaccharides and oligofructose derived from inulin; galacto-oligosaccharides and lactulose derived from lactose; and milk oligosaccharides [123, 154, 155].

1.8.1 Fructo-oligosaccharides

Inulin is a plant derived oligosaccharide and can be extracted from the roots of plants such as chicory (*Cichorium intybus)* [156]. When extracted, inulin is comprised of a family of identical linear structures that differ in their degree of polymerisation (DP), with an average DP of 10. DP refers to the number of repeat units in an oligomer or polymer chain [157]. Oligofructose is obtained by partial enzymatic hydrolysis of inulin, and whilst it is composed of the same analysis of gene expression section monomers as inulin it has a lower DP ranging from 2 to 9. This group can be further sub-divided into short-chain (DP of 2 to 4) and medium chain (DP 5 to 9) and referred to as fructo-oligosaccharide and oligofructose respectively [158]. In the colon, fermentation of inulin and oligofructose with different chain lengths by lactic acid producing bacteria occurs at different rates according to their DP. High DP inulin-type fructans are fermented slowly, which encourages beneficial saccharolytic activity in distal parts of the colon [158]. In contrast, low DP inulin-type fructans are fermented rapidly and are known to drastically modify the composition of the colonic microbiota, a phenomenon known as the bifidogenic effect [158]. The inclusion of oligofructose long chain inulin in the diet of rats with

either a simple (*Bacteroides vulgatus* and *B. longum*) or complex (human faecal microbiota) microbiota beneficially increased crypt depth in the colon and stimulated the formation of sulphomucins as the predominant type of mucin [159]. Inulin, in conjunction with a complex microbiota, increased mucin concentrations in faecal and ceacal contents of rats and increased the ceacal mucus layer [160].

1.8.2 Galacto-oligosaccharides

Lactose is the building block of both galacto-oligosaccharides (GOS) and lactulose [161]. GOS which have been synthesised using β-galactosidases are produced from whey-derived lactose (a by-product of the dairy industry) [162] but they are also naturally occurring and are present in human milk [163]. Lactulose does not occur naturally but is instead synthesised from lactose through isomerisation [161, 164]. Lactulose is used in the food industry as a prebiotic food additive and often included in infant formula and health foods [165].

A prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth, activity, or both of one or a limited number of bacterial species already resident in the colon [123]. For a food ingredient to be classified as a prebiotic, it must: neither be hydrolysed nor absorbed in the upper part of the gastrointestinal tract; be a selective substrate for one or a limited number of potentially beneficial commensal bacteria in the colon, thus stimulating the bacteria to grow, become metabolically active, or both; and be able as a consequence to alter the colonic microbiota toward a more healthier composition [166].

1.8.3 Milk oligosaccharides

Milk oligosaccharides, composed of 3 to 10 monosaccharide residues, are either neutral or acidic, containing *N*-acetylneuraminic acid (sialic acid) [167]. The oligosaccharide profile of milk from different mammalian species varies; human milk contains 3 to 13 g/L, whilst goat milk contains substantially less at 0.25 to 0.30 g/L, but is 5-8 times more than cow's milk (0.03 to 0.06 g/L) and 10 times more than sheep's milk (0.02 to 0.04 g/L) [168]. Goat milk has been shown to contain a variety of neutral and acidic oligosaccharide structures (Table 1-4).

Table 1-4 Neutral and acidic oligosaccharide structures found in goat milk [168]

Oligosaccharides	Concentration(mg/mL)
Acidic	
6'-sialyl-lactose	50-70
3'-sialyl-lactose	30-50
Disialyl-lactose	1-5
N-glycolylneuraminyl-lactose	40-60
Sialyl-lacto-*N*-hexoase	Trace
Sialyl-*N*-glycolylneuraminyl-lactose	Trace
Sialyl-hexosyl-lactose	Trace
N-glycolylneuraminyl-hexosyl-lactose	Trace
Sialyl-*N*-glycolylneuraminyl- hexosyl-lactose	Trace
Disialyl- hexosyl-lactose	Trace
Di-*N*-glycolylneuraminyl-lactose	Trace
Sialyl-dihexosyl-lactose	Trace
Di-*N*-glycolylneuraminyl-dihexosyl-lactose	Trace
Neutral	
3'-galactosyl-lactose	30-50
Lacto-*N*-hexose	1-5
N-acetylglucosaminyl-lactose	20-40
N-acetylglucosaminyl-lacto-*N*-hexose	Trace
Di-*N*-acetylglucosaminyl-lactose	Trace
N-acetylglucosaminyl-dihexosyl-lactose	Trace
N-acetylglucosaminyl-hexosyl-lactose	Trace

The neutral oligosaccharide fraction of goat milk predominantly contains linkages of galactose or *N*-acetylglucosamine (GlcNAc), although α-glycosidic linkages of galactose and *N*-acetylgalactosamine have been detected at the non-reducing terminus [169]. These oligosaccharides have been suggested to aid in the development of the colonic microbiota [169], especially GlcNAc containing oligosaccharides which are able to enhance the growth of *B. bifidum* [170].

Of the acidic oligosaccharide structures, sialic acid (also known as N-acetyl-neuraminic acid; Neu5Ac) can often be found at the non-reducing end [171]. In contrast to human milk, both Neu5Ac and *N*-glycolylneuraminic acid (Neu5Gc) are present in goat milk [168, 169]. Both neutral and acidic oligosaccharides have been shown to reduce infectious events from bacterial pathogens [168]. For example, acidic oligosaccharides prevent the adhesion of pathogens by acting as soluble receptor analogues [169] to those present on intestinal epithelial cells [172] whilst neutral oligosaccharides inhibit the toxicity of heat stable toxin of *E. coli* [173, 174].

Although, human milk oligosaccharides have been demonstrated to selectively promote the growth of highly specific strains of bifidobacteria [28], to date only a limited number of studies have investigated the effects of milk oligosaccharides on the colonic microbiota and the mucus layer. One study showed that the addition of oligosaccharides from goat milk in the diet increased the concentration of lactobacilli and bifidobacteria in the colonic contents of male Sprague-Dawley rats and normalised the expression levels of *Muc1*, *Muc2*, *Muc3*, and *Muc5* in a dextran-sodium-sulfate (DSS)-induced rat model of colitis [30]. In contrast, another study described the inclusion of goat milk oligosaccharides in the diet of rats prior to the chemical induction of colitis with trinitrobenzenesulphonic acid (TNBS) as having limited effects on *Muc2* and *Muc3* expression in treated animals [29]. In addition, pretreatment of rats with dietary goat milk oligosaccharides resulted in higher body weight gain and lower anorexia compared to TNBS challenged animals [29]. Taken together these results indicate that changes

in mucin expression may not be uniform between different experimental models of colitis [29].

1.9 Dietary modulation of bacterial fermentation

Dietary components are also able to alter concentrations of bacterial fermentation products. Intestinal bacteria produce hydrolytic enzymes that digest complex dietary carbohydrates and proteins that are not absorbed in the small intestine. These complex molecules are depolymerised by bacterial polysaccharidases, glycosidases, proteases, and peptidases to smaller oligomers as well as their component sugars and amino acids, which can be assimilated and metabolised by the microbiota. Since carbohydrate entering the colon mainly occurs in the form of polysaccharides, the rate at which these substances are depolymerised determines the availability of fermentable substrate, which in turn affects the types of fermentation end products that can be formed [175]. The basic fermentative reactions in the colon are hydrolysis of polysaccharides, oligosaccharides, and disaccharides to their monomeric sugars, which yields metabolisable energy for microbial growth and maintenance [176].

The end products of colonic bacterial fermentation are high concentrations (up to 140 mM for butyrate) of SCFAs (lactate, acetate, succinate, propionate, butyrate), resulting in a physiological luminal pH of 5.5–6.5. SCFAs (mainly acetate, propionate and butyrate) have major physiological effects on the colonic mucosa. For example, butyrate constitutes the major energy source for colonocytes and is preferentially and rapidly metabolised by the colonic epithelium, providing 60–70% of the energy needs of isolated colonocytes [17, 176, 177]. Lactate is formed by a number of intestinal bacteria, but this does not accumulate in the large intestine of healthy people because it is used as a fermentation substrate by cross-feeding species in the microbiota, and rapidly assimilated by the mucosa [175]. SCFAs also modulate epithelial cell differentiation and proliferation, a biological effect ascribed to histone deacetylase activity [178] as well as modulating intestinal immunity [176, 177]. Immunomodulatory effects include suppression of inflammatory cytokine secretion as well as blocking up-regulation of pro-inflammatory mediators IL-8 and also repression of the

transcriptional activator NF-κB [176, 177].

The chemical composition of growth substrates and different bacterial communities (adherent/non-adherent) can influence the SCFAs produced. For example, mixed communities (adherent/non-adherent) of faecal bacteria principally produce acetate and butyrate from starch utilisation [179]. The non-adherent faecal community primarily produce butyrate irrespective of the substrate provided, although oligosaccharides are more rapidly fermented, and produce increased levels of acetate when compared to biofilm communities [136].

SCFAs can stimulate mucin secretion with butyrate appearing to be the most effective in modulating mucin synthesis and release [91] both at the gene and protein level, [17, 177] leading to an increase in the thickness of the mucus layer [159]. Butyrate increases the expression levels of *MUC2, MUC3, MUC5AC* and *MUC5B* in HT29 cells, whereas acetate and propionate have no effect on these genes [17]. In another study, an increase in the thickness of the mucus layer was observed in rats [180] but there was no correlation with the expression of *Muc2* or *Muc3*, nor butyrate production or other SCFAs in the colon. However, this same study did highlight that diets containing different non-digestible carbohydrates could increase *Muc3* expression in the caecum which was associated with increased concentrations of butyric acid. This suggests that the effects of diet and SCFAs on mucin gene expression vary in different parts of the intestinal tract.

1.10 Models used to investigate mucin dynamics

Determining whether mucin dynamics are affected as a consequence of diet alone or the result of an interaction between the microbiota and food components is most often investigated using *in vitro* cell culture models representing the gastrointestinal tract, or by using germ-free animals.

1.10.1 *In vitro* – mono-culture cellular models

Mucin secreting *in vitro* cellular culture models of the human colon utilise different intestinal epithelial cell lines such as HT29 (and its many sub-clones), LS180, LS174T, HM3, LIM 2463,

COLO205 and T84 [5]. Although these cell lines all produce mucus they do not express the same mucins. For example, T84 cells express *MUC1* and *MUC2*, while LS180 cells express *MUC2, MUC5AC, MUC5B* and *MUC6* [5]. The HT29 cell line, originally isolated from a human carcinoma of the colon by Fogh and Trempe [181] forms a multilayer of predominantly undifferentiated cells in culture [182]. However, when these cells are cultured under various metabolic stress conditions (glucose [183-185] and glutamine deprivation, or sodium butyrate [186], 5-fluorouracil [187], or methotrexate (MTX) treatment [182, 188]) the cells form polarised monolayers of either absorptive or mucus-secreting cells [182]. The mucus secreting cell populations produced from these adaptation processes result in clones which are able to express different mucin genes. For example, the HT29-MTX population of cells (adapted with 10^{-6}M of MTX) are known to express *MUC1, MUC2, MUC3A, MUC3B, MUC4, MUC5AC* [189], *MUC5B* [190], and (*MUC11*)/*MUC12*, [51, 150] whilst the HT29 clone Cl.16E (created from treatment with sodium butyrate) express only *MUC1, MUC2, MUC3A, MUC3B* [191] *MUC5AC,* and *MUC5B* [17].

The HT29-MTX population of cells have been used as an *in vitro* intestinal model due to expression of an extensive range of different mucins. These cells were used to determine that bacterial adhesion of *Lactobacillus johnsonii* to mucins is pH dependent [192]; adhesion of both *L. acidophilus* LA1 [125] and four different bifidobacteria species inhibits the attachment of enteropathogenic bacterial species [193]; and adhesion of *L. plantarum* 299v results in an increase in *MUC2* and *MUC3* expression and also inhibits the adhesion of enteropathogenic *E. coli* [127]. In addition, the milk opioid peptide β-casmorphin-7 was shown to elicit an increase in the abundance of *MUC5AC* mRNA with a concurrent rise in its secretion [194], and that goat milk oligosaccharides modulate mucin production by decreasing *MUC2* and *MUC3* expression levels [195].

1.10.2 *In vitro* – co-culture cellular models

Although mucin dynamics in response to different stimuli can be investigated using mucin secreting cells cultured alone, there are limitations with this type of model as it does not accurately simulate the intestinal epithelium *in vivo*. As mentioned previously, the human intestinal epithelium is composed of four different epithelial cell types; the most abundant of which are absorptive enterocytes. A more accurate *in vitro* model of the intestinal epithelium would incorporate goblet cells and absorptive enterocytes at ratios which are more representative to those found *in vivo*. To this end, co-cultures of HT29-MTX and Caco-2 cells, a colonic epithelial cell line which does not produce mucus, have also been used as an *in vitro* intestinal epithelial cell model [196-199].

Caco-2 cells, in culture, spontaneously differentiate into polarised enterocyte-like cells, with an apical and basolateral surface, a well-developed apical brush border [200] and are characterised by the formation of tight junctions [201]. Tight junctions are a multifunctional complex that forms a seal between adjacent epithelial cells near the apical surface [202]. Caco-2 cells are widely used as an *in vitro* model of absorptive enterocytes to evaluate absorption of nutrients and drugs [196]. In addition, when cultured on permeable filter supports, Caco-2 cell monolayers have been used to investigate intestinal barrier permeability [203]. In recent years, the least invasive method of determining barrier permeability is to measure the Trans-epithelial electrical resistance (TEER). TEER is an inverse measure of ion permeability across the epithelium and reflects the tightness of the barrier, thus the higher the TEER, the less permeable the epithelium is to the passive flow of ions [204]. Unfortunately, for hydrophilic molecules that are transported through cell junctions by the paracellular pathway, the permeability of Caco-2 monolayers is up to 100 times lower than that of the human intestine *in vivo* [196].

Co-cultures of HT29-MTX and Caco-2 cells represent the two major cell types (absorptive enterocytes and goblet) found in the intestinal epithelium. In previous studies, the epithelial

cell co-culture model has been used to investigate the effects of a mucus layer on the transport and absorption of different molecules [198, 199, 205]. Walter *et al.* [206], used Caco-2 and HT29-MTX co-cultures to generate human and rat *in vivo-in vitro* drug permeability correlations, and Hilgendorf *et al.* [46] studied different seeding ratios of Caco-2 and HT29-MTX for *in vitro* drug absorption and intestinal permeability applications. Mahler *et al.* [207] incorporated HT29-MTX cells with Caco-2 cells to create an *in vitro* digestion/cell culture model to predict iron bioavailability. In all of these studies Caco-2:HT29-MTX co-cultures were able to form monolayers with junctional complexes between the cells, with a layer of mucus present at the apical surface and, had altered permeability when compared to Caco-2 mono-cultures. In addition, Laparra and Sanz [208] investigated the adhesion ability of intestinal bacteria to Caco-2:HT29-MTX co-cultures as well as to respective mono-cultures. The adherence of two *Escherichia coli* strains was different between Caco-2 and HT29-MTX mono-cultures, but not between the HT29-MTX mono-culture and the Caco-2:HT29-MTX co-culture, suggesting that the *E. coli* strains exhibited contrasting properties to adhere to cellular components and mucins present in the different models.

1.10.3 *In vivo* models

Although *in vitro* models of the intestinal epithelium are an important tool for studying impacts that different components have on mucin production and bacterial adherence; a potential disadvantage is that these cells are commonly derived from cancer cells and as such, may have different cellular structures and regulatory mechanisms compared to healthy cells. For example, altered level and pattern of mucin gene expression and altered glycosylation in cancer cells affect their biologic properties, such as cell proliferation, adhesion, motility and invasion [4]. Consequently, results obtained from *in vitro* investigations can only be used as a guide to *in vivo* activities.

Due to these limitations an alternate approach is the use of animals. However, in conventionally raised animals it is difficult to assess the contributions of treatments and

resident microbiota to any observed effects. Germ-free animals are reared in an environment in which bacteria are absent. Their use allows for the introduction of selected bacterial strains and different food components, so an assessment of their effects on biological functions can be determined. For example, using germ-free rats Kleessen *et al.* [159] determined that the inclusion of fructans in the diet had no effect on mucin dynamics nor on epithelial morphometry, while conversely Fontaine *et al.* [209] reported that the inclusion of inulin in the diet increased the presence of sulfomucins in the colon. The introduction of bacteria to germ-free mice was shown to modulate mucin gene expression but was dependent upon the origin of the bacterial sample. In one study germ-free mice colonised with a murine-derived microbiota resulted in elevated mucin expression while the introduction of a human-derived microbiota had limited effects [210]. However, there are some differences in colonic cellular morphology, the mucus layer, and mucin synthesis between germ-free and conventionally raised animals. Colonic goblet cells in germ-free rodents were less numerous and smaller in size relative to those of conventionally raised animals [211] possibly due to the absence of fermented metabolites from the colonic microbiota resulting in decreased growth and maturation of goblet cells [212]. Accompanied by a decreased number and size of goblet cells, there are contradicting reports of a reduction in the adherent mucus layer thickness [159, 213] and an increase in mucin protein synthesis in germ-free mice [210]. The mucin composition of germ-free animals was found to vary when compared to animals that had been conventionally raised in that an increased presence of sialylated mucins as opposed to sulfomucins was observed [214].

Overall, models of the intestine are very important research tools as they allow experiments that are difficult, if not impossible, in human volunteers [215]. In addition to studying mucin dynamics these models are very useful for assessing factors such as, nutrient absorption [216], drug delivery and toxicity [196, 217], and host-microbe interactions [218] including challenge experiments with pathogens [219]. Advantages of *in vitro* models over *in vivo* models include

increased throughput, reduced cost (media and plate costs compared to housing and diet for animals), and a considerable decrease in the amount of test compound required for assessment [220].

1.11 Conclusion and future prospectives

There is evidence the mucus layer composition and thickness in the intestine can be influenced and/or regulated by intestinal bacteria and specific dietary components such as oligosaccharides. These dietary components are likely to influence the type and relative abundance of specific bacterial species present in the large intestine and as a consequence alter the end-products of bacterial fermentation. Altered bacterial fermentation and the production of changed levels of SCFAs may lead to elevated levels of bacterial attachment to mucosal surfaces or food particles within the colon and may promote increased levels of substrate degradation when compared to free-floating planktonic bacterial cells, which in turn regulates mucus production. Further investigations into the interplay between bacteria, mucin production and oligosaccharides may provide new knowledge and insights for overall improvement in large bowel function through the incorporation of oligosaccharide components in the diet.

1.12 Hypothesis and aims

The large intestine provides the ideal environment for the growth of many bacterial species and their colonisation of food residues, the mucus layer and mucosa. Multiple interactions occur between the host and the intestinal microbiota of the large intestine and fermentable carbohydrates which transit relatively intact through the small intestine. Consequently, the overall aim of this study was to investigate the interactions between bacteria and readily fermentable oligosaccharides, using an *in vitro* cell co-culture model of the human intestinal epithelium, to ascertain their individual or combined beneficial effects on barrier integrity and mucin gene/protein expression, all of which are essential components of the intestinal barrier. Therefore, the main hypotheses of this study are:

1) Co-cultures of human mucin-secreting cells and absorptive enterocytes have parameters of epithelial barrier integrity that are more representative of conditions found in the human gastrointestinal system than the commonly used mono-culture models.
2) Readily fermentable oligosaccharides differentially modulate epithelial barrier integrity, mucin gene and mucin protein levels of human intestinal epithelial cells.
3) Bacterial strains that utilise an oligosaccharide-enriched fraction (OEF) from goat whey for growth when cultured in 5% CO_2 atmospheric conditions will have an increased adherence to intestinal epithelial cell cultures when the OEF is included in the culture, compared to bacterial strains which are unable to use this substrate for growth under these conditions.
4) Individual bacterial strains when in combination with readily fermentable oligosaccharides are more effective in enhancing epithelial barrier integrity, mucin gene and mucin protein abundance of human intestinal epithelial cells, than when limited to the individual components alone.

The specific aims of this study are:

1) To adapt the HT29 intestinal epithelial cell line with methotrexate and characterise their growth and mucin gene and mucin protein profiles overtime.
2) Develop and characterise an *in vitro* intestinal epithelial cell co-culture model using the newly adapted mucin secreting HT29-MTX cell line and absorptive enterocytes represented by the Caco-2 cell line.
3) Investigate the effect of a semi-purified OEF obtained from goat whey on the metabolic activity, epithelial barrier integrity using TEER, mucin gene expression and mucin protein abundance of the intestinal epithelial cell mono- and co-cultures.
4) Assess the growth profiles of bacterial strains in the presence of selected carbohydrates, and investigate the adhesion abilities of selected bacterial strains to

the intestinal epithelial cell mono- and co-cultures in the presence and absence of an OEF from goat whey.

5) Assess the impact of selected bacterial strains, in the presence and absence of the OEF from goat whey on TEER, mucin gene expression and mucin protein abundance.

A flowchart describing the structure of the dissertation is presented in Figure 1-8.

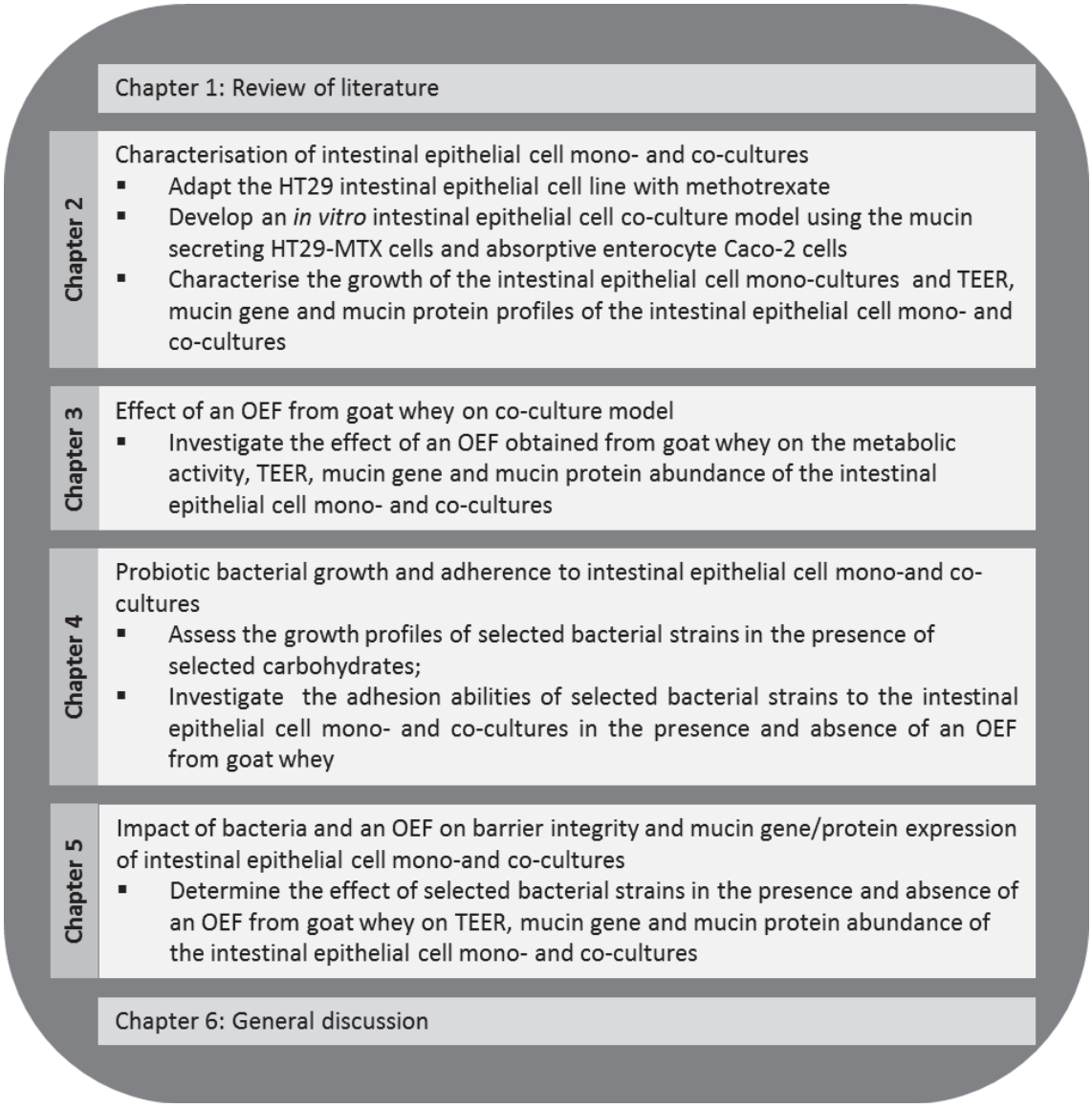


Figure 1-8 Structure of the dissertation

A flow chart describing the outline of the dissertation and its experimental chapters.

Chapter 2: Culture and characterisation of HT29-MTX, Caco-2 and co-cultures of Caco-2:HT29-MTX cells

2.1 Introduction

The gastrointestinal epithelium is covered by a layer of mucus which provides protection both chemically and physically, to the underlying epithelium. Mucins, which are high-molecular weight glycoproteins, are a major structural component of mucus and can be either secreted or membrane-bound. Secreted mucins are produced by specialised epithelial cells known as goblet cells and their secretion is generally restricted to this cell type. However, the production of membrane-bound mucins are not restricted to specific cell types but can instead be found at the surface of other epithelial cell types such as enterocytes and are thought to form part of the cell glycocalyx. In addition to mucin expression being cell type-specific it is also tissue specific. For example, in the human small intestine, *MUC2* and *MUC3* which code for a secretory mucin and a membrane-bound mucin respectively, are the major expressed mucins, whilst in the large intestine *MUC2* is the main mucin expressed [221]. The increased presence of secreted mucins in the large intestine may be directly related to the epithelial cell type; in the small intestine the epithelium is comprised of approximately 90% enterocytes and 10% goblet cells, but in the large intestine goblet cell numbers increase to approximately 25%.

Cell culture offers a means to investigate cell type-specific mucin expression. The human colon carcinoma Caco-2 cell line exhibits characteristics of foetal ileal epithelial cells from the small intestine. In culture, these cells are able to spontaneously differentiate into polarised monolayers with enterocyte-like characteristics with the development of an apical brush border, the formation of tight junctions between cells and the presence of key drug and nutrient transporters [222, 223]. Caco-2 cultures have previously been used as *in vitro* models to study intestinal barrier integrity and paracellular transport [201, 203, 224], but have also been used to characterise cell type-specific mucin gene expression. Van Klinken *et al* [221] determined Caco-2 cells have limited *MUC2* gene expression (virtually absent) and although *MUC4 and MUC5AC* were expressed at higher levels relative to tissue from small intestine, the expression levels of these genes were low and as a consequence protein secretion of these

specific mucins was not determined. In another study, Caco-2 cells were identified as having lower *MUC2* gene expression levels than another colon cancer cell line HT29, whilst the reverse was shown for the expression of the *MUC5AC* gene with lower levels being detected in HT29 cells [225].

The HT29 cell line was originally established from a human colon carcinoma. In culture, these cells form a heterogeneous multilayer of predominantly undifferentiated cells, of which, only a small proportion (<5%) of cells are absorptive enterocytes and mucus-secreting cells [182]. When cultured under conditions of metabolic stress, such as methotrexate (MTX) treatment, HT29 cells are able to adapt and grow under these new conditions. The growth adaptation process results in a differentiated cell population of either absorptive enterocytes or mucus-secreting cell types [188]. Adaptation arises after MTX enters the cell via reduced folate carriers and inhibits multiple folate dependent enzyme targets, such as thymidylate synthase and enzymes involved in purine synthesis. Consequently, MTX-treated cancer cells, both *in vitro* and *in vivo*, have reduced rates of DNA synthesis and cellular proliferation [226].

HT29 cells adapted with $10^{-7}M$ MTX form a polarised monolayer of mixed cell phenotypes, consisting of 50% absorptive enterocytes and 50% mucus-secreting cells. However, by increasing the MTX concentration from 10^{-7} *M* to 10^{-6} *M*, polarised monolayers are comprised predominantly of mucus-secreting goblet cells, with only a few absorptive enterocytes (<0.01%). Reverting the culture medium of these adapted cell populations to drug-free medium, creates differentiated populations of cells that are stable (do not revert back to mixed cell phenotype populations) and have stable growth kinetics [182].

The mucus-secreting goblet cells adapted with 10^{-6} *M* MTX have previously been shown to express a variety of mucin genes [189], with their expression differing according to the stage (age) of the culture. Qualitative expression of *MUC4* and *MUC2* transcripts from HT29-MTX cells adapted with 10^{-5} *M* MTX were shown to reach maximal levels with the induction of differentiation but decrease after late post confluence (day 21 post-seeding). *MUC5AC* was

present in cultures from day four and shown to be consistent throughout a 21 day culture period. The abundance of secreted MUC2 and MUC5AC protein was detectable from day 9 post-seeding. However, MUC2 was noted to decrease from day 17 post-seeding whilst MUC5AC protein levels were consistent through 21 days of culture. The relationship between *MUC4* gene expression and MUC4 protein was not determined in this study [51].

The introduction of Caco-2 enterocyte-like cells into culture with mucus-secreting HT29-MTX cells to create a Caco-2:HT29-MTX co-culture model of the intestinal epithelium has previously been used to investigate drug transport [46] and adherence of selected bacterial strains [208]. In addition, the thickness of the mucus layer covering the surface of co-cultures has been characterised [207], and one study undertaken by Lai *et al* [223] used the Caco-2:HT29-MTX co-culture model to determine the effects on cellular protein expression when exposed to low concentrations of functionalised carbon nanotubes (f-CNT). MUC5AC, one of the most abundant proteins detected, was shown to be decreased upon incubation. In another study, undertaken by Wan *et al* [227], it was shown that in co-cultures of Caco-2 and HT29-MTX cells, lower MUC5AC and MUC5B mRNA transcription, protein and total mucin-like glycoprotein secretion occurred following exposure to *Fusarium* mycotoxins. However, this study did not quantify or compare the abundance of mucin proteins produced by the Caco-2 and HT29-MTX cells. Consequently, to date, limited characterisation of mucin gene expression and mucin protein abundance of the Caco-2:HT29-MTX co-culture model has been undertaken.

2.2 Hypothesis and aims

The main hypothesis for the research in this chapter is that mucin mRNA levels and mucin protein abundance is greater in HT29-MTX mono-cultures compared to all other cell cultures because they are formed from mucin secreting goblet cells. In addition, the hypothesis that mucin protein abundance of the co-cultures is the sum of mucin protein abundance of the individual cell types present in the cell co-cultures will be examined. Additionally, epithelial barrier integrity of co-cultures is more representative of *ex vivo* intestinal tissue than the

commonly used mono-cultures.

The aim of the research reported in this chapter is to characterise a predominantly mucin-secreting population of HT29-MTX cells obtained after adaptation with 10^{-6} *M* MTX, and to utilise the newly adapted cells (in association with the Caco-2 cells) to culture an intestinal epithelial cell co-culture model that will more closely simulate the cell proportions found *in vivo*. Epithelial barrier integrity as measured by TEER, gene expression and protein abundance of selected mucins will be characterised for the epithelial cell co-culture models in addition to mono-cultures of Caco-2 and HT29-MTX cells, to determine if co-culturing of the two different epithelial cell types together influences intestinal epithelial barrier integrity and mucin production.

2.3 Methods and Materials

2.3.1 Culture of intestinal epithelial cells

The human colon adenocarcinoma cell line HT29 (HTB-38; American Type Culture Collection (ATCC)) previously adapted with 10^{-7} *M* MTX was kindly supplied by Dr Rachel Anderson (AgResearch Ltd, Grasslands, Palmerston North, NZ) at passage 9 and used in experiments at passages 12 to 18. HT29-MTX cells adapted with 10^{-6} *M* MTX were used in experiments from passage 18 to 31. The Caco-2 cell line (HTB-37), another human colorectal adenocarcinoma cell line, was obtained from ATCC at passage 18 and used in experiments at passage 28 to 33. It was critical to use cells within defined passage numbers, as phenotypes of Caco-2 cells can change over time [228]. HT29-MTX cells, Caco-2 cells and all cell culture media and reagents were handled using aseptic technique in a laminar flow hood (class II biological safety cabinet).

2.3.2 Maintenance of intestinal epithelial cells

The intestinal epithelial cells were maintained in cell culture medium (Table 2-1) under standard conditions (37°C; 5% CO_2 incubator). Cells were routinely cultured in 75 cm^2 rectangular canted-neck cell culture flasks with vent caps (Corning, Lindfield, New South Wales

Table 2-1 Cell culture media components and volumes required to prepare 50 mL, and their uses

Medium 1: Adaptation of HT29-MTX cells with methotrexate	
Component	Volume
Foetal Bovine Serum (FBS; Life Technologies)	4.5 mL
Penicillin-Streptomycin (10,000 units/mL Penicillin and 10 mg/mL Streptomycin; Sigma-Aldrich)	0.5 mL
McCoy's 5A medium (Life Technologies)	45 mL

Medium 2: Culture of Caco-2 and HT29-MTX mono-cultures (growth kinetic investigation only)	
Component	Volume
Foetal Bovine Serum (FBS; Life Technologies)	4.5 mL
Penicillin-Streptomycin (10,000 units/mL Penicillin and 10 mg/mL Streptomycin; Sigma-Aldrich)	0.5 mL
Medium 199 (M199; Life Technologies)	45 mL

Medium 3: Culture of Caco-2, HT29-MTX and co-cultures of Caco-2:HT29-MTX cells	
Component	Volume
Foetal Bovine Serum (FBS; Life Technologies)	4.5 mL
Penicillin-Streptomycin (10,000 units/mL Penicillin and 10 mg/mL Streptomycin; Sigma-Aldrich)	0.5 mL
Dulbecco's modified eagle's medium (DMEM low glucose; Life Technologies)	45 mL

Australia) containing 15 mL of cell culture medium. The minimum and maximum volumes of cell culture medium used in different culture vessels was calculated using Equation 2.1 [229]. The maximum volume limit was set by gaseous diffusion through the liquid layer, whilst the optimum volume of medium to use depended upon the oxygen requirement of the cells.

Equation 2.1 $$Minimum\ volume = \ Surface\ area\ of\ culture\ vessel\ (cm^2)\ X\ 0.2\ mL$$
$$Maximum\ volume = Surface\ area\ of\ culture\ flask\ (cm^2)\ X\ 0.5\ mL$$

The cell culture medium in the flask was initially replaced after 24 hours with subsequent medium changes every 2 to 3 days. The cells were passaged weekly at a 1:5 dilution as described in Section 2.3.4. To reduce undue stress on the cells, cell culture medium and other reagents were pre-warmed to 37°C.

2.3.3 Harvesting intestinal epithelial cells

Cells were dissociated from the flask (75 cm^2 flask) using 4.0 mL TrypLE Express (Life Technologies, Penrose, Auckland, NZ), a trypsin analogue, and incubated at 37°C for 12 to 15 minutes for Caco-2 cells and 10 minutes for HT29-MTX cells. The flask was inspected microscopically using an inverted light microscope to ensure that the cells had detached, and the flask rocked gently to aid detachment of loosely adherent cells and to reduce clumping of cells. The cell suspension was mixed with 8.0 mL cell culture medium to inactivate the TrypLE Express enzyme, centrifuged for 3 minutes at 240 x g, and cells resuspended in 5 mL fresh culture medium.

2.3.4 Passaging of intestinal epithelial cells

Passaging is the process of sub-culturing cells and involves splitting the cells and transferring (with or without dilution) into a new culture vessel [230]. Passaging should occur before cells become confluent due to differences in cell plating efficiency of exponentially growing cells and confluent cells [231]. Caco-2 and HT29-MTX cultures were passaged weekly, when they reached 80% confluence. Cells were harvested as described in Section 2.3.3. For a 1:5 dilution, 1 mL of the cell suspension was added to a new 75 cm^2 culture flask containing 14 mL pre-

warmed cell culture medium

2.3.5 Long term storage of intestinal epithelial cells

Frozen stocks of HT29-MTX cells were maintained in liquid nitrogen in cryopreservation medium. Cryopreservation medium consisted of 0.5 mL of dimethylsulphoxide (DMSO; Sigma-Aldrich, Auckland, New Zealand) and 9.5 mL of tissue culture medium. DMSO acts as a cryoprotective agent that increases dehydration of the cells and reduces the risk of damage to cells from ice crystal formation [232]. Cells harvested from one 75 cm^2 culture flask were resuspended in 4 mL of cryopreservation medium, and 1 mL transferred to cryogenic vials (Corning, Lindfield, New South Wales, Australia). Vials were stored overnight at -80°C inside a Nalge Nunc Cryo 1°C Mr Frosty Freezing Container (Thermo Fisher Scientific, Scoresby, Victoria, Australia) filled with 100% isopropyl alcohol, and then transferred into a liquid nitrogen cell Dewar.

Frozen stocks were recovered after thawing the cryogenic vial at 37°C and mixing with 2 mL of cell culture medium. Cells were pelleted by centrifugation for 3 minutes at 240 x g, and the supernatant containing DMSO was discarded. Although DMSO at low temperatures can act as a preservative, at ambient temperatures it is toxic to cells. The cells were resuspended in 8 mL of warm cell culture medium and added to a 25 cm^2 rectangular canted-neck cell culture flask (Corning, Lindfield, New South Wales, Australia). After 24 hours incubation at 37°C in a 5% CO_2 incubator the cells were examined under the light microscope to ensure cells had adhered to the flask, had normal morphology and lacked contamination. The medium in the flask was replaced with fresh cell culture medium. When cells had reached 80% confluence in the flask they were passaged and transferred without dilution to a 75 cm^2 flask (Section 2.3.4).

2.3.6 Cell counting

A 50 µL sample of cell suspension from harvested cells was mixed with 50 µL of Trypan Blue (0.4% solution, Life Technologies, Auckland, NZ). Trypan Blue is only absorbed by non-viable cells, which appear blue and asymmetrical when viewed under the microscope; healthy, viable

cells are refractory to the dye and appear rounded. Approximately 30 µL of the stained cell suspension was loaded onto a counting chamber of an improved Neubauer haemocytometer. The counting chamber is divided into nine large squares each of which has a volume of 0.1 mm^3. The number of cells in one corner square is equivalent to the number of cells x 10^4/mL. Thus to determine the number of cells per mL of stained cell suspension, the number of viable cells in the four corner squares was averaged and multiplied by 10^4. This number was multiplied by two to take into account the 1:2 dilution with Trypan Blue to calculate the concentration of cells in the undiluted cell suspension.

2.3.7 General culture conditions for intestinal epithelial cells

For all experiments Caco-2, HT29-MTX and co-cultures of Caco-2:HT29-MTX cells were seeded at a density of 6.3 x 10^4 cells per cm^2 in all culture vessels used (refer to Table 2-2 for all cell culture vessel information). Co-cultures were grown to simulate the difference in the epithelial composition of the small and large intestine and provide a more relevant culture system to mono-cultures. Thus, Caco-2 and HT29-MTX cells were cultured separately in T75 flasks until cells were at 80% confluence. Cells were detached from flasks and counted as described in Sections 2.3.3 and 2.3.6 respectively.

For co-cultures, Caco-2 and HT29-MTX cells were mixed together prior to seeding to yield cell ratios of 90:10 (to simulate the small intestine [46]) and 75:25 (to simulate the large intestine [46]) Caco-2:HT29-MTX cells respectively. In addition, 100% Caco-2 and HT29-MTX mono-cultures were used to allow for comparisons to be made to the co-cultures. Cell culture medium was changed 24 hours after initial seeding to remove unattached cells, with additional cell culture medium changes every two days throughout their culture.

Table 2-2 Cell culture vessels, volume of media and number of cells used in experiments

Epithelial cells (Caco-2 and HT29-MTX) were seeded at a density of 6.3 x 10^4 cells/cm^2 for all experiments. All cell culture dishes and flasks were purchased from Corning (Corning, Lindfield, New South Wales, Australia). Polyester (PET) Transwell inserts (Corning) with a diameter of 12 mm and pore size of 0.4 μm^2 were used for trans-epithelial electrical resistance (TEER) assays. 'Cells per well, flask or insert' denotes the number of cells seeded in a given size. 'Volume per well or flask' denotes the volume of cell culture medium used for each individual well or flask.

Cell culture vessel	Growth area (cm^2)	Cells/well, flask or insert	Volume/well or flask (mL)
96 well plate	0.32 cm^2	2 x 10^4	0.1 mL
24 well plate	1.9 cm^2	1.2 x 10^5	0.6 mL
12 well plate	3.8 cm^2	2.4 x 10^5	1.2 mL
6 well plate	9.5 cm^2	6.0 x 10^5	3.0 mL
T25 flask	25 cm^2	1.6 x 10^6	7.9 mL
PET Transwell	1.12 cm^2	7.0 x 10^4	0.35 mL (insert) 1.5 mL (well)

2.3.8 Adaptation of HT29 cells with methotrexate

HT29 cells were adapted with MTX according to the method of Lesuffleur *et al* [182]. Briefly, HT29-MTX cells previously adapted with 10^{-7} *M* MTX were seeded into 25 cm^2 rectangular canted-neck cell culture flasks (Corning, Lindfield, New South Wales, Australia) in cell culture medium 1 (Table 2-1) at a density of 6.3 x 10^4 cells/cm^2. Medium from flasks was removed after 24 hours and replaced with fresh cell culture medium containing MTX at a final concentration of 1 µM. The medium was changed every two days with MTX supplemented media and growth kinetics determined over a 28 day period. In addition, the growth kinetics of HT29-MTX cells previously adapted with 10^{-7} *M* MTX was determined.

2.3.9 Growth kinetics of Caco-2 and HT29-MTX epithelial cells

Triplicate 25 cm^2 flasks of Caco-2 and HT29-MTX cells were harvested and cells counted using the methods as described previously (Section 2.3.3 and Section 2.3.6 respectively). To calculate the impact of MTX on HT29-MTX cell viability, the non-viable, stained cells were counted in addition to the viable, unstained cells. Cell suspensions were used to re-seed new 25 cm^2 flasks to ensure propagation of the cells, and were in turn used to produce additional growth curves. When stable growth kinetics for the newly adapted HT29-MTX cells was obtained, cells were reverted to drug-free medium. The growth kinetics of HT29-MTX and Caco-2 cells in different media (Table 2-1) was determined in an attempt to obtain comparable growth rates for the individual cell types when cultured in the same media. M199 medium (medium 2, Table 2-1) has previously been shown to be a suitable culture medium for Caco-2 cells and offers increased buffering capacities for this cell line when cultured in the presence of bacteria [203]. Growth kinetics of cells was calculated as: 1) the total number of cell divisions to occur during the exponential growth period (Equation 2.2), 2) the number of cell divisions/hour during the exponential growth period (Equation 2.3), and 3) the generation time of cells during the exponential period (Equation 2.4).

Equation 2.2 $$number\ of\ divisions\ (n) = \frac{log_{10}(N) - log_{10}(N_0)}{log_{10}(2)}$$

Equation 2.3 $$number\ of\ divisions/hour\ (v) = \frac{n}{t} = \frac{log_{10}\ (N) - log_{10}\ (N_0)}{log_{10}\ (2)\ \mathrm{x}\ (t - t_0)}$$

Equation 2.4 $$generation\ time = g = \frac{t}{n} = \frac{1}{v}$$

Where *n* = number of generations; *N* = number of cells, N_0 = initial number of cells; *v* = division rate (number of divisions/hour); t = time; t_0 = initial time; and *g* = generation time (time required for the doubling of cell number)

2.3.10 Trans-epithelial electrical resistance measurements

The TEER assay is one of the simplest and least invasive methods of measuring the integrity of tight junctions between intestinal epithelial cells [203]. It is a measure of passive ion flow across the epithelium and reflects the `tightness' of the barrier [204].

Caco-2, HT29-MTX and co-cultures of Caco-2:HT29-MTX cells were seeded onto 12 mm diameter, 0.4 μm^2 pore size, PET Transwell inserts (Corning, Lindfield, New South Wales, Australia) in cell culture medium 3 (Table 2-1) at a density of 6.3 x 10^4 cells/cm^2. The resistance across each monolayer was measured using EndOhm culture cup (World Precision Instruments, Sarasota, Florida, USA) connected to an EVOM voltohmmeter (World Precision Instruments). The TEER was calculated from the resistance (Equation 2.5) where R is the resistance (Ω) across the cell monolayers, $R_{background}$ is the resistance (Ω) across the membrane without cell monolayers, and A is the area (cm^2) of the membrane.

Equation 2.5 $$TEER = \left(R - R_{background}\right) x\ A$$

2.3.11 Preparation of cultures

Twenty-four hours prior to assay, cell cultures were carefully removed from the incubator and placed in the biosafety cabinet. The culture medium was gently removed and replenished with non-supplemented (no FBS or Penicillin-Streptomycin) culture medium in each well with the liquid flowing slowly over the surface of the monolayers. The culture medium was replaced with serum- and antibiotic-free medium to starve the cells and to eliminate any interference from extraneous proteins or hormones [233]. Cell culture plates were returned to the incubator for 10 minutes after which time the cell culture medium was removed (wash 1) and

replenished with fresh medium. Cultures were returned to the incubator for one hour, and then the culture medium was again gently removed, and replenished with fresh culture medium (wash 2). Two additional gentle washes were undertaken and after the fourth wash, warm cell culture medium was replenished and monolayers returned to the incubator. After an additional 24 hours of continuous culture the spent media (SM) was removed and monolayers washed gently four times with PBS. Pre-warmed non-supplemented (no FBS or Penicillin-Streptomycin) cell culture medium was gently added to the monolayers and cultures returned to the incubator.

2.3.12 Analysis of gene expression

HT29-MTX, Caco-2 and co-cultures of HT29-MTX/Caco-2 cells were seeded into 12 well cell culture plates in cell culture medium 3 (Table 2-1) and cultured as described previously (Section 2.3.7).

2.3.12.1 RNA extraction

At designated time points (day 7, 14, 21 and 28 for HT29-MTX mono-cultures; and day 21 for all cell cultures at 0, 3, 12 and 24 hours post wash) monolayers were lysed with 1 mL of Tri-reagent (Invitrogen, Auckland, NZ) and samples homogenised by pipetting. Homogenised samples were centrifuged 12,000 x g for 10 minutes at 4°C and 100 µL bromochloropropane (BCP; Sigma-Aldrich, Auckland, New Zealand) added. After mixing by vortex (maximum speed for 15 seconds) and 5 minutes incubation at room temperature samples were centrifuged 12,000 x g for 10 minutes at 4°C. The colourless, upper aqueous phase (400 µL) was transferred to a new tube and 200 µL of 100% ethanol added. Samples were mixed immediately by vortex (maximum speed for 5 seconds) and samples passed through a filter cartridge (RiboPure kit; Invitrogen, Auckland, NZ) as per the manufacturer's protocol. After centrifuging (12,000 x g for 30 seconds at room temperature) and washing twice with wash buffer (wash buffer as supplied in RiboPure kit) the RNA was eluted with 50 µL of nuclease-free water and stored at -80°C. The quantity of RNA was determined using NanoDrop (ND-1000 UV-

Vis Spectrophotometer, Thermo Fisher Scientific) to ensure the A_{260}/A_{280} and A_{260}/A_{230} values were >1.8 and >2.0 respectively. A ratio of ~2.0 is generally accepted as pure for RNA while a lower ratio may indicate the presence of protein, phenol or other contaminants that absorb at or near 280 or 230 nm. Samples were also assessed for RNA quality using RNA 6000 NanoLabChip kit with the Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, California, USA), to ensure samples had an RNA integrity number (RIN) above 8.0 prior to downstream analysis. RIN is an estimate of the integrity of total RNA samples as determined by the entire electrophoretic trace of the RNA sample rather than by the ratio of the 28S:18S ribosomal RNA alone [234].

2.3.12.2 Quantitative polymerase chain reaction

For quantitative polymerase chain reaction (qPCR) analysis 1.5 µg of total RNA was reverse transcribed using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, California, USA). For each 20 µL reaction, 10 µL of 2x reverse transcriptase (RT) buffer, 1 µL of 20x RT enzyme mix and 10 µL of total RNA/nuclease-free water combined were used. The mix was incubated for 60 minutes at 37°C, followed by five minutes at 95°C in a Rotor-Gene 6000 real-time thermal cycler (Corbett Life Science, Concord, New South Wales, Australia). cDNA samples were stored at -20°C prior to gene expression analysis. Expression levels of each gene were determined using manufacturer-optimised primer/probe mixes, PrimeTime qPCR assays (20x; Integrated DNA Technologies (IDT), Coralville, Iowa, USA; Table 2-3) and a Kapa Probe Fast qPCR kit (Kapa Biosytems, Woburn, Massachusetts, USA) on a Rotor-Gene 6000 real-time thermal cycler. Expression levels of the target genes (*MUC4*, *MUC2* and *MUC5AC*) were assessed relative to the Hypoxanthine Phosphoribosyltransferase (*HPRT1*) reference gene.

Table 2-3 Genes selected for quantitative polymerase chain reaction analysis

Type	Gene	Gene symbol	Transcription location	IDT assay
Target gene	Mucin 2	*MUC2*	NM_002457 Exon location 26-28	Hs.PT.56a.26485553
Target gene	Mucin 4	*MUC4*	NM_004532 Exon location 5-6	Hs.PT.56a.5039491
Target gene	Mucin 5AC	*MUC5AC*	XM_003960847 Exon location 19-21	Hs.PT.56a.25473826
Reference gene	Hypoxanthine Phosphoribosyltransferase 1/-+*/0687	*HPRT1*	NM_000194 Exon location 6-8	Hs.PT.39a.22214821

All qPCR analyses (no-template controls, calibrator samples and test samples) were prepared as triplicate 10 µL reactions comprising a 9.0 µL aliquot of master mix (5.0 µL of Kapa Probe Fast Universal 2x qPCR Master Mix, 0.5 µL of PrimeTime qPCR assay for the gene of interest, 3.5 µL of nuclease-free water), and 1 µL of cDNA template. To the no-template control samples 1 µL of nuclease-free water was added in place of sample cDNA template. The thermal profile used was an initial enzyme activation step (95°C, 3 minutes) followed by 40 cycles of denaturation (95°C, 3 seconds) and annealing/extension/acquisition (60°C, 30 seconds).

2.3.13 Quantification of MUC2, MUC4 and MUC5AC mucin proteins

HT29-MTX, Caco-2 and co-cultures of HT29-MTX/Caco-2 cells were grown in 12 well cell culture plates using cell culture medium 3 (Table 2-1) and cultured as described previously (Section 2.3.7). MUC2, MUC4 and MUC5AC protein in cell lysate (CL) and SM was measured by indirect enzyme linked immunosorbent assay (ELISA).

The total protein concentration in SM and CL samples were determined using the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Albany, North Shore, NZ) according to manufacturer's instructions and using bovine serum albumin (BSA) as standards.

After 20 days of culture, monolayers were carefully washed using the method described in Section 2.3.11. After an additional 24 hours of culture (day 21 post-seeding) monolayers were gently washed four times with PBS, wells replenished with pre-warmed non-supplemented cell culture medium and cultures returned to the incubator.

To determine mucin protein abundance in CL samples at time 0, one triplicate set of each cell monolayer type (Caco-2 and HT29-MTX mono-culture as well as the 90:10 and 75:25 co-culture monolayers) were lysed immediately with lysis buffer (1% Triton X-100; Sigma-Aldrich and proteinase inhibitors in PBS; Complete mini (EDTA-free); Roche, Indianapolis, USA) for 10 minutes. At the end of the designated incubation periods (3, 12 and 24 hours post wash) SM samples were collected and proteinase inhibitors (10x concentrate in PBS) added to the samples. Cell monolayers were lysed with lysis buffer for 10 minutes and samples were

collected and centrifuged at 4,000 *g* for five minutes at 4°C and stored at -80°C until analysis.

2.3.14 Indirect enzyme linked immunosorbent assay of MUC2, MUC4 and MUC5AC

For the enzyme linked immunosorbent assay (ELISA), 50 µL of SM sample was serially diluted with carbonate-bicarbonate buffer, pH 9.6, in enzyme immunoassay/radioimmunoassay (EIA/RIA) medium protein-binding, flat bottom, 96-well plates (*in vitro* Technologies, Auckland, NZ) and incubated overnight at 4°C. For CL samples, total protein was diluted in carbonate-bicarbonate buffer and 50 µL (at 5 ng/µL) was added to EIA/RIA plates. Again these plates were incubated overnight at 4°C. After incubation samples were decanted, and wells blocked with 150 µL of filter sterilised (0.22 µm filters; Millipore Australia Pty Ltd, NSW, Australia) 3% BSA fraction V (Sigma-Aldrich, Auckland, New Zealand) in PBS for 1 hour at room temperature.

The blocking agent was decanted and 50 µL of either MUC2 mouse mono-clonal antibody (clone 4A4, 1:250; Creative Biomart, New York, USA), MUC4 mouse mono-clonal antibody (clone 5B12, 1:500; Abnova, Taipei City, Taiwan) or MUC5AC mouse mono-clonal antibody (clone 2H7, 1: 250; Abnova, Taiwan) in 1% BSA was added to wells and plates incubated at room temperature for 1 hour.

Primary antibody was decanted and all plates were washed once with 0.1% PBS-Tween (PBST; Tween 20; Sigma-Aldrich, Auckland, New Zealand) followed by an additional three washes with PBS. After all washing steps, 50 µL horseradish peroxidase-rabbit anti-mouse immunoglobulin G conjugate (Abcam, Cambridge, UK; 1:5,000 dilution for MUC2 and MUC5AC and 1:10,000 dilution for MUC4) in 1% BSA was dispensed into each well. Titration plates were undertaken to determine optimum concentrations of both primary and secondary antibody to use in the ELISA assay.

After 1 hour, plates were washed once with 0.05% Tween 20 (Sigma-Aldrich, Auckland, New Zealand) in PBS (PBST) and three times with PBS. Colour reaction was developed with 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase solution (100 µL/well; Invitrogen, Auckland, NZ) for 30 minutes and stopped with 2N H_2SO_4 (Reagent grade sulphuric acid; Sigma-Aldrich, Auckland,

New Zealand) (50 µL/well). Absorbance was read at 450 nm (FlexStation 3 Benchtop Multi-Mode Microplate Reader; Molecular Devices, Sunnyvale, California, USA) using the well scan option. The concentration of MUC2, MUC4 and MUC5AC present in all samples was calculated from standard curves using MUC2 (Creative Biomart, New York, USA), MUC4 (Abnova, Taiwan) and MUC5AC (Abnova, Taiwan) recombinant proteins as standards. There was no cross reactivity of the mucin mono-clonal antibodies to the different recombinant proteins.

The abundance of the individual mucin proteins was calculated as nano-grams of mucin protein per microgram of total protein as well as mucin content as a percentage of the total protein present in the SM or CL samples from each of the monolayers at all the time points investigated. From these values, it was possible to calculate the contribution each investigated mucin protein made to the overall mucin profile of the different monolayers and from which sample, either SM or CL, the majority of the mucin proteins were detected. The abundance levels of the individual mucin proteins (MUC4, MUC2 and MUC5AC) were calculated from three experiments (three successive passages of cells), each with three replicates per group. Each sample was analysed in duplicate by indirect ELISA.

2.3.15 Microscopic and histochemical analysis

To maintain the surface mucus layer of epithelial cell monolayers different fixation methods have been reported. However, fixation with formaldehyde or formalin fails to preserve the surface mucus gel layer, and although Carnoy's solution (an ethanol and acetic acid-based solution) enables the preservation of the surface mucus layer in paraffin sections [89] it has been shown to cause shrinkage of the mucus layer [86]. As an alternative, alcohol based fixatives have been recommended [235]. Consequently, visualising the surface mucus layer of Caco-2, HT29-MTX and co-cultures of Caco-2:HT29-MTX cells and the thickness of the mucus layer was determined utilising two different alcohol pre-fix (ethanol or methanol) and staining (alcian blue and Giemsa) methods. In addition, alkaline phosphatase that is expressed only on the brush border of differentiated Caco-2 cells was determined by cytochemistry and used as

an indicator of the Caco-2 cell proportion in the co-cultures.

Mono-cultures of Caco-2 or HT29-MTX and co-cultures of Caco-2:HT29-MTX cells (90:10 and 75:25 Caco-2:HT29-MTX respectively) were seeded onto 13 mm round Thermanox coverslips (Nunc, Roskilde, Denmark) in 24 well culture plates using culture medium 3 (Table 2-1) at a density of 6.3 x 10^4 cells/cm^2. After 20 days of culture SM was removed and the cells were washed four times with PBS. Wells were replenished with fresh non-supplemented media and cultures returned to the incubator for an additional 24 hours of culture.

Secreted mucins localised to HT29-MTX cells were stained with alcian blue (pH 2.5; 10 mg/mL in 3% glacial acetic acid; alcian Blue 8GX, Sigma-Aldrich, Auckland, New Zealand). Alcian blue stains both sulfated and carboxylated mucopolysaccharides and sulfated and carboxylated sialomucins (glycoproteins), by forming salt linkages with the acid groups of acid mucopolysaccharides. After removal of the culture medium and rinsing once with PBS, cells were pre-fixed in 70% (v/v) ethanol for 10 minutes. Monolayers were subsequently stained with alcian blue for 30 minutes at room temperature. To remove excess stain, cells were washed with distilled water until washes ran clear.

Giemsa stain was used as an alternative approach to traditional mucin staining methods, such as alcian blue or periodic acid Schiff. Giemsa stain is a mixture of methylene blue, azure, and eosin compounds. As such this stain is typically used in cytogenetics to identify chromosomal aberrations. However, Giemsa can also be used as a differential stain and is often used to study the adherence of bacteria to human cells. In addition, this stain is used in the determination of mucinous carcinomas of the breast where mucinous material is readily seen owing to the metachromatic staining of mucin [236]. SM from monolayers cultured on Thermanox coverslips was removed and cells washed gently with PBS (four times). Monolayers were fixed in 100% methanol for 5 minutes and then 70% (v/v) methanol for a further 5 minutes. Monolayers were stained with 10% Giemsa (BDH, London, UK) for 15 minutes at room temperature, rinsed with distilled water until washes ran clear, and then allowed to air

dry.

To visualise the mucus layer thickness, thin sections of the stained Thermanox coverslips were cut transversely using a tool made from two razor blades glued onto each side of a microscope slide [237]. Images of thin sections were recorded with a Leica DM2500 microscope equipped with DFC420 C camera (Leica Microsystems, Heerbrugg, Switzerland). Image analysis was conducted using ImageJ software (National Institutes of Health, USA). In ImageJ, colour images were adjusted to grey scale and the thickness of the mucus layer was measured at regular intervals (10 measurements per image) with three monolayers being used for each experiment.

Alkaline phosphatase is a membrane-bound enzyme localised to the brush border of enterocytes in the human intestinal epithelium and has been used previously to monitor the differentiation of enterocyte-like cells [238]. Caco-2, HT29-MTX and co-cultures of Caco-2:HT29-MTX cells were seeded on 13 mm round Thermanox coverslips. After 21 days of culture, the medium was aspirated and cells washed once with PBS. Cells were stained with Fast-Red TR/Naphthol AS-MX (Sigma-Aldrich, Auckland, New Zealand) substrate and incubated at 37°C until red staining became obvious. The reaction was stopped by washing once with water, and monolayers were visualised by microscopy.

2.3.16 Statistical analysis

For qPCR analysis, data was normalised to the reference gene (*HPRT1*) and analysed for expression level changes using Relative Expression Software Tool (REST) 2009 software (version 2.0.13; Qiagen). All other data analysis was undertaken using the Minitab statistical package (Minitab 16 Statistical Software (2010) State College, PA Minitab, Inc.). ELISA data were first evaluated for normality and homogeneity of variance with the Anderson-Darling and Levene's tests. Data were not normally distributed and had heterogeneous variances. Non-parametric tests were therefore used to assess data by one-way analysis of variance (ANOVA) with the Kruskal-Wallis test, followed by the Mann-Whitney U test. Comparisons between

TEER measurements were determined using two-way ANOVA. Differences were considered statistically significant when *p*-values were less than 0.05.

2.4 Results

2.4.1 Methotrexate adaptation of HT29-MTX cells

HT29-MTX cells previously adapted with 10^{-7} *M* MTX had a generation time of 45.4 hours (± 1.8 hours) during the exponential growth period (between day 2 and day 7), reached 100% confluency after 10 days, and were stationary after 14 days. Cells cultured in the presence of 10^{-6} *M* MTX had the same rate of growth in the first 48 hours and then showed an increase in cell mortality (Figure 2-1A). Microscopic examination of cultures showed significant debris floating in the culture medium and fewer cells attached to the surface of the flask. The attached cells formed islands rather than a dense mat as observed for the 10^{-7} *M* MTX cultures. Cell counts indicated there was a significant reduction ($P< 0.01$) in cell number compared to the initial seeding density.

Minimal growth was observed for 10^{-6} *M* MTX treated cultures between days 7 to 14 (25% increase in cell number) after which point cell growth resumed at a low rate (Figure 2-1A). By day 28, although total cell number had increased by 81% compared to day 14 values, cultures were not confluent. However, 97% of the cells present in these cultures were viable cells.

The surviving cells were passaged weekly and seeded in the presence of 10^{-6} *M* MTX (Figure 2-1B). After 21 days of continuous culture (passage 3) the cell number was increased by 19% compared to initial seeding values. Cell growth was still `patchy' and only 50% of the flask surface was covered. There was increased coverage (approximately 75%) of the flask surface with passage 4 cells after 30 days of continuous culture and the growth curve was sufficiently stable for the cells to be reverted to drug-free medium. At passage 5 cells were cultured in drug free medium. After 7 days, there was a 5 fold increase in cell number, with a further 2 fold increase by day 14 and cultures were approximately 100% confluent in the flask. Weekly passaging of cells in drug-free media resulted in stabilisation of the growth curve, which

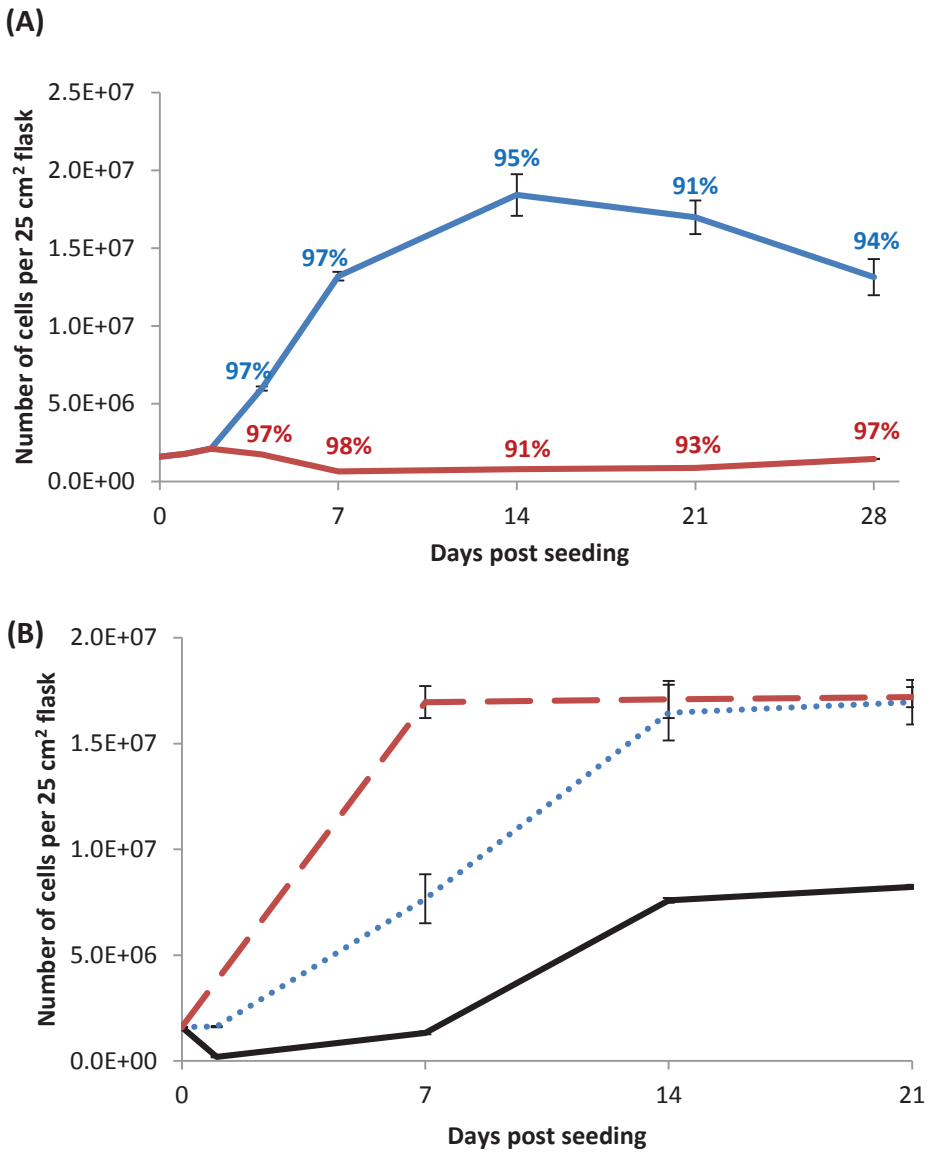


Figure 2-1 Growth and percentage viability of HT29-MTX cells over a 28 day culture period

Panel A: HT29-MTX cells previously adapted with 10^{-7} *M* MTX (blue line) and cultured in the presence of 10^{-6} *M* MTX (red line) and the percentage viability of cultures at each time point.

Panel B: Growth curves of HT29-MTX cells cultured in the presence of 10^{-6} *M* MTX (passage 3; black line), reverted to drug-free medium (passage 5 drug free; blue dotted line) and subsequent weekly passages (passage 11 to 14; red dashed line). Values represent mean ± standard error of the mean.

remained consistent throughout all subsequent passages. These stable cultures (passages 11 to 14) averaged (± SEM) 3.01 ± 0.03 divisions during the exponential period (day 2 to day 7), which related to 0.025 divisions/hour, and a generation time of 39 hours 42 minutes (± 20 minutes). Confluency was obtained by day 6 and cultures were stationary from day 10.

2.4.2 Growth kinetics of Caco-2, HT29-MTX and Caco-2:HT29-MTX co-cultures

The generation times of both Caco-2 and HT29-MTX cells in M199 medium was determined to assess if comparable growth kinetics could be achieved. The average (± SEM) generation time of Caco-2 cells cultured in this medium was 63.1 hours ± 1.1 hours and for HT29-MTX cells it was 50 hours ± 2.0 hours. Due to the contrasting generation times of the cell types another cell culture medium was tested. Low glucose DMEM Glutamax (medium 3, Table 2-1) was considered to be a suitable alternative medium and due to its low glucose content would offer additional attributes in further downstream applications when the effects of different carbohydrates on cell growth were to be determined. The average (± SEM) generation time of Caco-2 and HT29-MTX cells in this medium was 49.3 hours ± 0.46 hours and 44 hours ± 0.3 hours respectively and thus was considered to be a suitable medium for the co-culturing of the two cell types. The total number of cells in confluent Caco-2 and HT29-MTX monolayers when cultured in low glucose DMEM Glutamax was calculated from the growth curve data and determined to be 6.67×10^5 and 6.88×10^5 cells/cm^2 for Caco-2 and HT29-MTX cells respectively.

2.4.3 Trans-epithelial electrical resistance measurements

The TEER across monolayers of Caco-2, HT29-MTX and Caco-2:HT29-MTX cells was monitored over time with measurements recorded at days 1, 7, 14, 21 and 28 days post-seeding (Figure 2-2). Caco-2 mono-cultures obtained the highest average (± SEM) TEER (1070 ± 25 Ω.cm^2) of all monolayers with similar values obtained at 14, 21 and 28 days post-seeding. The 90:10 and 75:25 co-cultures obtained maximal TEER values (272 ± 7.26 and 163 ± 2.10 Ω.cm^2 respectively) at day 21 which were significantly lower ($P < 0.01$) than that recorded for Caco-2

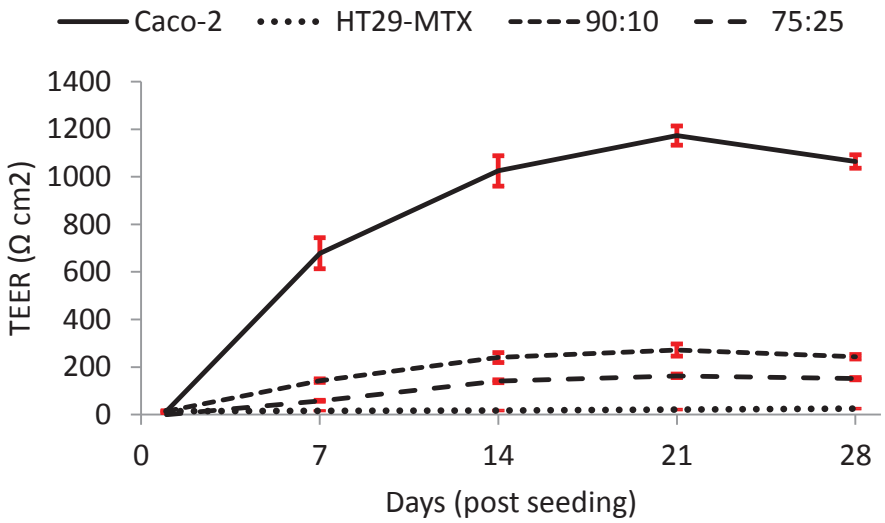


Figure 2-2 TEER measurements of Caco-2, HT29-MTX and Caco-2:HT29-MTX monolayers

Cells were cultured on Transwell PET membranes. Values plotted are the means of 12 monolayers and the error bars show the standard error of the mean.

mono-cultures. After 28 days of culture HT29-MTX cultures had obtained only minimal TEER values (21 ± 0.29 $\Omega.cm^2$), which were just above background.

2.4.4 Temporal changes in the transcription of mucin genes

Relative expression levels of *MUC2, MUC4*, and *MUC5AC* genes to the reference gene *HPRT1*, from HT29-MTX cells adapted with 10^{-6} *M* MTX were analysed by qPCR at days 7, 14, 21 and 28. Expression levels of the target genes *MUC2* and *MUC5AC* were consistent for all time points investigated (Table 2-4). Expression of *MUC4* was significantly increased ($P<0.05$) at days 21 and 28 when compared to day 7 and 14 although there was no significant difference in transcription of this gene between day 7 and 14 or between day 21 and 28.

Based on these data, the relative expression levels of the target genes *MUC2, MUC5AC* and *MUC4* to the reference gene *HPRT1* were determined in 21 day old cultures of HT29-MTX and Caco-2 cell mono-cultures as well as co-cultures of Caco-2:HT29-MTX cells. Day 21 post-seeding was selected because *MUC4* mRNA was shown to be significantly increased ($P< 0.05$) at this time point in HT29-MTX cells (Table 2-4).

After comparative analysis, the expression levels of the individual target genes (*MUC4, MUC2* and *MUC5AC*) for the individual epithelial cell cultures were not significantly different between the time points investigated of cultures at day 21 post-seeding (Table 2-5). However, expression levels of the target genes (*MUC4, MUC2* and *MUC5AC*) were significantly decreased ($P< 0.01$) in both co-cultures and Caco-2 mono-cultures when compared to the HT29-MTX mono-cultures at each time point. For example, expression levels of *MUC4* mRNA for Caco-2 mono-cultures was significantly lower ($P< 0.001$) when compared to the HT29-MTX mono-cultures at all the time points (Figure 2-3). In addition, expression levels of *MUC2* and *MUC5AC* mRNA, which code for secreted mucins were significantly lower ($P< 0.001$) in Caco-2 mono-cultures when compared to both of the co-cultures (Figure 2-4).

Table 2-4 Temporal analysis of *MUC4*, *MUC2* and *MUC5AC* mRNA levels from HT29-MTX cells by quantitative polymerase chain reaction

HT29-MTX cells were cultured for 7, 14, 21 and 28 days, total RNA was extracted, and *MUC2*, *MUC4* and *MUC5AC* mRNAs were quantified by qPCR as described in Section 2.3.12.2. The data are expressed as the mean relative expression level change (± SEM) of three replicates across two independent experiments. Genes with a relative expression level change (compared to reference samples which have an arbitrary value of 1.00 and highlighted in red) and statistically different (P< 0.05) are indicated by *.

Gene	Reference cells (days post-seeding)	Expression level change and days post-seeding			
		7	14	21	28
MUC4	7	1.00	0.96 (0.11)	1.78 (0.27)*	2.01 (0.25)*
	14	1.06 (0.15)	1.00	1.75 (0.27)*	1.98 (0.24)*
	21	0.58 (0.09)*	0.59 (0.09)*	1.00	1.15 (0.22)
	28	0.51 (0.06)*	0.51 (0.06)*	0.91 (0.17)	1.00
MUC5AC	7	1.00	1.09 (0.09)	1.01 (0.08)	1.04 (0.08)
	14	0.93 (0.07)	1.00	0.93 (0.07)	0.96 (0.08)
	21	1.0 (0.08	1.08 (0.09)	1.00	1.04 (0.08)
	28	0.97 (0.07	1.05 (0.08)	0.97 (0.07)	1.00
MUC2	7	1.00	0.84 (0.18)	0.88 (0.20)	1.02 (0.28)
	14	1.25 (0.26)	1.00	1.09 (0.11)	1.21 (0.23)
	21	1.20 (0.27)	0.93 (0.09)	1.00	1.16 (0.23)
	28	1.09 (0.30)	0.85 (0.16)	0.90 (0.18)	1.00

Table 2-5 Temporal analysis of *MUC4*, *MUC2* and *MUC5AC* mRNA levels from Caco-2, HT29-MTX and Caco-2:HT29-MTX monolayers by quantitative polymerase chain reaction

Cells were cultured for 21 days, total RNA was extracted, and *MUC2*, *MUC4* and *MUC5AC* mRNAs were quantified by qPCR as described in Section 2.3.12.2. The data are expressed as the mean relative expression level change (± SEM) of three replicates across two independent experiments. Relative expression level changes for all genes were compared to respective reference samples (0h for respective cell culture).

Cell monolayer	Reference sample	Gene	Expression level change and time (h)		
			3	12	24
Caco-2	0h	*MUC4*	0.98 (0.04)	0.96 (0.24)	1.00 (0.14)
		MUC2	0.98 (0.03)	1.06 (0.15)	1.03 (0.20)
		MUC5AC	1.00 (0.06)	1.03 (0.18)	0.99 (0.26)
90:10	0h	*MUC4*	1.01 (0.07)	1.02 (0.11)	1.03 (0.12)
		MUC2	1.01 (0.06)	0.98 (0.04)	1.04 (0.12)
		MUC5AC	1.03 (0.14)	1.00 (0.10)	1.02 (0.17)
75:25	0h	*MUC4*	1.03 (0.07)	0.98 (0.02)	1.02 (0.12)
		MUC2	1.01 (0.04)	0.99 (0.05)	1.00 (0.07)
		MUC5AC	1.01 (0.03)	1.02 (0.06)	1.02 (0.11)
HT29-MTX	0h	*MUC4*	0.99 (0.03)	1.03 (0.11)	0.98 (0.07)
		MUC2	0.98 (0.07)	0.99 (0.14)	1.02 (0.06)
		MUC5AC	0.97 (0.14)	0.98 (0.28)	0.99 (0.05)

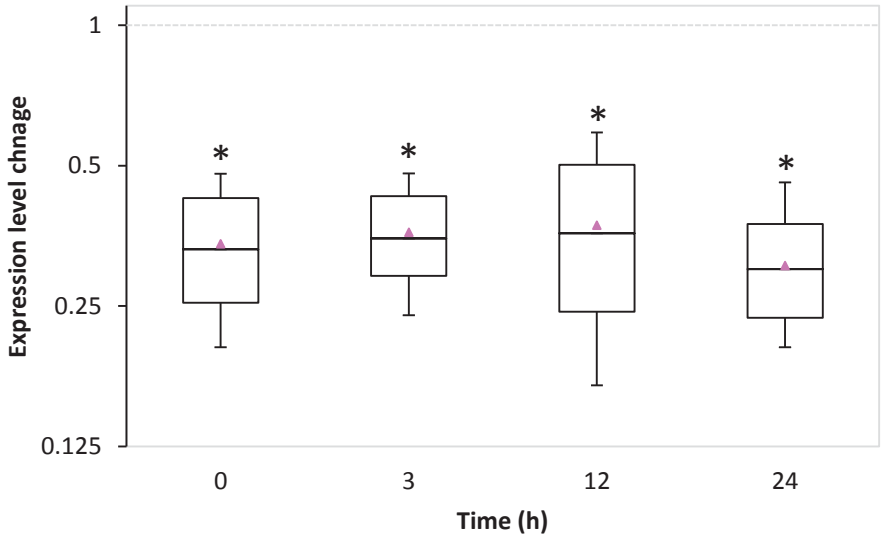


Figure 2-3 Temporal analysis of expression levels of *MUC4* gene from Caco-2 cell mono-cultures compared to the HT29-MTX mono-cultures by quantitative polymerase chain reaction

Data are expressed as the mean relative expression fold change (± SEM) of Caco-2 mono-cultures (21 days post-seeding) compared to respective reference samples (HT29-MTX) at the same time point (hours) which have an arbitrary value of 1 (represented by dotted line). In the graph the pink triangle represents mean gene expression change, the central line in the box represents median gene expression change, and the upper and lower boxes represent the minimum and maximum gene expression level changes. The error bars represent the 95% confidence intervals. *Significantly different compared to respective reference cultures $P < 0.05$

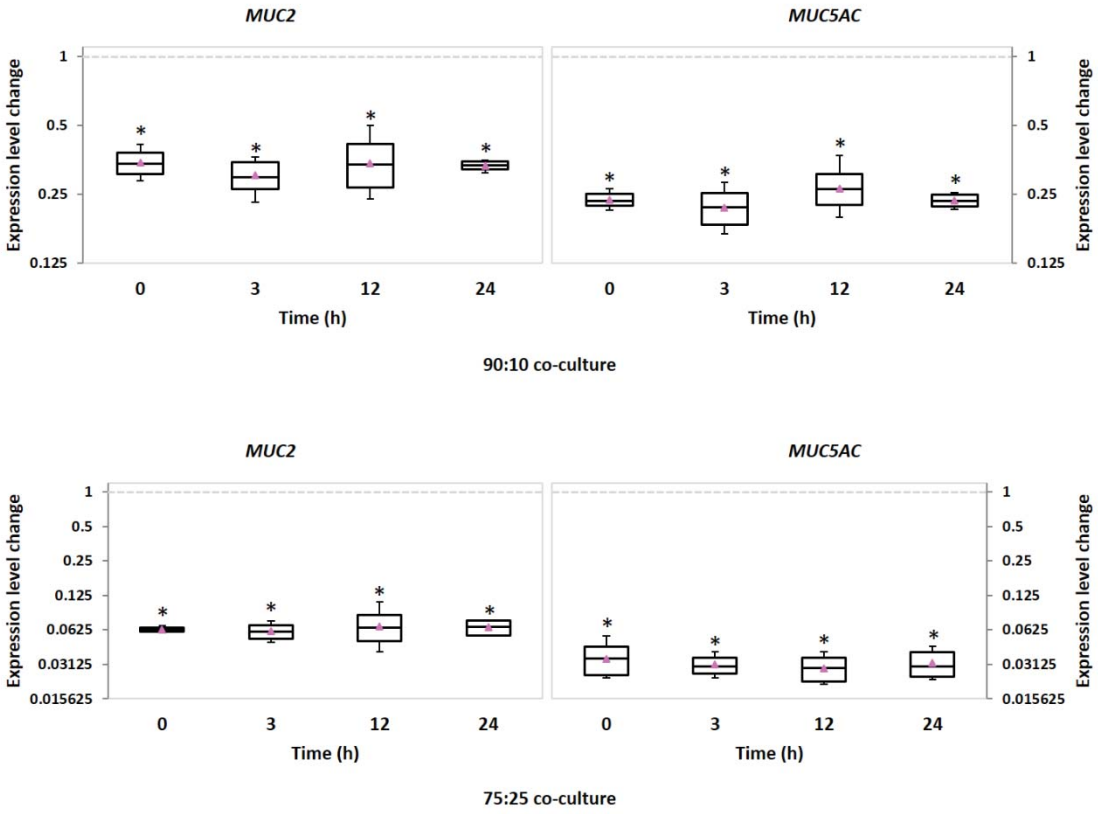


Figure 2-4 Temporal analysis of expression levels of *MUC2* and *MUC5AC* gene from Caco-2 cell mono-cultures by quantitative polymerase chain reaction

MUC2 and *MUC5AC* gene expression in Caco-2 mono-cultures (21 days post-seeding) compared to the 90:10 or 75:25 co-cultures (Caco-2:HT29-MTX respectively). Data are expressed as relative expression level change in Caco-2 mono-cultures compared to either 90:10 or 75:25 co-cultures (at the same time point (hours) which have an arbitrary value of 1 and represented by a dotted line). In the graph the pink triangle represents mean gene expression change, the central line in the box represents median gene expression change, and the upper and lower boxes represent the minimum and maximum gene expression level changes. The error bars represent the 95% confidence intervals. *Significantly different compared to respective reference cultures $P < 0.05$

In contrast, there was no difference in the expression levels of *MUC4* mRNA between Caco-2 mono-cultures and both of the co-cultures at any of the time points (Figure 2-5).

2.4.5 Mucin protein abundance

The abundance of MUC2, MUC4 and MUC5AC protein as ng/µg of total protein, and the total mucin content as a percentage of the total protein from 21 day old monolayers of Caco-2 and HT29-MTX mono-cultures and Caco-2:HT29-MTX co-cultures, was determined by indirect ELISA as described in Section 2.3.14.

2.4.5.1 Abundance of individual mucin proteins in spent media and cell lysate samples of cell cultures

The membrane-bound MUC4 mucin protein was detected in SM and CL samples from all cell cultures (Table 2-6). In Caco-2 mono-cultures MUC4 protein abundance in CL samples was significantly increased ($P< 0.05$) at successive time points with an overall increase of 12.10 (± SEM 0.14) ng/µg of total protein from time 0 to 24 hours. On average there was an increase of 0.5 (± SEM 0.04) ng/µg of total protein/hour over the 24 hour time period; although the majority of this increase occurred between 3 and 12 hours (average increase of 0.55 (± SEM 0.02) ng/µg of total protein/hour).

From CL samples of the 90:10 co-cultures there was no difference in MUC4 mucin abundance between time 0 and 3 hours or between 12 and 24 hours. However, there was a total increase of 8.26 (± SEM 1.31) ng/µg of total protein from time 0 to 24 hours. Similar to that seen for the Caco-2 mono-culture, the majority of this increase occurred between 3 and 12 hours where there was an average increase in MUC4 abundance of 0.71 (± SEM 0.15) ng/µg of total protein/hour.

MUC4 protein abundance from time 0 to 3 hours was similar for the 75:25 co-cultures. However, between 3 and 12 hours and 12 and 24 hours MUC4 protein abundance was significantly increased ($P< 0.05$). Similar to the Caco-2 mono-cultures there was an increase of 0.5 (± SEM 0.2) ng/µg of total protein/hour in CL samples but unlike the Caco-2 mono-cultures

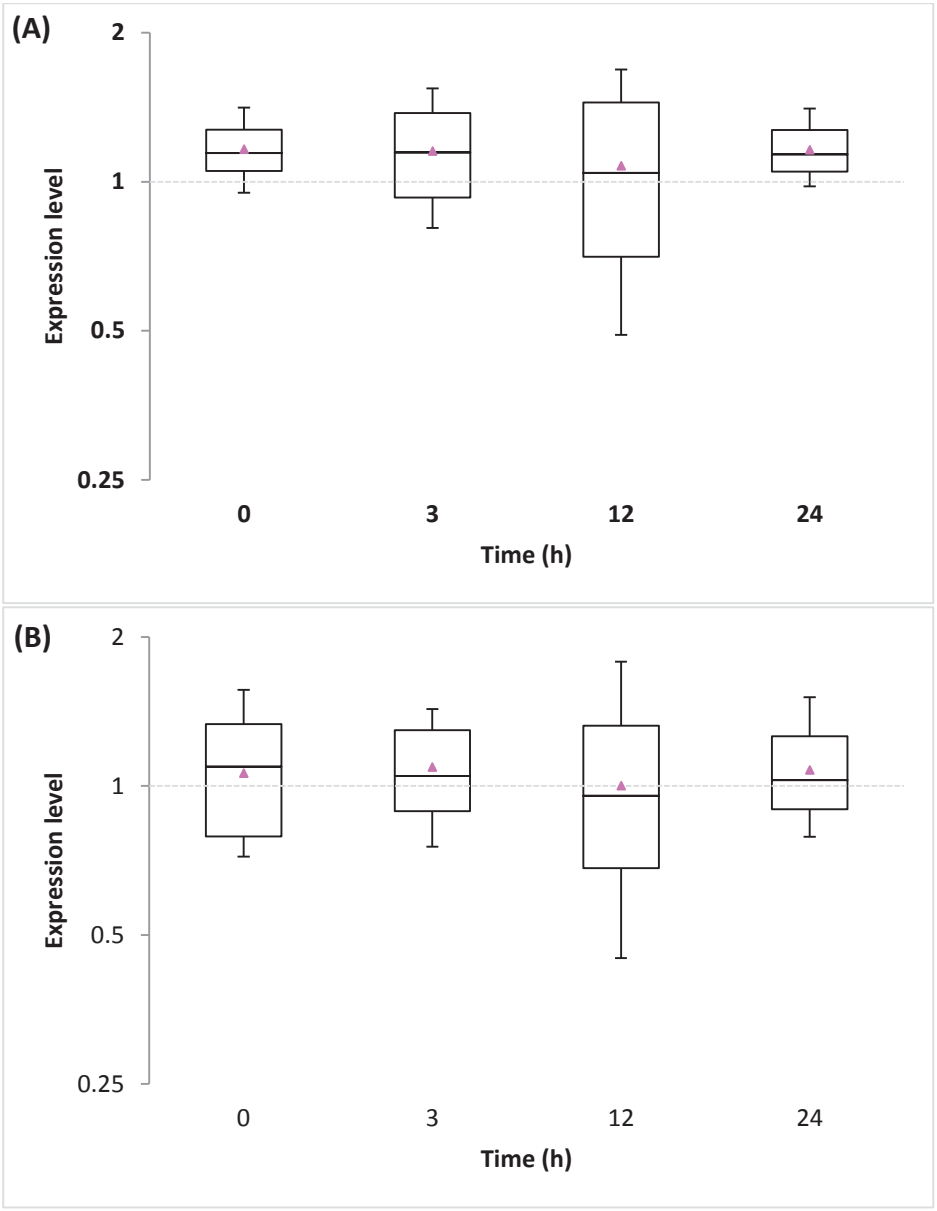


Figure 2-5 Temporal analysis of expression levels of *MUC4* gene from Caco-2 cell mono-cultures compared to the Caco-2:HT29-MTX co-cultures by quantitative polymerase chain reaction

MUC4 gene expression in Caco-2 mono-cultures compared to the 90:10 (A) or 75:25 (B) co-cultures (Caco-2:HT29-MTX respectively). Data are expressed as relative expression levels with either 90:10 or 75:25 co-cultures (given an arbitrary value of 1 for each time point and represented by a dotted line) using *HPRT1* as reference gene. In the graph the pink triangle represents mean gene expression, the central line in the box represents median gene expression, and the upper and lower boxes represent the minimum and maximum gene expression levels. The error bars represent the 95% confidence intervals.

Table 2-6 The abundance of MUC4 protein from Caco-2 and HT29-MTX mono- and co-cultures

The abundance of MUC4 protein (ng/µg of total protein) from Caco-2 and HT29-MTX mono-cultures and Caco-2:HT29-MTX co-cultures (90:10 and 75:25 Caco-2:HT29-MTX cells respectively). Cell lysate (CL) and spent media (SM) samples were collected over a 24 hour time period commencing on day 21 post-seeding and the concentration of MUC4 proteins determined by indirect ELISA. Values shown are the median, numbers in parenthesis are ranges and numbers in brackets are the mean. Values for the individual cell cultures that do not share a letter are significantly different $P < 0.05$.

Cell culture	Sample	Time			
		0h	3h	12h	24h
Caco-2	SM	ND	0.22 (0.20 – 0.23) [0.21] *a*	0.20 (0.19-0.21) [0.20] *a*	0.22 (0.21-0.22) [0.22] *a*
	CL	29.42 (29.27-29.61) [29.43] *b*	30.92 (30.71-31.04) [30.88] *c*	35.88 (35.77-35.93) [35.87] *d*	41.53 (41.37-41.68) [41.53] *e*
90:10	SM	ND	0.18 (0.17-0.21) [0.18] *a*	0.17 (0.16-0.18) [0.17] *a*	0.26 (0.25-0.27) [0.26] *b*
	CL	22.27 (20.68-23.88) [22.28] *c*	22.97 (21.35-24.55) [22.96] *c, d*	29.66 (26.20-32.02) [29.38] *e*	30.54 (30.48-30.60) [30.54] *e*
75:25	SM	ND	0.16 (0.14-0.17) [0.15] *a*	0.13 (0.12-0.15) [0.13] *a*	0.15 (0.14-0.16) [0.15] *a*
	CL	13.69 (13.40-16.26) [14.26] *b*	15.16 (14.86-17.75) [15.73] *b*	20.16 (18.28-22.67) [20.32] *c*	26.14 (26.07-26.22) [26.14] *d*
HT29-MTX	SM	ND	0.01 (0.01-0.01) [0.01] *a*	0.06 (0.05-0.07) [0.06] *b*	0.08 (0.06-0.08) [0.07] *b*
	CL	9.29 (8.98-9.60) [9.29] *c*	10.10 (9.80-10.40) [10.10] *d*	20.03 (18.96-20.37) [19.85] *e*	20.44 (20.39-20.49) [20.44] *f*

(ND = not determined)

this increase was shown to be consistent over the 24 hour time period.

Abundance of MUC4 protein in CL samples from the HT29-MTX mono-cultures was significantly increased ($P< 0.05$) at successive time points. Similar to Caco-2 mono-cultures and both co-cultures, MUC4 protein abundance increased in CL samples of the HT29-MTX mono-cultures between 3 and 12 hours with an average increase of 1.08 (± SEM 0.04) ng/µg of total protein/hour. The hourly increase in MUC4 protein abundance during this time period was significantly greater ($P< 0.01$) in comparison to all the other epithelial cell cultures.

In Caco-2 mono-cultures abundance of MUC4 protein was similar ($P> 0.05$) at all the time points in SM samples. For SM samples from the 90:10 co-cultures MUC4 abundance was significantly increased ($P< 0.05$) between the 12 and 24 hour time points while there was no difference between 3 and 12 hours. Similar to the Caco-2 mono-cultures, MUC4 in SM samples of the 75:25 co-cultures did not change over time. For the SM samples of the HT29-MTX mono-cultures MUC4 protein abundance was significantly increased ($P<0.05$) at 12 and 24 hours when compared to the 3 hour sample but were not significantly different to each other.

The secreted mucin MUC2 was not detected in either the CL or SM samples from Caco-2 mono-cultures, but was detected in both the SM and CL samples from all the other epithelial cell cultures with the highest total abundance (SM and CL sample totals combined) of MUC2 protein being recorded for the HT29-MTX mono-cultures at all the time points (Table 2-7). In this cell culture (HT29-MTX mono-culture) MUC2 protein abundance was significantly increased ($P< 0.05$) with each successive time point for SM samples and for CL samples except between 12 and 24 hours for CL samples which had comparable values. Similar to MUC4 mucin protein the majority of the increase occurred in the initial 12 hour time period. For SM samples there was an average increase of 0.12 (± 0.01) ng/µg of total protein/hour over this time period and for CL samples there was a total increase of 14.03 (± 0.12) ng/µg of total protein, which was significantly higher ($P< 0.01$) compared to that observed in the latter 12 hour period (3.31 (± 2.12) ng/µg of total protein).

Table 2-7 The abundance of MUC2 protein from Caco-2 and HT29-MTX mono- and co-cultures

The abundance of MUC2 protein (ng/µg of total protein) from Caco-2 and HT29-MTX mono-cultures and Caco-2:HT29-MTX co-cultures (90:10 and 75:25 Caco-2:HT29-MTX cells respectively). Cell lysate (CL) and spent media (SM) samples were collected over a 24 hour time period commencing on day 21 post-seeding and the concentration of MUC4 proteins determined by indirect ELISA. Values shown are the median and numbers in parenthesis are ranges. Values for the individual cell cultures that do not share a letter are significantly different P< 0.05.

Cell culture	Sample	Time			
		0h	3h	12h	24h
Caco-2	SM	ND	ND	ND	ND
	CL	ND	ND	ND	ND
90:10	SM	ND	0.34 (0.32-0.36) [0.34] *a*	0.39 (0.38-0.40) [0.39] *b*	0.61 (0.47-0.75) [0.61] *c*
	CL	6.70 (6.63-6.75) [6.70] *d*	6.95 (6.90-7.02) [6.95] *e*	6.73 (6.62-6.85) [6.73] *d*	8.12 (8.06-8.19) [8.12] *f*
75:25	SM	ND	1.30 (1.23-1.32) [1.29] *a*	1.31 (1.26-1.35) [1.31] *a*	1.37 (1.31-1.42) [1.37] *a*
	CL	10.76 (10.46-11.61) [10.90] *b*	15.69 (15.41-16.52) [15.83] *c*	34.60 (34.05-36.00) [34.81] *d*	53.01 (52.91-53.11) [53.01] *e*
HT29-MTX	SM	ND	0.93 (0.93-0.94) [0.93] *a*	1.99 (1.86-2.09) [1.98] *b*	3.12 (2.90-3.33) [3.21] *c*
	CL	47.75 (47.62-47.87) [47.75] *d*	49.33 (49.24-49.43) [49.33] *e*	61.23 (60.12-64.54) [61.78] *f*	65.34 (64.22-64.45) [65.09] *f*

(ND = not determined)

In CL samples of the 90:10 co-cultures, when compared to time 0, MUC2 protein abundance was significantly increased ($P< 0.05$) at 3 and 24 hours but was not significantly different at 12 hours. In addition, when compared to the 3 hour CL sample, MUC2 protein abundance was significantly decreased ($P< 0.01$) at 12 hours. Unlike HT29-MTX mono-cultures where MUC2 abundance was increased in the initial 12 hour period, the increase observed for the 90:10 co-cultures occurred between the 12 and 24 hour time points with an average increase of 0.12 (± 0.01) ng/µg of total protein/hour for CL samples. There was a significant increase ($P< 0.05$) in MUC2 protein abundance in SM samples at successive time points for this co-culture.

MUC2 protein abundance in CL samples of 75:25 co-cultures was significantly increased ($P< 0.05$) with each successive time point. There was an average increase of 23.92 (± SEM 0.55) ng/µg of total protein in the initial 12 hour period which was significantly higher ($P< 0.01$) compared to the increase observed in the latter 12 hour time period (18.19 (± SEM 0.86) ng/µg of total protein). However, MUC2 abundance in SM samples did not change over time, but instead; an average abundance of 1.32 (± SEM 0.06) ng/µg of total protein was detected at each time point.

Similar to MUC2, the other secreted mucin, MUC5AC, was not detected in either SM or CL samples from Caco-2 mono-cultures. The highest abundance of MUC5AC protein was recorded for the HT29-MTX mono-cultures in both the SM and CL samples, at all the time points when compared to the other epithelial cell co-cultures (Table 2-8). In SM samples from these mono-cultures there was an average increase of 0.57 (± SEM 0.14) ng/µg of total protein/hour which was consistent over the entire time period investigated. However, there was no difference in MUC5AC abundance between any of the CL samples of this mono-culture at any of the time points; instead an average MUC5AC protein abundance of 132.39 (± SEM 1.70) ng/µg of total protein was recorded at each of the time points.

Table 2-8 The abundance of MUC5AC protein from Caco-2 and HT29-MTX mono- and co-cultures

The abundance of MUC5AC protein (ng/µg of total protein) from Caco-2 and HT29-MTX mono-cultures and Caco-2:HT29-MTX co-cultures (90:10 and 75:25 Caco-2:HT29-MTX cells respectively). Cell lysate (CL) and spent media (SM) samples were collected over a 24 hour time period commencing on day 21 post-seeding and the concentration of MUC4 proteins determined by indirect ELISA. Values shown are the median and numbers in parenthesis are ranges. Values for the individual cell cultures that do not share a letter are significantly different P< 0.05.

Cell culture	Sample	Time			
		0h	3h	12h	24h
Caco-2	SM	ND	ND	ND	ND
	CL	ND	ND	ND	ND
90:10	SM	ND	1.03 (0.99-1.11) [1.04] *a*	0.96 (0.87-1.09) [0.97] *a*	1.08 (1.07-1.09) [1.11] *a*
	CL	24.14 (23.59-25.47) [24.34] *b*	25.89 (25.54-27.01) [26.08] *c*	32.95 (32.49-33.04) [32.86] *d*	39.29 (38.23-40.35) [39.29] *e*
75:25	SM	ND	0.80 (0.78-0.88) [0.83] *a*	0.81 (0.78-0.83) [0.81] *a*	0.85 (0.82-0.89) [0.85] *a*
	CL	62.67 (54.89-66.36) [61.65] *b*	66.84 (58.24-69.72) [65.41] *b*	109.32 (106.40-111.86) [109.32] *c*	112.44 (110.35-114.53) [112.44] *c*
HT29-MTX	SM	ND	2.27 (1.99-2.42) [2.24] *a*	2.87 (2.85-2.92) [2.88] *b*	3.38 (3.31-3.46) [3.38] *c*
	CL	131.64 (130.51-131.99) [131.45] *d*	131.86 (130.56-132.38) [131.67] *d*	134.21 (131.62-136.95) [134.25] *d*	132.19 (131.34-133.03) [132.19] *d*

(ND = not determined)

Unlike HT29-MTX mono-cultures, CL samples of the 90:10 co-cultures had significant increases ($P< 0.05$) in MUC5AC protein abundance at each successive time point. In the initial 3 hours (from time 0) abundance was increased by 1.75 (± SEM 0.82) ng/µg of total protein, and by the 12 hour time point this had increased again by 6.78 (± SEM 0.64) ng/µg of total protein. Overall this equated to an increase of 0.71 (± SEM 0.07) ng/µg of total protein/hour during this initial 12 hour time period. Although there was a subsequent increase in the next 12 hour period (from 12 to 24 hours) the average increase during this time period was only 0.62 (± SEM 0.03) ng/µg of total protein/hour which was a significantly lower ($P< 0.01$) increase to that observed in the initial 12 hour period. Although there were successive increases in MUC5AC abundance at the different time points for CL samples for this co-culture, the abundance of this mucin in SM samples of 90:10 co-cultures and also 75:25 co-cultures did not change over time. Although abundance of MUC5AC increased over time in CL samples from the 75:25 co-cultures it was not significantly different between time 0 and 3 hours or between 12 and 24 hours. However, abundance was significantly increased ($P< 0.05$) between 3 and 12 hours and 3 and 24 hours with an average increase of 4.87 (± SEM 0.56) and 3.22 (± SEM 0.00) ng/µg of total protein/hour during these time points respectively.

2.4.5.2 Total mucin protein abundance as a percentage of total protein

In addition to determining the abundance of the individual mucin proteins in SM and CL samples, the total abundance of mucin proteins as a percentage of the total protein was also determined. From these values it was possible to calculate the percentage contribution each individual mucin made to the total mucin protein profile and from which sample type (either SM or CL). In the 90:10 co-cultures after 24 hours the total mucin protein detected as a percentage of the total protein content (SM and CL samples combined) was 8% (Table 2-9).

Table 2-9 Mucin protein abundance as a percentage of total protein for 90:10 Caco-2:HT29-MTX co-cultures

The abundance of MUC4, MUC2 and MUC5AC mucin proteins was calculated as a percentage of the total protein detected in spent media (SM) and cell lysate (CL) samples from 90:10 Caco-2:HT29-MTX co-cultures. Mucin protein and total protein abundance was determined by indirect ELISA and BCA protein assay respectively. Values shown are the median, numbers in parenthesis are ranges and numbers in brackets are means. Values at individual time points that do not share a letter are significantly different $P< 0.05$.

Mucin protein as a percentage of total protein					
Mucin	Sample	Time			
		0h	3h	12h	24h
MUC4	SM	ND	0.02 (0.02-0.02)[0.02] *a*	0.02 (0.02-0.02) [0.02] *a*	0.03 (0.03-0.03) [0.03] *a*
	CL	2.22 (2.07-2.40) [2.23] *a*	2.30 (1.99-2.62) [2.30] *e*	2.96 (2.72-3.10) [2.93] *e*	3.05 (3.05-3.06) [3.05] *e*
	Total	2.22 (2.07-2.40) [2.23] *a*	2.32 (2.01-2.64) [2.32] *e*	2.98 (2.72-3.12) [2.95] *e*	3.08 (3.07-3.09) [3.08] *i*
MUC2	SM	ND	0.03 (0.03-0.04) [0.03] *b*	0.04 (0.04-0.04) [0.04] *b*	0.06 (0.05-0.08) [0.06] *b*
	CL	0.67 (0.67-0.67) [0.67] *b*	0.70 (0.65-0.74) [0.70] *f*	0.68 (0.64-0.70) [0.67] *f*	0.81 (0.81-0.82) [0.81] *f*
	Total	0.67 (0.67-0.67) [0.67] *b*	0.73 (0.68-0.77) [0.73] *f*	0.71 (0.68-0.74) [0.71] *f*	0.87 (0.58-0.89) [0.87] *j*
MUC5AC	SM	ND	0.10 (0.10-0.11) [0.10] *c*	0.09 (0.09-0.12) [0.10] *c*	0.11 (0.11-0.11) [0.11] *c*
	CL	2.38 (2.34-2.62) [2.40] *a*	2.57 (2.34-2.95) [2.61] *e*	3.34 (2.99-3.48) [3.29] *e*	3.93 (3.82-4.04) [3.93] *g*
	Total	2.38 (2.34-2.62) [2.40] *a*	2.67 (2.45-3.06) [2.71] *e*	3.45 (3.08-3.57) [3.39] *e*	4.04 (3.92-4.15) [4.04] *g*
Total mucin protein	SM	ND	0.15 (0.15-0.17) [0.15] *d*	0.15 (0.14-0.17) [0.16] *d*	0.20 (0.19-0.22) [0.20] *d*
	CL	5.26 (5.10-5.69) [5.30] *c*	5.56 (4.98-6.31) [5.61] *g*	6.96 (6.39-7.27) [6.89] *g*	7.80 (7.68-7.91) [7.79] *h*
	Total	5.26 (5.10-5.69) [5.30] *c*	5.71 (5.15-6.47) [5.76] *g*	7.12 (6.53-7.41) [7.05] *g*	8.00 (7.89-8.09) [7.99] *h*

ND = Not determined

Of this, an average of 3.1%, 0.9% and 4.0% was attributed to MUC4, MUC2 and MUC5AC respectively. In addition, only 0.2% of the total mucin protein detected was present in the SM samples, and thus for this epithelial cell co-culture mucin protein of the SM samples was significantly lower ($P< 0.05$) than that of CL samples. The abundance of MUC5AC protein in CL samples was similar to MUC4 at both time 0 and 12 hours, although by comparison MUC5AC was significantly increased ($P< 0.05$) at 3 and 24 hours. MUC2 had the lowest abundance ($P< 0.05$) in CL samples of this co-culture at all the time points when compared to MUC5AC and MUC4 mucin proteins.

For the Caco-2 mono-cultures (Table 2-10), although the total percentage of mucin content increased at successive time points the percentage in the SM sample did not change and only represented 0.02% of the total mucin content at each of the time points.

Similar to the Caco-2 mono-cultures the percentage of total mucin proteins detected in SM samples of the 75:25 co-cultures did not change between time points but consistently represented 0.02% of the total mucin content (Table 2-11). However, unlike the 90:10 co-cultures where the MUC5AC protein was the major contributor in the SM fraction, for the 75:25 co-cultures MUC2 was the most abundant mucin protein detected ($P> 0.05$) while MUC4 protein was the least ($P< 0.05$) at all the time points. In addition, MUC4 was the least abundant mucin protein in CL samples while MUC5AC was the most abundant mucin protein in these samples at all the time points.

The membrane-bound mucin MUC4 in SM samples from HT29-MTX mono-cultures represented only 0.001% of the total mucin detected at 3 hours, and although this was increased at 12 and 24 hours this mucin still only represented 0.01% of the total mucin detected in SM samples (Table 2-12). The secreted mucin MUC5AC was the most abundant mucin protein in SM samples for the HT29-MTX mono-cultures at both 3 and 12 hours, and was significantly higher ($P< 0.05$) than both MUC4 and MUC2.

Table 2-10 Mucin protein abundance as a percentage of total protein from Caco-2 mono-cultures

The abundance of MUC4, MUC2 and MUC5AC mucin proteins was calculated as a percentage of the total protein detected in spent media (SM) and cell lysate (CL) samples from Caco-2 mono-cultures. Mucin protein and total protein abundance was determined by indirect ELISA and BCA protein assay respectively. Values shown are the median, numbers in parenthesis are ranges and numbers in brackets are means. Values at individual time points that do not share a letter are significantly different $P< 0.05$.

Mucin protein as a percentage of total protein					
Mucin	Sample	Time			
		0h	3h	12h	24h
MUC4	SM	ND	0.02 (0.02-0.02) [0.02] *a*	0.02 (0.02-0.02) [0.02] *a*	0.02 (0.02-0.02) [0.02] *a*
	CL	2.94 (2.91-2.98) [2.94] *a*	3.09 (2.95-3.23) [3.09] *b*	3.57 (3.54-3.68) [3.59] *b*	4.15 (4.14-4.17) [4.15] *b*
	Total	2.94 (2.91-2.98) [2.94] *a*	3.11 (2.97-3.25) [3.11] *b*	3.59 (3.56-3.70) [3.61] *b*	4.17 (4.16-4.19) [4.17] *b*
MUC2	SM	ND	ND	ND	ND
	CL	ND	ND	ND	ND
	Total	ND	ND	ND	ND
MUC5AC	SM	ND	ND	ND	ND
	CL	ND	ND	ND	ND
	Total	ND	ND	ND	ND
Total mucin protein	SM	ND	0.02 (0.02-0.02) [0.02] *a*	0.02 (0.02-0.02) [0.02] *a*	0.02 (0.02-0.02) [0.02] *a*
	CL	2.94 (2.91-2.98) [2.94] *a*	3.09 (2.95-3.23) [3.09] *b*	3.57 (3.54-3.68) [3.59] *b*	4.15 (4.14-4.17) [4.15] *b*
	Total	2.94 (2.91-2.98) [2.94] *a*	3.11 (2.97-3.25) [3.11] *b*	3.59 (3.56-3.70) [3.61] *b*	4.17 (4.16-4.19) [4.17] *b*

ND = Not determined

Table 2-11 Mucin protein abundance as a percentage of total protein from 75:25 Caco-2:HT29-MTX co-cultures

The abundance of MUC4, MUC2 and MUC5AC mucin proteins was calculated as a percentage of the total protein detected in spent media (SM) and cell lysate (CL) samples from 75:25 Caco-2:HT29-MTX co-cultures. Mucin protein and total protein abundance was determined by indirect ELISA and BCA protein assay respectively. Values shown are the median, numbers in parenthesis are ranges and numbers in brackets are means. Values at individual time points that do not share a letter are significantly different $P< 0.05$.

Mucin protein as a percentage of total protein					
Mucin	Sample	Time			
		0h	3h	12h	24h
MUC4	SM	ND	0.02 (0.01-0.02) [0.02] *a*	0.01 (0.01-0.01) [0.01] *a*	0.01 (0.01-0.02) [0.01] *a*
	CL	1.36 (1.35-1.64) [1.43] *a*	2.02 (1.92-2.16) [2.03] *e*	2.00 (1.87-2.25) [2.03] *e*	2.61 (2.61-2.62) [2.61] *e*
	Total	1.36 (1.35-1.64) [1.43] *a*	2.03 (1.93-2.18) [2.05] *e*	2.01 (1.89-2.27) [2.04] *e*	2.63 (2.62-2.64) [2.63] *e*
MUC2	SM	ND	0.13 (0.12-0.13) [0.13] *b*	0.13 (0.13-0.14) [0.13] *b*	0.14 (0.13-0.14) [0.14] *b*
	CL	1.07 (1.04-1.17) [1.09] *b*	1.57 (1.47-1.73) [1.58] *e*	3.47 (3.41-3.58) [3.48] *f*	5.30 (5.29-5.31) [5.30] *f*
	Total	1.07 (1.04-1.17) [1.09] *b*	1.70 (1.59-1.86) [1.71] *e*	3.60 (3.54-3.71) [3.61] *f*	5.44 (5.42-5.45) [5.44] *i*
MUC5AC	SM	ND	0.08 (0.08-0.09) [0.08] *c*	0.08 (0.07-0.09) [0.08] *c*	0.09 (0.08-0.09) [0.09] *c*
	CL	6.09 (5.20-7.32) [6.18] *c*	6.53 (6.21-6.82) [6.52] *f*	10.93 (9.99-11.81) [10.92] *g*	11.24 (11.00-11.49) [11.24] *g*
	Total	6.09 (5.20-7.32) [6.18] *c*	6.61 (6.29-6.91) [6.60] *f*	11.01 (10.08-11.89) [11.00] *g*	11.33 (11.09-11.57) [11.33] *g*
Total mucin protein	SM	ND	0.23 (0.22-0.23) [0.23] *d*	0.22 (0.22-0.24) [0.23] *d*	0.24 (0.23-0.24) [0.24] *d*
	CL	8.56 (7.64-10.01) [8.69] *d*	10.06 (9.86-10.54) [10.13] *g*	16.36 (15.36-17.64) [16.43] *h*	19.16 (18.90-19.42) [19.16] *h*
	Total	8.56 (7.64-10.01) [8.69] *d*	10.29 (10.08-10.77) [10.36] *g*	16.58 (15.59-17.86) [16.65] *h*	19.40 (19.13-19.66) [19.40] *h*

ND = Not determined

Table 2-12 Mucin protein abundance as a percentage of total protein from HT29-MTX mono-cultures

The abundance of MUC4, MUC2 and MUC5AC mucin proteins was calculated as a percentage of the total protein detected in spent media (SM) and cell lysate (CL) samples from HT29-MTX mono-cultures. Mucin protein and total protein abundance was determined by indirect ELISA and BCA protein assay respectively. Values shown are the median, numbers in parenthesis are ranges and numbers in brackets are means. Values at individual time points that do not share a letter are significantly different P< 0.05.

Mucin protein as a percentage of total protein					
Mucin	Sample	Time			
		0h	3h	12h	24h
MUC4	SM	ND	*0.01* (0.01-0.01) [0.01] *a*	*0.01* (0.01-0.01) [0.01] *a*	*0.01* (0.01-0.01) [0.01] *a*
	CL	0.93 (0.90-0.96) [0.93] *a*	1.01 (0.98-1.04) [1.01] *e*	2.00 (1.92-2.01) [1.98] *e*	2.04 (2.04-2.05) [2.04] *d*
	Total	0.93 (0.90-0.96) [0.93] *a*	1.01 (0.98-1.04) [1.02] *e*	2.01 (1.93-2.02) [1.99] *e*	2.05 (2.05-2.06) [2.05] *d*
MUC2	SM	ND	0.09 (0.08-0.09) [0.09] *b*	0.20 (0.19-0.21) [0.20] *b*	0.31 (0.29-0.33) [0.31] *b*
	CL	4.77 (4.76-4.79) [4.77] *b*	4.93 (4.92-4.94) [4.93] *f*	6.12 (5.94-6.53) [6.18] *f*	6.53 (6.42-6.55) [6.51] *e*
	Total	4.77 (4.76-4.79) 4.77] *b*	5.03 (5.02-5.04) [5.03] *i*	6.32 (6.15-6.72) [6.38] *f*	6.84 (6.76-6.86) [6.82] *h*
MUC5AC	SM	ND	0.23 (0.20-0.24) [0.22] *c*	0.29 (0.25-0.32) [0.29] *c*	0.35 (0.27-0.37) [0.34] *b*
	CL	13.17 (12.62-13.63) [13.15] *c*	13.19 (12.67-13.63) [13.17] *g*	13.48 (12.86-13.89) [13.43] *g*	13.22 (13.17-13.27) [13.22] *f*
	Total	13.17 (12.62-13.63) [13.15] *c*	13.41 (12.91-13.83) [13.39] *g*	13.74 (13.17-14.21) [13.72] *g*	13.57 (13.44-13.65) [13.56] *i*
Total mucin protein	SM	ND	0.32 (0.29-0.34) [0.32] *d*	0.49 (0.45-0.54) [0.49] *d*	0.67 (0.57-0.72) [0.66] *c*
	CL	18.87 (18.28-19.37) [18.85] *d*	19.13 (18.57-19.61) [19.11] *h*	21.60 (21.31-21.84) [21.59] *h*	21.76 (21.73-21.83) [21.77] *g*
	Total	18.87 (18.28-19.37) [18.85] *d*	19.45 (18.91-19.91) [19.43] *h*	22.07 (21.81-22.38) [22.08] *h*	22.42 (22.33-22.55) [22.43] *j*

ND = Not determined

However, at 24 hours there was no difference in MUC5AC and MUC2 mucin proteins as a percentage of the total mucin detected in the SM samples. Similar to 75:25 co-cultures, MUC4 and MUC5AC were the least and most abundant mucin proteins respectively at all the time points in CL samples.

2.4.5.3 Comparison of total mucin as a percentage of total protein between cell cultures

Caco-2 mono-cultures had the lowest total abundance of mucin protein detected ($P < 0.05$) as a percentage of total protein at all the time points when compared to all the other epithelial cell cultures (Table 2-13). In addition, total mucin protein abundance increased as the percentage of HT29-MTX cells in the monolayers increased. For example, at 24 hours the total mucin proteins detected (as a percentage of total protein) from the HT29-MTX mono-cultures was 22.5% (± SEM 0.09) which, by comparison, was significantly higher ($P < 0.05$) than that detected for the 90:10 and 75:25 co-cultures.

2.4.5.4 Comparison between hypothetical and observed total mucin proteins detected in samples

It was originally hypothesised that mucin protein abundance of the co-cultures would be the sum of protein abundance of the individual cell types present in the cell co-cultures. Thus it was expected that the mucin proteins detected in the 90:10 co-cultures would represent 90% of that detected from the Caco-2 mono-culture and 10% of that detected in the HT29-MTX mono-culture with a similar result being obtained by the 75:25 co-cultures (except the percentage contribution from the different cell types would change to reflect the total percentage of each cell type present in the monolayer). For example (Table 2-14), at time 0 the observed total abundance of MUC4 protein was 7.21 ng and 8.66 ng in the HT29-MTX and Caco-2 mono-cultures respectively. Thus, the expected abundance of the MUC4 mucin protein at time 0 for the 90:10 co-cultures was 8.52 ng (0.721 ng from HT29-MTX cells and 7.794 ng from the Caco-2 cells) which was similar to that observed (8.93 (±0.52) ng).

Table 2-13 Total mucin protein abundance as a percentage of total protein

The total abundance of mucin proteins as a percentage of the total protein detected in samples from Caco-2 and HT29-MTX mono-cultures and co-cultures (90:10 and 75:25 Caco-2:HT29-MTX cells respectively). Mucin protein and total protein abundance was determined by indirect ELISA and BCA protein assay respectively. Values shown are the median, numbers in parenthesis are ranges and numbers in brackets are means. Values that do not share a letter are significantly different $P < 0.05$.

Total mucin protein abundance as a % of total protein				
Cell culture	Time			
	0h	3h	12h	24h
Caco-2	2.94 (2.91-2.98) [2.94] *a*	3.11 (2.97-3.25) [3.11] *a*	3.59 (3.56-3.70) [3.61] *b*	4.17 (4.16-4.19) [4.17] *c*
90:10	5.26 (5.10-5.69) [5.33] *d*	5.71 (5.15-6.47) [5.76] *d*	7.12 (6.53-7.41) [7.05] *e*	8.00 (7.89-8.09) [7.99] *f*
75:25	8.56 (7.64-10.01) [8.69] *f*	10.29 (10.08-10.77) [10.36] *g*	16.58 (15.59-17.86) [16.65] *h*	19.40 (19.13-19.66) [19.40] *i*
HT29-MTX	18.87 (18.28-19.37) [18.85] *i*	19.45 (18.91-19.91) [19.43] *i*	22.07 (21.81-22.38) [22.08] *j*	22.42 (22.33-22.55) [22.43] *j*

Table 2-14 The expected and observed abundance of individual and total mucin proteins (µg) from 90:10 and 75:25 (Caco-2:HT29-MTX) co-cultures

The observed values reflect the total abundance (± SEM) of mucin proteins as detected by indirect ELISA for each of the cell cultures. The expected mucin protein value for the co-cultures was calculated as the contribution of mucin protein that could be attributed to each cell type (Caco-2 or HT29-MTX) present in the cell co-cultures. The difference between the expected and observed abundance of mucin proteins for the co-cultures was calculated and P values attributed.

Protein	Time	Observed HT29-MTX mono-culture	Observed Caco-2 mono-culture	Expected 90:10 co-culture	Observed 90:10 co-culture	Difference 90:10 co-culture	Expected 75:25 co-culture	Observed 75:25 co-culture	Difference 75:25 co-culture
MUC4	0h	7.21 (0.27)	8.66 (0.04)	8.52 (0.06)	8.93 (0.52)	NS	8.3 (0.10)	5.61 (0.53)	P< 0.05
	3h	7.96 (0.27)	11.21 (0.05)	10.89 (0.07)	9.75 (0.55)	NS	10.4 (0.11)	6.71 (0.57)	P< 0.05
	12h	14.77 (0.50)	16.42 (0.04)	16.26 (0.09)	18.68 (1.54)	NS	16.01 (0.15)	13.68 (1.21)	NS
	24h	14.51 (0.04)	17.06 (0.07	16.81 (0.07)	17.68 (0.04)	P< 0.05	16.42 (0.06)	16.67 (0.06)	P< 0.05
MUC2	0h	37.08 (0.09)	ND	3.71 (0.01)	2.68 (0.02)	P< 0.05	9.27 (0.02)	4.29 (0.22)	P< 0.05
	3h	39.12 (0.09)	ND	3.91 (0.01)	3.02 (0.03)	P< 0.05	9.78 (0.02)	7.04 (0.23)	P< 0.05
	12h	46.14 (1.55)	ND	4.61 (0.16)	4.36 (0.07)	NS	11.54 (0.39)	23.67 (0.58)	P< 0.05
	24h	47.03 (0.37)	ND	4.7 (0.04)	4.8 (0.07)	NS	11.76 (0.09)	34.03 (0.09)	P< 0.05
MUC5AC	0h	102.07 (0.51)	ND	10.21 (0.05)	9.75 (0.33)	NS	25.52 (0.13)	24.26 (1.93)	NS
	3h	104.33 (0.60)	ND	10.43 (0.06)	11.28 (0.27)	P< 0.05	26.08 (0.15)	27.95 (2.13)	NS
	12h	100.5 (1.79)	ND	10.05 (0.18)	21.07 (0.18)	P< 0.05	25.13 (0.45)	73.54 (1.59)	P< 0.05
	24h	94.68 (0.72)	ND	9.47 (0.07)	22.88 (0.70)	P< 0.05	23.67 (0.18)	71.76 (1.54)	P< 0.05
Total mucin	0h	146.35 (0.75)	8.66 (0.04)	22.43 (0.11)	21.36 (0.43)	P< 0.05	43.08 (0.22)	34.17 (2.09)	P< 0.05
	3h	151.41 (0.82)	11.21 (0.05)	25.23 (0.13)	24.06 (0.40)	P< 0.05	46.26 (0.24)	41.7 (2.32)	NS
	12h	161.69 (1.02)	16.42 (0.04)	30.95 (0.14)	44.12 (1.71)	P< 0.05	52.74 (0.29)	110.89 (1.69)	P< 0.05
	24h	156.23 (0.54)	17.06 (0.07	30.98 (0.12)	45.36 (0.67)	P< 0.05	51.85 (0.19)	122.46 (1.69)	P< 0.05

ND = not detected; NS = not significant

However, at 24 hours the observed abundance of MUC4 protein in the 90:10 co- cultures was significantly higher ($P< 0.05$) than the expected values. In the 75:25 co-cultures the observed MUC4 protein detected was significantly lower ($P< 0.05$) than the expected abundance of MUC4 protein at time 0 and 3 hours; but at the 12 hour time point there was no difference between observed and expected values. At 24 hours there was a significant increase ($P< 0.05$) in the observed MUC4 protein detected which was similar to that seen for the 90:10 co-cultures.

Although the two secreted mucin proteins MUC2 and MUC5AC were not detected in the Caco-2 mono-cultures it was unknown what effect this may have on the abundance of these proteins in the co-cultures. At time 0 and 3 hours the observed MUC2 protein abundance was significantly lower ($P< 0.05$) than that expected for both the co-cultures; and although there was no difference in expected and observed values at 12 and 24 hours for the 90:10 co-cultures there was a significant increase ($P< 0.05$) at the same time points for the 75:25 co-cultures. There was no difference in the expected and observed values of MUC5AC protein in either of the co-cultures at time 0, or at 3 hours for the 75:25 co-cultures. However, at 12 and 24 hours there was a significant increase ($P< 0.05$) between expected and observed MUC5AC protein abundance for both co-cultures.

The observed total mucin protein abundance was significantly lower ($P< 0.05$) in both co-cultures at time 0, and were still significantly lower ($P< 0.05$) than the expected at 3 hours. At the 12 and 24 hour time points, the observed total mucin proteins detected were significantly higher ($P< 0.05$) than the expected values for both the 90:10 and 75:25 co-cultures.

2.4.6 Visualisation of the mucus layer and alkaline phosphatase activity

The intensity of alcian blue staining of monolayers pre-fixed with ethanol increased as the number of HT29-MTX cells in the cultures increased, with HT29-MTX mono-cultures staining more intensely than all other monolayers (Figure 2-6). There was limited background staining when monolayers were pre-fixed with either ethanol or methanol, although pre-fixing

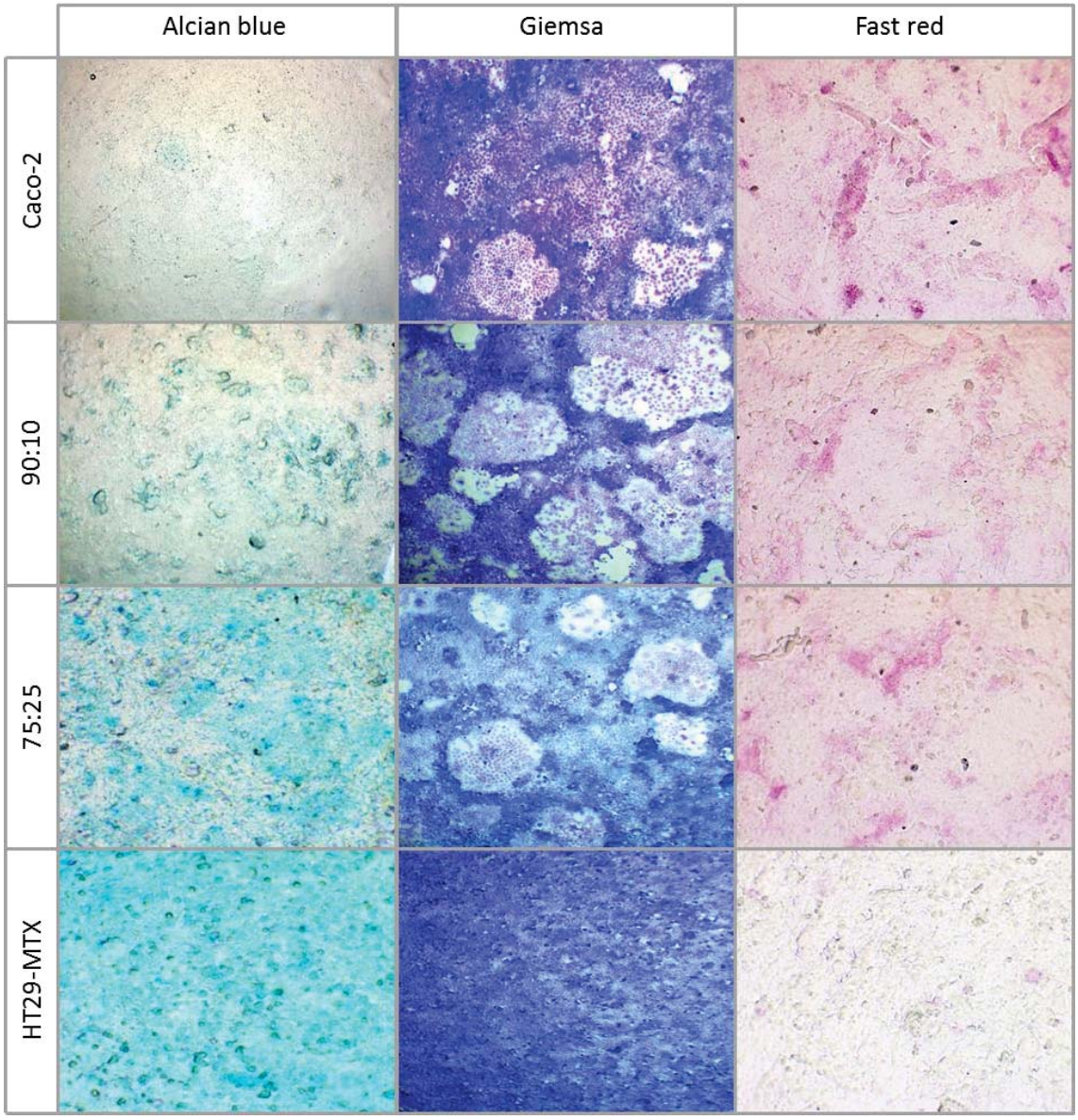


Figure 2-6 Visualisation of mucin glycoproteins and alkaline phosphatase enzyme activity

Monolayers of Caco-2, HT29-MTX and co-cultures (90:10 and 75:25; Caco-2:HT29-MTX respectively), were stained with alcian blue (pH 2.5) or Giemsa to characterise the mucus layer; or Fast red to characterise alkaline phosphatase enzyme activity after 21 days of culture. The intensity of alcian blue staining increased as the number of HT29-MTX cells in the cultures increased but in contrast, the intensity of red staining associated with alkaline phosphatase activity decreased (x4 magnification).

monolayers with methanol prior to Giemsa staining allowed for both the mucus layer and cell morphology to be visualised (Figure 2-6). The intensity of purple staining diminished as the number of HT29-MTX cells increased in the monolayers. For co-cultures, lightly stained purple/blue islands were visible which were surrounded by more intensely stained blue areas. This staining pattern was not visible in the HT29-MTX monolayers.

Alkaline phosphatase activity was detected in cell culture monolayers by cytochemistry, and used as an indicator of the Caco-2 cell proportion in the co-culture models. Alkaline phosphatase activity was restricted to the enterocyte-like Caco-2 cells with more intense staining occurring as the number of Caco-2 cells in the cultures increased (Figure 2-6). There was minimal staining of HT29-MTX monolayers.

The thickness of the mucus layer was visualised microscopically from thin transverse sections cut from the alcian blue and Giemsa stained monolayers. Although the mucus layer was clearly visible using both staining methods, the mucus layer and the cell monolayer were more clearly defined with the Giemsa staining method, with the mucus layer being more intensely stained than the cell layer (Figure 2-7). There was no mucus layer present on the surface of Caco-2 mono-cultures, and for the 90:10 co-cultures there were discontinuous, irregular patches of mucus over the surface which was approximately 2-10 µm thick. There was a continuous mucus layer covering the surface of both the 75:25 co-cultures and the HT29-MTX mono-cultures, with mucus layer thickness ranging from 5-15 µm for the 75:25 co-cultures and 7-28 µm for the HT29-MTX mono-cultures.

2.5 Discussion

The aim of the studies undertaken in this chapter was to adapt and characterise a mucin secreting population of HT29-MTX cells and incorporate them with enterocyte Caco-2 cells to cultivate an intestinal epithelial cell co-culture model that more closely simulates the cell composition and function found *in vivo*.

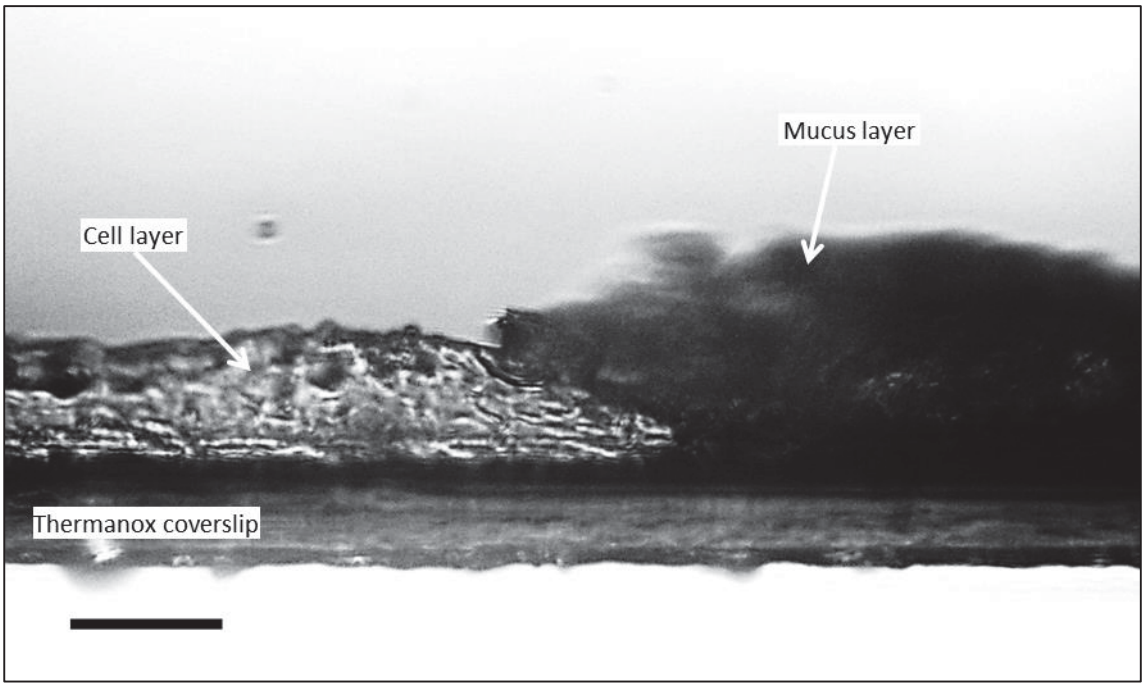


Figure 2-7 The cell and mucus layer of 90:10 co-cultures

A co-culture of 90:10 Caco-2:HT29-MTX cells grown on Thermanox coverslips for 21 days and stained with Giemsa stain. Giemsa weakly stained the cell monolayer but stained the mucus layer more intensely. The thickness of the mucus layer over the surface of these cell co-cultures was shown to be irregular, but was approximately 2-10 µm thick. Scale bar = 10 µm.

Initial investigations concentrated on characterising the growth kinetics of the Caco-2 cells and newly adapted HT29-MTX cells to determine whether comparable growth rates could be obtained with the use of different culture media. It was noted in M199 medium supplemented with 10% FBS and 1% Penicillin-Streptomycin, growth rates of the two cell types were not comparable; Caco-2 cells required an additional 13.18 ± 1.32 hours to undergo one doubling event. However, when both cell types were cultured in DMEM Glutamax supplemented with 10% FBS and 1% Penicillin-Streptomycin, Caco-2 cells only required an additional 5.30 ± 0.23 hours to undergo one doubling event. In addition, both cell mono-cultures had obtained confluency by day six and were stationary by day 10. Although doubling rates were not directly comparable, this medium provided the best option of all culture media investigated. Cell ratios of the Caco-2:HT29-MTX co-cultures at confluence were not determined in this study, but other investigators have reported that co-culturing Caco-2 and HT29-MTX cells under standard conditions resulted in a viable and relative proportionate mixture of cells from both cell lines [197].

Culture collections recommend different cell lines are cultured in different media [239]. For example, the recommended medium for the culture of Caco-2 cells is Eagle's Minimum Essential Medium, supplemented with 20% foetal bovine serum (FBS); for the HT29 cell line the recommended medium is McCoy's 5a Medium Modified, supplemented with 10% FBS; and for the HT29-MTX cell line, DMEM supplemented with 2mM Glutamine, 1% non-essential amino acids (NEAAs) and 10% FBS is the recommended medium [240]. A major difference in these culture media is the concentration of amino acids, vitamins and glucose.

2.5.1 Mucin gene and protein expression

In this study HT29-MTX mono-cultures had consistent expression levels of *MUC2* and *MUC5AC* mucin genes over a 28 day period. However, it has previously been reported that *MUC2* and *MUC5AC* are only expressed at low levels in 7 day old mono-cultures [189]. In addition, it was noted *MUC2* and *MUC5AC* mRNA from HT29-MTX mono-cultures had similar transcription

levels and this may have been related to the fact that MUC2 and MUC5AC are encoded within the same cluster at chromosome location 11p15.5 [241]. The expression levels of *MUC4* from HT29-MTX cells adapted with 10^{-6} *M* MTX over time has not previously been reported, but in this study *MUC4* expression was low in early cultures but had increased by 80% by day 21.

Little work has been undertaken to examine the relative expression of mucin genes in different cell lines and cell models and limited comparisons have been made between Caco-2, HT29-MTX and Caco-2:HT29-MTX co-cultures [225, 227]. It was originally hypothesised that mucin mRNA levels would be lower in the Caco-2 cell line in comparison to the HT29-MTX cell line, as the Caco-2 cell line is an enterocyte and not a mucin secreting goblet cell. This hypothesis was shown to be correct because expression levels of all the selected mucin genes were indeed decreased in Caco-2 mono-cultures compared to the HT29-MTX mono-cultures at all the time points investigated. This is in accordance with previous reports that indicate confluent cultures of Caco-2 cells have only very minimal, almost undetectable levels of mucin gene expression [221]. In addition, levels of mucin gene expression were lower in both the 90:10 and 75:25 co-cultures compared to the HT29-MTX mono-cultures, although it should be noted that expression levels of all target genes did not vary between the time points for any of the individual cell cultures.

In this study the secreted mucin proteins MUC2 and MUC5AC were not detected in samples from Caco-2 mono-cultures. In contrast, the MUC5AC mucin protein was recently reported to in SM and CL samples from Caco-2 mono-cultures by Wan et al [227]. Why this mucin protein was not detected in the cultures in this study may be related to the antibody used. In this study the MUC5AC antibody used was raised against a partial recombinant MUC5AC mucin protein and recognises an amino acid sequence in the C-terminal domain of the MUC5AC protein which is downstream of the GDPH cleavage site. By comparison Wan et al [227] used a MUC5AC antibody [1-13M1] that was raised against the peptide core of mucin isolated from the fluid of a human ovarian cyst and recognises the M1-a epitope of gastric mucins [242].

Although the antibodies used in the two studies recognise different regions of the MUC5AC mucin protein, the rationale for the antibody used in this study was the availability of a recombinant protein that could be utilised to quantify the abundance of this protein from the cell samples. The mono-clonal antibody used in this study may not detect MUC5AC protein from Caco-2 mono-cultures because: 1) of an absence of MUC5AC in the Caco-2 mono-cultures; 2) MUC5AC was only present at levels below the sensitivity of the ELISA; or 3) the MUC5AC mucin epitope of Caco-2 cells was not recognised by this mono-clonal antibody.

As anticipated, the total abundance of mucin proteins from the HT29-MTX mono-cultures was greater than other cell cultures. It was originally hypothesised that mucin protein abundance of the co-cultures would be the sum of protein abundance of the individual cell types present in the cell co-cultures. At 0 and 3 hours there was either no difference in the observed mucin protein abundance in the co-cultures or mucin protein abundance was considerably lower than expected values extrapolated from the individual or total mucin protein. However, at 24 hours, (except for MUC2 mucin protein in the 90:10 co-cultures) the observed mucin protein abundance (either individual mucin protein or total mucin protein) was considerably higher than expected for both the co-cultures. The increase between expected and observed values at this time point was not associated with an increase in the presence of mucin proteins in the SM samples alone. However, the contribution by Caco-2 cells to the total mucin profile of co-cultures may be enhanced when compared to the Caco-2 mono-cultures. Whether the increase in mucin protein abundance arises from only one of the cell types in the co-culture or is from an interaction between both cell types has also not been determined.

2.5.2 Barrier integrity as measured by trans-epithelial electrical resistance

In this study TEER values for Caco-2 mono-cultures did not increase from day 14 post-seeding. However, in previous studies TEER values for these cell cultures stabilise between days 3 to 10 post-seeding [201]. In addition, TEER values for both the 90:10 and 75:25 co-cultures had also stabilised by day 14 post-seeding, but were associated with a decrease in TEER with the

incorporation of HT29-MTX cells in the co-cultures in comparison to the Caco-2 mono-cultures [196, 243]. HT29-MTX mono-cultures obtained TEER values which were only just above background (19.7 ± 3.6 Ω cm^2) for all the time points investigated.

TEER values associated with Caco-2 mono-cultures may vary as much as 20 fold (80 – 1420 Ω cm^2) [220], therefore it was not possible to directly compare the TEER values obtained for the cell cultures in this study to those of published reports. Variations in TEER measurements may arise due to differing culture parameters; the passage number of the cells; the age and at what stage of differentiation the cells are at; the type of culture medium used; the density at what cells are seeded at and the type of support the cells are cultured on [244]. Despite this, previous studies have indicated that the incorporation of HT29-MTX cells into cultures of Caco-2 cells decreases TEER [46, 196, 244] and that Caco-2 mono-cultures are associated with TEER values which far exceed those found *ex vivo* [245-248].

Most of the information relating to TEER values of the human intestinal epithelium has been obtained from surgical specimens that have been removed from patients undergoing treatment for a variety of intestinal cancers [246, 249, 250]. In most instances TEER was measured in Ussing chambers from sections of the specimens which were adjacent to localised tumours. One exception recorded TEER values from jejunal samples removed from patients which were undergoing Roux-en-Y gastric bypass for morbid obesity [247]. The TEER values obtained from small intestine specimens were either from jejunal or ileal samples while the precise site of colon samples was not stated [246, 250]. Like *in vitro* TEER measurements, *ex vivo* TEER data varies for both human small intestine (8.6 - 203 Ω cm^2) [247, 250] and human large intestine (12 to 400 Ω cm^2) [248, 249], although TEER values in the human small intestine are lower than those observed in the large intestine [248].

The TEER values for the Caco-2 mono-cultures obtained in this study (average of 1067.1 ±53.5 Ω cm^2 between days 14 and 28 post-seeding) are much higher than those of the published *ex vivo* TEER values [200]. However, average TEER values (between days 14 to 28 post-seeding)

obtained for the 90:10 and 75:25 co-cultures (252 (± 24) and 151 (± 11) Ω cm^2 respectively) were comparable to *ex vivo* TEER values (8.6 to 400 Ω cm^2 [247-250]) which was in accordance with the original hypothesis. The 90:10 co-cultures, representative of the epithelial cell composition of the small intestine, had higher TEER values than the 75:25 co-cultures (cell composition representative of the large intestinal epithelium), which was in contrast to the observed *ex vivo* TEER values. The TEER values of the 90:10 co-cultures were hypothesised to be higher than that of the 75:25 co-cultures due to the presence of an increased percentage of Caco-2 cells alone. However, the discrepancy between the co-culture TEER results obtained in this study compared to published data for human small and large intestine may be due to the fact that although the cellular composition of the intestinal epithelium can be replicated *in vitro*, the architecture of the tissue itself cannot easily be modelled.

A major contrast between the *in vitro* and *in vivo* architecture is the presence of intestinal crypts and villi. What impact architecture has on TEER was not investigated in this study. However, in studies using novel 3 dimensional villous platforms (which impart the authentic size and shape of intestinal villi to *in vitro* cultures) it has been determined that TEER of Caco-2 mono-cultures are decreased when compared to the conventional 2 dimensional inserts used in most studies [251]. It was suggested TEER values were lower as a consequence of an increased number of non-differentiated Caco-2 cells along the lower section of the 3 dimensional scaffolds than were present at the tip. This cell arrangement is also known to occur in the intestinal epithelium *in vivo* where the epithelial cells originate from stem cells present in the base of the crypts and undergo proliferation and differentiation as they migrate to the intestinal surface [33]. For this study, it could be hypothesised that if there were a larger proportion of non-differentiated Caco-2 cells present in the co-cultures (as occurs in the lower parts of the crypts *in vivo*) the overall TEER may be reduced to levels observed for *ex vivo* intestinal tissue.

2.5.3 Mucus layer

In this study, staining of the epithelial cultures showed that the thickness of the surface mucus layer was different for each of the cell cultures, but in general, as the percentage of HT29-MTX cells present in the cultures increased so also did the thickness of the mucus layer. The depth of the mucus layer of the 90:10 co-cultures, seeded to mimic the cell ratio of the small intestine, was 2 to 10 µm and was shown to be discontinuous over the surface of the co-culture. This observation is in contrast to that of Mahler *et al* [207] who reported a continuous mucus layer present at the surface of similar co-cultures of Caco-2:HT29-MTX cells, although different fixation and staining methods were employed in the two studies.

The mucus layer thickness (5 to 15 µm) of the 75:25 co-cultures was greater than that of the 90:10 co-cultures but less than the mucus layer associated with the human large intestine *ex vivo* (26 to 100 µm) [86, 89, 90]. However, it has been reported that the firmly attached inner mucus layer of the human large intestine is considerably thinner than the outer loose layer [89]. For example, in the human ascending colon the thickness of the inner and outer mucus layers were measured at 4.7 and 34.4µm respectively, while in the rectum the thickness of the inner and outer mucus layer increased to 12.7 and 88.8 µm respectively [89]. Thus, the mucus layer thickness (5 to 15 µm) of the 75:25 co-cultures in this study was more similar to the reported thickness of the inner mucus layer of *ex vivo* samples [89]. This observation may indicate that the washing and fixation steps prior to the staining process of the co-cultures in this study may have resulted in the removal of the outer loose mucus layer.

2.6 Conclusion

A mucin secreting population of HT29-MTX cells has been successfully and characterised after adaptation with MTX. These cells have been incorporated with Caco-2 cells into cell co-cultures that more closely simulated the cell composition found *in vivo* as reported in the literature. The optimal time to undertake assays in which mono-cultures or co-cultures of HT29-MTX and Caco-2 cells were to be used was established as 21 days post-seeding due to cell cultures being

in stationary phase and increased expression levels of selected mucin genes of the HT29-MTX cells in comparison to younger cultures. In addition, at 21 days post-seeding the Caco-2 cells were suitably differentiated and the mucin proteins secreted by the HT29-MTX cells contributed to the formation of a mucus layer at the surface of the epithelial monolayers. TEER measurements for all cultures were stable at 21 days post-seeding and values obtained for Caco-2:HT29-MTX co-cultures were more representative of *ex vivo* studies than were Caco-2 mono-cultures. Although the thickness of the mucus layer of co-cultures was not directly comparable to the total mucus layer found *ex vivo* it was representative of the inner layer which is firmly attached to the epithelium. In conclusion it has been demonstrated that a co-culture system that more closely simulates that of *in vivo* has been successfully developed. These co-culture models could be utilised to investigate some of the interactions that occur between the intestinal epithelium and bacteria or food component, either individually or in combination, and would provide additional benefits, as compared to the mono-cultures, because resistance and mucus secretion could be studied simultaneously.

Chapter 3: Effect of a semi-purified oligosaccharide-enriched fraction from goat whey on intestinal epithelial barrier integrity and mucin production

3.1 Introduction

The major components of human breast milk are proteins, fats, carbohydrates and water and in one litre of human milk there is approximately 12 g (± 0.5) of protein, 32 g (± 2.0) of fat and 75 g (± 2.2) of carbohydrates [252, 253]. The most abundant carbohydrate in human milk is the disaccharide lactose, the concentration of which varies over lactation from 44 g/L (± 3.2) at four days postpartum, to 57 g/L (± 2.4) at 19 days postpartum [254]. In addition to lactose, the other significant carbohydrates are human milk oligosaccharides (HMOs). The concentration of HMOs also changes depending on the stage of lactation. For example, at day four postpartum the HMO concentration was 23.8 g/L (± 5.2), but at 3 months postpartum the HMO concentration had reduced to 14 g/L (± 3.2) [253]. However, others have estimated this range to be much lower at 3 to 13 g/L [254, 255]. HMOs are either neutral or acidic. Neutral oligosaccharides are comprised of a lactose core with attached galactose, glucose, fucose, *N*-acetylglucosamine or *N*-acetylgalactosamine monomers. Acidic oligosaccharides are comprised of the same monomers but have *N*-acetylneuraminic acid, or more rarely, *N*-glycolylneuraminic acid attached [256]. Although HMOs are only minimally digested by enzymes of the upper gastrointestinal tract [257] they are partially absorbed and appear in the urine of breast-fed infants [258, 259]. It has been demonstrated that neutral HMOs use transcellular as well as paracellular pathways to cross the intestinal epithelium, whereas acidic HMOs only cross via the paracellular pathway [260].

Due to structural similarities to host cell surface receptors and mucin proteins, HMOs can prevent bacterial adhesion to intestinal cells and may protect breast-fed infants against microbial infections [174]. HMOs may also interfere with host protein-carbohydrate interactions such as those of galectins (galactoside-binding lectins), and may ultimately disrupt cell growth, proliferation and apoptosis, as well as cell-cell and cell-matrix interactions [261].

Neutral and acidic HMOs can effect cell proliferation of Caco-2 and HT29 intestinal cells [262]. For example, neutral HMOs reduced proliferation rates of non-confluent, non-differentiated

Caco-2 and HT29 cells at concentrations of 0.34 and 2.33 mg/mL respectively after 72 hours of incubation; while acidic HMOs inhibited Caco-2 and HT29 cell growth at concentrations of 0.71 and 0.29 mg/mL [262]. In another study, caspase-3 activity (used as a marker of apoptosis) of post-confluent, differentiated Caco-2 cultures was increased after incubation with the HMO 6' sialyl lactose and the neutral HMO lacto-N-neotetraose [263]. In addition, epithelial barrier integrity of Caco-2 mono-cultures was reduced after 90 minutes incubation with 5 mg/mL neutral and acidic HMOs [260].

Although some HMOs can be chemically synthesised, an alternative source of natural lactose-derived oligosaccharides is from ruminant milk [168]. The composition of ruminant milk varies between different species and breeds, and also throughout lactation [264]. Goat milk contains substantially less oligosaccharides than human milk at 0.25 to 0.30 g/L but has 5-8 times more than cow's milk (0.03 to 0.06 g/L) and 10 times more than sheep's milk (0.02 to 0.04 g/L) [168]. Although milk from these ruminant species contains a variety of neutral and acidic oligosaccharide structures, the overall oligosaccharide profile of goat milk is more similar to human milk than either cow's or sheep's milk [168].

Oligosaccharides extracted from goat milk have normalised the expression levels of selected mucin genes in a dextran-sodium-sulfate (DSS)-induced rat model of colitis [30]. In contrast, another study determined oligosaccharides obtained from goat milk could modulate the expression of selected mucin genes and trefoil factors in HT29-MTX mono-cultures [195]. However, these studies did not investigate the effects of these milk constituents on mucin protein abundance and thus the relationship between mucin gene expression and mucin protein abundance was not characterised. Although previous *in vitro* studies have investigated the effects of oligosaccharides on cellular proliferation, transport mechanisms and mucin gene expression, the cellular models used were only representative of one cell type and thus may not be a true reflection of the impact oligosaccharides have on the intestinal epithelium *in vivo*.

3.2 Hypothesis and Aims

The main hypothesis for the research presented in this chapter is that an OEF from goat whey will enhance epithelial barrier integrity, mucin mRNA levels and mucin protein abundance and therefore have the potential to improve the physical intestinal barrier by increasing barrier integrity and the surface mucus layer.

In order to address the hypothesis two specific aims have been devised using the Caco-2 and HT29-MTX intestinal epithelial cell mono-cultures and co-cultures characterised in the previous chapter. The first aim is to characterise the impact of OEF on the metabolic activity and epithelial barrier integrity as measured by TEER of intestinal epithelial cell mono- and co-cultures, with the overall aim of selecting a concentration of OEF to use in further *in vitro* assays. The second aim of the research is to characterise the modulatory effects of OEF at the selected concentration on the levels of mucin gene expression and mucin protein abundance of the intestinal epithelial cell mono- and co-cultures.

3.3 Methods and Materials

3.3.1 Composition of oligosaccharide-enriched fraction

The OEF was isolated from goat whey (Over the Moon Dairy Company, Putaruru, Waikato, New Zealand) using the following method (Figure 3-1). Briefly, excess solids (proteins and fats) were removed from liquid goat whey by centrifugation (25,000 x g, 10 minutes 4°C). After removal of additional proteins by ultrafiltration using a 10 kDa membrane (Kvick lab self-contained unit, polyethersulfone membrane with 10,000 select pore size, GE Healthcare Limited, Auckland, New Zealand) the preparation was treated with β-galactosidase enzyme from *Aspergillus oryzae* (Sigma-Aldrich, St Louis, Missouri, USA) to hydrolyse lactose.

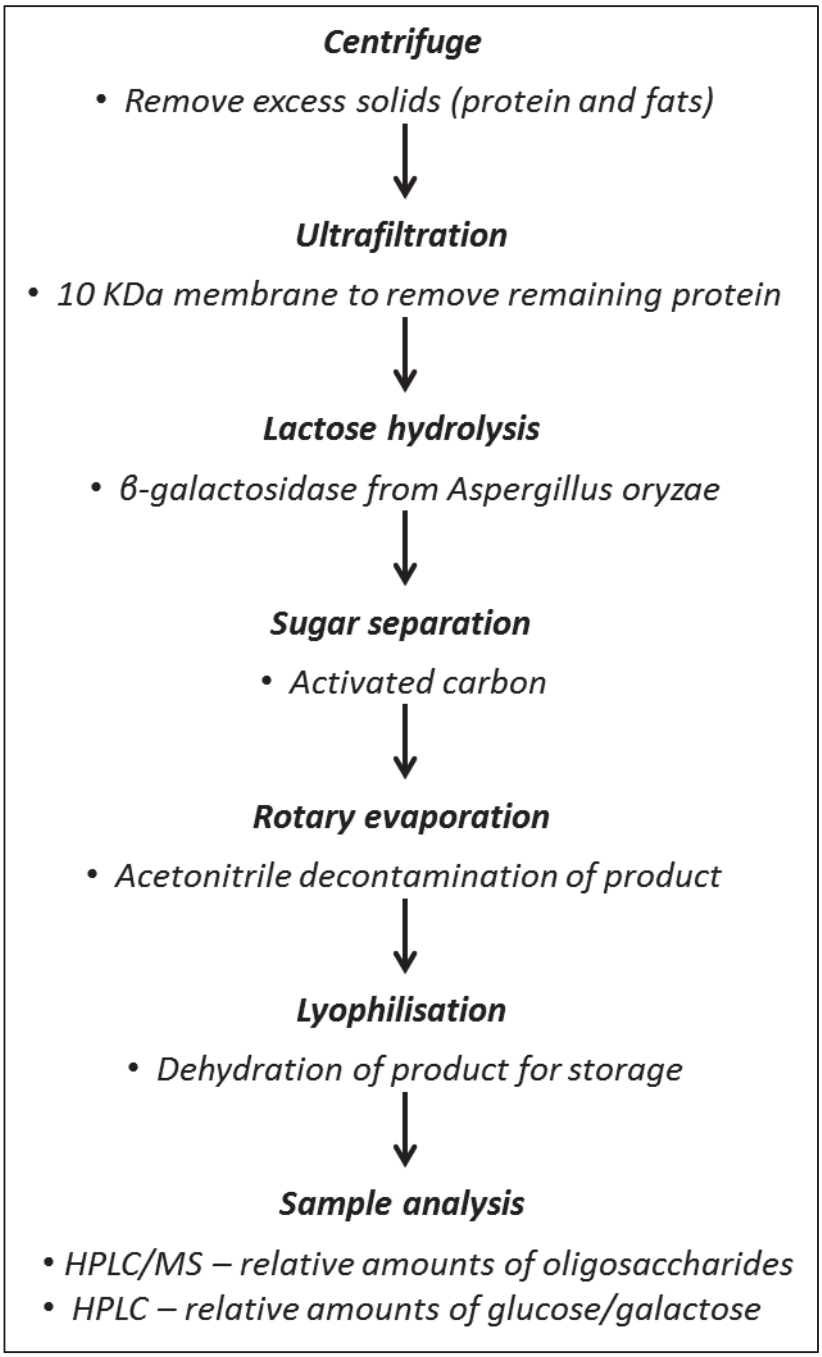


Figure 3-1 Flow chart depicting the main steps involved in the isolation of oligosaccharides from liquid goat whey

The β-galactosidase enzyme was added to the filtered preparation (2400 units/L (~270 mg/L, 8.9 units/mg)) and placed on a rotary shaker at 110 rpm for 24 hours at 40°C. After the preparation had cooled to room temperature the pH was increased to pH 7.5 with 500 μM sodium hydroxide (NaOH). After additional cooling to 4°C, the preparation was again centrifuged (25,000 x g for 10 minutes at 4°C), the supernatant containing mono- and oligosaccharides was collected and the pellet discarded. Glucose and galactose produced from lactose hydrolysis were separated from the oligosaccharides in the preparation utilising a column made from the union of a Pyrex® Brand 36060 fritted 3L funnel and a 2L Schott Duran bottle loaded with activated carbon media (activated charcoal organic refill trap (Grace Davison, Alltech no: 8124) Thermo Fisher Scientific New Zealand Limited, Albany, North Shore City, New Zealand) (Figure 3-2). The oligosaccharides bind to the activated carbon media, while glucose and galactose do not. The bound oligosaccharides were removed from the carbon media with 40% acetonitrile (Merck, Palmerston North, New Zealand), and after removal of the acetonitrile by rotary evaporation the fraction was lyophilised.

The carbohydrate composition of goat whey prior to purification and the OEF after purification (Table 3-1) and the oligosaccharide profile of the enriched fraction (Table 3-2) were determined by high-performance liquid chromatography (HPLC) analysis and HPLC-mass spectrometry.

3.3.2 High-performance liquid chromatography and mass spectrometry

For HPLC/MS analysis the protocol as described in [265] was used. Samples (20 μL) were injected using a high throughput system prep and load (HTS PAL) autosampler with a 25 μL syringe and a 15,000-psi injection valve (CTC Analytics, Zwingen, Switzerland). Two high pressure gradient JASCO X-LC 3085PU HPLC pumps (JASCO International, Tokyo, Japan) were used, pumping MilliQ water (solvent A) and acetonitrile (HPLC-grade; Merck, Palmerston North, New Zealand) (solvent B).

Figure 3-2 Charcoal media and vacuum system utilised to separate sugars

The sugars glucose and galactose produced from lactose hydrolysis were separated from the oligosaccharides in the preparation utilising a column made from the union of a 3L funnel and a 2L Duran bottle loaded with activated carbon media.

Table 3-1 Carbohydrate composition of goat whey and the oligosaccharide-enriched fraction obtained from goat whey

The carbohydrate composition, as a percentage of total carbohydrates, of goat whey and an oligosaccharide-enriched fraction obtained from goat whey before and after purification. Oligosaccharide content was determined by LC/MS analysis while glucose and galactose content was determined from HPLC analysis.

Carbohydrate	Before (%)	After (%)
Galacto-oligosaccharide (GOS)	5.8	0.4
Lactose	89.0	46.1
Glucose	0.0	12.0
Galactose	4.4	15.9
Oligosaccharides	0.8	25.6

Table 3-2 Abundance of oligosaccharides as a percentage of total oligosaccharides present in the enriched fraction obtained from goat whey

Oligosaccharide common name	Type of oligo	Percentage
α-3′-Galactosyl-lactose or β-6′-Galactosyl-lactose	Neutral	15
6′-N-acetylglucosaminyl-lactose	Neutral	3
6'-Sialyl-lactose or 3'-Sialyl-lactose	Acidic	22
6'-Glycolyl-neuraminyl-lactose	Acidic	42
Disialyl-N-lactose	Acidic	18

Oligosaccharides were separated on a Thermo Hypercarb column (100 X 2.1 mm, 5 μm particle size, Thermo Fisher Scientific, Waltham, MA, USA), running the following program at a flow rate of 0.4 mL/min: initial 1% B hold for 0.5 min, to 10% B after 1 min, to 16% B at 6 min, to 20% B at 12 min, to 30% B at 16 min, to 90% B at 18 min, hold to 20 min, and back to initial condition for 5 minute equilibration.

Mass spectrometry (MS) analyses were performed using a linear trap quadrupole (LTQ) ion trap mass spectrometer (Thermo Fisher Scientific) with electrospray ionisation in negative mode. Data was collected in profile data acquisition mode over the mass range from 300 to 2000 and was processed using the Xcalibur software package provided by the manufacturer.

Samples were analysed for monosaccharides using a Shimadzu RID 10A HPLC (Shimadzu Oceania Ltd., Auckland, New Zealand) fitted with a Bio-Rad Aminex HPX 87H HPLC column (column maintained at 45˚C). The mobile phase was sulfuric acid (5 mM) using an isocratic elution with a flow rate of 0.8 mL/minute. Injected volumes were 50 μL and a run time of 20 minutes between injections. Monosaccharides in samples were quantified on the basis of known amounts of glucose and galactose standards (Shimadzu Oceania, Pickering Laboratories, USA), using LC Solution ver. 1.22 SP1 software (Shimadzu, Kyoto, Japan).

3.3.3 Stock solutions of oligosaccharide-enriched fraction for intestinal epithelial cell based assays

The OEF for all epithelial cell culture experiments was suspended in phosphate buffered saline (PBS) pH 7.2, and filter sterilised (0.22 μm filters; Millipore Australia Pty Ltd, NSW, Australia). In these stock solutions the oligosaccharide component was present at a concentration of 50 mg/mL. For cell based assays the stock OEF solution was diluted with tissue culture medium until the desired concentration was obtained for the assay. In each assay, for control, unstimulated cells an equal volume of PBS (pH 7.2) to that found in treated samples was added to the culture medium.

3.3.4 Measurement of metabolic activity of intestinal epithelial cell cultures

The metabolic activity of Caco-2, HT29-MTX and co-cultures of Caco-2:HT29-MTX cells were determined by 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3 benzene disulfonate (Wst-1) colourimetric assay (Roche, Auckland, New Zealand). This assay is based on the cleavage of the tetrazolium salt Wst-1 to formazan by mitochondrial dehydrogenases (part of the respiratory chain) in viable cells. The amount of formazan dye produced is a measure of respiration and metabolic rate. Wst-1 reagent is light sensitive, and cell metabolism is temperature sensitive, so care was taken to reduce light incubation and cultures were maintained at 37°C throughout. The yellow-orange coloured fraction, stable soluble formazan, was quantified by its absorbance at 450 nm with a reference wavelength of 650 nm (FlexStation 3 Benchtop Multi-Mode Microplate Reader; Molecular Devices, Sunnyvale, California, USA).

Caco-2 and HT29-MTX cells were harvested from 75 cm^2 flasks as described in Section 2.3.3. After the concentration of cells in the suspensions was determined (Section 2.3.6) cells were seeded into 96-well plates at a density of 6.3 x 10^4 cells/cm^2. Non-confluent (2 days post-seeding) mono-cultures of Caco-2 and HT29-MTX cells and confluent (21 days post-seeding) monolayers of Caco-2, HT29-MTX and co-cultures of Caco-2:HT29-MTX cells were incubated with increasing concentrations of OEF at 0.1, 0.25, 0.5 and 1 mg/mL. Control groups had culture media supplemented with PBS at an equal volume to OEF-exposed cultures or media supplemented with 5% (v/v) DMSO. DMSO was used as a negative control as it induces cell-lysis [266]. All cells were cultured in the presence of test solutions for 24 or 72 hours at 37°C in an atmosphere of 5% CO_2 in air. In the last hour of incubation the medium was replaced with medium containing Wst-1 reagent (1:10 medium: Wst-1 reagent). Additional wells in each plate containing media and Wst-1 reagent only (without cells) were processed in parallel and used as reference blanks [224, 267]. Absorbance was read at 450 nm with a reference wavelength of 650 nm. Cell metabolic activity was expressed as a percentage of the control

PBS supplemented group. Metabolic activity was calculated from three experiments (three successive passages of cells), each with three replicates per group.

3.3.5 Calculation of coefficient of variation

The intra plate percentage coefficient of variation (CV) was calculated using Equation 3.1 and reflected the within plate variation. The inter plate variation was calculated using the same equation but was the mean and standard deviation for the three replicate plates per time point for each test compound and concentration. The recommended acceptance criteria of variability for *in vivo* or cell based assays is 20 to 50% which is higher than that for enzyme assays (recommended < 10%) [268]. In this study the acceptance criteria for the intra and inter assay % CV was 20%.

Equation 3.1 $$\% CV = \left(\frac{SD}{AVG}\right) \text{x } 100$$

Where SD = standard deviation, and AVG = average

3.3.6 Trans-epithelial electrical resistance assay

Caco-2 and HT29-MTX cells were harvested (Section 2.3.3), and cells counted (Section 2.3.6). Caco-2, HT29-MTX and co-cultures of Caco-2:HT29-MTX cells (90:10 and 75:25 respectively) were seeded onto 12 mm diameter, 0.4 µm^2 pore size, PET Transwell inserts (Corning, Lindfield, New South Wales, Australia) at a density of 6.3 x 10^4 cells/cm^2 in low glucose DMEM Glutamax supplemented with 10% FBS and 1% Penicillin-Streptomycin.

Confluent monolayers were prepared 24 hours before (day 20 post-seeding) the TEER assay by removing the media, washing with PBS (pH 7.2) and adding non-supplemented low glucose DMEM Glutamax (without FBS and penicillin-streptomycin) to the basal and apical compartments. After 24 hours incubation in non-supplemented media, initial resistance readings were obtained for all monolayers, after which point the media was replaced with PBS supplemented media (control), or OEF at increasing concentrations (0.1, 0.25, 0.5 and 1 mg/mL) suspended in culture medium. The resistance across each cell monolayer was measured and TEER calculated as described in Section 2.3.10; while the percentage change in

TEER compared to initial TEER for each insert was calculated using Equation 3.2

Equation 3.2 $$\% \, Change \, in \, TEER = \left((TEER \div initial \, TEER) \times 100 \right) - 100$$

TEER was calculated from three experiments (three successive passages of cells), each with three replicates per treatment group.

3.3.7 Mucin gene expression of intestinal epithelial cell cultures

Caco-2 and HT29-MTX cells were harvested (Section 2.3.3), and cells counted (Section 2.3.6). Caco-2, HT29-MTX and co-cultures of Caco-2:HT29-MTX cells (90:10 and 75:25 respectively) were seeded into 12 well cell culture plates at a density of 6.3 x 10^4 cells/cm^2 in low glucose DMEM Glutamax supplemented with 10% FBS and 1% Penicillin-Streptomycin.

Twenty days post-seeding monolayers were prepared as described in Section 2.3.11. After an additional 24 hours culture in non-supplemented medium the SM was removed and monolayers washed gently four times with PBS. Pre-warmed, PBS-supplemented (control) culture medium or medium supplemented with 1 mg/mL OEF was gently added to the monolayers and cultures returned to the incubator. At designated time points (3 and 12 hours) RNA was extracted as described in Section 2.3.12.1. cDNA synthesis and qPCR analysis was completed as described in Section 2.3.12.2. Changes in mucin gene expression was calculated from three experiments (three successive passages of cells), each with three replicates per treatment group. Each sample was analysed in triplicate.

3.3.8 Mucin protein abundance as determined by indirect ELISA

The total production of mucins consists of both mucins in the CL and SM. Therefore, in the present studies, the total production of MUC2, MUC4 and MUC5AC mucin protein was calculated as the sum of the mucin protein in CL and SM.

Caco-2 and HT29-MTX cells were harvested (Section 2.3.3), and cells counted (Section 2.3.6). Caco-2, HT29-MTX and co-cultures of Caco-2:HT29-MTX cells (90:10 and 75:25 respectively) were seeded into 12 well cell culture plates at a density of 6.3 x 10^4 cells/cm^2 in low glucose DMEM Glutamax supplemented with 10% FBS and 1% Penicillin-Streptomycin.

Twenty days post-seeding, confluent, differentiated, monolayers were prepared as described in Section 2.3.11. After an additional 24 hours culture in non-supplemented medium (no FBS and no Penicillin-Streptomycin) the SM was removed and monolayers washed gently four times with PBS. Pre-warmed, PBS supplemented (control) culture medium or medium supplemented 1 mg/mL OEF was gently added to the monolayers and cultures returned to the incubator. After 3 and 12 hours incubation, SM and CL samples were collected. The total protein concentration in samples was determined using the BCA protein assay kit (Thermo Fisher Scientific New Zealand Limited, Albany, North Shore City, New Zealand) according to manufacturer's instructions and using BSA as standards. The abundance of MUC2, MUC4 and MUC5AC mucin protein abundance was measured by ELISA as described in Section 2.3.14. Differences in the mean mucin protein abundance (± standard error) were calculated as the percentage change compared to untreated control monolayers. The abundance levels of the individual mucin proteins (MUC4, MUC2 and MUC5AC) were calculated from three experiments (three successive passages of cells), each with three replicates per treatment group. Each sample was analysed in duplicate by indirect ELISA.

3.3.9 Statistical analysis

The qPCR data was normalised to the reference gene (*HPRT1*) and analysed for expression level changes using Relative Expression Software Tool (REST) 2009 software (version 2.0.13; Qiagen). All other data analysis was undertaken using the Minitab statistical package (Minitab 16 Statistical Software (2010) State College, PA Minitab, Inc.). Data from the Wst-1 assay and ELISA were first evaluated for normality and homogeneity of variance with the Anderson-Darling and Levene's tests. One-way analysis of variance (ANOVA) with the Kruskal-Wallis test, followed by the Mann-Whitney U test, was used to identify significant differences for non-parametric data (all Wst-1 data except when oligo fraction was included and all ELISA data). The results were expressed as the median ± inter quartile range (IQR) of three individual experiments. One-way ANOVA with Tukey's multiple comparison test was used for analysing

parametric data (Wst-1 comparison of non-confluent and confluent monolayers incubated with OEF). The results were expressed as mean ± standard error of mean (SEM) of three individual experiments. Comparisons of TEER measurements between treatments were determined using two-way ANOVA. Differences were considered statistically significant when probability values were less than 0.05 ($P < 0.05$)

3.4 Results

3.4.1 Metabolic activity of intestinal epithelial cell monolayers

Non-confluent, non-differentiated (day 2 post-seeding) HT29-MTX and Caco-2 cells and confluent, differentiated (day 21 post-seeding) monolayers of HT29-MTX, Caco-2 and co-cultures of HT29-MTX/Caco-2 cells were incubated with increasing concentrations of OEF for 24 and 72 hours and the metabolic activity of the cells determined by the Wst-1 assay.

3.4.1.1 Metabolic activity of non-confluent Caco-2 and HT29-MTX mono-cultures

There was no significant difference in absorbance and consequently metabolic activity between the time points for non-confluent, control Caco-2 and HT29-MTX mono-cultures. However, the absorbance of Caco-2 mono-cultures was significantly lower ($P < 0.05$) than that observed for the HT29-MTX mono-cultures at 24 and 72 hours (Figure 3-3). As expected, incubation with 5% (v/v) DMSO significantly decreased ($P < 0.05$) the metabolic activity of Caco-2 mono-cultures at both time points compared to control untreated cells. However, the metabolic activity of HT29-MTX mono-cultures was only significantly decreased ($P < 0.05$) after 72 hours incubation with 5% DMSO (Figure 3-3). The intra and inter plate percentage coefficient of variation for non-confluent Caco-2 and HT29-MTX mono-cultures was below the 20% variation acceptance level for all the time points and cultures.

For Caco-2 mono-cultures metabolic activity was significantly increased ($P < 0.05$) after 72 hours incubation with the OEF at all concentrations except 0.25 mg/mL (Figure 3-4A). Incubation with the OEF at all concentrations for 24 or 72 hours had no significant effect on metabolic activity of the HT29-MTX mono-cultures when compared to control (Figure 3-4B).

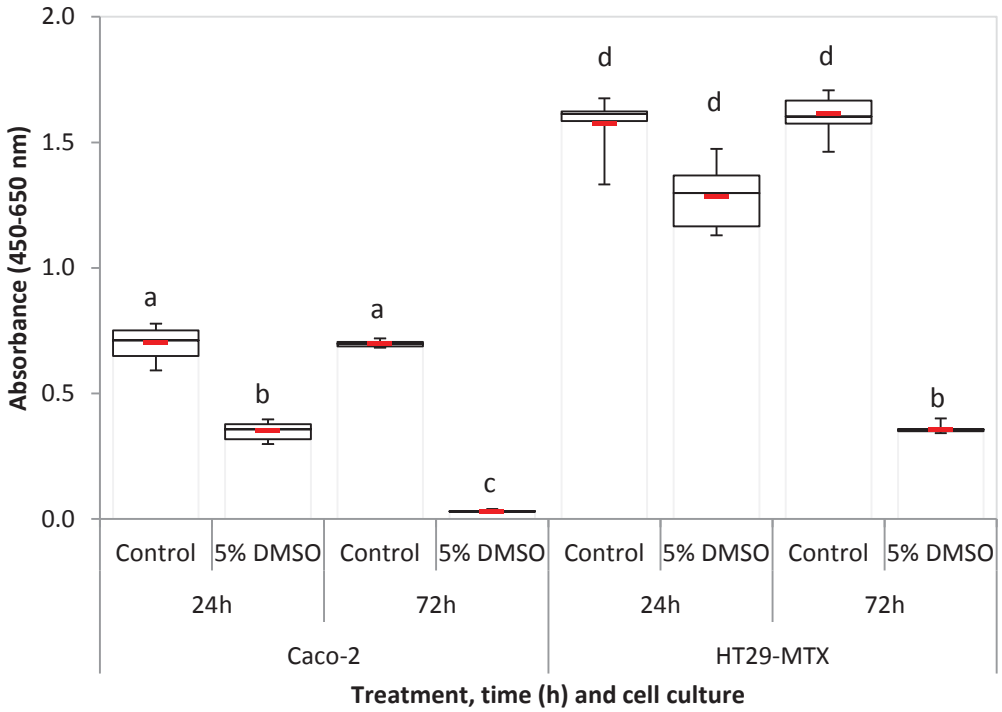


Figure 3-3 Temporal change in absorbance of non-confluent, control HT29-MTX and Caco-2 mono-cultures

The absorbance of non-confluent (2 days post-seeding) Caco-2 and HT29-MTX mono-cultures in PBS control non-supplemented medium or medium supplemented with 5% dimethylsulphoxide (DMSO) measured after Wst-1 assay. In the graph the red line represents mean absorbance; the central line in the box represents the median absorbance, and the upper and lower boxes represent the upper and lower interquartile range. The whiskers (error bars) represent the minimum and maximum absorbance values. Values which do not share a letter are significantly different ($P < 0.05$).

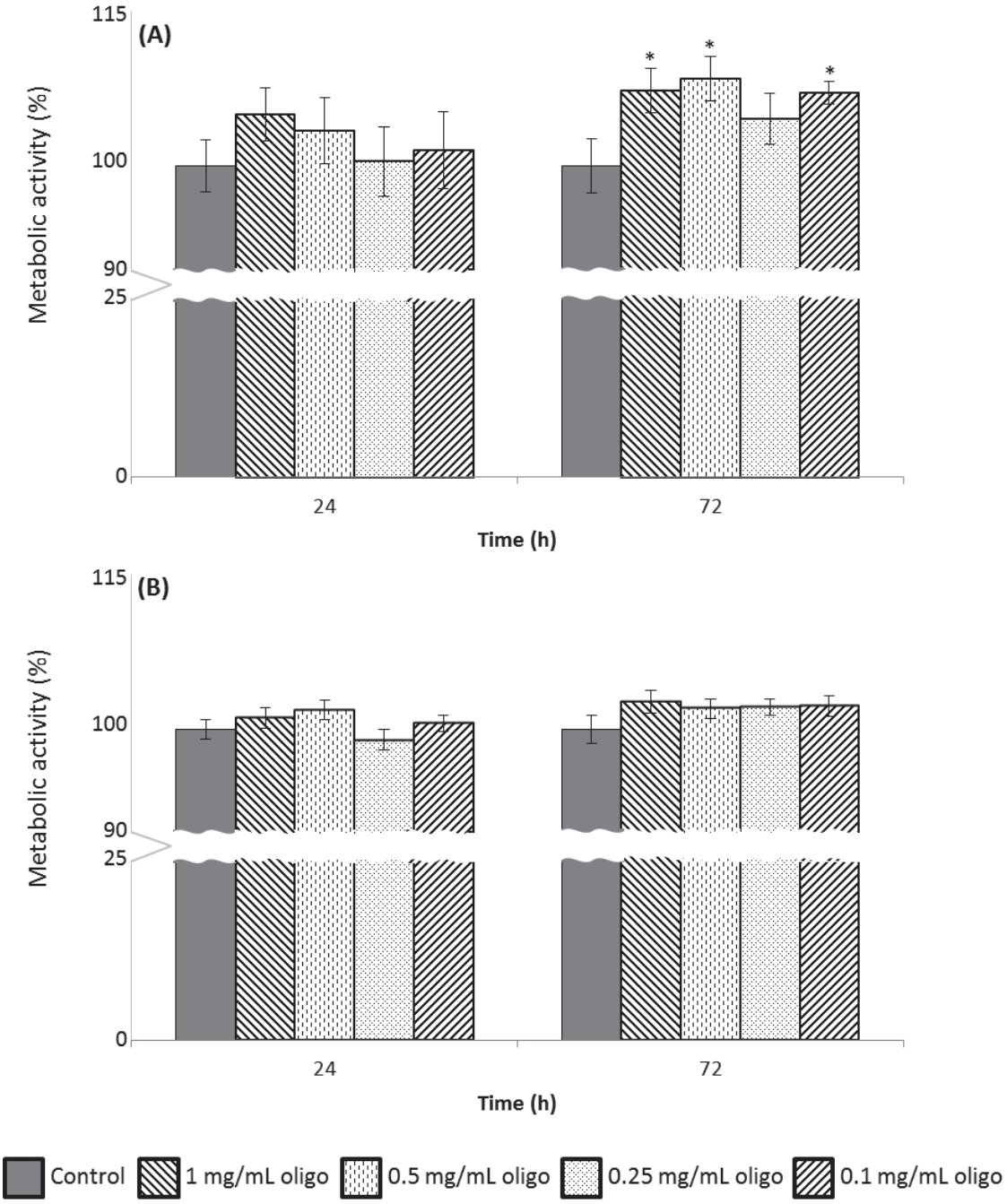


Figure 3-4 Effect of incubation with an oligosaccharide-enriched fraction on metabolic activity of non-confluent Caco-2 and HT29-MTX mono-cultures

The metabolic activity of non-confluent (2 days post-seeding) Caco-2 (A) and HT29-MTX (B) mono-cultures as a percentage of control (control has an arbitrary value of 100%) measured after Wst-1 assay. To visualise parts of the data in more detail the y axis is broken. Values are means (± SEM) for 3 experiments (n = 12; 4 samples per treatment per experiment). *Different from respective control at each time point $P< 0.05$.

3.4.1.2 Metabolic activity of confluent mono-and co-cultures of Caco-2 and HT29-MTX cells

There was no significant difference in metabolic activity as measured by absorbance between the time points for the individual confluent monolayers regardless of the cell composition (Caco-2, HT29-MTX and Caco-2:HT29-MTX cell co-cultures) (Figure 3-5). However, absorbance was significantly higher ($P< 0.05$) for confluent, control, HT29-MTX mono-cultures compared to all the other control cell cultures at the same time points (Figure 3-5). Incubation with 5% DMSO (v/v) significantly decreased ($P< 0.05$) the metabolic activity of confluent cultures at each time point compared to control cultures, except HT29-MTX mono-cultures after 24 hours incubation. The intra and inter plate/assay percentage CV for all confluent monolayers of Caco-2 mono-cultures, 90:10 and 75:25 co-cultures was less than 8% at any time point, while for HT29-MTX mono-cultures there was less than 3% variation.

There was no significant difference in the metabolic activity of confluent mono-cultures of Caco-2 and HT29-MTX cells and co-cultures of Caco-2:HT29-MTX after 24 hours incubation with any concentration of the OEF compared to control (Figure 3-6). After 72 hours metabolic activity was significantly increased ($P< 0.05$) for Caco-2 mono-cultures incubated with OEF at concentrations more than or equal to 0.5 mg/mL compared to control.

3.4.1.1 Comparison of the metabolic activity of non-confluent and confluent mono-cultures

The decrease in metabolic activity of non-confluent mono-cultures incubated with 5% DMSO was significantly greater ($P< 0.05$) than confluent mono-cultures at successive time points (Figure 3-7). For example, the metabolic activity of non-confluent Caco-2 mono-cultures incubated with 5% DMSO for 24 hours was approximately 50% that of control cells, which in contrast was significantly lower ($P< 0.05$) than confluent Caco-2 mono-cultures (71%) (Figure 3-7). A similar observation was made for HT29-MTX mono-cultures and after 72 hours incubation with 5% DMSO, metabolic activity was 22% and 92% for non-confluent and

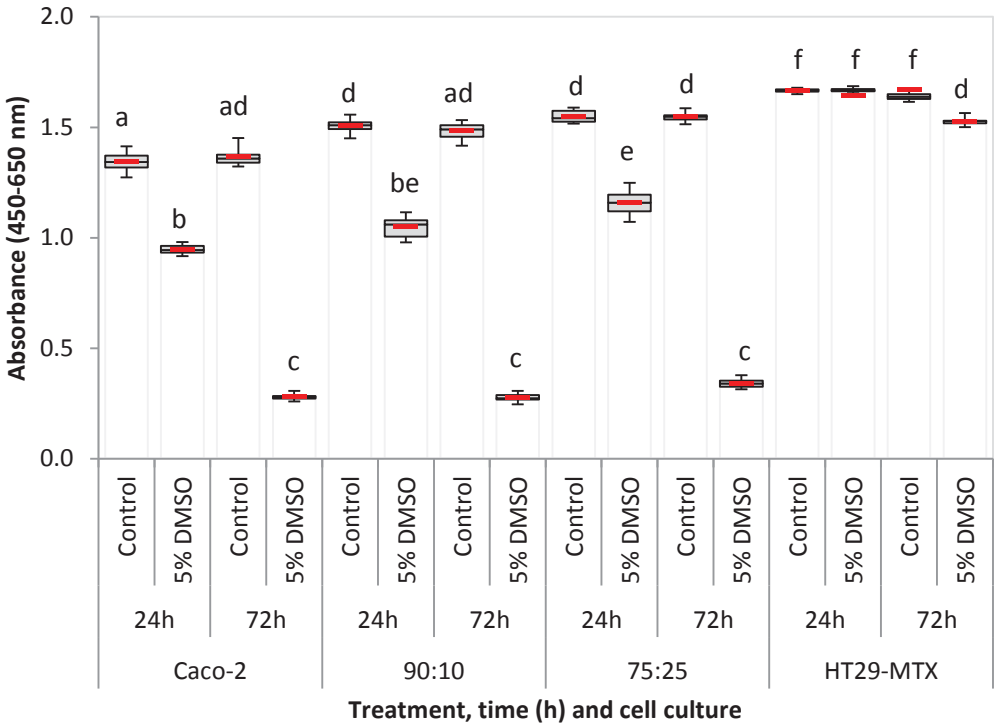


Figure 3-5 Temporal change in absorbance of confluent monolayers of Caco-2, HT29-MTX and Caco-2:HT29-MTX cultures

The absorbance of confluent (21 days post-seeding) Caco-2 and HT29-MTX mono-cultures and Caco-2:HT29-MTX co-cultures (90:10 and 75:25 Caco-2:HT29-MTX cells respectively) in PBS control non-supplemented medium or medium supplemented with 5% dimethylsulphoxide (DMSO) measured after Wst-1 assay. In the graph the red line represents mean absorbance; the central line in the box represents the median absorbance, and the upper and lower boxes represent the upper and lower interquartile range. The whiskers (error bars) represent the minimum and maximum absorbance values. Values which do not share a letter are significantly different ($P < 0.05$).

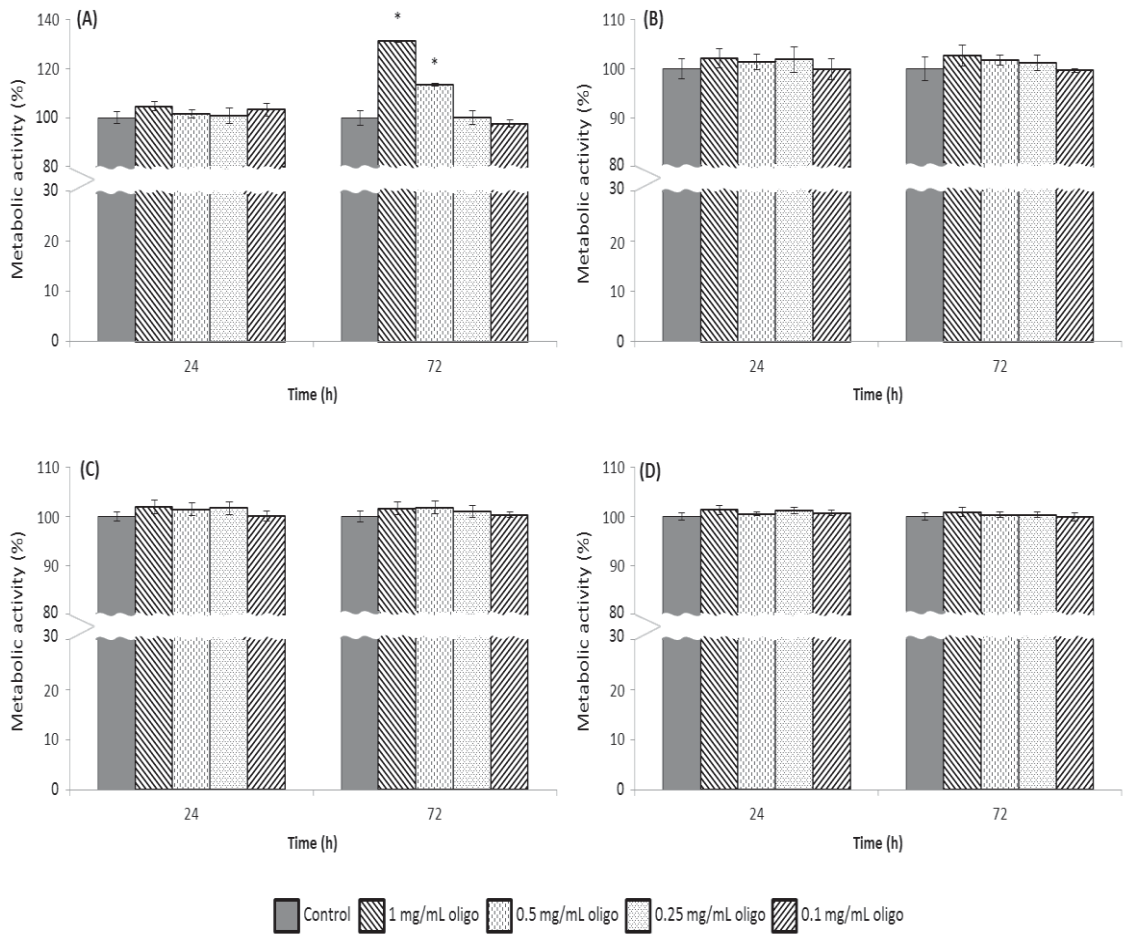


Figure 3-6 Effect of incubation with an oligosaccharide-enriched fraction on metabolic activity of confluent Caco-2 and HT29-MTX mono- and co-cultures

The metabolic activity of confluent, differentiated Caco-2 (A); 90:10 and 75:25 (Caco-2:HT29-MTX) co-cultures (B and C respectively) and HT29-MTX (D) mono-cultures (21 days post-seeding) as a percentage of control (control had an arbitrary value of 100%) measured after Wst-1 assay. To visualise parts of the data in more detail the y axis is broken. Values are means (± SEM) for 3 experiments (n = 12; 4 samples per treatment per experiment). *Different from respective control at each time point P< 0.05.

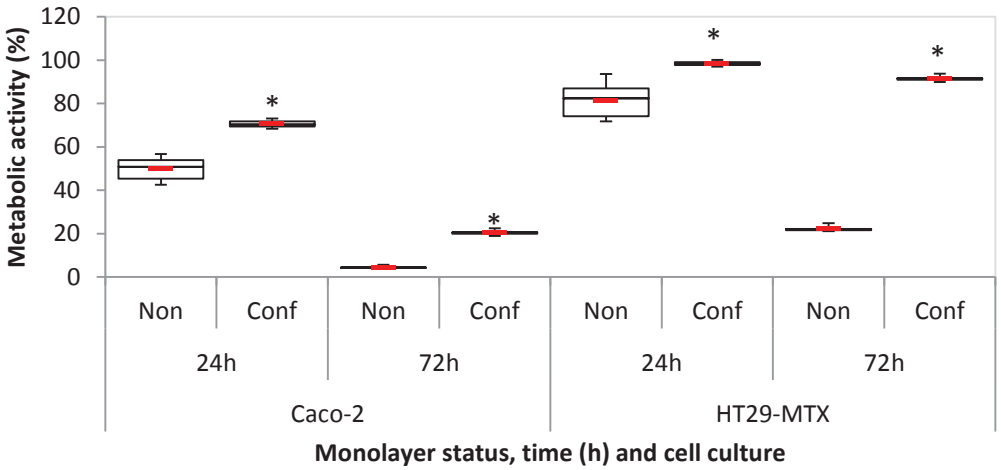


Figure 3-7 Comparison of metabolic activity of non-confluent and confluent Caco-2 and HT29-MTX mono-cultures after incubation with dimethylsulphoxide

The metabolic activity of non-confluent (Non; 2 days post-seeding) and confluent (Conf; 21 days post-seeding) Caco-2 and HT29-MTX mono-cultures incubated with 5% (v/v) dimethylsulphoxide (DMSO) as a percentage of control (control cultures had an arbitrary value of 100%). In the graph the red line represents mean metabolic activity; the central line in the box represents the median metabolic activity, and the upper and lower boxes represent the upper and lower interquartile range. The whiskers (error bars) represent the minimum and maximum values. *Different to respective non-confluent mono-culture P< 0.05.

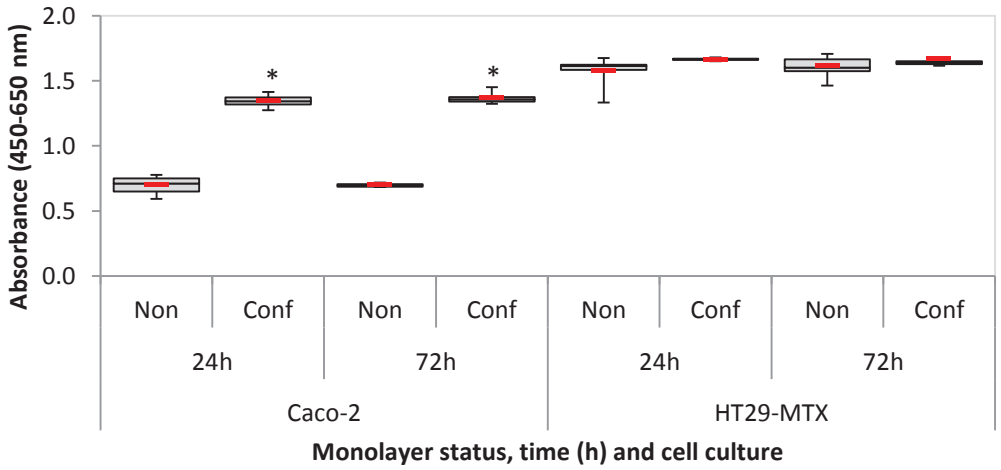


Figure 3-8 Comparison of metabolic activity as measured by absorbance of non-confluent and confluent mono-cultures

The absorbance of non-confluent (Non; 2 days post-seeding) and confluent (Conf; 21 days post-seeding) mono-cultures of Caco-2 or HT29-MTX cells after the Wst-1 assay. In the graph the red line represents mean absorbance; the central line in the box represents the median absorbance, and the upper and lower boxes represent the upper and lower interquartile range. The whiskers (error bars) represent the minimum and maximum absorbance values. *Different to respective non-confluent mono-culture P< 0.05.

confluent HT29-MTX mono- cultures respectively compared to control.

In comparison to non-confluent monolayers, confluent mono-cultures of Caco-2 cells had an average (± SEM) absorbance increase at each time point of 47% (± 2.9). In contrast, the absorbance values of HT29-MTX mono-cultures were similar for non-confluent and confluent cultures (Figure 3-8).

3.4.2 Trans-epithelial electrical resistance measurements of confluent monolayers of intestinal epithelial cells

The TEER of confluent monolayers was measured over a 12 hour time period after 21 days of continuous culture for Caco-2 and HT29-MTX mono-cultures and Caco-2:HT29-MTX co-cultures at ratios of 90:10 and 75:25 respectively. The TEER of HT29-MTX mono-cultures was significantly less ($P< 0.05$) than any other cell culture at any time point, although values were similar between all the time points (Table 3-3). TEER of Caco-2 mono-cultures was significantly higher ($P< 0.05$) than any other cell culture at any time point. However, TEER was significantly lower ($P< 0.05$) at 3, 6 and 9 hours compared to time 0 and 12 hours, although at 9 hours TEER was significantly increased ($P< 0.05$) compared to 3 and 6 hours.

Although TEER of both co-cultures were significantly lower ($P< 0.05$) than Caco-2 mono-cultures, the TEER of the 75:25 co-cultures was also significantly lower ($P< 0.05$) than the 90:10 co-culture at all the time points. However, the TEER of 90:10 co-cultures was significantly increased ($P< 0.05$) after 9 and 12 hours compared to 3 and 6 hours, while for 75:25 co-cultures TEER was significantly increased ($P< 0.05$) after 12 hours compared to time 0, 3 and 6 hours.

Table 3-3 Trans-epithelial electrical resistance measurements across confluent monolayers of intestinal epithelial cells

TEER (Ω cm^2) of confluent (21 days post-seeding) Caco-2 and HT29-MTX mono-cultures and Caco-2:HT29-MTX co-cultures (90:10 and 75:25 Caco-2:HT29-MTX cells respectively). Values represent the mean ± SEM, n = 12. Means that do not share a letter are significantly different P< 0.05.

Cell culture	Time (h) and TEER (Ω cm^2)				
	0h	3h	6h	9h	12h
Caco-2 mono-culture	1190 (5) *a*	986 (19) *b*	1007 (28) *b*	1108 (12) *c*	1228 (15) *a*
90:10 co-culture	283 (3) *d, e*	275 (3) *d*	279 (2) *d*	290 (2) *e, f*	298 (3) *f*
75:25 co-culture	165 (3) *g*	164 (3) *g*	167 (3) *g*	170 (3) *g, h*	179 (2) *h*
HT29-MTX mono-culture	24 (1) *i*	24 (1) *i*	24 (1) *i*	24 (1) *i*	24 (1) *i*

3.4.2.1 Impact of the oligosaccharide-enriched fraction on trans-epithelial electrical resistance

The OEF at increasing concentrations was used in the TEER assay to assess its effect on the integrity of the tight junctions between the intestinal epithelial cells of the different monolayers (Figure 3-9). There was a significant decrease ($P< 0.05$) in TEER at 3 hours compared to initial TEER for all OEF-exposed and control Caco-2 mono-cultures (Figure 3-9A). However, TEER was significantly increased ($P< 0.05$) when incubated with 1 mg/mL OEF in comparison to the control and all other OEF-exposed monolayers at all the time points. The change in TEER of Caco-2 mono-cultures incubated with the OEF at concentrations equal to or less than 0.5 mg/mL were not different to the control monolayers at any time point.

TEER of 90:10 co-cultures incubated with 1 mg/mL OEF was significantly increased ($P< 0.01$) compared to time 0 and also significantly ($P< 0.05$) increased compared to control at all the time points (Figure 3-9B). Similar to that observed for Caco-2 mono-cultures, the TEER of both 90:10 and 75:25 co-cultures incubated with the OEF at concentrations less than 1 mg/mL were similar to their respective control at all the time points. Except cultures incubated with 1 mg/mL OEF which had significantly increased ($P< 0.05$) TEER at all the time points there was no change in TEER compared to initial TEER for all 75:25 co-cultures until 12 hours (Figure 3-9C). There was no change in TEER at any time point for HT29-MTX mono-cultures in response to the OEF at any concentration (Figure 3-9D).

3.4.3 Mucin gene expression of epithelial cell cultures in response to an oligosaccharide-enriched fraction

The expression of selected mucin genes was determined in epithelial cell cultures incubated with 1 mg/mL OEF for 3 and 12 hours (Figure 3-10). This concentration of OEF was selected because it was shown to increase TEER compared to control at all the time points for cell cultures containing Caco-2 cells (Figure 3-9). The 3 hour time point was selected because TEER of control, unstimulated cultures containing Caco-2 cells was either lower or comparable to

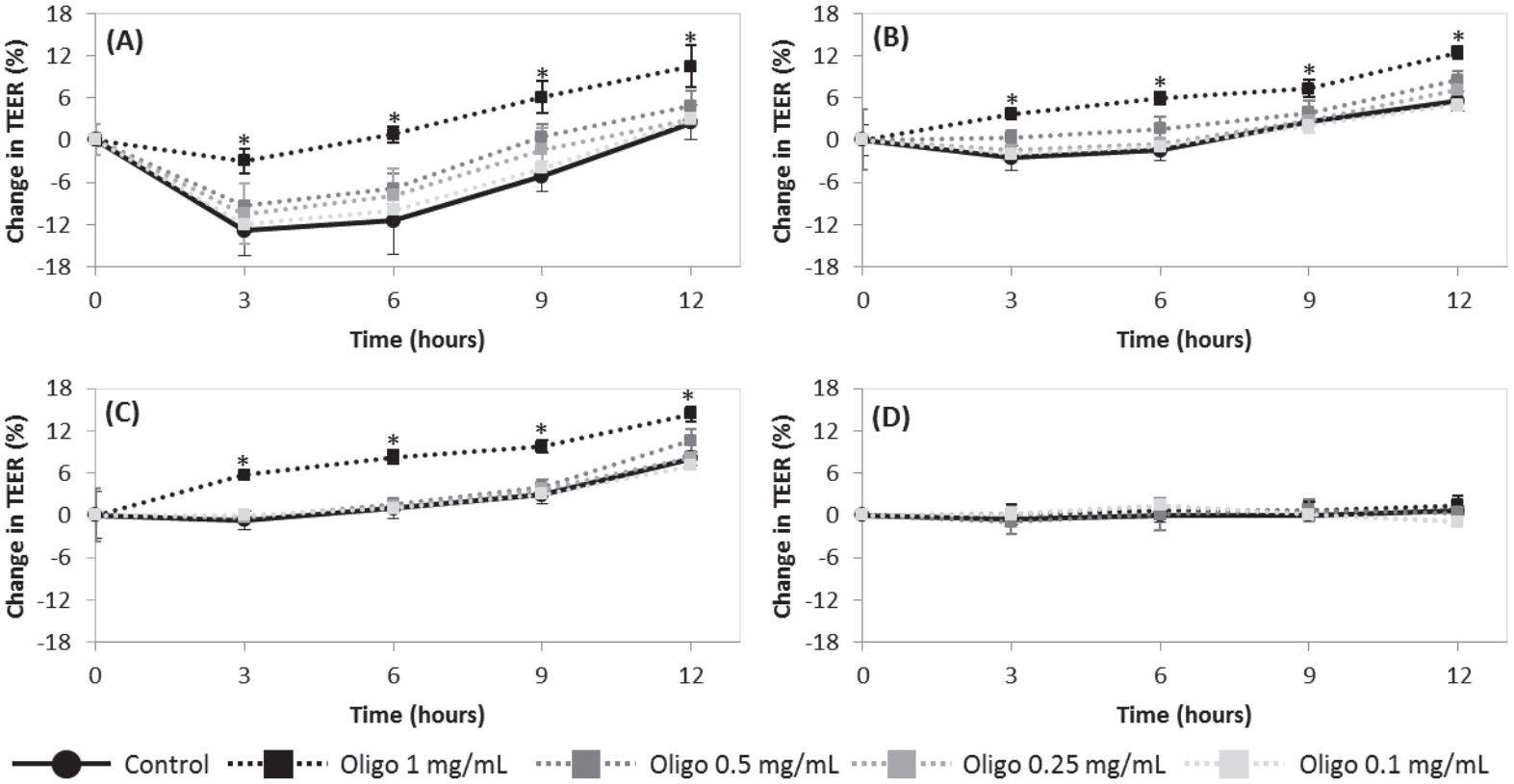


Figure 3-9 Impact of the oligosaccharide-enriched fraction on trans-epithelial electrical resistance of confluent monolayers

The change in TEER as the percentage change compared with the initial TEER for each monolayer (Caco-2 (A) and HT29-MTX (D) mono-cultures in addition to Caco-2:HT29-MTX co-cultures (90:10 (B) and 75:25 (C) respectively). Values are means (± SEM) n = 9 (3 samples per treatment per experiment). *Significantly different to control at P< 0.05.

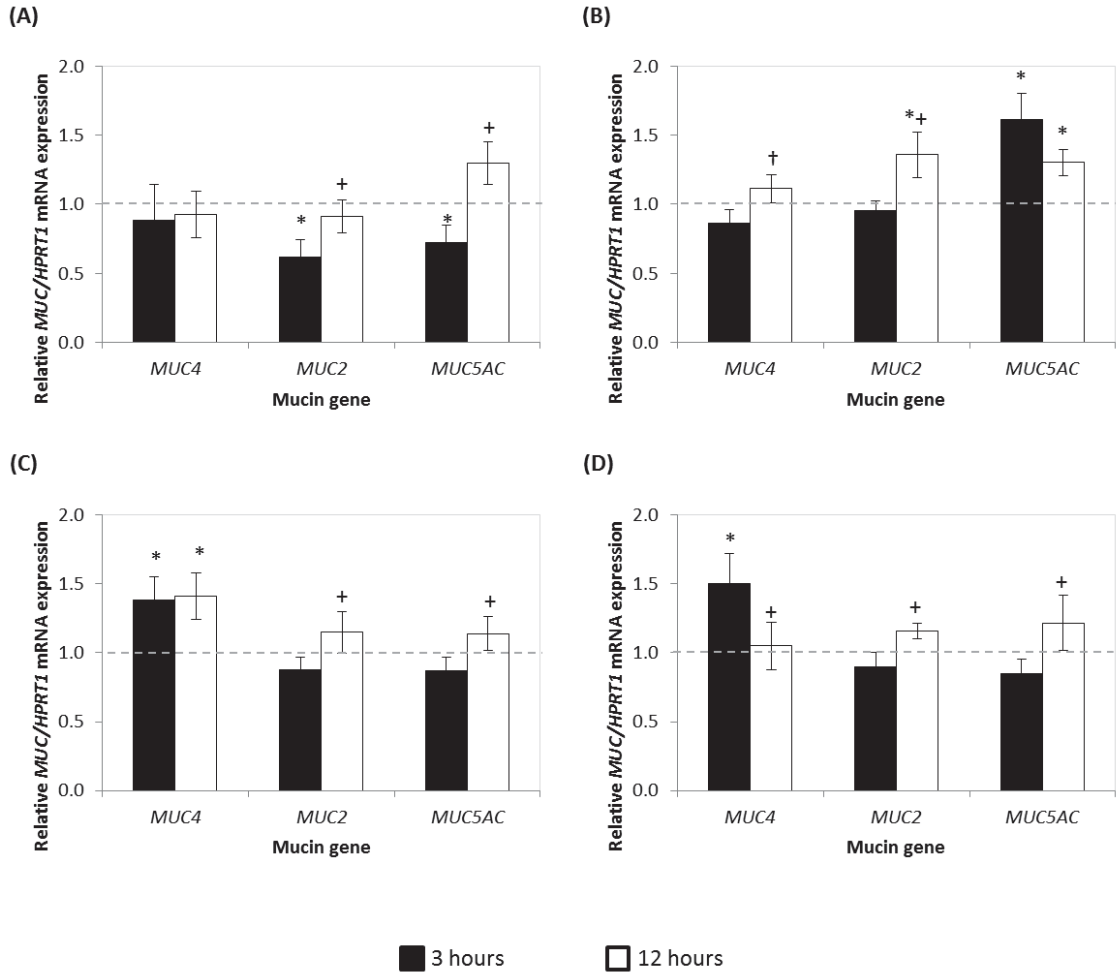


Figure 3-10 Temporal changes in the relative abundance of *MUC* mRNA from epithelial cell cultures incubated with an oligosaccharide-enriched fraction

The relative abundance of MUC4, MUC2 and MUC5AC mRNA from Caco-2 mono-cultures (A), 90:10 Caco-2:HT29-MTX co-cultures (B), 75:25 Caco-2:HT29-MTX co-cultures (C) and HT29-MTX mono-cultures (D) after 3 or 12 hour incubation with 1 mg/mL oligosaccharide-enriched fraction (OEF). Results are the mean (± SEM) expression levels of *MUC/HPRT1* mRNA and calculated as relative expression to unstimulated control cells which have an arbitrary value of 1 (represented by a dotted line). * Significantly different ($P< 0.05$) to respective unstimulated control cells. + Significantly different to respective 3 hour OEF sample ($P< 0.05$).

TEER at time 0, while TEER at 12 hours was either comparable or higher than at time 0.

Expression levels of *MUC2* and *MUC5AC* genes were significantly lower ($P < 0.05$) in Caco-2 mono-cultures after 3 hours incubation with 1 mg/mL OEF compared to control (Figure 3-10A). There was no difference in expression levels for mucin genes between control and OEF-exposed monolayers after 12 hours. However, expression levels of *MUC2* and *MUC5AC* were significantly increased ($P < 0.05$) in monolayers incubated with the OEF for 12 hours compared to those incubated with the OEF for 3 hours.

For 90:10 co-cultures only expression levels of *MUC5AC* was significantly increased ($P < 0.05$) after 3 hours incubation with 1 mg/mL OEF but after 12 hours incubation expression levels of both *MUC2* and *MUC5AC* were significantly increased ($P < 0.05$) compared to respective controls (Figure 3-10B). Although expression levels of *MUC4* was similar to control after 3 and 12 hours incubation with the OEF, there was a significant increase ($P < 0.05$) in *MUC4* and *MUC2* expression levels observed in 12 hour OEF-exposed monolayers compared to 3 hour OEF-exposed cultures.

In 75:25 co-cultures only expression levels of *MUC4* were significantly increased ($P < 0.0$) after 3 and 12 hours incubation with the OEF compared to control, although expression levels of *MUC2* and *MUC5AC* were significantly increased ($P < 0.05$) after 12 hours incubation with the OEF compared to 3 hour OEF exposed cultures (Figure 3-10C).

In HT29-MTX mono-cultures, after 3 hours incubation with the OEF, the expression level of *MUC4* was significantly increased ($P < 0.05$) compared to control (Figure 3-10D). The expression level of *MUC4* was significantly lower ($P < 0.05$) in 12 hour OEF-exposed cultures compared to 3 hour OEF-exposed monolayers, while in contrast expression of *MUC2* and *MUC5AC* was significantly higher ($P < 0.05$).

3.4.4 Mucin protein abundance of epithelial cell cultures in response to oligosaccharide-enriched fraction

Caco-2 mono-cultures incubated with 1 mg/mL OEF for 3 hours were shown to have no change

in the transcription of the *MUC4* gene (Figure 3-10A). However, total MUC4 protein abundance was significantly higher ($P < 0.01$) as a percentage compared to control at 3 hours with significant increases ($P < 0.01$) in both SM and CL samples (Figure 3-11). Abundance of MUC4 protein was not significantly different ($P > 0.05$) between control and OEF-exposed monolayers after 12 hours.

The total mucin protein detected in 90:10 Caco-2:HT29-MTX co-cultures was significantly increased ($P < 0.05$) in monolayers incubated with 1 mg/mL OEF for 3 hours compared to control (Figure 3-12). However, only MUC5AC protein was significantly increased ($P < 0.05$) compared to control at this time point which was consistent with that observed for *MUC5AC* transcription levels (Figure 3-10B). After 12 hours there was no difference ($P > 0.05$) in total mucin abundance between control monolayers and those incubated with the OEF. However, the abundance of the secreted mucin proteins MUC2 and MUC5AC were significantly increased ($P < 0.05$) in CL samples, but in contrast, both of these mucins were significantly decreased ($P < 0.05$) in SM samples.

Incubation with 1 mg/mL OEF for 3 hours significantly increased ($P < 0.01$) total mucin protein abundance for 75:25 co-cultures with significant increases ($P < 0.05$) observed for all mucin proteins (Figure 3-13). However, abundance of MUC5AC protein in SM samples was significantly less ($P < 0.05$) for OEF-exposed monolayers. The total abundance of mucin proteins were significantly increased ($P > 0.05$) for OEF-exposed monolayers after 12 hours. The total abundance of MUC4 was significantly increased ($P < 0.05$) compared to control which was consistent with the observed increase in *MUC4* transcription levels (Figure 3-10C). Total mucin protein abundance was significantly lower ($P < 0.05$) in SM samples of OEF-exposed monolayers likely as a result of significantly lower ($P < 0.05$) abundance of MUC5AC mucin protein.

The total abundance of mucin protein of control, unstimulated HT29-MTX mono-cultures and those incubated with the OEF for 3 and 12 hours were similar ($P > 0.05$) at both time points (Figure 3-14). However, the total abundance of MUC4 protein was significantly increased ($P <$

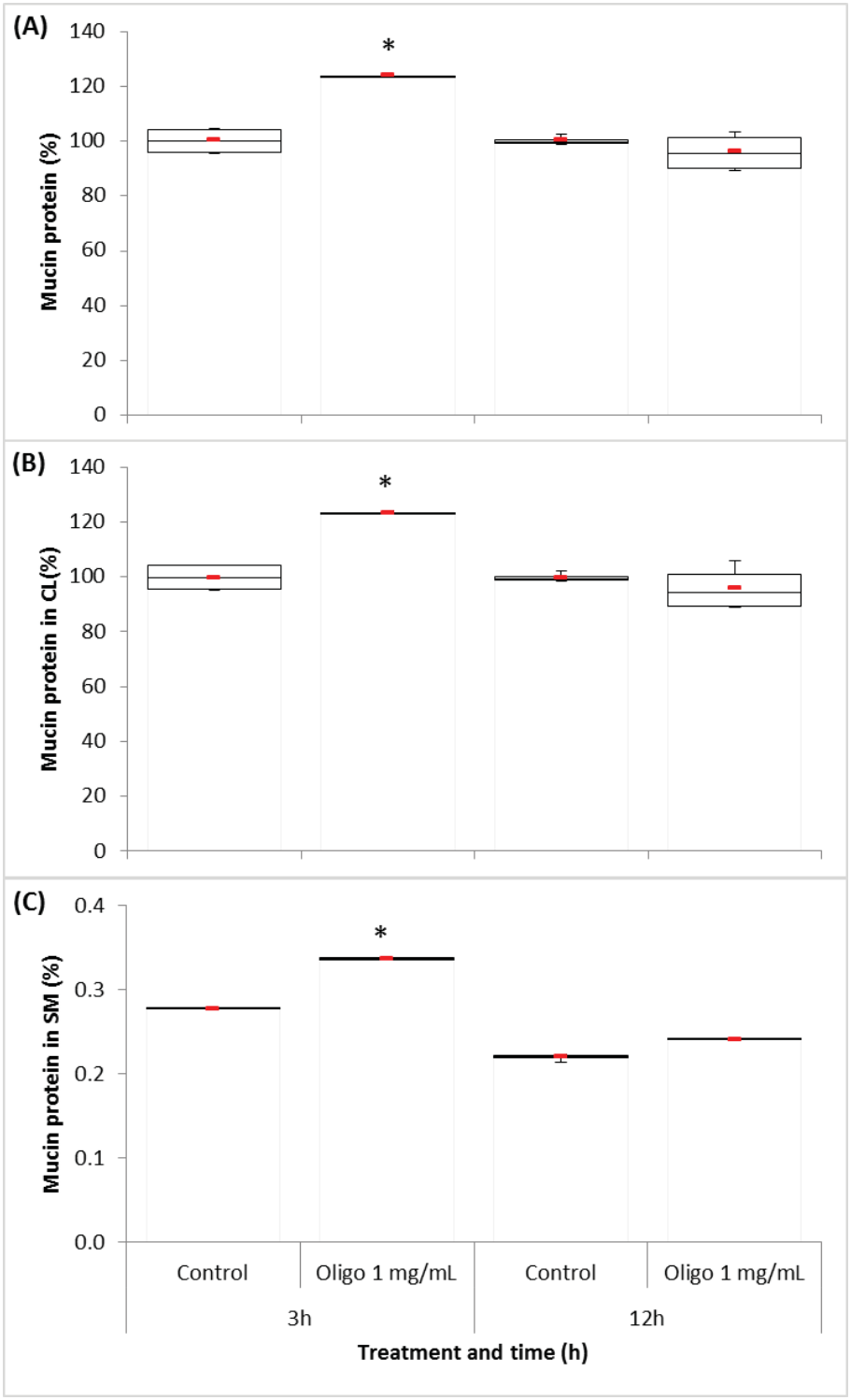


Figure 3-11 Abundance of MUC4 mucin protein from Caco-2 mono-cultures

The total abundance of MUC4 protein from control Caco-2 mono-cultures and those incubated with 1 mg/mL oligosaccharide-enriched fraction for 3 or 12 hours (panel A). The contribution to the total MUC4 protein abundance from cell lysate (CL, panel B) and spent media (SM, panel C) samples. Results are expressed as median percentage change compared to control. Box = 25th and 75th percentiles; bars = min and max values and red line = mean. * P< 0.01 compared to respective control samples.

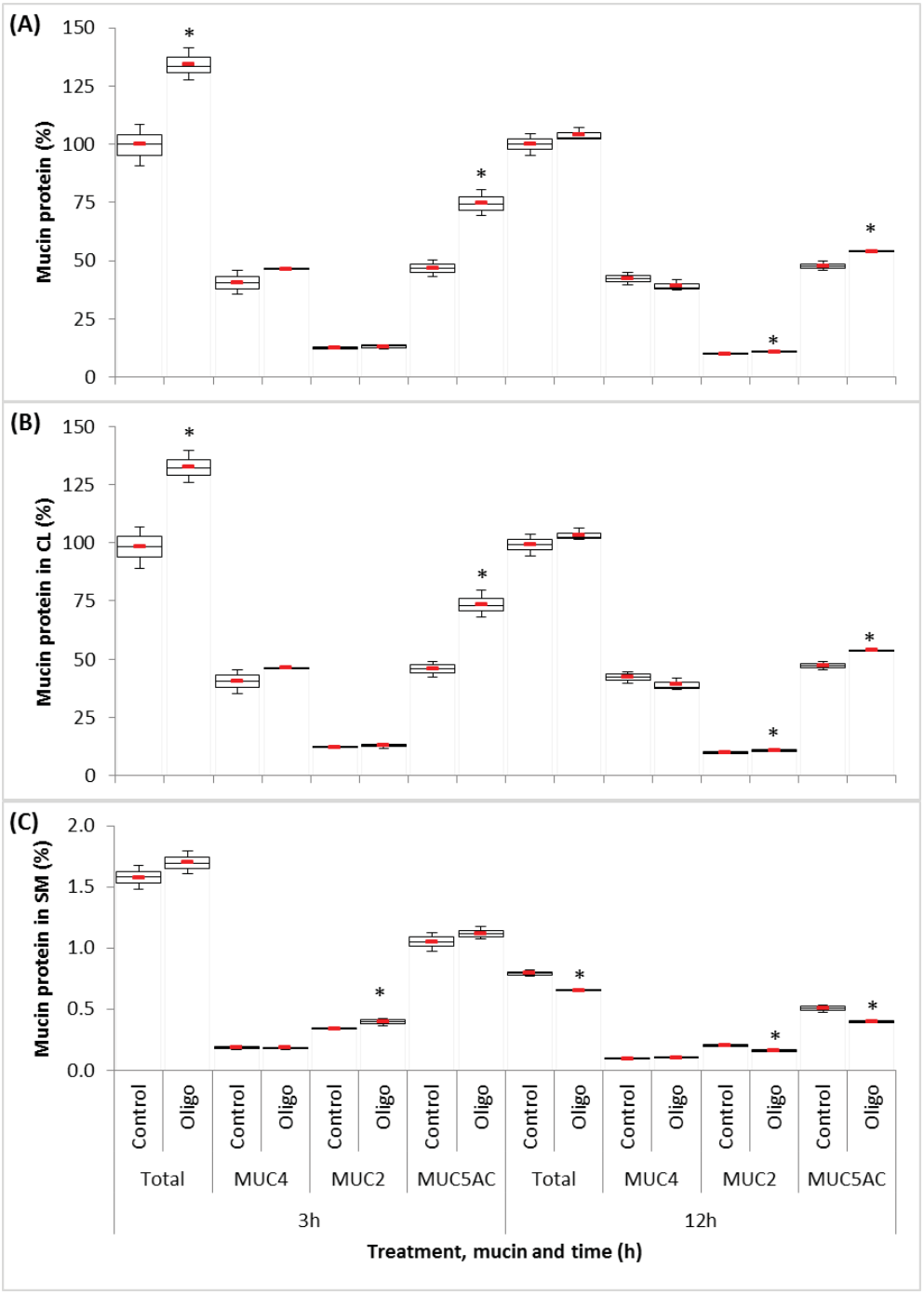


Figure 3-12 Abundance of MUC4, MUC2 and MUC5AC mucin proteins from 90:10 Caco-2:HT29-MTX co-cultures

The total abundance of mucin proteins from control 90:10 co-cultures and those incubated with 1 mg/mL oligosaccharide-enriched fraction for 3 or 12 hours (panel A). The contribution to the total protein abundance from cell lysate (CL, panel B) and spent media (SM, panel C) samples and the contribution of the individual mucin proteins (MUC4, MUC2, and MUC5AC) to the total mucin protein abundance. Results are expressed as median percentage change compared to control. Box = 25th and 75th percentiles; bars = min and max values and red line = mean. * P< 0.05 compared to respective control.

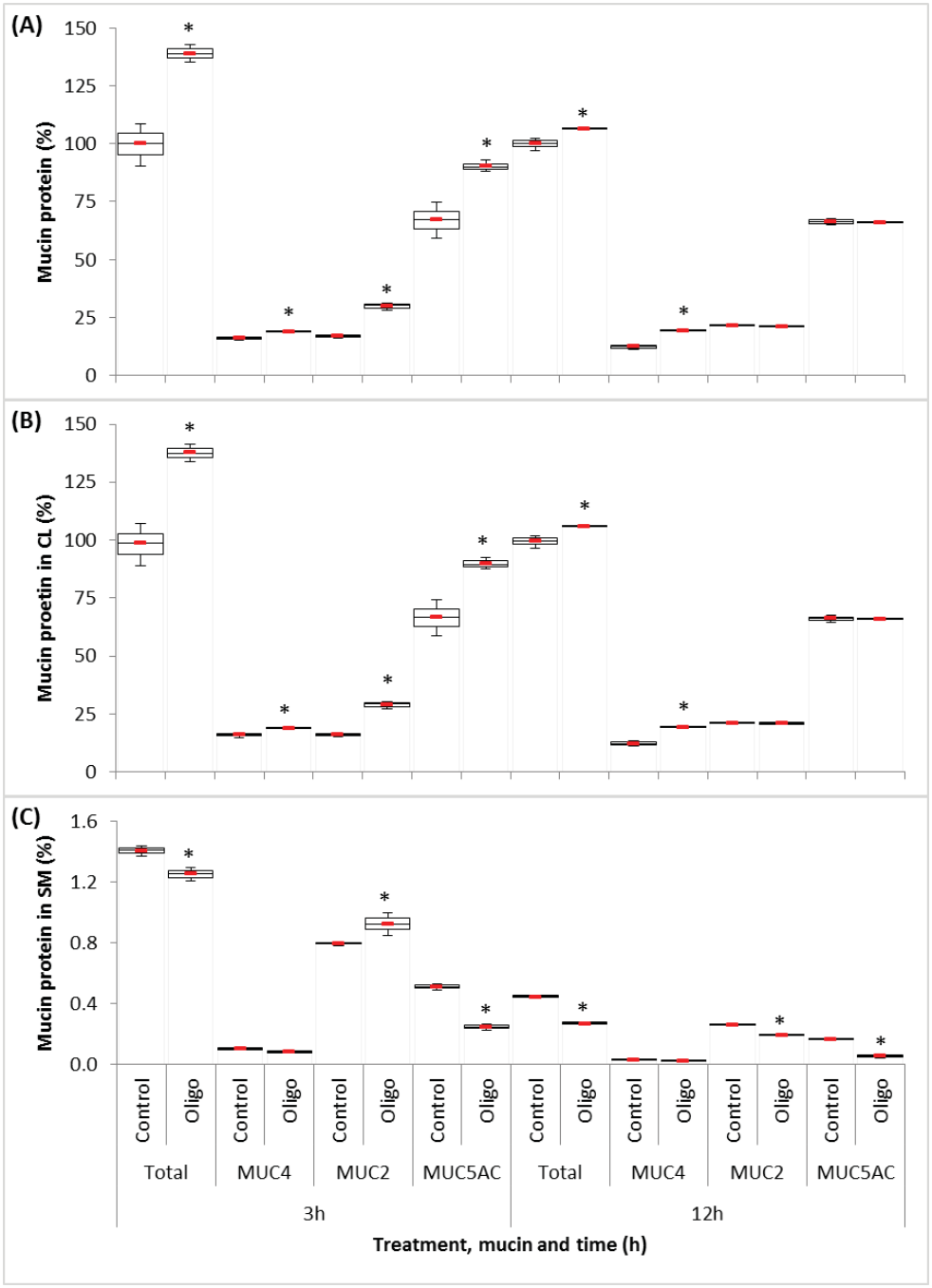


Figure 3-13 Abundance of MUC4, MUC2 and MUC5AC mucin proteins from 75:25 Caco-2:HT29-MTX co-cultures

The total abundance of mucin proteins from control 75:25 co-cultures and those incubated with 1 mg/mL oligosaccharide-enriched fraction for 3 or 12 hours (panel A). The contribution to the total protein abundance from cell lysate (CL, panel B) and spent media (SM, panel C) samples and the contribution of the individual mucin proteins (MUC4, MUC2, and MUC5AC) to the total mucin protein abundance. Results are expressed as median percentage change compared to control. Box = 25th and 75th percentiles; bars = min and max values and red line = mean. * P< 0.05 compared to respective control.

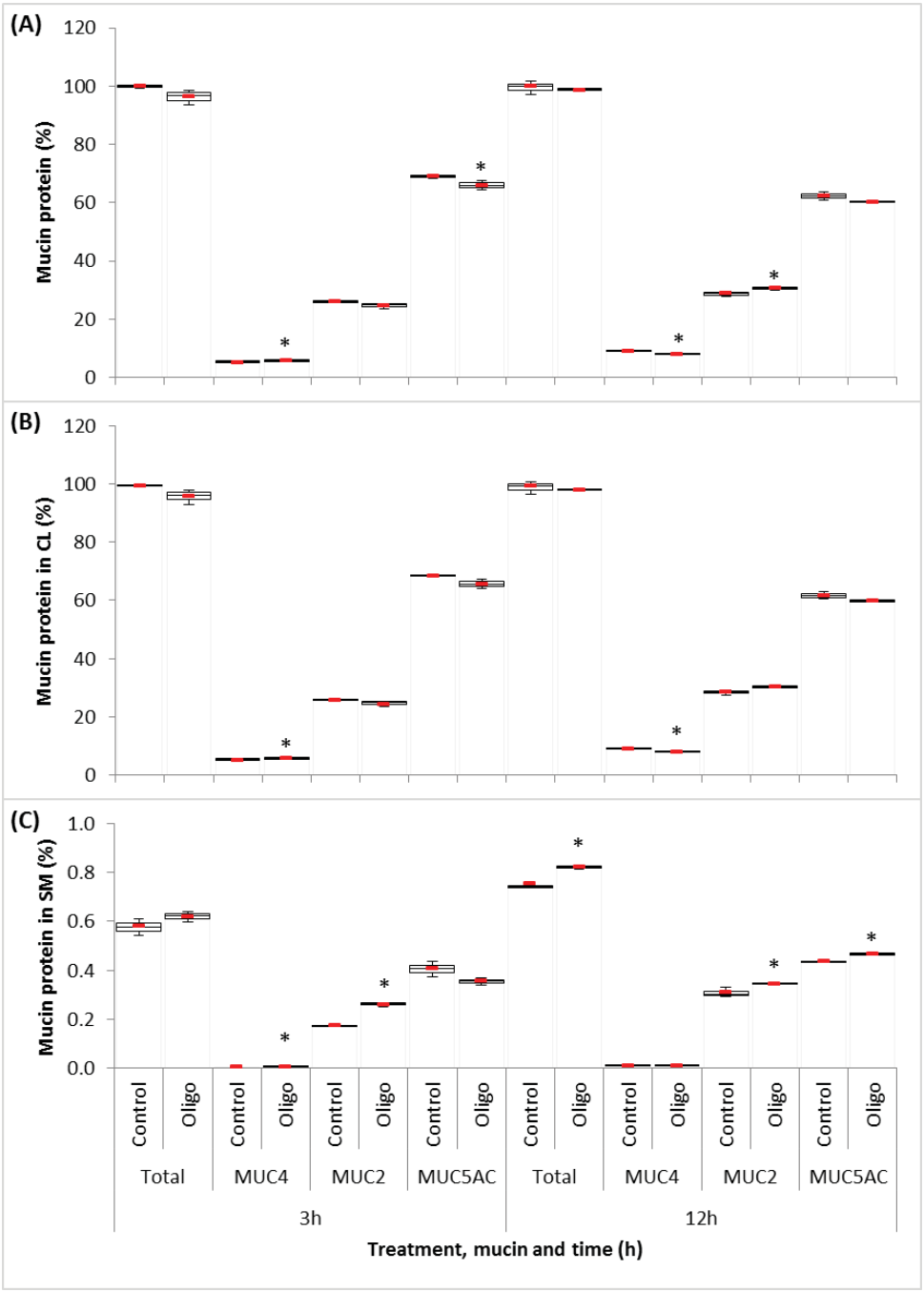


Figure 3-14 Abundance of MUC4, MUC2 and MUC5AC mucin proteins from HT29-MTX mono-cultures

The total abundance of mucin proteins from control HT29-MTX mono-cultures and those incubated with 1 mg/mL oligosaccharide-enriched fraction for 3 or 12 hours (panel A). The contribution to the total protein abundance from cell lysate (CL, panel B) and spent media (SM, panel C) samples and the contribution of the individual mucin proteins (MUC4, MUC2, and MUC5AC) to the total mucin protein abundance. Results are expressed as median percentage change compared to control. Box = 25th and 75th percentiles; bars = min and max values and red line = mean. * P< 0.05 compared to respective control.

0.05) after 3 hours but significantly decreased (P< 0.05) after 12 hours in OEF-exposed monolayers compared to control. After 12 hours incubation with the OEF MUC2 and MUC5AC in SM samples was significantly higher (P< 0.05) than control. A summary of the results are shown in Table 3-4.

3.5 Discussion

This is the first study to investigate the effect of an OEF obtained from goat whey on the metabolic activity, TEER, mucin mRNA expression and mucin protein production of Caco-2 and HT29-MTX mono-cultures and co-cultures (90:10 and 75:25 Caco-2:HT29-MTX cells respectively).

The OEF obtained from goat whey used in this study was composed of a combination of different oligosaccharides in addition to other monosaccharides, disaccharides and galacto-oligosaccharides. Of the oligosaccharides present in the fraction 82% were acidic and 18% were neutral. However, it is possible that other oligosaccharides may have been present in the OEF but to date, have only been poorly characterised [269]. The inclusion of this OEF in the culture medium of intestinal epithelial cell cultures at the maximum concentration tested (1 mg/mL), although substantially less than would be found in human breast milk (3 to 13 mg/mL) [254], was shown to increase TEER and enhance mucin gene expression and mucin protein abundance, which was in accordance with the original hypothesis.

This is the first study to investigate the impact of an OEF on metabolic activity of differentiated intestinal epithelial cells (e.g. confluent co-cultures), which have a similar cell composition to human small and large intestine *in vivo*. The OEF at the concentrations tested was shown tohave no detrimental effects on the metabolic activity of non-confluent and confluent Caco-2 or HT29-MTX mono-cultures after 24 or 72 hours incubation. In addition, the OEF also had no detrimental effects on the metabolic activity of confluent co-cultures (90:10 and 75:25 Caco-2:HT29-MTX cells respectively). However, previous studies have reported that non-confluent HT29 cell cultures had reduced proliferation rates after incubation with neutral and acidic

Table 3-4 Summary of results for individual epithelial cell cultures after incubation with oligosaccharide-enriched fraction

Change in status of epithelial cell cultures incubated with 1 mg/mL OEF compared to respective controls for the different parameters investigated. Increase compared to control (↑), decrease compared to control (↓), no significant change (P> 0.05) compared to control (–) and not determined (ND).

Cell culture	Parameter	Variable	Time (h)	Status
Caco-2	Metabolic activity	Non-confluent, non-differentiated	24h	–
			72h	↑
		Confluent, differentiated	24h	–
			72h	↑
	TEER		3h	↑
			12h	↑
	Mucin gene	MUC4	3h	–
			12h	–
		MUC2	3h	↓
			12h	–
		MUC5AC	3h	↓
			12h	–
	Mucin protein	MUC4	3h	↑
			12h	–
		MUC2	3h	**ND**
			12h	**ND**
		MUC5AC	3h	**ND**
			12h	**ND**
90:10	Metabolic activity	Non-confluent, non-differentiated	24h	–
			72h	–
		Confluent, differentiated	24h	–
			72h	–
	TEER		3h	↑
			12h	↑
	Mucin gene	MUC4	3h	–
			12h	–
		MUC2	3h	–
			12h	↑
		MUC5AC	3h	↑
			12h	↑
	Mucin protein	MUC4	3h	–
			12h	–
		MUC2	3h	–
			12h	↑
		MUC5AC	3h	↑
			12h	↑

Cell culture	Parameter	Variable	Time (h)	Status
75:25	Metabolic activity	Non-confluent, non-differentiated	24h	–
			72h	–
		Confluent, differentiated	24h	–
			72h	–
	TEER		3h	↑
			12h	↑
	Mucin gene	MUC4	3h	↑
			12h	↑
		MUC2	3h	–
			12h	–
		MUC5AC	3h	–
			12h	–
	Mucin protein	MUC4	3h	↑
			12h	↑
		MUC2	3h	↑
			12h	–
		MUC5AC	3h	↑
			12h	–
HT29-MTX	Metabolic activity	Non-confluent, non-differentiated	24h	–
			72h	–
		Confluent, differentiated	24h	–
			72h	–
	TEER		3h	–
			12h	–
	Mucin gene	MUC4	3h	↑
			12h	–
		MUC2	3h	–
			12h	–
		MUC5AC	3h	–
			12h	–
	Mucin protein	MUC4	3h	↑
			12h	↓
		MUC2	3h	–
			12h	↑
		MUC5AC	3h	↓
			12h	–

oligosaccharides (0.2 mg/mL and 0.4 mg/mL respectively) from human milk, although Caco-2 brush border expressing (Caco-2Bbe) cultures were unaffected at these concentrations [263]. In another study proliferation rates of non-confluent HT29 and Caco-2 were reduced after incubation with neutral oligosaccharides (2.3 and 0.2 mg/mL respectively), and also acidic oligosaccharides (0.3 mg/mL and 0.7 mg/mL respectively) [262]. In contrast, there were no changes in proliferation when primary human foetal intestinal cells (FHs-74 cell line) were incubated with 1 mg/mL lactose and tri-galactose, however incubation with the neutral HMO Lacto-N-neotetraose at the same concentration reduced proliferation after 72 hours [270]. The anti-proliferative effect of both neutral and acidic oligosaccharides on non-confluent cell cultures was found to be related to changes in the cell cycle, with an accumulation of cells in the G2/M stage of the cell cycle and a decreased number of cells in G0/G1 phase [270, 271]. However, cell cycle analysis was not investigated in this study.

Several findings associated with this study differ from other previously published work. For example, in this study the OEF was obtained from goat whey while in the other studies the preparations were obtained from human milk samples [262, 263]. It is known that the oligosaccharide structure in milk from different species varies [272]. For example, in human milk the major neutral oligosaccharides are fucosylated [254]. However, to date, these oligosaccharide structures have not been shown to exist in goat milk [168] although recent research has identified their presence in bovine milk [273]. Another significant difference between this study and other studies was the composition of the OEF. Here the fraction used was comprised of a combination of acidic and neutral oligosaccharides in addition to other saccharides, while in the other studies [262, 263] the preparations represented either neutral or acidic oligosaccharides as separate preparations with limited contamination of samples with other saccharide components.

The OEF used in this work differentially modulated TEER in a dose dependent manner when added to Caco-2 mono-cultures or both co-cultures (Figure 3-9). No effect was observed on

TEER upon addition of the OEF to HT29-MTX mono-cultures. Although inclusion of the OEF was shown to decrease TEER of Caco-2 monolayers after 3 hours, the decrease was considerably less than that observed for control Caco-2 mono-cultures. Thus it could be hypothesised, that the inclusion of the OEF in the culture medium had a beneficial impact on TEER of Caco-2 mono-cultures. However, the beneficial effects at this time point may not be due to the OEF having an effect on the cells, but may just be due to the background resistance of the treatment being different, and therefore may change how the current passes through the media. However, an initial decrease in TEER of Caco-2 mono-cultures upon addition of both acidic and neutral oligosaccharides has been observed previously [260]. In addition, the introduction of non-digestible saccharides such as fructo-oligosaccharides induced an 80% reduction in TEER after 2 hours incubation [274].

In this study incubation with the OEF differentially modulated mucin gene expression levels and mucin protein abundance in cell cultures, although changes in mucin gene expression and protein production were not simply a consequence of increased metabolism.

A noteworthy observation was that after 3 hours incubation with the OEF, *MUC4* transcription levels were increased in monolayers containing increased numbers of HT29-MTX cells. This may suggest a possible interaction between the OEF and HT29-MTX cells which does not occur with the Caco-2 cells. However, increased expression levels of *MUC4* can be associated with a loss of interaction between neighbouring cells, resulting from of a re-localisation of E-cadherin from adherens junctions at the lateral membrane of the cells to the apical membrane [275]. Although in the 75:25 co-cultures, where there was an increase in *MUC4* expression after incubation with the OEF, TEER was also increased compared to control. This suggests that although *MUC4* expression was increased there were no detrimental effects on the junctions between cells.

Similar transcriptional responses for both *MUC2* and *MUC5AC* were observed in the individual cell cultures following incubation with the OEF. This suggests a possible common regulatory

mechanism between *MUC2* and *MUC5AC* may exist. This could be related to the location of both of these genes on chromosome 11p15.5 in a 400-kb region that also includes the genes for *MUC5B* and *MUC6* [142].

In both 90:10 and 75:25 Caco-2:HT29-MTX co-cultures similar expression patterns were observed between mucin mRNA transcripts and mucin protein abundance after incubation with the OEF. A relationship between mucin protein abundance and gene expression has been observed previously and may be related to the process involved in mucin secretion [276, 277]. For example, unregulated, constitutive secretion of mucins is dependent upon the continuous movement of mucin granules from the golgi to the apex of the cell and is coupled with the corresponding increase in *MUC* gene expression to replenish the intracellular mucin pool [140, 233]. A similar action can be found in other secretory cells such as pancreatic β cells, which respond to changes in blood glucose by first secreting insulin and next increasing insulin biosynthesis [233].

In 90:10 co-cultures expression of both *MUC2* and *MUC5AC* genes were increased and was associated with an increase in their respective secreted protein fractions. Gene and protein expression levels of the membrane-bound mucin MUC4 were unchanged in this co-culture. In contrast, for 75:25 co-cultures, MUC2 and MUC5AC gene and protein levels were unchanged while there was an increase in the expression levels of *MUC4* mRNA and its associated protein fraction. However, the abundance of MUC4 protein was higher than that of MUC2 protein in the 90:10 co-cultures, but in contrast, was lower than MUC2 in the 75:25 co-cultures. The increased abundance of MUC4 in comparison to MUC2 mucin protein of 90:10 co-cultures, and the decreased abundance of MUC4 compared to MUC2 in 75:25 co-cultures observed here, may be related to the relative abundance of the different cell types present in the monolayers, and also the differentiation status of the Caco-2 cells. MUC4 is expressed in both human intestinal absorptive cells and mucus secreting goblet cells and highly expressed in normal human colon [278, 279]. However, MUC4 is only minimally expressed in poorly differentiated

or non-differentiated carcinoma cell lines such as HT29, while its expression is increased in differentiated epithelia [280]. Although MUC2 is the major colonic mucin in the healthy, human foetal and adult intestine, its expression is restricted to goblet cells [278, 280]. Thus, in the 90:10 co-cultures, where there were an increased number of differentiated Caco-2 cells, the abundance of MUC4 mucin protein was also increased. While in 75:25 co-cultures a decreased relative abundance of Caco-2 and more HT29-MTX cells was associated with the respective decrease of MUC4 and increase in MUC2 mucin protein abundance observed.

3.6 Conclusion

The results indicate that intestinal epithelial cell cultures respond differently to incubation with an OEF obtained from goat whey. Metabolic activity of non-confluent and confluent monolayers was unaffected after 24 hours when incubated with the highest concentration (1 mg/mL) of OEF. In addition, the OEF differentially modulated TEER in a dose dependent manner, and was shown to have the greatest beneficial effect on intestinal barrier integrity by enhancing TEER only at the maximum concentration (1 mg/mL). In cell cultures which represented differentiated enterocytes, mucin secreting goblet cells or epithelial cell compositions similar to human small and large intestine *in vivo*, incubation with the OEF enhanced intestinal epithelial barrier integrity by increasing TEER and enhanced mucin gene expression and mucin protein abundance, all of which are essential components of host mucosal function.

In conclusion, these observations have important implications for the use of goat milk oligosaccharides as potential functional food supplements for improving intestinal health, and in combination with the co-culture system that more closely simulates that of *in vivo*, further hypotheses testing and experimentation can be undertaken.

Chapter 4: Effects of an oligosaccharide-enriched fraction from goat whey on bacterial growth and adherence to intestinal epithelial cells

4.1 Introduction

Human milk oligosaccharides (HMOs) are not digested in the proximal human intestine and are only minimally absorbed [257, 258]. Consequently, the majority of ingested oligosaccharides pass into the large intestine where mutualistic symbiotic bacteria can use them as a source of energy [281]. HMOs are specific growth factors that enrich intestinal bacteria and are often termed as bifidogenic carbohydrates [255, 282]. As such breast-fed infants have a microbiota uniquely rich in bifidobacteria, with *Bifidobacterium infantis* and *B. bifidum* being the major utilisers of HMOs found in breast-fed infant faeces [281].

Bifidogenic carbohydrates are important components of functional food ingredients and are often referred to as prebiotics [283]. Similar to HMOs, prebiotics are non-digestible dietary factors that stimulate the growth and/or activity of a limited number of bacteria in the large intestine, conferring benefits upon host health [123, 284]. Current research on prebiotic oligosaccharides is focused on specific effects to achieve a microbiota dominated by bifidobacteria and lactobacilli and thus the effect of prebiotics is often considered to be the same as the bifidogenic effect of HMOs [285].

In vitro utilisation of HMOs, FOS and GOS by lactobacilli and bifidobacteria has been previously examined [286, 287]. *B. bifidum* ATCC 29521 released monosaccharides from HMO polymers but did not ferment fucose, sialic acid and *N*-acetylglucosamine. In contrast, *B. breve* ATCC 27539 was able to ferment the HMO monosaccharide constituents individually but was unable to access these monosaccharides from the HMO polymers [286]. In addition, *in vitro* digestion of HMOs by *Lactobacillus gasseri* UCD 235, *L. acidophilus* NCFM, and *Lactococcus lactis* IL1403 was low in comparison with *B. infantis* ATCC 15697 [287-289]. Although, individual strains of lactobacilli have been reported to ferment GOS, *L. rhamnosus* DR20 preferentially degraded GOS with a lower degree of polymerisation (mono and disaccharides) than more complex saccharides (tri- and tetra-saccharides) [290]. The inability of lactobacilli to grow on HMOs is in contrast to the metabolic capabilities of bifidobacteria, which are highly adapted to utilise

HMOs as a carbon source for growth [291].

In addition to the beneficial effects exerted by prebiotics on the indigenous intestinal microbiota (e.g. bifidobacteria and lactobacilli), prebiotics are generally considered to improve the function of probiotics [292]. Probiotics have been defined as "live microbes that, when administered in adequate amounts, confer a health benefit to the host" [19]. Certain activities define microbes as probiotics including; acid and bile tolerance; survival through the intestinal tract; and an ability to adhere to epithelial cells and mucus, which enables their persistence and proliferation in the intestine [121, 293]. The use of probiotics in conjunction with specific prebiotic substrates (e.g. a FOS with a bifidobacteria or lactitol with lactobacilli), are referred to as synbiotics [294]. It is thought that the addition of a readily fermentable growth substrate to a probiotic preparation improves its survival, colonisation and subsequent proliferation, which results in advantages to the host that the live micro-organism and prebiotic offer [166].

The adherence of probiotic bacteria to mono-cultures of Caco-2 cells [203], mono-cultures of HT29 (and various sub-clones) [218], and co-cultures of Caco-2:HT29-MTX cells [208] have previously been investigated. To date, only a limited number of studies have determined what influence prebiotics have on the adherence of probiotic strains to intestinal epithelial cells [292, 294, 295]. One study reported adherence of *L. acidophilus* CCDM 79, *Enterococcus durans* CCDM 500 and *B. animalis* subsp. *lactis* CCDM 74 to Caco-2:HT29-MTX co-cultures was decreased in the presence of the prebiotics inulin (Orafti GR) and oligofructose (Orafti Synergy) [292]. Conversely, in the same study, the adherence of each of these probiotic strains was increased in the presence of an inulin/OF prebiotic combination (Orafti P95) and also a GOS/lactose/glucose/galactose combination (Vivinal) [292]. Although it was hypothesised that adherence was lower in Orafti GR than any other substrate as a consequence of the bacteria redirecting their energy to metabolic processes, the growth of these strains in the different prebiotic substrates was not examined. In a different study, both the growth and adherence of selected bacterial strains was investigated in response to the proposed prebiotic substrate

tagatose [294]. Of the 385 strains investigated (275 strains from human or animal faeces and 110 strains from fermented milks), 39 strains showed positive consumption of tagatose, but only 5 strains obtained high levels of growth in the presence of this substrate. Two of these strains, *L. rhamnosus* GG and *L. casei* strain 01, had increased adherence to mono-cultures of HT29 cells when cultured in the presence of this substrate, which suggested a synergistic interaction between the different components [294]. However, one limitation of this study was that only the adherence of strains that had achieved high levels of growth in the same substrate was investigated.

4.2 Hypothesis and aims

The hypothesis tested in the research presented in this chapter is that (candidate) probiotic bacterial strains that utilise an OEF from goat whey for growth, have increased adherence to intestinal epithelial cell cultures when the OEF is included in the culture media, than bacterial strains that do not.

In order to address this hypothesis, two specific aims were devised. The first aim was to characterise the impact of the OEF on the growth of selected probiotic bacterial strains under various atmospheric culture conditions. The second aim was to examine the adherence of the selected probiotic bacterial strains to Caco-2 and HT29-MTX mono-cultures and Caco-2:HT29-MTX co-cultures (90:10 and 75:25 Caco-2:HT29-MTX respectively) characterised previously (Chapter 2), in the presence and absence of the OEF, to determine the modulatory effects of the OEF on this adherence.

4.3 Methods and materials

4.3.1 Bacterial strains and culture conditions

The sources of the bacterial strains used in this study are described in Table 4-1. Eight commercially used probiotics were chosen on the basis that there were published data showing their efficacy in various *in vitro* and *in vivo* models of the intestine [203]. In addition, *Bifidobacterium angulatum* (DSM 200 98) was included in this study as it has been reported to

Table 4-1 Bacterial strains

Bacterial strain	Company
Lactobacillus rhamnosus HN001 also known as *L. rhamnosus* HN001 (DR20)	Danisco New Zealand Ltd, Auckland, New Zealand
Lactobacillus rhamnosus Goldin & Garbach (GG)	Valio, Helsinki, Finland
Lactobacillus plantarum 299v	Probi AB, Lund, Sweden (DSM9843)
Lactobacillus plantarum AGR1526	Procalf Plus Donaghys, Christchurch, New Zealand
Lactobacillus casei Shirota	Yakult New Zealand, Auckland, New Zealand
Lactobacillus acidophilus NCFM (North Carolina Food Microbiology)	Danisco New Zealand Ltd, Auckland, New Zealand
Bifidobacteria animalis subsp. *lactis* Bb-12	Chr Hansen Pty Ltd, Melbourne, Australia
Bifidobacteria bifidum	DSM20456
Bifidobacteria angulatum	DSM20098

DSM (Deutsche Sammlung von Mikroorganismen)

strongly adhere to starch granules but their adherence to intestinal epithelial cells has yet to be determined [296].

4.3.2 Preparation of bacterial culture media and agar

4.3.2.1 Anaerobic broth

De Man, Rogosa and Sharp (MRS) broth was prepared by mixing 55 g of lactobacilli MRS broth powder (Acumedia, Michigan, USA) in 1 L of distilled water. The solution was heated to aid the dissolving of the powder and to eliminate dissolved oxygen. The solution was allowed to cool in an ice-water bath while being bubbled with oxygen-free CO_2. Once cooled to room temperature, the broth was dispensed into Hungate tubes (16 mm diameter, 125 mm long; BellCo glass, Vineland, New Jersey, USA) filled with oxygen-free CO_2 in 10 mL aliquots using a pipette flushed with oxygen-free CO_2. The tubes were sealed with butyl rubber stoppers and perforated plastic caps with a headspace of 100% CO_2 and autoclaved at 121°C and 15 psi for 20 minutes.

4.3.2.2 Defined semi-synthetic basal broth

A defined semi-synthetic medium was used to determine the fermentation capabilities of bacterial strains [297]. All components listed in Table 4-2, except cysteine hydrochloride were dissolved in 1 L distilled water. The solution was heated to aid the dissolving of the powder and to eliminate dissolved oxygen. The solution was allowed to cool in an ice-water bath while being bubbled with oxygen-free CO_2. Once cooled to room temperature cysteine hydrochloride was added and the pH of the solution was adjusted to 7.0 ± 0.2 using 1M NaOH while maintaining the stream of CO_2. The solution was dispensed as described in Section 4.3.2.1 except only 4.5 mL was added to each Hungate tube. The tubes were sealed with butyl rubber stoppers and perforated plastic caps (anaerobic cultures) or Teflon screw caps (5% CO_2 culture) with a headspace of 100% CO_2 and autoclaved for 20 minutes 121°C and 15 psi.

Table 4-2 Defined, semi-synthetic medium recipe

Media components and amount required to prepare 1 L of defined, semi-synthetic basal medium.

Media component	Weight
Bactopeptone (Becton Dickinson)	10 g
Yeast extract (Becton Dickinson)	5 g
Dipotassium phosphate (Fisher BioReagents)	2 g
Sodium acetate (Fisher BioReagents)	5 g
Ammonium citrate (Fisher Chemical)	2 g
Magnesium sulfate (Fisher Chemical)	0.2 g
Manganese sulfate (Unilab - Ajax)	0.05 g
Cysteine hydrochloride (Acros Biochemicals)	0.5 g
Distilled water	1 L

4.3.2.3 Defined broth supplemented with porcine gastric mucin

Defined semi-synthetic basal broth was prepared as described in Section 4.3.2.2 with 0.3% (w/v) of porcine gastric mucin (PGM; Sigma-Aldrich, Auckland, New Zealand) and autoclaved at 121°C and 15 psi for 20 minutes.

4.3.2.4 Agar plates

MRS broth was prepared by mixing 55 g of lactobacilli MRS broth powder (Acumedia, Michigan, USA) in 1 L of distilled water and heated to aid the dissolving of the powder. The solution was added directly to a high pressure Schott bottle containing 15 g of bacteriological Agar (Oxoid, Hampshire, UK). The bottle was sealed with a butyl rubber stopper and perforated Schott cap, prior to being autoclaved at 121°C and 15 psi for 20 minutes. The agar was poured into Petri dishes and once set were stored in plastic bags at 4°C. Prior to inoculation, plates were pre-equilibrated in an anaerobic atmosphere overnight.

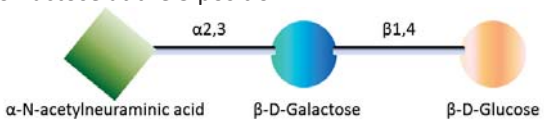
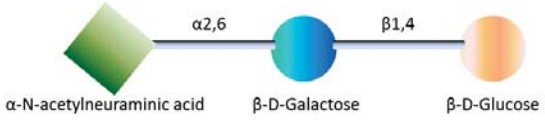
4.3.2.5 Agar slopes

MRS broth was prepared as described in Section 4.3.2.1 with 1.5% (w/v) of Bacteriological Agar and bubbled with oxygen-free CO_2. The solution was dispensed into Hungate tubes containing 150 mg of bacteriological agar and filled with oxygen-free CO_2 in 10 mL aliquots using a pipette flushed with oxygen-free CO_2. The tubes were sealed with butyl rubber stoppers and perforated plastic caps with a headspace of 100% CO_2 and autoclaved at 121°C and 15 psi for 20 minutes. After autoclaving and prior to solidification, the tubes were gently inverted to redistribute agar and placed on the workbench with the plastic caps supported on a piece of rubber tubing at an angle of approximately 15° and agar allowed to set.

4.3.3 Carbohydrates for bacterial fermentation

The carbohydrate substrates used are shown in Table 4-3. Of these, two different fructo-oligosaccharide (FOS) preparations were used: 1) Orafti oligofructose (OF) and 2) Orafti inulin.

Table 4-3 Carbohydrates used to investigate bacterial growth

Carbohydrate	Comments
Glucose (BDH, Global Science, Auckland, New Zealand) 1% w/v and 0.12% w/v	
Lactulose (Carbosynth, Berkshire, UK.) 1% w/v	Lactulose (4-*O*-*β*-D-galactopyranosyl-D-fructose) does not occur naturally but is instead synthesised from lactose through isomerisation.
Oligofructose (OF) (Orafti) 1% w/v	Consists of oligofructose and the natural sugars glucose, fructose and sucrose (95% oligofructose and 5% glucose/fructose/sucrose; DP 2-8)
Inulin (Orafti) 1% w/v	A mixture of oligo- and polysaccharides composed of fructose units connected by β-(1→2) linkages. DP of ≥23.
3' sialyl lactose (Carbosynth, Berkshire, UK.) 1% w/v	The acetylneuraminyl unit is connected to the galactosyl unit of lactose at the 3 position. 
6' sialyl lactose (Carbosynth, Berkshire, UK.) 1% w/v	The acetylneuraminyl unit is connected to the galactosyl unit of lactose at the 6 position. 
Oligosaccharide-enriched fraction (OEF) from goat whey 1% w/v	Composition of this product is as follows: Oligosaccharides: 25.64%; Galactose: 15.92%; Glucose: 12.02%; Galacto-oligosaccharide (GOS): 0.35% and Lactose: 46.07%
Sugar combination – (combo) 0.74% w/v	Equivalent concentrations of sugars to the OEF product (minus the oligosaccharides).
Lactose (BDH, Global Science, Auckland, New Zealand) 0.46% w/v	Equivalent concentration to that found in the OEF
Galactose (BDH, Global Science, Auckland, New Zealand) 0.16% w/v	Equivalent concentration to that found in the OEF
No carbohydrate (No CHO)	Basal (non-supplemented), defined, semi synthetic medium.

OF consists of OF and the neutral sugars glucose, fructose and sucrose (95% OF and 5% glucose/fructose/sucrose content), where the fructose units are connected by β-(1→2) linkages. The total number of fructose (or glucose) units (degree of polymerisation or DP) is between 2 and 8. Orafti inulin is a mixture of oligo and polysaccharides composed of fructose units also connected by β-(1→2) linkages. Whereas OF molecules may have terminal units of glucose, inulin is predominantly terminated with glucose and has a DP greater or equal to 23.

In addition to the OEF from goat whey, a comparable sugar combination (minus the oligosaccharide fraction) as well as the individual monosaccharides (glucose and galactose) and disaccharide (lactose) present at the same concentration as those found in the OEF, were included in bacterial fermentation studies. Also, two acidic oligosaccharides, 3' and 6' sialyl lactose were included to assess their utilisation. These sugars only differ from one another by the bond between the *N*-acetylneuraminic acid and galactose units. In 3' sialyl lactose this bond is in position α2,3 whilst for 6' sialyl lactose it is in the α2,6 position.

Carbohydrates were suspended in distilled water (10 g/L) gassed with CO_2, filter sterilised (0.22 μm filters; Millipore Australia Pty Ltd, NSW, Australia) and introduced to autoclaved, nitrogen pressurised Wheaton glass serum bottles (Sigma-Aldrich, Auckland, New Zealand) until use.

4.3.4 Maintenance and propagation of bacterial cultures

4.3.4.1 Preservation with glycerol

Freezer maintenance medium was prepared by mixing 5.5 g of MRS powder to 66 mL of water and adding 33 mL of glycerol and mixing thoroughly. 1 mL aliquots were prepared in cryogenic vials and autoclaved at 121°C and 15 psi for 20 minutes. Two to three fresh colonies of each bacterial strain from agar plates was added per vial, mixed well, and frozen at -80°C.

4.3.4.2 Propagation of bacterial cultures

Bacterial strains, from glycerol stocks, were plated onto agar plates and grown overnight at 37°C in an atmosphere of 10% CO_2, 10% H_2 in N_2 (anaerobic). A single colony was inoculated into anaerobic MRS broth (Section 4.3.2.1) using a sterile syringe flushed with anaerobic CO_2.

Cultures were grown at 37°C until cultures had obtained stationary growth phase (as determined by absorbance measurement at 600 nm). Probiotic bacteria are commonly grown to stationary phase to achieve maximal cell numbers and tolerance to processing before cells are harvested [298].

4.3.4.3 Preservation on agar slopes

MRS agar slopes were prepared as described in Section 4.3.2.5. Bacterial strains were cultured in MRS broth until stationary growth (Section 4.3.4.2) and using a sterile syringe flushed with CO_2, 20 µL of bacterial culture was gently inoculated down the agar slope. Inoculated tubes were cultured for 24 hours at 37°C and then stored at -80°C as frozen stocks.

4.3.4.4 Growth of bacterial cultures in MRS broth

The growth of each bacterial strain in MRS broth was characterised prior to their use in any other downstream investigation. Stock bacterial cultures maintained on agar slopes were thawed at 37°C. Using a sterile syringe flushed with CO_2 gas, 200 µL of bacterial culture was removed from agar slopes, inoculated into Hungate tubes containing MRS broth (Section 4.3.2.1) and cultured for 24 hours at 37°C.

Thirty µL of bacteria from these broth cultures was inoculated into fresh MRS broth (each bacterial strain was investigated in triplicate). The growth of each bacterial strain was examined using an Ultraspec 1100 pro photometer (Amersham Biosciences, Auckland, New Zealand) set at 600 nm. Immediately after inoculation with bacterial culture, the absorbance of each tube was recorded to represent time zero. The Hungate tubes were incubated at 37°C, and the OD_{600} was measured at hourly intervals until stationary phase. Prior to absorbance measurements the incubated tubes were inverted three times to suspend sedimented cells. The growth of each bacterial strain was assessed based on OD_{600} measurements and used to determine the time required for each bacterial strain to obtain stationary phase, and consequently the optimum time at which to harvest the bacterial cells for all other downstream investigations.

4.3.5 Bacterial fermentation investigations

4.3.5.1 Broth media

The fermentation characteristics of each bacterial strain was assessed in triplicate using Hungate tubes containing 4.5 mL of defined basal broth (Section 4.3.2.2), and supplemented with 0.5 mL of the selected carbohydrate substrates to give a final concentration of 1% (w/v). For the sugar combination (excluding oligosaccharides), lactose, galactose and glucose these were added to defined basal broth at comparable concentrations to that found in the OEF, e.g. sugar combination = 0.74% (w/v); lactose = 0.46% (w/v); galactose = 0.16% (w/v) and glucose = 0.12% (w/v). Glucose was also assessed at a final concentration of 1% (w/v). For non-carbohydrate supplemented tubes an equal volume (0.5 mL) of autoclaved distilled water was used.

4.3.5.2 Dulbecco's modified eagles medium

The OEF was prepared as described in Section 3.3.4 and diluted with low glucose DMEM Glutamax until the oligosaccharide component was present at a final concentration of 1 mg/mL. Sterile Hungate tubes with Teflon screw caps were prepared with 5 mL oligosaccharide supplemented or non-supplemented low glucose DMEM Glutamax.

4.3.5.3 Measurement of bacterial growth in anaerobic media

4.3.5.4 Defined media

Thirty µL of bacteria from each sub-cultured stationary phase bacteria from broth cultures (Section 4.3.4.4) was inoculated into defined basal broth supplemented with the selected carbohydrates, and MRS broth (positive control). Growth of each bacterial strain and fermentable carbohydrate was examined in triplicate based on optical density (OD) measurements set at 600 nm. Immediately after inoculation with bacterial culture the absorbance of each tube was recorded to represent time zero. The Hungate tubes were incubated at 37°C and the OD_{600} was measured at hourly intervals from time zero to 12 hours post inoculation with a final reading at 24 hours. The arithmetical median was calculated from

all single OD readings and absorbance values at time zero were subtracted from each time point for respective Hungate tubes. The ability of test strains to utilise the selected carbohydrates for growth was assessed after 24 hours. Levels of growth were determined by their absorbance values and included: minimal growth (range 0.0 to 0.599); low growth (range 0.6 to 1.199); moderate growth (range 1.2 to 1.799); moderate-high growth (range 1.8 to 2.399) and high growth (range 2.4 to 3.00).

4.3.5.5 Bacterial mucin utilisation assay

The ability of the test strains to utilise mucin as a growth substrate was tested in liquid cultures as described previously [299]. Defined basal broth was prepared (Section 4.3.2.2), supplemented with porcine gastric mucin (Section 4.3.2.3), and was inoculated with 30 µL of sub-cultured stationary phase bacterial broth cultures. Growth was monitored by measuring absorbance at 600 nm. The arithmetical median was calculated from all single OD readings after 24 hour incubation.

4.3.5.6 Measurement of bacterial growth in tissue culture media

The growth of bacterial strains cultured in non-supplemented and OEF-supplemented low glucose DMEM Glutamax (intestinal epithelial cell culture medium; Section 4.3.5.2) was assessed in a 5% CO_2 atmosphere. Thirty µL of each sub-cultured stationary phase bacteria from broth cultures was inoculated into the prepared tubes. The Hungate tubes with loosely sealed screw caps were incubated at 37°C in 5% CO_2 and OD_{600} measured at hourly intervals from time zero to 12 hours post inoculation.

4.3.5.7 Measurement of bacterial growth in contrasting atmospheric conditions and culture media

The growth of bacterial strains in non-supplemented and OEF-supplemented (1% w/v) defined broth (Section 4.3.2.2) and low glucose DMEM Glutamax (Section 4.3.5.2) was assessed in both anaerobic and 5% CO_2 atmospheric conditions.

All tubes were inoculated with 30 µL of sub-cultured stationary phase bacteria from broth

cultures as described in Section 4.3.4.4, and incubated at 37°C in a 5% CO_2 environment. The anaerobic cultures remained tightly sealed, while the screw caps of the other cultures were only loosely attached. Optical density at 600 nm was measured at hourly intervals from time zero to 12 hours post inoculation, and growth assessed after 12 hours. Growth was determined by absorbance as described in Section 4.3.5.4.

4.3.6 Bacterial cell adherence to intestinal epithelial cells

4.3.6.1 Preparation of bacterial cultures for co-culture assays with intestinal epithelial cells

The OEF was prepared as described in Section 3.3.4 and diluted with low glucose DMEM Glutamax until the oligosaccharide component was present at a final concentration of 1 mg/mL. Bacterial strains (as indicated in Table 3-2) were cultured in MRS broth as described in Section 4.3.4.4. Bacteria from stationary phase culture (200 µL) were added to 1 mL of PBS and pelleted by centrifugation at 2492 x g for 5 minutes (11180/13190 rotor, Sigma 3-18K centrifuge). The supernatant was removed and bacterial cells resuspended in 10 mL of either OEF-supplemented or non-supplemented low glucose DMEM culture medium (no FBS or Penicillin-Streptomycin were present in the media).

4.3.6.2 Enumeration of bacteria – colony forming units

A 10 times serial dilution of each bacterial strain was made by adding 20 µL of the neat bacterial preparation (Section 4.3.6.1) to 180 µL PBS, mixing and then with fresh pipette tips taking 20 µL of the 10^{-1} dilution and adding to a further 180 µL of PBS. Dried, pre-reduced MRS agar plates (Section 4.3.2.4) were divided into 4 quadrants, each of which corresponded to a single dilution factor. For each sample of bacterial suspension, 3 drops of 20 µL from each dilution was spotted onto the appropriate quadrant. Once the PBS drops had soaked into the agar the plates were incubated for 24-48 hours upside down inside the anaerobic workstation (Concept Plus, Ruskinn Technology Ltd) at 37°C until bacterial colonies were visible. Quadrants with colony numbers of 30 to 300 per 20 µL spot were counted, and multiplied by the

appropriate dilution factor to determine the total number of colony-forming units (CFUs)/ mL. Only quadrants containing 30-300 colonies per 20 µL spot were considered statistically valid.

4.3.6.3 Number of bacterial cells per mammalian cell in monolayer

The number of bacterial cells per mammalian cell in the monolayer was calculated as the number of CFU in 0.5 mL inoculum divided by the number of mammalian cells present in the monolayer. The number of bacterial cells present in the bacteria-supplemented tissue culture medium was calculated as described in Section 4.3.6.2 while the number of mammalian cells present in confluent monolayers was determined previously (Section 2.4.2).

4.3.6.4 Intestinal epithelial cell culture

Caco-2 and HT29-MTX cells were harvested (Section 2.3.3), and cells counted (Section 2.3.6). Caco-2, HT29-MTX and co-cultures of Caco-2:HT29-MTX cells (90:10 and 75:25 respectively) were seeded at a density of 6.3 x 10^4 cells/cm^2 in low glucose DMEM Glutamax supplemented with 10% FBS and 1% Penicillin-Streptomycin. Epithelial cells were cultured as described in Section 2.3.7.

4.3.6.5 Bacterial adhesion to intestinal epithelial cells

Bacterial adhesion of the selected strains to mono- and co-cultures of Caco-2 and HT29-MTX cells was investigated. For adhesion assays intestinal epithelial cells were seeded into 24-well tissue culture plates. After 20 days of continuous culture monolayers were washed and prepared as described in Section 2.3.11. Prior to bacterial inoculation (day 21 post-seeding) media was aspirated from all wells, and monolayers washed gently 4 times with PBS. To each well, 0.5 mL bacteria-supplemented tissue culture medium (Section 4.3.6.1) was added and plates incubated at 37° in a 5% CO_2 atmosphere for 3 hours. Following incubation and removal of media, monolayers were washed gently four times with PBS. Each strain was assessed for adherence to each of the different mammalian epithelial monolayers in triplicate.

Adherent lactobacilli and bifidobacteria were enumerated on MRS agar plates as described in Section 4.3.6.2. After washing with PBS, cell monolayers were lysed with 1% (v/v) Triton X-100

in PBS (1 mL/well) for 15 minutes at room temperature to release adherent bacteria. The detached cells were repeatedly aspirated to make a homogenous suspension then serially diluted and plated on MRS agar. After incubation for 24 to 48 hours colonies were counted (B_1 cfu/mL). Bacterial cells present in the initial inoculum were also counted (B_0 cfu/mL) and the adhesion percentage was calculated as: $\% \, adhesion = (B_1|B_0) \times 100$.

4.3.7 Statistical analysis

All data analysis was undertaken using the Minitab statistical package (Minitab 16 Statistical Software (2010) State College, PA Minitab, Inc.). Data were first evaluated for normality and homogeneity of variance with the Anderson-Darling and Levene's tests. Data were not normally distributed and had heterogeneous variances. Non-parametric tests were therefore used to assess all data by one-way analysis of variance (ANOVA) with the Kruskal-Wallis test, followed by the Mann-Whitney U test and expressed as median ± median absolute deviation (MAD). Differences were considered statistically significant when *p*-values were less than 0.05.

4.4 Results

4.4.1 Bacterial growth

All bacterial strains reached stationary phase within 24 hours post inoculation when cultured anaerobically in MRS broth (Figure 4-1). *L. plantarum* 299v, *L. casei* Shirota and *L. rhamnosus* HN001 reached stationary phase between 9 and 13 hours post-inoculation. In contrast, an extended culture period (18 to 24 hours post inoculation) was required for the other bacterial strains to reach stationary phase (Figure 4-1). These data were used to determine the duration of growth for individual strains prior to any assay, and to identify the optimum time at which to harvest bacterial cells.

As defined in Section 4.3.5.4 growth was minimal (OD_{600} < 0.600) for all bacterial strains in non-supplemented defined, basal broth (Figure 4-2). Similarly, supplementation of the media with porcine gastric mucin, 3' sialyl lactose, 6' sialyl lactose, and inulin resulted in minimal growth for all bacteria except *B. angulatum*, which exhibited moderate growth (OD_{600} 1.2 to 1.799) in

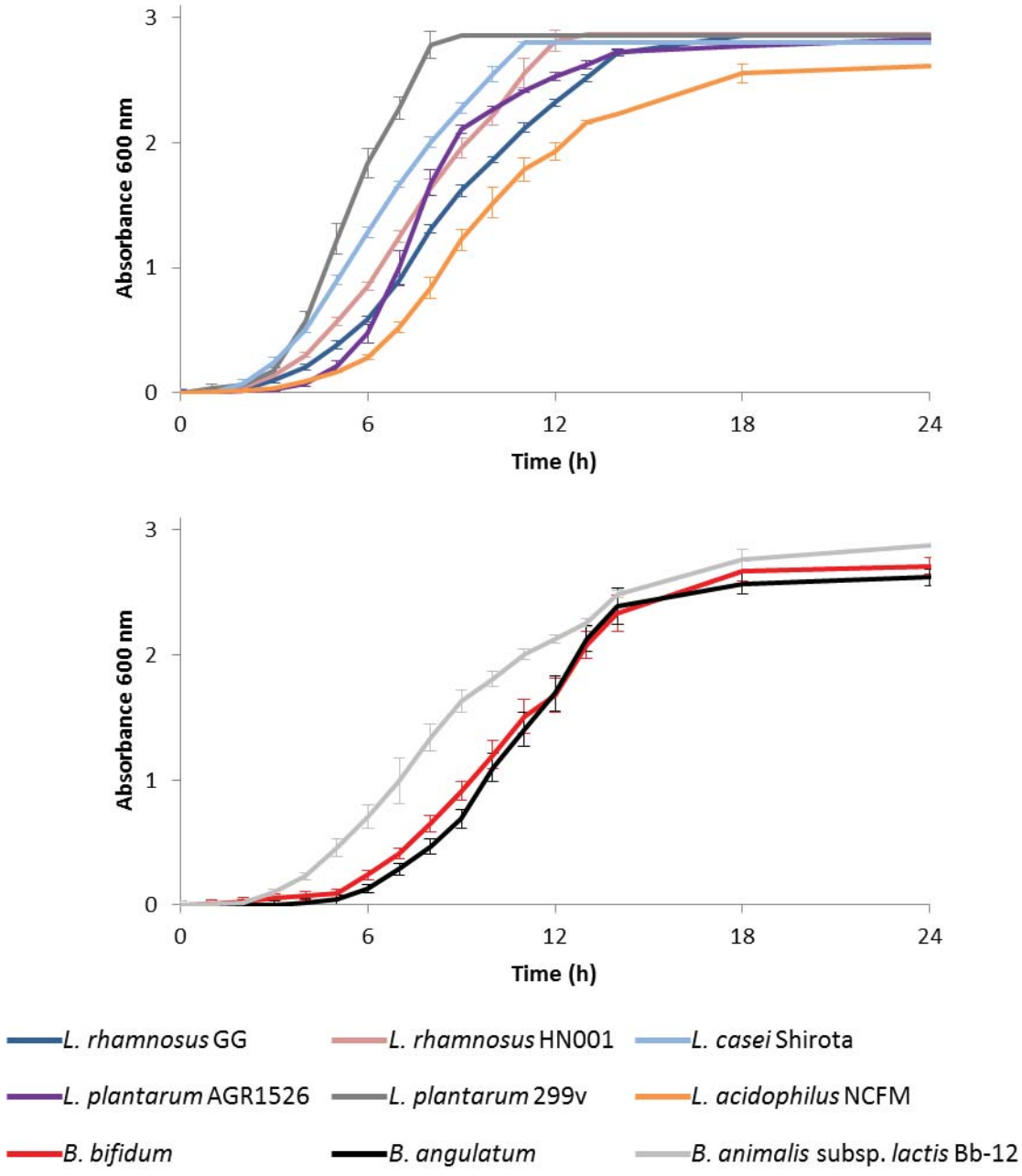


Figure 4-1 Growth of selected bacteria in MRS broth

Each growth curve shows the mean (± standard deviation) absorbance (600 nm) over time (n = 6). All bacteria were cultured in MRS broth anaerobically at 37°C.

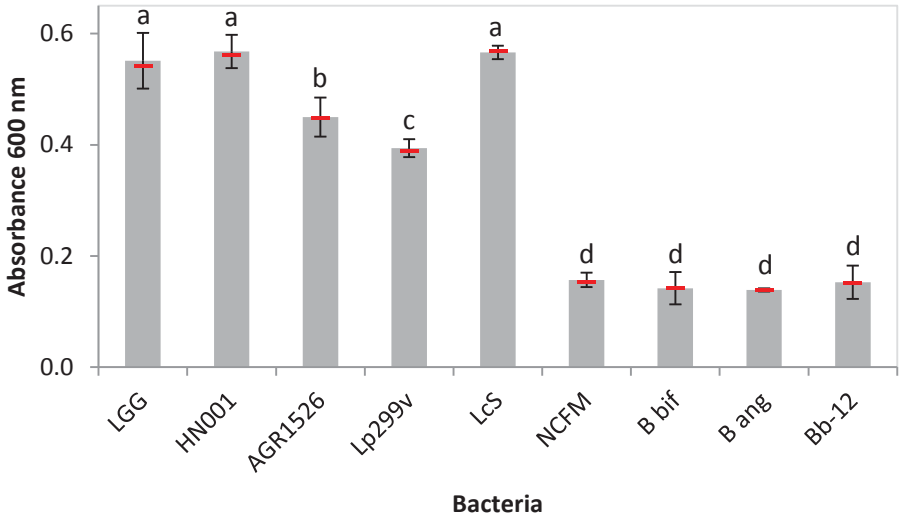


Figure 4-2 Bacterial growth in defined basal broth

All strains were cultured in a defined, basal broth without carbohydrate under anaerobic conditions at 37°C. Values represent the median absorbance (600 nm) after 24 hours, error bars are the median absolute deviation and the red line represents the mean. Values which do not share a letter are significantly different (P< 0.05). LGG = *L. rhamnosus* GG; HN001 = *L. rhamnosus* HN001; AGR1526 = *L. plantarum* AGR1526; Lp299v = *L. plantarum* 299v; LcS = *L. casei* Shirota, NCFM = *L. acidophilus* NCFM; B bif = *B. bifidum*; B ang = *B. angulatum* and Bb-12 = *B. animalis* subsp. *lactis* Bb-12.

inulin-supplemented media (Figure 4-3). Minimal growth was also observed for *L. casei* Shirota, *L. acidophilus* NCFM, *B. bifidum* and *B. lactis* Bb-12 in lactulose-supplemented media. Conversely, all the other bacterial strains investigated had high levels of growth (OD_{600} 2.4 to 3.0) in this media (Figure 4-3).

Supplementation with OF resulted in low levels of growth (OD_{600} 0.6 to 1.199) for *L. rhamnosus* GG, *L. rhamnosus* HN001, *L. plantarum* 299v and *L. casei* Shirota. In contrast, there was moderate growth for *B. lactis* Bb-12 and *B. angulatum* and there was a high level of growth of *L. plantarum* AGR1526 in the same media (Figure 4-3). Additionally, high levels of growth were obtained in media supplemented with either glucose (1% w/v), or the OEF by most bacterial strains investigated, except *L. acidophilus* NCFM, *B. bifidum* and *B. angulatum* (Figure 4-3).

The impact of selected carbohydrates at comparable concentrations to that found in the OEF on bacterial growth was also assessed after 24 hours (Figure 4-3). Although *L. casei* Shirota had high and moderate-high levels of growth in the OEF and combo-supplemented media respectively, this strain had a low level of growth in lactose-supplemented media (Figure 4-3). However, all other bacterial strains that exhibited high levels of growth in media supplemented with the OEF also had high levels of growth in combo- and lactose-supplemented media (Figure 4-3).

In glucose (0.12% w/v) and galactose-supplemented media, minimal levels of growth were observed for both *L. acidophilus* NCFM and *B. bifidum*. Minimal growth in glucose-supplemented media (0.12% w/v) was also observed for *B. angulatum*, but in galactose-supplemented media this bacteria had low levels of growth. For all other bacterial strains a moderate level of growth was observed. However, for each of these strains, growth in glucose-supplemented media (0.12% w/v) was significantly lower ($P < 0.05$) compared to galactose-supplemented media (Figure 4-3).

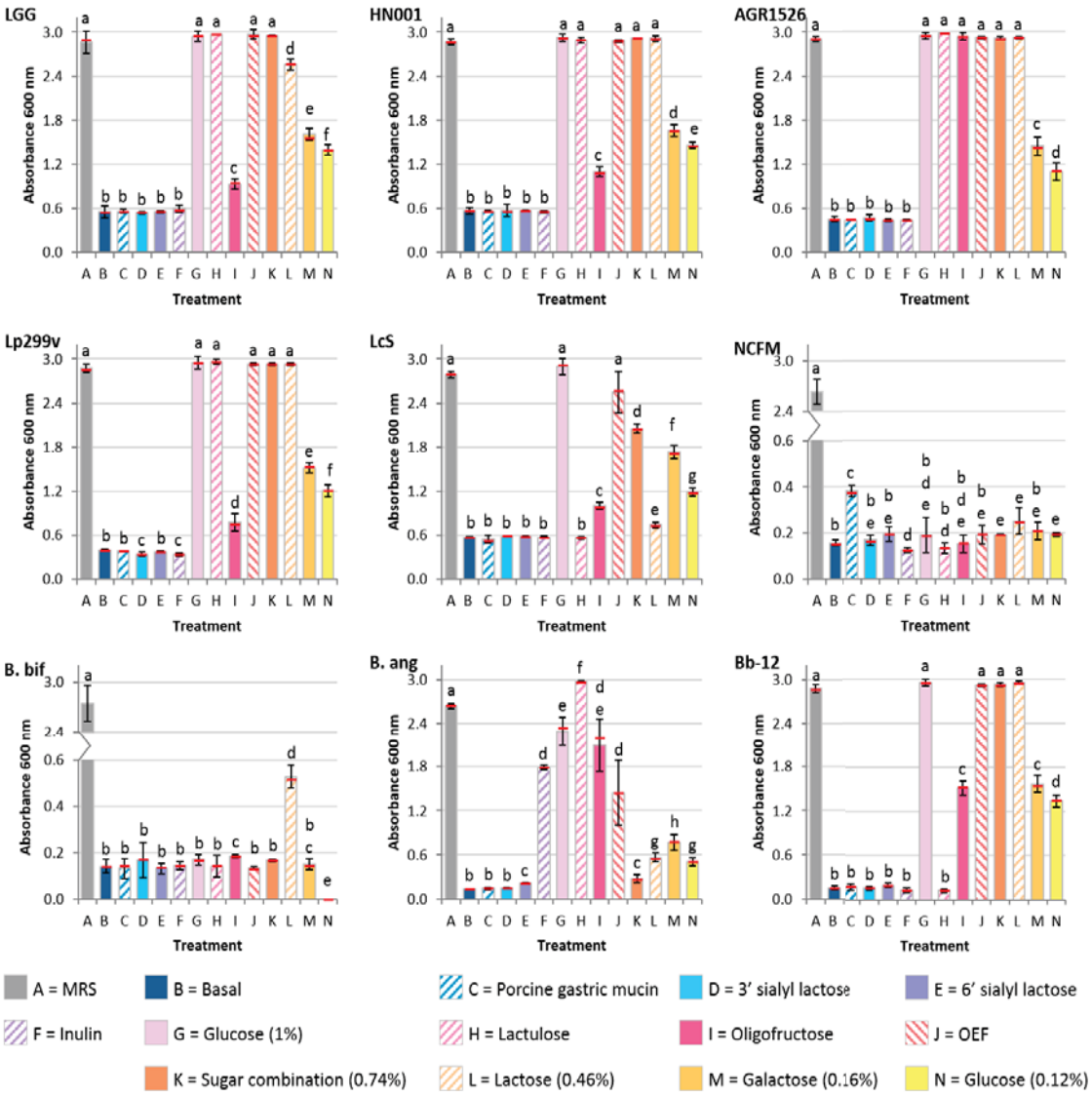


Figure 4-3 Bacterial growth in defined basal broth supplemented with selected carbohydrates

All strains were cultured in defined basal broth supplemented with selected carbohydrates at 1% w/v (unless otherwise indicated) under anaerobic conditions at 37°C for 24 hours. Values represent median absorbance (600 nm), error bars are median absolute deviations and red line represents the mean. To visualise parts of the data in more detail the y axis is broken for panels NCFM and B. bif. Values which do not share a letter are significantly different (P< 0.05). OEF= oligosaccharide-enriched fraction, LGG= *L. rhamnosus* GG; HN001= *L. rhamnosus* HN001; AGR1526= *L. plantarum* AGR1526; Lp299v= *L. plantarum* 299v; LcS= *L. casei* Shirota; NCFM = *L. acidophilus* NCFM; B. bif = *B. bifidum*; B. ang = *B. angulatum* and Bb-12 = *B. animalis* subsp. *lactis* Bb-12. Levels of growth (absorbance): minimal growth (range 0.0-0.599); low growth (range 0.6-1.199); moderate growth (range 1.2-1.799); moderate-high growth (range 1.8-2.399); and high growth (range 2.4-3.00).

4.4.2 Impact of atmospheric conditions and culture media on bacterial growth

Minimal growth (OD_{600} < 0.6) was observed for all strains in basal (non-supplemented) defined broth, and basal (non-supplemented) DMEM media, when cultured for 12 hours anaerobically or in 5% CO_2 atmospheric conditions (Figure 4-4). Additionally, in defined broth and DMEM media supplemented with the OEF (1% w/v), minimal growth was observed for *L. acidophilus* NCFM and *B. bifidum*, irrespective of the atmospheric culture conditions. Conversely, when cultured anaerobically in MRS broth (positive control), bacterial strains had levels of growth ranging from moderate (OD_{600} 1.2 to 1.799) to high (OD_{600} 2.4 to 3.0).

In OEF-supplemented defined broth, *B. angulatum* had low levels of growth (OD_{600} 0.6 to 1.119) when cultured anaerobically. However, when cultured in 5% CO_2 atmospheric conditions in the same media, and also OEF-supplemented DMEM, only minimal growth was observed. All other bacterial strains, in OEF-supplemented media (defined and DMEM), had low levels of growth when cultured in 5% CO_2 atmospheric conditions (Figure 4-4). This growth was significantly lower ($P < 0.05$) than when these strains were cultured in defined anaerobic broth supplemented with the OEF.

4.4.1 Bacterial cell adherence to Caco-2, HT29-MTX and co-culture cell monolayers

Using the data obtained from the growth curves generated from each of the bacterial strains in MRS broth, the optimum time to harvest bacterial cells from stationary cultures and their use in all assays was determined (Section 4.4.1 and Figure 4-1). The number of bacteria, as determined using viable counts inoculated into wells containing tissue culture cells, varied from strain to strain but there was approximately one bacterial cell for every mammalian cell (Table 4-4). This result allowed for the adherence ability of the different strains to the different epithelial cell cultures to be compared.

Bacterial adhesion to mono-cultures and co-cultures varied with 0.1% of inoculated *B. angulatum* adherent to 90:10 co-cultures to 38% of inoculated *L. plantarum* AGR1526 adherent to 75:25 co-cultures (Figure 4-5). Bacterial adherence to Caco-2 mono-cultures varied

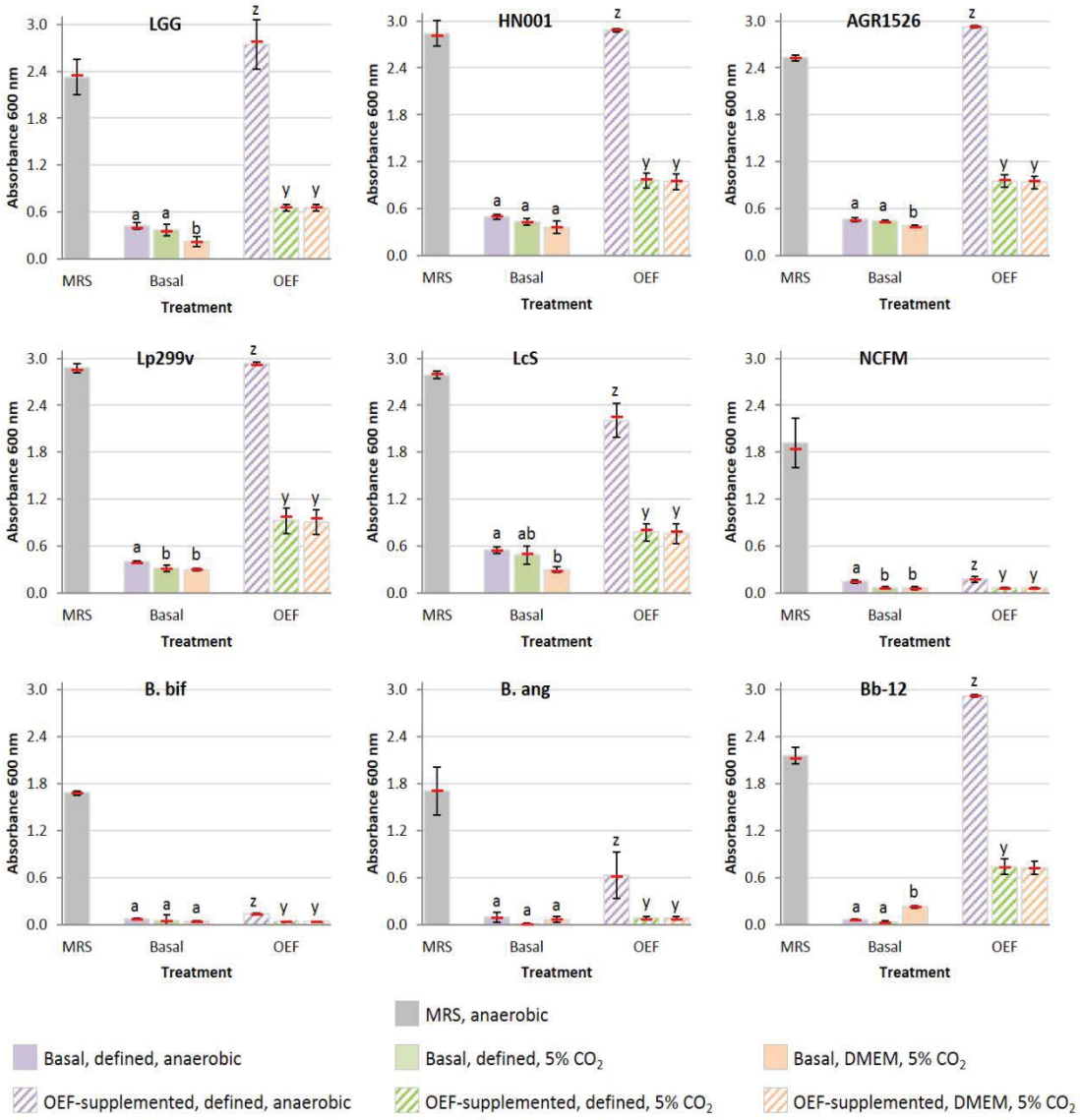


Figure 4-4 Bacterial growth in response to oligosaccharide-enriched culture media and atmospheric conditions

Bacterial growth (absorbance, 600 nm) in MRS (positive control), defined basal broth or basal DMEM. Oligosaccharide-enriched fraction (1% w/v) was added as a fermentable carbohydrate source as indicated (OEF) and all broths were cultured for 12 hours anaerobically or in 5% CO_2 atmospheric conditions. Values represent median absorbance, error bars are median absolute deviation, and red line represents the mean. Values for individual bacteria and treatments which do not share a letter are significantly different $P< 0.05$. LGG = *L. rhamnosus* GG; HN001 = *L. rhamnosus* HN001; AGR1526 = *L. plantarum* AGR1526; Lp299v = *L. plantarum* 299v; LcS = *L. casei* Shirota; NCFM = *L. acidophilus* NCFM; B. bif = *B. bifidum*; B. ang = *B. angulatum* and Bb-12= *B. animalis* subsp. *lactis* Bb-12. Levels of growth (absorbance): minimal growth (range 0.0 to 0.599); low growth (range 0.6 to 1.199); moderate growth (range 1.2 to 1.799); moderate-high growth (range 1.8 to 2.399); and high growth (range 2.4 to 3.00).

Table 4-4 Numbers of bacteria inoculated into wells

Viable counts of bacteria inoculated into wells containing intestinal epithelial cell monolayers as determined from overnight plate counts; and the respective number of bacterial cells in the media suspension per mammalian cell in the monolayer. Values are medians, numbers in parenthesis are ranges and numbers in brackets are means. Values which do not share a letter are significantly different.

Bacterial strain	***Bacteria in supplemented media (log_{10} CFU)***	***Number of bacteria/mammalian cell***
L. rhamnosus GG	6.16 (6.15-6.17) [6.16] *a*	1.07 (1.05-1.08) [1.07] *b*
L. rhamnosus HN001	6.16 (6.15-6.19) [6.17] *a*	1.07 (1.04-1.14) [1.08] *b*
L. plantarum AGR1526	6.15 (6.11-6.17) [6.14] *a*	1.04 (0.94-1.09) [1.02] *b*
L. plantarum 299v	6.16 (6.15-6.17) [6.16] *a*	1.07 (1.04-1.09) [1.07] *b*
L. casei Shirota	6.16 (6.15-6.17) [6.16] *a*	1.07 (1.04-1.09) [1.07] *b*
L. acidophilus NCFM	6.15 (6.06-6.16) [6.12] *a*	1.03 (0.85-1.07) [0.98] *b*
B. bifidum	6.16 (6.13-6.18) [6.16] *a*	1.07 (1.00-1.11) [1.06] *b*
B. angulatum	6.13 (6.11-6.17) [6.14] *a*	1.00 (0.96-1.09) [1.01] *b*
B. lactis Bb-12	6.16 (6.15-6.17) [6.16] *a*	1.07 (1.05-1.08) [1.07] *b*

Average (1.36×10^6) number of mammalian cells in confluent monolayers after 21 days continuous culture in 24 well plates: HT29-MTX = 1.38×10^6 and Caco-2 = 1.33×10^6.

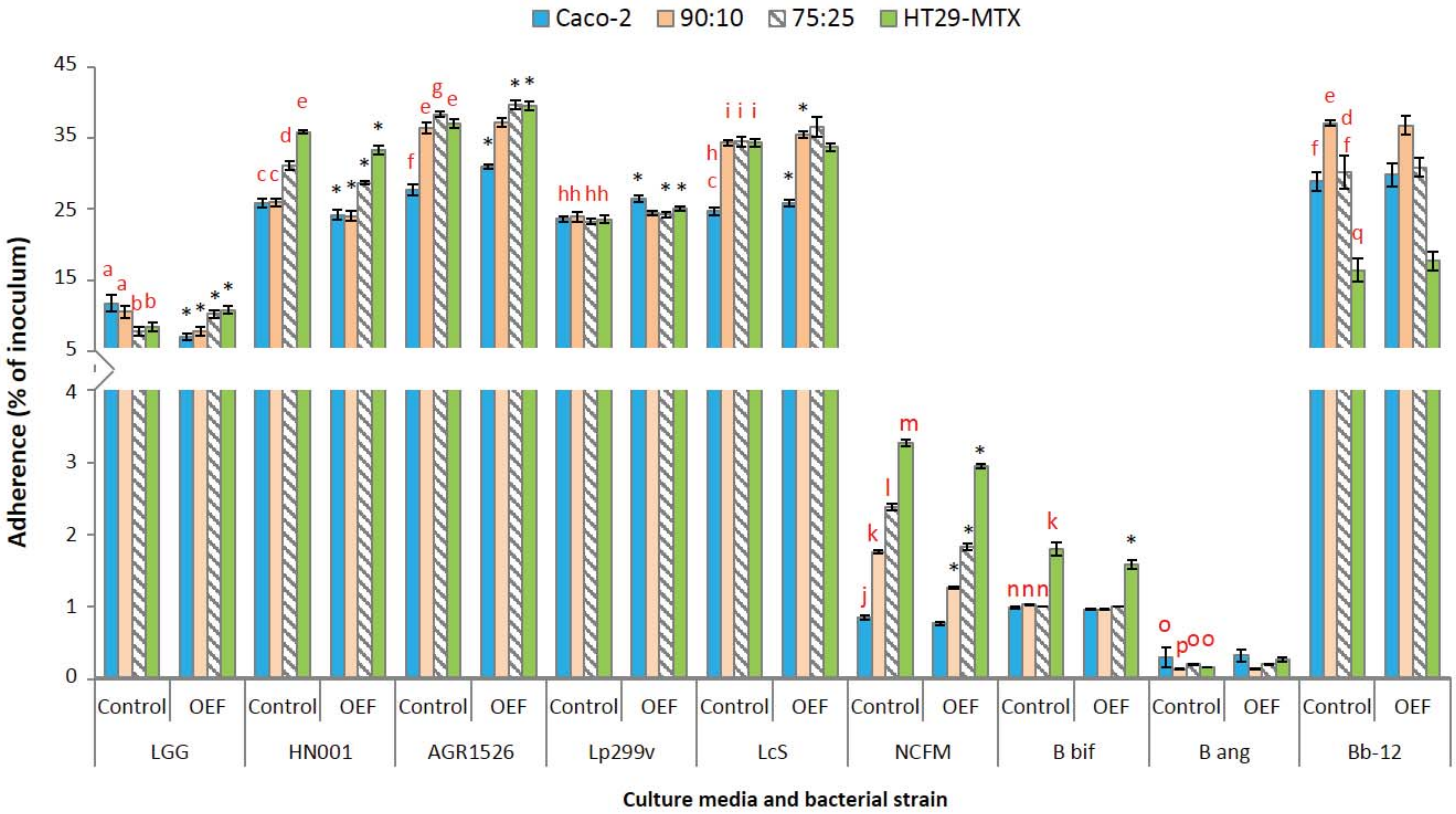


Figure 4-5 Bacterial adherence to Caco-2 and HT29-MTX mono-cultures and Caco-2:HT29-MTX co-cultures

Bacteria (percentage of inoculum) adherent to Caco-2 and HT29-MTX mono-cultures and Caco-2:HT29-MTX co-cultures (90:10 and 75:25 Caco-2:HT29-MTX cells respectively) after 3 hours incubation in control (basal) and oligosaccharide-enriched fraction (OEF) supplemented (1 mg/mL) DMEM media. To visualise parts of the data in more detail the y axis is broken. Values are medians and error bars are the median absolute deviation. Control values which do not share a letter are significantly different ($P< 0.05$). * Significantly different to respective control ($P< 0.05$). LGG = *L. rhamnosus* GG; HN001 = *L. rhamnosus* HN001; AGR1526 = *L. plantarum* AGR1526; Lp299v = *L. plantarum* 299v; LcS = *L. casei* Shirota, NCFM = *L. acidophilus* NCFM; B bif = *B. bifidum*; B ang = *B. angulatum* and Bb-12 = *B. animalis* subsp. *lactis* Bb-12.

from 0.3% to 29% of inoculum with *B. lactis* Bb-12 (29%) and *L. plantarum* AGR1526 (28%) having the highest adhesion percentages. The adhesion of *L. rhamnosus* HN001 (26%), *L. plantarum* 299v (24%) and *L. casei* Shirota (25%) were significantly higher ($P<0.05$) than those of *L. rhamnosus* GG (11.7%), *L. acidophilus* NCFM (0.9%), *B. bifidum* (1.0%), and *B. angulatum* (0.3%); although the latter three strains did not have adhesion values greater than 4% to any epithelial cell culture (Figure 4-5).

Adhesion to 90:10 co-cultures was significantly increased ($P< 0.05$) for *L. plantarum* AGR1526 (37%), *L. casei* Shirota (34%), *L. acidophilus* NCFM (2%) and *B. lactis* Bb-12 (37%) when compared to the adhesion of these strains to Caco-2 mono-cultures (28%, 25%, 1% and 29% respectively). In contrast, adhesion of strain *B. angulatum* was significantly decreased ($P< 0.05$) to 90:10 co-cultures (0.1%) than to Caco-2 mono-cultures (0.3%) and was the lowest adhesion of any strain to any epithelial cell culture (Figure 4-5).

The highest adhesion percentage of any bacterial strain to any epithelial cell culture was obtained by *L. plantarum* AGR1526 to the 75:25 co-cultures (38.3%). Adhesion values of *L. rhamnosus* GG were significantly decreased ($P< 0.05$) to 75:25 co-cultures than to both Caco-2 and 90:10 monolayers (8%, 12% and 11% respectively). In contrast, adhesion of *L. rhamnosus* HN001 to 75:25 co- cultures (31%) was significantly increased ($P< 0.05$) compared to both Caco-2 (26%) and 90:10 (26%) monolayers (Figure 4-5).

Adhesion of *B. lactis* Bb-12 to 75:25 co-cultures (30%) was significantly lower ($P< 0.05$) than adherent to 90:10 co-cultures (37%). However, adhesion of this strain was significantly lower ($P< 0.05$) to HT29-MTX mono-cultures (16.4%) than to any other epithelial cell culture (Figure 4-5). In contrast, adhesion of *L. rhamnosus* HN001 (36%), *L. acidophilus* NCFM (3%) and *B. bifidum* (2%) were significantly increased ($P< 0.05$) to HT29-MTX mono-cultures than to any other epithelial cell culture. However, there were similarities ($P> 0.05$) in the adhesion of *L. rhamnosus* HN001 (36%) to HT29-MTX mono-cultures; *L. plantarum* AGR1526 to both 90:10 co-cultures (37%) and HT29-MTX mono-cultures (37%); and also *B. lactis* Bb-12 (37%) to 90:10

co-cultures (Figure 4-5).

In comparison to respective controls, the inclusion of the OEF in the media significantly reduced ($P< 0.05$) the adhesion of *L. rhamnosus* HN001 to all epithelial monolayers (Figure 4-5). In addition, the adhesion of *L. rhamnosus* GG was also significantly reduced ($P> 0.05$) to Caco-2 mono-cultures (5%) and 90:10 co-cultures (3%). In contrast, adhesion of this strain was significantly increased ($P< 0.05$) to 75:25 co-cultures (2%) and HT29-MTX mono-cultures (2%) in OEF-supplemented media (Figure 4-5). In comparison to respective controls, adhesion of *L. plantarum* AGR1526 and *L. plantarum* 299v was significantly increased ($P< 0.05$) to all intestinal epithelial monolayers except 90:10 co-cultures in OEF-supplemented media. Conversely, adhesion of *L. acidophilus* NCFM was significantly reduced ($P< 0.05$) to all epithelial cell cultures except Caco-2 mono-cultures in OEF-supplemented media compared to controls (Figure 4-5). Supplementation of the media with the OEF had no impact on percentage adhesion of *B. angulatum* and *B. lactis* Bb-12 to any epithelial cell culture. A summary of the results are shown in Table 4-5.

4.5 Discussion

In this study it was shown for the first time that an OEF from goat whey stimulated the growth and activity of some probiotic bacterial strains with proven intestinal benefits. Moreover, the OEF improved the adhesion of some probiotic strains to intestinal epithelial cells, hence a synbiotic effect. However, of the probiotic strains that utilised the OEF as a growth substrate, not all had increased adherence to intestinal epithelial cell cultures when OEF was included in the media, which was in contrast to the hypothesis of this study.

Of the nine probiotic bacterial strains investigated in this study, 7 (*L. rhamnosus* strain GG and HN001, *L. plantarum* strain AGR1526 and 299v, *L. casei* Shirota, *B. angulatum* DSM 20098, and *B. animalis* subsp. *lactis* Bb-12) were able to utilise the OEF for growth when cultured anaerobically in a defined semi-synthetic broth. Of these, only *L. casei* Shirota and *B. angulatum* DSM 20098 had higher levels of growth in OEF-supplemented media when

Table 4-5 Summary of results for bacterial growth in oligosaccharide-enriched fraction from goat whey and their adherence to epithelial cell cultures

Growth levels (as indicated) of selected bacteria in OEF-supplemented broth/media in different atmospheres after 12 hours. Adherence (values as indicted) of selected bacteria to intestinal epithelial cell cultures after 3 hours in basal media and the change in adherence in OEF-supplemented media. Increase compared to control (↑), decrease compared to control (↓) and no significant change (P> 0.05) compared to control (–).

Parameter	Variable		Bacterial strain								
Growth	Atmosphere	Media	LGG	HN001	AGR1526	Lp299v	LcS	NCFM	B bif	B ang	Bb-12
	Anaerobic	Defined broth	High	High	High	High	Mod-high	Min	Min	Low	High
	5% CO_2	Defined broth	Low	Low	Low	Low	Low	Min	Min	Min	Low
	5% CO_2	DMEM	Low	Low	Low	Low	Low	Min	Min	Min	Low

Levels of growth as determined by absorbance 600 nm

Minimal 0.0 – 0.599	Low 0.6 – 1.199	Moderate 1.2 – 1.799	Moderate-high 1.8 – 2.399	High 2.4 – 3.000

Adherence	Cell culture	Media	LGG	HN001	AGR1526	Lp299v	LcS	NCFM	B bif	B ang	Bb-12
	Caco-2	Basal	++	+++	++++	+++	+++	+	+	+	++++
		OEF	↓	↓	↑	↑	↑	–	–	–	–
	90:10 Caco-2:HT29-MTX	Basal	++	+++	++++	+++	++++	+	+	+	++++
		OEF	↓	↓	–	–	↑	↓	–	–	–
	75:25 Caco-2:HT29-MTX	Basal	++	++++	++++	+++	++++	+	+	+	++++
		OEF	↑	↓	↑	↑	–	↓	–	–	–
	HT29-MTX	Basal	++	++++	++++	+++	++++	+	+	+	+++
		OEF	↑	↓	↑	↑	–	↓	↓	–	–

Levels of adherence

+ 0 – 5%	++ 5.01 – 15%	+++ 15.01 – 30%	++++ 30.1%+

LGG = *L. rhamnosus* GG; HN001 = *L. rhamnosus* HN001; AGR1526 = *L. plantarum* AGR1526; Lp299v = *L. plantarum* 299v; LcS = *L. casei* Shirota, NCFM = *L. acidophilus* NCFM; B bif = *B. bifidum*; B ang = *B. angulatum* and Bb-12 = *B. animalis* subsp. *lactis* Bb-12.

compared to the sugar combination. This suggested that the presence of the oligosaccharide component in the OEF was responsible for the increased growth of these strains, but not for the other strains.

For both *L. casei* Shirota and *B. angulatum* DSM 20098 growth was increased in galactose-supplemented media when compared to lactose-supplemented, which was in contrast to all other bacterial strains investigated in this study. Reduced growth of these strains in lactose-supplemented media may be related to the alternative pathways in which lactose can be metabolised by different bacteria. For example, lactose transport, concomitant phosphorylation and hydrolysis by β-phospho-galactosidase generates galactose 6-phosphate and glucose from lactose which are utilised simultaneously, whereas an alternative pathway transports lactose into the cell prior to hydrolysis by β-galactosidase which results in preferential metabolism of glucose and excretion of galactose [291, 300, 301]. Thus reduced growth of *L. casei* Shirota and *B. angulatum* DSM 20098 in lactose-supplemented media may suggest that lactose transport and/or modification systems may be deficient.

L. acidophilus NCFM and *B. bifidum* DSM 20456 had only minimal levels of growth in the defined broth supplemented with the OEF. However, for both of these strains only minimal levels of growth were observed in the defined broth supplemented with any carbohydrate substrate. It is probable that the minimal growth observed for both of these strains in the defined broth may be related to its chemical composition. When cultured anaerobically in the commercial lactobacilli MRS broth, both of these strains had high levels of growth within 20 hours of inoculation. One component absent in the defined medium used here but present in the commercial medium was polysorbate 80. Polysorbate is a surfactant, and surfactants can increase enzyme activity of some micro-organisms and also facilitate the uptake of nutrients by some lactobacilli [302]. When included in culture medium polysorbate has previously been shown to improve growth rates of lactobacilli and enhance bacterial acid resistance for both lactobacilli and bifidobacteria [303], although its inclusion in culture media was shown to have

no beneficial effects on the growth of *L. casei* strain YIT 9018 (used in the manufacture of Yakult in Korea and thus similar to *L. casei* Shirota) [304]. Polysorbate was not included in the defined broth used in this study due to its suppression of some analytes during downstream applications such as liquid chromatography/mass spectrometry [305]. Although these analytical methods were not fully employed in this study their use was provisionally considered and consequently polysorbate was omitted from the defined medium.

4.5.1 Utilisation of fructo-oligosaccharide substrates

The utilisation patterns of other carbohydrate compounds such as inulin (DP ≥ 23) and oligofructose (DP 2 to 8) were heterogeneous for most strains, with differences in growth response values being more pronounced for oligofructose samples than any other substrates. The results in this study indicated bifidobacteria had greater utilisation of this substrate than lactobacilli with only *B. angulatum* obtaining moderate levels of growth in the presence of inulin.

Differences in the utilisation of oligofructose substrates by bifidobacteria and lactobacilli may be associated with the localisation of oligofructose transporters and the enzymes responsible for its degradation [156]. For example, the enzyme β-fructofuranosidase is located intracellularly in bifidobacteria and can be inducible, but only after oligofructose has gained entry into the cell [156]. For some strains of bifidobacteria a specific permease type of transporter for oligofructose has been reported, although an adenosine tri-phosphate (ATP)-binding cassette (ABC) type or phosphotransferase-type transporter is more common for other bacterial genera and have been identified in some lactobacilli species [156, 297]. Consequently, preferential metabolism of oligofructose by some bifidobacterial strains indicates that uptake may occur first and induces the enzymes necessary for hydrolysis and subsequent metabolism of fructose monomers. This is the case for *B. infantis* and *B. animalis* [306], although other studies have noted the inability of some *Bifidobacterium* to metabolise the larger fructan polymers such as those present in inulin [283]. In other bacterial species

such as lactobacilli it is thought that oligofructose degradation occurs extracellularly, making them less competitive when growing in this substrate [307].

4.5.2 Utilisation of milk oligosaccharides and mucin

Utilisation of the HMOs 3' sialyl lactose and 6' sialyl lactose was minimal for all strains investigated in this study (Figure 4-3). Others have previously shown that there was minimal utilisation of HMOs by lactobacilli strains, and although some strains of *Bifidobacterium* such as *B. infantis* were able to utilise these components for growth not all bifidobacteria possess the required enzymes (1,2-α-$_L$-fucosidase and endo-α-*N*-acetylgalactosaminidase) to metabolise these sugars [301, 308, 309]. In addition, it was also shown in this study that there was limited utilisation of porcine gastric mucin for growth by all bacterial strains investigated. Porcine gastric mucin has similar structures and chemical properties to human gastric mucin [310] and is therefore often used to investigate the mucin degrading phenotype of bacteria. At physiological concentrations (equal to or greater than 20 mg/mL) mucins generally form a gel-like structure, which is important for protecting the epithelial layer. However at more dilute concentrations (less than 1 mg/mL) mucins do not exhibit such extensive aggregation which make these concentrations more suitable for investigating the mucin degrading abilities of bacteria [311]. Human intestinal glycans are similar to the structures of HMOs, containing *N*-acetylglucosamine, galactose, fucose, sialic acid and *N*-acetylygalactosamine [312]. Although some bacteria such as *Bacteroides fragilis* are known to utilise host-derived glyco-conjugates found in the mucus layer of the intestine as a growth substrate [287, 313], an ability to degrade mucin has been suggested as a virulence determinant for a number of enteropathogens, including *Campylobacter jejuni, Vibrio cholerae*, and *Yersinia enterocolitica* [314, 315]. Consequently, mucin degrading properties are considered to be an undesirable trait in probiotic bacteria [314].

4.5.3 Bacterial adherence to intestinal epithelial cell cultures

Adherence to intestinal epithelial cells after 3 hours was variable among strains. A 3 hour

incubation period was selected because previous reports have indicated that adherence does not substantially increase with longer incubation periods [295]. The adhesion of *L. acidophilus* NCFM, *B. bifidum* and *B. angulatum* was lower to all intestinal cell cultures compared to other bacterial strains. However, these three bacterial strains grew poorly when cultured in a 5% CO_2 environment regardless of media type, suggesting that low adherence (less than 4% of inoculum) was as a consequence of the culture conditions rather than their respective adherence characteristics. Bifidobacteria are generally considered to be obligate anaerobes that will lose viability in the presence of atmospheric levels of oxygen; although some species such as *B. animalis* subsp. *lactis* Bb-12 will tolerate low oxygen concentrations [316]. Adherence values of *L. acidophilus* NCFM were low to all intestinal epithelial cell cultures (< 4%); but was increased as the percentage of HT29-MTX cells in the cell monolayer also increased. This may suggest a greater affinity of this bacterial strain to the HT29-MTX cells compared to Caco-2 cells. However, it has previously been reported that adherence of this strain to HT29 intestinal epithelial cells (2.1%), which are a predominantly undifferentiated population of cells which do not produce mucin, was similar to that observed to HT29-MTX cells [299]. In contrast, it has also been reported that *L. acidophilus* HN017 has increased adherence capability to HT29-MTX cells than to either Caco-2 or HT29 cells and that adherence to Caco-2 cells was greater than that to HT29 cells [317]. This would suggest bacterial adhesion to intestinal epithelial cells is both strain and species specific [193, 318], but may also be affected by the growth conditions, media components and the differentiation status of the epithelial cells.

Additionally, adherence values for some of these strains may have been lower because of the methods employed to determine adherence values. In this study the conventional plating and colony counting method was used. However, this method of enumeration requires that individual bacteria are released from the epithelial cultures and remain culturable after the release process giving rise to a single colony from each individual cell. An alternative approach

would have been to metabolically radiolabel the bacteria with [5'-^{3}H] thymidine and determine the percentage adhesion of bacteria to the different epithelial cell cultures by liquid scintillation [319].

In this study adherence of *L. plantarum* AGR1526, *L. rhamnosus* HN001, and *L. casei* Shirota was increased when the mucus secreting HT29-MTX cell line was present in the co-cultures. Although increased adherence of strain *L. rhamnosus* HN001 to HT29-MTX mono-cultures than to either HT29 or Caco-2 mono-cultures has been reported previously [317]. It is possible that this result may be due to the physical entrapment of cells in the mucus layer rather than a higher specific affinity of these strains to this cell line. However, some strains of lactobacilli possess mucus binding proteins which are known as one of the effector molecules involved in the adherence of lactobacilli to the host [320]. An extracellular mucus binding protein which has characteristics typical of cell surface proteins of Gram positive bacteria (a C-terminal sortase recognition site targeting the protein for covalent attachment to the peptidoglycan layer at the outside of the bacterial cells and N-terminal signal peptide targeting the protein for secretion) was identified in *L. reuteri* 1063 [10]. A similar domain was identified in *L. plantarum* WCFS [321]. *L. acidophilus* NCFM, *L. gasseri*, and *L. johnsonii* have known or proposed mucus binding proteins (as determined from comparative analysis of multiple amino acid sequence alignments) [11], while the presence of a mucus binding protein associated with the pili of *L. rhamnosus* GG mediated the persistence of this strain in the human intestinal tract, compared to *L. rhamnosus* LC705 where the mucus-binding pili were absent [322]. Mucus-binding proteins were not observed in the genomes of *B. infantis* ATCC 15697 [288, 323], or *B. animalis* subsp. *lactis* [324]. Instead, other putative cell surface proteins have been identified for *B. animalis* subsp. *lactis* that could be involved in interactions with human intestinal epithelial cells and include: two putative collagen adhesion proteins, an elastin-binding protein and a fibronectin-binding protein [324].

4.5.4 Modulation of bacterial adherence

Not all of the strains (*L. rhamnosus* strain GG and HN001, *L. plantarum* strain AGR1526 and 299v, *L. casei* Shirota and *B. animalis* subsp. *lactis* Bb-12) which were shown to utilise the OEF for growth when cultured in 5% CO_2 atmospheric conditions had increased adherence to intestinal epithelial cell cultures when the OEF was included in the culture media, which was in contrast to the hypothesis stated for this study. Of these strains, only adherence of *B. animalis* subsp. *lactis* Bb-12 to any of the epithelial cell cultures was unchanged when the OEF was included in the culture media; while for others (*L. rhamnosus* GG, *L. plantarum* strain AGR1526 and 299v and *L. casei* Shirota) adherence was increased to some of the epithelial monolayers. Previously in this study (Chapter 3, Section 3.4.4) some epithelial cell cultures exhibited increased mucin protein abundance after 3 hours incubation with the OEF. In particular the membrane bound mucin MUC4 protein was increased in all the cell cultures except 90:10 co-cultures. In this current study there was an increased level of adherence of *L. plantarum* AGR1526 and 299v to the intestinal epithelial cell cultures, except 90:10 co-cultures, when the OEF was present in the culture media. Whether there is an association with an increase in MUC4 protein abundance in the same intestinal epithelial cell cultures remains to be determined, but it could be hypothesised that a potential relationship between MUC4 mucin protein and *L. plantarum* adhesion exists. Previous work has suggested an interaction between another lactic acid bacteria, *Lactococcus lactis* subsp. *lactis* BGKP1 and MUC3 and MUC5AC proteins, which aids in the adherence of this bacterial species to the mucus layer [325].

Although the inclusion of the OEF in the culture media increased the adherence of *L. rhamnosus* GG to 75:25 co-cultures and HT29-MTX mono-cultures, adherence of this strain to Caco-2 mono-cultures and 90:10 Caco-2:HT29-MTX co-cultures was decreased. Adherence was also decreased for *L. rhamnosus* HN001 to all intestinal epithelial monolayers when the OEF was included in the culture media. Whether the decrease in adherence was as a consequence of changes in the epithelial cell cultures, such as changes in mucin expression was not

determined in this study. However, it could be hypothesised that changes in the mucus layer as a consequence of altered mucin gene expression and/or protein abundance may result in changes in the adherence ability of some bacterial strains. Alternatively, it could be suggested that the OEF may have acted as molecular receptor decoys or anti-adhesives. A similar effect is observed for some pathogenic bacteria such as enteropathogenic *Escherichia coli* (EPEC) [326]. For example, when exposed in the presence of galacto-oligosaccharides, adherence of EPEC strain E2348/69 (O127:H6) to Hep-2 and Caco-2 cells is decreased. This reduced adherence was related to the anti-adhesive nature of the prebiotic substrate, and not to a change in the expression of the bundle-forming-pilus protein which are involved in local adherence of this bacterial strain [326].

In addition, it was interesting to note that of the probiotic strains that did not utilise the OEF for growth (*L. acidophilus* NCFM, *B. bifidum* DSM 20456 and *B. angulatum* DSM 20098) when cultured in 5% CO_2 atmospheric conditions, adherence to the intestinal epithelial cultures could still be modulated. For example, when the OEF was included in the culture media there was decreased adherence of *L. acidophilus* NCFM to all epithelial cell cultures except Caco-2 mono-cultures, while adherence was decreased for *B. bifidum* DSM 20456 to HT29-MTX mono-cultures. Although this result was not expected, it was in accordance with a previous report that indicated that although *B. longum* subsp. *infantis* ATCC 15697 was unable to utilise either of the HMOs 3' and 6' sialyl lactose for growth, pre-exposure to these substrates could modulate the adherence ability of this strain to both HT29 and Caco-2 intestinal epithelial cells [295].

In general, the effect of the OEF on adherence was considered to be strain specific with positive, neutral and negative modulatory effects observed for different bacterial strains to different intestinal epithelial cell cultures. Similar observations have been reported previously. For example, tagatose had positive modulatory effects of on the adherence of both *L. casei* 01 and *L. rhamnosus* GG to HT29 cell mono-cultures [294], HMOs had positive modulatory effects

on adherence of *B. infantis* to the same intestinal epithelial cell mono-cultures [295]. In contrast, a selection of commercially used prebiotics had negative modulatory effects on the adherence of several probiotic and dairy cultures to Caco-2:HT29-MTX co-cultures (90:10 Caco-2:HT29-MTX respectively) [292].

4.6 Conclusion

In conclusion this study identified 6 bacterial strains (*L. rhamnosus* strain GG and HN001, *L. plantarum* strain AGR1526 and 299v, *L. casei* Shirota and *B. animalis* subsp. *lactis* Bb-12) which could utilise the OEF from goat whey for growth when cultured in 5% CO_2 atmospheric conditions. Of these, all could adhere to intestinal epithelial cells and remain sufficiently viable after their culture in the same atmospheric conditions. Additionally, for some of these strains, adherence to various intestinal epithelial cell cultures could be modulated in the presence of the OEF. Thus, it could be inferred that the OEF together with selected bacteria may provide synergistic effects on the intestinal health. Of the bacterial strains investigated in this study two strains, *L. acidophilus* NCFM and *B. bifidum* DSM 20456, were unable to utilise OEF for growth irrespective of culture media or atmospheric conditions. These strains along with *B. angulatum* were also poorly adherent (< 4% of inoculated cells) to the intestinal epithelial cell cultures both in the presence or absence of the OEF. This adherence phenotype was likely as a consequence of culture conditions, especially for the bifidobacterial strains, rather than their inability to adhere to the intestinal epithelial cells *per se*.

Thus, from these results it could be suggested that the OEF increases the viability of selected probiotic bacteria and reinforces their probiotic functions such as improving their adhesion to intestinal cells. Therefore, the OEF and selected probiotic bacteria may provide functions that are of importance to the health and well-being of the host and contribute in a number of ways to the functional improvements of foods.

Chapter 5: Interactions between probiotic bacteria, an oligosaccharide-enriched fraction from goat whey and intestinal epithelial cell cultures

5.1 Introduction

In the human intestine many complex interactions occur between the host, bacteria and food components. For example, a diet high in starch, non-starch polysaccharides or non-digestible oligosaccharides can cause significant changes in the growth of colonic bacteria, which in turn, can alter the population sizes of specific bacterial species such as bifidobacteria and lactobacilli [136]. Diet also plays an important role in intestinal bacterial colonisation. The intestinal microbiota of full-term breast-fed infants and formula-fed infants differs with bifidobacteria and lactobacilli predominating in breast-fed infants, and coliforms, enterococci and *Bacteroides* predominating in formula-fed infants [109]. Additionally, dietary components are also able to alter the concentrations of bacterial fermentation products such as lactate, acetate, succinate, propionate and butyrate in the large intestine. These short chain fatty acids (SCFAs) (mainly acetate, propionate and butyrate) have major physiological effects on the colonic mucosa. For example, butyrate constitutes the major energy source for colonocytes and is preferentially and rapidly metabolised by the colonic epithelium, providing 60–70% of the energy needs of isolated colonocytes [17, 176, 177]. SCFAs also modulate epithelial cell differentiation and proliferation, a biological effect ascribed to histone deacetylase activity [178] in the intestinal tract. Consequently the interactions between food and bacteria are also important for the optimal functioning of the intestinal epithelium [212].

Certain bacterial taxa with probiotic properties are naturally part of the intestinal microbiota and have a number of different mechanisms by which they are proposed to improve health, such as inhibition of pathogenic bacteria, altering the host's immune response, and improving epithelial barrier function [203]. Recent evidence indicates that specific probiotic organisms can regulate intestinal barrier function and promote mucosal recovery during pathological conditions such as sepsis, as a result of direct effects on epithelial permeability or anti-inflammatory effects [327]. The mode of action, by which probiotics provide protective effects for the intestinal epithelial barrier, may be as a consequence of: actively secreting soluble

mediators [328]; enhancing the production of carbohydrate fermentation by-products [329]; facilitating tight junction formation [330]; inducing mucin gene expression and changes in mucus layer integrity [212].

Clinical benefits from the consumption of prebiotics are obtained through their ability to improve the colonisation of beneficial probiotics and promoting the growth of beneficial colonic bacteria [166]. The fermentation of dietary fibres, such as inulin and oligofructose, by the colonic microbiota increases the production of SCFAs (primarily acetate, butyrate and propionate), which leads to a decrease in lumen pH that is likely to contribute to the protection against pathogens [123]. It is also assumed that such modifications in the colonic microbiota induced by prebiotics, such as fructo-oligosaccharides, may ultimately affect immune function [331]. For example, administration of a synbiotic combination of probiotic *Lactobacillus acidophilus* and prebiotic inulin attenuated the secretion and expression of pro-inflammatory cytokines [332]. There is also some evidence to indicate that the addition of readily fermentable substrates such as OF, inulin and GOS to the diet can cause an increase in mucin production [159, 160, 333]. The increase in mucin production may occur in response to the decreased pH accompanying the production of SCFAs [334] or may also be in a direct response to the bacteria [18]. In addition, fermentation products of certain pro- and prebiotics may be effective for improved epithelial barrier function [329].

Several *in vitro* studies have investigated the impact of selected probiotic bacterial strains on barrier integrity and function of intestinal epithelial cell monolayers by measuring TEER [203, 335, 336], mucin gene expression and/or protein abundance [18, 337]. However, few of these studies have examined the impact of selected probiotics on TEER, mucin gene expression and protein abundance in the same study. One such study, identified that the probiotic bacterial strain *Escherichia coli* Nissle 1917 induced minimal changes in TEER of T84 epithelial cell cultures, and no change in the expression levels of *MUC2*, *MUC3* and *MUC5AC* mucin genes or the abundance of their respective protein products of HT29 cell cultures [19]. Additionally, the

impact of selected prebiotic substrates on TEER, mucin gene expression and protein abundance has also previously been investigated [195, 260, 274]; and like that for the bacterial studies, not all parameters were investigated in the same study. However, in Chapter 3 of this dissertation inclusion of the OEF from goat whey was shown to increase TEER, and mucin gene/protein expression of intestinal epithelial cell cultures, while in Chapter 4 the OEF supported the growth of selected bacterial strains and could alter their adherence to epithelial cells. It has previously been demonstrated that the survival and colonisation of probiotic bacteria in the intestinal tract are increased in the presence of a suitable prebiotic, however, there is no evidence of any *in vitro* studies that have investigated the impact of such combinations on intestinal epithelial barrier integrity and levels of mucin gene and protein expression.

5.2 Hypothesis and Aims

The hypothesis of the research described in this chapter is that individual probiotic bacterial strains when in combination with the OEF are more effective in enhancing intestinal epithelial barrier integrity and mucin gene/protein expression than when limited to the individual components alone.

Consequently, the aim of the research in this chapter is to characterise the enhancing effect of six probiotic bacteria (selected from Chapter 4), either alone or in combination with the OEF from goat whey (Chapter 3) on intestinal barrier integrity and mucin production of intestinal epithelial cell cultures representing the composition of enterocytes and goblet cells in the small and large intestine (validated in Chapter 2). Intestinal epithelial barrier integrity as measured by TEER and mucin gene/protein expression will be characterised for the epithelial cell cultures after 3 and 12 hours, as related to possible incubation times to the components in the human small and large intestine, to determine if selected probiotic bacterial strains in combination with the OEF have greater enhancing effects on intestinal epithelial barrier integrity and mucin production than either component alone.

5.3 Methods and materials

5.3.1 Bacterial strains and culture conditions

Bacterial strains (as indicated in Table 5-1) were cultured in MRS broth as described in Section 4.3.4.2 and Section 4.3.4.4 and prepared as described in Section 4.3.6.1.

5.3.2 Intestinal epithelial cell culture

Caco-2 and HT29-MTX cells were harvested (Section 2.3.3), and cells counted (Section 2.3.6). Caco-2, HT29-MTX and co-cultures of Caco-2:HT29-MTX cells (90:10 and 75:25 respectively) were seeded at a density of 6.3 x 10^4 cells/cm^2 in low glucose DMEM Glutamax supplemented with 10% FBS and 1% Penicillin-Streptomycin. Epithelial cells were cultured as described in Section 2.3.7 for 20 days at which point cultures were washed and prepared as described in Section 2.3.11.

5.3.3 Trans-epithelial electrical resistance assay

Caco-2, HT29-MTX and co-cultures of Caco-2:HT29-MTX cells (90:10 and 75:25 respectively) were seeded onto 12 mm diameter, 0.4 μm^2 pore size, PET Transwell inserts (Corning, Lindfield, New South Wales, Australia) at a density of 6.3 x 10^4 cells/cm^2 in low glucose DMEM Glutamax supplemented with 10% FBS and 1% Penicillin-Streptomycin.

Confluent monolayers were prepared 24 hours before (day 20 post-seeding) the TEER assay as described in Section 3.3.7. After 24 hours incubation in non-supplemented media (no FBS and no Penicillin-Streptomycin), initial resistance readings were obtained for all monolayers. The media was removed from the basal compartment and replenished with non-supplemented low glucose DMEM Glutamax. To the apical compartment 0.5 mL of non-supplemented media (control), OEF-supplemented media (Section 3.3.4), bacteria-supplemented media (Section 4.3.6.1) or bacteria and OEF- supplemented media (Section 4.3.6.1) was added and plates incubated at 37°C in a 5% CO_2 atmosphere. The resistance across each cell monolayer was measured and TEER calculated as described in Section 2.3.10; while the percentage change in TEER compared to initial TEER for each insert was calculated as described in section 3.3.7.

Table 5-1 Selected bacterial strains

Bacterial strain	Company
Lactobacillus rhamnosus HN001 also known as *L. rhamnosus* HN001 (DR20)	Danisco New Zealand Ltd, Auckland, New Zealand
Lactobacillus rhamnosus GG	Valio, Helsinki, Finland
Lactobacillus plantarum 299v	Probi AB, Lund, Sweden
Lactobacillus plantarum AGR1526	Procalf Plus Donaghys, Christchurch, New Zealand
Lactobacillus casei Shirota	Yakult New Zealand, Auckland, New Zealand
Bifidobacteria animalis subsp. *lactis* Bb-12	Chr Hansen Pty Ltd, Melbourne, Australia

5.3.4 Impact of bacteria on mucin dynamics

Intestinal epithelial cells were seeded into 12 well cell culture plates at a density of 6.3 x 10^4 cells/cm^2 in low glucose DMEM Glutamax supplemented with 10% FBS and 1% Penicillin-Streptomycin. Twenty days post-seeding monolayers were prepared as described in section 2.3.11. After an additional 24 hours culture, spent media (SM) was removed and monolayers washed gently four times with PBS. Pre-warmed, non-supplemented media (control), OEF-supplemented media (Section 3.3.4), bacteria-supplemented media (Section 4.3.6.1) or bacteria and OEF-supplemented media (Section 4.3.6.1) was added and plates incubated at 37° in a 5% CO_2 atmosphere.

5.3.5 Mucin gene expression

RNA was extracted as described in Section 2.3.12.1 from monolayers at designated time points (3 and 12 hours). cDNA synthesis and qPCR analysis were completed as described in Section 2.3.12.2. Changes in the expression levels of mucin genes were calculated from three experiments (three successive passages of cells), each with three replicates per treatment group. Each sample was analysed in triplicate.

5.3.6 Mucin protein abundance

Caco-2, HT29-MTX and co-cultures of Caco-2:HT29-MTX cells (90:10 and 75:25 respectively) were seeded into 12 well cell culture plates at a density of 6.3 x 10^4 cells/cm^2 in low glucose DMEM Glutamax supplemented with 10% FBS and 1% Penicillin-Streptomycin.

Twenty days post-seeding monolayers were prepared as described in Section 2.3.11. After an additional 24 hours culture in non-supplemented medium (no FBS and no Penicillin-Streptomycin) the SM was removed and monolayers washed gently four times with PBS. Pre-warmed, non-supplemented media (control), OEF-supplemented media (Section 3.3.4), bacteria-supplemented media (Section 4.3.6.1) or bacteria and OEF-supplemented media (Section 4.3.6.1) was added and plates incubated at 37°C in a 5% CO_2 atmosphere. After 3 and 12 hours incubation, SM and CL samples were collected. The total protein concentration in

samples was determined using the BCA protein assay kit (Thermo Fisher Scientific New Zealand Limited, Albany, North Shore City, New Zealand) according to manufacturer's instructions and using BSA as standards. The abundance of MUC2, MUC4 and MUC5AC protein abundance was measured by ELISA as described in Section 2.3.14. Differences in mucin protein abundance were calculated as the percentage change compared to untreated control monolayers. The abundance levels of the individual mucin proteins (MUC4, MUC2 and MUC5AC) were calculated from three experiments (three successive passages of cells), each with three replicates per treatment group. Each sample was analysed in duplicate by indirect ELISA.

5.3.7 Statistical analysis

The qPCR data was normalised to the reference gene (*HPRT1*) and analysed for expression level changes using Relative Expression Software Tool (REST) 2009 software (version 2.0.13; Qiagen). All other data analysis was undertaken using the Minitab statistical package (Minitab 16 Statistical Software (2010) State College, PA Minitab, Inc.). Data from the TEER assay and ELISA were first evaluated for normality with the Ryan-Joiner and Anderson-Darling variance homogeneity test. Data were not normally distributed and had heterogeneous variances. Non-parametric tests were therefore used to assess all data by one-way analysis of variance (ANOVA) with the Kruskal-Wallis test, followed by the Mann-Whitney U test. Differences were considered statistically significant when *p*-values were less than 0.05.

5.4 Results

5.4.1 Impact of bacteria alone and in combination with an oligosaccharide-enriched fraction from goat whey on trans-epithelial electrical resistance

In comparison to the control Caco-2 monolayers with no bacteria added, the decrease in TEER was significantly greater ($P < 0.05$) for monolayers incubated with *L. rhamnosus* HN001, *L. plantarum* 299v and *L. casei* Shirota after 3 hours (Figure 5-1A). In contrast, the decrease in TEER was significantly less ($P < 0.05$) than control after incubation with *B. lactis* Bb-12. TEER of Caco-2 mono-cultures incubated with *L. rhamnosus* HN001 in combination with OEF was

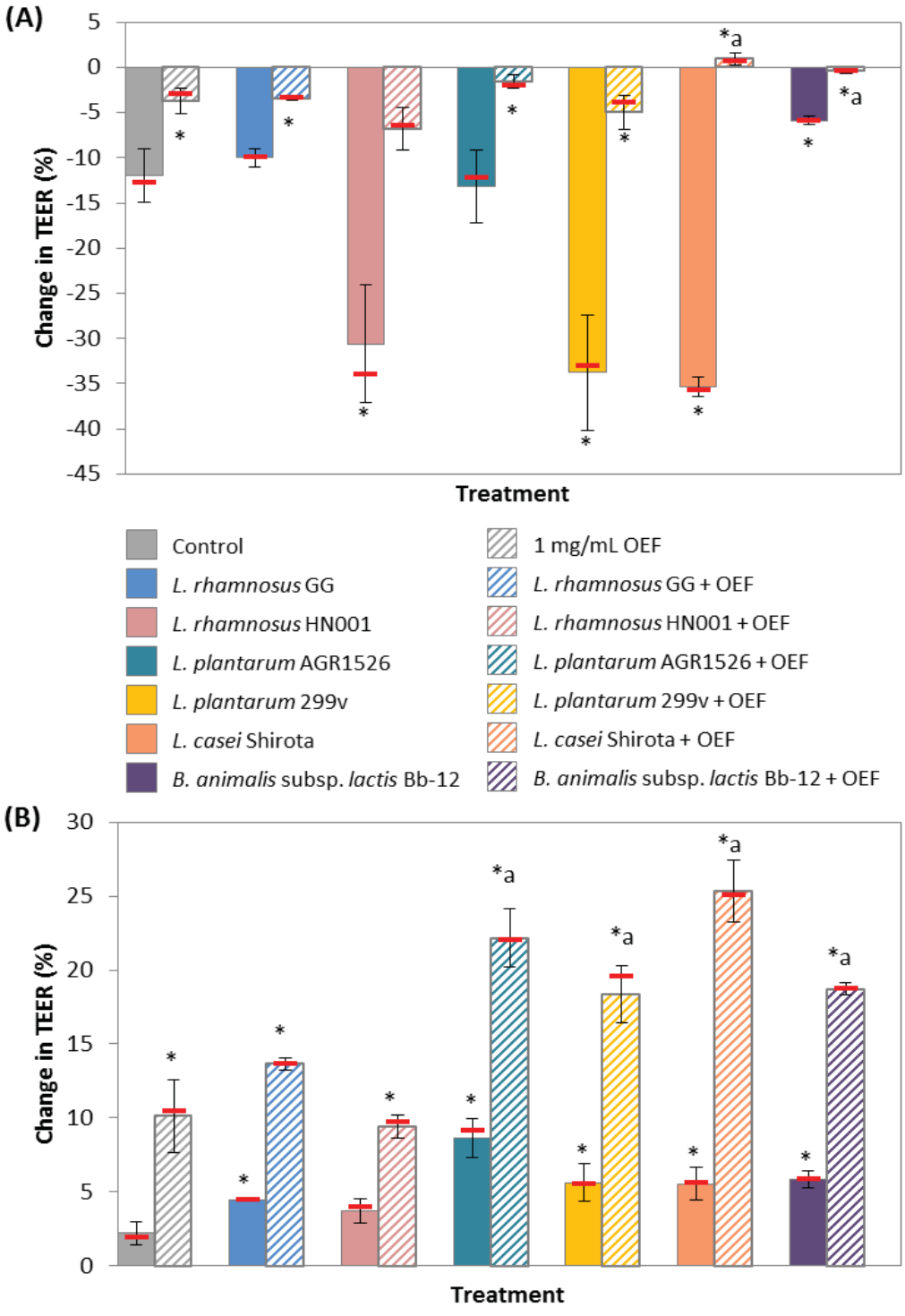


Figure 5-1 Change in TEER of Caco-2 mono-cultures over time in response to bacteria alone, an oligosaccharide-enriched fraction alone, or bacteria in combination with an oligosaccharide-enriched fraction

Change in TEER after 3 hours (A) and 12 hours (B). Values shown are the median, error bars are the median absolute deviation and red line represents the mean. *= significantly different (P< 0.05) to control, and a = significantly different (P< 0.05) to monolayers incubated with 1 mg/mL OEF alone.

similar (P> 0.05) to that of control, while for all other bacteria and OEF combinations, the decrease in TEER was significantly less (P< 0.05). Compared to Caco-2 monolayers incubated with the OEF alone, *L. casei* Shirota in combination with OEF had significantly increased (P< 0.05) TEER after 3 hours. The decrease in TEER of monolayers incubated with *B. lactis* Bb-12 in combination with OEF was significantly less (P< 0.05) than Caco-2 monolayers incubated with the OEF alone; while all other individual bacteria and OEF combinations were shown to have a reduced TEER that was not significantly different (P> 0.05) to that of Caco-2 monolayers incubated with the OEF alone (Figure 5-1A).

Compared to control Caco-2 mono-cultures after 12 hours, TEER was significantly increased (P< 0.05) after incubation with all treatments, except for monolayers incubated with *L. rhamnosus* HN001 which had similar values (P> 0.05) (Figure 5-1B). In comparison to monolayers incubated with the OEF alone, TEER was significantly increased (P< 0.05) after incubation with all individual bacteria and OEF combinations except those incubated with the *L. rhamnosus* GG and OEF and *L. rhamnosus* HN001 and OEF combinations which had similar values (Figure 5-1B).

Compared to control, TEER of all 90:10 co-culture monolayers was significantly increased (P< 0.05) upon incubation with any treatment after 3 hours (Figure 5-2A). In comparison to 90:10 monolayers incubated with the OEF alone, TEER was significantly increased (P< 0.05) after incubation with all individual bacteria and OEF combinations except those incubated with the *L. rhamnosus* GG and OEF combination that were not significantly different TEER values (P> 0.05) (Figure 5-2A).

Similar to that observed at 3 hours, TEER of all 90:10 co-culture monolayers were significantly increased (P< 0.05) upon incubation with any treatment after 12 hours compared to control (Figure 5-2B). Monolayers incubated with the *L. rhamnosus* GG and OEF and *B. lactis* Bb-12 and OEF combinations had similar (P> 0.05) TEER values to monolayers incubated with the OEF alone; but for all other bacteria and OEF combinations TEER values were significantly increased

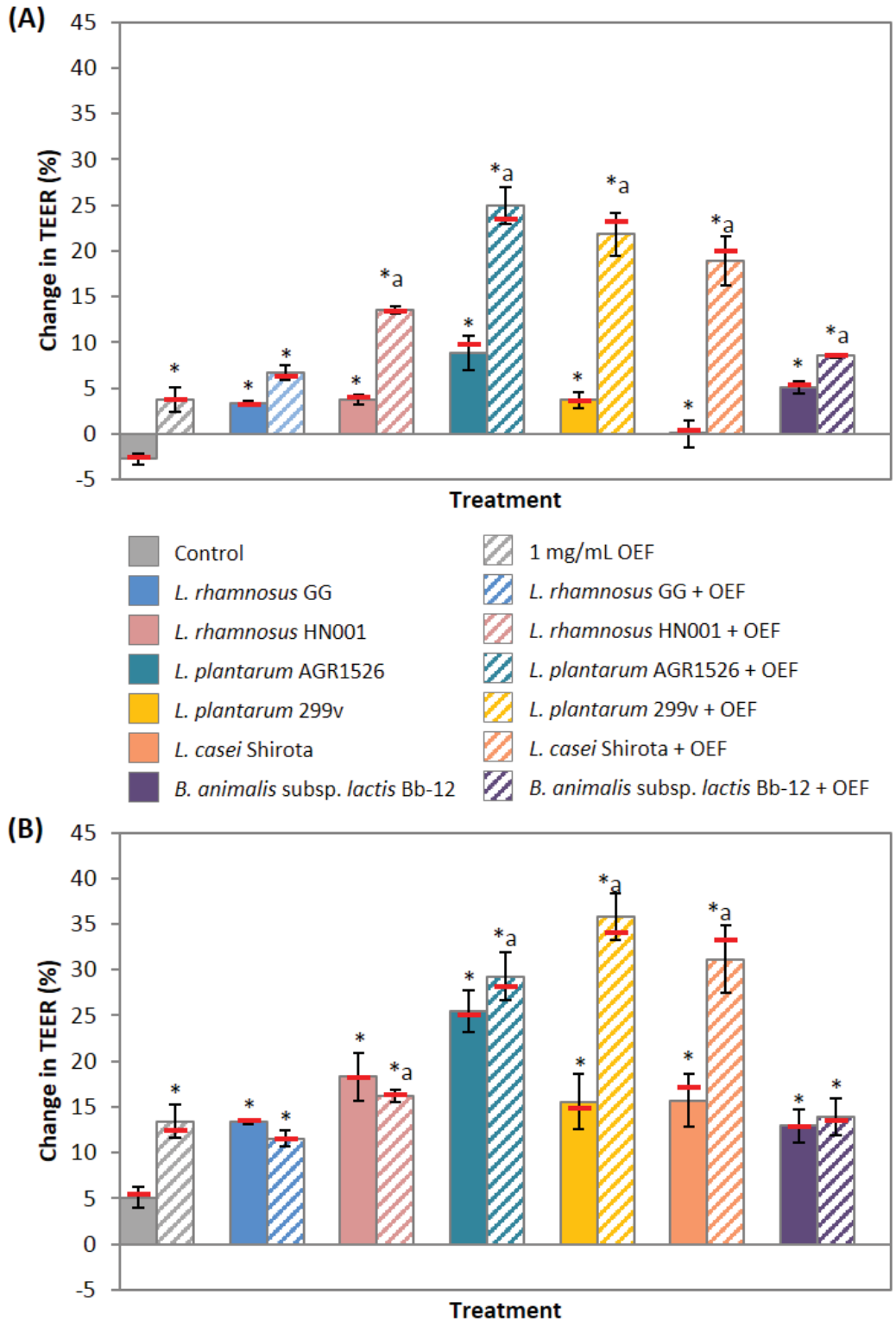


Figure 5-2 Change in TEER of 90:10 Caco-2:HT29-MTX co-cultures over time in response to bacteria alone, an oligosaccharide-enriched fraction alone, or bacteria in combination with an oligosaccharide-enriched fraction

Change in TEER after 3 hours (A) and 12 hours (B). Values shown are the median, error bars are the median absolute deviation and red line represents the mean. *= significantly different (P< 0.05) to control, and a = significantly different (P< 0.05) to monolayers incubated with 1 mg/mL OEF alone.

(P< 0.05) (Figure 5-2B).

Similar to 90:10 co-cultures, TEER of all 75:25 co-culture monolayers were significantly increased (P< 0.05) upon incubation with any treatment after 3 hours (Figure 5-3A) and 12 hours (Figure 5-3B) compared to control monolayers. However, in comparison to 75:25 monolayers incubated with the OEF alone, TEER was significantly lower (P< 0.05) after 3 hours incubation with the *B. lactis* Bb-12 and OEF combination, but was similar after 12 hours. In contrast, TEER of all other individual bacteria and OEF combinations were significantly increased (P< 0.05) after 12 hours (Figure 5-3B). The TEER of HT29-MTX mono-cultures was similar between all treatments and controls after 3 and 12 hours incubation (Figure 5-4A and Figure 5-4B respectively).

5.4.1 Impact of bacteria alone and in combination with an oligosaccharide-enriched fraction on mucin gene expression

5.4.1.1 Caco-2 mono-cultures

In comparison to control Caco-2 mono-cultures, *MUC2* mRNA was significantly increased (P< 0.05) in monolayers incubated with *L. rhamnosus* HN001, but in contrast was significantly decreased (P< 0.05) upon incubation with *B. lactis* Bb-12 and *L. plantarum* 299v for 3 hours (Figure 5-5A). In addition, expression levels of *MUC4* and *MUC5AC* mRNA were also significantly reduced (P< 0.05) upon incubation with *L. plantarum* 299v. The expression level of *MUC4* mRNA was also significantly reduced (P< 0.05) after incubation with *L. casei* Shirota compared to control Caco-2 monolayers. *MUC4* mRNA expression levels were significantly reduced (P< 0.05) after 3 hours incubation with all individual bacteria and OEF combinations, except the *L. casei* Shirota and OEF combination which had similar (P> 0.05) levels to control Caco-2 mono-cultures (Figure 5-5B). Similarly, all individual bacteria and OEF combinations resulted in significantly decreased (P< 0.05) *MUC2* mRNA expression levels. Incubation with *L. plantarum* AGR1526, *L. plantarum* 299v and *L. casei* Shirota in combination with the OEF resulted in significantly decreased (P< 0.05) *MUC5AC* expression levels compared to control

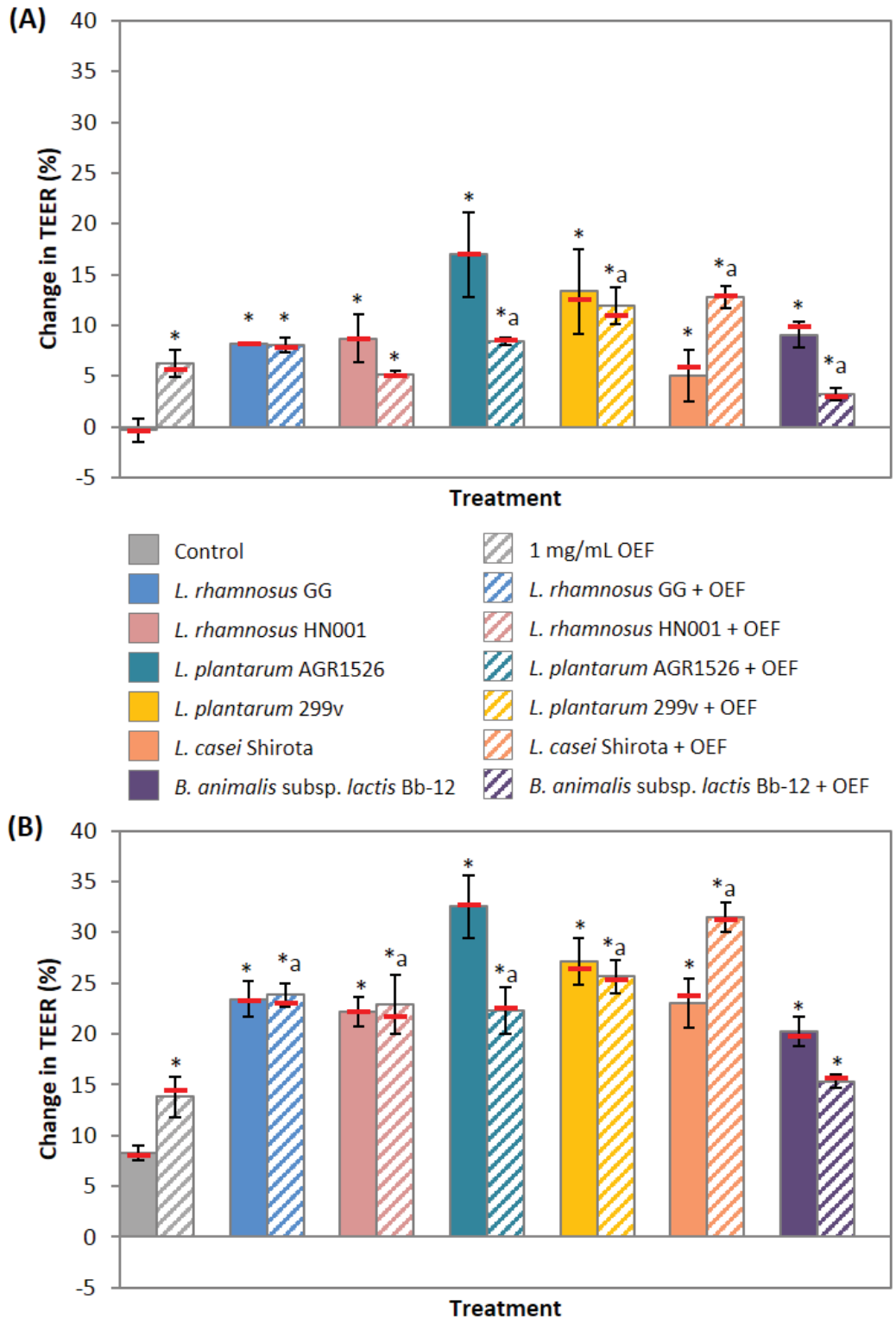


Figure 5-3 Change in TEER of 75:25 Caco-2:HT29-MTX co-cultures over time in response to bacteria alone, an oligosaccharide-enriched fraction alone, or bacteria in combination with an oligosaccharide-enriched fraction

Change in TEER after 3 hours (A) and 12 hours (B). Values shown are the median, error bars are the median absolute deviation and red line represents the mean. *= significantly different (P< 0.05) to control, and a = significantly different (P< 0.05) to monolayers incubated with 1 mg/mL OEF alone.

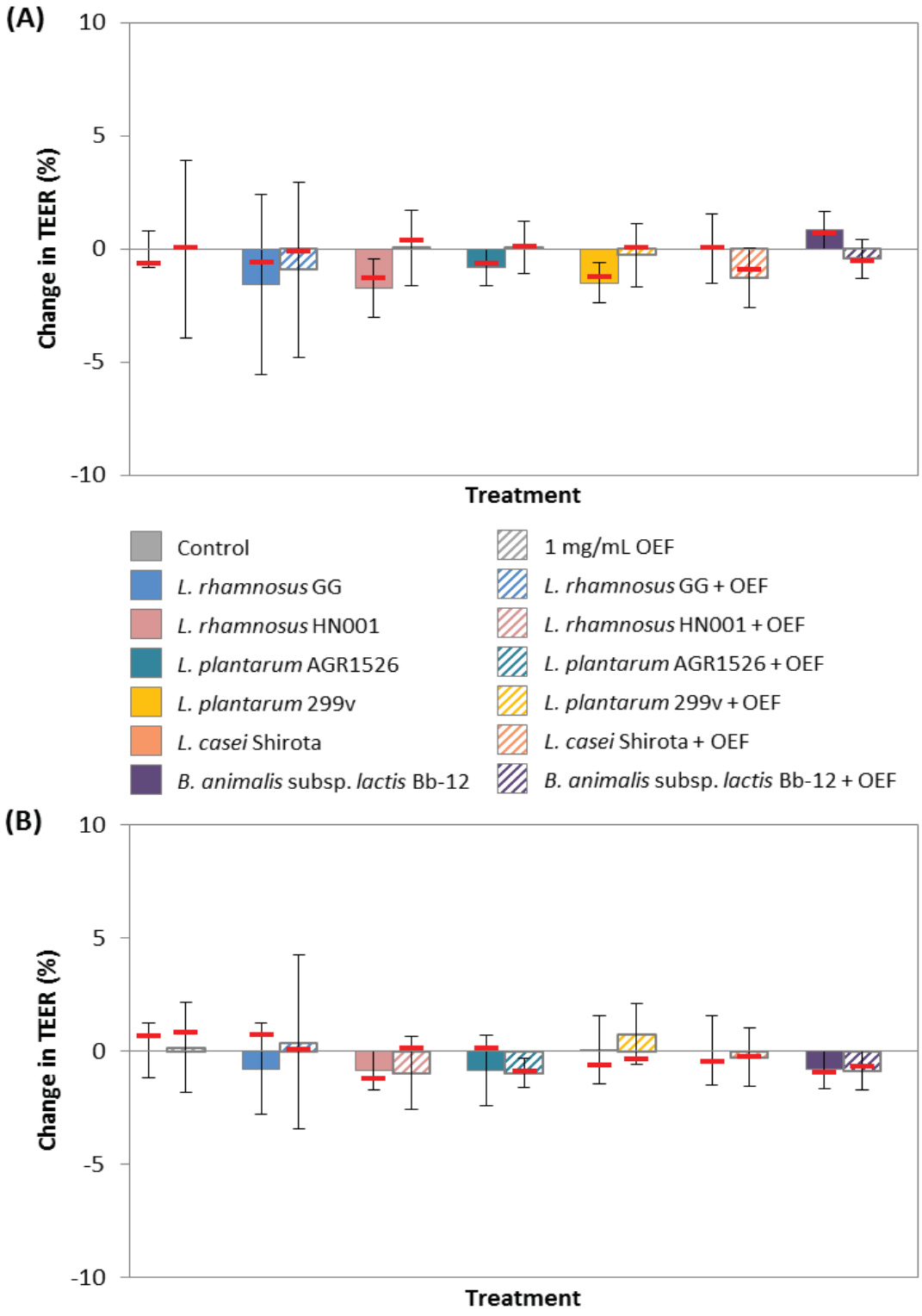


Figure 5-4 Change in TEER of HT29-MTX mono-cultures over time in response to bacteria alone, an oligosaccharide-enriched fraction alone, or bacteria in combination with an oligosaccharide-enriched fraction

Change in TEER after 3 hours (A) and 12 hours (B). Values shown are the median, error bars are the median absolute deviation and red line represents the mean. *= significantly different (P< 0.05) to control, and a = significantly different (P< 0.05) to monolayers incubated with 1 mg/mL OEF alone.

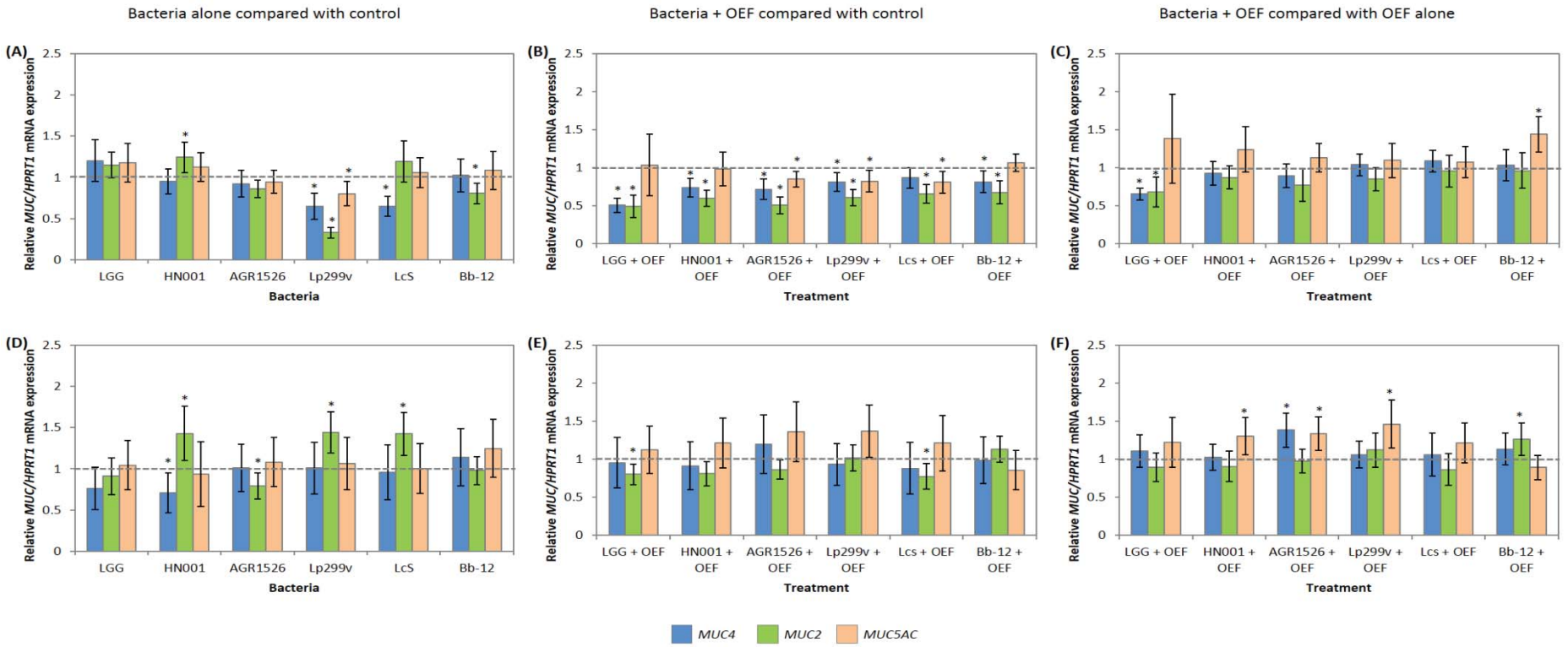


Figure 5-5 Temporal changes in the relative abundance of *MUC* mRNA from Caco-2 mono-cultures incubated with bacteria alone and in combination with an oligosaccharide-enriched fraction from goat whey

The relative abundance of *MUC4*, *MUC2* and *MUC5AC* mRNA after 3 hours (A, B and C) and 12 hours (D, E and F) incubation with bacteria alone (A and D) and in combination with an oligosaccharide-enriched fraction (OEF) (B and E) compared to control; and compared to monolayers incubated with the OEF alone (C and F). Results are the mean (± SEM) expression levels of *MUC/HPRT1* mRNA and calculated as relative expression to respective controls which have an arbitrary value of 1 (dotted line). * Significantly different ($P < 0.05$) to respective control. LGG = *L. rhamnosus* GG; HN001 = *L. rhamnosus* HN001; AGR1526 = *L. plantarum* AGR1526; Lp299v = *L. plantarum* 299v; LcS = *L. casei* Shirota and Bb-12 = *B. lactis* Bb-12.

Caco-2 monolayers.

In comparison to Caco-2 monolayers incubated with the OEF alone, incubation with the *L. rhamnosus* GG and OEF combination resulted in a significant decrease ($P< 0.05$) in the expression levels of *MUC4* and *MUC2* mRNA; but in contrast, incubation with the *B. lactis* Bb-12 and OEF combination resulted in a significant increase ($P< 0.05$) in the expression level of *MUC5AC* mRNA (Figure 5-5C).

In comparison to control Caco-2 monolayers, 12 hours incubation with *L. rhamnosus* HN001 resulted in a significant decrease ($P< 0.05$) in the expression of *MUC4* mRNA but significantly increased ($P< 0.05$) the expression of *MUC2* mRNA (Figure 5-5D). *MUC2* mRNA was also significantly increased ($P< 0.05$) after 12 hours incubation with *L. plantarum* 299v and *L. casei* Shirota, but was significantly decreased ($P< 0.05$) after incubation with *L. plantarum* AGR1526. Additionally, *MUC2* mRNA was significantly decreased ($P< 0.05$) after 12 hours incubation with *L. rhamnosus* GG and *L. casei* Shirota when in combination with the OEF compared to control Caco-2 monolayers (Figure 5-5E). In comparison to Caco-2 monolayers incubated with OEF alone, incubation with *L. plantarum* AGR1526 in combination with OEF resulted in significant increases ($P< 0.05$) in the expression levels of *MUC4* and *MUC5AC* mRNA (Figure 5-5F). *MUC5AC* mRNA was also significantly increased ($P< 0.05$) after incubation with the *L. rhamnosus* HN001 and OEF and *L. plantarum* 299v and OEF combinations. The expression level of *MUC2* mRNA was only significantly increased ($P< 0.05$) after 12 hours incubation with *B. lactis* Bb-12 in combination with the OEF compared to Caco-2 monolayers incubated with OEF alone.

5.4.1.2 90:10 Caco-2:HT29-MTX co-cultures

In comparison to control 90:10 co-culture monolayers, incubation with *L. plantarum* 299v, *L. casei* Shirota and *B. lactis* Bb-12 resulted in a significant decrease ($P< 0.05$) in the expression levels of all mucin genes (*MUC4, MUC2* and *MUC5AC*) investigated after 3 hours (Figure 5-6A). In contrast, incubation with *L. rhamnosus* GG resulted in a significant increase ($P< 0.05$) in the

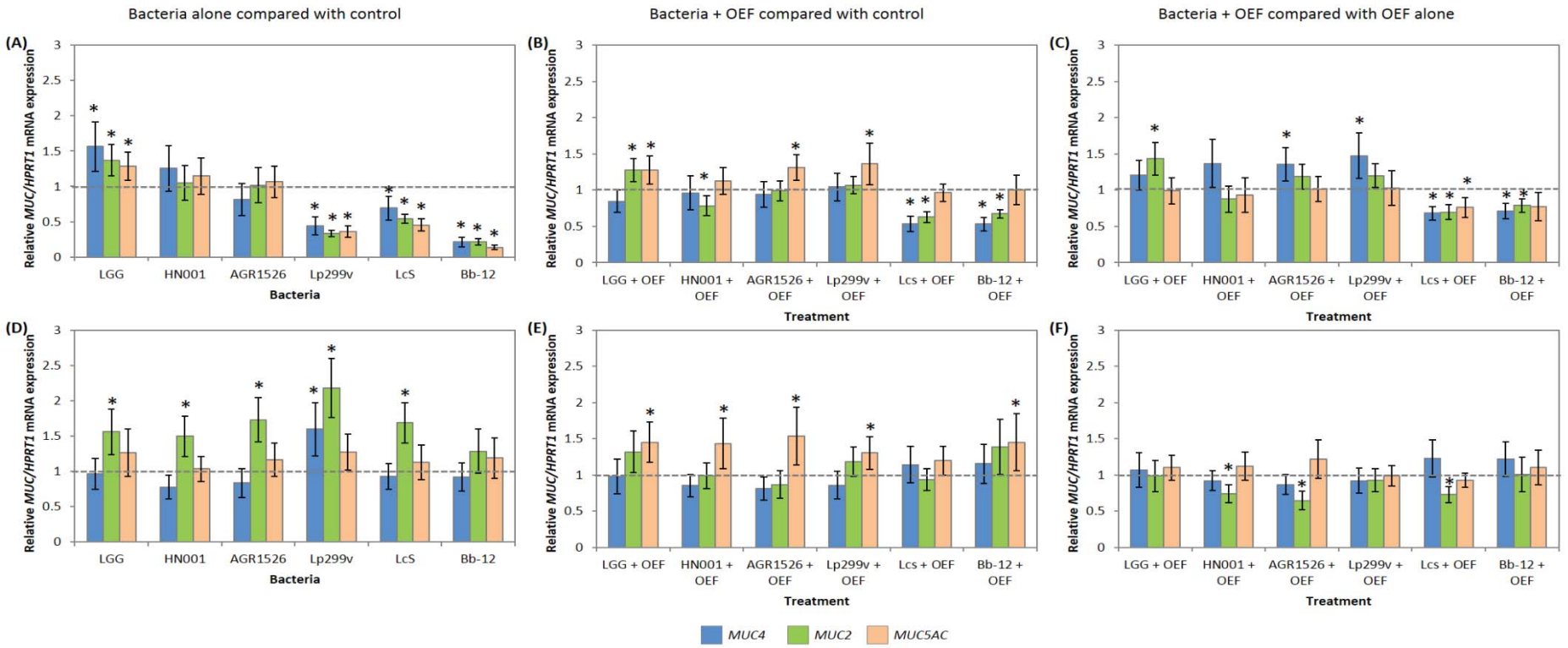


Figure 5-6 Temporal changes in the relative abundance of *MUC* mRNA from 90:10 Caco-2:HT29-MTX co-cultures incubated with bacteria alone and in combination with an oligosaccharide-enriched fraction from goat whey

The relative abundance of *MUC4*, *MUC2* and *MUC5AC* mRNA after 3 hours (A, B and C) and 12 hours (D, E and F) incubation with bacteria alone (A and D) and in combination with an oligosaccharide-enriched fraction (OEF) (B and E) compared to control; and compared to monolayers incubated with the OEF alone (C and F). Results are the mean (± SEM) expression levels of *MUC/HPRT1* mRNA and calculated as relative expression to respective controls which have an arbitrary value of 1 (dotted line). * Significantly different ($P < 0.05$) to respective control. LGG = *L. rhamnosus* GG; HN001 = *L. rhamnosus* HN001; AGR1526 = *L. plantarum* AGR1526; Lp299v = *L. plantarum* 299v; LcS = *L. casei* Shirota and Bb-12 = *B. lactis* Bb-12.

expression levels of all mucin genes. Additionally, incubation with *L. rhamnosus* GG in combination with the OEF resulted in a significant increase (P< 0.05) in *MUC2* and *MUC5AC* mRNA expression levels (Figure 5-6B). *MUC5AC* mRNA was also significantly increased (P< 0.05) after 3 hours incubation with the *L. plantarum* AGR1526 and OEF and *L. plantarum* 299v and OEF combinations compared to control. In contrast, *MUC2* mRNA was significantly decreased (P< 0.05) after incubation with *L. rhamnosus* HN001 when in combination with the OEF. Similarly, both *MUC2* and *MUC4* mRNA were significantly decreased (P< 0.05) after 3 hours incubation with the *L. casei* Shirota and OEF and *B. lactis* Bb-12 and OEF combinations (Figure 5-6B).

In comparison to 90:10 co-culture monolayers incubated with the OEF alone, incubation with the *L. casei* Shirota and OEF and *B. lactis* Bb-12 and OEF combinations resulted in significant reductions (P< 0.05) in the expression levels of both *MUC4 and MUC2* mRNA after 3 hours (Figure 5-6C). In addition, incubation with the *L. casei* Shirota and OEF combination also significantly decreased (P< 0.05) *MUC5AC* mRNA expression levels. In contrast, *MUC2* mRNA expression was significantly increased (P< 0.05) after incubation with the *L. rhamnosus* GG and OEF combination, and *MUC4* mRNA expression was significantly increased (P< 0.05) after incubation with both the *L. plantarum* AGR1526 and OEF and *L. plantarum* 299v and OEF combinations compared to 90:10 co-culture monolayers incubated with the OEF alone (Figure 5-6C).

Levels of *MUC2* mRNA were significantly increased (P< 0.05) after 12 hours incubation with all individual bacterial strains except *B. lactis* Bb-12, which had similar values to control 90:10 co-cultures (Figure 5-6D). The expression level of *MUC4* mRNA was only significantly increased (P< 0.05) in monolayers incubated with *L. plantarum* 299v. Additionally, all individual bacteria and OEF combinations were shown to significantly increase (P< 0.05) *MUC5AC* mRNA, except the *L. casei* Shirota and OEF combination which had similar levels to control 90:10 co-culture monolayers (Figure 5-6E). In comparison to 90:10 co-culture monolayers incubated with the

OEF alone, monolayers incubated with the *L. rhamnosus* HN001 and OEF, *L. plantarum* AGR1526 and OEF and *L. casei* Shirota and OEF combinations for 12 hours had significantly lower (P< 0.05) *MUC2* mRNA expression levels (Figure 5-6F).

5.4.1.3 75:25 Caco-2:HT29-MTX co-cultures

In comparison to control 75:25 co-culture monolayers, 3 hours incubation with *B. lactis* Bb-12 resulted in a significant decrease (P< 0.05) in the expression of all mucin genes investigated (*MUC4*, *MUC2*, and *MUC5AC*) (Figure 5-7A). *MUC5AC* mRNA level was also significantly reduced (P< 0.05) after 3 hours incubation with *L. casei* Shirota. In contrast, levels of *MUC2* and *MUC4* mRNA were significantly increased (P< 0.05) after incubation with *L. rhamnosus* HN001 and *L. plantarum* 299v respectively. Similarly, *MUC5AC* mRNA levels were significantly increased (P< 0.05) after 3 hours incubation with the *L. rhamnosus* HN001 and OEF and *B. lactis* Bb-12 and OEF combinations when compared to control 75:25 co-culture monolayers (Figure 5-7B). In contrast, incubation with the *L. rhamnosus* GG and OEF, *L. plantarum* AGR1526 and OEF and *L. casei* Shirota and OEF combinations resulted in a significant decrease (P< 0.05) in the expression level of *MUC2* mRNA. In comparison to 75:25 co-culture monolayers incubated with the OEF alone, only *MUC5AC* mRNA expression was significantly increased (P< 0.05) after incubation with the *L. rhamnosus* HN001 and OEF, *L. casei* Shirota and OEF and *B. lactis* Bb-12 and OEF combinations after 3 hours (Figure 5-7C).

Twelve hours incubation of 75:25 co-culture monolayers with *L. plantarum* AGR1526, *L. casei* Shirota and *B. lactis* Bb-12 resulted in a significant increase (P< 0.05) in *MUC4* mRNA compared to control monolayers (Figure 5-7D). Conversely, incubation with *L. casei* Shirota was also shown to significantly decrease (P< 0.05) *MUC5AC* mRNA expression levels. The expression of all mucin genes (*MUC4 MUC2* and *MUC5AC*) were significantly increased (P< 0.05) after 12 hours incubation with the *L. plantarum* AGR1526 and OEF combination when compared to control 75:25 co-culture monolayers (Figure 5-7E). Additionally, *MUC4* and *MUC2* mRNA levels were also significantly increased (P< 0.05) after incubation with the *L. plantarum* 299v and

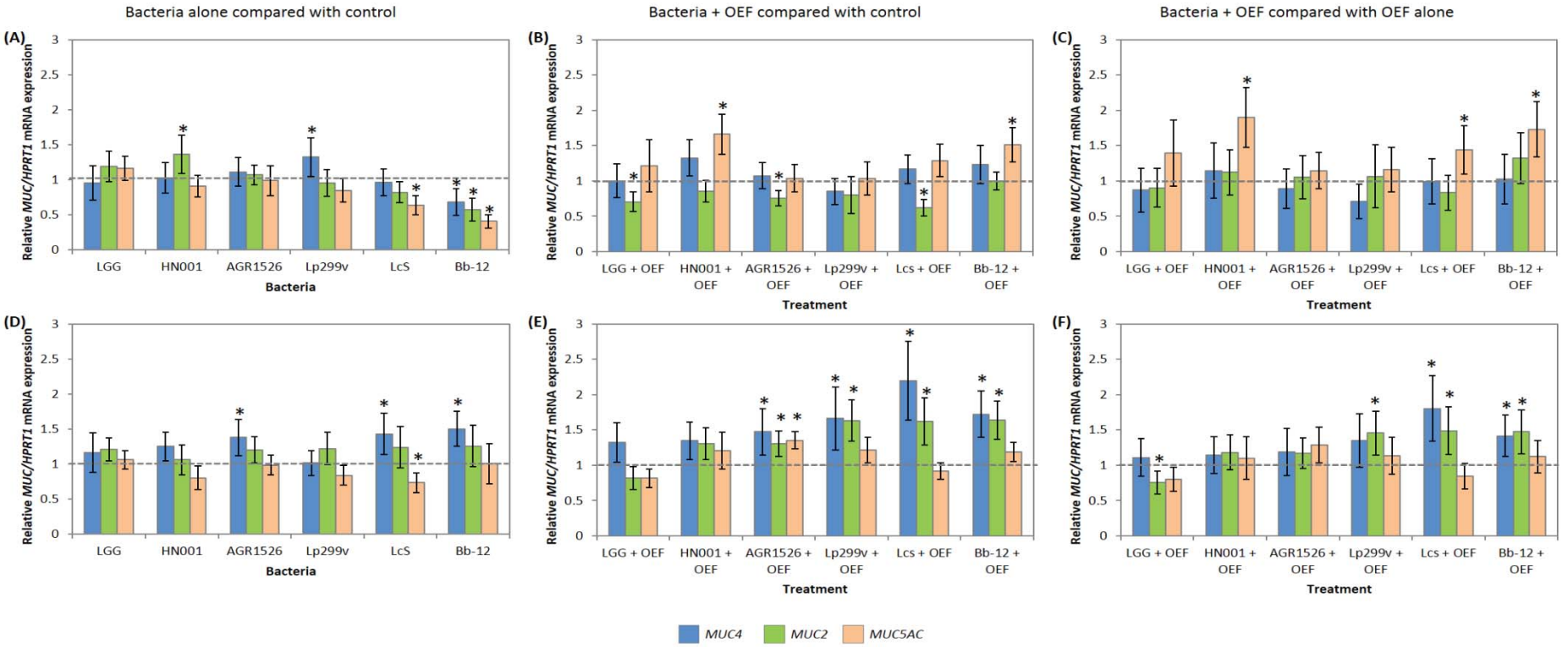


Figure 5-7 Temporal changes in the relative abundance of *MUC* mRNA from 75:25 Caco-2:HT29-MTX co-cultures incubated with bacteria alone and in combination with an oligosaccharide-enriched fraction from goat whey

The relative abundance of *MUC4*, *MUC2* and *MUC5AC* mRNA after 3 hours (A, B and C) and 12 hours (D, E and F) incubation with bacteria alone (A and D) and in combination with an oligosaccharide-enriched fraction (OEF) (B and E) compared to control; and compared to monolayers incubated with the OEF alone (C and F). Results are the mean (± SEM) expression levels of *MUC/HPRT1* mRNA and calculated as relative expression to respective controls which have an arbitrary value of 1 (dotted line). * Significantly different (P< 0.05) to respective control. LGG = *L. rhamnosus* GG; HN001 = *L. rhamnosus* HN001; AGR1526 = *L. plantarum* AGR1526; Lp299v = *L. plantarum* 299v; LcS = *L. casei* Shirota and Bb-12 = *B. lactis* Bb-12.

OEF, *L. casei* Shirota and OEF and *B. lactis* Bb-12 and OEF combinations. Similarly, when compared to 75:25 co-culture monolayers incubated with the OEF alone, there was a significant increase (P< 0.05) in the expression of *MUC4* and *MUC2* mRNA after incubation with the *L. casei* Shirota and OEF and *B. lactis* Bb-12 and OEF combinations (Figure 5-7F). In addition, *MUC2* mRNA was also significantly increased (P< 0.05) after 12 hours incubation with the *L. plantarum* 299v and OEF combination, but in contrast, was significantly reduced (P< 0.05) after incubation with the *L. rhamnosus* GG and OEF combination.

5.4.1.4 HT29-MTX mono-cultures

In comparison to control HT29-MTX mono-cultures the levels of *MUC4* and *MUC5AC* mRNA were significantly increased (P< 0.05) after 3 hours incubation with *L. plantarum* AGR1526 (Figure 5-8A). In contrast, the levels of both *MUC2* and *MUC5AC* mRNA were significantly decreased (P< 0.05) after incubation with *L. casei* Shirota and *B. lactis* Bb-12. The expression of *MUC2* mRNA was also significantly decreased (P< 0.05) after 3 hours incubation with the *L. rhamnosus* GG and OEF, *L. rhamnosus* HN001 and OEF, *L. casei* Shirota and OEF and *B. lactis* Bb-12 and OEF combinations (Figure 5-8B). In contrast, *MUC4* and *MUC5AC* mRNA expression levels were both significantly increased (P< 0.05) after 3 hours incubation with the *L. plantarum* AGR1526 and OEF and *L. plantarum* 299v and OEF combinations; while incubation with the *L. rhamnosus* GG and OEF and *B. lactis* Bb-12 and OEF combinations significantly increased (P< 0.05) *MUC5AC* and *MUC4* mRNA levels respectively compared to control HT29-MTX mono-cultures. In comparison to HT29-MTX monolayers incubated with the OEF alone, the expression levels of all mucin genes investigated (*MUC4, MUC2* and *MUC5AC*) were significantly increased (P< 0.05) after 3 hours incubation with the *L. plantarum* 299v and OEF combination (Figure 5-8C). Similarly, *MUC2* and *MUC5AC* mRNA expression levels were also significantly increased (P< 0.05) as a result of incubation with the *L. plantarum* AGR1526 and OEF combination. In contrast the expression of *MUC4* mRNA was significantly reduced (P< 0.05) as a result of incubation with the *L. casei* Shirota and OEF combination.

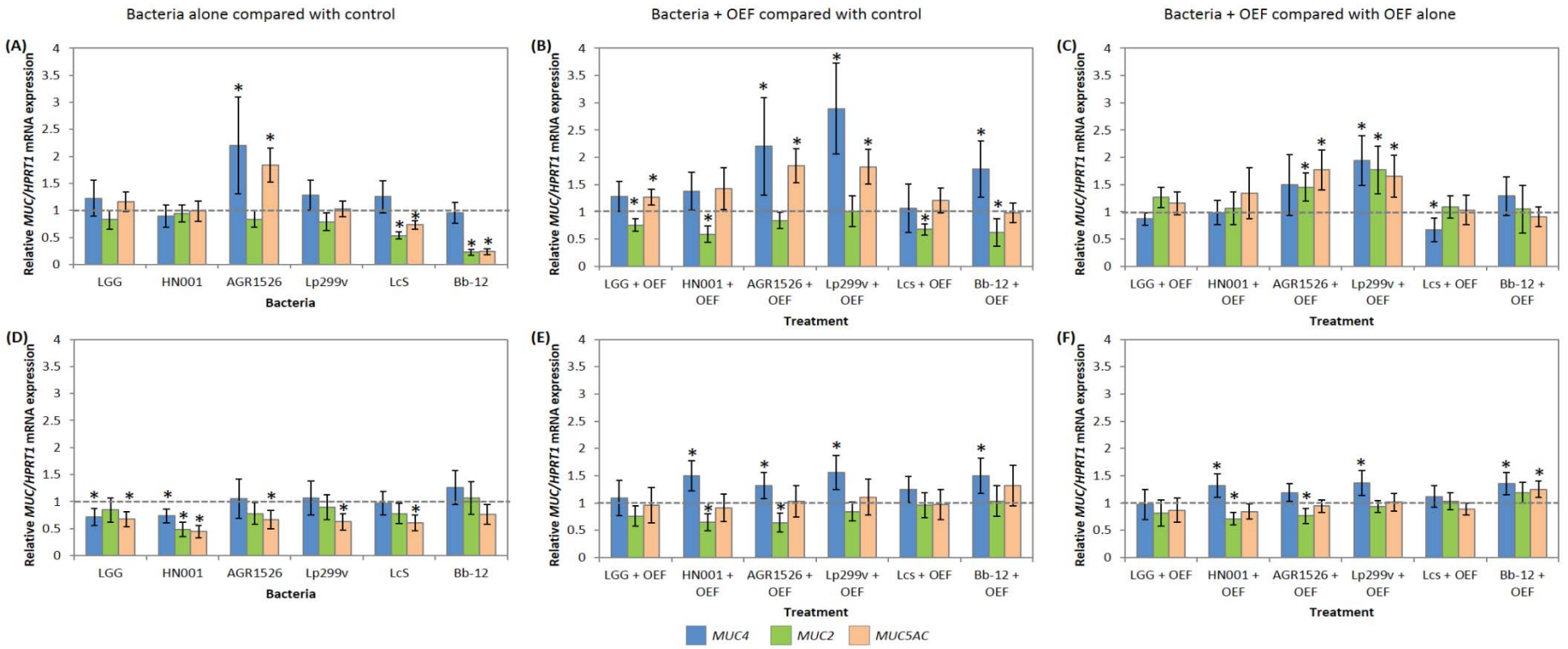


Figure 5-8 Temporal changes in the relative abundance of *MUC* mRNA from HT29-MTX mono-cultures incubated with bacteria alone and in combination with an oligosaccharide-enriched fraction from goat whey

The relative abundance of *MUC4*, *MUC2* and *MUC5AC* mRNA after 3 hours (A, B and C) and 12 hours (D, E and F) incubation with bacteria alone (A and D) and in combination with an oligosaccharide-enriched fraction (OEF) (B and E) compared to control; and compared to monolayers incubated with the OEF alone (C and F). Results are the mean (± SEM) expression levels of *MUC/HPRT1* mRNA and calculated as relative expression to respective controls which have an arbitrary value of 1 (dotted line). * Significantly different (P< 0.05) to respective control. LGG = *L. rhamnosus* GG; HN001 = *L. rhamnosus* HN001; AGR1526 = *L. plantarum* AGR1526; Lp299v = *L. plantarum* 299v; LcS = *L. casei* Shirota and Bb-12 = *B. lactis* Bb-12.

HT29-MTX monolayers had a significant reduction (P< 0.05) in *MUC5AC* mRNA expression levels after 12 hours incubation with all individual bacterial strains except *B. lactis* Bb-12, which had similar expression levels to control HT29-MTX monolayers (Figure 5-8D). Similarly, the levels of both *MUC4* and *MUC2* mRNA were significantly reduced (P< 0.05) after incubation with *L. rhamnosus* HN001, while *MUC4* mRNA levels were significantly reduced (P< 0.05) after incubation with *L. rhamnosus* GG. Although incubation with the *L. rhamnosus* GG and OEF and *L. casei* Shirota and OEF combinations did not affect *MUC4* mRNA levels, incubation with all other HT29-MTX monolayers had a significant reduction (P< 0.05) in *MUC5AC* mRNA expression levels after 12 hours incubation with all individual bacterial strains except *B. lactis* Bb-12, which had similar expression levels to control HT29-MTX monolayers (Figure 5-8D). Similarly, the levels of both *MUC4* and *MUC2* mRNA were significantly reduced (P< 0.05) after incubation with *L. rhamnosus* HN001, while *MUC4* mRNA levels were significantly reduced (P< 0.05) after incubation with *L. rhamnosus* GG. Although incubation with the *L. rhamnosus* GG and OEF and *L. casei* Shirota and OEF combinations did not affect *MUC4* mRNA levels, incubation with all other individual bacteria and OEF combinations for 12 hours resulted in a significant increase (P< 0.05) in *MUC4* mRNA levels (Figure 5-8E). In contrast, *MUC2* expression levels were significantly reduced (P< 0.05) after incubation with the *L. rhamnosus* HN001 and OEF and *L. plantarum* AGR1526 and OEF combinations compared to control HT29-MTX monolayers. Expression levels of *MUC4* mRNA were significantly increased (P< 0.05) as a result of incubation with the *L. rhamnosus* HN001 and OEF, *L. plantarum* 299v and OEF and *B. lactis* Bb-12 and OEF combinations when compared to HT29-MTX monolayers incubated with the OEF alone (Figure 5-8F). In addition, *MUC5AC* mRNA levels were significantly increased (P< 0.05) after incubation with the *B. lactis* Bb-12 and OEF combination. In contrast, *MUC2* mRNA expression levels were significantly reduced (P< 0.05) after incubation with the *L. rhamnosus* HN001 and OEF and *L. plantarum* AGR1526 and OEF combinations.

5.4.2 Impact of bacteria alone and in combination with an oligosaccharide-enriched fraction on mucin protein abundance

5.4.2.1 Caco-2 mono-cultures 3 and 12 hours

It has previously been demonstrated (Section 2.4.5.1) that the secreted mucin proteins MUC2 and MUC5AC were not detected in either the SM or CL samples taken from Caco-2 mono-cultures. Consequently the impact of different components (bacteria, OEF and individual bacteria and OEF in combination) on the abundance of these proteins from Caco-2 mono-cultures was not determined.

In comparison to control Caco-2 monolayers, the abundance of MUC4 mucin protein was significantly increased ($P< 0.05$) after 3 hours incubation with *L. plantarum* AGR1526, *L. plantarum* 299v, and *B. lactis* Bb-12 (10%, 19% and 17% respectively) (Figure 5-9A). Similarly, MUC4 mucin protein was significantly increased ($P< 0.05$) after incubation with all individual bacteria and OEF combinations except the *L. plantarum* 299v and OEF and *L. casei* Shirota and OEF combinations, which had similar values to control (Figure 5-9B). In comparison to Caco-2 monolayers incubated with the OEF alone, incubation with all individual bacteria and OEF combinations significantly decreased ($P< 0.05$) MUC4 mucin protein abundance, except those incubated with the *L. plantarum* AGR1526 and OEF combination which had similar levels to control (Figure 5-9C).

There was no difference in MUC4 protein abundance of Caco-2 mono-cultures after 12 hours incubation with any of the individual bacterial strains tested (Figure 5-9D). In contrast, incubation with the *L. plantarum* 299v and OEF combination resulted in a significant increase ($P< 0.05$) in MUC4 protein abundance (Figure 5-9E). In comparison to Caco-2 mono-cultures incubated with the OEF alone, 12 hours incubation with all individual bacteria and OEF combinations significantly decreased ($P< 0.05$) MUC4 mucin protein abundance, except those incubated with the *L. plantarum* 299v and OEF combination which had similar levels to Caco-2 mono-cultures incubated with the OEF alone (Figure 5-9F).

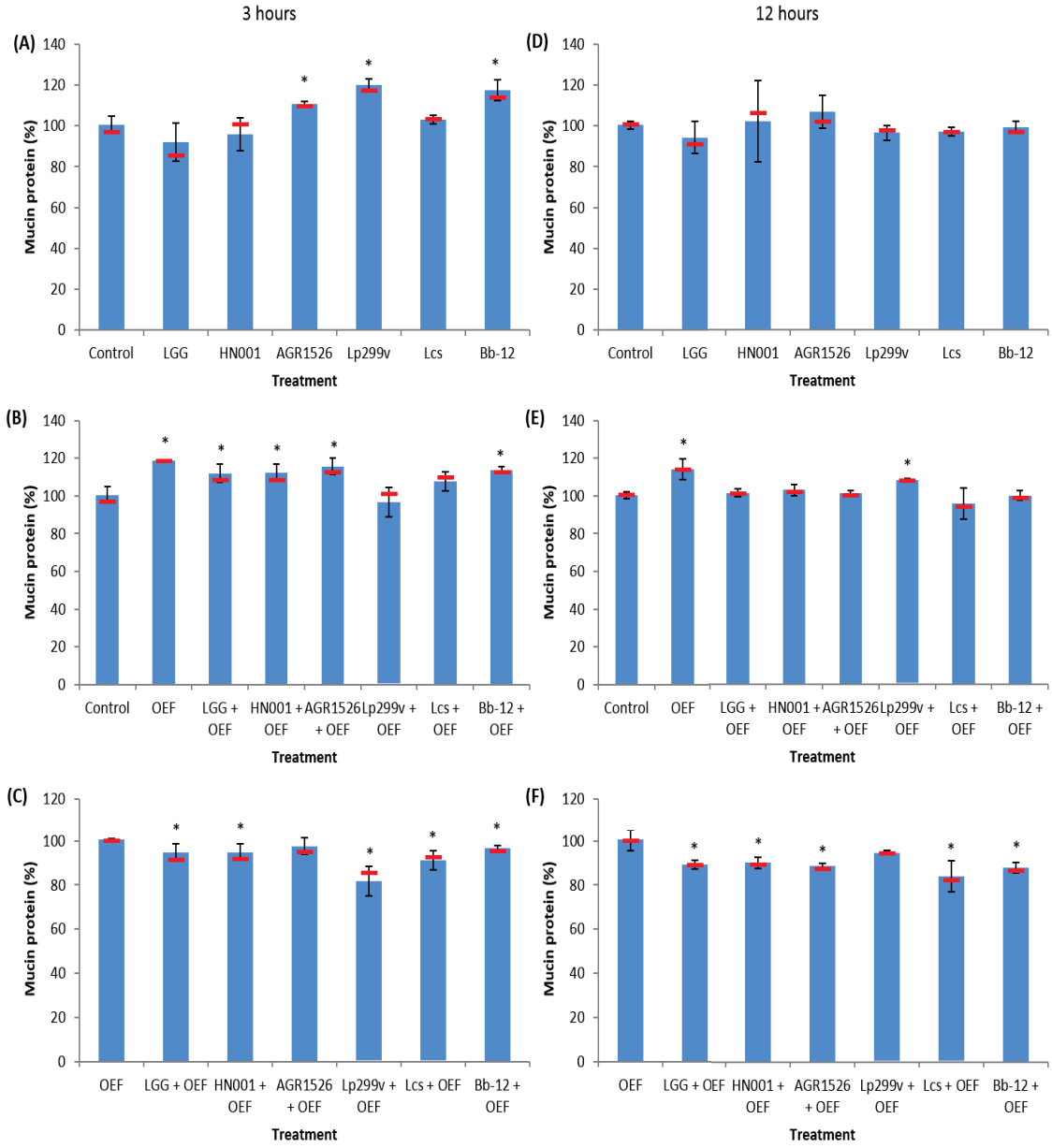


Figure 5-9 Abundance of MUC4 protein from Caco-2 mono-cultures after 3 and 12 hours incubation with bacteria alone and in combination with an oligosaccharide-enriched fraction from goat whey

The total abundance of MUC4 protein from Caco-2 mono-cultures after 3 (A – C) and 12 (D – F) hours incubation with bacteria alone (A and D) and in combination with an oligosaccharide-enriched fraction (OEF) (B and E) compared to control; and compared to monolayers incubated with the OEF alone (C and F). Values shown are the median, error bars are the median absolute deviation and red line represents the mean. * P< 0.05 compared to respective control. LGG = *L. rhamnosus* GG; HN001 = *L. rhamnosus* HN001; AGR1526 = *L. plantarum* AGR1526; Lp299v = *L. plantarum* 299v; LcS = *L. casei* Shirota and Bb-12 = *B. lactis* Bb-12.

5.4.2.1 90:10 Caco-2:HT29-MTX co-cultures 3 hours incubation

In comparison to control 90:10 co-culture monolayers the total abundance of mucin protein was significantly increased ($P< 0.05$) after 3 hours incubation with *L. plantarum* AGR1526 and *L. plantarum* 299v (32% and 39% respectively), which was associated with a significant increase ($P< 0.05$) in the abundance of MUC5AC for both of these strains (Figure 5-10A). Although the total abundance of mucin protein was also significantly increased ($P< 0.05$) after 3 hours incubation with *L. rhamnosus* GG (24%), there was a significant increase ($P< 0.05$) in MUC5AC abundance but a significant decrease ($P< 0.05$) in MUC2 abundance (29% and 5% respectively). Additionally, although the total abundance of mucin protein was unaffected, incubation with *L. rhamnosus* HN001 and *L. casei* Shirota significantly reduced ($P< 0.05$) the abundance of MUC2 protein (5% and 5% respectively). In contrast the abundance of MUC5AC protein was significantly increased ($P< 0.05$) after incubation with *B. lactis* Bb-12 (9%) but this increase was not sufficient to increase the overall abundance of mucin proteins detected (Figure 5-10A).

The total abundance of mucin proteins of 90:10 co-culture monolayers was significantly increased ($P< 0.05$) after incubation with all individual bacteria and OEF combinations compared to control 90:10 monolayers after 3 hours (Figure 5-10B). Significant increases ($P< 0.05$) in the abundance of MUC4, MUC2, and MUC5AC proteins (11%, 5% and 25% respectively) contributed to the increase observed after incubation with the *L. plantarum* 299v and OEF combination. For monolayers incubated with the *L. rhamnosus* GG and OEF, *L. rhamnosus* HN001 and OEF and *L. plantarum* AGR1526 and OEF combinations, the increase in total mucin protein abundance was associated with a significant increase ($P< 0.05$) in MUC5AC (17%, 12% and 25% respectively) (Figure 5-10B). Although the abundance of MUC5AC protein was also significantly increased ($P< 0.05$) for monolayers incubated with the *L. casei* Shirota and OEF combination (10%), there was also a significant decrease ($P< 0.05$) in the abundance of MUC2 protein (5%) for this combination. Similarly, MUC2 protein abundance was

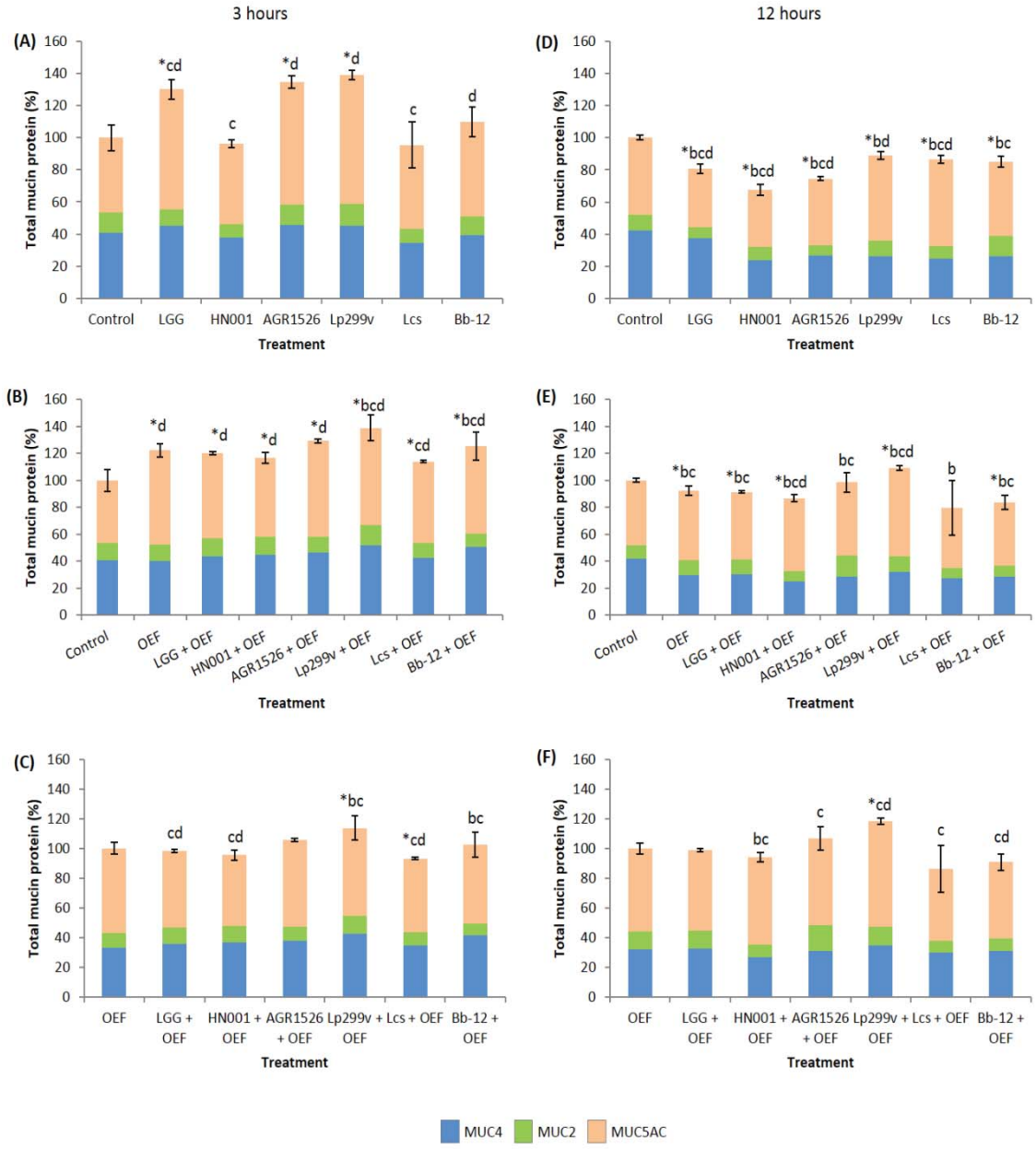


Figure 5-10 Abundance of MUC4, MUC2 and MUC5AC protein from 90:10 Caco-2:HT29-MTX co-cultures after 3 and 12 hours incubation with bacteria alone and in combination with an oligosaccharide-enriched fraction from goat whey

The contribution of the individual mucin proteins (MUC4, MUC2 and MUC5AC) to the total mucin protein abundance from 90:10 co-cultures after 3 (A – C) and 12 (D – F) hours incubation with bacteria alone (A and D); and in combination with an oligosaccharide-enriched fraction (OEF) compared to control (B and E); and compared to monolayers incubated with the OEF alone (C and F). Values shown are the median and error bars are the median absolute deviation. Significantly different ($P < 0.05$) compared to respective control or OEF exposed monolayers: * = total mucin protein; b = MUC4 protein; c = MUC2 protein and d = MUC5AC protein. LGG = *L. rhamnosus* GG; HN001 = *L. rhamnosus* HN001; AGR1526 = *L. plantarum* AGR1526; Lp299v = *L. plantarum* 299v; LcS = *L. casei* Shirota and Bb-12 = *B. lactis* Bb-12.

significantly lower (P< 0.05) for monolayers incubated with the *B. lactis* Bb-12 and OEF combination compared to control (5%), but the abundance of MUC4 and MUC5AC (10% and 18% respectively) mucin proteins were significantly increased (P< 0.05) which resulted in a significantly increased (P< 0.05) abundance of total mucin proteins (25%) compared to control 90:10 monolayers (Figure 5-10B).

In comparison to 90:10 co-culture monolayers incubated with the OEF alone, total mucin protein abundance was significantly increased (P< 0.05) for monolayers incubated with the *L. plantarum* 299v and OEF combination (14%) (Figure 5-10C). This was associated with significant increases (P< 0.05) in the abundance of MUC4 and MUC2 mucin proteins (9% and 5% respectively). In contrast, the total abundance of mucin protein was significantly decreased (P< 0.05) after incubation with the *L. casei* Shirota and OEF combination (7%) with significant decreases (P< 0.05) in the abundance of both the secreted mucin proteins, MUC2 and MUC5AC. Incubation with the *L. rhamnosus* GG and OEF and *L. rhamnosus* HN001 and OEF combinations resulted in a significant increase (P< 0.05) in the abundance of MUC2 protein, but a significant decrease (P< 0.05) in the abundance of MUC5AC protein (5% and 9% respectively). The total abundance of mucin proteins after incubation with the *B. lactis* Bb-12 and OEF combination was similar to 90:10 co-culture monolayers incubated with the OEF alone, although there was a significant increase (P< 0.05) in the abundance of the MUC4 protein (8%), but a significant decrease (P< 0.05) in the total abundance of MUC2 (5%) (Figure 5-10C).

5.4.2.2 90:10 Caco-2:HT29-MTX co-cultures 12 hours incubation

Compared to control 90:10 co-culture monolayers, there was a significant decrease (P< 0.05) in the total abundance of mucin protein after 12 hours incubation with each of the individual bacterial strains (Figure 5-10D). For monolayers incubated with *L. rhamnosus* GG, *L. rhamnosus* HN001 and *L. plantarum* AGR1526 the decrease in total mucin protein (18%, 33% and 26% respectively) was associated with significant decreases (P< 0.05) in the abundance of all

individual mucin proteins. Although incubation with *L. plantarum* 299v resulted in an overall significant decrease ($P< 0.05$) in total mucin protein abundance (11%) when compared to control there was a significant increase ($P< 0.05$) in MUC5AC protein abundance (5%) (Figure 5-10D).

In comparison to control 90:10 co-culture monolayers, the total abundance of mucin protein was significantly increased ($P< 0.05$) after 12 hours incubation with the *L. plantarum* 299v and OEF combination (10%) (Figure 5-10E). In contrast, incubation with all other individual bacteria and OEF combinations (except the *L. plantarum* AGR1526 and OEF combination which had similar levels to control) resulted in a significant decrease ($P< 0.05$) in the total abundance of mucin protein, which was associated with a significant decrease ($P< 0.05$) in the abundance of MUC4 protein. Although total mucin protein abundance was significantly decreased ($P< 0.05$) after incubation with the *L. rhamnosus* GG and OEF combination, there was a significant increase ($P< 0.05$) in the abundance of MUC2 mucin protein. Conversely, there was a significant decrease ($P< 0.05$) in the abundance of MUC2 after incubation with the *L. rhamnosus* HN001 and OEF and *B. lactis* Bb-12 and OEF combinations compared to control 90:10 co-culture monolayers (Figure 5-10E).

In comparison to 90:10 co-culture monolayers incubated with the OEF alone, there was a significant increase ($P< 0.05$) in the abundance of total mucin protein after 12 hours incubation with the *L. plantarum* 299v and OEF combination (17%) (Figure 5-10F). This was associated with a significant increase ($P< 0.05$) in the abundance of MUC2 and MUC5AC mucin proteins (5% and 12% respectively). All individual and total mucin protein abundance values were similar ($P> 0.05$) to 90:10 co-culture monolayers after 12 hours incubation with the *L. rhamnosus* GG and OEF combination. However, although incubation with all other individual bacteria and OEF combinations was shown to alter the abundance levels of individual mucin proteins, there was no significant change ($P> 0.05$) in the overall total abundance of mucin proteins.

5.4.2.3 75:25 Caco-2:HT29-MTX co-cultures 3 hours incubation

Compared to control 75:25 co-culture monolayers there was a significant decrease ($P< 0.05$) in the abundance of total mucin protein detected from monolayers incubated with *L. rhamnosus* GG and *L. rhamnosus* HN001 (41% and 31% respectively) for 3 hours (Figure 5-11A). The decrease in total mucin protein abundance after incubation with *L. rhamnosus* GG was associated with a significant decrease ($P< 0.05$) in the abundance of MUC4 and MUC5AC mucin protein (3% and 38% respectively), while the decrease in total mucin protein after incubation with *L. rhamnosus* HN001 was associated with a significant decrease ($P< 0.05$) in the abundance of MUC5AC (31%) mucin protein. In contrast, incubation with *L. plantarum* AGR1526, *L. plantarum* 299v and *B. lactis* Bb-12 resulted in a significant increase ($P< 0.05$) in the abundance of total mucin protein (33%, 37% and 67% respectively) compared to control monolayers, which was associated with a significant increase ($P< 0.05$) in the abundance of MUC2 (13%, 5% and 5% respectively) and MUC5AC (20%, 34% and 61% respectively). Additionally, incubation with *L. plantarum* 299v led to a significant increase ($P< 0.05$) in the total abundance of MUC4 protein (5%) compared to control monolayers. The total abundance of mucin protein, and the abundance of the individual mucin proteins was similar ($P> 0.05$) to control 75:25 co-cultures after 3 hours incubation with *L. casei* Shirota (Figure 5-11A).

Compared to control 75:25 co-culture monolayers, the total abundance of mucin protein was significantly increased ($P< 0.05$) after 3 hours incubation with all individual bacteria and OEF combinations (except the *L. rhamnosus* GG and OEF combination which had similar levels to control) (Figure 5-11B). MUC2 protein abundance was significantly increased ($P< 0.05$) after incubation with all individual bacteria and OEF combinations; while MUC4 protein abundance was significantly increased ($P< 0.05$) for all bacteria and OEF combinations excluding the *L. rhamnosus* HN001 and OEF combination which had similar values to control. Additionally, except monolayers incubated with the *L. plantarum* 299v and OEF combination, the abundance of MUC5AC protein was also significantly increased ($P< 0.05$) for all other individual

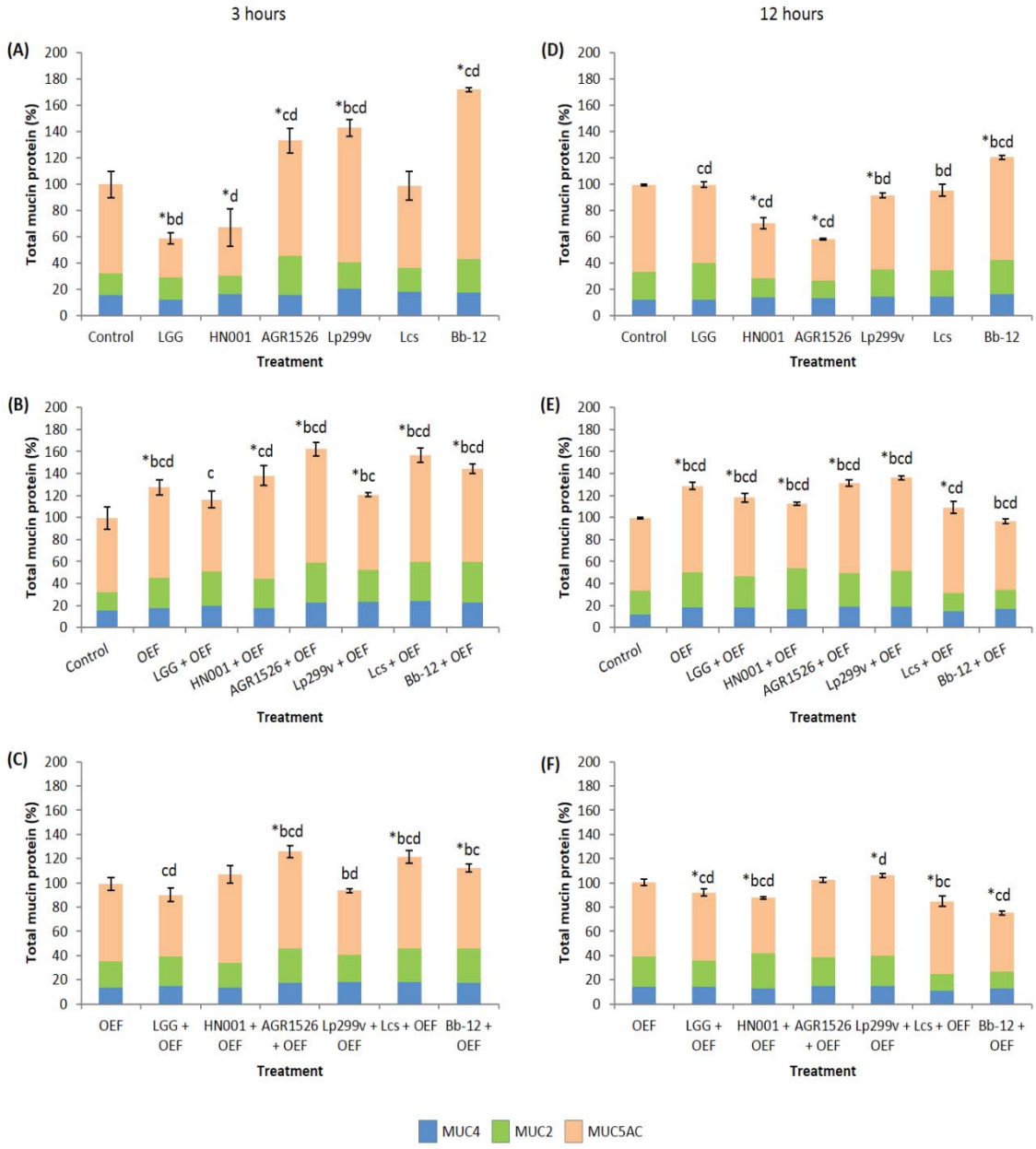


Figure 5-11 Abundance of MUC4, MUC2 and MUC5AC protein from 75:25 Caco-2:HT29-MTX co-cultures after 3 and 12 hours incubation with bacteria alone and in combination with an oligosaccharide-enriched fraction from goat whey

The contribution of the individual mucin proteins (MUC4, MUC2 and MUC5AC) to the total mucin protein abundance from 90:10 co-cultures after 3 (A – C) and 12 (D – F) hours incubation with bacteria alone (A and D); and in combination with an oligosaccharide-enriched fraction (OEF) compared to control (B and E); and compared to monolayers incubated with the OEF alone (C and F). Values shown are the median and error bars are the median absolute deviation. Significantly different ($P < 0.05$) compared to respective control or OEF exposed monolayers: * = total mucin protein; b = MUC4 protein; c = MUC2 protein and d = MUC5AC protein. LGG = *L. rhamnosus* GG; HN001 = *L. rhamnosus* HN001; AGR1526 = *L. plantarum* AGR1526; Lp299v = *L. plantarum* 299v; LcS = *L. casei* Shirota and Bb-12 = *B. lactis* Bb-12.

bacteria and OEF combinations (Figure 5-11B).

The abundance of total mucin protein was significantly increased (P< 0.05) after 3 hours incubation with the *L. plantarum* AGR1526 and OEF, *L. casei* Shirota and OEF and *B. lactis* Bb-12 and OEF combinations (28%, 22% and 11% respectively) compared to 75:25 co-culture monolayers incubated with the OEF alone (Figure 5-11C). For each of these, there was a significant increase (P< 0.05) in the abundance of MUC4 and also the MUC2 protein (7%, 6% and 7% respectively).

In addition, monolayers incubated with the *L. plantarum* AGR1526 and OEF and *L. casei* Shirota and OEF combinations were also noted to have a significant increase (P< 0.05) in MUC5AC abundance (17% and 11% respectively). Although there was a significant increase (P< 0.05) in MUC2 abundance for monolayers incubated with the *L. rhamnosus* GG and OEF combination, this was coupled with a significant decrease (P< 0.05) in MUC5AC (13%) protein levels (Figure 5-11C). For monolayers incubated with the *L. plantarum* 299v and OEF combination there was a significant increase (P< 0.05) in MUC4 (5%) protein, but a significant decrease (P< 0.05) in MUC5AC (11%) protein abundance compared to 75:25 co-culture monolayers incubated with the OEF alone.

5.4.2.4 75:25 Caco-2:HT29-MTX co-cultures 12 hours incubation

Incubation of 75:25 co-culture monolayers with *B. lactis* Bb-12 for 12 hours significantly increased (P< 0.05) the total abundance of mucin protein (20%) compared to control (Figure 5-11D), with a significant increase (P< 0.05) in MUC4 (5%), MUC2 (5%) and MUC5AC (10%) protein. In contrast, the total abundance of mucin protein detected in samples incubated with *L. rhamnosus* HN001, *L. plantarum* AGR1526 and *L. plantarum* 299v was significantly reduced (P< 0.05) after 12 hours (31%, 43% and 6% respectively) compared to control monolayers. Incubation with each of these bacterial strains significantly reduced (P< 0.05) MUC5AC protein abundance (24%, 35% and 8% respectively), while incubation with *L. rhamnosus* HN001 and *L. plantarum* AGR1526 resulted in a significant reduction (P< 0.05) in the abundance of MUC2

protein (7% and 8% respectively). Conversely, the abundance of MUC4 protein was significantly increased (P< 0.05) after incubation with *L. plantarum* 299v (2%) (Figure 5-11D).

Compared to control 75:25 co-culture monolayers, there was a significant increase (P< 0.05) in the total abundance of mucin protein after 12 hours incubation with all individual bacteria and OEF combinations except those incubated with the *B. lactis* Bb-12 and OEF combination (Figure 5-11E). Incubation with the *L. rhamnosus* GG and OEF, *L. plantarum* AGR1526 and OEF and *L. plantarum* 299v and OEF combinations resulted in significant increases (P< 0.05) in MUC4 (6%, 7% and 7% respectively), MUC2 (7%, 9% and 11% respectively) and MUC5AC (6%, 16% and 19% respectively) proteins.

For 75:25 monolayers incubated with the *B. lactis* Bb-12 and OEF combination were shown to have similar total mucin protein abundance levels to control. However, there was a significant increase (P< 0.05) in MUC4 (9%) protein but significant decreases (P< 0.05) in MUC2 and MUC5AC (4% and 5% respectively) proteins.

Compared to 75:25 co-culture monolayers incubated with the OEF alone, there was a significant increase (P< 0.05) in the total abundance of mucin protein (6%) after 12 hours incubation with the *L. plantarum* 299v and OEF combination (Figure 5-11F), which was associated with a significant increase (P< 0.05) in total MUC5AC protein abundance (6%). Although the total abundance of mucin protein was significantly reduced (P< 0.05) after 12 hours incubation with the *L. rhamnosus* GG and OEF, *L. rhamnosus* HN001 and OEF, *L. casei* Shirota and OEF and *B. lactis* Bb-12 and OEF combinations, there were differences in the abundance levels of the individual mucin proteins. For monolayers incubated with both the *L. rhamnosus* GG and OEF and *B. lactis* Bb-12 and OEF combinations there were significant decreases (P< 0.05) in the MUC2 and MUC5AC protein abundance levels. Additionally, MUC4 protein abundance were significantly lower (P< 0.05) after incubation with both the *L. rhamnosus* HN001 and OEF and *L. casei* Shirota and OEF combinations.

5.4.2.5 HT29-MTX mono-cultures 3 hours incubation

In comparison to control HT29-MTX mono-cultures incubation with any of the individual bacterial strains for 3 hours resulted in a significant increase ($P< 0.05$) in total mucin protein abundance (Figure 5-12A). The observed increases were associated with a significant increase ($P< 0.05$) in MUC5AC protein for all strains, but also a significant decrease ($P< 0.05$) in the abundance of MUC4 protein. Additionally, except monolayers incubated with *L. rhamnosus* GG, there was also a significant decrease ($P< 0.05$) in the total abundance of MUC2 protein after incubation with the individual bacterial strains. For HT29-MTX monolayers incubated with the *L. rhamnosus* GG and OEF combination there was a significant decrease ($P< 0.05$) in total mucin protein abundance compared to control monolayers (Figure 5-12B). This was associated with significant decreases ($P< 0.05$) in the abundance of each of the individual mucin proteins investigated. In contrast, incubation with all other individual bacteria and OEF combinations resulted in a significant increase ($P< 0.05$) in total mucin protein abundance compared to control monolayers, with significant increases ($P< 0.05$) in the abundance of MUC5AC protein. However, incubation with the *L. rhamnosus* HN001 and OEF and *L. plantarum* AGR1526 and OEF combinations resulted in a significant reduction ($P< 0.05$) in MUC2 protein (8% and 4% respectively) but after incubation with the *B. lactis* Bb-12 and OEF combination there was a significant increase ($P< 0.05$) in the abundance levels of the same mucin protein.

In comparison to HT29-MTX monolayers incubated with the OEF alone, monolayers incubated with the *L. rhamnosus* GG and OEF combination for 3 hours had significantly lower ($P< 0.05$) total abundance of mucin protein (Figure 5-12C). This resulted from significantly decreased ($P< 0.05$) levels of both MUC2 and MUC5AC (12% and 7% respectively) proteins. Incubation with all other individual bacteria and OEF combinations resulted in a significant increase ($P< 0.05$) in total mucin protein abundance, which was associated with significantly increased ($P< 0.05$) levels of MUC5AC protein. However, for monolayers incubated with the *L. rhamnosus* HN001

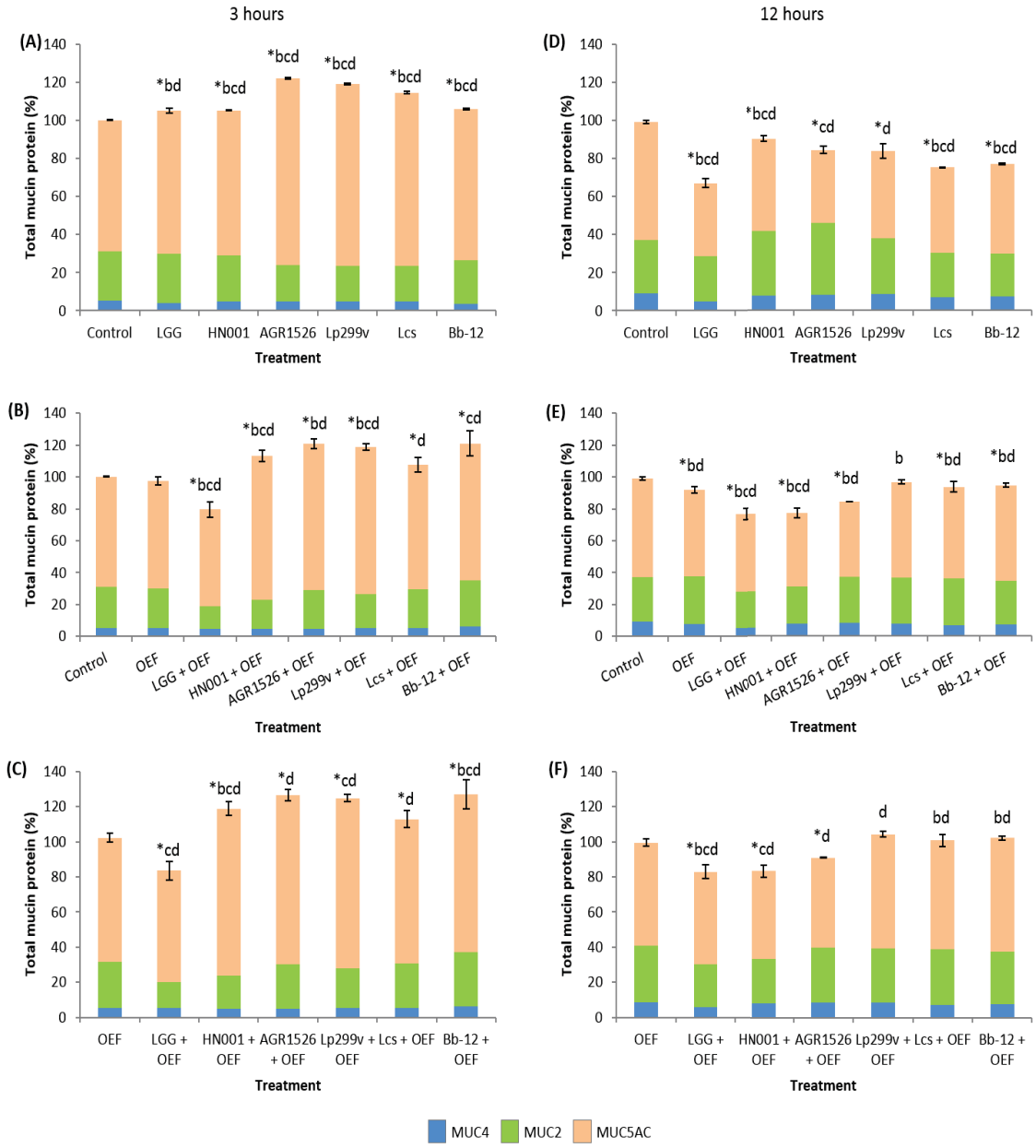


Figure 5-12 Abundance of MUC4, MUC2 and MUC5AC protein from HT29-MTX mono-cultures after 3 and 12 hours incubation with bacteria alone and in combination with an oligosaccharide-enriched fraction from goat whey

The contribution of the individual mucin proteins (MUC4, MUC2 and MUC5AC) to the total mucin protein abundance from 90:10 co-cultures after 3 (A – C) and 12 (D – F) hours incubation with bacteria alone (A and D); and in combination with an oligosaccharide-enriched fraction (OEF) compared to control (B and E); and compared to monolayers incubated with the OEF alone (C and F). Values shown are the median and error bars are the median absolute deviation. Significantly different ($P < 0.05$) compared to respective control or OEF exposed monolayers: * = total mucin protein; b = MUC4 protein; c = MUC2 protein and d = MUC5AC protein. LGG = *L. rhamnosus* GG; HN001 = *L. rhamnosus* HN001; AGR1526 = *L. plantarum* AGR1526; Lp299v = *L. plantarum* 299v; LcS = *L. casei* Shirota and Bb-12 = *B. lactis* Bb-12.

and OEF and *L. plantarum* 299v and OEF combinations there was a significant decrease (P< 0.05) in the abundance of MUC2 protein (7% and 4% respectively).

5.4.2.6 HT29-MTX mono-cultures 12 hours incubation

In comparison to control HT29-MTX monolayers, after 12 hours incubation with all individual bacterial strains tested, there was a significant reduction (P< 0.05) in total mucin protein abundance (Figure 5-12D). For each individual bacterial strain this reduction was associated with a significant decrease (P< 0.05) in total MUC5AC mucin protein abundance. In addition, for monolayers incubated with *L. rhamnosus* GG, *L. casei* Shirota and *B. lactis* Bb-12 there was a significant decrease (P< 0.05) in the abundance levels of MUC4 (4%, 5% and 5% respectively) and MUC2 (4%, 5% and 6% respectively) proteins. In contrast, MUC2 levels were significantly increased (P< 0.05) for HT29-MTX monolayers incubated with *L. rhamnosus* HN001 and *L. plantarum* AGR1526 (6% and 10% respectively) (Figure 5-12D).

Except for monolayers incubated with the *L. plantarum* 299v and OEF combination, which had similar values to control, total mucin protein abundance was significantly lower (P< 0.05) than control HT29-MTX mono-culture monolayers after 12 hours for all other individual bacteria and OEF combinations (Figure 5-12E). Of these, all were shown to have significantly reduced (P< 0.05) abundance levels of MUC4 and MUC5AC proteins. In addition to the decrease in MUC4 and MUC5AC protein levels, there was a significant decrease (P< 0.05) in MUC2 protein abundance after incubation with the *L. rhamnosus* GG and OEF and *L. rhamnosus* HN001 and OEF combinations.

In comparison to HT29-MTX monolayers incubated with OEF alone, the abundance of total mucin protein was significantly reduced (P< 0.05) after incubation with the *L. rhamnosus* GG and OEF, *L. rhamnosus* HN001 and OEF and *L. plantarum* AGR1526 and OEF combinations (14%, 15% and 7% respectively) (Figure 5-12F). This decrease was associated with a significant decrease (P< 0.05) in total MUC5AC protein levels for each of these individual bacteria and OEF combinations (6%, 8% and 7% respectively). Additionally, total MUC2 abundance was also

significantly decreased (P< 0.05) after incubation with both *L. rhamnosus* GG and *L. rhamnosus* HN001 in combination with the OEF (8% and 7% respectively). Samples from HT29-MTX monolayers exposed for 12 hours to *L. plantarum* 299v, *L. casei* Shirota and *B. lactis* Bb-12 in combination with OEF had significantly increased (P< 0.05) abundance levels of total MUC5AC protein compared to control (6%, 5% and 6% respectively). A summary of the results obtained in this study can be viewed in Table 5-2.

5.1 Discussion

This is the first study to investigate the effects of probiotic bacterial strains, either individually or in combination with OEF obtained from goat whey on intestinal barrier integrity as measured by TEER and mucin mRNA expression and mucin protein production in Caco-2 and HT29-MTX mono- and co-cultures. In this study it was shown that selected probiotic bacterial strains had a beneficial effect on intestinal barrier integrity by enhancing TEER and also mucin gene and mucin protein levels. In addition, probiotic bacteria in combination with the OEF also increased TEER, mucin gene and mucin protein levels. However, although some individual bacteria and OEF combinations were able to elicit a response greater than that observed for the individual components (bacteria or OEF alone), especially in regards to TEER, other individual bacteria and OEF combinations did not, which was in contrast to the hypothesis tested in this study. However, one combination, that of *L. plantarum* 299v when in combination with the OEF was shown to consistently enhance TEER and mucin gene/protein expression of both the 90:10 and 75:25 Caco-2:HT29-MTX co-cultures.

Table 5-2 Summary of results for individual epithelial cell cultures after incubation with individual bacteria alone or in combination with an oligosaccharide-enriched fraction from goat whey

Change in status compared to respective controls for the different parameters investigated. Increase (↑), decrease (↓), no significant change (P> 0.05) (–) and not determined (ND).

Caco-2 mono-culture

Control/ standard	Treatment	Bacterial strain	Time (h)	Parameter measured							
				TEER	Mucin gene			Mucin protein			
					MUC4	*MUC2*	*MUC5AC*	MUC4	MUC2	MUC5AC	Total
Control no addition	Bacteria	LGG	3h	–	–	–	–	–	ND	ND	–
			12h	↑	–	–	–	–	ND	ND	–
		HN001	3h	↓	–	↑	–	–	ND	ND	–
			12h	–	↓	↑	–	–	ND	ND	–
		AGR1526	3h	–	–	–	–	↑	ND	ND	↑
			12h	↑	–	↓	–	–	ND	ND	–
		Lp299v	3h	↓	↓	↓	↓	↑	ND	ND	↑
			12h	↑	–	↑	–	–	ND	ND	–
		LcS	3h	↓	↓	–	–	–	ND	ND	–
			12h	↑	–	↑	–	–	ND	ND	–
		Bb-12	3h	↑	–	↓	–	↑	ND	ND	↑
			12h	↑	–	–	–	–	ND	ND	–
Control no addition	*Bacteria and OEF*	LGG	3h	↑	↓	↓	–	↑	ND	ND	↑
			12h	↑	–	↓	–	–	ND	ND	–
		HN001	3h	–	↓	↓	–	↑	ND	ND	↑
			12h	↑	–	–	–	–	ND	ND	–
		AGR1526	3h	↑	↓	↓	↓	↑	ND	ND	↑
			12h	↑	–	–	–	–	ND	ND	–
		Lp299v	3h	↑	↓	↓	↓	–	ND	ND	–
			12h	↑	–	–	–	↑	ND	ND	↑
		LcS	3h	↑	–	↓	↓	–	ND	ND	–
			12h	↑	–	↓	–	–	ND	ND	–
		Bb-12	3h	↑	↓	↓	–	↑	ND	ND	↑
			12h	↑	–	–	–	–	ND	ND	–
OEF	*Bacteria and OEF*	LGG	3h	–	↓	↓	–	↓	ND	ND	↓
			12h	–	–	–	–	↓	ND	ND	↓
		HN001	3h	–	–	–	–	↓	ND	ND	↓
			12h	–	–	–	↑	↓	ND	ND	↓
		AGR1526	3h	–	–	–	–	–	ND	ND	–
			12h	↑	↑	–	↑	↓	ND	ND	↓
		Lp299v	3h	–	–	–	–	↓	ND	ND	↓
			12h	↑	–	–	↑	–	ND	ND	–
		LcS	3h	↑	–	–	–	↓	ND	ND	↓
			12h	↑	–	–	–		ND	ND	
		Bb-12	3h	↑	–	–	↑	↓	ND	ND	↓
			12h	↑	–	↑	–	↓	ND	ND	↓

LGG = *L. rhamnosus* GG; HN001 = *L. rhamnosus* HN001; AGR1526 = *L. plantarum* AGR1526; Lp299v = *L. plantarum* 299v; LcS = *L. casei* Shirota and Bb-12 = *B. lactis* Bb-12.

90:10 Caco-2:HT29-MTX co-culture

Control/ standard	Treatment	Bacterial strain	Time (h)	Parameter measured							
				TEER	Mucin gene			Mucin protein			
					MUC4	*MUC2*	*MUC5AC*	MUC4	MUC2	MUC5AC	Total
Control no addition	Bacteria	LGG	3h	↑	↑	↑	↑	–	↓	↑	↑
			12h	↑	–	↑	–	↓	↓	↓	↓
		HN001	3h	↑	–	–	–	–	↓	–	–
			12h	↑	–	↑	–	↓	↓	↓	↓
		AGR1526	3h	↑	–	–	–	–	–	↑	↑
			12h	↑	–	↑	–	↓	↓	↓	↓
		Lp299v	3h	↑	↓	↓	↓	–	–	↑	↑
			12h	↑	↑	↑	–	↓	–	↑	↓
		LcS	3h	↑	↓	↓	↓	–	↓	–	–
			12h	↑	–	↑	–	↓	↓	↑	↓
		Bb-12	3h	↑	↓	↓	↓	–	–	↑	–
			12h	↑	–	–	–	↓	↑	–	↓
Control no addition	*Bacteria and OEF*	LGG	3h	↑	–	↑	↑	–	–	↑	↑
			12h	↑	–	–	↑	↓	↑	–	↓
		HN001	3h	↑	–	↓	–	–	–	↑	↑
			12h	↑	–	–	↑	↓	↓	↑	↓
		AGR1526	3h	↑	–	–	↑	–	–	↑	↑
			12h	↑	–	–	↑	↓	↑	–	–
		Lp299v	3h	↑	–	–	↑	↑	↑	↑	↑
			12h	↑	–	–	↑	↑	↑	↑	↑
		LcS	3h	↑	↓	↓	–	–	↓	↑	↑
			12h	↑	–	–	–	↓	–	–	–
		Bb-12	3h	↑	↓	↓	–	↑	↓	↑	↑
			12h	↑	–	–	↑	↓	↓	–	↓
OEF	*Bacteria and OEF*	LGG	3h	–	–	↑	–	–	↑	↓	–
			12h	–	–	–	–	–	–	–	–
		HN001	3h	↑	–	–	–	–	↑	↓	–
			12h	↑	–	↓	–	↓	↓	–	–
		AGR1526	3h	↑	↑	–	–	–	–	–	–
			12h	↑	–	↓	–	–	↑	–	–
		Lp299v	3h	↑	↑	–	–	↑	↑	–	↑
			12h	↑	–	–	–	–	↑	↑	↑
		LcS	3h	↑	↓	↓	↓	–	↓	↓	↓
			12h	↑	–	↓	–	–	↓	–	–
		Bb-12	3h	↑	↓	↓	–	↑	↓	–	–
			12h	–	–	–	–	–	↓	↓	–

LGG = *L. rhamnosus* GG; HN001 = *L. rhamnosus* HN001; AGR1526 = *L. plantarum* AGR1526; Lp299v = *L. plantarum* 299v; LcS = *L. casei* Shirota and Bb-12 = *B. lactis* Bb-12.

75:25 Caco-2:HT29-MTX co-culture

Control/ standard	Treatment	Bacterial strain	Time (h)	Parameter measured							
				TEER	Mucin gene			Mucin protein			
					MUC4	*MUC2*	*MUC5AC*	MUC4	MUC2	MUC5AC	Total
Control no addition	Bacteria	LGG	3h	↑	–	–	–	↓	–	↓	↓
			12h	↑	–	–	–	–	↑	↓	–
		HN001	3h	↑	–	↑	–	–	–	↓	↓
			12h	↑	–	–	–	–	↓	↓	↓
		AGR1526	3h	↑	–	–	–	–	↑	↑	↑
			12h	↑	↑	–	–	–	↓	↓	↓
		Lp299v	3h	↑	↑	–	–	↑	↑	↑	↑
			12h	↑	–	–	–	↑	–	↓	↓
		LcS	3h	↑	–	–	↓	–	–	–	–
			12h	↑	↑	–	↓	↑	–	↓	–
		Bb-12	3h	↑	↓	↓	↓	–	↑	↑	↑
			12h	↑	↑	–	–	↑	↑	↑	↑
Control no addition	*Bacteria and OEF*	LGG	3h	↑	–	↓	–	–	↑	–	–
			12h	↑	–	–	–	↑	↑	↑	↑
		HN001	3h	↑	–	–	↑	–	↑	↑	↑
			12h	↑	–	–	–	↑	↑	↓	↓
		AGR1526	3h	↑	–	↓	–	↑	↑	↑	↑
			12h	↑	↑	↑	↑	↑	↑	↑	↑
		Lp299v	3h	↑	–	–	–	↑	↑	–	↑
			12h	↑	↑	↑	–	↑	↑	↑	↑
		LcS	3h	↑	–	↓	–	↑	↑	↑	↑
			12h	↑	↑	↑	–	–	↓	↑	↑
		Bb-12	3h	↑	–	–	↑	↑	↑	↑	↑
			12h	↑	↑	↑	–	↑	↓	↓	–
OEF	*Bacteria and OEF*	LGG	3h	–	–	–	–	–	↑	↓	–
			12h	↑	–	↓	–	–	↓	↓	↓
		HN001	3h	–	–	–	↑	–	–	–	–
			12h	↑	–	–	–	↓	↑	↓	↓
		AGR1526	3h	↑	–	–	–	↑	↑	↑	↑
			12h	↑	–	–	–	–	–	–	–
		Lp299v	3h	↑	–	–	–	↑	–	↓	–
			12h	↑	–	↑	–	–	–	↑	↑
		LcS	3h	↑	–	–	↑	↑	↑	↑	↑
			12h	↑	↑	↑	–	↓	↓	–	↓
		Bb-12	3h	↓	–	–	↑	↑	↑	–	↑
			12h	–	↑	↑	–	–	↓	↓	↓

LGG = *L. rhamnosus* GG; HN001 = *L. rhamnosus* HN001; AGR1526 = *L. plantarum* AGR1526; Lp299v = *L. plantarum* 299v; LcS = *L. casei* Shirota and Bb-12 = *B. lactis* Bb-12.

HT29-MTX mono-culture

Control/ standard	Treatment	Bacterial strain	Time (h)	Parameter measured							
				TEER	Mucin gene			Mucin protein			
					MUC4	*MUC2*	*MUC5AC*	MUC4	MUC2	MUC5AC	Total
Control no addition	Bacteria	LGG	3h	–	–	–	–	↓	–	↑	↑
			12h	–	↓	–	↓	↓	↓	↓	↓
		HN001	3h	–	–	–	–	↓	↓	↑	↑
			12h	–	↓	↓	↓	↓	↑	↓	↓
		AGR1526	3h	–	↑	–	↑	↓	↓	↑	↑
			12h	–	–	–	↓	–	↑	↓	↓
		Lp299v	3h	–	–	–	–	↓	↓	↑	↑
			12h	–	–	–	↓	–	–	↓	↓
		LcS	3h	–	–	↓	↓	↓	↓	↑	↑
			12h	–	–	–	↓	↓	↓	↓	↓
		Bb-12	3h	–	–	↓	↓	↓	↓	↑	↑
			12h	–	–	–	–	↓	↓	↓	↓
Control no addition	*Bacteria and OEF*	LGG	3h	–	–	↓	↑	↓	↓	↓	↓
			12h	–	–	–	–	↓	↓	↓	↓
		HN001	3h	–	–	↓	–	↓	↓	↑	↑
			12h	–	↑	↓	–	↓	↓	↓	↓
		AGR1526	3h	–	↑	–	↑	↓	↓	↑	↑
			12h	–	↑	↓	–	↓	–	↓	↓
		Lp299v	3h	–	↑	–	↑	–	↓	↑	↑
			12h	–	↑	–	–	↓	–	–	–
		LcS	3h	–	–	↓	–	–	–	↑	↑
			12h	–	–	–	–	↓	–	↓	↓
		Bb-12	3h	–	↑	↓	–	–	↑	↑	↑
			12h	–	↑	–	–	↓	–	↓	↓
OEF	*Bacteria and OEF*	LGG	3h	–	–	–	–	–	↓	–	↓
			12h	–	–	–	–	↓	↓	↓	↓
		HN001	3h	–	–	–	–	↓	↓	↑	↑
			12h	–	↑	↓	–	–	↓	↓	↓
		AGR1526	3h	–	–	↑	↑	–	–	↑	↑
			12h	–	–	↓	–	–	–	↓	↓
		Lp299v	3h	–	↑	↑	↑	–	↓	↑	↑
			12h	–	↑	–	–	–	–	↑	↑
		LcS	3h	–	↓	–	–	–	–	↑	↑
			12h	–	–	–	–	↓	–	↑	–
		Bb-12	3h	–	–	–	–	↑	↑	↑	↑
			12h	–	↑	–	↑	↓	–	↑	–

LGG = *L. rhamnosus* GG; HN001 = *L. rhamnosus* HN001; AGR1526 = *L. plantarum* AGR1526; Lp299v = *L. plantarum* 299v; LcS = *L. casei* Shirota and Bb-12 = *B. lactis* Bb-12.

5.1.1 Changes in intestinal barrier integrity

Incubation of Caco-2 mono-cultures and both co-cultures with probiotic bacteria increased TEER, although this increase was strain-dependent. Caco-2 mono-cultures, after 12 hours incubation with *L. rhamnosus* GG had increased TEER, but in contrast, *L. rhamnosus* HN001 was unable to obtain values greater than that of control. This result is in accordance with others that have shown *L. plantarum* strain 299 had a greater positive effect on TEER of Caco-2 cells than *L. plantarum* strain 299v [203]. Although the mechanisms by which probiotic bacteria exert their beneficial effects on TEER was not investigated here, previous studies have suggested that changes in the expression of individual tight junction proteins and activation of signalling pathways may be involved [335]. For example *L. rhamnosus* GG accelerated intestinal barrier maturation by upregulation of claudin 3 expression in neonatal mice [338].

In comparison to control monolayers, all bacterial strains when in combination with the OEF enhanced TEER of Caco-2 mono-cultures and both Caco-2:HT29-MTX co-cultures. However, when the impact on TEER of the different individual bacteria and OEF combinations were compared to those of monolayers incubated with the OEF alone, results were variable. Incubation with the *L. plantarum* AGR1526 and OEF, *L. plantarum* 299v and OEF and *L. casei* Shirota and OEF combinations were shown to consistently enhance TEER to a greater extent than that of co-culture monolayers incubated with the OEF alone. A previous study, using Caco-2 mono-cultures, determined that there were definite species- and sugar-dependent effects with fermentation of Raftiline and Raftilose (both of which are inulin based oligofructose with DP values of 22 to 25 and 3 to 7 respectively) with the probiotics *B. lactis* Bb-12 and *L. rhamnosus* GG exerting the most beneficial effects [329]. In another study, the synbiotic combination of resistant starch and *B. lactis* (strain unknown), which protected against the development of colorectal cancer in rats, was greater than the benefit of either component alone [339]. Thus it could be suggested that a possible synbiotic relationship exists between some bacteria and the OEF which results in an increase in TEER greater than that

observed for either component alone, but that it is strain specific.

5.1.2 Changes in mucin dynamics

Additionally, bacteria either alone or in association with the OEF had varied impacts on mucin gene transcription and protein expression, another important aspect of the protective capacity of the intestinal barrier.

Inoculation with *L. rhamnosus* GG for 3 hours increased *MUC4*, *MUC2*, and *MUC5AC* mRNA transcripts of 90:10 co-cultures, but this was not the case for the other epithelial cell cultures. In contrast to these results *L. rhamnosus* GG has previously been shown to increase *MUC2* gene expression in Caco-2 cells after 3 hours incubation and the abundance of its protein product in CL samples [118]. In another study it was reported that mucin gene expression (*MUC3*) was increased in HT29 mono-cultures after one hour incubation with the probiotic bacteria *L. plantarum* 299v and *L. rhamnosus* GG, which was coupled with an increase of its respective mucin protein product in SM [18]. However, exposure to *B. lactis* Bb-12 decreased mucin gene expression levels but increased mucin protein abundance of the 75:25 co-cultures. These differences may be explained by post-transcriptional or translational regulatory mechanisms linked to mucin molecule synthesis and secretion [227]. It is possible that changes to mRNA stability following exposure to bacteria may occur and has been reported with other stimuli [227, 340]. Additionally, differences between mucin gene expression levels and mucin protein abundance may relate to detection thresholds for either mRNA or protein, of which the methods for quantifying mRNA transcript levels are more sensitive than those for protein identification and quantification [341].

In this study it was shown that when in combination with the OEF the impact of some of the individual bacterial strains on reducing mucin gene/protein expression was reduced. Incubation with *L. plantarum* 299v in combination with the OEF for 3 hours reversed the reduced levels of *MUC4* mRNA transcription seen with this bacterial strain alone in 90:10 co-cultures. Additionally, the abundance of MUC2 mucin protein was increased, the increase of

which was greater than that observed in monolayers incubated with either component alone. Other bacteria and OEF combinations such as *L. casei* Shirota and OEF and *B. lactis* Bb-12 and OEF combinations were shown to reduce the levels of *MUC4* and *MUC2* mRNA transcription in 90:10 co-cultures after 3 hours, the decrease of which was greater than that of the individual components. In contrast, incubation with the same bacteria and OEF combinations was shown to increase *MUC4* and *MUC2* mRNA transcription in 75:25 co-cultures after 12 hours, the increase of which was greater than either component alone. Thus, it could be hypothesised that the combination of bacteria and OEF can modify the reduced transcription or translation events caused by incubation with the bacteria alone, and may suggest a synergistic relationship between the OEF and specific bacterial strains.

It is recognised that an increase in mucin expression and secretion such as occurred for 75:25 co-cultures incubated with *L. plantarum* 299v for 3 hours, could be of benefit to the bacteria by increasing its ability to colonise the intestinal tract [212]. However, increased mucin synthesis, if it directly relates to an increase in mucus layer thickness, could reduce the absorption of both dietary and endogenous macromolecules and ions [342]. A previous study indicated that inclusion of a 5-probiotic bacteria combination (*Lactobacillus reuteri* DSM 16350, *Enterococcus faecium* DSM 16211, *Bifidobacterium animalis* DSM 16284, *Pediococcus acidilactici* DSM 16210 and *Lactobacillus salivarius* DSM 16351) to the diet of broiler chickens increased the thickness of the mucus layer in the small intestine and this increase was associated with increased body weight gain [343, 344]. Another report has indicated that although there were enhanced rates of mucin synthesis during periods of starvation of chickens (fasting for 48 hours) this was associated with an accumulation of mucin within the goblet cells, and a depletion of the mucus layer [342]. Consequently, because the mucus layer thickness was not determined here any correlation or association between increased mucin production and increased thickness of the mucus layer could not be made. However, increased mucin protein abundance as observed after incubation with bacteria alone or in combination

with OEF would potentially be associated with an increase in the mucus layer.

5.1.3 Potential health benefits of synbiotics

The results in this study highlight that the effects of individual bacteria and OEF combinations on mucin gene transcription and protein expression differed as a consequence of culture duration and the epithelial cell model being investigated. However, one combination, that of *L. plantarum* 299v and OEF consistently enhanced TEER, and mucin gene/mucin protein expression of both co-cultures. For 90:10 co-cultures, TEER, the expression of *MUC4* gene and the abundance of all mucin proteins were increased, after 3 and 12 hours incubation with the *L. plantarum* 299v and OEF combination. After 12 hours incubation with the same bacteria and OEF combination 75:25 co-cultures had an increase in the same parameters except *MUC5AC* expression which was unchanged. Although the *L. plantarum* AGR1526 and OEF combination also increased these parameters of the 75:25 co-cultures after 12 hours, the response in the 90:10 co-cultures, both at 3 and 12 hours, was more variable than that observed for the *L. plantarum* 299v and OEF combination. Although some of the increases in these parameters in response to bacteria in combination with the OEF could be directly attributed to the inclusion of the OEF, others could not; suggesting that a synergistic interaction between the bacteria and OEF was responsible for the observed increases.

Whether the same response would be observed *in vivo* was not determined in this study; although it could be suggested that the effects of some of these combinations on the 90:10 and 75:25 co-cultures, would be similar to those of the small and large intestine *in vivo*. An essential aspect to consider would be the transit time of digesta through the different regions of the intestine. For example, transit time through the human small intestine ranges from 15 minutes to 5 hours [345] which is substantially faster than that of the human large intestine (8.7 hours to 56 hours) [346]. Consequently the response of the 90:10 and 75:25 co-cultures (seeded at similar cell compositions to that of the small and large intestine respectively), after 3 and 12 hours respectively would match a biologically relevant time period *in vivo*. Regional

and temporal differences in mucus dynamics have been observed previously in response to probiotic combinations [343]. For example the inclusion of a 5-probiotic bacteria combination to the diet from day 1 of male broilers was shown to increase the thickness of the mucus layer of the duodenum but not the caecum after 14 days. However, an extended treatment period (42 days in total) was shown to have no effect on the thickness of the mucus layer in either region of the poultry intestine [343].

The enhanced effect of synbiotics compared with either their probiotic or prebiotic components have been investigated in animal and human studies. Synbiotic products containing *B. longum* and lactulose or inulin reduced the incidence and size of aberrant crypt foci in rats challenged with the carcinogen azoxymethane [347-349]. Another study determined that rats fed a mixture of high and low molecular weight inulin (Synergy 1), with the probiotic strains *L. rhamnosus* GG and *B. lactis* Bb-12, developed fewer colonic tumours after challenge with azoxymethane, than rats fed the probiotic strains alone [350]. Few studies have been undertaken in humans on the effectiveness of synbiotics [351-354]. A synbiotic mix containing inulin and *Bifidobacterium* spp. increased bifidobacterial numbers in faecal samples of healthy volunteers, but the increase was not attributed to the inclusion of the prebiotic component [353]. The addition of the synbiotic (*Bifidobacterium longum* and psyllium) to the diet of patients with Ulcerative Colitis improved their health-related quality of life (as assessed by the total Inflammatory Bowel Disease questionnaire) when compared to probiotic or prebiotic treatment alone [355]. However, a systematic review of eight randomised studies including 999 adult intensive care patients concluded that probiotics and synbiotics lacked beneficial effects in respect to established outcome criteria including length of stay in the intensive care unit, hospital mortality and the incidence of nosocomial infections such as pneumonia [327, 356]. Consequently, synbiotic products have the potential for enhanced health promotion over either probiotics or prebiotics alone but require further investigations [349].

5.2 Conclusion

In conclusion this study identified that individual probiotic bacterial strains when in combination with the OEF can have a beneficial impact on intestinal barrier integrity (as measured by an increase in TEER) and mucin gene/protein expression. However, beneficial effects only occurred with defined bacteria and OEF combinations. Of these, *L. plantarum* 299v in combination with the OEF was shown to have the greatest enhancing effects on barrier integrity and mucin gene/protein expression in both the 90:10 and 75:25 Caco-2:HT29-MTX co-cultures. Collectively, it could be concluded that the combination of *L. plantarum* 299v and OEF has the potential to enhance intestinal barrier integrity to a greater extent than other bacteria and OEF combinations. However, the fact that differences occur between certain combinations highlights the need for further investigations into other bacterial strains as potential probiotic/prebiotic/synbiotic formulations.

Chapter 6: General discussion

6.1 Background

Many complex interactions between food, host and bacteria occur in the human intestine. As our knowledge of the interactions between these components increases, so too does the potential to develop functional foods for increased health and wellness benefits. Unravelling the interactions between these components is difficult when using whole systems i.e. humans or animals. Consequently, an alternative, reductionist approach is the use of *in vitro* cellular models.

To date many *in vitro* epithelial models have concentrated on the use of mono-cultures of two types of epithelial cells found in the gastrointestinal tract; absorptive enterocytes or mucus-secreting goblet cells. Although these cellular models have their usefulness, these mono-cultures have some characteristics that may be a drawback. Mono-cultures of absorptive cells have tighter intercellular junctions in comparison to those *in vivo* [357] and lack a mucus layer. Alternatively mono-cultures of goblet cells have the benefit of the mucus layer but do not form intercellular junction complexes and as such, have altered intestinal barrier integrity [3, 357].

Co-culturing of both absorptive and mucus-secreting cells together overcomes some of the restrictions of the epithelial mono-cultures. Although the development of such co-cultures is not new, their use has previously been restricted to studies investigating drug transport, bacterial adhesion or bacterial pathogenesis/infection. Thus to develop a deeper understanding of the interactions between food, host and bacteria, it is essential that co-culture models of the intestinal epithelium are characterised.

A major aim of this research was to characterise and utilise intestinal epithelial co-cultures that more closely simulated the intestinal epithelial layer found *in vivo*; and to determine the impact of probiotic bacteria and the OEF from goat whey, either individually or in combination, on intestinal barrier integrity.

The epithelial co-cultures that have been validated can be used to gain insights into the mechanisms of interactions between the food, intestinal cells and the bacteria and new

knowledge generated using these co-cultures will assist in the development of food-based strategies that improve intestinal barrier function.

6.2 Summary

This dissertation presents *in vitro* co-culture models that have been developed to simulate the cellular composition and function of the epithelial layer of the human small and large intestine *in vivo*. These co-culture models were used to elucidate some of the interactions that occur between the host (co-cultures), bacteria (selected probiotic strains) and food (OEF from goat whey) and their impact, either individually or in combination, on intestinal epithelial barrier integrity and mucin gene/protein expression.

An essential element of this dissertation was to utilise *in vitro* models that were more representative of the human intestine *in vivo*. As mentioned in the introduction and Chapter 2, the cellular composition of the epithelial layer in the human small and large intestine varies in respect to the numbers of absorptive enterocytes and mucus-secreting goblet cells. Although exact numbers are unknown for the different cell types in the human intestine it has been proposed that the proportion of goblet cells among epithelial cell types increase from the duodenum (4%) to the distal colon (16-24%) [4, 33, 34, 46]. Consequently, *in vitro* models that simulate this cellular composition would be expected to have more similar functions to that of *in vivo* intestinal tissue.

In Chapter 2 the major aims were to characterise epithelial co-cultures which were representative of the human small and large intestine in terms of their cellular composition and to determine, whether as a consequence of similar cellular compositions, these cultures would be more representative of the function of *ex vivo/in vivo* intestinal epithelia as reported in the literature, when compared to the respective mono-cultures.

The cell lines used throughout this dissertation for the establishment of co-cultures were the well characterised Caco-2 (absorptive enterocytes) and HT29-MTX (mucus-secreting goblet cells) cells. These cell lines were selected because when cultured together, they have

previously been shown to form monolayers with junctional complexes between the cells and a layer of mucus present at the apical surface [46, 207].

The co-cultures developed in Chapter 2 were shown to have some properties which were similar to those published for *ex vivo* and *in vivo* human data [89, 247, 250]. For example, TEER of the co-cultures was more similar to values of *ex vivo* intestinal tissue of human small and large intestine than either of the two mono-cultures [200, 248]. Additionally, the thickness of the mucus layer present at the apical surface of 75:25 co-cultures (cellular composition representative of the large intestine) was similar to the thickness of the inner mucus layer of the human large intestine [89].

Although mucin dynamics were determined in Chapter 2 for both the mono- and co-cultures, abundance values of the different mucin proteins were not compared to *in vivo* data. Instead the abundance of mucin proteins from the co-cultures were compared to the individual mono-cultures. There was no relationship between the abundance of mucin proteins in the co-cultures and those of the mono-cultures, but the results obtained were limited to a certain extent because the secreted mucin proteins MUC2 and MUC5AC were not determined in the Caco-2 mono-cultures. This may have been due to an inability of the selected antibodies used here, to recognise the epitopes of the mucins produced by Caco-2 cells. The rationale for using the selected antibodies was the availability of recombinant proteins that could be utilised to quantify the abundance of these proteins from the cell samples. In future investigations the suitability of different antibodies that recognise alternate epitopes of the mucin proteins could be investigated.

Chapter 3 focused on the interaction between the OEF and the epithelial cell cultures. The composition of the OEF was a combination of different oligosaccharides, in addition to other mono, disaccharides and galacto-oligosaccharides. Introduction of the OEF to the epithelial cell cultures characterised in Chapter 2 was shown to have no detrimental effects on metabolic activity and could differentially modulate TEER in a dose-dependent manner. This was contrary

to previous studies which have observed that oligosaccharides from human milk can interfere with host protein-carbohydrate interactions and may disrupt cell growth as well as cell-cell and cell-matrix interactions [261]. However, results from this work may have differed to that of others because of the varied composition of the OEF (mono, disaccharides and galacto-oligosaccharides, in addition to oligosaccharides) whereas the other studies examined the impact of pure oligosaccharide fractions.

Although the maximum concentration of the oligosaccharide component in the OEF tested (1 mg/mL) was less than would be found in human breast milk (3 to 13 mg/mL) [254], this concentration was shown to increase TEER and enhance mucin gene/protein expression of both the co-culture models. Although the increase in mucin mRNA transcripts and the respective protein products were variable at the different time points for the different co-culture models, similar expression patterns were observed between mucin mRNA transcripts and mucin protein abundance for the same co-culture model. A relationship between mucin protein abundance and gene expression has been observed previously and may be related to the process involved in mucin secretion [276, 277]. However, increased expression levels of MUC4 protein can be associated with a loss of interaction between neighbouring cells, resulting from a re-localisation of E-cadherin from adherens junctions at the lateral membrane of the cells to the apical membrane [275]. Although in the 75:25 co-cultures, where there was an increase in MUC4 protein expression after incubation with the OEF, TEER was also increased compared to control. Thus it could be argued that although MUC4 expression was increased there were no detrimental effects on the junctions between cells, although this was not measured directly. Barrier integrity was evaluated by TEER in this work, which is a measure of ion permeability. Alternative methods for the functional assessment of intestinal barrier integrity are based on actively measuring paracellular intestinal leakage of administered test substrates such as mannitol or lactulose [358]. An increase in the passage of such substrates from the apical to the basolateral domains of cell cultures would be an indicator of decreased

barrier integrity and a reduction in TEER.

While Chapter 3 concentrated on the interaction between the OEF and epithelial cells, investigations in Chapter 4 examined first the impact of food on bacterial growth. Interactions between food and bacteria can lead to increased numbers of specific bacterial species within the human large intestine. Additionally, dietary components are also able to alter the concentrations of bacterial fermentation products such as lactate, acetate, succinate, propionate and butyrate, although this was not determined in this thesis.

The bacteria used in this thesis were selected because their probiotic attributes, such as acid and bile tolerance and beneficial effects on various immune related functions had previously been characterised [203]. Of the bacterial strains investigated, two strains, *L. casei* Shirota and *B. angulatum* DSM 20098, preferentially used the OEF for growth when compared to a sugar combination comprised of equivalent concentrations of the individual carbohydrates present in the OEF (minus the oligosaccharide fraction). Thus it could be suggested that these bacteria utilise specific components which are present within the OEF, such as the oligosaccharides, although both of these strains had only minimal utilisation of the milk oligosaccharides 3' and 6' sialyl lactose. For *B. angulatum* DSM 20098 minimal utilisation of 3' and 6' sialyl lactose can be explained by a lack of *exo*-α-sialidase (EC: 3.2.1.18) enzyme activity that hydrolyses linkages of terminal sialic acid residues in sugar chains, such as those of HMOs and mucin molecules [359]. To date, the presence of this enzyme has not been reported for *L. casei* Shirota, but it could be suggested that, as a consequence of minimal utilisation of these substrates, *L. casei* Shirota also lacks this enzyme.

When cultured in 5% CO_2 atmospheric conditions; the same atmospheric conditions as the intestinal epithelial cell cultures; only 6 of 9 probiotic strains were able to utilise the OEF for growth. Of these, all were able to adhere to the different epithelial cell cultures although at varying degrees of efficiency. The mechanisms by which the different probiotics adhered to the epithelial cultures was not examined in this thesis, however the presence of known

effector molecules such as mucus-binding proteins may have been responsible for the variation in the observed adherence phenotypes displayed by the strains [320].

The inclusion of the OEF was shown to modulate the adherence of some bacterial strains to the different epithelial cultures. Decreased adherence may have been related to the oligosaccharides in the OEF being structurally similar to receptor sites of the epithelial cells to which specific bacteria recognise and adhere. In this way the oligosaccharides would act as molecular receptor decoys that can inhibit bacterial adherence similar to that observed for other bacterial species when cultured in the presence of galacto-oligosaccharides [326]. Consequently, rather than binding to host cell surface oligosaccharides the bacteria would bind to the decoy oligosaccharides present in the OEF. Adherence may also have been altered in response to the OEF as a consequence of a change in the expression of genes/proteins involved in adherence. For example, incubation of *B. longum* subsp. *infantis* ATCC 15697 to 3' and/or 6' sialyl lactose led to an increase in the expression of genes responsible for the formation and assembly of pili and a subsequent increase in the adherence of this bacterium to Caco-2 cells [295]. Other factors which are known to affect adherence of probiotic bacteria to epithelial cells include growth temperature, pH of the cultures and the specific growth phase of the bacteria themselves [360, 361]. Future studies could examine the surface architecture of bacteria using electron microscopy to determine whether growth in the presence of the OEF changes the expression of pili/fimbriae.

In Chapter 5 the interactions between bacteria, the OEF and epithelial cells were further examined. The expression levels of mucin genes and the abundance of mucin proteins for the each of the epithelial cell cultures, in response to the bacteria alone and in combination with the OEF were determined. There were varied impacts associated with the bacteria in combination with the OEF on mucin gene transcription and mucin protein expression. The combined effect of specific bacteria and OEF combinations, such as *L. plantarum* 299v and OEF, on mucin dynamics of specific co-cultures (mainly the 90:10 and 75:25 co-cultures), was

greater than for the individual components alone suggesting a possible synergistic relationship between certain bacterial strains and the OEF. Additionally in Chapter 5 TEER was shown to be enhanced in both co-cultures as a consequence of incubation with all individual bacterial strains. TEER was further enhanced when the individual bacterial strains were in combination with the OEF. For some of these bacteria and OEF combinations the increase in TEER was greater than that observed for either of the individual components alone, further suggesting a synergistic relationship between defined bacterial strains and the OEF.

Such a relationship was evident between the combinations of *L. plantarum* 299v and OEF with both the 90:10 and 75:25 co-culture models. When in combination with the 90:10 co-culture TEER, *MUC5AC* mRNA expression and the abundance of total mucin protein as well as the individual mucin proteins investigated were all increased at both 3 and 12 hours. When in combination with the 75:25 co-culture TEER, *MUC4* and *MUC2* gene expression as well as the abundance of all mucin proteins were increased after 12 hours incubation. Thus it could be suggested that when in combination with the OEF *L. plantarum* 299v enhanced intestinal epithelial barrier integrity and mucin gene/protein expression, all of which are essential components of host mucosal immunity and function.

Although the exact mechanisms by which *L. plantarum* 299v in combination with the OEF increased mucin gene/protein expression and TEER was not determined, it could be suggested that the fermentation of the OEF by the bacteria led to the production of SCFAs such as acetate or propionate which were responsible for the observed mucin gene/protein expression increases [362]. When in combination acetate and propionate are also effective in modulating mucin synthesis and release both at the gene and protein level [17, 91, 177] and has the capacity to increase TEER by increasing the expression of tight junction protein products [31]. Future studies could examine whether the fermentation profiles (individual SCFAs and their relative ratios) of the different bacterial strains changed when cultured in the OEF.

6.3 Limitations and possible alternatives

The co-cultures characterised in Chapter 2 offer advantages over the mono-cultures, not only because they have a cellular composition more similar to that of the *in vivo* situation but also have TEER and mucus layer properties more akin to that of *ex vivo/in vivo*. However, there are limitations with the co-cultures characterised here, the most prominent of which is that the cell lines used were derived from tumour origins and as such have different cellular structures and regulatory mechanisms compared to normal/non-diseased cells. For example, altered levels and pattern of mucin gene expression and altered glycosylation in cancer cells affect their biologic properties, such as cell proliferation, adhesion, motility and invasion [4]. More specifically, Caco-2 cells exhibit physiological and biochemical properties of small intestinal absorptive enterocytes although they were derived from a human colon [363]. An important aspect in terms of the studies undertaken here is the expression of mucins. In the healthy human foetal and adult large intestine, MUC2 is the main secreted mucin making up the mucus layer. However, in a diseased state such as colon cancer, MUC2 levels are decreased while there is an increase in the abundance of MUC5AC in the large intestine. Consequently any results obtained from *in vitro* investigations using cell lines which have similar mucin profiles, such as the HT29-MTX cell line, can only be used as a guide to *in vivo* activities. An alternative approach would be to utilise cell lines which have mucin profiles more similar to that found in healthy cells. Primary cell cultures are such an alternative, although these are also associated with inherent difficulties such as their extraction and maintaining as viable cells [364].

Another aspect of the co-cultures characterised here which is different to the *in vivo* tissue is the lack of intestinal crypts and villi. Consequently more complex systems, such as 3-dimensional models could be considered a viable option to overcome these limitations. One such model utilising hydrogel scaffolds to create 3-dimensional villous platforms, which impart the authentic size and shape of intestinal villi to *in vitro* cultures, has been developed [251].

This model demonstrated the importance of the 3-dimensional microenvironment on cell behaviour especially when related to differentiation and TEER. Although, to date this model has only been used in conjunction with Caco-2 cells, this model could potentially be further developed to include additional cell types such as mucus-secreting goblet cells or immature dendritic cells [357, 365, 366] to be more representative of the intestinal epithelium *in vivo*. The co-culture models characterised in Chapter 2 provide a suitable starting point on which to build, but as our knowledge of these co-cultures improves, adding in additional cell types and increasing the complexity of such cell cultures will increase our understanding of interactions between intestinal epithelial cells, food and bacteria.

Another limitation of the epithelial models characterised in this thesis is the static nature of the cultures. As eluded to in Chapter 5, digesta flows through the intestinal tract at different rates. As the digesta flows through these regions not only does it move ingested material but it is also responsible for removing some of the mucus layer, especially the outer mucus layer. This removal process allows for a replenishment of the mucus layer from newly secreted mucins but also removes some of the bacteria which have been able to colonise this outer layer. Consequently, an *in vitro* model of the intestine that could incorporate this physiological process would further enhance the use of these co-cultures. A model that can emulate peristalsis has been developed, which is composed of a microchip with microfluidic channels lined by Caco-2 cells, and utilise vacuum chambers to mimic peristalsis [367]. This model allowed for the co-culture with the bacterial strain *L. rhamnosus* GG for extended periods (over a week) without compromising the viability of either cell type. Incorporation of mucus-secreting goblet cells would enhance this model and could potentially be further developed to investigate interactions between intestinal epithelial cells, food and bacteria.

6.4 Future perspectives

Inclusion of the OEF used in this thesis was shown to have a direct impact on the epithelial co-cultures by enhancing TEER and mucin gene/protein expression. In addition, the OEF was

shown to support the growth of different probiotic bacterial strains. However the OEF was only preferentially used by two bacterial strains, *L. casei* Shirota and *B. angulatum* DSM 20098, when compared to a sugar combination comprised of equivalent concentrations of the individual carbohydrates present in the OEF (minus the oligosaccharide fraction). When included in combination with different bacteria the OEF could modulate bacterial adherence to epithelial cell cultures, TEER and mucin gene/protein expression.

Whether the effects on these parameters would be the same *in vivo* is unknown. However it was suggested in Chapter 5 that the effects of the OEF observed in the 90:10 and 75:25 co-cultures would be similar to those of the small and large intestine *in vivo*. However, because the OEF used here was a heterogeneous mix of oligosaccharides (25.6%), monosaccharides (glucose 12% and galactose 15.9%), disaccharide (lactose 46.1%) and galacto-oligosaccharides (0.4%), it could be suggested that the mono and disaccharides of the OEF would be preferentially utilised in the small intestine and would unlikely reach the large intestine. Consequently the main component of the OEF that would reach the large intestine relatively intact would be the oligosaccharides. Thus further purification of the OEF to remove or limit the mono and disaccharide components could be considered an important aspect. The lactose component could be reduced by increasing the concentration of the β-galactosidase in the lactose hydrolysis step, followed by sugar separation using the activated carbon. Nano-filtration of samples could potentially remove the monosaccharides [368] prior to evaporation and lyophilisation.

An alternative approach to investigate the impact of the OEF on cellular models of the intestine, in its current composition, would be to utilise the 90:10 co-cultures as a first pass, and then transfer the supernatant/SM from these co-cultures to the 75:25 co-cultures. For example, the OEF-supplemented media could be introduced to the 90:10 co-cultures for a designated period of time that reflects the transit time of digesta through the small intestine *in vivo*, thus 15 minutes to 5 hours. After which point the SM could be transferred from the 90:10

co-cultures to the 75:25 co-cultures and again incubated for a designated period of time to reflect the transit time of digesta through the large intestine (8.7 to 56 hours). It could be hypothesised that the impact of the OEF on barrier integrity and mucin gene/protein expression of the different co-cultures at the end of such experiments would be more similar to that of what would occur *in vivo*. To increase the complexity of such *in vitro* models bacteria could also be introduced to examine the combined effects of bacteria and OEF. The time at which the bacteria were introduced, and to which co-culture model could also be varied. However, a limitation of such a model is the build-up of acids and waste products in the SM, which is known to be detrimental to epithelial cell viability over extended periods of time. Thus a better model would be if the two different co-cultures could be seeded into separate compartments, but be connected in some way with a gated divider that could be switched to allow for the flow of media from one section to another. An additional gated switch would allow for the SM to be removed from the 75:25 co-cultures at a designated flow rate. Although the exact requirements and specifics of such an *in vitro* model have not been determined, it could be a potential way of further investigating the interaction between the OEF, bacteria and intestinal epithelial cells.

The studies undertaken here have identified that when in combination with the OEF *L. plantarum* 299v has a beneficial impact on barrier integrity and mucin gene/protein expression. However it was shown in Chapter 4 that *L. plantarum* 299v was able to grow when cultured in a 5% CO_2 atmosphere, while that of *B. angulatum* DSM 20456 was unable to do so. Similarly, the majority (99%) of the microbiota of the large intestine would also be unable to grow or even survive in such atmospheric conditions, because they are obligate anaerobes. Consequently a major limitation of the co-cultures characterised here is the restrictive nature of the culture atmosphere which limits the bacterial strains that can be investigated to those that can tolerate a 5% CO_2 atmosphere.

A model that has already been developed, but could be further adapted to investigate the

interaction between bacteria and the OEF, is the apical anaerobic model of the intestine [47]. This model utilises a dual-environment co-culture chamber in which Caco-2 cells are cultured so that the apical (luminal) side are exposed to an anaerobic environment, while maintaining an aerobic basal side. This model could be developed into a more complex system with the introduction of mucin-secreting HT29-MTX cells, but would provide the additional benefit of being able to culture other bacterial strains which are unable to survive in the 5% CO_2 atmosphere.

Introduction of the strain *L. plantarum* 299v in combination with the OEF into such a model may change the interactions that occur between the OEF, bacteria and the co-cultures. Would the different atmospheric conditions relate to a change in the rate at which the bacteria grow or utilise the OEF? As indicated in Chapter 4, when cultured anaerobically, there was increased fermentation of the OEF by *L. plantarum* 299v when compared to growth in the 5% CO_2 atmosphere. It was suggested previously that the beneficial impact of this strain in combination with the OEF on barrier integrity was related to the production of SCFAs. If this was the reason for the increase in TEER and mucin dynamics, how different would this impact be when the bacteria are cultured in an environment which is more similar to the large intestine *in vivo*? It is conceivable that because fermentation of the OEF was increased, there would also be an increase in the production of SCFAs. However, would the fermentation products produced stay the same or would they also be altered? Would there be a greater increase in lactic acid production in a shorter period of time, which would limit the duration of investigation? The use of such a model would introduce additional physiological attributes which are lacking in the co-cultures characterised here. However preliminary research would be required to determine whether the HT29-MTX cells would be able to survive under such growth conditions, which could then be further extended to that of the co-cultures.

The impact of the OEF in combination with bacteria on intestinal barrier integrity and mucin gene/protein expression could further be investigated using animals such as mice, rats or pigs.

However, to distinguish the impact of the OEF on these parameters, separate from those of the bacteria, would require the use of germ-free animals. However, there are some differences in the colonic cellular morphology, the mucus layer and mucin synthesis between germ-free and conventionally raised animals. Colonic goblet cells in germ-free rodents are less numerous and smaller in size relative to those of conventionally raised animals [211] possibly due to the absence of fermented metabolites from the colonic microbiota resulting in decreased growth and maturation of goblet cells [212]. Accompanied by a decreased number and size of goblet cells, there are contradicting reports of a reduction in the adherent mucus layer thickness [159, 213] and an increase in mucin protein synthesis in germ-free mice [210]. The mucin composition of germ-free animals also varies when compared to animals that have been conventionally raised, with an increased presence of sialylated mucins as opposed to sulfomucins [214]. Consequently, although *in vivo* models would allow for barrier integrity and mucin dynamics to be investigated, they too are associated with some limitations.

6.5 Concluding remarks

In vitro co-cultures incorporating absorptive enterocytes (Caco-2) and mucus-secreting goblet cells (HT29-MTX) are important models of the intestinal epithelial layer because they more closely simulate the cellular composition and function of that *in vivo*. Aspects of these co-cultures, such as TEER and mucus layer thickness, are more representative of *in vivo/ex vivo* published data than either of the two mono-cultures which were also characterised. As such, the use of co-cultures to evaluate interactions between bacteria, food components and intestinal cells provide additional benefits, as compared to the mono-cultures, by providing reliable and suitable *in vitro* models that better approach the physiological characteristics of the *in vivo* situation. However the potential drawbacks of the co-culture models should not be overlooked. Importantly because the human intestinal cells used were from tumour origins, it may not be appropriate to extrapolate data generated using these models to the *in vivo* situation. Thus, when drawing any and all conclusions using these models the limitations of the

models should be considered first.

Nonetheless these co-cultures allowed for the simultaneous study of both intestinal barrier integrity and mucus secretion and provided suitable models of the intestine for evaluating bacterial adhesion ability and as such, could further help to elucidate the type of interactions that mediate the host-microbe interactions. Using the co-culture models it was demonstrated that an OEF from goat whey exerted promoting effects on intestinal epithelial function. Additionally, individual probiotic bacterial strains when in combination with the OEF had increased adherence to the epithelial models and a beneficial impact on intestinal barrier integrity (as measured by an increase in TEER) and mucin gene/protein expression, but only defined bacteria and OEF combinations could elicit a response greater than that of the individual components alone. Although some of the increases in these parameters in response to bacteria in combination with the OEF could be directly attributed to the inclusion of the OEF, others could not; suggesting that a synergistic interaction between the bacteria and OEF was responsible for the observed increases. Thus it could be suggested that the OEF from goat whey has important implications as a potential functional food supplement, not only for improving intestinal health, but also for increasing the viability of selected probiotic bacteria and reinforcing their probiotic function, such as improving their adhesion to intestinal cells and mucus.

Of these, *L. plantarum* 299v in combination with the OEF was shown to have the greatest enhancing effects on barrier integrity and mucin gene/protein expression of both the 90:10 and 75:25 Caco-2:HT29-MTX co-cultures. Thus it could be suggested that an interaction exists between some probiotic bacteria, the OEF from goat whey and intestinal epithelial cells that have the potential for enhanced health promotion over either probiotics or prebiotics alone and could be of benefit to intestinal function.

Collectively, it could be concluded that the OEF from goat whey has important implications as a potential functional food supplement for improving intestinal health and in combination with

L. plantarum 299v has the potential of enhancing intestinal barrier integrity and mucin gene/protein expression greater than that of other bacteria and OEF combinations. However, the fact that differences occur between certain probiotic bacteria and OEF combinations highlights the need for further investigations into other bacterial strains as potential probiotic/prebiotic/synbiotic formulations for improving host mucosal function and to better understand both the positive and negative interactions that may occur between bacteria, food components and intestinal cells *in vivo*.

References

1. Savage, D.C., *Microbial Ecology of the Gastrointestinal Tract.* Annual Review of Microbiology, 1977. **31**(1): p. 107-133.
2. Lievin-Le Moal, V. and A.L. Servin, *The Front Line of Enteric Host Defense against Unwelcome Intrusion of Harmful Microorganisms: Mucins, Antimicrobial Peptides, and Microbiota.* Clinical Microbiology Reviews, 2006. **19**(2): p. 315-337.
3. Grootjans, J., G. Thuijls, F. Verdam, J.P. Derikx, K. Lenaerts, and W.A. Buurman, *Non-invasive assessment of barrier integrity and function of the human gut.* World Journal Of Gastrointestinal Surgery, 2010. **2**(3): p. 61-69.
4. Kim, Y. and S. Ho, *Intestinal Goblet Cells and Mucins in Health and Disease: Recent Insights and Progress.* Current Gastroenterology Reports, 2010. **12**(5): p. 319-330.
5. Deplancke, B. and H.R. Gaskins, *Microbial modulation of innate defense: goblet cells and the intestinal mucus layer.* American Journal of Clinical Nutrition, 2001. **73**(6): p. 1131S-1141.
6. Coppa, G.V., L. Zampini, T. Galeazzi, and O. Gabrielli, *Prebiotics in human milk: a review.* Digestive and Liver Disease, 2006. **38, Supplement 2**: p. S291-S294.
7. Westerbeek, E.A.M., A. van den Berg, H.N. Lafeber, J. Knol, W.P.F. Fetter, and R.M. van Elburg, *The intestinal bacterial colonisation in preterm infants: A review of the literature.* Clinical nutrition (Edinburgh, Scotland), 2006. **25**(3): p. 361-368.
8. Harmsen, H.J., A.C. Wildeboer-Veloo, G.C. Raangs, A.A. Wagendorp, N. Klijn, J.G. Bindels, and G.W. Welling, *Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods.* Journal of Pediatric Gastroenterology and Nutrition, 2000. **30**(1): p. 61-67.
9. Van den Abbeele, P., C. Grootaert, S. Possemiers, W. Verstraete, K. Verbeken, and T. Van de Wiele, *In vitro model to study the modulation of the mucin-adhered bacterial community.* Applied Microbiology and Biotechnology, 2009. **83**(2): p. 349-359.
10. Roos, S. and H. Jonsson, *A high-molecular-mass cell-surface protein from Lactobacillus reuteri 1063 adheres to mucus components.* Microbiology, 2002. **148**(2): p. 433-442.
11. Boekhorst, J., Q. Helmer, M. Kleerebezem, and R.J. Siezen, *Comparative analysis of proteins with a mucus-binding domain found exclusively in lactic acid bacteria.* Microbiology, 2006. **152**(1): p. 273-280.
12. Buck, B.L., E. Altermann, T. Svingerud, and T.R. Klaenhammer, *Functional Analysis of Putative Adhesion Factors in Lactobacillus acidophilus NCFM.* Applied and Environmental Microbiology, 2005. **71**(12): p. 8344-8351.
13. Lebeer, S., J. Vanderleyden, and S.C.J. De Keersmaecker, *Genes and Molecules of Lactobacilli Supporting Probiotic Action.* Microbiology and Molecular Biology Reviews, 2008. **72**(4): p. 728-764.
14. Shipman, J.A., J.E. Berleman, and A.A. Salyers, *Characterization of Four Outer Membrane Proteins Involved in Binding Starch to the Cell Surface of Bacteroides thetaiotaomicron.* Journal of Bacteriology, 2000. **182**(19): p. 5365-5372.
15. Imamura, Y., K. Yanagihara, Y. Mizuta, M. Seki, H. Ohno, Y. Higashiyama, Y. Miyazaki, K. Tsukamoto, Y. Hirakata, K. Tomono, J.-i. Kadota, and S. Kohno, *Azithromycin Inhibits MUC5AC Production Induced by the Pseudomonas aeruginosa Autoinducer N-(3-Oxododecanoyl) Homoserine Lactone in NCI-H292 Cells.* Antimicrobial Agents and Chemotherapy, 2004. **48**(9): p. 3457-3461.
16. Dharmani, P., V. Srivastava, V. Kissoon-Singh, and K. Chadee, *Role of Intestinal Mucins in Innate Host Defense Mechanisms against Pathogens.* Journal of Innate Immunity, 2009. **1**(2): p. 123-135.
17. Gaudier, E., A. Jarry, H.M. Blottiere, P. de Coppet, M.P. Buisine, J.P. Aubert, C. Laboisse, C. Cherbut, and C. Hoebler, *Butyrate specifically modulates MUC gene*

expression in intestinal epithelial goblet cells deprived of glucose. American Journal of Physiology: Gastrointestinal and Liver Physiology, 2004. **287**(6): p. G1168-1174.
18. Mack, D.R., S. Ahrne, L. Hyde, S. Wei, and M.A. Hollingsworth, *Extracellular MUC3 mucin secretion follows adherence of Lactobacillus strains to intestinal epithelial cells in vitro.* Gut, 2003. **52**(6): p. 827-833.
19. Otte, J.-M. and D.K. Podolsky, *Functional modulation of enterocytes by gram-positive and gram-negative microorganisms.* American Journal of Physiology: Gastrointestinal and Liver Physiology, 2004. **286**(4): p. G613-626.
20. Forder, R.E.A., *An in vivo model to study mucin-bacterial interactions during early post-hatch development of broiler chickens.*, in *School of Agriculture, Food and Wine: Agriculture and Animal Science*2007, University of Adelaide: Adelaide. p. 188.
21. Meslin, J.-C., N. Fontaine, and C. Andrieux, *Variation of mucin distribution in the rat intestine, caecum and colon: effect of the bacterial flora.* Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology, 1999. **123**(3): p. 235-239.
22. Hooper, L.V. and J.I. Gordon, *Commensal Host-Bacterial Relationships in the Gut.* Science, 2001. **292**(5519): p. 1115-1118.
23. Shen, L., *Functional Morphology of the Gastrointestinal Tract*, in *Molecular Mechanisms of Bacterial Infection Via the Gut*, C. Sasakawa, Editor 2009, Springer-Verlag: Berlin Heidelberg. p. 1-35.
24. O'Hara, A.M. and F. Shanahan, *The gut flora as a forgotten organ.* Embo Reports, 2006. **7**(7): p. 688-693.
25. Lidbeck, A. and C.E. Nord, *Lactobacilli and the Normal Human Anaerobic Microflora.* Clinical Infectious Diseases, 1993. **16**: p. S181-S187.
26. Borriello, S.P., W.P. Hammes, W. Holzapfel, P. Marteau, J. Schrezenmeir, M. Vaara, and V. Valtonen, *Safety of probiotics that contain lactobacilli or bifidobacteria.* Clinical Infectious Diseases, 2003. **36**(6): p. 775-780.
27. Katayama, T., K. Fujita, and K. Yamamoto, *Novel bifidobacterial glycosidases acting on sugar chains of mucin glycoproteins.* Journal of Bioscience and Bioengineering, 2005. **99**(5): p. 457-465.
28. German, J.B., S.L. Freeman, C.B. Lebrilla, and D.A. Mills, *Human Milk Oligosaccharides: Evolution, Structures and Bioselectivity as Substrates for Intestinal Bacteria*, in *Personalized Nutrition for the Diverse Needs of Infants and Children*, D.M. Bier, J.B. German, and B. Lonnerdal, Editors. 2008. p. 205-222.
29. Daddaoua, A., V. Puerta, P. Requena, A. Martinez-Ferez, E. Guadix, F.S. de Medina, A. Zarzuelo, M.D. Suarez, J.J. Boza, and O. Martinez-Augustin, *Goat milk oligosaccharides are anti-inflammatory in rats with hapten-induced colitis.* Journal of Nutrition, 2006. **136**(3): p. 672-676.
30. Lara-Villostada, F., E. Debras, A. Nieto, A. Concha, J. Galvez, E. Lopez-Huertas, J. Boza, C. Obled, and J. Xaus, *Oligosaccharides isolated from goat milk reduce intestinal inflammation in a rat model of dextran sodium sulfate-induced colitis.* Clinical Nutrition, 2006. **25**(3): p. 477-488.
31. Guzman, J.R., V.S. Conlin, and C. Jobin, *Diet, Microbiome, and the Intestinal Epithelium: An Essential Triumvirate?* BioMed Research International, 2013. **2013**: p. 12.
32. Barker, N., M. van de Wetering, and H. Clevers, *The intestinal stem cell.* Genes & Development, 2008. **22**(14): p. 1856-1864.
33. Brittan, M. and N.A. Wright, *The gastrointestinal stem cell.* Cell Proliferation, 2004. **37**(1): p. 35-53.
34. Mahida, Y.R., *Epithelial cell responses.* Best Practice & Research Clinical Gastroenterology, 2004. **18**(2): p. 241-253.
35. *Intestines%20compared*. 2013 [cited 2013 30 October 2013]; Available from: http://www.vetmed.vt.edu/education/curriculum/vm8054/Labs/Lab19/IMAGES/INTESTINES%20COMPARED.jpg.

36. Crosnier, C.c., D. Stamataki, and J. Lewis, *Organizing cell renewal in the intestine: stem cells, signals and combinatorial control.* Nature Reviews: Genetics, 2006. **7**(5): p. 349-359.
37. Chopra, D.P., K.Y. Yeh, and R.W. Brockman, *Isolation and characterisation of epithelial-cell types from the normal rat colon* Cancer Research, 1981. **41**(1): p. 168-175.
38. Sancho, E., E. Batlle, and H. Clevers, *Live and let die in the intestinal epithelium.* Current Opinion in Cell Biology, 2003. **15**(6): p. 763-770.
39. Porter, E.M., C.L. Bevins, D. Ghosh, and T. Ganz, *The multifaceted Paneth cell.* Cellular and Molecular Life Sciences CMLS, 2002. **59**(1): p. 156-170.
40. van der Flier, L.G. and H. Clevers, *Stem Cells, Self-Renewal, and Differentiation in the Intestinal Epithelium.* Annual Review of Physiology, 2009. **71**: p. 241-260.
41. Verburg, M., I.B. Renes, H.P. Meijer, J.A.J.M. Taminiau, H.A. Buller, A.W.C. Einerhand, and J. Dekker, *Selective sparing of goblet cells and Paneth cells in the intestine of methotrexate-treated rats.* American Journal of Physiology: Gastrointestinal and Liver Physiology, 2000. **279**(5): p. G1037-1047.
42. Verdugo, P., *Goblet Cells Secretion and Mucogenesis.* Annual Review of Physiology, 1990. **52**(1): p. 157-176.
43. Katz, J.P., N. Perreault, B.G. Goldstein, C.S. Lee, P.A. Labosky, V.W. Yang, and K.H. Kaestner, *The zinc-finger transcription factor Klf4 is required for terminal differentiation of goblet cells in the colon.* Development, 2002. **129**(11): p. 2619-2628.
44. Forstner, G., *Signal-transduction, packaging and secretion of mucins* Annual Review of Physiology, 1995. **57**: p. 585-605.
45. Specian, R.D. and M.G. Oliver, *Functional biology of intestinal goblet cells.* American Journal of Physiology: Cell Physiology, 1991. **260**(2): p. C183-193.
46. Hilgendorf, C., H. Spahn-Langguth, C.G. Regårdh, E. Lipka, L. Amidon Gordon, and P. Langguth, *Caco-2 versus caco-2/HT29-MTX co-cultured cell lines: Permeabilities via diffusion, inside- and outside-directed carrier-mediated transport.* Journal of Pharmaceutical Sciences, 2000. **89**(1): p. 63-75.
47. Ulluwishewa, D., *Interactions between commensal obligate anaerobes and human intestinal cells*, 2013, Massey University,Manawatu, New Zealand: Palmerston North.
48. Ivanov, A.I., I.C. McCall, C.A. Parkos, and A. Nusrat, *Role for Actin Filament Turnover and a Myosin II Motor in Cytoskeleton-driven Disassembly of the Epithelial Apical Junctional Complex.* Molecular Biology of the Cell, 2004. **15**(6): p. 2639-2651.
49. Ulluwishewa, D., R.C. Anderson, W. Young, W.C. McNabb, P. van Baarlen, P.J. Moughan, J.M. Wells, and N.C. Roy, *Live Faecalibacterium prausnitzii in an apical anaerobic model of the intestinal epithelial barrier.* Cellular Microbiology, 2015. **17**(2): p. 226-240.
50. Cone, R.A., *Barrier properties of mucus.* Advanced Drug Delivery Reviews, 2009. **61**(2): p. 75-85.
51. Gouyer, V., A. Wiede, M.-P. Buisine, S. Dekeyser, O. Moreau, T. Lesuffleur, W. Hoffmann, and G. Huet, *Specific secretion of gel-forming mucins and TFF peptides in HT-29 cells of mucin-secreting phenotype.* Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2001. **1539**(1-2): p. 71-84.
52. McGuckin, M.A., R. Eri, L.A. Simms, T.H. Florin, and G. Radford-Smith, *Intestinal barrier dysfunction in inflammatory bowel diseases.* Inflammatory Bowel Diseases, 2009. **15**(1): p. 100-113.
53. Johansson, M.E.V., K.A. Thomsson, and G.C. Hansson, *Proteomic Analyses of the Two Mucus Layers of the Colon Barrier Reveal That Their Main Component, the Muc2 Mucin, Is Strongly Bound to the Fcgbp Protein.* Journal of Proteome Research, 2009. **8**(7): p. 3549-3557.
54. *HGNC HUGO Gene Nomenclature Committee*. [cited 2014 17/06]; Available from: http://www.genenames.org/.

55. van Tassell, M.L. and M.J. Miller, *Lactobacillus adhesion to mucus.* Nutrients, 2011. **3**(5): p. 613-636.
56. Moniaux, N., F. Escande, N. Porchet, J.P. Aubert, and K.B. Surinder, *Structural Organisation and Classification of the Human Mucin Genes.* Frontiers in Bioscience, 2001. **6**: p. 1192-1206.
57. Gendler, S.J. and A.P. Spicer, *Epithelial Mucin Genes.* Annual Review of Physiology, 1995. **57**(1): p. 607-634.
58. Hollingsworth, M.A. and B.J. Swanson, *Mucins in cancer: Protection and control of the cell surface.* Nature Reviews Cancer, 2004. **4**(1): p. 45-60.
59. Corfield, A.P., D. Carroll, N. Myerscough, and C.S.J. Probert, *Mucins in the gastrointestinal tract in health and disease.* Frontiers in Bioscience, 2001. **6**: p. D1321-D1357.
60. Roberton, A.M. and D.P. Wright, *Bacterial glycosulphatases and sulphomucin degradation.* Canadian Journal of Gastroenterology, 1997. **11**(4): p. 361-366.
61. Robbe, C., C. Capon, E. Maes, M. Rousset, A. Zweibaum, J.-P. Zanetta, and J.-C. Michalski, *Evidence of Regio-specific Glycosylation in Human Intestinal Mucins.* Journal of Biological Chemistry, 2003. **278**(47): p. 46337-46348.
62. Lai, S.K., Y.-Y. Wang, D. Wirtz, and J. Hanes, *Micro- and macrorheology of mucus.* Advanced Drug Delivery Reviews, 2009. **61**(2): p. 86-100.
63. Shaoul, R., Y. Okada, E. Cutz, and M.A. Marcon, *Colonic Expression of MUC2, MUC5AC, and TFF1 in Inflammatory Bowel Disease in Children.* Journal of Pediatric Gastroenterology and Nutrition, 2004. **38**(5): p. 488-493.
64. Barnett, A.M., N.C. Roy, W.C. McNabb, and A.L. Cookson, *The interactions between endogenous bacteria, dietary components and the mucus layer of the large bowel.* Food & Function, 2012. **3**(7): p. 690-699.
65. Wiggins, R., S.J. Hicks, P.W. Soothill, M.R. Millar, and A.P. Corfield, *Mucinases and sialidases: their role in the pathogenesis of sexually transmitted infections in the female genital tract.* Sexually Transmitted Infections, 2001. **77**(6): p. 402-408.
66. Gum, J.R., J.J.L. Ho, W.S. Pratt, J.W. Hicks, A.S. Hill, L.E. Vinall, A.M. Roberton, D.M. Swallow, and Y.S. Kim, *MUC3 human intestinal mucin - Analysis of gene structure, the carboxyl terminus, and a novel upstream repetitive region.* Journal of Biological Chemistry, 1997. **272**(42): p. 26678-26686.
67. Moniaux, N., S. Nollet, N. Porchet, P. Degand, A. Laine, and J.P. Aubert, *Complete sequence of the human mucin MUC4: a putative cell membrane-associated mucin.* Biochemical Journal, 1999. **338**: p. 325-333.
68. Parry, S., F.G. Hanisch, S.-H. Leir, M. Sutton-Smith, H.R. Morris, A. Dell, and A. Harris, *N-Glycosylation of the MUC1 mucin in epithelial cells and secretions.* Glycobiology, 2006. **16**(7): p. 623-634.
69. Byrd, J. and R. Bresalier, *Mucins and mucin binding proteins in colorectal cancer.* Cancer and Metastasis Reviews, 2004. **23**(1): p. 77-99.
70. Crawley, S.C., J.R. Gum, J.W. Hicks, W.S. Pratt, J.-P. Aubert, D.M. Swallow, and Y.S. Kim, *Genomic Organization and Structure of the 3' Region of Human MUC3: Alternative Splicing Predicts Membrane-Bound and Soluble Forms of the Mucin.* Biochemical and Biophysical Research Communications, 1999. **263**(3): p. 728-736.
71. Williams, S.J., M.A. McGuckin, D.C. Gotley, H.J. Eyre, G.R. Sutherland, and T.M. Antalis, *Two Novel Mucin Genes Down-Regulated in Colorectal Cancer Identified by Differential Display.* Cancer Research, 1999. **59**(16): p. 4083-4089.
72. Williams, S.J., D.H. Wreschner, M. Tran, H.J. Eyre, G.R. Sutherland, and M.A. McGuckin, *MUC13, a Novel Human Cell Surface Mucin Expressed by Epithelial and Hemopoietic Cells.* Journal of Biological Chemistry, 2001. **276**(21): p. 18327-18336.
73. Itoh, Y., M. Kamata-Sakurai, K. Denda-Nagai, S. Nagai, M. Tsuiji, K. Ishii-Schrade, K. Okada, A. Goto, M. Fukayama, and T. Irimura, *Identification and Expression of Human*

Epiglycanin/MUC21: a Novel Transmembrane Mucin.* Glycobiology, 2008. **18**(1): p. 74-83.

74. Rachagani, S., M.P. Torres, N. Moniaux, and S.K. Batra, *Current status of mucins in the diagnosis and therapy of cancer.* BioFactors, 2009. **35**(6): p. 509-527.
75. Baruch, A., M.L. Hartmann, M. Yoeli, Y. Adereth, S. Greenstein, Y. Stadler, Y. Skornik, J. Zaretsky, N.I. Smorodinsky, I. Keydar, and D.H. Wreschner, *The breast cancer-associated MUC1 gene generates both a receptor and its cognate binding protein.* Cancer Research, 1999. **59**(7): p. 1552-1561.
76. Baruch, A., M.L. Hartmann, S. ZrihanLicht, S. Greenstein, M. Burstein, I. Keydar, M. Weiss, N. Smorodinsky, and D.H. Wreschner, *Preferential expression of novel MUC1 tumor antigen isoforms in human epithelial tumors and their tumor-potentiating function.* International Journal of Cancer, 1997. **71**(5): p. 741-749.
77. Hartman, M.L., A. Baruch, I. Ron, Y. Aderet, M. Yoeli, O. Sagi-Assif, S. Greenstein, Y. Stadler, M. Weiss, E. Harness, M. Yaakubovits, I. Keydar, N.I. Smorodinsky, and D.H. Wreschner, *MUC1 isoform specific monoclonal antibody 6E6/2 detects preferential expression of the novel MUC1/Y protein in breast and ovarian cancer.* International Journal of Cancer, 1999. **82**(2): p. 256-267.
78. Higuchi, T., T. Orita, S. Nakanishi, K. Katsuya, H. Watanabe, Y. Yamasaki, I. Waga, T. Nanayama, Y. Yamamoto, W. Munger, H.W. Sun, R.J. Falk, J.C. Jennette, D.A. Alcorta, H.P. Li, T. Yamamoto, Y. Saito, and M. Nakamura, *Molecular cloning, genomic structure, and expression analysis of MUC20, a novel mucin protein, up-regulated in injured kidney.* Journal of Biological Chemistry, 2004. **279**(3): p. 1968-1979.
79. Pallesen, L.T., L. Berglund, L.K. Rasmussen, T.E. Petersen, and J.T. Rasmussen, *Isolation and characterization of MUC15, a novel cell membrane-associated mucin.* European Journal of Biochemistry, 2002. **269**(11): p. 2755-2763.
80. Parry, S., H.S. Silverman, K. McDermott, A. Willis, M.A. Hollingsworth, and A. Harris, *Identification of MUC1 proteolytic cleavage sites in vivo.* Biochemical and Biophysical Research Communications, 2001. **283**(3): p. 715-720.
81. Schmid, B.C., M. Rudas, G. Fabjani, P. Speiser, K. Kaserer, S. Leodolter, and R. Zeillinger, *Evaluation of MUC1 splice variants as prognostic markers in patients with ductal carcinoma in situ of the breast.* Oncology Reports, 2003. **10**(6): p. 1981-1985.
82. Williams, S.J., M.A. McGuckin, D.C. Gotley, H.J. Eyre, G.R. Sutherland, and T.M. Antalis, *Two novel mucin genes down-regulated in colorectal cancer identified by differential display.* Cancer Research, 1999. **59**(16): p. 4083-4089.
83. Williams, S.J., D.J. Munster, R.J. Quin, D.C. Gotley, and M.A. McGuckin, *The MUC3 gene encodes a transmembrane mucin and is alternatively spliced.* Biochemical and Biophysical Research Communications, 1999. **261**(1): p. 83-89.
84. Perez-Vilar, J. and R.L. Hill, *The Structure and Assembly of Secreted Mucins.* Journal of Biological Chemistry, 1999. **274**(45): p. 31751-31754.
85. Linden, S.K., T.H.J. Florin, and M.A. McGuckin, *Mucin Dynamics in Intestinal Bacterial Infection.* Plos One, 2008. **3**(12).
86. Jordan, N., J. Newton, J. Pearson, and A. Allen, *A novel method for the visualization of the in situ mucus layer in rat and man.* Clinical Science, 1998. **95**(1): p. 97-106.
87. Newton, J.L., N. Jordan, L. Oliver, V. Strugala, J. Pearson, O.F.W. James, and A. Allen, *Helicobacter pylori in vivo causes structural changes in the adherent gastric mucus layer but barrier thickness is not compromised.* Gut, 1998. **43**(4): p. 470-475.
88. Newton, J.L., N. Jordan, J. Pearson, G.V. Williams, A. Allen, and O.F.W. James, *The adherent gastric antral and duodenal mucus gel layer thins with advancing age in subjects infected with Helicobacter pylori.* Gerontology, 2000. **46**(3): p. 153-157.
89. Matsuo, K., H. Ota, T. Akamatsu, A. Sugiyama, and T. Katsuyama, *Histochemistry of the surface mucous gel layer of the human colon.* Gut, 1997. **40**(6): p. 782-789.
90. Pullan, R.D., G.A. Thomas, M. Rhodes, R.G. Newcombe, G.T. Williams, A. Allen, and J.

Rhodes, *Thickness of adherent mucus gel on colonic mucosa in humans and its relevance to colitis.* Gut, 1994. **35**(3): p. 353-359.
91. Finnie, I.A., A.D. Dwarakanath, B.A. Taylor, and J.M. Rhodes, *Colonic mucin syntheis is increased by sodium-butyrate* Gut, 1995. **36**(1): p. 93-99.
92. Schreiber, O., *Microcirculation, Mucus and Microbiota in Inflammatory Bowel Disease*, in *Faculty of Mecicine*2010, Uppsala University: Uppsala.
93. Phillipson, M., M.E.V. Johansson, J. Henriksnas, J. Petersson, S.J. Gendler, S. Sandler, A.E.G. Persson, G.C. Hansson, and L. Holm, *The gastric mucus layers: constituents and regulation of accumulation.* American Journal of Physiology: Gastrointestinal and Liver Physiology, 2008. **295**(4): p. G806-812.
94. Jintapattanakit, A., V.B. Junyaprasert, and T. Kissel, *The Role of Mucoadhesion of Trimethyl Chitosan and PEGylated Trimethyl Chitosan Nanocomplexes in Insulin Uptake.* Journal of Pharmaceutical Sciences, 2009. **98**(12): p. 4818-4830.
95. Jonsson, L., X. Liu, B.-A. Jonsson, M. Ljungberg, and S.-E. Strand, *A Dosimetry Model for the Small Intestine Incorporating Intestinal Wall Activity and Cross-Doses.* Journal of Nuclear Medicine, 2002. **43**(12): p. 1657-1664.
96. Johansson, M.E.V., J.M. Holmén Larsson, and G.C. Hansson, *The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host–microbial interactions.* Proceedings of the National Academy of Sciences, 2010.
97. Johansson, M.E.V., M. Phillipson, J. Petersson, A. Velcich, L. Holm, and G.C. Hansson, *The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria.* Proceedings of the National Academy of Sciences, 2008. **105**(39): p. 15064-15069.
98. Andrianifahanana, M., N. Moniaux, and S.K. Batra, *Regulation of mucin expression: Mechanistic aspects and implications for cancer and inflammatory diseases.* Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, 2006. **1765**(2): p. 189-222.
99. Atuma, C., V. Strugala, A. Allen, and L. Holm, *The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo.* American Journal of Physiology: Gastrointestinal and Liver Physiology, 2001. **280**(5): p. G922-929.
100. Holzapfel, W.H., P. Haberer, J. Snel, U. Schillinger, and J.H.J. Huis in't Veld, *Overview of gut flora and probiotics.* International Journal of Food Microbiology, 1998. **41**(2): p. 85-101.
101. Hawrelak, J.A. and S.P. Myers, *The causes of intestinal dysbiosis: A review.* Alternative Medicine Review, 2004. **9**(2): p. 180-197.
102. Ericsson, C.D., C. Hatz, and A.W. DuPont, *Postinfectious Irritable Bowel Syndrome.* Clinical Infectious Diseases, 2008. **46**(4): p. 594-599.
103. Sekirov, I., S.L. Russell, L.C.M. Antunes, and B.B. Finlay, *Gut Microbiota in Health and Disease.* Physiological Reviews, 2010. **90**(3): p. 859-904.
104. Qin, J., R. Li, J. Raes, M. Arumugam, K.S. Burgdorf, C. Manichanh, T. Nielsen, N. Pons, F. Levenez, T. Yamada, D.R. Mende, J. Li, J. Xu, S. Li, D. Li, J. Cao, B. Wang, H. Liang, H. Zheng, Y. Xie, J. Tap, P. Lepage, M. Bertalan, J.-M. Batto, T. Hansen, D. Le Paslier, A. Linneberg, H.B. Nielsen, E. Pelletier, P. Renault, T. Sicheritz-Ponten, K. Turner, H. Zhu, C. Yu, S. Li, M. Jian, Y. Zhou, Y. Li, X. Zhang, S. Li, N. Qin, H. Yang, J. Wang, S. Brunak, J. Dore, F. Guarner, K. Kristiansen, O. Pedersen, J. Parkhill, J. Weissenbach, P. Bork, S.D. Ehrlich, and J. Wang, *A human gut microbial gene catalogue established by metagenomic sequencing.* Nature, 2010. **464**(7285): p. 59-65.
105. Hooper, L.V., T. Midtvedt, and J.I. Gordon, *How host-microbial interactions shape the nutrient environment of the mammalian intestine* Annual Review of Nutrition, 2002. **22**(1): p. 283-307.
106. Satokari, R.M., E.E. Vaughan, C.F. Favier, J. Doré, C. Edwards, and W.M.d. Vos, *Diversity of Bifidobacterium and Lactobacillus spp. in Breast-Fed and Formula-Fed Infants as Assessed by 16S rDNA Sequence Differences.* Microbial Ecology in Health and Disease,

2002. **14**(2): p. 97-105.
107. Jimenez, E., L. Fernandez, M.L. Marin, R. Martin, J.M. Odriozola, C. Nueno-Palop, A. Narbad, M. Olivares, J. Xaus, and J.M. Rodriguez, *Isolation of commensal bacteria from umbilical cord blood of healthy neonates born by cesarean section.* Current Microbiology, 2005. **51**(4): p. 270-4.
108. Jimenez, E., M.L. Marin, R. Martin, J.M. Odriozola, M. Olivares, J. Xaus, L. Fernandez, and J.M. Rodriguez, *Is meconium from healthy newborns actually sterile?* Research in Microbiology, 2008. **159**(3): p. 187-93.
109. France de La Cochetiere, M., C. Rouge, D. Darmaun, J. Christophe Roze, G. Potel, and C. Gras Leguen, *Intestinal Microbiota in Neonates and Preterm Infants: A Review.* Current Pediatric Reviews, 2007. **3**(1): p. 21-34.
110. Hopkins, M.J., R. Sharp, and G.T. Macfarlane, *Age and disease related changes in intestinal bacterial populations assessed by cell culture, 16S rRNA abundance, and community cellular fatty acid profiles.* Gut, 2001. **48**(2): p. 198-205.
111. Dethlefsen, L., P.B. Eckburg, E.M. Bik, and D.A. Relman, *Assembly of the human intestinal microbiota.* Trends in Ecology & Evolution, 2006. **21**(9): p. 517-523.
112. Schwarz, R., A. Kaspar, J. Seelig, and B. Künnecke, *Gastrointestinal transit times in mice and humans measured with 27Al and 19F nuclear magnetic resonance.* Magnetic Resonance in Medicine, 2002. **48**(2): p. 255-261.
113. Xu, X., P. Xu, C. Ma, J. Tang, and X. Zhang, *Gut microbiota, host health, and polysaccharides.* Biotechnology Advances, 2013. **31**(2): p. 318-337.
114. Goldin, B. *The Micoenvironment of the Colonic cell and Bacterial Flora*. 2008 17 May 2011]; Available from: http://www.tummywise.com/summitsummary1.html.
115. Villarreal, M.R. *Digestive system diagram en.svg*. 2007 10 May 2011]; Available from: http://commons.wikimedia.org/wiki/File:Digestive_system_diagram_en.svg.
116. Macfarlane, S. and J.F. Dillon, *Microbial biofilms in the human gastrointestinal tract.* Journal of Applied Microbiology, 2007. **102**(5): p. 1187-1196.
117. Cummings, J.H. and G.T. Macfarlane, *Role of intestinal bacteria in nutrient metabolism.* Clinical Nutrition, 1997. **16**(1): p. 3-11.
118. Mattar, A. Mattar, D. Teitelbaum, Drongowski, R. Drongowski, Yongyi, F. Yongyi, Harmon, C. Harmon, Coran, and A. Coran, *Probiotics up-regulate MUC-2 mucin gene expression in a Caco-2 cell-culture model.* Pediatric Surgery International, 2002. **18**(7): p. 586-590.
119. Mayo, B., D.v. Sinderen, and M. Ventura, *Genome analysis of food grade lactic acid bacteria: From the basics to applications.* Current Genomics, 2008. **9**(3): p. 169-183.
120. Holzapfel, W.H., P. Haberer, R. Geisen, J. Bjorkroth, and U. Schillinger, *Taxonomy and important features of probiotic microorganisms in food and nutrition.* American Journal of Clinical Nutrition, 2001. **73**(2): p. 365S-373.
121. Ouwehand, A.C., E.M. Tuomola, S. Tölkkö, and S. Salminen, *Assessment of adhesion properties of novel probiotic strains to human intestinal mucus.* International Journal of Food Microbiology, 2001. **64**(1-2): p. 119-126.
122. Sherman, P.M., J.C. Ossa, and K. Johnson-Henry, *Unraveling Mechanisms of Action of Probiotics.* Nutrition in Clinical Practice, 2009. **24**(1): p. 10-14.
123. Gibson, G.R. and M.B. Roberfroid, *Dietary Modulation of the Human Colonic Microbiota: Introducing the Concept of Prebiotics.* Journal of Nutrition, 1995. **125**(6): p. 1401-1412.
124. Johnson-Henry, K.C., K.E. Hagen, M. Gordonpour, T.A. Tompkins, and P.M. Sherman, *Surface-layer protein extracts from Lactobacillus helveticus inhibit enterohaemorrhagic Escherichia coli O157:H7 adhesion to epithelial cells.* Cellular Microbiology, 2007. **9**(2): p. 356-367.
125. Bernet, M.F., D. Brassart, J.R. Neeser, and A.L. Servin, *Lactobacillus acidophilus LA 1 binds to cultured human intestinal cell lines and inhibits cell attachment and cell*

invasion by enterovirulent bacteria. Gut, 1994. **35**(4): p. 483-489.
126. Bezkorovainy, A., *Probiotics: determinants of survival and growth in the gut.* American Journal of Clinical Nutrition, 2001. **73**(2): p. 399S-405.
127. Mack, D.R., S. Michail, S. Wei, L. McDougall, and M.A. Hollingsworth, *Probiotics inhibit enteropathogenic E. coli adherence in vitro by inducing intestinal mucin gene expression.* American Journal of Physiology: Gastrointestinal and Liver Physiology, 1999. **276**(4): p. G941-950.
128. Gusils, C., O. Oppezzo, R. Pizarro, and S. Gonzalez, *Adhesion of probiotic lactobacilli to chick intestinal mucus.* Canadian Journal of Microbiology, 2003. **49**(7): p. 472-478.
129. Kirjavainen, P.V., A.C. Ouwehand, E. Isolauri, and S.J. Salminen, *The ability of probiotic bacteria to bind to human intestinal mucus.* FEMS Microbiology Letters, 1998. **167**(2): p. 185-189.
130. Cohen, P.S., D.C. Laux, and J.D.a.I.O. Ron, *Bacterial adhesion to and penetration of intestinal mucus in vitro*, in *Methods in Enzymology*1995, Academic Press. p. 309-314.
131. Macfarlane, S., E.J. Woodmansey, and G.T. Macfarlane, *Colonization of Mucin by Human Intestinal Bacteria and Establishment of Biofilm Communities in a Two-Stage Continuous Culture System.* Applied and Environmental Microbiology, 2005. **71**(11): p. 7483-7492.
132. Ruas-Madiedo, P., M. Gueimonde, M. Fernandez-Garcia, C.G. de los Reyes-Gavilan, and A. Margolles, *Mucin Degradation by Bifidobacterium Strains Isolated from the Human Intestinal Microbiota.* Applied and Environmental Microbiology, 2008. **74**(6): p. 1936-1940.
133. Willis, C.L., J.H. Cummings, G. Neale, and G.R. Gibson, *In Vitro Effects of Mucin Fermentation on the Growth of Human Colonic Sulphate-Reducing Bacteria: ECOLOGY.* Anaerobe, 1996. **2**(2): p. 117-122.
134. Costerton, J.W., Z. Lewandowski, D.E. Caldwell, D.R. Korber, and H.M. Lappinscott, *Microbial Biofilms.* Annual Review of Microbiology, 1995. **49**: p. 711-745.
135. Hall-Stoodley, L., J.W. Costerton, and P. Stoodley, *Bacterial biofilms: from the Natural environment to infectious diseases.* Nature Reviews: Microbiology, 2004. **2**(2): p. 95-108.
136. Macfarlane, S. and G.T. Macfarlane, *Composition and Metabolic Activities of Bacterial Biofilms Colonizing Food Residues in the Human Gut.* Applied and Environmental Microbiology, 2006. **72**(9): p. 6204-6211.
137. Lebeer, S., T.L.A. Verhoeven, M. Perea Velez, J. Vanderleyden, and S.C.J. De Keersmaecker, *Impact of Environmental and Genetic Factors on Biofilm Formation by the Probiotic Strain Lactobacillus rhamnosus GG.* Applied and Environmental Microbiology, 2007. **73**(21): p. 6768-6775.
138. Alander, M., R. Satokari, R. Korpela, M. Saxelin, T. Vilpponen-Salmela, T. Mattila-Sandholm, and A. von Wright, *Persistence of Colonization of Human Colonic Mucosa by a Probiotic Strain, Lactobacillus rhamnosus GG, after Oral Consumption.* Applied and Environmental Microbiology, 1999. **65**(1): p. 351-354.
139. Linden, S.K., P. Sutton, N.G. Karlsson, V. Korolik, and M.A. McGuckin, *Mucins in the mucosal barrier to infection.* Mucosal Immunology, 2008. **1**(3): p. 183-197.
140. McCool, D.J., J.F. Forstner, and G.G. Forstner, *Regulated and unregulated pathways for MUC2 mucin secretion in human colonic LS180 adenocarcinoma cells are distinct* Biochemical Journal, 1995. **312**: p. 125-133.
141. Stanley, C.M. and T.E. Phillips, *Selective secretion and replenishment of discrete mucin glycoforms from intestinal goblet cells.* American Journal of Physiology: Gastrointestinal and Liver Physiology, 1999. **277**(1): p. G191-200.
142. Hong, D.H., G. Petrovics, W.B. Anderson, J. Forstner, and G. Forstner, *Induction of mucin gene expression in human colonic cell lines by PMA is dependent on PKC-epsilon.* American Journal of Physiology: Gastrointestinal and Liver Physiology, 1999. **277**(5): p.

G1041-1047.
143. Theodoropoulos, G. and K.L. Carraway, *Molecular signaling in the regulation of mucins.* Journal of Cellular Biochemistry, 2007. **102**(5): p. 1103-1116.
144. Roumagnac, I. and C. Laboisse, *A mucus-secreting human colonic epithelial cell line responsive to cholinergic stimulation* Biology of the Cell, 1987. **61**(1-2): p. 65-68.
145. Vieira, M.A.M., T.A.T. Gomes, A.J.P. Ferreira, T. Knobl, A.L. Servin, and V. Lievin-Le Moal, *Two Atypical Enteropathogenic Escherichia coli Strains Induce the Production of Secreted and Membrane-Bound Mucins To Benefit Their Own Growth at the Apical Surface of Human Mucin-Secreting Intestinal HT29-MTX Cells.* Infection and Immunity, 2010. **78**(3): p. 927-938.
146. McNamara, N. and C. Basbaum, *Signaling networks controlling mucin production in response to Gram-positive and Gram-negative bacteria.* Glycoconjugate Journal, 2001. **18**(9): p. 715-722.
147. Dharmani, P., J. Strauss, C. Ambrose, E. Allen-Vercoe, and K. Chadee, *Fusobacterium nucleatum Infection of Colonic Cells Stimulates MUC2 Mucin and Tumor Necrosis Factor Alpha.* Infection and Immunity, 2011. **79**(7): p. 2597-2607.
148. Khailova, L., K. Dvorak, K.M. Arganbright, M.D. Halpern, T. Kinouchi, M. Yajima, and B. Dvorak, *Bifidobacterium bifidum improves intestinal integrity in a rat model of necrotizing enterocolitis.* American Journal of Physiology: Gastrointestinal and Liver Physiology, 2009. **297**(5): p. G940-949.
149. Coconnier, M.-H., E. Dlissi, M. Robard, C.L. Laboisse, J.-L. Gaillard, and A.L. Servin, *Listeria monocytogenes Stimulates Mucus Exocytosis in Cultured Human Polarized Mucosecreting Intestinal Cells through Action of Listeriolysin O.* Infection and Immunity, 1998. **66**(8): p. 3673-3681.
150. Liévin-Le Moal, V., G. Huet, J.-P. Aubert, J. Bara, M.-E. Forgue-Lafitte, A.L. Servin, and M.-H. Coconnier, *Activation of mucin exocytosis and upregulation of MUC genes in polarized human intestinal mucin-secreting cells by the thiol-activated exotoxin listeriolysin O.* Cellular Microbiology, 2002. **4**(8): p. 515-529.
151. Liévin-Le Moal, V., A.L. Servin, and M.H. Coconnier-Polter, *The increase in mucin exocytosis and the upregulation of MUC genes encoding for membrane-bound mucins induced by the thiol-activated exotoxin listeriolysin O is a host cell defence response that inhibits the cell-entry of Listeria monocytogenes.* Cellular Microbiology, 2005. **7**(7): p. 1035-1048.
152. Nelson, D.L. and M.M. Cox, *Lehninger- Principles of Biochemistry*. 4 ed. 2005, New York: W H Freeman and company.
153. Moncada, D.M., S.J. Kammanadiminti, and K. Chadee, *Mucin and Toll-like receptors in host defense against intestinal parasites.* Trends in Parasitology, 2003. **19**(7): p. 305-311.
154. Boehm, G., M. Lidestri, P. Casetta, J. Jelinek, F. Negretti, B. Stahl, and A. Marini, *Supplementation of a bovine milk formula with an oligosaccharide mixture increases counts of faecal bifidobacteria in preterm infants.* Archives of Disease in Childhood. Fetal and Neonatal Edition, 2002. **86**(3): p. F178-81.
155. Fanaro, S., G. Boehm, J. Garssen, J. Knol, F. Mosca, B. Stahl, and V. Vigi, *Galacto-oligosaccharides and long-chain fructo-oligosaccharides as prebiotics in infant formulas: a review.* Acta Paediatrica. Supplement, 2005. **94**(449): p. 22-6.
156. Van der Meulen, R., L. Makras, K. Verbrugghe, T. Adriany, and L. De Vuyst, *In Vitro Kinetic Analysis of Oligofructose Consumption by Bacteroides and Bifidobacterium spp. Indicates Different Degradation Mechanisms.* Applied and Environmental Microbiology, 2006. **72**(2): p. 1006-1012.
157. Kelly, G., *Inulin-Type Prebiotics - A Review: Part 1.* Alternative Medicine Review, 2008. **13**(4): p. 315-329.
158. Bosscher, D., J.V. Loo, and A. Franck, *Inulin and oligofructose as prebiotics in the*

prevention of intestinal infections and diseases. Nutrition Research Reviews, 2006. **19**(02): p. 216-226.
159. Kleessen, B., L. Hartmann, and M. Blaut, *Fructans in the diet cause alterations of intestinal mucosal architecture, released mucins and mucosa-associated bifidobacteria in gnotobiotic rats.* British Journal of Nutrition, 2003. **89**(05): p. 597-606.
160. Van den Abbeele, P., P. Gérard, S. Rabot, A. Bruneau, S. El Aidy, M. Derrien, M. Kleerebezem, E.G. Zoetendal, H. Smidt, W. Verstraete, T. Van de Wiele, and S. Possemiers, *Arabinoxylans and inulin differentially modulate the mucosal and luminal gut microbiota and mucin-degradation in humanized rats.* Environmental Microbiology, 2011. **13**(10): p. 2667-2680.
161. Olano, A. and N. Corzo, *Lactulose as a food ingredient.* Journal of the Science of Food and Agriculture, 2009. **89**(12): p. 1987-1990.
162. Macfarlane, G.T., H. Steed, and S. Macfarlane, *Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics.* Journal of Applied Microbiology, 2008. **104**(2): p. 305-344.
163. Angus, F., S. Smart, and C. Shortt, *Prebiotic Ingredients with Emphasis on Galacto-Oligosaccharides and Fructo-Oligosaccharides*. Probiotic Dairy Products. 2007: Blackwell Science Ltd. 120-137.
164. Aider, M. and D. de Halleux, *Isomerization of lactose and lactulose production: review.* Trends in Food Science & Technology, 2007. **18**(7): p. 356-364.
165. Paseephol, T., D.M. Small, and F. Sherkat, *Lactulose production from milk concentration permeate using calcium carbonate-based catalysts.* Food Chemistry, 2008. **111**(2): p. 283-290.
166. Collins, M.D. and G.R. Gibson, *Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut.* American Journal of Clinical Nutrition, 1999. **69**(5): p. 1052S-1057.
167. Raynal-Ljutovac, K., G. Lagriffoul, P. Paccard, I. Guillet, and Y. Chilliard, *Composition of goat and sheep milk products: An update.* Small Ruminant Research, 2008. **79**(1): p. 57-72.
168. Martinez-Ferez, A., S. Rudloff, A. Guadix, C.A. Henkel, G. Pohlentz, J.J. Boza, E.M. Guadix, and C. Kunz, *Goats' milk as a natural source of lactose-derived oligosaccharides: Isolation by membrane technology.* International Dairy Journal, 2006. **16**(2): p. 173-181.
169. Boehm, G. and B. Stahl, *Oligosaccharides from Milk.* Journal of Nutrition, 2007. **137**(3): p. 847S-849.
170. Kunz, C. and S. Rudloff, *Health promoting aspects of milk oligosaccharides.* International Dairy Journal, 2006. **16**(11): p. 1341-1346.
171. Mills, S., R.P. Ross, C. Hill, G.F. Fitzgerald, and C. Stanton, *Milk intelligence: Mining milk for bioactive substances associated with human health.* International Dairy Journal. **21**(6): p. 377-401.
172. Nakamura, T. and T. Urashima, *The milk oligosaccharides of domestic farm animals.* Trends in Glycoscience and Glycotechnology, 2004. **16**(88): p. 135-142.
173. Asakuma, S., T. Urashima, M. Akahori, H. Obayashi, T. Nakamura, K. Kimura, Y. Watanabe, I. Arai, and Y. Sanai, *Variation of major neutral oligosaccharides levels in human colostrum.* European Journal of Clinical Nutrition, 2008. **62**(4): p. 488-494.
174. Newburg, D.S., *Do the Binding Properties of Oligosaccharides in Milk Protect Human Infants from Gastrointestinal Bacteria?* The Journal of Nutrition, 1997. **127**(5): p. 980S-984S.
175. Macfarlane, G.T. and S. Macfarlane, *Bacteria, Colonic Fermentation, and Gastrointestinal Health.* Journal of AOAC International, 2012. **95**(1): p. 50-60.
176. Topping, D.L. and P.M. Clifton, *Short-Chain Fatty Acids and Human Colonic Function: Roles of Resistant Starch and Nonstarch Polysaccharides.* Physiological Reviews, 2001.

81(3): p. 1031-1064.
177. Gaudier, E., M. Rival, M.P. Buisine, I. Robineau, and C. Hoebler, *Butyrate Enemas Upregulate Muc Genes Expression but Decrease Adherent Mucus Thickness in Mice Colon.* Physiological Research, 2009. **58**(1): p. 111-119.
178. Blottiere, H.M., B. Buecher, J.P. Galmiche, and C. Cherbut, *Molecular analysis of the effect of short-chain fatty acids on intestinal cell proliferation.* Proceedings of the Nutrition Society, 2003. **62**(1): p. 101-106.
179. Englyst, H.N. and G.T. Macfarlane, *Breakdown of resistant and readily digestible starch by human gut bacteria.* Journal of the Science of Food and Agriculture, 1986. **37**(7): p. 699-706.
180. Hedemann, M.S., P.K. Theil, and K.E. Bach Knudsen, *The thickness of the intestinal mucous layer in the colon of rats fed various sources of non-digestible carbohydrates is positively correlated with the pool of SCFA but negatively correlated with the proportion of butyric acid in digesta.* British Journal of Nutrition, 2009. **102**(01): p. 117-125.
181. Fogh, J. and G. Trempe, *New human tumor cell lines*. 1 ed. Human tumor cells *in vitro*, ed. J. Fogh. 1975, New York: Plenum Press.
182. Lesuffleur, T., A. Barbat, E. Dussaulx, and A. Zweibaum, *Growth Adaptation to Methotrexate of HT-29 Human Colon Carcinoma Cells Is Associated with Their Ability to Differentiate into Columnar Absorptive and Mucus-secreting Cells.* Cancer Research, 1990. **50**(19): p. 6334-6343.
183. Huet, C., C. Sahuquillo-Merino, E. Coudrier, and D. Louvard, *Absorptive and mucus-secreting subclones isolated from a multipotent intestinal cell line (HT-29) provide new models for cell polarity and terminal differentiation.* Journal of Cell Biology, 1987. **105**(1): p. 345-357.
184. Pinto, M., M.D. Appay, P. Simonassmann, G. Chevalier, N. Dracopoli, J. Fogh, and A. Zweibaum, *Enterocytic differentiation of cultured human-colon cancer-cells by replacement of glucose by galactose in the medium* Biology of the Cell, 1982. **44**(2): p. 193-196.
185. Zweibaum, A., M. Pinto, G. Chevalier, E. Dussaulx, N. Triadou, B. Lacroix, K. Haffen, J.L. Brun, and M. Rousset, *Enterocytic differentiation of a subpopulation of the human colon tumor cell line HT-29 selected for growth in sugar-free medium and its inhibition by glucose.* Journal of Cellular Physiology, 1985. **122**(1): p. 21-29.
186. Augeron, C. and C.L. Laboisse, *Emergence of Permanently Differentiated Cell Clones in a Human Colonic Cancer Cell Line in Culture after Treatment with Sodium Butyrate.* Cancer Research, 1984. **44**(9): p. 3961-3969.
187. Lesuffleur, T., A. Kornowski, C. Augeron, E. Dussaulx, A. Barbat, C. Laboisse, and A. Zweibaum, *Increased growth adaptability to 5-fluorouracil and methotrxate of HT-29 subpopulations selected for their commitment to differentiation* International Journal of Cancer, 1991. **49**(5): p. 731-737.
188. Lesuffleur, T., A. Barbat, C. Luccioni, J. Beaumatin, M. Clair, A. Kornowski, E. Dussaulx, B. Dutrillaux, and A. Zweibaum, *Dihydrofolate reductase gene amplification-associated shift of differentiation in methotrexate-adapted HT-29 cells.* Journal of Cell Biology, 1991. **115**(5): p. 1409-1418.
189. Lesuffleur, T., N. Porchet, J.P. Aubert, D. Swallow, J.R. Gum, Y.S. Kim, F.X. Real, and A. Zweibaum, *Differential expression of the human mucin genes MUC1 to MUC5 in relation to growth and differentiation of different mucus-secreting HT-29 cell subpopulations.* Journal of Cell Science, 1993. **106**(3): p. 771-783.
190. Pigny, P., I. Van Seuningen, J.L. Desseyn, S. Nollet, N. Porchet, A. Laine, and J.P. Aubert, *Identification of a 42-kDa Nuclear Factor (NF1-MUC5B) from HT-29 MTX Cells That Binds to the 3' Region of Human Mucin GeneMUC5B.* Biochemical and Biophysical Research Communications, 1996. **220**(1): p. 186-191.

191. Velcich, A., L. Palumbo, A. Jarry, C. Laboisse, J. Racevskis, and L. Augenlicht, *Patterns of expression of lineage-specific markers during the in vitro-induced differentiation of HT29 colon carcinoma cells.* Cell Growth and Differentiation, 1995. **6**(6): p. 749-757.
192. Granato, D., G.E. Bergonzelli, R.D. Pridmore, L. Marvin, M. Rouvet, and I.E. Corthesy-Theulaz, *Cell Surface-Associated Elongation Factor Tu Mediates the Attachment of Lactobacillus johnsonii NCC533 (La1) to Human Intestinal Cells and Mucins.* Infection and Immunity, 2004. **72**(4): p. 2160-2169.
193. Bernet, M.F., D. Brassart, J.R. Neeser, and A.L. Servin, *Adhesion of human bifidobacterial strains to cultured human intestinal epithelial cells and inhibition of enteropathogen-cell interactions.* Applied and Environmental Microbiology, 1993. **59**(12): p. 4121-4128.
194. Zoghbi, S., A. Trompette, J. Claustre, M.E. Homsi, J. Garzon, G. Jourdan, J.-Y. Scoazec, and P. Plaisancie, *beta-Casomorphin-7 regulates the secretion and expression of gastrointestinal mucins through a {micro}-opioid pathway.* American Journal of Physiology: Gastrointestinal and Liver Physiology, 2006. **290**(6): p. G1105-1113.
195. Martinez-Augustin, O., V. Puerta, A. Marti´nez-Fe´rez, L. Baro´, E. Lo´pez-Huertas, and M. Suarez, *Goat's milk oligosaccharides modulate mucin and trefoil factors production in the mucus producing intestinal cell line HT29/MTX.* Clinical Nutrition, 2003. **22**(Supplement 1): p. S42-S42.
196. Calatayud, M., M. Vázquez, V. Devesa, and D. Vélez, *In Vitro Study of Intestinal Transport of Inorganic and Methylated Arsenic Species by Caco-2/HT29-MTX Cocultures.* Chemical Research in Toxicology, 2012. **25**(12): p. 2654-2662.
197. Chen, X.-M., I. Elisia, and D.D. Kitts, *Defining conditions for the co-culture of Caco-2 and HT29-MTX cells using Taguchi design.* Journal of Pharmacological and Toxicological Methods, 2010. **61**(3): p. 334-342.
198. Laparra, J.M., R.P. Glahn, and D.D. Miller, *Different responses of Fe transporters in Caco-2/HT29-MTX cocultures than in independent Caco-2 cell cultures.* Cell Biology International, 2009. **33**(9): p. 971-977.
199. Walter, E., S. Janich, B.J. Roessler, J.M. Hilfinger, and G.L. Amidon, *HT29-MTX/Caco-2 cocultures as an in vitro model for the intestinal epithelium: In vitro-in vivo correlation with permeability data from rats and humans.* Journal of Pharmaceutical Sciences, 1996. **85**(10): p. 1070-1076.
200. Mar Contreras, M., A. Sancho, I. Recio, and C. Mills, *Absorption of Casein Antihypertensive Peptides through an In Vitro Model of Intestinal Epithelium.* Food Digestion, 2012. **3**(1-3): p. 16-24.
201. Hidalgo, I.J., T.J. Raub, and R.T. Borchardt, *Characterization of the human -colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability.* Gastroenterology, 1989. **96**(3): p. 736-749.
202. Ulluwishewa, D., R.C. Anderson, W.C. McNabb, P.J. Moughan, J.M. Wells, and N.C. Roy, *Regulation of Tight Junction Permeability by Intestinal Bacteria and Dietary Components.* The Journal of Nutrition, 2011. **141**(5): p. 769-776.
203. Anderson, R.C., A.L. Cookson, W.C. McNabb, W.J. Kelly, and N.C. Roy, *Lactobacillus plantarum DSM 2648 is a potential probiotic that enhances intestinal barrier function.* FEMS Microbiology Letters, 2010. **309**(2): p. 184-192.
204. McCormick, B.A., *The use of transepithelial models to examine host-pathogen interactions.* Current Opinion in Microbiology, 2003. **6**(1): p. 77-81.
205. Wils, P., A. Warnery, V. Phung-Ba, and D. Scherman, *Differentiated intestinal epithelial cell lines as in vitro models for predicting the intestinal absorption of drugs.* Cell Biology and Toxicology, 1994. **10**(5): p. 393-397.
206. Walter, E. and T. Kissel, *Heterogeneity in the human intestinal cell line Caco-2 leads to differences in transepithelial transport.* European Journal of Pharmaceutical Sciences, 1995. **3**(4): p. 215-230.

207. Mahler, G.J., M.L. Shuler, and R.P. Glahn, *Characterization of Caco-2 and HT29-MTX cocultures in an in vitro digestion/cell culture model used to predict iron bioavailability.* The Journal of Nutritional Biochemistry, 2009. **20**(7): p. 494-502.
208. Laparra, J.M. and Y. Sanz, *Comparison of in vitro models to study bacterial adhesion to the intestinal epithelium.* Letters in Applied Microbiology, 2009. **49**(6): p. 695-701.
209. Fontaine, N., J.C. Meslin, S. Lory, and C. Andrieux, *Intestinal mucin distribution in the germ-free rat and in the heteroxenic rat harbouring a human bacterial flora: effect of inulin in the diet.* British Journal of Nutrition, 1996. **75**(06): p. 881-892.
210. Comelli, E.M., R. Simmering, M. Faure, D. Donnicola, R. Mansourian, F. Rochat, I. Corthesy-Theulaz, and C. Cherbut, *Multifaceted transcriptional regulation of the murine intestinal mucus layer by endogenous microbiota.* Genomics, 2008. **91**(1): p. 70-77.
211. Kandori, H., K. Hirayama, M. Takeda, and K. Doi, *Histochemical, lectin-histochemical and morphometrical characteristics of intestinal goblet cells of germfree and conventional mice.* Experimental Animals, 1996. **45**(2): p. 155-160.
212. Caballero-Franco, C., K. Keller, C. De Simone, and K. Chadee, *The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells.* American Journal of Physiology: Gastrointestinal and Liver Physiology, 2007. **292**(1): p. G315-322.
213. Petersson, J., O. Schreiber, G.C. Hansson, S.J. Gendler, A. Velcich, J.O. Lundberg, S. Roos, L. Holm, and M. Phillipson, *Importance and regulation of the colonic mucus barrier in a mouse model of colitis.* American Journal of Physiology: Gastrointestinal and Liver Physiology, 2011. **300**(2): p. G327-G333.
214. Forder, R.E.A., G.S. Howarth, D.R. Tivey, and R.J. Hughes, *Bacterial Modulation of Small Intestinal Goblet Cells and Mucin Composition During Early Posthatch Development of Poultry.* Poultry Science, 2007. **86**(11): p. 2396-2403.
215. Rastall, R.A., *Functional Oligosaccharides: Application and Manufacture.* Annual Review of Food Science and Technology - (new in 2010), 2010. **1**(1): p. 305-339.
216. Musatti, A., V. Devesa, M. Calatayud, D. Vélez, M. Manzoni, and M. Rollini, *Glutathione-enriched baker's yeast: production, bioaccessibility and intestinal transport assays.* Journal of Applied Microbiology, 2014. **116**(2): p. 304-313.
217. Behrens, I., P. Stenberg, P. Artursson, and T. Kissel, *Transport of Lipophilic Drug Molecules in a New Mucus-Secreting Cell Culture Model Based on HT29-MTX Cells.* Pharmaceutical Research, 2001. **18**(8): p. 1138-1145.
218. Coconnier, M.H., T.R. Klaenhammer, S. Kerneis, M.F. Bernet, and A.L. Servin, *Protein-mediated adhesion of Lactobacillus acidophilus BG2FO4 on human enterocyte and mucus-secreting cell lines in culture.* Applied and Environmental Microbiology, 1992. **58**(6): p. 2034-2039.
219. Alemka, A., M. Clyne, F. Shanahan, T. Tompkins, N. Corcionivoschi, and B. Bourke, *Probiotic Colonization of the Adherent Mucus Layer of HT29MTXE12 Cells Attenuates Campylobacter jejuni Virulence Properties.* Infection and Immunity, 2010. **78**(6): p. 2812-2822.
220. Balimane, P.V. and S. Chong, *Cell culture-based models for intestinal permeability: A critique.* Drug Discovery Today, 2005. **10**(5): p. 335-343.
221. Van Klinken, B.J.W., E. Oussoren, J.J. Weenink, G.J. Strous, H.A. Buller, J. Dekker, and A.W.C. Einerhand, *The human intestinal cell lines Caco-2 and LS174T as models to study cell-type specific mucin expression.* Glycoconjugate Journal, 1996. **13**(5): p. 757-768.
222. Loo, Y., C.L. Grigsby, Y.J. Yamanaka, M.K. Chellappan, X. Jiang, H.-Q. Mao, and K.W. Leong, *Comparative study of nanoparticle-mediated transfection in different GI epithelium co-culture models.* Journal of Controlled Release, 2012(0).
223. Lai, X., B.L. Blazer-Yost, J.W. Clack, S.L. Fears, S. Mitra, S.A. Ntim, H.N. Ringham, and

F.A. Witzmann, *'Protein expression profiles of intestinal epithelial co-cultures: effect of functionalised carbon nanotube exposure'.* International Journal of Biomedical Nanoscience and Nanotechnology, 2013. **3**(1/2): p. 127-162.
224. Konsoula, R. and F.A. Barile, *Correlation of in vitro cytotoxicity with paracellular permeability in Caco-2 cells.* Toxicology In Vitro, 2005. **19**(5): p. 675-684.
225. Bu, X.-D., N. Li, X.-Q. Tian, and P.-L. Huang, *Caco-2 and LS174T cell lines provide different models for studying mucin expression in colon cancer.* Tissue and Cell, 2011. **43**(3): p. 201-206.
226. Singh, R., A.A. Fouladi-Nashta, D. Li, N. Halliday, D.A. Barrett, and K.D. Sinclair, *Methotrexate induced differentiation in colon cancer cells is primarily due to purine deprivation.* Journal of Cellular Biochemistry, 2006. **99**(1): p. 146-155.
227. Wan, L.-Y.M., K.J. Allen, P.C. Turner, and H. El-Nezami, *Modulation of Mucin mRNA (MUC5AC and MUC5B) Expression and Protein Production and Secretion in Caco-2/HT29-MTX Co-cultures Following Exposure to Individual and Combined Fusarium Mycotoxins.* Toxicological Sciences, 2014. **139**(1): p. 83-98.
228. Briske-Anderson, M.J., J.W. Finley, and S.M. Newman, *The Influence of Culture Time and Passage Number on the Morphological and Physiological Development of Caco-2 Cells.* Experimental Biology and Medicine, 1997. **214**(3): p. 248-257.
229. Freshney, R.I., *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications*. 6th ed. 2010, Hoboken, New Jersey: John Wiley & Sons Inc.
230. Masters, J.R. and G.N. Stacey, *Changing medium and passaging cell lines.* Nature Protocols, 2007. **2**(9): p. 2276-2284.
231. Olejnik, A., M. Lewandowska, W. Grajek, and K. Czaczyk, *New rapid method of Caco-2 cell differentiation.* Polish Journal of Food and Nutrition Sciences, 2003. **12**(SI 1): p. 60-64.
232. Simione, F.P., Jr., *Key issues relating to the genetic stability and preservation of cells and cell banks.* Journal of parenteral science and technology : a publication of the Parenteral Drug Association, 1992. **46**(6): p. 226-32.
233. Martínez-Maqueda, D., B. Miralles, S. De Pascual-Teresa, I. Reverón, R. Muñoz, and I. Recio, *Food-Derived Peptides Stimulate Mucin Secretion and Gene Expression in Intestinal Cells.* Journal of Agricultural and Food Chemistry, 2012. **60**(35): p. 8600-8605.
234. Schroeder, A., O. Mueller, S. Stocker, R. Salowsky, M. Leiber, M. Gassmann, S. Lightfoot, W. Menzel, M. Granzow, and T. Ragg, *The RIN: an RNA integrity number for assigning integrity values to RNA measurements.* BMC Molecular Biology, 2006. **7**: p. 3.
235. Corfield, A.P., *Glycoprotein. Methods and Protocols: The Mucins*. Methods in Molecular Biology, ed. J.M. Walker. Vol. 125. 2000, Bristol: Humana Press.
236. Laucirica, R., J.S. Bentz, W.E. Khalbuss, A.C. Clayton, R.J. Souers, and A.T. Moriarty, *Performance Characteristics of Mucinous (Colloid) Carcinoma of the Breast in Fine-Needle Aspirates: Observations From the College of American Pathologists Interlaboratory Comparison Program in Nongynecologic Cytopathology.* Archives of Pathology and Laboratory Medicine, 2011. **135**(12): p. 1533-1538.
237. Wikman, A., J. Karlsson, I. Carlstedt, and P. Artursson, *A Drug Absorption Model Based on the Mucus Layer Producing Human Intestinal Goblet Cell Line HT29-H.* Pharmaceutical Research, 1993. **10**(6): p. 843-852.
238. Nollevaux, G., C. Deville, B. El Moualij, W. Zorzi, P. Deloyer, Y.-J. Schneider, O. Peulen, and G. Dandrifosse, *Development of a serum-free co-culture of human intestinal epithelium cell-lines (Caco-2/HT29-5M21).* BMC Cell Biology, 2006. **7**(1): p. 20.
239. *The Global Resource Centre*. [cited 2014 17/06/]; Available from: www.atcc.org.
240. *General Cell Collection: HT29-MTX*. [cited 2014 17/06/]; Available from: www.phe-culturecollections.org.uk.
241. Perrais, M.l., P. Pigny, M.-C. Copin, J.-P. Aubert, and I. Van Seuningen, *Induction of*

*MUC2 and MUC5AC Mucins by Factors of the Epidermal Growth Factor (EGF) Family Is Mediated by EGF Receptor/Ras/Raf/Extracellular Signal-regulated Kinase Cascade and Sp1**. Journal of Biological Chemistry, 2002. **277**(35): p. 32258-32267.

242. Nollet, S., M.E. Forgue-Lafitte, P. Kirkham, and J. Bara, *Mapping of two new epitopes on the apomucin encoded by MUC5AC gene: Expression in normal GI tract and colon tumors.* International Journal of Cancer, 2002. **99**(3): p. 336-343.
243. Wood, K.M., G.M. Stone, and N.A. Peppas, *The effect of complexation hydrogels on insulin transport in intestinal epithelial cell models.* Acta Biomaterialia, 2010. **6**(1): p. 48-56.
244. Sambuy, Y., *The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics.* Cell Biology and Toxicology, 2005. **21**(1): p. 1-26.
245. Wikman-Larhed, A. and P. Artursson, *Co-cultures of human intestinal goblet (HT29-H) and absorptive (Caco-2) cells for studies of drug and peptide absorption.* European Journal of Pharmaceutical Sciences, 1995. **3**(3): p. 171-183.
246. Soler, A.P., R.D. Miller, K.V. Laughlin, N.Z. Carp, D.M. Klurfeld, and J.M. Mullin, *Increased tight junctional permeability is associated with the development of colon cancer.* Carcinogenesis, 1999. **20**(8): p. 1425-1432.
247. Bjorkman, E., A. Casselbrant, S. Lundberg, and L. Fandriks, *In vitro assessment of epithelial electrical resistance in human esophageal and jejunal mucosae and in Caco-2 cell layers.* Scandinavian Journal of Gastroenterology, 2012. **47**(11): p. 1321-1333.
248. Fleisher, D., *Biological Transport Phenomena in the Gastrointestinal Tract*, in *Transport Processes in Pharmaceutical Systems*1999, Informa Healthcare. p. 147-184.
249. Rubas, W., M.E.M. Cromwell, Z. Shahrokh, J. Villagran, T.N. Nguyen, M. Wellton, T.H. Nguyen, and R.J. Mrsny, *Flux measurements across Caco-2 monolayers may predict transport in human large intestinal tissue.* Journal of Pharmaceutical Sciences, 1996. **85**(2): p. 165-169.
250. Fernandez Miyakawa, M.E., V. Pistone Creydt, F.A. Uzal, B.A. McClane, and C. Ibarra, *Clostridium perfringens Enterotoxin Damages the Human Intestine In Vitro.* Infection and Immunity, 2005. **73**(12): p. 8407-8410.
251. Yu, J., S. Peng, D. Luo, and J.C. March, *In vitro 3D human small intestinal villous model for drug permeability determination.* Biotechnology and Bioengineering, 2012. **109**(9): p. 2173-2178.
252. de Halleux, V. and J. Rigo, *Variability in human milk composition: benefit of individualized fortification in very-low-birth-weight infants.* American Journal of Clinical Nutrition, 2013. **98**(2): p. 529S-535S.
253. Coppa, G.V., O. Gabrielli, P. Pierani, C. Catassi, A. Carlucci, and P.L. Giorgi, *Changes in Carbohydrate Composition in Human Milk Over 4 Months of Lactation.* Pediatrics, 1993. **91**(3): p. 637-641.
254. Kunz, C., S. Rudloff, W. Baier, N. Klein, and S. Strobel, *Oligosaccharides in Human Milk: Structural, Functional, and Metabolic Aspects.* Annual Review of Nutrition, 2000. **20**(1): p. 699-722.
255. Kunz, C. and S. Rudloff, *Biological functions of oligosaccharides in human milk.* Acta Pædiatrica, 1993. **82**(12): p. 903-912.
256. Meyrand, M., D.C. Dallas, H. Caillat, F. Bouvier, P. Martin, and D. Barile, *Comparison of milk oligosaccharides between goats with and without the genetic ability to synthesize α_{s1}-casein.* Small Ruminant Research, 2013. **113**(2-3): p. 411-420.
257. Gnoth, M.J., C. Kunz, E. Kinne-Saffran, and S. Rudloff, *Human Milk Oligosaccharides Are Minimally Digested In Vitro.* Journal of Nutrition, 2000. **130**(12): p. 3014-3020.
258. Bode, L., *Recent Advances on Structure, Metabolism, and Function of Human Milk Oligosaccharides.* Journal of Nutrition, 2006. **136**(8): p. 2127-2130.
259. Goehring, K.C., A.D. Kennedy, P.A. Prieto, and R.H. Buck, *Direct Evidence for the*

Presence of Human Milk Oligosaccharides in the Circulation of Breastfed Infants. PLoS ONE, 2014. **9**(7): p. e101692.
260. Gnoth, M.J., S. Rudloff, C. Kunz, and R.K.H. Kinne, *Investigations of the in Vitro Transport of Human Milk Oligosaccharides by a Caco-2 Monolayer Using a Novel High Performance Liquid Chromatography-Mass Spectrometry Technique.* Journal of Biological Chemistry, 2001. **276**(37): p. 34363-34370.
261. Perillo, N.L., M.E. Marcus, and L.G. Baum, *Galectins: versatile modulators of cell adhesion, cell proliferation, and cell death.* Journal of Molecular Medicine, 1998. **76**(6): p. 402-412.
262. Kuntz, S., S. Rudloff, and C. Kunz, *Oligosaccharides from human milk influence growth-related characteristics of intestinally transformed and non-transformed intestinal cells.* British Journal of Nutrition, 2008. **99**(3): p. 462-471.
263. Holscher, H.D., S.R. Davis, and K.A. Tappenden, *Human Milk Oligosaccharides Influence Maturation of Human Intestinal Caco-2Bbe and HT-29 Cell Lines.* Journal of Nutrition, 2014. **144**(5): p. 586-591.
264. Russ, A., M. Barnett, W. McNabb, R. Anderson, G. Reynolds, and N. Roy, *Post-weaning effects of milk and milk components on the intestinal mucosa in inflammation.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2010. **690**(1-2): p. 64-70.
265. Harrison, S.J., K. Fraser, G.A. Lane, S. Villas-Boas, and S. Rasmussen, *A reverse-phase liquid chromatography/mass spectrometry method for the analysis of high-molecular-weight fructooligosaccharides.* Analytical Biochemistry, 2009. **395**(1): p. 113-115.
266. Wang, G., Y. Gong, F.J. Burczynski, and B.B. Hasinoff, *Cell lysis with dimethyl sulphoxide produces stable homogeneous solutions in the dichlorofluorescein oxidative stress assay.* Free Radical Research, 2008. **42**(5): p. 435-441.
267. Tajima, A., T. Iwase, H. Shinji, K. Seki, and Y. Mizunoe, *Inhibition of Endothelial Interleukin-8 Production and Neutrophil Transmigration by Staphylococcus aureus Beta-Hemolysin.* Infection and Immunity, 2009. **77**(1): p. 327-334.
268. BioSciences Research Associates, I. *Assay development*. 2008 [cited 2014 27 May]; Available from: http://www.bsrlabs.com/Assay-Development.html.
269. Albrecht, S., J.A. Lane, K. Marino, K.A. Al Busadah, S.D. Carrington, R.M. Hickey, and P.M. Rudd, *A comparative study of free oligosaccharides in the milk of domestic animals.* British Journal of Nutrition, 2014. **111**(07): p. 1313-1328.
270. Hester, S.N. and S.M. Donovan, *Individual and combined effects of nucleotides and human milk oligosaccharides on proliferation, apoptosis and necrosis in a human fetal intestinal cell line.* Food and Nutrition Sciences, 2012. **3**(11): p. 1567-1576.
271. Kuntz, S., C. Kunz, and S. Rudloff, *Oligosaccharides from human milk induce growth arrest via G2/M by influencing growth-related cell cycle genes in intestinal epithelial cells.* British Journal of Nutrition, 2009. **101**(9): p. 1306-1315.
272. Urashima, T., E. Taufik, K. Fukuda, and S. Asakuma, *Recent Advances in Studies on Milk Oligosaccharides of Cows and Other Domestic Farm Animals.* Bioscience, Biotechnology, and Biochemistry, 2013. **77**(3): p. 455-466.
273. Mehra, R., D. Barile, M. Marotta, C.B. Lebrilla, C. Chu, and J.B. German, *Novel high-molecular weight fucosylated milk oligosaccharides identified in dairy streams.* PLoS ONE, 2014. **9**(5): p. e96040-e96040.
274. Suzuki, T. and H. Hara, *Various Nondigestible Saccharides Open a Paracellular Calcium Transport Pathway with the Induction of Intracellular Calcium Signaling in Human Intestinal Caco-2 Cells.* Journal of Nutrition, 2004. **134**(8): p. 1935-1941.
275. Pino, V., V.P. Ramsauer, P. Salas, C.A.C. Carraway, and K.L. Carraway, *Membrane Mucin Muc4 Induces Density-dependent Changes in ERK Activation in Mammary Epithelial and Tumor Cells: Role in Reversal of Contact Inhibition* Journal of Biological Chemistry, 2006. **281**(39): p. 29411-29420.

276. Homsi, M.E., R. Ducroc, J. Claustre, G. Jourdan, A. Gertler, M. Estienne, A. Bado, J.-Y. Scoazec, and P. Plaisancie, *Leptin modulates the expression of secreted and membrane-associated mucins in colonic epithelial cells by targeting PKC, PI3K, and MAPK pathways.* American Journal of Physiology: Gastrointestinal and Liver Physiology, 2007. **293**(1): p. G365-373.
277. Smirnova, M.G., L. Guo, J.P. Birchall, and J.P. Pearson, *LPS up-regulates mucin and cytokine mRNA expression and stimulates mucin and cytokine secretion in goblet cells.* Cellular Immunology, 2003. **221**(1): p. 42-49.
278. Buisine, M.P., L. Devisme, T.C. Savidge, C. Gespach, B. Gosselin, N. Porchet, and J.P. Aubert, *Mucin gene expression in human embryonic and fetal intestine.* Gut, 1998. **43**(4): p. 519-524.
279. Jonckheere, N. and I. Van Seuningen, *The membrane-bound mucins: From cell signalling to transcriptional regulation and expression in epithelial cancers.* Biochimie, 2010. **92**(1): p. 1-11.
280. Alos, L., B. Lujan, M. Castillo, A. Nadal, M. Carreras, M. Caballero, C. de Bolos, and A. Cardesa, *Expression of Membrane-Bound Mucins (MUC1 and MUC4) and Secreted Mucins (MUC2, MUC5AC, MUC5B, MUC6 and MUC7) in Mucoepidermoid Carcinomas of Salivary Glands.* American Journal of Surgical Pathology, 2005. **29**(6): p. 806-813.
281. Yu, Z.-T., C. Chen, and D.S. Newburg, *Utilization of major fucosylated and sialylated human milk oligosaccharides by isolated human gut microbes.* Glycobiology, 2013. **23**(11): p. 1281-1292.
282. Moro, G., I. Minoli, M. Mosca, S. Fanaro, J. Jelinek, B. Stahl, and G. Boehm, *Dosage-Related Bifidogenic Effects of Galacto- and Fructooligosaccharides in Formula-Fed Term Infants.* Journal of Pediatric Gastroenterology and Nutrition, 2002. **34**(3): p. 291-295.
283. Kneifel, W., *In vitro growth behaviour of probiotic bacteria in culture media with carbohydrates of prebiotic importance.* Microbial Ecology in Health and Disease, 2000. **12**(1): p. 27 - 34.
284. Roberfroid, M., *Prebiotics: The Concept Revisited.* The Journal of Nutrition, 2007. **137**(3): p. 830S-837S.
285. Kunz, C., S. Kuntz, and S. Rudloff, *Intestinal Flora*, in *Breast-Feeding: Early Influences on Later Health*, G. Goldberg, et al., Editors. 2009, Springer Science and Business Media.
286. Ward, R.E., M. Niñonuevo, D.A. Mills, C.B. Lebrilla, and J.B. German, *In vitro fermentability of human milk oligosaccharides by several strains of bifidobacteria.* Molecular Nutrition & Food Research, 2007. **51**(11): p. 1398-1405.
287. Marcobal, A., M. Barboza, J.W. Froehlich, D.E. Block, J.B. German, C.B. Lebrilla, and D.A. Mills, *Consumption of Human Milk Oligosaccharides by Gut-Related Microbes.* Journal of Agricultural and Food Chemistry, 2010. **58**(9): p. 5334-5340.
288. Sela, D.A., J. Chapman, A. Adeuya, J.H. Kim, F. Chen, T.R. Whitehead, A. Lapidus, D.S. Rokhsar, C.B. Lebrilla, J.B. German, N.P. Price, P.M. Richardson, and D.A. Mills, *The genome sequence of Bifidobacterium longum subsp. infantis reveals adaptations for milk utilization within the infant microbiome.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(48): p. 18964-9.
289. Ward, R.E., M. Ninonuevo, D.A. Mills, C.B. Lebrilla, and J.B. German, *In Vitro Fermentation of Breast Milk Oligosaccharides by Bifidobacterium infantis and Lactobacillus gasseri.* Applied and Environmental Microbiology, 2006. **72**(6): p. 4497-4499.
290. Gopal, P.K., P.A. Sullivan, and J.B. Smart, *Utilisation of galacto-oligosaccharides as selective substrates for growth by lactic acid bacteria including Bifidobacterium lactis DR10 and Lactobacillus rhamnosus DR20.* International Dairy Journal, 2001. **11**(1-2): p. 19-25.
291. Ganzle, M.G. and R. Follador, *Metabolism of oligosaccharides and starch in lactobacilli:*

a review. Frontiers in Microbiology, 2012. **3**: p. 340-340.
292. Kadlec, R. and M. Jakubec, *The effect of prebiotics on adherence of probiotics.* Journal of Dairy Science, 2014. **97**(4): p. 1983-1990.
293. Parvez, S., K.A. Malik, S.A. Kang, and H.Y. Kim, *Probiotics and their fermented food products are beneficial for health.* Journal of Applied Microbiology, 2006. **100**(6): p. 1171-1185.
294. Koh, J.H., S.H. Choi, S.W. Park, N.-J. Choi, Y. Kim, and S.H. Kim, *Synbiotic impact of tagatose on viability of Lactobacillus rhamnosus strain GG mediated by the phosphotransferase system (PTS).* Food Microbiology, 2013. **36**(1): p. 7-13.
295. Kavanaugh, D.W., J. O'Callaghan, L.F. Butto, H. Slattery, J. Lane, M. Clyne, M. Kane, L. Joshi, and R.M. Hickey, *Exposure of Bifidobacterium longum subsp. infantis to Milk Oligosaccharides Increases Adhesion to Epithelial Cells and Induces a Substantial Transcriptional Response.* PLoS ONE, 2013. **8**(6): p. 1-13.
296. Crittenden, R., A. Laitila, P. Forssell, J. Mättö, M. Saarela, T. Mattila-Sandholm, and P. Myllärinen, *Adhesion of Bifidobacteria to Granular Starch and Its Implications in Probiotic Technologies.* Applied and Environmental Microbiology, 2001. **67**(8): p. 3469-3475.
297. Barrangou, R., E. Altermann, R. Hutkins, R. Cano, and T.R. Klaenhammer, *Functional and comparative genomic analyses of an operon involved in fructooligosaccharide utilization by Lactobacillus acidophilus.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(15): p. 8957-8962.
298. Laakso, K., K. Koskenniemi, J. Koponen, M. Kankainen, A. Surakka, T. Salusjärvi, P. Auvinen, K. Savijoki, T.A. Nyman, N. Kalkkinen, S. Tynkkynen, and P. Varmanen, *Growth phase-associated changes in the proteome and transcriptome of Lactobacillus rhamnosus GG in industrial-type whey medium.* Microbial Biotechnology, 2011. **4**(6): p. 746-766.
299. Turpin, W., C. Humblot, M.-L. Noordine, M. Thomas, and J.-P. Guyot, *Lactobacillaceae and Cell Adhesion: Genomic and Functional Screening.* PLoS ONE, 2012. **7**(5).
300. de Vos, W.M. and E.E. Vaughan, *Genetics of lactose utilization in lactic acid bacteria FEMS microbiology reviews 15 (1994) 217–237.* FEMS Microbiology Reviews, 1995. **16**(1): p. 77-77.
301. Schwab, C. and M. Ganzle, *Lactic acid bacteria fermentation of human milk oligosaccharide components, human milk oligosaccharides and galactooligosaccharides.* FEMS Microbiology Letters, 2010. **315**(2): p. 141-148.
302. Goto, M., H. Bae, S.S. Lee, M.S. Yahaya, S. Karita, K. Wanjae, and K.J. Cheng, *Effects of surfactant Tween 80 on forage degradability and microbial growth on the in vitro rumen mixed and pure cultures.* Asian-Australasian Journal of Animal Sciences, 2003. **16**(5): p. 672-676.
303. Corcoran, B.M., C. Stanton, G.F. Fitzgerald, and R.P. Ross, *Growth of probiotic lactobacilli in the presence of oleic acid enhances subsequent survival in gastric juice.* Microbiology, 2007. **153**(1): p. 291-299.
304. Oh, S., S. Rheem, J. Sim, S. Kim, and Y. Baek, *Optimizing conditions for the growth of Lactobacillus casei YIT 9018 in tryptone-yeast extract-glucose medium by using response surface methodology.* Applied and Environmental Microbiology, 1995. **61**(11): p. 3809-14.
305. Shou, W.Z. and W. Naidong, *Post-column infusion study of the 'dosing vehicle effect' in the liquid chromatography/tandem mass spectrometric analysis of discovery pharmacokinetic samples.* Rapid Communications in Mass Spectrometry, 2003. **17**(6): p. 589-597.
306. Van der Meulen, R., L. Avonts, and L. De Vuyst, *Short Fractions of Oligofructose Are Preferentially Metabolized by Bifidobacterium animalis DN-173 010.* Applied and Environmental Microbiology, 2004. **70**(4): p. 1923-1930.

307. Falony, G., K. Lazidou, A. Verschaeren, S. Weckx, D. Maes, and L. De Vuyst, *In Vitro Kinetic Analysis of Fermentation of Prebiotic Inulin-Type Fructans by Bifidobacterium Species Reveals Four Different Phenotypes.* Applied and Environmental Microbiology, 2009. **75**(2): p. 454-461.
308. LoCascio, R.G., M.R. Ninonuevo, S.R. Kronewitter, S.L. Freeman, J.B. German, C.B. Lebrilla, and D.A. Mills, *A versatile and scalable strategy for glycoprofiling bifidobacterial consumption of human milk oligosaccharides.* Microbial Biotechnology, 2008. **2**(3): p. 333-342.
309. LoCascio, R.G., P. Desai, D.A. Sela, B. Weimer, and D.A. Mills, *Broad Conservation of Milk Utilization Genes in Bifidobacterium longum subsp. infantis as Revealed by Comparative Genomic Hybridization.* Applied and Environmental Microbiology, 2010. **76**(22): p. 7373-7381.
310. Allen, A. and D. Snary, *Structure and function of gastric mucus* Gut, 1972. **13**(8): p. 666-672.
311. Lee, S., M. Muller, K. Rezwan, and N.D. Spencer, *Porcine Gastric Mucin (PGM) at the Water/Poly(Dimethylsiloxane) (PDMS) Interface: Influence of pH and Ionic Strength on Its Conformation, Adsorption, and Aqueous Lubrication Properties.* Langmuir, 2005. **21**(18): p. 8344-8353.
312. Marcobal, A., A.M. Southwick, K.A. Earle, and J.L. Sonnenburg, *A refined palate: Bacterial consumption of host glycans in the gut.* Glycobiology, 2013. **23**(9): p. 1038-1046.
313. Nakayama-Imaohji, H., M. Ichimura, T. Iwasa, N. Okada, Y. Ohnishi, and T. Kuwahara, *Characterization of a gene cluster for sialoglycoconjugate utilization in Bacteroides fragilis.* The Journal of Medical Investigation, 2012. **59**(1,2): p. 79-94.
314. Zhou, J.S., P.K. Gopal, and H.S. Gill, *Potential probiotic lactic acid bacteria Lactobacillus rhamnosus (HN001), Lactobacillus acidophilus (HN017) and Bifidobacterium lactis (HN019) do not degrade gastric mucin in vitro.* International Journal of Food Microbiology, 2001. **63**(1-2): p. 81-90.
315. McGuckin, M.A., S.K. Lindén, P. Sutton, and T.H. Florin, *Mucin dynamics and enteric pathogens.* Nat Rev Micro, 2011. **9**(4): p. 265-278.
316. Jungersen, M., A. Wind, E. Johansen, J. Christensen, B. Stuer-Lauridsen, and D. Eskesen, *The Science behind the Probiotic Strain Bifidobacterium animalis subsp. lactis BB-12®.* Microorganisms, 2014. **2**(2): p. 92-110.
317. Gopal, P.K., J. Prasad, J. Smart, and H.S. Gill, *In vitro adherence properties of Lactobacillus rhamnosus DR20 and Bifidobacterium lactis DR10 strains and their antagonistic activity against an enterotoxigenic Escherichia coli.* International Journal of Food Microbiology, 2001. **67**(3): p. 207-216.
318. Candela, M., G. Seibold, B. Vitali, S. Lachenmaier, B.J. Eikmanns, and P. Brigidi, *Real-time PCR quantification of bacterial adhesion to Caco-2 cells: Competition between bifidobacteria and enteropathogens.* Research in Microbiology, 2005. **156**(8): p. 887-895.
319. Vesterlund, S., J. Paltta, M. Karp, and A.C. Ouwehand, *Measurement of bacterial adhesion in vitro evaluation of different methods.* Journal of Microbiological Methods, 2005. **60**(2): p. 225-233.
320. MacKenzie, D.A., F. Jeffers, M.L. Parker, A. Vibert-Vallet, R.J. Bongaerts, S. Roos, J. Walter, and N. Juge, *Strain-specific diversity of mucus-binding proteins in the adhesion and aggregation properties of Lactobacillus reuteri.* Microbiology, 2010. **156**(11): p. 3368-3378.
321. Pretzer, G., J. Snel, D. Molenaar, A. Wiersma, P.A. Bron, J. Lambert, W.M. de Vos, R. van der Meer, M.A. Smits, and M. Kleerebezem, *Biodiversity-Based Identification and Functional Characterization of the Mannose-Specific Adhesin of Lactobacillus plantarum.* Journal of Bacteriology, 2005. **187**(17): p. 6128-6136.

322. Kankainen, M., L. Paulin, S. Tynkkynen, I. von Ossowski, J. Reunanen, P. Partanen, R. Satokari, S. Vesterlund, A.P.A. Hendrickx, S. Lebeer, S.C.J. De Keersmaecker, J. Vanderleyden, T. Hamalainen, S. Laukkanen, N. Salovuori, J. Ritari, E. Alatalo, R. Korpela, T. Mattila-Sandholm, A. Lassig, K. Hatakka, K.T. Kinnunen, H. Karjalainen, M. Saxelin, K. Laakso, A. Surakka, A. Palva, T. Salusjarvii, P. Auvinen, and W.M. de Vos, *Comparative genomic analysis of Lactobacillus rhamnosus GG reveals pili containing a human- mucus binding protein.* Proceedings of the National Academy of Sciences, 2009. **106**(40): p. 17193-17198.
323. Garrido, D., Jae Han Kim, J.B. German, H.E. Raybould, and D.A. Mills, *Oligosaccharide binding proteins from Bifidobacterium longum subsp. infantis reveal a preference for host glycans.* PLoS ONE, 2011. **6**(3): p. e17315 [13].
324. Barrangou, R., E.P. Briczinski, L.L. Traeger, J.R. Loquasto, M. Richards, P. Horvath, A.-C. Coute-Monvoisin, G. Leyer, S. Rendulic, J.L. Steele, J.R. Broadbent, T. Oberg, E.G. Dudley, S. Schuster, D.A. Romero, and R.F. Roberts, *Comparison of the Complete Genome Sequences of Bifidobacterium animalis subsp. lactis DSM 10140 and Bl-04.* Journal of Bacteriology, 2009. **191**(13): p. 4144-4151.
325. Lukic, J., I. Strahinic, B. Jovcic, B. Filipic, L.a. Topisirovic, M. Kojic, and J. Begovic, *Different Roles for Lactococcal Aggregation Factor and Mucin Binding Protein in Adhesion to Gastrointestinal Mucosa.* Applied and Environmental Microbiology, 2012. **78**(22): p. 7993-8000.
326. Shoaf, K., G.L. Mulvey, G.D. Armstrong, and R.W. Hutkins, *Prebiotic Galactooligosaccharides Reduce Adherence of Enteropathogenic Escherichia coli to Tissue Culture Cells.* Infection and Immunity, 2006. **74**(12): p. 6920-6928.
327. Versalovic, J., *Probiotics: Intestinal gatekeeping, immunomodulation, and hepatic injury.* Hepatology, 2007. **46**(3): p. 618-621.
328. Madsen, K., A. Cornish, P. Soper, C. McKaigney, H. Jijon, C. Yachimec, J. Doyle, L. Jewell, and C. De Simone, *Probiotic Bacteria Enhance Murine and Human Intestinal Epithelial Barrier Function.* Gastroenterology, 2001. **121**(3): p. 580-591.
329. Commane, D.M., C.T. Shortt, S. Silvi, A. Cresci, R.M. Hughes, and I.R. Rowland, *Effects of Fermentation Products of Pro- and Prebiotics on Trans-Epithelial Electrical Resistance in an In Vitro Model of the Colon.* Nutrition and Cancer, 2005. **51**(1): p. 102-109.
330. Resta-Lenert, S. and K.E. Barrett, *Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive Escherichia coli (EIEC).* Gut, 2003. **52**(7): p. 988-997.
331. Benyacoub, J., F. Rochat, K.-Y. Saudan, I. Rochat, N. Antille, C. Cherbut, T. von der Weid, E.J. Schiffrin, and S. Blum, *Feeding a diet containing a fructooligosaccharide mix can enhance Salmonella vaccine efficacy in mice.* Journal of Nutrition, 2008. **138**(1): p. 123-129.
332. Foye, O.T., I.F. Huang, C.C. Chiou, W.A. Walker, and H.N. Shi, *Early administration of probiotic Lactobacillus acidophilus and/or prebiotic inulin attenuates pathogen-mediated intestinal inflammation and Smad 7 cell signaling.* FEMS Immunology and Medical Microbiology, 2012. **65**(3): p. 467-480.
333. Leforestier, G., A. Blais, F. Blachier, A. Marsset-Baglieri, A.-M. Davila-Gay, E. Perrin, and D. Tomé, *Effects of galacto-oligosaccharide ingestion on the mucosa-associated mucins and sucrase activity in the small intestine of mice.* European Journal of Nutrition, 2009. **48**(8): p. 457-464.
334. Schley, P.D. and C.J. Field, *The immune-enhancing effects of dietary fibres and prebiotics.* British Journal of Nutrition, 2002. **87**: p. S221-S230.
335. Sultana, R., A.J. McBain, and C.A. O'Neill, *Strain-Dependent Augmentation of Tight-Junction Barrier Function in Human Primary Epidermal Keratinocytes by Lactobacillus and Bifidobacterium Lysates.* Applied and Environmental Microbiology, 2013. **79**(16):

p. 4887-4894.
336. Seth, A., F. Yan, D.B. Polk, and R.K. Rao, *Probiotics ameliorate the hydrogen peroxide-induced epithelial barrier disruption by a PKC- and MAP kinase-dependent mechanism*. Vol. 294. 2008. G1060-G1069.
337. Hafez, M., *Upregulation of Intestinal Mucin Expression by the Probiotic Bacterium E. coli Nissle 1917.* Probiotics and Antimicrobial Proteins, 2012. **4**(2): p. 67-77.
338. Patel, R.M., L.S. Myers, A.R. Kurundkar, A. Maheshwari, A. Nusrat, and P.W. Lin, *Probiotic Bacteria Induce Maturation of Intestinal Claudin 3 Expression and Barrier Function.* American Journal of Pathology, 2012. **180**(2): p. 626-635.
339. Le Leu, R.K., Y. Hu, I.L. Brown, R.J. Woodman, and G.P. Young, *Synbiotic intervention of Bifidobacterium lactis and resistant starch protects against colorectal cancer development in rats.* Carcinogenesis, 2010. **31**(2): p. 246-251.
340. Chorley, B.N., A.L. Crews, Y. Li, K.B. Adler, M. Minnicozzi, and L.D. Martin, *Differential Muc2 and Muc5ac secretion by stimulated guinea pig tracheal epithelial cells in vitro.* Respiratory Research, 2006. **7**.
341. Greenbaum, D., C. Colangelo, K. Williams, and M. Gerstein, *Comparing protein abundance and mRNA expression levels on a genomic scale.* Genome Biology, 2003. **4**(9).
342. Smirnov, A., D. Sklan, and Z. Uni, *Mucin dynamics in the chick small intestine are altered by starvation.* The Journal of Nutrition, 2004(4): p. 736.
343. Tsirtsikos, P., K. Fegeros, C. Balaskas, A. Kominakis, and K.C. Mountzouris, *Dietary probiotic inclusion level modulates intestinal mucin composition and mucosal morphology in broilers.* Poultry Science, 2012. **91**(8): p. 1860-1868.
344. Mountzouris, K.C., P. Tsitrsikos, I. Palamidi, A. Arvaniti, M. Mohnl, G. Schatzmayr, and K. Fegeros, *Effects of probiotic inclusion levels in broiler nutrition on growth performance, nutrient digestibility, plasma immunoglobulins, and cecal microflora composition.* Poultry Science, 2010. **89**(1): p. 58-67.
345. Kim, S.K., *Small intestine transit time in the normal small bowel study* American Journal of Roentgenology, 1968. **104**(3): p. 522-524.
346. Bouchoucha, M., G. Devroede, E. Dorval, A. Faye, P. Arhan, and M. Arsac, *Different segmental transit times in patients with irritable bowel syndrome and normal colonic transit time: is there a correlation with symptoms?* Techniques in Coloproctology, 2006. **10**(4): p. 287-296.
347. Burns, A.J. and I.R. Rowland, *Anti-carcinogenicity of probiotics and prebiotics.* Current Issues in Intestinal Microbiology, 2000. **1**(1): p. 13-24.
348. Pool-Zobel, B.L., C. Neudecker, I. Domizlaff, S. Ji, U. Schillinger, C. Rumney, M. Moretti, I. Vilarini, R. Scassellati-Sforzolini, and I. Rowland, *Lactobacillus- and Bifidobacterium-mediated antigenotoxicity in the colon of rats.* Nutrition and Cancer, 1996. **26**(3): p. 365-380.
349. Tuohy, K.M., H.M. Probert, C.W. Smejkal, and G.R. Gibson, *Using probiotics and prebiotics to improve gut health.* Drug Discovery Today, 2003. **8**(15): p. 692-700.
350. Femia, A.P., C. Luceri, P. Dolara, A. Giannini, A. Biggeri, M. Salvadori, Y. Clune, K.J. Collins, M. Paglierani, and G. Caderni, *Antitumorigenic activity of the prebiotic inulin enriched with oligofructose in combination with the probiotics Lactobacillus rhamnosus and Bifidobacterium lactis on azoxymethane-induced colon carcinogenesis in rats.* Carcinogenesis, 2002. **23**(11): p. 1953-1960.
351. Schaafsma, G., W.J.A. Meuling, W. van Dokkum, and C. Bouley, *Effects of a milk product, fermented by Lactobacillus acidophilus and with fructo-oligosaccharides added, on blood lipids in male volunteers.* European Journal of Clinical Nutrition, 1998. **52**(6): p. 436-440.
352. Kiessling, G., J. Schneider, and G. Jahreis, *Long-term consumption of fermented dairy products over 6 months increases HDL cholesterol.* European Journal of Clinical

Nutrition, 2002. **56**(9): p. 843-849.
353. Bouhnik, Y., B. Flourie, C. Andrieux, N. Bisetti, F. Briet, and J.C. Rambaud, *Effects of Bifidobacterium sp fermented milk ingested with or without inulin on colonic bifidobacteria and enzymatic activities in healthy humans.* European Journal of Clinical Nutrition, 1996. **50**(4): p. 269-273.
354. Furrie, E., S. Macfarlane, A. Kennedy, J.H. Cummings, S.V. Walsh, D.A. O'Neil, and G.T. Macfarlane, *Synbiotic therapy (Bifidobacterium longum/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial.* Gut, 2005. **54**(2): p. 242-249.
355. Fujimori, S., K. Gudis, K. Mitsui, T. Seo, M. Yonezawa, S. Tanaka, A. Tatsuguchi, and C. Sakamoto, *A randomized controlled trial on the efficacy of synbiotic versus probiotic or prebiotic treatment to improve the quality of life in patients with ulcerative colitis.* Nutrition, 2009. **25**(5): p. 520-525.
356. Watkinson, P.J., V.S. Barber, P. Dark, and J.D. Young, *The use of pre- pro- and synbiotics in adult intensive care unit patients: Systematic review.* Clinical Nutrition, 2006. **26**(2): p. 182-192.
357. Araujo, F. and B. Sarmento, *Towards the characterization of an in vitro triple co-culture intestine cell model for permeability studies.* International Journal of Pharmaceutics, 2013. **458**(1): p. 128-134.
358. Barboza Jr, M.S., T.M.J. Silva, R.L. Guerrant, and A.A.M. Lima, *Measurement of intestinal permeability using mannitol and lactulose in children with diarrheal diseases.* Brazilian Journal of Medical and Biological Research, 1999.
359. Kiyohara, M., K. Tanigawa, T. Chaiwangsri, T. Katayama, H. Ashida, and K. Yamamoto, *An exo-α-sialidase from bifidobacteria involved in the degradation of sialyloligosaccharides in human milk and intestinal glycoconjugates.* Glycobiology, 2011. **21**(4): p. 437-447.
360. Cook, R., R. Harris, and G. Reid, *Effect of culture media and growth phase on the morphology of lactobacilli and on their ability to adhere to epithelial cells.* Current Microbiology, 1988. **17**(3): p. 159-166.
361. Deepika, G., E. Karunakaran, C. Hurley, C. Biggs, and D. Charalampopoulos, *Influence of fermentation conditions on the surface properties and adhesion of Lactobacillus rhamnosus GG.* Microbial Cell Factories C7 - 116, 2012. **11**(1): p. 1-12.
362. van Zanten, G.C., A. Knudsen, H. RÃ¶ytiÃ¶, S. Forssten, M. Lawther, A. Blennow, S.J. Lahtinen, M. Jakobsen, B. Svensson, and L. Jespersen, *The Effect of Selected Synbiotics on Microbial Composition and Short-Chain Fatty Acid Production in a Model System of the Human Colon.* PLoS ONE, 2012. **7**(10): p. e47212.
363. Pinto, M., S. Robineleon, M.D. Appay, M. Kedinger, N. Triadou, E. Dussaulx, B. Lacroix, P. Simonassmann, K. Haffen, J. Fogh, and A. Zweibaum, *Enterocyte-like differentiation and polarization of the human-colon carcinoma cell-line Caco-2 in culture* Biology of the Cell, 1983. **47**(3): p. 323-330.
364. Roig, A.I., U. Eskiocak, S.K. Hight, S.B. Kim, O. Delgado, R.F. Souza, S.J. Spechler, W.E. Wright, and J.W. Shay, *Immortalized Epithelial Cells Derived From Human Colon Biopsies Express Stem Cell Markers and Differentiate In Vitro.* Gastroenterology, 2010. **138**(3): p. 1012-1021.e5.
365. Antunes, F., F. Andrade, F. AraÃºjo, D. Ferreira, and B. Sarmento, *Establishment of a triple co-culture in vitro cell models to study intestinal absorption of peptide drugs.* European Journal of Pharmaceutics and Biopharmaceutics. **83**(3): p. 427-435.
366. Butler, M., C.-Y. Ng, D.A. van Heel, G. Lombardi, R. Lechler, R.J. Playford, and S. Ghosh, *Modulation of dendritic cell phenotype and functionin an in vitro model of the intestinal epithelium.* European Journal of Immunology, 2006. **36**(4): p. 864-874.
367. Kim, H.J., D. Huh, G. Hamilton, and D.E. Ingber, *Human gut-on-a-chip inhabited by microbial flora that experiences intestinal peristalsis-like motions and flow.* Lab on a

Chip, 2012.
368. Goulas, A.K., A.S. Grandison, and R.A. Rastall, *Fractionation of oligosaccharides by nanofiltration.* Journal of the Science of Food and Agriculture, 2003. **83**(7): p. 675-680.