

# MICROBIAL CONTRIBUTIONS TO GUT DEVELOPMENT IN THE NEONATAL PIG

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## ABSTRACT

The commensal intestinal microbiota contributes substantially to intestinal development in the early neonatal period by mechanisms that are not yet elucidated but could contribute to novel strategies to improve intestinal health. A series of gnotobiotic experiments using isolator-reared caesarian section-derived piglets inoculated at 1 d of age with selected bacteria and euthanized at 14 or 15 days of age were performed to investigate intestinal morphology, inflammation and digestive function. In Experiment 1, piglets were maintained germfree (GF), mono-associated with *Escherichia coli* (EC), mono-associated with *Lactobacillus fermentum* (LF) or conventionalized with sow feces (CV). Increased ( $P<0.05$ ) gene expression of Fas ligand (FasL) and tumor necrosis factor (TNF $\alpha$ ) in EC and CV as compared to LF and GF pigs coincided with increased apoptotic and proliferative activity. Toll-like receptors (TLR) 2, 4 and 9 were differentially regulated ( $P<0.05$ ) by colonizing species. In Experiment 2 using the same animals as Exp. 1, increased turnover of brush border enzymes was indicated by reduced ( $P<0.05$ ) specific activity of aminopeptidase N (APN) and lactase (LPH) and increased expression of APN in CV and EC as compared to GF and LF pigs. Reduced enzyme activity to gene expression ratio corresponded with an *in vitro* assay of microbial inactivation of APN. In Experiment 3, probiotic *Lactobacillus sp.*, L3777, and *Bifidobacteria sp.*, B5445, did not induce expression of inflammatory cytokines in mono-association but di-association with *E. coli* increased ( $P<0.05$ ) inflammatory and anti-inflammatory mediators and resulted in a high rate of sepsis (50%) relative to *E. coli* mono-association. Induced expression of inflammatory cytokines by commensal bacteria

through TLR and other means, appear to play a substantial role in microbially-induced enterocyte turnover. Enterocyte immaturity did not account for reduced enzyme activity associated with inflammation as increased expression of APN in response to microbial colonization was observed, suggesting a host response pathway enabling effective competition with the intestinal microbiota for available peptide nutrients. Probiotic bacteria were relatively benign in mono-association but may have facilitated increased translocation of *E. coli* in di-association. Gnotobiotic animal models are essential to demonstrate outcomes of host response characterized by communication among numerous cell types, although are of significant technical difficulty.

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## LIST OF ABBREVIATIONS

A:G	activity:gene expression
ANOVA	analysis of variance
AP-1	activator protein 1
Apaf-1	apoptotic protease-activating factor 1
APN	aminopeptidase N
ATBF1-A	AT motif-binding factor 1A
AZ	ornithine decarboxylase antizyme
BB	brush border
Bb	Mono-association with B5445
Bb*	Bb contaminated
BbE	B5445 and <i>Escherichia coli</i>
BSA	bovine serum albumin
CAMP	commensal-associated molecular pattern
casp3	caspase 3
cDNA	complimentary deoxyribonucleic acid
CFU	colony forming units
cpn60	chaperonin 60
cpnDB	chaperonin 60 database
CV	conventional
DISC	death-inducing signaling complex
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EC	<i>Escherichia coli</i>
EDTA	ethylene diamine tetra acetate
FADD	Fas-associated death domain
FasL	Fas ligand
FLIP	Fllice inhibitory protein
GAPDH	glyceraldehydes-phosphate dehydrogenase
GF	germfree
HNF-1	hepatocyte nuclear factor 1
HSP	heat shock protein
IAP	inhibitor of apoptosis
IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
IEL	intra-epithelial lymphocyte
IFN $\gamma$	interferon gamma
IgA	immunoglobulin A
IL	interleukin
IRF3	interferon regulatory factor 3
La	Mono-association with L3777
LAB	lactic acid bacteria
LaE	L3777 and <i>Escherichia coli</i>
LF	<i>Lactobacillus fermentum</i>
LFKP	<i>Lactobacillus fermentum</i> and <i>Klebsiella pneumoniae</i>

LPH	lactase phlorizin hydrolase
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MAMP	microbiota-associated molecular pattern
MHC	major histocompatibility complex
MLN	mesenteric lymph node
mRNA	messenger ribonucleic acid
NF- $\kappa$ B	nuclear factor kappa B
NGF	nerve growth factor
Nods	nucleotide-binding oligomerization domains
ODC	ornithine decarboxylase
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline + tween
PCR	polymerase chain reaction
PCNA	proliferating cell nuclear antigen
PMSF	phenylmethanesulfonyl fluoride
PP	Peyer's patches
PPAR $\gamma$	peroxisome proliferators activated receptor gamma
PSA	polysaccharide A
PYY	peptide YY
qPCR	quantitative PCR
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SCFA	short chain fatty acid
SE	standard error
SGLT-1	sodium glucose cotransporter
SI	small intestine
SOCS	suppressor of cytokine signaling
sprr2a	small praline-rich protein-2a
TGF $\beta$	transforming growth factor beta
TIR	toll/interleukin01 receptor
TLR	toll-like receptor
TNF $\alpha$	tumor necrosis factor alpha
Tollip	toll-interacting protein
UT	universal target
ZO-1	zona occludens 1

## 1.0 INTRODUCTION

The term ecological development was recently coined to support the idea that development is a product of both genetics of the host as well as the interactions with a resident microbiota (Hooper, 2004). Even though the role of a resident microbiota in health was first hypothesized by Pasteur over a century ago (Sears, 2005), the multiplicity of physiological changes induced by commensal bacteria has only recently been recognized (Hooper et al., 2001). The hygiene hypothesis, the role of bacteria in inflammatory bowel diseases and recent evidence implicating microbial composition in obesity (Breves et al., 2000; Turnbaugh et al., 2006) have drawn considerable attention to the human microbiota. In animal production, increased interest in understanding the intestinal microbiota and the underlying mechanisms for affecting the host is largely related to increased pressure to reduce use of antibiotics, thus the need to develop alternatives to promote health and rapid growth.

Although there is limited research demonstrating a clear mechanism by which they improve health and performance, probiotics and prebiotics have become commonplace. Examples of probiotic effects demonstrated *in vivo* include increased nutrient transport (Breves et al., 2000) and non-specific immune response (Hatcher and Lambrecht, 1993); however it is unclear as to whether these host responses are a direct response to the probiotic organism, or a response to changes in the microbial composition.

It is clear that bacteria significantly contribute to intestinal development, yet the bacteria responsible for these changes have not been identified. Identifying the bacteria that contribute to particular aspects of development and the mechanism by which physiological changes are induced is impossible in a conventional animal because of the highly diverse microbiota, composed of over 400 species (Savage, 1977). To define the relationship of individual organisms with the host, many have used *in vitro* culture of cell lines along with bacteria; however, *in vitro* systems usually only use a single cell type and are confounded by the nature of immortal cell lines. Using a gnotobiotic (germfree) animal model overcomes the challenge of the microbial diversity while allowing the study of developmental processes that require interactions between the diverse cell types present in the host-microbial interface.

Our lab has recently developed a gnotobiotic pig model, providing a tool to identify mechanisms that will allow for improved swine production while providing an excellent model for human neonatal research. Shirkey et al. (2006) reported that mono-association of pigs with commensal, early colonizing bacteria representing the major cell wall divisions, non-pathogenic *Escherichia coli* (Gram-negative) and *Lactobacillus fermentum* (Gram-positive), differentially affect villus structure and expression of pro-inflammatory cytokines, IL-1 $\beta$  and IL-6.

Based on these findings, we hypothesize that the composition of early colonizing bacteria in the neonatal pig plays an important role in directing intestinal development. The objective of the first experiment was to evaluate whether bacteria differentially affect proliferative and apoptotic activity as well as to identify a contributing mechanism for bacteria to affect cell turnover. The objective of the

second experiment was to investigate how microbial colonization contributes to intestinal maturation specifically pertaining to digestive function. Finally, the objective of the third experiment was to determine how the host responds to probiotic bacteria alone and in di-association with a bacterium previously shown to induce inflammation.

## 2.0 LITERATURE REVIEW

It has long been recognized that colonization of commensal microbiota in the intestinal tract is advantageous to the host. Microbial metabolism increases the host's digestive capabilities, creates nutrients that would otherwise not be present (e.g. vitamins, volatile fatty acids) and excludes harmful pathogens. More recently an appreciation for the impacts of the commensal microbiota on the physiological responses by the host has been established. Physiological effects on the host not only include development of the adaptive immune response, but many parameters of innate immunity, digestive function, metabolism of absorbed nutrients and even systemic effects (Tanida et al., 2005). Relatively little is understood about the mechanisms by which the microbiota induce host changes, although recent reports have begun to identify components of specific responses pathways. Elucidating the mechanisms by which the host recognizes the commensal microbiota and the associated physiological outcomes will provide opportunities to develop strategies to specifically modify these outcomes with the objective of improving health, nutrition and performance in animals. A brief overview is presented on the composition of the intestinal microbiota and microbial impacts on host physiology. However, the focus of the review will be on the identified and speculated host pathways through which microbial products are recognized. Although not exclusively, we will give particular consideration for observations in the pig.



## 2.1 Intestinal Microbiology

### 2.1.1 Composition and succession

Understanding the composition and location of bacteria in the digestive tract is important in understanding their contribution to normal intestinal development, digestive and protective functions. This is particularly true since host responses may be specific to a particular broad (family, genus) or narrow (species, strain) taxonomic group. The potential to modulate intestinal microbial ecology to manipulate normal development or alleviate abnormal physiology may offer an important prophylactic or therapeutic approach.

Microbial colonization succession in the pig has received limited attention and in large part has focused on the feces or distal colon due to ease of sample accessibility. Early colonization in pigs and humans is remarkably similar (Mackie et al., 1999). At birth the pig leaves the sterile environment of the uterus and is immediately introduced to a population of bacteria in the vaginal tract and subsequently in the surrounding environment, particularly sow feces (Tannock et al., 1990a,b).

Based on conventional microbial plating techniques the distal colon of the pig is colonized with  $10^9$  to  $10^{10}$  bacteria per gram of digesta within 12 hours of birth (Swords et al., 1993). This population is initially dominated by facultative anaerobes, predominantly coliforms, but is quickly overrun by obligate anaerobes. It has been suggested that these facultative anaerobes create a reduced environment favorable for anaerobic species (Stark and Lee, 1982). *Clostridium spp.* become dominant after 5 days of age and begin to decline at weaning and are eventually supplanted by

*Bacteroides spp.*, the dominant adult colonic microbiota in the pig (Swords et al., 1993). The abundance of *Lactobacillus spp.* in the colon peaks at 2 days of age. The diversity and activity of lactobacilli also changes through time, particularly at weaning (Janczyk et al., 2007).

Whether microbial succession patterns in proximal small intestine mirror those observed in colon and feces is not clear, however, molecular based profiling studies in the pig have indeed demonstrated marked differences in microbial composition between proximal and distal intestinal locations (Hill et al., 2005; Konstantinov et al., 2004; Richards et al., 2005). In a recent 16s rDNA-based clone library study, Konstantinov (2006) examined post-natal development of the porcine microbiota in ileal digesta collected at 2 days of age, weaning and postweaning and identified *E. coli*, *Shigella flexneri* and *L. sobrius*, *L. reuteri* and *L. acidophilus* as the early dominant species.

The bacterial population of the gastrointestinal tract increases in density and complexity from proximal to distal regions, with the exception of the mouth, and changes composition through location and time. The stomach and duodenum have a relatively low microbial density with  $10^3$ - $10^5$  colony forming units (cfu) per gram of contents and a population dominated by the *Lactobacillus*, *Streptococcus* and *Enterococcus* genera. This population is limited in density and diversity by the acidic environment as well as bile secretions. The ability to attach to and colonize the mucosal layer seems to be important in the proximal SI as rate of passage is rapid. Rate of passage slows and bacterial counts increase to  $10^8$  to  $10^9$  cfu per gram contents in the distal SI, with phylogenetic representation increasing to include those

genera indicated above plus genera within the Clostridiales and Bacteroidetes taxonomic families (Hill et al., 2004; Richards et al., 2005). Complexity of the microbial population is highest in the hindgut and density increases to  $10^{10}$  to  $10^{12}$  cfu per gram, dominated by the low G+C Gram-positive groups including *Clostridium*, *Bacillus*, *Ruminococcus* and *Fusobacterium* (Hill et al., 2002; Leser et al., 2002). Fermentation of carbohydrates and amino acids becomes more prominent in distal locations (Cummings and Macfarlane, 1991). Variation within bacterial species among locations also contributes substantially to overall microbial diversity (Dixit et al., 2004).

#### 2.1.2 Factors Affecting Microbiota

Attempts made at manipulating bacterial populations have included changes in diet with the inclusion of unabsorbable carbohydrates (prebiotics) and live bacteria (probiotics). Several factors have emerged as important in microbial succession. In infants, diet plays a significant role as bifidobacteria are dominant in feces of breast-fed infants compared to a more diverse fecal microbiota with more *Bacteroides* spp. in formula-fed infants (Harmsen et al., 2000; Stark and Lee, 1982). Other factors affecting succession patterns include method of delivery (vaginal versus Caesarean section), postnatal hygiene, environment and antibiotic use (Gronlund et al., 1999; Lofmark et al., 2006; Schwartz et al., 2003). Components of human milk contain non-absorbable oligosaccharides, nucleotides and gangliosides, which affect bifidobacteria colonization (Balmer et al., 1994; Kunz and Rudloff, 1993; Rueda et al., 1998). The major factors affecting microbial number and composition along the

length of the intestine appear to be age, diet composition and genotype (Apajalahti et al., 2001; Hill et al., 2005; Van Der Wielen et al., 2000). While bacterial number and complexity may be comparatively low in the small intestine, this is obviously the primary location for nutrient digestion and absorption. In addition, using ATP concentration in intestinal contents as an indicator, Jensen and Jorgensen (1994) demonstrated considerable microbial-origin metabolic activity along the entire small intestine reaching levels in the distal ileum equivalent to the cecum.

The diversity of intestinal microbial populations between individuals is surprisingly high even with similar diets and lifestyles (Moore et al., 1978). The interaction between microbe and host is clearly bi-directional as the extent of host control on microbial population has been demonstrated by reciprocal transplants of gut microbiota between mice and zebrafish where abundance of microbial lineages shifted to resemble the normal microbial population of that species (Rawls et al., 2006).

### 2.1.3 Localization

Bacteria survive within four microhabitats in the digestive tract including the intestinal lumen, the mucus layer lining the villus, the mucus layer in the crypts as well as attached to the surface of enterocytes (Mackie et al., 1999). One of the main selection criteria for probiotic bacteria is their ability to adhere to intestinal epithelial cells. Attachment to the mucosal cell has been shown to be dependent on a variety of ligands on bacterial surface including collagen binding protein (Sillanpaa et al., 2000), an unidentified high molecular mass cell protein (Roos and Jonsson, 2002) as

well as specific receptors such as sugar chains of glycolipids or glycoproteins produced by host cells expressed luminally. *Lactobacillus johnsonii* uses elongation factor Tu (EF-Tu), an outer surface protein, to bind directly to intestinal epithelial cells *in vitro* (Granato et al., 2004). The carbohydrate chains from ABO antigen expressed on the surface of erythrocytes are also expressed in colonic mucin (Nakayama et al., 1987) and some lactobacilli have a strong binding affinity for human blood type-A antigen (Uchida et al., 2006). The ability of bifidobacteria to adhere to Caco-2 cells as well as the effect of pH on adhesion is strain dependent suggesting that adhesion is achieved through more than one mechanism (Riedel et al., 2006).

Direct adherence to the microvilli of Caco-2 cells *in vitro* by *L. acidophilus* has been observed using a scanning electron micrograph (Coconnier et al., 1992), however the level to which bacteria bind directly to enterocytes *in vivo* has recently come into question. Interestingly, direct association of bacteria to the epithelial surface was not observed using 16S rRNA fluorescent based probes on ileal and colon biopsies suggesting that bacteria live in a suspension in the mucous layer (van der Waaij et al., 2005). The existence of multiple receptor like molecules on Caco-2 cells and the direct attachment of mucus adhesion promoting protein (MapA) of *L. reuteri* to Caco-2 cells would suggest that direct contact is likely (Miyoshi et al., 2006). Although potentially not in continuous direct contact with the intestinal epithelium, substantial populations live in close proximity in the mucin layer and likely have intermittent direct contact. The abundance of bacteria in close proximity to the intestinal epithelium has been demonstrated by measuring microbial

populations present in homogenized whole tissue after intestinal contents have been washed out (Frece et al., 2005). The discrepancy observed between attachment *in vitro* and *in vivo* may be a consequence of the static nature of *in vitro* cell lines and the fact that they lack other important host factors that keep the intestinal microbiota at bay. Conversely the recent observation that bacteria are not directly associated with the intestinal epithelium may be a consequence of tissue collection conditions and assay sensitivity. Although the extent to which commensal bacteria come in direct contact with the intestinal epithelium is not clear, these bacteria clearly have major effects on the host.

## **2.2 Microbial Effects on the Host**

Substantial advances in the understanding of microbial contributions to intestinal and general health have occurred since Pasteur first postulated the importance of a resident flora in our health, culminating in the most recent report implicating intestinal microbial composition in obesity (Turnbaugh et al., 2006). Early reports comparing germfree and conventional animals showed many differences including microbially dependent changes in organ weights and intestinal structure (Gordon et al., 1966; Wostmann, 1981). More recent investigations have begun to examine the host-microbial interaction at a closer level using genomic and proteomic (Danielsen et al., 2007) techniques combined with gnotobiology to define the physiological effects of a single or groups of organisms on specific cell populations *in vivo*.

The response to a single organism, *Bacteroides thetaiotaomicron*, leads to differential transcription of genes responsible for nutrient absorption, mucosal barrier fortification, xenobiotic metabolism, angiogenesis and intestinal maturation (Hooper et al., 2001). A similar response observed in zebrafish to the same organism indicates an evolutionary conservation of host-microbial interactions (Rawls et al., 2004). Although some similarities have been observed in host response to different bacteria, there were also many differences suggesting that changing the composition of the gut microbiota can have specific physiological effects (Hooper et al., 2001a).

#### 2.2.1 Specific Immunity

Development of the mucosal specific immune system is largely dependent on an intestinal microbiota. This is demonstrated by poorly developed Peyer's patches and mesenteric lymph nodes in germfree animals (Miniats and Valli, 1973; Pabst et al., 1988; Rothkotter, 1991). The effects of microbiota are not limited to organized tissues as germfree animals have a reduced development of the lamina propria as well as reduced infiltration of immune cells into the lamina propria and intraepithelial space (Guygrand et al., 1978). Colonization not only affects the number of immune cells in the intestinal epithelium, it is also necessary for a balanced T-helper cell population, with germfree animals having a disproportionate Th2:Th1 ratio (Mazmanian et al., 2005). Interaction with bacteria also increased antigen sampling by M cells (Gebert et al., 2004).

### 2.2.2 Barrier Function and Innate Immunity

Although the intestinal epithelium is only one cell layer thick, rapid turnover, tight junctions between cells, production of mucins and secretion of anti-microbials make it a relatively impermeable barrier. Colonization of commensal bacteria substantially contributes to the fortification of this barrier. Bacteria can also inhibit inflammation (Kelly et al., 2004), which may be protective to the host as excessive inflammation can lead to disruption of the epithelial barrier.

At birth tight junctions between enterocytes have begun to form however, bacterial colonization leads to a much stronger barrier by enforcement of tight junctions. The expression of small proline-rich protein-2 (sprr2a), an important component of the cornified cell envelope and a bridging protein in tight junctions, (Steinert and Marekov, 1999) is upregulated over 100-fold when mice are colonized as compared to germfree mice (Hooper et al., 2001). Tight junction protein, Zona occludens 1 (ZO-1), is also upregulated by bacterial recognition (Cario et al., 2004).

Mucin secretion plays an important role in host defense and is also highly affected by bacterial colonization. *Lactobacillus casei* GG increases mucin gene, MUC2, expression in intestinal epithelial cell monolayers as compared to untreated cells (Mattar et al., 2002), while other bacteria have been shown to increase the expression of both MUC2 and MUC3 by goblet cells (Mack et al., 1999, 2003). Bacteria can also alter the pattern of the glycoconjugate repertoire expressed by absorptive enterocytes (Hooper et al., 1999). Before weaning rodents express mostly sialylated surface oligosaccharides (Torrespinedo and Mahmood, 1984) and an increase in fucosylation after weaning is in large part due to effects of changes in



bacterial population including an increase in *Bacterioides* spp. representation (Hooper et al., 1999). Bacteria can also reduce the expression of glycoconjugates as the expression of Gal $\alpha$ 1,3Gal on the surface of enterocytes is lower in conventional zebrafish (Bates et al., 2006).

Another important mechanism of innate defense that is highly affected by microbial colonization is turnover of the intestinal epithelium. In a conventional animal turnover of the entire intestinal epithelium occurs every 3-5 days, with a balance being maintained, at least in the healthy state, between cell proliferation and cell loss. Bacteria impact regulatory signals controlling epithelial cell mitosis, differentiation and maturation. It has been shown that germfree animals have a 2 fold decrease in intestinal epithelial cell (IEC) proliferation as compared to animals colonized with enteric bacteria (Heneghan et al. 1984). In a germfree animal the rate of replacement is much slower with enterocytes taking twice as long to migrate from the crypt to the villus tip (Savage et al., 1981). *B. thetaiotaomicron* increases epithelial proliferation in both mice and zebrafish as indicated by the increased expression in mono-associated animals of 15 genes involved in DNA replication and cell division (Rawls et al., 2004).

The substantially shorter villi in conventional as compared to germfree animals indicates a substantial bacterial contribution to apoptosis and cell sloughing. Apoptosis or programmed cell death is the means by which old, damaged or unwanted cells are eliminated without marked inflammation. The process is highly controlled by a sequence of steps regulating nuclear condensation and fragmentation, cytoplasmic blebbing and cell shrinkage (Kerr et al., 1972). This is in contrast to cell

necrosis where direct injury to the cell leads to cell membranes being permeabilized and rupture ensues, leading to inflammation in surrounding tissue. The role of apoptosis in the regulation of cell number in the intestinal epithelium is not clearly understood. Original observations of intestinal tissues suggested that the main mechanism by which the epithelial cell number was regulated was through sloughing from the villus tip (Creamer et al., 1961; Tsubouchi and Leblond, 1979). This is contrary to more recent evidence that there are enough apoptotic events along the villus axis to account for the majority of the large population of cells migrating onto the villus each day (Hall et al., 1994). Apoptosis is not randomly distributed, but is concentrated at the distal end of the migration route (Hall et al., 1994). However, apoptosis is not limited to cells of the villus and is observed also at a low level in proliferative crypt cells, which is increased by genotoxic insults (Merritt et al., 1994; Potten et al., 1990).

Induction of apoptosis by bacteria can be induced by an intrinsic pathway (mitochondrial pathway) and by an extrinsic pathway activated by death ligands of the TNF family. Enterocytes express death receptors for death ligands, Fas ligand (FasL) and TNF $\alpha$ , on the basolateral surface (Strater and Moller, 2000). When ligation between death ligand and death receptor occurs, a death domain in the cytoplasmic tail of the receptor recruits the death-inducing signaling complex (DISC), which in turn activates initiator caspases 8 and 10 (Strater and Moller, 2000). Initiator caspases then cleave executioner caspases (caspase 3, 6 and 7), which activate enzymes required for the controlled degradation of the nucleus and other cell components (Gupta, 2003).

The intrinsic apoptosis pathway is induced by a variety of stimuli including cytotoxic agents and irradiation (DNA damage) and cellular stress by reactive oxygen and nitrogen species (Gupta, 2003). In the presence of these stimulators the mitochondrial membrane becomes permeable and cytochrome C is released. Cytochrome C then recruits Apaf-1 (Apoptotic protease-activating factor) and procaspase 9 to form an apoptosome, leading to the activation of caspase 9, which activates executioner caspases 3, 6 and 7 again leading to apoptosis (Gupta, 2003).

Two other important mechanisms of innate defense mediated by microbial colonization are the infiltration of non-specific phagocytic immune cells (Coates, 1975) as well as the expression of antimicrobial peptides. Microbial colonization of mice induces the expression of RegIII $\gamma$ , an anti-microbial lectin protein that binds to peptidoglycan carbohydrate in paneth cells (Cash et al., 2006). Although pigs do not have paneth cells, enterocytes also produce several anti-microbial peptides (Oswald, 2006; Veldhuizen et al., 2007).

### 2.2.3 Digestive Function

The absorptive and digestive function of enterocytes is also affected by colonization. *B. thetaiotaomicron* increases the expression of some proteins important for host nutrient absorption including Na<sup>+</sup>/glucose cotransporter and fatty acid binding protein while reducing expression of lactase-phlorizin hydrolase (Hooper et al., 2001a). Conventionalization of mice leads to reduced activities of brush border enzymes lactase, alkaline phosphatase and  $\gamma$ -glutamylultranspeptidase, while increasing sucrase and glucoamylase (Kozakova et al., 2001). This shift in

activities indicates that bacteria accelerate maturation of enterocytes. Bacteria and their products have also been shown to affect intestinal motility and ion transport (Cuche et al., 2000; Yajima, 1985, 1988).

## **2.3 Mechanisms of Host Response**

Over thousands of years of co-evolution, the host has developed means to recognize and respond appropriately to its resident microbiota. In this section we will consider the known mechanisms by which bacteria drive the physiological changes in the host that we have briefly discussed above. Defining the mechanisms by which bacteria exert their effects has been challenging, however, observations using mutant strains, purified microbial components and metabolites have led to some new insights. Many of the host responses are receptor mediated while others are a response to damaging or nutritive effects induced by bacteria or their sometimes beneficial and sometimes toxic metabolites, with only some of the effects being dependent on live organisms (Bates et al., 2006).

### **2.3.1 Host Sampling of Bacterial Antigens**

Bacteria and other antigens present in the lumen of the digestive tract are sampled and processed by the gut-associated lymphoid tissue (GALT). Peyer's patches the sampling portion of GALT and are segmented throughout the jejunum, but continuous in the ileum of the pig (Keren, 1992). Bacteria are sampled by specialized epithelial cells known as M cells that are located directly above Peyer's

patches (PP). M cells do not express major histocompatibility complex (MHC) class II antigens (Keren, 1992) and their role is simply to transport bacteria, and other antigens to the PP. Antigens are then engulfed by MHC class II bearing cells including macrophages, dendritic cells and B cells that process and present antigens to lymphocytes triggering an immune response. This process is moderated by a cascade of inflammatory and regulatory cytokines and leads to expansion and organization of PP's as well as the trafficking of activated lymphocytes via lymphatic and blood vessels into the lamina propria and intraepithelial space along the length of the intestine.

The nature of microbial components sampled is important for appropriate development of the specific mucosal immune system. Mazmanian et al. (2005) found a bacterial polysaccharide (PSA) that directs cellular and physical maturation of the developing immune system. Comparing germfree and conventional animals they observed differences in Th1/Th2 response, and further that association of germfree mice with wild-type *Bacteroides fragilis* corrected systemic T cell deficiencies and Th1/Th2 imbalances, while a PSA mutant of *B. fragilis* did not. It was also established that PSA is taken up and presented by dendritic cells which activate CD4+ T cells and elicit an appropriate cytokine release.

### 2.3.2 Receptor Mediated Recognition of Microbiota-associated Molecular Patterns

Cells of the intestinal epithelium including dendritic cells, M cells and enterocytes are able to detect structural signatures of prokaryotes through a variety of receptor families including Toll-like receptors (TLR), nucleotide-binding

oligomerization domains (Nods) and galectins. The ligands identified by these receptors were originally identified as pathogen-associated molecular patterns (PAMPs), however commensal bacteria also express these ligands leading others to suggest that commensal-associated molecular patterns (CAMPs) is a more appropriate term (Cario et al., 2002). Since both the terms are exclusive to the alternate group we will adopt the term microbiota-associated molecular patterns (MAMPS).

There are 11 TLRs in the human genome (Collier-Hyams and Neish, 2005), however all of their ligands have not been identified. Identified ligands that activate TLR2, 4, 5 and 9 are lipoteichoic acid and lipoprotein (Gram-positive bacteria), lipopolysaccharide (LPS) (Gram-negative bacteria), flagellin and CpG-containing DNA, respectively. The expression of TLRs 1-6, 8 and 9 have been demonstrated in isolated human colonic epithelial cells (Otte et al., 2004), but little evaluation of TLR expression has been done in porcine intestinal epithelium (Tohno et al., 2005, 2006).

TLRs are composed of two main parts including an extracellular portion necessary for ligand recognition and the toll/interleukin-1 receptor (TIR) that resides in the cytoplasmic domain and is required for activation of signal-transduction proteins (Kalliomaki and Walker, 2005). When receptors are activated by MAMPs the TIR domain initiates a signaling cascade that ends with activation of transcription factors NF- $\kappa$ B, activator protein 1 (AP-1) and interferon regulatory factor (IRF)3 (Kalliomaki and Walker, 2005). These transcriptional factors induce the expression of pro-inflammatory, anti-inflammatory and anti-apoptotic factors. Activation of TLRs also stimulates apoptotic signals independent of NF- $\kappa$ B since inhibition of

lipoprotein-induced NF- $\kappa$ B activation downstream of MyD-88 potentiates apoptosis (Aliprantis et al., 2000). Thus, eukaryotic cells respond to MAMPs by inducing a controlled inflammation and/or programmed cell death, thus increasing cellular integrity or eliminating infected cells (Collier-Hyams and Neish, 2005).

Activation of TLRs leads to increased expression of inflammatory cytokines including IL-1 $\beta$ , IL-6, IL-8 and TNF $\alpha$  (Krutzik et al., 2001) and the influx of immune cells including macrophages, intraepithelial lymphocytes and monocytes into the intestinal epithelium and adjacent lamina propria. To balance the inflammatory effects of TLR activation, increased expression of regulatory cytokines IL-10 and TGF $\beta$ , occurs in response to microbial activation of the intestinal epithelium (Haller and Jobin, 2004). There are also intrinsic regulators important in preventing excessive inflammation, which include: toll-interacting protein (Tollip) which is highly expressed in enterocytes, suppressor of cytokine signaling (SOCS), peroxisome proliferator activated receptor  $\gamma$  (PPAR $\gamma$ ) to name a few (for review see Haller and Jobin, 2004).

Activation of TLR2 also leads to a pro-apoptotic signal, which is mediated by an interaction between MyD88 and Fas-associated death domain protein (FADD) leading to activation of caspase 8. However, anti-apoptotic mediators expressed in response to NF- $\kappa$ B activation include IAP (inhibitor of apoptosis) family (cIAP1, cIAP2 and XIAP) and FLIP (Flice inhibitory protein) (Collier-Hyams and Neish, 2005). These mediators bind to various caspases involved in both the intrinsic and extrinsic pathway thus blocking the caspase cascade (Collier-Hyams and Neish, 2005). Conversely, apoptotic activity in human gingival epithelial cells induced by

*Porphyromonas gingivalis* (a Gram-negative anaerobe) is dependent on NF- $\kappa$ B activation and NF- $\kappa$ B dependent expression of FasL with apoptosis being FasL-mediated (Brozovic et al., 2006). As indicated above, bacteria induce the infiltration of IELs which express death ligands FasL and TNF $\alpha$  (Merger et al., 2002) thus indicating one mechanism by which bacteria regulate apoptosis. Cytoprotective effects of TLRs are demonstrated by the increased apoptotic activity induced by an aflegellate *Salmonella* as compared to its isogenic parent strain (Vijay-Kumar et al., 2006).

TLR expression and localization in the intestinal epithelial cell has not been characterized for all TLRs, however some groups have reported that TLR5 is only expressed in the basolateral membrane, indicating a need for flagellated bacteria such as salmonella, to cross through tight junctions to stimulate a host response (Gewirtz et al., 2001). However, others observed TLR5 expression on the apical surface (Sierro et al., 2001). TLR3, 8 and 9 are not expressed on the cell surface and thus survey only the intracellular environment. TLRs 2 and 4 have been shown to be expressed on the surface of the intestinal epithelium, however in some cases at limited levels and limited expression of accessory protein MD-2 and co-receptor CD14 (Abreu et al., 2001; Melmed et al., 2003). This limited expression is not consistent in all experiments, yet indicates a means by which the host limits the inflammatory response to the continuous barrage of bacteria. It has also been shown that TLRs 2



and 4 can relocate to the Golgi apparatus as a result of continuous stimulation (Cario et al., 2002; Hornef et al., 2002). In human colonic tissue the expression of TLRs 2 and 4 falls off as cells move out of the crypt (Furrie et al., 2005), again reducing ligand exposure.

Epithelial cells also express Nod1 and Nod2 intracellularly to monitor cytoplasm for microbial ligands. Nod1 and Nod2 proteins contain caspase activation and recruitment domains acting similar to TIR of TLRs allowing activation of signaling cascades (Inohara and Nunez, 2003). Nod1 recognizes the diaminopimelic acid component of peptidoglycan (found in Gram-negative bacteria) while Nod2 recognizes all bacterial cell walls (Kalliomaki and Walker, 2005).

The family of galectins recognizes non-self carbohydrates expressed on the surface of bacteria, viruses and parasites (Kilpatrick, 2002). Galectin-3 is expressed on the surface of macrophages, dendritic cells, and epithelial cells and has recently been identified as a pattern recognition receptor that recognizes  $\beta$ -1,2-linked oligomannans (Kohatsu et al., 2006). Recognition of oligosaccharide MAMPs by galectins induces broad host defense responses including opsonization, activation of complement, phagocytosis, inflammation and interestingly, galectin-3 has been shown to have direct fungicidal activity (Janeway and Medzhitov, 2002; Kohatsu et al., 2006). Because of the diversity of primary structures recognized (Kilpatrick, 2002) the role of galectins in host response to commensal bacteria deserves attention.

As well as induction of the inflammatory response bacterial ligands can also affect intestinal function. Interestingly, LPS is sufficient to increase alkaline phosphatase (classic marker of enterocyte maturation) expression in germfree animals (Bates et al., 2006). Furthermore, TLR activation and NF- $\kappa$ B transactivation is involved in enterocyte defensin production (Vora et al., 2004).

### 2.3.3 Host Response to Bioactive Fermentation Products

#### 2.3.3.1 *Short Chain Fatty Acids*

Short chain fatty acids (SCFAs) are produced by bacterial fermentation of undigested carbohydrate and fiber (Karaki et al., 2006) and accordingly SCFA production is highly affected by diet. Feeding gluconic acid, a fermentable carbohydrate, substantially increases SCFA production and most prominently butyric acid (Biagi et al., 2006). The SCFAs produced by breast-fed infant flora are predominantly acetic and lactic acid, while formula-fed infant microbiota produce acetic and propionic acid (Edwards et al., 1994).

Of the SCFAs produced by the intestinal microbiota, butyrate has been implicated for contributing the most to physiological effects on the host. Butyrate provides a major energy source for colonic enterocytes; however its effects on the intestinal epithelium go beyond increased metabolic activity. Enteroendocrine cells present in the intestinal epithelial layer, and mucosal mast cells present in the lamina propria, express a SCFA receptor GPR43 (Karaki et al., 2006). Activation of this receptor is responsible for the release of peptide YY (PYY) and 5-hydroxytryptamine (5-HT). PYY is produced by enteroendocrine cells in the distal ileum and colon

(Lundberg et al., 1982), and acts to slow transit in more proximal regions (Cuche et al., 2000). Butyrate also induces transcriptional up-regulation of epithelial sodium channel (ENaC) through transcription factor Sp-3, however, the mechanism by which Sp-3 is activated is not yet clear (Zeissig et al., 2007).

The effects of short chain fatty acids on cell replacement in the intestinal epithelium, particularly butyrate, have been inconsistent. While *in vitro* studies show inhibition of proliferation (Singh et al., 1997), *in vivo* studies demonstrate the induction of proliferation by butyrate (Sakata, 1987). Until recently few of the *in vivo* experiments had been based on physiologically relevant intra-colonic butyrate supply. Recent studies have clearly shown that infusion of physiological levels of butyrate into the cecum leads to an increase in cell proliferation (Kien et al., 2007). Intestintrophic effects of butyrate are likely due, at least in part, to the induction of GLP-2 expression by enteroendocrine cells (Tappenden et al., 2003). Butyrate also increases functional properties in the gut by increasing glucose absorption through increased expression of glucose transporter, GLUT-2 (Tappenden et al., 1997). Although GLP-2 increases GLUT-2 expression, butyrate can independently increase the expression of GLUT-2 in differentiated Caco-2BBE cells (Mangian et al., 2006).

Microbial production of butyrate also helps preserve intestinal viability by the regulation of heat shock proteins. Modifying the intestinal microbiota using metronidazole, which affects anaerobic bacteria, or feeding a diet lacking fermentable fiber decreases heat shock protein (HSP) expression, while infusion of butyrate increases HSP expression (Arvans et al., 2005). Although receptors for butyrate have been identified, it is likely that there are other undiscovered receptors and regulatory

pathways activated by its presence in the intestinal lumen, based on the diverse physiological effects. Whether specific receptors for other SCFA exist in the intestine is unknown.

#### 2.3.3.2 *Bioactive Amines*

A variety of bioactive amines are produced by bacterial decarboxylation of amino acids, including histamine, putrescine, tyramine and cadaverine (Allison and Macfarlane, 1989). Histamine has diverse physiological effects on the intestine including increased mucosal blood flow, goblet cell secretion and smooth muscle contraction (Gaskins, 2001). Histamine also has mitogenic effects on the proliferative crypt cells through a histamine H<sub>3</sub> receptor present on intestinal epithelia and increases proliferating cell number in the distal small intestine and colon of rats (Grandi et al., 2006).

Polyamines, spermidine, spermine, and their precursor putrescine are essential for mammalian cell growth and viability as they play an important role in DNA replication (Mitchell et al., 2002). Enterocytes can derive polyamines through absorption from the lumen and by synthesis in the cells with ornithine decarboxylase (ODC) as the rate limiting enzyme. Bacteria have a major effect in conjunction with diet on polyamine levels in cecal contents and mucosa. Noack et al. (2000) showed that the amounts of polyamines synthesized in the cecum of di-associated rats consuming a pectin diet were 84-fold higher for putrescine and four-fold higher for spermidine than the amounts consumed in the diet.

Hooper et al. (2001a) observed an increase in mRNA expression of ornithine decarboxylase antizyme (AZ) in mice mono-associated with *B. thetaiotaomicron* as compared to germfree. AZ is a key regulator of cellular polyamine levels as it targets the cellular producer of polyamines, ornithine decarboxylase, to degradation by the 26S proteasome as well as suppresses cellular uptake of polyamines (Hascilowicz et al., 2002). When cellular polyamine levels increase, AZ transcription is induced by a polyamine dependent translational frameshift. In an experiment where putrescine levels in the intestine in rats were increased to 10  $\mu$ M, ODC activity was undetectable (Gill et al., 2002).

Delzenne et al. (2000) showed that rats fed oligofructose had elevated putrescine levels that coincided with increased tissue weight. It was suggested that dietary fermentable substrates that cause increased mucosal proliferation in the colon are potentially doing so because of bacterial production of polyamines. This is supported by experiments showing that suckling rats ingesting additional spermine show increased maturation of the small intestine (Peulen et al., 2001). Polyamines also play an important role in apoptosis and cell sloughing. *In vitro*, polyamines have induced the release of soluble mitochondrial intermembrane proteins (SIMP) from liver mitochondria, which is important in activation of apoptosis (Mather and Rottenberg, 2001). Peulen et al. (2001) showed that shortly after ingestion of spermine there was increased elimination at the villus tip. Receptors for polyamines have not been identified, however, it is clear that the host tightly regulates intracellular levels and that they induce increased cell turnover.

#### 2.3.3.3 Toxic Metabolites

The intestinal microbiota produces a variety of metabolites such as  $\text{NH}_3$ ,  $\text{H}_2\text{S}$ , deconjugated bile and phenolics, which are toxic locally in the small intestine and systemically. Microbial deamination of amino acids leads to the production of ammonia, a toxic catabolite that has been considered in the etiology of inflammatory bowel disease (IBD). This was hypothesized because the microbiota of IBD patients produce more ammonia than those from healthy individuals *in vitro* (Van Nuenen et al., 2004) and because of its toxic properties. Perfusion of ammonia into the rat colon shows that the life span of colon cells is shortened by ammonia and it also induces proliferation of mucosa (Lin and Visek, 1991). Ammonia concentration in the feces can be reduced by feeding fermentable carbohydrate (Awati et al., 2006) as ammonia is used as a preferential nitrogen source by bacteria fermenting carbohydrate (Bryant, 1974). Chronic exposure of *Channa punctatus* (fish) to ammonia deteriorated villus structure and eventually led to lesions in the submucosal layer (Banerjee and Bhattacharya, 1995). Increased apoptosis associated with elevated ammonia is mediated through mitochondrial cell death as indicated by increased activation of caspase 9 (Suzuki et al., 2002). The effects of ammonia appear to be related to their toxic effects on the cell although may also be mediated by cell receptors that have yet to be discovered.

Hydrogen sulfide ( $\text{H}_2\text{S}$ ) is produced by bacteria as a result of the fermentation of sulfur-containing amino acids, as well as other dietary and host derived sulfur sources including sulfur containing mucins (Florin et al., 1991; Magee et al., 2000).  $\text{H}_2\text{S}$  reduces overall metabolic activity of the intestinal epithelial cell leading to

reduction of glutamine, butyrate and acetate oxidation (Leschelle et al., 2005). This reduction in overall metabolic activity and slowdown of all cell cycle phases leads to a reduction in mitotic activity. Although previously hypothesized, the genotoxicity of H<sub>2</sub>S was recently demonstrated, as exposure led to observable DNA damage using single-cell gel electrophoresis when DNA repair was inhibited (Attene-Ramos et al., 2006).

Lactobacilli are responsible for the majority of bile salt hydrolysis, although *Bacteroides*, *Bifidobacterium* and *Clostridium* spp. all have bile salt hydrolase activity (Gaskins, 2001). Deconjugation of bile may affect fat digestion and fat soluble vitamin absorption (Knarreborg et al., 2002) and production of lithocholic acid may be toxic to enterocytes (Knarreborg et al., 2002; Wanitschke and Ammon, 1978).

Major producers of phenolic compounds from aromatic amino acids are *Bacteroides*, *Lactobacillus*, *Clostridium* and *Bifidobacterium* species (Macfarlane and Macfarlane, 1995). Microbial production of phenolics is highly variable between individuals, which is most likely associated with microbes present (Van Nuenen et al., 2004). The metabolism of tyrosine leads to the production of several toxic compounds including p-cresol (Bakke and Midtvedt, 1970). p-cresol is absorbed and conjugated to p-cresylsulphate, which has been shown to increase the percentage of leukocytes displaying oxidative burst activity (Schauser and Larsson, 2005). It has been suggested that microbially produced phenolics reduce growth in weanling pigs and, further, that antibiotic treatment reduced urinary and fecal excretion of aromatic compounds (Yokoyama et al., 1982).

#### 2.3.3.4 Bioactive Peptides

Bacteria along with digestive enzymes break down dietary proteins converting them into peptides, some of which are bioactive. Many bioactive peptides are produced through pre-prandial fermentation by bacteria, and the extent to which they are produced *in vivo* by luminal bacteria has yet to be directly studied. Their effects on the host are broad, ranging from local effects in the intestine, such as mucin production, to systemic effects, such as reduced blood pressure. Many of the effects of bioactive peptides are receptor mediated and some of those receptors have been identified, however, there is still a lot to be defined. Interestingly some attempts to isolate the bioactive fermentation products of *L. helveticus* have lead to confusing results, as all 3 peptide fractions collected had the same bioactive effects in the host (Leblanc et al., 2002).

$\beta$ -casomorphin-7, a product of milk fermentation, causes a dramatic increase in mucin production in rat intestinal explants (Trompette et al., 2003). This bioactive peptide acts directly on the intestinal epithelium after absorption through  $\mu$ -opioid receptors present on the basolateral side of goblet cells (Zoghbi et al., 2006). This interaction likely leads to improved intestinal protection and may have implications for improved intestinal health. Similar observations have been made *in vivo*, with goblet cell hypertrophy and hyperplasia of Paneth and goblet cells being induced with the administration of yoghurt fermented with *L. casei* (Thoreux et al., 1998). Fermentation products of *L. rhamnosus* also suppress the release of prostaglandins (mediators of acute and chronic inflammation in response to IL-1 $\beta$ ) through an opioid receptor mediated response in intestinal epithelial cells (Fiander et al., 2005).



*Lactobacillus johnsonii* appears to produce a metabolite that lowers blood pressure via a histaminergic receptor present in the intestinal epithelium (Tanida et al., 2005). A microbially produced histamine-like molecule was implicated as the effects of *L. johnsonii* infusion were eliminated with the addition of a histaminergic H3-receptor antagonist.

Bioactive peptide production is not limited to milk products. Fermentation of fish proteins also leads to potentially bioactive peptides (Duarte et al., 2006). Consumption of bioactive peptides produced from the fermentation of milk and fish leads to an increased number of IgA(+) B cells in the lamina propria (Duarte et al., 2006; Perdigon et al., 1999). Fermented fish protein also leads to increased production of pro- and anti-inflammatory cytokines IL-4, IL-6, IL-10, IFN $\gamma$  and TNF $\alpha$ .

#### 2.3.4 Microbial-Origin Effector Molecules

In contrast to host pathways designed to recognize and orchestrate appropriate host responses to microbiota, bacteria can produce specific effector molecules whose function is to regulate targeted host functions to benefit the bacteria. The use of effector molecules by pathogenic bacteria has been intensely studied and serve a variety of functions in pathogen establishment, while relatively little is understood about effector molecules secreted by commensal bacteria. Enteropathogenic *E. coli*, for example, inject an effector protein into enterocytes causing actin rearrangement in the host leading to the formation of a pedestal-like structure allowing intimate microbial host contact. Commensal bacteria secrete effector molecules that have

been shown to stimulate the host to provide a nutrient source, as well as inhibit the inflammatory response (Hooper and Gordon, 2001a,b; Sougioultzis et al., 2003).

*Saccharomyces boulardii* reduces the inflammatory response by secreting a small heat stable, water soluble factor that blocks I $\kappa$ B $\alpha$  degradation thus reducing NF- $\kappa$ B binding to DNA and subsequently reduces IL-8 expression (Sougioultzis et al., 2003). *B. thetaiotaomicron* also reduces inflammatory induction by a mechanism that involves shuttling RelA (NF- $\kappa$ B) from the nucleus and targeting it to ubiquitination (Kelly et al., 2004).

*Bacteroides thetaiotaomicron* is able to “request” that the host manufacture fucosylated glycans in place of sialylated glycans because it uses fucose as a main energy source. Segmented filamentous bacteria and a normal microflora are also able to induce the expression of this glycolipid (Umesaki et al., 1995). The mechanism by which fucosylated glycan production is upregulated is not yet clear, although the signaling molecule produced by *B. thetaiotaomicron* has been found and termed control of signal production (csp) and it has been established that transcription of fucosyltransferase genes are activated (Hooper and Gordon, 2001b).

## **2.4 Conclusion**

Science has gotten as far as recognizing what bacterial genes are required to activate fucosylated glycan production and identified specific microbial components required to activate inflammatory responses, yet many of the physiological effects induced by microbial colonization are still unclear. When developing strategies to create an “optimal” intestinal microbiota that supports maximization of health and

growth, we must consider all of the mechanisms by which bacteria affect the host. It is apparent that microbial effects are tremendous in scope and it is likely that the mechanisms of host-microbial interactions that have been described represent only a fraction of the mechanisms by which commensal bacteria affect their host. Elucidation of mechanisms will continue to be challenged by the complexity of the microbiota as well as the variety of fermentation products. Identifying specific host response pathways and outcomes, which can subsequently be modulated, either thru microbial community modulation or pharmacological development offers substantial opportunity for improved animal health and performance.

### 3.0 ENTEROCYTE PROLIFERATION AND APOPTOSIS IN THE DISTAL SMALL INTESTINE IS INFLUENCED BY THE COMPOSITION OF COLONIZING COMMENSAL BACTERIA IN THE NEONATAL GNOTOBIOTIC PIG

#### 3.1 Abstract

We have previously observed marked differences in small intestinal morphology, including changes in crypt depth and villus height, following inoculation of germfree pigs with different bacterial species. In an attempt to identify the mechanisms governing changes in villus morphology associated with bacterial colonization two gnotobiotic experiments were performed. In each experiment 16 piglets were allocated to 4 treatment groups including germfree (GF), mono-association with *Lactobacillus fermentum* (LF) or *Escherichia coli* (EC) or conventionalized with sow feces (CV). Piglets were reared under gnotobiotic conditions until 14 days of age at which time whole intestinal tissue and enterocytes were collected for histological, gene expression and protein analysis. Proliferating cell nuclear antigen (PCNA), tumor necrosis factor (TNF) $\alpha$ , Fas ligand (FasL), caspase 3 (casp3) and toll-like receptors (TLR) 2, 4 and 9 expression were measured by quantitative PCR (qPCR). Activated casp3 was measured by Western-blot. Increased abundance ( $P<0.05$ ) of activated casp3 and transcripts encoding PCNA, TNF $\alpha$  and FasL was observed in CV and EC treatment groups as compared to GF and LF. TLR2 expression was increased in the CV treatment and tended to be higher in EC relative to LF and GF. Results indicate that conventional bacteria and *E. coli*

but not *L. fermentum* increase overall cell turnover by stimulating increased apoptosis through the expression of Fas ligand and TNF $\alpha$  and by increasing cell proliferation. The differential regulation of TLR expression indicates microbially induced changes may be mediated in part by these receptors. Induced expression of inflammatory mediators and activation of apoptosis through death receptors appears to play a significant role in microbially induced enterocyte turnover.

### 3.2 Introduction

At birth the gastrointestinal tract of the pig quickly changes from a sterile state, to one harbouring a diverse and abundant microbial population. Coliforms are the first dominant organism, but are quickly replaced by *Clostridium* and *Lactobacillus* species (Swords et al., 1993). In the early postnatal period, concurrent to changes in microbial composition, the gastrointestinal tract undergoes dramatic changes. Early structural changes include rapid increases in intestinal length, mucosal weight and overall surface area occurring in conjunction with changes in digestive brush border enzyme functions (Zhang et al., 1997). In the first week there is an increase in villus height (Smith and Jarvis, 1978), which is followed by a gradual reduction most prominently observed at weaning. At birth the mucosal immune system is also relatively naïve and void of lymphoid cells, but over the first few weeks the intestine is rapidly colonized with lymphoid cells and Peyer's patches begin to organize (Stokes et al., 2004).

It has been established that the host requires an indigenous microbial population for many aspects of gut development including glycoconjugate repertoire

(Hooper, 2004), villus capillary networks (Stappenbeck et al., 2002), development of Peyer's patches (Shirkey et al., 2006) and maintenance of intestinal epithelial homeostasis (Rakoff-Nahoum et al., 2004). It has also been shown that the composition of the early microbial population has long term effects on health (Kalliomaki et al., 2001), thus driving the effort to understand the roles that different bacteria play in intestinal development. Because of the complex nature of the intestinal microbiota (Savage, 1977) identifying the roles of individual organisms in regulating host physiology has been difficult. However, with the use of strict rearing conditions and sterilized diets, germfree animals can be generated and purposefully colonized with specific bacteria. Examining the expression profile of mice mono-associated with different prominent pre-weaning ileal microbiota, Hooper et al. (2001a) observed that host responses are specific to colonizing bacterial species. To investigate the effects of early colonizing bacteria on intestinal development we established a gnotobiotic model in the pig. This model allows for the study of developmental processes that require interaction between the numerous cell types found in the intestine and provides a valuable model for human neonatal research. In our first experiments we reported marked effects of microbial status on distal small intestinal morphology (Shirkey et al., 2006).

The replacement of the intestinal epithelium is a process that occurs rapidly with cells proliferating in the crypts, maturing along the villus axis and being exfoliated from the villus tip with a turnover rate of 3 to 5 days (Potten et al., 1997). While this desquamation and renewal limits the opportunity for pathogenic bacteria from colonizing (Potten and Loeffler, 1990) it comes at a substantial metabolic cost.

With amino acids being used as both building blocks for endogenous secretions and as an energy source, fractional rates of protein synthesis can exceed 100% per day (Nyachoti et al., 2000), and estimates of endogenous ileal protein losses ranges from 10.5 to 17.1 g/kg dry matter intake (For review see Jansman et al., 2002).

There are numerous ways in which microbial populations have been shown to affect villus morphology, including the production of volatile fatty acids (VFA), most prominently butyrate (Sakata, 1987). Other microbial metabolites including ammonia and sulphur compounds have been shown to have damaging effects on the survival of epithelial cells. One specific means by which the host recognizes and responds to bacteria is through toll-like receptors (TLR). TLR2, TLR4, TLR5 and TLR9 have been shown to respond to Gram-positive bacteria, Gram-negative bacteria, bacterial flagella and unmethylated CpG DNA pathogen associated molecular patterns (PAMPs) respectively (Medzhitov, 2001). The activation of TLR by their specific ligands activate a host response most prominently depicted by the activation of pro-inflammatory transcription factor NF- $\kappa$ B and the production of classic pro-inflammatory cytokines, including TNF $\alpha$ , IL-1, IL-6 and IL-8 (Krutzik et al., 2001). *In vitro* work has shown that intestinal epithelial cells show a growth response to activation with lipopolysaccharide (LPS) that is mediated by TNF $\alpha$  (Ruemmele et al., 2002) and *in vivo* that TLRs are essential in intestinal homeostasis (Rakoff-Nahoum et al., 2004). There is limited research on *in vivo* expression of TLR's in the small intestine, and even less on TLR expression in the pig (Guzylack-Piriou et al., 2006; Raymond and Wilkie, 2005; Shinkai et al., 2006).

We hypothesize that changes in commensal intestinal microbial composition may have significant consequences with respect to enterocyte. To test this hypothesis we examined intestinal morphology and measured indicators of cell proliferation and apoptosis in gnotobiotic pigs mono-associated with model bacterial species known to colonize the neonatal digestive tract and representing the major Gram-negative and Gram-positive cell wall divisions; namely *Escherichia coli* and *Lactobacillus fermentum*. Because of their potential role in this response we examined the expression of TLRs 2, 4 and 9 as well as death ligand, FasL, and inflammatory cytokine TNF $\alpha$ .

### **3.3 Materials and Methods**

#### **3.3.1 Derivation and Rearing of Gnotobiotic Piglets**

Experimental protocols were approved by the Animal Care Committee of the University of Saskatchewan and were performed in accordance with recommendations of the Canadian Council on Animal Care (1993).

Derivation of germfree piglets and preparation of isolators was performed as described in a previous gnotobiotic study (Shirkey et al., 2006). Briefly, 16 piglets (>800g BW, Large White X White Duroc) were derived via caesarian section from 2 sows and transferred into a HEPA-filtered transfer unit through a betadine filled dip tank. The pigs were then transported to the gnotobiotic facility where they were fed, weighed, and allocated to one of four treatment groups balanced for litter of origin and weight. For the first 24 hours the piglets were fed a 1:1 mixture of sterile porcine



serum (Gibco, Burlington, ON) and canned infant formula (Similac®, Abbot Laboratories, Abbott Park, IL) to simulate immunoglobulins normally obtained from colostrum. Subsequently they were ad libitum-fed a 2:1 Similac®:water mixture 3 times daily for the remainder of the experiment. Similac® is composed of dry skim milk, lactose, oil (safflower, sunflower, coconut and soy oil) and whey protein concentrate and contains 136 Cal, 2.8 g protein, 7.3 g fat, 14.6 g carbohydrate per 100 mL.

### 3.3.2 Experimental Design

In two replicate experiments 16 piglets were allocated to 4 treatment groups including pigs that remained germfree, those that were mono-associated with non-pathogenic *Escherichia coli* (EC) or with *Lactobacillus fermentum* (LF) or were conventionalized with sow feces along with doses of EC and LF (CV). The inoculating feces sample was freshly collected, separately for each experiment, from a clinically healthy sow that had recently farrowed in a research swine herd (Prairie Swine Centre Inc., Saskatoon, SK). *E. coli* and *L. fermentum* inoculants were isolated from the cecum of a healthy pig. 18 hour cultures of *E. coli* in tryptic soy broth (BBL, Sparks, MD) and *L. fermentum* in Lactobacilli MRS broth (Difco Laboratories, Sparks, MD) were prepared, sub sampled for enumeration and used to orally inoculate piglets at 24 and 30 hours in their feed. By mixing into their feed and bottle feeding mono-associated piglets received 2 ml of their appropriate inoculants and CV piglets received 1g of feces mixed with 1 ml of sterile peptone and 2 ml of LF and EC inoculants, making a total volume of 6 ml.

### 3.3.3 Tissue Collection

At 14 days of age piglets were removed from isolators, weighed and killed by submersion in CO<sub>2</sub> and exsanguination at which time digesta from the jejunum, ileum and cecum were taken to confirm treatment status and to enumerate culturable bacteria. The small intestine was carefully dissected from the mesentery and length recorded. A 2 cm segment obtained at 75% of the small intestinal length was placed in 10% buffered formalin for 24 hours and subsequently transferred to 70% ethanol before embedding in paraffin and staining with hematoxylin and eosin for histological analysis. Two 10 cm segments for mRNA and protein analysis were obtained at 75% of the small intestinal length, snap frozen and stored at -80°C.

### 3.3.4 Enterocyte Isolation

Enterocytes were obtained using the distended intestinal sac method modified from Fan et al. (2001). Briefly an 80 cm segment immediately proximal to 75% of the intestinal length was initially rinsed with a pre-incubation buffer (PBS with 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiothreitol (DTT), pH 7.4). The distal end was then clamped and the segment filled with isolation buffer (PBS with 1.5 mM ethylene diamine tetra acetate (EDTA), 0.2 mM PMSF, 0.5 mM DTT, pH 7.4) until fully distended. The proximal end was then clamped and the distended segment placed in a saline bath at 37 °C for 30 minutes. The contents of the segment, including isolated cells, were then emptied into a conical tube and centrifuged at 400 g for 3 min at 4 °C. The supernatant was discarded and the cell pellet was resuspended in ice cold PBS, repelleted and snap frozen in liquid nitrogen. The

isolation procedure was repeated 6 times to obtain cells along the entire villus-crypt axis. Cell recovery was confirmed by evaluation of hematoxylin and eosin stained cross sectional tissues taken through the course of enterocyte isolation procedure. Enterocyte isolates were either pooled within pig (1 sample per pig) or pooled by location within treatment (1 location per treatment).

### 3.3.5 Confirmation of Treatment Status and Microbial Enumeration

Swabs were taken peri-anally from GF pigs daily and placed in brain heart infusion broth (Difco Laboratories, Sparks, MD) with 0.5% cysteine hydrochloride to confirm bacteria free status through the course of the experiment. Before harvesting tissues, digesta from the ileum and cecum were collected in sterile peptone, diluted  $10^{-1}$ ,  $10^{-2}$  and  $10^{-4}$  and plated using the Autoplate<sup>®</sup> 4000 (Spiral Biotech Inc., Bethesda, MD) onto blood agar base (BBL, Sparks, MD) with 5% defibrinated sheep blood and cultured aerobically and anaerobically. Contents of LF, EC and CV pigs were also plated on MacConkey's agar (Difco Laboratories) cultured aerobically, and on MRS agar (BBL) cultured anaerobically. The plates were incubated for 24 to 48 hours at 37 °C prior to assessment of colony morphology and enumeration of ileal contents. Selected colonies from EC and LF treatments were Gram-stained to confirm cell wall type and morphology. Colonies or cells with morphology inconsistent with the inoculated organism were isolated and identified by sequencing of chaperonin 60 UT sequence (Hill et al., 2002).

### 3.3.6 Intestinal morphology

Using hematoxylin and eosin stained cross sections, villus height and crypt depth were measured by a blinded observer in 10, well oriented, villi for each pig using an Axiostar plus light microscope (Carl Zeiss Canada Ltd., Toronto, ON) and analyzed using AxioVision 3.1 measurement software (Carl Zeiss Canada Ltd.).

### 3.3.7 Caspase 3 Western Blot

A Western blot assay was performed on enterocyte homogenates. 200 mg of tissue was homogenized in 5 mL of lysis buffer with the Brinkman Homogenizer at low speed for 1 minute. Lysis buffer contained 50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1% Triton X-100, 500 IU/ml aprotinin, and 1 mM PMSF (pH 7.5). The homogenate was centrifuged for 5 minutes at 3000g and 1 mL of supernatant transferred to a microfuge tube and centrifuged again for 3 min at 17,000 g. A small aliquot of the supernatant was diluted 1:100 with ddH<sub>2</sub>O to measure protein content using the Bio-Rad protein microassay procedure (Bio-Rad). The remainder of the supernatant was frozen at -80 °C until the blot was performed. 15 µg of protein was diluted in loading buffer (4:1, loading buffer: sample), and heated to 97 °C for 6 minutes then loaded into an 18% acrylamide pre-cast gel (Assorted Ready Gel<sup>®</sup>, Bio-Rad) and run at 150V until the dye front reached the bottom of the gel. A positive control of human casp3 (active subunit 17kDa) recombinant protein (Catalog number: CC119, Chemicon International, Temecula, CA) and a standard of 15 µg of pooled protein from CV enterocytes was included in each gel. Protein was transferred to a 0.2 µm polyvinylidene difluoride (PVDF) membrane (Immuno-

Blot<sup>TM</sup>, Bio-Rad) at 100V for 1 hour using the Mini Trans-Blot<sup>®</sup> Electrophoretic Transfer Cell. PVDF membrane was then rinsed with ddH<sub>2</sub>O (3x5min), then blocked with 5% non-fat dry milk (Blotting Grade Non-Fat Dry Milk, Bio-Rad) in PBS-T for 2 hours. The membrane was then washed in PBS-T (3x5min) before applying the primary antibody, rabbit-anti-active human casp3 polyclonal antibody (Chemicon International) diluted at 1:2000 in PBS-T (0.1% BSA) for 90 min, washed again in PBS-T (6x5min) before the secondary antibody, goat-anti-rabbit conjugated with horseradish peroxidase (Opti-4CN<sup>TM</sup> Substrate Kit) diluted at 1:8000 in PBS-T (0.1% BSA), was applied for 90 min. Colorimetric detection was performed using Opti-4CN Substrate Kit (Bio-Rad). Band intensity was measured using Scion Image software (Frederick, MD) and band intensity was standardized between gels to the pooled CV homogenate. Preliminary tests using a dilution series of CV homogenate showed a linear relationship between casp3 concentration and optical density.

### 3.3.8 PCNA Immunohistochemistry

Formalin fixed, paraffin embedded cross sections from the 75% location of SI length were subjected to immunohistochemical staining for PCNA using a streptavidin-biotin complex technique as previously described (Ellis et al., 1998; Haines and Chelack, 1991). Immunohistochemical staining was performed by Prairie Diagnostic Services (Saskatoon, SK) using a rabbit polyclonal antibody for human Ki-67 (PCNA) (abcam Inc., Cambridge, MA) diluted 1:100, and secondary antibody goat anti rabbit (Vector lab, Burlingame, CA) diluted 1:400. Tissues were observed using an Axiostar plus light microscope (Carl Zeiss Canada Ltd.).

### 3.3.9 Gene Expression Analysis

Whole intestinal segments and enterocyte pellets were ground using a mortar and pestle and total RNA was extracted from 20-30 mg of tissue using an RNeasy<sup>®</sup> Mini Kit (Qiagen, Mississauga, ON). Genomic DNA was removed from RNA using RNase-free DNase (Qiagen). RNA was quantified by optical density at 260/280 nm using a spectrophotometer (Ultrospec<sup>®</sup> 2000, Pharmacia Biotech, Baie d'Urfe, PQ) and 1 µg of RNA was used to generate first strand cDNA using i-Script<sup>®</sup> cDNA Synthesis Kit (Bio-Rad). Porcine PCNA sequence was not available thus a segment of the gene was sequenced by amplification with primers designed based on human sequence (GenBank accession BC062439). The resulting sequence (GenBank accession DQ473295) showed 93% identity to human PCNA transcript. Primers (Table 3.1) were designed for TNF $\alpha$ , FasL, PCNA, casp3, CD3 $\epsilon$  and TLRs 2, 4 and 9, and where possible were designed to land on known or predicted intron/exon splice regions. Primers for TLRs were not intron spanning due to the lack of introns. Primers were designed using Oligo 6 (Molecular Biology Insights, Inc., Cascade, CO) and Beacon Designer (PREMIER Biosoft International, Palo Alto, CA) software. Transcript abundance was measured by qPCR using SYBR Green<sup>®</sup> detection (iCycler iQ Real-Time PCR detection system, Bio-Rad). CD3 $\epsilon$  and PCNA expression were measured in villus fractions along the villus axis to indicate whether IEL's were the source of proliferative activity. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as the internal control. Relative gene expression was corrected for PCR efficiency as according to Pfaffl (2001).

Table 3.1 Quantitative real-time PCR primers for all genes.

Gene	Forward (5'-3')	Reverse (5'-3')
GAPDH	GTTTGTGATGGGCGTGAAC	ATGGACCGTGGTCATGAGT
PCNA	TACGCTAAGGGCAGAAGATAATG	CTGAGATCTCGGCATATACGTG
TNF $\alpha$	TGGCCCAAGGACTCAGATCAT	TCGGCTTTGACATTGGCTACA
FasL	AAGAAGAAGAGGGACCACAATG	CTTTGGCTGGCAGACTCTCT
Casp3	ACCCAAACTTTTCATAATTCA	ACCAGGTGCTGTAGAATATGC
TLR2	GACTGGCCGGAGAACTACCT	TGAGCAGGAGCAGCAGGAA
TLR4	TCACTACAGAGACTTCATTCCCG	GGTCTGGGCAATCTCATACTCAA
TLR9	GTGGAAGTGTGTTTGGCATC	CACAGCACTCTGAGCTTTGT
CD3 $\epsilon$	TTATACCTGCACAGTCGGAGAG	CGGATGGGCTCATAGTCTGG

### 3.3.10 Statistical Analysis

Data were analyzed separately for each experiment as a one-way ANOVA using the general linear model procedure (SPSS program, Chicago, IL). Treatment means were separated using least squares difference with significance of  $P < 0.05$ . Correlation coefficients between parameters were determined by the Pearson correlation procedure (SPSS).

## 3.4 Results

### 3.4.1 Microbial Colonization

Swabs taken prior to the removal of pigs from isolators and culture of ileal and cecal contents indicated that in both experiments all treatment groups were maintained successfully as germfree or mono-associated with the exception of the LF treatment group in experiment 2. This group was contaminated with a *Klebsiella pneumoniae* making the treatment group di-associated, and subsequently denoted as LFKP. The distinct colony morphology associated with *K. pneumoniae* was not observed on EC or GF MacConkey plates indicating that this was the only contaminated treatment. Culture of ileal contents from EC pigs on MacConkey agar indicated colonization ranges of 8.5 to 9.0 and 8.0 to 9.0 log cfu/g of contents in experiments 1 and 2, respectively. In LF pigs, ileal counts on MRS agar ranged from 6.1 to 7.5 log cfu/g of contents. In LFKP, total anaerobes enumerated on blood agar ranged between 6.9 to 9.2 log cfu/g consistent with growth of *Klebsiella pneumoniae* whereas lactobacilli counts on MRS agar ranged from 6.4 to 6.9 log cfu/g. Culture of



CV ileal contents resulted in diverse colony morphologies with aerobic blood agar counts ranging between 6.3 to 8.2 and 7.0 to 8.2 log cfu/g, anaerobic blood agar counts of 6.2 to 8.8 and 7.2 to 9.0 log cfu/g for experiments 1 and 2, respectively. CV inoculants were obtained fresh from different sows thus although colonization levels were similar the composition of colonizing bacteria in CV treatment groups were likely different.

### 3.4.2 Villus Morphology

Conventionalisation reduced villus height ( $P<0.05$ ) and increased ( $P<0.05$ ) crypt depth relative to GF (Fig. 3.1). Intestinal morphology in LF pigs was similar to GF whereas villus height and crypt depth in EC pigs was intermediate between CV and LF treatments. *K. pneumoniae* contamination of the LF group in experiment 2 significantly reduced ( $P<0.05$ ) villus height and increased crypt depth as compared to GF in contrast to LF mono-association in experiment 1.

### 3.4.3 Western Blot for Active Caspase 3

Compared to GF activated casp3 abundance in enterocytes was increased ( $P<0.05$ ) by conventional microbiota in both experiments (Fig. 3.2). In EC activated casp3 abundance was intermediate to CV and GF, while LF elicited a similar response as GF. LFKP, similar to CV, induced greater levels ( $P<0.05$ ) of activated

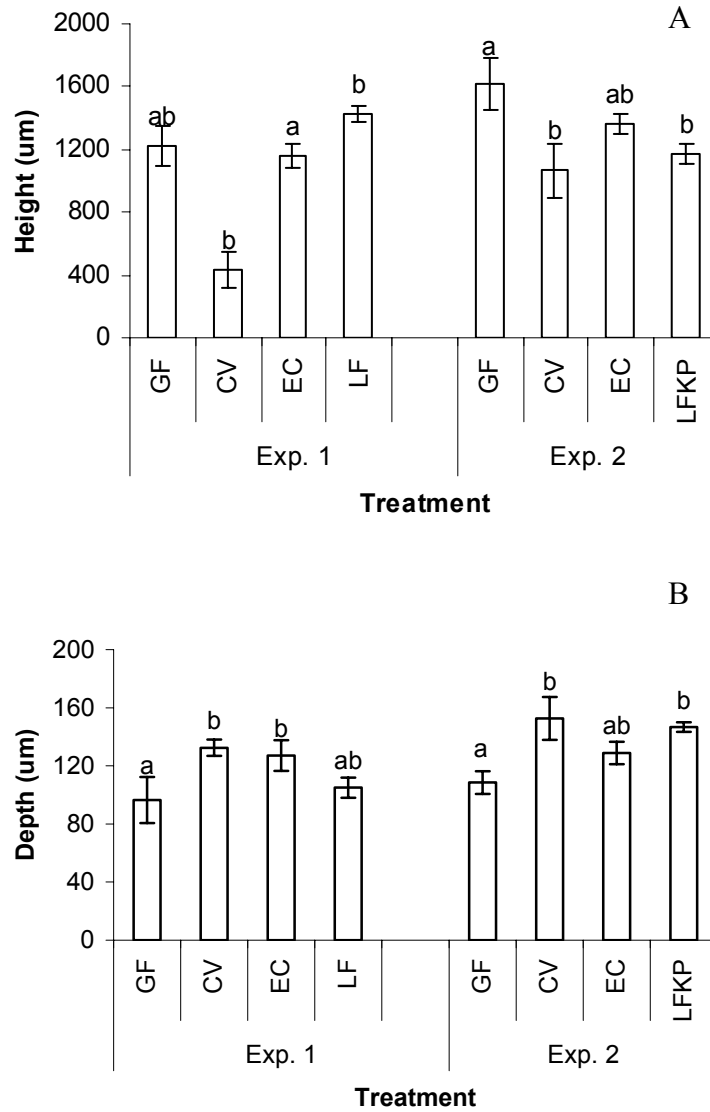


Figure 3.1 Mean villus height (A) and crypt depth (B) at the 75% location of the small intestine as measured from the pyloric sphincter for Experiments 1 and 2 in germfree (GF), *L. fermentum* (LF) or *E. coli* (EC) mono-associated, LF and *K. pneumonia* (LFKP) di-associated and conventionalized (CV) pigs. Vertical bars represent SE. <sup>a,b,c</sup>Means within the same experiment with a different superscript are different ( $P < 0.05$ ).

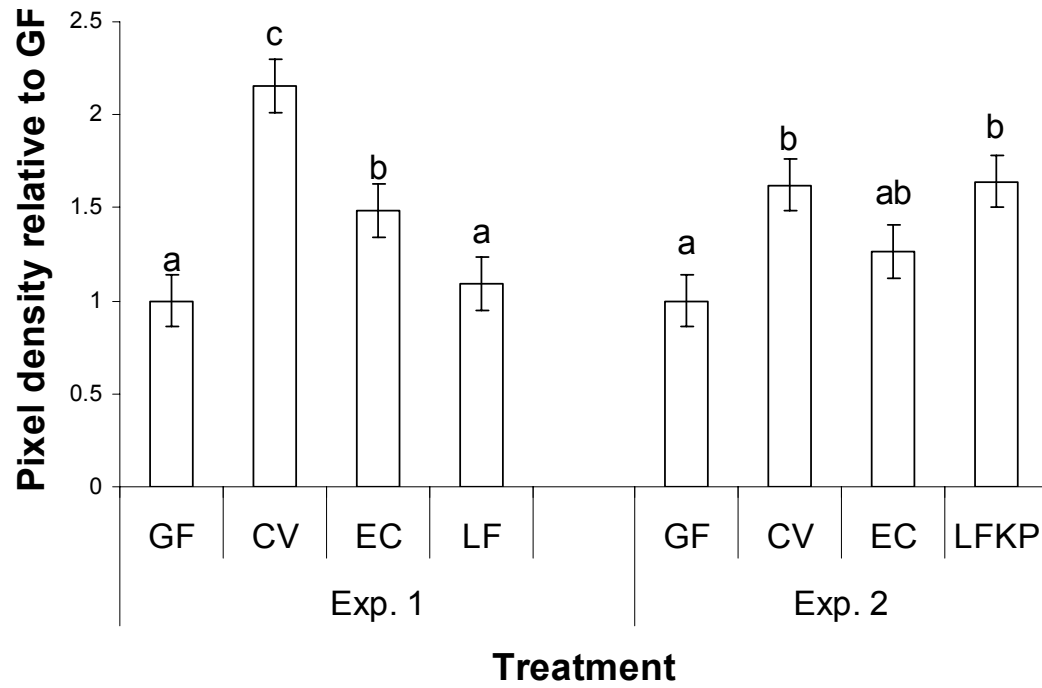


Figure 3.2 Activated caspase 3 abundance in isolated small intestinal enterocytes for experiments 1 and 2 in germfree (GF), *L. fermentum* (LF) or *E. coli* (EC) mono-associated, LF and *K. pneumonia* (LFKP) di-associated and conventionalized (CV) pigs. Vertical bars represent SE. <sup>a,b,c</sup>Means within the same experiment with a different superscript are different ( $P < 0.05$ ).

casp3 as compared to GF. Casp3 gene expression in enterocytes was unaffected by treatment in both experiments (Table 3.2). Active casp3 was negatively correlated ( $P<0.01$ ) to villus height ( $R^2 = -0.732$ ) and positively correlated ( $P<0.02$ ) with FasL (Exp. 1,  $R^2 = 0.880$ ; Exp. 2:  $R^2 = 0.587$ ).

#### 3.4.4 PCNA Immunohistochemistry

The majority of cells positive for PCNA staining in the intestinal epithelium were in the villus crypt (Fig. 3.3 and 3.4). There were few positive cells within the epithelial cell layer, whereas in the submucosa, lamina propria and particularly the Peyer's patches (Fig. 3.5) numerous PCNA-positive cells were evident.

#### 3.4.5 PCNA Expression

PCNA was measured in isolated enterocytes as an indicator of proliferative activity. In experiment 1 conventionalisation significantly increased PCNA transcript abundance, as compared to GF (Table 3.2), while LF pigs were similar to GF and EC pigs had intermediate ( $P<0.05$ ) PCNA expression falling between GF/LF and CV. In experiment 2 the same trends were seen although differences were not significant. PCNA gene expression was also positively correlated ( $P<0.01$ ) with crypt depth ( $R^2=0.602$ ) measured at 75% of SI length.

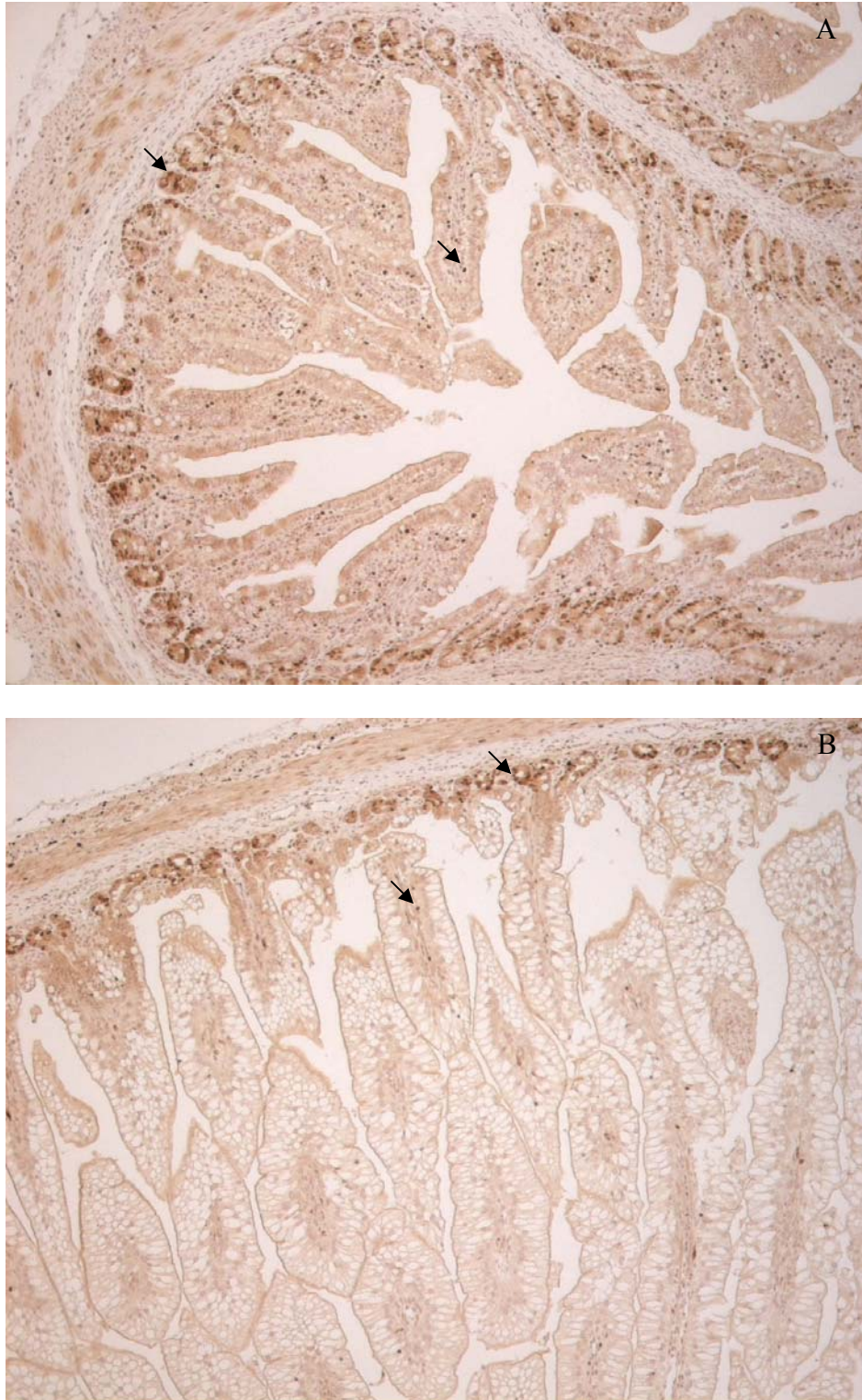


Figure 3.3 Representative light micrograph of an intestinal cross section taken at 75% of the length of the small intestine from a conventionalized (A) or germfree (B) pig, stained for PCNA. Black arrows show selected PCNA positive cells. 100X magnification.



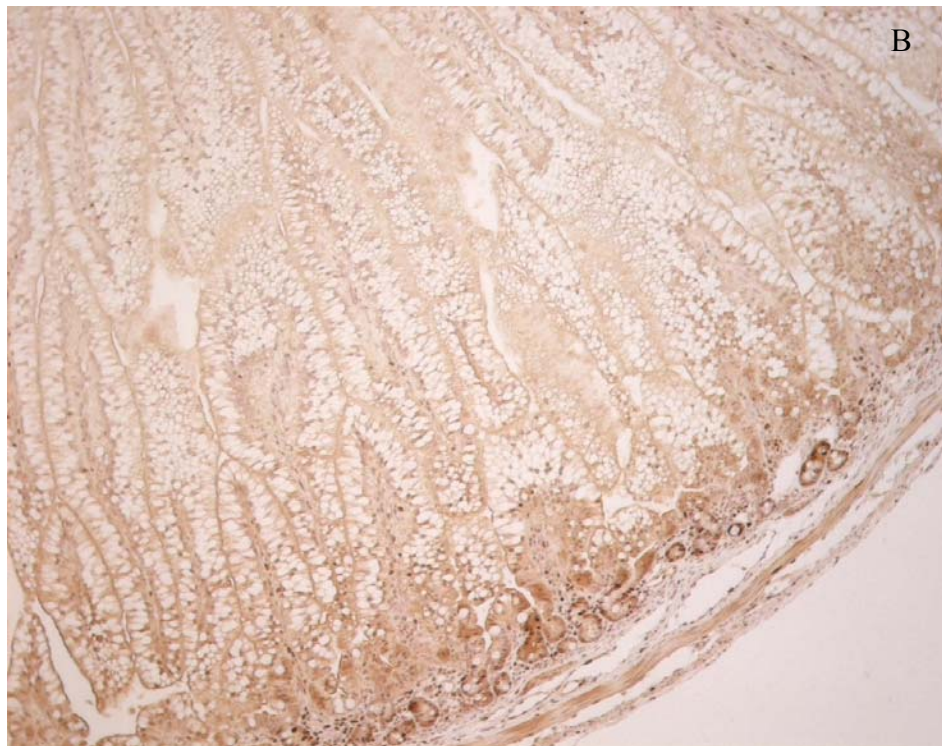
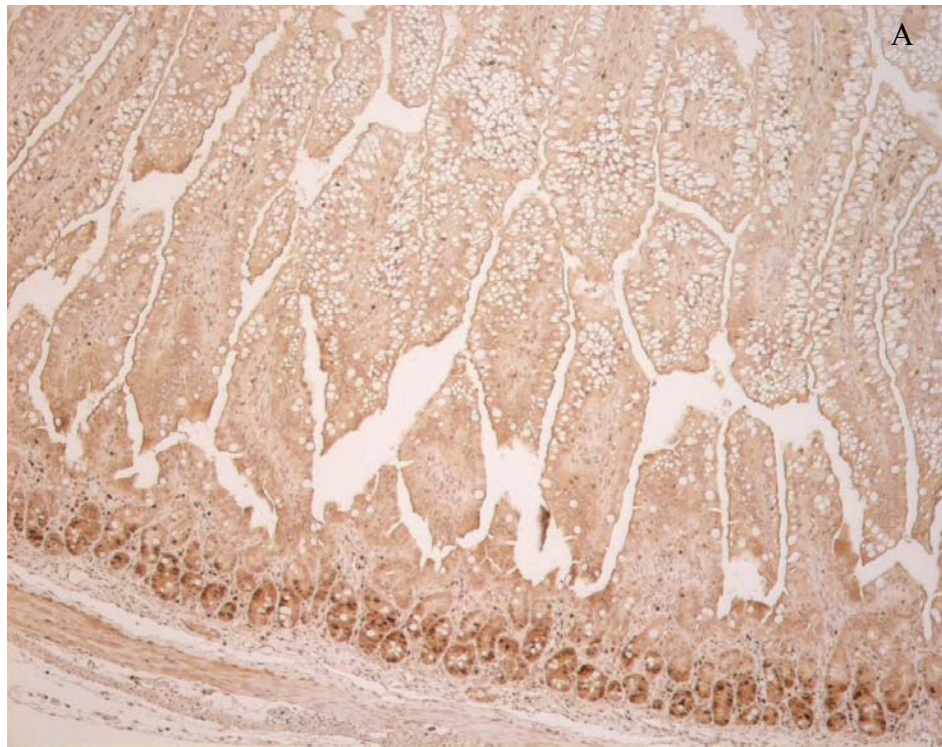


Figure 3.4 Representative light micrograph of an intestinal cross section taken at 75% of the length of the small intestine from a pig mono-associated with *E. coli* (A) or *L. fermentum* (B) stained for PCNA. 100X magnification.



Figure 3.5 Light micrograph of an intestinal cross section taken at 75% of the length of the small intestine from a conventional pig containing a Peyer's patch showing numerous PCNA-positive cells. 100X magnification.

Table 3.2 Mean fold change for TNF $\alpha$ , FasL, PCNA, Casp3, TLR2, TLR4, TLR9 relative to GF in enterocytes isolated from an 80cm segment between 50 and 75% of small intestinal length in 14 day old gnotobiotic pigs.

Treatment Group	Gene of interest						
	PCNA	FasL	TNF $\alpha$	Casp3	TLR2	TLR4	TLR9
Experiment 1							
GF	1.03 <sup>a</sup>	1.02 <sup>a</sup>	1.01 <sup>a</sup>	1.03	1.24 <sup>a</sup>	1.15 <sup>a</sup>	1.03 <sup>a</sup>
CV	2.42 <sup>c</sup>	5.66 <sup>c</sup>	3.59 <sup>b</sup>	1.17	3.03 <sup>b</sup>	1.62 <sup>ab</sup>	1.84 <sup>ab</sup>
EC	1.72 <sup>b</sup>	3.49 <sup>b</sup>	3.52 <sup>b</sup>	1.07	1.72 <sup>ab</sup>	3.43 <sup>b</sup>	3.68 <sup>b</sup>
LF	0.98 <sup>a</sup>	0.78 <sup>a</sup>	1.01 <sup>a</sup>	0.86	1.08 <sup>a</sup>	1.02 <sup>a</sup>	0.74 <sup>a</sup>
Pooled SEM	0.176	0.629	0.494	0.132	0.475	0.568	0.703
Experiment 2							
GF	1.01	1.07 <sup>a</sup>	1.13 <sup>a</sup>	1.01	1.23 <sup>a</sup>	1.10 <sup>a</sup>	1.03 <sup>a</sup>
CV	1.94	5.71 <sup>c</sup>	3.62 <sup>b</sup>	1.04	3.74 <sup>ab</sup>	4.69 <sup>b</sup>	7.1 <sup>b</sup>
EC	1.95	2.75 <sup>b</sup>	3.34 <sup>b</sup>	1.03	4.89 <sup>b</sup>	2.54 <sup>ab</sup>	3.1 <sup>ab</sup>
LFKP	1.42	3.59 <sup>b</sup>	2.71 <sup>ab</sup>	0.91	3.72 <sup>ab</sup>	1.52 <sup>a</sup>	1.8 <sup>a</sup>
Pooled SEM	0.533	0.524	0.537	0.144	0.906	0.935	1.359

<sup>a,b,c</sup> Means within the same column and experiment with different superscripts are different ( $P < 0.05$ )



Table 3.3 Mean fold change for TNF $\alpha$ , FasL, TLR2, TLR4, TLR9 relative to GF in whole intestinal tissue collected at the location identified as 75% of SI length as in 14 day old gnotobiotic pigs.

Treatment Group	Gene of interest				
	FasL	TNF $\alpha$	TLR2	TLR4	TLR9
Experiment 1					
GF	1.02 <sup>a</sup>	1.03 <sup>a</sup>	1.10 <sup>a</sup>	1.02 <sup>a</sup>	1.04
CV	3.69 <sup>b</sup>	2.69 <sup>b</sup>	4.72 <sup>b</sup>	2.30 <sup>b</sup>	1.77
EC	1.97 <sup>a</sup>	2.12 <sup>b</sup>	2.16 <sup>a</sup>	1.59 <sup>ab</sup>	1.53
LF	0.88 <sup>a</sup>	1.14 <sup>a</sup>	1.00 <sup>a</sup>	1.10 <sup>a</sup>	0.80
Pooled SEM	0.629	0.494	0.475	0.261	0.275
Experiment 2					
GF	1.04 <sup>a</sup>	1.04	1.01 <sup>a</sup>	1.00	1.03
CV	2.73 <sup>b</sup>	1.70	2.46 <sup>b</sup>	1.20	1.10
EC	1.46 <sup>a</sup>	1.38	2.04 <sup>ab</sup>	1.08	0.81
LFPK	2.00 <sup>ab</sup>	1.40	2.47 <sup>b</sup>	0.89	0.88
Pooled SEM	0.326	0.176	0.360	0.190	0.175

<sup>a,b,c</sup> Means within the same column and experiment with different superscripts are different ( $P < 0.05$ )

#### 3.4.6 TLR Expression

Expression of TLRs 2, 4 and 9 was observed in both isolated enterocytes (Table 3.2) and whole intestinal tissue (Table 3.3). Generally, expression of TLRs was increased in CV and EC pigs relative to GF whether examined in enterocytes or whole tissue. LF did not show increased TLR expression relative to GF in enterocytes or whole tissue. In the LFKP group, TLR expression was similar to EC and CV. With the exception of TLR2, increases in TLR expression associated with bacterial colonization were higher in enterocytes versus whole tissue.

#### 3.4.7 TNF $\alpha$ Expression

Expression of inflammatory cytokine TNF $\alpha$  was induced over 3-fold ( $P < 0.05$ ) in enterocyte isolates by both CV and EC as compared to GF in both experiments (Table 3.2). LFKP induced TNF $\alpha$  expression that was intermediate to GF and CV but not significantly different from either, while LF pigs had TNF $\alpha$  expression equal to GF. The response observed in homogenates of whole tissue (Table 3.3) showed a similar, although reduced response compared to enterocyte isolates. TNF $\alpha$  transcript abundance was positively correlated (Exp. 1:  $R^2 = 0.820$  Exp. 2:  $R^2 = 0.666$  ( $P < 0.01$ )) with PCNA transcript abundance.

#### 3.4.8 Fas Ligand Expression

Transcript abundance of death ligand, FasL, in isolated enterocytes was increased ( $P<0.05$ ) over 5-fold in CV, intermediate in EC and LFKP ( $P<0.05$ ) and unaffected by LF as compared to GF (Table 3.2). The response was similar in whole tissue although less pronounced with the only significant increase in expression being observed in CV pigs in both experiments (Table 3.3).

#### 3.4.9 CD3 $\epsilon$ and PCNA Expression along the Villus Axis

The expression of T-cell marker CD3 $\epsilon$  and PCNA in isolated enterocytes from conventionalized pigs pooled according to the villus tip to crypt axis is shown in Figure 3.6. PCNA expression increased from villus tip to crypt, while CD3 $\epsilon$  expression was consistent through all fractions indicating that T-cell proliferation did not explain increased proliferative activity in CV pigs.

### 3.5 Discussion

The replacement of the intestinal epithelium plays an important role in keeping the abundant intestinal microflora at bay (Oswald, 2006b), but high cell turnover comes at a significant metabolic cost (Le Floc'h and Seve, 2000). In this experiment we demonstrate that the commensal intestinal microbiota play a role in the regulation of enterocyte turnover rate in the distal intestine and that host response is dependent on the colonizing organism. Previously we have observed that intestinal structure as well as pro-inflammatory cytokine gene expression is dependent on

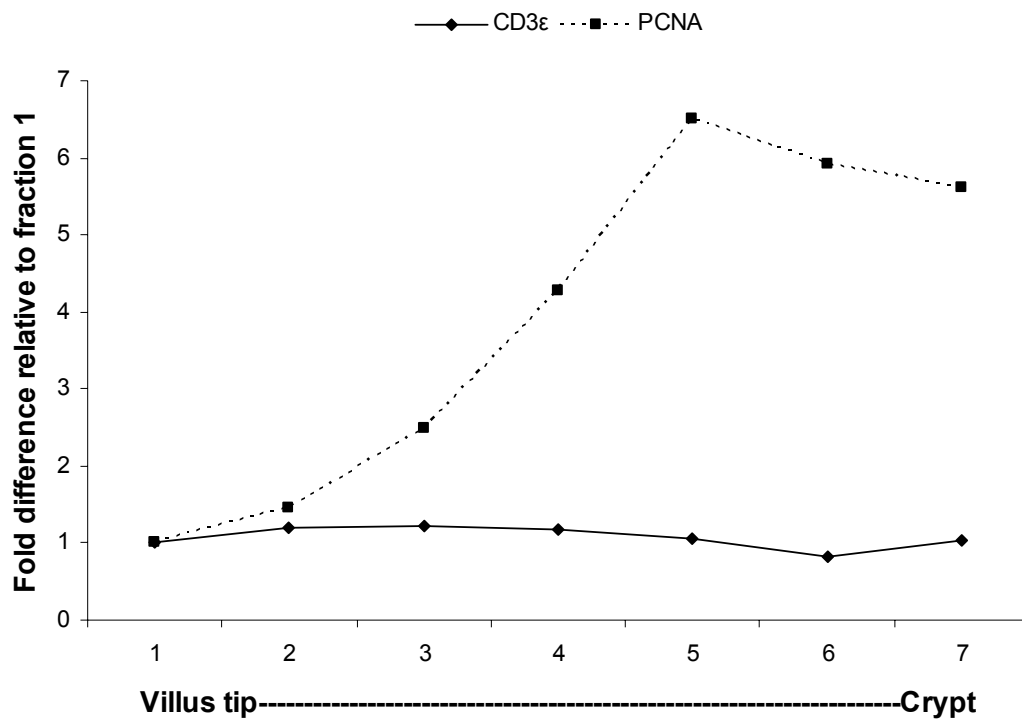


Figure 3.6 CD3ε and PCNA expression in enterocytes harvested along the villus tip (fraction 1) to crypt (Fraction 7) axis in conventionalized (CV) pigs.

microbial colonization and varies with colonizing species (Shirkey et al., 2006). Using our established gnotobiotic neonatal pig model we measured microbial dependent modifications in villus morphology, including reduced villus length and increased crypt depth, that agree with our previous findings as well as observations of others in germfree pigs (Thompson and Trexler, 1971), chickens (Furuse and Okumura, 1994) and rats (Coates, 1973). We further show that these differences are attributed to both apoptotic and proliferative activity. Early comparisons of germfree and ex-germfree mice demonstrated that a full intestinal microbiota stimulates enterocyte turnover (Savage et al., 1981), yet the effort to unravel the complex relationship between bacteria and host, and how bacteria affect cell turnover is still in its infancy.

We chose to isolate and examine host response in epithelial cell fraction in order to specifically focus on the cell type which directly interfaces between the bacteria and mediates nutrient digestion and absorption. For comparison, we also measured expression in whole intestinal tissue. Interestingly, the expression of FasL and TNF $\alpha$  were similarly affected in whole tissue and isolated enterocytes, however, the response in CV and EC pigs was more pronounced in isolated enterocytes. The muted response in whole tissue may have been a result of dilution by other cell types associated with the muscle and submucosa layers and/or that host responses may be more robust in cells directly exposed to luminal bacteria. We have therefore given primary consideration to responses observed isolated enterocytes.

The role of apoptosis in the regulation of cell number in the intestinal epithelium of the mouse accounts for the majority of cell loss in adult tissues (Hall et

al., 1994). This supports the strong negative correlation between activated casp3 abundance and villus height observed in this experiment. While it is possible that apoptosis may occur without participation of casp3 and thus would not be identified in this study, casp3 is a primary executioner caspase and its activation has correlated well with other apoptotic indicators in bacterial infection models (Schauser et al., 2005). In fact, the 2-fold increase in activated casp3 activity associated with CV pigs may be an underestimate of the rate of cell apoptosis as apoptotic cells are quickly cleared within 1-2 hours through phagocytosis by neighboring parenchymal cells (Coles et al., 1993).

The difference in apoptotic activity between EC and LF pigs agree with the differences observed in villus morphology and suggests that *E. coli* either produces a toxic metabolite that *L. fermentum* does not, or that the difference is associated with their ability to induce an inflammatory response. Apoptosis induced by bacteria in the intestinal epithelium can be caused by two means. The first is a direct effect associated with the production of toxic metabolites such as ammonia (Suzuki et al., 2002), hydrogen sulphide (Roediger and Babidge, 1997) and deconjugated bile acids (Leschelle et al., 2002). The second is indirect, through the induction of an inflammatory response, including increased expression of death ligands, TNF $\alpha$  and FasL. Receptors for both of these death ligands are expressed in enterocytes and are important in the regulation of apoptosis (for review see Strater and Moller, 2000). The increased expression of both TNF $\alpha$  and FasL in CV, EC but not LF and GF pigs indicates that the induction of an inflammatory response likely plays a major role in the ability of *E. coli* and conventional bacteria to induce apoptosis. The apoptotic

response may not be exclusive to inflammation as the presence of luminal toxins was not measured in this experiment.

FasL is expressed by lymphocytic cells including intraepithelial lymphocytes (IEL) and increased expression in CV and EC pigs in enterocyte isolates may have been a product of both increased infiltration of IEL, a product of inflammation, into the mucosal surface as well as increased expression within these cells. We have previously observed increased IEL infiltration in the proximal intestine in response to a conventional microbiota but not the same *E. coli* strain (Shirkey et al., 2006) suggesting that increased expression of FasL by IEL may have played a role in *E. coli*-associated pigs. Inflammatory conditions markedly enhance enterocyte apoptosis induced by FasL (Ruemmele et al., 1999) thus infiltration of cells expressing FasL is not sufficient to induce apoptosis in enterocytes alone.

Enterocyte proliferation as indicated by immunohistochemistry and PCNA gene expression mirrored apoptotic activity in response to microbial treatments, with highest proliferation in CV intermediate in EC as compared to LF and GF. Increased intestinal epithelial proliferation has also been shown in conventional as compared to germfree mice (Abrams et al., 1963). In accordance with our findings, Savage et al. (1981) found that a strain of indigenous *Lactobacillus sp.* did not stimulate transit rate in mono-associated ex-germfree mice as indicated by the accumulation of [<sup>3</sup>H]TdR. A good correlation between PCNA transcript abundance and crypt depth indicated variation in proliferative response to different bacteria. Although PCNA protein abundance by immunohistochemistry is a more common method for assessing proliferative activity, PCNA transcript abundance as reported here is considerably

more quantitative. The pattern of PCNA-positive epithelial cells observed in cross sections at 75% SI length and PCNA transcript abundance in isolated enterocytes was comparable across treatments and along the villus tip to crypt axis in agreement with previous observations in the mouse enterocytes (Mariadason et al., 2005). Since CD3ε transcript abundance tended to decline along the villus tip to crypt axis whereas PCNA transcript abundance increased dramatically, it is very unlikely that IEL contributed to differences in PCNA expression observed between treatment groups.

The enterocyte proliferative response to *E. coli* and conventional microbiota may be a response to reduced number of mature enterocytes as well as enterotrophic factors produced by these bacteria. It has been shown that short chain fatty acids have a trophic effect on the intestinal epithelium (Sakata, 1987) and may account for increased expression of the enterotrophic factor proglucagon, observed in mono-associated pigs by Siggers et al. (2003). Providing non-digestible fructooligosaccharides (FOS) to the microbial population has been shown to increase n-butyrate concentrations and subsequently increase the number of mitotic enterocytes (Tsukahara et al., 2002). A 2.4 fold increase in the amount of ornithine decarboxylase antizyme mRNA in the enterocytes of mice colonized with *Bacteroides thetaiotaomicron* as compared to GF (Hooper et al., 2001a) indicates a potential role of polyamines produced by bacteria. Enterotoxic products of bacterial metabolism such as ammonia may also contribute to altered enterocyte replacement rate associated with different species of commensal bacteria (Suzuki et al., 2002) as ammonia has been shown to increase epithelial cell turnover by affecting intermediary metabolism and DNA synthesis (Vissek, 1978).



Gram-negative-induced host responses were greater than for Gram-positive bacteria and may have been mediated by highly immunostimulatory lipopolysaccharide abundant in Gram-negative cell wall. Indeed, increased expression of inflammatory cytokines has been observed in conventional and *E. coli*-associated pigs as indicated by IL-1 $\beta$  and IL-6 expression (Shirkey et al., 2006). Since pro-inflammatory cytokines have been shown to enhance enterocyte renewal (Corredor et al., 2003) this is one mechanism by which enterocyte replacement rate may have been affected. The family of toll-like receptors recognizes a number of distinct bacterial products and likely mediates at least in part enterocyte responses to different bacterial species (Rakoff-Nahoum et al., 2004).

The growth modulatory effects of LPS in the IEC-6 cell line are dependent on endogenously produced TNF $\alpha$  and act in an autocrine/paracrine way (Ruemmele et al., 2002). The correlation between TNF $\alpha$  and cell proliferation as indicated by PCNA is supported by an experiment where blocking TNF $\alpha$  with antagonistic antibodies directed at p55 TNF receptor abolished the effects of LPS on IEC growth (Ruemmele et al., 2002). The importance of TLRs in gut repair and intestinal homeostasis has been demonstrated (Rakoff-Nahoum et al., 2004). The induced expression of inflammatory cytokines and reparative factors IL-6 and TNF $\alpha$  is dependent on the presence of commensal bacteria and an intact TLR pathway (Rakoff-Nahoum et al., 2004).

In this experiment we show that TLRs 2, 4 and 9 are expressed in enterocyte isolates as well as in whole SI tissue in the neonatal pig, and furthermore that their expression is affected by microbial colonization. Increased expression of TLR2 in

murine alveolar macrophages treated with LPS (Oshikawa and Sugiyama, 2003) agrees with the increase in TLR2 expression associated with EC and CV pigs. Although human colonic epithelial cells seem to be fairly unresponsive to LPS (Abreu et al., 2002) small intestinal enterocytes are believed responsive to LPS by clathrin-dependent mechanisms where TLR4 is present in the Golgi. However, we did not identify the location of TLR4 protein within the cell in this experiment. Gene expression of TLR's 2, 4 and 9 in mouse dendritic cells is stimulated by LPS and CpG (An et al., 2002), and this response is suppressed by inhibition of NF- $\kappa$ B activation. They also found that TLRs are differentially regulated as blocking p38 reduced TLR2 and TLR4 expression while it increased TLR9 expression. Microbial metabolites have also been shown to affect TLR expression as Saegusa et al. (2004) found that treating Caco-2 cells with butyric acid induced an increased expression of TLR 1 and 6, although it had no effect on TLR2 expression. The implications of differential TLR expression, based on microbial colonization, are not clear, but the downstream effects of inflammatory response including cytokine production and eventual recruitment of IEL suggests that they play an important role in host responses to luminal bacteria.

The immunostimulatory response to Gram-negative and Gram-positive bacteria vary greatly and do not necessarily separate based on cell wall structure (Aderem and Ulevitch, 2000). In support *E. coli* LPS induces apoptosis in a primary culture of guinea-pig gastric mucous cells at 1/1000 the concentration of *Helicobacter pylori* LPS (Durkin et al., 2006). Similarly, it should also be noted that lipoteichoic acid (LTA) of all gram-positive organisms is not equal in its ability to activate TLR

and thus the inability of *L. fermentum* to induce cell death and proliferation should not be transferred to all gram-positive organisms. Galdeano et al. (2006) found that probiotic bacteria, *Lactobacillus casei*, increased the infiltration of CD-206 and TLR2 positive immune cells into the lamina propria in a conventional mouse. They also observed an increase in IL-6 producing cells after 7 days administration of *L. casei*, while no specific antibodies to *L. casei* were observed. Although *L. helveticus* and *L. casei* were shown to stimulate IL-6 production in large intestinal epithelial cells above levels observed in control, *E. coli* induced double the response at the same cell concentration (Vinderola et al., 2005). Our results are supported by Erickson and Hubbard (2000) who state that PG may need to be 1,000 fold the concentration LPS to induce similar cytokine secretion and by the relative inability of *L. johnsonii* to induce inflammatory cytokine production as compared to *E. coli* in cultured HT-29 cells (Delneste et al., 1998).

*K. pneumoniae* is a Gram-negative bacterium in the enterobacteriaceae family similar to *E. coli*. Interestingly, *K. pneumoniae* contamination of *L. fermentum* mono-associated pigs resulted in a response profile similar to *E. coli*. Since we have no data on the effect of *K. pneumoniae* in mono-association we cannot conclude the *L. fermentum* was unable to ameliorate or that it exacerbated the effect of *K. pneumoniae*, however, the result does seem to support a relatively benign effect of *L. fermentum* on host response.

Here we present evidence that intestinal bacteria differentially affect enterocyte turnover by increasing both apoptotic and proliferative activity. Microbial induced expression of inflammatory cytokines and increased expression of death

ligands appear to play a significant role in increased apoptosis and turnover. A possible implication of these findings is that even in a low pathogen environment manipulation of the commensal microbial population colonizing the intestine could improve growth efficiency by improving digestive function and reducing the metabolic cost of replacing the intestinal epithelium. Although we report here that *E. coli* increases metabolic costs by increasing cell turnover, sufficient activation of the intestinal epithelium in forming tight junctions and maintaining adequate cell renewal is important. Further investigation of the role of early colonizing commensal bacteria on intestinal development is required.

## 4.0 INTESTINAL MICROBIOTA DIFFERENTIALLY AFFECT BRUSH BORDER ENZYME ACTIVITY AND GENE EXPRESSION

### 4.1 Abstract

To study microbial influence on intestinal development pertaining to nutrient digestion two separate gnotobiotic experiments were performed, each with 16 piglets allocated to 4 treatment groups: germfree (GF), mono-association with *Escherichia coli* (EC), mono-association with *Lactobacillus fermentum* (LF) or conventionalization (CV). At day 14 enterocytes were collected from a segment proximal to 75% of the small intestinal length. Enzyme activity and gene expression of lactase-phlorizin hydrolase (LPH) and aminopeptidase N (APN) were measured in isolated enterocytes using specific substrates and quantitative PCR (qPCR), respectively. CV pigs had reduced APN activity, but had increased gene expression relative to GF, making the specific activity/mRNA (A:G) ratio dramatically lower ( $P<0.05$ ). Similarly, LPH A:G ratio was significantly reduced ( $P<0.05$ ) in CV pigs as compared to GF, although gene expression was not up-regulated. LF pigs had A:G ratios that were similar to GF while the EC pigs were more similar to CV. Co-incubation of LF, EC and faecal bacteria with APN indicate a direct relationship between enzyme inactivation and specific A:G ratio in enterocytes. Increased APN expression in CV pigs did not appear to be explained by reduced expression of AT motif binding factor 1-A (ATBF1-A), which negatively regulates APN transcription. We conclude that enterocyte up-regulation of APN expression occurs as either a

direct response to microbial colonization or as a feedback mechanism in response to reduced enzyme activity through microbial degradation. This mechanism may play a role in ensuring effective competition of the host with the intestinal microbiota for available nutrients.

## **4.2 Introduction**

The gastrointestinal tract goes through substantial structural and functional changes in the early postnatal period (for review see Walthall et al., 2005). Concurrent to these changes, the intestinal microbial population is established and continues to change in composition until maturity (Swords et al., 1993). We and others have shown that the maturation of the intestine is not only dependent on both host factors, such as hormones (Petersen et al., 2002; Sangild et al., 1995), and environmental factors including diet composition (Tivey et al., 1994; Zhang et al., 1998) but is dramatically affected by the intestinal microbiota (Hooper et al., 2001; Kozakova et al., 2001; Shirkey et al., 2006). One important functional change that occurs as the pig ages is the expression (Adeola and King, 2006) and kinetics (Fan et al., 2002a) of brush border (BB) digestive enzymes. Each BB enzyme shows a specific developmental pattern as the animal ages (Simon et al., 1979) and these changes have been associated with the maturation of enterocytes. Specifically, changing disaccharidase activities has been used as an indicator of intestinal maturation with a suckling animal having high LPH and low sucrase activities with this pattern reversing at weaning (Cummins et al., 1988).

The intestinal microbiota has been shown to affect BB enzyme expression, as the intestine of a germfree mouse has a different pattern of BB enzymes than a conventional mouse (Kozakova et al., 2001). Furthermore, individual bacteria can stimulate a similar response as mono-association of gnotobiotic mice such that *Bifidobacteria bifidum* mono-association induces a shift in enzyme activity to a pattern similar to that of a conventional mouse (Kozakova et al., 2001). The regulation of BB enzymes that give absorptive cells the ability to digest a given nutrient is regulated at many levels including transcription (Krasinski et al., 1994), synthesis (Seetharam et al., 1980), post-translational glycosylation (Beaulieu et al., 1989) and turnover (Seetharam et al., 1980). The mechanism by which bacteria induce these changes or which bacteria are responsible has not been elucidated. Understanding how bacteria contribute to the host's ability to digest and absorb nutrients is of considerable interest. Functional implications are demonstrated by the greater ability of the germfree intestine to absorb xylose than the conventional intestine (Heneghan, 1963). Disaccharidase activities are higher in the small intestine of the germfree as compared to conventional rats (Reddy and Wostmann, 1966) and colonization of gnotobiotic mice with *Bacteroides thetaiotaomicron* increases Na<sup>+</sup>/glucose cotransporter (SGLT-1) and colipase mRNA expression in ileal tissues while causing a reduction in the expression of LPH (Hooper et al., 2001). As indicated these microbially induced changes have been observed at the level of activity and expression, but not concurrently.

Earliest colonizers of the pig digestive tract include a large group of coliforms, dominated by *E. coli*, which are quickly supplanted within 48 hours by

*Clostridium* and *Lactobacillus* species (Swords et al., 1993). Microbially induced changes are not limited to digestive function, as indicated by their importance in the regulation of immune development (Mazmanian et al., 2005), glycoconjugate repertoire (Nanthakumar et al., 2005) and villus morphology (Shirkey et al., 2006) and are dependent on colonizing species. Previously we have reported that early colonizing non-pathogenic *E. coli* and *L. fermentum* differentially affect villus structure (Shirkey et al., 2006), and enterocyte replacement (Chapter 3), thus we wanted to investigate the effect of these organisms on intestinal maturation specifically pertaining to digestive function. Using our established gnotobiotic pig model (Shirkey et al., 2006) we tested this hypothesis by comparing APN and LPH gene expression and enzyme activity in isolated enterocytes from germfree and conventionalized pigs as well as pigs mono-associated with *E. coli* or *L. fermentum*.

## **4.3 Materials and Methods**

### **4.3.1 Experimental Design and Animal Rearing**

The animals used for this experiment are the same as those used in Chapter 3. Animal use was approved by the Animal Care Committee of the University of Saskatchewan and was performed in accordance with recommendations of the Canadian Council on Animal Care (1993). In two replicate experiments 16 germfree piglets were derived by caesarean section, as previously described (Shirkey et al., 2006), balanced for birth weight and litter, and allocated to 4 treatment groups. Piglets were inoculated at 24 and 30 hours of age with either an 18 hour tryptic soy



(BBL, Sparks, MD) broth culture of non-pathogenic *Escherichia coli* (treatment denoted EC), an 18 hour Lactobacilli MRS (Difco Laboratories, Sparks, MD) broth culture of *Lactobacillus fermentum* (treatment denoted LF), a combination of 1 gram of fresh feces (conventional microbiota) derived from a clinically healthy sow, 1 mL of peptone and 2 mL of both *E. coli* and *L. fermentum* cultures (treatment denoted CV), or were maintained germfree (treatment denoted GF). LF and EC inoculants were originally isolated from a healthy pig and 18 hour cultures contained 8.6 to 8.9 log cfu/mL. Piglets were bottle fed a mixture of 1 part infant formula (Similac®) to 1 part sterile porcine serum for the first 24 hours. Subsequently piglets were fed *ad libitum* in a trough a 2:1 mixture of infant formula and water replenished 3 times daily for the remainder of the trial.

#### 4.3.2 Tissue Collection

Pigs were killed by submersion in CO<sub>2</sub> and exsanguination at 14 days of age at which time the SI and caecum were tied off and removed. Digesta from the ileum and cecum were taken to confirm treatment status and to enumerate culturable bacteria. SI length was determined and an 80 cm segment proximal to the 75% location was removed and subjected to an enterocyte isolation process.

#### 4.3.3 Enterocyte Isolation

The distended sac method modified from Fan et al. (2001) was used to remove enterocytes. The 80 cm segment was initially rinsed with a pre-incubation buffer (PBS with 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiothreitol (DTT), pH 7.4). The segment was then filled with isolation buffer (PBS with 1.5 mM ethylene diamine tetra acetate (EDTA), 0.2 mM PMSF, 0.5 mM DTT, pH 7.4) by clamping the distal end filling until fully distended and subsequent clamping of the proximal end. The distended segment was then placed in a saline bath at 37 °C for 30 min. Isolated cells were collected by centrifugation at 400g for 3 min at 4 °C, and the resulting cell pellet resuspended in ice cold PBS to remove buffer, repelleted at 400g and snap frozen in liquid nitrogen after discarding the supernatant. The intestinal segment was refilled and subjected to 6 consecutive washes to allow removal of enterocytes along the entire villus-crypt axis. A 1 cm segment was collected after each wash, fixed in 10% buffered formalin and stained for hematoxylin and eosin to allow for the assessment of enterocyte removal. Enterocyte isolates were either pooled within pig (1 sample per pig) or pooled by location within treatment (1 location per treatment). Specific removal intestinal of epithelium was confirmed by examination of tissue cross sections before and after enterocyte isolation (Fig. 4.1).

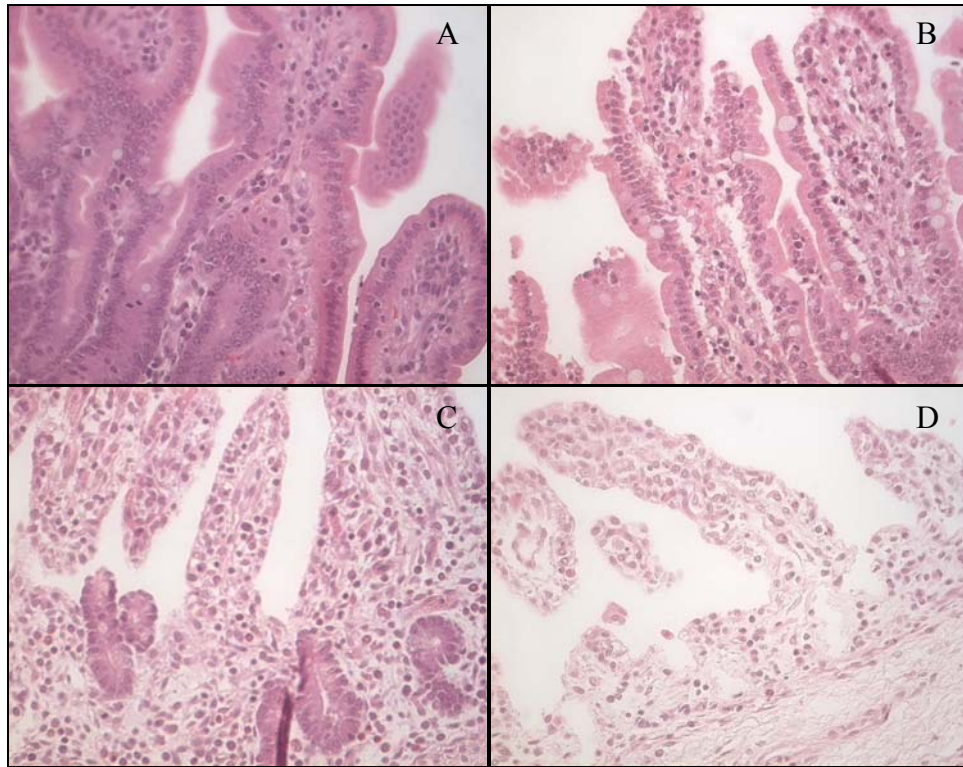


Figure 4.1 Representative light micrographs of hematoxylin and eosin-stained cross sections of SI tissue taken before (A), after 2 (B), 4 (C) and 6 (D) sequential EDTA washes using the distended sac method. Integrity of lamina propria and sequential removal of enterocytes from tip to crypt can be observed. Magnification 400X.

#### 4.3.4 Confirmation of Treatment Status

Bacteria free status of GF pigs was confirmed through the course of the trial by peri-anal swabs taken daily, culture in brain heart infusion broth with 0.5% cysteine HCl and evaluation of turbidity. At the end of the study digesta was collected from the jejunum, ileum and cecum, diluted to  $10^{-1}$ ,  $10^{-2}$  and  $10^{-4}$  in peptone water and plated using the Autoplate<sup>®</sup> 4000 (Spiral Biotech Inc., Bethesda, MD) onto blood agar base (BBL, Sparks, MD) with 5% defibrinated sheep blood and cultured aerobically and anaerobically. Digesta were also plated on MacConkey's agar (Difco Laboratories) cultured aerobically, and on MRS agar (BBL) cultured anaerobically. Plates were incubated for 24-48 hours at 37 °C prior to colony enumeration. Gram stains were performed on colonies from EC and LF treatments and viewed microscopically to confirm appropriate morphology and cell wall type. The identity of colonies with inconsistent colony and/or cell morphology was determined by colony isolation, sequencing of chaperonin 60 UT and comparison with typed strain data in the cpnDB (Hill et al., 2002).

#### 4.3.5 Gene Expression

Total RNA was extracted from 20-30 mg of isolated enterocytes and subject to RNase-free DNase digestion using the RNeasy<sup>®</sup> Mini Kit (Qiagen, Mississauga, ON). RNA was quantified by optical density at 260/280 nm using a spectrophotometer (Ultrospec 2000; Pharmacia Biotech, Baie d'Urfe, QUE, Canada) and 1 µg of RNA was used to generate first strand cDNA using i-script<sup>®</sup> kit (Biorad). Quantitative PCR using an iCycler iQ Real-Time PCR detection system

(BioRad) was performed on resulting cDNA using SYBR<sup>®</sup> Green detection. Primers for APN, LPH, PCNA, ATBF1-A, and housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) are shown in Table 4.1. For quantification of transcript copy number standard curves were established for target and housekeeping genes. Standard curves (range  $10^1$ - $10^7$  copies target gene) were generated by cloning the appropriate RT-PCR product using the pGEM-T<sup>®</sup> Easy Vector System II (Promega Co., Madison, WI), purifying plasmid DNA (Qiagen Plasmid Mini Kit, Qiagen) and quantification of plasmid DNA using PicoGreen DNA Quantitation Kit (Molecular Probes, Eugene, OR). Transcript copy numbers are reported per 1000 copies of GAPDH.

#### 4.3.6 Protein and Enzyme Assays

Isolated enterocytes (200 mg) were homogenized in 10 mL of 1% Triton X-100 with a Brinkman Homogenizer and centrifuged at 1500g for 5 min. The supernatant was used for enzyme activity assays for LPH and APN. Enzyme activities were reported per minute per gram of protein determined using the Bio-Rad protein microassay procedure (Bio-Rad Laboratories) with a bovine serum albumin standard (Bradford, 1976).

The method of assaying APN activity was modified from Maroux et al. (1973). Substrate for the APN assay was 10 mM L-alanine-4-nitroanilide with 50 mM TRIS HCl at pH 7.3. The standards included a range from 6.25 to 200  $\mu$ M of 4-nitroaniline with 10  $\mu$ l boiled protein homogenate, with the blank containing 200  $\mu$ l substrate and 10  $\mu$ l deactivated protein. 200  $\mu$ l of substrate was added to 10  $\mu$ l of homogenate and the color development measured at 405 nm after 5 minutes at 37 °C.

Table 4.1 Primers designed for quantitative RT-PCR.

Gene	Forward (5'-3')	Reverse (5'-3')
APN	CAATATGCCGCCCAAAGGTTT	CCGGATCAGGACGCCATTT
LPN	CCAAGTTCTACGCCTCCATAGTC	TCCAAGAAGCAGAAGAGCAAAGA
PCNA	TACGCTAAGGGCAGAAGATAATG	CTGAGATCTCGGCATATACGTG
GAPDH	GTTTGTGATGGGCGTGAAC	ATGGACCGTGGTCATGAGT
ATBF1-A	AGTTAAAGAGAGCCCTCAAGAG	GTTACTGCCACTGGTACTCAC

Lactase activity assay was modified from Dahlqvist (1964) and was determined by measuring the amount of glucose liberated from lactose. 20  $\mu$ L of 0.1 M  $\beta$ -lactose was added to 20  $\mu$ L of homogenate and incubated in a 96 well plate for 30 minutes at room temperature. Glucose liberated was then measured using the Wako Glucose Assay Kit (Wako Bioproducts, Richmond, VA).

#### 4.3.7 *In Vitro* Microbial Deactivation of APN

Microbial degradation of APN was determined by incubating 0.5 units of rat aminopeptidase N (Calbiochem, La Jolla, CA) in 1 mL of PBS containing  $10^8$ - $10^9$  CFU of either *L. fermentum*, *E. coli*, conventional bacteria obtained from a fresh pig fecal sample or no bacteria for 2 hours at 37°C on a shaker. Bacteria were then removed by centrifugation at 5000g for 1 minute and supernatant was used for determination APN activity as described above.

#### 4.3.8 Statistical Analysis

All results were analyzed as a one-way ANOVA using the general linear model procedure (SPSS program, Chicago, IL). Treatment means were separated using least squares difference with significance of  $P < 0.05$ . Experiments were analyzed separately.

## 4.4 Results

### 4.4.1 Microbial Colonization

Assessment of culture morphology, Gram-stain and *cpn60* UT sequence of bacteria isolated from ileal and cecal contents indicated that the LF treatment in trial 2 was contaminated with *Klebsiella pneumoniae* making this treatment di-associated, thus denoted LFKP. No other contaminants were identified in any other treatment group. Enumeration of *E. coli* on MacConkey agar indicated colonization ranges of 8.5-9.0 and 8.0-9.0 log cfu/g of contents in EC pigs in trials 1 and 2, respectively. Ileal counts of *L. fermentum* on MRS agar in LF pigs (trial 1) ranged from 6.1-7.5 log cfu/g of contents. LFKP treatment (trial 2) had 6.9-9.2 log cfu/g total aerobes enumerated on blood agar indicating growth of *Klebsiella pneumoniae* whereas lactobacilli counts on MRS agar ranged from 6.4-6.9 log cfu/g. Diverse colony morphologies were observed in cultures of ileal contents from CV pigs with aerobic blood agar counts ranging between 6.3-8.2 and 7.0-8.2 log cfu/g, anaerobic blood agar counts of 6.2-8.8 and 7.2-9.0 log cfu/g for trials 1 and 2, respectively. No colony growth was observed for GF pigs.

### 4.4.2 Enzyme Activity and Gene Expression

For all parameters examined, response trends among the 3 treatment groups common to both trials (GF, CV and EC) were similar. APN activity (Fig. 4.2a) was lowest ( $P<0.05$  for trial 1) in CV as compared to GF and EC pigs, which were not different. In contrast, APN gene expression (Fig. 4.3a) was highest ( $P<0.05$  trial 1



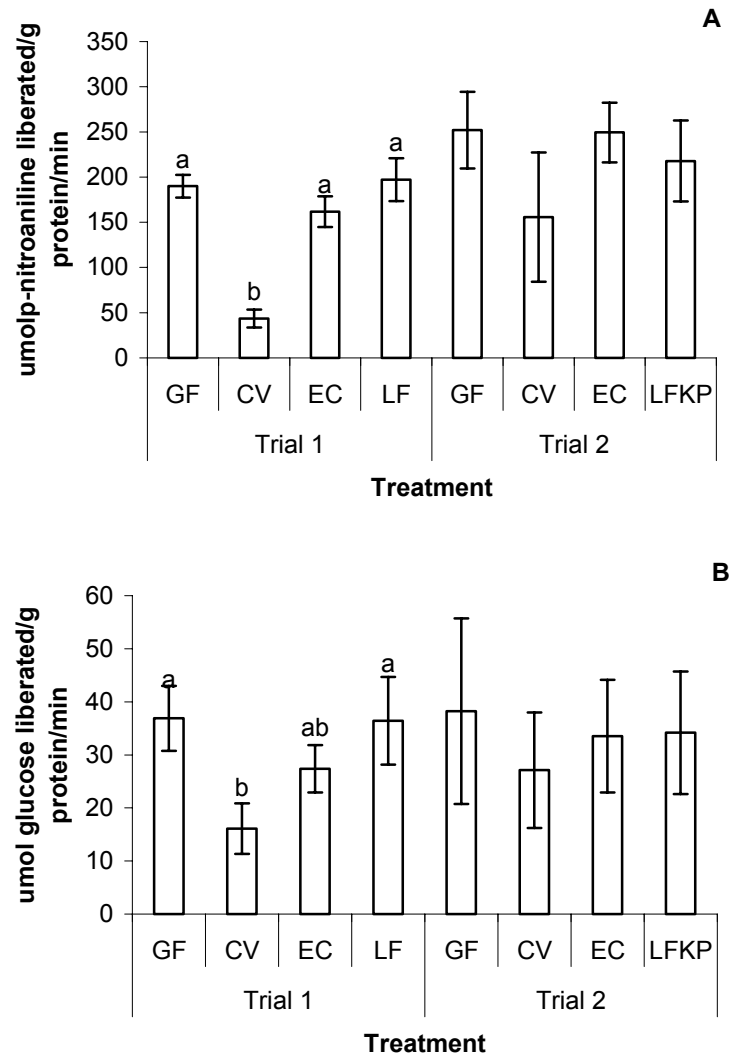


Figure 4.2 Enzyme activities of APN (a) and LPH (b) in enterocyte isolates from GF, LF, EC, LFKP and CV pigs. Vertical bars represent SE. <sup>ab</sup>Bars within the same experiment without a common letter are significantly different ( $P < 0.05$ )

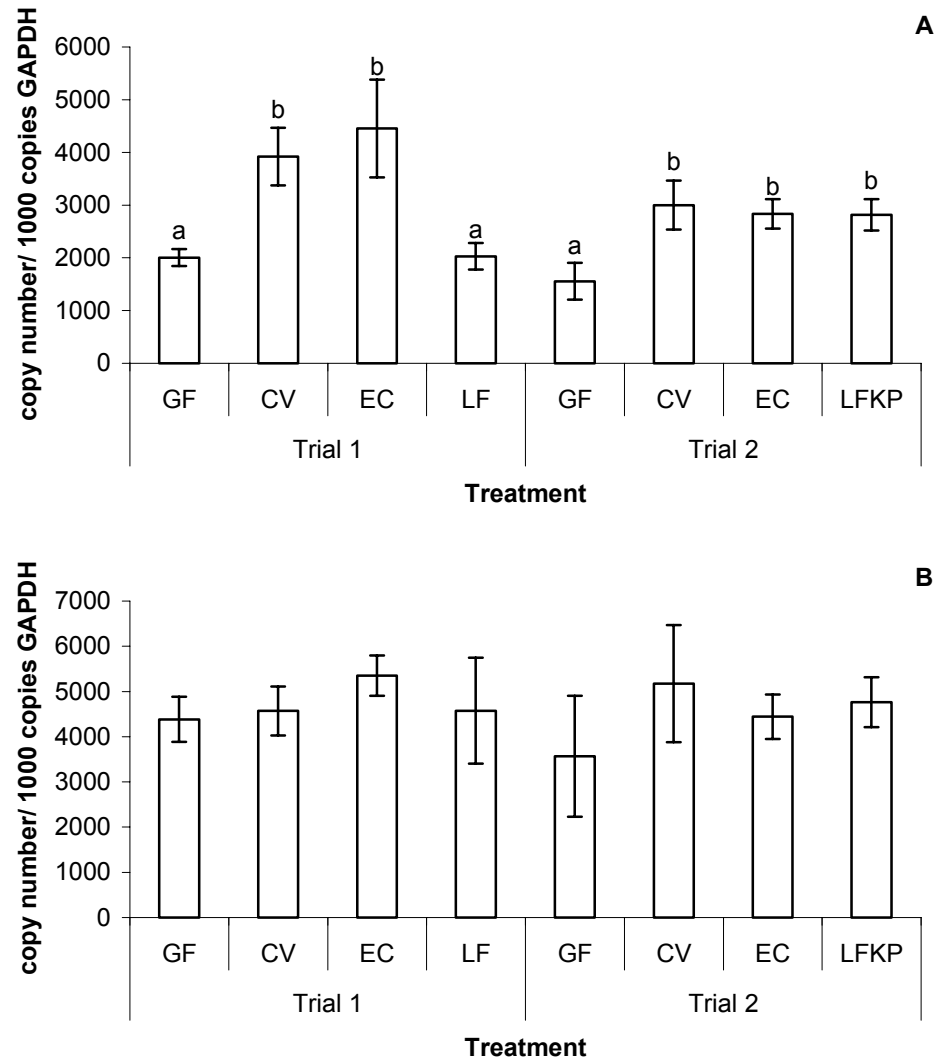


Figure 4.3 Gene expression of APN (A) and LPH (B) in enterocyte isolates from GF, LF, EC, LFKP and CV pigs. Vertical bars represent SE. <sup>ab</sup>Bars within the same trial without a common letter are significantly different ( $P < 0.05$ )

and 2) in CV, and EC compared with GF. As a result, APN A:G ratio (Fig. 4.4a) was highest ( $P<0.05$  trial 1 and 2) in GF compared with CV and EC pigs. The APN A:G ratio was higher, but not significantly different, in EC compared with CV pigs in both trials. For trial 1, LF pigs had similar APN activity to EC and GF groups, while APN gene expression was lower ( $P<0.05$ ) than EC and similar to GF. The APN A:G ratio for the LF group was thus similar to GF and higher ( $P<0.05$ ) than CV or EC. For trial 2, *K. pneumoniae* contamination of the LF group (LFKP) resulted in an APN activity and expression profile very similar to the EC group.

LPH activity (Fig. 1b) showed a similar pattern of activity to APN in all treatment groups where the lowest ( $P<0.05$  for trial 1) activity was observed for CV pigs. There were no differences in LPH gene expression (Fig. 4.3b) among treatment groups. The resulting LPH activity to gene expression ratio (Fig. 4.4b) was lower in CV ( $P<0.05$  trial 1 and 2) and EC (trial 1 only) pigs compared with GF. Similar to the APN A:G ratio, the LPH A:G ratio in LF pigs was similar to GF whereas in LFKP pigs the ratio was similar to EC.

#### 4.4.3 APN and LPH Gene Expression along the Villus-crypt Axis

APN gene expression was highest in the enterocyte isolates representing the villus tip as compared to the crypt in both GF and CV pigs (Fig. 4.5). In contrast, PCNA expression (Fig. 4.5) was lowest in villus tip cells and highest in crypt cells consistent with high proliferative activity in crypt cells. APN mRNA expression was

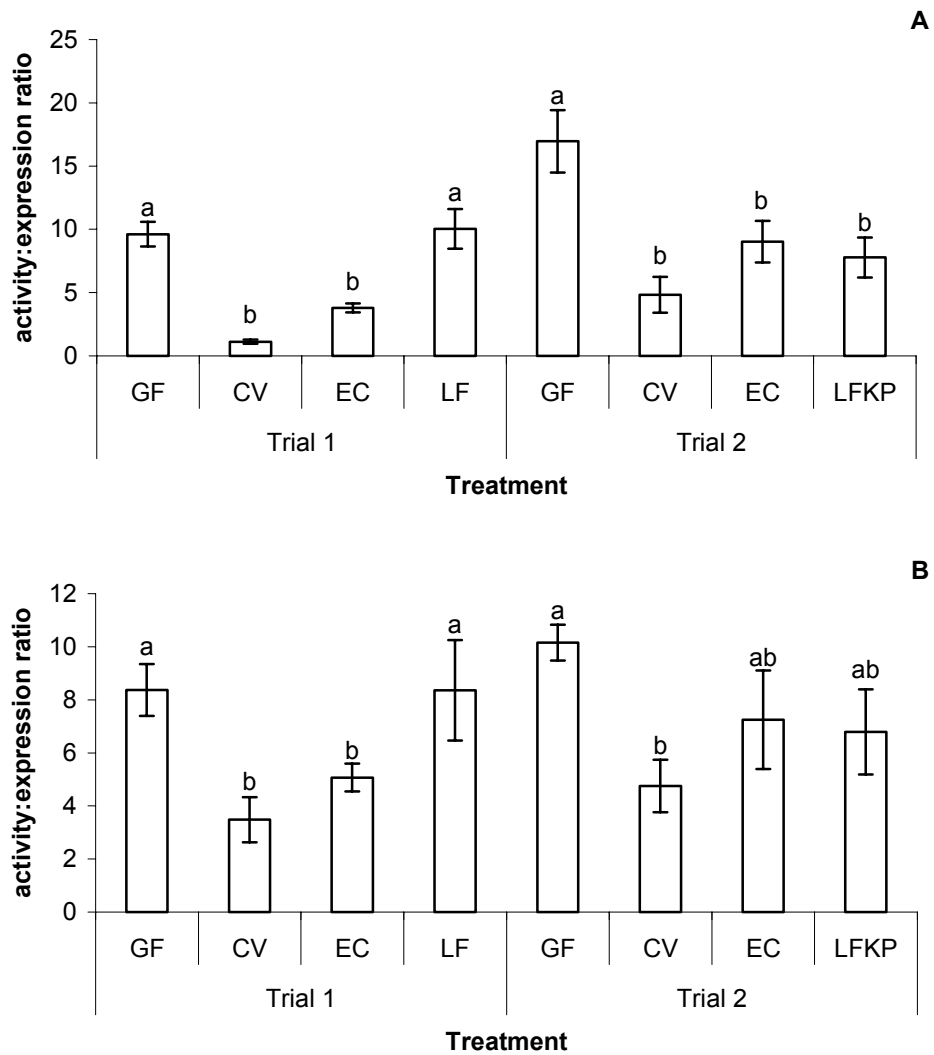


Figure 4.4 Enzyme activity to gene expression ratio for APN (A) and LPH (B) in enterocyte isolates of GF, LF, LFKP, EC and CV pigs. Vertical bars represent SE. <sup>ab</sup>Bars within the same experiment without a common letter are significantly different ( $P < 0.05$ )

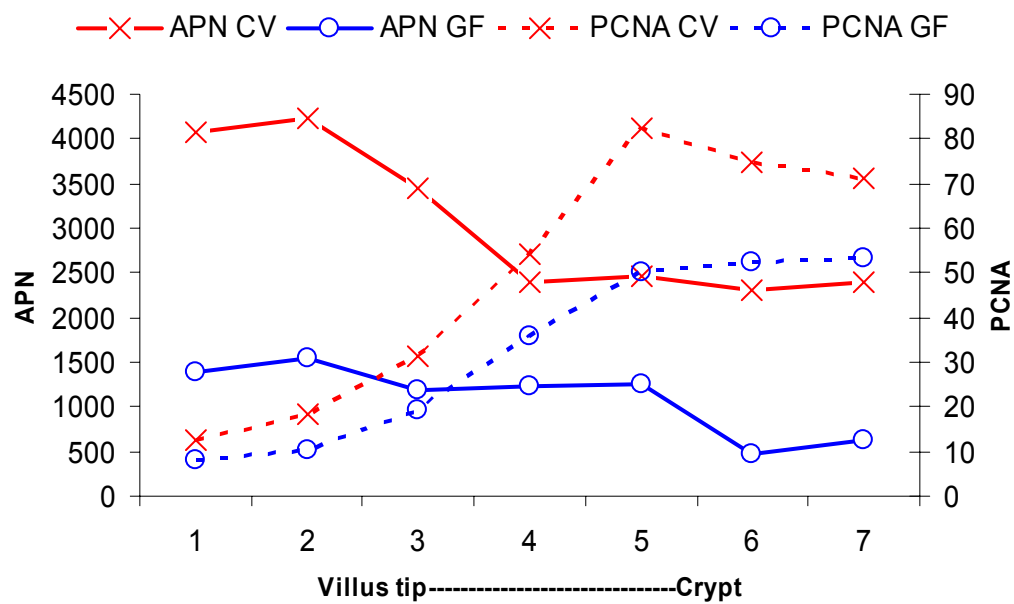


Figure 4.5 mRNA expression of APN and PCNA per 1000 copies GAPDH in enterocytes sequentially isolated from the villus tip (fraction 1) to crypt (fraction 7) in germfree (GF) and conventionalized (CV) pigs (Trial 2).

higher in CV as compared to GF pigs in all enterocyte fractions. LPH expression in CV and GF groups was similar, although LPH expression appeared to fall off in the villus tip of GF pigs (Fig. 4.6).

#### 4.4.4 Bacterial Degradation of APN

Co-incubation of purified rat APN with bacteria in PBS reduced APN enzyme activity in subsequent APN assay after bacteria were removed. APN activity was reduced 28% ( $P<0.05$ ) by conventional bacteria, 17% ( $P<0.05$ ) by *E. coli* and not by *L. fermentum*. The expression of ATBF1-A was unaffected by treatment group (data not shown).

#### 4.5 Discussion

It is well recognized that the intestinal microbiota plays a significant role in development of intestinal immunity (Hooper, 2004; Hooper et al., 2001a; Mazmanian et al., 2005; Shirkey et al., 2006), yet relatively little attention has been paid to the effects of commensal bacteria on digestive function. In rodents, microbially dependent modifications in BB enzyme profile have been observed at both the mRNA (Hooper et al., 2001a) and protein levels (Kozakova et al., 2001), but to our knowledge they have not been examined concurrently.

In the present study, enzyme activity and gene expression were measured in isolated enterocytes obtained using the distended sac method, as successive EDTA washes. The method has been used previously in rodents and pigs to study BB

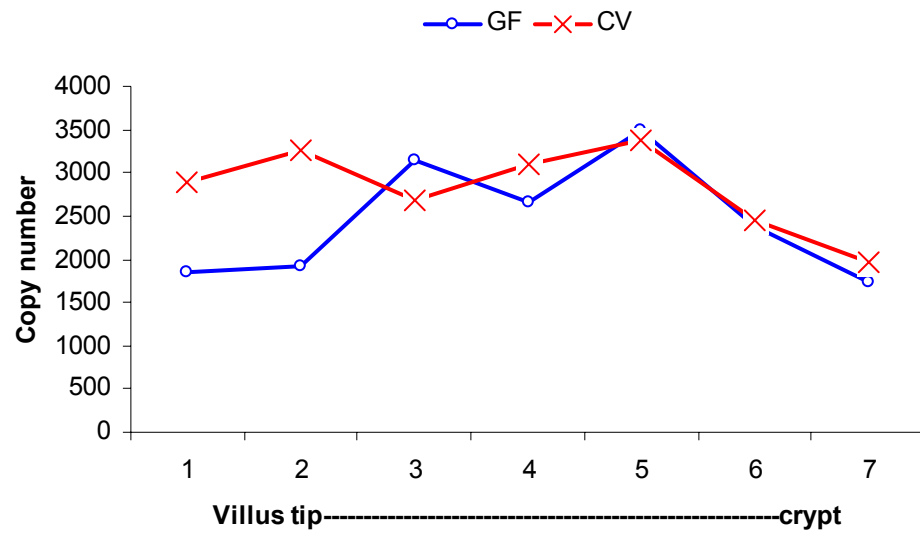


Figure 4.6 mRNA expression of LPH per 1000 copies GAPDH in enterocytes sequentially isolated from the villus tip (fraction 1) to crypt (fraction 7) in germfree (GF) and conventionalized (CV) pigs (Trial 2).

enzyme activity and shown to improve homogeneity of tissue analyzed between treatments over whole tissue (Raul et al., 1977). BB enzyme activity and mRNA expression observed here in enterocytes were similar to whole tissue obtained from 75% of SI length (data not shown) and gene expression profile along the villus-crypt axis were consistent with an expected predominance of proliferative activity in crypts and enterocyte digestive maturity at villus tips.

Conventionalization in pigs reduced enterocyte BB enzyme activity compared to GF without a concomitant reduction in gene expression in the case of LPH and with a paradoxical increase in gene expression in the case of APN. For both enzymes the ratio of activity to mRNA abundance was markedly reduced by conventionalization. Because of the reduced villus height and increased enterocyte replacement rate observed in conventional as compared to germfree animals (Coates, 1973; Furuse and Okumura, 1994; Thompson and Trexler, 1971) it has been postulated that the higher disaccharidase activity in the small intestine of germfree as compared to conventional rats (Reddy and Wostmann, 1966) is due to an increased number of mature enterocytes.

In this experiment reduced villus height (Fig. 3.1a) was observed in CV pigs, thus reduced enzyme activity may partly be explained by reduced cell maturity or mature cell number. However, the paradoxical increase in APN expression suggested that other mechanisms are involved. Expression of APN is distinct in that it is blocked in the villus crypt by AT motif-binding factor (ATBF1-A) and upregulated by hepatocyte nuclear factor 1 (HNF-1) in the villus (Ihara et al., 2003). APN expression was highest in the villus tip fraction and fell off in the crypt cells in both



GF and CV pigs, which concurs with transcriptional regulation and parallels enzyme activity in cell fractions along the villus crypt axis (Danielsen, 1984; Fan et al., 2001). The expression pattern of APN along the villus axis and the consistently higher expression observed in all fractions in CV pigs suggests that the reduced activity in conventional pigs is not entirely explained by variation in the ratio of mature and immature enterocytes.

The fact that decreased activity occurs in the face of increased APN expression suggests that there is either reduced transcript stability, reduced enzyme kinetics or reduced enzyme stability. We cannot rule out transcript stability, however, APN Vmax and Km increase as the pig matures (Fan et al., 2002) and the conventional animal has previously been shown to express a more mature pattern of BB enzyme activity (Kozakova et al., 2001) suggest that a reduced enzyme kinetics is unlikely responsible. We propose that reduced protein stability occurs due to microbial BB enzyme deactivation. In support, *E. coli* and conventional bacteria deactivated purified APN *in vitro* consistent with the *in vivo* data. Bacteria degrade mucus glycoproteins produced by the intestinal epithelium using a variety of glycoside hydrolases, depending on the organism (Carlstedtduke et al., 1986; Hoskins et al., 1985). Furthermore, chymotrypsin activity is higher in the ileal contents of the germfree than the conventional rabbit (Malis et al., 1976) indicating that bacteria metabolize or deactivate the secreted enzyme. Similar to the degradation of secreted digestive enzymes, it is likely that BB enzymes are not equally deactivated, as chymotrypsin and elastase are more sensitive to bacterial inactivation than trypsin (Malis et al., 1976), however, we did see a similar reduction in activity for LPH and

APN. In contrast to pancreatic enzymes, APN is anchored to the membrane via hydrophobic interaction in the N-terminal region of the polypeptide chain (Semenza, 1986), thus predominantly resides in enterocyte apical surface. Because of the bound nature of these enzymes, bacteria would likely have to come in close proximity to the brush border to cause damage to the enzyme. Differences observed between *E. coli* and *L. fermentum* may be explained by their ability to penetrate the protective mucus layer and reach the epithelial layer, however *in vitro* results indicate *L. fermentum* lacked metabolic capacity to deactivate APN.

The lack of, or minimal change of LPH gene expression in response to deactivation as compared to APN is not necessarily surprising as it has been shown that they are differentially regulated. APN and LPH show different mRNA accumulation patterns (Freund et al., 1990) suggesting different regulatory mechanisms of expression. The response may also be associated with the location in the intestine and resulting substrate available. LPH is more highly expressed in the proximal intestine, while APN is distributed more evenly through the length of the SI (Freund et al., 1990).

At this point it is unclear whether the increase in APN expression was the result of a feedback response to enzyme deactivation, or a direct response to microbial colonization. Microbial butyrate production could represent one mechanism by which APN expression was directly induced, as butyrate reduces ATBF-1 expression (Kataoka et al., 2000) thus allowing expression of APN. Luminal butyrate was not measured in this experiment, however, its role is somewhat suspect since *E. coli* is not a large producer of butyrate but induced a similar increase

in APN expression as observed in conventionalized pigs. The expression of ATBF1-A was also not affected by treatment group. Since bacterial metabolism may have changed the amino acid and carbohydrate supply at the enterocyte surface, and since these nutrients can affect expression of digestive brush border enzymes (Cui et al., 2004), this mechanism could also contribute to increased APN gene expression in the current study.

CD13, which is identical to aminopeptidase N, is also expressed on monocytes, basophils, eosinophils and neutrophils (Olsen et al., 1988). An increased inflammatory response would result in infiltration of these cells into the lamina propria. However, to explain the increased expression, these cells would have to express APN at greater levels than enterocytes, while having limited or no activity. Myeloperoxidase activity, an enzyme uniquely expressed by these immune cells, was nearly undetectable in CV tissues (data not shown) indicating that CD13 bearing cells minimally contaminated the enterocyte isolates, and cannot explain the increased APN expression levels observed in CV and EC pigs.

One previously observed commonality between CV and EC pigs is their increased inflammatory response including IL-1 and IL-6 expression (Shirkey et al., 2006). Enterocytes have been shown to respond functionally, at a posttranscriptional level, to inflammatory cytokines including IL-1 $\alpha$ , IL-6 and IL-8 by increasing glucose absorption without increases in SGLT-1 content (Hardin et al., 2000). However, we are unaware of any data suggesting inflammatory cytokines can upregulate BB enzyme expression.

EC and LF pigs did not have significantly different BB enzyme activities, however, there was increased APN expression in EC and LFKP as compared to LF pigs. Increased gene expression in EC as compared to LF pigs suggests that these two early colonizing bacteria play different roles in the regulation of brush border enzymes.

In this experiment we indicate one mechanism by which bacteria regulate brush border enzyme activity and further show that the response is dependent on the colonizing bacteria. It has been suggested that most strains of *E. coli* are harmless commensals in mammals (Hartl and Dykhuizen, 1984). Although not causing overt disease, *E. coli* increased the turnover of brush-border enzymes, imposing a substantial metabolic cost on the host. We conclude that enterocyte up-regulation of brush border enzyme expression occurs as either a direct response to microbial colonization or as a feedback mechanism in response to reduced enzyme activity through microbial degradation. This mechanism may play a role in ensuring effective competition of the host with the intestinal microbiota for available nutrients.

## 5.0 A COMPARISON OF MODE OF ACTION FOR TWO PROBIOTIC ORGANISMS IN A GNOTOBIOTIC PIG MODEL

### 5.1 Abstract

Probiotics are becoming commonplace, yet there is still relatively little understanding of their modes of action. A gnotobiotic pig experiment was conducted to characterize physiological responses in the intestine to each of two Chr Hansen probiotic organisms, a *Bifidobacterium* sp. Chr Hansen Collection (CHCC) 5445 (B5445) and a *Lactobacillus* sp. CHCC 3777 (L3777), alone and in conjunction with a commensal *Escherichia coli* that was previously shown to induce expression of inflammatory cytokines in mono-associated pigs. Twenty-four caesarean section derived piglets from 3 litters were allocated to 4 treatment groups including mono-association with L3777 (La), mono-association with B5445 (Bb), di-association with L3777 and *E. coli* (LaE) and di-association with B5445 and *E. coli* (BbE). Pigs were killed at 15 d of age and tissues were taken for histological, gene expression and protein analysis from the location identified at 75% of the small intestinal (SI) length. Gene expression of inflammatory cytokines IL-1 $\beta$ , TNF $\alpha$  and IL-8 and anti-inflammatory mediators, nerve growth factor (NGF), IL-10 and TGF $\beta$  were measured. Caspase 3 like activity and mRNA abundance of proliferating cell nuclear antigen (PCNA) were measured in homogenized tissue as indicators of apoptotic and proliferative activity, respectively. Mono-association with L3777 and B5445 did not appear to induce pro or anti-inflammatory responses in the intestinal epithelium.

Three of 5 and 2 of 5 pigs, respectively, succumbed to sepsis when di-associated with *E. coli* and L3777 or B5445, whereas mono-association with *E. coli* in previous experiments rarely presented with sepsis. In surviving pigs, di-association led to reduced ( $P<0.05$ ) villus:crypt ratios, increased expression of both pro-inflammatory (IL-1 $\beta$  and TNF $\alpha$ ) and anti-inflammatory (IL-10) cytokines as compared to mono-associated pigs. *E. coli* induction of IL-8 and FasL expression was greater in LaE as compared BbE suggesting greater inflammatory protection with B5445. These results indicate that L3777 and B5445 in mono-association are well tolerated in the neonatal pig. A high prevalence of sepsis induced by di-association with *E. coli* suggests these bacteria may induce anti-inflammatory mechanisms; however the regulatory mechanism remains unclear. The importance of an appropriate in vivo model to assess probiotic mode of action is also indicated.

## 5.2 Introduction

Lactic acid bacteria (LAB) are the most commonly used probiotic organisms and include a diverse group of *Lactobacilli* and *Bifidobacteria* species (Quigley and Quera, 2006), some strains of which have been shown to reduce or prevent the colonization and damaging effects of specific enteric pathogens (Saavedra, 1995). Many mechanisms of action have been proposed to mediate the beneficial effects of probiotic bacteria including competing with pathogens for binding sites and available nutrients, creating a hostile environment by the production of bacteriocins and reduction of pH and by neutralizing bacterial toxins (Castagliuolo et al., 1999; Dixit et al., 2004; Fooks and Gibson, 2002; Silva et al., 1987). More recently attention has

focused on the ability of probiotics to stimulate an appropriate immune response (Erickson and Hubbard, 2000; Galdeano and Perdigon, 2006; Mazmanian et al., 2005) and conversely to stimulate an anti-inflammatory response to mitigate the effects of pathogens or chronic inflammatory conditions (Ma et al., 2004; O'hara et al., 2006). The importance of anti-inflammatory mediators in maintaining intestinal homeostasis is demonstrated by the lack of tolerance to bacterial exposure in IL-10 knockout mice (Sydora et al., 2003).

Because of the complex nature of the intestinal microbiota (Savage, 1977), it is unclear as to whether the effects of probiotics are due to a direct interaction with the host or are attributed to the effect of the probiotic organism on other microbial species. For example, the probiotic *Lactobacillus rhamnosus* has been reported to reduce IL-1 and TNF $\alpha$  (Lu et al., 2004) by changing intestinal microbial composition, however, a direct effect of *L. rhamnosus* on cytokine expression cannot be excluded. In the case of *Lactobacillus reuteri*, a direct anti-inflammatory effect on cultured enterocytes through stimulation of IL-10 and NGF has been demonstrated requiring the presence of live organisms (Ma et al., 2004). In addition, metabolites produced by *Lactobacillus* species can inhibit TNF- $\alpha$  production in macrophages (Pena and Versalovic, 2003) and *Bifidobacteria* BB-12 and *Lactobacillus* LA-5 are strong inducers of TNF $\alpha$ , IL-6 and IL-10 production in peripheral blood mononuclear cells (PBMC) (Miettinen et al., 1996). Thus, at least in simplified in vitro systems, direct immunomodulatory effects of probiotics have been demonstrated.

Because of the numerous cell types involved in intestinal function and homeostasis, in vitro models have limited applicability for the screening of probiotic

organisms. Yet, when assessing mechanisms, the *in vivo* model creates confusion because of the diversity and complexity of the microbiota. Using a gnotobiotic pig model we are able to assess the effects of probiotic organisms on the host alone and with a defined flora. Previously we have observed that mono-association of a gnotobiotic pig with commensal *E. coli* stimulates a substantial inflammatory response as compared to germfree or mono-association with *L. fermentum* (Savage, 1977; Shirkey et al., 2006). To elucidate probiotic mediation of host inflammatory responses we examined expression of pro and anti-inflammatory cytokines following mono-association with the probiotic strains L3777 and B5445. In addition these responses were investigated following stimulation of inflammation by di-association with *E. coli*.

## **5.3 Materials and Methods**

### **5.3.1 Experimental Design and Animal Rearing**

Animal use in this experiment was approved by the Animal Care Committee of the University of Saskatchewan and was performed in accordance with recommendations of the Canadian Council on Animal Care (1993). Twenty four piglets from 3 sows were derived by caesarean section as previously described (Shirkey et al., 2006), balanced for birth weight (1.07-1.45 kg) and litter, and allocated to 4 treatment groups. In the first 8 hours of life piglets received four 25 mL doses of irradiated colostrum (total 100 mL) by gastric tube. They were subsequently fed a mixture containing 2 parts infant formula (Similac®, Abbott Park,



IL) to 1 part water. Piglets were fed *ad libitum* by bottle for the first 30 hours and were subsequently weaned to a trough replenished 3 times daily. 18 hour cultures ( $10^9$  CFU) were used to inoculate piglets at 24 and 30 hours of age. The inoculated *Bifidobacterium* sp. B5445 (Chr Hansen Collection number 5445) and *Lactobacillus* sp. L3777 (Chr Hansen Collection number 3777) cultures were obtained from Chr Hansen (Hørsholm, Denmark) and were prepared in MRS broth (Difco Laboratories, Sparks, MD) containing cysteine HCl while the *E. coli* culture was prepared in tryptic soy broth (BBL, Sparks, MD). The *E. coli* inoculant was a non-haemolytic isolate obtained from feces of a clinically healthy pig and utilized in previous investigations (Shirkey et al. 2006).

### 5.3.2 Tissue Collection

At 15 days of age pigs were killed by submersion in CO<sub>2</sub> and exsanguination then the small intestine, cecum, liver and spleen were removed. Proximal and distal SI contents were collected separately as well as cecal contents to confirm treatment status and to enumerate culturable bacteria. A 30 cm segment was collected from the location identified as 75% of the SI length and mesentery was carefully removed. From this segment two 10 cm segments were snap frozen for later gene expression analysis and a 1 cm section was collected and preserved in 10% buffered formalin for histological examination.

### 5.3.3 Confirmation of Treatment Status

Conventional microbial culture and molecular identification were used to enumerate bacteria and confirm treatment status. Bacteria free status of pigs was confirmed by peri-anal swabs placed in brain heart infusion broth with 0.5% cysteine HCl prior to inoculation at 24 hours of age. Mono and di-association were confirmed by collecting digesta from the proximal SI, distal SI and cecum in peptone tubes, diluted  $10^{-1}$ ,  $10^{-3}$  and  $10^{-5}$  and plating using the Autoplate<sup>®</sup> 4000 (Spiral Biotech Inc., Bethesda, MD) onto blood agar base (BBL, Sparks, MD) with 5% defibrinated sheep blood and cultured aerobically and anaerobically. For the enumeration of inoculant bacteria diluted intestinal contents were also plated on MacConkey's agar (Difco Laboratories) cultured aerobically, and on MRS agar with CysHCl (BBL) cultured anaerobically. Plates were incubated for 24-48 hours at 37 °C prior to colony enumeration. Gram-stains were performed on colonies from all treatments and viewed microscopically to confirm appropriate morphology and cell wall type. The identity of colonies with inconsistent cell or colony morphology was determined by sequencing of chaperonin 60 UT sequence (Hill et al., 2002). Species specific cpn60 primers were designed for contaminant organisms in Bb treatment, *Staphylococcus simulans* (forward 5'AGGCGAAGCGGTACAAGC3' reverse 5'TCTGTGTTGAAGCCGTTTGATTC3') and *Corynebacterium* (forward 5'CGACGCCTTGCTCTCTTCC3' reverse 5'CGACGCCGAAGGTGTTGG3'), to assess whether contamination was limited to this treatment group.

#### 5.3.4 Villus Morphology

Villus morphology was assessed on hematoxylin and eosin stained cross sections from the location identified at 75% of intestinal length. Villus height and crypt depth were measured in 10 well oriented villi by a blinded observer on an Axiostar plus light microscope (Carl Zeiss Canada Ltd., Toronto, ON).

#### 5.3.5 Caspase 3 Activity

Caspase 3 like enzyme activity was measured in homogenized tissue using the EnzChek<sup>®</sup> Caspase 3 Assay Kit (Molecular Probes, Inc., Eugene, OR) based on fluorescence emission following Asp-Glu-Val-Asp (DEVD) specific cleavage. Briefly, a 5 mg sub sample of a pulverized small intestinal segment (10 cm) was lysed in 200  $\mu$ L of lysis buffer (10 mM TRIS, pH 7.5, 0.1 M NaCl, 1 mM EDTA, 0.01% TRITON<sup>™</sup> X-100) using a freeze thaw cycle in liquid nitrogen. The lysed cells were then centrifuged (5000 rpm for 5 minutes) and 50  $\mu$ L of the resulting lysate was added in duplicate to a 96 well plate. To insure that fluorescence signal was due to caspase 3 like proteases a control was included by adding Ac-DEVD-CHO inhibitor to lysed cells. A standard curve was prepared using amino-4-methylcoumarin (AMC) ranging from 3.125-100  $\mu$ M. 50  $\mu$ L of 2X reaction buffer (20 mM PIPES, pH 7.4, 2 mM EDTA, 0.2% CHAPS, 0.02 mM Z-DEVD-AMC) was added to each sample well and incubated for 10 min at room temperature. Fluorescence was read using a Fluoroskan Ascent fluorometer (Thermo Labsystems, Helsinki, Finland). Assay specificity was confirmed by addition of the specific inhibitor, Ac-DEVD-CHO. Linearity was confirmed using serial 2-fold dilutions of cell lysate.

### 5.3.6 Gene Expression

Total RNA was extracted using the RNeasy<sup>®</sup> Mini Kit (Qiagen, Mississauga, ON) from a 20-30 mg subsample after pulverizing a 10 cm segment of intestinal tissue under liquid N<sub>2</sub> with a mortar and pestle. Optional on column DNase digestion was performed using the RNase-free DNase Set (Qiagen). RNA was quantified using Ribogreen<sup>®</sup> (Ultrospec 2000; Pharmacia Biotech, Baie d'Urfe, QUE, Canada) and 1 µg of RNA was used to generate first strand cDNA using SuperScript<sup>™</sup> III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Quantitative PCR using an iCycler iQ Real-Time PCR detection system (BioRad) was performed on resulting cDNA using SYBR<sup>®</sup> Green detection. Primers for IL-1β, IL-8, IL-10, NGF, TGF-β, FasL, TNFα, PCNA and GAPDH are shown in Table 5.1. For quantification of transcript copy number standard curves were established for target and housekeeping genes. Standard curves (range 10<sup>1</sup>-10<sup>7</sup> copies target gene) were generated either by using quantified gel extracted PCR product or by cloning the appropriate RT-PCR product using the pGEM-T<sup>®</sup> Easy Vector System II (Promega Co., Madison, WI), purifying plasmid DNA (Qiagen Plasmid Mini Kit, Qiagen) and quantification of plasmid DNA using PicoGreen DNA Quantitation Kit (Molecular Probes, Eugene, OR). Transcript copy numbers are reported per 10,000 copies of GAPDH.

### 5.3.7 Statistical Analysis

All results were analyzed as a one-way ANOVA using the general linear model procedure (SPSS program, Chicago, IL). Treatment means were separated using REGWF with significance of  $P < 0.05$ . Correlations were analyzed using the Pearson correlation coefficient.

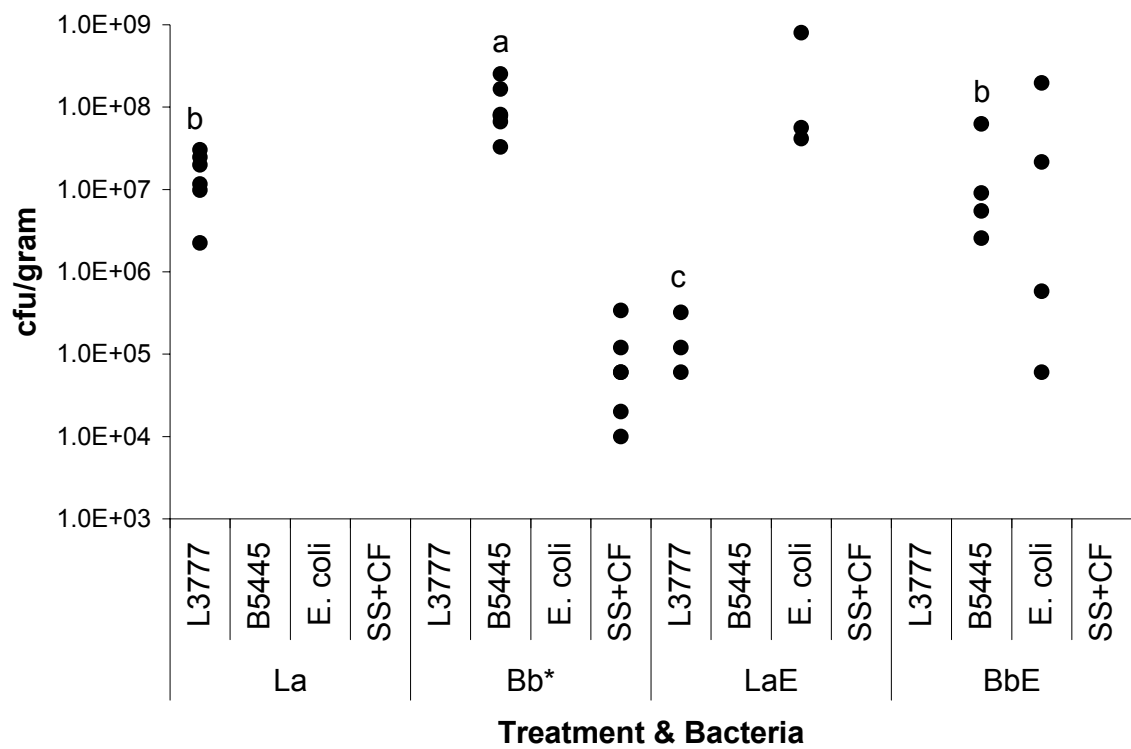
Table 5.1 Quantitative real-time PCR primers for all genes.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
FasL	AAGAAGAAGAGGGACCACAATG	CTTTGGCTGGCAGACTCTCT
GAPDH	GTTTGTGATGGGCGTGAAC	ATGGACCGTGGTCATGAGT
IL-1 $\beta$	CTCCCATTTCTCAGAGAACCAAG	GTGATGGCTAACTACGGTGACAA
IL-8	GGACCAGAGCCAGAGAGAC	GGTGTGGAATGCGTATTTATGC
IL-10	TGAGAACAGCTGCATCCACTTC	TCTGGTCCTTCGTTTGAAAGAAA
NGF	GAGCAAGCGGTCGTCATC	CTGTTGTTGATATTCACCTCTCC
TGF $\beta$	CGGAACCTGTATTGCTCTCTC	GACATCAAAGGACAGCCACTC
TNF $\alpha$	CCAGGGCTCCAGGAGGTG	CCAGAGGGTTGATGCTCAAGG
PCNA	TACGCTAAGGGCAGAAGATAATG	CTGAGATCTCGGCATATACGTG

## 5.4 Results

### 5.4.1 Microbial Colonization

Anal swabs taken prior to inoculation at 24 hours of age and cultured in BHI broth containing cysteine HCl indicated that all pigs were germfree. All swabs taken 48 hours after inoculation suggested microbial colonization had occurred. Colony morphology of cultured digesta samples taken from the proximal SI, distal SI and cecum indicated that the Bb treatment was contaminated and subsequently denoted Bb\*. Sequencing of *cpn60* UT sequence for suspect contaminating colonies indicated 2 contaminating organisms, namely; *Staphylococcus simulans* and an organism 89% similar to *Corynebacterium flavescens*. Both of the contaminant organisms grew on blood agar aerobically whereas B5445 did not. Total colonization of the two contaminant organisms in the distal intestine ranged from 4.0 to 5.5 log cfu/g (Fig. 5.1). Culture analysis indicated mono-association of the La pigs and di-association of the LaE pigs. B5445 colonized at higher ( $P < 0.05$ ) levels than L3777 in mono-associated and di-associated treatments, and *E. coli* reduced colonization levels ( $P < 0.05$ ) of both L3777 and B5445 as compared to mono-association ( $P < 0.05$ ) (Fig. 5.1). *E. coli* colonization ranged from 4.78-8.29 and 7.61-8.9 log cfu/g for BbE and LaE treatments respectively (Fig. 5.1). To confirm that the *S. simulans* and *Corynebacterium* sp. contaminants were not present in other treatment groups, standard PCR was performed on DNA isolated from distal SI contents using species specific *cpn60* primers. Contamination of all Bb pigs with *Staphylococcus* and



*Corynebacterium* was indicated by the presence of an appropriately sized PCR product. No PCR product was observed in samples from pigs in remaining treatment groups.

#### 5.4.2 Animal Health and Performance

Over the first 8 days piglets in all treatments performed well with no signs of illness or diarrhea. Three piglets in LaE treatment were euthanized, one each on days 10, 11 and 15, and 2 piglets in BbE treatment were euthanized, one each on days 12 and 14 after acute onset of clinical signs including rapid respiration, shivering, lethargy and anorexia. Post-mortem examination indicated systemic sepsis including peritoneal and pericardial fluid accumulation and fibrinosis. Liver swabs were positive for *E. coli* and negative for L3777 or B5445. All La and Bb\* piglets were healthy through the course of the experiment. Of the BbE pigs appearing clinically normal at the end of the experiment two of 4 clinically normal surviving BbE pigs and 2 of 3 clinically normal surviving LaE pigs presented with mild pericardial fibrinosis on euthanasia at the end of the experiment.

#### 5.4.3 Villus Morphology

The surviving di-associated pigs showed reduced villus:crypt ratios ( $P < 0.05$ ) as compared to their respective mono-associated and contaminated mono-associated treatments, although there were no significant effects on villus height (Fig. 5.2). BbE pigs had deeper crypts ( $P < 0.05$ ) than Bb\* and La treatment groups.



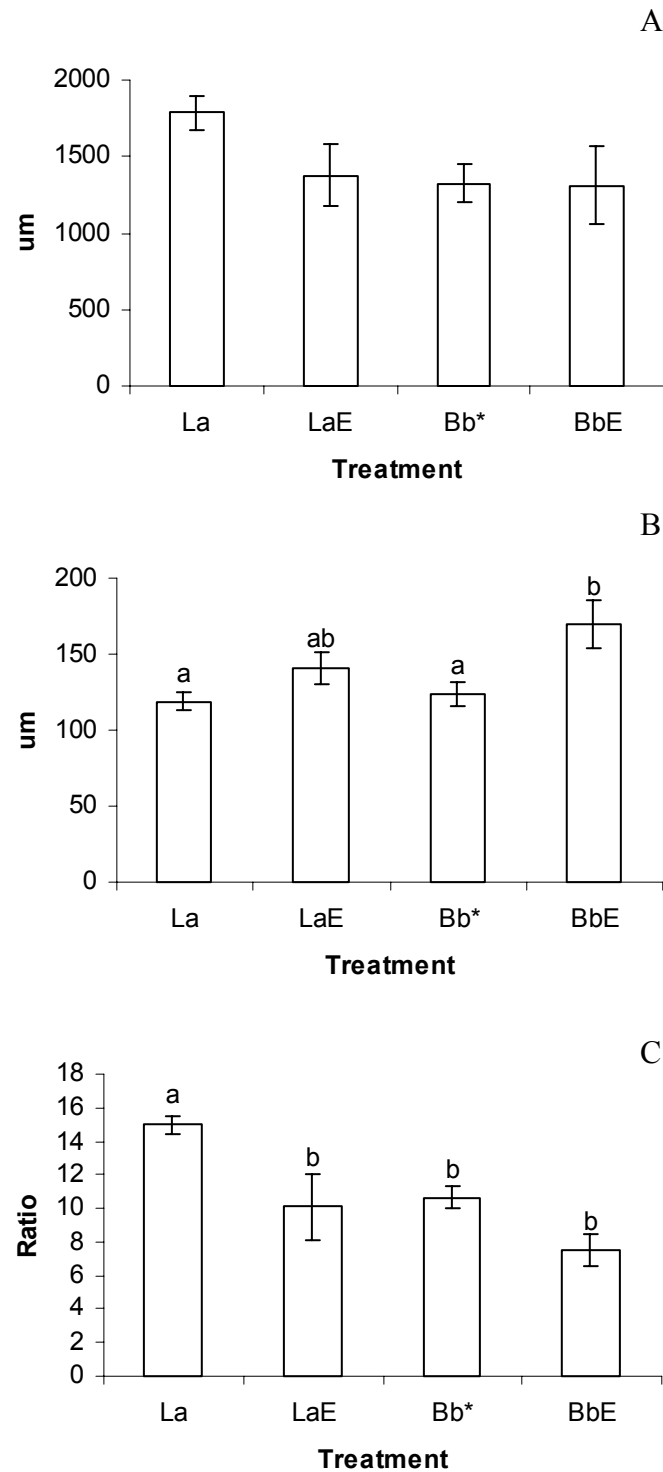


Figure 5.2 Villus height (A), crypt depth (B) and villus crypt ratio (C) in 15 day old pigs. Vertical bars represent SE. <sup>a,b</sup>Means with a different superscript are different ( $P<0.05$ ).

#### 5.4.4 Proliferation and Apoptosis

Caspase 3 like specific activity was reduced 2 fold for each 2-fold dilution of lysate. No activity was detected in samples incubated with a specific inhibitor. Caspase 3 like activity was increased ( $P < 0.05$ ) in LaE, BbE, and Bb\* as compared to La pigs (Fig. 5.3) and was positively correlated ( $R^2 = 0.580$ ,  $P < 0.05$ ) with expression of death ligand, FasL. Proliferative activity as indicated by PCNA was not significantly different between treatment groups (Table 5.2).

#### 5.4.5 TNF $\alpha$ , IL-1 $\beta$ , IL-8 and FasL Gene Expression

The expression of inflammatory cytokines (Table 5.2), IL-1 $\beta$  and TNF $\alpha$ , was increased ( $P < 0.05$ ) in di-associated pigs, while La and Bb\* pigs showed similarly low expression. IL-8 expression was significantly higher ( $P < 0.05$ ) in LaE pigs as compared to BbE as well as in all other treatment groups with La pigs showing the lowest IL-8 expression. The expression of death ligand FasL was higher ( $P < 0.05$ ) in LaE pigs than all other treatments indicating increased T-cell infiltration. Again La treatment showed the lowest expression of FasL. The expression of IL-8 and FasL were positively correlated ( $R^2 = 0.658$ ,  $P < 0.05$ ).

#### 5.4.6 IL-10 and TGF- $\beta$ Gene Expression

The expression of anti-inflammatory cytokine IL-10 (Table 2) was quite low in intestinal homogenates, however expression was higher ( $P < 0.05$ ) in di-associated pigs than La pigs while Bb\* showed intermediate expression. The expression of IL-

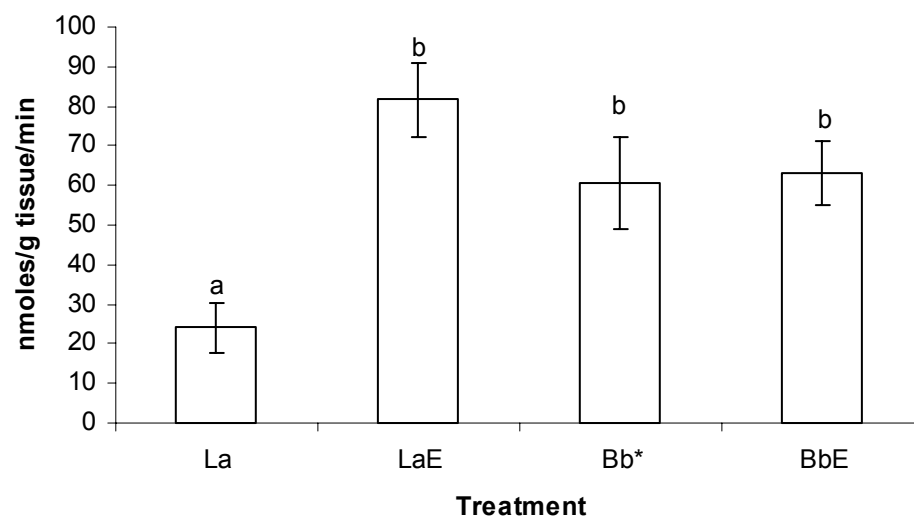


Figure 5.3 Caspase 3 like activity in homogenized intestinal tissue from the 75% location in 15 day old pigs. Vertical bars represent SE. <sup>a,b</sup>Bars with a different superscript are different ( $P<0.05$ )

Table 5.2 Mean number of IL-1 $\beta$ , IL-8, TNF $\alpha$ , IL-10, TGF- $\beta$ , NGF, and FasL transcripts per 10,000 copies of GAPDH in homogenized tissue at 75% of small intestinal length in 15 day old gnotobiotic pigs.

Treatment Group	Gene of interest							
	IL-1 $\beta$	IL-8	TNF $\alpha$	IL-10	TGF- $\beta$	NGF	FasL	PCNA
La	3.67 <sup>a</sup>	5.60 <sup>a</sup>	4.62 <sup>a</sup>	.12 <sup>a</sup>	48.02	1.33	12.4 <sup>a</sup>	379.1
Bb*	3.54 <sup>a</sup>	8.13 <sup>a</sup>	6.88 <sup>a</sup>	.32 <sup>ab</sup>	61.53	1.41	21.8 <sup>ab</sup>	387.5
LaE	15.15 <sup>b</sup>	11.80 <sup>b</sup>	13.66 <sup>b</sup>	.50 <sup>b</sup>	74.50	1.48	42.8 <sup>c</sup>	533.8
BbE	11.87 <sup>b</sup>	7.49 <sup>a</sup>	11.33 <sup>b</sup>	.49 <sup>b</sup>	67.16	1.32	22.1 <sup>b</sup>	447.2
Pooled SEM	1.72	1.61	1.23	.08	11.25	0.18	9.4	55.4

<sup>a,b,c</sup> Means within the same column with different superscripts are different ( $P < 0.05$ )

\* Treatment contaminated with *S. simulans* and *Corynebacterium* sp.

10 was positively correlated with  $\text{TNF}\alpha$  ( $R^2 = 0.795$ ,  $P < 0.05$ ) and  $\text{IL-1}\beta$  ( $R^2 = 0.709$ ,  $P < 0.05$ ).  $\text{TGF-}\beta$  was highly expressed in the intestinal tissue although expression was not significantly affected by treatment. NGF was unaffected by microbial treatment.

## 5.5 Discussion

Administration of probiotic organisms and probiotic cocktails, including a variety of *Lactobacillus* and *Bifidobacterium* species, have proven beneficial in human and animal health, both by potentiating the innate and adaptive immune systems (Hatcher and Lambrecht, 1993; Linkamster et al., 1994; Schiffrin et al., 1995) and conversely by stimulating anti-inflammatory responses (Matsumoto and Benno, 2006; Osman et al., 2006). Defining the mode of action in vivo is challenged by the complexity of the intestinal microbiota, while in vitro experiments lack the complex interactions between cell types involved in the immune response.

*Lactobacillus* and *Bifidobacterium* species are common Gram-positive commensal bacteria found in the digestive tract of both humans and pigs. Consistent with our previous comparison of a representative Gram-positive bacteria (*L. fermentum*) and representative Gram-negative bacteria (*E. coli*) we observed that mono-association with L3777 and B5445 (plus contaminants) did not significantly stimulate an inflammatory response as compared to pigs associated with Gram-negative *E. coli* plus L3777 or B5445. Di-association with *E. coli* and either L3777 or B5445 in the current study resulted in induction of inflammatory cytokine expression and a high incidence of sepsis. Previously we have observed increased

expression of TNF $\alpha$  and IL-1 $\beta$  (Shirkey et al., 2006) in *E. coli* mono-associated pigs but without sepsis. In fact, among 20 pigs mono-associated with this organism only a single case of sepsis was observed (unpublished). It is unfortunate that isolator space limitations did not allow us to incorporate an *E. coli* mono-associated group in the current study to permit a direct comparison.

The effect of bacterial association on enterocyte turnover was investigated by examination of several parameters including mucosal morphology, caspase 3-like activity as an indicator of apoptosis, PCNA expression as an index of cell proliferation, and FasL expression as a death receptor ligand. In each case these parameters were in agreement and suggested that L3777 had the lowest impact on enterocyte turnover. These parameters suggested that enterocyte turnover was somewhat higher in Bb\* group, however, this may have reflected colonization by the contaminating organisms rather than a response to B5445. Enterocyte turnover indicators were also consistent in suggesting that *E. coli* di-association markedly increased enterocyte turnover. Enterocyte turnover indicators were similar for *E. coli* di-association with L3777 or B5445, although the FasL response was lower in B5445 di-associated pigs and crypt depth was paradoxically higher.

The lack of IL-8 expression induced by L3777 agrees with findings that *L. fermentum* did not induce NF- $\kappa$ B activation and IL-8 secretion in intestinal epithelial cells (Frick et al., 2007). Consistent with our results Lammers et al. (2002) found that Gram-positive probiotic bifidobacteria and lactobacilli were unable to induce IL-8 in epithelial cell monolayers, while probiotic *E. coli* Nissle induced IL-8 in a dose dependent manner.

IL-1 $\beta$  and TNF $\alpha$  were higher in surviving di-associated pigs but interestingly IL-8 was significantly increased only after L3777 di-association and not B5445. IL-1 $\beta$  and TNF $\alpha$  were also numerically lower in the B5445 di-associated pigs relative to L3777 di-association. This could indicate a level of inflammatory protection by B5445. In agreement, Riedel et al. (2006), found that 6 of 8 bifidobacteria strains inhibited LPS induced NF- $\kappa$ B activation in HT-29 intestinal epithelial cells. They observed a dose dependent decrease in IL-8 mRNA with bifidobacteria administration as well as a decrease in TNF $\alpha$ . Jijon et al. (2004) also found that *Bifidobacteria* inhibit basal IL-8 secretion in epithelial cells.

As well as inducing the expression of inflammatory cytokines and chemokines, *E. coli* induced the expression of anti-inflammatory cytokine IL-10, which is consistent with the ability of LPS to induce IL-10 in monocytes (Hessle et al., 2000). Expression of IL-10 in La pigs was particularly low and is contrary to reports of IL-10 induction by administration of *Lactobacillus sp.* probiotics to bone marrow derived macrophages (Diaz-Ropero et al., 2007), supporting the requirement for an in vivo model for probiotic assessment. It has been suggested that NGF may play an important role in prevention of uncontrolled inflammation in the intestine (Ma et al., 2003) however, NGF expression was not affected in this experiment. We did not observe an increase in the expression of NGF in response to increased IL-10 expression, as has been previously observed in intestinal epithelial cells (Ma et al., 2003).

Probiotic *Bifidobacteria* and *Lactobacilli* have been shown to be strong inducers of TNF $\alpha$ , IL-6 and IL-10 production in peripheral blood mononuclear cells (PBMC) (Miettinen et al., 1996). However, we did not observe a similar induction of cytokine production in vivo with B5445 or L3777. Of course, exposure of PBMC to bacteria requires translocation across the intestinal epithelium, thus this difference may not be surprising. Also, dendritic cells, which reside in the intestinal epithelium and may have direct contact with bacteria in the intestinal lumen, respond quite differently to Gram-positive and Gram-negative bacteria than do monocytes in peripheral blood (Karlsson et al., 2004). Monocytes produce more IL-12 and TNF $\alpha$  when exposed to *L. plantarum* than when exposed to *E. coli*, whereas monocyte-derived dendritic cells respond robustly to *E. coli* and minimally to *L. plantarum* by expression of inflammatory cytokines (IL-6, IL-12 and TNF $\alpha$ ) and IL-10 (Karlsson et al., 2004). Our results are consistent with the observation in dendritic cells, indicating the importance of cell choice in evaluation of probiotic organisms.

*Bifidobacteria* and *Lactobacilli* probiotics have demonstrated the ability to increase non-specific immune response in a macrophage-like cell line enhancing phagocytosis of Salmonella (Hatcher and Lambrecht, 1993). Feeding of products fermented with *Bifidobacteria* and *Lactobacilli* probiotic strains increased the ability of blood leukocytes to phagocytose *E. coli* (Schiffrin et al., 1995). *Bifidobacteria* have also been shown to potentiate the immune response to *Salmonella typhi* by increasing IgA titer when administered in conjunction with attenuated *S. typhi* (Linkamster et al., 1994). In this experiment we do not see a potentiation of the



immune system as pigs associated with both *E. coli* and L3777 or B5445 were surprisingly susceptible to sepsis.

Evidence of sepsis was indicated in the majority of di-associated pigs indicating translocation of *E. coli* across the epithelial barrier. Indigenous bacteria translocate continuously at low levels in healthy hosts but are rapidly cleared in mesenteric lymph nodes (MLN). There are three main mechanisms by which translocation is promoted including damage to the mucosal barrier, a compromised immune system and bacterial overgrowth (Berg, 1995). In this experiment we observe bacterial overgrowth ( $>10^8$  *E. coli* in cecum) in animals with limited immune development evidenced by limited organization of Peyer's patches (Shirkey et al., 2006). However, *E. coli* overgrowth was similar to, or lower than in our previous mono-association experiments (8-9 log cfu/g) without significant incidence of sepsis. Translocation to the mesenteric lymph node complex (MLN) occurs at elevated levels in mice mono-associated with *E. coli* (Berg and Garlington, 1979). However *E. coli* are undetectable in MLN when an indigenous microbiota is introduced, suppressing *E. coli* colonization below  $10^8$  in the cecum (Berg and Owens, 1979), suggesting that translocation would not be a concern in a conventional animal.

Overt signs of sepsis were not observed until 8 days of age. In a mouse sepsis model, induced by oral administration of antibiotics and an immunosuppressive agent, translocating bacteria are first cultured from MLNs, but with time continue to the liver, spleen, kidney, peritoneal cavity and finally the bloodstream (Berg et al., 1988). The number of bacteria at these sites increased with time eventually leading to

lethal sepsis in all mice by day 14, agreeing with the delayed period before clinical signs of sepsis in this experiment.

The level of translocation is directly related to level of colonization for individual bacteria, but different bacteria have varying abilities to translocate, with *E. coli* having a great aptitude for translocation (Steffen et al., 1988). The ability of *Lactobacilli* to inhibit or displace enteropathogens is species specific and it appears that L3777 does not displace this *E. coli* (Gueimonde et al., 2006). Translocation in the case of bacterial over-growth is through intracellular passage (through epithelial cells) and not by physical damage or disruption of tight junctions (Berg, 1995), which agrees with our morphological observation of tissues, as we saw no evidence of barrier damage. Translocation of LAB has been reported (Soleman et al., 2003), however swabs taken from the liver were negative for L3777 and B5445, but positive for *E. coli*.

The reason we observed sepsis in this experiment and not previously with *E. coli* mono-association may be a result of increased stress. Non-stressed mice show limited bacterial translocation as indicated by levels of bacteria in lymph nodes, spleen and liver as compared to mice stressed with social disruption or chronic restraint (Bailey et al., 2006). In this experiment pigs were housed in groups of 3 whereas in previous experiments they have been housed individually. However, the pigs were not mixed after initial grouping and very little fighting or competition for food was observed. The argument could also be made that group housing reduced stress.

Increased sepsis may also have been related to changes in the activation of immune function. Both CD4<sup>+</sup> and CD8<sup>+</sup> T cells and macrophages are important in preventing translocation (Berg, 1995; Gautreaux et al., 1995). The probiotic organisms may have reduced the initial inflammatory response associated with *E. coli* colonization thus reducing infiltration of immune cells required to clear the translocating bacteria. Both *L. fermentum* and *L. reuteri* reduce acute inflammatory response as evidenced by reduced myeloperoxidase activity, TNF $\alpha$  expression and inducible NO synthase (iNOS) expression in a model of rat colitis (Peran et al., 2007). This theory would agree with the reduced ability of aflagellate *Salmonella* mutants to induce pro-inflammatory and anti-apoptotic factors during early infection, but increase apoptotic activity leading to a delayed but exacerbated mucosal inflammation and elevated systemic and intestinal bacterial load (Vijay-Kumar et al., 2006).

*L. acidophilus* isolated from a newborn, inoculated into a germfree mouse improves macrophage ability to clear intravenously injected *E. coli* (Neumann et al., 1998). Although macrophages and polymorphonuclear leukocytes are important in host defense it has also been suggested that they engulf bacteria at the mucosal layer and transport them to lymph nodes and other sites (Harmsen et al., 1985). It has been considered that the difference observed between experiments may have been a result of genetic difference, however translocation of commensal Enterobacteriaceae does not seem to be affected by genotype as 14 inbred strains of mice show no differences in translocation (Maejima et al., 1984) and we observed sepsis in pigs from all three litters.

*L. salivarius* and *B. infantis* have proven effective in reducing inflammatory cytokine production in IL-10 knockout mice while having no effect on anti-inflammatory cytokine TGF $\beta$  (McCarthy et al., 2003) suggesting that there are other anti-inflammatory mediators involved. *L. salivarius* and *B. lactis* alone strongly stimulate IL-10 production in PBMC in vitro while having little effect on IL-12 production (Drouault-Holowacz et al., 2006). *B. infantis* has been shown to reduce myeloperoxidase activity in a rat colitis model as well as IL-1 $\beta$  expression, while IL-10 and TGF- $\beta$  were unaffected (Osman et al., 2006). The fact that acute inflammation was reduced without any subsequent increase in these anti-inflammatory mediators suggests that there are other mechanisms by which inflammation is suppressed in this model.

The results of this experiment indicate the importance of experimental model when assessing the effectiveness of probiotic organisms. Relative to *E. coli* both L3777 and B5445 have a limited ability to stimulate an inflammatory response suggesting that the immune potentiation previously observed may be due to modification of the microbiota. The incidence of sepsis observed here relative to our previous data suggests both probiotics may have anti-inflammatory effects allowing enhanced *E. coli* translocation. However, cytokine expression profiling did not indicate a clear anti-inflammatory mechanism. Evaluation of the inflammatory and anti-inflammatory response earlier in the colonization period may give some more insight as to why sepsis occurred in this experiment. Certainly, an anti-inflammatory mode of action may be of benefit for some probiotic indications (e.g. ileitis). The

results of this experiment also indicate a need for caution in the use of probiotic organisms that may suppress an inflammatory response in immuno-compromised animals or those not harbouring a complete intestinal microflora.

## 6.0 GENERAL DISCUSSION AND CONCLUSIONS

There have been several significant contributions of this work as set out by the hypotheses and objectives posed at the outset (p. 2). In Chapter 3, we found that members of the early colonizing commensal microbiota, namely, *E. coli* and *L. fermentum* have substantially different effects on both proliferative and apoptotic activity and that this response is associated with the induced expression of an inflammatory cytokine and death ligands. While enterocyte replacement has previously been well described in response to pathogenic bacteria we extend findings to members of the commensal microbiota. In Chapter 4, we found that a conventional microbiota substantially affects digestive function of the host, and indicate that microbial deactivation of BB enzymes plays a substantial role. Although not fully elucidated we present evidence of a compensatory host mechanism to maintain digestive function associated with microbial competition for nutrients. In Chapter 5, we found that probiotic bacteria B5445 and L3777, have limited ability to induce the expression of inflammatory cytokines *in vivo*, indicating the importance of an *in vivo* model when assessing probiotic mode of action. Overall these results indicate that the composition of the early colonizing microbiota plays an important role in intestinal development and function and emphasizes the need to understand the mechanisms by which commensal and probiotic bacteria exert their effects on host physiology.

The study of microbial effects on host physiology and the mechanisms involved in the host-microbial interaction poses many challenges. A single mutation within a bacterial species can have a dramatic affect on the host response to colonization (Mazmanian et al., 2005), thus considering how a population of over 400 species, each expressing thousands of genes, affects the host is unfathomable. Using germfree or gnotobiotic models allows for the study of how individual or groups of microbes affect host physiology, alleviating the challenge of microbial diversity, while providing some insights that cannot be observed *in vitro*.

*In vitro* study of the host-microbial interaction is advantageous in that it allows for the study of cell specific responses and for the study of numerous bacteria, however results observed *in vitro* often cannot be replicated *in vivo* and vice versa. An example of the deficiency of using cell lines is their lack of Cdx-2 expression, an important transcription factor in chemokine expression, thus the ability of bacteria to induce expression of chemokines observed *in vivo* may not be observed *in vitro* (Ericsson et al., 2004).

Another important deficiency of *in vitro* models is that they lack the variety of cell types present in and adjacent to the intestinal epithelium. The many cell types including absorptive enterocytes, goblet cells, enteroendocrine cells, and intra-epithelial lymphocytes present in the intestinal epithelium interact with each other, as well as with cells in the lamina propria including a variety of immune cells. This communication is essential in how the host as a whole responds to microbial colonization. There are many examples of interactions between cells and I will only mention one. As discussed in Chapter 2, many bacteria produce butyrate as a product

of carbohydrate fermentation. Enteroendocrine cells respond to butyrate by increasing the expression of GLP-2. Increased release of GLP-2 by enteroendocrine cells then induces a variety of responses in neighboring absorptive cells (Tappenden et al., 2003). This important interaction would be missed *in vitro* because of the absence of enteroendocrine cells in intestinal epithelial cell lines.

In the gnotobiotic model we are able to identify specific *in vivo* outcomes in response to bacteria, but often have difficulty in identifying the pathways involved due to the number of possibilities, or the inability to examine specific cell types without interference. *In vitro* studies more readily allow for the control or manipulation of regulatory pathways. Determining that the growth regulatory effect of LPS on epithelial cells is dependent on the expression of TNF $\alpha$  was made clear by *in vitro* work (Ruemmele et al., 2002).

Certainly, there are opportunities to combine *in vivo* and *in vitro* approaches. One approach is to verify the results of *in vitro* studies *in vivo*, while a second approach is to discover effects *in vivo* and then try to determine the mechanism responsible *in vitro*. For instance, to identify the mechanism by which APN was upregulated in this experiment, examining the effect of microbial components or metabolites such as LPS or butyrate on IEC *in vitro* may allow pathways to be identified. If the response was an indirect response through increased inflammation, exposing IEC to inflammatory cytokines could be revealing. It might also be intriguing to observe whether the *E. coli* used in this experiment translocates across a cell layer, and if so, whether translocation increases with co-incubation with probiotic organisms.



Gnotobiotic research at the *in vivo* level comes with its own challenges including maintaining gnotobiotic conditions, providing an appropriate sterile diet, and microbial overgrowth in the absence of competing bacteria. In early gnotobiotic studies, using autoclaved feed, germfree animals performed quite poorly and high mortality rates were observed because nutrients were destroyed or made unavailable by the sterilization process (Wostmann, 1975). We have overcome this challenge by using canned infant formula; however this creates a different concern. Although piglets grew reasonably well (~90 g/day), it has been shown that the composition of feeds can also affect gene expression (Schmitz and Langmann, 2006). In infant formula, the main energy source is carbohydrate (lactose), while in sows' milk it is fat (Veum and Odle, 2001). This diet may have induced physiological changes in the host; however, diet was consistent in all treatments thus microbial effects are independent of diet.

Another major challenge is establishing a consistent 'conventional' inoculant. To ensure that piglets received a normal flora we used fresh fecal samples for each of the experiments. Although we observed similar results in both trials (Chapters 3 and 4), the differences in villus height were less pronounced in conventional pigs in experiment 2 as compared to conventional pigs in experiment 1. This difference may well have been attributed to differences in microbial composition in the inoculant. Using frozen fecal samples was considered, however, was not used because many bacteria do not tolerate freezing, limiting the ability of a frozen inoculant to reflect a conventional flora. It is also questionable whether only two fecal inoculations, as we used here in the very early postnatal period, allow for natural microbial succession.

In nature piglets are continually exposed to fecal inoculants, whereas in gnotobiotic isolators viable bacteria in the inoculant may have died before microbial succession created a niche allowing their growth. Regardless, the physiological differences observed between conventional pigs in the two experiments support the hypothesis that there may be opportunity to improve growth efficiency by manipulating microbial composition in the early neonatal period.

In the gnotobiotic model the lack of normal microbial-microbial competition for nutrients and space allow some bacteria to colonize at much higher levels than in a conventional animal. Microbial overgrowth can exacerbate the effects that a given bacteria will have on the host, thus results should be interpreted carefully. The converse may also be true as many bacteria are dependent on their co-colonizers for the environment they create or metabolites produced, thus may colonize at lower levels than in a conventional environment or produce a different array of fermentation products.

To characterize physiological changes induced by early colonizing bacteria we compared pigs mono-associated with *L. fermentum* or *E. coli* to pigs that were maintained germfree and pigs conventionalized with sow feces. *L. fermentum* and *E. coli* were selected because they are dominant early colonizing bacteria and as representatives of the major cell wall division (Gram-negative and Gram-positive).

In Chapter 3 we measured gene expression in both isolated enterocytes as well as whole tissue. Results were similar for genes that were measured in both tissues; however, we believe that by measuring certain responses in enterocyte isolates gives deeper insight into host response. We were able to identify proliferating enterocytes

as the main contributor to increased PCNA expression in CV and EC pigs, whereas this would not have been clear if we had measured PCNA in whole tissue as cells of Peyer's patches would have been a major contributor to PCNA abundance. The use of laser capture microdissection would have further aided in interpretation as responses in individual cell types could have been evaluated. The question of whether mRNA and protein abundance changed during the process of isolation would have been avoided, although villus-crypt data suggest that this was not significant, at least for the parameters we investigated.

In Chapter 3 we demonstrated that intestinal structure of the conventional animal is dependent on the composition of early colonizing microbiota. Two distinct commensal bacteria elicited substantially different results with *E. coli* increasing both apoptotic and proliferative activities. The mechanism by which the host responded to these bacteria is still not clear, however, the differential regulation of TLRs indicates that they may play a substantial role. Because of the increased expression of death ligand, FasL, and its correlation to apoptotic activity, it appears that induction of inflammatory responses and activation of apoptosis through death receptors play a significant role in microbially induced enterocyte turnover. Increased proliferative activity in CV and EC pigs strongly correlated with TNF $\alpha$  expression and agrees with findings *in vitro* where the growth stimulatory effects of LPS on IEC have been shown to be dependent on TNF $\alpha$ , which is induced by TLR4 activation (Ruemmele et al., 2002).

One might consider the minimal turnover associated with *L. fermentum* colonization to be beneficial, while increased inflammatory response and turnover associated with *E. coli* to be detrimental. This may be the case in an entirely pathogen free situation as cell turnover comes at a significant metabolic cost (Nyachoti et al., 2000). However, it must be recognized that cell turnover plays an important role in host defense (Oswald, 2006). In targeting an ‘optimal’ microbiota in the early neonatal period one might target a microbiota that induces an intermediate level of turnover, optimizing growth efficiency while maintaining intestinal health.

In examining the effect of these same bacteria on digestive function (Chapter 4), we showed that a conventional microbiota, and to a lesser extent *E. coli* increase turnover of brush border enzymes. The increased deactivation of secreted enzymes in conventional as compared to gnotobiotic animals has previously been demonstrated (Malis et al., 1976), however this has not previously been demonstrated in BB enzymes. The results were intriguing, because reduced APN activity was observed in the face of increased gene expression. Others have reported that colonization may increase digestive capacity based on gene expression results (Hooper et al., 2001a). Had we approached this experiment in the same way we would have concluded that a conventional microbiota increases the ability of the pig to digest peptides, whereas the observed decrease in APN activity suggests the opposite. The increased gene expression of APN in CV and EC pigs may be the result of direct response to microbial colonization, or alternatively may be a response to microbial deactivation of the enzyme. Regardless of the mechanism inducing increased expression, the host

may use this as a means to ensure effective competition with the intestinal microbiota for nutrients. These results also point to the value of looking at both mRNA and protein levels.

In the final experiment (Chapter 5) we examined the effect of probiotic bacteria, L3777 and B5445, alone and in combination with *E. coli*. When co-inoculated with these probiotic bacteria, *E. coli* had inflammatory effects similar to those previously observed. Unfortunately an *E. coli* mono-associated group could not be included in this experiment because of a limited number of isolators. The probiotic bacteria alone had little effect on pro or anti-inflammatory mediators. These organisms have both been shown to be pro-inflammatory when exposed to PBMC, indicating a discrepancy between *in vitro* and *in vivo* results and supporting the need for an *in vivo* model when investigating probiotic mode of action. Interestingly co-inoculation of probiotic bacteria with *E. coli* increased host susceptibility to *E. coli* translocation and sepsis. This result suggests that these probiotic bacteria may inhibit the early inflammatory response, thus preventing the host from mounting a sufficient response to clear *E. coli*. Examining the effect of these probiotic bacteria on inflammatory and anti-inflammatory parameters earlier in the colonization process may give some insight as to why sepsis occurred, and would be of considerable interest.

In the parameters measured in these experiments, the host appeared to respond more to *E. coli* than other bacteria including *L. fermentum*, L3777 and B5445. *L. fermentum* has, however, been shown to induce physiological parameters in the pig intestine including increased expression of GLP-2 (Siggers et al., 2003), down-

regulation of metallo endopeptidase and up-regulation of galectin-3 (Danielsen et al., 2007) as compared to *E. coli*, indicating that this bacteria is not entirely benign. *K. pneumoniae* induced similar responses to *E. coli*. The commonality of outer cell wall and LPS between these two organisms suggests the importance of outer membrane in inducing these physiological responses. Examining the effects of ingested purified portions of bacteria including LPS, LTA and other MAMPs might be very informative. Because coliforms and lactobacilli are both early colonizers, it is of interest as to whether these differences observed in the early postnatal period have long term effects.

Further understanding of mechanisms through which the host responds to microbiota might include the use of knockout animals, receptor antagonists and even small interfering RNA (siRNA) in a gnotobiotic setting. Also, comparing the effects of isogenic bacteria with a single mutation will help identify microbial components or products that are recognized by the host or induce physiological change.

Although individually evaluating the effects of each microbe that colonizes the pig intestine is not feasible, understanding the diverse mechanisms by which bacteria affect the host will allow for the development of the 'optimal' microbiota. Understanding these mechanisms will also allow for the development of specific desirable microbial manipulations through the use of pre and probiotics as well as pharmacological applications by blocking or activating host response pathways identified in the host-microbial interaction. In developing strategies to improve animal health and performance consideration must be given to a balance between healthy immune activation and growth efficiency.

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