Peptide Transport in the Neonatal Yucatan Miniature Pig

By

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Abstract

The H^+-coupled transporter peptide transporter 1 (PepT1) is found primarily in the intestine and is capable of transporting dietary di- and tripeptides as well as peptides produced by bacteria. However, little is known about the ontogeny of PepT1 in the piglet. The first part of this thesis describes the investigation of the effects of development and diet on peptide transport in the intestine of the Yucatan miniature piglet. Dipeptide transport was significantly higher in the ileal section in the youngest age group (1 week) compared to the other suckling groups ($p<0.05$); however, all suckling piglet groups demonstrated lower ileal transport compared to post-weaned pigs. These results suggest that peptide transport in the small intestine is important during the first week of suckling and again with diet transition following weaning. The objective of the second part of this thesis was to determine the impact of enterally delivered dipeptide-containing diets on indices of intestinal adaptation in neonatal piglets after intestinal resection, as PepT1 is preferentially maintained over free amino acid transporters in situations of gut stress such as short bowel syndrome. In this model no evidence was found that enteral dipeptides provide specific adaptive benefits compared to constituent amino acids. However, the dipeptide-containing diets reduced pro-inflammatory cytokine concentrations in the mucosa ($p<0.05$). One dipeptide in particular, cysteinyl-glycine, supported greater villus height compared to all other dipeptides and greater crypt depth compared to alanyl-glutamine yet no dipeptide diet altered intestinal morphology compared to the free amino acid control diet. This study demonstrated that while there was no explicit morphological benefit of enteral dipeptides over their constituent free amino acids, there was the potential for the amelioration of intestinal inflammation by reducing pro-inflammatory cytokines. As PepT1 is also capable of transporting bacterial peptides were then investigated intestinal response to a pro-inflammatory peptide, formyl-

methionyl-leucyl-phenylalanine (fMLP) alone or in combination with cysteinyl-glycine in a model of intestinal atrophy. Piglets received parenteral nutrition (PN) for 4 d to induce atrophy while littermates remained with the sow. In both dietary treatments, intestinal segments exposed to fMLP had higher mucosal pro-inflammatory cytokines with this inflammatory effect being attenuated when cysteinyl-glycine was co-perfused with this bacterial peptide ($p<0.05$). Morphologically, fMLP exposure did not alter villus height or crypt depth in sow-fed animals; in contrast, intestinal segments from PN-fed piglets exposed to fMLP had reduced villus height compared to unexposed loops. Inclusion of cysteinyl-glycine was effective at attenuating a bacterial peptide-induced inflammatory response in the injured SI. This may be due to efficient dipeptide uptake in a situation of impaired free amino acid absorption, and/or competitive inhibition of fMLP uptake. Through the use of *in vivo* piglet models, these studies have contributed to the understanding of peptide transport in health and disease states in addition to demonstrating the potential benefits of enteral dipeptide provision.

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List of Abbreviations

Å	Angstrom
AA	Amino acid
AQ	Alanyl-glutamine
AQ+CG	Alanyl-glutamine, cysteinyl-glycine
ANOVA	Analysis of variance
Arg	Arginine
BBMV	Brush-border membrane vesicle
BCA	Bicinchoninic acid assay
BrdU	Bromodeoxyuridine
cDNA	Complementary DNA
CG	Cysteinyl-glycine
CP	Crude protein
Cys	Cysteine
DAB	3,3′-Diaminobenzidine
DEPC	Diethylpyrocarbonate
Dpm	disintegrations per minute
DSS	Dextran sodium sulfate
E. coli	*Escherichia coli*
EN	Enteral
fMLP	formyl-methionyl-leucyl-phenylalanine
GkPOT	*Geobacillus kaustophilus* proton oligopeptide transporter
GlpT	Glycerol-3-phosphate transporter
Glu	Glutamine
Gly	Glycine
GSH	Glutathione (γ-Glu-Cys-Gly)

GSSG	Glutathione disulfide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hPepT1	Human peptide transporter 1
HPT1	Hexose-phosphate transporter 1
IBD	Inflammatory bowel disease
IC_{50}	Half maximal inhibitory concentration
Iκβ	Inhibitor of Nuclear factor kappa beta
IFN-γ	Interferon gamma
IFN-γR1	Interferon gamma receptor 1
IFN-γR2	Interferon gamma receptor 2
IL-1	Interleukin 1
IL-2	Interleukin 2
IL-10	Interleukin 10
IL-10R1	Interleukin 10 receptor 1
IL-10R2	Interleukin 10 receptor 2
IU	International units
JAK	Janus activated kinase
K_i	Inhibition constant
kDa	Kilodalton
KRB	Krebs-Ringer buffer
LacY	Lactose permease
M cell	Microfold cell
MHC-1	Major histocompatibility complex class 1
MPO	Myeloperoxidase
mRNA	Messenger RNA
NEC	Necrotizing enterocolitis
NFκβ	Nuclear factor kappa beta

NHE3	Sodium-hydrogen exchanger 3
PCR	Polymerase chain reaction
PepT1	Peptide transporter 1
PepT2	Peptide transporter 2
$PepT_{So}$	*Shewanella oneidensis* peptide transporter
$PepT_{St}$	*Streptococcus thermophiles* peptide transporter
PHT 1	Peptide/histidine transporter 1
PHT 2	Peptide/histidine transporter 2
PITC	Phenylisothiocyanate
PMSF	Phenylmethanesulfonyl fluoride
PN	Parenteral nutrition
POT	Proton oligopeptide transporter
PTR2	Peptide transporter R2
qPCR	Quantitative polymerase chain reaction
RIP	Death domain kinase receptor-interacting protein
RT-PCR	Reverse transcriptase polymerase chain reaction
SAA	Sulfur amino acids
SBS	Short-bowel syndrome
SI	Small intestine
STAT	Signal transducer and activator of transcription
SRA	Specific radioactivity
TBST	Tris-buffered saline and Tween
TNF-α	Tumor necrosis factor alpha
TNF-R1	Tumor necrosis factor alpha receptor 1
TNF-R2	Tumor necrosis factor alpha receptor 2
Tri-DAP	L-Ala-γ-D-Glu-meso-diaminopimelic acid
V_{max}	Maximum velocity of reaction

Chapter 1: Literature Review

1.1 *Protein digestion and amino acid transport*

Protein is an essential part of a complete daily diet. A typical Western diet usually contains 70-100 g protein per day. Of this total protein, about 95-98% is completely digested and absorbed (Erickson et al., 1995). After initiation of digestion in the stomach, dietary products are moved into the small intestine where pancreatic enzymes continue to break down complex compounds into absorbable products. Through this gastrointestinal processing, protein is broken down into di/tri-peptides, comprising roughly 80% of the total protein, with the remaining 20% being reduced to free amino acids (Ganapathy V, 2006). These resulting products of protein digestion are absorbed from the lumen of the small intestine via specific transport mechanisms found on enterocytes, epithelial cells primarily responsible for nutrient uptake. The mechanism by which an individual amino acid is absorbed by the intestine varies depending on the amino acid. Free amino acid transporters can be classified into five different groups: 1) neutral amino acids, 2) cationic amino acids, 3) anionic amino acids, 4) proline, hydroxyproline, lysine and 5) taurine, β-amino acids (Broer, 2008). Certain amino acids may be transported by more than one system, thereby providing redundancy (Broer, 2008). The activity of the transporters depends on the concentration and variety of amino acids present. While these systems are capable of transporting the free amino acid products of protein digestion, another transporter is required for the removal of di/tripeptides from the lumen of the small intestine (Daniel, 2004).. There are multiple benefits to the transport of peptides over free amino acids. Uptake of di/tripeptides by an apical peptide transporter, PepT1, requires the same amount of energy that is required to transport a single free amino acid (Daniel, 2004). Additionally, it is more efficient to transport amino acids in

small peptide form compared to transporting their constituent free amino acids because peptides demonstrate faster rates of uptake while requiring the same amount of energy (Gilbert et al., 2008b). Investigation of PepT1 has begun to reveal a new facet of amino acid uptake, as well as the evolutionarily conserved nature of peptide transport.

1.2 *Introduction to Proton Oligopeptide Transporters*

Proteins capable of moving small peptides across membranes have been grouped into a transporter superfamily known as proton oligopeptide transporter, or POT. Members of this family are found in most organisms with a significant degree of evolutionary conservation through bacteria, yeast, plants and animals (Daniel et al., 2006). All members of this family of transport proteins are believed to utilize a proton gradient to drive the uptake of their substrates across cell membranes. Currently there are 40 members of this superfamily, however many of those predicted to transport oligopeptides in *C. elegans*, *A. thaliana* and *E. coli* have yet to experimentally demonstrate peptide transport (Meredith and Boyd, 2000). The inclusion of these putative peptide transporters is based on the detection of a specific protein sequence, PTR2, currently used as an identifier for potential peptide transporters. There are currently five peptide transporters identified in mammals, varying in tissue distribution but with similarities in substrate specificity (Daniel and Kottra, 2004). Classed predominantly as members of the SLC15 solute carrier family of transporters, they are SLC15A1, also known as PepT1, SLC15A2/PepT2, SLC15A3/ PHT 2 and SLC15A4/ PHT 1 (Daniel and Kottra, 2004). The fifth peptide transporter, HPT1, has greater similarity to cadherins rather than its fellow peptide transporters (Dantzig et al., 1994).

1.2.1 *Peptide Transporter 1 (SLC15A1) and Peptide Transporter 2 (SLC15A2)*

The first identified peptide transporter, PepT1 (SLC15A1), was cloned from a rabbit intestinal cDNA library (Fei et al., 1994). Shortly thereafter the peptide transporter PepT2 was first isolated from a human kidney cDNA library (Liu et al., 1995). Of the members of the POT superfamily, PepT1 and PepT2 have received the most investigative attention. Using immunohistochemistry, PepT1 was localized to the apical membrane of enterocytes (Ogihara et al., 1996). PepT2 was not found in the intestine, but rather in the epithelium of the kidney, lung, mammary glands as well as regions of the central nervous system (Shen et al., 1999, Rubio-Aliaga and Daniel, 2002). PepT1 and PepT2 have many similarities in protein structure and sequence. Both share putative sequences illustrating the potential for 12 trans-membrane domains while PepT2 shares 50% identity and 70% similarity to the previously identified PepT1 (Liu et al., 1995). Significantly, the sequence similarities are much higher in the trans-membrane regions than in the amino- and carboxy-terminus suggesting conservation of functionally important residues. Despite these similarities there are important differences found between the substrate specificities of these peptide transporters. PepT2 showed a higher affinity for dipeptides compared to PepT1 in rabbit and human samples (Amasheh et al., 1997, Ramamoorthy et al., 1995). PepT1 is considered a low-affinity/high-capacity peptide transporter whereas PepT2 is high-affinity/low-capacity. The primary function of PepT1 is the absorption of dipeptides from the nutritionally rich intestinal lumen, whereas PepT2 removes peptides at relatively low concentration from the tubules of the kidney; as such, this particular adaptation is physiologically beneficial. Early *in situ* studies employed the everted jejunal ring model and exposed the tissue to hydrolysis-resistant oligopeptides of two, three or four amino acid residues to describe the structure affinity relationships of PepT1. It was determined that only di/tripeptides were transported across the epithelial barrier of the small intestine; tetrapeptides

were not transported in this model (Daniel and Kottra, 2004). Drugs that are structurally similar to small peptides are also transported by PepT1 and computational modeling has been extensively used to study this activity (Bolger et al., 1998, Irie et al., 2005). Studies attempting to determine the distinct substrate specificities of PepT1 have revealed that almost all di/tripeptides constructed from physiological amino acids are potential substrates for transport (Vig et al., 2006). While no similar study has been performed on PepT2, numerous peptides have been investigated as potential substrates and similar results have been obtained (Liu et al., 1995, Ramamoorthy et al., 1995). Both PepT1 and PepT2 have also demonstrated the capacity to transport compounds other than amino acids such as β-lactam antibiotics (Han et al., 1998, Terada et al., 1997), while PepT1 is also involved in the movement of bacterial peptides across the intestinal epithelium (Merlin et al., 1998). This transport of non-physiological substrates is of interest as it provides another potential avenue for drug targeting, and it may be a causative factor in intestinal inflammation.

1.2.1.1 *Introduction to PepT1/PepT2 Structure/Function*

PepT1 consists of 12 trans-membrane domains (Figure 1), with each domain and loop unit associated with one of the 23 exons responsible for its coding (Urtti et al., 2001). The transport of short peptides requires protons to provide a motive force, as well as sodium to maintain the proton gradient. NHE3, a sodium/proton antiporter, is responsible for providing the proton gradient necessary for intestinal PepT1 function (Ganapathy and Leibach, 1985). PepT1 has the capability to bind a large number of different substrates and the structure-affinity relationship of this transporter is an area of interest (Brandsch et al., 2004). With amino acids lengths of 710 and 729 respectively, PepT1 and PepT2 have several putative

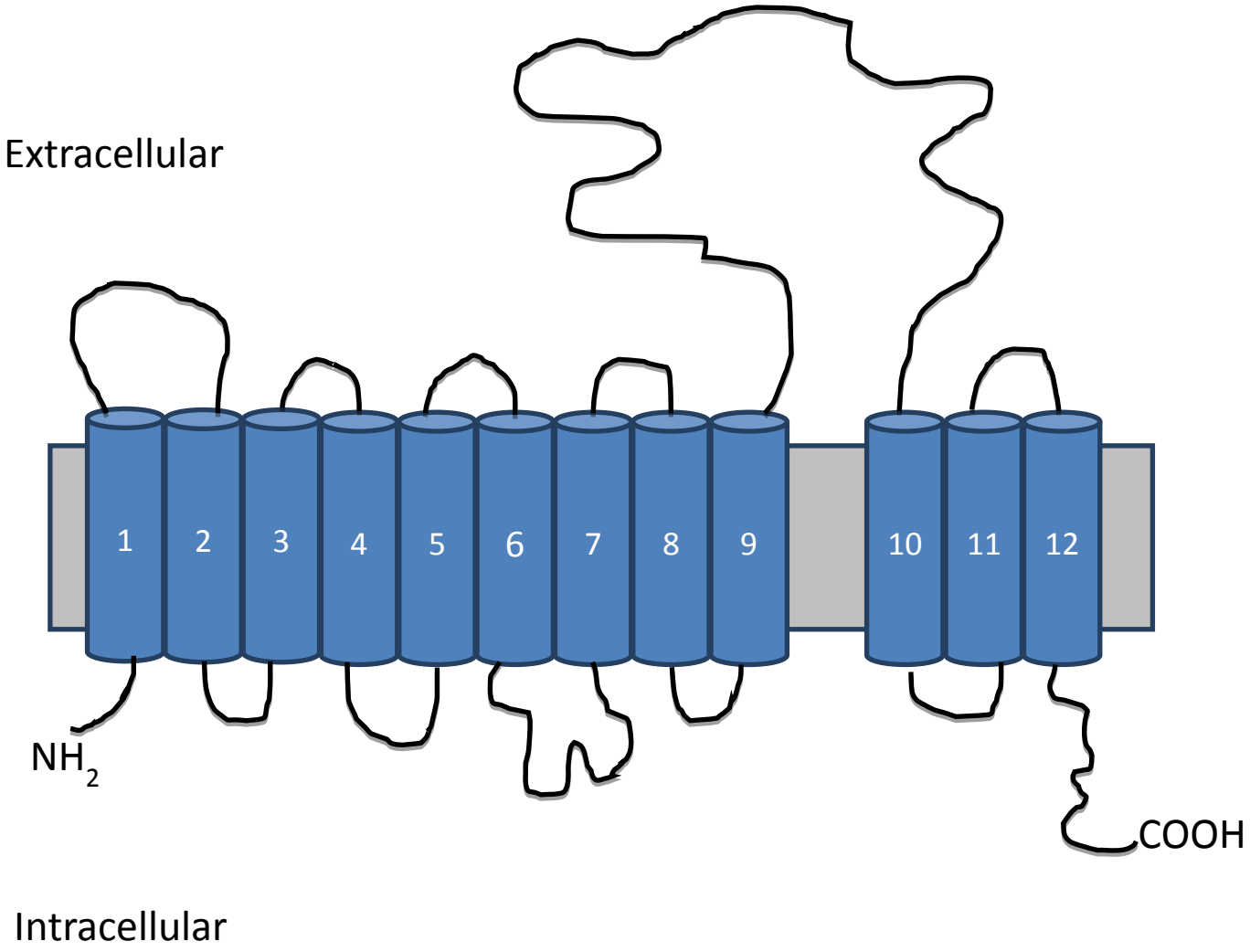

Figure 1.1: Proposed structure of PepT1

phosphorylation and glycosylation sites. Hydropathy analysis demonstrated that the peptide transporters likely contain 12 trans-membrane domains with cytosolic amino and carboxy termini (Fei et al., 1994). In order to confirm the number and position of these trans-membrane domains, epitope mapping of human PepT1 was performed (Covitz et al., 1998). This analysis confirmed the cytosolic location of the carboxy terminus and the number of trans-membrane domains. Additionally, the experiment revealed that the loop between domains 3 and 4 was extracellular and a large extracellular loop between domains 9 and 10 was present. The exact location of the amino terminus was not determined due to complications arising from epitope insertions between domains 1 and 3. During these particular epitope insertions, hPepT1 function was disturbed, suggesting that this region is important for folding or transporter function.

1.2.1.2 *Recent Developments in the Structure of PepT1*

Although a large amount of data has been gathered through computational modeling and mutagenic studies, the structure of PepT1 is still under discussion. Greater understanding of the protein structure, and thereby an understanding of substrate binding residues, would lead to predictions of binding affinity for novel therapeutic substrates. Currently there is no complete structure of mammalian PepT1, however crystals structures have been obtained for numerous similar bacterial peptide transporters.

Comparisons of PepT1 with the structure of LacY and GlpT, due to their predicted similarity in structure (Saier et al., 2006), aided in the development of a structural model of rabbit PepT1 (Meredith and Price, 2006). This structural model was the first to directly challenge the hydropathy plot which predicted the 12 trans-membrane domains mentioned above (Fei et al., 1994). Through the use of a more modern structural prediction program (MEMSAT3) (McGuffin et al., 2000), the predicted location of trans-membrane domain 1 shifted to residues

24 to 42 rather than 7 to 75, as predicted by hydropathy (Meredith and Price, 2006, Fei et al., 1994). This shift would provide an explanation for the complications found in the structural analysis via epitope mapping (Covitz et al., 1998). The model proposed by Meredith and Price (2006) also positions a number of essential residues facing the central pore thereby providing a structural explanation for their importance.

Recently the crystal structure of several prokaryotic peptide transporters has been solved (Newstead et al., 2011, Solcan et al., 2012, Doki et al., 2013). $PepT_{So,}$ initially isolated from the bacterium *Shewanella oneidensis,* shares 30% identity within the trans-membrane regions of PepT1 and PepT2 including many of the previously identified significant residues (Newstead et al., 2011). Unlike the previous models, $PepT_{So}$ contains 14 trans-membrane domains at a resolution of 3.6 Å. Transport of glycyl-sarcosine by $PepT_{So}$ was similar to that of hPepT1 and experimentation with free amino acids, di/tripeptides and tetra-peptides suggested similar substrate affinity as other peptide transporters. Reliance on the proton motive force was also demonstrated in $PepT_{So}$. Shortly after the publication of this structure the Newstead lab reported a higher resolution, 3.3 Å, structure of a peptide transporter from *Streptococcus thermophilus*, $PepT_{St}$ (Solcan et al., 2012). The functional characterization of this transporter revealed a hinge movement in the C-terminal region of the transporter, domains 10 and 11, with salt bridge interactions that alternate during the act of substrate transport. The structure of a peptide transporter from *Geobacillus kaustophilus* (GkPOT) has been resolved at the highest resolution at1.9Å for ligand-free and 2.0 Å for sulphate bound form (Doki et al., 2013). The structure of GkPOT was determined to be similar to that of $PepT_{So}$ and $PepT_{St}$. This was the first study to investigate the functional impact of site directed mutagenesis at this resolution. Through the study of mutated forms of the protein it was shown that glutamate 310 is responsible for binding

the carboxyl group of the peptide substrate. After substrate release, it is the interaction between glu310 and arg43 which causes a transition returning the transporter to a state ready to accept additional extracellular substrates. Although the determination of this structure is an important step in understanding the direct mechanism of action of peptide transport, it is important to consider that it only represents a static moment in the action of this transporter. Since there is only a 30% identity between $PepT_{So}$ and the mammalian PepT1 and PepT2, any direct comparisons on transport mechanisms or methods of substrate recognition must take this fact into consideration.

1.2.1.3 *Importance of Histidine, Arginine and Tyrosine Residues in Peptide Transport*

Previous work has demonstrated that transport systems which co-transport hydrogen ions rely on histidyl residues for their activity (Ganapathy et al., 1987). Considering that POT family members use these ions as the primary motive force, histidyl residues were studied as potentially vital components of both PepT1 (Kramer et al., 1988) and PepT2 (Miyamoto et al., 1986). In order to specifically investigate the function of the histidyl residues, diethylpyrocarbonate (DEPC) was used in combination with brush-border membrane vesicles (BBMV) isolated from rabbit kidney (Miyamoto et al., 1986) or rabbit small intestine (Kramer et al., 1988). The compound DEPC is capable of specifically altering the structure of histidine residues without modifying other residues in the protein. The work by Miyamoto et al. (1986) demonstrated that functional histidyl residues were essential for PepT2 function, and suggested that thiol groups present around the binding site are also important. They did not determine whether the histidine residues were present in the dipeptide-binding site or if they were involved with H^+ binding. Treatment of BBMV with DEPC inhibited the transport of β-lactam antibiotics that contained an

α-amino group, contributing to the theory that histidine residues are also important for PepT1 function (Kramer et al., 1988).

In order to understand the mechanisms controlling the binding capacity of these transporters, attention was directed towards the identification of specific histidine residues that were functionally significant. Through sequence analysis it was determined that histidine 57 and 121 were highly conserved in PepT1 and predicted to be present in trans-membrane domains 2 and 4 respectively (Terada et al., 1996). Therefore site directed mutagenesis was performed replacing histidine 57, 121, or both, with glutamine and then measuring uptake of glycyl-sarcosine, a hydrolysis resistant dipeptide, in *Xenopus* oocytes. Alteration of either or both of these residues resulted in a dramatic reduction in glycyl-sarcosine transport, thereby indicating involvement in the activity of PepT1 through either substrate binding or other important functions. Further research using a cell culture model transfected with either rat PepT1 or PepT2 investigated the interaction of antibiotics with histidyl residues in an attempt to delineate the specific function of these residues (Terada et al., 1998). It was established that the α-amino group of both dipeptides and specific antibiotics interacted with the histidine residues; whether it was interaction with histidine 57 or 121 was unknown. Study of these two residues has been ongoing, with certain results highlighting the significance of histidine 57 while suggesting that histidine121 is of little to no importance (Fei et al., 1997, Chen et al., 2000). Other studies have maintained the necessity of two histidine residues, one for proton coupling and the other for peptide coupling (Steel et al., 1997).

Molecular and computational modelling of PepT1 has been very important in determining the potential importance of other conserved residues (Irie et al., 2005, Bolger et al., 1998, Meredith and Price, 2006). A positively charged amino acid residue, varying between

arginine or lysine depending on the animal being studied, is present in trans-membrane domain 7 of all studied mammalian isoforms of PepT1 (Meredith, 2004). There is a similar residue found in PepT2 indicating potential functional significance. Investigation of the importance of arginine 282 via mutagenesis into alanine slightly modified the uptake of glycyl-sarcosine in human embryonic kidney cells transfected with hPepT1 (Bolger et al., 1998). Found in the seventh trans-membrane domain of PepT1 (Meredith and Boyd, 2000), mutation of this residue into glutamate uncoupled the transport of peptides from protons (Meredith, 2004). This alteration from active to facilitated transport was investigated further in *Xenopus* oocytes transfected with wild-type and mutated rabbit PepT1 to determine the effect of the amino acid charge present at position 282 on transport of phenylalanyl-glutamine (Pieri et al., 2008). Mutation of arginine 282 to lysine, another positively charged amino acid, did not impact the efficacy of peptide transport. Mutation of this position to histidine also exhibited similar activity as wild-type, potentially due to a positive charge on the histidine thus enabling it to function like arginine or lysine. Double mutations revealed an interaction between arginine 282 with an aspartic acid residue at position 341 on trans-membrane domain 8 as charge swapping between these two residues (R282E/D341R) did not impact peptide transport.

A similar sequence of investigations has occurred with particular tyrosine residues in PepT1. Beginning with sequence analysis, tyrosine 167 was identified as a conserved residue from bacteria to humans (Graul and Sadee, 1997). Following its identification, tyrosine167 underwent site-directed mutagenesis to alanine, phenylalanine, serine or histidine all of which abolished uptake of glycyl-sarcosine in cell culture (Yeung et al., 1998). The sensitivity of transporter activity to the mutation of this residue, combined with the well conserved nature at this position, indicated the importance of this tyrosine residue. Continued experimentation

revealed that tyrosine 56 is also important for peptide uptake as mutation to phenylalanine or alanine dramatically reduced transport of glycyl-leucine (Pieri et al., 2009, Chen et al., 2000). The tyrosine present at position 91was identified as potentially interacting with protons (Bolger et al., 1998) and mutation of that tyrosine reduced transport capacity (Links et al., 2007). Mutation of this residue uncoupled the transport of dipeptides from the proton motive force, strengthening the belief that tyrosine 91 was interacting with protons. Further study has identified a potential interaction between tyrosine 91 and histidine 57, located adjacent to tyrosine 91 on trans-membrane domain 2 (Pieri et al., 2009). As these residues are vital for proton coupling of peptide transport, this interaction may be involved in regulating pH stimulation of peptide transport

1.3 *PepT1 in the Intestine and Kidney*

1.3.1 *Gastrointestinal Tract*

PepT1 was first discovered in intestinal samples (Erickson et al., 1995); thus, the gastrointestinal tract has undergone intense investigation to determine the distribution of this peptide transporter. Most studies have reported no detection of PepT1 in the oesophagus, stomach, cecum or rectum (Ogihara et al., 1996, Freeman et al., 1995); however, one study detected weak expression of PepT1 mRNA in the stomach of rodents (Lu and Klaassen, 2006). In the intestine, the transporter is localized to the apical membrane of the villi, facilitating access to digesta passing through the lumen (Ogihara et al., 1996). Both PepT1 protein and mRNA have been detected in the small intestine of a variety of animal species and the concentration of the transporter varies depending on intestinal location and species studied. Black bears showed the highest concentration of PepT1 in the middle of the small intestine (Gilbert et al., 2007b)

while studies in humans have demonstrated the greatest presence in the duodenum/jejunum (Terada et al., 2005, Herrera-Ruiz et al., 2001). In contrast, Chen *et al.* showed a consistent amount of PepT1 expression across the entire length of the small intestine of mature sheep, dairy cows, pigs, and chickens (Chen et al., 1999). A study performed in 8-week old rats and mice showed no significant differences in the amount of PepT1 mRNA along the length of the small intestine (Lu and Klaassen, 2006). Developmental changes in the location and concentration of PepT1 along the small intestine have been reported in rats (Shen et al., 2001), chickens (Chen et al., 2005) and pigs (Wang et al., 2009). Shen *et al*. (2001) reported that PepT1 presence in the colon was transient; by day seven no PepT1 mRNA was found in the colon of developing rats. PepT1 has been found in the colon of rats at later time points (Shi et al., 2006a), but its presence has been primarily related to a state of intestinal injury such as gut resection and therefore has been a source of controversy. A study in rats demonstrated increased PepT1 protein in the colon of animals with intestinal resection that were fed chow (Shi et al., 2006a), whereas rats fed an elemental diet did not express any PepT1 mRNA in colonic tissues (Lardy et al., 2006). A recent study demonstrated that there is a greater concentration of PepT1 mRNA in the distal colon of mice, rather than proximal, and that this signal was lost in PepT1 knockout mice (Wuensch et al., 2013). Assessment of glycyl-sarcosine transport combined with immunofluorescence indicated that PepT1 protein was present in the distal colon of wild-type mice and that it was fully functional. A similar condition has been demonstrated in humans, as PepT1 has been detected in the colon of humans that have undergone intestinal resection (Ziegler et al., 2002). When compared to control subjects, PepT1 mRNA and protein isolated from colonic samples were higher in patients with massive bowel resections. When combined, these distribution patterns

indicate that while PepT1 is ubiquitously expressed in the small intestine of numerous animals, colonic PepT1 is only present in early life or in disease states.

1.3.2 *Kidney*

Although PepT2 is considered to be the primary peptide transporter in the kidney, expression profiling of the kidney has revealed the presence of both PepT1 and PepT2 (Lu and Klaassen, 2006, Smith et al., 1998, Shen et al., 2001). Analysis of specific regions of rat kidney revealed that there is a gradient of expression, from proximal to distal nephron, in both PepT1 and PepT2 (Smith et al., 1998). PepT1 was found solely in the kidney cortex with PepT2 being expressed in the cortex and the medulla. While PepT2 was more abundant in rat kidney compared to PepT1, the latter was more specific for the early segments of the proximal tubule, S1, with the former having a greater concentration in the later segments, S2 and S3. This, when combined with the low-affinity/high-capacity aspect of PepT1 and the high-affinity/low-capacity aspect of PepT2, indicates that the reabsorption of peptides in the kidney is handled in a sequential manner. It has been suggested that PepT2 is primarily responsible for the transport of peptides, as glycyl-sarcosine was reabsorbed from the late portions of the proximal tubule (Silbernagl et al., 1987). The relative importance of PepT1 in renal peptide transport has yet to be determined; however as it is a high-capacity transporter its role may be significant.

1.4 *Regulation of PepT1 in Health and Disease*

1.4.1 *Dietary Factors*

There is a growing body of evidence demonstrating that PepT1 expression and activity is at least partially substrate driven. Rats fed a high protein diet had greater uptake of carnosine than rats fed a low protein diet (Ferraris et al., 1988). As carnosine is a substrate for PepT1, this

result suggested that diet may play a role in regulating PepT1; however the mechanism had yet to be determined. Although the transporter was identified along the length of the small intestine, a high protein diet fed to rats induced PepT1 transcription in the proximal small intestine only (Erickson et al., 1995). Greater abundance of PepT1 transporters was also observed in rats fed a standard diet supplemented with a single dipeptide compared to controls fed free amino acids (Shiraga et al., 1999). The quality of protein ingested may also affect PepT1 expression. A study performed in broiler chicks investigated whether PepT1 expression differed if corn-based diets were supplemented with soybean meal or gluten meal (Gilbert et al., 2008a). Chicks fed soybean meal had greater expression of PepT1 than those fed gluten, similar to the trend found in the $b^{o,+}$ AT, a neutral and basic free amino acid transporter. Incubation of Caco-2 cells with a dipeptide resulted in alteration of the expression of PepT1 (Walker et al., 1998). Caco-2 cells were transfected with human PepT1 cDNA and incubated with the dipeptide glycyl-glutamine. This dipeptide exposure increased transport of glycyl-sarcosine with increased expression of PepT1 mRNA and a concomitant increase in PepT1 protein.

Increasing evidence suggests that PepT1 expression and/or activity can be altered by manipulating nutritional status or health. In situations of gut stress such as malnutrition, intestinal failure or surgical intervention, PepT1 expression is maintained or increased, in contrast with other nutrient transporters which typically decline in number (Satoh et al., 2003). Humans fasted for 14 days demonstrated a significant decrease in the transport of amino acids but peptide transport was maintained (Vazquez et al., 1985). Changes were described in the transporter population in rats that were either food deprived for 4 days, food restricted to 50% of a control group intake, or nourished completely by parenteral nutrition (PN) (Ihara et al., 2000). The food deprived rats demonstrated an increase in PepT1 mRNA in the proximal gut by 179%

compared to controls. The PN-fed rats with atrophy of the small intestine (SI) responded similarly to the food deprived animals. Similar responses were observed in broiler chicks undergoing feed restriction (Chen et al., 2005, Gilbert et al., 2008a). In one study, chickens were assigned to diets containing 12, 18 or 24 % crude protein with sampling performed during the first 35 days post-hatch (Chen et al., 2005). Dietary intake of chickens receiving 18% or 24% crude protein (CP) was restricted to that consumed by those receiving the 12% diet with an additional group receiving free access to the 24% CP diet. Chickens on 12% CP showed lower expression of PepT1, but the animals receiving a higher protein diet but restricted intake had higher expression of PepT1 compared to those receiving unlimited access to the higher protein diet. Animals with free access to the diet containing 24% CP had declining expression of PepT1 mRNA during the first two weeks of the study with increasing expression afterwards; however animals undergoing diet restriction still had greater expression of PepT1. Interestingly, high luminal concentrations of some free amino acids may impair dipeptide uptake (Himukai et al., 1982), although this finding varies between *in vitro* model systems (Daniel, 2004). This suggests that dietary factors, such as protein content in the diet, alter the expression of PepT1.

1.4.2 *Development*

Differences in the location and concentration of PepT1 mRNA along the small intestine during development have been reported in rats (Shen et al., 2001), chickens (Chen et al., 2005, Gilbert et al., 2007a) and pigs (Wang et al., 2009). In rats, there is a dramatic shift in cellular localization of PepT1 between 18 days gestation and immediately after birth at 21 days gestation (Hussain et al., 2002). Following birth there is a movement of PepT1 protein from the brush border membrane of the enterocytes to the cytoplasm and the basolateral membrane. The transient nature and functional importance of this basolateral Pept1 has yet to be elucidated.

Early work on the developmental changes in rats showed that intestinal PepT1 mRNA was highest at 4 days of age and continued to decline until reaching concentrations found in adulthood at 28 days of age (Miyamoto et al., 1996). Similarly, Shen *et al.* (2001) demonstrated that although PepT1 mRNA expression was at its highest point 3-5 days after birth in a neonatal rat model, it declined rapidly at all locations in the small intestine during the suckling period. Comparable to rats, chickens also demonstrated variance in the location and concentration of PepT1 mRNA during development (Chen et al., 2005, Gilbert et al., 2007a). In a study of broilers, chickens raised for meat production, PepT1 mRNA increased linearly with age (Chen et al., 2005). Further investigation into the differences among intestinal positions corroborated this result and revealed that the greatest expression of PepT1 mRNA was found in the duodenum (Gilbert et al., 2007a). Microarray analysis has been performed on chicken intestinal samples taken at varying stages of development from late stage embryos to two weeks post-hatch (Li et al., 2008). In agreement with the previous studies, there was a linear increase in PepT1 mRNA in all regions of the chick intestine sampled from embryonic day 18 to post-hatch day 20 and at a greater quantity in the duodenum and jejunum compared to the ileum. In Tibetan piglets, PepT1 mRNA increased in the duodenum and jejunum from birth to the middle of the suckling period, after which the expression decreased in these intestinal regions (Wang et al., 2009). In these animals the distal jejunum had greater expression of PepT1 mRNA than the other intestinal regions tested. D'Inca et al. determined that there was a dramatic decrease in piglet PepT1 mRNA expression within the first 48 hours after birth; however, intrauterine growth restriction delayed this adaptation (D'Inca et al., 2011). This suggests that PepT1 and peptide transport are potentially important in the early life of the suckling neonate.

1.4.3 *Insulin*

Insulin is a key metabolic regulatory hormone that has been demonstrated to regulate amino acid transport. Incubation with insulin was shown to rapidly increase dipeptide transport in a Caco-2 cell line (Thamotharan et al., 1999). Inclusion of genistein, an inhibitor of receptor tyrosine kinases, in the incubation medium prevented this transport increase thereby demonstrating that the effect was due to insulin signalling. A shift in the V_{max} of PepT1 confirmed an increase in the transporter population rather than an effect on substrate affinity. Curiously, a concomitant increase in PepT1 protein without an increase in PepT1 mRNA was observed. Disturbance of the Golgi apparatus by brefeldin treatment did not disrupt the effect of insulin, while depolymerizing the microtubules through colchicine treatment abolished the increase in peptide uptake. This indicated that insulin was inducing the movement of a pre-existing cytosolic pool of PepT1 to the apical surface, rather than *de novo* transporter synthesis. A study on the impact of insulin treatment on the transport of cephalexin (an antibiotic substrate of PepT1) in Caco-2 cells co-incubated with genistein and/or colchicine had similar results (Watanabe et al., 2004). Taken together, it is possible that circulating insulin interacts with receptors on the basolateral membrane of enterocytes to mobilize cytosolic stores of PepT1 to the apical membrane thereby facilitating increased uptake of substrates present in the intestinal lumen. This was confirmed by studies in Caco-2 cells (Nielsen et al., 2003). Similar to previous studies, brefeldin and colchicine inhibited the insulin stimulated uptake of the test dipeptide, in this case glycyl-sarcosine. Basolateral stimulation of Caco-2 cells with insulin resulted in greater uptake of glycyl-sarcosine than either control or apical stimulation, with no difference detected between apical stimulation and control samples.

1.4.4 *Short Bowel Syndrome*

Short bowel syndrome (SBS) is a clinical condition induced through the surgical removal of intestinal tissue. Causes for intestinal resection are varied and include inflammatory disorders such as Crohn's disease and colitis, tumors, physical trauma and infection such as necrotizing enterocolitis (NEC) (Goulet et al., 1991). Intestinal resection results in a loss of absorptive capacity (Sukhotnik et al., 2002) potentially leading to a requirement for long term parenteral nutrition. In newborn and pre-term infants, SBS is commonly the result of congenital malformations or necrotizing enterocolitis (Sodhi et al., 2008). NEC is an aggressive, anaerobic infection that develops rapidly in the gastrointestinal tract in approximately 10% of all very low birth weight infants, with up to a 34% mortality rate in the lowest birth weight category (Fitzgibbons et al., 2009). If SBS occurs as a consequence of intestinal disease, then a number of strategies have been employed in an attempt to improve nutrient absorption, intestinal motility and subsequently, adaptation (Miller and Burjonrappa, 2013). Elucidation of adaptive responses after intestinal resection, including alteration in peptide transport, could lead to dietary treatments that are designed to optimize nutrient availability in the compromised SI, and ultimately enhance adaptation in the remaining intestine.

1.4.5 *PepT1 and Intestinal Adaptation in SBS*

Whether or not PepT1 expression occurs in the colon after short bowel surgery is controversial. After an 80% small intestinal resection in rats, changes were quantified in the colon one week post-operatively (Lardy et al., 2006). There was no difference in PepT1 expression compared to controls; however, this may have been due to the post-resection feeding of an elemental diet, which did not provide the stimulus necessary to induce the transporter. In contrast, Shi *et al*. (2006a) developed a rat model that used SI resection in rats to induce colonic

PepT1 (Shi et al., 2006a). This resected rat model was used to study the role of PepT1 in colonic inflammation. This study clearly identified PepT1 protein in the colon of SI resected but not control rats, when killed 2 weeks post-operatively. Rats were fed a liquid diet initially, and rat chow for the final week. Therefore the induction of PepT1 in the colon may require the luminal presence of dipeptides. One study investigated whether colonic PepT1 was expressed in patients with SBS (Ziegler et al., 2002). Mucosal biopsy specimens were obtained from 13 adult patients with SBS and 33 controls. There was no difference in PepT1 expression in the ileum of SBS patients compared to controls; however, there was a fivefold higher expression of the transporter in samples from the colon of SBS patients. Although there has been much research on colonic adaptation, currently no study has investigated the impact of enterally delivered dipeptides on small intestinal adaptation in a piglet model of short bowel syndrome.

1.4.6 *Inflammatory Bowel Disease*

Inflammatory bowel disease (IBD) is an umbrella term used to describe chronic inflammation of the large and/or small intestine. Although there are many types of inflammatory bowel diseases the most prominent, and most extensively studied, are Crohn's disease and ulcerative colitis (Baumgart and Sandborn, 2012). Crohn's disease was first described in patients suffering inflammation of the ileum, but inflammation can be found in any area of the gastrointestinal tract including the colon. Ulcerative colitis, however, is an intermittent disease where inflammation occurs primarily in colonic tissue. In humans, colonic expression of PepT1 has been reported in patients with inflammatory bowel disease (Merlin et al., 2001). Evidence from both human and rat studies has indicated that PepT1 is capable of transporting of bacterial peptides, which may exacerbate the inflammatory process in Crohn's disease and ulcerative colitis (Merlin et al., 2001, Shi et al., 2006c). Initial studies in Caco2-BBE cells, a variant of the

Caco2 cell line with greater homogeneity of brush border proteins, demonstrated both the presence of human isoform of PepT1 and its capacity to transport the bacterial peptide formyl-methionyl-leucyl-phenylalanine (fMLP) (Merlin et al., 1998). Further investigation revealed that hPepT1 is expressed in the colon of patients with chronic ulcerative colitis, but not normal colonic tissue, and that transport of fMLP may affect immune response by stimulating expression of MHC-1 molecules (Merlin et al., 2001). One theory suggests that in IBD, there is an over production of proinflammatory peptides by the colonic microflora. These peptides then act as substrates to induce PepT1 expression in the colon (Adibi, 2003). Exploration of the interaction between PepT1, bacteria and the immune system may result in a greater understanding of peptides in the intestinal inflammatory response.

1.5 *Substrates for PepT1*

1.5.1 *Di/tripeptides*

To understand the potential impact of peptide transport, it is important to consider the variable affinity of PepT1 substrates. Alanyl-alanine, for example, has a high affinity for PepT1 (K_i of 0.08 mM) whereas glycyl-sarcosine is classified as a medium affinity substrate with a K_i of 1.1 mM, determined in Caco-2 cells (Brandsch et al., 1999, Brandsch et al., 1998). Other sarcosine containing dipeptides have K_i's ranging from 0.13 mM for phenylalanyl-sarcosine to 2.5 mM for sarcosyl-proline, with sarcosyl-sarcosine showing no affinity for PepT1 (Brandsch et al., 1999). Another study has reported an apparent K_i for sarcosyl-sarcosine as 15.9 mM; however affinities higher than 15 mM are typically considered to be non-substrates (Daniel et al., 1992).

Dipeptides containing proline, in either the N-terminal or C-terminal position have been extensively studied in both kidney BBMV and Caco-2 cells (Thwaites et al., 1994, Daniel et al., 1992, Brandsch et al., 1999). In the case of prolyl-glycine, transport was undetectable in the kidney (Daniel et al., 1992) while an excess of the dipeptide reduced the uptake of glycyl-sarcosine by only 59% in Caco-2 cells (Thwaites et al., 1994). In an investigation of the transport of twelve different X-pro dipeptides in Caco-2 cells, all dipeptides were able to reduce uptake of glycyl-sarcosine (Brandsch et al., 1999). Affinity for PepT1 ranged from 0.15 mM for alanyl-proline to 1.2 mM for prolyl-proline. A concurrent experiment was performed by replacing the C-terminal proline with alanine, for example alanyl-proline would become alanyl-alanine. Interestingly, most X-ala dipeptides investigated showed greater affinity for PepT1 than X-pro, with the exception of prolyl-alanine which had a K_i of 9.5 mM compared to the 1.2 mM of prolyl-proline. This investigation determined that in the case of X-pro dipeptides, the affinity for PepT1 is positively correlated with the percentage of *trans* conformation present, with greater *cis* conformation reducing transporter affinity.

In recent years it was suggested that K_i ranges should be used to describe PepT1 substrates as high affinity (K_i <0.5 mM), medium affinity (K_i 0.5-5 mM) and low affinity (K_i >5 mM) with non-substrates having an affinity > 15 mM (Brandsch et al., 2004). This would enable easier classification of PepT1 substrates and potentially reduce the variability currently in the literature pertaining to affinity, as literature K_i values are inconsistent. The most thorough investigation into dipeptide transport to date was published in 2006 (Vig et al., 2006). This study quantified the IC_{50}, the half maximal inhibitory concentration, of 81 different di/tripeptides in MDCK cells expressing hPepT1. Although IC_{50} is not directly indicative of the K_i, the two are related where the lower the IC_{50} the greater the affinity of the substrate for the transporter of

interest. Similar to the classification system proposed by Brandsch *et al.* (2004), the study by Vig *et al.* (2006) attempted to group the substrates investigated as either non/poor, intermediate, good or best substrates for PepT1. In this system the best substrates for PepT1 include ala-ala, ala-phe, ala-tyr, leu-leu, phe-ala, phe-gly, phe-phe, phe-tyr, trp-ala, trp-val, tyr-ala, tyr-tyr, and val-val. In contrast, certain dipeptides tested were not substrates for PepT1. This challenges the long held theory that all di/tripeptides are substrates for PepT1. These studies centering on the elucidation of peptide affinities provide indirect evidence regarding the binding pockets present in PepT1 thereby providing important information regarding the structure of this transporter.

1.5.2 *Bacterial peptides*

Bacterial colonization is more prolific in the colon than the small intestine. Certain bacterial peptides are substrates for PepT1, two examples of which are formyl-methionyl-leucyl-phenylalanine (Shi et al., 2006c, Carlson et al., 2007, Buyse et al., 2001) and l-Ala-γ-d-Glu-meso-DAP, Tri-DAP (Dalmasso et al., 2010). *Escherichia coli* produces chemotactic compounds, however the major neutrophil chemotactic substance produced is formyl-methionyl-leucyl-phenylalanine (fMLP) (Marasco et al., 1984). Transport of fMLP by PepT1 has been demonstrated in cell culture (Merlin et al., 1998) and in rats (Buyse et al., 2002, Shi et al., 2006a). In Caco-2 cells, uptake of fMLP was inhibited by the presence of known substrates of PepT1 (Merlin et al., 1998, Foster and Zheng, 2007). Creation of an inwardly directed proton gradient increased the uptake of fMLP in this model, thereby providing further support that PepT1 is responsible for the transport of this peptide. Transport of fMLP was shown to induce neutrophil migration across the epithelial monolayer, an activity which was abolished if fMLP uptake was inhibited. Transport of fMLP has also been measured in rats using intestinal perfusion (Buyse et al., 2002). Differences in the inflammatory response to fMLP were found

based on gut location. Perfusion of fMLP in the jejunum, where there is a high expression of PepT1, resulted in neutrophil invasion and altered morphology while colonic perfusion produced no indices of inflammation. Perfusion of fMLP into the colon of rats with SI resection (the model used to up-regulate PepT1 expression in the colon) resulted in greater myeloperoxidase activity and damage to the colonic mucosa compared to controls (Shi et al., 2006a, Shi et al., 2006c). The mechanism by which fMLP can induce a pro-inflammatory cytokine response is through the interaction with TNF-α leading to an increase in NFκβ (Pan et al., 2010, Pan et al., 2000). It is through this signalling cascade that fMLP is able to induce intestinal inflammation. As PepT1 expression is negligible in a healthy colon, bacterially produced peptides have little access to this transporter which minimizes the potential for transport. Abnormal expression of Pept1 in the colon, overgrowth of bacteria in the small intestine or increased intestinal permeability due to atrophy provide opportunities for transport or translocation of bacterial peptides. Competitive inhibition or direct regulation of PepT1 expression may ameliorate intestinal inflammation in cases of excessive bacterial peptide exposure to PepT1. To date there have been no studies quantifying the effect of fMLP in a state of intestinal atrophy.

1.5.3 *Cysteinyl-glycine*

Cysteine is a conditionally essential amino acid in neonates. It can be synthesized via trans-sulfuration from methionine; however, cysteine is classified as conditionally essential as adequate methionine is necessary to support cysteine synthesis. Additionally, there is evidence that the enzyme cystathionase, which is involved in cysteine synthesis (Rao et al., 1990), is immature in preterm infants and cannot supply sufficient cysteine to meet whole body requirements (Zlotkin and Anderson, 1982) if dietary cysteine is inadequate. Cysteine is a component of proteins and is also precursor for taurine and glutathione synthesis, both of which

function as antioxidants (Beetsch and Olson, 1998). Rapid cell growth and turnover, and maintenance of the redox status in the mucosa are both vital to maintenance of the integrity of the intestinal barrier. As such, adequate cysteine availability is important to the intestine. This was demonstrated in a study during which piglets were fed an enteral diet free of sulfur amino acids (Bauchart-Thevret et al., 2009). The authors reported reduced cellular proliferation, lower numbers of goblet cells and reduced villus height in the small intestine. Given that it was a diet free of methionine, protein synthesis was reduced leading to intestinal atrophy. Further, with the absence of dietary methionine, cysteine could not be synthesized. Cysteine is necessary for protein synthesis, but also has been shown to have direct stimulatory effects on cellular proliferation, via influencing the movement from the G1 to S phase of the cell cycle (Noda et al., 2002). Thus, a deficit in sulfur amino acids in the intestinal mucosa has profound multifactorial effects on growth and proliferation. Cysteine alone is part of the oxidative stress control system (Jones, 2006); however, it is also a residue in the tri-peptide glutathione (γ-Glu-Cys-Gly, GSH) which contributes to controlling cellular redox states (Wu et al., 2004). Limiting the availability of cysteine led to lower concentrations of glutathione in the plasma and intestinal mucosa of rats (Nkabyo et al., 2006). Conversely, when diets were supplemented with sulfur amino acids (218% of requirement), rats had greater plasma redox potential. Studies in humans with inflammatory bowel disorders requiring surgical resection have demonstrated compromised redox status thereby influencing cellular signalling/metabolism and potentially aiding in the development of inflammation (Sido et al., 1998).

Cysteinyl-glycine (CG) is a product of glutathione degradation (Cappiello et al., 2004) that can be hydrolyzed by a number of different peptidases; however, the stability of the dipeptide in plasma has not been as clearly delineated (Cappiello et al., 2004). Although there

have been no studies specifically investigating whether CG is a substrate for PepT1, certain characteristics of dipeptides can be used to determine their affinity for transport via PepT1. A study involving a variety of small peptides detailed the effect of peptide size, hydrophobicity, composition and charge on dipeptide transport (Vig et al., 2006). Cysteine containing dipeptides were not tested in that study, but the results can be used to make inferences regarding the bioavailability of cysteinyl-glycine. All X-gly dipeptides were transported via PepT1 and neutral dipeptides resulted in higher activation than charged peptides thereby suggesting that CG is a viable substrate for PepT1. An investigation of peptide transport in astroglia-rich primary cultures, a cell type containing PepT2, demonstrated that the uptake of CG was inhibited in the presence of alanyl-alanine, a known substrate for both PepT1 and PepT2 (Dringen et al., 1998). This result supports the theory that cysteinyl-glycine is a substrate for PepT1. For individuals with compromised SI function, the inclusion of cysteine-containing peptides in enteral diets may enhance cysteine availability due to the stability of PepT1 expression and efficiency of peptide transport. Improved cysteine availability may, in turn, enhance cellular proliferation and/or the generation of GSH, leading to greater redox capability and better recovery of the injured gut.

1.5.4 *Alanyl-glutamine*

One of the more clinically studied dietary substrates of PepT1 is the glutamine-containing dipeptide alanyl-glutamine (AQ). There are known physiological benefits when glutamine is provided as a dipeptide rather than its free amino acid form. Glutamine is much more stable as a dipeptide, and the concentration of plasma glutamine was greater when glutamine was provided to healthy men as alanyl-glutamine versus L-glutamine (Harris et al., 2012). The impact of this dipeptide on intestinal health has been studied *in vitro* using cell culture (Alteheld et al., 2005) and *in vivo* via PN infusion into piglets (Burrin et al., 1994); as well, a number of human trials

have employed both enteral and parenteral provision of this dipeptide (Eroglu, 2009, Luo et al., 2008, Lima et al., 2007). Parenteral provision of AQ has been shown to increase antioxidant capacity and plasma glutamine concentrations, while intraperitoneal injection of AQ reduced the number of IFN-γ producing cells in a mouse model of dextran sodium sulfate-induced colitis (Chu et al., 2012). A comparative study of enteral versus parenteral alanyl-glutamine in critically ill patients revealed that plasma glutamine was higher when the dipeptide was provided parenterally (Luo et al., 2008). Other studies determining organ specific removal of dipeptides from human plasma observed that the liver, kidneys, muscle and intestine were responsible for removing 60% of the plasma alanyl-glutamine (Vazquez et al., 1993). When provided as the free amino acid, enteral glutamine supressed pro-inflammatory cytokine production during an *E. coli* challenge in piglets (Ewaschuk et al., 2011); however the beneficial effect of enteral glutamine-containing dipeptides has yet to be determined (Luo et al., 2008, Eroglu, 2009, Ligthart-Melis et al., 2009). Greater understanding of the potential beneficial or harmful effects of enterally provided glutamine peptides such as alanyl-glutamine would provide the basis for more effective delivery of glutamine in situations where regular intestinal function is compromised.

1.6 *Intestinal immune system and cytokine response*

1.6.1 *Epithelial barrier*

One of the roles of the epithelium of the small intestine, outside of nutrient transport, is to act as a barrier between the contents of the lumen and the circulatory system. In a healthy individual the intestinal barrier is intact, preventing both paracellular transport of foreign or infectious material and interaction of luminal bacteria with the intestinal cells (Turner, 2009). In cases of intestinal inflammation this barrier function of the intestine fails, leading to the

recruitment of immune cells and the propagation of the inflammatory response (Groschwitz and Hogan, 2009). A multifaceted defense, the intestinal barrier is comprised of both extracellular and intracellular components.

The most notable part of the extracellular barrier is the protein family known as mucins (McGuckin et al., 2009). Mucins, produced by goblet cells, are heavily glycosylated proteins which have multiple functions including the prevention of direct bacterial contact with enterocytes (Johansson et al., 2008). The thickness of the mucin layer varies depending on intestinal location, but has been found to be upwards of 800 μm thick in the colon of rats (Atuma et al., 2001). Besides reducing bacterial contact with the epithelium, this mucous layer also contains a number of antimicrobial compounds including immunoglobulins, lectins and antimicrobial peptides such as defensins and cathelicidins (McGuckinet al., 2009). These antimicrobials can be maintained at a much higher concentration by being present in the mucous layer than if they were secreted directly into the lumen of the intestine, thereby increasing their efficacy.

The intercellular component of the intestinal barrier involves regulation of paracellular transport. The apical junction complex is comprised of the tight junction proteins including claudins and occludins, and the adherens junction, involving E-cadherin and catenins (Laukoetter et al., 2006). This complex regulates the movement of solutes, and bacteria, through the paracellular pathway. Any disruption of the apical junction complex results in a reduction of epithelial barrier integrity, increasing the risk of infection and inflammation. In addition to the epithelial barrier provided by the enterocytes, the lymphoid tissue found within the intestine provides a wide range of immune cells, and biochemical signals, responsible for aiding in the prevention of infection and disease.

1.6.2 *Mucosa-associated lymphoid tissue*

The mucosa-associated lymphoid tissues comprise the largest immune organ in the body (Turner, 2009); however there are site-specific differences between the various mucosal surfaces in the body. Gut-associated lymphoid tissue is comprised of Peyer's patches, organized lymphoid nodules, and mesenteric lymph nodes (Wershil and Furuta, 2008). Peyer's patches are found underneath a specific layer of columnar cells referred to as the follicle associated epithelium which contains a specialized epithelial cell type known as a microfold or M cell (Neutra, 1999). The function of these M cells is to transport antigens from the lumen of the intestine directly to the lymphoid tissues and present them to dendritic cells. Another method of inducing the immune response in the intestine occurs solely through dendritic cells, without the assistance of M cells (Rescigno, 2010). Certain classes of dendritic cells are capable of sampling the contents of the intestinal lumen directly thereby obtaining antigens to present to naïve T-cells and B-cells resulting in their activation. Whether through the action of M cells or through dendritic cells alone, presentation of the foreign antigen is the first step in the initiation of the immune response, which can act through signalling compounds known as cytokines.

1.6.3 *Pro-inflammatory and anti-inflammatory cytokines*

1.6.3.1 *TNF-α*

Tumor necrosis factor α (TNF-α) is a potent pro-inflammatory cytokine that plays a vital role in chronic inflammatory diseases such inflammatory bowel disease (Pedersen et al., 2014) but is also involved in the inflammatory response to infection (Surbatovic et al., 2013). The primary cell type responsible for the production of TNF-α is activated macrophages, a subset of macrophages that have been exposed to interferon-γ in combination with either TNF-α or an infectious agent such as bacteria (Mosser, 2003). It is important to note that while macrophages

are the primary producers of TNF-α, lymphocytes, as well as other cell types such as mast cells and neutrophils, are also capable of producing TNF-α albeit to a lesser extent (Kriegler et al., 1988, Luettig et al., 1989).

Initially TNF-α is produced as a 26 kDa transmembrane protein that is capable of acting as a ligand, interacting with TNF-α receptors directly, or as a receptor by transmitting extracellular information into the cell (Eissner et al., 2004). This transmembrane TNF-α can then be acted upon by a TNF-α converting enzyme to release a 17 kDa product, the soluble form of TNF-α (Black et al., 1997). Both the transmembrane and soluble forms of TNF-α are homotrimers and this trimeric structure is required in order to mediate the biological activity of this cytokine (Tang et al., 1996).

There are two primary receptors for TNF-α, TNF-R1 and TNF-R2 (Hohmann et al., 1989) however these receptors are regulated via different mechanisms (Vandenabeele et al., 1995). Expression of TNF-R1 is regulated by a house-keeping promoter that cannot be stimulated by other cytokines such as TNF-α or IFN-γ (Rothe et al., 1993). The regulation of TNF-R2 is more complex and differs based on cell type. Macrophages, for example, show increased expression of TNF-R2 after exposure to bacterial products such a lipopolysaccharide (Tannenbaum et al., 1993) whereas B-cells require exposure to mitogens in order to begin increasing expression of this receptor (Erikstein et al., 1991). While the extracellular portion of the TNF-α receptors are very similar, the intracellular domains show little sequence homology indicating significantly different methods of action (Ledgerwood et al., 1999).

Capable of being activated by both the membrane bound and soluble forms of TNF-α, the signalling pathway of TNFR-1 is both well characterized and intricate (Grell et al., 1995). After activation, TNFR-1 is bound by the TNF-receptor associated death domain protein which then

activates either the pro-apoptotic pathway, via the FAS-associated death domain protein, or the pro-inflammatory pathway via TNF receptor-associated factor 2 (TRAF2) and activation of NFκβ (Ting et al., 1996). Unlike TNFR-1, TNFR-2 is only able to be activated via the transmembrane form of TNF-α (Grellet al., 1995). Interestingly TNFR-2 is capable of inducing most of the same signalling pathways as TNFR1, despite the differences between their cytoplasmic domains (Rothe et al., 1994). Unlike TNFR-1, TNFR-2 lacks the death domain binding capability and so the primary signalling route is through the TRAF2 pathway; the pathway which results in the activation of NFκβ.

1.6.3.2 *Interferon-γ*

Interferon-γ (IFN-γ) is a vital component of the innate and adaptive immune responses. This cytokine is involved in defending against infections (Filipe-Santos et al., 2006), viral diseases (Sedger et al., 1999), and tumor prevention (Ikeda et al., 2002). In the innate immune response, the primary cells responsible for the production of IFN-γ are the natural killer cells, a type of cytotoxic lymphocyte, and natural killer T cells, a group of cells sharing the properties of both T cells and natural killer cells (Stetson et al., 2003). These cell types are able to respond to infection through the rapid production of IFN-γ. In the adaptive immune system the $CD8^+$ cytotoxic lymphocytes and $CD4^+$ Th1 effector T cells are capable of producing IFN-γ however this process takes several days (Bach et al., 1997).

The membrane bound IFN-γ receptor is comprised of two primary components, the ligand binding IFN-γ R1 chain, 90 kDa, and the accessory IFN-γ R2 chain, 60 kDa, both of which contain extracellular and cytosolic domains (Pestka et al., 1997). Binding of IFN-γ to its receptor requires dimerization of the cytokine (Ealick et al., 1991), while transduction of the appropriate signals into the cell requires four subunits, two each of the R1 and R2 chains

(Pestkaet al., 1997). If only IFN-γ R1 is present, signal transduction does not occur (Rashidbaigi et al., 1986) and without IFN-γ R1, the R2 chain is unable to crosslink or bind IFN-γ (Kotenko et al., 1995). Signal transduction through the IFN-γ receptor complex also requires the recruitment of kinases. The intracellular domain of IFN-γR1 contains the binding motifs necessary for the recruitment of Janus tyrosine kinase (JAK) 1 as well as the recruitment of signal transducer and activator of transcription (STAT) 1 (Schroder et al., 2004). Similarly, IFN-γR2 is able to recruit a JAK2 kinase which then undergoes autophosphorylation allowing it to activate the JAK1 kinase on IFN-γR1 via transphosphorylation. This kinase, JAK1, then phosphorylates tyrosine residues on IFN-γR1 leading to the recruitment, and subsequent phosphorylation, of STAT1. This phosphorylated STAT1 is now active and can translocate to the nucleus.

The genes that are regulated by IFN-γ vary significantly (Schroderet al., 2004). This cytokine is capable of influencing the transcription of genes involved in the antigen presentation pathway, antiviral response, and is both anti-proliferative and apoptotic. In the presence of IFN-γ chemoattractants for T cells, monocytes and macrophages are produced, nitrous oxide is produced to dilate blood vessels and there is an increase in adhesion molecules on endothelial cells and leukocytes. These responses increase the extraversion of leukocytes at the site of the inflammatory response leading to phagocytosis of any foreign objects, antibody production and cytokine release.

1.6.3.3 *Interleukin-10*

Unlike TNF-α and IFN-γ, interleukin-10 (IL-10) functions to limit the inflammatory response. Multiple immune cell types are capable of producing IL-10; however the primary source is macrophages (Ruffell et al., 2014, Sonderegger et al., 2012). Interestingly, macrophages are also the cell population most influenced by IL-10 suggesting at least some auto-

regulation (Moore et al., 2001). Structurally IL-10 is similar to IFN-γ as it is a homodimer (Zdanov et al., 1995). The biological activities of IL-10 include down regulation of major histocompatibility complex class II proteins on macrophages (Bogdan et al., 1991) as well as inhibition of TNF-α (de Waal Malefyt et al., 1991) and IFN-γ (Ito et al., 1999) production. These activities of IL-10 allow the host immune response to be modulated in order to prevent unnecessary damage to the healthy tissue at the site of inflammation.

As the structure of IL-10 is similar to that of IFN-γ, it is no surprise that their respective receptors also share structural similarity. Like IFN-γ, the IL-10 receptor complex is comprised of two chains, IL-10R1 and IL-10R2 (Kotenko et al., 1997). Unlike IFN-γ, the IL-10 receptor is lacking a soluble form as it has only been detected as a membrane associated receptor *in vivo (Mooreet al., 2001).* A study performed in a JAK1 knockout mouse model demonstrated that macrophages lacking JAK1 do not respond to the presence of IL-10 (Rodig et al., 1998). Further studies in mutant mouse macrophage cells found that STAT3, a transcription factor, was also required in order to induce macrophage proliferation through IL-10 stimulation (O'Farrell et al., 1998). This situation is similar to that of the JAK/STAT pathway previously discussed and the proposed method of IL-10 signaling closely mimics that of IFN-γ (Kotenkoet al., 1997). It is through the binding of IL-10 to either IL-10R1, which then recruits IL-10R2, or the complete IL-10R complex, that the autophosphorylation of the tyrosine kinases and transphosphorylation of JAK1 can occur. This phosphorylation of JAK1 then recruits STAT3 which, after undergoing its own phosphorylation by JAK1, is able to translocate to the nucleus and begin altering the transcription activity of the cell.

As with most cytokines the biological activities of IL-10 varies depending on the properties of the cell upon which it is acting. In the case of cells such as macrophages, IL-10 is

responsible for the inhibition of IL-1, TNF-α as well as numerous other cytokines and colony stimulating factors, which are glycoproteins capable of inducing differentiation and proliferation of hematopoietic stem cells (Mooreet al., 2001). This inhibition of IL-1 and TNF-α is key to the anti-inflammatory properties of Il-10. The prevention of nitric oxide synthesis, and thereby the reduction in vasodilation, is another property of IL-10; however, rather than being a direct action upon nitric oxide synthases, it is the down regulation of the upstream signalling messengers TNF-α and IFN-γ that is responsible for the reduction in nitric oxide synthesis (Flesch et al., 1994).

1.6.3.4 *Nuclear factor κβ*

Essential in T-cell and B-cell differentiation, as well as having anti-apoptotic and pro-inflammatory effects, the transcription factor NFκβ plays a vital role in the inflammatory response (Baeuerle and Henkel, 1994). There are five different members of the NFκβ family of proteins, RelA (p65), c-Rel, RelB, NFκβ1 (p50) and NFκβ2 (p52) (Liou, 2002). NFκβ1 and NFκβ2 contain a c-terminal domain that must be removed by proteolytic processing to become active.

As a potent transcription factor, the activation of NFκβ is tightly regulated through another family of proteins called inhibitors of NFκβ, or IκB (Auphan et al., 1995). The common sequence between the IκB family and NFκβ1/NFκβ2 is known as an ankyrin repeat. Typically, NFκβ exists in the cytoplasm as a p50/p65 heterodimer which is bound by IκB. In order for NFκβ to perform its action as a transcription factor, the IκB must be degraded. This degradation is accomplished through the activity of the IκB kinase complex (Baeuerle and Baltimore, 1996). This complex phosphorylates the appropriate region of the inhibitor leading to its polyubiquitination and degradation. Activation of NFκB is only one part of the immune

signaling cascade and cytokines such as TNF- α, IFN-γ and IL-10 have demonstrated the capability to alter the activation of this essential transcription factor.

The activation of NFκβ via TNF-α requires the destruction of any bound IκB and the mechanism may be different depending upon whether TNF-R1 or TNF-R2 is considered (Bradley, 2008, Devin et al., 2000). In the case of TNF-R1 two components are required for activation of the IκB kinase complex, TRAF2 and the death domain kinase receptor-interacting protein (RIP) (Devinet al., 2000). Initially TRAF2 brings the IκB kinase complex to TNF-R1 and RIP activates the kinase complex leading to the degradation of the inhibitor and thereby the activation of NFκβ. Although the activation of NFκβ through TNF-R2 is not as well characterized, it appears to also occur through an interaction with TRAF2 (Bradley, 2008). Should this be the case, the remaining signalling cascade would be similar to that of TNF-R1.

In addition to its capacity to signal through STAT3, IFN-γ is also capable of inducing expression of NFκβ, albeit not by itself (Cheshire and Baldwin, 1997, Rimbach et al., 2000). A study investigating the interaction between macrophages and human endothelial cells determined that when macrophages were exposed to IFN-γ they, in turn, increased NFκβ activation and DNA binding (Rimbachet al., 2000). Although this is not a direct effect of IFN-γ, it demonstrates that this cytokine is capable of inducing the activation of NFκβ. It appears that IFN-γ must act indirectly, as in the case of macrophage assisted endothelial NFκβ production, or in combination with other cytokines such as TNF- α in order to elicit activation of NFκβ (Cheshire and Baldwin, 1997). When endothelial cells or pre-neuronal cells were co-stimulated with IFN-γ and TNF- α, activation of NFκβ occurred even at concentrations where the individual cytokines would have had no effect. This indicated a synergistic effect between these cytokines

for the activation of this transcription factor and it was determined that in endothelial cells this synergism occurred due to increased degradation of IκB.

Anti-inflammatory cytokines, such as IL-10, are capable of repressing activation of NFκB (Wang et al., 1995). This study, conducted in human monocytes, found that IL-10 was able to inhibit NFκB in a dose dependent manner. Further investigation of IL-10 and its impact on NFκB activation revealed that IL-10 is capable of preventing activation of the IκB kinase complex and directly inhibits DNA binding of activated NFκB (Schottelius et al., 1999). This inhibition of NFκB would prevent transcription of many pro-inflammatory cytokines and chemokines making IL-10 a powerful anti-inflammatory signal.

1.7 *Surgical methods*

1.7.1 *Gut Loop Model*

The ligated loop model, or gut loop model, has been used in pigs to investigate amino acid metabolism of specific regions of the small intestine (Adegoke et al., 1999a, Adegoke et al., 1999b). This model has also been used to demonstrate PepT1-mediated transport of substrates including glycyl-sarcosine (Pan et al., 2002, Hindlet et al., 2007) as well as pharmaceutical compounds (Yang and Smith, 2013, Chu et al., 2001) and bacterial peptides (Wu and Smith, 2013, Buyse et al., 2002). Differences among the studies described in the literature include both intestinal positioning and length of the loops, but the methodology was similar in all studies. In all cases, the intestine was exposed and the gut segment to be perfused was isolated and cannulated to create a closed system. The mesentery was undisturbed, allowing for an *in vivo* assessment of intestinal activity. The advantages of this model for investigating intestinal substrate transport include the ability to isolate and compare specific intestinal segments (*i.e.*

jejunum versus ileum). Furthermore, it allows for careful control of the composition of luminal contents presented to the intestinal mucosa. Finally, it permits the implantation of multiple loops in one animal thereby reducing inter-animal variability. The ligated loop model had been used previously (Nichols and Bertolo, 2008), and is ideal for investigations of the ontogeny of peptide transport in the Yucatan piglet and to study the impact of fMLP on markers of inflammation in healthy and atrophied intestine.

1.7.2 *Intestinal Resection*

Animal models have contributed important information about the adaptive capacity of the small intestine in response to resection, but most of the data were derived from mature rodent models which may not be of relevance to neonates. Morphological and biochemical changes have been described post-resection in rat models treated with enteral feeding (Vanderhoof et al., 1992, Cronk et al., 2000). Fewer studies have been conducted in a clinically relevant piglet model, but include both enteral (Heemskerk et al., 1999) and parenteral feeding strategies (Bartholome et al., 2004). A Yucatan miniature piglet model of intestinal adaptation with an 80% jejuno-ileal resection was characterized by my supervisors research group (Dodge et al., 2012). The study employed a combined protocol of enteral and parenteral feeding, and demonstrated profound early adaptive capacity in the distal ileum in piglets, compared to parenteral- or sham-treated controls. Piglets had greater intestinal length with greater cellular proliferation index and ornithine decarboxylase activity in the distal ileum after receiving enteral feeding. In that study, the enteral diet was elemental, containing free amino acids. The evidence that PepT1 is maintained with intestinal injury (Satoh et al., 2003) and that transporter regulation is partially substrate driven (Walker et al., 1998) makes it a worthy target to investigate whether dipeptides might provide a nutritional advantage over free amino acids to support nitrogen

uptake. Our piglet model is well-suited to investigate the potential effects of enterally provided dipeptides on intestinal adaption in neonates.

1.8 Problem of Investigation

1.8.1 Background

Differences in the location and concentration of PepT1 mRNA along the small intestine during development have been reported in rats (Shen et al., 2001), chickens (Chen et al., 2005, Gilbert et al., 2007a) and pigs (Wang et al., 2009). In Tibetan piglets, PepT1 mRNA increased in the duodenum and jejunum from birth to the middle of the suckling period, after which the expression decreased in these intestinal regions. This suggests that PepT1 and peptide transport are potentially important in the early life of the suckling neonate. Characterizing the ontogeny of PepT1 in the neonate is necessary for the development of effective feeding strategies for both healthy and sick infants. Dipeptide transport capacity via PepT1 across the length of the developing intestine has not been previously reported in our piglet model.

Short bowel syndrome induced through intestinal resection reduces absorptive capacity (Sukhotnik et al., 2002) resulting in nutrient deficits and prolonged medical care. A piglet model of short bowel syndrome was characterized which demonstrated greater adaptive response in animals receiving enteral nutrition (Dodge et al., 2012). PepT1 expression is maintained during intestinal stress while certain free amino acid transporters are reduced in number (Satoh et al., 2003) and expression is at least partially substrate driven (Ferraris et al., 1988). With PepT1 expression maintained during intestinal injury and the increased adaptive response of the intestine in enterally fed piglets, I investigated the adaptive benefits of enterally provided dipeptides in our piglet model of short bowel syndrome.

Escherichia coli produces many chemotactic compounds, but the major neutrophil chemotactic substance produced is formyl-methionyl-leucyl-phenylalanine (fMLP) (Marasco et al., 1984). Transport of fMLP by PepT1 has been demonstrated in cell culture (Merlin et al., 1998) and in rats using intestinal perfusion (Buyse et al., 2002). Intestinal perfusion in rat jejunum induced invasion of neutrophils into the mucosa and altered villus morphology, signs of intestinal inflammation. Transport of fMLP has been implicated in exacerbating inflammatory bowel disease, with increased transport being correlated with increased expression of PepT1. Although it has been demonstrated that presence of dipeptides can prevent transport of fMLP via PepT1, the question of whether parenteral nutrition and associated gut atrophy increases the susceptibility to bacterially induced inflammation has yet to be addressed.

1.8.2 Questions to be addressed

Although the importance of peptide transport has been noted in a wide range of organisms, little information is available on peptide absorption in the piglet. The piglet is a key model organism for neonatal development in humans, and swine are vital to the agricultural industry in Canada. As such, information on peptide uptake in piglets has important applications in human health and swine production. The research programme outlined in this thesis was performed in order to further elucidate peptide transport in Yucatan miniature pigs and to delineate potential benefits of enteral dipeptides.

1.8.3 Specific Objectives

1) Identify the potential for peptide transport in the piglet small intestine and determine if there were any differences in peptide transport due to developmental changes or dietary alterations (Chapter 2)

2) Study the potential ameliorative effects of enterally-delivered dipeptides in a surgically shortened intestine using a piglet model of short-bowel syndrome (SBS) (Chapter 3).

3) Investigate the impact of a bacterial peptide, formyl-methionyl-leucyl-phenylalanine (fMLP) on intestinal inflammation in a model of gut atrophy (Chapter 4).

<u>Chapter 2:</u> Ontogeny of dipeptide uptake and peptide transporter 1 (PepT1) expression along the gastrointestinal tract in the neonatal Yucatan miniature pig

The work presented in this chapter was funded in part by a grant from the Natural Sciences and Engineering Research Council of Canada. It represents work that was presented at Experimental Biology 2008 in San Diego, CA, U.S.A and was published in the British Journal of Nutrition in 2013 (M.G. Nosworthy, R.F.P Bertolo and J.A. Brunton**.** Ontogeny of dipeptide uptake and PepT1 expression along the gastrointestinal tract in the neonatal Yucatan miniature pig. Br J Nutr. 2013 12:1-7). JAB and RFP were responsible for designing the study, MGN carried out the animal work in addition to the laboratory and statistical analyses.

A significant proportion of dietary amino acids are absorbed as small peptides through the activity of the intestinal di/tripeptide transporter PepT1. The characterization of PepT1 in the developing neonate is of tremendous importance for the advancement of effective feeding strategies for both healthy and sick infants. In this study, we determined PepT1 transport capabilities and relative quantities of PepT1 mRNA at specific locations along the gastrointestinal tract of piglets during suckling and post-weaning.

Hypothesis: All intestinal regions studied will demonstrate the capacity to transport dipeptides. Additionally, the intestinal capacity for peptide uptake will change during development.

2.1 Abstract

The H^+-coupled transporter, PepT1 is responsible for the uptake of dietary di- and tri-peptides in the intestine. Using an *in vivo* continuously perfused gut loop model in Yucatan miniature pigs, we measured dipeptide disappearance from four 10 cm segments placed at equidistant sites along the length of the small intestine. Pigs were studied at 1, 2, 3 (suckling) and 6 weeks (post-weaning) post-natal age. Transport capability across the PepT1 transporter was assessed by measuring the disappearance of ^{3}H-glycyl-sarcosine; real time RT-PCR was also used to quantify PepT1 mRNA. Each of the regions of intestine studied demonstrated the capacity for dipeptide transport. There were no differences among age groups in transport rates measured in the most proximal intestine segment. Transport of ^{3}H-glycyl-sarcosine was significantly higher in the ileal section in the youngest age group (1 week) compared to the other suckling groups; however, all suckling piglet groups demonstrated lower ileal transport compared to the post-weaned pigs. Colonic PepT1 mRNA was maximal in the earliest weeks of development and decreased to its lowest point by week 6. These results suggest that peptide transport in the small intestine may be of importance during the first week of suckling and again with diet transition following weaning.

2.2 Introduction

Dietary protein is absorbed from the intestinal lumen as its constituent amino acids as well as small peptides containing two or three residues. The protein responsible for dietary di- and tri-peptide transport is PepT1, a member of the proton-coupled oligopeptide transporter (POT) super family of proteins (Daniel, 2004). PepT1 is localized to the apical membrane of intestinal villi, allowing it access to the digesta passing through the lumen. Studies that have investigated PepT1 mRNA at distinct regions along the intestine have reported variable results (Chen et al., 1999, Li et al., 2008, Wang et al., 2009), likely due to different animal models. In rats and mice, the amount of PepT1 mRNA does not change across the length of the small intestine (Lu and Klaassen, 2006, Rome et al., 2002, Erickson et al., 1995); however in the developing chick intestine, the highest quantities were found in the duodenum and jejunum (Li et al., 2008). In adult humans and rats, higher concentrations of PepT1 mRNA in the duodenum and jejunum have also been reported (Terada et al., 2005, Herrera-Ruiz et al., 2001). In contrast, Chen *et al.* reported similar expression of PepT1 mRNA across the entire length of the small intestine of mature sheep, dairy cows, pigs and chickens (Chen et al., 1999).

Few studies have reported developmental changes in PepT1. Differences in the location and concentration of PepT1 mRNA along the small intestine with increasing age have been reported in rats (Shen et al., 2001) and chickens (Chen et al., 2005, Gilbert et al., 2007a). PepT1 protein increased linearly with age in chickens, but there was a decrease in PepT1 mRNA in rats between postnatal days 4 and 50. Shen *et al.* demonstrated that even though PepT1 mRNA expression was at its highest point 3-5 days after birth in a neonatal rat model, it declined rapidly at all locations in the small intestine during the following days of suckling, suggesting that PepT1 and peptide transport are potentially important in the early life of the suckling neonate

(Shen et al., 2001). In piglets, D'Inca *et al.* determined that there was a dramatic decrease in PepT1 mRNA expression within the first 48 hours after birth; however, intrauterine growth restriction delayed this adaptation, further supporting the importance of peptide transport in the newborn (D'Inca et al., 2011).

In adult humans, a significant proportion of amino acids are absorbed as di- and tripeptides, and dipeptides are absorbed faster than free amino acids in the jejunum due to the high capacity for uptake by PepT1 (Ganapathy V, 1994). A high turnover rate and expression level of PepT1 contributes to the rapid uptake of peptides (Steinhardt and Adibi, 1986). The efficiency in uptake of small peptides combined with the lower osmolarity of peptide solutions support the use of peptides rather than free amino acids in therapeutic enteral formulas (Boza et al., 2000). In neonates, proteolytic capacity is not as well developed as in adults, due to significantly lower production of pepsin (Henderson et al., 2001). Compared to adults, a higher proportion of amino acids would be present in the neonatal gut as small peptides. As such, the capacity for peptide transport may be high to support optimal nutritional status and growth. Indeed, alanyl-glutamine is currently being studied as a potential dietary supplement, and has been reported to increase the nutritive status and intestinal barrier function in human neonates (Lima et al., 2007) as well as to aid in the maintenance of cellular glutathione concentrations during oxidative stress (Alteheld et al., 2005). Although the absorption of small peptides is important in the healthy individual, the bioavailability of peptides in times of intestinal duress may be of particular importance. In addition, the added benefits of stability, hypotonicity and palatability (Silk et al., 1982) support the importance and usefulness of dipeptide preparations for modern clinical treatments.

Characterizing the ontogeny of PepT1 in the neonate is of tremendous importance for the development of effective feeding strategies for both healthy and sick infants. The piglet is an excellent model for the human neonate, and especially preterm infants (Shulman, 1993). Neonatal pigs share similar patterns of intestinal development with humans, as well as similar nutritional requirements. Dipeptide transport capacity via PepT1 and the effects of development have not been previously reported in this model. In this study, we determined PepT1 transport capabilities and relative quantities of PepT1 mRNA at specific locations along the gastrointestinal tract of piglets during suckling and post-weaning.

2.3 Experimental Methods

2.3.1 In situ *perfusion (gut loop model)*

Sixteen Yucatan miniature piglets (N = 4 per group) were obtained from the breeding herd at Memorial University of Newfoundland (average birthweight 1.01 ± 0.03 kg; average growth rate from birth to 1 month of age: 45 ± 3 g/kg bodyweight/d). All procedures were approved by the Institutional Animal Care Committee, and were in accordance with the guidelines of the Canadian Council on Animal Care. Piglets were studied at 1, 2, 3 or 6 weeks of age. Piglets in this herd are weaned at 4 weeks old to a standard pelleted grower pig diet based on wheat, barley, oats and canola meal (15.6% crude protein, 3.75% crude fat, 12.1 MJ/kg digestible energy) (Eastern Farmers Co-op, St. John's, NL); preweaning piglets were exclusively suckled by sows fed a typical lactation diet with 14% crude protein and 3% crude fat. To avoid fasting (which may alter PepT1 expression or function (Ma et al., 2012)), the in situ study was initiated within 2 h of separating the piglets from the sows at 1, 2 or 3 weeks of age and within 2 h of last feed for 6 week old piglets. Anaesthesia was induced with an intramuscular injection of

22 mg/kg ketamine hydrochloride (Bimeda-MTC, Cambridge, ON) plus 0.5 mg/kg acepromazine (Vétoquinol Canada Inc., Lavaltrie, QC). General anaesthesia was maintained with 1-2% isoflurane (Abbot Laboratories Ltd., Montreal, QC) delivered with 1.5 L/min oxygen. Body temperature was maintained via a homeothermic blanket system that automatically adjusted heat output in response to a rectal temperature probe (Harvard Apparatus, Saint-Laurent, QC). A mid-line incision was made in the abdomen to open the peritoneum. The complete length of the small intestine (SI) was measured and four equidistant regions were marked for loop placement. The most proximal jejunal loop (0%) was defined as 15 cm from the ligament of Treitz. Closed loops of intestine consisted of 10-cm sections of intestine with inlet and outlet cannulas (ID, 1/16 in.; OD, 1/8 in., Watson Marlow, Cornwall, UK) inserted through a small perforation at both ends of the 10 cm. In total, four loops were placed at 0, 25, 50 and 75% of SI length. Each isolated gut loop was perfused with a dipeptide-containing buffer that was re-circulated through the loop for the duration of the study by a multi-channel peristaltic pump (Watson Marlow, Cornwall, UK). The perfusate (65 ml) contained 5 mM glycyl-sarcosine (gly-sar) with 37 kBq ^{3}H-glycyl-sarcosine (Moravek, Brea, CA) in PBS (144.6 mM NaCl, 15.9 mM Na_2HPO_4, 1.2 mM NaH_2PO_4), pH 6.0, and was maintained at 37°C for the duration of the study. Perfusions through each closed loop continued for 120 min. A 1 mL sample of perfusate from each closed loop was removed every 10 min, to determine isotope disappearance. During the procedure the exposed intestines were kept moist with warmed saline and covered with gauze and plastic wrap. Heart rate and blood oxygenation were monitored via pulse-oximetry (Surgivet, Dublin, OH) throughout the experiment. At the end of the perfusion period the loops were excised by cautery, flushed with cold 0.9% saline, placed on ice, cut longitudinally and scraped with a microscope slide to remove the mucosa. Samples were also removed from the

apex of the spiral colon and flushed, and all tissues were immediately flash frozen in liquid nitrogen and stored at -80°C until further analyses.

2.3.2 Disappearance of ^{3}H-glycylsarcosine

Sampled perfusate (100 µL) was added to 4 mL Scintiverse (Fisher Scientific, Ottawa, ON) for liquid scintillation counting. The specific radioactivity (SRA) was calculated as the mean disintegrations per minute (dpm) per µmol of glycyl-sarcosine present in the perfusate. SRA was calculated at baseline (prior to perfusion) and in each of the perfusate samples taken over the course of the 2-h study. The SRA was then used to determine the total quantity of glycyl-sarcosine. Rate of disappearance of glycyl-sarcosine was determined via area under the curve using GraphPad Prism 4.0. Perfusates were also derivatized with phenylisothiocyanate and analyzed using HPLC (Bidlingmeyer et al., 1984). Complete fraction collection was used to determine whether the isotope was associated with glycyl-sarcosine, glycine or some other metabolite.

2.3.3 Real-time RT-PCR

PepT1 mRNA was measured in mucosa sampled from each of the perfused intestinal loops. Samples of colon were also analyzed. RNA was extracted using the Qiagen RNEasy Mini kit (Qiagen Inc., Mississauga, ON) according to the manufacturer's protocol. Relative concentration and purity were determined by measuring the absorbance at 260 and 280 nm using a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE). RNA integrity was visualized via the agarose gel visualization of the 28S:18S ratio. cDNA was created according to the protocol outlined in the QuantiTect (Qiagen Inc., Mississauga, ON) reverse transcriptase manual. 1 µg of total RNA was used in the reverse transcription reaction. Taqman probes were utilized

in the PCR reaction. The sequences of the primers and probes were as follows: PepT1 forward primer 5' d CTGGAGTTCTCCTATTCTCA 3', reverse primer 5' d AACAGCCACGGTCAACAG 3', probe sequence for PepT1 5' d BHQ-2-TCCTTCCAACATGAAGTCGGTGC-Pulsar 650 3'. β-actin was used as an internal control with the following sequences: forward primer 5' d CCCAGCACGATGAAGA 3', reverse primer 5' d CGATCCACACGGAGTC 3', probe 5' d FAM-TCAAGATCATCGCGCCTCCAGA-BHQ-1 3'. The accession numbers for the template sequences were AY180903.1 for PepT1[23] and AY55069 for beta-actin. The Lightcycler (Roche, Indianapolis, IN) was set to the following conditions: 15 min at 95°C, 40 cycles of 1 min at 95°C and 1 min at 55°C, and a 40°C incubation for 1 min. Reaction efficiency for PepT1 was 0.94 ± 0.03 and β-actin reaction efficiency was 0.96 ± 0.06. Each sample was run in triplicate and analyzed using the Livak method (2^{-ddCt})(Livak and Schmittgen, 2001).

2.3.4 Statistical analyses

Data were analyzed by one-way (colonic PepT1 mRNA) or two-way repeated measures ANOVA (overall uptake of glycyl-sarcosine) with Bonferroni's protected means separation test. The variables analyzed in the two-way ANOVA were age, intestinal location and their interaction. Uptake data over time were tested for linearity using least squares regression. Sample size was N = 4 piglets per age group and differences were noted as significant if $p < 0.05$ (GraphPad Prism 4.0, La Jolla, CA).

2.4 Results

Throughout the perfusion studies, all piglets remained stable, well oxygenated and maintained a core body temperature above 37.5˚C.

2.4.1 Dipeptide disappearance

The proportion of ^{3}H associated with glycyl-sarcosine accounted for greater than 90% of the radioactivity in the perfusates at baseline, determined through HPLC fraction collection, while glycine contained less than 5% of the label. This suggests that the dipeptide did not degrade intraluminally and was available for transport. Glycyl-sarcosine transport occurred linearly in all intestinal loops in all animals studied, except in the most distal loop (75%) in week 1 and 3 piglets, and in the 0% loop in piglets at week 6 (Fig.2.1). In order to assess how dipeptide uptake might change along the longitudinal axis of the small intestine as the animal ages, we compared glycyl-sarcosine uptake between age groups at the four sites of the small intestine (Fig. 2.2). Overall, there was a significant effect of age ($p < 0.0001$), intestinal location ($p < 0.005$) and age-by-location interaction ($p < 0.0005$). When compared by intestinal location, dipeptide uptake was remarkably consistent at the most proximal site (0%) across all age groups. At the mid-jejunum site (25%), glycyl-sarcosine uptake in 1 week old pigs was twice that in pigs 2 and 3 weeks old ($p < 0.05$), with 6 week old pigs intermediate. In the distal jejunum (50%), greater uptake occurred in the post-weaning animals (6 weeks) compared to the week 2 animals ($p < 0.05$), with uptake in piglets at 1or 3 weeks of age intermediate. The uptake of glycyl-sarcosine in the ileum (75%) for week 6 animals was about twice that for piglets aged 2 and 3 weeks ($p < 0.001$), and 30% greater than piglets at 1 week ($p < 0.05$). Within age groups, 1 week old animals had greatest uptake in mid-jejunum and ileum ($p < 0.05$). In 6 week old animals, there was a notable gradient of glycyl-sarcosine uptake with the lowest uptake in the proximal jejunum up to a 3-fold higher uptake in the ileum (Fig. 2.2).

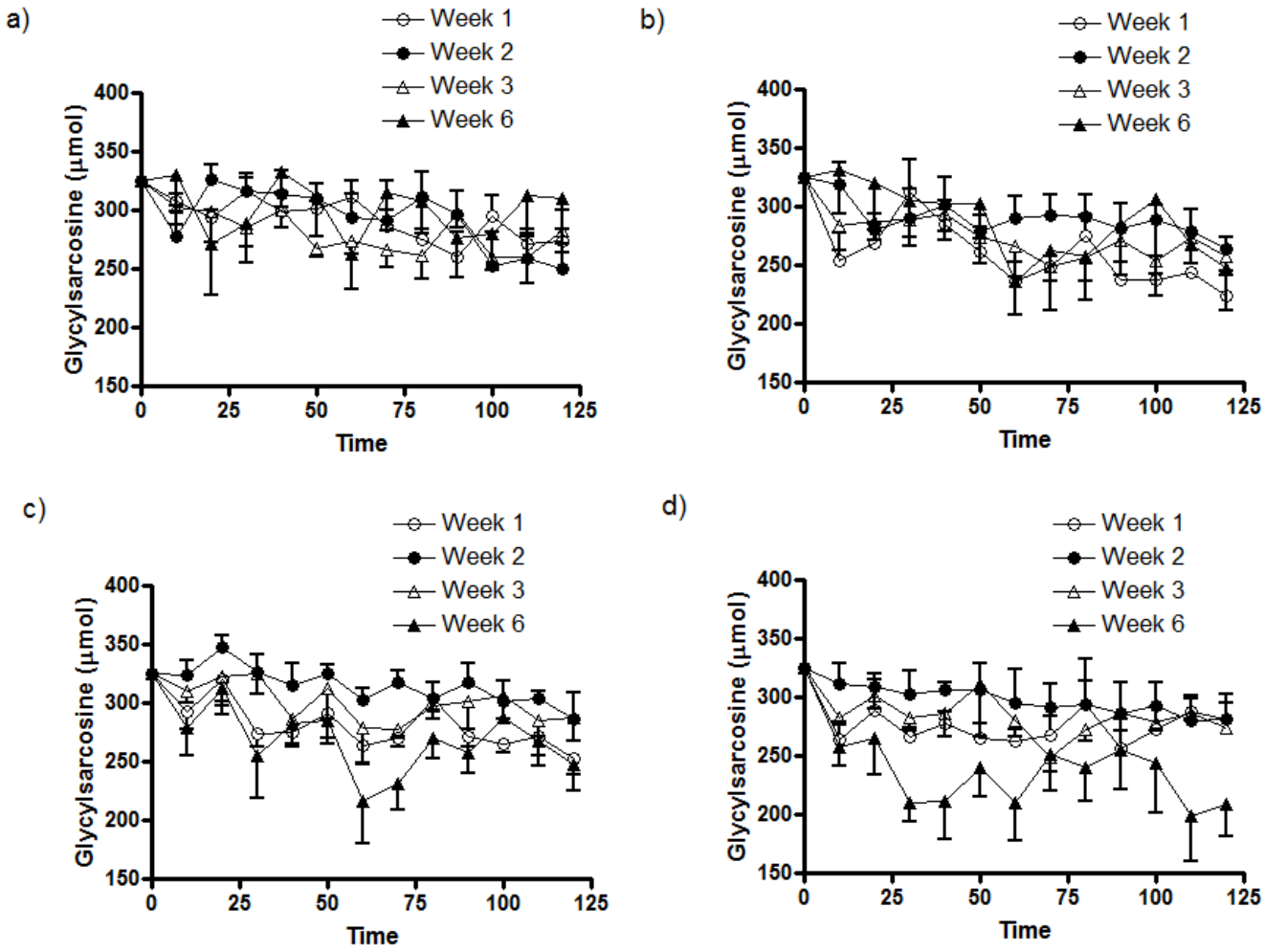

Fig. 2.1 Total glycylsarcosine disappeared from closed loops of small intestine during the 2 h perfusion study. Position of each intestinal loop is indicated by its relative position from the ligament of Treitz: (a) 0, (b) 25, (c) 50 and (d) 75 %. Values are means with their standard errors (N=4 for each group).

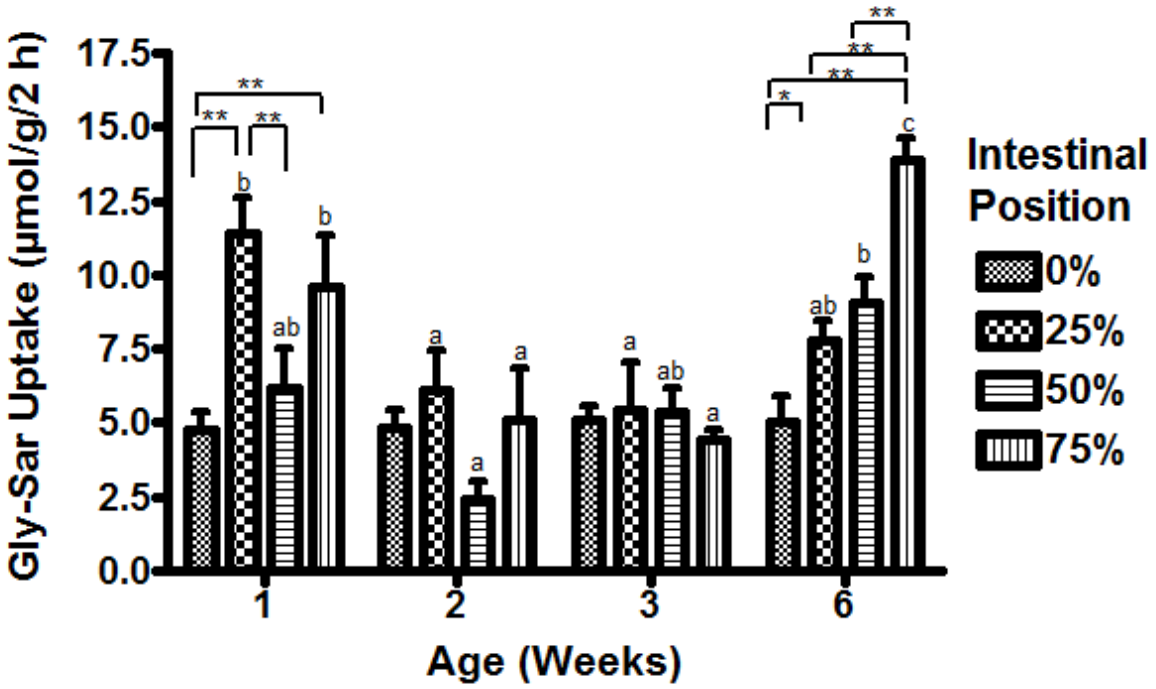

Fig. 2.2 Rate of glycyl-sarcosine (Gly-Sar) uptake from closed loops of small intestine. Intestinal position is given as distance from the ligament of Treitz as a percent of the total length of the intestine. Uptake was determined by measuring the disappearance of ^{3}H-glycyl-sarcosine. Data are expressed as mean ± SD (N = 4 for each bar) and were analyzed by two-way ANOVA. Significant effects of age ($p < 0.0001$), intestinal location ($p < 0.005$) and age-by-location interaction ($p < 0.0005$) were observed. Lines represent significant differences between intestinal sections *within* an age group (*$p < 0.05$, **$p < 0.01$). Differing letters represent significant differences ($p < 0.05$) within each specific intestinal site, compared among age groups.

2.4.2 Quantification of PepT1 mRNA in the intestine

The PCR results from the small intestinal tissue samples were highly variable, thus no significant differences in PepT1 mRNA were detected (Fig. 2.3). PepT1 mRNA was detected in the colon of all animals (Fig. 2.4); however, the quantity was ~10% of that measured in the proximal jejunum of piglets at 1 week of age, and ~2% of that found in the proximal jejunum of post-weaning animals ($p < 0.05$). Although present in similar abundance at ages 1, 2 and 3 weeks, colonic PepT1 mRNA in post-weaned pigs was only 10% of that seen than in suckling pigs ($p < 0.05$) (Fig. 2.4).

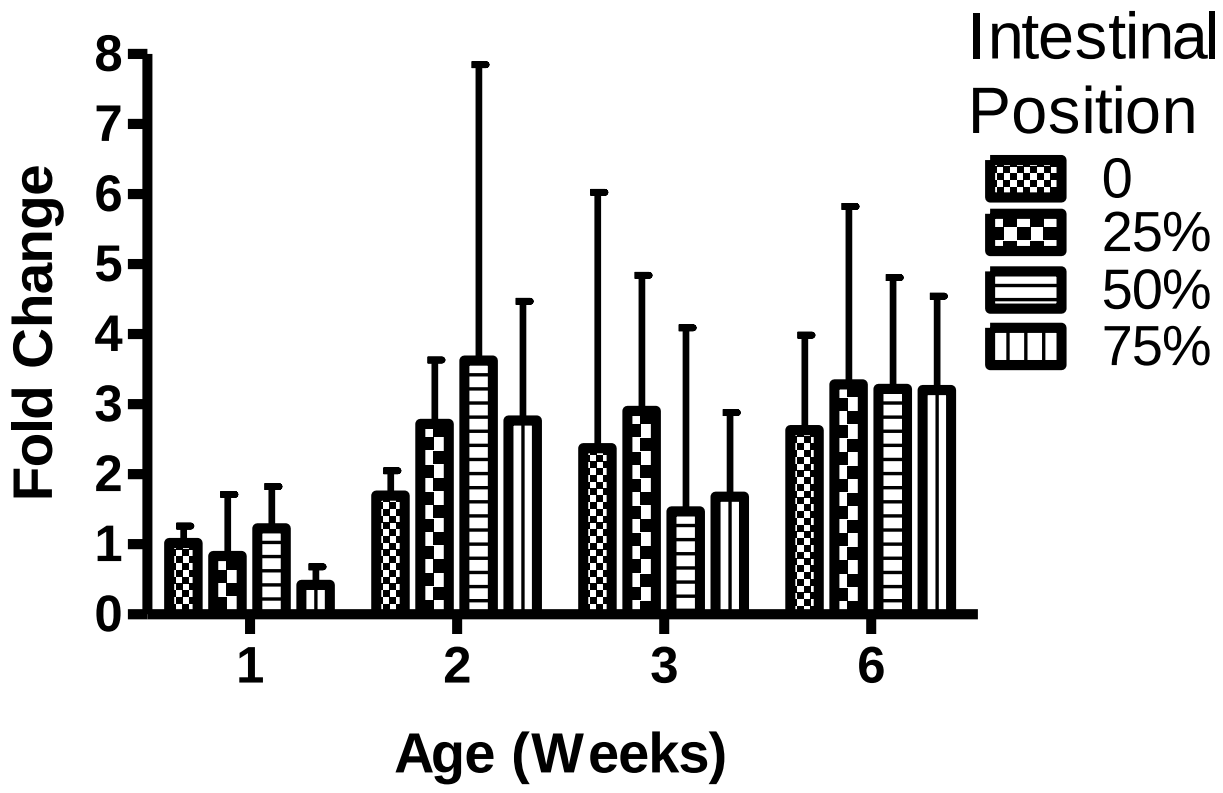

Fig. 2.3 Real time RT-PCR analysis of PepT1 mRNA closed loops of small intestine. Intestinal position is given as distance from the ligament of Treitz as a percent of the total length of the intestine. Week 1 samples were set at 1 for comparison purposes (Livak and Schmittgen 2001). Data are expressed as mean ± SD (N = 4 for each bar).

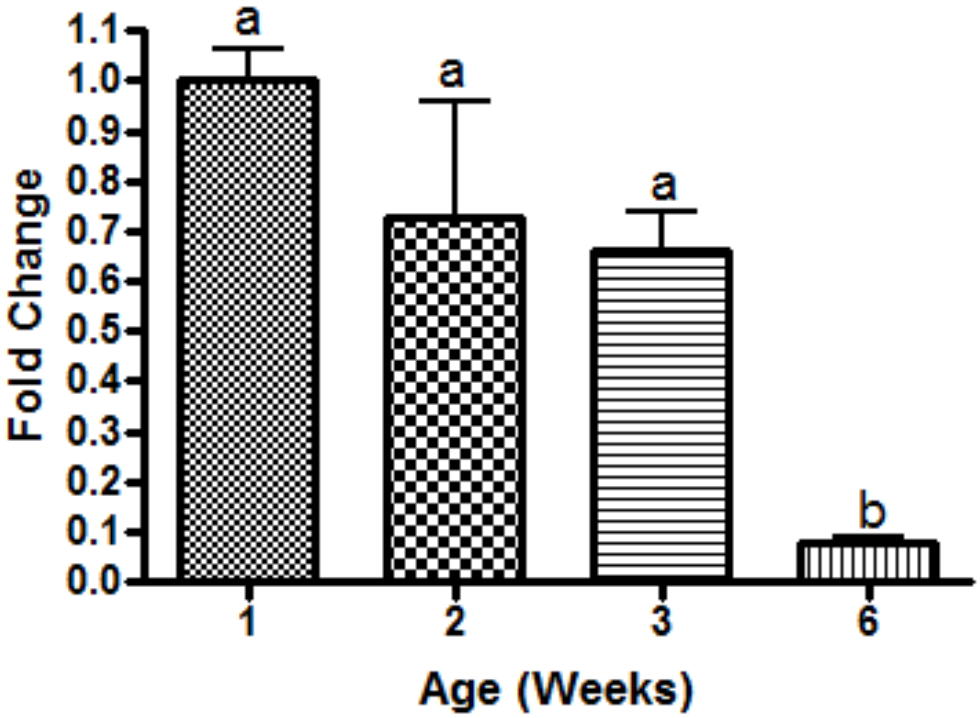

Fig. 2.4 Real time RT-PCR analysis of PepT1 mRNA in the colon at four ages. Week 1 samples were set at 1 for comparison purposes (Livak and Schmittgen 2001). Data are expressed as mean ± SD (N = 4 for each bar) and were analyzed by one-way ANOVA. Bars with differing letters are significantly different, $p < 0.05$.

2.5 Discussion

Although the porcine peptide transporter has been cloned and characterized (Klang et al., 2005), there has been little investigation into the ontogeny of porcine PepT1. The objective of this study was to evaluate the ontogenic pattern in peptide transport capability and PepT1 mRNA quantity in the small intestine of the Yucatan miniature pig as it develops from suckling to a post-weaned state. Clinically, such information is necessary to appropriately design feeding regimens for infants. This objective was accomplished through the use of a hydrolysis resistant dipeptide tracer, ^{3}H-glycyl-sarcosine, and real time RT-PCR for mRNA analysis. From these techniques it was determined that there was little variation in uptake of glycyl-sarcosine at any location in the small intestine during the suckling period; however in the post-weaning period, there was a distinct gradient in rate of uptake which was greatest in the distal small intestine. Small intestinal PepT1 mRNA was extremely variable at all locations measured in the small intestine at each age, whereas colonic PepT1 abundance declined dramatically after the animals were weaned.

In this study, small intestinal loops were perfused with a dipeptide-containing solution and uptake was calculated from the disappearance of ^{3}H-glycyl-sarcosine. This *in situ* model is ideal to isolate specific regions of the intestine for study by allowing multiple loops per animal, limiting systemic effects of the perfusate and still maintaining first-pass metabolism (Nichols and Bertolo, 2008, Adegoke et al., 1999a, Adegoke et al., 1999b). For these reasons, this model was selected to examine dipeptide uptake in the piglet model.

Absorption of glycyl-sarcosine and PepT1 mRNA was detected in the loops at all four locations in the small intestine. There was a significant effect of intestinal location on the uptake

of the dipeptide glycyl-sarcosine, particularly in post-weaning pigs (Fig. 2.2). Uptake of glycyl-sarcosine was relatively consistent in all intestinal locations examined in the older suckling animals (weeks 2 and 3). So once an animal was no longer suckling and was weaned onto a solid grain-based diet, there was a greater capacity for dipeptide transport in the ileum. Components of sow milk are readily digested and absorbed in the proximal parts of the small intestine (Mavromichalis et al., 2001, Buddington et al., 2001), so it is likely that the dietary stimulus for PepT1 upregulation in more distal portions of the ileum may not be present in luminal contents. In contrast, products of protein hydrolysis in grain-based diets with lower digestibility consumed by weaned animals would likely reach the ileum before absorption (Low, 1979), providing a substrate for PepT1, and perhaps a stimulus for PepT1 upregulation in the distal intestine of the post-weaned piglet. In this respect, the distal ileum may be important for optimal nitrogen absorption in the period immediately following a diet transition. Alternatively, the transition to solid feeding can be considered a period of intestinal injury and inflammation for the milk-fed piglet (Moeser et al., 2007, Pie et al., 2004) and such injury could lead to a stimulation of peptide uptake capacity (Vavricka et al., 2006). With respect to age, dipeptide uptake tended to follow a U-shaped curve with higher uptake in 1 week old and post-weaned piglets.

Although PepT1 is present in the small intestine, we have also identified its presence in the colon of the developing piglet. Our results demonstrated that PepT1 mRNA is present in colonic tissues until after weaning occurs (week 6). This may be an adaptive response that occurs post-weaning; as the distal small intestine increases its capacity for oligopeptide absorption, the colon reduces the transcription of PepT1 mRNA. Shen *et al*. reported that PepT1 presence in the colon was transient, because by day seven no PepT1 mRNA was found in the

colon of developing rats (Shen et al., 2001). PepT1 has been found in the colon of rats at later time points (Shi et al., 2006b), but its presence has been primarily related to a state of intestinal injury such as gut resection. PepT1 has also been detected in the colon of humans that have undergone intestinal resection (Ziegler et al., 2002). PepT1 is present in the colon of humans with inflammatory bowel disease (Merlin et al., 2001) and it has been shown that PepT1 is capable of transporting fMLP, which is a bacterial peptide. As such, PepT1 may be responsible for the exacerbation of inflammatory bowel disease. Colonic PepT1 mRNA and protein were also higher in patients with massive bowel resections, when compared to control subjects (Ziegler et al., 2002). A study in rats demonstrated an increase in PepT1 protein in the colon of animals with intestinal resection that were fed chow (Shi et al., 2006b), whereas rats fed an elemental diet did not express any PepT1 mRNA in colonic tissues (Lardy et al., 2006). Findings such as these underscore the importance of peptide presence in the lumen of the colon for the induction of PepT1. Although colonic peptide transport is extremely low when compared to the whole small intestine, in the event of intestinal injury or malnutrition, capacity for oligopeptide uptake may be increased through the up-regulation of PepT1, thereby recovering nutritionally valuable substrates. Whether this also leads to greater uptake of pathogenic bacterial peptides remains to be determined.

To understand the potential impact of peptide transport, it is important to consider the variable affinity of PepT1 substrates. Alanyl-alanine, for example, is a high affinity substrate for PepT1 (K_i of 0.08 ± 0.01 mM) whereas glycyl-sarcosine is classified as a medium affinity substrate with a K_i of 1.1 ± 0.1 mM, determined in Caco-2 cells (Brandsch et al., 1998, Brandsch et al., 1999). Previous work on glycyl-sarcosine transport in porcine jejunum, using an Ussing chamber system, demonstrated dipeptide uptake of approximately 600 nmol/cm^2/2 h (modified

from Winckler *et al*) (Winckler et al., 1999). In our study, using the more physiological ligated loop model, we found that the overall average glycyl-sarcosine uptake was 424 ± 226 nmol/cm^2/2 h. Thus, the data from our *in situ* model are comparable to previous *in vitro* findings with the same non-nutritional dipeptide and likely underestimate the transport rates of dietary dipeptides with higher PepT1 transporter affinity.

There are two possible mechanisms for the intestinal transport of oligopeptides: PepT1-mediated transcellular transport (Daniel, 2004) and paracellular transport (McCollum and Webb, 1998). The role of these pathways in intestinal oligopeptide transport is not yet fully understood, and there is increasing evidence that the paracellular pathway may be of some importance in absorption of small peptides and peptidomimetic drugs (Lafforgue et al., 2008, Menon and Barr, 2003). It has been well documented that the small intestine of suckling animals demonstrates greater paracellular transport at younger ages (Udall et al., 1981, Weaver et al., 1984). Thus, it is possible that paracellular transport could mask low PepT1 activity during suckling. We also observed the greatest disappearance of glycyl-sarcosine in the ileum of the post-weaning animals. Overall, if paracellular transport had significant impact on the transport of glycyl-sarcosine in our piglets, it would have been most prominent at the younger ages (weeks 1, 2 and 3) rather than the post-weaning state.

In summary, we have examined the ontogenic changes of the peptide transporter PepT1 in a piglet model of the developing intestine. These results have demonstrated that the capacity for dipeptide transport is present in both the proximal and distal small intestine, with the colon potentially being able to transport peptides during the suckling state. By post-weaning, the ileum is the site of highest dipeptide uptake. The importance of dipeptides to the nutritional status of the developing infant is still unknown; however, when therapeutic formulas are necessary, it is

likely that the provision of all or part of the nitrogen as small peptides conveys advantages over preparations of free amino acids, and this may be of particular importance when compromised intestinal function is present.

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Chapter 3:Enterally delivered dipeptides induce changes in small intestinal morphology in a piglet model of intestinal resection

The work presented in this chapter was funded in part by a grant from the Ajinomoto Amino Acid Research Program. It represents work that was presented at Experimental Biology 2011 in Washington, D.C., U.S.A and will be submitted for publication in Clinical Nutrition. The co-authors of this work are Matthew G. Nosworthy, M. Elaine Dodge, Robert F. Bertolo and Janet A. Brunton. All authors were responsible for designing the study and conducting the surgeries, MGN carried out the laboratory analysis with assistance from MED with respect to the western blots and BrdU, MGN performed the statistical analyses.

Intestinal resection is an important issue in neonates leading to a reduction in absorptive capacity, with 2.2% of neonatal intensive care unit admissions resulting in short bowel syndrome, a condition with a 37.5% fatality rate. As PepT1 is preferentially maintained over free amino acid transporters during gut stress, provision of dipeptides would result in increased nutrient availability thereby potentially enhancing the adaptive response of a surgically shortened intestine. By selecting the dipeptides cysteinyl-glycine and alanyl-glutamine as test dipeptides, this study also established whether these dipeptides were beneficial in inducing adaptation in a piglet model of short bowel syndrome.

Hypothesis: Dipeptides (alanyl-glutamine, cysteinyl-glycine, alanyl-alanine) delivered into a surgically shortened gut will induce an increase in PepT1 mRNA and protein in the residual intestine. Greater efficiency of uptake of glutamine and cysteine as dipeptides (compared to

alanyl-alanine or free amino acids) will result in structural and functional enhancements of the SI mucosa, greater rates of mucosal protein synthesis and improved redox status.

3.1 Abstract

PepT1, a di/tripeptide transporter, is preferentially maintained over free amino acid transporters in situations of gut stress. Therefore our objective was to determine the impact of enterally delivered dipeptide-containing diets on indices of intestinal adaptation in neonatal piglets after intestinal resection.

Methods: Piglets (N = 25, 10 ± 1 d old) underwent an 80% jejuno-ileal resection and were randomized to either 1) a control diet containing free amino acids, or the same diet but with equimolar amounts of free amino acids replaced by 2) alanyl-alanine, 3) alanyl-glutamine , 4) cysteinyl-glycine or 5) both alanyl-alanine and cysteinyl-glycine. Outcome measures included plasma and mucosal amino acid concentrations, morphological and histological differences in the remnant intestine, PepT1 mRNA and protein expression, and mucosal cytokine concentrations.

Result: Intestinal length, organ weight and protein synthesis rates were similar between groups after 4 d of enteral feeding. All of the dipeptide-containing diets reduced pro-inflammatory cytokine concentrations in the mucosa (TNF-α, IFN-γ). The cysteinyl-glycine diet supported greater villus height compared to all other dipeptides and greater crypt depth compared to alanyl-glutamine; however, none of the dipeptide diets altered intestinal morphology compared to the free amino acid control diet.

Conclusions: This study showed that while there was no explicit morphological benefit of enteral dipeptides over their constituent free amino acids, there was the potential for the amelioration of

intestinal inflammation by reducing pro-inflammatory cytokines. Enteral provision of dipeptides impacted intestinal adaptation, but the response was dipeptide-specific.

3.2 Introduction

Peptide transporter 1 (PepT1) is responsible for absorption of small peptides, two or three residues, from the lumen of the small intestine. Increasing evidence suggests that PepT1 population and/or activity can be altered by manipulating the nutritional status or health of the animal (Daniel, 2004). In situations of gut stress such as malnutrition, intestinal failure or surgical intervention, PepT1 expression is maintained or increased, in contrast with other nutrient transporters which typically decline in number (Satoh et al., 2003). Humans fasted for 14 days demonstrated a significant decrease in the transport of amino acids but peptide transport was maintained (Vazquez et al., 1985). Ihara *et al.* (Ihara et al., 2000) reported a 179% increase in PepT1 mRNA over control samples in the jejunum of rats after a 4 day fast. In addition to being preserved during gut stress, PepT1 expression is also substrate driven. The presence of exogenous peptides in the culture media resulted in an increase in PepT1 expression in Caco-2 cells (Walker et al., 1998). Dietary provision of peptides has also demonstrated the same result in rats (Erickson et al., 1995, Shiraga et al., 1999). When the uptake of dietary protein is compromised due to intestinal injury such as inflammation or surgery, it may be advantageous to provide small peptides rather than free amino acids in the diet, to stimulate the population of PepT1 at the brush border and exploit the capacity for nitrogen transport.

Short bowel syndrome (SBS) is a clinical condition induced through the surgical removal of intestinal tissue. Causes for intestinal resection are varied, and include inflammatory disorders such as Crohn's disease and colitis, tumors, physical trauma and infection such as necrotizing enterocolitis (NEC) (Goulet et al., 1991). Intestinal resection results in a loss of

absorptive capacity (Sukhotnik et al., 2002) leading to a requirement for long term parenteral nutrition (PN). In newborn and pre-term infants, SBS is commonly the result of congenital malformations or NEC (Sodhi et al., 2008). NEC is an aggressive, anaerobic infection that develops rapidly in the gastrointestinal tract in approximately 10% of all very low birth weight infants, with up to a 34% mortality rate in the lowest birth weight category (Fitzgibbons et al., 2009).

A recent study conducted in my supervisors lab demonstrated that enteral feeding (EN) of an elemental diet in combination with PN, induced intestinal adaptation in a piglet model of SBS. This led to greater cell mass and intestinal length compared to PN alone (Dodge et al., 2012). Due to the substrate driven expression of PepT1, we hypothesized that providing enteral amino acids as peptides may stimulate the up-regulation of PepT1, leading to greater amino acid transport potential.

Certain amino acids such as glutamine and cysteine are involved in intestinal barrier function and the regulation of oxidative stress. Glutamine may be necessary for the localization of tight junction proteins in Caco-2 cells, thereby linking glutamine directly to intestinal integrity (Li et al., 2004). Decline in the B0 transporter after surgical resection (Satoh et al., 2003) may prevent adequate absorption of glutamine, which could interfere with the maintenance of the intestinal barrier. With PepT1 potentially up-regulated following surgery, and single amino acid transporters possibly depressed, the provision of enteral glutamine as a dipeptide may be particularly advantageous to the remaining intestine.

Cysteine is a conditionally essential amino acid as it can be synthesized via trans-sulfuration from methionine. Cysteine has been linked to increased cellular proliferation via

transition from the G1 phase to S phase of the cell cycle (Noda et al., 2002). Piglets that were fed an enteral diet free of sulfur amino acids presented with intestinal atrophy as demonstrated by reduced cellular proliferation, lower numbers of goblet cells and reduced villus height (Bauchart-Thevret et al., 2009). Cysteine is also one of the amino acid residues in glutathione (γ-Glu-Cys-Gly, GSH), and contributes to controlling cellular redox states (Wu et al., 2004). Limiting the availability of cysteine led to lower concentrations of glutathione in the plasma and intestinal mucosa of rats (Nkabyo et al., 2006). Studies in humans with inflammatory bowel disorders requiring surgical resection have demonstrated compromised redox status (Sido et al., 1998). Inclusion of cysteine-containing peptides in enteral diets may enhance cysteine availability and increase the generation of GSH, leading to improved recovery and intestinal adaptation.

In this study we utilized a Yucatan miniature piglet model of intestinal adaptation which was previously characterized in my supervisors laboratory (Dodge et al., 2012) to investigate the potential benefits of alanyl-glutamine and cysteinyl-glycine when provided enterally to piglets with an 80% proximal resection of the small intestine.

3.3 Materials and Methods

3.3.1 Surgical procedures

Twenty-five (25) Yucatan miniature piglets, 10-12 days of age, were obtained from a breeding herd at Memorial University of Newfoundland (St John's, NL) and randomized to one of five experimental groups. All experimental procedures were approved by the Institutional Animal Care Committee in accordance with guidelines of the Canadian Council of

Animal Care. Anesthesia was induced with an intramuscular injection of ketamine hydrochloride (22 mg/kg; Bimeda Canada, Cambridge, ON) and acepromazine (0.5 mg/kg; Vetoquinol Canada Inc**,** Lavaltrie, QC). After atropine sulfate injection (0.05 mg/kg; Rafter Dex Canada, Calgary, AB), the piglets were intubated and maintained under anesthesia with 1.0 - 1.5% isoflurane (Abbott Laboratories Ltd, Montreal, QC) mixed with oxygen at a flow rate of 1.5 L/min. In each piglet, 2 venous catheters were surgically implanted. One catheter was introduced into the femoral vein and advanced to the inferior vena cava for blood sampling and drug delivery. A second catheter was introduced into the jugular vein and advanced to the superior vena cava for the delivery of parenteral nutrition. The abdomen was opened, and approximately 80% of the proximal SI was resected, leaving 100 cm of the distal ileum proximal to the ileocecal valve intact. Continuity of the SI was restored using an end-to-end anastomosis. Animals also underwent implantation of a gastric catheter to allow for infusion of the enteral diet.

After surgery, piglets received intravenous antibiotics (20 mg of trimethoprim and 100 mg of sulfadoxine; Borgal, Intervet Canada Ltd, Kirkland, ON) and analgesic (0.03 mg/kg of buprenorphine hydrochloride; Temgesic, Schering-Plough, Kirkland, ON). Borgal was given daily and Temgesic every 12 h for the first 3 days postoperatively. Piglets were fitted with jackets secured to a tether-swivel system with dual-infusion ports (Lomir Biomedical, Notre-Dame-De-L'Ile-Perrot, QC), allowing for the continuous infusion of both parenteral and enteral fluids. Piglets were housed in individual circular metabolic cages (1 m diameter), which allowed visual and aural contact with other piglets; toys were also provided. Lighting was maintained on a 12-h light:dark cycle, and room temperature was maintained between 23°C and 28°C with supplemental heat provided by heat lamps. Piglet weights were taken daily beginning 48 h after surgery.

3.3.2 Parenteral/Enteral Diets

Following surgery, infusion of parenteral diet was initiated via the jugular vein at 50% of targeted intake. On the morning of day 1, the rate of infusion was increased to 75% for 12 h and then to 100% by the end of day 1 (13.5 $mL \cdot kg^{-1} \cdot d^{-1}$). The complete parenteral diet provided 1.1 MJ of metabolizable $energy \cdot kg^{-1} \cdot d^{-1}$ with glucose (24.5 $g \cdot kg^{-1} \cdot d^{-1}$) and lipid (20% Intralipid, Pharmacia, Stockholm, Sweden) each supplying 50% of non-protein energy and 15 $g \cdot kg^{-1} \cdot d^{-1}$ of protein, supplied as free amino acids. The amino acid composition was as follows (per gram of total L-amino acids): alanine, 107 mg; arginine, 67 mg; aspartate, 61 mg; cysteine, 14 mg; glutamate, 105 mg; glycine, 27 mg; histidine, 31 mg; isoleucine, 46 mg; leucine, 104 mg; lysine-HCl, 102 mg; methionine, 19 mg; phenylalanine, 55 mg; proline, 83 mg; serine, 56 mg; taurine, 5 mg; threonine, 41 mg; tryptophan, 21 mg; tyrosine, 8 mg; and valine, 53 mg (Dodge et al., 2012). Prior to feeding, vitamins (Multi-12K1 Pediatric, Sabex Boucherville, QC), trace minerals, 200% of NRC recommendations, (NRC, 1998), lipid, and iron dextran (Fe, 3.0 mg/kg; Vetoquinol Canada Inc, Lavaltrie, QC**)** were added to the diet. On day 2, the presence of ileus was tested by infusing a 10-mL bolus of the complete parenteral diet into the stomach via the gastric catheter. If gastric emptying was evident, then enteral feeding was initiated on day 3, and increased over the following 24 h to achieve 50% of total nutritional intake with the balance being maintained via parenteral nutrition. The diets were continuously infused intravenously and enterally by pressure sensitive peristaltic pumps.

The piglets were randomized to one of 5 experimental enteral diets (N = 5 per group). The experimental diets were based on the elemental diet used for parenteral nutrition, with the majority of the nitrogen provided as free L-amino acids. The dietary manipulations included 1) free amino acids as a control diet (CON), or the equimolar replacement of free amino acids with

the following dipeptides; 2) alanyl-alanine (AA), 3) alanyl-glutamine (AQ), 4) cysteinyl-glycine (CG) or 5) the combination of alanyl-glutamine and cysteinyl-glycine (AQ+CG) (Dipeptides were purchased from Bachem, Torrance, CA). All enteral diets were isonitrogenous. The amino acid composition of the enteral diets is given in Table 3.1.

3.3.3 Necropsy and Tissue Collection

Animals were injected intravenously with 50 mg/kg of 5-bromo-2′-deoxyuridine (BrdU) (Sigma Aldrich, Oakville, ON) 4 hours before necropsy. Thirty minutes prior to necropsy animals were injected intravenously with 1.5 mmol/kg phenylalanine, of which 0.15 mmol/kg was labelled with ^{3}H-phenylalanine. At necropsy, the site of anastomosis was identified and the small intestine removed from the site of anastamosis to the ileocecal valve. The weight and length of this portion of the small intestine were measured and considered the "remnant intestine". A 3-cm segment of remnant intestine immediately distal to the site of anastomosis was also immersed in neutral buffered 10% formalin (Fisher Scientific, Pittsburgh, PA) for histologic analyses. The following 50 cm of remnant intestine was isolated and weighed, and the mucosa was harvested from the this intestinal section which was slit longitudinally and placed on a glass plate on ice. The mucosal tissue was scraped from the underlying muscle using a glass slide with even pressure, weighed and flash frozen in liquid nitrogen. Liver and kidney weights were also measured.

3.3.4 Histological Analysis

3.3.4.1 Preparation of Slides

After fixation in 10% buffered formalin (Fisher Scientific, Pittsburgh, PA), samples of intestine were dehydrated in ethanol, cleared in xylene, embedded in paraffin wax, and sliced into 5-µm sections.

Table 3.1: Amino acid composition of the enteral diets (g/L)

Amino Acid	Control	AA	AQ	CG	AQ+CG
alanine	4.4	0	1.03	4.4	1.03
arginine	3.59	3.59	3.59	3.59	3.59
aspartate	3.27	3.27	3.27	3.27	3.27
cysteine	1.1	1.1	1.1	0	0
glutamate	0	0	0	0	0
glycine	1.47	1.47	1.47	0.8	0.8
histidine	1.67	1.67	1.67	1.67	1.67
isoleucine	2.48	2.48	2.48	2.48	2.48
leucine	5.61	5.61	5.61	5.61	5.6
lysine	5.58	5.58	5.58	5.58	5.58
methionine	1.04	1.04	1.04	1.04	1.04
phenylalanine	2.95	2.95	2.95	2.95	2.95
proline	4.46	4.46	4.46	4.46	4.46
serine	5.19	5.19	5.19	5.19	5.19
taurine	0.24	0.24	0.24	0.24	0.24
tryptophan	1.14	1.14	1.14	1.14	1.14
tyrosine	0.42	0.42	0.42	0.42	0.41
valine	2.86	2.86	2.86	2.86	2.86
threonine	2.18	2.18	2.18	2.18	2.18
glutamine	5.5	5.5	0	5.5	0
alanyl-glutamine	0	0	8.87	0	8.87
alanyl-alanine	0	4.4	0	0	0
cysteinyl-glycine	0	0	0	1.77	1.77

3.3.4.2 Crypt Depth/Villus Height

Sections were stained with hematoxylin and eosin (Fisher Scientific, Pittsburgh, PA). Villus height and crypt depth were measured with a Zeiss Axiostar microscope (Carl Zeiss Toronto, ON). Images were captured with an Infinity 1 camera and Infinity Analyze software (Lumenera Corporation Nepean, ON) Ten measurements of villus height and crypt depth were performed per animal. All histological measurements were performed in a blinded manner by a single investigator (MGN), see figure 3.1 for a representative image.

3.3.4.3 Cellular Proliferation Index Using 5-Bromo-2'-deoxyuridine

Immunohistologic analyses were performed to measure incorporation of BrdU into proliferating cells of small intestinal crypts (BD Biosciences Pharmingen, Mississauga, ON) with visualization based on DAB substrate (Vector Laboratories, Burlington, ON). Data were expressed as the number of cells labeled with BrdU per total number of cells in an individual crypt (10 crypts per animal).

3.3.5 Tissue Protein Synthesis

Fractional rates of protein synthesis were measured by infusing a flooding dose of labeled and unlabeled phenylalanine (Garlick et al., 1980). Briefly, 4 days after initiation of enteral feeding and 30 min prior to necropsy, piglets were given an i.v. bolus of ^{3}H-phenylalanine (37 MBq per kg body weight) in 150 mmol/L phenylalanine (10 mL/kg body weight). Thirty minutes after the initiation of the phenylalanine bolus, piglets were anesthetized, and samples of liver and mucosa from the remnant intestine were taken for analysis of rates of protein synthesis. Specific radioactivities of the tissue free phenylalanine and the protein-bound phenylalanine were determined as described previously (Brunton et al., 2012).

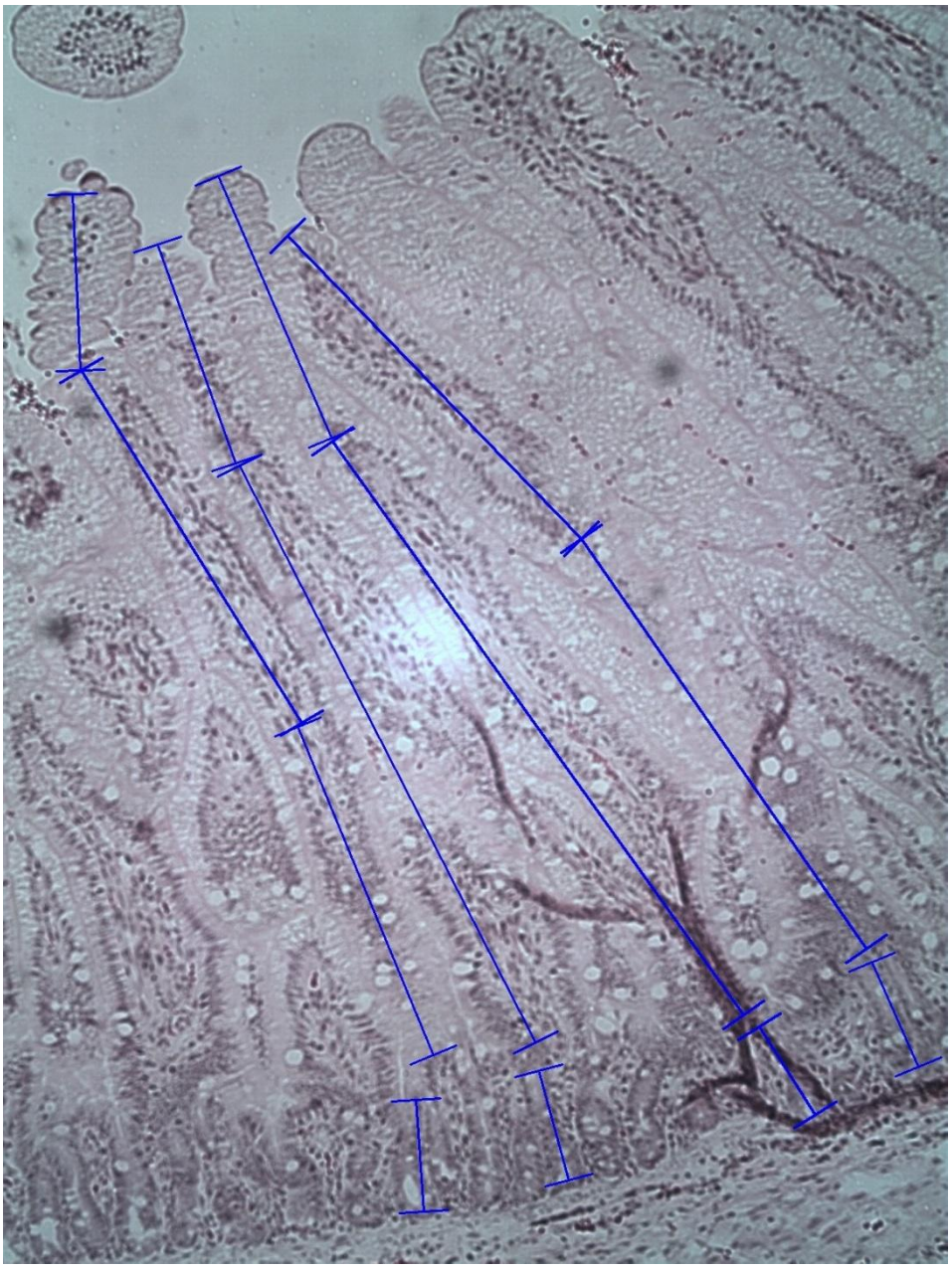

Figure 3.1 Representative image of remnant intestine villus height and crypt depth measurements.

3.3.6 Tissue and Plasma Amino Acid Determination

Free amino acid concentrations in plasma and tissue samples were analyzed using PITC derivatization (Bidlingmeyer et al., 1984) with norleucine as the internal standard. Phenylalanine fractions were collected and the radioactivity associated with these fractions was determined by scintillation counting.

3.3.7 Real-time RT-PCR

PepT1 mRNA was measured in small intestinal mucosa. RNA was extracted using the Qiagen RNEasy Mini kit (Qiagen Inc. Montréal, QC) according to the manufacturer's protocol. Concentration, purity and integrity were determined via the Agilent RNA Nano chip (Agilent Technologies, Mississauga, ON). cDNA was generated according to the protocol outlined in the QuantiTect (Qiagen Inc. Montréal, QC, Canada) reverse transcriptase manual. 800 ng of total RNA was used in the reverse transcription reaction. Roche Faststart DNA master Sybr Green I kit (Roche, Laval, QC) was used for the qPCR reaction. The sequences of the primers were as follows: PepT1 forward primer 5' d CTGGAGTTCTCCTATTCTCA 3', reverse primer 5' d AACAGCCACGGTCAACAG 3', β-actin was used as an internal control with the following sequences: forward primer 5' d CCCAGCACGATGAAGA 3', reverse primer 5' d CGATCCACACGGAGTC 3'. The accession numbers for the template sequences were AY180903.1 for PepT1 (Klang *et al.* 2005) and AY55069 for beta-actin. The qPCR machine (Eppendorf Mastercycler, Eppendorf Mississauga, ON) was set to the following conditions: 10 min at 95°C, 40 cycles of 15 sec at 95°C and 15 at 58°C, and 63°C incubation for 15 sec. Reaction efficiency for PepT1 was 0.87 ± 0.04 and β-actin reaction efficiency was 0.89 ± 0.04.

Each sample was run in duplicate and analyzed using 2^{-ddCt} method (Livak and Schmittgen, 2001).

3.3.8 PepT1 Protein Analysis

3.3.8.1 Brush Border Membrane Vesicle (BBMV) Preparation

Brush border membrane vesicles were isolated from mucosal scrapings. Briefly, tissues were homogenized in 100 mM mannitol, 2 mM HEPES/Tris pH 7.1 and 0.1 mM PMSF and 2 mL of the homogenate was removed for protein and marker enzyme analysis. The remaining sample was centrifuged at 500 g for 12 min. Then 1 M $MgCl_2$ was added to the supernatant to a final concentration of 10 mM, this solution was incubated on ice for 20 minutes prior to centrifugation at 3,000 g for 15 min. The supernatant was collected and centrifuged at 30,000 g for 30 min. The pellet was then resuspended in 20 ml of 100 mM mannitol, 2 mM HEPES/Tris pH 7.4 and 1 mM $MgSO_4$ and centrifuged at 30,000 g for 30 min to isolate BBMV's. The final pellet was resuspended in 300 mM mannitol, 20 mM HEPES/Tris pH 7.4 and 0.1 mM $MgSO_4$ (400 µl/g of wet tissue) and stored at -80°C.

3.3.8.2 Western Blot

BBMV's were analyzed for protein content using the BCA protein assay (Pierce Chemicals, Rockford, IL). Equivalent amounts of protein (50 µg) were electrophoresed on 8% SDS-polyacrylamide gels. After transfer to nitrocellulose membrane, blots were stained with Ponceau Red (Sigma Aldrich, Oakville, ON) to assess the equivalency of protein loading. Blots were blocked in 3% milk-TBST (Tris-buffered saline and Tween 20 at 0.2% V/V) for 45 min at room temperature and incubated with primary antibodies overnight at 4 °C. Primary antibodies

used were PepT1 (rabbit polyclonal 1:600, gift provided by E.A. Wong, Department of Animal and Poultry Sciences, Virginia Tech, Blacksburg, VA), and β-Actin (1:600 Sigma Aldrich, Oakville, ON). Blots were visualized using the Immun-Star WesternC Kit (Bio Rad, Montreal, QC) and images obtained using aa Alpha Innotech Chemiimager Gel Documentation System. Band intensity was analyzed using AlphaVIEW SA (ProteinSimple, Toronto, ON) and PepT1 expression was assessed relative to β-actin for each sample.

3.3.8.3 Sucrase Enrichment of Brush Border Membrane Vesicles

Brush border membrane vesicles (BBMV's) were analysed for sucrase enrichment as per Dahlqvist (1968). In brief, 40 µg of protein from tissue homogenate or BBMV's were incubated with 0.056 M sucrose at 37 °C for 20 min in triplicate. One triplicate was heat inactivated via immersion in boiling water for 5 min prior to incubation (blank). After incubation an assay reagent consisting of o-Dianisidine, glucose oxidase and peroxidase (Sigma Aldrich, Oakville, ON) was added to each sample and incubated for an additional 20 min at 37 °C. The enzymatic reaction was stopped by boiling the samples for 5 min. The absorbance of the resulting solution was measured at 420 nm in a BioMate 3 spectrophotometer (Fisher Scientific, Pittsburgh, PA) and total glucose release was calculated through comparison to a standard curve after subtraction of the sample blank. Overall sucrase enrichment was calculated by the ratio of sucrose release per µg of total protein from BBMV suspension to that of the tissue homogenate.

3.3.9 Tissue and plasma glutathione

Plasma and intestinal tissue concentrations of reduced and total glutathione were measured using the Biovision Glutathione assay kit (Biovision, Milpitas, CA) according to their

protocol. In brief, 60 µl of plasma was deproteinized via the addition of 20 µl of perchloric acid. This mixture was centrifuged at > 10,000 g for 2 minutes at 4 ˚C and 10 µl of the supernatant was added to the microplate. Assay buffer was used to increase the sample volume to either 90 µl to measure reduced glutathione or 80 µl + 10 µl of reducing reagent to detect total glutathione. 10 µl of the o-phthalaldehyde probe was added to each well and incubated at room temperature for 40 min. Plates were read in a Powerwave XS microplate reader (Biotek, Winooski, VT) with Ex/Em=340/450. Concentration of GSSG was calculated by subtracting the concentration of GSH from the total glutathione concentration.

3.3.10 TNF-α and IFN-γ

Mucosal TNF-α and IFN-γ concentrations were determined via porcine ELISA kits (Pierce, Rockford, IL). The kits utilized anti-human antibodies that cross react with porcine cytokines. Tissue supernatants were prepared by homogenizing tissue in PBS containing Protease Inhibitor Cocktail III (Calbiochem, San Diego, CA) and 1 mM PMSF (Sigma Aldrich, Oakville, ON). Homogenates were then centrifuged at > 10,000 g for 5 minutes at 4 ˚C to allow for analysis of tissue supernatants according to the protocol provided by the supplier. Absorbance of the enzyme-substrate product was determined by subtracting the calculated value at 550 nm from that determined at 450 nm. Linear regression was used to calculate the final concentration of cytokine in the supernatant which was reported as concentration per gram of mucosa.

3.3.11 Statistics

All results were expressed as mean ± standard deviation for each group of animals. Data were analyzed by one-way ANOVA with Bonferroni's protected means separation test. Sample

size was N = 5 piglets per dietary treatment and differences were noted as significant if $p < 0.05$ (GraphPad Prism 4.0, La Jolla, CA).

3.4 Results

3.4.1 Morphologic measurements

Body weights did not differ amongst treatment groups (initial: 2.23 ± 0.30 kg; final control 3.27 ± 0.46 kg, AA 3.28 ± 0.26 kg, AQ 3.20 ± 0.49 kg, CG 2.85 ± 0.32 kg, AQ+CG 3.42 ± 0.38 kg). Also, no differences were found in weight gain per kilogram per day (determined for the period after initiation of EN), and the percentage increase in body weight was similar among all treatment groups (Table 3.2). No significant differences were determined in plasma amino acid concentrations amongst treatment groups (Table 3.3). At necropsy, total liver and kidney weights were also not different amongst treatment groups (Table 3.2). Protein synthesis rates in the liver and intestinal mucosa were also similar across dietary treatments (Table 3.2). Length of the remnant intestine increased in all groups after one week of enteral feeding (Table 3.2).

There was no effect of diet on the total weight in the remnant small intestine (Table 3.2), but the alanyl-glutamine and AQ+CG treatment resulted in lower mucosa weight in the proximal 50 cm of the remnant intestine when compared to the control diet (Figure 3.2).

3.4.2 Histology

Analysis of intestinal morphology was performed on sections taken distal to the site of anastomosis (Figure 3.3). Villus height was similar between CG and control treatments (CG: 906 ±119 µm, control: 801 ± 49 µm); however villus height in the CG group was significantly

Table 3.2: Comparison of morphological and metabolic changes in piglets receiving different enteral diets.

	Control	AA	AQ	CG	AQ+CG
Remnant Intestinal Length (cm)	185 ± 21	196 ± 15	170 ± 36	174 ± 31	180 ± 25
Remnant Intestinal Weight (g/cm)	0.31 ± 0.07	0.23 ± 0.04	0.26 ± 0.08	0.31 ± 0.10	0.28 ± 0.09
Kidney Weight (g/kg)	3.67 ± 0.41	3.54 ± 0.72	4.40 ± 0.89	4.37 ± 0.75	3.70 ± 0.38
Liver Weight (g/kg)	37.36 ± 3.89	38.85 ± 3.71	40.14 ± 7.00	41.77 ± 7.92	38.26 ± 2.54
Mucosal Protein Synthesis (%/day)	78 ± 27	90 ± 13	85 ± 23	98 ± 14	83 ± 14
Liver Protein Synthesis (%/day)	114 ± 38	71 ± 19	75 ± 30	88 ± 35	80 ± 14

N = 5 piglets per group, values are mean ± SD.

Table 3.3: Plasma Amino Acid Concentrations (μmol/L)

Amino Acid	Control	AA	AQ	CG	AQ+CG
Alanine	455 ± 100	543 ± 57	515 ± 57	572 ± 54	484 ± 122
Arginine	126 ± 34	118 ± 32	134 ± 40	94 ± 24	137 ± 38
Aspartate	25 ± 12	23 ± 11	25 ± 8	32 ± 19	18 ± 7
Cysteine	260 ± 35	229 ± 52	285 ± 62	226 ± 59	229 ± 53
Glutamate	161 ± 62	192 ± 60	201 ± 51	217 ± 75	150 ± 55
Glutamine	230 ± 40	205 ± 63	224 ± 31	245 ± 70	233 ± 22
Glycine	920 ± 302	927 ± 245	985 ± 213	961 ± 228	970 ± 496
Isoleucine	148 ± 21	171 ± 12	164 ± 37	167 ± 20	150 ± 45
Leucine	286 ± 47	280 ± 88	262 ± 94	273 ± 61	284 ± 50
Lysine	370 ± 71	330 ± 185	392 ± 168	410 ± 196	365 ± 98
OH-Proline	64 ± 11	66 ± 25	48 ± 18	67 ± 22	58 ± 7
Phenylalanine	181 ± 44	182 ± 27	190 ± 32	175 ± 8	182 ± 57
Proline	510 ± 80	500 ± 98	528 ± 110	549 ± 87	513 ± 83
Serine	480 ± 87	476 ± 34	450 ± 53	509 ± 98	443 ± 65
Taurine	143 ± 20	154 ± 49	137 ± 18	161 ± 21	132 ± 50
Threonine	245 ± 48	221 ± 6	227 ±24	319 ± 95	230 ± 61
Valine	267 ± 39	315 ± 51	248 ± 66	257 ± 49	269 ± 69

N = 5 piglets per group, values are mean ± SD.

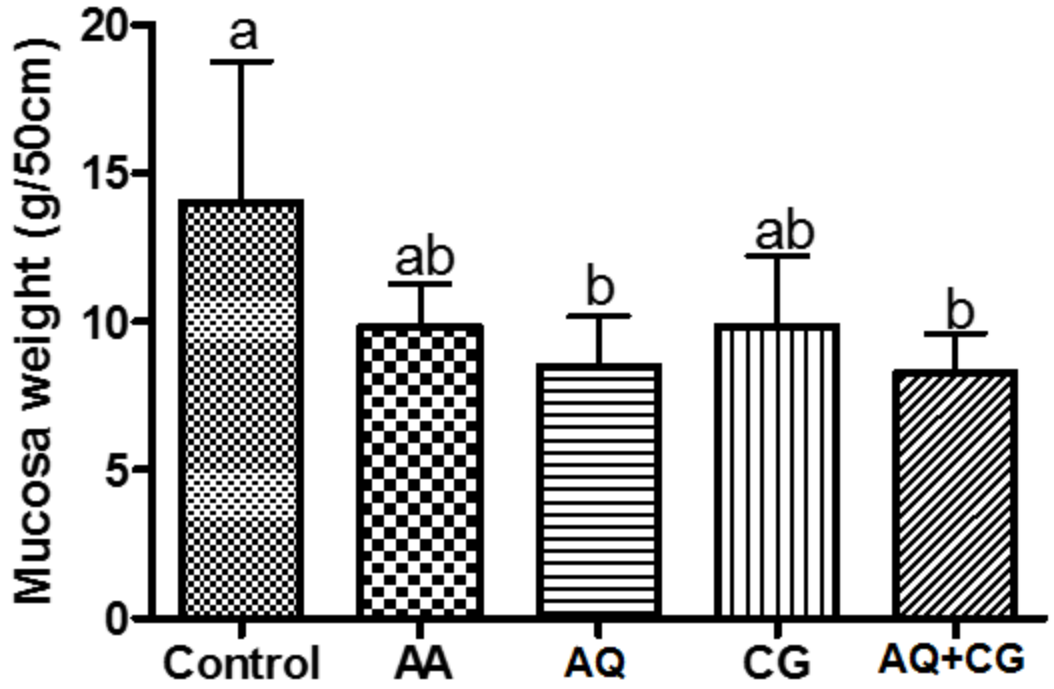

Figure 3.2: Weight of mucosa from the proximal 50 cm of the remnant intestine in piglets fed diets containing either all free amino acids (Control), or one of alanyl-alanine (AA), alanyl-glutamine (AQ), cysteinyl-glycine (CG) or both AQ and CG (AQ+CG). N = 5 piglets per group, values are mean ± SD. Data were analyzed by 1-way ANOVA with Bonferroni's protected means separation test for post hoc analysis. Bars with differing letters are significantly different $p < 0.05$.

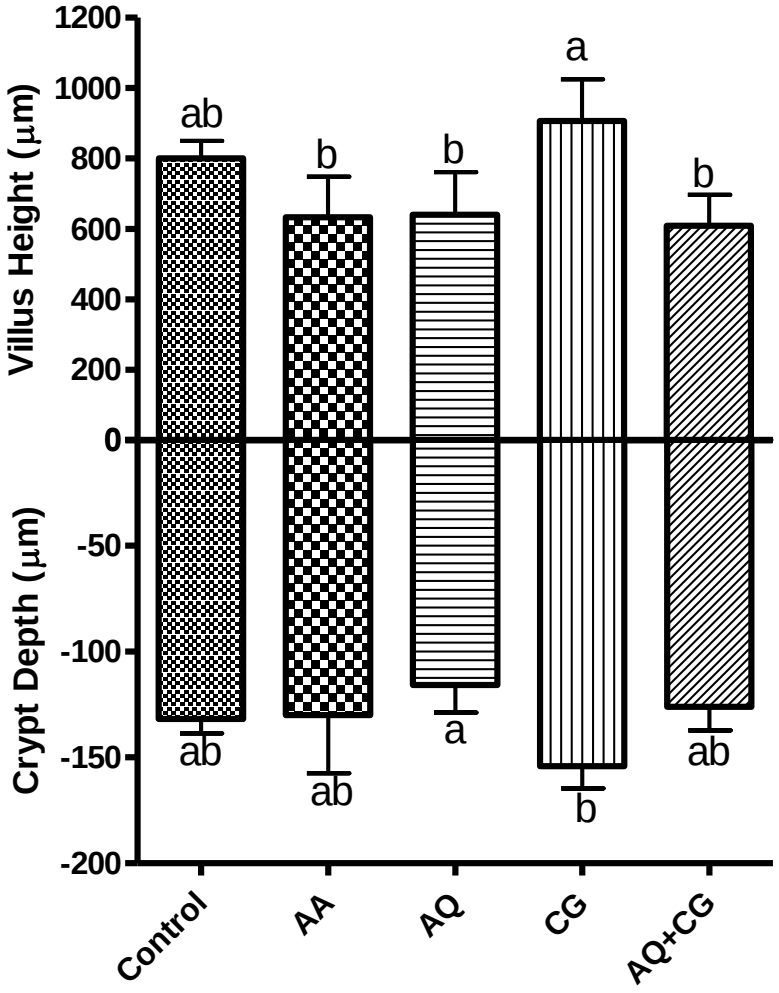

Figure 3.3: Villus height and crypt depth distal to the site of anastamosis in piglets fed diets containing either all free amino acids (Control), or one of alanyl-alanine (AA), alanyl-glutamine (AQ), cysteinyl-glycine (CG) or both AQ and CG (AQ+CG). N = 5 piglets per group, values are mean ± SD. Data were analyzed by 1-way ANOVA with Bonferroni's protected means separation test. Bars with differing letters are significantly different $p < 0.05$.

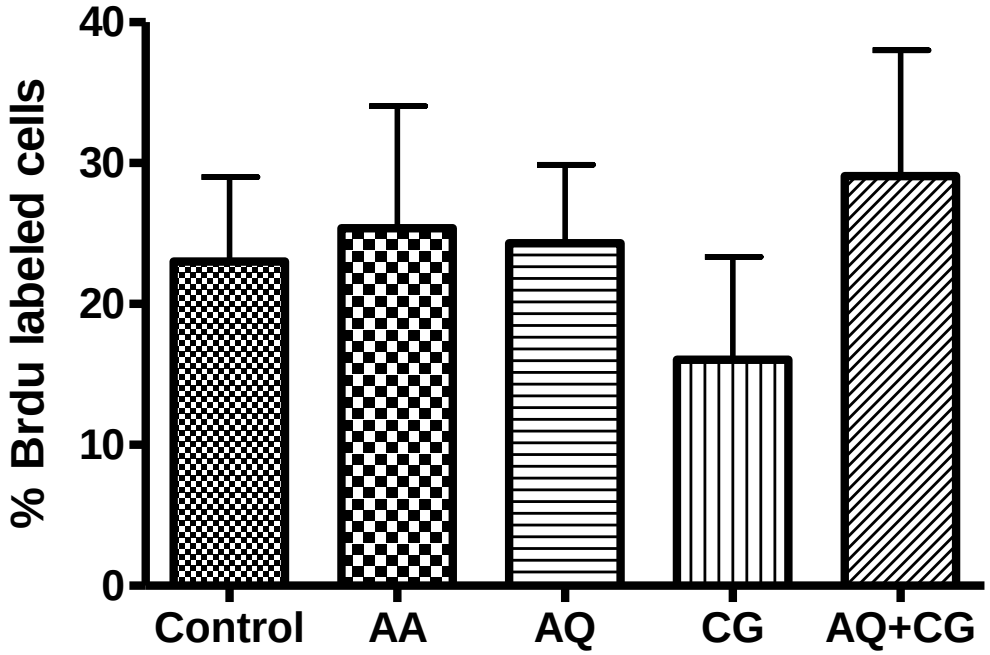

Figure 3.4: BrdU incorperation into the intestinal crypts of piglets fed diets containing either all free amino acids (Control), or one of alanyl-alanine (AA), alanyl-glutamine (AQ), cysteinyl-glycine (CG) or both AQ and CG (AQ+CG). Data are presented as percent of total cells labelled with BrdU. N = 5 piglets per group, values are mean ± SD.

higher than any other dipeptide group ($p < 0.05$) (Figure 3.2). Enteral CG also resulted in significantly greater crypt depth when compared to AQ (CG 154 ± 11 µm vs AQ 116 ± 13 µm) ($p < 0.05$) (Figure 3.3). Provision of enteral dipeptides did not alter cellular proliferation, as determined by BrdU incorporation (Figure 3.4).

3.4.3 GSH, TNF-α and IFN-γ

Total and reduced glutathione was quantified in both plasma and mucosal tissue (Figure 3.5); no significant differences amongst treatments were detected. The inclusion of AQ or CG, or both, in the enteral diets significantly reduced the concentration of IFN-γ to less than 40% of control ($p < 0.05$) (Figure 3.6). The inclusion of any of the dipeptides in the diets resulted in a dramatic reduction in TNF-α, to less than 27% of control ($p < 0.01$) (Figure 3.7)

3.4.4 PepT1 mRNA/Protein Expression

Samples of mucosa taken from the remnant intestine were used to determine PepT1 mRNA and protein expression. No significant difference was found in PepT1 mRNA (Figure 3.8) or protein expression (Figure 3.10) between any of the dietary regimens.

3.5 Discussion

In a previous study (Dodge et al., 2012), it was demonstrated that early provision of an elemental enteral diet in tandem with parenteral nutrition resulted in massive adaptive responses in a piglet model of short bowel syndrome. We used this model to investigate potential trophic effects of dipeptides, some of which were composed of metabolically important amino acids. The most intriguing outcome was the substantial influence of dipeptides on the mucosal concentration of pro-inflammatory cytokines. Surprisingly, in contrast to this observation, the

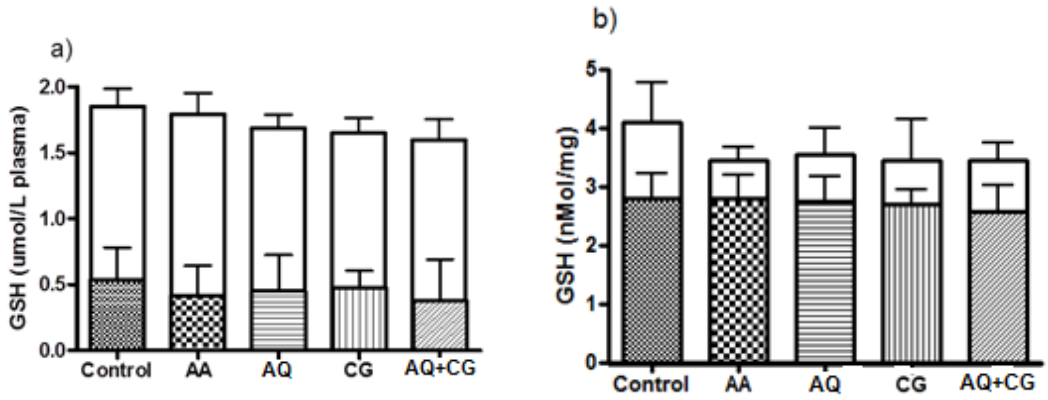

Figure 3.5: Total (white bar) and reduced (patterned bar) glutathione concentrations in plasma (a) and mucosa (b) in piglets fed diets containing either all free amino acids (Control), or one of alanyl-alanine (AA), alanyl-glutamine (AQ), cysteinyl-glycine (CG) or both AQ and CG (AQ+CG). N = 5 piglets per group, values are mean ± SD. Data were analyzed by 1-way ANOVA.

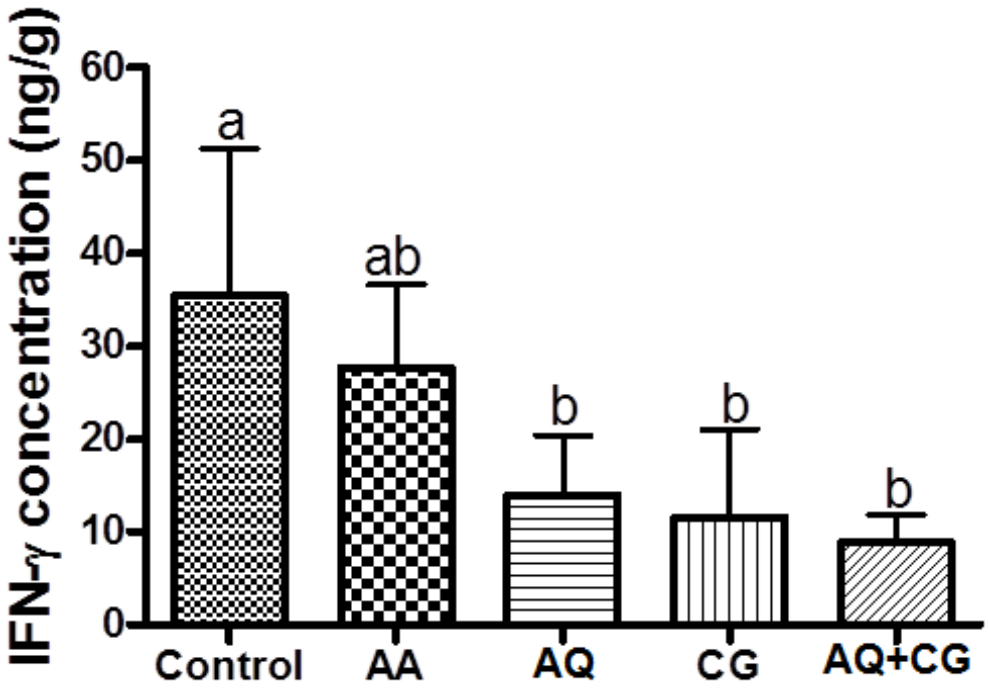

Figure 3.6: Concentration of IFN-γ in intestinal mucosa of piglets fed diets containing either all free amino acids (Control), or one of alanyl-alanine (AA), alanyl-glutamine (AQ), cysteinyl-glycine (CG) or both AQ and CG (AQ+CG). N = 5 per group. Values are mean ± SD. Data were analyzed by 1-way ANOVA with Bonferroni's protected means separation test for post-hoc analysis. Bars with differing letters are significantly different $p < 0.05$.

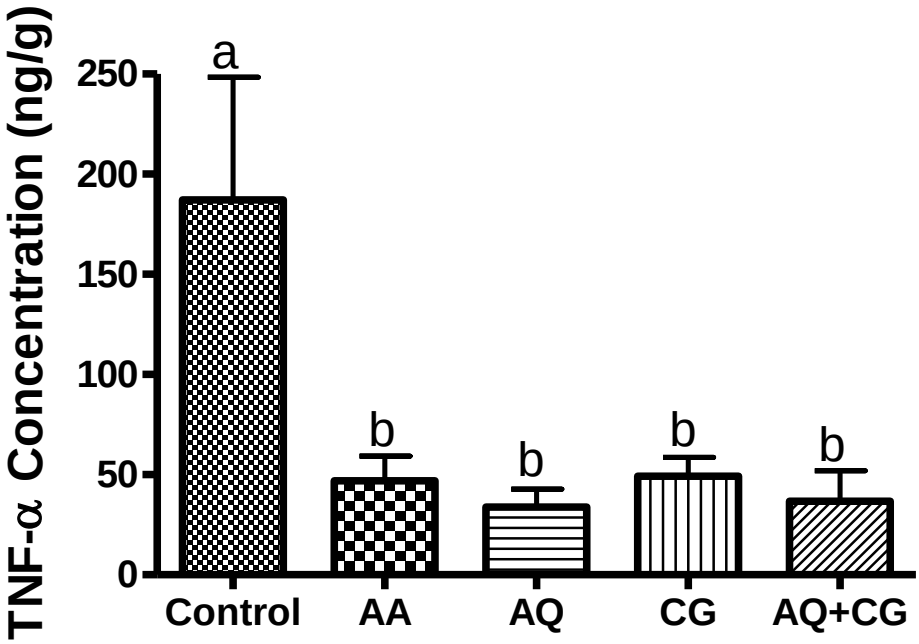

Figure 3.7: Concentration of TNF-α in intestinal mucosa of piglets fed diets containing either all free amino acids (Control), or one of alanyl-alanine (AA), alanyl-glutamine (AQ), cysteinyl-glycine (CG) or both AQ and CG (AQ+CG). N = 5 per group. Values are mean ± SD. Data were analyzed by 1-way ANOVA Bonferroni's protected means separation test for post-hoc analysis. Bars with differing letters are significantly different $p < 0.05$.

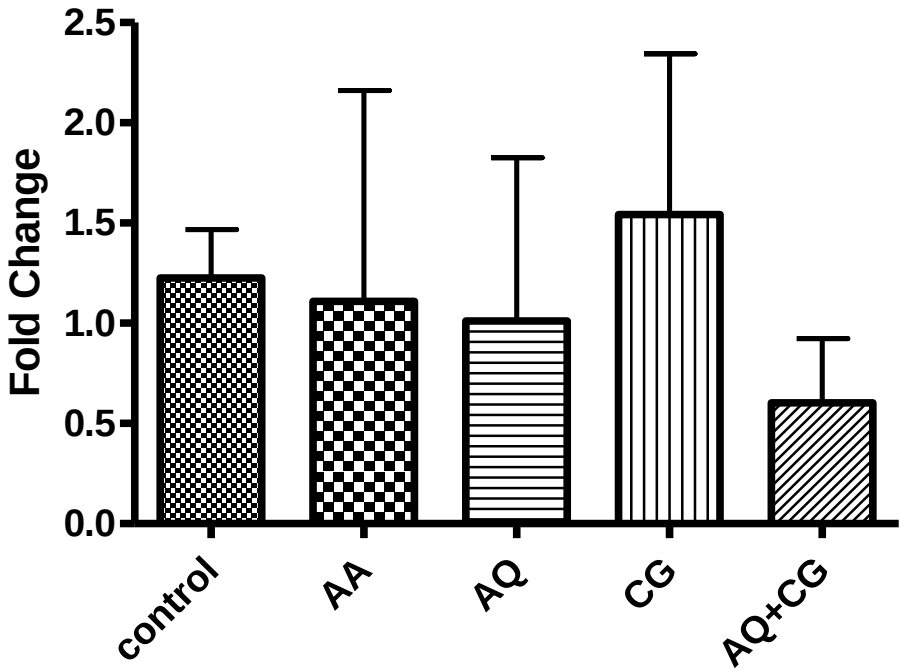

Figure 3.8: Fold change of PepT1 mRNA in intestinal mucosa of piglets fed diets containing either all free amino (Control), or one of alanyl-alanine (AA), alanyl-glutamine (AQ), cysteinyl-glycine (CG) or both AQ and CG (AQ+CG). N = 5 per group. Values are mean ± SD.

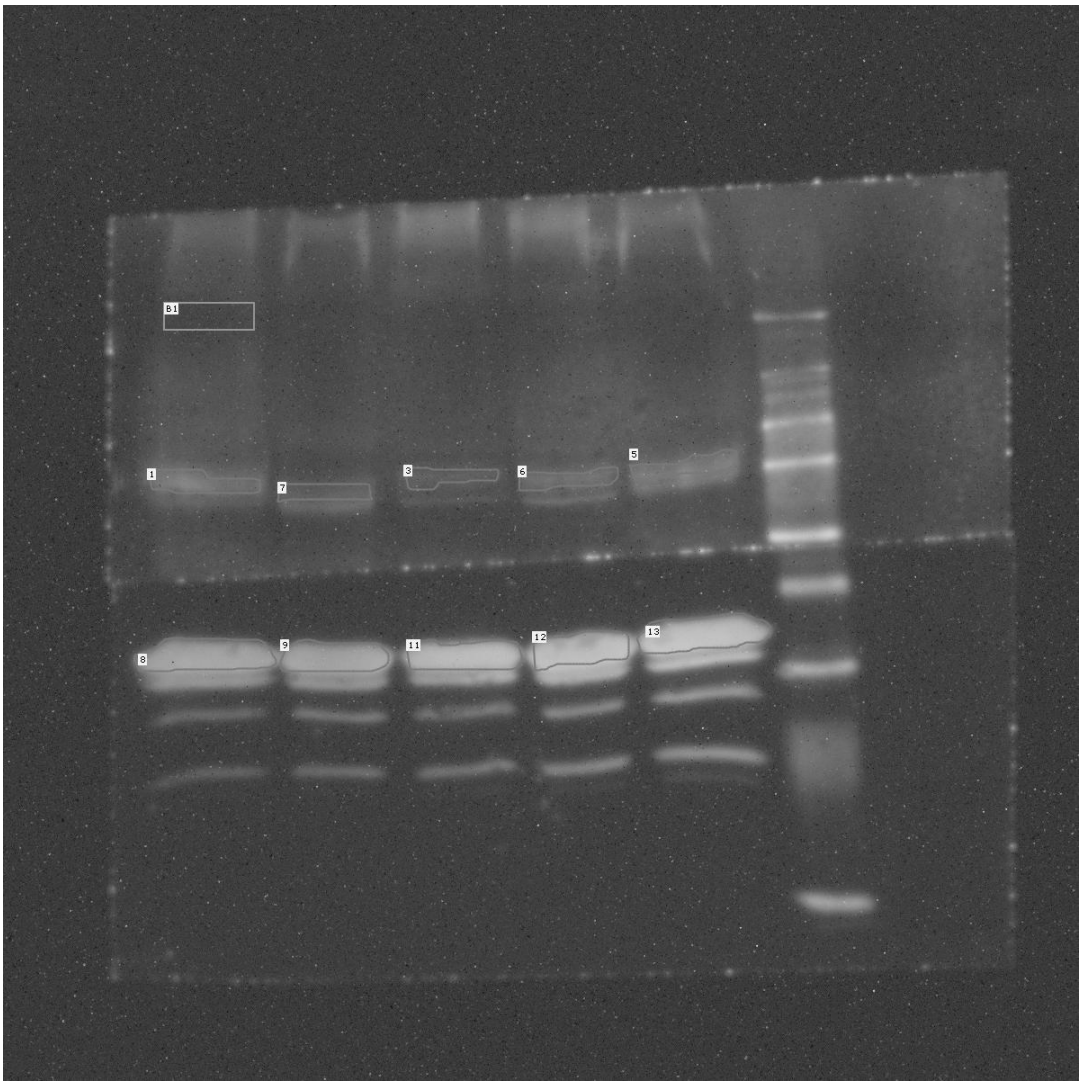

Figure 3.9: Representative western blot of PepT (top) and β-actin (bottom). Circled bands were selected for analysis on the basis of anticipated size. Lanes are as follows: AA, AQ+CG, CG, AQ and control.

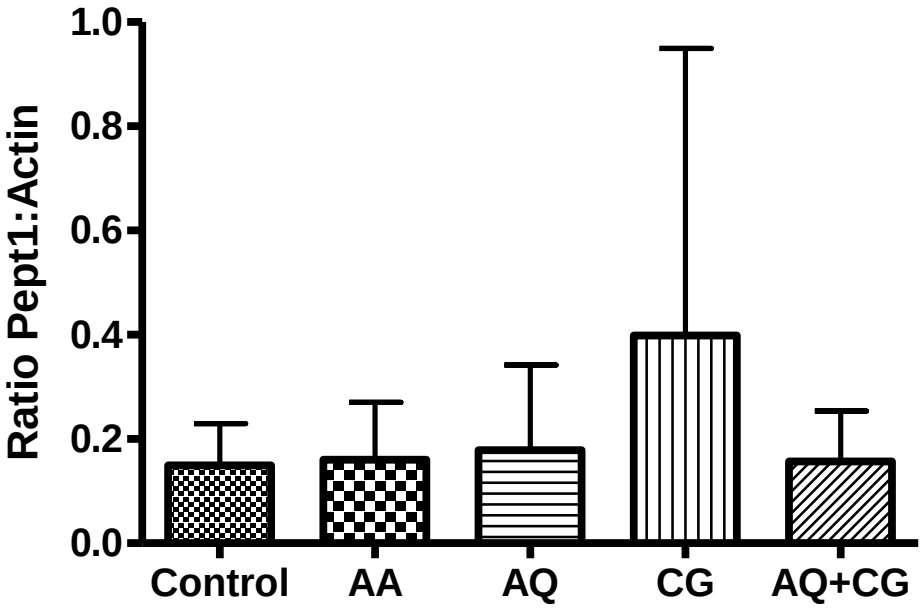

Figure 3.10: Ratio of PepT1 protein to β-actin in intestinal mucosa of piglets fed diets containing either all free amino acids (Control), or one of alanyl-alanine (AA), alanyl-glutamine (AQ), cysteinyl-glycine (CG) or both AQ and CG (AQ+CG). N = 5 per group. Values are mean ± SD.

data also suggest that inclusion of alanyl-glutamine in the enteral diet may actually be detrimental to mucosal growth in this surgical model.

Differences in the cytokine concentrations in mucosa isolated from piglets demonstrate that the form of the dietary amino acids can alter the inflammatory state of the small intestine. Small intestinal bacterial overgrowth is a common and potentially serious complication of short bowel syndrome in infants that dramatically increases the risk of systemic infection (Cole et al., 2010). In a study of 10 infants with SBS, the concentration of circulating pro-inflammatory cytokines was inversely correlated with enteral intake; however, the form of the enteral diets was not described (Cole et al., 2010). In our study, mucosal IFN-γ concentration was lower than control in the treatment groups that received CG, AQ or both, but not in the group fed AA. Thus, there is an underlying mechanism at work that responds to specific dipeptides. The impact of alanyl-glutamine on intestinal health has been studied *in vitro* using cell culture (Alteheld et al., 2005), *in vivo* via PN infusion into piglets (Burrin et al., 1994) and in a number of human trials that have employed both enteral and parenteral provision of this dipeptide (Eroglu, 2009, Luo et al., 2008, Lima et al., 2007). Parenteral provision of AQ has been shown to increase antioxidant capacity and plasma glutamine concentrations, while intraperitoneal injection of AQ reduced the number of IFN-γ producing cells in a mouse model of DSS-induced colitis (Chu et al., 2012). When provided as the free amino acid, enteral glutamine supressed pro-inflammatory cytokine production during an *E. coli* challenge in piglets (Ewaschuk et al., 2011); however the beneficial effect of enteral glutamine-containing dipeptides has yet to be established (Luo et al., 2008, Eroglu, 2009, Ligthart-Melis et al., 2009). We have demonstrated that while enteral AQ does reduce pro-inflammatory cytokines, it has no other significant benefits over provision of free amino acids.

Mucosal TNF-α was also dramatically lower in the dipeptide-treated piglets compared to the control group. Unlike IFN-γ, however, the presence of any of the test dipeptides resulted in a reduction of TNF-α. Bacterial peptides, substrates for PepT1, can induce a pro-inflammatory cytokine response in monocytes through NFκβ signalling (Pan et al., 2010). l-Ala-γ-d-Glu-meso-DAP (Dalmasso et al., 2010) and formyl-methionyl-leucyl-phenylalanine (Shi et al., 2006b, Carlson et al., 2007) are examples of bacterially derived pro-inflammatory substrates for PepT1. NF-κβ is a ubiquitous transcription factor and is highly involved in regulation of the immune system and its activation requires the phosphorylation and subsequent degradation of its inhibitor, Iκβ (Baeuerle and Henkel, 1994). Once active, NF-κβ can act on the promoter region for pro-inflammatory cytokines such as TNF-α (Baeuerle and Henkel, 1994) and IFN-γ (Sica et al., 1997). TNF-α is also capable of stimulating activation of NF-κβ by activating Iκβ kinase, the enzyme responsible for marking Iκβ for degradation. This can result in a positive feedback loop and the propagation of the inflammatory response (Grell et al., 1995). Interestingly, both thiols such as n-acetyl-cysteine, and glutamine are capable of blocking NFκβ activation (Singleton et al., 2005,). The mechanism by which thiols prevent NFκβ activation is believed to be the prevention of Iκβ degradation, either through the improper folding of required kinases or other inhibition of Iκβ phosphorylation (Staal et al., 1990). For TNF-α, it is possible that the dipeptide effect of competitive inhibition for fMLP transport reduced NFκβ activation by the bacterial peptide, subsequently resulting in reduced transcription of the cytokine. Conversely for IFN-γ, the lower concentration of IFN-γ found in AQ, CG and AQ + CG animals may be due to the presence of thiols, in the form of CG, or glutamine, in the form of AQ. These compounds could have disrupted the phosphorylation of Iκβ, with the similar result of preventing the activation of NFκβ and IFN-γ transcription.

In Caco-2 cells, supplementation of the media with IFN-γ resulted in an increased mRNA expression of PepT1 (Foster et al., 2009, Vavricka et al., 2006). This was not reflected in our findings as PepT1 mRNA concentration was not altered despite differing concentrations of IFN-γ. Similarly, previous work demonstrated the capacity for substrate induced expression of PepT1 in Caco-2 cells and increased mRNA in a rodent model (Walker et al., 1998, Shiraga et al., 1999). We did not detect any differences in either PepT1 mRNA or protein among any of the dietary regimens. Whether this is due to a lack of stimulation of PepT1 mRNA transcription, a reduction in protein trafficking to the brush border, or a missed temporal window of enterocyte response is undetermined.

An interesting finding of this study was that both cysteinyl-glycine and free amino acids demonstrated structural advantages over alanyl-glutamine. It is possible that enteral glutamine is less accessible for use by the intestine as a dipeptide due to inefficient intracellular hydrolysis, and that alanyl-glutamine is exported intact into the circulatory system. A comparative study of enteral versus parenteral alanyl-glutamine in critically ill patients revealed that plasma glutamine was higher when the dipeptide was provided parenterally (Luo et al., 2008). Other studies determining organ specific removal of dipeptides from human plasma reported that the intestine was responsible for removing only 13% of alanyl-glutamine present in the plasma (Reviewed in Vazquez et al., 1993). When glycyl-leucine and glycyl-glycine were assayed, it was found that the intestine was responsible for contributing the lowest rate of plasma clearance compared to the other tissues studied. This indicates that the intestine does not significantly utilize circulating dipeptides compared to other tissues. Although enterocytes are able to utilize plasma glutamine, the intestine preferentially utilizes enteral glutamine compared with intravenously provided glutamine for arginine synthesis (Ligthart-Melis et al., 2009). If alanyl-glutamine is being

transported into the circulatory system intact, the glutamine would be less accessible for use by the intestine and therefore not the best form of glutamine for an injured gut.

We ascertained that supplementation with either free cysteine or cysteinyl-glycine did not affect the concentration of cysteine in either plasma or intestinal mucosa. This is similar to the findings of Shyntum *et al.* who determined that the plasma pool of cysteine was not modulated through dietary intake in a rat model of bowel resection (Shyntum et al., 2009). Mucosal and plasma glutathione concentrations were similarly unaffected by altering the enteral diets potentially due to the fact that no difference in amino acid availability was determined. Cysteine alone is part of the oxidative stress control system; however, it is also an essential part of glutathione (GSH), the primary regulator of oxidative stress (Jones, 2006). Nkabyo *et al.* demonstrated greater redox potential with sulphur amino acid (SAA) supplementation (218% of control diet SAA) in a model of bowel resection in rats (Nkabyo et al., 2006). Although this demonstrated that SAA supplementation results in an improved redox status, we have not demonstrated any additional advantages in redox status of this surgical model when cysteine was supplied as the dipeptide cysteinyl-glycine. It is possible that supplementing with additional CG rather than providing only the nutritional requirements of cysteine as a dipeptide could have led to greater redox potential in our model. Additionally, the bioavailability of this dipeptide to enterocytes, or lack thereof, is also a potential reason for the lack of response in our model.

In this study, enteral dipeptides had no impact on protein synthesis or intestinal length when compared to free amino acids. Piglets receiving CG had significantly greater villus height compared with either AQ or AQ+CG, and the CG treatment also resulted in greater crypt depth when compared to piglets receiving AQ. However, there was no detectable difference in cellular proliferation at necropsy. This discrepancy between the morphological data and cellular growth

data could be due to the time at which the samples were taken for analysis. In this model, samples were removed approximately one week after surgery. Previous work in our lab (Dodge et al., 2012) using an identical surgical model demonstrated that within 24 h of initiating enteral feeding there is a period of rapid cellular proliferation and a high rate of protein synthesis, that was not detectable one week later. This study determined potential adaptive benefits of enteral dipeptides and therefore necessitated extended enteral feeding before necropsy; therefore it is likely that the period of rapid cellular proliferation occurred prior to sample removal.

An important aspect to consider in this study is the bioavailability of these dipeptides. Stability and clearance of plasma cysteinyl-glycine have not been as clearly delineated as alanyl-glutamine. However, as CG is a product of GSH degradation, numerous peptidases such as leucyl amino peptidase and alanyl peptidase have been shown to hydrolyse CG (Cappiello et al., 2004). Certain characteristics of dipeptides may predict their affinity for transport via PepT1. A study by Vig *et al.* detailed the effect of peptide size, hydrophobicity, composition and charge on dipeptide transport (Vig et al., 2006). Although cysteine containing dipeptides were not used in that study, the results can be used to make inferences regarding the bioavailability of cysteinyl-glycine. All X-glycine dipeptides were transported via PepT1 and neutral dipeptides resulted in greater transport than charged peptides thereby suggesting that CG is a viable substrate for PepT1.

The objective of this study was to determine the impact of enteral dipeptides on indices of intestinal adaptation in a piglet model of short bowel syndrome. Enteral dipeptides induced a marked reduction in pro-inflammatory cytokines when compared to free amino acids, while there was no effect on either PepT1 mRNA or protein expression. These results demonstrated that while there was no explicit morphological benefit of enteral dipeptides over their constituent free

amino acids, there is the potential for the amelioration of intestinal inflammation by reducing pro-inflammatory cytokines.

3.6 References

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<u>Chapter 4:</u> Cysteinyl-glycine reduces mucosal pro-inflammatory cytokine response to fMLP in a piglet model of intestinal atrophy.

The work presented in this chapter was funded in part by a grant from the Canadian Institutes of Health Research. This work was presented at Experimental Biology 2013 in Boston, MA, U.S.A and will be submitted for publication in the Journal of Nutrition. The co-authors of this work are Matthew G. Nosworthy and Janet A. Brunton. MGN and JAB were responsible for designing the study, MGN carried out the animal work in addition to the laboratory and statistical analyses.

Substrates for PepT1 are widely varied, including not only dietary peptides and peptidomimetic drugs but also bacterially produced peptides such a formyl-methionyl-leucyl-phenylalanine (fMLP). This peptide has been demonstrated to induce intestinal inflammation and is the primary chemotactic factor produced by *Escherichia coli.* As parenteral nutrition increases both the risk of bacterial infection and PepT1 expression in the distal intestine, this study determined whether parenterally-fed neonatal piglets were more susceptible to fMLP-induced inflammation than their sow-fed siblings. Additionally, these experiments investigated whether the dipeptide cysteinyl-glycine could attenuate the inflammation stimulated by the bacterial peptide.

Hypothesis: Parenteral feeding will increase the susceptibility of the intestine to fMLP-induced inflammation while inclusion of cysteinyl-glycine will ameliorate indices of inflammation in both dietary treatments to a greater extent than the free amino acids cysteine and glycine.

4.1 Abstract

PepT1 is an intestinal di/tripeptide transporter also capable of transporting bacterial peptides. We measured the mucosal inflammatory response to a pro-inflammatory peptide, formyl-methionyl-leucyl-phenylalanine (fMLP), when delivered with cysteinyl-glycine in a model of intestinal atrophy. Pigs (N = 6, 10 d) received parenteral nutrition (PN) for 4 d to induce atrophy of the small intestine; littermates (N = 6) remained with the sow. Subsequently, five 10 cm loops of the distal SI were isolated and perfused for 3 h with one of: 1) 5 mM cysteine + 5 mM glycine 2) 5 mM cysteinyl-glycine 3) 10 µM fMLP 4) 5 mM cys + gly + 10 µM fMLP 5) 5 mM cysteinyl-glycine + 10 µM fMLP. In both dietary treatments, intestinal segments exposed to fMLP had higher mucosal TNF-α and IFN-γ compared to unexposed loops (p <0.001). IFN-γ was higher in parenterally fed piglets compared to sow-fed pigs (p < 0.01). Co-perfusion of fMLP and cysteinyl-glycine resulted in a lower IFN-γ response in both sow-fed and parenterally fed piglets (p < 0.05), but neither group responded significantly to free cys + gly. Interestingly, free cys + gly reduced the TNF-α response in sow-fed pigs (p < 0.001), but not in the PN-fed group. Loops exposed to cysteinyl-glycine and fMLP had lower TNF-α concentrations compared to fMLP alone in both diet groups (p < 0.001) and in sow-fed piglets the response was significantly more abated than with cys + gly (p < 0.001). Interleukin-10, an anti-inflammatory cytokine, was lower in animals undergoing parenteral nutrition compared to sow-fed (p < 0.05), but did not differ between loop treatments. Morphologically, fMLP exposure did not alter villus height or crypt depth in sow-fed animals; in contrast, intestinal segments from PN-fed piglets exposed to fMLP had reduced villus height compared to unexposed loops (p < 0.05). Inclusion of cysteinyl-glycine was effective at attenuating a bacterial peptide-induced inflammatory response in the injured SI; this may be due to efficient dipeptide uptake in a situation of impaired free amino acid absorption, and/or competitive inhibition of fMLP uptake.

4.2 Introduction

The products of protein digestion that are absorbed by the small intestinal epithelium include free amino acids and small peptides of two to three residues in length. These di/tri-peptides are removed from the nutrient rich intestinal lumen by a H^+/peptide symporter, *pep*tide *t*ransporter 1 or PepT1 (Vig et al., 2006). This transporter is localized to the apical surface of the intestinal villi and has broad substrate specificity. It is important to note that potential substrates for PepT1 include almost all possible dietary di/tripeptides (Vig et al., 2006), some antibiotics (Terada et al., 1997, Terada et al., 1998, Zhang et al., 2009) and pro-inflammatory bacterial peptides (Shi et al., 2006c, Carlson et al., 2007, Buyse et al., 2001, Dalmasso et al., 2010).

The primary neutrophil chemotactic substance produced by *Escherichia coli* is formyl-methionyl-leucyl-phenylalanine (fMLP) (Marasco et al., 1984). This tripeptide is the most predominant N-formylated peptide present in the colonic lumen of humans (Marasco et al., 1984). PepT1-mediated transport of fMLP has been demonstrated in cell culture (Merlin et al., 1998, Foster and Zheng, 2007) and *in vivo* in rats (Buyse et al., 2002, Shi et al., 2006a). Uptake of fMLP was inhibited by the presence of known substrates of PepT1 in cell culture studies (Merlin et al., 1998, Foster and Zheng, 2007). Further, the presence of fMLP induced neutrophil migration across an epithelial monolayer, an activity which was abolished if fMLP uptake was inhibited. Uptake of fMLP has been investigated in rats using an intestinal perfusion approach (Buyse et al., 2002). Marked inflammatory response occurred in jejunal segments perfused with fMLP, an intestinal position known to have high expression of PepT1. This inflammation was accompanied by an increase in DNA binding by NFκβ, a transcription factor involved in the regulation of pro-inflammatory cytokines. The transcription factor NFκβ is a key regulator of the immune response (Baeuerle and Henkel, 1994). This transcription factor it capable of inducing

transcription of TNF-α, a potent pro-inflammatory cytokine involved in inflammatory disease. Similarly, NFκβ is capable of promoting the transcription of IFN-γ (Sica et al., 1997), another pro-inflammatory cytokine. Therefore the induction of NFκβ by fMLP may also increase the concentration of IFN-γ and TNF-α. Competitive inhibition of fMLP transport or direct regulation of PepT1 expression may have the potential to ameliorate intestinal inflammation in pathological situations where there is an abnormally high exposure of bacterial peptides to PepT1.

The necessity for parenteral nutrition (PN) support represents a pathological situation for the gut that is characterized by greater intestinal permeability with potential for intestinal atrophy (Buchman et al., 1995). Furthermore, PN modulates the immune response of the intestine leading to higher risk of infection (Omata et al., 2013, Heneghan et al., 2013). This risk is potentiated via the suppression of the bactericidal response of the small intestine (Omata et al., 2013) and a reduction in Paneth cell function leading to an inability to replenish lost enterocytes (Heneghan et al., 2013). A study measuring mRNA of amino acid transporters of parenterally and orally fed adult rats noted an increase in PepT1 mRNA in the distal small intestine (Howard et al., 2004). This combination of sustained PepT1 with bacterial overgrowth in the small intestine, or bacterial infection due to parenteral nutrition, may facilitate greater uptake of bacterial peptides leading to the development of intestinal inflammation.

We used a piglet model of PN in combination with a ligated loop model of intestinal perfusion to investigate changes in cytokine response and intestinal morphology after perfusion with fMLP alone or in combination with a competitor for PepT1, the dipeptide cysteinyl-glycine. The purpose of this study was to quantify the inflammatory response to fMLP in the distal small intestine of piglets with PN-induced intestinal changes, compared to healthy sow-fed littermates.

Further, we determined whether the mucosal response to fMLP was altered when presented to the small intestine with a dipeptide (cysteinyl-glycine), or the constituent free amino acids (L-cysteine and glycine). The selection of cysteinyl-glycine as the dipeptide for this study was based on the anti-inflammatory capacity of cysteine (Jones, 2006), as well as previous work having demonstrated that cysteinyl-glycine was able to reduce the mucosal concentration of pro-inflammatory cytokines in a piglet model of short bowel syndrome (Chapter 3)

4.3 Materials and Methods

4.3.1 Study design

All experimental procedures were approved by the Institutional Animal Care Committee in accordance with guidelines of the Canadian Council of Animal Care. Yucatan miniature piglets were randomized to either parenteral nutrition (PN, N = 6) or sow-feeding (N = 6) as littermate pairs. The littermates assigned to PN were removed from the sow at 10 d of age and underwent surgical insertion of jugular and femoral catheters, as previously described (Ch 3, pg 64). The catheters were implanted for blood sampling and delivery of the parenteral solution. PN was initiated immediately following surgery and continued until day four post-operatively as short term parenteral feeding (48 hrs) is capable of inducing intestinal atrophy (Niinikoski et al. 2004). The sow-fed group remained with the sow during this time. The complete parenteral diet provided 1.1 MJ of metabolizable energy$\cdot kg^{-1}\cdot d^{-1}$ with glucose (24.5 g$\cdot kg^{-1}\cdot d^{-1}$) and lipid (20% Intralipid, Pharmacia) each supplying 50% of non-protein energy and 15 g$\cdot kg^{-1}\cdot d^{-1}$ of protein, supplied as free amino acids. The amino acid composition was as follows (per gram of total L-amino acids): alanine, 107 mg; arginine, 67 mg; aspartate, 61 mg; cysteine, 14 mg; glutamate, 105 mg; glycine, 27 mg; histidine, 31 mg; isoleucine, 46 mg; leucine, 104 mg; lysine-HCl, 102

mg; methionine, 19 mg; phenylalanine, 55 mg; proline, 83 mg; serine, 56 mg; taurine, 5 mg; threonine, 41 mg; tryptophan, 21 mg; tyrosine, 8 mg; and valine, 53 mg (Dodge et al., 2012). Prior to feeding, vitamins (Multi-12K1 Pediatric, Sabex, St Boucherville, QC) trace minerals at 200% of NRC recommendations, (NRC, 1998), lipid, and iron dextran (Fe, 3.0 mg/kg; Vetoquinol Canada Inc, Saint-Hyacinthe, QC) were added to the diet.

4.3.2 In situ *perfusion (gut loop model)*

On d 4 of study (d 4 post-op for PN piglets), sow-fed and PN piglets were brought to the laboratory to undergo an *in situ* perfusion study. The piglets were pre-anesthetized with an IM injection of ketamine (20 mg/kg) plus acepromazine (0.5 mg/kg). Subsequently, the piglets were intubated and maintained under general anesthesia using 0.6-1.0% isoflurane (Abbott Laboratories Ltd, Montreal, QC) mixed with oxygen at a flow rate of 1.5 L/min. A laparotomy was performed to expose the small intestine. The sites for the five intestinal loops were located along the length of the distal small intestine (i.e., ileum). Closed loops of intestine consisted of 10 cm sections of intestine with inlet and outlet cannulas (inner diameter, 0.2 cm; outer diameter, 0.3 cm, Watson Marlow Pumps Group, Wilmington, MA) inserted through a small perforation at both ends of the 10 cm. A suture was placed around the tube and intestine, occluding flow of intestinal contents into that section. The loop was gently flushed of luminal contents using warmed (37°C) KRB (constituents in g/L: D-glucose 1.8, magnesium chloride (anhydrous) 0.0468, potassium chloride 0.34, sodium chloride 7.0, sodium phosphate dibasic (anhydrous) 0.1 and sodium phosphate monobasic (anhydrous) 0.18) buffer until the effluent ran clear. Loops were separated by 30 cm of intestine with the last loop being placed 50 cm from the ileocecal valve. Piglets were kept warm by a homeothermic blanket and the exposed intestines were kept

moistened with warmed saline and covered with gauze and plastic wrap. Heart rate, body temperature and blood oxygenation were monitored throughout the 180 min experiment, after which the loops were excised by cautery and flushed with cold 0.9% saline. A 2-cm segment of loop tissue was immersed in neutral buffered 10% formalin (Fisher Scientific, Pittsburgh, PA) for histologic analyses. The remaining segment was placed on ice, cut longitudinally and scraped with a microscope slide to remove the mucosa which was then frozen in liquid nitrogen and stored at -80°C for further analysis.

4.3.3 Perfusates

Five different loop treatments (perfusates) were randomly assigned to intestinal position with treatments matched for location between littermates. The treatments contained: 1) 5 mM cysteine + glycine; 2) 5 mM cysteinyl-glycine; 3) 10 µM fMLP; 4) 5 mM cysteine +glycine + 10 µM fMLP; 5) 5 mM cysteinyl-glycine + 10 µM fMLP. The concentration of dipeptide was selected due to the work of Klang et al. (2005) while the concentration of fMLP was selected due to previously published work by Buyse et al. (2002). All perfusates were constructed in KRB. The fMLP treatment also included ^{3}H-fMLP and all loops contained ^{14}C-mannitol to assess intestinal permeability. The disappearance of ^{3}H-fMLP and ^{14}C-mannitol from the perfusates was determined by adding 100 µL of perfusate to 4 ml Scintiverse (Fisher Scientific, Pittsburgh, PA) for liquid scintillation counting. The specific radioactivity was calculated as the mean dpm/mmol of ^{3}H-fMLP or ^{14}C-mannitol present in the perfusate. Specific radioactivity was calculated at baseline (prior to perfusion) and in each of the perfusate samples taken over the course of the 3 h study. The specific radioactivity was then used to determine the total quantity of fMLP or mannitol remaining in the perfusates at the time points studied.

4.3.4 TNF-α, IFN-γ and IL-10

Mucosal TNF-α, IFN-γ and IL-10 concentrations were determined via porcine ELISA kits (Pierce, Rockford, IL). The kits utilized anti-human antibodies that cross react with porcine cytokines. Tissue supernatants were prepared by homogenizing tissue in PBS containing Protease Inhibitor Cocktail III (Calbiochem, Etobicoke, ON) and 1 mM PMSF (Sigma Aldrich, Oakville, ON). Homogenates were then centrifuged at > 10,000 *g* for 5 minutes at 4 ˚C to allow for analysis of tissue supernatants according to the protocol provided by the supplier. Absorbance of the enzyme-substrate product was determined by subtracting the calculated value at 550 nm from that determined at 450 nm. Linear regression was used to calculate the final concentration of cytokine in the supernatant which was reported as pg per gram of mucosa.

4.3.5 Myeloperoxidase (MPO) Assay

Intestinal tissue samples (50-100 mg) were homogenized on ice in 0.5% hexadecyltrimethylammonium bromide (Sigma Aldrich, Oakville, ON) in 50 mM KPO_4. Homogenates underwent three rapid freeze/thaw cycles (-80°C/37°C) and were then centrifuged at12,800 *g* for 15 minutes at 4°C. The supernatant containing MPO was assayed spectrophotometrically after the addition of 50 mM KPO_4 containing 0.53 mM O-dianisidine dihydrochloride and 0.15 mM hydrogen peroxide. Changes in absorbance were measured at 460 nm for 2 minutes with readings taken every 15 seconds (BU-530, Beckman Coulter, Mississauga, ON). MPO activity was reported as IU/g wet tissue where one IU was defined as the quantity of enzyme able to convert 1 µmol of hydrogen peroxide to water in 1 min at room temperature.

4.3.6 Histological analysis

4.3.6.1 Preparation of slides

After fixation in 10% buffered formalin (Fisher Scientific, Pittsburgh, PA), samples of intestine were dehydrated in ethanol, cleared in xylene, embedded in paraffin wax, and sliced into 5-µm sections.

4.3.6.2 Crypt Depth/Villus Height

Sections were stained with hematoxylin and eosin (Fisher Scientific, Pittsburgh, PA). Villus height and crypt depth were measured with a Zeiss Axiostar microscope (Carl Zeiss, Toronto, ON). Images were captured with an Infinity 1 camera and Infinity Analyze software (Lumenera Corporation, Nepean, ON). Ten measurements of villus height and crypt depth were performed per sample. All histological measurements were performed in a blinded manner by a single investigator (MGN).

4.3.7 Statistical Analysis

For all analyses a mixed model two-way ANOVA was used, with loop treatment as the repeated measure within pigs and diet as the second variable. Piglets receiving parenteral feeding were matched to sow-fed littermates of the same gender. Differences were determined to be significant if $p < 0.05$ (Graphpad Prism 5.0, La Jolla, CA)

4.4 Results

Throughout the perfusion studies all piglets remained stable, well-oxygenated and maintained a core body temperature between 37-39°C.

4.4.1 Mucosal Cytokines

Parenteral nutrition did not affect mucosal IFN-γ concentrations (Fig. 4.1), as the concentrations were similar to the sow-fed group when not exposed to fMLP. Perfusion of intestinal loops with fMLP alone significantly increased the concentration of IFN- γ. Co-perfusion of fMLP with CG resulted in significantly lower concentrations of IFN- γ in PN fed piglets; however this effect was not detected when fMLP was co-perfused with free cysteine and glycine. Similar to the IFN-γ response, there was no effect of parenteral feeding on mucosal TNF-α concentrations (Fig.4.2). Perfusion of fMLP resulted in a greater concentration of TNF-α compared to the control loops, regardless of the presence of either cysteine + glycine or cysteinyl-glycine. Treatment with fMLP and amino acids demonstrated that there was a significant effect of the form of amino acids. Compared to fMLP alone, the dipeptide resulted in lower TNF-α concentrations stimulated by fMLP in both sow-fed and parenterally-fed piglets. Sow-fed piglets had lower TNF-α responses to fMLP compared to PN animals when either free cysteine and glycine or the dipeptide were added to the perfusate. The mucosal concentration of Il-10 was not affected by any loop treatment; however, piglets receiving parenteral nutrition had lower concentrations of the anti-inflammatory cytokine than their sow-fed littermates (Fig. 4.3).

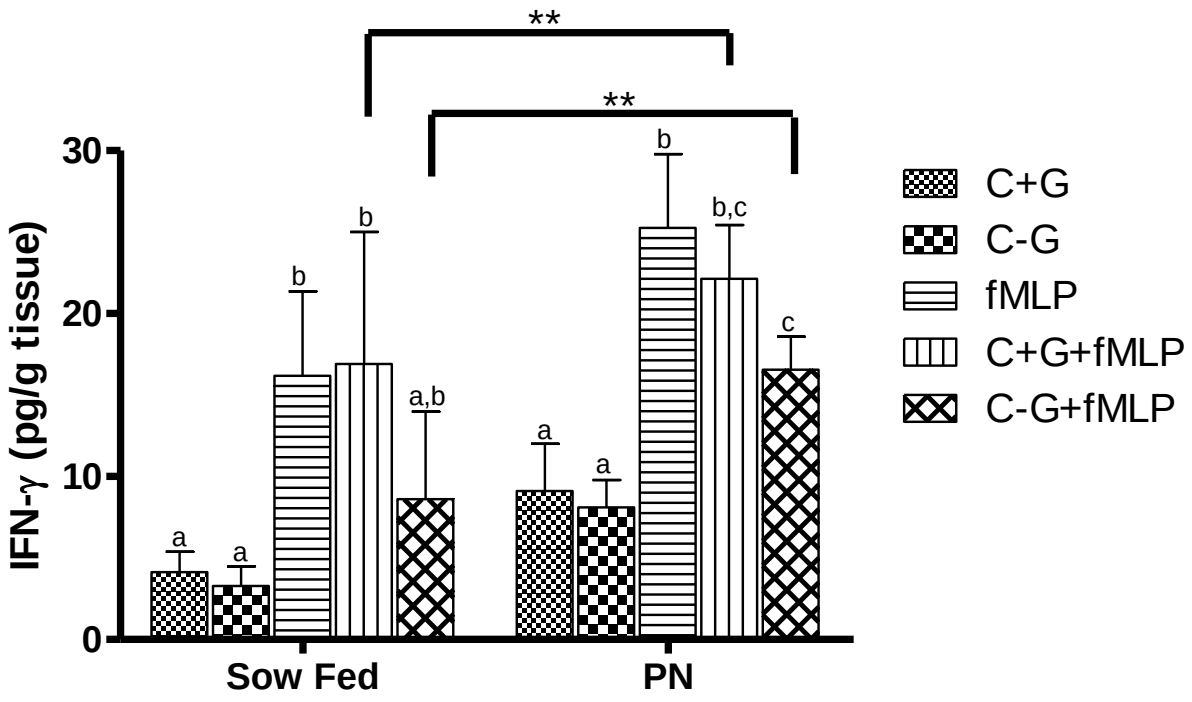

Figure 4.1: Mucosal IFN-γ concentration in sow-fed and PN-fed piglets sampled from ligated loops perfused with cysteine + glycine (C+G), cysteinyl-glycine (C-G), formyl-methionyl-leucyl-phenylalanine (fMLP), a combination of cysteine + glycine + fMLP (C+G+fMLP) or cysteinyl-glycine + fMLP (C-G+fMLP). Lines represent significant differences between diet treatments (** $p < 0.01$). Differing letters indicate differences amongst loop treatments within the diet treatment ($p < 0.05$). Data were analyzed via mixed model 2-way ANOVA with Bonferroni post-hoc analysis. N = 6 per group. Values are mean + SD.

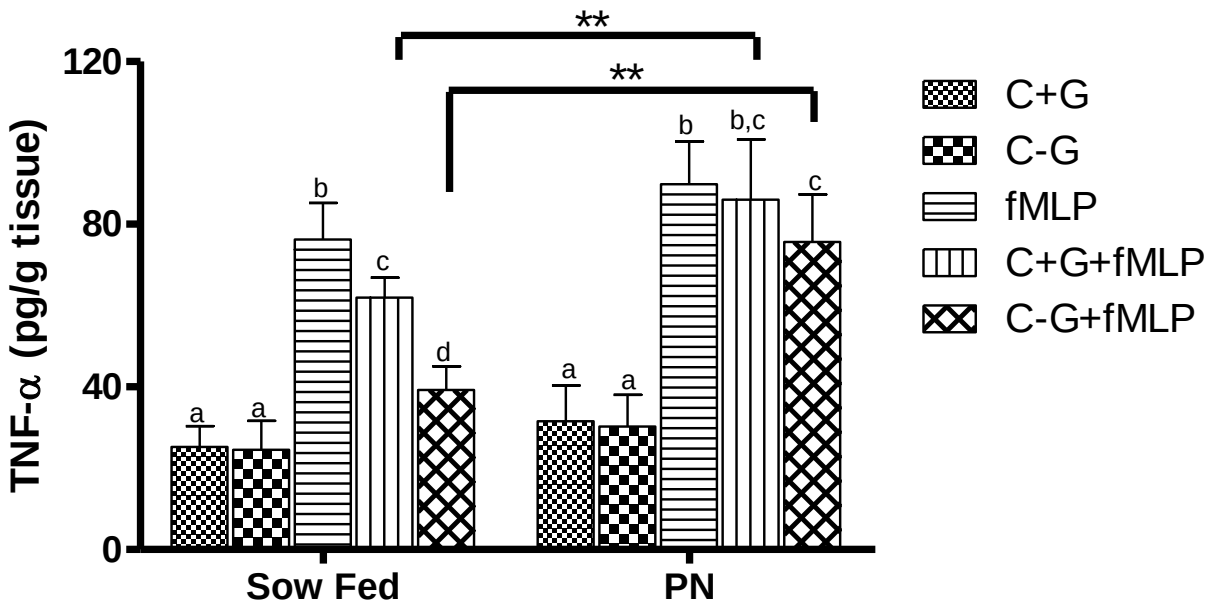

Figure 4.2: Mucosal TNF-α concentration in sow-fed and PN-fed piglets sampled from ligated loops perfused with cysteine + glycine (C+G), cysteinyl-glycine (C-G), formyl-methionyl-leucyl-phenylalanine (fMLP), a combination of cysteine + glycine + fMLP (C+G+fMLP) or cysteinyl-glycine + fMLP (C-G+fMLP). Lines represent significant differences between diet treatments (** $p < 0.01$). Differing letters indicate differences amongst loop treatments within the diet treatment ($p < 0.05$). Data were analyzed via mixed model 2-way ANOVA with Bonferroni post-hoc test. N = 6 per group. Values are mean + SD.

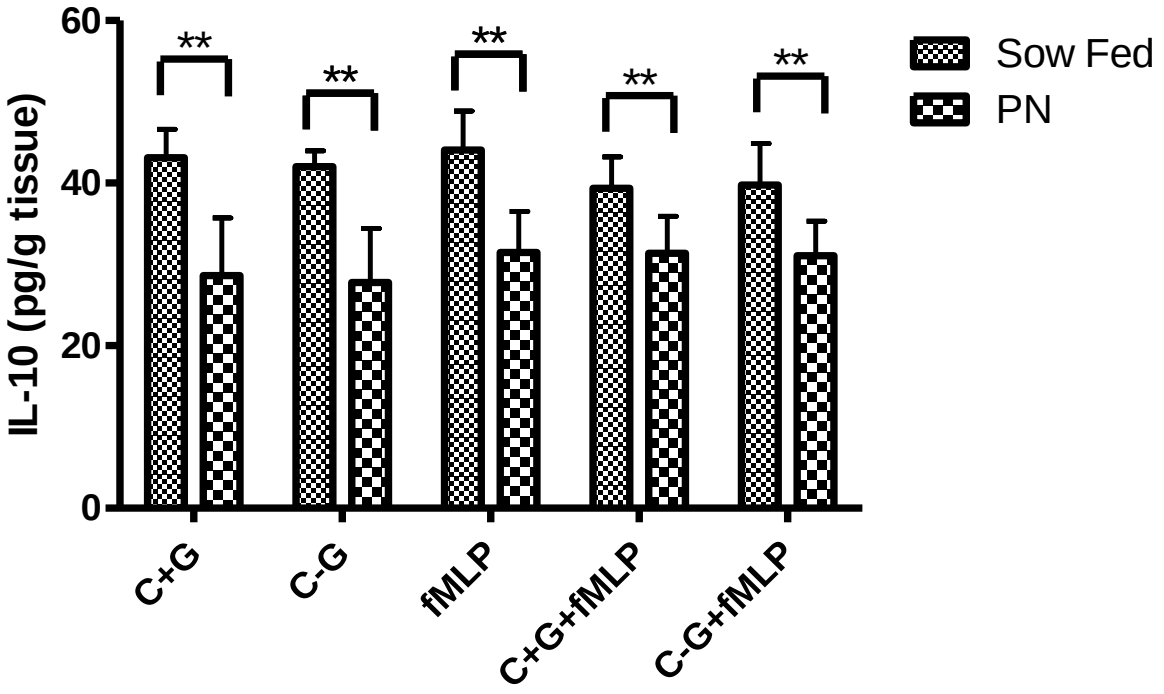

Figure 4.3: Mucosal IL-10 concentration in sow-fed and PN-fed piglets sampled from ligated loops perfused with cysteine + glycine (C+G), cysteinyl-glycine (C-G), formyl-methionyl-leucyl-phenylalanine (fMLP), a combination of cysteine + glycine + fMLP (C+G+fMLP) or cysteinyl-glycine + fMLP (C-G+fMLP). Lines represent significant differences between diet treatment (** $p < 0.01$). Data were analyzed via mixed model 2-way ANOVA with Bonferroni post-hoc test. N = 6 per group. Values are mean + SD.

4.4.2 Disappearance of ^{3}H-fMLP or ^{14}C-mannitol

Perfusates sampled throughout the 3-hour procedure were analyzed to determine disappearance of ^{3}H-fMLP (Fig. 4.4), as a marker for fMLP transport and ^{14}C-mannitol (Fig. 4.5), an indicator of paracellular transport. Scintillation counting of the perfusate samples revealed highly variable results for the transport of ^{3}H-fMLP in all loops, both in PN and sow-fed piglets, with no significant differences found either between dietary regimen or perfusate contents. Analysis of ^{14}C-mannitol disappearance found no change in mannitol concentration with any perfusate or in either sow-fed or PN fed piglets.

4.4.3 Myeloperoxidase activity

Myeloperoxidase activity was measured in the intestinal mucosa as an indicator of neutrophil migration. There was no effect of route of feeding on basal myeloperoxidase activity (Fig. 4.6). Perfusion of intestinal segments with fMLP generated greater MPO activity in both groups, regardless of dietary regimen. The inclusion of cysteine + glycine with fMLP had no impact on MPO activity, but co-perfusion of cysteinyl-glycine with fMLP resulted in lower MPO activity that was similar to that of the control samples in both sow-fed and PN piglets.

4.4.4 Intestinal morphology

There was no effect of route of feeding or loop treatment on crypt depth (Figure 4.7). In the sow-fed animals, villus height was unaffected by exposure to fMLP. In the PN group, however, villi were significantly shorter in the loops perfused with fMLP, and co-perfusion with either cysteine and glycine or cysteinyl-glycine were intermediate between the control and fMLP

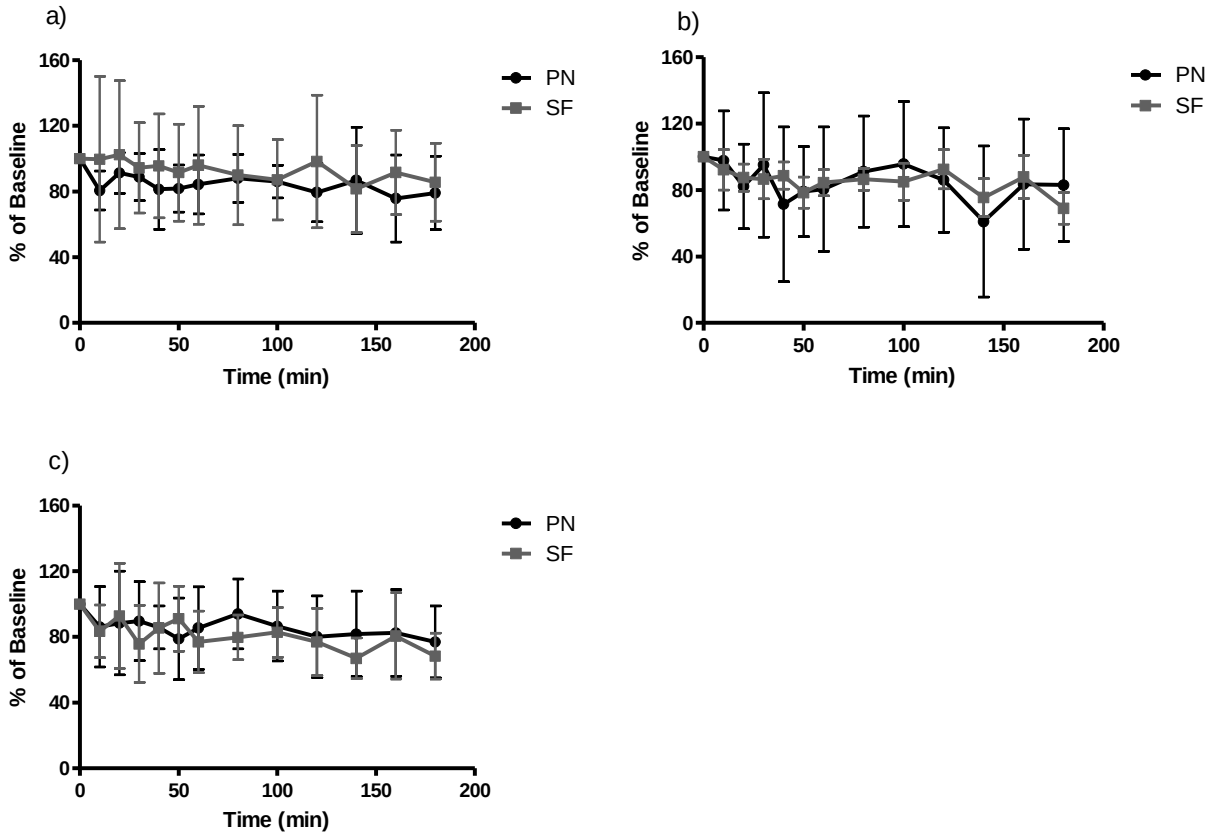

Figure 4.4: Disappearance of fMLP in in sow-fed and PN-fed piglets with intestinal loops perfused with either a) formyl-methionyl-leucyl-phenylalanine (fMLP), b) a combination of cysteine + glycine + fMLP (C+G+fMLP), or c) cysteinyl-glycine + fMLP (C-G+fMLP). Data were analyzed via mixed model 2-way ANOVA with Bonferroni post-hoc test. N = 6 per group. Mean ± SD

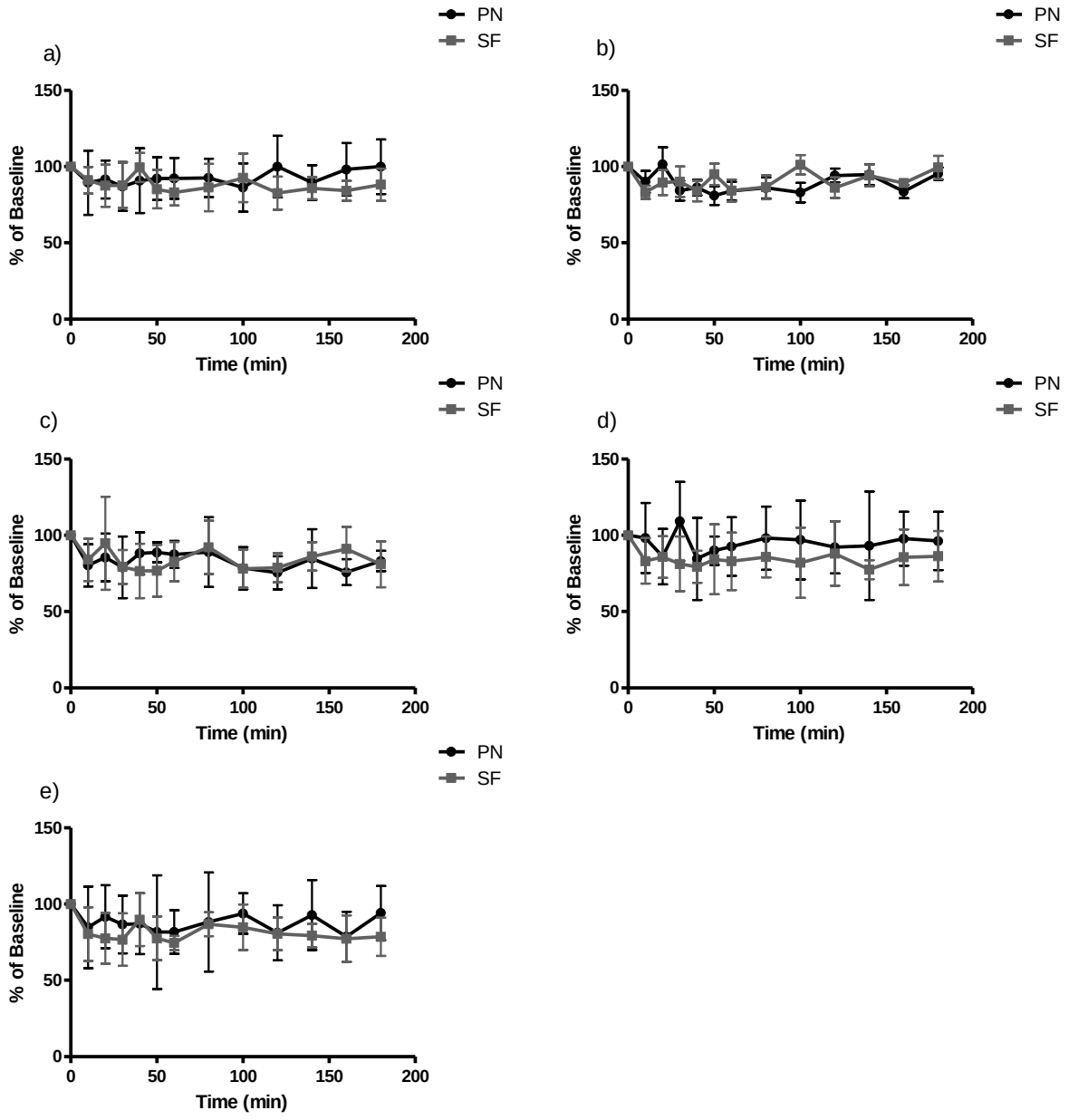

Figure 4.5: Disappearance of mannitol in in sow-fed and PN-fed piglets with intestinal loops perfused with either a) cysteine + glycine (C+G) b) cysteinyl-glycine (C-G) c)formyl-methionyl-leucyl-phenylalanine (fMLP) d) a combination of cysteine + glycine + fMLP (C+G+fMLP) e) cysteinyl- glycine + fMLP (C-G+fMLP). Data were analyzed via mixed model 2-way ANOVA with Bonferroni post-hoc. N = 6 per group. Mean ± SD

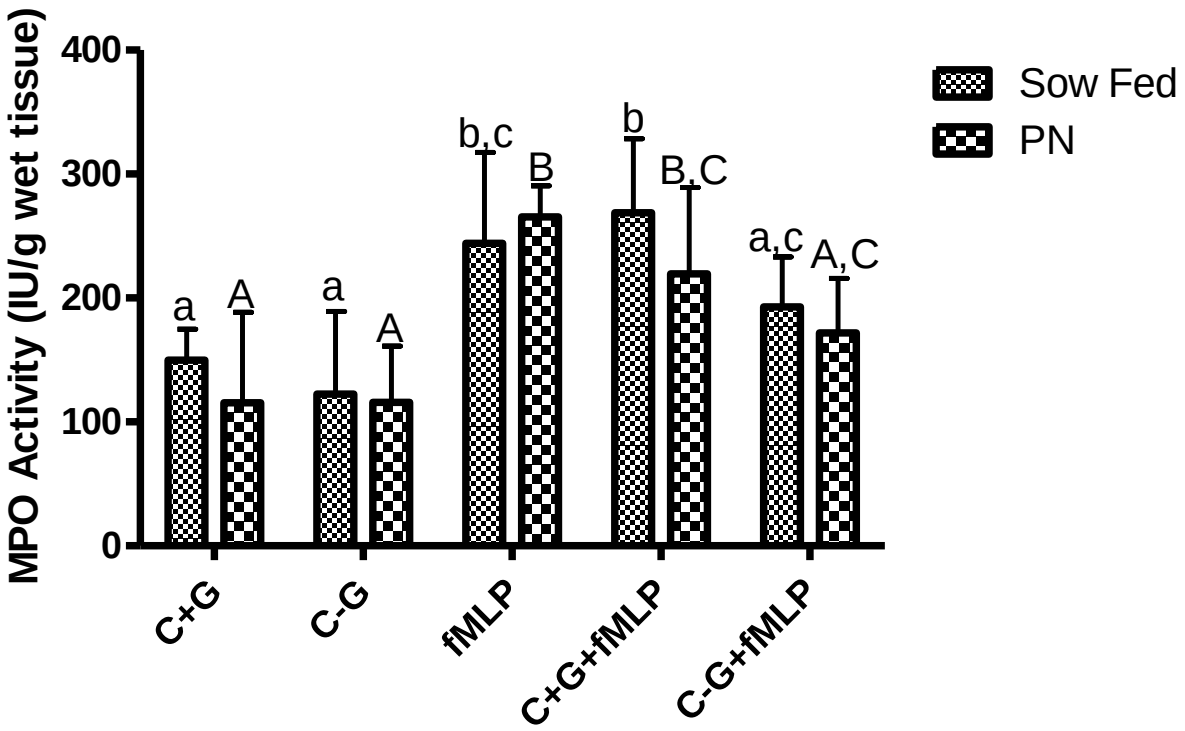

Figure 4.6: Mucosal myeloperoxidase activity in ligated loops in sow-fed and PN-fed piglets sampled from ligated loops perfused with cysteine + glycine (C+G), cysteinyl-glycine (C-G), formyl-methionyl-leucyl-phenylalanine (fMLP), a combination of cysteine + glycine + fMLP (C+G+fMLP) or cysteinyl-glycine + fMLP (C-G+fMLP). Differing letters represent significant differences between loop treatments within a feeding group ($p < 0.05$). Data were analyzed via mixed model 2-way ANOVA with Bonferroni post-hoc. N = 6 per group. Values are mean + SD.

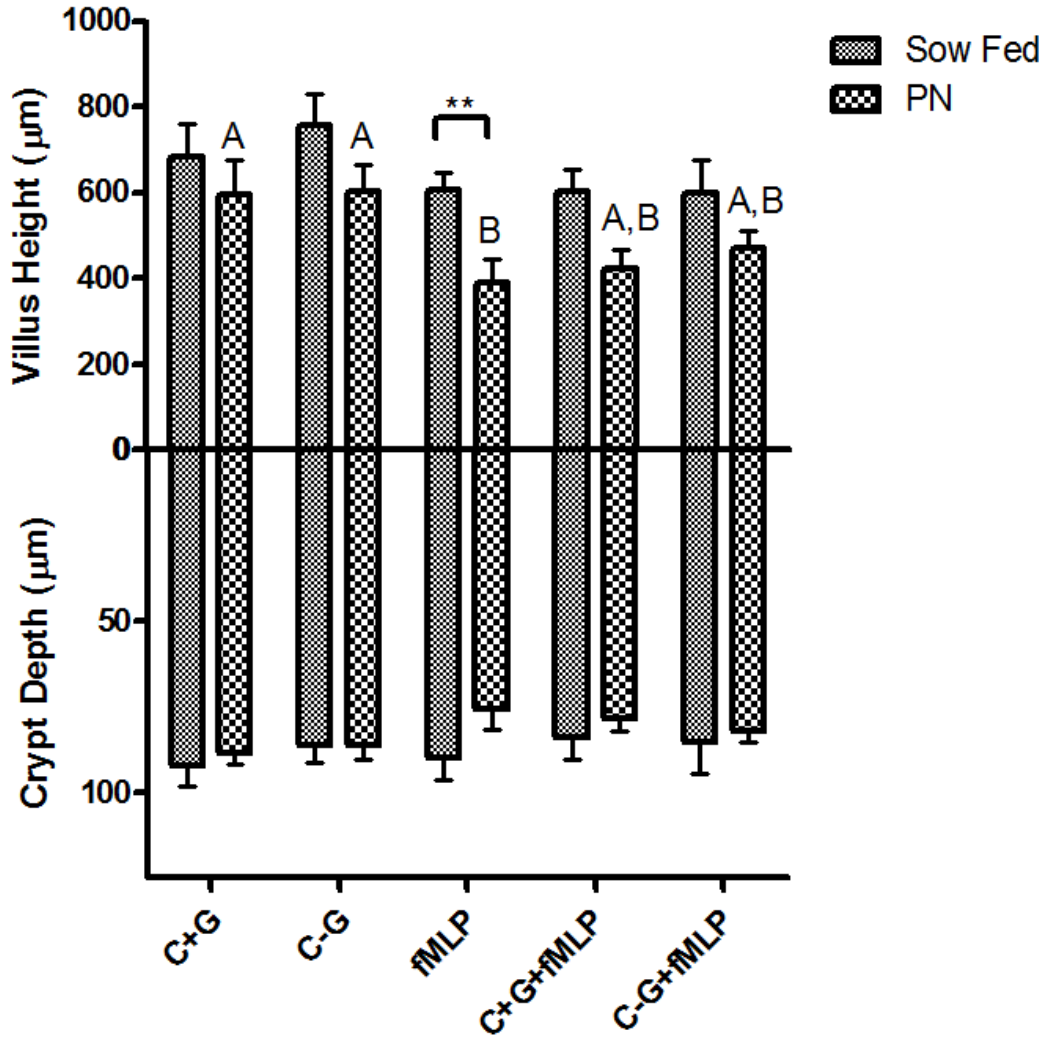

Figure 4.7: Villus height (>0) and crypt depth (<0) of ligated loops in sow-fed and PN-fed piglets sampled from ligated loops perfused with cysteine + glycine (C+G), cysteinyl-glycine (C-G), formyl-methionyl-leucyl-phenylalanine (fMLP), a combination of cysteine + glycine + fMLP (C+G+fMLP) or cysteinyl-glycine + fMLP (C-G+fMLP). Differing letters represent significant differences between loop treatments within parenterally fed animals ($p < 0.05$). Lines represent significant differences between diet treatment (** $p < 0.01$). Data were analyzed via mixed model 2-way ANOVA with Bonferroni post-hoc test. N = 6 per group. Values are mean ± SEM.

segments. Interestingly, villus damage in response to fMLP occurred only in the PN animals compared to identical conditions in sow-fed piglets.

4.5 Discussion

The objectives of this study were to investigate the mucosal response to fMLP-induced inflammation in healthy versus compromised gut and to determine the impact of cysteinyl-glycine on fMLP-induced inflammation. These objectives were accomplished through quantification of the cytokine response to fMLP, as determined by myeloperoxidase activity and intestinal histology. Not surprisingly, we determined that parenterally-fed animals were more susceptible to fMLP-induced inflammation than their sow-fed littermates. A novel finding is that cysteinyl-glycine was more effective at attenuating fMLP-induced inflammation, through the reduction of pro-inflammatory cytokines, compared to equimolar amounts of the constituent free amino acids.

Intestinal mucosa exposed to fMLP had greater concentrations of IFN-γ and TNF-α than loops perfused with either free amino acids or cysteinyl-glycine alone. Previous work on other cell types such as neurons (Cianciulli et al., 2009), myeloid cells (Browning et al., 1997) and peripheral blood monocytes (Pan et al., 2000) demonstrated that fMLP exposure induces expression of NF-κβ. In turn, NF-κβ can act on the promoter regions for pro-inflammatory cytokines such as IFN-γ and TNF-α (Baeuerle and Henkel, 1994, Sica et al., 1997). In a positive feedback loop, TNF-α is also capable of inducing NF-κβ activation resulting in the propagation of the inflammatory response (Grell et al., 1995). The function of IFN-γ is to induce the production of chemoattractants for leukocytes; additionally, IFN-γ is both anti-proliferative and apoptotic (Schroder et al., 2004). Although we did not directly measure NF-κβ, the conserved nature of the response to fMLP exposure across numerous cell types combined with

the fMLP-induced increase of pro-inflammatory cytokines in the intestine suggests that enterocytes may respond to fMLP in a similar fashion.

Inclusion of a dipeptide as cysteinyl-glycine ameliorated fMLP induced production of IFN-γ and TNF-α, potentially through the reduction of PepT1-mediated fMLP uptake via competitive inhibition. It has been well demonstrated that transport of fMLP is inhibited in the presence of other PepT1 substrates (Buyse et al., 2002, Shi et al., 2006c, Foster and Zheng, 2007). Perfusion of fMLP resulted in a greater concentration of pro-inflammatory cytokines, while co-perfusion with cysteinyl-glycine acted as a competitive inhibitor of uptake. If the concentrations of luminal substrates for PepT1 were increased, this could potentially lead to further competition for transport and thereby further reduce the inflammatory response induced by fMLP.

Interestingly, co-perfusion of fMLP with free cysteine and glycine significantly reduced TNF-α only in sow-fed piglets, not their PN-fed littermates. Cysteine is part of the oxidative stress control system, independent of its role as a component of glutathione (GSH) (Jones, 2006). Both cysteine and glycine have been demonstrated to exhibit anti-inflammatory effects in arterial endothelial cells through the reduction of NF-κβ activation (Hasegawa et al., 2012). The differing responses by diet treatment may be related to impairment of amino acid uptake secondary to PN-induced intestinal atrophy; thus, the tempered response to free cysteine and glycine could be due to reduced amino acid availability.

To determine whether anti-inflammatory cytokines were also affected by route of feeding or fMLP exposure, we quantified mucosal concentration of interleukin-10 (IL-10). Unlike the pro-inflammatory cytokines measured, IL-10 concentration was not altered by exposure to

fMLP. Piglets receiving parenteral nutrition, however, had lower mucosal IL-10 compared to their sow-fed littermates. In a mouse model of parenteral nutrition, intestinal epithelial lymphocytes produced less IL-10, leading to an overall lower concentration of mucosal IL-10 compared to mice undergoing enteral feeding (Fukatsu et al., 2001, Sun et al., 2008). This lack of IL-10 synthesis was accompanied by a reduction in tight junction proteins and greater epithelial permeability. Altered intestinal permeability would allow for additional bacterial translocation thereby inducing or exacerbating the immune response and intestinal inflammation.

In order to determine if there was altered ileal paracellular permeability in our piglet model, we measured mannitol disappearance from the perfusate during intestinal perfusion. In a Caco-2 cell model, exposure to fMLP reduced intestinal barrier function as indicated by elevated mannitol movement (Foster and Zheng, 2007). The piglet model used in the current study, however, studied only the distal intestine rather than whole SI or colon. Studies in rats have shown that the ileum has much lower permeability than either the jejunum or Caco-2 cells, with ileal permeability being similar between rats and humans (Artursson et al., 1993, Kim, 1996). Parenteral nutrition has also been shown to affect gut barrier function with greater permeability to macromolecules in the small intestine (Illig et al., 1992, Iiboshi et al., 1994); however, these studies did not directly investigate ileal permeability. One study in rats did demonstrate greater permeability in the ileum after seven days of parenteral nutrition (Mosenthal et al., 2002). In our piglet model, four days of parenteral feeding did not alter permeability in the ileum; however, it is unknown if longer duration of PN would result in greater ileal permeability thereby potentiating the intestinal inflammatory response to bacteria.

Quantification of the disappearance of fMLP using a radiolabelled substrate was conducted; however, there were no significant differences detected among any of the perfusates

or between dietary treatments. There are two possible explanations for this result. One is that there was no difference in the uptake of fMLP from the perfusate. Alternatively, the method we used to quantify fMLP disappearance was not sufficiently sensitive. Indirect evidence of fMLP uptake was provided by the differing concentrations of mucosal cytokines in the presence of the bacterial peptide. Similarly, different mucosal responses depending on whether fMLP was perfused alone, with free amino acids or with cysteinyl-glycine suggest that there was potential inhibition when a dipeptide was present. This suggests that the detection method is not sensitive enough to quantify the movement of ^{3}H-fMLP. If the quantity of radiolabelled fMLP in the perfusate were reduced, the ability to detect significance in smaller variations of dpm would be increased resulting in a more sensitive assay. As a surrogate indicator of fMLP transport, and its resultant impact on intestinal inflammation, mucosal myeloperoxidase activity was measured.

Myeloperoxidase activity is useful as a measure of intestinal inflammation. In this study, MPO activity was not affected by intestinal atrophy induced by parenteral nutrition. However cysteinyl-glycine was able to attenuate fMLP induced MPO activity in both sow-fed and PN animals. Previous studies have investigated MPO activity in the small intestine and colon of rats (Buyse et al., 2002, Shi et al., 2006c). These investigations demonstrated that exposure to fMLP stimulated MPO activity in the small intestine but only in colonic tissue of rats with PepT1 expression induced by small bowel resection. We have demonstrated that supplying a competitive inhibitor for fMLP uptake, cysteinyl-glycine, prevents any significant stimulatory effect of fMLP on MPO activity. Although studies have shown competitive inhibition of fMLP transport with dipeptides or other substrates of PepT1 (Buyse et al., 2002, Shi et al., 2006c, Foster and Zheng, 2007), whether there is an additional benefit due to the presence of a particular dipeptide has yet to be determined. Inclusion of a hydrolysis resistant dipeptide, such as glycyl-

sarcosine, could potentially further reduce fMLP-induced MPO activity through its stability in the lumen of the intestine.

After four days of parenteral feeding, there was no deleterious effect on the villus architecture compared to the sow-fed littermates. When the challenge of fMLP exposure was added, it was apparent that parenteral feeding increased susceptibility to villus damage. Destruction of intestinal villi by bacterial peptides would result in reduced nutritional absorptive capacity, leading to reduced protein deposition and prolonged recovery for individuals requiring parenteral nutrition. This effect is not limited to the ileum as a study in rats investigated the inflammatory effect of fMLP and found similar villus damage in the jejunum after perfusion with fMLP (Buyse et al., 2002). In the current study, the villi damage induced by fMLP was ameliorated when either cysteine + glycine or cysteinyl-glycine was included in the perfusate.

Although the inflammatory effects of bacterial peptides in colonic tissues in cases of intestinal injury have been previously investigated (Shi et al., 2006a, Shi et al., 2006c, Merlin et al., 2001), this study was the first analysis of fMLP-induced inflammation in a parenterally-fed animal model. We found that parenteral feeding sensitized the ileum to fMLP-induced inflammation and that the inclusion of a dipeptide, cysteinyl-glycine, in the lumen of the intestine ameliorated this response. These findings are of particular importance when long-term parenteral nutrition is required in neonates, as reduction of pro-inflammatory cytokines could reduce risk for intestinal damage.

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Chapter 5: Summary and Conclusions

5.1 *General Overview*

The series of studies presented in this thesis set out to investigate intestinal dipeptide transport via PepT1 in the neonatal Yucatan miniature pig with particular focus on developmental changes, intestinal injury and bacterial peptide induced inflammation. This was accomplished through *in vivo* experimentation using ligated intestinal loops for the study of developmental changes and fMLP-induced inflammation. An 80% jejuno-ileal resection was performed to study the ameliorative potential of alanyl-glutamine and cysteinyl-glycine in a model of intestinal injury. The piglet is a model organism for human neonatal development and nutrient requirements, with swine also being vital to the agricultural industry in Canada. As such, information on peptide uptake in piglets has important applications in both human health and swine husbandry.

5.2 *Investigation of the ontogeny of peptide transport in the piglet*

5.2.1 *Overview of results*

The objectives for my first study were to identify the potential for peptide transport in the piglet small intestine and determine if there were any differences in peptide transport due to developmental changes or dietary alterations. To accomplish this goal, I quantified the disappearance of radiolabelled glycyl-sarcosine after perfusion through isolated equidistant segments of small intestine from both suckling and post-weaned piglets. PepT1 mRNA was also analysed from the small intestinal loops, as well as samples from the spiral colon, to determine if there were any developmental changes in the expression of this peptide transporter. Through this model, I determined that the capacity for dipeptide transport is present in both the proximal and

distal small intestine. By post-weaning, the ileum was the site of highest dipeptide uptake compared to jejunal segments. Analysis of PepT1 mRNA revealed that while PepT1 mRNA was present in colonic samples in both suckling and post-weaning animals, after weaning PepT1 expression was dramatically reduced.

5.2.2 *Implications and modifications*

This study was the first to use the ligated loop model to investigate developmental effects on peptide uptake at various locations in the small intestine in piglets. Characterizing the capacity for peptide transport in the neonatal piglet was a vital step in my research programme. Knowing the intestinal location where dietary peptides are primarily transported could influence surgical decisions such as prioritizing which region of intestine to spare during resection. Similarly, armed with the knowledge that peptide transport is possible throughout the intestine, dietary regimens could be altered to include di/tripeptides over free amino acid due to favorable factors such as increased stability and solubility. The fact that colonic expression of PepT1 mRNA was lost after weaning was also an interesting finding. It is possible that in early life there is a greater potential for uptake of harmful bacterial peptides from the lumen of the colon than after weaning.

One of the findings of this work was greater transport in the ileum of post-weaned piglets. Unfortunately it cannot be determined whether this occurs through a developmental change, a dietary change or the combination of both. The study design could have been altered to include another group piglets kept on sow's milk for six weeks. This would have provided a clearer image of the true ontogeny of this transporter; however it would not have been as physiologically relevant. Additionally, direct investigation of the PepT1 protein would have been beneficial. I found highly variable quantities of PepT1 mRNA in the small intestine and

relatively small quantities in the colon. Direct visualization or quantification of PepT1, through western blots or immunohistochemistry, would have provided direct information regarding the quantity of PepT1 protein present as mRNA content does not always equate with protein expression.

Overall I believe that this initial project was well designed and an excellent launching point for the studies which followed. The use of the ligated loop model allowed for investigation of peptide transport at multiple locations in the same animal, dramatically reducing the variability. The choice of substrate, glycyl-sarcosine, controlled for hydrolysis at the brush border and inclusion of the radiolabel provided an easy way to track its disappearance.

As I completed this initial study a piglet model of short-bowel syndrome was being characterized. This model removed the majority of the small intestine but left 100 cm of the ileum intact. As I had previously determined that the ileum was important in the transport of dipeptides I was interested in moving my research into this new model. Was it possible that inclusion of dipeptides in an enteral diet would result in increased intestinal adaptation compared to equivalent free amino acids in this model of short-bowel syndrome?

5.3 *Investigation of the adaptive benefits of enteral peptides in a surgically shortened gut*

5.3.1 Overview of results

Continuing my work in peptide transport, my objective was to study the potential ameliorative effects of enterally-delivered dipeptides in a surgically shortened intestine using a piglet model of short-bowel syndrome. The piglets underwent surgery at ten days of age as the surgical procedure was quite invasive and younger piglets may not have survived the entire study. This piglet model involved the removal of 80% of the small intestine, leaving 100 cm

proximal from the ileocecal valve, and performing an anastomosis to the remaining jejunal tissue. After recovery, enteral feeding of dipeptides was initiated and maintained for four days. The dipeptides used in this study were alanyl-alanine, as a control for the effect of dipeptides alone, alanyl-glutamine (AQ), as a stable form of glutamine, and cysteinyl-glycine (CG), a dipeptide providing two residues required for glutathione synthesis as well as amino acids that have previously demonstrated anti-inflammatory characteristics. Using control groups provided with equimolar free amino acid diets, and a synergistic dietary regimen of a combination of alanyl-glutamine and cysteinyl-glycine (AQ+CG), I was able to determine that there is no explicit morphological benefit of enteral dipeptides compared to equimolar free amino acids. However, provision of enteral dipeptides did reduce mucosal concentrations of the pro-inflammatory cytokines TNF-α and IFN-γ. Inclusion of CG alone did result in greater morphological adaptation, as villus height was greater than either AQ or AQ+CG while crypt depth was greater than AQ alone. Other outcomes measured, including cellular proliferation, protein synthesis as well as mucosal and plasma amino acid concentrations, did not differ amongst any dietary regimen.

5.3.2 *Implications and modifications*

Surgical resection is a common procedure for neonates and the development of a novel dietary regimen that increases intestinal adaptation would be extremely beneficial. While the dipeptides I investigated, AQ and CG, did not stimulate any greater adaptive response than free amino acids, the impact on cytokines was an unexpected beneficial result generated through post-hoc analysis. These dipeptides could be of use as supplements to individuals suffering from inflammatory bowel disorders to reduce in severity of the inflammatory response.

With a study of this complexity there were complications as well as additional outcomes which would have clarified some of the results. Initially I intended to ensure that each block of treatments would be tested simultaneously on littermates, thereby increasing the power of my analysis. Due to complications with litter sizes this, unfortunately, was not possible. For the outcomes that were measured, I theorized that one of the reasons why I did not see adaptive responses was due to a missed window of adaptation. Samples were taken after 4 days of enteral feeding, so early adaptive responses may have been missed by this study design. Altering the design to include additional cohorts where sampling could be done at earlier time points would have provided the necessary data to confirm this theory but would have required significantly more animals.

Perhaps the aspect of this study which would require the most modification would be the investigation of the cytokine response. Although I determined that TNF-α and IFN-γ concentrations were lower in piglets receiving AQ and CG I did not determine the mechanism by which this occurred. The hypothesis that this occurs is through modulation of NFκβ is sound however without additional measurements this theory remains unproven and it is likely that these dipeptides also alter other cellular signals. There are a number of additional experiments which would have provided the necessary details to clarify the mechanism. A microarray, followed by qPCR validation, would have revealed increases or decreases in a variety of cytokine signalling pathway intermediates, while a cytokine array would have given a clearer image of the cytokine status after enteral feeding.

Bacterial overgrowth was also proposed as a potential explanation for the cytokine response. Biopsy of a mesenteric lymph node and culturing the bacteria present would have revealed any increase in bacterial translocation. However production of bacterial peptides could

also have induced the inflammatory response. This ties into the dual nature of PepT1, capable of transporting both dietary as well as bacterial peptides. Is it possible that the inflammation I detected, and the subsequent reduction in the presence of enteral dipeptides, was directed through transport of bacterial peptides?

5.4 *Investigation of intestinal susceptibility to bacterial inflammation after parenteral feeding*

5.4.1 *Overview of results*

As my second study had created more questions than answers, the objective I had in mind for my third study was to investigate the impact of a bacterial peptide, formyl-methionyl-leucyl-phenylalanine on ileal inflammation in a model of gut atrophy in the presence of cysteinyl-glycine. This would provide a better understanding of the interaction between a bacterial peptide and CG, potentially explaining some of the results found in my second study. After four days of parenteral nutrition, distal segments of the small intestine were isolated and perfused with combinations of fMLP, free cysteine and glycine or the dipeptide cysteinyl-glycine. Similar procedures were performed on sow-fed littermates to compare any impact of parenteral feeding on intestinal inflammation. Perfusion of the ligated loops with fMLP led to the induction of pro-inflammatory cytokines in both sow and PN-fed piglets, with PN feeding potentiating the inflammation induced by fMLP as demonstrated by villus damage only in piglets undergoing parenteral feeding. Mucosal concentration of anti-inflammatory IL-10 also was lower in PN-fed piglets compared to sow fed littermates. Co-perfusion of fMLP with CG resulted in lower concentrations of these pro-inflammatory cytokines than fMLP alone.

5.4.2 *Implications and modifications*

The design of this study developed out of the results of my previous work, hence the focus on the distal intestine, the location of the remaining intestine after resection. Similar to the first study, using the gut loop model allowed for many different solutions to be investigated in one animal, removing the potential for inter-animal variability and giving the analysis additional power. Although increases in bacterial population and translocation following parenteral nutrition has been well documented, the finding that parenteral nutrition sensitizes the intestine to bacterial peptide-based inflammation is novel. These findings indicate that inflammation due to bacterial peptides is of greater concern for individuals undergoing parenteral nutrition and that enteral provision of a dipeptide, cysteinyl-glycine, is advantageous for its capability to reduce or inhibit the production of pro-inflammatory cytokines. Similarly, in cases of bacterial overgrowth in the small intestine, enteral provision of cysteinyl-glycine would be beneficial to prevent unwanted transport of bacterial peptides.

Moving from a surgically shortened intestine to a gut loop model was necessary for a number of reasons. Most importantly the fragility of the intestine after resection would cause complications for insertion and maintenance of a three hour ligated loop perfusion, and the lack of a suitable length of intestine in which to place the loops would make this study impossible to complete in the previous model. Unfortunately, the results of this study do not directly answer all the questions raised from the short-bowel work. I was able to demonstrate that fMLP could induce inflammation in the distal intestine and inclusion of CG attenuated this inflammation. Whether the mechanism by which CG affects fMLP-induced inflammation is through competitive inhibition, direct alteration of cellular signalling or a combination of both was not determined by this study design. Similar to the second study, use of modern genomic/proteomic

analysis would have provided the necessary information to elucidate the underlying mechanism by which CG was reducing the immune response to fMLP.

Working within the gut loop model, a few minor alterations to the study design would have provided additional information, although not all of it tied directly to the primary objective. Although four days of parenteral nutrition has been shown to induce intestinal atrophy, I did not demonstrate atrophy in my study. Implantation of additional loops in the jejunum would have provided information regarding potential increases in paracellular transport while histological sampling of the proximal intestine would have indicated any alteration in intestinal architecture confirming intestinal atrophy. While the initial objective of the study was to determine the impact in the distal intestine, investigation of the proximal intestine would have been relatively simple. Under pathological conditions the ileum would undergo bacterial overgrowth to a greater extent than the jejunum. If parenteral nutrition induces greater atrophy in the jejunum the impact of bacterial peptides in that region may be noteworthy in cases where the intestine had to be surgically shortened thereby exposing jejunal tissue to an increased pathogen load. During the loop excision process if measurements of the exact length and width of each loop were taken, the total area of absorption could have been calculated leading to a more precise measurement of fMLP and mannitol disappearance.

5.5 *Final thoughts*

I believe that future work following from the studies presented here should focus on two particular areas. Having shown that there are developmental differences in intestinal peptide transport, the nutritional relevance and metabolic impact of intact dietary di/tripeptides during neonatal development should be elucidated. Certain dipeptides, such as alanyl-glutamine, have

been used in therapeutic formulas for a number of years but there has been little work towards understanding the potential benefits of replacing free amino acids with di/tripeptides in neonates.

The contribution of PepT1 to intracellular signalling should also be investigated. As substrates for this transporter can include bacterial compounds, further understanding of the downstream signalling mechanisms of these substrates could provide targets for future therapeutic interventions. It has already been suggested that fMLP acts through NFκβ, but there is the potential for dietary substrates of PepT1 to influence cellular signalling either due to an activity of the intact dipeptide or simply be increasing intracellular concentration of amino acids. Ultimately, a more detailed understanding of PepT1 will directly contribute to the development of novel dietary therapies and the enhancement of modern nutrition in both health and disease.

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