

**The Interaction between Lysyl-lysine and Arginine in the Neonatal Yucatan Miniature Pig  
Small Intestine**

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## **Abstract**

Using an *in situ* intestinal perfusion model in sow-fed neonatal piglets, our objectives were to determine whether the lysyl-lysine enhanced arginine absorption led to a functional benefit of greater mucosal protein synthesis, and whether it was mediated through the mTOR pathway. Three segments of piglet's small intestine were isolated, and were continuously perfused with: arginine, arginine + lysyl-lysine, or arginine + L-lysine. At 90 min, buffers containing the same amino acids plus phenylalanine were initiated for 30 min to measure mucosal protein synthesis. Six additional piglets underwent the identical protocol, but one hour before the intestinal perfusion, rapamycin was delivered intravenously. Co-perfusing arginine with lysyl-lysine resulted in greater arginine uptake, and higher mucosal protein synthesis. Moreover, both protein synthesis and the phosphorylation of mTOR were inhibited by rapamycin. Thus, lysyl-lysine led to a greater mucosal protein synthesis, which was likely mediated through enhanced arginine absorption.

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## **Abbreviations**

AA- amino acid

Arg- arginine

Lys-lys - lysyl-lysine

Lys- lysine

PepT1- peptide transporter 1

rBAT/b<sup>0,+</sup> - basic amino acid transporter

rapa- rapamycin

NO- nitric oxide

OAT- ornithine aminotransferase

P5CS- pyrroline-5-carboxylate synthase

TPN- total parenteral nutrition

GIT- gastrointestinal tract

mTOR- mammalian target of rapamycin

PI3K- phosphatidylinositol (PI) 3-kinase

PIKK- phosphatidylinositol 3-kinase related kinase family

Raptor- the regulatory associated protein of mTOR

PRAS40- proline-rich Akt substrate 40 kDa

mLST8- G-protein  $\beta$  -subunit-like protein

mSin1- mammalian stress-activated protein kinase-interacting protein 1

riCTOR- the rapamycin-insensitive companion of mTOR

S6K1- P70 ribosomal S6 kinase 1

IECs – Intestinal epithelial cells

4E-BP1- the eukaryotic initiation factor 4E (eIF4E)- binding protein 1

hVps34- human vacuolar protein-sorting associated protein 34

Rag- GTPase heterodimers

MAP4K3- the mitogen-activated protein kinase

Vps15- vacuolar protein sorting-associated protein 15

PtdIns (3) p- phosphatidylinositol 3 kinase phosphate

Rheb- Ras-homolog enriched in brain

MAP4K3- the mitogen-activated protein kinase

eIF4E- the eukaryotic translation initiation factor 4E

mRNA- messenger RNA

eEF2- the eukaryotic elongation factor 2 kinase

eIF4G- the eukaryotic translation initiation factor 4G

FKBP12- the 12-kDa FK506-binding protein

B<sub>0</sub>- the neutral system

X<sub>AG</sub> - the acidic system

IMINO- the iminoglycine system

β- the β-amino acid system

PKC- protein kinase C

PKA- protein kinase A

Phe-Gly- phenylalanyl-glycine

Tyr-Gly- tyrosyl-glycine

Trp-Gly- tryptophyl-glycine

Gly-Gly- glycyl-glycine

Gly-Tyr- glycyl-tyrosine

Gly-Phe- glycyl-phenylalanine

Gly-Asp- glycyl aspartic acid

Gly-Glu- glycyl-glutamic acid

Gly-Gln- glycyl-glutamine

BBMVs- brush-border membrane vesicles

PBS- phosphate buffered saline buffer

DMSO- dimethyl sulfoxide

BSA- bovine serum albumin protein standard

SDS-PAGE- sodium dodecyl sulfate polyacrylamide gel electrophoresis

TBST- tris buffered saline 0.05% Tween-20

ECL- immobilon western chemiluminescent HRP substrate

P-mTOR- phosphorylated mTOR.

IPEC-J2- neonatal porcine intestinal epithelial cells

tRNA- transfer RNA

NaCl- sodium chloride

Na<sub>2</sub>HPO<sub>4</sub>- disodium phosphate

NaH<sub>2</sub>PO<sub>4</sub>- monosodium phosphate

HCL- hydrochloric acid

K<sub>2</sub>CO<sub>3</sub>- potassium carbonate

DPM- disintegrations per minute

TFA- trifluoroacetic acid

MeOH- methanol

TEA- triethylamine

PITC- phenylisothiocyanate.

Ks- fractional rate of protein synthesis, %/d.

SRA<sub>bound</sub>- specific radioactivity of bound phenylalanine in tissue, DPM/ $\mu$ mol.

SRA<sub>free</sub>- specific radioactivity of phenylalanine in the precursor pool, DPM/ $\mu$ mol.

## **Chapter 1: Literature Review.**

### **1.1 Arginine.**

#### **1.1.1. Arginine nutrition.**

In the body, arginine is derived from protein turnover, diet, and *de novo* synthesis (Morris, 2006). Arginine has many important metabolic roles other than as a component of proteins; arginine serves as a precursor for important metabolites such as nitric oxide (NO), a molecule that regulates vascular relaxation and influences the immune response, polyamines which are important for cellular growth, and creatine which is involved in cellular energy metabolism (Wu & Morris, 1998; Wyss & Kaddurah-Daouk, 2000). Arginine is also an intermediate in the urea cycle, and is necessary for the disposal of ammonia through the production of urea (Wu & Morris, 1998). In neonates, arginine is a conditionally essential amino acid, as a dietary source is required for optimal growth (Wu, Knabe, & Kim, 2004). Arginine is also conditionally essential in adults; however, the need for a supplement is linked to the individual's health (Jacobs, 2011).

#### **1.1.2. *De novo* arginine synthesis.**

The organs involved in *de novo* arginine synthesis change in relative importance with development (Wu, Jaeger, Bazer & Rhoads, 2004 a). During early neonatal life, the small intestine is responsible for the synthesis of arginine. Amino acid precursors, predominantly proline, are converted into ornithine and citrulline and then to arginine (Bertolo, Brunton, Pencharz & Ball, 2003). A previous study demonstrated that ornithine aminotransferase (OAT) and proline dehydrogenase were both expressed in the neonatal piglet small intestine which facilitated the conversion of proline to arginine through ornithine (Bertolo et al., 2003). Also, negligible

expression of arginase in the small intestine resulted in the release of newly synthesized arginine into the portal blood (Bertolo et al., 2003). This intestinal metabolism is tremendously important for normal arginine homeostasis because most other organs, including the kidney, are not capable of producing sufficient arginine to meet the whole body needs (Cynober, Boucher & Vasson, 1995). As a result, arginine synthesis in enterocytes is essential and it compensates for the low arginine concentration in mammary milk (Wu, Knabe & Kim, 2004 b).

In piglets, it has been demonstrated that as the animal ages, arginine synthesis is reduced in enterocytes due to an increase in the expression of arginase (Blachier, M'rabet-touil, Posho, Darcy-Vrillon & Duée, 1993). This change affects the ability of enterocytes to synthesize and release arginine; instead, the amino acid precursors are converted to, and released as citrulline (Cynober et al., 1995). The direct precursor of citrulline in the small intestine is ornithine, which is synthesized in enterocytes from glutamine and proline (Marini, Stoll, Didelija & Burrin, 2012). The citrulline is released into the portal blood, and is taken up by the kidneys, where it is converted into argininosuccinate and then arginine in the proximal tubules (Jacobs, 2011; Morris, 2006).

In the liver, arginine is also produced in the urea cycle; however, due to the high cytosolic arginase activity, most of the newly formed arginine is hydrolyzed to release urea and ornithine (Flynn, Meininger, Haynes & Wu, 2002). As a result, the predominant source of endogenously synthesized arginine is the intestine in early life (Bertolo et al., 2003). After weaning, citrulline is released from the gut and arginine is formed by the intestinal-renal axis (Cynober et al., 1995; Windmueller & Spaeth, 1981).

### **1.1.3. Advantages of supplementing arginine to neonates.**

The arginine concentration in mammal's milk, such as pigs, humans and cows, is considerably low, and is estimated in pigs to supply only 40% of the required arginine at one week of age (Jacobs, 2011; Wu et al., 2007). As a result, in young milk-fed animals, growth may be limited, particularly if *de novo* synthesis is impaired for some reason (Wu et al., 2007). Supplementing arginine in the diet of early weaned pigs for two weeks, at concentrations of 0.2% and 0.4% improved the daily weight gain by 28% and 66%, respectively, resulting in final body weights that were 15% and 32% greater than controls. These results suggest that the arginine content in sow milk is too low to maximize growth and that supplementing arginine may be beneficial for commercial swine production purposes (Kim, McPherson & Wu, 2004).

Weaning in piglets is a stressful event which can negatively affect food intake and growth. Weaning also affects gut morphology resulting in reduced nutrient absorption and transport (Lallès et al., 2004). Yao et al. (2011) conducted a study that supplemented weaned piglets with 0 or 1% arginine added to a basal maize and soybean meal diet. After only 7 days, the daily weight gain was 38% greater in the 1% arginine group, and the small intestine's relative weight was 33% higher compared to 0% arginine group. Furthermore, 1% arginine increased both the villus height, and the expression of the vascular endothelial growth factor along the entire length of the small intestine. Hence, supplementing arginine to the diet may be a beneficial practice during stress events in piglets such as gastrointestinal infections or weaning (Yao et al., 2011). Additionally, arginine has a role in activating the mTOR signaling pathway which contributes towards promoting neonatal growth (Yao et al., 2008). In a separate study by Yao and colleagues (2008), piglets were fed sow milk replacer supplemented with arginine at 6% between the age of 7 and 14 days. They reported enhanced daily body weight gain and the skeletal muscle protein synthesis

with the arginine supplement (Yao et al., 2008).

#### **1.1.4. The role of the gastrointestinal tract in arginine deficiency.**

Many factors may contribute to arginine deficiency in the neonate. The most common factors in human infants include a reduced metabolic mass of intestinal tissue such as gut atrophy due to prolonged parenteral feeding, acute intestinal failure due to congenital malformations or infections. More rare causes include genetic anomalies such inborn errors of metabolism (e.g. pyrroline-5-carboxylate synthase (P5CS) deficiency), or transporter defects (Baumgartner et al., 2000; Kamada et al., 2001; Piton, Manzon, Cypriani, Carbonnel & Capellier, 2011).

Gut atrophy as the result of prolonged parenteral nutrition (PN) use in preterm infants may be common. PN is used in a high proportion of preterm infants, and is necessary when normal feeding into the gastrointestinal tract (GIT) is contraindicated (Hay, 2008). Gut atrophy occurs due to a lack of enteral stimulation such as nutrients in the intestinal lumen which may affect anabolic hormones. The alteration in these hormones results in gut atrophy which affects the small intestinal metabolic capacity, and protein turnover (Bertolo, Chen, Pencharz & Ball, 1999). Gut atrophy that is caused by parenteral feeding may disrupt whole body nitrogen metabolism; delivering diet through parenteral nutrition to neonatal piglets resulted in the reduction of nitrogen retention (Bertolo et al., 1999). According to Bertolo and colleagues (1999), in piglets that were supported by a parenteral diet, the weight of the total small intestine and the mucosal mass were reduced by 40%; intravenously-fed piglets also demonstrated atrophied villous heights with decreased crypt depth compared to intragastrically-fed piglets. This in turn, may have impaired arginine synthesis which negatively influenced protein deposition (Bertolo et al., 1999).



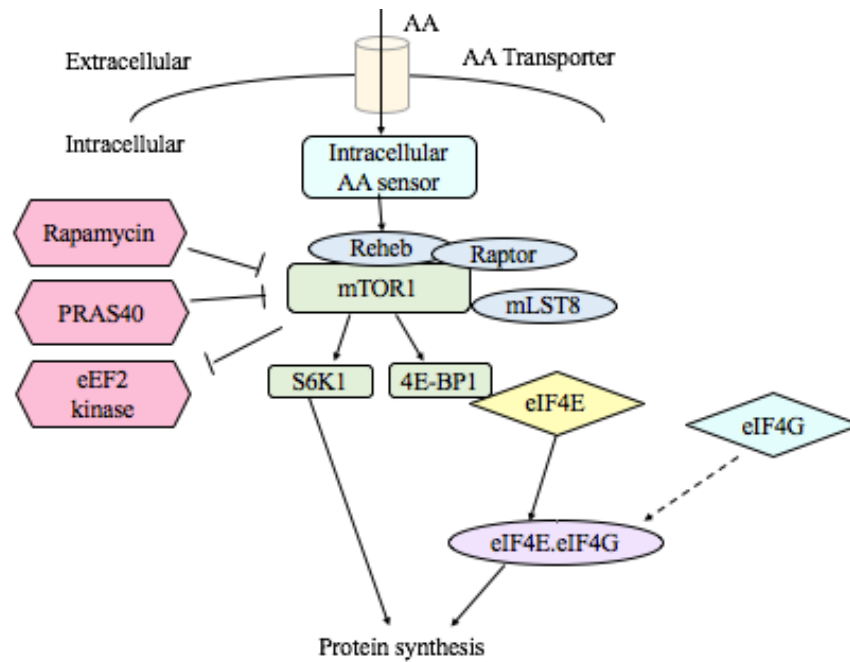
Indeed, in neonates the gastrointestinal tract has an important role in arginine metabolism, and several common factors including PN feeding or severe GI infection such as necrotizing enterocolitis may affect the function of the gastrointestinal tract, resulting in arginine deficiency.

## **1.2. mTOR pathway activation.**

### **1.2.1. mTOR structure.**

The mammalian or mechanistic target of rapamycin (mTOR), which is a serine/ threonine protein kinase, functions as a regulator for the growth, proliferation, and metabolism of the cell. mTOR controls anabolic and catabolic processes in the cell by sensing the levels of nutrients and energy. Because the catalytic domain at the C-terminus of mTOR is similar to PI3K, mTOR belongs to the phosphatidylinositol (PI) 3-kinase (PI3K) related kinase (PIKK) family (Zhou, Luo, & Huang, 2010). mTOR has two different signaling protein complexes, mTORC1 and mTORC2, and each has different subunits, and both are inhibited by rapamycin. mTORC1 is composed of four proteins: the catalytic subunit, the regulatory associated protein of mTOR (Raptor), proline-rich Akt substrate 40 kDa (PRAS40), and G-protein  $\beta$ -subunit-like protein (mLST8) (Figure 1-1). mTORC2 shares some similarities with mTORC1 in that both consist of the mTOR catalytic subunit and mLST8. However, mTORC2 has two different proteins that replace proteins in mTORC1. These proteins are the mammalian stress-activated protein kinase-interacting protein 1 (mSin1), and the rapamycin-insensitive companion of mTOR (rictor). The major function of mTORC1 is to control the proliferation, survival and growth of the cell by activating its substrates S6K1 and 4E-BP1. However, mTORC2 serves as a regulator for the actin cytoskeleton and activator for Akt, which controls cell cycle, and growth (Zhou et al., 2010).

The specific role of each of the mTORC1 protein compartments is not fully determined yet (Laplante & Sabatini, 2009). Raptor, for example, is suggested to act as a regulator for mTORC1 complex assembly (Hara et al., 2002). In addition, Raptor recruits protein substrates to mTOR through the interaction of Raptor with mTOR-signaling (TOS) motifs (Nojima et al., 2003). TOS motifs are short amino acid sequence motifs located on mTOR substrates (Fenton & Gout, 2011). mLST8 protein's function is difficult to define. This is because the mTORC1 signaling was not disrupted by the elimination of mLST8 protein (Guertin et al., 2006). As mTORC1 activity decreases, PRAS40 becomes important. PRAS40 serves as a negative controller of mTORC1 activity by binding to mTORC1 (Sancak et al., 2007; Wang, Harris, Roth & Lawrence, 2007). This binding prevents the interaction between mTORC1 and its substrates resulting in inhibition of mTORC1. When mTORC1 is activated, the association with PRAS40 is decreased through mTORC1 phosphorylating PRAS40 (Wang et al., 2007).



**Figure 1- 1:** The proposed mechanism for mTORC1 activation by amino acids. After amino acids are transported inside the cell, the mTOR1 is activated via the amino acid sensors (Kim, 2009). When mTORC1 is activated, it phosphorylates the downstream targets: the P70 ribosomal S6 kinase 1 (S6K1), and the eukaryotic initiation factor 4E (eIF4E)- binding protein 1 (4E-BP1). When mTOR phosphorylates 4EBP1, it results in the release of eIF4E from 4EBP1. This allows eIF4E to bind to other translation factors. Thus, mTOR1 activation results in stimulating protein synthesis. Abbreviations: AA, amino acids, PRAS40, proline-rich Akt substrate 40 kDa, eEF2 kinase, eukaryotic elongation factor2, Reheb, Ras-homolog enriched in brain, Raptor, regulatory associated protein of mTOR, PRAS40, proline-rich Akt substrate 40 kDa, mLST8, G-protein  $\beta$ -subunit-like protein, eIF4E, eukaryotic translation initiation factor 4E.

### 1.2.2. Proposed Intracellular amino acids sensors for mTORC1 activation.

It is known that amino acids stimulate the mTORC1 pathway for protein synthesis. However, the exact mechanism of amino acid activation of mTORC1 is not fully understood due to the different amino acid structures and routes of metabolism (Kim, 2009). It was suggested that the cell is capable of sensing amino acids levels, which control protein synthesis and breakdown, via amino acids sensors. These sensors are either on the cell membrane, or within the cell (Kim, 2009). Three proteins have been proposed as amino acid sensors located inside the cell: hVps34, Rag GTPase, and MAP4K3. Each of these proteins plays a role in mTORC1 activation (Findlay, Yan, Procter, Mieulet & Lamb, 2007; Gulati et al., 2008; Sancak et al., 2008). The Vps34 protein, human vacuolar protein-sorting associated protein 34, belongs to class III PI3K and reacts with Vps15 protein kinase to produce PtdIns (3) P (Gulati et al., 2008). Upon amino acids sensing, the activity of both Vps34 and PtdIns (3) P are elevated. *In vitro*, it has been reported that Vps34 is activated by amino acids through enhancing the  $Ca^{2+}$  influx (Gulati et al., 2008). In addition to Vps34, Rag protein was proposed to have a role in amino acids sensing. Rag activates mTOR1 through binding to Raptor which relocates mTORC1 to a region where the positive controller for mTOR activation, Rheb (Ras-homolog enriched in brain), is located in the intracellular region (Sancak et al., 2008). Another more recent study suggested that the Rag GTPase/ Ragulator complex is important for activating mTORC1 following translocation to the lysosomal membrane where mTOR1 interacts with the activator Rheb (Sancak et al., 2010). Furthermore, MAP4K3 protein, which is the mitogen-activated protein kinase, was suggested to have a role in amino acids signaling (Findlay et al., 2007). According to Findlay et al., 2007, it has been reported that amino acids acts as a regulator for MAP4K3 protein and the overexpression of MAP4K3 contributes towards enhancing the S6K phosphorylation which stimulates cell growth (Findlay et al., 2007).

In conclusion, the mechanism of amino acids sensing and mTORC1 activation is not fully understood, and most of the experimental results are controversial (Findlay, et al., 2007; Kim, 2009).

### **1.2.3. Mechanism for mTORC1 activation by amino acids.**

The initiation of the mTORC1 signaling pathway is stimulated via upstream signals such as nutrients (arginine and leucine), growth factors, oxygen and energy levels, and hormones (Laplante & Sabatini, 2009; Long, Lin, Ortiz-Vega, Yonezawa & Avruch, 2005). In the case of depletion of any of these factors, the mTORC1 signaling pathway is inhibited (Laplante & Sabatini, 2009). When mTORC1 is activated, it phosphorylates the downstream targets, P70 ribosomal S6 kinase 1 (S6K1), and the eukaryotic initiation factor 4E (eIF4E)- binding protein 1 (4E-BP1) (Fingar, Salama, Tsou, Harlow & Blenis, 2002). Thus, mTORC1 activation results in stimulating protein synthesis (Bodine et al., 2001). Because of the TOS motif found in S6K1 N-terminal and 4EBP1 C-terminal, mTORC1 is able to phosphorylate these substrates by associating Raptor with TOS motif (Schalm & Blenis, 2002). The activated S6K1 is responsible of stimulating the translation of mRNA (Ma, Yoon, Richardson, Jülich & Blenis, 2008). The un-phosphorylated 4EBP1 is considered a repressor for translation as it restrains the eukaryotic translation initiation factor 4E (eIF4E) from interacting with other factors that promote protein synthesis (Bodine et al., 2001; Gingras, Kennedy, O’Leary, Sonenberg & Hay, 1998). When mTOR phosphorylates 4EBP1, it results in the release of eIF4E from 4EBP1. This allows eIF4E to bind to other translation factors (Bodine et al., 2001). mTOR is not only responsible for stimulating ribosomal biogenesis, but also

stimulating elongation (Kim, 2009). This is achieved by inhibiting the function of the eukaryotic elongation factor2 (eEF2) kinase, which is the translation inactivating kinase, through the phosphorylation of eEF2 by S6K1 (Figure 1-1) (Redpath, Foulstone & Proud, 1996).

#### **1.2.4. Arginine and protein synthesis in neonates.**

Yao et al. (2008) suggested that supplementing the diet with arginine increases piglet growth by activating the mTOR signaling pathway. They reported that arginine supplemented at 6% to a milk replacer diet enhanced the daily body weight gain and the skeletal muscle protein synthesis. The stimulation of skeletal protein synthesis was via the phosphorylation of mTOR and 4E-BP1 which in turn increased the association of eIF4G with eIF4E to stimulate protein synthesis (Yao et al., 2008). Furthermore, arginine has been shown to stimulate protein synthesis in the neonatal small intestine. The effect of arginine on growth and protein synthesis was studied *in vitro* in neonatal porcine intestinal columnar epithelial cells (IPEC-J2). At physiological concentrations (0.1–0.5 mmol/l), arginine increased the protein synthesis in cells via the stimulation of the mTOR pathway and the phosphorylation of downstream targets P70S6K and 4E-BP. Moreover, when IPEC-J2 cells were treated with rapamycin, protein synthesis was inhibited by 42%, suggesting that protein synthesis in the intestine was partially controlled by the mTOR pathway (Bauchart-Thevret, Cui, Wu & Burrin, 2010). Another study evaluated the effect of arginine on intestinal protein synthesis in neonatal piglets with rotavirus enteritis. Arginine was supplied to a standard milk replacer diet at 0.4 g. k<sup>-1</sup>. d<sup>-1</sup> with or without rapamycin and compared to controls. The intestinal protein synthesis was increased by 1.3 fold with arginine; also, arginine stimulated the activation of mTOR/p70S6k throughout the villus. However, the intestinal protein synthesis was inhibited when rapamycin was included, suggesting that arginine may be responsible for the

intestinal protein synthesis by activating p70S6k (Corl et al., 2008).

In summary, arginine has a key role in stimulating protein synthesis via activating the mTOR pathway. As such, adequate arginine available to the intestinal enterocytes is of significant importance, particularly during intestinal injury in neonates.

#### **1.2.5. Rapamycin as inhibitor for protein synthesis.**

Rapamycin is used as an anticancer agent, and is used to treat a number of different diseases such as coronary restenosis and rheumatoid arthritis (Laplante & Sabatini, 2009). The macrocyclic lactone rapamycin is derived from the bacterial species Streptomyces hygroscopicus which is found in soil. The first use of rapamycin was as an anti-fungal agent; however, recent studies suggest that rapamycin is also effective as an immunosuppressor (Zhou et al., 2010). Studies demonstrated that rapamycin forms a complex by binding to the intracellular receptor FKBP12. The formed complex then binds to the FKBP12-rapamycin binding domain at the TOR proteins (Chen, Zheng, Brown & Schreiber, 1995; Choi, Chen, Schreiber & Clardy, 1996; Helliwell et al., 1994; Kunz & Hall, 1993). Thus, the signaling of TOR protein is inhibited which in turn prevents the phosphorylation of the downstream substrates (Zhou et al., 2010). This inhibitory effect may be caused by disrupting the binding of raptor to mTORC1 which inhibits substrate binding (Oshiro et al., 2004) (Figure 1-1). Thus, rapamycin is a useful tool to study factors which may alter the activity of the mTOR pathway, and influence the rate of protein synthesis in the small intestine.

### **1.3. Protein digestion and absorption.**

#### **1.3.1. Protein digestion process.**

Protein digestion is initiated in the stomach by the action of proteases. The predominant protease is pepsin, which is released from gastric cells in the form of a zymogen, pepsinogen, and is hydrolyzed to pepsin in the acidic gastric environment (Hinsberger & Sandhu, 2004; Kiela & Ghishan, 2016). Proteolytic activity produces a small amount of free amino acids, with the majority of the protein leaving the stomach and enters the duodenum as polypeptides (Hinsberger & Sandhu, 2004). The pancreas also contributes to protein digestion by secreting pancreatic enzymes in an inactive form. Pancreatic proteases are activated once they reach the duodenum by the brush border enzyme enteropeptidase. Furthermore, enteropeptidase activates trypsin, which in turn activates the other pancreatic proteases including elastase, chymotrypsin, and carboxypeptidases A and B. The main function of these enzymes is to hydrolyze the specific peptide bonds, resulting in free amino acids and oligopeptides with two to six amino acids residues. Mucosa-bound peptidases further hydrolyze the short peptides (Erickson & Kim, 1990). The end products of protein digestion are free amino acids, and oligopeptides (dipeptides, and tripeptides), which then are transported into the intestinal enterocyte by specific transporters on the brush border (Erickson & Kim, 1990; Hinsberger & Sandhu, 2004).

#### **1.3.2. Amino acids absorption at small intestine.**

Nutrient absorption takes place over the length of the small intestine. However, the main location for peptide and free amino acids uptake occurs in the proximal jejunum (Bröer, 2008; Kiela & Ghishan, 2016). The uptake of amino acids by intestinal enterocytes requires transporting across the brush-border membrane by a passive or an active transporter (Kiela & Ghishan, 2016;



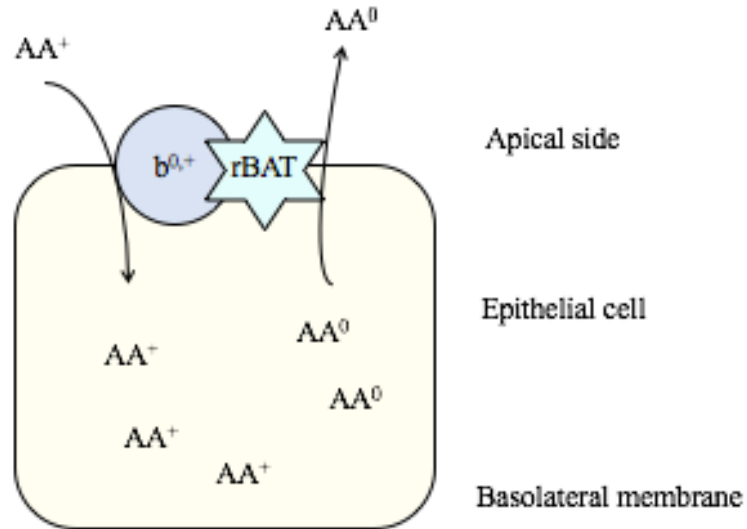
Stevens, Kaunitz & Wright, 1984). Amino acids may be utilized by the cell, or transported out of the cell through the basolateral membrane into the portal blood (Stevens et al., 1984). Once in the portal blood, amino acids may be picked up by the liver or distributed to peripheral tissues (Bröer, 2008; Elwyn, Parikh & Shoemaker, 1968).

### **1.3.3. Transporting free amino acids at brush border membrane.**

The process of transporting amino acids in the brush border membrane is complex as it involves multiple transporters with different affinities to amino acids (Kiela & Ghishan, 2016). The brush border membrane has different amino acid transporter systems which include the neutral system ( $B^0$ ), the basic system ( $b^{0,+}$ ), the acidic system ( $X^-_{AG}$ ), the iminoglycine system (IMINO), and the  $\beta$ -amino acid system ( $\beta$ ) (Bröer, 2008). The neutral system ( $B^0$ ) is  $Na^+$  dependent, and is responsible for transporting neutral L-amino acids such as glycine, isoleucine, leucine, methionine, valine, phenylalanine, tryptophan and tyrosine (Bröer, 2008; Kiela & Ghishan, 2016; Sloan & Mager, 1999). The basic system ( $b^{0,+}$ ) is a  $Na^+$ -independent transporter that carries neutral and cationic amino acids such as lysine, arginine, ornithine as well as cysteine. Additionally, the acidic system ( $X^-_{AG}$ ) is a  $Na^+$ -and  $H^+$ -dependent carrier that transports anionic amino acids, especially aspartate and glutamate. Furthermore, the iminoglycine system (IMINO) is a  $Na^+$ - and  $Cl^-$ -dependent transporter and it is responsible for transporting imino acids including proline, hydroxyproline, and glycine. The  $\beta$ -amino acid system ( $\beta$ ) is a  $Na^+$ - and  $Cl^-$ -dependent transporter that carries  $\beta$ -alanine and taurine (Bröer, 2008 ; Kiela & Ghishan, 2016).

### **1.3.3.1. rBAT/b<sup>0,+</sup> for transporting lysine, arginine, ornithine, and cysteine.**

The main locations of the b<sup>0,+</sup>, and neutral and basic amino acid transporter (rBAT) proteins are the apical membrane of the epithelial cells of the small intestine and renal proximal tubules (Feliubadaló et al., 1999; Furriols et al., 1993; Pickel et al.1993; Mizoguchi et al., 2001). The b<sup>0,+</sup> transporter mediates the obligatory exchange of dibasic amino acids. This can be explained by the greater amino acid efflux binding affinity compared to the amino acid influx (Torras-Llort et al., 2001). The association of b<sup>0,+</sup> with another amino acid transporter rBAT to form heterodimeric complex is necessary to fully activate b<sup>0,+</sup> for transporting cystine and dibasic amino acids (Sakamoto et al. 2009) (Figure 1-2). The heavy chain rBAT and light chain b<sup>0,+</sup> are connected by disulfide bonds (Chairoungdua et al., 1999). The heavy chain belongs to type II membrane protein, and it has one transmembrane domain and a large extracellular domain (Palacín & Kanai, 2004). The light chain such as b<sup>0,+</sup> AT, on the other hand, forms 12 putative transmembrane domains. The main function of the heavy chain is to facilitate the heterodimeric complexes targeting by the plasma membrane (Verrey et al., 2004). In contrast, the light chain forms the catalytic subunits, which function in substrate binding and transporting (Reig et al., 2002). Therefore, at the brush border of the small intestine, arginine and lysine are transported into the epithelial cells via rBAT/b<sup>0,+</sup> to be used by the cell or to cross the basolateral membrane for distribution by the portal vein.



**Figure 1- 2:** The association of  $b^{0,+}$  with  $rBAT$  to form heterodimeric complex. The heterodimeric complex is necessary to fully activate  $b^{0,+}$  for transporting cystine and dibasic amino acids through an obligatory exchange system. Abbreviation:  $AA^+$ , dibasic free amino acids,  $AA^0$ , neutral free amino acids.

#### 1.4. Oligopeptides absorption at the small intestine.

##### 1.4.1. Oligopeptides transporting mechanisms.

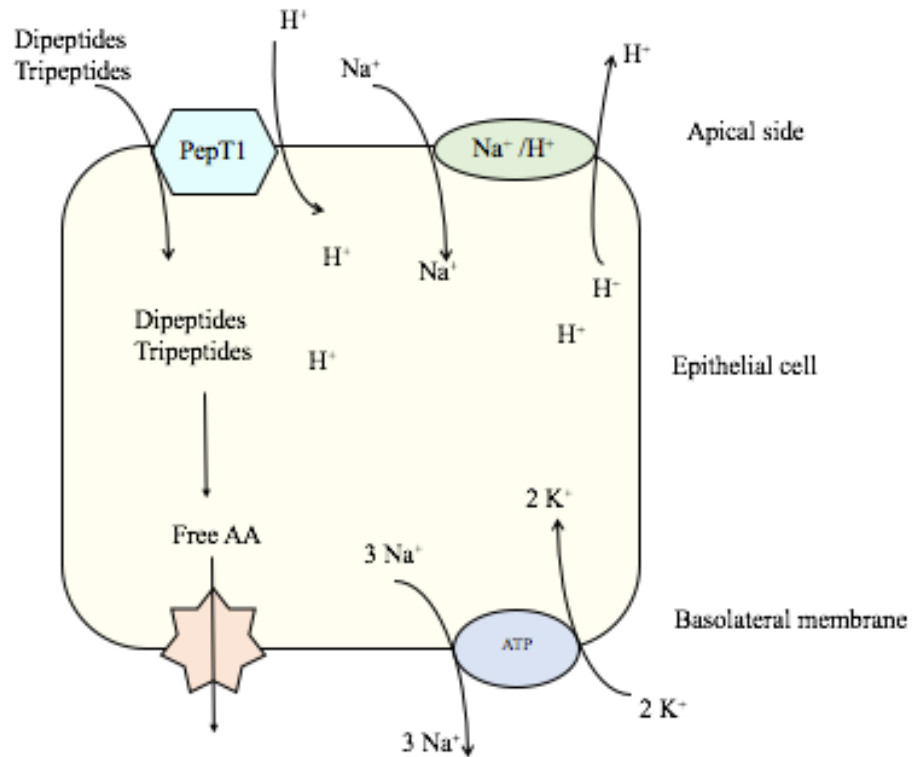
The intestinal uptake of oligopeptides occurs through one of the three transport pathways which include the paracellular pathway, the cell penetration peptides pathway, and the PepT1 mediated pathway (Gilbert, Wong & Webb, 2008). The paracellular pathway is defined as the movement of particles between cells, through the tight junctions (Gilbert et al., 2008). The cell

penetration peptides (CPPs) refer to the short sequences of peptide, whose function is to transport cargo in the form of peptides from the lumen to inside the cell (Gilbert et al., 2008; Munyendo, Lv, Benza-Ingoula, Baraza & Zhou, 2012). The paracellular pathway, and the cell penetration peptides pathway will not be discussed in this thesis.

#### **1.4.2. PepT1- Mediated Pathway.**

Peptide transporter 1 (PepT1) (SLC15A1) belongs to the proton-coupled oligopeptide transporter superfamily (Gilbert et al., 2008). PepT1 is located on the apical surface of intestinal epithelial cells (Saito & Inui, 1993). It is responsible for transporting di- and tri-peptides into the cells (Freeman, Bentsen, Thwaites & Simmons, 1995). PepT1 is proton ( $H^+$ ) coupled transporter, and as such, the  $H^+$  gradient affects the activity of the transporter; an inward  $H^+$  gradient results in movement of oligopeptides. Moreover, PepT1 activation is influenced by the intracellular membrane potential. In other words, negative intracellular membrane potential activates PepT1, while a positive membrane potential deactivates it (Leibach & Ganapathy, 1996). When peptides along with  $H^+$  are transported into the enterocyte, the proton ( $H^+$ ) is pumped out of the cell through a  $Na^+ /H^+$  exchanger. This  $Na^+ /H^+$  exchanger is also located on the intestinal brush border membrane, and it exchanges the intracellular  $H^+$  for extracellular  $Na^+$ .  $Na^+$  inside the cell is transported across the basolateral membrane via  $Na^+ /K^+$  ATPase pump. This assists in the exchange of three intracellular  $Na^+$  molecules for two extracellular  $K^+$ , which in turn recovers the electrochemical gradient. When peptides are taken up by the intestinal cell, some are subjected to hydrolysis through intracellular peptidases to produce free amino acids. Free amino acids are then transported through amino acid transporters on the basolateral membrane. Alternatively, peptides that are resistant to hydrolysis are also transported at the basolateral membrane, but via a “carrier-

mediated mechanism” for peptide transport, that has not yet been clearly described (Gilbert et al., 2008) (Figure 1-3).



**Figure 1- 3:** PepT1- Mediated Pathway. PepT1 is proton (H<sup>+</sup>) coupled transporter, and it is responsible for transporting di- and tri-peptides into the cells. Abbreviation: AA, amino acid.

#### 1.4.2.1. PepT1 structure.

PepT1 is a 127 kDa protein that has histidyl groups as a catalytic domain (Daniel, 1996; Daniel, Spanier, Kottra, & Weitz, 2006; Fei et al., 1997). The histidyl catalytic domain is required for PepT1 activity in which the histidine side chain imidazole residue acts as a proton acceptor/donor (Leibach & Ganapathy, 1996). According to the hydropathy prediction, PepT1 has 12 transmembrane domains including large extracellular hydrophilic loops within domains nine

and ten (Daniel, 1996). In the luminal side, six putative N-glycosylation sites are located on the extracellular loop (N50, N406, N439, N510, N515, N532) (Stelzl, Baranov, Geillinger, Kottra & Daniel, 2016). In addition, the phosphorylation locations for protein kinase C (PKC) and A (PKA) are in the intracellular loop, in which phosphorylation of these promotes the highest rate of transport (Brandsch, Miyamoto, Ganapathy & Leibach, 1994; Van, Pan, Webb & Wong, 2005). Each terminus in PepT1 serves a significant function in substrate binding. One to six transmembrane domains form the N-terminus half which is involved in pH changes when substrate binds and peptide is transport through PepT1 (Terada, Saito, Sawada, Hashimoto & Inui, 2000). Furthermore, seven to nine transmembrane domains form the C-terminus and controls and set the substrate affinity (Fei et al., 1998).

#### **1.4.2.2. Transporter Affinity for Specific Peptides.**

There are some features that are necessary for substrate binding to PepT1 (Brandsch, Knütter & Leibach, 2004). The charge on a peptide appears to affect its affinity for transport via PepT1. Neutral peptides have a higher affinity for PepT1 compared to positively or negatively charged peptides (Eddy, Wood, Miller, Wilson & Hidalgo, 1995). This was illustrated in Caco-2 cell monolayers in which the affinity for the cationic dipeptide lys-lys was lower than neutral dipeptides (Eddy et al., 1995). Furthermore, the intestinal PepT1 transporter is stereospecific, and it has much higher affinity for lys-lys dipeptides (or lys-lys-lys-tripeptides) than for peptides with D-amino acids (Brandsch et al., 2004). Lister, Sykes, Bailey, Boyd & Bronk, (1995) conducted an experiment using isolated rat jejunum perfused with dipeptides that were combinations of D- or L- alanine and/ or phenylalanine. They observed that dipeptides containing L-alanine at the N-terminus position were transported efficiently even with D-phenylalanine in the C-terminus

position; however, if both amino acids were D-isomers, transport was negligible. This suggests that the chirality of amino acids is a contributing factor for PepT1 affinity, but transport of D-amino acids appears possible as part of a peptide in the C-terminus position (Lister et al., 1995). Also, large hydrophobic amino acids at the N-terminus have high affinity for PepT1 (Vig et al., 2006). For example, the binding affinity for Phe-Gly, Tyr-Gly, and Trp-Gly were higher compared to Gly-Gly. Similarly, when the C-terminal was a large hydrophobic amino acid, the dipeptide affinity for PepT1 was enhanced. For instance, enhancing Gly-X dipeptides affinity to PepT1 was linked to increasing the bulkiness and hydrophobicity on position 2. As a result, the affinities of Gly-Tyr and Gly-Phe were higher compared to Gly-Gly, Gly-Asp, and Gly-Glu (Vig et al., 2006). Aromatic amino acids, and proline on the C-terminus were also known to increase the binding affinity with PepT1 (Vig et al., 2006).

#### **1.4.2.3. PepT1 Distribution.**

PepT1 distribution varies within the small intestine among species (Gilbert et al., 2008). PepT1 was found to be expressed throughout the length of the small intestine in mature dairy cows, sheep, pigs, and chickens (Chen, Wong, & Webb, 1999). In chickens, duodenum had the highest PepT1 expression, while in pigs, PepT1 was expressed in jejunum, and in ruminants, both jejunum and ileum expressed PepT1 (Chen et al., 1999). Such observations may be the result of high rates of protein digestion and absorption at these sites of the small intestine (Chen et al., 1999). In a human, PepT1 was found to be expressed along the length of digestive system with high expression in duodenum and jejunum (Terada et al., 2005). This is attributed to the fact that the majority of the digested protein in the intestinal lumen is in the form of oligopeptides (Terada et al., 2005). In addition, the cytoplasm of the mucosal cells is the major site where the cleavage of di/tripeptides

to free amino acids occurs (Kim, Birtwhistle & Kim, 1972; Terada et al., 2005). As a result, the high PepT1 expression in the upper small intestine illustrates the significance of peptide absorption from digested protein to sustain protein nutrition. (Terada et al., 2005). In rabbit small intestine, the expression of PepT1 m-RNA was mostly located in duodenum and jejunum while ileum had the lowest expression. PepT1 expression is also found in the colon; however, it was very low compared to the small intestine (Freeman et al., 1995). In rats, the highest PepT1 expression was observed in four day old rats and declined by 28 days. Furthermore, at four days of age, PepT1 mRNA expression was 3.6 fold higher than in adult rats, which indicates the importance of peptide transportation during neonate life (Miyamoto et al., 1996). In young Yucatan piglets, PepT1 expression and dipeptide uptake were reported during suckling age (1,2,3 weeks), and after weaning (6 weeks) (Nosworthy, Bertolo and Brunton, 2013). Piglets at suckling age showed the ability to transport dipeptide in both proximal and distal small intestines. Also, at suckling age, the colon expressed PepT1 mRNA. This adaptation is important since intestinal peptide absorption is crucial in the suckling period. Interestingly, post-weaning, the capacity for dipeptide uptake was high in the ileum relative to pre-weaned piglets. The change in the digestibility of the diet likely contributed to the expression of PepT1 in the ileum. Compared to sow milk, feeding a grain-based diet was much less digestible, so the lower gut was exposed to the protein hydrolysis products which may promote PepT1 expression (Nosworthy et al., 2013).

#### **1.4.2.4. Factors affecting PepT1 function and activity.**

There are a variety of health or environmental factors that may affect PepT1 activity in the small intestinal epithelia such as diet, hormones, age, and malnutrition. Diet is one of the influencing factors that affects PepT1 expression in the small intestine. A high protein meal



produces large amounts of di- and tripeptides following digestion. The high concentration of luminal di- and tripeptides enhances the PepT1 gene expression, resulting in greater di- and tripeptides transportation into the epithelial cells (Adibi, 2003). An experiment was conducted by Erickson and his colleagues (1995) to compare PepT1 expression in rat intestines following low and high protein diets (Erickson, Gum, Lindstrom, McKean & Kim, 1995). They reported a 1.5- 2 fold increase in PepT1 expression in the high protein diet group compared to the low protein group (Erickson et al., 1995). These results indicate that the high protein diet functioned as a substrate which induced PepT1 expression (Erickson et al., 1995).

Hormones may also contribute to increasing peptide uptake by intestinal epithelial cells (IECs). In an *in vitro* study using Caco-2 cells, exposure to insulin triggered the uptake of glycyl-glutamine when insulin was provided at physiological concentrations. The authors suggested that the elevation of PepT1 population in the epithelial apical membrane was via the enhancement of the translocation of PepT1 from a preformed cytoplasmic pool (Thamotharan, Bawani, Zhou, & Adibi, 1999 b).

The nutritional status of the animal may alter the expression of PepT1 in the small intestine (Thamotharan, Bawani, Zhou & Adibi, 1999 a). The effects of fasting on PepT1 expression was assessed in a study on rats. Rats were divided into two groups that were fed a standard diet or fasted for one day, and then brush-border membrane vesicles (BBMVs) were prepared from the harvested rat jejunum to measure glycyl-glutamine (Gly-Gln) absorption. Even though short term fasting (one day) resulted in a reduction in the mucosal cell mass, the uptake (Gly-Gln) was

enhanced by two fold. Also, the PepT1 population and gene expression were three-fold higher in tissue from the fasted compared to the fed rats (Thamotharan et al., 1999 a).

Total parenteral nutrition (TPN) has also been studied as a factor that affects the intestinal PepT1 expression (Ihara, Tsujikawa, Fujiyama & Bamba, 2000). The effect of a TPN diet was examined by Ihara and his colleagues (2000) who fed Sprague-Dawley rats by TPN for 10 days. Results indicated that the TPN diet enhanced the PepT1 mRNA expression by 161% in jejunum, regardless of the intestinal atrophy that was caused by TPN feeding. They attributed the rise in PepT1 expression in the intestine to the inadequate delivery of nutrition by this mode of feeding (Ihara et al., 2000).

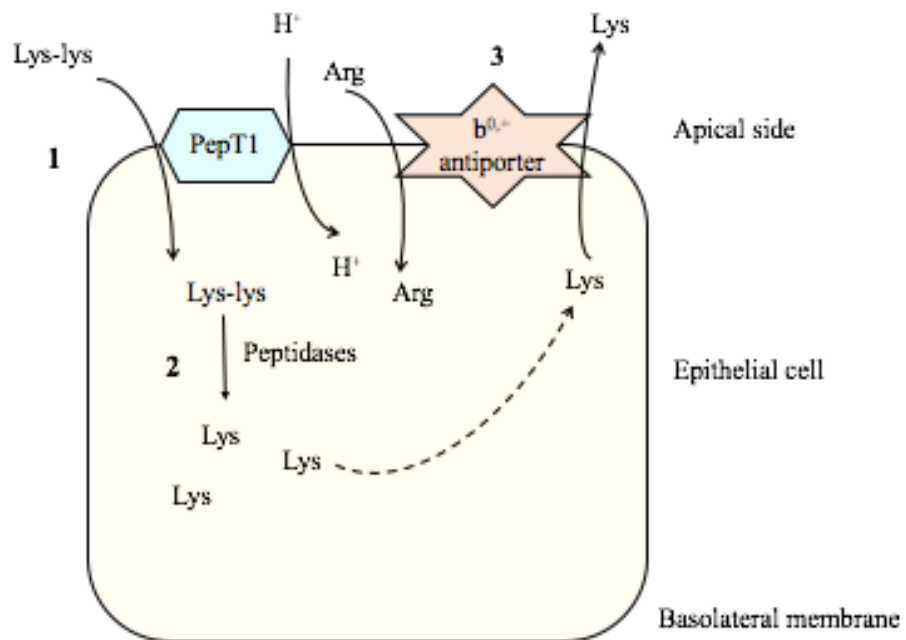
#### **1.4.3. Nutritional significance of small chain amino acids absorption.**

Compared to the free amino acids transporters, PepT1 is an effective method for transporting amino acids. The energy required for the transport of two or three amino acids into a cell is equal to that utilized for transporting a single amino acid. Moreover, the time required for transporting an equivalent amount of nitrogen as di- and tripeptides is less than that for the same composition of free amino acids, due to the rapid rate of absorption (Gilbert et al., 2008). A particular benefit of peptide transport is that patients with free amino acids transporter defects are capable of absorbing essential amino acids through PepT1 which avoids amino acids deficiencies (Adibi, 1997). A common example is Hartnup disease, which is characterized by a disorder in the neutral amino acids transporter located in the small intestine and kidney. Patients with Hartnup disease are able to sustain a good nutritional state, despite the neutral amino acids transporter defect (Leonard et al., 1976).

### **1.5. Role of PepT1 in trans-stimulation of $b^{0,+}$ antiporter.**

Early evidence investigating amino acid and peptide transport suggested that the presence of peptides inhibited free amino acids uptake in the small intestinal cells (Fairclough et al., 1977). This was concluded based on a study that used a human intestinal perfusion model to measure free amino acids uptake by enterocytes when provided with high concentration of dipeptide. A thirty centimeter isolated segment of jejunum was perfused with either free amino acids mixture (24.9 mmol/l) alone, or with high concentration of glycyl-glycine (100 mmol/l). During the perfusion process, samples from the perfused buffers were taken to measure amino acids and peptide concentrations. Researchers observed that free amino acids uptake was lower when provided with Gly-Gly compared to when provided alone. Although free amino acids and peptides were transported via different systems, Gly-Gly was able to inhibit the uptake of free amino acids (Fairclough et al., 1977). The inhibitory effect of the dipeptide Gly-Gly on free amino acids uptake was attributed to the free glycine that was released from Gly-Gly at the time of perfusion. When the researchers analyzed the lumenally perfused buffers, they observed that Gly-Gly was partially hydrolyzed to free glycine which resulted in 18.6 mmol/l of free glycine and 60.7 mmol/l of the dipeptide Gly-Gly. They were unable to determine if the generated free glycine was from the actions of brush border or cytoplasmic peptidase. The concentration of free glycine from Gly-Gly was much higher than the concentration of free amino acids; therefore, the high free glycine concentration may have caused the inhibitory effect on free amino acids uptake (Fairclough et al., 1977). However, recent studies have not supported this conclusion. A study by Wenzel, Meissner, Döring & Daniel, (2001) assessed the interaction between cationic amino acid transport and PepT1 by using human intestinal Caco-2 cells. This group measured the uptake of the free L-arginine (10 mmol/l) when incubated with specific dipeptides (10 mmol/l). In these experimental conditions,

arginine absorption was not inhibited by dipeptides. Actually, the arginine uptake into cells was greater in the presence of dipeptides. They ascribed the findings to an interesting mechanism called trans-stimulation of the  $b^{0,+}$  antiporter. The mechanism involves peptide transporter 1 (PepT1), which transports dipeptides into the enterocytes. Intracellularly, the dipeptide may be hydrolyzed to free amino acids, increasing the concentrations of free amino acid inside the cell. This, in turn, stimulates the  $b^{0,+}$  antiporter, which initiates the exchange of the intracellular amino acid for luminal amino acid (Figure 1-2). The rate limiting step in trans-stimulation was the dipeptide hydrolysis inside the cell (Wenzel et al., 2001). This was determined when cells were pre-incubated with a hydrolysis resistant dipeptide, and the effect of trans-stimulation was inhibited, such that arginine uptake was not enhanced (Wenzel et al., 2001). Hence, PepT1 can provide greater amino acid absorption in two ways when compared to the other amino acids transporters.



**Figure 1- 4:** Trans-stimulation of  $b^{0,+}$  antiporter. (1) Peptide transporter 1 (PepT1) facilitates the uptake of lys-lys into enterocytes. (2) Lys-lys may be hydrolyzed to free lysine, increasing its concentration inside the cell. (3) This in turn stimulates  $b^{0,+}$  antiporter which initiates the exchange of the intracellular lysine for luminal arginine.

Initially, PepT1 rapidly transports peptides that contain essential amino acids; as a result, it reduces competition among amino acids for a common transporter. Then, the intracellular hydrolysis of peptides increases the free amino acid concentration which initiates the trans-stimulation process, enabling greater free amino acid uptake (Wenzel et al., 2001). Another study also measured the permeability of gabapentin, which is an amino acid-like drug, using an *in vivo* intestinal perfusion model in rats. Nguyen and colleagues (2007) observed that the permeability of gabapentin was

enhanced when it was perfused with glycyl-glutamate (Gly-Glu) dipeptide. The authors suggested that when Gly-Glu is taken into the cell through PepT1, it may be hydrolyzed to glycine and glutamate. Glutamate may be converted into glutamine which may have been responsible for activating the trans-stimulation of  $b^{0,+}$  antiporter. Thus, glutamine may be exchanged with luminal gabapentin (Nguyen, Smith & Fleisher, 2007).

### **1.5.1. Trans-stimulation of $b^{0,+}$ antiporter in a neonatal model.**

Most of the experiments that investigated the interaction between dipeptides and free amino acids were conducted *in vitro* (Wenzel et al., 2001). The trans-stimulation effect of the  $b^{0,+}$  antiporter was also studied by our research group *in vivo* using a piglet model (Tennakoon, Bertolo & Brunton, 2011). That research assisted in understanding the interactions between dipeptides and free amino acids in a physiologically relevant model for human infants. By using an *in situ* gut loop perfusion model in Yucatan miniature piglets, L- arginine absorption was measured when perfused alone or with different concentrations of either lysyl-lysine, or lysyl-glycine (Tennakoon et al., 2011). Tennakoon et al. (2011) hypothesized that when arginine was perfused simultaneously with lysyl-lysine, arginine uptake by jejunal intestinal cells would be greater compared to when perfused alone or with free lysine. Results demonstrated that perfusing 10 mmol/l arginine with 20 mmol/l lysyl-lysine resulted in 181% increase in arginine uptake by intestinal cells compared to when arginine was perfused alone. They attributed the increase in arginine uptake to the trans-stimulation of  $b^{0,+}$  antiporter which facilitated the exchange of intracellular lysine for luminal arginine. However, compared to arginine alone, arginine uptake was not significantly improved (139%) when only 10 mmol/l lysyl-lysine was perfused with 10 mmol/l arginine. They suggested that an important element for the trans-stimulation of  $b^{0,+}$

<sup>+</sup>antiporter was the intracellular free amino acids concentration; 10 mmol/l lysyl-lysine may not have raised the intracellular free amino acids concentration above that threshold. Similarly, 50 mmol/l lysyl-lysine did not result in the enhancement of arginine uptake (131%) by the intestinal cells. They observed that during the perfusion process, a significant amount of the lysyl-lysine underwent intraluminal hydrolysis producing high amounts of free lysine. Arginine and the high free lysine concentration are believed to have competed for the same transporter which could have resulted in undermining the enhancement of arginine uptake. The lysine concentration from lysyl-lysine hydrolysis in the 10 mmol/l and 20 mmol/l lysyl-lysine conditions was low and likely not sufficient to compete with arginine (Tennakoon et al., 2011). Lysyl-glycine dipeptide was also used in the perfusion procedure to determine whether the cationic free amino acids concentrations had an effect on triggering the trans-stimulation of the antiporter  $b^{0,+}$ . Enhanced arginine absorption was not observed when lysyl-glycine at different concentrations was perfused with arginine. The glycine concentration in mucosal tissue did not increase in parallel with the perfused lysyl-glycine concentration. This result may be attributed to the hydrolysis resistant lysyl-glycine dipeptide which effectively would lower the intracellular free lysine concentration and prevent the trans-stimulation of antiporter  $b^{0,+}$  (Tennakoon et al., 2011).

To investigate the effects of both the intracellular hydrolysis of transported dipeptide and the intracellular concentration of free amino acid, the gut was perfused with 10  $\mu$ mol/l amastatin which is an aminopeptidase inhibitor. Under the same experimental conditions but with the inhibitor, the enhanced uptake of arginine with 20 mmol/l lysyl-lysine did not occur. The inhibition of the enhanced arginine uptake with the peptidase inhibitor supports the role of dipeptides in inducing trans-stimulation of the antiporter  $b^{0,+}$  (Tennakoon et al., 2011).

In summary, simultaneous delivery of 10 mmol/l arginine with 20 mmol/l lysyl-lysine resulted in enhanced arginine uptake by the intestinal cells via the trans-stimulation of antiporter b<sup>0</sup> +. Delivering dietary amino acids as a combination of peptides and free amino acids may be beneficial for neonates to facilitate greater uptake by the intestinal mucosa, particularly for important amino acids such as arginine.

### **1.6. Arginine and lysine antagonism.**

Lysine and arginine antagonism may arise when lysine or arginine is provided in the diet in excess. Providing excess arginine or lysine may influence the metabolism of the other amino acid resulting in higher requirements (Ball, Urschel & Pencharz, 2007). Lysine is an essential amino acid, and in standard pig feeds, lysine is the first limiting amino acid due to the low concentration of lysine in cereal grain diets (Ball et al., 2007; Liao, Wang & Regmi, 2015). Lysine deficiency will negatively affect growth rate, and body weight (Chang & Wei, 2005). Lysine is involved in the synthesis of protein, peptides, non-peptide molecules, and carnitine (Liao et al., 2015; Wu, 2013). Also, lysine has a key role in regulating the metabolism of other amino acids (Shikata et al., 2007). Therefore, providing an ideal concentration of lysine is of particular importance in swine nutrition.

Lysine-arginine antagonism has been reported for many different animals including rats, chicks, dogs, and guinea pigs (Austic & Scott, 1975; Baker, 1987; Czarnecki, Hirakawa & Baker, 1985; Ulman, Kari, Hevia & Visek, 1981). Lysine-arginine antagonism was attributed to the competition between lysine and arginine for uptake in the kidney and the intestine (Czarnecki et al., 1985). All



observations demonstrated that supplementing the diet with excess lysine caused lower growth rate which was reversed when arginine was supplemented (Czarnecki et al., 1985; Ulman et al., 1981). Interestingly, lysine-arginine antagonism is controversial in young pigs. Supplementing excess arginine to pigs had adverse effects on lysine in term of decreased feed intake (Hagemeier, Libal & Wahlstrom, 1983). Furthermore, supplementing a large amount of arginine to piglet diets had a negative effect on feed intake and weight gain. Moreover, adding lysine to the diet with excess arginine did not improve the negative effect of arginine (Southern & Baker, 1982). Baker (1987) also investigated the effect of supplementing lysine above the basal level on weight gain and feed efficiency. The basal diet was composed of 1.15% lysine and 0.53% arginine, which were within the range of requirement levels; lysine was added at 1.15%, 2.3% or 3.45% to the basal diet. Weight gain and feed intake were decreased in the basal diet supplemented with 2.3% and 3.45%, while it was unaffected in the 1.15% diet when compared to the basal diet. However, the feed efficiency was similar among all groups supplemented with different lysine levels. Even though supplementing excess lysine resulted in an increase in plasma lysine concentration, it had no effect on plasma concentration of arginine, ornithine, and histidine. This indicates that lysine-arginine imbalance is the main cause for growth depression, and it is not due to the lysine-arginine antagonism. Another study that was conducted by Tennakoon et al., (2011) examined the effect of perfusing 10 mmol/l arginine with 20 mmol/l free lysine in a gut loop model in piglets. Arginine uptake was not impeded when compared to perfusing arginine alone, which suggests that lysine did not antagonize arginine. Even when arginine was perfused with 50 mmol/l of lysyl-lysine which had undergone significant hydrolysis, it did not reduce arginine uptake compared to when arginine was perfused alone. Thus, no lysine-arginine antagonism was apparent (Tennakoon et al., 2011).

While competition between lysine and arginine for uptake from the intestinal lumen may or may not be a problem for rapidly growing neonates, both are very important amino acids; a deficiency in either would interfere with optimal protein synthesis and growth. In the preterm human infant, the risk of arginine deficiency is high, and can have potentially devastating consequences. In the weaning piglet, the change from sow milk to solid feeding represents a period of intestinal injury, and growth faltering and diarrheal diseases are common at this time. Providing arginine or lysine as free amino acids in the diet may be beneficial, but is expensive and providing one in excess may create negative effects on the metabolism of the other amino acids. To that end, providing lysine as a dipeptide may enhance arginine uptake while optimizing overall nitrogen retention. A specific advantage of this combined approach in situations of intestinal injury in neonates, such as during infection or following IV feeding, may be enhanced uptake by the mucosa, leading to improved GI recovery and growth.

### **1.7. The piglet as a research model.**

Piglets are one of the best non-primate models to study neonatal nutrition (Puiman & Stoll, 2008). Pigs and humans share similar anatomy of the gastrointestinal tract, with similar metabolic processes and proportionately similar nutrient requirements (Guilloteau, Zabielski, Hammon & Metges, 2010; Shulman, Henning & Nichols, 1988). Also, the distribution and activities of intestinal enzymes in the neonatal piglet small intestine resembles those in human infants, all of which supports the use of the neonatal pig to study human intestinal development (Shulman et al., 1988). With respect to nutrient uptake, pigs and human share many of the same intestinal transporters. Specific to amino acid transport, Zhi et al. (2008) found that higher b<sup>0,+</sup> AT mRNA

expression occurred in the jejunum of piglets between 1 to 26 days of age, and it shared 88.9% amino acid homology with the human transporter (Zhi et al., 2008). Moreover, PepT1 expression was reported in piglets at suckling and after weaning throughout the small intestine (Nosworthy et al., 2013). Because neonatal piglets and human infants share the same amino acids transporters in the GIT, the piglet is a suitable model to study amino acid uptake in human infants.

### **1.8. The *in vivo* intestinal perfusion model.**

The gut loop model was developed to investigate the interaction between nutrients, transporters and gut functions. Adegoke, McBurney & Baracos, (1999) validated the gut loop model in a study that isolated multiple intestinal segments in six week old piglets. Intestinal segments were exposed to different nutrients via the lumen to study the effect on mucosal protein synthesis. A study from our lab adapted the *in situ* gut loop model to measure PepT1 expression and the disappearance of the dipeptide glycyl-sarcosine from isolated small intestinal segments at suckling and post-weaned ages (Nosworthy et al., 2013). The gut loop model was also used to investigate the interaction between arginine and lysyl-lysine in the intestinal mucosal in miniature pigs (Tennakoon et al., 2011). The gut loop model involves isolating segments of small intestine by ligature, with inlet and outlet cannulas inserted for continuous perfusion of a nutrient solution; the blood supply remains intact (Tennakoon et al., 2011). The isolated loops in this model represent only a small portion of the animal's total absorptive capacity; therefore, the perfused treatments should not have large systemic effects. The gut loop model permits the comparison of a number of varying treatments within the same animal, as multiple treatments are perfused concurrently. Therefore, a lower number of animals is required for each study, and each animal can serve as its

own control (Adegoke et al., 1999). In conclusion, the gut loop model has several advantages, and was validated as an appropriate model for the study of gut functions.

### **1.9. Flooding dose to measure the rate of protein synthesis.**

The flooding dose technique has been widely used to measure protein synthesis. The name “flooding dose” was devised because the method involves a “rapid injection of a large dose of tracee amino acid along with tracer amino acid” (El-Khoury, 1999). The large dose of amino acid results in flooding the free amino acid pool which serves to decrease the disparity between the free amino acid specific radioactivity in the extracellular and intracellular pools, as well as the amino acid acylated tRNA (El-Khoury, 1999). In other words, the flooding dose will flood every potential precursor pool (Garlick, Mcnurlan & Preedy, 1980). The utilization of the flooding dose eliminates the need to estimate the tracer’s specific radioactivity in the aminoacyl tRNA which is the “true precursor pool for protein synthesis” (Schaefer & Scott, 1993). One advantage of the flooding dose is that the specific radioactivity of the precursor pool is assumed to remain stable during the period when labeled amino acid is being incorporated into protein. The flooding dose technique is considered suitable for measuring short-term protein synthesis in rapidly turning over tissues such as the intestine (El-Khoury, 1999). Several amino acids have been used as the tracer for the flooding dose procedure, including tyrosine, valine, leucine, and phenylalanine (Schaefer & Scott, 1993). Adegoke et al. (1999) successfully used a flooding dose of L-[2,6-<sup>3</sup>H] phenylalanine in a gut loop model to investigate the effect of nutrients on mucosal protein synthesis in six week old piglets. Luminal delivery of <sup>3</sup>H-phenylalanine as the flooding dose tracer was also used in piglets to measure mucin and protein synthesis in the intestine (Nichols & Bertolo, 2008). Phenylalanine has been validated for use as the flooding dose tracer because changes in phenylalanine

concentration in the plasma will not affect the protein synthesis rate during the incorporation time (El-Khoury, 1999). Indeed, the flooding dose is a reliable technique to measure the rate of protein synthesis, especially in rapidly turning over tissues.

## **Chapter 2: Rationale, objective and hypothesis.**

### **2.1. Rationale.**

Arginine and lysine are nutritionally important amino acids in neonates. Arginine serves as a precursor for important metabolites such as nitric oxide (NO), polyamines, and creatine (Wu & Morris, 1998; Wyss & Kaddurah-Daouk, 2000) and is conditionally essential in neonates (Brunton et al., 1999). Furthermore, arginine has a role in activating the mTOR signaling pathway which contributes towards promoting neonatal growth (Yao et al., 2008). Lysine is an essential amino acid, and it is the first limiting amino acid due to the low concentration of lysine in cereal grain diet (Ball et al., 2007). Providing arginine or lysine as free amino acids in the diet may be beneficial, but providing one in excess may negatively influence the metabolism of the other amino acids. It would be advantageous to supplement amino acids in the form of dipeptides due to the low required energy and rapid absorption through PepT1 (Gilbert et al., 2008). We speculate that supplementing the diet with amino acids as peptides improves the absorption and the utilization of amino acids by the small intestine which assists piglets in growth during stressful events. Previously, we demonstrated in neonatal gut loops that simultaneous delivery of arginine with lysyl-lysine resulted in enhanced arginine uptake compared to the control condition (Tennakoon et al., 2011). Based on the results obtained in the previous study, the current study will determine if there are functional benefits to the greater arginine uptake by the small intestine, specifically as greater protein synthesis.

### **2.2. Objectives and hypothesis.**

The objectives of this research were:

- 1) to confirm that lysyl-lysine (lys-lys) enhances arginine absorption
- 2) to determine whether enhanced arginine availability within the intestinal enterocytes leads to functional benefits, as demonstrated by the rate of mucosal protein synthesis
- 3) to determine if greater mucosal protein synthesis in response to arginine uptake is mediated through the mTOR pathway.

Hypothesis:

The presence of the lysyl-lysine with L-arginine in the small intestinal lumen will lead to the functional advantage of greater mucosal protein synthesis via activation of mTOR, which is likely mediated through enhanced arginine absorption.

## Chapter 3: Materials and Methods

### 3.1. *In vivo* intestinal perfusion studies.

#### 3.1.1. Surgical preparation for the perfusion studies.

An *in situ* intestinal perfusion experiment was performed on sow-fed Yucatan miniature piglets between the ages of 16 and 21 days old (n = 12) which were obtained from Animal Care Services at Memorial University of Newfoundland (St. John's, Canada). All procedures were approved by the Institutional Animal Care Committee (Memorial University of Newfoundland) and were in accordance with the guidelines of the Canadian Council on Animal Care. The experimental procedures were started immediately after the piglets were delivered to the animal facility. This was done to prevent a fasting effect which might alter the function or expression of PepT1.

Piglets (n=6) were pre-anaesthetized with an intramuscular (IM) injection of ketamine hydrochloride (20 mg/kg; Bimeda Canada, Cambridge, ON, Canada) and acepromazine (0.5 mg/kg; Vetoquinol, Quebec, Canada). In order to reduce the airway secretions, an IM injection of atropine sulfate (0.05 mg/kg; Rafter Dex Canada, Calgary, AB) was also given to the animals. Finally, an IM injection of buprenorphine hydrochloride (0.03 mg/kg; Temgesic, Reckitt Benckiser Healthcare, UK) was administered as an analgesic before starting the experimental procedure. Subsequently, an endotracheal tube was inserted into the trachea to maintain the airway. General anaesthesia was employed throughout the experiment using 0.8-1.5 % isoflurane (Abbott Laboratories Inc., Canada) delivered by a flow for oxygen at rate of 1.5 l/min. To ensure that we maintain the piglets' body temperature at 37 °C, the rectal temperature was measured every 15 minutes, and heat was delivered via a heating pad placed under the piglet. Similarly, heart



rate and the blood oxygen saturation were monitored during the experimental procedure. To prevent dehydration during surgery, a catheter was inserted in the jugular vein which allowed for 0.9% saline administration at a rate of 13.5 ml/kg/h for the entire time of the surgery. When the piglet was anaesthetized and stable, the small intestine was exposed by laparotomy, and the ligament of Treitz was identified, which is considered to be the distal end of duodenum. The small intestine was measured longitudinally starting at the ligament of Treitz. The first loop segment was initiated at 15 cm distal to the ligament, and measured 10 cm in length. The segment had inlet and outlet cannulas inserted at both ends (ID, 1/16 in.; OD 1/8 in., Watson Marlow, Cornwall, UK), and the cannulas were secured by silk ligatures tied around the entire small intestine and cannula, which interrupted the continuity of the gut and prevented chyme from entering the isolated segment. Subsequently, five more segments of the proximal small intestine were similarly isolated with 50 cm between each segment, for a total of six loops per piglet. The mesenteric blood supply remained intact. The loops were flushed with warmed phosphate buffered saline (PBS) buffer (described below) to remove residual digesta. Once all of the loops were in place, the intestines were covered with gauze soaked in warm saline and plastic wrap to prevent insensible water losses.

### **3.1.2. Perfusion study procedure.**

The recirculating intestinal perfusion model was facilitated by a multi-channel peristaltic pump (Watson Marlow, Cornwall, UK) that was capable of pumping six different buffers simultaneously. The treatment buffers were kept warm during the perfusion procedure by a water bath set at 37 °C. Each bottle containing the six treatment buffers had an inlet cannula that moved fluid from the bottle to the intestinal segment, and an outlet cannula returned buffer from the intestinal segment back into the bottle. Segments were perfused with one of the treatments buffers

(described below Table 3-1.) at rate of 1 ml/min for 120 minutes. For the first piglet in the experiment, the treatment buffers were randomly assigned to each loop; in subsequent experiments, the distribution of the treatment buffers was assigned to ensure an equal distribution of treatments by loop placement, to avoid the effect of location. During the experimental procedure, the treatment buffers were sampled before initiating the perfusion, and then every 15 minutes during perfusion study. The perfusion study continued for 120 minutes. At the end of the perfusion period, the 10 cm intestinal segments were excised by cautery and flushed with cold saline. The intestinal segments were placed on a glass plate on ice, cut lengthwise, and the mucosa was acquired by scraping the segments with a glass microscope slide. The weight of mucosa samples was measured and samples were frozen in liquid nitrogen, and stored at -80 °C for further analyses.

### **3.1.3. Perfused treatments.**

The treatments for each loop were prepared in PBS buffer (NaCl 144.6 mmol/l, Na<sub>2</sub>HPO<sub>4</sub> 15.9 mmol/l, NaH<sub>2</sub>PO<sub>4</sub> 1.2 mmol/l, PH 7.4) (Sigma-Aldrich Canada Ltd, Oakville, ON). Segments were perfused with one of the three treatment buffers: 10 mmol/l L-arginine (Ajinomoto North America, Inc, Raleigh, NC), 10 mmol/l arginine + 20 mmol/l lysyl-lysine (Bachem Americas, Torrance, CA), or 10 mmol/l arginine + 40 mmol/l L-lysine (Ajinomoto North America, Inc, Raleigh, NC) (Table 3-1). Segments perfused with 10 mmol/l arginine alone were considered the control condition. Each of the treatments were repeated in two loops within one pig. In one of the two replicates, <sup>3</sup>H-arginine (60 µCi [2.22 MBq] per 60 ml) was included to measure arginine disappearance. The second replicate was used to measure mucosal protein synthesis. To facilitate this measurement, the buffers were perfused without radioactive amino acid for the first 90

minutes. Subsequently, the perfusion was stopped at 90 minutes and the buffers were replaced with treatments containing the same amino acids plus <sup>3</sup>H-phenylalanine (60 µCi [2.22 MBq] per 60 ml) (Moravek Biochemicals, CA, USA) and 2 mmol/l L-phenylalanine (Ajinomoto North America, Inc, Raleigh, NC). The labeling period with phenylalanine-containing buffers continued for 30 minutes.

Six additional piglets underwent the identical protocol as described above; however, after piglets were anaesthetized, 0.75 mg/kg rapamycin (LC Laboratories, MA, USA) was delivered intravenously (IV) one hour prior to the intestinal perfusion study. The 0.75 mg/kg rapamycin was dissolved in 5% dimethyl sulfoxide (DMSO) (Sigma-Aldrich Canada Ltd, Oakville, ON) and saline for IV delivery (Kimball et al., 2000).

**Table 3- 1:** Amino acid composition of the treatment buffers.

<b>Amino Acids</b>	<b>Treatment 1</b>	<b>Treatment 2</b>	<b>Treatment 3</b>	<b>Treatment 4</b>	<b>Treatment 5</b>	<b>Treatment 6</b>
<b>Arginine</b>	10 mmol/l	10 mmol/l	10 mmol/l	10 mmol/l	10 mmol/l	10 mmol/l
<b>Lysyl-lysine</b>		20 mmol/l			20 mmol/l	
<b>Lysine</b>			40 mmol/l			40 mmol/l
<b>Phenylalanine</b>				2 mmol/l*	2 mmol/l*	2 mmol/l*
<b><sup>3</sup>H-Arginine</b>	1 µCi/l	1 µCi/l	1 µCi/l			
<b><sup>3</sup>H-Phenylalanine</b>				1 µCi/l*	1 µCi/l*	1 µCi/l*

\*Perfused during the last 30 minutes with the same amino acids treatments.

## 3.2. Analyses.

### 3.2.1. <sup>3</sup>H-arginine disappearance from luminal perfusates.

The <sup>3</sup>H-arginine disappearance from luminal perfusate samples was measured by liquid scintillation counting to provide an estimate of <sup>3</sup>H-arginine absorption by the intestinal cells. 100 µl of perfusate sampled from the buffers at each time point was mixed with 5 ml ScintiVerse (Fisher Scientific, ON, Canada). The samples were then kept in the dark overnight. The following day, the disintegrations per minute (DPM) were measured for 10 minutes using a TriCarb 2810 TR liquid scintillation counter (Perkin Elmer Life and Analytical Sciences, Downers Grove, IL). Finally, the arginine disappearance from perfusate samples was determined from the following formula:

$$\text{Arginine disappearance} = \frac{(DPM_{t0} - DPM_{t120})}{\frac{DPM_{t0}}{n_0}}$$
$$n_0 = \frac{\text{initial arginine concentration}}{\text{initial volume}}$$

Where:

DPM<sub>t0</sub> is the DPM in luminal perfusate sample prior to the perfusion procedure.

DPM<sub>t120</sub> is DPM in luminal sample at the end of the perfusion procedure.

n<sub>0</sub> is the arginine concentration in luminal perfusate samples prior to perfusion

### 3.2.2. Amino acids concentrations in perfused buffers.

Perfusate samples were analyzed using the high performance liquid chromatography (HPLC) (Waters Inc., Mississauga, ON) to measure amino acids concentrations. The perfusate samples were diluted to 1:10 in order to bring the sample concentrations within the loading capacity of HPLC column. Then, 100 µl of the diluted samples were mixed with 20 µl of 2.5 mmol/l norleucine

internal standard (Sigma-Aldrich Canada Ltd, Oakville, ON). To allow any protein in the perfusate samples to precipitate, the perfusate samples were mixed with 1 ml of 0.5% trifluoroacetic acid (TFA) (Sigma-Aldrich Canada Ltd, Oakville, ON) in methanol (MeOH) (Fisher Scientific, Whitby, ON). Then the samples were centrifuged at speed of 4200 X g for 3 minutes at 4 °C (Eppendorf, NY, USA) in order to isolate the supernatant. The supernatant was poured into another labeled tube, and was frozen in liquid nitrogen and then vacuum dried (Thermo Fisher Scientific, MA, USA) overnight. The following day, 50 µl of fresh 20% triethylamine (TEA) (Sigma-Aldrich Canada Ltd, Oakville, ON), 20% methanol (MeOH) (Fisher Scientific, Whitby, ON), and 60% HPLC water was added to each sample. Then the samples were frozen with liquid nitrogen, and vacuum dried for one hour. After drying the samples, 20 µl of a derivatizing solution was added, that contained 10% water, 10% TEA, 70% MeOH, 10% phenylisothiocyanate (PITC) (Thermo Scientific, USA) and the samples were left for 35 minutes at room temperature to facilitate the labelling process. This resulted in PITC binding to the primary amine group of the free amino acids which produces phenylthiocarbamyl amino acids. After 35 minutes, the reaction was stopped by freezing the samples in liquid nitrogen and then the samples were vacuum dried overnight. On the following day, the samples were re-suspended in 200 µl of sample diluent containing 5 mmol/l Na<sub>2</sub>HPO<sub>4</sub> (Sigma-Aldrich Canada Ltd, Oakville, ON), and 10% acetonitrile (Fisher Scientific, Whitby, ON) at pH 7.4. The samples were centrifuged at 3000 X g for 5 minutes at 4 °C. The resulting supernatant was transferred to 0.5 ml Eppendorf tube, and was centrifuged again at 3000 X g for 5 minutes. After centrifuging for the second time, the supernatant was pipetted into Eppendorf which was placed into an HPLC vial.

Perfusate samples were analyzed by using the Pico-Tag method with HPLC. 100  $\mu$ l of the derivatized sample was injected onto a reverse phase C18 column (Water, Mississauga, Ontario, Canada). The temperature of the column was maintained at 46 °C to optimize amino acids separation. UV detector was set at a wavelength of 254 nm to detect the phenylthiocarbamyl (PTC) derivatives. The running time of samples was 112 minutes since amino acids of interest such as lysine and lysyl-lysine have high retention times. The flow rate was 1 ml/min, and the buffer flow gradient protocol for lysyl-lysine separation is listed in table 3-2. Buffers A and B were prepared according to table 3-3, and 3-4. The eluent associated with arginine peak was fraction-collected in 3 minute time windows by using Water Fraction Collector II (Water, Mississauga, ON). The 3 ml fractions were mixed with 5 ml ScintiVerse (Fisher Scientific, ON, Canada), and the disintegrations per minute (DPM) were measured for 10 minutes using a TriCarb 2810 TR liquid scintillation counter (Perkin Elmer Life and Analytical Sciences, Downers Grove, IL) to measure the radioactivity associated with amino acid. By using Empower 3 Software (Water, Mississauga, ON), the amino acids peaks were integrated. The amino acid concentrations were determined by comparing the area of amino acid to the area of the internal standard, norleucine.

### **3.2.3. Tissue free amino acids concentrations in intestinal mucosa.**

Amino acid concentrations in the mucosal tissue precursor pool were determined by HPLC. 0.25 g of mucosa samples were homogenized with 1 ml of 2% perchloric acid. The samples were then centrifuged at 3000 X g for 15 minutes at 4 °C. The resulting supernatant was transferred to labeled scintillation vials. The homogenizing and centrifuging steps were repeated three times, and the final volume of supernatant was 3 ml. The resulting pellet was stored at -20 °C for subsequent analysis of protein-bound amino acid concentrations. For each supernatant, 25  $\mu$ l norleucine

standard (25 mmol/l) was added, and the supernatant was vortexed. To neutralize the supernatant, 1 ml was mixed with 125  $\mu$ l of 2 mol/l  $K_2CO_3$ . Then it was centrifuged at 3000 X g for 3 minutes, and the resultant supernatant was then frozen in liquid nitrogen and freeze dried overnight. The following day, samples were derivatized and analyzed with HPLC analysis by following the same steps described in the previous section 3.2.2.

**Table 3- 2:** Buffer flow gradient protocol for lysyl-lysine separation.

<b>Time (min)</b>	<b>Flow (ml/min)</b>	<b>%A</b>	<b>%B</b>	<b>Curve</b>
<b>0.01</b>	1.0	100.0	0.0	6
<b>13.5</b>	1.0	97.0	3.0	11
<b>24.0</b>	1.0	96.0	4.0	8
<b>30.0</b>	1.0	91.0	9.0	5
<b>50.0</b>	1.0	66.0	34.0	6
<b>66.0</b>	1.0	66.0	34.0	6
<b>92.0</b>	1.0	0.0	100.0	6
<b>102.0</b>	1.0	0.0	100.0	6
<b>102.5</b>	1.0	100.0	0.0	6
<b>112.0</b>	1.0	100.0	0.0	6



**Table 3- 3:** Composition of buffer A.

<b>Ingredients</b>	<b>Quantity</b>
<b>HPLC water</b>	4.0 l
<b>Sodium acetate</b>	38 g
<b>Acetonitrile</b>	100 ml

\*pH was adjusted to 6.55

**Table 3- 4:** Composition of buffer B.

<b>Ingredients</b>	<b>Quantity</b>
<b>HPLC water</b>	1.6 l
<b>Methanol</b>	600 ml
<b>Acetonitrile</b>	1.8 l

#### **3.2.4. Mucosal protein synthesis rate.**

To determine the mucosal protein synthesis rate, mucosal free and protein-bound amino acids were quantified by HPLC. The method of analysis of the mucosal free amino acid concentrations was described in the previous section 3.2.3.

To the protein pellet that resulted from tissue free amino acids preparation, 100 µl norleucine (25 mmol/l) was added. After that, 1 ml of 6 mol/l HCl was added, and the pellet was disrupted with a glass rod. Samples were then transferred to labeled Pyrex tubes with screw caps. The tube which contained the homogenized pellet was rinsed with another 1.5 ml of 6 mol/l HCl to ensure that the entire sample was completely removed, and added to the Pyrex tube. The protein pellet was hydrolyzed at 110 °C for 24 hours in a drying oven. After 24 hours, the tubes were removed from the drying oven and were transferred to the fume hood to cool overnight. Once cooled, the hydrolysates were transferred to pre-weighed Erlenmeyer flasks. The Pyrex tubes were rinsed twice with HPLC water, and the final volume was brought to 6.3 ml. Samples were filtered using a 0.45 µm syringe filter into labeled vials. 1 ml of the filtered sample was placed in the vacuum oven to dry overnight.

For both mucosal free and protein-bound amino acid analyses, 100 µl of fresh 20:20:60 TEA: MeOH: water was added to each sample. Samples were then frozen in liquid nitrogen and freeze dried for one hour. Then to each sample, 50 µl of derivatizing solution (10% water, 10 % TEA, 70% MeOH, 10% PITC) was added, and samples were frozen in liquid nitrogen and freeze dried overnight. Before samples were injected onto the HPLC, they were re-suspended in 300 µl sample diluent (described above).

Amino acid concentrations in the samples were analyzed by the Pico-Tag method with HPLC. Derivatized sample was injected onto a reverse phase C 18 column (Water, Mississauga, Ontario, Canada), using the same method and buffers as described above but with a run time of 90 minutes (Table 3-5) The eluent associated with the phenylalanine peak was fraction-collected in a 3 minute time window (Water Fraction Collector II, Water, Mississauga, ON) and scintillation counted as described above to measure the radioactivity associated with phenylalanine.

The fractional synthesis rates of protein in the mucosa samples were calculated as follows:

$$Ks = (SRA_{\text{bound}}/SRA_{\text{free}}) \times (1440/\text{time}) \times 100$$

Where  $SRA = \frac{\text{DPM in phenylalanine}}{\text{phenylalanine concentration in tissue}}$

**Ks**= Fractional rate of protein synthesis, %/d.

**SRA<sub>bound</sub>**= Specific radioactivity of bound phenylalanine in tissue, DPM/ $\mu$ mol.

**SRA<sub>free</sub>**= Specific radioactivity of phenylalanine in the precursor pool, DPM/ $\mu$ mol.

**Time**= Incorporation time in minutes.

**Table 3- 5:** Buffer flow gradient protocol, 90 minutes.

<b>Time (min)</b>	<b>Flow (ml/min)</b>	<b>%A</b>	<b>%B</b>	<b>Curve</b>
<b>0.01</b>	1.0	100.0	0.0	6
<b>13.5</b>	1.0	97.0	3.0	11
<b>24.0</b>	1.0	94.0	6.0	8
<b>30.0</b>	1.0	91.0	9.0	5
<b>50.0</b>	1.0	66.0	34.0	6
<b>66.0</b>	1.0	66.0	34.0	6
<b>66.5</b>	1.0	0.0	100.0	6
<b>78.5</b>	1.0	0.0	100.0	6
<b>79.0</b>	1.0	100.0	0.0	6
<b>90.0</b>	1.0	100.0	0.0	6

### **3.2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting.**

Western blotting was used to detect the presence of mTOR protein in mucosa samples. Western blotting consists of three steps: gel electrophoresis to facilitate protein separation by molecular weight, protein transfer to a membrane, and incubation with antibodies to detect the protein of interest (Mahmood & Yang, 2012).

#### **3.2.5.1. Proteins extraction.**

The cytosolic proteins in mucosa samples were extracted by using the lysis and extraction buffer, radioimmunoprecipitation assay (RIPA<sup>++</sup>) buffer (Mahmood & Yang, 2012). RIPA<sup>++</sup> was prepared by first diluting the RIPA stock solution 1:10 (Cells Signalling Technology, Danvers, USA). Then protease inhibitors, phosphatase inhibitor and antioxidant agents (Sigma-Aldrich, St. Louis, MO) were added to the RIPA solution to make the RIPA<sup>++</sup> buffer (Table 3-6). To initiate the extraction process, 400  $\mu$ l of RIPA<sup>++</sup> was first added to 1.5 ml labeled Eppendorf tubes on ice. 0.15 g of mucosa was added to each Eppendorf. After adding samples to the RIPA<sup>++</sup> buffer, they were allowed to thaw on ice for at least 20 minutes. The samples were then vortexed and homogenized with a micropestle while on ice (Geneaid, New Taipei, Taiwan) and then placed on a shaker (Boekel Scientific, PA, USA) at 4 °C for 30 minutes. The samples were then centrifuged at 10000 X g for 10 minutes at 4°C. The resultant supernatant was transferred to another labeled Eppendorf, and the pellet was kept and stored at -20 °C. Before storing the samples, the protein concentrations were determined as described in the following section.

**Table 3- 6:** Preparation of the lysis buffer RIPA<sup>++</sup>

<b>Reagents</b>	<b>Function</b>	<b>Prepared stock concentration</b>	<b>Working dilution</b>
<b>Sodium Orthovanadate</b>	Phosphatase inhibitor	200 mmol/l	1/200
<b>Dithiothreitol (DTT)</b>	Antioxidant	1 mol/l	1/1000
<b>Leupeptin</b>	Phosphatase inhibitor	10 µg/µl	1/100
<b>Aprotinin</b>	Protease inhibitor	Diluted to 1/100	1/100
<b>Sodium fluoride (NaF)</b>	Phosphatase inhibitor	2.5 mol/l	1/50
<b>Beta-Glycerophosphate</b>	Phosphatase inhibitor	1 mol/l	1/50
<b>Phenylmethane sulfonyl fluoride (PMSF)</b>	Protease inhibitor	200 mmol/l	1/200

### **3.2.5.2. Determining the mucosal protein concentrations.**

A standard curve was constructed to determine the mucosal protein concentrations. The purpose of determining the mucosal protein concentrations was to ensure equal protein loading into the electrophoresis gel. To construct the standard curve, different concentrations of standards were prepared from a stock bovine serum albumin (BSA) protein standard (200 mg/ml) (Sigma-Aldrich, St. Louis, MO). Standard concentrations that were prepared were 0, 0.5, 1, 2, 3, 4 and 5 mg/ml. The Pierce™ bicinchoninic acid assay (BCA) protein assay kit was used (Thermo Scientific, Illinois, USA). A working solution was made by mixing proprietary Reagent A with Reagent B from BCA protein assay kit in 50:1 dilution, and 200 µl was added to each well in the 96 well microplate. All samples were run in duplicate. To each well, 5 µl of each of the prepared standards was added and mixed with the pipette. Before adding the mucosa samples to the reagent in the wells, the samples were first diluted to 1:10. The plate was covered with parafilm and incubated for 30 minutes at 37 °C. After that, it was cooled to room temperature. The absorbances were read by spectrophotometry (BioTek, VT, USA). Absorbances from the standards were used to construct the standard curve, which was then used to calculate the protein concentration in each sample.

### **3.2.5.3. Preparing SDS-PAGE gel and gel electrophoresis.**

After determining mucosa sample protein concentrations, the gel electrophoresis technique was used to separate proteins by molecular weight. Two gel cassette sandwiches were assembled by combing a short plate with a spacer plate. The gel cassette sandwiches were then inserted into the casting frame, and placed in the casting stand apparatus (Mini-protean 3, Bio-Rad Laboratories, CA, USA). 15 ml of the separating gel was prepared (Table 3-7), which was enough to make two

1 mm gels. The separating gel was pipetted in between the short plate and the spacer plate, and 1 cm was left as a space on the top of the gel. Water was poured on the top of the separating gel space. Afterwards, the separating gel was allowed to polymerize for one hour. After the one hour, the water was removed by moving small piece of filter paper in the space above the separating gel. The stacking gel was then prepared (Table 3-8), and was pipetted on the top of the separating gel. The 15 well comb was placed on the stacking gel, and then it was left to polymerize for 10 minutes. The electrophoresis apparatus was assembled, and filled with 700 ml of the running buffer which was made of 25 mmol/l Tris base (Thermo Scientific, Illinois, USA), 190 mmol/l glycine (Ajinomoto North America, Inc, Raleigh, NC), and 3.5 mmol/l sodium dodecyl sulfate (Sigma-Aldrich, St. Louis, MO). The protein amount that was loaded onto the gel was 60 µg. To ensure equal volume for all samples, the final volume of samples was adjusted by the addition of RIPA<sup>++</sup>, and the 5x gel loading buffer. The added amount of RIPA<sup>++</sup>, and the 5x gel loading buffer depended on the highest calculated value of protein concentration in the sample set in which it was used in the equation to determine the amount of both the RIPA<sup>++</sup>, and the 5x gel loading buffer.

Before loading samples into the gel, they were heated on a temperature block (Lanline Instruments, USA) at 90 °C for 3 minutes to denature the proteins. Then the BLUelf prestained protein ladder (FraggaBio, ON, Canada), and mucosa protein samples were loaded into the gel using sample loading pipette tips. The protein ladder is used as a reference to assist in estimating the molecular weight of the protein of interest. After loading the samples, the power supply was connected, set to 200 V, and run for 35 minutes. When the running was completed, the two gel cassette sandwiches were removed from electrophoresis tank, and by using the gel wedge (Bio-Rad Laboratories, CA, USA), the gels were removed by separating the glass plates. Then the stacking



gel was eliminated by the gel wedge, and the separating gel was soaked for 15 minutes in a cold transfer buffer which was made of 25 mmol/l Tris base without methanol (Thermo Scientific, Illinois, USA) and 190 mmol/l glycine (Ajinomoto North America, Inc, Raleigh, NC).

**Table 3- 7:** Preparation of the 10% separating gel

<b>Chemical</b>	<b>Quantity</b>
<b>30 % Acrylamide</b>	5 ml
<b>4X Resolving Gel Buffer pH 8.8</b>	3.75 ml
<b>Deionized H<sub>2</sub>O</b>	6.25 ml
<b>Tetramethylethylenediamine (TEMED)</b>	12 µl
<b>10 % Ammonium persulfate (APS)</b>	110 µl

**Table 3- 8:** Preparation of 6 ml of 3.5% stacking gel

<b>Chemical</b>	<b>Quantity</b>
<b>30 % Acrylamide</b>	0.7 ml
<b>4X Stacking Gel Buffer pH 6.7</b>	1.5 ml
<b>Deionized H<sub>2</sub>O</b>	3.8 ml

#### **3.2.5.4. Western blot.**

Before assembling the transfer sandwiches, fiber pads/sponge, Whatman 3 mm papers (Bio-Rad Laboratories, CA, USA), and 0.2 µm nitrocellulose membranes papers (Bio-Rad Laboratories, CA, USA) were soaked in a transfer buffer with methanol which was made with 25 mmol/l Tris base (Thermo scientific, Illinois, USA), 190 mmol/l glycine (Ajinomoto North America, Inc, Raleigh, NC), and 20% methanol (Fisher Scientific, ON, Canada). The transfer sandwiches were assembled in the following order: soaked fiber pad first, the Whatman 3 mm papers, then the gel, membrane, Whatman 3 mm papers, and finally the pad. A roller was used to roll over the transfer sandwiches in order to remove any air bubbles. The transfer sandwiches were placed in the tank with ice, and the tank was filled with the transfer buffer with methanol. The transfer process was one hour long, and took place at 4 °C at 100 volts, and 350 mV. The transfer step allows the transfer of proteins from the gel to the membranes.

#### **3.2.5.5. Incubation with antibodies.**

After completing the transfer process, the membranes were blocked with 3% milk in Tris buffered saline 0.05% Tween-20 (TBST) which was made of 5 mol/l NaCl (AFC, QC , Canada), 1 mol/l Tris HCl (Thermo Scientific, Illinois, USA) at pH 7.8, and 20 % Tween-20 (Fisher Scientific, ON, Canada), at 55 °C for one hour. The membranes were then washed with TBST two times for three minutes. Then, the protein ladder was used as a reference to predict the position of the proteins of interest, and based on that the membranes were cut. The membranes were incubated with two antibodies: P-mTOR (Ser2448) rabbit pAb (Cells Signalling Technology, Danvers, USA) at 1/1000 dilution in 5% OmniPur® BSA, Fraction V Heat Shock Isolation (Millipore Ltd, ON,

Canada) in TBST, and actin rabbit (Sigma-Aldrich Canada Ltd, Oakville, ON) antibody at dilution 1/7500 in 3% milk in TBST. The membranes were incubated overnight in the shaker at 4 °C. The following day, the membranes were washed with TBST 5 times for 5 minutes each, and then incubated at room temperature for one hour with the anti rabbit antibody secondary antibody conjugated to Horseradish Peroxidase (HRP) (Santa Cruz Biotechnology, Inc., TX, USA) at dilution of 1/7500 in 3% milk in TBST at room temperature. After incubating with the secondary antibody, the membranes were washed with TBST 6 times for 5 minutes each time. During the incubation time, immobilon western chemiluminescent HRP substrate (ECL) (EMD Millipore, MA, USA) was prepared by adding HRP substrate peroxide solution with HRP substrate luminal reagent at dilution of 1:1, and was kept at room temperature to warm up. Before visualizing the protein bands, the membranes were placed in ECL for 5 minutes, and then dried with filter paper. The protein bands were visualized by ImageQuant LAS 4000 (GE Healthcare, QC, Canada). The membranes were then stripped with a stripping buffer for re-probing. The stripping process was conducted in a water bath at 55 °C for one hour. Then, the membranes were washed with TBS (5 mol/l NaCl, 1 mol/l Tris HCl) 7 times for 5 minutes, and blocked again with 3% milk in TBST at 55 °C for one hour. The membranes were incubated overnight in the shaker at 4 °C with mTOR rabbit pAb (Cells Signalling Technology, Danvers, USA) at dilution 1/1500 in 5% BSA in TBST. The following day, the membranes were washed with TBST, and incubated with the secondary antibody, and visualized by ImageQuant LAS 4000. During visualization of the bands, the ImageQuant LAS 4000 software automatically corrected for the background. The visualized bands were quantified by using Image Studio Lite software.

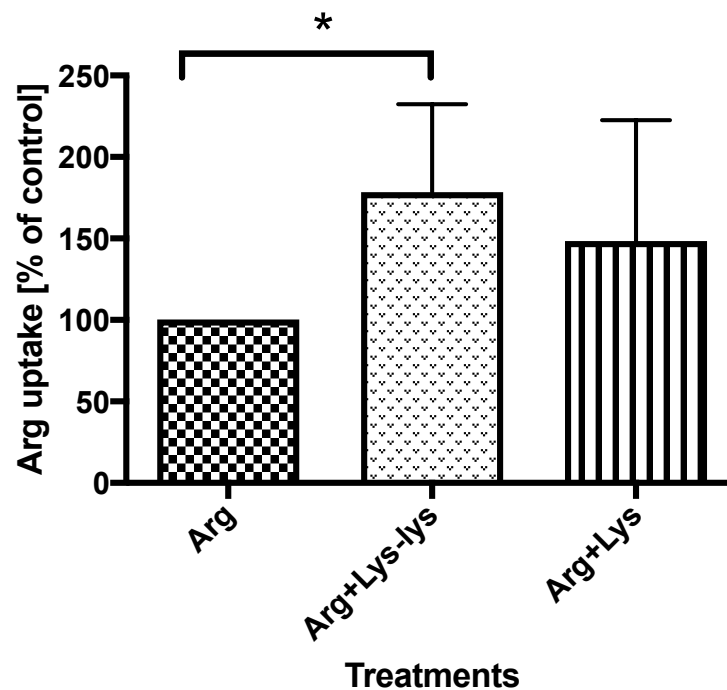
### 3.3. Statistical analyses.

Data were analyzed by repeated measures one-way ANOVA followed by Tukey's multiple comparisons test using Graph Pad Prism 7 (GraphPad Software, Inc., CA, USA). For comparison of the luminal <sup>3</sup>H-arginine uptake in the control condition (loops perfused with arginine alone) with and without rapamycin, data were analyzed by two tailed unpaired t-test using Graph Pad Prism 7. Similarly, differences in the mucosal protein synthesis rate, and the ratio of the phosphorylated/un-phosphorylated mTOR protein, among different treatments in both animal groups treated with and without rapamycin were analyzed by using two tailed unpaired t-test using Graph Pad Prism 7. A t-test was considered suitable for these rapamycin comparisons because we were interested in comparing only the same treatment between both animal groups: arginine vs. arginine + rapamycin, arginine + lysyl-lysine vs arginine +lysyl-lysine + rapamycin, and arginine + free lysine vs arginine + free lysine + rapamycin. Also, the two-tailed unpaired t-test was used instead of two-way ANOVA because when all treatments are compared together in both animal groups, this resulted in a loss of statistical power, therefore, affecting the ability of the test to detect differences. For all tests, the mean values were considered significantly at P-values less than 0.05 (P <0.05).

## Chapter 4: Results

### 4.1. Luminal <sup>3</sup>H-arginine disappearance.

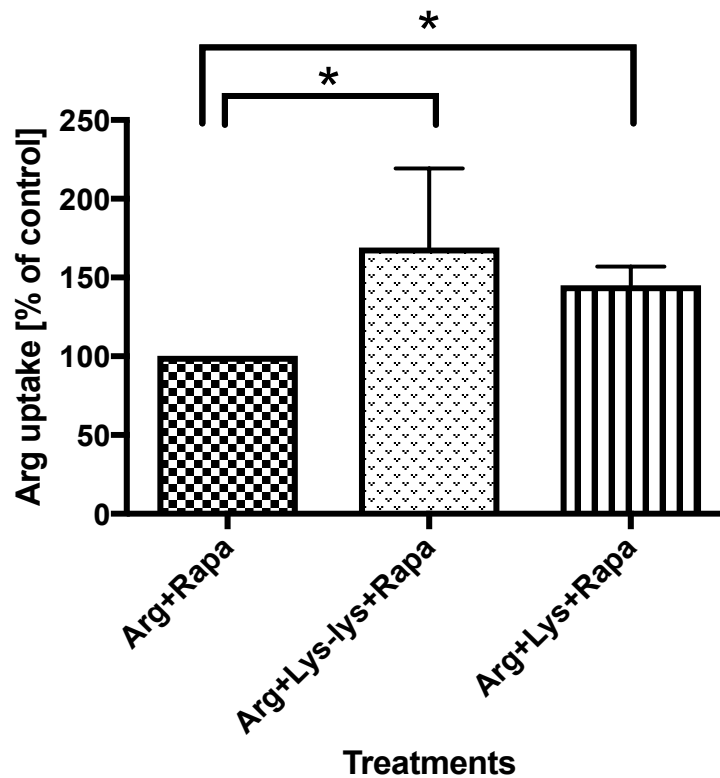
Arginine uptake by the intestinal mucosa in the gut loop perfusion model was calculated from the disappearance of <sup>3</sup>H-arginine. Compared to the control condition (10 mmol/l arginine alone), arginine uptake was 78% higher when co-perfused with 20 mmol/l lysyl-lysine (Figure 4-1). However, there was no significant difference in arginine uptake between the control treatment and free lysine treatment, or between the lysyl-lysine and free lysine treatments (Figure 4-1).



**Figure 4- 1: Luminal arginine uptake.** Data were expressed as the percentage of control, which was the arginine disappearance from the luminal buffer when perfused alone (Arg). Values are mean  $\pm$  SD, n=6 per treatment. Data were analyzed by repeated measures one-way ANOVA followed by Tukey's multiple comparisons test, \*P < 0.05. Abbreviations: Arg, Arginine, Lys-lys, Lysyl-lysine, Lys, Lysine.

#### 4.2. Effect of rapamycin on luminal <sup>3</sup>H-arginine disappearance.

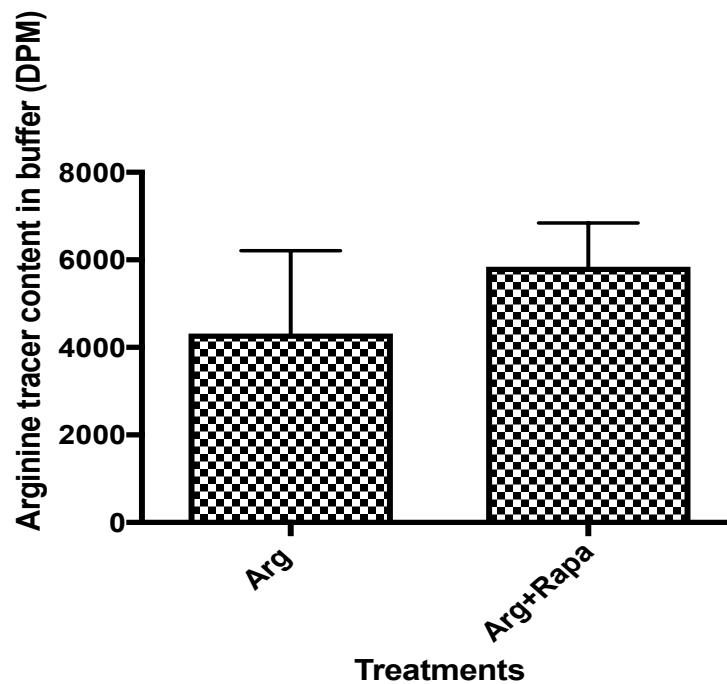
Arginine disappearance from the perfused buffer was also measured in the piglets pre-treated with IV rapamycin. In this experiment, co-perfusion of arginine with lysyl-lysine or free lysine resulted in significantly greater uptake of arginine compared to the control ( $P < 0.05$ ) (Figure 4-2).



**Figure 4- 2: Luminal arginine uptake following pre-treatment with rapamycin.** Data were expressed as the percentage of control, which was the arginine disappearance from the luminal buffer when perfused alone (Arg). Values are mean  $\pm$  SD,  $n=6$  per treatments. Data were analyzed by repeated measures one-way ANOVA followed by Tukey's multiple comparisons test,  $*P < 0.05$ . Abbreviations: Arg, Arginine, Lys-lys, Lysyl-lysine, Lys, Lysine, Rapa, Rapamycin.

### 4.3. Comparison between luminal <sup>3</sup>H-arginine disappearance in loops perfused with arginine alone with and without rapamycin.

Arginine tracer disappearance in the control loop was compared in piglets treated and not treated with rapamycin, to determine whether rapamycin affects arginine uptake. Arginine disappearance was not significantly different between the two experimental conditions, suggesting that rapamycin does not influence arginine uptake into the mucosa ( $P > 0.05$ ) (Figure 4-3).



**Figure 4- 3: Comparison of luminal <sup>3</sup>H-arginine disappearance in loops perfused with arginine alone.** Arginine tracer content in the control condition buffers (100  $\mu$ l) following the perfusion study in piglets treated with and without rapamycin (n=6 per group). Values are mean  $\pm$  SD and were analyzed by two tailed unpaired t-test,  $P > 0.05$ . Abbreviations: Arg, Arginine, Rapa, Rapamycin.



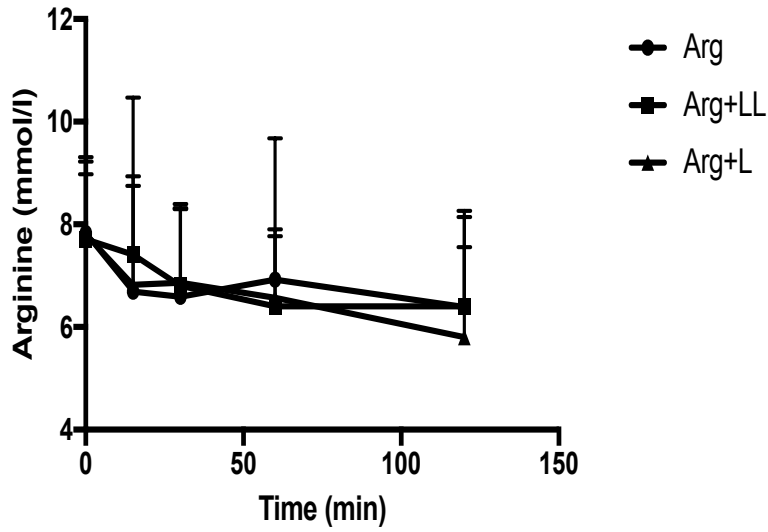
#### **4.4. Amino acid concentrations in the perfusion buffers.**

Perfusate samples collected at time 0, 15, 30, 60, and 120 minutes were analyzed to assess the changes in amino acids concentrations over time. Because no differences in arginine disappearance was apparent in piglets treated with rapamycin, data from both sets of piglets were pooled for some outcomes.

Arginine concentration in perfusion buffer decreased over time in all three treatments, and there was no significant difference between treatments ( $P > 0.05$ ) (Figure 4-4).

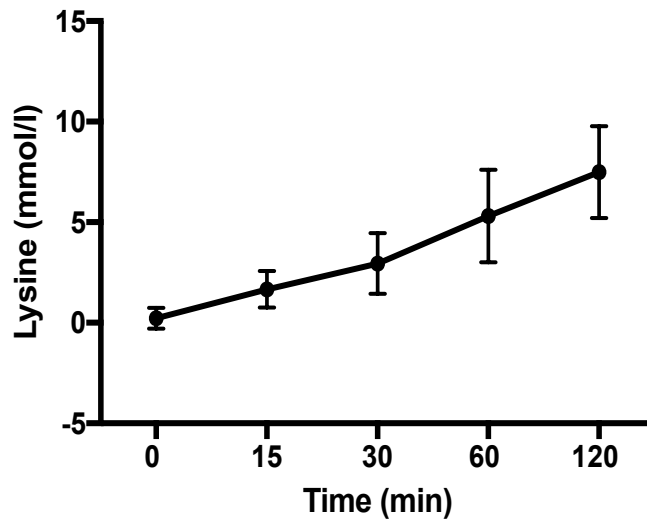
Lysyl-lysine was subjected to hydrolysis in intestinal loops that were perfused with the dipeptide. This was demonstrated by the increased concentrations of free lysine over time ( $P > 0.05$ ) (Figure 4-5).

The lysine concentration did not decrease with time in buffers that contained arginine and free lysine at the initiation of the study ( $P > 0.05$ ) (Figure 4-6).

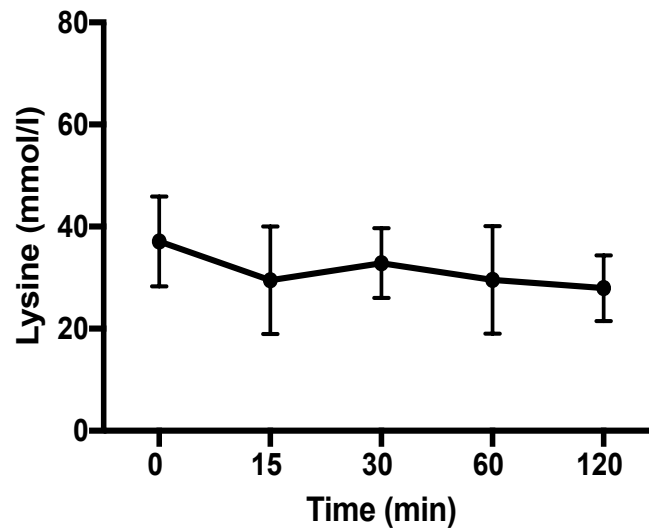


**Figure 4- 4: Arginine concentration over time in perfused buffers containing differing forms and concentrations of lysine.** Values are means + SD (n=12). Data were analyzed by repeated measures one-way ANOVA followed by Tukey’s multiple comparisons test ( $P > 0.05$ ).

Abbreviations: Arg, Arginine, Lys-lys, Lysyl-lysine, Lys, Lysine.



**Figure 4- 5: Lysine concentration over time from lysyl-lysine hydrolysis in perfused buffers.** Values are means  $\pm$  SD (n=12). Data were analyzed by repeated measures one-way ANOVA followed by Tukey’s multiple comparisons test ( $P > 0.05$ ).



**Figure 4- 6: Lysine concentration in perfused buffers.** Values are means  $\pm$  SD (n=12). Data were analyzed by repeated measures one-way ANOVA followed by Tukey's multiple comparisons test ( $P > 0.05$ ).

#### 4.5. Free amino acid concentrations in intestinal tissue.

Mucosa samples that were obtained at the end of perfusion study were used to determine the amino acid concentrations in that tissue. Segments that were perfused with  $^3\text{H}$ -arginine with either arginine, lysyl-lysine, or free lysine were used to measure free concentrations of tissue arginine, ornithine, citrulline, glutamate, glutamine, proline and lysine.

Although we measured higher arginine disappearance in intestinal segments co-perfused with lysyl-lysine, the tissue arginine concentration was not different, nor were any of the amino acids that may be synthesized from arginine (Table 4-1). However, there was a significant difference ( $P < 0.05$ ) in lysine concentration in mucosa samples from loops that were perfused with either lysyl-

lysine or free lysine, compared to the control loop that was not exposed to a luminal source of lysine (Table 4-1).

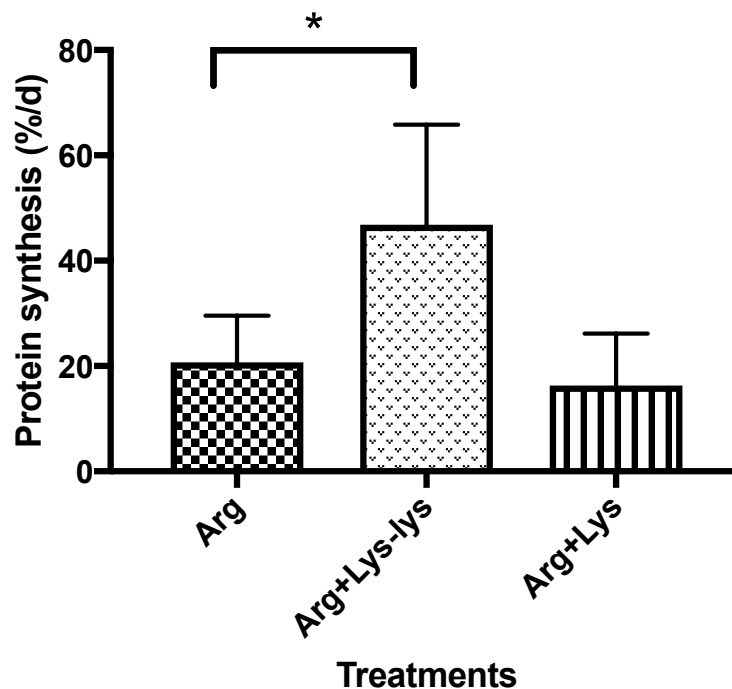
**Table 4- 1: Tissue free arginine, ornithine, citrulline, proline, glutamate, glutamine and lysine concentrations in intestinal mucosa.**

<b>Amino Acid</b>	<b>Arginine (10 mmol/l)</b>	<b>Arginine + lysyl-lysine (10 mmol/l+ 20 mmol/l)</b>	<b>Arginine + Lysine (10 mmol/l+ 40 mmol/l)</b>
<b>Arginine (μmol/g)</b>	0.985 ± 0.68	1.129 ± 0.73	1.122 ± 0.69
<b>Ornithine (μmol/g)</b>	0.092 ± 0.025	0.085 ± 0.016	0.125 ± 0.043
<b>Citrulline (μmol/g)</b>	0.685 ± 0.55	0.662 ± 0.59	0.823 ± 0.74
<b>Proline (μmol/g)</b>	0.503 ± 0.22	0.476 ± 0.20	0.571 ± 0.18
<b>Glutamate (μmol/g)</b>	0.986 ± 0.14	0.986 ± 0.11	1.061 ± 0.14
<b>Glutamine (μmol/g)</b>	0.177 ± 0.06	0.220 ± 0.09	0.283 ± 0.12
<b>Lysine (μmol/g)</b>	0.52± 0.11	<b>3.3±1.55*</b>	<b>4.0±1.65*</b>

Values are mean ± SD (n=6). Data were analyzed by repeated measures one-way ANOVA followed by Tukey's multiple comparisons test \*p < 0.05.

#### 4.6. Mucosal protein synthesis.

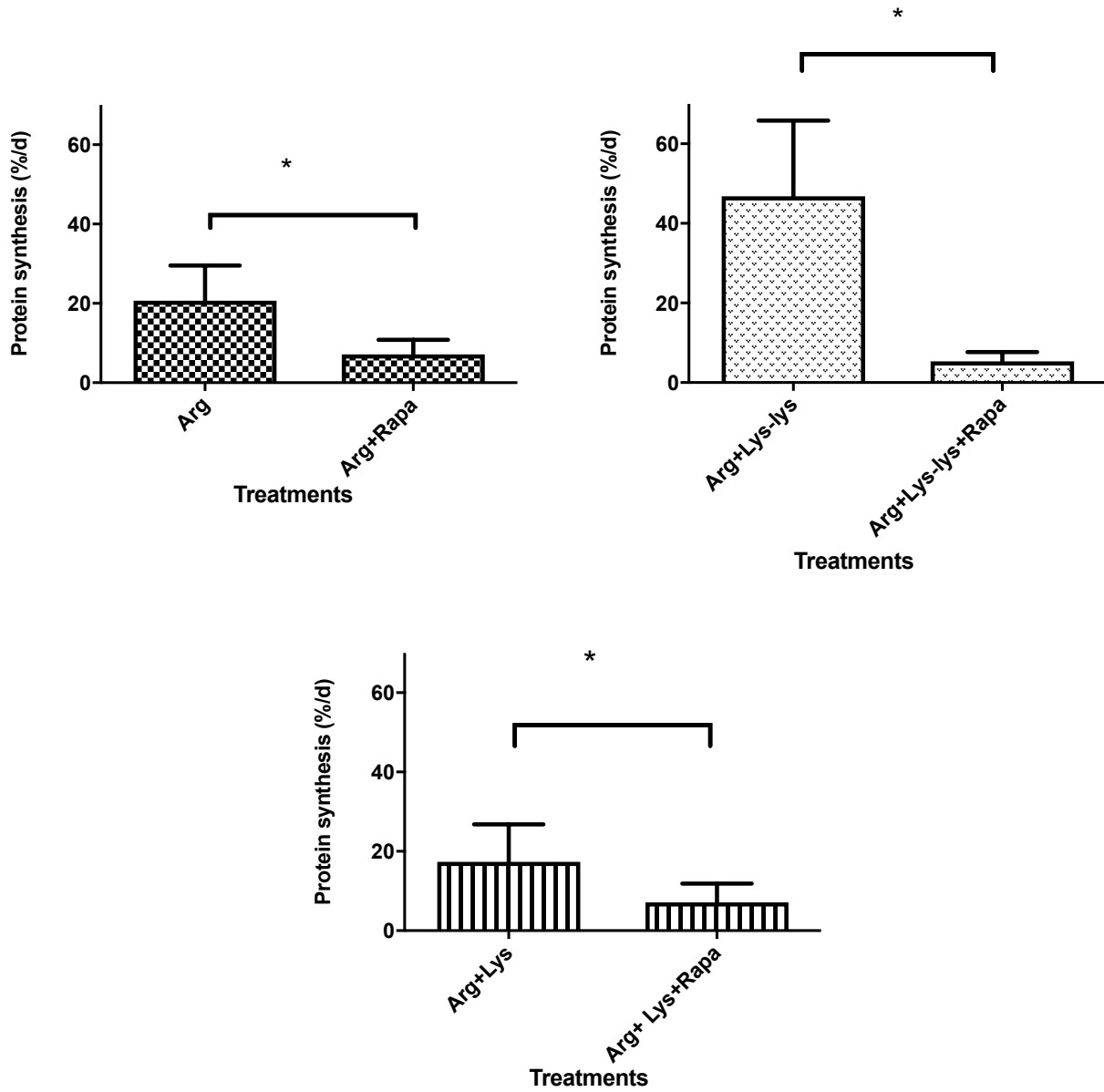
The rate of mucosal protein synthesis was measured in segments that were perfused with  $^3\text{H}$ -phenylalanine in addition to arginine, lysyl-lysine, or free lysine. The lysyl-lysine treatment resulted in significantly higher mucosal protein synthesis compared to the control treatment of arginine alone ( $P < 0.05$ ) (Figure 4-7). Interestingly, the rate of mucosal protein synthesis tended to be different between the dipeptide and free lysine treatments ( $P = 0.055$ ) (Figure 4-7). However, there was no significant difference in protein synthesis between the control condition arginine alone and the free lysine condition (Figure 4-7).



**Figure 4- 7: Mucosal fractional protein synthesis rates.** Values are mean  $\pm$  SD, n=6 per treatment. Data were analyzed by repeated measures one-way ANOVA followed by Tukey's multiple comparisons test, \*  $P < 0.05$ . Abbreviations: Arg, Arginine, Lys-lys, Lysyl-lysine, Lys, Lysine.

#### **4.7. Effect of rapamycin on mucosal protein synthesis.**

The mucosal protein synthesis rate was also measured in the small intestine in piglets pre-treated with IV rapamycin. Mucosal protein synthesis was lowered by more than 60% in all amino acid treatments when rapamycin was given prior to the perfusion study (Figure 4-8).



**Figure 4- 8: Mucosal fractional protein synthesis rates with and without rapamycin pre-treatment.** Values are mean  $\pm$  SD, n=6 per treatment. \*P < 0.05 by two tailed unpaired t-test. Abbreviations: Arg, Arginine; Lys-lys, Lysyl-lysine; Lys, Lysine; Rapa, Rapamycin.

#### **4.8. Effect of rapamycin on mTOR pathway activation in the small intestine.**

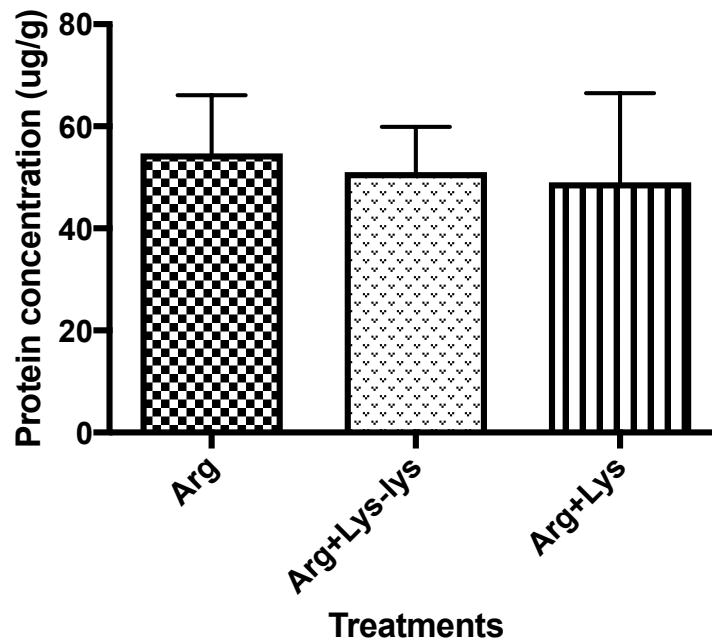
Western blotting was used to determine mTOR protein activation in mucosa samples that were obtained at the end of the perfusion procedure from the intestinal segments perfused with <sup>3</sup>H-arginine (i.e. three segments per piglet). Before proceeding to western blot analysis, protein concentration in mucosa samples was measured to ensure equal protein loading onto the gel. There were no significant differences in mucosal protein concentration among the treatments (Figure 4-9).

After running the gel electrophoresis and incubating with the primary and secondary antibodies, protein bands were visualized (Figure 4-10). The three membranes (Figure 4-10) represent phosphorylated mTOR (A), total un-phosphorylated mTOR protein (B), and actin (C), which is used as internal control. These bands represent the three treatments with and without rapamycin. The phosphorylation of mTOR protein was inhibited in all treatments in the group injected with rapamycin (Figure 4-10).

Rapamycin significantly inhibited the mTOR activation pathway in both 10 mmol/l arginine (A) and 10 mmol/l arginine with 40 mmol/l free lysine (C) ( $P < 0.05$ ) treated loops (Figure 4-11), with a tendency for a lower ratio of the phosphorylated: nonphosphorylated mTOR in the rapamycin-treated lysyl-lysine loop (B) ( $P = 0.09$ ) (Figure 4-11).

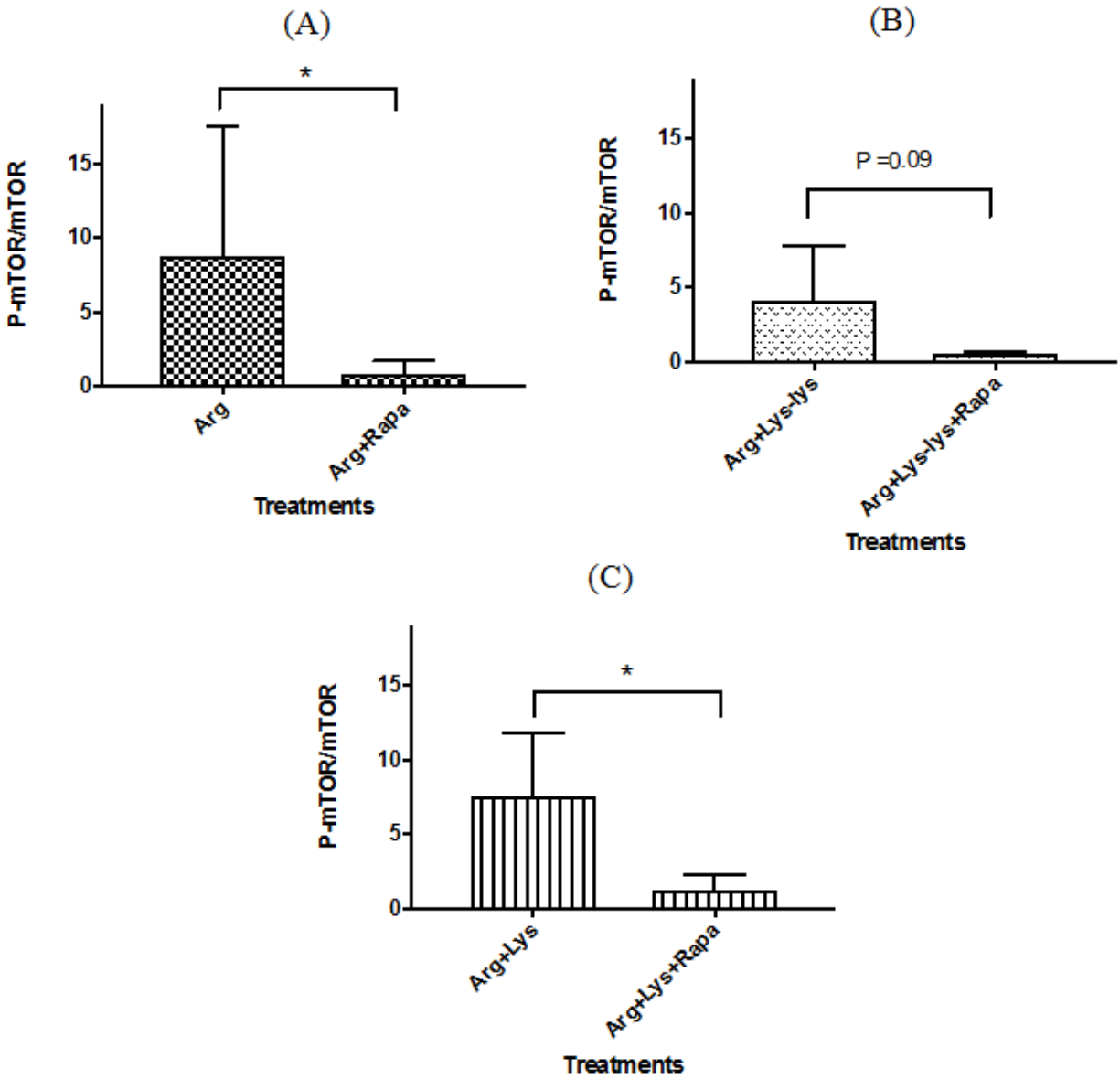
We expected that the intensity of mTOR band should be similar in both groups: with and without rapamycin. However, we observed low intensity band of mTOR in the rapamycin treated group when compared to non rapamycin treated group.





**Figure 4- 9: Mucosal protein concentration.** Values are means + SD (n=12) and data were analyzed by repeated measures one-way ANOVA followed by Tukey's multiple comparisons  $P > 0.05$ . Abbreviations: Arg, Arginine; Lys-lys, Lysyl-lysine; Lys, Lysine.





**Figure 4- 11: The ratio of phosphorylated to nonphosphorylated mTOR with and without rapamycin.** Values are mean  $\pm$  SD, n=6 per treatment. \*P < 0.05 by two tailed unpaired t-test. Abbreviations: Arg, Arginine; Lys-lys, Lysyl-lysine; Lys, Lysine; Rapa, Rapamycin.

## Chapter 5: Discussion

### 5.1. Luminal <sup>3</sup>H-arginine uptake in intestinal loops perfused with lysyl-lysine.

We confirmed the findings by Tennakoon et al. (2013) that lysyl-lysine enhanced arginine absorption in the neonatal piglet's small intestine. Our results indicate that the luminal <sup>3</sup>H-arginine uptake in miniature piglets was significantly higher in intestinal loops perfused with 10 mmol/l arginine and 20 mmol/l lysyl-lysine (178%) compared to the control condition of arginine alone (Figure 4-1). The enhanced arginine uptake by the dipeptide, lysyl-lysine, may be explained by the trans-stimulation of the b<sup>0</sup>,<sup>+</sup> antiporter. PepT1 facilitates the uptake of lysyl-lysine into enterocytes. Lysyl-lysine can then be hydrolyzed by intracellular peptidases to free lysine, increasing its concentration inside the cell. This free lysine in turn may stimulate the b<sup>0</sup>,<sup>+</sup> antiporter which initiates the exchange of the intracellular lysine for luminal arginine, resulting in enhanced arginine uptake. Also, we observed the same results under different experimental conditions, when rapamycin was infused intravenously prior to the perfusion study. Under those conditions, co-perfusing 20 mmol/l lysyl-lysine with arginine again resulted in the highest arginine uptake (179%) compared to the control condition (Figure 4-2). The results from these two experiments were very similar to what was observed previously in a study that was done in our lab (Tennakoon et al., 2011). When 20 mmol/l lysyl-lysine was co-perfused with 10 mmol/l arginine in an *in situ* gut loop model in miniature piglets, arginine absorption by intestinal cells was enhanced compared to the control condition (Tennakoon et al., 2011). In addition, a similar observation was reported in human intestinal Caco-2 cells (Wenzel et al., 2001). When Caco-2 cells were incubated with lysyl-lysine (10 mmol/l) or free amino acids, the uptake of the free L-arginine was greater with the presence of dipeptides (Wenzel et al., 2001).

We also measured the mucosal tissue free amino acid concentrations in intestinal segments that were perfused with arginine with or without lysyl-lysine. We found that lysine concentration was almost six times higher in segments exposed to lysyl-lysine compared to the control condition with no lysine or dipeptide (Table 4-1). This finding supports the concept that high intracellular lysine generated from lysyl-lysine hydrolysis was a factor in initiating the trans-stimulation of  $b^{0,+}$  antiporter. Similar results were reported in the study by Tennakoon et al. (2011). In that study, when arginine was perfused with different concentrations of lysyl-lysine (10 or 20 mmol/l), the lysine concentration in tissue was only significantly higher in the 20 mmol/l condition compared to the control condition. The authors suggested that the concentration of intracellular lysine was the main factor in initiating the trans-stimulation of  $b^{0,+}$  antiporter. As a result, they only observed higher arginine uptake by mucosa cells in the 20 mmol/l lysyl-lysine condition, but not the 10 mmol/l. They attributed the absence of enhanced arginine uptake with the 10 mmol/l lysyl-lysine to the possibility of the low lysine concentration which was unable to stimulate the trans-stimulation of  $b^{0,+}$  antiporter (Tennakoon et al., 2011).

We also observed in the current study that free lysine increased in the buffer in the lysyl-lysine treatment. By the end of the perfusion process, lysine concentration in the buffer was  $\sim 7.5 \pm 2.3$  mmol/l (initial concentration was  $\sim 0$  mmol/l). The free lysine may have come from the transport of intracellular lysine to the lumen by the  $b^{0,+}$  antiporter to exchange for arginine.

In conclusion, co-perfusing lysyl-lysine with arginine likely stimulates the trans-stimulation of  $b^{0,+}$  antiporter which results in greater arginine uptake by mucosal cells. It would be interesting to determine if other dipeptide/free amino acid combinations also exhibit the same beneficial effect,

or if this arginine/lysyl-lysine relationship exists in other circumstances, such as during intestinal injury.

## **5.2. Luminal <sup>3</sup>H-arginine uptake in intestinal loops perfused with free lysine.**

Lysine-arginine antagonism describes the competition between lysine and arginine for transport in the intestine (Czarnecki et al., 1985). Lysine-arginine antagonism has been documented in a number of different animals including rats, chicks, dogs, and guinea pigs (Austic & Scott, 1975; Baker, 1987; Czarnecki et al., 1985; Ulman et al., 1981). However, in young pigs, lysine-arginine antagonism is controversial (Baker, 1987; Hagemeyer et al., 1983). In this study, we did not observe lysine-arginine antagonism. Co-perfusing 10 mmol/l arginine with 40 mmol/l free lysine did not inhibit arginine uptake. Indeed, arginine uptake when delivered with free lysine was almost 50% greater than the control condition (Figure 4-1), although not significantly different. In the second experiment when piglets were pretreated with rapamycin, the delivery of arginine with 40 mmol/l free lysine significantly enhanced arginine uptake compared to the control condition (Figure 4-2). There was less variability in the response to the free lysine in the rapamycin experiment, which likely facilitated the statistical difference.

In the study by Tennakoon et al. (2011), they also did not observe lysine-arginine antagonism using the gut loop model and delivering 20 mmol/l free lysine with arginine (Tennakoon et al., 2011). Similar to our study, perfusing arginine with free lysine resulted in more arginine uptake (i.e. ~ 123-130% of the control condition), but in that study, it was not significantly different from the control condition (Tennakoon et al., 2011). The absence of lysine-arginine antagonism in the

*situ* model may be attributed to the activity of another transporter that is responsible for transporting cationic amino acids in intestinal piglets (Deves & Boyd, 1998).

We also measured the concentration of free lysine in mucosal tissue, after the tissue was exposed to lysine during the perfusion study. Not surprisingly, the lysine concentration was significantly higher when 40 mmol/l of free lysine was co-perfused with arginine compared to the control condition (Table 4-1). Although there was a high lysine concentration in the mucosal tissue, we did not observe enhanced arginine uptake when compared to the control condition in the first experiment. We anticipated that the presence of high lysine concentration in the buffer may compete with arginine for the same  $b^{0,+}$  transporter for uptake, but this was not the case in all conditions. Indeed, we did not observe any effect of perfusing lysine with arginine, and lysine did not impair arginine uptake in miniature pig's small intestine.

### **5.3. Mucosal protein synthesis.**

We hypothesized that the presence of the lysyl-lysine with arginine in the small intestinal lumen will lead to the functional advantage of greater mucosal protein synthesis, which is likely mediated through enhanced arginine absorption. We measured the rate of mucosal protein synthesis in samples that were acquired from intestinal segments which were perfused with  $^3\text{H}$ -phenylalanine. The delivery of lysyl-lysine with arginine resulted in the highest rate of mucosal protein synthesis (47%/d) compared to the other two treatments (Figure 4-7). Although not statistically different, the arginine uptake with free lysine tended to be  $\sim 50\%$  greater than the control; however, this did not translate into greater protein synthesis, which was similar between free lysine (16%/d) and the control condition (21%/d) (Figure 4-7). These rates of mucosal protein

synthesis tend to be lower than literature values for fed piglets (Bauchart-Thevret et al, 2010). This was likely due to the model wherein only arginine and lysine were perfused through the gut rather than a balance of amino acids; this would limit protein synthesis. The higher rate of protein synthesis with the lysyl-lysine may be due to greater arginine availability for incorporation into proteins, or greater activation of the mTOR pathway (Bauchart-Thevret et al., 2010); however, the differences in rate of protein synthesis are much larger than the differences measured in arginine uptake in the various experimental conditions. An alternate explanation involves a direct effect of the lysyl-lysine dipeptide on mTOR activation. A recent study measured the effects of lysyl-lysine in IPEC-J2 cells harvested from neonatal piglet jejunum. Exposure to different concentrations of lysine and lysyl-lysine were used to measure mTOR activation (Yin et al., 2016). The authors reported that high lysine concentration (5 mmol/l) lowered the phosphorylation of mTOR, while exposure to high lysyl-lysine (5 mmol/l) enhanced mTOR activation (Yin et al., 2016). The dipeptide lysyl-lysine may have a role in mTOR pathway activation and nutrient sensing, thus directly modifying protein synthesis in the small intestine (Yin et al., 2016).

#### **5.4. Mucosal protein synthesis with rapamycin.**

One of the objectives of this study was to determine if the mucosal protein synthesis in response to arginine uptake was mediated through the mTOR pathway. The rate of mucosal protein synthesis was measured in neonatal piglets after they were pretreated with rapamycin inhibitor. As predicted, rapamycin significantly inhibited the mucosal protein equally amongst all treatments (Figure 4-8). A similar observation was reported in an *in vitro* study which used neonatal porcine intestinal epithelial cells (IPEC-J2) (Bauchart-Thevret et al., 2010). When the cells were exposed to varying arginine concentrations (0, 0.1, 0.5, 1.0 mmol/l), there was a stimulation of the mTOR



pathway, and the rate of protein synthesis increased by 76% compared to the control condition. However, the highest protein synthesis rate was at the physiological concentrations of arginine, 0.1– 0.5 mmol/l. Moreover, when IPEC-J2 cells were treated with rapamycin at different concentrations (1-1000 nmol/l), protein synthesis was inhibited compared to the control condition. However, protein synthesis was significantly inhibited by 42% after treating the cells with rapamycin at concentrations of 1-10 nmol/l. These results suggest that protein synthesis in the intestine was partially controlled by the mTOR pathway (Bauchart-Thevret et al., 2010).

In summary, we observed that the intestinal proteins synthesis was equally inhibited among all treatments after pre-treating piglets with IV rapamycin. Our results support previous findings that the protein synthesis in the small intestine mucosa is stimulated by arginine availability.

### **5.5. mTOR pathway inhibition by rapamycin.**

To further clarify the roles of arginine and mTOR in stimulating protein synthesis, western blotting was used to assess the activation status of mTOR pathway intermediates in the presence and absence of rapamycin. Rapamycin significantly inhibited the mTOR pathway activation in the arginine alone and arginine with free lysine conditions (Figure 4-11). For the arginine plus lysyl-lysine condition, ratio of phosphorylated/nonphosphorylated mTOR was not significantly different, despite an 87% lower ratio with rapamycin (Figure 4-11).

We expected that the intensity of mTOR band would be similar in both groups, with and without rapamycin. Because the intensity of the mTOR band was low in the rapamycin treated group when compared to the non-rapamycin group, we may have overestimated the ratio of p-mTOR/mTOR

in rapamycin treated group (Figure 4-10) (Figure 4-11). Despite this potential error, we still observed significant inhibition. One possible reason that may have contributed to the low intensity mTOR band was that rapamycin may have changed the antibody binding site on- mTOR.

The decrease of the ratio of phosphorylated/nonphosphorylated mTOR follows the same pattern of the mucosal protein synthesis inhibition data. Rapamycin lowered the level of phosphorylated/nonphosphorylated mTOR by 80% or more when piglets were pre-treated with IV rapamycin. Similar results were reported in an *in vitro* experiment which demonstrated that arginine (0.5 mmol/l) activated mTOR signaling in neonatal porcine IEC (IPEC-J2) cells, and the addition of rapamycin (10 nmol/l) affected mTOR and its downstream target activation by decreasing the phosphorylation levels which also in turn decreased the mucosal protein synthesis (Bauchart-Thevret et al., 2010). Moreover, another study that was done in arginine-supplemented neonatal piglets with rotavirus enteritis demonstrated that rapamycin decreased the relative phosphorylation level of mTOR/p70S6k by 80% in the small intestine which was also reflected in the mucosal protein synthesis (Corl et al., 2008).

We expected that mTOR activation would parallel the stimulation of mucosal protein synthesis. In other words, since the protein synthesis in the small intestine is mediated by the mTOR signaling pathway, the level of the activated mTOR should be mirrored by the level of protein synthesis stimulated in the small intestine. According to our protein synthesis data (in the absence of rapamycin), we assumed that we would find higher mTOR activation in loops perfused with arginine and lysyl-lysine compared to arginine alone or free lysine. However, results were unexpected, and what we observed was completely the opposite of the protein synthesis data. The

mTOR activation was higher with arginine alone and free lysine. As a result, the pattern of mTOR activation among different treatments without rapamycin (Figure 4-11) did not parallel the mucosal protein synthesis data (Figure 4-7). This may be because the relationship between protein synthesis and mTOR is not linear and that mTOR was already maximally activated. Another reason that may be that the amino acids that were readily available in the mucosa were not sufficient to support any greater protein synthesis, because we did not perfuse the full profile of amino acids through the loops.

We also attempted to measure other proteins in the mTOR pathway which are the downstream targets P70S6K and 4E-BP1; however, we did not report the results. During western blot analysis, we encountered multiple methodological problems. After incubating with the primary P70S6K and 4E-BP1 and secondary antibodies, we did not visualize any bands on the membrane; this may be because of a failure of antibody binding to the protein in the membrane. The reason for not visualizing any bands could be related to the sample concentrations or the antibody concentrations used. However, we tried multiple sample concentrations and antibody concentrations, and we still did not produce any bands. Another reason could be the antibody itself, which is not specifically for porcine protein detection; as a result, it may not have bound to the protein on the membrane. It is important in the future to compare the the antigenic epitopes used for the generation of P70S6K and 4E-BP1 antibodies with the porcine sequences, to verify whether or not there is cross reactivity between antibodies and the porcine proteins. Eventually, attempts to quantify P70S6K and 4E-BP1 were abandoned due to time and funding constraints.

In summary, piglets pre-treated with IV rapamycin demonstrated a decrease in the ratio of phosphorylated/nonphosphorylated mTOR protein among all treatments when compared to the non-rapamycin-treated group. The decrease in mucosal protein synthesis in the neonatal piglet small intestinal mucosa is likely due to the mTOR inhibition by rapamycin. Our results demonstrated that when more arginine substrate was available, protein synthesis was stimulated. Nevertheless, our results failed to demonstrate that arginine stimulates mTOR since we did not include zero or low arginine treatment.

#### **5.6. Limitations and modifications.**

The gut loop model was used in piglets to measure intestinal amino acid uptake. The gut loop model is an excellent design to investigate multiple experimental conditions in one animal by having multiple intestinal segments perfused with different treatments simultaneously. The main purpose of this study was to define the amino acids interaction at the level of the mucosa in the small intestine which would ultimately assist in designing optimal diets to support piglet growth. Perfusing multiple treatments at the same time is also considered one of the limitations of the study design because it is difficult to determine how each treatment will contribute to overall piglet performance. In order to be able to interpret the effect of each treatment on growth, protein synthesis, and overall amino acids metabolism in tissues, it would be necessary to supply only one treatment to one animal. Moreover, by perfusing multiple treatments in the same animal, it is impossible to measure plasma amino acids profile. Measuring the plasma amino acids profile would assist in supporting the reported results, in terms of greater amino acids uptake by intestinal cells being reflected in the blood. Also, a single treatment would facilitate determining the form

of amino acids that enters the circulation (dipeptide vs. free amino acid) after exiting the basolateral side of the intestinal cells.

Finally, the perfused buffers were composed of only PBS and arginine, arginine and lysyl-lysine, or arginine and free lysine, which is likely not the ideal nutrient profile to support mucosal protein synthesis. Interestingly, the highest rate of protein synthesis measured in this study (46%/d) was only marginally lower than that reported by Nichols et al. (~53%) in a perfusion study with neonatal piglets that included a balance of amino acids in the buffers (Nichols et al., 2008). Future studies could utilize buffers that were formulated with a full amino acid profile similar to sow milk.

## **5.7. Conclusion.**

In isolated small intestinal segments, the delivery of lysyl-lysine with arginine resulted in greater arginine uptake and higher mucosal protein synthesis compared to arginine alone. The enhanced arginine uptake with lysyl-lysine may have been due to the trans-stimulation of  $b^{0,+}$  antiporter that facilitated greater arginine uptake into enterocytes. Arginine is known to increase mucosal protein synthesis via the stimulation of the mTOR pathway (Bauchart-Thevret et al., 2010), which is involved in cellular growth and protein synthesis; activation of the pathway occurs via phosphorylation of mTOR downstream targets P70S6K and 4E-BP1. Arginine is not the only amino acid that stimulates the mTOR pathway; relevant to this study, a recent study suggested that the dipeptide lysyl-lysine may also have a role in the mTOR pathway activation which may explain some of our findings in this study (Yin et al., 2016). In the current study, when piglets were pre-exposed to the mTOR pathway inhibitor, rapamycin, prior to the perfusion studies, mucosal protein

synthesis was inhibited by greater than 60%, and was not different amongst treatments. Moreover, rapamycin treatment inhibited the phosphorylation of mTOR, interfering with its activation.

In the swine industry, it is becoming a common practice to add free amino acids to the diet while decreasing the crude protein content. This practice is adopted to lower both the nitrogen excretion and ammonia released in the animal's waste, which are considered an environmental hazard (Hayes et al., 2004). Also, it was reported that lowering the crude protein in piglet diets can lower post-weaning diarrhea (Heo et al., 2008). However, the addition of free amino acids to the diet may affect the uptake of other amino acids which may have a negative consequence on piglet growth or recovery. As such, it may be more beneficial to add peptides to the diet with free amino acids to maximize the uptake by the small intestine, especially during stressful events.

In situations of intestinal injury in neonates, such as weaning, infection, or following IV feeding, the small intestinal capacity for transporting free amino acids may be compromised; however, it may not be the case for peptides. As a result, supplying dietary amino acids as a combination of peptides and free amino acids may enhance uptake by the mucosa, which assists in improving GI recovery and overall performance. The results of the current study contribute information towards the design of an optimal diet which enhances amino acid absorption by the small intestine during stressful events and ultimately may contribute to optimizing growth.

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