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The protein composition of endogenous losses in the human gut.

A thesis presented in partial fulfillment of the requirements for the degree
of Doctor of Philosophy in Human Nutrition at Massey University,
Palmerston North, New Zealand

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2012

Abstract

During the digestion of food there is a significant amount of endogenous proteinaceous material secreted into the gut. A dynamic equilibrium exists in the gastrointestinal tract (GIT) between dietary protein intake and the secretion of endogenous proteinaceous material into the lumen with the concomitant absorption from the gut of digested materials, both exogenous and endogenous in origin. There have been no systematic studies of the protein composition (i.e. sources of protein) of ileal digesta in humans. The objectives of this study were to quantify the endogenous protein components of ileal digesta collected from the terminal ileum of both humans and pigs (the growing pig was used as an animal model for the adult human).

Preliminary work was undertaken to ascertain the best methods for quantifying mucin and bacterial protein in digesta from the terminal ileum. Preferred methods for the determination of mucin utilised the markers N-acetylglucosamine and N-acetylgalactosamine, and diaminopimelic acid as a marker for bacterial protein.

Of the total nitrogen lost at the terminal ileum of pigs fed a casein-based diet nearly 73% was proteinaceous; nearly 45% originated from bacteria, 13% from soluble free protein and 11% from mucin. Of the non-protein nitrogen 11% was ammonia and 5% urea. Only 8.3% of the total nitrogen remained unidentified. Mucin was the single most abundant truly endogenous component (13% of total dry matter). In humans fed the same casein-based diet 86% of total nitrogen at the terminal ileum was proteinaceous; nearly 60% originated from bacterial protein, 15% from mucin and 6% from soluble free protein. Of the non-protein nitrogen 5% was ammonia and 4% urea. Only 4% of the total nitrogen remained unidentified. Once again mucin was the single most abundant truly endogenous component (13% of total dry matter). In a final study the ileal endogenous protein components were determined in human subjects given three isonitrogenous diets, which differed only in the form that nitrogen was supplied. In comparison with a synthetic amino acid based-diet casein and enzyme hydrolysed casein increased endogenous nitrogen losses, including both the concentration and flow of mucin in the terminal ileal digesta. The form of dietary protein did not affect the proportion of bacterial protein in the ileal effluent.

Acknowledgements

The work reported in this dissertation was undertaken at the Riddet Institute (Massey University, Palmerston North, New Zealand under the supervision of Distinguished Professor Paul Moughan. The collection of digesta from human subjects was carried out in collaboration with the UMR914 Nutrition Physiology and Ingestion Behaviour group (INRA/AgroParisTech, Paris, France).

I would like to express my sincere gratitude to Professor Moughan my chief supervisor, for his excellent tutorage, generously giving me guidance and encouragement over the years of this study. I would also like to sincerely thank the late Dr Malcolm Fuller who helped me through the process of drafting and publishing research material from this study.

I would also like to offer my gratitude to Dr Amélie Deglaire for her assistance with the acquisition of samples from both pigs and human subjects that are the foundation of this study. I wish to thank Dr Gordon Reynolds for expertly undertaking the surgery to install the PVTC's at the commencement of the pig trial and Dr Robert Benamouzig for his expertise in managing the installation of the naso-ileal tubes at the start of the human trial in his hospital service. I should also like to thank Dr Shane Rutherford and Maggie Zou for their assistance with some of the methodologies used in this study. Also the staff of the Nutrition Laboratory at the Institute of Food Nutrition and Human Health at Massey University, particularly Mrs Fliss Jackson and Ms Leiza Turnbull for sample analysis and their time and patience they unselfishly gave me.

Special gratitude must go to my wife Lynda who has supported me spiritually and financially throughout this study, as without her patience and generosity I could not have completed it. Thanks must also go to both my daughters, Cheryl and Corinne, who have given me valuable encouragement throughout this study.

“To see is to see a better way; to perceive any problem clearly is to begin to create its solution. All we need is the wisdom and patience to keep looking.”

Laurence Boldt.

“If I can see at all it is because I have stood on the shoulders of giants.”

Warren Miner-Williams.

An adaptation of a remark made by Isaac Newton in a letter to Robert Hooke
February 5, 1676.

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Chapter I

Review of Literature

I. Structure and function of the gastrointestinal tract and the mucosal layer

I.1 Introduction

Dietary nutrients are the materials necessary to support life. However, such exogenous nutrients present in food cannot enter the body in their dietary form. They must first be digested; deconstructed into simpler molecules that can be absorbed by the epithelial cells that line the alimentary canal. Although the principal functions of the gastrointestinal tract (GIT) are the chemical breakdown of exogenous dietary macromolecules and the absorption of the resultant simpler products, there is also a simultaneous movement of nitrogenous (endogenous) materials into the lumen of the GIT (Fuller and Reeds, 1998). Such substances enter the GIT from accessory organs in a variety of digestive secretions. Salivary, gastric, hepatic, pancreatic, and intestinal secretory cells, all secrete endogenous proteins into the lumen of the gut (Jansman et al., 2002) that are vital for digestion and absorption (Wapnir and Teichberg, 2002). Much of the endogenous material secreted into the GIT is also subject to digestion and reabsorption, such that only a small fraction of the total nitrogen secreted into the lumen is lost in the faeces (Fuller and Reeds 1998). To fully describe the dynamic flux between dietary protein intake and the secretion of endogenous proteinaceous material into the intestinal lumen, together with the concomitant absorption of both exogenous and endogenous materials from the gut, it is necessary to briefly describe the anatomy and physiology of the GIT.

I.2. General character of the wall of the alimentary canal

Formed embryologically from the endoderm folding to form a tube, the alimentary canal extends from the mouth to the anus (**Figure 1**). Accessory organs that develop from buds along the embryonic alimentary canal include the salivary glands, pancreas, liver and gall bladder. All of these organs release secretions into the alimentary canal that are pivotal to the digestive process.

The wall of the alimentary canal has four distinct layers. Though some regions have specialist functions the four layers are common throughout its length:

i. Mucosa

On the inner surface of the lumen the mucosa is formed from the surface epithelia and underlying connective tissue, the lamina propria. Cylindrical invaginations of the mucosal cells form crypts that secrete fluid and electrolytes. The function of the mucosal layer is:

- a) secretion
- b) absorption and
- c) protection of the layers beneath,

ii. Submucosa

The submucosa is the vascular layer beneath the mucosa formed from the connective tissue that contains blood vessels, lymphatic vessels, glands and nerves. The purpose of the submucosa is to perfuse the surrounding tissues with nutrients and to remove absorbed materials

iii. Muscular layer

The muscle coat is formed from two types of smooth muscle that are under autonomic control:

- a) Circular muscle that contracts the diameter of the tube, and
- b) Longitudinal muscle that on contraction shortens the tube.

The rhythmic contractions of the circular muscle propel material through the GIT by the process of peristalsis.

iv. Serosa

The outer covering of the alimentary canal is composed of epithelial tissue that covers the underlying connective tissue. The epithelia secrete serous fluid that

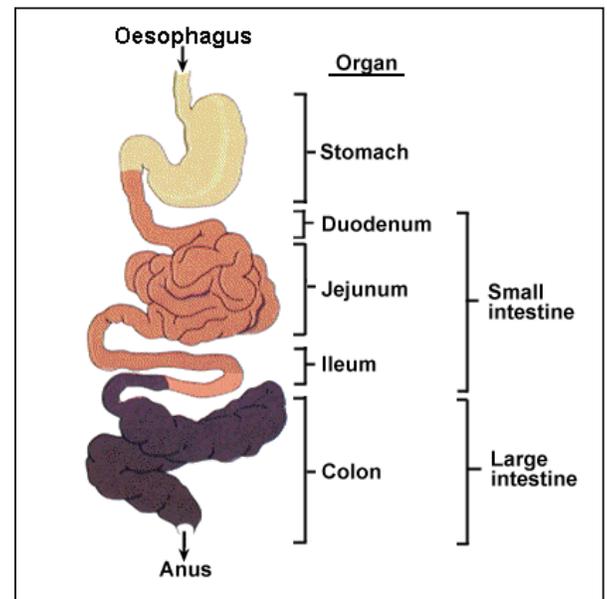


Figure 1. The organs of the alimentary canal.*

* Illustration from the University of Florida in the public domain.

moisten and lubricate the outer surface so that organs contained within the peritoneal cavity can move freely against one another.

I.3. Structure and function of the gastrointestinal mucosa.

The primary function of the GIT is to digest the macromolecules of food and absorb the resultant nutrients from the complex environment of the gut lumen into the hepatic portal system (Field and Frizzel, 1991).

The intestinal lumen is a particularly noxious environment. As the alimentary canal is effectively open at both ends the epithelia of gastrointestinal mucosa are directly exposed to the hazards of the external environment, food, anti-nutritional factors and potentially damaging secretions (including bile salts, acids, digestive enzymes, food toxins, and pathogenic bacteria) (Bevins *et al.*, 1999; Sanderson and Walker, 1999). As a direct result of this the mucosal epithelia are rapidly and continuously renewed, a process which is essential to ensure the effective digestion and absorption of nutrients, together with reducing the colonisation of pathogenic microorganisms. In the small intestine the epithelial surface is formed into finger like projections or villi that are 0.5 to 1.5 mm in length, though their size varies in different regions of the gut. At the base of each villus there are 5-10 tubular intestinal glands, the crypts of Lieberkühn, which secrete fluid and electrolytes into the lumen. The anatomical characterisation of the mucosal epithelia is illustrated in **Figure 2**.

Pluripotent stem cells within the crypts of Lieberkühn give rise to daughter cells that differentiate to form four cell types as they migrate on to the villus or to the base of the crypt. Once the migrating cells reach the tip of the villus they are sloughed off. The rapid turnover of mucosal cells renews the intestinal epithelial lining every three to six days (Bajaj-Elliott and Sanderson, 2004).

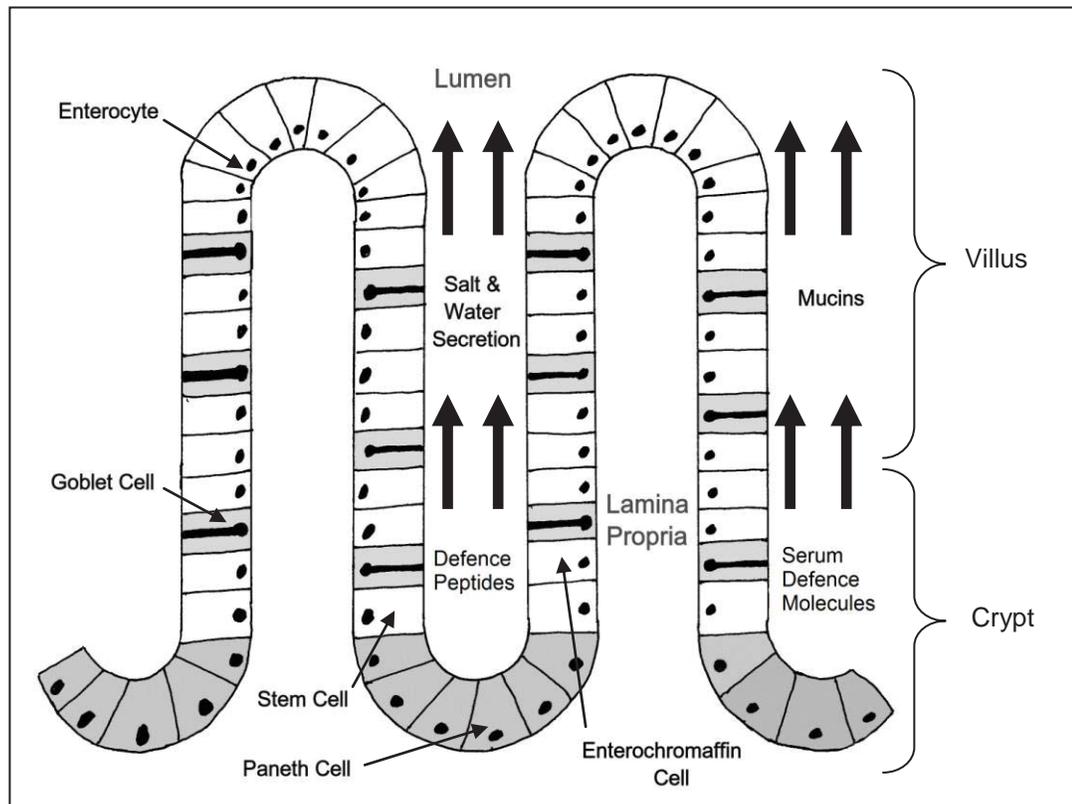


Figure 2. The anatomical distribution of the major cell types of the lamina propria and the epithelium of the small intestine. Modified from Bajaj-Elliott and Sanderson (2004).

There are four major types of epithelial cell in the small intestine:

a) Columnar enterocytes.

As crypt cells migrate upward towards the tip of the villus (**Figure 3**) they differentiate from secretory cells into absorptive enterocytes. One of the major morphological changes occurs at the apical surface of the cells with the formation of microvilli (**Figure 4**) that are often described as the brush border (Moosker, 1985). Digestive enzymes,

such as peptidases and intestinal lipase embedded within the membranes of the brush border break down food molecules just before absorption occurs. The villi, together with the microvilli of the brush border, greatly increase the total

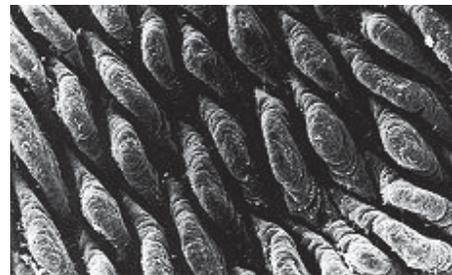


Figure 3 Villi carpeting the inner surface of the small intestine. Permission granted by Keith R. Porter (From Kimball, 1978).

absorptive area of the gut to aid the absorption of nutrients from the lumen. The absorptive enterocytes make up 90-95% of the cells of the mucosa that cover the surface villi (Madara and Trier, 1994).

b) Goblet cells.

Goblet cells throughout the small intestine share a similar morphology being distinguished by the appearance of a “brandy goblet.” The principal function of the goblet cells is the secretion of mucin that acts as a barrier

to protect the underlying mucosa from harmful substances that may be present in the lumen (Bansil *et al.*, 1995; Cheng and Leblond, 1974). Several studies have shown that certain intra-luminal antigens such as microorganisms (e.g. *E. coli*) or peptides (e.g. β casomorphin-7) stimulate a protective response that includes the secretion of mucin (Bansil *et al.*, 1996; Claustre *et al.*, 2002; Moon *et al.*, 1971).

c) Enterochromaffin cells.

Common throughout the alimentary canal enterochromaffin cells most commonly occur in the crypts of the small intestine. Although these cells are similar to the columnar enterocytes their morphology differs slightly. They are narrower than enterocytes and their microvilli are fewer in number and are more irregularly spaced. There are as many as eight different subclasses of these cells depending on the nature of the peptides they secrete. These subclasses include: gastrin, serotonin, cholecystokinin, neurotensin, and secretin-like immunoreactive cells.

d) Paneth cells.

As part of the mucosal barrier, paneth cells synthesise and secrete proteins (which are associated with innate immunity and host defence) into the lumen of the crypts of Lieberkühn of the small intestine (Bajaj-Elliott and Sanderson, 2004; Bevins *et al.*, 1999; Ouellette, 1999). Paneth cells are found in most

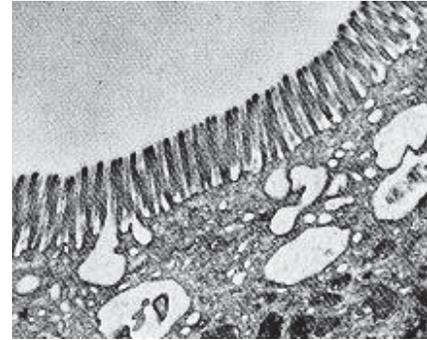


Figure 4 Microvilli of the mouse intestinal cell. Permission granted by Dr. Samuel L. Clark (From Kimball, 1978).

mammals with the exception of cats, dogs and pigs. Anti-microbial proteinaceous secretions from these cells include secretory phospholipase A2 (Mulherkar *et al.*, 1993) anti-trypsin (Molmenti *et al.*, 1993) and peptides of the α -defensin family (Jones and Bevins, 1992). In mice, high levels of lysozyme, an enzyme known to hydrolyse the glycoprotein coat of bacterial cell walls, is present in the Paneth cell granules and suggests that the cells are involved with host defence,(Ghoos and Vantrappen, 1971).

The apical surface of the mucosal epithelium forms a selective barrier between the cells of the underlying lamina propria and the external environment (Bajaj-Elliott and Sanderson, 2004; Sanderson and Walker, 1999). Highly regulated, selective absorption takes place by either a transcellular or paracellular route. Transcellular absorption is via passive diffusion through the enterocytes, endocytosis or carrier mediated transport systems e.g. PEPT1.

The high metabolic cost of tissue turnover strongly determines the nutrient requirements of the gut wall. This may be as much as 50% of the total nutrient requirement of the gut wall and is a relatively high percentage of the whole-body protein turnover. As much as 40% of total heat production of the GIT may be attributed to tissue turnover. The remainder may be attributed to other synthetic and maintenance processes, 50% of which can be attributed to transport of ions and nutrients (Baldwin, 1995).

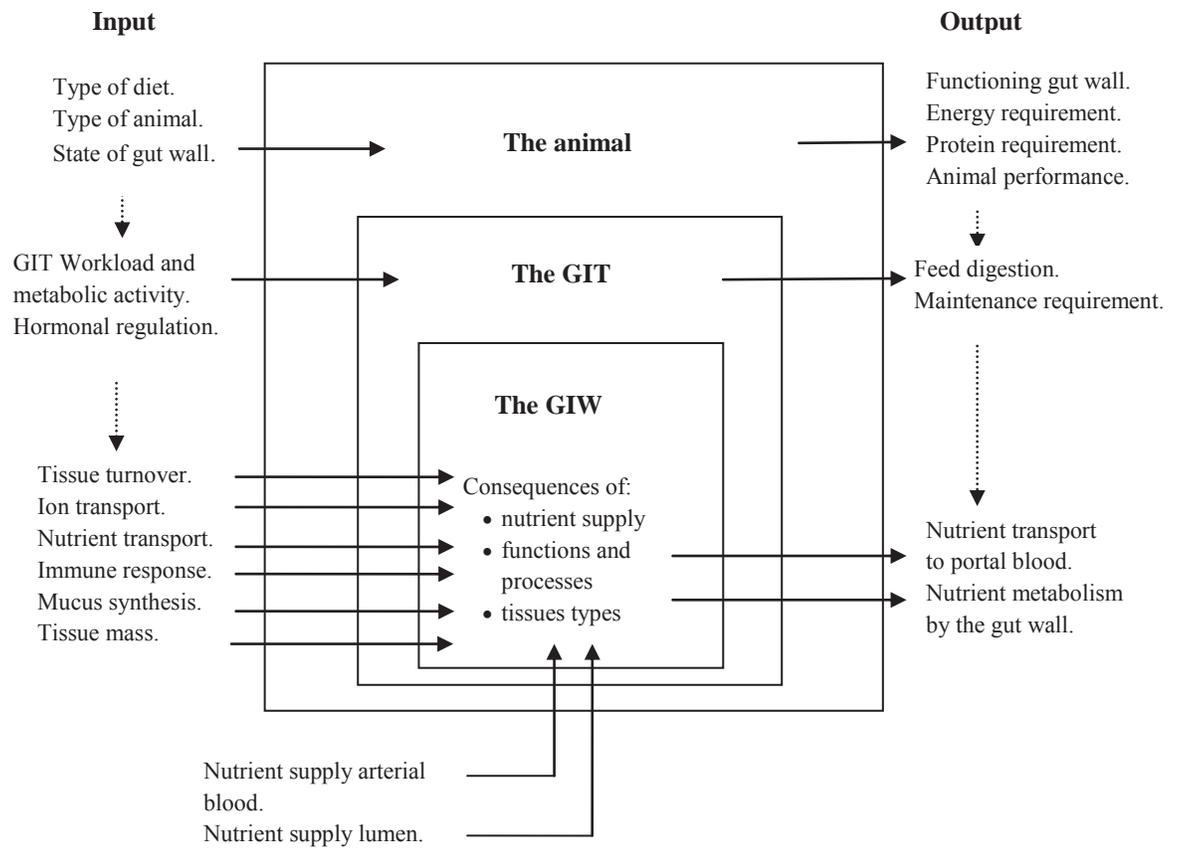
The GIT is also an integral part of the body's immune system (Goddeeris *et al.*, 2002) and the majority of the total immune cells in the whole body are located in the GIT. Intra-epithelial lymphocytes, the first-line of defence against dietary or pathogenic antigens represent 15% of the cells in the GIT epithelium (Dugan *et al.*, 1994). The energy costs associated with an immune response of the GIT may be significantly more than in the remainder of the body and as such contribute significantly to the nutrient requirements of the gut wall. The most important immunoglobulin secreted by the GIT IgA is synthesised by specialised cells within the Peyer's patches, is highly specific and a central element of the immune response (Alverdy, 1990). Such immunoglobulins are highly resistant to enzymatic degradation, changes in temperature and pH, and effectively prevent the colonisation of microorganisms in the

mucosal wall (Bannink et al., 2006). Although strictly not part of the mucosal epithelia, Peyer's patches are noteworthy and appear as small swellings on the mucosal epithelial surface, they contain clusters of lymphoid cells that are covered by follicle associated epithelium (FAE). FAE contain membranous cells or *M*-cells that play an important role in the immune mechanism of the mucosal barrier by antigen sampling (Kraehenbuhl and Neutra, 2000; Neutra *et al.*, 1987; Neutra *et al.*, 1996). The apical surface of *M*-cells differs from the surface of columnar enterocytes as they lack the typical brush border. When luminal antigens bind to the glycocalyx surface of *M*-cells they are absorbed by transcellular endocytosis (Neutra *et al.*, 1988). The endocytotic activity of *M*-cells is much greater than that of the columnar enterocyte. The abundance of vesicles within the cytoplasm of the *M*-cells facilitates the encapsulation and transport of antigens, attached to microdomains on the apical membrane, to the underlying lymphoid tissue (Bhalla and Owen, 1983; Kerneis *et al.*, 1996; Neutra *et al.*, 1988). The ontogeny of *M*-cells is uncertain, though it is likely that they originate from the epithelial stem cells.

Maintaining the integrity of the mucosal barrier is paramount for gut homeostasis and immunological defence as breaches of this system have been implicated in a number of inflammatory diseases (Sanderson and Walker, 1993). Other mechanisms that supplement the security of the mucosal barrier are: gut motility, the secretion of mucus, cell turnover and the probiotic microflora (Alderberth *et al.*, 1999).

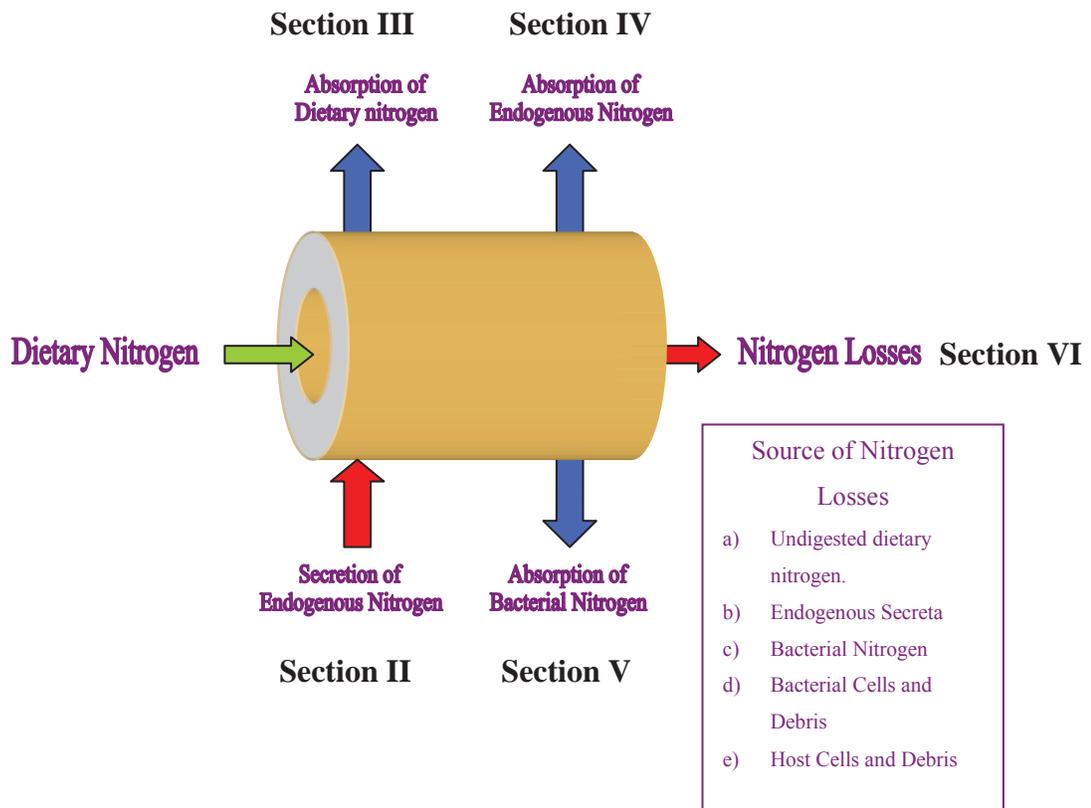
The physiological and metabolic functions of the GIT affect the nutrient requirement of the tissues of the gut wall. The effect of gut wall metabolism on whole-body metabolism is profound with respect to protein turnover, ion transport, and costs of the immune response of the animal (Bannink et al., 2006). The relative contribution of each of these functions or processes, together with that of mucus and enzyme secretions, depends on the nutritional and physiological state of the GIT and may change dramatically with altered conditions. A schematic representation of the digestive system at different levels of organisation is given in **Figure 5**. For an improved quantification of the metabolic activity of the gut wall, the kinetics of the digestive processes, of the intraluminal microbial activity, of the absorption of nutrients, of the arterial supply of nutrients, and of the specific metabolic functions of the gut wall must all be studied (Bannink et al., 2006).

Figure 5 A schematic representation of the digestive system at different levels of organisation: the **animal**, the **gastrointestinal tract (GIT)** and tissue of the **gastrointestinal wall (GIW)**.



Modified from Bannink *et al.* (2006).

Figure 6. A key to sections that review the passing of nitrogenous materials into the lumen of the GIT, nitrogenous materials being absorbed from the lumen and the loss of nitrogenous materials from the small intestine.



II. Secretion of endogenous nitrogen: An overview of materials passing into the small intestine.

Although the principal functions of the GIT are the chemical breakdown of exogenous dietary macromolecules and the absorption of the resultant smaller and simpler products, there are significant amounts of material secreted into the gut. Indeed the mass of the endogenous proteinaceous secretions has been estimated to be equal to that of ingested protein (Nasset and Ju, 1961). The secretions of the salivary, gastric, hepatic, pancreatic and intestinal secretory cells (predominantly endogenous proteins), are vital for both digestion and absorption (Wapnir and Teichberg, 2002). For the pig the estimates of daily nitrogen contained within secreted materials are: 3-5g N/day from the pancreas (Corring and Saucier, 1972); 2g N/day from the gall bladder (Sambrook, 1981); 0.3-0.6g N/day from salivary and gastric secretions (Zebrowska *et al.*, 1983); together with 3-5g N/day from the duodenal mucosa (Leibholz, 1982). The protein content of the digesta is thus a dynamic equilibrium between dietary intake and the secretion of endogenous material into the lumen on the one hand and on the other hand the concomitant absorption from the gut of digested materials, both exogenous and endogenous in origin.

Within the GIT a number of productive functions are characterised by the synthesis of secreted materials (e.g. enzymes, mucus, immune secretions, e.g. immunoglobulins) by rapid cell turnover and the synthesis of cellular components, together with the generation of endocrine responses to the intraluminal conditions (Bannink *et al.*, 2006 Burrin *et al.*, 2000). Intensive metabolic processes within the mucosa have a high demand on nutrients such as; glucose, acetate, propionate, butyrate, amino acids, and marginally long-chain fatty acids (Britton and Krehbiel, 1993). These processes involve ATP and NADH as units of metabolic energy and reducing power, the respiratory gases, O₂, CO₂, together with the metabolites formed e.g. β -hydroxybutyrate and lactate (Bannink *et al.*, 2006). Compared to other body tissues, these processes, together with intensive ion and nutrient transport across cell membranes generate a high demand for metabolic energy, and amino acids are an important source of this energy as dietary glucose plays virtually no part in intestinal metabolism (Reeds and Burrin, 2000). For the rat it is suggested that the total energy

utilisation of the portal-drained viscera (PDV) ranges between 20 to 35% of the whole body energy utilization (McNurlan and Garlick, 1980) although more recently, for the pig, the range was estimated to be between 10 to 20% (van Goudoever *et al.*, 2000), which is still high when the PDV is just 6% of the total body weight (Bannink *et al.*, 2006).

Approximately 90% of the total protein synthesis in the mucosa is related to the synthesis of materials secreted into the lumen: digestive enzymes, mucus and immunoglobulins together with sloughed mucosal cells (Gaskins, 2003). Without the digestion and reabsorption of such endogenous proteinaceous secretions the magnitude of whole body amino acid requirements would be untenable. Irrespective of the large proportion of the secreted material being recycled the whole process has a considerable metabolic cost.

The majority of the chemical processes involved in digestion are autocatalytically activated by enzymes secreted from a number of tissues associated with the GIT (see **Table 1**). Secreted proteins, such as pancreatic digestive enzymes, are synthesized on mRNA molecules bound to the endoplasmic reticulum. Following post-translational modification in the endoplasmic reticulum and Golgi complex the nascent proteins are transported as zymogen granules to the apical membrane and secreted via regulated exocytosis (Cook *et al.*, 1996).

Protein digestion is initiated in the stomach with the release of pepsins and hydrochloric acid. Pepsinogen, the zymogen precursor of pepsin, is secreted from gastric chief cells and is activated by the acidic environment of the stomach. The hydrolytic products of this digestive stage may influence a number of gastric functions that are under hormonal control, such as the secretion of acid and pepsinogen, and the rate of gastric emptying. The rate of gastric emptying is modulated by both the volume and the nature of ingested food, which together with its energy content, not only influences the extent of gastric protein digestion but also its digestion and absorption in the small bowel and the metabolic fate of the component endogenous amino acids (Dangin *et al.*, 2001; Fouillet *et al.*, 2001; Mahe *et al.*, 1992; Mariotti *et al.*, 2000).

Table 1. The major digestive enzymes secreted into the human GIT.

Enzyme	Source of secretion	Digestive action
Salivary Enzyme		
α -Amylase	Salivary glands	Initial carbohydrate digestion hydrolysing starch and glycogen to disaccharides
Gastric Enzymes		
Pepsin	} Gastric glands	Initial digestion of protein
Lipase		Initial hydrolysis of fat
Pancreatic Enzymes		
α -Amylase	} Pancreas	Hydrolysis of starch and glycogen into disaccharides
Lipase		Digestion of water insoluble fats into monoglycerides and fatty acids
Proteolytic Enzymes		
Trypsin	} Pancreas	Hydrolysis of proteins or peptides into peptides
Chymotrypsin A, B and C		
Elastase 1 and 2		
Carboxypeptidase		Further hydrolysis of peptides into amino acids
Nucleases		Breakdown of nucleic acids into nucleotides
Intestinal Enzymes		
Peptidases	} Mucosal cells	Hydrolysis of peptides into amino acids
Sucrase, maltase, lactase		Hydrolysis of disaccharides into monosaccharides
Lipase		Further breakdown of fats into fatty acids and glycerol
Enterokinase		Converts trypsinogen into trypsin

(Shier et al. 1999)

In the small intestine the cells of the crypts of Lieberkühn secrete large amounts of watery fluid and electrolytes, which are rapidly reabsorbed when the columnar enterocytes absorb the nutrient products of digestion. The fluid from these crypts is

effectively pH neutral and contains no digestive enzymes. In addition to the mucin secreting goblet cells of the crypts and villous epithelium, there are specialised mucus secreting glands in the submucosa of the proximal duodenum called Brunner's glands. These glands secrete a glutinous alkaline mucin that contains bicarbonate ions to neutralise the acidic gastric fluids and forms a slippery gel that lubricates the mucosa of the proximal intestinal tract. The unique capacity of this mucus layer to protect the delicate underlying epithelial surfaces is due primarily to the gel-forming properties of its glycoprotein molecules (Krause, 2000). Thought to be the product of the MUC6 gene, the class III mucin from the human Brunner's gland consists of O-linked oligosaccharides that are attached to a central core of glycoprotein (Wapnir and Teichberg, 2002). The mucin also contains a number of other compounds including: epidermal growth factor, trefoil peptides, bactericidal factors, proteinase inhibitors, and surface-active lipids (Krause, 2000). Such compounds, when incorporated into the mucus layer, guard against its degradation and protect the underlying mucosa from gastric acid and pancreatic enzymes. The Brunner's glands also provide both active and passive immunological defence mechanisms, promote cellular proliferation and differentiation, as well as raising the pH of the luminal contents by promoting the secretions of the intestinal mucosa, pancreas and gall bladder (Krause, 2000).

Powered by osmotic pressure, water is absorbed from the lumen into the enterocytes and the intercellular spaces, in exchange for sodium ions, until isotonicity between the lumen and the mucosa is achieved (Madara and Pappenheimer, 1987; Powell, 1986; Schafer and Andreoli, 1986). The consistency of the chyme and digesta is essential for the efficient digestion and absorption of nutrients. The secretion and absorption of substances across the physiological mucosal barrier is under hormonal control; hormones that are synthesised in both neural and enterochromaffin cells (Cooke, 1994). Two of the most studied vectors that control the secretion of material into the lumen are vasoactive intestinal peptide (VIP) (Cooke, 2000; Ekblad and Sundler, 1997) and serotonin (5-hydroxytryptamine) (Graf and Sarna, 1997). While serotonin affects the synthesis of cyclic nucleotides and inhibits the absorption of sodium ions into the mucosal enterocyte, VIP, a peptide of twenty amino acids, has been shown to affect the tone of the ileal longitudinal muscle in the rat (Ekblad and Sundler, 1997; Grishina *et al.*, 1998; Spiller, 2001). Both serotonin and VIP are known to affect gut motility (Steadman *et al.*, 1992) as does the glycoprotein, Substance-P. Found

primarily in the small intestine, Substance-P is a short peptide chain of just 11 amino acids and is related to the tachykinins. Known to be a vector of secretory regulation since the 1930's Substance-P affects both gut motility and water exchange (McFadden *et al.*, 1986). Other gastrointestinal hormones and peptides that effect secretions into the gut are; cyclic guanosine monophosphate (cGMP), cholecystokinin (CCK), galanin and guanylin/ uroguanylin (Bedecs *et al.*, 1995; Cuthbert, 2001; Farthing, 2000; Forte and Hamra, 1996).

Hydrogen receptors in the small intestine involved in the regulation of secretions into the gut are affected by histamine (Del Valle and Gantz, 1997; Hardcastle and Hardcastle, 1987). Interestingly, Kurek *et al.* (1996) and Stepnik and Kurek (2002) have demonstrated that β casomorphin-7 and α -casein (90-95) are both histamine releasing agents. Acting on opioid receptors in the gut, they postulate that these casein-derived, opioidergic peptides may cause mast cells, within the GIT, to release histamine following the ingestion of milk. Both they and Wapnir *et al.* (2002) report that the release of histamine is dependent upon the availability of Ca^{2+} ions, which in turn cause the release of chloride ions into the lumen. Hardcastle and Hardcastle (1987) has demonstrated that the removal of serosal Ca^{2+} ions, using calcium channel blocking drugs, has a strong anti-secretory effect. Other researchers of bioactive peptides state that a number of casein derived phosphopeptides play a major role in the paracellular absorption of calcium from the distal small intestine (Kitts and Yuan, 1992; Meisel, 1997a). Thus milk derived bioactive peptides may indirectly influence the secretion of substances into the gut. Wapnir and Teichberg (2002) suggest that one of the most important dietary bioactive peptides is carnosine (β -alanyl-L-histidine), a dipeptide with vasodilatory action mediated by enhanced cGMP production (Ririe, Roberts, Shouse, and Zaloga, 2000), and with additional effects on cardiac contractions and intracellular calcium concentration (Zaloga *et al.*, 1997).

The GIT also plays an important role as a barrier and defence against damaging influences from the external environment of the animal as well as the toxic milieu of the intestinal lumen. The GIT is inhabited by a multitude of non-pathogenic microflora which exert an influence on the tissues of the GIT (Bannink *et al.*, 2006). As a consequence the metabolic activity of the GIT is strongly related to the microbial status of the host. Most endemic microorganisms found in the small intestine colonise

the surface mucus layer and may inhibit the colonisation of other new species by competition for suitable attachment sites and nutrients, by the secretion of anti-microbial factors or by changing actively the local growth conditions (Gaskins, 2003).

Although enteric microflora compete with the host for nutrients in the GIT and strongly influence the metabolic activity of the gut wall, they have a protective as well as a nutritional role for the host (Gaskins, 2003). The endemic microbial population may release nutrients from materials that are difficult to digest, such as mucus and digestive enzymes, making them available for absorption. Microorganisms may also produce metabolites that have a potentially negative effect on the gut wall, including; amines, ammonia, phenols and indols, that are the end products of partial amino acid fermentation. They may also inhibit fat digestion due to the microbial degradation of bile salts (Gaskins, 2003; Bannink et al., 2006). Simultaneously, the host invests heavily in keeping microorganisms outside the mucosa and reacts with an acute immune response if microorganisms break through the mucosal defensive mechanisms. Microorganisms may also stimulate cell turnover in the mucosa and affect the rates of secretion (Bannink et al., 2006). Defence against pathogenic microorganisms may also activate a series of physiological functions such as bleeding into the lumen and the secretion of water and ions, causing increased motility of the GIT and the rate of digesta passage (Chang and Rao, 1994).

III. Absorption of dietary nitrogen from the small intestine

For absorption to be effective, homeostatic regulatory mechanisms need to balance the absorption and secretion of materials across the mucosal barrier. Control mechanisms that regulate conditions in the lumen of the GIT are initiated by: the distension of the stomach and intestinal wall by the volume of food ingested; the osmolarity and pH of the chyme; the concentration of specific products of digestion (e.g. the absorption of amino acids from the lumen is dependent upon their luminal concentration). The regulation of motility and secretion is achieved via a concordant system of long reflexes via the central nervous system (CNS), together with short reflexes from the enteric nervous system and reflexes from gastrointestinal peptides.

Sensory information directly from the digestive system may be received, integrated and acted upon by the enteric system alone. Local short reflexes, independent of the CNS, regulate muscles that surround the intestinal lumen and secretions of the associated exocrine glands. The myenteric plexus, the major nerve supply of the GIT, is located between the longitudinal and circular layers of the muscularis externa and provides motor innervation to both muscle layers and secretomotor innervation to the mucosa. The submucosal plexus, supplying the innermost group of neurons regulates the configuration of the luminal surface; glandular secretions; alters electrolyte and water transport across the mucosa; and also regulates local blood flow. Both the myenteric plexus (parasympathetic and sympathetic input) and submucosal plexus (parasympathetic input), receive sensory information from the gut lumen and/or the CNS (Bowen, 2005). Long reflexes, through the CNS via sympathetic and parasympathetic nerves, also affect the GIT. Stimuli from the digestive tract itself or from other sources (feedforward reflexes) may invoke gastrointestinal responses and may include; reactions to food, or danger-triggering effects (Wood *et al.*, 1999).

Digestive function is also regulated by hormones produced by endocrine glands which are scattered throughout the gastrointestinal epithelia. The GIT is the largest endocrine organ in the body and forms what is referred to as the enteric endocrine system. Endocrinocytes in the enteric epithelia continually sample the luminal environment, and responding to chemical changes, they release enteric hormones into the bloodstream. Three of the most notable enteric hormones are:

- a) **Gastrin**: Secreted from the stomach plays an important role in the control of gastric acid secretion.
- b) **Cholecystokinin**: Secreted by I-cells of the duodenum stimulates the secretion of pancreatic enzymes and bile.
- c) **Secretin**: Secreted by S-cells of the duodenum stimulates the secretion of bicarbonate-rich fluids from the pancreas and liver.

There are three phases of gastrointestinal control (Kellow and Malcolm, 1996; Wood et al., 1999):

- a) **Cephalic phase** is initiated by sight, smell, taste, mastication, and emotional states that stimulate receptors in the brain. Reflexes mediated by both sympathetic and parasympathetic nerves activate secretory and contractile activity.
- b) **Gastric phase** is initiated by distension, acidity, and the presence of both amino acids and peptides in the stomach. This phase is mediated by both short and long reflexes and activates gastrin secretion.
- c) **Intestinal phase** is initiated by distension, acidity, and the osmolarity of digestive products in the intestine. This phase is mediated by enteric hormones and both short and long neural reflexes.

The nutrient cost involved in the transport of nutrients and ions is dependent upon the different routes of transport. The important mechanisms responsible for the absorption of nutrients across the apical membrane into the enterocyte are as follows:

a) **Amino acids**

Amino acids are absorbed via specific facilitated Na^+ -independent amino acid transporters such as those shown in **Table 2**.

b) **Peptides**

Potential mechanisms for the enterocytic uptake of peptides from the intestinal lumen are illustrated in **Figure 7**. Di- and tri-peptides are absorbed across the apical membrane by the tertiary active process of the H^+ /di- and tri-peptide symporter, PEPT1 (Adibi, 1997; Brandsch and Brandsch, 2003; Terada *et al.*, 2005). [The PEPT1 transporter is described in greater detail in the section dealing with the absorption of bioactive peptides.] Once inside the enterocyte, such small peptides are usually hydrolysed to free amino acids in the cytoplasm by various intracellular peptidases (Vermeirssen *et al.*, 2004). However, the extent to which di- or tripeptides are transported into the enterocyte followed by complete intracellular hydrolysis is not known (Daniel, 2004).

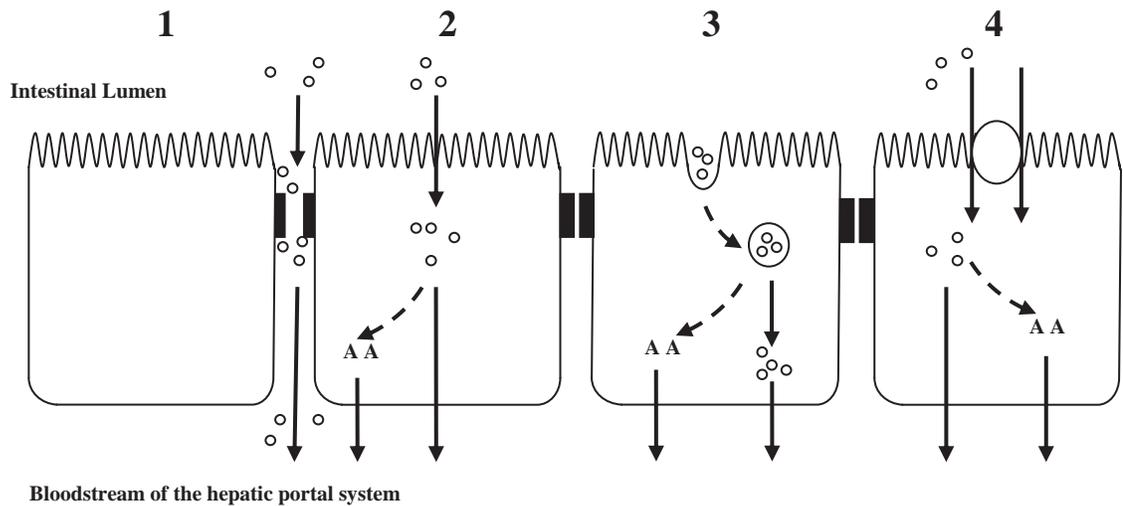
Table 2. Amino acid transporter mechanisms of the GIT.

Transporter	Enterocyte location	Ion dependency	Substrate
y^+	Apical	Na^+ -independent	Cationic amino acids
X^-_{AG}	Apical	Na^+ -dependent	Neutral amino acids
b^{0+}	Apical	Na^+ and Cl^- -dependent	Glutamate, aspartate
B^0	Apical	Na^+ -independent	Neutral and cationic amino acids
$B^{0,+}$	Apical	Na^+ -dependent	Most neutral amino acids
IMINO	Apical	Na^+ and Cl^- -dependent	Neutral and cationic amino acids
β	Apical	H^+ -dependent	Small, neutral, unbranched, Proline
		Na^+ and H^+ -dependent inward with K^+ outward	β -alanine, taurine, hypotaurine
L	Basolateral	Na^+ -independent	Branched chain/aromatic, neutral amino acids
A^*	Basolateral	Na^+ -dependent	Small aliphatic, neutral amino acids: serine, alanine, glutamine and imino acids
ASC^*	Basolateral	Na^+ -dependent	Small aliphatic amino acids; alanine, serine, cysteine
y^+L	Basolateral	Na^+ -independent	Cationic
T	Basolateral	Na^+ -dependent	Neutral *
		Na^+ and H^+ -independent	Aromatic amino acids: tryptophane, tyrosine, phenylalanine
Gly	Basolateral	Na^+ and Cl^- -dependent	Glycine and sarcosine
Asc^*	Basolateral	Na^+ -independent	Alanine, serine, cysteine, D-serine

Data taken from Brandsch and Brandsch (2003) Castagna *et al.* (1997) Kilberg *et al.* (1993); Metzner *et al.* (2008); Thwaites and Anderson (2007); van Winkle (1993).

*Transport is from the blood plasma into the enterocyte.

Figure 7. Potential mechanisms of enterocytic uptake of peptides.



Data taken from: Brandsch and Brandsch, 2003; Terada and Inui, 2004; Vermeirssen *et al.*, 2004.

Notes:

1. **Paracellular**; increased permeability of tight junctions may permit the passage of peptides.
2. **Passive diffusion**; cell penetrating peptides are capable of transporting peptides as cargo.
3. **Endocytosis**; followed by the endosomal release of the peptides.
4. **Carrier mediated transport**; transport via the intestinal peptide transporter PEPT1. Inside the enterocyte peptides can be hydrolysed into their constituent amino acids before being transported across the basolateral membrane by specific amino acid transporters. It is thought that the transport of peptides across the basolateral membrane is mediated through other transporters such as those suggested by Terada *et al.* (1999; 2004), Shepherd *et al.* (2002) and Irie *et al.* (2004).

Abbreviations: AA; amino acids. PEPT1; the H⁺/di- and tri-peptide symporter

Although the presence of intracellular peptidases has been demonstrated, the complete hydrolysis of all dietary protein-derived peptides into their constituent amino acids remains uncertain and the identity of the basolateral peptide transporter remains elusive (Gilbert *et al.*, 2008).

c) Carbohydrates

The active transport of dietary sugars, D-glucose and D-galactose, across the brush-border membrane is via the sodium dependent glucose co-transporter SGLT1. It is well known that the concentration of these two sugars within the lumen has a regulatory effect on the activity of the intestinal SGLT1 mechanism. Glucose moves through the cytosol and exits the enterocyte via the basolateral membrane and thus into the blood via facilitated diffusion catalyzed by the glucose carrier GLUT-2 (which belongs to the major family of transport facilitators GLUTs). The GLUTs are energy independent, unlike active transporters which often require the presence of ATP to drive their translocation mechanism, and stall if the ATP/ADP ratio drops too low. Galactose uses the same transport system. Fructose crosses the apical membrane of the enterocyte, using GLUT-5 and is thought to cross the basolateral membrane into blood capillaries using one of the other GLUT transporters. Butyrate is the principal source of energy for colonic epithelial cells and effects their proliferation, differentiation and apoptosis. The transport of butyrate across the colonocyte luminal membrane is mediated by the monocarboxylate transporter 1 (MCT1) (Cuff *et al.*, 2002).

d) Ions

The uptake of ions including sodium, calcium, magnesium, and iron typically occurs through active transport. Ion transport takes a substantial part of the total energy utilisation of the tissues of the gut wall. It is estimated that ion and Na⁺-dependent nutrient transport together represent nearly a quarter of whole-body energy utilisation, and more than a third of the GIT (Bannink *et al.*, 2006; Summers *et al.*, 1988)

e) Water

Water uptake follows the osmotic gradient established by Na^+/K^+ ATPase on the surface of the basolateral membrane. This can occur by transcellular or paracellular mechanisms.

f) Lipids

Lipids are broken down by pancreatic lipase and bile, then diffuse through the apical membrane into the enterocytes. Smaller lipids are transported into intestinal capillaries, while larger lipids are processed by the Golgi and smooth endoplasmic reticulum into lipoprotein chylomicra and exocytosed into the lacteals.

g) Bile salts

Bile salts not used in emulsification of lipids are reabsorbed in the ileum.

The absorption of amino acids and di/tri-peptides such as in the healthy large intestine (discussed in greater detail later) is understood to be minimal and of little nutritional benefit (Darragh *et al.*, 1994). However, the colonic absorption of these proteinaceous compounds cannot be completely excluded as a number of researchers have demonstrated the presence of amino acid and di/tri-peptide transporters within the colon (Blachier *et al.*, 2007; Ford *et al.*, 2003; Gaudichon *et al.*, 2002; Ugawa *et al.*, 2001; Ziegler *et al.*, 2002). It is also known that microbially derived amino acids are absorbed and these may consequently appear in the systemic circulation; (Metges *et al.*, 1999a; Torrallardona *et al.*, 1996) however, the site of such absorption is not known with any certainty (Torrallardona *et al.*, 2003a). The nutritional significance of putative colonic amino acid absorption has yet to be determined (Blachier *et al.*, 2007).

It is worthy of note that the PEPT1 apical membrane peptide transporter expressed in the small intestine has little or no expression in the normal colon although it is expressed during chronic inflammatory disease of the colon (Dalmaso *et al.*, 2008; Hang *et al.*, 2009; Joly *et al.*, 2009; Shi *et al.*, 2006).

III.1. Amino acid homeostasis

The GIT plays an important role in amino acid homeostasis, which is critical in supporting gut intermediary metabolism, secretion, absorptive and protective functions (Reeds *et al.*, 1999). Furthermore, amino acid metabolism, within the GIT and accessory organs, makes a disproportionate contribution to amino acid turnover, having an important bearing on the availability of amino acids necessary for other bodily functions such as growth and reproduction (Reeds and Burrin, 2000). Research measurements of portal mass balance combined with enteral or intravenous infusions of labelled amino acids has established that the first-pass metabolism of dietary amino acids is largely a function of metabolic activity in the gut itself (Lobley *et al.*, 1996; Stoll *et al.*, 1997; Yu *et al.*, 1992). The utilization of amino acids in humans suggests that 25% of total protein intake is used in the splanchnic bed, the majority of which being in the gut wall, and that metabolism in the GIT substantially modifies the availability of amino acids to the organism (Biolo *et al.*, 1992; Cayol *et al.*, 1997; Hoerr *et al.*, 1991, 1993; Matthews *et al.*, 1993). Although there is a simultaneous utilization of both arterial and luminal dietary indispensable amino acids by the intestinal tissues the majority are supplied from the mesenteric artery and most of these are channelled towards protein synthesis (Bouteloup-Demange *et al.*, 1998; Stoll *et al.*, 1997; Stoll *et al.*, 1998). However, dietary amino acids are utilized by the GIT for energy generation (Stoll *et al.*, 1999), and the synthesis of glutathione and other amino acids (Murphy *et al.*, 1996; Reeds *et al.*, 1997). In disease and with protein restricted diets the unavailability of some dietary indispensable amino acids (e.g. proline and threonine) required for protein synthesis in peripheral tissues is due to the almost complete utilization of dietary amino acids by the gut itself (Reeds and Burrin, 2000). Perhaps as much as 65% of all protein synthesis in the small intestinal mucosa is devoted to the production of secretory proteins (Reeds and Burrin, 2000). With amino acid utilization by the GIT accounting for half that of the whole body, the synthesis of secretory proteins has a substantive effect on amino acid homeostasis (Reeds and Burrin, 2000). The amino acid requirements, for the integrity and physiologic function of the GIT, have a disproportionate influence on whole body amino acid availability.

The source of dietary protein influences the kinetics of protein digestion and amino acid absorption, and thereby the type and the quantity of amino acids appearing in portal blood. Small peptides as well as free amino acids may be absorbed into the enterocytes (Webb and Bergman, 1991) although the bulk of the peptides are hydrolysed by the gut wall and metabolised or appear in portal blood as free amino acids (Bannink et al., 2006). Amino acid metabolism by the gut wall is not limited to dietary dispensable amino acids as it has been shown that extensive catabolism of dietary indispensable amino acids occurs (Windmueller and Spaeth, 1980).

III.2. Absorption of amino acids from the large intestine

Although the absorption of amino acids from the colon has been demonstrated in newborn pigs (Smith and James, 1976) this ability diminishes as the animal matures (James and Smith, 1976; Sepulveda and Smith, 1979). This age related decline is thought to result from the increased microbial colonisation of the GIT as maturation progresses. From experiments where the distal ileum or caecum of fistulated pigs has been infused with protein, such protein was digested, and although there was no significant increase in faecal nitrogen excretion reported there was an increase in urinary nitrogen excretion (Gargallo and Zimmerman, 1981; Zebrowska, 1973b, 1975). There was no significant improvement in body nitrogen balance, thus prompting the conclusion that although the large intestine may have the physiological capacity to absorb amino acids this makes no contribution to the amino acid economy of the animal (Fuller and Reeds, 1998). When Heine and colleagues (1987) infused ^{15}N labelled yeast protein directly into the colon of infants most of the labelled nitrogen was retained in the body; from this it was concluded that the protein was degraded by microbial hydrolases into ammonia which was then absorbed by the colonic epithelia and utilized for the *de novo* synthesis of amino acids. This appears to be corroborated by reports of the enrichment of plasma lysine with ^{15}N following the ingestion of ^{15}N labelled ammonium salts or urea (Metges *et al.*, 1996; Patterson *et al.*, 1995). Fuller and Reeds (1998) suggest that, given lysine does not transaminate, the appearance of labelled lysine in the plasma and tissues is presumptive evidence that the labelled lysine (along with other amino acids) was synthesized by gastrointestinal microflora and subsequently absorbed by the host. The radioisotope studies of Torrallardona *et al.*

(1994) using ^{14}C , demonstrated that the distribution of radioactivity was across all body amino acids, thus confirming that such *de novo* synthesis is not confined to lysine. Whether such microbially synthesised lysine is absorbed in the colon or small intestine is unclear. However, in another study by Torrallardona *et al.* (1996) in which ileal digesta from pigs fed a diet containing ^{15}N labelled ammonium chloride was infused into the caecum of other pigs, fed a similar, though unlabelled diet, they determined that 75% of the plasma ^{15}N enriched lysine was absorbed proximal to the ileo-caecal junction and 25% distal to this point. Similar experiments with humans given ^{15}N labelled ammonium chloride have also demonstrated the enrichment of lysine in the body tissues (Metges *et al.*, 1997).

A later study by Torrallardona *et al.* (2003b), using a digesta transfer protocol to determine the site of absorption of lysine synthesized by the gastrointestinal microflora of pigs, demonstrated that >90% of the absorption of microbial lysine occurred in the small intestine. Libao-Mercado *et al.* (2009) noted that the utilisation of amino acids and protein synthesized by enteric microbiota can only provide a net positive contribution to the host's amino acid supply if the substrates are of no further value to the host (i.e. non-protein nitrogen from urea and ammonia, amino acids from indigestible protein or surplus dietary dispensable amino acids). Any amino acids derived from dietary or endogenous protein that could have been absorbed directly will not be of any net benefit to the host.

Libao-Mercado *et al.* (2009) demonstrated that more than 70% of the ammonia found in the ileal digesta of growing pigs was produced from non-urea sources and originated from the fermentation of both dietary and endogenous protein in the upper GIT. However, how much of this ammonia constitutes fermentative amino acid losses remains to be answered, as the quantification of this would require the determination of the rate of ammonia generation in the upper gut to explain both the ammonia-nitrogen flow at the distal ileum and the absorption and usage of ammonia for microbial synthesis.

Based on the contributions of urea, endogenous protein and dietary protein to microbial valine, Libao-Mercado *et al.* (2009) estimated that the incorporation of amino acids from both dietary and endogenous protein, into microbial protein, in the

upper part of the digestive tract was greater than 90%. They suggested that the contribution to the host's amino acid supply is small, although it remains uncertain how much of those dietary and endogenous proteins would have been digested and absorbed without the intervention of the microbiota present in the upper GIT. Interestingly they found that lumen-associated microbes used more amino acids from dietary protein than mucosa-associated microbes (mucosa-associated microbes predominantly utilize amino acids from endogenous protein).

In summary although amino acids synthesized by enteric microbiota are absorbed predominantly from the small intestine (Torrallardona et al., 2003b) their nutritional benefit to the host animal remains uncertain. It has not been ascertained whether the dietary and endogenous protein, from which such amino acids are predominantly derived, would have been digested and absorbed without the action of the microflora (Libao-Mercado et al., 2009).

III.3. The quality of dietary protein

Information on the digestibility of dietary proteins for man becomes particularly important when the daily intake of protein is low, as often occurs in developing countries or for hospitalized or chronically-ill people (Rowan *et al.*, 1994).

Traditionally digestibility has been determined as the difference between what is supplied by the diet and what remains unabsorbed. The utilisation of a dietary amino acid for protein synthesis is modulated by many variables that may be difficult to control experimentally (Moughan, 2003). Such variables may include:

a) dietary variables,

- energy supply,
- vitamins,
- minerals,
- dietary amino acid imbalance,
- fibre,
- heat treatments of dietary protein and
- anti-nutritional factors such as plant lectins as well as,

b) non-dietary variables;

- gender genotype,
- thermal environment,
- body mass,
- processing conditions,
- variety of cereal grain used,
- fertiliser application and
- microbial de novo synthesis of amino acids.

Amino acid digestibility was defined by Mosenthin (2000) as being the percentage intake of an amino acid that does not appear in the digesta or faeces. Such a definition is in itself unremarkable although it is subject to many of the variables above, any or all of which may account for the intra-laboratory, systemic variation, in the apparent ileal digestibility values of protein and amino acids, among different samples of the same food (Mosenthin 2000). The degree of endogenous nitrogen flux must be accurately determined and corrected for if dietary amino acid and protein digestibility values are to be determined unconfounded by endogenous compounds.

IV. The absorption of endogenous nitrogen from the small intestine

The term endogenous loss relates to compounds in the digesta which leave the small intestine, which are not directly of food origin. This fraction contains: digestive enzymes, mucin, immunoglobulins, microbial protein and sloughed mucosal cells that have resisted complete degradation (Jansman *et al.*, 2002). It has long been realised that the mass of nitrogenous materials leaving the ileum represents the net balance between nitrogen intake and secretion minus the absorption of dietary nitrogen and reabsorption of endogenous nitrogen (Moughan, 2003).

Knowledge of the composition of digesta at the terminal ileum of humans is therefore important to enable an understanding of the dynamic flux of dietary protein intake and the secretion of endogenous proteinaceous material into the lumen together with the concomitant absorption of both exogenous and endogenous materials from the GIT. The proteinaceous flux in the gut, the absorption and secretion of nitrogenous materials in the small intestine is illustrated in **Figure 8**. Nitrogenous compounds are the basic building blocks of life. The amino acids, together with the purines and pyrimidines are the core compounds in proteins and DNA, two of the four major macromolecules of all living things (Franklin and Gosling, 1953; Pauling and Corey, 1951a; Pauling, Corey, and Branson, 1951b; Watson and Crick, 1953).

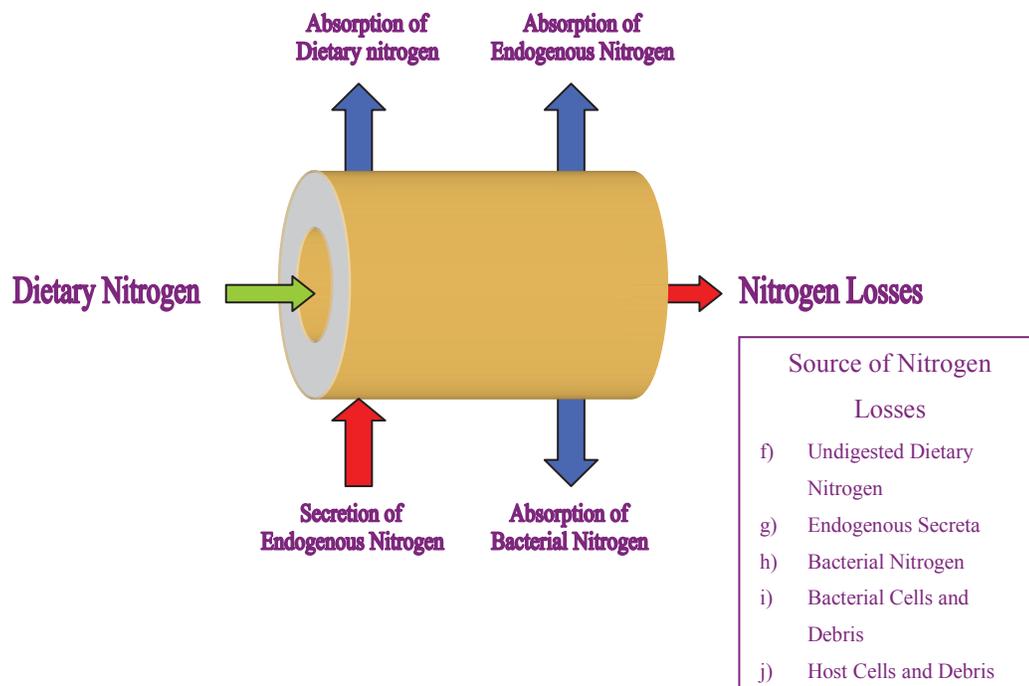


Figure 8. An illustration of the absorption and secretion of nitrogenous materials in the small intestine.

The lack of quantitative knowledge on the effect of gut wall metabolism and of recycling between the mucosa and the lumen is seen as a major limitation to understanding the contribution of gut wall metabolism to endogenous protein losses and to whole-animal amino acid metabolism (Reeds et al., 1999). Research indicates that 35% to 60% of the ileal outflow of nitrogen is of endogenous origin (Bannink et al., 2006). Although digesta contain a myriad of both organic and inorganic

compounds, this review concentrates on quantifying the more important nitrogen-containing compounds.

Souffrant (1991) defined endogenous nitrogen as the amount of nitrogenous constituents in terminal ileal digesta or faeces that does not originate from the diet. Most of the endogenous protein is itself hydrolysed by proteolytic enzymes that are either free within the lumen or bound to the apical membrane of the columnar enterocytes or are produced by microbes, before being absorbed prior to reaching the terminal ileum. This process is known as nitrogen cycling (Fuller and Reeds, 1998; Grala *et al.*, 1998), an example of which Leterme *et al.* (1996b) demonstrated using ^{15}N labelled tracer, showing that amino acids absorbed in the upper GIT may reappear in pancreatic secretions within 50 minutes of ingestion.

Mosenthin and Sauer (2000) characterised endogenous crude protein and amino acids as either non-specific basal or specific. Non-specific basal endogenous crude protein was later defined as being: the amount of endogenous crude protein at the terminal ileum of a physiologically normal functioning animal; fed a protein-containing diet that does not contain factors which increase the flow of specific endogenous crude protein. The ileal flow of basal endogenous crude protein and amino acids is dietary dry matter and bodyweight (Leterme and Thewis, 2004) dependent and the nitrogen and amino acid losses for the animal are considered to be minimal and are often expressed as grams of crude protein or amino acids /kg food dry matter intake.

Whilst non-specific basal flow is independent of dietary composition, the flow of specific endogenous crude protein is affected by dietary factors such as:

- The amount and type of dietary protein and dietary fibre (Schulze *et al.*, 1995) (Examples are given in **Table 3**).
- The presence of anti-nutritional factors such as protease inhibitors (Jansman *et al.*, 1994) lectins (Schulze *et al.*, 1995) condensed tannins (Jansman *et al.*, 1995).
- Damage to peptides and amino acids in heat-treated foodstuffs, cross linkage and Maillard reactions. (de Vrese *et al.*, 2000; Hurrell and Finot, 1985; Moughan *et al.*, 1996).
- The presence of bioactive peptides (Wilkinson, 1974).

Table 3. Examples of foodstuffs in which diet specific factors alter the endogenous nitrogen (EN) flows in the ileal digesta of pigs fed different proteins. ¹

Protein source	Dietary protein g EN/100 g protein
Skim milk	1.3
Wheat	3.1
Soya protein isolate	3.3
Barley	4.0
Phaseolus beans	10.8

¹ Adapted from Darragh and Hodgkinson (2000).

IV.1. The absorption of peptides

The possibility that small peptides may be absorbed intact from the GIT into the bloodstream was first demonstrated by Newey and Smyth (1959) more than fifty years ago, and was corroborated by the work of Matthews *et al.* (1968; 1969) and Adibi *et al.* (1971) some ten years later. Interestingly there is substantial evidence that peptides are absorbed from the GIT more rapidly than free amino acids (Adibi, 1971; Burston *et al.*, 1972; Cheng, Navab *et al.*, 1971; Craft *et al.*, 1968). The more rapid absorption of peptides over amino acids suggests that independent peptide transport systems exist and it was quickly determined that such peptide transport is limited to di- and tri-peptides (Craft *et al.*, 1968; Matthews *et al.*, 1968). Although there is some evidence supporting the absorption of larger peptides (Chabance *et al.*, 1998) the likelihood of the large-scale transport of such peptides seems minimal (Adibi and Morse, 1977; Korhonen and Pihlanto, 2006; Webb, 1990). Although independent active transport carriers facilitate the absorption of small peptides, those peptides absorbed may not leave the cell intact (owing to the presence of cytosolic peptide hydrolases) as attempts to quantify the appearance of such peptides in the mesenteric or portal circulation have been largely unsuccessful. Those di- and tri-peptides resistant to cytosolic hydrolysis may also be broken down by circulatory peptidases in the plasma (Haque *et al.*, 2009; Meisel, 1997b; Silva and Malcata, 2005). Indeed the half life of many peptides in the plasma is very short (Gardner, 1998; Vermeirssen *et al.*, 2004)

e.g. 9 minutes (van der Pijl *et al.*, 2008). Proline containing di- and tri-peptides, especially those with a C-terminal proline or hydroxyl proline are generally resistant to degradation by digestive enzymes (Matsufuji *et al.*, 1994; Vermeirssen *et al.*, 2004).

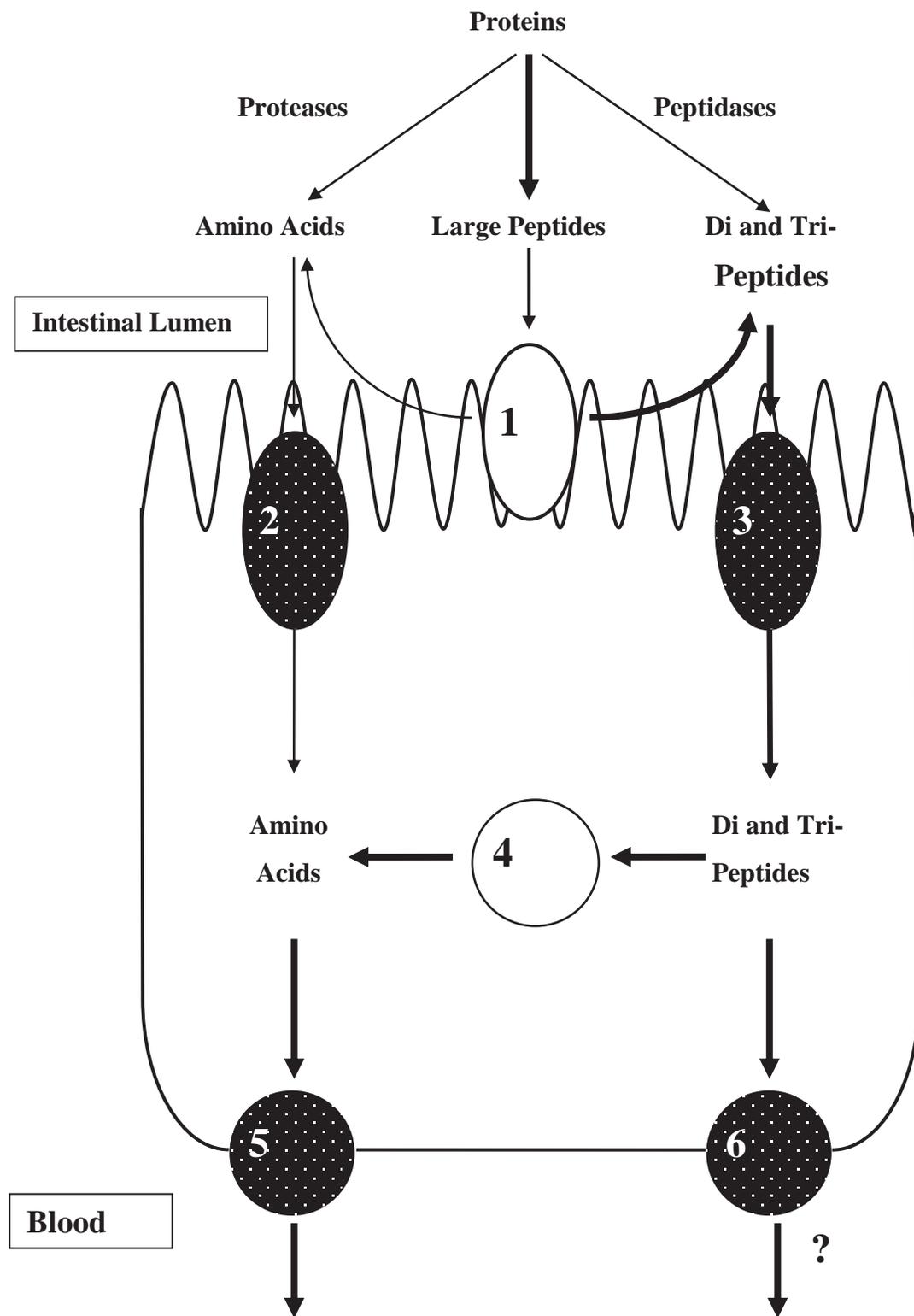
The possible mechanisms for the absorption of peptides from the intestinal lumen (illustrated in Figure 7) include:

- Paracellular pathways, via the tight junctions.
- Passive diffusion through the enterocytes.
- Endocytosis.
- Carrier mediated transport systems e.g. PEPT1.

The luminal digestion of proteins is primarily undertaken by both gastric and pancreatic proteases, the resulting large peptides are then hydrolysed further by peptidases present in the enterocytic brush border. At this stage amino acids are held primarily in small peptides rather than in their free form (see **Figure 9**). Free amino acids are absorbed by the enterocytes by specific amino acid transport systems, detailed in Table 2. One example of this is the B⁰ system, a Na⁺ dependent and Cl⁻ independent transporter that is responsible for the uptake of most neutral amino acids at the brush border membranes of the enterocytes (Munck and Munck, 1994; Stevens *et al.*, 1982).

Di- and tri-peptides are absorbed across the apical membrane by the tertiary active process of the H⁺/di- and tri-peptide symporter, PEPT1 (Adibi, 1997; Brandsch and Brandsch, 2003; Terada *et al.*, 2005). The PEPT1 (see **Figure 10**) operates as an electrogenic proton/peptide symporter with a broad substrate specificity to transport virtually all di- and tri-peptides. Transport is enantio-selective and involves a variable proton-to-substrate stoichiometry for the uptake of neutral and mono- or polyvalently charged peptides. Neither free amino acids nor peptides containing four or more amino acids are accepted as substrates (Daniel, 2004; Vermeirssen *et al.*, 2004; Yang *et al.*, 1999).

Figure 9 Digestion and absorption of protein in the mammalian small intestine (taken from Brandsch and Brandsch 2003).

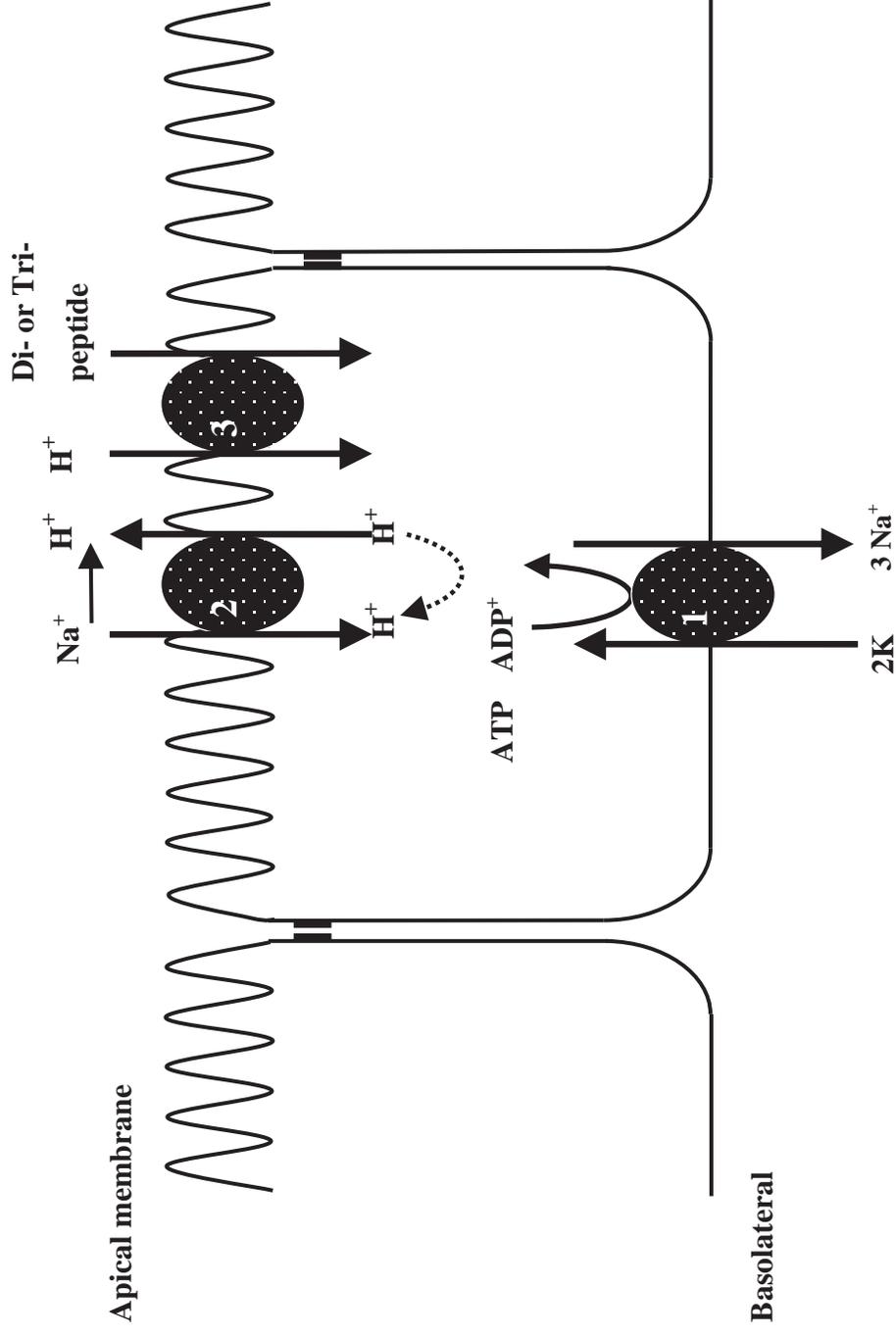


At the Apical Membrane: 1. Peptidases; 2. Amino acid transport systems, such as the B⁰ system;

3. Peptide transporter; 4. Cytosolic peptidases.

At the basolateral membrane: 5. Amino acid transport systems; 6. Peptide transport system.

Figure 10 PEPT1 as a tertiary active H^+ /peptide symporter.



Modified from Brandsch and Brandsch (2003) and Daniel (2004). **1.** $Na^+-K^+-ATPase$, **2.** Na^+/H^+ antiporter, **3.** PEPT1.

Terada *et al.* (2005) found a reciprocal axial gradient in the mRNA expression of PEPT1 and B⁰AT1 in the GIT. The expression of the PEPT1 mRNA peaked in the duodenum and gradually decreased towards the ileum while the expression gradient for B⁰AT1 mRNA was the inverse of this. The digestion of proteins by membrane bound peptidases is much higher in the ileum than in the jejunum (Das and Radhakri, 1974), therefore the concentrations of free amino acids increases while the concentration of small peptides decreases as the luminal contents pass along the GIT (Terada *et al.*, 2005).

Once absorbed by the enterocytes, highly active cytosolic peptidases rapidly hydrolyse most of the peptides to generate further free amino acids. Such free amino acids are then utilised for cell metabolism within the enterocyte or undergo basolateral efflux via more amino acid transport systems. Hydrolysis resistant peptides may then be transported intact into the blood although little is known about the putative basolateral peptide transporter(s) (Brandsch and Brandsch, 2003).

However, it is accepted by most researchers that the transport of peptides across the basolateral membrane is only a small fraction of that which crosses the apical membrane and that such a transport mechanism contributes little to the total protein absorptive process (Meredith and Boyd, 2000; Brandsch and Brandsch, 2003).

The concept that larger peptides are absorbed is not universally accepted, although there are claims that peptides of 10-51 amino acid residues can be absorbed through the intestinal wall to produce physiological effects (Erdmann *et al.*, 2008). However, the physiological potency of such peptides seems inversely proportional to their chain length (Roberts *et al.*, 1999; Vermeirssen *et al.*, 2004).

Calf κ -caseinglycopeptide (cCGP) and human κ -caseinglycopeptide (hCGP) released from κ -casein have been detected in the plasma of neonates (Chabance *et al.*, 1995). In a later study Chabance *et al.* (1998) found that the plasma concentration of hCGP in adults was much lower than that found in the plasma of neonates, suggesting that the neonate gut is more permeable to such peptides than those of adults (Beach *et al.*, 1982; Sturner and Chang, 1988; Chabance *et al.*, 1998); a logical physiological adaptation as milk is the primary source of protein for neonates but not of adults. Although Chabance *et al.* (1998) detected 2 fragments of α S1casein (containing 23 and 21 amino acid

residues) in the plasma of adults 1 hour after the ingestion of milk, the passage in adults of intact peptides from the intestinal lumen and into the blood circulation is exceptional, as it is usually prevented by digestion processes, the intestinal barrier, and hydrolysis in both the enterocytes and plasma

IV.2. Exogenous bioactive peptides and their physiological effects

Inextricably enmeshed in the debate of whether large peptides can be absorbed intact by the GIT is the notion of bioactive peptides, their absorption from the GIT and their physiological effects. To obtain a fuller understanding of peptide absorption by the gut it is necessary to review the current literature regarding bioactive peptides and their physiological effects.

Traditionally the principal consideration in the evaluation of dietary protein has been its nutritional value and the availability of nitrogen from constituent amino acids; more recently though has been the discovery that specific protein fragments have a physiological effect and may influence body health (Kitts and Weiler, 2003). As a result the physiological activity of peptides released from exogenous, dietary precursor proteins during digestive enzymatic proteolysis (the so called bioactive peptides) have been of interest to researchers since their discovery in the 1970's (Wilkinson, 1974). Both the physiological effects of bioactive peptides and the foods in which they are found are vast, and as a result the scientific journals are replete with studies describing a multitude of bioactive peptides that have been shown to be contained (among other foods) within:

- a) Milk, both bovine (Meisel and Fitzgerald, 2003) and human (Lonnerdal, 2003).
- b) Rice (Flamand *et al.*, 1996).
- c) Spinach (Yang *et al.*, 2001).
- d) Soya (Gibbs *et al.*, 2004; Lo and Li-Chan, 2005).
- e) Gluten from cereals (Fanciulli *et al.*, 2005; Fukudome and Yoshikawa, 1992).
- f) Eggs (Watanabe *et al.*, 1998).
- g) Fish (Fujita and Yoshikawa, 1999; Matsufuji *et al.*, 1994).

- h) Haemoglobin from meat (Dubois *et al.*, 2005; Ivanov *et al.*, 1997; Zhao *et al.*, 1997a).

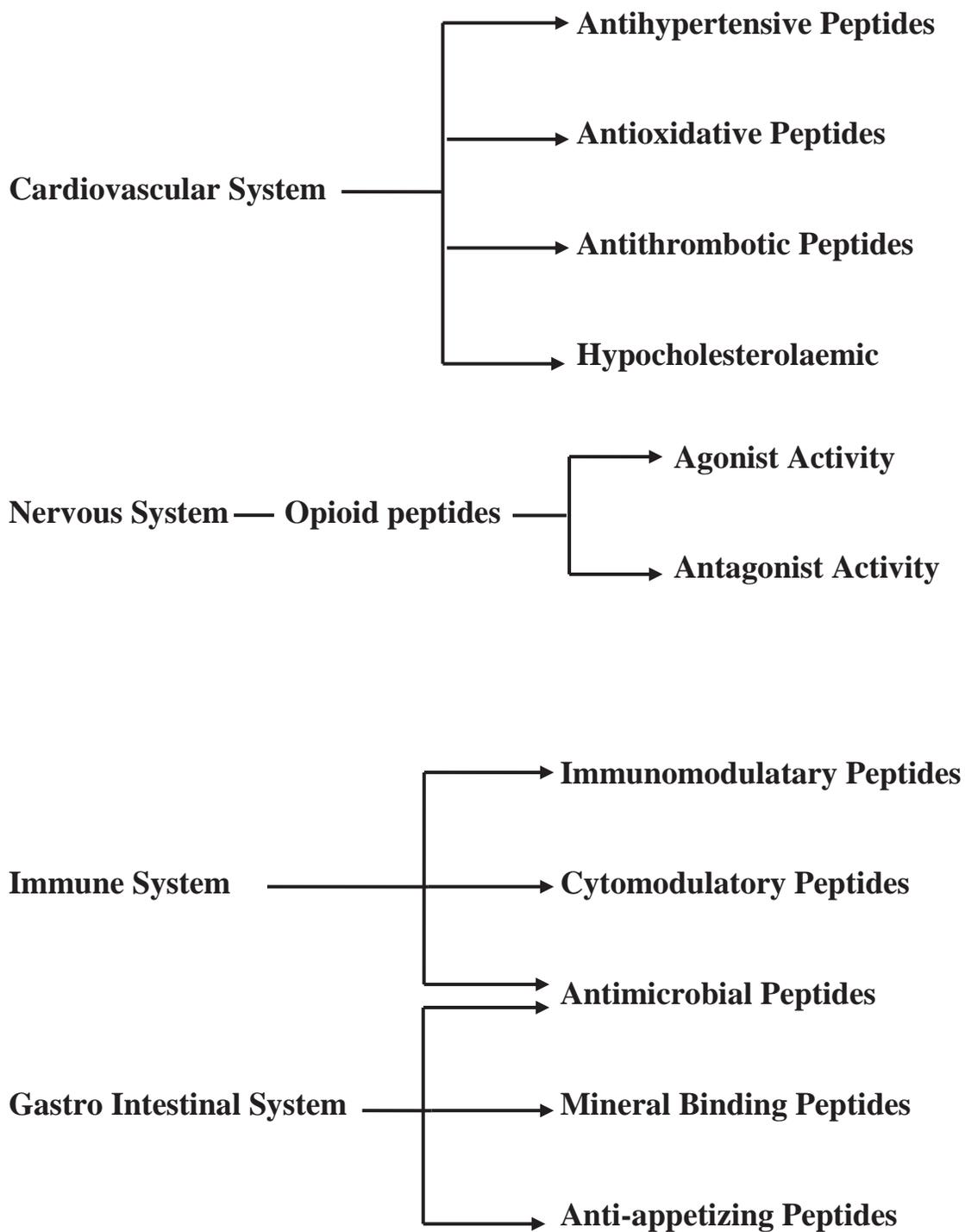
Following the early research by Wilkinson (1974), where the immunological significance of casein molecules was observed, many researchers have investigated the possibility that other milk derived peptides can evoke a physiological effect. The classification of bioactive peptides according to their physiological effect is given in **Figure 11**. Such bioactive molecules are cryptic and latent (inactive), within the amino acid sequence of the original protein, though once liberated by enzymatic proteolysis, they can exhibit regulatory effects, such as those described in **Table 4**. Such molecules, it is thought, are resistant to further enzymatic hydrolysis and may bind to gastric intra-epithelial receptors responsible for a specific physiological response (Gill *et al.*, 2000; Meisel, 1997b).

IV.2.1. Bioactive compounds found within milk and their physiological effect

The physiological properties and structural composition of the many milk-derived bioactive peptides have been comprehensively reviewed (Clare and Swaisgood, 2000; Lonnerdal, 2003; Shah, 2000) and are summarised in **Table 5**.

The nutritional value of mammalian milk for infants is beyond dispute; after all it is the only source of nutriment available to the developing mammalian neonate. However, in addition to the nutritional value of milk the bioactivity of some milk proteins, and peptides, have intrigued researchers for almost thirty years. There are many studies describing the effects of a multitude of bioactive peptides contained within milk proteins; both bovine and human (see Table 5). Such bioactive components of milk are encrypted within the major milk protein precursors and are released during the digestive process by enzymatic proteolysis. These bioactive peptides have a role within the mother, the GIT of the newborn or, following digestion, within the body of the infant. A large number of studies have focussed on the opioid properties that many of these peptides exhibit and the physiological effects these molecules might have (see **Table 6**).

Figure 11 The classification of bioactive peptides according to their physiological effect.



Modified from Korhonen and Pihlanto (2006)

Table 4. Reported physiological effects of bioactive peptides.

Physiological Effect	Dietary Source	Peptide	References
Modulation of gastrointestinal transit time and anti-diarrhoeal action.	1. Milk Casein Hydrolysate	1. β -casomorphin	1. Daniel, Vohwinkel <i>et al.</i> , 1990a Schulte-Frohlinde <i>et al.</i> , 1994 Froetschel 1996 Mihatsch <i>et al.</i> , 2001
Stimulation of secretions:			
1. Prolactin	1. Wheat Gluten.	1. Gluten Exorphin B5.	1. Fanciulli, Dettori <i>et al.</i> , 2005
2. Prolactin	2. Milk Casein Hydrolysate	2. β -casomorphins	2. Yen <i>et al.</i> , 1985
3. Oxytocin	3. Milk Casein Hydrolysate	3. β -casomorphins	3. Teschemacher <i>et al.</i> , 1994
4. Insulin.	4. Milk Casein Hydrolysate	4. β -casomorphins	4. Schusdziarra <i>et al.</i> , 1983a
5. Somatostatin.	5. Milk Casein Hydrolysate	5. β -casomorphins	5. Schusdziarra <i>et al.</i> , 1983b
Mineral Binding	1. α_{s1} - casein	1. Casein Phosphopeptide (f43-58)	1. Vegarud <i>et al.</i> , 2000
	2. α_{s1} - casein	2. Casein Phosphopeptide (f59-79)	2. Schlimme and Meisel 1995
	3. β - casein	3. Casein Phosphopeptide (f1-25)	3. Berrocal <i>et al.</i> , 1989
Antimicrobial Properties	1. Lactoferrin	1. Lactoferricin (f17-41)	1. Clare <i>et al.</i> , 2003
	2. α_{s1} - casein	2. Isracidin (f1-23)	2. Lahov and Regelson 1996
	3. α_{s2} - casein	3. Casocidin-I (f165-203)	3. Zucht <i>et al.</i> , 1996
Immunomodulation	1. α -lactalbumin	1. Immunopeptide (f50-51) and (f18-19),	1. Kayser and Meisel 1996
	2. κ -casein	2. Immunopeptide (f18-20),	2. Mullally <i>et al.</i> , 1996
	3. β -casein	3. Immunopeptide (f63-68),	3. Migliore-Samour <i>et al.</i> , 1989
	4. β -casein	4. Immunopeptide (f191-193),	4. Meisel 1997a and 1997b
	5. α -casein	5. α_{s1} -Casokinin-6	5. Migliore-Samour <i>et al.</i> , 1989
Antithrombotic Activity	1-4. κ -casein (caseinoglycomacropeptide)	1. Casoplatelin (f106-116)	1. Jolles <i>et al.</i> , 1986
		2. Thrombin inhibitory peptide (f106-112)	2. Mazoyer <i>et al.</i> , 1992
		3. Casoplatelin (f112-116)	3. Meisel 1997b
		4. Casopiastrin (f106-110)	4. Clare and Swaisgood 2000
Anti-hypertensive: Inhibition of Angiotensin Converting Enzyme	1. β - casein	1. β -Casokinin-7	1-4. Maruyama <i>et al.</i> , 1985;
	2. α_{s1} - casein	2. α_{s1} -Casokinin-5	Meisel 1997a
	3. α_{s1} - casein	3. α_{s1} -Casokinin-6	
Analgesia	1. β -casein	1. β -casomorphins	1-2 Zioudrou <i>et al.</i> , 1979;
	2. α -casein	2. α - casomorphins	Matthies <i>et al.</i> , 1984; Paroli 1988
	3. β -casein	3. Morphiceptin	3. Chang <i>et al.</i> , 1982

Table 5 Examples of bioactive peptides derived from bovine milk proteins.

	Bioactive Peptide	Protein Precursor	Amino Acid Segment	Peptide Sequence	Bioactivity
Bioactive Peptides Derived From Casein Precursors					
1	α -Casein exorphin	α_{S1} -Casein	f90–96	RYLGYLE	Opioid Agonist
2	α -Casein exorphin	α_{S1} -Casein	f90–95	RYLGYL	Opioid Agonist
3	α -Casein exorphin	α_{S1} -Casein	f91–96	YLGYLE	Opioid Agonist
4	β -Casomorphin-11	β -Casein	f60–70	YFPFGPIPNSL	Opioid Agonist
5	β -Casomorphin-7	β -Casein	f60–66	YFPFGPI	Opioid Agonist, Immunomodulatory and ACE inhibitor
6	β -Casomorphin-5	β -Casein	f60–64	YFPFG	Opioid Agonist
7	Casoxin 6	κ -Casein	f33–38	SRYPST · OCH ₃	Opioid Antagonist
8	Casoxin A	κ -Casein	f35–42	YPSYGLNY	Opioid Antagonist
9	Casoxin B	κ -Casein	F58–61	YPPY	Opioid Antagonist
10	Casoxin C	κ -Casein	f25–34	YIPIQYVLSR	Opioid Antagonist
11	Casoplatelin	κ -Casein	f106–116	MAIPPKKNQDK	Antithrombotic
12	α_{S1} -Casokinin-5	α_{S1} -Casein	f23–27	FFVAP	ACE inhibitor
13	α_{S1} -Casokinin-6	α_{S1} -Casein	f194–199	TTMPLW	Immunomodulatory and ACE inhibitor
14	α_{S1} -Casokinin-7	α_{S1} -Casein	f28–34	FPEVFGK	ACE inhibitor
15	β -Casokinin-7	β -Casein	f177–183	AVPYPQR	ACE inhibitor
16	β -Casokinin-10	β -Casein	f193–202	YQQPVLGPVR	Immunomodulatory and ACE inhibitor
17	Immunopeptide	β -Casein	f63–68	PGPIPNS	Immunomodulatory
18	Immunopeptide	β -Casein	f191–193	LLY	Immunomodulatory
19	Casein Phosphopeptide	α_{S1} -Casein	F43–58	DIGS*ES*TEDQ AMEDIM	Calcium Binding and Transport
20	Casein Phosphopeptide	α_{S1} -Casein	F59–79	QMEAES*IS*S* S*EEIVPNS*VE QK	Calcium Binding and Transport
21	Casein Phosphopeptide	β -Casein	f1–25	RELEELNVPGEI VES*LS*S*S*EE SITR	Calcium Binding and Transport
Bioactive Peptides Derived From Whey Protein Precursors					
22	Serorphin	Bovine Serum Albumin	f399–404	YGFQNA	Opioid Agonist
23	α -Lactorphin	α -Lactalbumen	f50–53	YGLF · NH ₂	Opioid Agonist and ACE inhibitor
24	β -Lactorphin	β -Lactoglobulin	f102–105	YLLF · NH ₂	Opioid Agonist and ACE inhibitor
25	Lactoferricin	Lactoferrin [⊗]	f17–41	FKCRRWNRMK KLGAPSIT- CVRRAF	Immunomodulatory and Antimicrobial
26	β -Lactotensin	α -Lactoglobulin	f146–149	HIRL	Ileum Contraction
27	Immunopeptide	α -Lactalbumen	f50–51 f18–19	YG	immunopotential
28	Immunopeptide	α -Lactalbumen	f18–20	YGG	immunopotential
29	Albutensin A	Bovine Serum Albumin	F208–216	ALKAWSVAR	Ileum Contraction and ACE inhibitor

^a The one letter amino acid codes were used; Phosphoserin = S*

[⊗] Lactoferrin is a neutrophil-derived glycoprotein found in secreted mammalian fluids (Teschmacher 1997). ACE = angiotensin-converting enzyme.

Table 6 Exorphins: peptides derived from milk having opioid properties.

	Bioactive Peptide	Protein Precursor	Opioid Receptor	Bioactivity	Physiological Effect
1	α -Casein exorphin	α_{S1} -Casein	δ	Opioid Agonist	Increases in Intestinal Transit Time ¹ , Amino Acid Uptake ² , and Water Balance ³
2	α -Casein exorphin	α_{S1} -Casein	δ	Opioid Agonist	
3	α -Casein exorphin	α_{S1} -Casein	δ	Opioid Agonist	Additionally in Neonates Analgesia ⁴ that Results in Calmness and Sleep ⁵
4	β -Casomorphin-11	β -Casein	μ	Opioid Agonist	
5	β -Casomorphin-7	β -Casein	μ	Opioid Agonist	
6	β -Casomorphin-5	β -Casein	μ	Opioid Agonist	
7	Serorphin	Bovine Serum Albumin	μ^*	Opioid Agonist	
8	α -Lactorphin	α -Lactalbumen	μ^*	Opioid Agonist	vasorelaxation and smooth muscle contraction ⁶ .
9	β -Lactorphin	β -Lactoglobulin	μ^*	Opioid Agonist	
10	Casoxin 4	κ -Casein	μ and κ	Opioid Antagonist	
11	Casoxin 6	κ -Casein	μ and κ	Opioid Antagonist	
12	Casoxin A	κ -Casein	μ and κ^{**}	Opioid Antagonist	
13	Casoxin B	κ -Casein	μ and κ^{**}	Opioid Antagonist	
14	Casoxin C	κ -Casein	μ and κ^{**}	Opioid Antagonist	
15	Casoxin D	α_{S1} -Casein	μ and δ^{**}	Opioid Antagonist	

* Represents opioid activity with low potency. ** Represents low affinity to this receptor

- Schulte-Frohlinde *et al.*, 1994; Teschemacher *et al.*, 1994; Froetschel 1996; Allescher *et al.*, 2000.
- Brandsch *et al.*, 1994.
- Daniel *et al.*, 1990a.
- Matthies, 1984; Taira *et al.*, 1990.
- Taira *et al.*, 1990.
- Yoshikawa *et al.*, 1994.

Dietary exogenous opioid molecules have been termed exorphins and as well as being in milk they have been found in a variety of other proteinaceous staple foods including; gluten in cereals (Fanciulli et al., 2005; Fukudome and Yoshikawa, 1992) and haemoglobin in meat (Ivanov et al., 1997; Zhao et al., 1997a). The nutritional and physiological functionality of milk and its constituents must lie with its target organism, the mammalian neonate. Mammalian milk has evolved as a complete, species specific food for the mammalian neonate and contains a number of important, physiologically active components that assist in the early development of the newborn. These include a wide range of antimicrobial factors, digestive enzymes, hormones, trophic factors, and growth modulators. However, as soon as the mammalian young are weaned, milk ceases to be a natural dietary component, and with the consequent reliance on a variety of solid foods the gut becomes almost impervious to such bioactive molecules and their endogenous physiological influence is almost entirely blocked. Humans evolved as omnivorous hunter-gatherers and milk has only been a dietary component since the domestication of a variety of herbivorous mammals. Milk, from their own species, is not a normal dietary component of adult mammals and in particular adult humans. Therefore a physiologically functional role of milk-derived bioactive peptides, in adult mammals outside the GIT, seems unlikely.

It may be postulated that as the adult diet varies, the concentration of these bioactive molecules within the diet would be random, thus rendering any role they may have in the homeostasis of the mammalian adult unreliable and open to interference from any exogenous bioactive peptides (Teschemacher *et al.*, 1994). It is suggested that, for adult mammals, milk is not a natural food source and such bioactive compounds have no natural endogenous physiological role. However, the affect that bioactive compounds have on the adult mammalian GIT warrants more detailed research to fully understand the exogenous physiological significance of these compounds.

IV.2.2. Exogenous bioactive peptides and their influence on gut function.

From as early as 1979 (Brantl *et al.*, 1979; Zioudrou *et al.*, 1979) many of the bioactive peptides derived from milk, and casein in particular, were demonstrated to have opioid

properties such as those described in Table 6. Opioid acting bioactive peptides are often referred to as exorphins; a corruption of *exogenous*, the dietary source and *morphine* the alkaloid compound that such peptides mimic (Klee *et al.*, 1978).

For exorphins to function as opioid peptides in the central nervous system in vivo they must (Froetschel, 1996; Zioudrou *et al.*, 1979):

- a) Be produced in the GIT (Svedberg *et al.*, 1985).
- b) Survive degradation by intestinal proteases (Daniel *et al.*, 1990b; Loukas *et al.*, 1983; Tome *et al.*, 1987).
- c) Be absorbed, without degradation, into the bloodstream.
- d) Cross the blood-brain barrier.
- e) Interact with opiate receptors in the brain.

Such functions are sequential and each one of them (b) to (e), is dependent upon its preceding concomitant partners. It is in this regard that function (c), the absorption of intact exorphins into the bloodstream, founders on the rocks of speculation as no intact transepithelial passage has been convincingly demonstrated for these peptides (Picariello *et al.*, 2010; Vermeirssen *et al.*, 2004). Any physiological effects might only be mediated through receptors on the intestinal wall (Korhonen and Pihlanto, 2006; Picariello *et al.*, 2010). The absorption of bioactive peptides is discussed in greater detail in section IV 2.7.

Although exorphins may not be absorbed intact, their physiological influence resulting from their interaction with opioid receptors in the GIT is in little doubt. Many studies report that small opiate acting peptides released during digestion by proteolytic hydrolysis can affect intestinal function (Meisel and Fitzgerald, 2000; Teschemacher, 2003; Teschemacher *et al.*, 1997).

IV.2.3. The Opioid Receptors.

The existence of three opioid receptors (μ , δ , and κ), were described simultaneously by three different groups of researchers in 1973 (Pert and Snyder, 1973; Simon *et al.*,

1973; Terenius, 1973) and have been exhaustively reviewed in the Handbook of Experimental Pharmacology (Mansour and Watson, 1993). It is supposed that the μ , δ , and κ opioid receptors belong to the G-protein coupled receptor family and have seven transmembrane helices which are characteristic of the group (Sato and Minami, 1995). All three of these receptors have been detected in the small intestine and are located, not on the brush border, but in the myenteric plexus (Karras and North, 1981; Nishimura *et al.*, 1986). The myenteric (Auerbach's) plexus is a network of ganglionated nerve fibres and neuron cell bodies that are located between the longitudinal and circular muscle layers of the tunica muscularis in the GIT. The smooth muscles of the tunica muscularis contract in response to the stimulation of these nerve fibres and thus play an important role in controlling gut motility. Other opioid receptors demonstrated recently are the ϵ -opioid receptor (Nock *et al.*, 1993) that had first been proposed by Schulz in (1979), and the Or11 receptor by Mollereau *et al.* (1994) both of which appear to be restricted to the central nervous system.

IV.2.4. Opioid Receptor Ligands.

a) The agonists.

There are two classes of agonistic opioid receptor ligands; the alkaloids which include the opiates such as morphine used in analgesia, and the peptides. Opioid receptor ligands are also classified into two groups 'typical' and 'atypical' designated by Teschemacher *et al.* (1994).

- i.** The 'typical' peptides all originate from three endogenous precursor proteins pro-opiomelanocortin (endorphins), pro-enkephalin (enkephalins), and pro-dynorphin (dynorphins) (Höllt, 1986). All the typical opioid peptides share the same N-terminal amino acid sequence, YGGF. Although they can bind to more than one type of receptor they usually have a greater affinity for just one, dynorphins for κ -receptors, the enkephalins for δ -receptors and the endorphins for both μ , δ , and ϵ -receptors (Paterson *et al.*, 1983).

- ii. The 'atypical' receptor ligands originate from a variety of precursor proteins either endogenous or exogenous, and although their N-terminal amino acid sequence may vary they all have a terminal tyrosine residue in common (all except opioid receptor ligands that originate from α -casein) and another aromatic amino acid residue, such as tyrosine or phenylalanine, in the third or fourth position. The biological activity of these peptides is dependent upon the terminal tyrosine residue as its absence eliminates all bioactivity (Chang *et al.*, 1981). A proline residue at position 2 also appears to be necessary for the correct orientation of the peptide when it binds to the opioid receptor (Mierke *et al.*, 1990).

b) The antagonists.

Opioid antagonists have much in common with the 'atypical' agonists though they do not usually have a similar N-terminal amino acid sequence. The antagonistic potency of naturally occurring milk borne peptides is relatively low though some synthetic derivatives have very high potency and receptor selectivity. The synthetic alkaloid agonists, naloxone and naltrexone are often used in opioid peptide research as inhibitory confirmation of the presence of an agonistic opioid receptor ligand.

IV.2.5. The occurrence of milk derived opioid receptor ligands.

Table 6 sets out the 'atypical' bioactive peptides that are found in the milk of most mammalian species, together with their precursor protein amino acid sequence and their reported physiological effect. Interestingly a recent report by De Noni and Cattaneo (2010) shows for the first time that some casomorphins may be found, in their free form, in some milk products. β -casomorphin-5 was detected in milk products such as yoghurt and infant formulas, whereas β -casomorphin-7 was only detected in cheeses. Both β -casomorphin-5 and β -casomorphin-7 were only previously detected by these researchers in the simulated digestion of milk based infant formulas (De Noni, 2008).

Table 6 is specific to the opioid receptor ligands found in milk and sets out their opioid receptor, their bioactivity as an opioid agonist or antagonist and their physiological effect. Milk-borne opioid receptor ligands have been extensively reviewed by Clare and Swaisgood (2000), and the collection of review papers, by various researchers, in the British Journal of Nutrition supplement in 2000 that was solely dedicated to the bioactivity of milk and its molecular components (Schrezenmeir *et al.*, 2000).

IV.2.6. The physiological effect of opioid receptor ligands on the gastrointestinal tract.

a) Gastrointestinal motility

The administration of a number of opioids, including those derived from milk, has been demonstrated to delay gastric emptying and increase intestinal transit time in a variety of species including: the rat; (Allescher *et al.*, 2000; Galligan and Burks, 1982; Nishimura *et al.*, 1986) the dog; (Burks *et al.*, 1982; Shook *et al.*, 1986) the guinea pig; (Shook *et al.*, 1986) the rabbit; (Tome *et al.*, 1987) and human (Schulte-Frohlinde *et al.*, 1994).

Froetschel (1996) suggests that gastrointestinal motility is modulated by neuro-mediated mechanisms in both the central and peripheral nervous systems where exogenous peptides stimulate an adrenergic response that subsequently causes an increase in intestinal transit time. Additionally opioid receptor ligands such as those from milk can directly affect gastrointestinal motility by binding to receptors on the myenteric plexus. It is postulated (Karras and North, 1981) that peristaltic activity of intestinal smooth muscle may then be inhibited when exogenous opioid receptor ligands cause hyper-polarisation of the myenteric neurons, preventing those neurons from firing and consequently inhibiting the release of acetylcholine. Small intragastric doses of casein (10 mg), or β -casomorphin-5 (5 mg), have demonstrably increased intestinal retention in rats, 40% longer than similar animals infused with whey protein or treated with the opioid antagonist naloxone (Froetschel, 1996).

b) Hormonal response.

Bioactive peptides may induce an endogenous hormonal response or act as exogenous hormones that directly elicit a physiological response.

i. Dietary peptides acting as food-borne hormones

Secretions into the intestinal lumen are a physiological process concurrent with digestion and absorption. Gastric, pancreatic and intestinal secretions are under both hormonal and neural control, both of which are influenced by dietary peptides. Although the significance of bioactive peptides acting as “food hormones” (Morley, 1982) is still unclear, it has been demonstrated that certain exorphins do exhibit hormone-like properties when acting, via opiate receptors in the gut, as exogenous regulators of gastrointestinal motility, permeability and hormone release (Morley, 1982; Schusdziarra *et al.*, 1981; Schusdziarra *et al.*, 1983b; Schusdziarra *et al.*, 1983c; Tome *et al.*, 1988).

An important physiological effect of bioactive exogenous peptides is that they are dietary secretagogues which signal to the enteric nervous system the need for gastrointestinal changes necessary for the effective digestion of specific dietary substrates. Such a mechanism would involve gastrointestinal peptide reflexes and short reflexes from the enteric nervous system that invoke hormonal responses (such as cholecystinin), that modify the activity of gastrointestinal secretions into the lumen (such as those from the pancreas, and bile), which aid the digestion of specific substrates and that concomitantly increase gastrointestinal protection (e.g. increased mucin secretion and epithelial cell proliferation).

ii. Hormonal responses to dietary bioactive peptides.

Orally administered β -casomorphins are known to modify the endocrine activity of the pancreas and influence postprandial metabolism by stimulating the secretion of insulin and somatostatin (Meisel and Schlimme, 1990; Schusdziarra *et al.*, 1983b; Schusdziarra *et al.*, 1983c). Bovine κ -casein glycomacropptide (GMP), was found to inhibit gastric secretions and diminish contractions of the stomach in dogs

(Vasilevskaya *et al.*, 1977). It has also been found to stimulate the release of the intestinal hormone cholecystokinin (CCK), which regulates gastrointestinal function (Beucher *et al.*, 1994). CCK is produced in the GIT (Liddle, 1995) in response to the ingestion of protein or fats. It is thought that GMP binds with the CCKA receptors that are located in the gut to stimulate the release of CCK. This implies that GMP may decrease feeding and thus have an effect on satiety (Bray, 2000; Lieveise *et al.*, 1995).

IV.2.7. The absorption of bioactive peptides

Although the absorption of di- and tri-peptides into the enterocytes has been demonstrated, there is conflicting evidence regarding the absorption of larger peptides. For example; Korhonen *et al.* (2006) quote the work of Chabance *et al.* (1998) stating that glycomacropeptide (GMP):

“can be absorbed both intact and partially digested into the blood circulation of adult humans after milk or yoghurt ingestion,”

Silva *et al.* (2005) state:

“the large GMP molecule cannot be absorbed as such, so it has to be broken down into smaller peptides before an effect on blood components arises.”

However, the evidence for the absorption of smaller peptides is more straightforward. Séverin and Xia (2005) reported that 2 tri-peptides (Val-Pro-Pro and Ile-Pro-Pro) from milk were able to cross the intestinal barrier, and post absorption, inhibit the production of angiotensin II in the bloodstream; this is not the case for all bioactive peptides as they later point out when referring to immunomodulating peptides from β -casein and α_{S1} casein:

“in order to function physiologically in the human body, the active peptides must be absorbed from the intestine in an active form. Di- and tri-peptides can be easily absorbed in the intestine; however, it is not clear that larger bioactive peptides containing in excess of three amino acids are absorbed from the

intestine and reach the target organ. Most of the claimed physiological properties of the casein based bioactive peptides have been carried out *in vitro* or in animal model systems and these hypothesized properties remain to be proven in humans.”

Although Masuda and colleagues (1996) reported that the two angiotensin-converting enzyme (ACE) inhibitory tri-peptides Val-Pro-Pro and Ile-Pro-Pro could be transported intact through the intestinal wall via paracellular routes, Satake *et al.* (2002) reported that significant amounts of these peptides are hydrolysed by cytosolic peptidases. Indeed a point made repeatedly by other researchers is that only a few of the great number of the milk peptides identified as having antihypertensive properties in *in vitro* experiments have so far been proven to be clinically effective in either animal or human studies (Fitzgerald *et al.*, 2004; Gobbetti *et al.*, 2004; Li *et al.*, 2004; Vermeirssen *et al.*, 2004; Yamamoto *et al.*, 2003). Korhonen and Pihlanto (2006) go further when they concur with Séverin and Xia (2005) stating that the majority of the known bioactive peptides are not absorbed from the GIT into the bloodstream, although the effect of some are mediated directly in the gut lumen or through receptors on the brush border membrane; e.g. the opioid acting peptides. The pharmacokinetics of three proline rich tri-peptides (Val-Pro-Pro, Ile-Pro-Pro and Leu-Pro-Pro) were studied by van der Pijl *et al.* (2008) who went to extraordinary lengths to determine the very low concentration of these intact peptides in the bloodstream following their intragastric infusion. Using liquid chromatography-mass spectrometry they found the absolute bioavailability of the three peptides to be approximately 0.1%. Having infused a single dose of Ile-Pro-Pro, [12 $\mu\text{mol/kg}$ bodyweight in pigs approximately 25kg in weight], they determined the plasma concentrations peaking at 4 nmol nearly ten minutes after the infusion was started and 1000 times lower than similar intravenous infusions. They determined the elimination half life of Ile-Pro-Pro to be just over 9 minutes. Commenting on the low plasma concentrations they speculated that this was due to peptidase activity in the lumen, the brush border membrane and the cytosol. With the effective plasma concentration for the inhibition of ACE being estimated by van Platerink *et al.* (2007) to be 5.6 μmol , the plasma concentrations determined by van der Pijl *et al.* (2008) were approximately 1000 fold less, and far below the effective concentration required to have any influence on lowering blood pressure.

Both Fitzgerald *et al.* (2004) and Foltz *et al.* (2007) reported that the previously reported ACE inhibitory bioactive peptides failed to lower blood pressure in *in vivo* studies, highlighting the intestinal breakdown of the so called stable proline rich tri-peptides (Matsufuji *et al.*, 1994; Vermeirssen *et al.*, 2004). The research of Nakamura *et al.* (1995) is often quoted as demonstrating that "Calpis", a Japanese sour milk or a preparation of the pure tri-peptides (Val-Pro-Pro and Ile-Pro-Pro), decreased the systolic blood pressure in spontaneously hypertensive rats 6 to 8 hours after their oral administration (Erdmann *et al.*, 2008; Escudero *et al.*, 2010; Fitzgerald and Murray, 2006; Foltz *et al.*, 2007; Haque *et al.*, 2009; Korhonen and Pihlanto, 2006; Miguel *et al.*, 2005; Mizuno *et al.*, 2005; Robert *et al.*, 2004; Severin and Xia, 2005; Vermeirssen *et al.*, 2004; Zaloga and Siddiqui, 2004). However, Nakamura *et al.* (1995) and others (Miguel *et al.*, 2005; Muguerza *et al.*, 2006) also showed that the Calpis sour milk and mixed tri-peptides did not change the systolic blood pressure of the normotensive strain of Wistar-Kyoto rats, important conflicting data that many of the researchers citing this paper fail to mention (Erdmann *et al.*, 2008; Escudero *et al.*, 2010; Haque *et al.*, 2009; Korhonen and Pihlanto, 2006; Severin and Xia, 2005).

Mizuno *et al.* (2005) experimenting with human volunteers, administered the two peptides orally, in tablet form containing four different dosages. They found that the reduction of systolic blood pressure was dose dependent and most effective in mildly hypertensive subjects. They also found that there was no significant reduction in diastolic blood pressure in all of their test groups or in comparison with the control group who received placebo. If such an antihypertensive effect can be sustained then it may be mediated through receptors on the intestinal wall (Korhonen and Pihlanto, 2006; Picariello *et al.*, 2010).

Although many *in vitro* studies have demonstrated that milk is a rich source of bioactive peptides (Clare *et al.*, 2003; Clare and Swaisgood, 2000; Korhonen and Pihlanto, 2003, 2006; Meisel, 1997a, 1997b, 2005; Meisel and Schlimme, 1990) few peptides have been shown to be biologically active *in vivo* following the ingestion of milk or fermented milk products; [i.e. it has not been ascertained whether the functional domains of milk proteins survive digestion and reach the blood in concentrations of any physiological significance (Picariello *et al.*, 2010)] For example,

Petrilli *et al.* (1984) demonstrated that β -casomorphins, the family of bioactive peptides derived from milk β -casein (Daniel and Hahn, 1990; Kreil *et al.*, 1983), do not survive digestive degradation. In addition Schmelzer *et al.* (2007) determined that no significant amounts of β -casomorphins or other known bioactive peptides were formed during the peptic digestion of bovine β -casein under simulated gastric conditions. Vermeirssen *et al.* (2004) also came to a similar conclusion, stating that no intact transepithelial passage has been detected for these peptides. However, there are many papers suggesting the physiological effects/functions of milk borne bioactive peptides [for reviews see Clare and Swaisgood, 2000; Kitts and Weiler, 2003; Korhonen and Pihlanto, 2003, 2006; Meisel, 2004, 2005; Meisel and Fitzgerald, 2000; Pihlanto-Leppala, 2000; Rutherfurd-Markwick and Moughan, 2005; Silva and Malcata, 2005; Teschemacher, 2003; Tome and Debabbi, 1998; Yamamoto *et al.*, 2003]. Current opinion is that the majority of the known bioactive peptides do not pass into the bloodstream and that any physiological effects might only be mediated through receptors on the intestinal wall (Korhonen and Pihlanto, 2006; Picariello *et al.*, 2010).

Although Picariello *et al.* (2010) reiterate the opinion of many researchers, that the majority of bioactive peptides do not pass into the bloodstream, they began their paper with reference to one of the most quoted/misquoted papers on this subject, that of Gardner (1988) (cited 117 times) stating that it has been observed that large peptides or proteolysis resistant proteins can enter the bloodstream albeit in small amounts. However, Gardner did not give any direct evidence for the absorption of intact protein in humans; instead he offered the following in support of the hypothesis that intact proteins and the macromolecular fragments of them are absorbed:

- a) That antibodies to many food proteins and their immune complexes have been detected in the circulation of healthy individuals (Bazin *et al.*, 1973; Cunningham-Rundles, 1987; Kenrick, 1970; Paganelli and Levinsky, 1980; Robertson *et al.*, 1982). However, he qualified this suggesting that such antibodies might arise through the intestinal immune system responding to luminal proteins rather than absorbed ones.

- b) Radioimmunoassay techniques by Husby *et al.* (1987), and Jakobssen *et al.* (1986), show the presence of orally administered proteins such as ovalbumin in the blood. However referring back to the researchers cited by Gardner, Husby *et al.* (1987) fed 10 children (aged 2½ -13 years), a test meal containing 2 mL of raw egg and 10 mL cow's milk per kg bodyweight through a gastric tube placed adjacent to the ligament of Treitz. Five of the children had been diagnosed with celiac disease and the five controls were also suspected of having celiac disease. Although they found ovalbumin in the plasma of three of the five celiac patients and all five of the controls, the feeding conditions and all the experimental subjects being children, with or suspected of suffering from celiac disease, mean their results should be treated with caution. With respect to Jakobssen *et al.* (1986) they did not find that the target protein was absorbed in all subjects. The protein α -lactalbumin purified from human milk was not detected ($<5 \mu\text{g/L}$) in the serum of adult men, non-pregnant women or in serum from formula-fed infants. However, α -lactalbumin was found in serum from pregnant women, cord blood and from newborn non-fed infants.
- c) That intact or largely intact horseradish peroxidase was found in the blood of carp and trout (McLean and Ash, 1986; 1987). Can the digestion of protein in fish be confidently extrapolated to include adult humans?
- d) They cited the work of Walker *et al.* (1975; 1976) who studied the *in vitro* absorption of dietary antigen and antigen-antibody complexes to corroborate their hypothesis. This will be discussed in more detail below, relative to the work of Roberts *et al.* (1999).

Another paper often quoted (cited 107 times) is that of Fiat *et al.* (1993) who cited Gardner *et al.* (1988) when they concluded their paper with the bold statement:

“Moreover, there is now irrefutable evidence that small amounts of intact peptides and proteins enter the circulation under normal circumstances after absorption *per os*.”

Statements such as this over simplify what is known about protein absorption and mislead the reader. It is true that Gardner did state in his summary:

“There is now no reasonable doubt that small quantities of intact proteins do cross the GIT in animals and adult humans,”

However, he concluded the same sentence stating:

“and that this is a physiologically normal process required for antigen sampling by sub-epithelial immune tissue in the gut.”

Then qualified this statement:

“It is too small to be nutritionally significant in terms of the gross acquisition of amino-nitrogen, but since it has important implications relating to dietary composition it must receive consideration from nutritionists.”

Gardner describes the GIT is a major site of immunological competence, as substantial numbers of lymphocytes and macrophages are found throughout the intestinal lamina propria. He hypothesises that absorption occurs predominantly by transcellular endocytosis in the M-cells, [or lymphoepithelial cells (Wolf *et al.*, 1981)] and that this allows subepithelial lymphocytes direct access to luminal antigens. Transcellular endocytosis occurs when protein molecules bind to receptors on the brush border membrane, which are then encapsulated into phagolysosomes. Proteolysis in the phagolysosomes minimises the entry of intact bioactive peptides into the circulation, which Gardner (1984) states are likely to be deleterious. So although antibodies to numerous food proteins may occur in the circulation of healthy individuals this should not be taken as evidence for the presence of the original peptide antigens within the blood. Gardner also states that:

“it is not yet possible to state with reliable accuracy what fraction of the Protein.....will enter the circulation in macromolecular form.”

Much has been done since Gardner wrote his paper in 1988 yet still little is known about the absorption of bioactive peptides. Teschemacher *et al.* (1997) stated that β -casomorphins and their precursors have not been identified in the cardiovascular compartment in any more than negligible concentrations and it is unlikely that they have any functional role in adult mammals outside the GIT. Teschemacher also states that enzymatic degradation in the intestinal wall and in the blood appear to prevent it (Mahe *et al.*, 1989; Read *et al.*, 1990; Teschemacher *et al.*, 1986). For example dipeptidyl-peptidase is one of the enzymes that effectively degrades β -Casomorphins in plasma (Kreil *et al.*, 1983) and in the intestinal brush border (Tiruppathi *et al.*, 1993). Meisel (2000) also stated that opioid casein fractions have not been detected in the plasma of adult mammals and that only in the neonate was the intestine permeable to casomorphins and their precursors. He confirmed what Gardner stated; that in adult mammals the brush border membrane appears to be the main site for the physiological effects of dietary opioid peptides. He went on to say that:

“As yet no meaningful application in human nutrition has been described.”

Clare and Swaisgood (2000), Gill (2000), and Shah (2000), all stated that there is no evidence that larger bioactive peptides can be absorbed from the GIT and that any hypothesised properties have yet to be proven in adult humans. Froetschel (1996) stated that there is no evidence to support the theory that opioid peptides, such as the beta-casomorphins, are transported into the bloodstream to the brain, or that they can cross the blood brain barrier. No bioactive peptides or their native proteins have any established physiological role though they do have a variety of physiological effects.

However, in order to demonstrate the physiological effects of the multitude of bioactive peptides researchers have introduced them directly into the blood (Bray, 2000) the CSF (Bakalkin *et al.*, 1992; Nyberg *et al.*, 1997; Zhao *et al.*, 1997a) and even directly into the brain. (Hedner and Hedner, 1987; Lin *et al.*, 1998; Yoshikawa *et al.*, 1986). Conclusive evidence for the functional significance of milk derived opioid peptides has not yet been presented (Teschemacher *et al.*, 1997) and the effects demonstrated by researchers for isolated or synthesized peptides derived from milk represents evidence of a pharmacological activity and not of a physiological role.

Roberts *et al.* (1999) studied the effect of amino acid chain length on the absorption of biologically active peptides from the GIT. Studying the absorption of just 3 peptides of differing chain length (thyrotropin-releasing hormone, a tripeptide; luteinizing hormone-releasing hormone, a decapeptide; and human insulin a 51-amino acid polypeptide), they concluded that “large peptides” (*sic*) as large as 51 amino acids generated in the diet can be absorbed intact through the intestine and produce biologic effects at the tissue level. This conclusion has been quoted by many other researchers, e.g. (Cheng *et al.*, 2008; Erdmann *et al.*, 2008; Escudero *et al.*, 2010; Robert *et al.*, 2004; van der Pijl *et al.*, 2008; Vermeirssen *et al.*, 2004; Zaloga and Siddiqui, 2004). For example:

“Biologically active peptides generated in the diet can be absorbed intact through the intestine and produce biological effects at the tissue level.” (Vermeirssen *et al.*, 2004).

“Larger peptides (10-51 amino acids) present in the diet can also be absorbed intact through the intestine and produce biological effects, although the potency of the peptides decreases as the chain length increases.” (Erdmann *et al.*, 2008).

“It is known that peptides of different length can be absorbed intact from the GIT” (Escudero *et al.*, 2010).

However, such claims cannot be justified as there are a number of points which limit such sweeping conclusions made by Roberts *et al.* (1999), and the other researchers who accept their conclusions *carte blanche*:

- a) The study was undertaken using rats. Although these animals are an accepted animal model for simple stomach mammals the results should be taken with caution as they may not be applicable to humans.
- b) The polypeptides were administered into the small intestine of the animal, distal to the pancreatic ducts and level with the ligament of Treitz, effectively bypassing both gastric and pancreatic degradative hydrolysis.

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- c) They stated that the absorptive capacity of the small intestine in the experimental animals was not assessed and that the animal received a surgical procedure which placed a feeding tube within the small intestine that “may have altered gut permeability and absorption.” For example it is known that such a naso-ileal tube affects gastric emptying (Fone *et al.*, 1991; Medhus *et al.*, 1999) increases intestinal transit time (Read *et al.*, 1983) can stimulate intestinal secretions (Hendrix and Bayless, 1970; Read *et al.*, 1983) and that the effect of a gastrointestinal tube on the absorption and secretion of other substances has not been fully investigated.
 - d) Although they measured serum insulin in their experimental animals they did not state if this compound was the human insulin administered enterally or native rat insulin. In addition they did not determine the serum levels of the other peptides administered to their experimental animals, only their physiological effect; i.e. for thyrotropin-releasing hormone by determining serum thyroid-stimulating hormone levels and for luteinizing hormone-releasing hormone they determined serum follicle-stimulating hormone levels. They also did not rule out that the physiological effects may have been mediated directly in the gut lumen or through receptors on the brush border membrane.
 - e) They tested the absorption of human insulin which has a different amino acid sequence to that of rat insulin, and in addition they used a very large enteral dose of 25 mg to produce a measurable physiological effect.

Roberts *et al.* (1999) extrapolate their conclusions too much; they assume that:

- a) The oral intake of dietary peptides would be the same as enteral administration.
- b) That gastric and pancreatic hydrolysis of such dietary peptides would be negligible.

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- c) That all dietary peptides resistant to digestive degradation would be absorbed from the small intestine the same as their experimental peptides (human insulin, thyrotropin-releasing hormone and luteinizing hormone-releasing hormone).

Interestingly Roberts *et al.* (1999) also misquote other researchers when they cite two much older papers, claiming that larger protein fragments may be absorbed via pinocytosis or through paracellular channels (Sugimoto *et al.*, 1981; Walker *et al.*, 1976). In the first paper by Sugimoto *et al.* they found that horseradish peroxidase was absorbed via absorptive cells of the small intestine in the bullfrog tadpole, that was detected in subapical multivesicular bodies which demonstrated that such vesicles are important sites of intracellular digestion. They did not determine that the horseradish peroxidase was absorbed through the basolateral membrane and into the portal bloodstream, or that the absorption of horseradish peroxidase in the bullfrog tadpole would be the same in humans. The second paper by Walker *et al.* (1976) studied the *in vitro* absorption of soluble antigen and antigen-antibody complexes; they concluded that after the ingestion of dietary protein antigens, digestive processes in the intestinal lumen both effectively and efficiently degrade these proteins, allowing only a few intact molecules to contact antibody molecules presumed to be present in the mucus coat of the intestinal surface (Walker *et al.*, 1975). The resultant complexes, formed in antibody excess, would be unlikely to be absorbed, thus limiting the access of proteinaceous antigens to the systemic circulation.

If the gut is permeable to all bioactive peptides, then the physiological response to the varying amounts that would be present in an adult human's ever changing diet, would be extremely variable and not conducive to efficient physiological homeostasis. The homeostatic mechanism would be subject to wildly different external influences that would require unpredictable increases in energy expenditure. Finally not all bioactive peptides are beneficial, some may even have a toxicological effect (Schaafsma, 2009; Picariello *et al.*, 2010).

IV.2.8. The influence of bioactive peptides on cell proliferation.

Gastrointestinal mucosal epithelia are directly exposed to the hazards of an external environment, the presence of bile salts, acids, digestive enzymes and pathogenic bacteria, making the intestinal lumen a particularly noxious environment (Bevins *et al.*, 1999; Sanderson and Walker, 1999). As a result the mucosal epithelia are rapidly and continuously renewed; a process essential to ensure effective digestion, absorption of nutrients and to reduce the colonisation of pathogenic micro-organisms.

It is thought that exogenous bioactive peptides may influence gastrointestinal epithelia proliferation (Kelly and Coutts, 1997). Certainly bioactive peptides influence the release of somatostatin, [mentioned previously (Schusdziarra *et al.*, 1983a)] and gastrin, both of which are known to influence epithelial proliferation in the gut (Ichikawa *et al.*, 1993). Mucin secretions in the gut contain epidermal growth factor that promotes epithelial proliferation. The casomorphins are known to affect the secretion of mucin, suggesting a protective response that also stimulates the production of epidermal growth factor and increased cell growth.

IV.2.9. The influence of bioactive peptides on mucus secretion

The entire length of the GIT is permanently covered by a strongly adherent layer of high molecular weight glycoproteins, secreted by specialised goblet cells within the mucosa. The mucus is a complex biofilm that contains proteins, fats and bacteria in a gel-like matrix (Lamont, 1992). The matrix may also contain other compounds including: bicarbonate ions, epidermal growth factor, trefoil peptides, bactericidal factors, protease inhibitors, and surface-active lipids (Krause, 2000). Such compounds, when incorporated into the mucus layer, guard against its degradation and protect the underlying mucosa from gastric acid and pancreatic enzymes. The matrix also acts as a barrier to enteric microorganisms and the toxins produced by them, as well as being a diffusion barrier for dissolved compounds of low molecular weight (Perez-Vilar and Hill, 1999).

In addition to the mucus secreting goblet cells of the crypts and villous epithelium, there are specialised mucus secreting glands in the submucosa of the proximal duodenum called Brunner's glands. These glands secrete a glutinous alkaline mucin that contains bicarbonate ions to neutralise the acidic gastric fluids and forms a slippery gel that lubricates the mucosa of the proximal intestinal tract. Thought to be the product of the MUC6 gene, the class III mucin from the human Brunner's gland consists of O-linked oligosaccharides that are attached to a central protein core (Wapnir and Teichberg, 2002). The Brunner's glands also provide; both active and passive immunological defence mechanisms, promote cellular proliferation and differentiation, as well as raising the pH of the luminal contents by promoting the secretions of the intestinal mucosa, pancreas and gall bladder (Krause, 2000).

The most important proteins within the matrix are mucins, a family of polydisperse molecules of high molecular weight and a high proportion of covalently-bound oligosaccharide side chains (Corfield *et al.*, 2001) which afford high resistance to the effects of acid and digestive enzymes. They may be characterised as secreted or membrane-bound. Secreted mucins, up to 2×10^6 Da (Montagne *et al.*, 2004) contain a central polypeptide core, of 1500 to 4500 amino acids in length, with 100-200 oligosaccharide side chains that contain 1 to 20 or more monosaccharides, oriented in a fashion similar to a bottlebrush; such oligosaccharides may account for 50-80% of the molecule's mass (Roussel and Delmotte, 2004). The highly glycosylated regions of the polypeptide are rich in threonine, serine and proline and may account for 70%-80% of the molecule, the poorly glycosylated regions contain less serine and threonine but are rich in cysteine, which allows the formation of disulphide bridges between mucin molecules to form very high molecular mass mucous polymers (Krause, 2000). The secreted mucins protect the delicate underlying mucosal surfaces by the polymerisation of mucin monomers to form viscoelastic gels (Krause, 2000). Membrane-bound mucins do not form gels; the glycosylated monomers stretch out from the epithelial surface and form the cell-surface membrane glycocalyx.

The oligosaccharide side chains are strongly hydrophilic, which:

- promotes the binding of water molecules and supports the formation of the gel matrix,
- prevents the degradation of the polypeptide chain by proteases from the pancreas and bacteria,
- binds pathogens, parasites and toxins within the gel matrix.

The hydrophobic areas may bind fats and promote protein–protein interactions. The most important mucosal produced mucins are rich in cysteine, threonine, proline and serine, and substantial changes in mucus secretion therefore have a measurable effect on the cysteine and threonine requirements of an animal (Reeds *et al.*, 1999).

Studies suggest that mucin production is regulated by the same hormonal control as other digestive processes, e.g. insulin (Tabuchi *et al.*, 1997) secretin (Tani *et al.*, 1997) and gastrin. (Ichikawa *et al.*, 1993) The physiological role of gastrointestinal mucins is summarised in **Table 7**.

Mucins are continuously being degraded, by both proteolysis and physical erosion at the apical surface; this is compensated for by continuous secretions by goblet cells of the mucosa. The density of goblet cells increases from the proximal duodenum to the distal rectum.

Table 7 The physiological role of gastrointestinal mucin.

Function in relation to gut physiology	Function in relation to gut health
Protection of the epithelium within the gut against acidic environment	Fixation of commensal bacteria permitting colonisation resistance
Selective diffusion barrier permeable to nutrients but not to macromolecules	Fixation of pathogens; bacteria, viruses, and parasites
Protection against endogenous and bacterial proteases	Component of the gut-associated lymphoid tissue
Lubrication of the gut epithelium	Substrate for bacterial fermentation

Adapted from Montagne *et al.* (2004).

Within the GIT, a dynamic equilibrium exists between the rate at which mucin is synthesised and secreted and the rate at which it is degraded. Moreover, the mucus is continuously transported through the GIT including the cellular fats, DNA, proteins, sloughed epithelial cells and micro-organisms captured by it (Bannink *et al.*, 2006). The quantity of mucins secreted into the GIT may total 50% to 65% of the endogenous protein secreted into the gut (Montagne *et al.*, 2004). Although the synthesis of mucus is a smaller metabolic burden than ion and nutrient transport and tissue turnover, the costs are nevertheless still significant [estimated to be 10% to 20% of the total energy costs of gastrointestinal wall metabolism (Allen and Flemstrom, 2005)]. Being continually degraded mucins are a significant proportion of endogenous protein losses and may total 5% to 11% of the endogenous protein leaving the terminal ileum (Lien *et al.*, 1997b; Montagne *et al.*, 2004). Because of the importance of mucin in the protection of the mucosa any mechanism that alters this defensive barrier has important physiological implications, especially in the control and management of inflammatory diseases of the bowel (Barcelo *et al.*, 2000).

The secretion of mucus may be increased in response to a variety of physiological and pathological stimuli, e.g. bacteria, bacterial toxins, inflammation mediators, chemical stimuli or neural stimuli (Bannink *et al.*, 2006). A number of dietary factors may also affect the secretion of mucin including the amount of fibre and anti-nutritional factors (Claustre *et al.*, 2002; Lien *et al.*, 1996; Montagne *et al.*, 2004; Satchithanandam *et al.*, 1996). It has also been reported that certain dietary components may alter the composition of mucin secreted into the GIT (Montagne *et al.*, 2004).

The hypothesis that dietary proteins and their hydrolysates, containing bioactive peptides, may affect mucin secretion has been studied by a number of researchers (Claustre *et al.*, 2002; Han *et al.*, 2008; Lien *et al.*, 2001; Montagne *et al.*, 2000). The effect of opioid peptides, specifically the β -casomorphins -7, -6, -4, -4NH₂ and -3 and a number of neuropeptides, on mucin secretion has been reported by Trompette *et al.* (2003) where the intra-luminal administration of the β -casomorphin-7 provoked a 500% increase (over the controls), in the secretion of mucin. β -casomorphin-7 seems unique in this respect as little or no increase in mucin secretion was observed from the other opioid peptides tested.

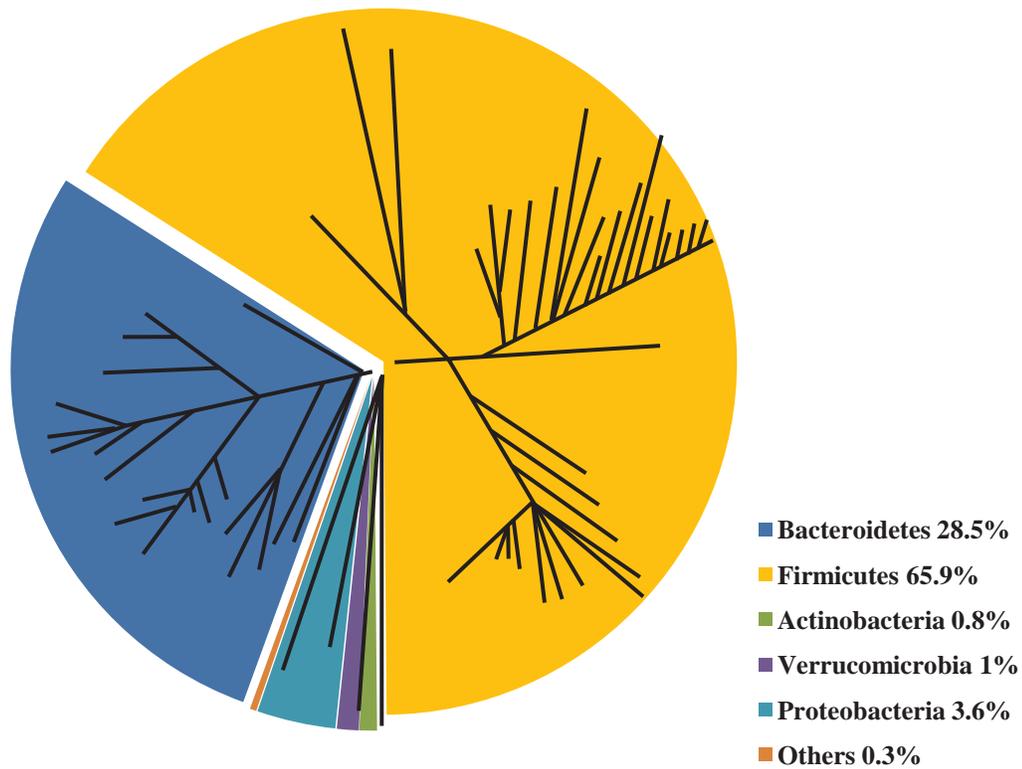
Perhaps here lies the key to the variability in the observed changes in endogenous protein secretion that have been reported by so many researchers (Claustre *et al.*, 2002; Han *et al.*, 2008; Lien *et al.*, 2001; Montagne *et al.*, 2000; Trompette *et al.*, 2003). The analysis of digesta samples taken from the terminal ileum of animals given casein hydrolysate compared to those given a milk protein or protein-free diets may reveal clearer information regarding the effects of opioid peptides on endogenous protein losses.

V. Bacterial nitrogen

The GIT of all mammals is colonised by a plethora of microbial species which in adult humans form a diverse community (Ley *et al.*, 2006) that profoundly influence many of the physiological processes of the host (MacDonald and Monteleone, 2005). The microflora inhabiting the GIT meet their own nutritional requirements by the degradation of both exogenous and endogenous substances and because these organisms possess enzymes their hosts do not, they can utilize substrates, such as complex carbohydrates and glycoproteins, that are resistant to mammalian digestive enzymes (Fuller and Reeds, 1998).

The role that microflora play in the physiology, nutrition and health of the GIT has gained much attention over the last decade (Bergen and Wu, 2009; Furrie, 2006). The host and intestinal microflora are in a state of symbiotic mutualism, forming what Goodacre (2007) terms a human-microbe hybrid where the human genome and the microbiome collectively define a “superorganism”. The microbial diversity of the human intestinal microbiota is illustrated by the phylogenetic tree of **Figure 12**. Over 400 species of bacteria (Gordon *et al.*, 1997) inhabit the human gut though they are not distributed uniformly either in number, species or metabolic activity, **Table 8**.

There is a growing body of evidence that microbes in the GIT play an important role in host protein and amino acid metabolism (Blachier *et al.*, 2010; Dai *et al.*, 2010, 2011).

Figure 12 Bacteria of the human gastrointestinal tract.

The phylogenetic tree of microbial diversity in the human intestinal microbiota is based on 16S rRNA bacterial sequence data generated by Eckburg *et al.* (2005) and from Turroni (2008). The percentage composition is based on data from Walker *et al.* (2008).

Table 8: Bacterial population of the human gastrointestinal tract. ²

Location	Environmental pH	Bacterial Count	Most Abundant Species
Stomach	1 - 5	$0 - 10^4$	Lactobacilli
Duodenum	5 - 7	$0 - 10^3$	Lactobacilli, Bacteroides
Jejunum	6 - 7	10^5	Enterobacteria, Bacteroides
Ileum	6 - 7.5	$10^6 - 10^9$	Bifidobacteria, Enterococci
Caecum/Colon	5.5 - 7	$10^{10} - 10^{12}$	Enterococci, Enterobacteria, Clostridia, Bacteroides

² Adapted from (Autenrieth, 1998; Hovgaard and Brondsted, 1996; Metges, 2000)

Previous studies (Metges, 2000; Torrallardona et al., 2003a, 2003b) have shown that dietary indispensable amino acids synthesized by the enteric microbiota are absorbed and contribute to the amino acid supply of humans and monogastric animals. Further, amino acids originating from both dietary and endogenous protein are diverted extensively to microbial protein in the small intestine (Libao-Mercado *et al.*, 2009). A number of studies have shown that many dietary indispensable amino acids, synthesized by luminal bacteria, are absorbed primarily from the small intestine (Metges, 2000; Torrallardona et al., 2003a, 2003b), and yet these results cannot explain the net high rate of amino acid utilization by the gut (van Goudoever et al., 2000).

In their studies, Libao-Mercado *et al.* (2009) found that more than 92% of valine in microbial protein was derived from endogenous and dietary protein, suggesting that the contribution of *de novo* synthesis to microbial amino acids, and thence to the host's amino acid supply, is small. How much of those endogenous and dietary proteins would have been digested and absorbed without the intervention of the microbiota remains uncertain. Protein and amino acids synthesised by the intestinal microbiota cannot be of nutritional value unless the substrates are valueless to the host, as in the case of non-protein nitrogen compounds such as ammonia, urea or surplus dietary dispensable amino acids.

Indeed the diversion of amino acids derived from dietary and endogenous protein into microbial protein, even if subsequently digested and absorbed, would not make a net contribution to the amino acid supply of the host (Libao-Mercado et al., 2009).

Using a novel gut-microbe-subculture approach Dai *et al.* (2010) reported that the significant sites of luminal metabolism of amino acids were:

- The duodenum for the luminal metabolism of glutamate and histidine.
- The jejunum and ileum for the luminal metabolism of dietary lysine, valine, threonine, arginine, leucine and isoleucine.

Both Dai *et al.* (2010) and Wu (1998) speculate that as the jejunum is the main site for amino acid absorption, high rates of catabolism of lysine and arginine in the lumen of the jejunum could limit their absorption into enterocytes.

Considering the length of the small intestine the bioavailability of dietary amino acids to extra-intestinal tissues may be compromised by the high rate of amino acid metabolism (Fuller and Reeds 1998; Dai *et al.*, 2010). Dai *et al.*, suggest compartmentalization of amino acid metabolism in the small intestine may exist at three levels:

- The different sites of the small intestine (gut segments, lumen, and mucosa).
- The different cell types (different species of bacteria and their functional redundancy).
- The different cellular sites (cell membrane, cytoplasm, mitochondria).

Based on their research Dai *et al.* (2010) estimated that around 50% of the amino acids utilized by the bacteria of the jejunum and ileum were for protein synthesis.

Interestingly they also showed that the variety and concentration of amino acids could regulate the populations of intestinal microbiota and thus play an important role in the prevention and control of infectious diseases of the GIT (Schaible and Kaufmann 2005; Wang *et al.*, 2009). The beneficial role that microbiota in the GIT play in the human host, by regulating genes related to the metabolism of glycans, amino acids, the biosynthesis of vitamins and isoprenoids, has been demonstrated by Gill *et al.* (2006) using metagenome analysis.

However, enteric microbiota also provoke metabolic costs that diminish any positive benefit to the host (Metges *et al.*, 2006; Libao-Mercado *et al.*, 2009) include:

- Enhanced secretion of mucus glycoproteins.
- Increased epithelial cell turnover.
- Increased amino acid needs resulting from immune system stimulation.
- Nutrient diversion to support the enteric microbiota, including fermentative amino acid catabolism.

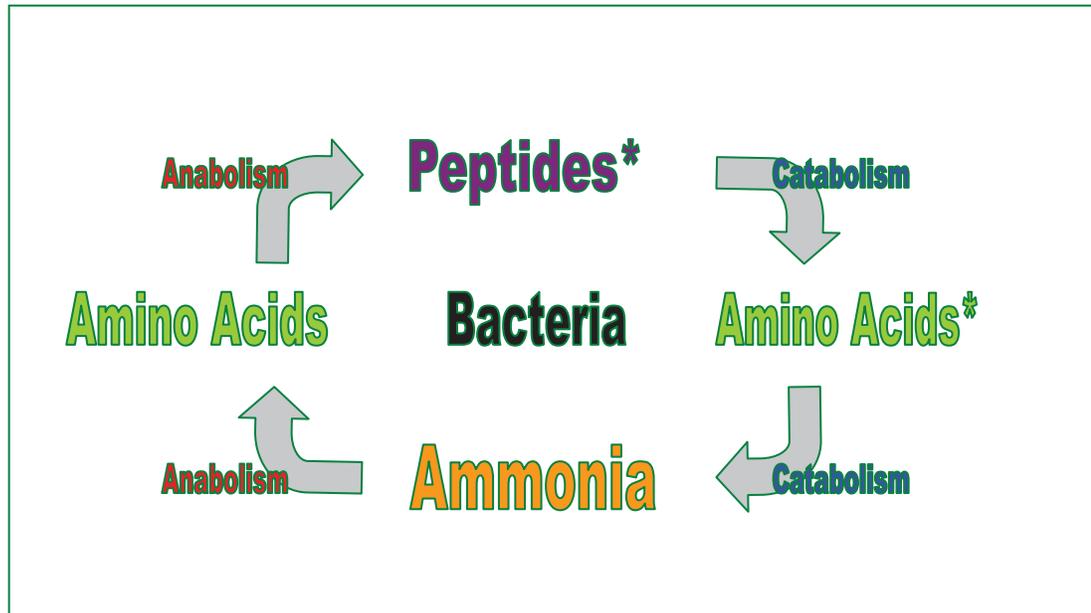
Many dietary dispensable, as well as branched-chain amino acids, arising from digestion in the small intestine are deaminated or degraded to form ammonia that may

then enter the portal vein, be utilised for synthetic or degradative metabolism in the enterocyte, or re-enter the small intestinal lumen to be utilized by microbial cells to synthesise amino acids and thence protein (Wu 1998; Chen, Yin *et al.*, 2007; Chen *et al.*, 2009). Studies with both pigs and humans indicate that ammonia is also generated in the intestinal lumen (primarily in the small intestine) by the microbial hydrolysis of urea; this typically accounts for 20% – 25% of the urea synthesized by the liver (Bergen *et al.*, 2009; Long *et al.*, 1978).

Utilising nitrogen from a mixture of ammonia (derived from the microbial degradation of proteinaceous material) and endogenous urea (secreted by pancreatic, biliary and mucosal epithelia), intestinal microflora can synthesise many amino acids which are then absorbed to become part of the free plasma amino acid pool. Torrallardona (1996) found that some 75% of microbial lysine absorption occurs in the small intestine. Krawielitzki *et al.* (1990) found that 90% of all endogenous nitrogen secreted into the gut is reabsorbed. This led Metges (2000) to suggest that microbial proteolytic activity plays an important role in the catabolism of mucins and other digestive secretions which are particularly resistant to digestion by mammalian digestive enzymes, a conclusion indirectly corroborated by Rutherford and Moughan (1998). Nitrogenous compounds in the digesta from the terminal ileum (2-3 g Nitrogen·d⁻¹) are reported to contain 10-15% urea/ammonia/nitrate and free amino acids, 48-51% protein and 34-42% peptides (Chacko and Cummings, 1988; Metges, 2000). The cyclic effect of the gut microbial activity is illustrated in **Figure 13**.

The ability of the gut microflora to utilise simple nitrogenous compounds such as urea and ammonia to synthesise amino acids and incorporate these amino acids into microbial cellular material (Mason, 1984; Metges, 2000) as well as transform and degrade them, has a direct and important bearing on the determination of amino acid digestibilities (Moughan, 2003). The fermentation of proteinaceous material in the large intestine is thought to provide little additional nourishment and amino acids do not appear to be absorbed as such by this part of the gut in any significant amount (Moughan, 2003).

Figure 13. Bacterially mediated nitrogen cycling within the gastrointestinal tract.



* Peptides and amino acids originating from endogenous, dietary or microbial sources.

VI. Endogenous nitrogen losses in humans

VI.1. Techniques for the collection and quantification of gut endogenous protein

The determination of endogenous nitrogen losses using faecal analysis, as developed by Kuiken and Lyman (1948) measured the difference between the concentrations of nitrogen in the diet with that found in the faeces of a test animal. However, many researchers have reported the inadequacy of measuring the proportion of unabsorbed nitrogenous compounds using faecal analysis, especially when 80% of faecal nitrogen is microbial in origin (Low and Zebrowska, 1989; Moughan, 2003; Rutherford and Moughan, 1998). It has been clearly demonstrated that any proteins or amino acids entering the large intestine have little or no nutritional value (Wunsche *et al.*, 1979; Zebrowska, 1973a).

Microbial action on digesta entering the large intestine is largely dependent upon the carbohydrate concentration as well as the nitrogenous composition (Fuller and Reeds 1998). The greater the amount of fermentable carbohydrate the greater the degradation of amino acids present within the digesta.

Nitrogenous material entering the large intestine is derived from a number of sources (**Table 9**), and as a result of microbial proteolytic, deaminative and decarboxylative enzymes proteinaceous material in the large intestine is mostly broken down to ammonia.

Ammonia released in this process is either absorbed by the epithelia of the hindgut, before being excreted as urea in the urine, recycled (Mosenthin et al., 2000), or incorporated into microbial protein (Mason, 1984).

Table 9 The source and nature of proteinaceous material entering the large intestine.

Undigested dietary sources	Endogenous sources	Microbial sources	Cellular material
Protein	Enzymes	Protein	Plant cells, i.e. plant fibre
Peptides	Mucins	Free D-amino acids e.g. D-alanine	Microbial cells see Table 4
Free amino acids	Free amino acids Protein, e.g. Albumin Urea	Diaminopimelic acid Amino-ethylphosphonic acid	Host cells and cell debris from all sources

Recent studies by Metges *et al.* (1999b) have shown that significant amounts of lysine and threonine (amino acids that are not transaminated by mammalian tissues), are synthesised *de novo* by gastrointestinal microflora and are absorbed into the plasma pool. As a result of amino acid degradation and/or their *de novo* synthesis, coupled with the changing concentration of fermentable carbohydrate, the nitrogenous flux within the large intestine renders faecal determination of nitrogen and endogenous nitrogen losses unreliable.

When Mosenthin *et al.* (2000) compared the apparent faecal and ileal digestibilities of raw and heat treated soy flakes, they concluded that faecal analysis is less sensitive and subject to greater error. They also stated that the greater sensitivity of ileal digesta analysis was particularly significant when comparing diets that contain poorer quality protein. Corroborative evidence of Mosenthin's conclusions are not hard to find (Darragh and Hodgkinson, 2000; Moughan, 2003), though data reported by Dierick *et al.* (1988) is particularly convincing. They reported that there was much greater correlation between weight gain and ileal digestibility than the corresponding faecal digestibility values ($r = 0.76$ vs. $R = 0.34$ respectively) an inadequacy emphasised by Moughan (2003) that has been reported as far back as 1946 (Crampton and Bell, 1946).

In conclusion, as the digestion and absorption of proteinaceous material is virtually complete by the time digesta reaches the terminal ileum and that microbial action in the large intestine cannot be predicted, protein and endogenous nitrogen losses are more reliably determined from ileal analysis than from corresponding faecal measurements.

VI.1.2. The collection of digesta from the terminal ileum of humans.

The collection of digesta from the terminal ileum of human subjects has been undertaken traditionally using two quite different techniques, i.e. total collection from ileostomates, or sampling via a naso-ileal tube.

VI.1.2.1. Ileostomates

Ileal digesta can be collected from subjects who have been ileostomised (Moughan *et al.*, 2005a; Rowan *et al.*, 1994) as a result of a pathological condition of the colon such as inflammatory bowel disease (ulcerative colitis), familial adenomatous polyposis syndrome and colorectal carcinoma (Christl and Scheppach, 1997). It is usual that following colectomy a stoma is formed where the final few centimetres of the ileum are attached to the abdominal wall.

The chemical composition of ileostomy effluent however may not be a true reflection of digesta from the terminal ileum of subjects with a normal intact digestive system.

Two major concerns in considering this form of digesta collection are:

- a) Normal digestive function may be altered by the primary pathological condition that resulted in the patient's colectomy. Also it is known that colectomy induces many adaptations in a number of physiological and metabolic processes (Christl and Scheppach, 1997; Stern *et al.*, 1999).

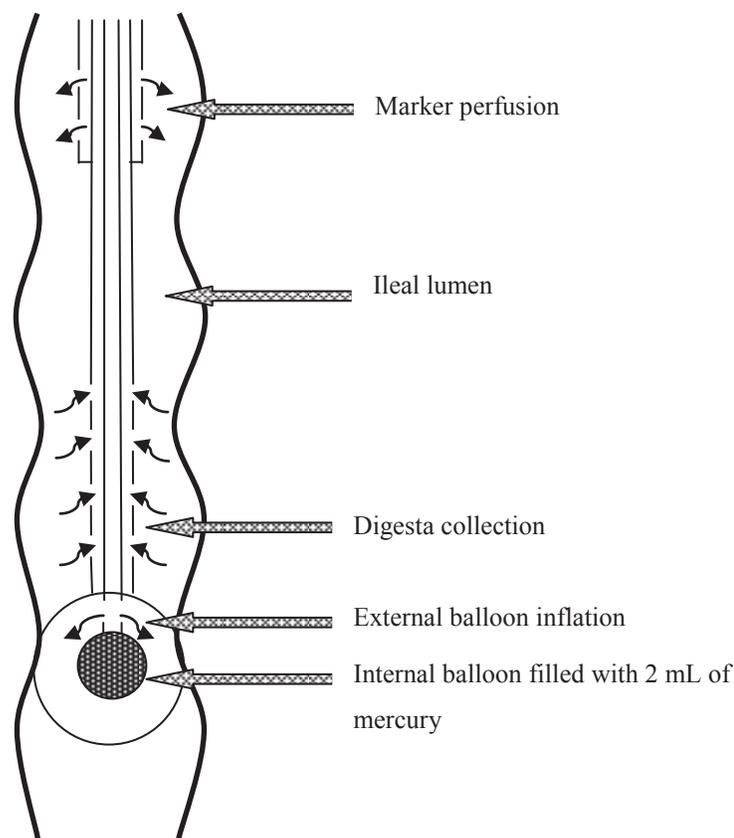
- b) It has long been established that both large numbers and varieties of microflora inhabit the terminal ileum of ileostomy patients, and that such an ecology equilibrates rapidly following surgery (Vince *et al.*, 1973). Although the bacterial counts in the neo-terminal ileum are higher (10^5 – 10^7 g⁻¹) than in the normal ileum (10^3 – 10^5 g⁻¹) they are much less than those found in normal faeces (10^{12} g⁻¹) (Christl and Scheppach, 1997). With lower concentrations of anaerobes and yeasts, plus higher numbers of aerobes (once established), both the numbers and variety of microflora present in the terminal ileum of ileostomy patients do not mimic the faecal microflora populations (Christl and Scheppach, 1997). With elevated populations of microorganisms deaminating peptones within the terminal ileum, the digesta collected from ileostomates may not be a true reflection of that from healthy patients with an intact digestive system. Indeed Fuller *et al.* (1994) estimated that amino acid flows may be reduced by up to 15%.

VI.1.2.2. Naso-ileal intubation

Naso-intestinal intubation for the collection of digesta was first used by Schedl and Clifton in 1961, and later modified by Sladen and Dawson in 1969 to include an inflatable balloon that would stimulate peristalsis and hasten the progression of the tube through the gut (Sladen and Dawson, 1969). Used to study the effects of intraluminal perfusion of the human intestine it was later developed by Modigliani and

colleagues (1973). With a distal balloon containing mercury to weigh them down these tubes required 12 to 30 hours to reach the ileum; although this time may be reduced if using a second inflatable balloon as suggested by Sladen and Dawson. This configuration was again modified by having the inflatable balloon outside that of the mercury filled balloon at the distal end of the tube, and has been used to determine the ileal digestibility of protein in humans (Champ *et al.*, 1998; Deglaire *et al.*, 2009; Mahe *et al.*, 1992) (**Figure 14**).

Figure 14. The triple lumen tube used for the collection of ileal digesta in humans.



Modified from Deglaire *et al.* (2009).

The advantages of the latter configuration are:

- a) The mercury balloon allows gravity to assist the rapid passage of the tube through the nose, oesophagus and stomach.

- b) The inflated terminal balloon stimulates peristalsis to accelerate the tube's progression to the ileo-caecal junction.

- c) Once in position the inflated terminal balloon partially blocks the ileum and allows the almost complete collection of digesta for the duration of the collection period. This can be corroborated by the recovery of an indigestible marker.

The naso-ileal tube method has been evaluated by comparing it with digesta collected via cannulation in animals (Noah *et al.*, 1998) and ileostomy effluent in humans (Langkilde, 1994); although neither study affords a perfect control it allows precise and accurate sampling of intestinal digesta and collected samples are representative of the fraction reaching the large intestine (Deglaire, 2008a). The validity of this digesta collection method has been further investigated by Deglaire *et al.* (2008b) using data from unpublished studies at the Human Nutrition Laboratory, INRA-AgroParisTech (France) and their own experimental data. By determining the recovery of a non-digestible marker, polyethylene glycol 4000 (added to test meals), they concluded that such a sampling method allows the precise and accurate sampling of digesta.

However, although gastrointestinal intubation has been used frequently for the recovery of digesta (Bos *et al.*, 2007; Champ *et al.*, 1998; Mahe *et al.*, 1992; Mariotti *et al.*, 1999) the effect of intubation on the normal gut processes is unclear. Fone *et al.* (1991) reported that ileal intubation has an important inhibitory influence on gastric emptying and antral motility, whilst more recently Medhus *et al.* (1999) reported that duodenal intubation delayed gastric emptying of a liquid meal.

Likewise, for a solid meal, Read *et al.* (1983) reported that gastrointestinal intubation slows gastric emptying and reduces total intestinal transit time. Although it has been reported that the transit rate of food through the digestive tract has little or no effect on amino acid absorption and thus on overall protein digestibility (Lien *et al.*, 1996; Mariotti *et al.*, 2000; Weber and Ehrlein, 1998; Zhao *et al.*, 1997b), the effect of a gastrointestinal tube on the absorption and secretion of other substances has not been fully investigated.

The presence of mechanoreceptors throughout the oesophagus, stomach, and small intestine are well documented (Paintal, 1957) and gastric emptying may be delayed by the stimulation of mucosal mechanoreceptors at all of these sites (Gregersen and Kassab, 1996; Phillips and Powley, 2000; Read et al., 1983). Indeed the mechanical stimulation of the pyloric region of the stomach induces the secretion of acid (Lim *et al.*, 1925; Read et al., 1983). Likewise the mechanical stimulation of the small intestine with a soft catheter has been used as a method for stimulating and collecting small intestinal secretions (Florey *et al.*, 1941; Hendrix and Bayless, 1970; Read et al., 1983). The physical abrasion/stimulation of the pylorus by a gastrointestinal tube may therefore stimulate the secretion of both soluble and membrane-bound gastric mucins. Similar stimulation of the small intestine may also stimulate the secretion of both soluble and membrane-bound small intestinal mucins. As mucins are an important source of endogenous nitrogen the presence of a gastrointestinal tube may affect the quantification of endogenous nitrogen.

Although the technique also relies heavily upon the inclusion of indigestible markers and is restricted to purified proteins and to acute studies, it is probably the best approach currently available for repeated sampling of ileal digesta in the conscious adult human.

VI.1.3. The pig model

Given the inherent difficulties in obtaining ileal digesta samples in human studies suitable animal models have been developed. The growing pig has been used widely as a digestive model for the adult human. Although the rat has traditionally been considered to be a suitable human model animal for humans (FAO, 1991) the pig has also been suggested as a suitable model for the investigation of protein digestibility in humans (Moughan *et al.*, 1992a; Moughan, Cranwell, Darragh, and Rowan, 1994). Indeed the pig has been shown to be a more suitable model for the investigation of protein digestibility in humans (Darragh and Moughan, 1995; Fleming and Wasilewski, 1984; Moughan *et al.*, 1992) as the GIT anatomy, physiology and metabolism of the pig are very similar to those of the human (Darragh and Hodgkinson, 2000; Miller and Ullrey, 1987; Moughan et al., 1994). When both Rowan

et al. (1994) and Deglaire *et al.* (2009) compared the ileal digestibility of amino acids in both the human and the pig they found little difference between them.

When the pig and human ileal digestibility dataset of Deglaire *et al.* (2009) was augmented by the inclusion of data previously reported by Rowan *et al.* (1994) the correlation between pig and human true ileal digestibility coefficients was high ($r = 0.94$) and close to significance ($P = 0.06$) for nitrogen from meals containing casein and hydrolysed casein, and was statistically significant ($r = 0.83$ and $P < 0.001$) for amino acids from meals containing casein and hydrolysed casein. The linear regression equations derived to allow predictions of human true ileal digestibility values from determined pig true ileal digestibility values were $y = 1.47x - 0.47$ and $y = 1.05x - 0.06$ for nitrogen ($R^2 = 0.88$) and for amino acids ($R^2 = 0.68$), respectively; data which indicate strong evidence in support of the use of the pig as a model for human protein digestibility (Darragh and Hodgkinson, 2000; Deglaire *et al.*, 2009).

Although there are no major arguments that contradict the suitability of using pigs in digestibility studies, there are concerns relating to: variability induced by cannulation techniques, feeding and collection strategies; (Boisen and Moughan, 1996) and the expense of running large animal trials (Boisen, 2007). More recent studies have shown that endogenous nitrogen losses are higher in weaned piglets than in growing pigs thus introducing a systematic age effect when determining ileal digestibilities (Mariscal-Landin and de Souza, 2006).

VI.1.4. The collection of digesta from the terminal ileum of pigs.

Since the early 1970's a variety of different digesta collection techniques have been used to determine ileal digestibilities in pigs. These have been reviewed by Sauer and Ozimek (1986), Fuller (1991), Yin and McCracken (1996) and include:

- Sampling under anaesthetic (or sometimes following euthanasia, and consequently called the slaughter technique).
- Ileo-rectal anastomosis, and
- Cannulation of the gut

With these techniques, some of which allow only samples of digesta to be collected once, indigestible marker are added to the diet given to the animal to allow relation back to the diet. Each collection method therefore has advantages as well as disadvantages that require review.

VI.1.4.1. The slaughter technique.

As the name of this technique implies the experimental animal is sacrificed for the digesta samples to be collected. First suggested by Payne *et al.* (1968) for the collection of ileal digesta from birds it was subsequently adapted for use in the rat and the pig by a number of researchers (Butts *et al.*, 1991; Donkoh *et al.*, 1994). This technique has a number of advantages over other methods:

- There is minimum disruption to the normal GIT.
- It allows digesta to be collected from a number of regions of the digestive tract.
- There are no restraints on the type or nature of the diet.
- Reduction of the time the test animals are subjected to experimental conditions.
- Lower ethical cost.
- Simple and less labour intensive and not requiring expensive surgical procedures.

However there are a number of possible disadvantages:

- The potentiality of not obtaining digesta samples that are representative of normal animal digestion (Fuller, 1988).
- The optimum sampling time may vary from one diet to another (Buraczewski *et al.*, 1971).
- Only a single terminal ileal digesta sample can be taken from the terminal ileum of each animal.

- Unless care is taken mucosal epithelial cells can be sloughed off at death or during sampling (Badawy *et al.*, 1957; Badawy *et al.*, Campbell *et al.*, 1958; Fell, 1961).

When Donkoh *et al.* (1994) compared the slaughter method with that of simple T-piece cannulation the low inter-animal variance for the determined amino acid digestibilities suggested that the sloughing of epithelial cells did not occur. Post-mortem autolysis and gastric mucosal cell shedding increases with time; Thorpe and Thomlinson (1967) found that there was only negligible loss of epithelial cells for up to ten minutes after death. Mindful of this Donkoh *et al.* (1994) took their samples within five minutes of the animal's death and shedding was further minimised by first anaesthetising the animals with halothane before killing them with an intracardial injection of barbiturate (sodium pentobarbitone), a technique recommended by Badawy (1964). However, in a study by Viljoen *et al.* (2000) comparing the slaughter technique, ileo-rectal anastomosis and simple T-cannulation, the digesta was collected by squeezing out the contents from the distal third of the ileum, as a result sloughing of epithelial cells would have been considerable and any observed variance may have been an aberration of squeezing the gut sample to extract the digesta.

In the work of Donkoh *et al.* (1994) there were no statistically significant differences in amino acid digestibility between the slaughter technique and simple T-cannulation of the terminal ileum for pigs given a diet containing meat and bone meal as the sole protein source. In addition to this they found that collecting the digesta samples nine hours after feeding gave the least variance in both sample size and nitrogen digestibility. In contrast to the work of Buraczewski *et al.* (1975) and Poppe and Meier (1977), Donkoh *et al.*, found that the digestibility values were not affected by the sampling site when it was between 0 and 140cm of the ileo-caecal valve. Their results concurred with both Kies *et al.* (1986) and van Barneveld *et al.* (1991) who both found that if the digesta samples were taken in the same region of the terminal ileum (i.e. between 0 to 140 cm of the ileo-caecal valve), the digestibility values were unaffected. The preferred site of sampling in the study by Donkoh *et al.* (1994) was found to be the terminal 20cm of the ileum.

VI.1.4.2. Ileo-rectal anastomosis.

To avoid interference from colonic microflora and to allow repeated sampling of digesta over time, ileo-rectal anastomoses (IRA), have in the past been used to collect pre-caecal digesta from the terminal ileum. Numerous IRA configurations have been tried since they were first proposed in the early 1980's (Fuller and Livingstone, 1982; Laplace *et al.*, 1985a; Picard *et al.*, 1984). Digestibility estimates obtained using these procedures varied depending on the configuration being used (Darcy-Vrillon and Laplace, 1990). There were four common configurations which are illustrated in **Figure 15**.

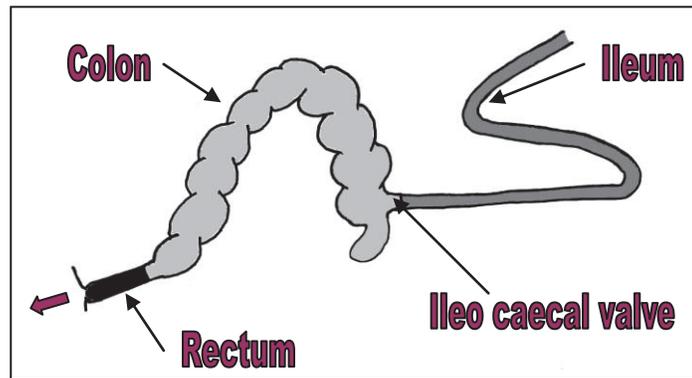
The two main differences are:

- a) End to side, where the ileum is cut before the ileo-caecal valve and then joined to the descending colon. This technique does not completely isolate the large intestine, which may retain some functionality (Köhler *et al.*, 1991; Laplace *et al.*, 1985a).
- b) End to end, where the ileum is cut before the ileo-caecal valve and then joined directly to the rectum. This technique isolates the large intestine completely (Laplace *et al.*, 1989; Laplace *et al.*, 1994).

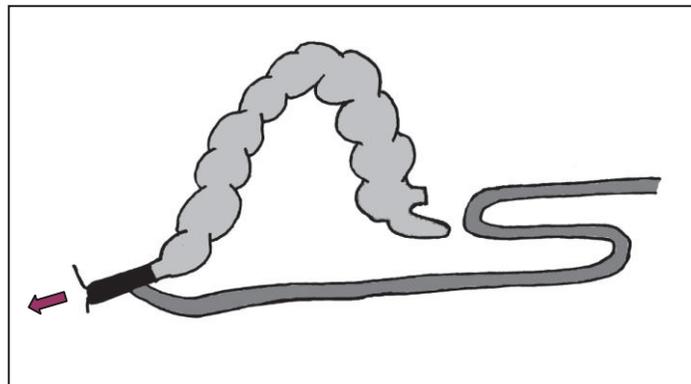
The ileo-caecal valve may be preserved in either technique to give four different configurations (Souffrant *et al.*, 1985). Although preservation of the ileo-caecal valve appeared to aid the digestion process in the small intestine, as the action of the valve slows the gastro-intestinal transit time, it did not appear to affect protein ileal digestibility values (Laplace *et al.*, 1994). In all of these ileo-rectal configurations the normal functions associated with the large intestine are interrupted, and will therefore affect the absorption of water and minerals (Köhler *et al.*, 1991). Although there were advantages of IRA there were also many disadvantages, these together with ethical considerations render these techniques obsolete.

Figure 15. A schematic diagram illustrating different techniques of ileo-rectal anastomoses, [adapted from (Sauer et al., 1989)].

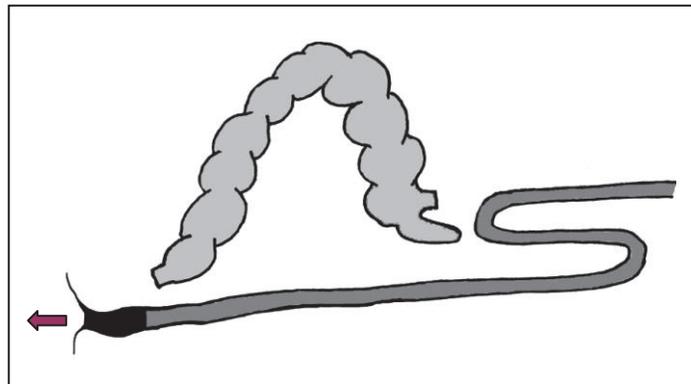
1. Unaltered.



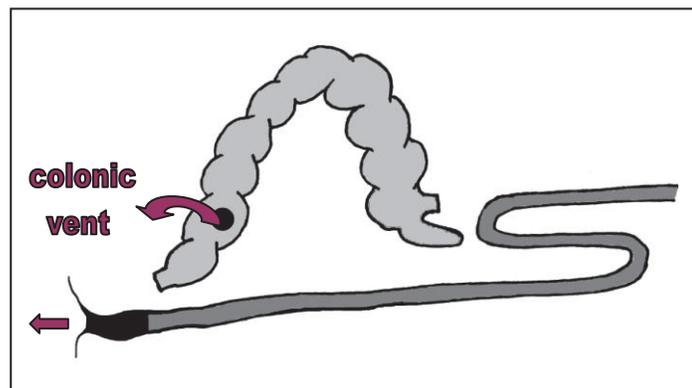
2. End to Side.



3. End to End.



4. End to end with colonic vent



VI.1.4.3. T-Cannulae.

Classically, methods to obtain ileal digesta samples have involved the use of cannulae, where the lumen of the distal ileum has been exteriorised.

Cannulation techniques can be broadly divided into two categories, those where the experimental animal is fitted with a single cannula and those fitted with two, the so called re-entrant variants. Double re-entrant cannulas are rarely used today because of the complex and expensive surgical procedures.

- a) Single cannula:
 - i. Simple “T” cannula (Livingstone *et al.*, 1977b).
 - ii. Post valve T-caecum cannula (van Leeuwen *et al.*, 1991).
 - iii. Steered ileo-caecal valve (Mroz *et al.*, 1996).

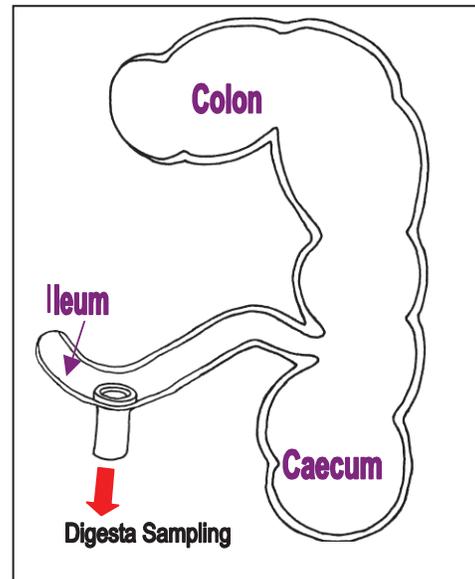
- b) Double re-entrant cannula,
 - i. Simple ileo-ileal re-entrant cannula (Braude *et al.*, 1969; Cunningham *et al.*, 1962).
 - ii. Ileo-caecal cannula (Easter and Tanksley, 1973).
 - iii. Ileo-colic post valve fistulation (Darcy *et al.*, 1980a).

Compared with single cannulation techniques, re-entrant type cannulation has been thought to give more reliable nutrient digestibility values since they allow the collection of total ileal digesta samples within the experimental time period, which obviates the need for an indigestible marker (Yin and McCracken, 1996). However, both approaches have been criticised on the grounds that such procedures have a modifying effect on the processes of digestion and absorption (Sauer and Ozimek, 1986), and that differences in the estimations of digestibility may occur depending on the technique used (Darcy-Vrillon and Laplace, 1990). Studies comparing the different techniques have been undertaken by a number of researchers who have all reported some disparity in the calculated digestibility coefficients (Donkoh *et al.*, 1994; Köhler

et al., 1990; Yin and McCracken, 1996; Yin *et al.*, 2000a and 2000b), though some of these differences may be attributable to other secondary factors including the composition of the diet; the amount of fibre; or the nature of the indigestible marker (Laplace *et al.*, 1994; Sauer and Ozimek, 1986; Yin *et al.*, 2000a and 2000b). Further discussion on the merits of these techniques is therefore required.

VI.1.4.3.1. Simple 'T' cannula.

First proposed by Livingstone *et al.* (1977b) this technique utilised a "T"-shaped cannula made of annealed Pyrex glass that had an internal diameter of just 16-20 mm. The cannula was secured from becoming internalised by one or more Perspex washers and a self locking nylon strap. The shank of the cannula was threaded to take an impact resistant plastic cap. The cannula was inserted 15 cm proximal to the ileo-caecal valve.

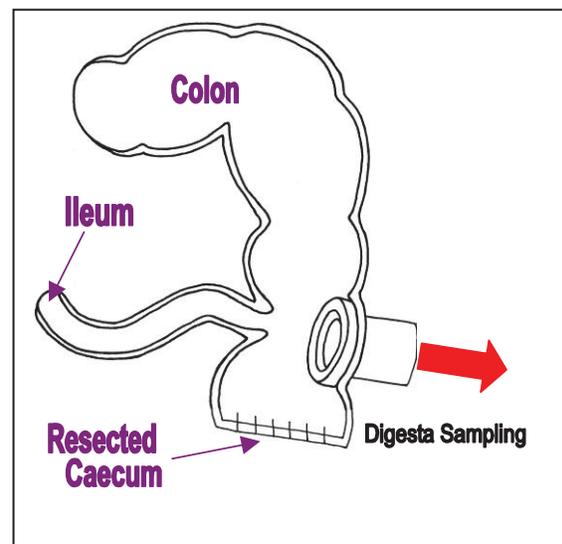


Livingstone reported that the cannulae remained intact and functional when fitted to pigs, housed in smooth-walled pens, growing to a live weight of 175 kg. Trauma from the surgery of fitting the cannulae was reported to be minimal and the intestinal activity, appetite and growth rate of the animals was unaffected by the cannulation. They concluded that the pigs fitted with the cannula were physiologically similar to those that had not been surgically treated (Livingstone, *et al.*, 1977a; Livingstone *et al.*, 1977b). As with all single cannula techniques, not all of the digesta flows through the cannula and the incorporation of an indigestible dietary marker to determine the total flow of digesta is essential. It is assumed that the composition of the digesta collected via the T-cannula is representative of the total amount flowing through the distal part of the ileum, and that no fractionation of its contents, particularly between the solid and liquid phases, occurs. However, this has been questioned by a number of researchers (Fuller *et al.*, 1994a; Sauer *et al.*, 1986; Yin *et al.*, 1996). Differences in the recovery of indigestible markers have indicated that phase separation may indeed

occur and that this may be one reason why greater variability in results were observed by Köhler *et al.* (1990) when they compared simple T-cannula with post valve T-caecum cannula and re-entrant cannulae. They suggested that a reduction in the homogeneity of the samples recovered through the T-cannula may be attributable to changes in pressure at the base of the open cannula, forcing a separation of coarse and fine insoluble particles (Schröder, 1988), and this might explain differences in the digestibility coefficients observed in diets rich in fibre. Although no incidence of the cannula blocking was reported by Livingstone *et al.* (1977a and b) this has been noted by Potkins *et al.* (1991), who had to change their experimental design owing to the frequency of blockages at the site of the cannula and the consequential inability to collect digesta when their experimental animals were fed a diet rich in bran fibre.

VI.1.4.3.2. Post valve T-caecum cannula.

Developed by van Leeuwen *et al.* (1988) in the late 1980's at the Institute for Animal Nutrition and Physiology in the Netherlands, the Post valve T-caecum cannula (PVTC) is made of medical grade silicon tubing, with an internal diameter of 25 mm and is secured by an exterior ring and self locking nylon straps. The technique involves



the removal of part of the caecum and the installation of the cannula opposite the ileo-caecal valve. One hour before the collection of digesta starts the cannula is opened to allow the ileo-caecal valve to protrude into the lumen of the cannula. Although this ensures that more of the digesta flowing from the ileum is collected, this technique cannot guarantee a complete quantitative collection of ileal digesta and so the inclusion of an indigestible dietary marker is advised (van Leeuwen *et al.*, 1991). Having studied more than 100 animals fitted with PVTC cannulae, within live weights ranging from 8-100 kg, van Leeuwen's group found that post operative recovery was swift and the animals displayed no signs of discomfort or a reduction in appetite, even over long

periods of time. Following the cecectomy and insertion of the cannula into what remains of the caecum the physiology of both the ileum and the colon appear to be normal. As the gut is not transected, there appears to be no interference with the myoelectric innervation of the intestines that may reduce intestinal motility, points that have been corroborated by a number of other researchers (Köhler *et al.*, 1990; Köhler *et al.*, 1992a; Köhler *et al.*, 1992b; Yin and McCracken, 1996; Yin *et al.*, 2000b). Although few digestibility studies have compared PVTC cannulae with other methods of collection those that have conclude that results, using the PVTC technique, are comparable with those obtained using simple T-cannula (Köhler *et al.*, 1990; Yin and McCracken, 1996; Yin *et al.*, 2000b).

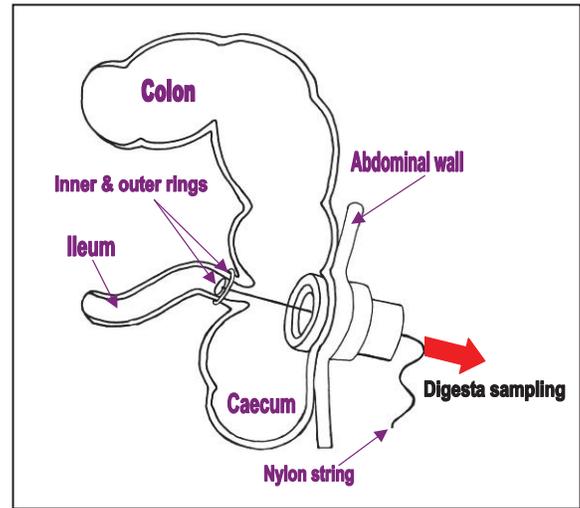
In one long term study by Kohler *et al* (1992a and 1992b) using 23 pigs (nine with IRA's, seven with PVTC's and six intact pigs), no differences were found in growth performance, body nitrogen retention, blood variables and mineral balances, between intact animals and those fitted with PVTC's. However, statistically significant differences were found for several variables between the experimental animals with IRA's and pigs with PVTC's.

Greater precision can be achieved using the PVTC method for most diets and this is thought to be attributable to greater consistency in the recovery of the indigestible marker, specifically titanium dioxide. However, with high fibre diets some variability in the amount of digesta recovered has been recorded, which confirms the necessity of using an indigestible marker. (Köhler *et al.*, 1991; Köhler *et al.*, 1990; van Leeuwen *et al.*, 1991; Yin *et al.*, 2000a)

VI.1.4.3.3. Steered ileo-caecal valve.

The normality of gut function in cannulated pigs and those with ileo-rectal anastomoses when compared to intact pigs has been called into question concerning total tract digestibility of proximate dietary nutrients by a number of researchers (Fuller, 1991; Köhler *et al.*, 1990; Mroz *et al.*, 1994; Mroz *et al.*, 1996; Radcliffe *et al.*, 2005; Viljoen *et al.*, 2000; Yin *et al.*, 2000b). With such contradictory evidence in mind Mroz *et al.* (1996) developed a new T-cannulation technique which they termed

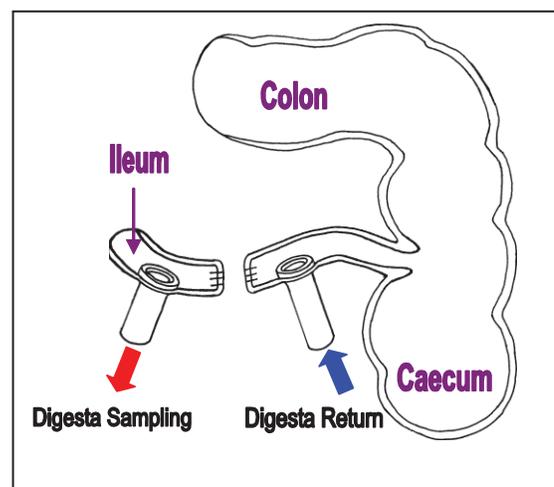
the steered ileo-caecal valve (SICV), cannulation. This technique permits the collection of digesta, with both low and high fibre diets, and maintains the normal physiological function of the gut as it does not require the removal or transection of any part of the GIT. Although this technique was thought to allow the quantitative collection of digesta the recovery of a chromium marker from the faeces of cannulated pigs was approximately 20% less than in intact pigs. Therefore under the conditions of their study the use of an inert marker was deemed advisable for the determination of total tract digestibility and ileal digestibility



of nutrients, as calculating these values from chromium ratios was deemed to be independent of the inevitable losses of feed, digesta and faeces; and resulted in greater precision. Changes proposed by Radcliffe *et al* (2005) resulted in a decrease in post-surgical complications, notably fewer and less severe adhesions, together with less leakage of digesta around the cannulae. The surgery for this technique is more complex than the PVTC method and it appears to be less prone to blockages consequently it offers greater reliability in obtaining a quantitative digesta sample as the ileo-caecal valve is kept in position, inside the lumen of the cannula, by the nylon cord.

VI.1.4.4.1. Simple ileo-ileal re-entrant cannula

Although the use of re-entrant fistulae have been used to study the digestive process in ruminants (Phillipson, 1952), Cunningham *et al.* (1962 and 1963), used a similar re-entrant cannula to compare the digestibility and rate of passage of a variety of purified and natural feed materials in the gut of the pig. Blockages would often occur in the

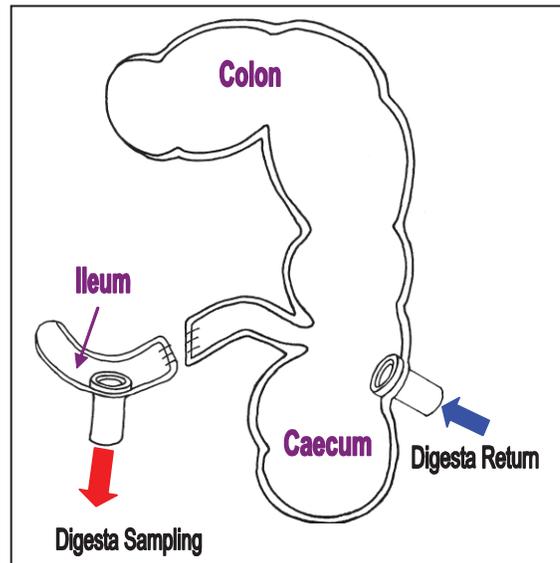


cannulae, particularly if coarsely ground feeds were administered to the animals. However, pigs fitted with this type of fistulae remained healthy some 5 months after the surgery when as many as fifty 12-hour experimental collection periods had been undertaken.

VI.1.4.4.2. Ileo-caecal cannula

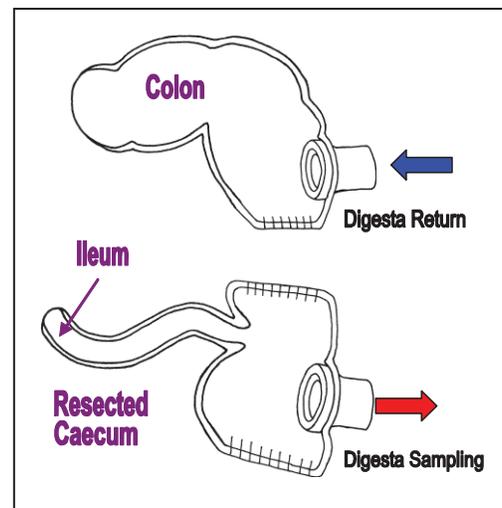
Both Cunningham *et al.* (1963) and later Cho *et al.* (1971) found the flow of digesta through the externally joined ileo-ileal re-entrant cannulae was not uniform. Easter and Tanksley (1972; 1973) believed that the surgically disrupted small intestine was not capable of forcing digesta through the externalised cannulae and the ileo-caecal valve. As the ileo-caecal valve was not essential to the digestion process

(Davenport, 1966) changing the configuration of the re-entrant cannula from ileo-ileal to ileo-caecal offered a bypass to this resistance. Although the use of single caecal cannulae in the digestion studies of pigs had been demonstrated by earlier researchers (Henderickx *et al.*, 1964; Redman *et al.*, 1964) this was the first time that a double, ileo-caecal re-entrant had been used.



VI.1.4.4.3. Ileo-colic post valve fistulation

First described by Darcy *et al.* (1980b), ileo-colic post valve fistulation (ICPV), which they considered to be a reference method, is surgically difficult and too time consuming for routine ileal digestibility measurement. Early suggestions that this method could be used in diets rich in fibre (Sauer and Ozimek, 1986) were recanted by Fuller *et al.* (1994a) who reported evidence to the contrary for diets rich



in barley. Differences between the digestibility values obtained by Fuller *et al.*, comparing the ICPV with simple T-cannulae and ileo-rectal anastomoses, were “somewhat inflated” by having to re-grind the barley based feedstock. They stated that such comparative results might have been closer if the barley rich diet could be administered without regrinding.

VI.1.4.5. Comparative summary of ileal digesta collection techniques.

There is little agreement between researchers as to which digesta collection technique is the best. Variations in determined ileal amino acid digestibility values have been noted due to differences in:

- Collection techniques (Fuller *et al.*, 1994a).
- Basal diets (Viljoen *et al.*, 2000; Yin and McCracken, 1996).
- Dietary fibre (Darcy-Vrillon and Laplace, 1985; Laplace *et al.*, 1985a).
- Laboratories (Radcliffe *et al.*, 2005; Yin and McCracken, 1996).

Indeed Fuller *et al.* (1994a), state that each method has its strengths and limitations such that no single method is suited for all purposes. Such variation is also compounded by the differing performance of indigestible markers necessary to quantify the total flow of digesta (Yin *et al.*, 2000b). A comparative summary of the advantages and disadvantages of the various collection techniques is given in **Tables 10 and 11**

Some digesta collection techniques are not suitable when the fibre content is high. In order to avoid potentially fatal intestinal blockages some researchers have overcome this problem by regrinding the feed to a smaller mesh size (Fuller *et al.*, 1994a). However in that study Fuller stated that as the purpose of feed evaluation is to ascribe nutritional values to dietary components in the same form as they appear in the feed; so if food containing medium to high fibre has to be reground for fear of causing fatal intestinal blockages, then the resulting nutritional information cannot be applied back to the original feedstock.

Table 10 Advantages and disadvantages of the different cannulation techniques.

Ileal digesta collection method	Advantages	Disadvantages
Simple “T” cannula, (Livingstone et al., 1977b)	<ul style="list-style-type: none"> a) Minimal disruption of normal gut activity.⁸ b) Minimal surgical trauma.⁸ c) Appetite and rate of growth unaffected.⁸ 	<ul style="list-style-type: none"> a) Indigestible marker required.⁴ b) Use of indigestible marker is likely to induce errors with heterogeneous digesta.¹ c) Possibility of unrepresentative sampling and marker recovery.^{1,3,4.} d) Cost of surgery.² e) Blockages with high fibre diets.⁵
Post valve T-caecum cannula, (van Leeuwen et al., 1991)	<ul style="list-style-type: none"> a) Semi-quantitative sampling.¹ b) Greater precision and less labour intensive than simple T-cannulation.⁶ c) Greater recovery of indigestible marker.⁴ d) No interference with gut wall, ileo-caecal valve or colon.¹ 	<ul style="list-style-type: none"> a) Involves the removal of gut tissue.⁵ b) High fibre diets cause blockages and interfere with the normal rate of digesta passing through the gut.⁵ c) Variable recovery of digesta confirms the need for an indigestible marker.⁶
Steered ileo-caecal valve, (Mroz et al., 1994)	<ul style="list-style-type: none"> a) Semi-quantitative sampling.⁵ b) Normal flow through the gut when not sampling.⁷ c) Minimal blockages.¹ 	<ul style="list-style-type: none"> a) Invasive, costly and complex surgery.¹ b) Possibility of adhesions causing fatal gut obstructions.⁸
Simple ileo-ileal re-entrant cannula, (Braude et al., 1969; Cunningham et al., 1962)	<ul style="list-style-type: none"> a) Quantitative collection of digesta samples.¹⁰ 	<ul style="list-style-type: none"> a) Assumption of 100% marker recovery incorrect.^{3,9} b) Transection of the gut interrupts migrating myoelectric complex necessary for the normal passage of digesta through the gut.^{6,11}
Ileo-caecal cannula, (Easter and Tanksley, 1973)	<ul style="list-style-type: none"> a) Quantitative collection of digesta samples.¹⁰ b) Placement of the distal cannula in the caecum reduces the number of blockages.¹⁰ 	<ul style="list-style-type: none"> a) Assumption of 100% marker recovery incorrect.^{3,9} b) Inhibition of the myoelectric complex.^{6,11}
Ileo-colic post valve fistulation, (Darcy et al., 1980b)	<ul style="list-style-type: none"> a) Fewer blockages.¹² b) Claims to be a reference method.¹² 	<ul style="list-style-type: none"> a) Surgical technique “too difficult and time consuming for routine measurements.¹² b) Inhibition of the myoelectric complex.^{6,11}

1. Yin and McCracken (1996)
2. Donkoh, *et al.* (1994)
3. Köhler *et al.* (1990)
4. van Leeuwen, *et al.* (1991)

5. Mroz, *et al.* (1996)
6. Yin, *et al.* (2000b)
7. Radcliffe, *et al.* (2005)
8. Livingstone, *et al.* (1977b)

9. Fuller, *et al.* (1994a)
10. Easter and Tanksley (1973)
11. Sauer and Ozimek (1986)
12. Darcy, *et al.* (1980a)

Table 11 The advantages and disadvantages of different ileal digesta collection techniques.

Ileal digesta collection method	Advantages	Disadvantages
Cannulation	<ul style="list-style-type: none"> a) Simple T-cannulae cause minimal disturbance to the digestion of protein throughout the digestive tract.⁶ b) Surgery straightforward and less invasive for simple T-cannulae.⁶ c) Less adverse affects upon gut physiology for simple T-cannulae.⁶ 	<ul style="list-style-type: none"> a) Use of a indigestible marker likely to introduce errors with heterogeneous digesta.² b) Re-entrant cannulation reduces gut motility which can cause blockages^{3, 4}. c) Cannot be used for diets rich in fibre unless particle size is reduced.⁹ d) Marker recovery less than 100%.³
Ileorectal anastomosis	<ul style="list-style-type: none"> a) Quantitative digesta collection via the anus.¹ b) Use of an indigestible marker not required.³ c) Minimal blockage and leakage¹. d) No dietary restrictions.¹⁰ 	<ul style="list-style-type: none"> a) Ethically less acceptable, prohibited in the Netherlands.^{5, 8} b) Complex surgery with longer animal recovery time.^{6, 7} c) Colon has no digestive/absorption role, therefore need for electrolyte replacement.⁸ d) Colonic bypass may affect animal physiology which leads to some compensatory adaptation.⁹ e) Increased microbial colonisation in gut.¹ f) Possibility of digesta reflux into colon and microbial fermentation.⁸
Slaughter Technique	<ul style="list-style-type: none"> a) Technique straightforward.⁶ b) Time required for study reduced.⁶ c) Ethically more acceptable.⁶ d) Suitable for small lab animals and birds. e) No dietary restrictions.⁶ 	<ul style="list-style-type: none"> a) Possible sloughing of epithelial tissue, although this can be avoided with the correct technique.⁶ b) Small volume of digesta recovered.⁶ c) Difficulty in pinpointing the optimal time for slaughter of test animals.⁶ d) Requires a frequent feeding regimen.

1. Köhler, *et al.* (1992a)
2. Mroz, *et al.* (1996)
3. Yin, *et al.* (2000b)
4. Sauer and Ozimek (1986)
5. Fuller, *et al.* (1994a)
6. Donkoh, *et al.* (1994)
7. Darcy, *et al.* (1980a)
8. Yin and McCracken (1996)
9. Kohler, *et al.* (1990)
10. Laplace, *et al.* (1994)

Köhler *et al.* (1992a) question whether experimental animals with re-entrant cannulae or ileo-rectal anastomoses (IRA), compared to those with intact gastrointestinal tracts have similar metabolic rates. MacRae *et al.* (1982) found that sheep fitted with re-entrant cannulae had a higher metabolic rate than those of intact animals. With the IRA technique, Köhler argues, an increased metabolic rate would result in a lower live weight gain for the same metabolizable energy intake.

Such differences in live weight gain were reported by Köhler (1992a), with IRA-animals gaining only 47% of the mass gained by intact animals. In contrast, animals fitted with PVTC cannulae gained 93% of that achieved by intact animals.

Using the Diaminopimelic Acid (DAPA), and Volatile Fatty Acid (VFA) levels, as a measure of microbial activity in the gut, Köhler (1992a) found the digesta concentration of VFA in IRA-animals was 3 to 8 times higher than in PVTC-animals and DAPA 1.3 to 2.3 times higher. Higher microbial digestive activity, he argued, might therefore affect the composition of digesta collected.

Although both re-entrant cannulae and ileo-rectal anastomoses are claimed to be quantitative methods of digesta collection, where the inclusion of an indigestible marker is unnecessary, both Kohler *et al.* (1990) and Fuller *et al.* (1994a) reported that when a marker is included less than 100% is recovered for both techniques. This may be a property of the marker itself, [e.g. the recovery of chromic III oxide (Cr_2O_3), appears to be less than that of titanium IV oxide (TiO_2)] or consequent to the digesta collection method used or the diet being studied e.g. (a) the percentage recovery of Cr_2O_3 is less for T-cannulae than for PVTC cannulae and (b) the recovery of Cr_2O_3 is less for diets high in fibre than those with less (Yin *et al.*, 2000b).

In conclusion, the chosen method of digesta collection for the studies reported in this dissertation is the PVTC cannula as it allows the semi-quantitative sampling of digesta (Köhler *et al.*, 1992a) with greater precision and is less labour intensive than simple T-cannulation (Donkoh *et al.*, 1994). There is proportionally a greater recovery of indigestible marker (Sauer and Ozimek, 1986) and there is no interference with the gut wall, ileo-caecal valve or colon (Köhler *et al.*, 1992a). Finally, it appears to interfere less with the determination of microbial activity in the GIT and the composition of the

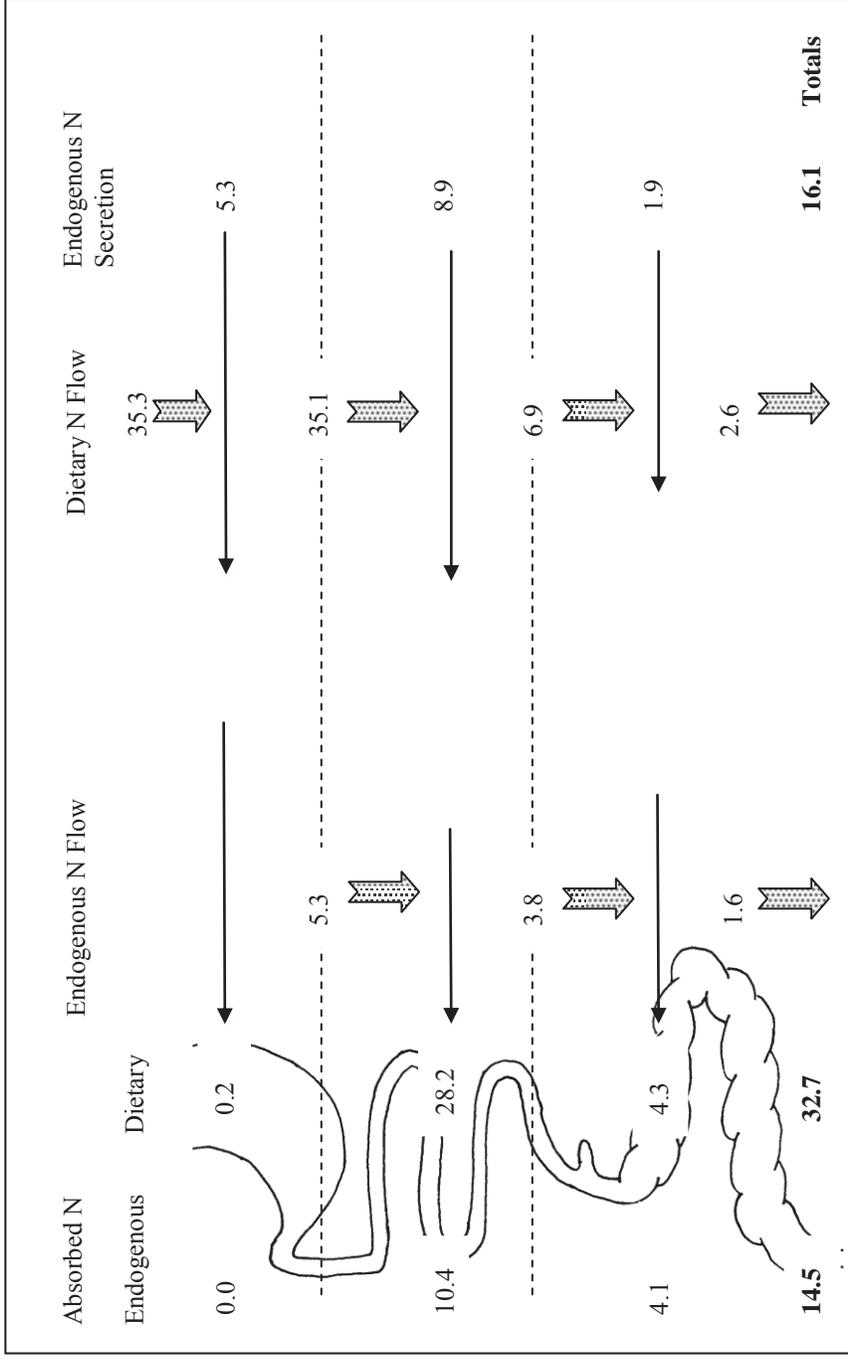
digest collected (Köhler et al., 1992a). The advantages of using the PVTC cannula far outweigh any disadvantages.

VII. Methods for determining gut endogenous protein losses.

The primary sources of endogenous nitrogen are from: salivary and gastric secretions, 0.3-0.6 g per day (Zebrowska et al., 1983); pancreatic secretions, 3-5 g per day (Corring and Saucier, 1972); bile secretions, 2 g per day (Sambrook, 1981); together with secretions from the small intestine and sloughed epithelial cells, 3-5 g per day (Leibholz, 1982). Although not strictly endogenous, microbial protein is commonly included in estimations of endogenous materials and may be the largest single contributor to the nitrogen pool at the terminal ileum (Caine *et al.*, 1999). The mass of endogenous proteinaceous material secreted into the gut is estimated to equal that of ingested dietary protein (Nasset and Ju, 1961). It has long been realised that the mass of nitrogenous materials leaving the ileum represents the net balance between nitrogen intake and secretion minus the absorption of dietary nitrogen and reabsorption of endogenous nitrogen (Moughan, 2003). Most of the endogenous amino acids and nitrogen are reabsorbed before the digesta leave the small intestine (Souffrant, 1991) and enter the large intestine where they may be metabolised by microbes or excreted unaltered in the faeces. Knowledge of the composition of digesta at the terminal ileum of humans is therefore important to enable an understanding of the dynamic flux of dietary protein intake and the secretion of endogenous proteinaceous material into the lumen together with the concomitant absorption of both exogenous and endogenous materials from the GIT. The nitrogen flux within the gastrointestinal system is illustrated in **Figure 16**.

It is important to accurately quantify the endogenous protein, amino acids and nitrogen lost from the digestive tract as they are an important component of the protein requirements of both animals and humans (Nyachoti *et al.*, 1997b; Tamminga *et al.*, 1995) and allow the factorial estimation of dietary protein requirements. Quantification of these losses allows the determination of true digestibility coefficients for application in describing the protein quality of foods.

Figure 16 The typical movement of nitrogen through three sections of the GIT.



Modified from Fuller and Reeds (1998) The flow, secretion and absorption of nitrogen is given as grams of nitrogen per day for a 30kg pig, estimated by ¹⁵N labelling and digesta exchange. (Krawielitzki *et al.*, 1994; Krawielitzki *et al.*, 1990)

VII.1. The protein-free diet

A traditional method for the determination of endogenous ileal nitrogen and amino acid flows has involved feeding humans or animals a diet devoid of protein. Under these conditions all of the amino acids present in the digesta at the terminal ileum must be of endogenous origin (Carlson and Bayley, 1970). However, this approach is considered to be unphysiological (Low, 1980) and will induce a negative body nitrogen balance (Darragh and Hodgkinson, 2000). Protein-free feeding has also been shown to result in a general decrease in the rate of protein synthesis both in the body and the gut (Millward *et al.*, 1976; Muramatsu, 1990), which may lead to a reduction of cell replication and cell protein turnover in the GIT (Millward *et al.*, 1976; Munro and Goldberg, 1964; Muramatsu, 1990; Simon 1989). Added to which there may be a reduction in the amount of protein secreted into the gut (Buraczewska, 1979; Corring and Saucier, 1972; Fauconneau and Michel, 1970; Hodgkinson *et al.*, 2000a; Rodriguez *et al.*, 1982; Schneeman, 1982; Snook and Meyer, 1964). Thus diets with low or no protein may lead to a decrease in both the volume and the composition of the gastrointestinal secretions which results in diminished endogenous protein losses. The metabolic effects due to protein-free feeding will be exacerbated the longer the duration of protein deficiency (Moughan *et al.*, 1998). The high concentration of proline in the endogenous protein losses of pigs fed a protein-free diet has been reported in the literature (De Lange *et al.*, 1989; Hodgkinson *et al.*, 2000a; Leterme *et al.*, 1996a) and appears to be the result of free proline secreted into the lumen of the GIT resulting from insufficient daily protein intake (Pedersen *et al.*, 2002). Therefore the evidence indicates that the determination of endogenous protein losses when administering a protein-free diet is untenable or misleading at best.

VII.2. Synthetic amino acid diet

To avoid the major criticisms of the protein-free diet method (i.e. the effect of a negative body nitrogen balance with the inevitable metabolic adaptation) Moughan, *et al.* (1998), researchers in New Zealand, developed the method of feeding experimental animals diets that contain a mixture of crystalline amino acids as the only source of nitrogen (Butts *et al.*, 1993; Darragh *et al.*, 1990; Skilton *et al.*, 1988). If certain

dietary amino acids are omitted from the diet and administered intravenously, then the endogenous amino acid losses for the omitted amino acid, as it appears in ileal digesta, can be determined directly and unambiguously (Moughan et al., 1998). Results from this technique highlighted once more that for proline and glycine the endogenous flows were substantially higher for the protein-free diet. In addition, this technique demonstrated that endogenous ileal amino acid flows, for the remaining amino acids, were very similar to those found for the protein-free diet; (Butts *et al.*, 1993; Darragh et al., 1990) thus providing unequivocal evidence that such diminished endogenous losses are not caused by a negative body nitrogen balance. These results supported the protein-free diet method, but the possibility remains that dietary protein or peptide secretagogues lead to higher endogenous losses (Moughan et al., 1998).

A variation to this technique is the combination of a protein-free diet supplemented by simultaneous intravenous amino acid infusion (De Lange et al., 1989; Leterme *et al.*, 1996a). Both of these studies confirmed that, above all other amino acids, proline endogenous losses were significantly higher, and that negative body nitrogen balance *per se* does not cause lowered ileal amino acid losses.

Evidence that protein and/or peptide secretagogues stimulate gastrointestinal secretions is provided by another innovative technique developed by the New Zealand group. Zein, a purified maize protein, is devoid of lysine and so the endogenous losses of this amino acid can be quantified directly if zein based diets are fed to experimental animals when accompanied by supplementary intravenous lysine infusion (Butts *et al.*, 1993). In their study, Butts *et al.* (1993) found that the endogenous lysine flows were significantly higher than when compared to the protein-free controls. Given that zein contains no anti-nutritional factors or fibre their study provides corroborative evidence that protein and/or its degradative products affect endogenous ileal amino acid losses.

VII.3.Linear regression

If experimental animals are fed increasing amounts of dietary protein, while keeping the intake of food dry matter constant, then the trend line of endogenous loss extrapolated back to zero dietary protein intake gives an estimate of endogenous

nitrogen loss. It was postulated that the ileal endogenous losses could be determined under normal conditions of protein alimentation using this approach (Furuya and Kaji, 1989; Furuya and Kaji, 1986). Moughan *et al.* (1998) have pointed out a number of drawbacks with this approach; primarily that the resulting endogenous ileal amino acid flows are constrained by the algorithms used, and that the linearity of response together with the constancy in endogenous amino acid loss, at differing quantities of dietary protein intake, is assumed. Although the studies of Fan *et al.* (1995) found statistically significant linear relationships ($P < 0.001$) between the dietary contents of apparent ileal digestible and total amino acids; irrespective of differences in the ranges of different graded dietary levels of amino acids, not all researchers have reported such definite linearity (Moughan *et al.*, 1998). Unless a wide range of dietary protein intakes is applied (4% – 24%) the standard errors are excessive (Fan *et al.*, 1995) especially at lower dietary protein intake levels when enhanced losses of proline and glycine can be anticipated. Because of the requirement for testing such a large range of dietary protein intake levels this method is inappropriate for the study of human endogenous ileal amino acid flows (Deglaire, 2008). Finally as the endogenous ileal amino acid flows between the regression technique and that of the protein-free technique have been found to be similar (Furuya and Kaji, 1989), and that they are significantly lower than those of other techniques using protein and/or peptide based diets (Donkoh *et al.*, 1995), the validity of regression estimates has been called into question. Mathematical error is inherent in the extrapolation beyond the data recorded (Moughan *et al.*, 1998).

VII.4.Homoarginine

This method first demonstrated by Hagemester and Erbersdobler in (1985) involves treating dietary protein with o-methyl-isourea to transform lysine to homoarginine, a derivative of lysine that does not occur naturally in the body tissue as it is not a substrate for protein synthesis (Moughan *et al.*, 1998). The homoarginine method can be applied in two ways:

- **Partial guanidination, the indirect method.** When dietary protein is partially guanidinated both the homoarginine and lysine present in the ileal digesta may

be quantified and the endogenous lysine flow can be calculated (Hagemeister and Erbersdobler 1985; Schmitz *et al.*, 1991).

- **Complete guanidination, the direct method.** Homoarginine does not occur naturally in proteins and therefore if all of the dietary lysine has been guanidinated any homoarginine detected in the digesta at the terminal ileum must be of dietary origin and any lysine must therefore be endogenous (Moughan and Rutherfurd, 1990b).

In the partial guanidination method the excretion of endogenous lysine is estimated by the difference between the true and apparent coefficients of homoarginine and lysine absorption, respectively (Rutherfurd and Moughan 1990). Relying on differences, small errors in the determination of either lysine or homoarginine can produce larger errors in the estimation of endogenous lysine flows.

Guanidination has been applied to a multitude of foodstuffs such as casein (Moughan and Rutherfurd, 1990b; Nyachoti *et al.*, 1997b) cotton seed, fish meal, soybean meal (Ravindran *et al.*, 1996) together with barley and canola meal (Nyachoti *et al.*, 1997a). However, the complete conversion of lysine to homoarginine may not be achieved for many proteins (Maga 1981; Rutherfurd and Moughan 1990). Gelatine, which has a low lysine content, has been used successfully as it can be guanidinated to near-complete (95%) conversion (Rutherfurd and Moughan 1990).

The method is dependent upon a number of assumptions, [discussed at length in Moughan *et al.* (1998)] which have by and large been shown to be tenable, especially for purified proteins (Hodgkinson and Moughan, 2000). The main advantage of this method is that it allows the determination of ileal endogenous losses during the feeding of dietary protein and that it can be applied to many sources of protein. However, the disqualifying disadvantages are that it can only be applied to the determination of lysine endogenous losses; whereas the endogenous losses of other amino acids must be calculated using nitrogen or amino acid:lysine ratios, an assumption which has been shown to be invalid (Deglaire, 2008). In addition the method may only be applied for short time intervals as the absorbed homoarginine, which is toxic, is not metabolised

and accumulates in the liver of the experimental animal. For this reason it cannot be used in human studies (Deglaire, 2008; Hodgkinson and Moughan, 2000).

VII.5. Enzyme hydrolysed protein

The enzyme hydrolysed protein method, also referred to as the ultrafiltration method or peptide alimentation method, was first proposed by Moughan *et al.* (1990a) and allows the simultaneous determination of both endogenous nitrogen and amino acid flows when the test animals are fed a diet containing peptides and amino acids as the only source of nitrogen. The dietary mixture contains peptides (none larger than 5kDa), and amino acids from hydrolysed casein that simulate the natural products of protein digestion. Enzyme hydrolysed casein is almost completely digested by the growing pig (Kies *et al.*, 1986; Moughan and Smith, 1984, 1985). Digesta collected from the test animal are centrifuged before being ultrafiltered to remove any compounds smaller than a filtration cut-off of 10kDa; thus removing any unabsorbed dietary amino acids and peptides. The retentate (>10kDa fraction of digesta) is added to the precipitate from the centrifugation step and this material is used for the determination of endogenous nitrogen and amino acids. The enzyme hydrolysed protein method has been applied to determine gut endogenous nitrogen losses in several species of simple-stomached animals including: the rat (Butts *et al.*, 1991; Darragh *et al.*, 1990); the pig (Butts *et al.*, 1993; Hodgkinson and Moughan, 2001; Moughan *et al.*, 1992); the cat (Hendriks *et al.*, 1996); the dog (Hendriks *et al.*, 2002); the chicken (Cowieson and Ravindran, 2007; Ravindran and Hendriks, 2004); and humans (Deglaire *et al.*, 2007; Moughan *et al.*, 2005b).

One of the main advantages of this method over other methods is that it allows the determination of total nitrogen, and all amino acids, directly, in experimental animals with a positive body nitrogen balance (Moughan *et al.*, 1998).

However, in addition to unabsorbed dietary peptides and amino acids the filtrate (< 10kDa) may also contain small peptides and free amino acids of endogenous origin, and not accounting for this may lead to an underestimation of endogenous nitrogen and amino acid flows. The amount of endogenous molecules < 10kDa has been estimated

by ultrafiltering digesta from animals fed a protein-free diet, where all protein, peptides and amino acids are endogenous. In the pig Moughan and Schuttert (1991) found that only 14% of the total nitrogen was from molecules that were < 10kDa. However, in the rat, Butts *et al.* (1992) found the percentage nitrogen from molecules less than 10kDa to be 33%. In another study Leterme *et al.* (1996a) found the total nitrogen present in the < 10kDa fraction was between 19% – 27% for pigs, fed pea fibre isolates; however, this percentage may have been overestimated as the pH of the digesta was not lowered sufficiently to prevent autolysis.

To improve the accuracy of the determination of endogenous flows calculated using this method and to reduce any underestimation, high quality ultrafiltration devices with a molecular weight cut-off of 3kDa are now available commercially and have been used by Hodgkinson and Moughan (2003) to estimate the difference in endogenous nitrogen and amino acid flows between the two ultrafiltration cut offs (i.e. 3kDa and 10kDa). They found that the endogenous ileal nitrogen was 17% greater when using < 3kDa ultrafiltration than for the < 10kDa ultrafiltration. The difference in endogenous ileal amino acid flows ranged from 1.7% for arginine and phenylalanine to 24.5% for glutamic acid and 26% for serine, with the mean amino acid increase being 12%. Interestingly serine and glutamic acid are two of the six amino acids present in the highest proportions in the polypeptide core of mucins. Hodgkinson and Moughan (2003) recommended that the molecular weight cut off for this assay be reduced to < 3kDa.

Leterme *et al.* (1996a) also used the 3kDa ultrafiltration cut off for discarding undigested dietary peptides/amino acids and suggested (although not quantified), that even at this low level a significant proportion of endogenous amino acids are discarded. They claimed that the observed increase in amino acid flows, when enzyme hydrolysed casein was fed to their experimental animals, was largely due to the presence of undigested dietary molecules < 1kDa. A flawed argument as these small peptides should have been discarded, if they had indeed used the ultrafiltration technique. They stated that the three main amino acid flow increases were observed for glutamic acid (accounting for one fifth of casein), followed by aspartic acid, and proline, these amino acids are three of the six most prolific amino acids in gastrointestinal mucins; the secretion of which is known to increase when

protein/peptides are present in the diet (Claustre *et al.*, 2002; Hodgkinson *et al.*, 2000b; Lien *et al.*, 1996; Montagne *et al.*, 2004).

In suggesting that the enzyme hydrolysed casein is not absorbed completely they cite Rouanet *et al.* (1990), stating that the activity of amino peptidases in the brush border is lower when a protein is given in a hydrolysed form. However, what Leterme *et al.* (1996a) fail to say is that Rouanet *et al.*, concluded that the hydrolysate, containing di- and tri-peptides, is utilized efficiently in healthy growing rats, although the efficacy is not higher than with intact dietary bovine plasma proteins. But Leterme *et al.* (1996a) miss the point as the dietary peptides do not need to be fully absorbed if they are being discarded in the residue left from ultrafiltration. When using different dietary concentrations of enzyme hydrolysed casein, to study its effect on the endogenous flow of amino acids in broiler chickens Ravindran *et al.* (2009) observed that increasing dietary peptide concentrations increased the flow of endogenous amino acid flow at the terminal ileum in a dose-dependent manner that also caused changes in the composition of endogenous protein. They concluded that such changes in the amino acid profile of endogenous protein may be reflective of changes in the output of one or more of the components of endogenous protein (Ravindran *et al.*, 2009). A conclusion which would seem to corroborate the notion that increasing the quantity of dietary proteins/peptides increases the secretion of gastrointestinal mucins.

Leterme *et al.* (1996a) also dispute the accuracy of ultrafiltration, stating that it is designed for the preparative purification of large proteins and not for the quantitative separations of proteins and peptides. Citing Amicon's Guide to Users, they state that the loss of proteins with a molecular mass of 12.4 kDa typically reaches 15% with the Centriprep-10 (cut-off: 10 kDa). However, the retention and recovery performance of the Centriprep-10 quoted by Millipore (Millipore, 2009) when used to filter the 12.4kDa cytochrome C is 96%; and for the Amicon[®] Ultra (Millipore, 2001) recovery ranges between 93% and 95% (for the Amicon Ultra-15 and Amicon Ultra-4 respectively), suggesting losses of just 5% – 7%, which is somewhat better than the performance cited by Leterme *et al.* (1996a).

Leterme *et al.* (1996a) conclude with the suggestion that nitrogen free diets should be supplemented with the inclusion of:

“40 to 50 g·kg⁻¹ dry matter intake of a highly digestible protein such as casein or egg yolk protein instead of 100 g kg⁻¹ dry matter intake of a hydrolysed form. The digestive secretions would be stimulated, the presence of dietary nitrogen (*sic*) molecules in the ileal digesta would be very limited, the total endogenous amino acid losses would be collected, and this would enable the flow of the endogenous amino acids under normal feeding conditions to be estimated easily and accurately.”

The notion that a whole protein such as casein is more efficiently utilized than its hydrolysate is untenable and contrary to the conclusion of Rouanet *et al.* (1990) discussed above.

When Jansman *et al.* (2002) reviewed different methods of determining the amino acid composition of basal endogenous crude protein in the pig, they reported that in two studies the enzyme hydrolysed casein method produced a higher mean value for the flow of basal endogenous crude protein and amino acids (17.1 ± 0.4 g crude protein per kg dry matter intake) when compared to other methods, including that of eleven studies using casein/wheat gluten. Such uncontrolled cross-study comparisons are fraught with difficulties. Deglaire *et al.* (2008) found no evidence of a heightened ileal endogenous protein loss due to the dietary peptides along the entire digestive tract from the stomach to the colon. Caution however, is still needed, as more research is required to compare directly the effect of feeding intact protein with its hydrolysed oligopeptides products in the same species.

In summary the enzyme hydrolysed protein method, employing ultrafiltration with a molecular mass cut off of 3kDa, may underestimate endogenous ileal nitrogen and amino acid flows, due to the presence of some endogenous amino acids and peptides in the discarded ultrafiltrate, but such an underestimation is suggested likely to be minimal.

VII.6. Isotope dilution

Stable isotopes such as ^{15}N and ^{13}C incorporated into amino acids and proteins can be utilised to determine both endogenous nitrogen and amino acid flows under protein alimentation, based upon the dilution of the isotope in the digesta (Deglaire, 2008). Stable isotopes may be used with both animal and human subjects, and can also be utilised to study the fate of both dietary and endogenous amino acids.

VII.6.1. Labelled endogenous protein

After the prolonged feeding of ^{15}N labelled protein/amino acids to experimental animals the labelled body nitrogen pool reaches equilibrium, after which (assuming minimal recycling of the label), the endogenous ileal nitrogen flow may be determined directly. Investigations into the absorption and secretion of nitrogen-containing endogenous compounds in the pig using ^{15}N labelled foods were first undertaken by Gebhardt *et al.*, in the late 1970's (Gebhardt *et al.*, 1978; Köhler *et al.*, 1978) and later by the intravenous infusion of ^{15}N labelled amino acids such as leucine (De Lange *et al.*, 1992; De Lange *et al.*, 1990; Gabert *et al.*, 1997; Krawielitzki *et al.*, 1996) and by pulse-oral dosage (Steendam *et al.*, 2004). Similar ^{15}N tracer studies in humans soon followed (Gaudichon *et al.*, 1994; Gaudichon *et al.*, 1996; Mahe *et al.*, 1994). Moughan *et al.* (1992b) reported that the dilution factor data within dietary treatments (a protein free diet, a synthetic amino acid diet or an enzyme hydrolysed casein diet) were highly variable, none of the pools examined (plasma free, plasma bound and small intestinal tissue) gave consistently reliable results and could be accepted as a valid precursor pool for the endogenous proteins. Although the plasma free amino acid pool is commonly accepted as a suitable precursor pool, more research is required as to which body pool or pools (e.g. small intestinal mucosa or cell and mucus fractions) should be sampled in order to accurately ascertain the specific activity of the undigested endogenous protein at the terminal ileum.

VII.6.2. Labeled dietary protein

The evolution of ^{15}N labelled fertilizers and salts has enabled researchers to develop a range of labelled proteins. One major advantage of using ^{15}N labelled dietary proteins is the simplicity by which endogenous and exogenous protein, amino acids and nitrogen can be identified, separated and quantified. However, the main disadvantage is the rapid recycling of the labelled nitrogen, as a large proportion of exogenous amino acids are metabolised by the enterocytes (Leterme *et al.*, 1996b; Souffrant *et al.*, 1993). Also, only a limited range of labelled food proteins has been developed.

VII.7. Miscellaneous factors that may affect the determination of endogenous losses.

Other factors able to modulate both the amount and composition of endogenous amino acid losses need to be considered when comparing basal endogenous flows of crude protein and amino acids. Factors that need to be considered include:

- a) The possible stimulating effects of bioactive peptides from hydrolyzed protein on the secretion of endogenous protein compounds into the gut (Boisen and Moughan, 1996; Claustre *et al.*, 2002; Yin *et al.*, 2004).
- b) Dietary fibre (Schulze *et al.*, 1994).
- c) Antinutritional factors such as protease inhibitors (Huisman *et al.*, 1992), lectins (Schulze *et al.*, 1995) and tannins (Jansman *et al.*, 1995).

VIII Estimates of the proteinaceous and nitrogenous components of terminal ileal digesta

Introduction

The nitrogenous flux in the GIT is outlined in Figure 16 and shows the proportions of dietary nitrogen entering the digestive system and the endogenous nitrogen secreted into the lumen together with the proportions of nitrogen absorbed, both dietary exogenous and endogenous. Fuller and Reeds (1998) suggest that the direct measurement of secretory outflow from specific organs fails to address three important questions crucial to understanding the dynamics of nitrogen flux in the GIT:

- What is the total secretion of nitrogenous substances from the mucosa?
- What fraction of the nitrogen flowing through the GIT is endogenous?
- What fraction of endogenous nitrogen is reabsorbed in the small intestine and what fraction enters the colon?

To answer these questions they suggest requires quantitative methods that distinguish endogenous protein in the digesta from undigested dietary protein. Although this cannot be achieved directly it can be undertaken using a variety of indirect methods, the assumptions and limitations of which have important implications for the interpretation of the results (Fuller and Reeds, 1998).

Digesta from the terminal ileum would be expected to contain the following nitrogenous materials:

1. Enzymes, mucus, hormones from salivary glands (Tenovuo, 2002).
2. Enzymes and hormones from the pancreas (Corring and Saucier, 1972).
3. Mucus, proteins and peptides (e.g. glutathione), bilirubin and biliverdin from the gall bladder (Esteller, 2008; Sambrook, 1981).
4. Enzymes, mucus and hormones from gastric and intestinal secretions (Zebrowska *et al.*, 1983).

5. Secreted proteins e.g. immunoglobulins (Alverdy, 1990; Tomasi, 1972) and serum albumin (Rambaud *et al.*, 1981) from the pancreas and mucosa.
6. Urea and ammonia (Fuller and Reeds, 1998).
7. Sloughed cells from the intestinal mucosa (Leibholz, 1982).
8. Bacterial proteins (Caine *et al.*, 1999).

The aim of this section of the review of literature is to briefly review methods used to determine some of these components in ileal digesta and examine the information available as to the contents of some of these material in digesta.

Referring to the list of endogenous nitrogen-containing materials found in the GIT, the following specific groups of nitrogenous components can be expected to be found:

- Total Protein and nitrogen.
- Bacterial protein and nitrogen.
- Mucin.
- Non-specific protein and nitrogen compounds such as; immunoglobulins, digestive enzymes, serum albumin, small protease-resistant peptides and undigested dietary protein fractions.
- Urea and Ammonia.
- DNA, bacterial and mucosal.
- Creatinine.
- Unidentifiable nitrogenous compounds; these would include: free amino acids, RNAs, amines, and the tetrapyrroles, bilirubin and biliverdin.

The nitrogenous materials leaving the ileum represent the net balance between secretion and reabsorption and therefore any unabsorbed proteinaceous materials, both exogenous and endogenous, arriving at the terminal ileum are thought to have no further nutritional value (Wunsche *et al.*, 1979). Although there may be small quantities of amino acids absorbed in the large intestine, such amounts are trivial and of little nutritional benefit to the host (Moughan, 2003).

Losses of endogenous nitrogen and amino acids in ileal effluent have previously been determined by administering a protein-free diet, because any proteinaceous material in the digesta at the ileo-caecal junction is assumed to be from endogenous sources. However, such a diet is physiologically unnatural (Darragh and Hodgkinson, 2000) and has been shown to decrease the rate of protein synthesis in gut tissues (Millward *et al.*, 1976) and of protein secretions into the gut; which leads to the underestimation of endogenous nitrogen losses (Hodgkinson and Moughan, 2003).

To determine endogenous nitrogen losses under conditions of protein alimentation a protein such as casein, which is easily digested and absorbed (Deglaire *et al.*, 2009) can be utilized. Although small amounts of dietary peptides and amino acids may remain unabsorbed at the terminal ileum when casein is fed to an experimental subject/animal, it is anticipated that these will have little bearing on the estimation of endogenous nitrogen containing compounds when measured directly.

Early attempts at quantifying the amounts of protein, peptides and amino acids in digesta were undertaken by Buraczewska (1979), Asche *et al.* (1989) and Moughan *et al.* (1990a). However, these studies have been criticised as precautions were not always undertaken to avoid auto-digestion in the digesta after collection. Moughan and Schuttert (1991) endeavoured to quantify the nitrogen-containing fractions in the terminal ileal digesta of pigs, while Butts *et al.* (1992) quantified the digesta of rats; when both animals were fed a protein-free diet. However, to date there have not been any comprehensive analyses particularly of the amounts of specific proteins in terminal ileal digesta collected from animals or humans under conditions of protein alimentation.

Before discussing what is known concerning the nitrogen-containing components of terminal ileal digesta, it is first necessary know how digesta samples need to be treated in order to prepare samples for chemical analysis, and to discuss methods developed for the determination of components. These are addressed in the following sections.

VIII.1. Digesta preparation

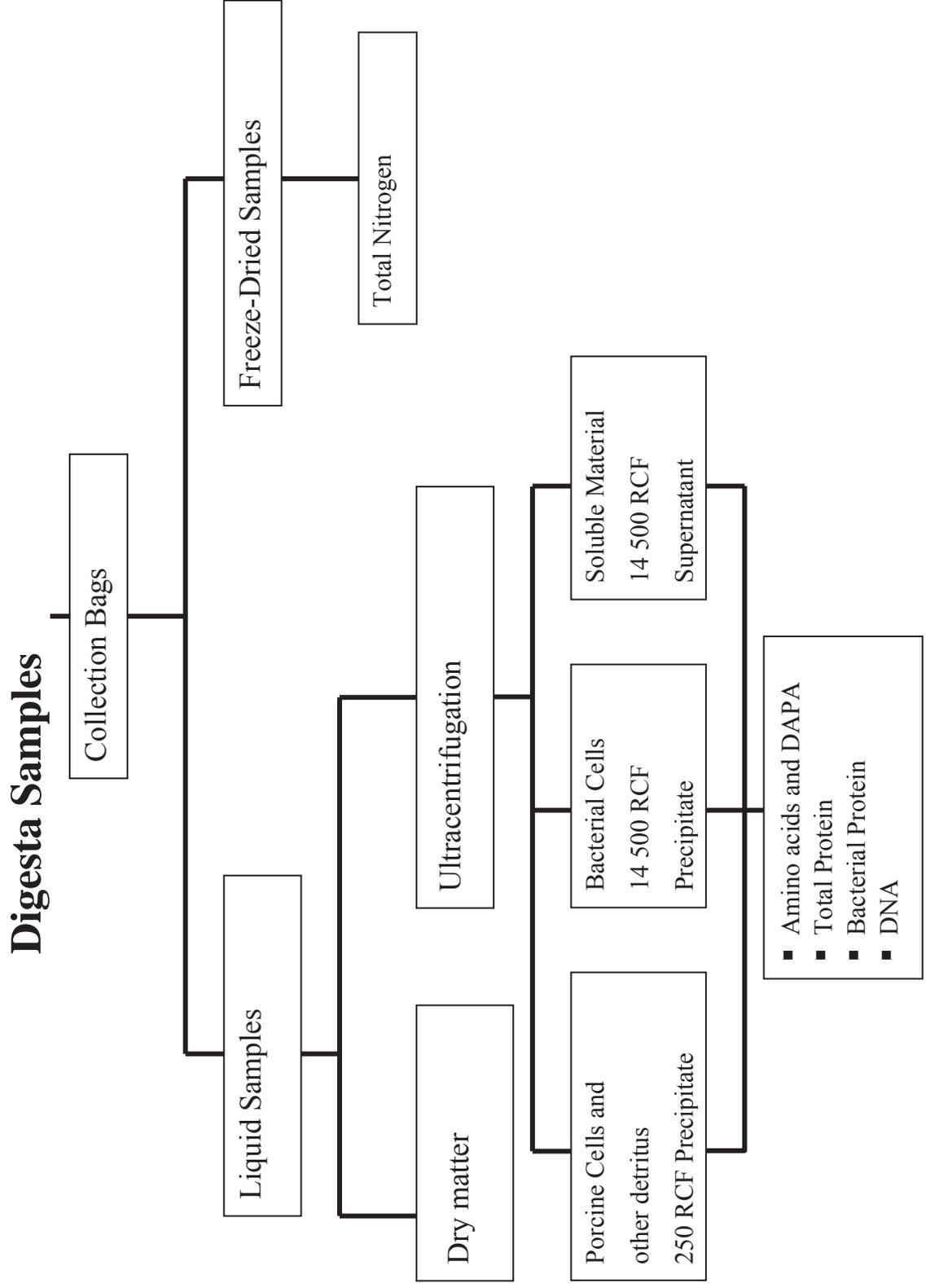
Following the method of Mason (1969), Laplace (1985), developed a centrifugation method for separating bacteria from faecal material in the pig. When researching the incorporation of urea and ammonia nitrogen into ileal microbial proteins in humans Metges *et al.* (1999b) adapted the same differential centrifugation method to separate the digesta components into three distinct fractions. Pooled digesta were centrifuged first at 250 RCF for 15 minutes at 4°C, giving a precipitate that was expected to contain food particles and mucosal cells; further centrifugation at 14 500 RCF for 30 minutes at 4°C, gave a precipitate that was expected to contain only microbial cells, and finally the 14500 RCF supernatant was expected to contain the remaining soluble components of the digesta: proteins, peptides, free amino acids, mucins, neutral sugars, urea, creatinine and ammonia. A summary of the centrifugation protocol is given in **Figure 17**. The differential centrifugation method of Metges *et al.* (1999b) is accepted in the work reported here, as a particularly useful method of treating ileal digesta, to prepare distinct fractions of digesta expected to contain different components.

VIII.2. Determination of mucin and estimates of amounts of mucin in terminal ileal digesta.

Mucin is a major component of endogenous protein losses from the digestive tract, and there are few studies that detail the quantification of gastrointestinal mucin because it is particularly difficult to assay (Piel *et al.*, 2004). Ileal digesta may contain several different types of mucins, originating from different parts of the GIT and the chemical composition of these mucins is known to vary with both their origin and with the animal species (Corfield *et al.*, 2001; Mantle and Allen, 1989). However, in ileal digesta it is expected that mucins originating directly, from the stomach and the small intestine would predominate.

The isolation of mucins often precedes quantification and this has been achieved using a variety of techniques. Mucins can be solubilised using aqueous buffers, such as the buffers used in immuno-precipitation.

Figure 17. Schematic diagram of the processing of the digesta samples.



However, the isolation of mucins is often time consuming; therefore, the inhibition of further degradation using high concentrations of protease inhibitors is essential. One method for mucin isolation was developed by Carlstedt *et al.* (1983) using 6M guanidinium–HCl. This homogenization buffer will solubilise most monomeric mucins (e.g. gastric mucins) effectively. However, it does not solubilise small intestinal and colonic mucins which are oligomeric (Carlstedt *et al.*, 1995). Being a strong denaturing agent, 6M guanidinium–HCl will denature all proteases and glycosidases present in the homogenate and protect the mucins from any further degradation. Oligomeric mucins may be solubilised if a reducing agent is added to the homogenization stage to disrupt any disulphide bridges between the mucin monomers. Gel filtration and density gradient centrifugation allow the isolation of mucins in high concentration guanidinium–HCl buffers. However, one key disadvantage of these procedures is the dilution of the samples (van Klinken *et al.*, 1998a). Concentrating solutions of mucins cannot be achieved effectively by ultrafiltration as the mucins clog the filtration pores. Lyophilising the homogenates is also not appropriate as the freeze-dried mucin samples are then difficult to re-solubilise (van Klinken *et al.*, 1998b). Isopycnic density centrifugation, using caesium chloride density gradients, can isolate mucins which, due to their high glycan content, are buoyant at $1.4 \text{ g}\cdot\text{ml}^{-1}$ (Mantle and Allen, 1981; Starkey *et al.*, 1974). The advantage of this technique is that it effectively concentrates the mucin samples rather than dilutes them as occurs with gel filtration (Tytgat *et al.*, 1996). However, it is worthy of note that both guanidinium chloride and caesium chloride invoke conformational changes to gastric mucoproteins (Snary *et al.*, 1974)

Gravimetric methods of precipitating mucins using high concentrations of ethanol have been undertaken for many years (Dekanski *et al.*, 1975; Tettamanti and Pigman, 1968). However, the specificity of this technique is questionable because digesta contain many types of protein. Ethanol has been used to precipitate a wide variety of different compounds; e.g., α -galactosidase from a fungus (Kotwal *et al.*, 1999); human-immunodeficiency-virus-inhibitory glycoprotein from aqueous extracts of a Caribbean sponge (O'Keefe *et al.*, 1997); and gonadotropins from the pituitary gland of halibut (Weltzien *et al.*, 2003). It is also suggested that high concentrations of ethanol denature protein by dehydration (Voet, 1995), which may interfere with hydrophobic interactions within the mucus gel, causing conformational changes in the secondary

structure of the glycoprotein (Lin *et al.*, 1997). Although the validity of ethanol precipitation, for the determination of human gastric mucin has been accepted by some (Azuumi *et al.*, 1993; Lien *et al.*, 1996), its effectiveness has been questioned by others (O'Keefe *et al.*, 1997; Piel *et al.*, 2004). Juntunen *et al.* (2001) combined the isolation of mucins using ethanol precipitation with the spectrophotometric chromophore alcian blue, a method developed by Hall *et al.* (1980) However, Stanley *et al.* (1983) warn that the heterogeneity of some types of mucin poses a potential problem, as different structural forms may react with the chromophore in different ways. This problem may be compounded by microbial degradation of mucins that occur along the whole length of the GIT, altering the conformational structure of the mucin subunits (Stanley *et al.*, 1986; Variyam and Hoskins, 1981). Interestingly, mucins have also been precipitated using aluminium (Exley, 1998) although the mucoid precipitate is also likely to be degraded.

Although all mucins are sulphated the degree of sulphation varies, making the chemical detection of sulphate esters very difficult and not commonly applied to the quantification of gastrointestinal mucins (Dodgson, 1961; Dodgson and Price, 1962). Several anti-sulphomucin antibodies have been developed, which may aid in the analysis of sulphated mucins (Amerongen *et al.*, 1998). In addition, lectins may be used to detect mucins (Ohara *et al.*, 1997). However, the disadvantage of both lectins and anti-sulphomucin antibodies is that these reagents may not recognize all mucins and especially degraded mucins, as their adhesion depends on the distribution of particular sulphated carbohydrate structures within the mucin matrix (van Klinken *et al.*, 1998b).

In mammals, mucins typically contain the carbohydrates; fucose, galactose, N-acetylglucosamine (GluNAc), N-acetylgalactosamine (GalNAc), sialic acid, and very low amounts of mannose (Forstner and Forstner, 1994). The quantification of mucin in ileal digesta is therefore often undertaken using the carbohydrate markers. The colorimetric analysis of mucins, based upon the histological stain (periodic acid/Schiff PAS) was adapted by Mantle and Allen (1978) from an ultracentrifugation method of (Jabbal *et al.*, 1975). Mantle and Allen's assay, minus the dialysis step featured in the method of Jabbal *et al.* (1975) is claimed to be an easy and sensitive assay for measuring the polysaccharides that are oxidised by periodate, and is particularly useful

at quantifying large numbers of glycoprotein samples obtained from column chromatography or density-gradient centrifugation. Other methods for quantifying glycoprotein by measuring the total hexose content of the digesta using the anthrone method (Seifter *et al.*, 1950) or orcinol method (Weimer and Moshin, 1953) lack sensitivity because of interference from free protein and nucleic acids (Mantle and Allen 1978). Measuring purified glycoproteins using the PAS method, is claimed to be three times more sensitive than the anthrone method and twenty times more sensitive than the orcinol method (Mantle and Allen 1978). As there is considerable variation in the oligosaccharide side chains of different mucins found within the GIT, and between different species, it is uncertain that all the sugar residues would be oxidised by the periodic acid (Clamp *et al.*, 1978). Such differences in composition may therefore affect the sensitivity of the PAS assay. Although the PAS staining of electrophoresis gels or histological specimens containing glycoproteins is a sensitive and widely used procedure, its use in the analysis of mucins in biological fluids does not appear to be common.

Phenol in the presence of sulphuric acid has been used for the quantitative colorimetric determination of sugars and their methyl derivatives, oligosaccharides, and polysaccharides (Dubois *et al.*, 1956). The method is simple and sensitive, and gives reproducible results. The reagent is inexpensive and stable; the colour produced is permanent and no special conditions are required. The Dubois method (cited over 22 000 times) is still in use today (Henare *et al.*, 2012; Lai and Liang, 2012). A scaled down version of the Dubois method developed for the detection of neutral sugars in glycoproteins was described by Beeley (1985). However, like the PAS reaction, this method cannot distinguish between the monosaccharides that are common to glycoproteins and there is no certainty that the extinction coefficients of these sugars are similar.

Sialic acid is a collective term for the acylated derivatives of neuraminic acid. In mammalian glycoproteins, sialic acids occur at the terminal end of the oligosaccharide side chains of the glycoconjugate and give the mucin molecules an electronegative charge. There are many methods available for the analysis of sialic acids, and all have been widely reviewed (Lacomba *et al.*, 2010; Manzi *et al.*, 1990). Such methods can be separated into two categories: those based on separation e.g., chromatography or

electrophoresis (Anumula, 2006), and those performed without separation, which are mostly spectrophotometric techniques (Aminoff, 1961; Schauer, 1982; Warren, 1959). The spectrophotometric methods are fairly straightforward and use relatively inexpensive equipment. However, there are many components that may interfere with the assays, and these can limit their application to complex matrices (Spichtig *et al.*, 2010). Methods based on separation technologies require the release of the sialic acids before analysis, either by enzymatic hydrolysis or acidic hydrolysis. The hydrolysis step is often a balance between complete release and the degradation of the analyte of interest; in most cases the hydrolytic conditions have to be optimized for the particular sample of interest (Spichtig *et al.*, 2010). After release the separation techniques for the analysis of the sialic acids may include; thin layer chromatography, liquid chromatography, gas chromatography, capillary electrophoresis, and mass spectrometry (Lacomba *et al.*, 2010).

Another set of carbohydrate markers of mucin are the hexosamines, GluNAc and GalNAc. The value of GalNAc as a mucin marker lies in its limited occurrence in dietary constituents (Lien *et al.*, 1997b). However, GluNAc is less specific because it is found in some dietary proteoglycans (Roden and Horowitz, 1978). Classic colorimetric assays for amino sugars are based on the method of Elson and Morgan (1933). Modifications include those by: Boas (1953), Ludowieg and Benmaman (1968) and Schloss (1951). However, most modern methodologies involve gas chromatography. Based upon the early method of Sweeley *et al.* (1963), the concentrations of both GlcNAc and GalNAc in ileal digesta have been estimated by gas chromatography using the methods of Combe *et al.* (1980) and Lien *et al.* (1995;1996). The method described by Lien quantifies the amino sugars as alditol acetates by gas-liquid chromatography, a method developed from the procedures of Blakeney *et al.* (1983) and Kraus *et al.* (1990). In ileal effluent, non-mucin glycoproteins, glycolipids and proteoglycans are not important sources of GalNAc as they are present in trace amounts and their GalNAc content is low (Clamp and Gough, 1991). The GluNAc:GalNAc ratio in gastric and intestinal mucin differs considerably (Lien *et al.*, 1997b; Mantle and Allen, 1989; Stanley *et al.*, 1983). Given the difference in the concentrations of the two amino sugars, the relative proportions of gastric and intestinal mucins in ileal digesta can be estimated (Lien *et al.*, 1997b). This is

particularly useful when investigating the effect of dietary components on the flow of glycoprotein in the GIT.

Using very low percentage gels (3%–4% acrylamide), sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting techniques can be used to analyze mucins (Dekker *et al.*, 1991). Using these techniques mucins can be distinguished from non-mucin glycoproteins, and although they have very low mobility they do stain very well with PAS (van Klinken *et al.*, 1998b). Mature mucins were found to separate well on 1% agarose electrophoresis. Increasing the sialylation of the mucins in the sample led to an increased mobility on agarose gel (Thornton *et al.*, 1995) and better resolution of various mature mucins in a single preparation using Western blotting to detect the separated mucins; (Thornton *et al.*, 1996) or by separation of specifically immunoprecipitated mucins (van Klinken *et al.*, 1998). Bolscher *et al.* (1995) also described the use of a density gradient electrophoresis apparatus to separate mucins based on their intrinsic charge. When mucins present at the terminal ileum are expected to be degraded, and may have undergone conformational changes due to microbial degradation, the resolution of such a mixture would not be easy. Further, these techniques do not lend themselves to the quantification of large batches of digesta samples containing a mixture of degraded mucins.

With the development of immunochemistry techniques, e.g. enzyme-linked immunosorbent assays (ELISA), have been devised for the quantification of mucins (Piel *et al.*, 2004). However, an important pitfall of this approach is that changes in the structure of the glycosylation and/or levels of glycan sulphation due to microbial degradation may lead to errors in the quantification of digesta mucins (van Klinken *et al.*, 1998b). Antibodies raised against antigenic peptides representing cysteine-rich sequences, which are not or very sparsely O-glycosylated in the mature mucin, may recognize degraded as well as the fully O-glycosylated mucin (van Klinken *et al.*, 1998a). Piel *et al.* (2004) used ethanol precipitation to concentrate the mucins prior to their ELISA (Lin *et al.*, 1997), and as high concentrations of ethanol are known to denature human gastric mucin, it is possible that such a pre-treatment may have affected their assay.

The mucin output ($\text{g}\cdot\text{day}^{-1}$) in terminal ileal digesta varies greatly with species, method of detection and diet (**Table 12**).

Table 12 Examples of the variation in mucin output ($\text{g}\cdot\text{day}^{-1}$) of terminal ileal digesta as affected by species, method of determination and diet.

Mucin output	Species	Marker	Diet	Reference
15	Human ileostomates	GlcNAc–GalNAc	Mixed	Clamp and Gough, 1991
1.1-33.7	Human ileostomates	GlcNAc–GalNAc	Soya fibre	Lien et al., 1996
1.0-1.3	Pig	GlcNAc–GalNAc	Pea fibre	Lien et al., 1997b
5.3-5.6	Pig	GlcNAc–GalNAc	Protein-free	Lien et al., 1997b
7.7-14.0	Pig	GlcNAc–GalNAc	Barley, legume	Lien <i>et al.</i> , 2001
4.4-5.8	Calf	GlcNAc–GalNAc	Skim milk	Montagne <i>et al.</i> , 2000
2.9-6.1 4.8-10.2 0.9-4.6 1.0-12.6 2.0-3.6 4.7-8.0	Weaned-piglet	GlcNAc–GalNAc	Casein and Chickpea	Piel <i>et al.</i> , 2004
		ELISA		
		Ethanol precipitation		

The composition of both secreted and membrane-bound mucins varies greatly with different tissues along the GIT that synthesize and secrete them. This is particularly so with the carbohydrate content of the oligosaccharide side chains. However, it would be expected that mucins found in the ileal effluent would be a mixture of predominantly gastric and intestinal mucins.

The relative proportions of these two mucins will vary with the type of diet consumed. Lien *et al.* (1997b) have demonstrated that mucin in the ileal digesta from pigs fed a protein-free diet was largely derived (approximately 75%) from the small intestine, whereas mucin found in the ileal digesta of pigs fed a wheat and pea fibre diet contained gastric and intestinal mucin in similar amounts (45%–50% gastric mucin). It is therefore imperative that in any comparative analysis of mucin a normal control must be part of the study. With so many variables affecting the quantities of mucin secreted throughout the GIT it is difficult to compare data from different studies reported in the literature.

VIII.3. Bacterial protein

Bacterial protein is often included in estimations of endogenous proteinaceous materials (Moughan, 2005), and although not strictly endogenous, bacterial protein may be the largest single contributor to the nitrogen content of terminal ileal digesta (Caine *et al.*, 1999), as approximately 50% of the dry mass of bacterial cells is protein (Bremer and Dennis, 1996).

Many methods have been developed for the determination of microbial nitrogen/protein and most methodologies can be characterised into two categories: methods for tracing bacterial protein or those based on the analysis of marker components believed to be uniquely microbial in origin. Methods for tracing the bacterial protein are not really appropriate for quantifying bacterial protein in ileal digesta. Such methods include labelling bacterial protein with ^{15}N (Firkins *et al.*, 1992; Siddons *et al.*, 1985), or ^{35}S (Whitelaw *et al.*, 1984) and bacterial phospholipids with ^{32}P (van Nevel and Demeyer, 1977).

Methodologies best suited to quantify bacterial protein in biological fluids involve the use of bacterial markers. The ideal bacterial marker should:

- be absent in the food;
- not be absorbed by the host;
- be biologically stable;

- have a relatively straightforward assay;
- be exclusively bacterial in origin;
- occur in similar proportions in the different species of bacteria colonizing the GIT;
- be in constant proportions throughout all stages of microbial cell growth;
- flow throughout the GIT at a similar rate in both free and bound forms.

In recent years a number of different bacterial markers have been utilised for the estimation of microbial protein; they include:

- Ribonucleic acids (RNA) (Smith and McAllan, 1970);
- Deoxyribosenucleic acids (DNA) Gaussères and Fauconneau (1965);
- Adenosine triphosphate (ATP) (Forsberg and Lam, 1977);
- 2,6-diaminopimelic acid (DAPA) (Czerkawski, 1974; Masson *et al.*, 1991; Purser and Buechler, 1966; Weller *et al.*, 1958);
- D-alanine (D-Ala) (Garrett *et al.*, 1987; Ueda *et al.*, 1989);
- D-aspartic acid (D-Asp) and D-glutamic acid (D-Glu) (Csapo *et al.*, 2001b).

Using concentrations of DNA in digesta, Gaussères and Fauconneau (1965) estimated the microbial contribution to digesta total nitrogen in ruminants, a method that has been adapted to the digesta of monogastric animals (Rowan *et al.*, 1992). In recent times commercial DNA extraction kits have become available although their use for the quantification of bacterial protein is not common (Johnson *et al.*, 2006; Smith *et al.*, 2003; Thanantong *et al.*, 2006).

Smith and McAllan (1970) proposed using the ratio of RNA to total bacterial nitrogen as a marker of bacterial and protozoan protein synthesis. However their method wrongly assumes that all of the dietary RNA is degraded in the rumen and overestimates the quantity of microbial protein particularly with feedstuffs containing high amounts of nucleic acids or heat-treated proteins (Buttery and Cole, 1977). Derivations of this assay that eliminate much of this error were developed by a number of researchers; including assays that estimate microbial nitrogen using the ratio of purines to bacterial nitrogen (Cecava *et al.*, 1990; Ushida *et al.*, 1985; Zinn and

Owens, 1986). However, Broderick and Merchen (1992) raised concerns about employing only the bacterial purine:N ratios as they may underestimate bacterial and protozoan protein yields. In recent years there has been renewed interest in using purines as markers of microbial protein (Chandrasekharaiah *et al.*, 2010; Reynal *et al.*, 2009; Reynal *et al.*, 2005), including the use of the quantitative polymerase chain reaction (PCR) (Belanche *et al.*, 2010; Sylvester *et al.*, 2004).

Estimating the quantity of ATP was proposed as a marker for microbial nitrogen by Forsberg and Lamm (1977) as the amount of ATP in microbes is very similar. Using bioluminescence, its determination is a relatively simple and reliable procedure. However, great variability was found in the ATP content of similarly prepared samples. Wallace and West (1982) established that the ATP content of microbial biomass varies and they concluded that, although ATP may be an approximate indicator of microbial biomass in digesta, continued hydrolysis of ATP precluded its use for estimating bacterial flow in the abomasum or small intestine. This method is therefore not applicable for the routine quantitative analysis of microbial protein (Csapo *et al.*, 2001a).

Although the cell walls of different bacterial species are structurally diverse they all contain peptidoglycans composed of polysaccharide strands cross-linked to short peptides (Schieber *et al.*, 1999). These short peptides are composed of alternating D- and L-amino acid isomers with a di-amino acid, most commonly D-Asp, D-Glu or DAPA. However, gram-positive bacteria contain between 30%–60% peptidoglycan whereas gram-negative bacteria have only 10% peptidoglycan (Schoenhusen *et al.*, 2008). Despite this, many researchers have quantified these bacterial markers to estimate the amount of bacterial nitrogen and/or protein in fluids taken from the GIT of both humans and animals (Broudiscou and Jouany, 1995; Csapo *et al.*, 2001a; Schonhusen *et al.*, 1995). However, the question of which of these markers is subject to the least error is moot and few can agree.

Work (1950) first reported the occurrence of an, as then unknown, amino acid, 2,6-diaminopimelic acid (DAPA), in a single bacterial species; *Corynebacterium diphtheriae*. Later DAPA was found to be a component of the oligopeptides in bacterial cell walls (Ling, 1990). Although Purser and Buechler (1966) found the

quantity of DAPA present in the cell wall to be strongly dependent on the species and size of bacteria (Dufva *et al.*, 1982; Ling and Buttery, 1978) DAPA:protein ratios of mixed bacteria samples were relatively constant (Czerkawski, 1974). Since it was first suggested as a marker of microbial nitrogen and protein (Synge, 1953), DAPA has become the most commonly used internal marker for estimating bacterial protein (Ling, 1990). The ratios of DAPA to bacterial nitrogen (Wunsche *et al.*, 1991) and to bacterial dry matter (Czerkawski, 1974) are well known and widely used by researchers. The microbial community within the GIT of experimental animals may be diverse but its population of limited species and bacterial density remain relatively stable within an individual for many months (Eckburg *et al.*, 2005; Ley *et al.*, 2006). Despite the number of researchers critical of the use of DAPA as a marker of bacterial nitrogen and protein there are just as many still using DAPA (Huang *et al.*, 2001; Jensen *et al.*, 2006; Karr-Lilienthal *et al.*, 2004; Rubio, 2003).

Horiguchi and Kandatsu (1959) were the first to isolate 2-aminoethylphosphonic acid (AEP) in unicellular protozoa and Ibrahim and Ingalls (1972) were the first to use it to quantify the protein synthesized by protozoa. However, the quantity of AEP varies with the species of protozoa and it can occur in substantial quantities in foods.

D-alanine (D-Ala) was suggested as a marker for bacterial protein by Garrett *et al.* (1987). Like DAPA, D-Ala is found in the peptidoglycan-polysaccharide complexes of the bacterial cell wall (Schleife and Kandler, 1967). However, just like DAPA the concentration of D-Ala in bacterial cells varies according to the cell type and the amount of peptidoglycan in the cells (Quigley and Schwab, 1988). Quigley and Schwab (1988) also found that the bacterial cell protein flow exceeded the total protein flow, a disadvantage for its continued use as a bacterial marker. When comparing DAPA and D-Ala as suitable markers for determining the quantity of bacterial protein Csapo *et al.* (2001b) found no difference between the two markers with respect to error, either in the analytical determination or in the estimation of protein of bacterial origin. However, while some researchers show that D-Ala is an effective bacterial marker (Garrett *et al.*, 1987; Ueda *et al.*, 1989) others claim the reverse (Quigley and Schwab, 1988; Schoenhusen *et al.*, 2008).

The other D-amino acids present in the bacterial cell wall that have been used as markers of bacterial protein are D-aspartic acid (D-Asp) and D-glutamic acid (D-Glu) (Csapo *et al.*, 2001b; Csapo *et al.*, 2002). Csapo *et al.* (2001b) claim that in comparison with the ion exchange column chromatography analysis of DAPA, the analysis of the D-amino acids by high pressure liquid chromatography can be completed quickly and with precision.

Several methods have been developed to determine amino acid enantiomers. Early methods using polarimetry and various enzymatic techniques found little favour with researchers due to their lack of sensitivity in estimating trace amounts of D-amino acids and the error associated with contamination from amino acids in reagent enzymes (Csapo *et al.*, 2001a). Based on a method by Einarsson *et al.* (1987) Csapo *et al.* (2001a) reported a procedure for the separation and determination of chiral amino acids using HPLC after precolumn derivatization with o-phthaldialdehyde and 1-thio-beta-D-glucose tetra-acetate). Using these methods very small quantities of D-amino acids can be detected and quantified alongside L-amino acids present in much larger quantities. A more recent technique was reported by Zahradníčková *et al.* (2007) involves trapping the chiral amino acids on a cation-exchange resin, following their derivatization with pentafluoropropyl chloroformate, and separating the amino acid enantiomers on a Chirasil-Val capillary column, in order that the D- and L-enantiomeric ratios can be determined.

However, a major problem with all determinations of D-amino acids is racemisation occurring during protein hydrolysis; as this will increase the quantities of D-Asp and D-Glu determined and lead to possible overestimation of the amount of bacterial protein. Using their method of hydrolysis, Csapo *et al.* (2001b) claim that only a very low degree of racemisation will take place. However, as the degree of epimerization cannot be quantified this is a source of error that casts doubt on the reliability of using D-Asp and D-Glu as markers of bacterial protein.

D-amino acids have been detected in foodstuffs which undergo bacterial fermentation (Jin *et al.*, 1999; Voss and Galensa, 2000) and food that has aged or undergone thermal treatment (Gandolfi *et al.*, 1994). Also in recent years, the ratio of D- to L-amino acids in the environment has increased as a result of water contamination with chemical

waste, fertilizers, sweeteners, cleansers and other artificial sources containing racemic mixtures of amino acids (Zahradníčková *et al.*, 2007).

Although detailed information on the microbiota that inhabit the mammalian GIT can be gained from the phylogenetic analysis of 16s ribosomal DNA (16s rDNA) this method is not ideal for the quantification of microbial protein, because in addition to having low sensitivity and being inconvenient to use, quantitative PCR is not straightforward. As DNA molecules are amplified during PCR, the initial quantity of the target DNA can only be estimated by assuming that such amplification is reproducible. Because of the exponential nature of this process any disturbance of the amplification efficiency may result in major PCR bias (Felske *et al.*, 1998). As with other microbial markers the 16s rDNA content of bacteria varies with different species and environment (Hopkins and Macfarlane, 2000). Additionally as bacteria become more metabolically active, their demand for protein synthesis increases and ribosome production is upregulated (Hopkins and Macfarlane, 2000). Finally the relationship between growth rate and 16s rDNA may not always be linear (Kaplan and Apirion, 1975).

The hybridization of bacteria with fluorescent probes targeting 16S rRNA coupled with fluorescence microscopy has become an essential technique for the analysis of multi-species bacterial samples (Amann *et al.*, 1995; Harmsen *et al.*, 2002; Sghir *et al.*, 2000). 16S rRNA-hybridization permits the analysis of unlysed bacteria that is impossible with quantitative PCR- methods (Malinen *et al.*, 2002). Probes can be designed for species-specific detection of a single or a large group of related species. It is estimated that just a few probes will detect most intestinal bacteria (Sghir *et al.*, 2000). Fluorescence in situ hybridization (FISH) coupled with general DNA-staining (or using a Eub 338 probe) will supposedly stain all bacterial cells present in the sample. When the DNA-stain and the probe fluoresce at different wavelengths, the proportion of the probe target bacteria in the sample can be calculated (Franks *et al.*, 1998; Harmsen *et al.*, 2002; Harmsen *et al.*, 2000; Langendijk *et al.*, 1995). However, microscopy-FISH techniques are slow and have poor reproducibility (Moore and Holdeman, 1974; Sghir *et al.*, 2000).

Automated flow cytometry methods for the analysis of 16S rRNA-hybridized bacteria have been developed (Fuchs *et al.*, 1998; Simon *et al.*, 1995; Wallner *et al.*, 1995; Wallner *et al.*, 1997) to improve upon the problems associated with microscopy-FISH. Coupled with DNA-staining and compared to hybridization with Eub 338 probe, the method becomes both rapid and reliable. Despite these advantages, only a few studies utilizing FCM, 16S rRNA hybridization and DNA staining in the analysis of intestinal samples have been published so far (Carmen *et al.*, 2007; Teran-Ventura *et al.*, 2010; Vaahtovuori *et al.*, 2007).

Isolating bacteria from the digesta using ultrafiltration (Pellegrino, 2000) or differential centrifugation (Metges *et al.*, 1999b) allows the direct determination of bacterial protein using conventional chemical analysis. As the bacterial isolate contains whole bacterial cells it is necessary to develop a cell lysis and protein solubilisation method that minimizes protein losses. Lysis of bacterial cells has been achieved using a number of techniques: Mickle cell disintegrator, freeze/thaw, sonication, and lysis buffers (Encheva *et al.*, 2006), or detergents (Wijey *et al.*, 2004), and diffusive mixing and dielectrophoretic trapping (Prinz *et al.*, 2002).

Lysis and the extraction of protein from bacterial cells is a critical prerequisite for bacterial protein quantification. Recovery of the protein and the stability of any necessary biological activity must be considered when selecting a lysis and extraction buffer system. A number of proprietary systems are available, e.g. the PE LB™ kit (bacterial protein extraction lysis buffer) from G-Biosciences, St Louis, MO, USA.

Souffrant (1991) has suggested that bacterial protein may be the largest single contributor to endogenous protein at the terminal ileum. In non-ruminants the microbial nitrogen has been reported to contribute between 2.5 to 55% of the ileal endogenous nitrogen (**Table 13**). The great variation in bacterial nitrogen values reported cannot be easily explained. Even allowing for differences in the animals studied, the methodology used to quantify bacterial nitrogen (and protein), together with the different experimental diets no consensus on the range of bacterial nitrogen present in the intestinal lumen can be found in the literature. For example, some researchers have reported that bacterial nitrogen is diet dependent (Wunsche *et al.* (1991) others report the contrary (Kluess *et al.* 2010).

Table 13 The proportion of endogenous nitrogen determined to be of bacterial origin in terminal ileal digesta as affected by species, method of determination and diet.

Percentage	Species	Marker	Reference
55-81*		RNA†	
51-71*	Sheep	³⁵ S	Ling and Buttery (1978)
25-68*		DAPA†	
23-39*	Pig	DAPA	Drochner (1984)
35	Calf	DAPA	Guilloteau <i>et al.</i> (1986)
37-53*	Pig	DAPA	Wunsche <i>et al.</i> (1991)
50	Pig	DAPA	Schulze <i>et al.</i> (1994)
46-55*	Cow	DAPA	
48-55*	(duodenum)	RNA	Robinson <i>et al.</i> (1996)
27	Pig	DAPA	Lien, Sauer, and Dugan (1997a)
17-32*	Pig	DAPA	Leterme <i>et al.</i> (1998)
36-54*	Pig	DAPA	Bartelt <i>et al.</i> (1999)
30-47*	Weaned-piglets	DAPA†	Caine <i>et al.</i> (1999)
14-34*	Pig	DAPA	Huang <i>et al.</i> (2001)
42-46*	Pig	Purine†	
42-55*	Pig	DAPA†	Partanen <i>et al.</i> (2007)
40	Pig	D-Alanine†	Schoenhusen <i>et al.</i> (2008)
35	Cattle	Meta-analysis	Marini <i>et al.</i> (2008)
2.5	Weaned Piglets	D-Alanine	Kluess <i>et al.</i> (2010)

* Variation of bacterial nitrogen ascribed to different experimental diets. † Methods involving the isolation of bacteria from the digesta.

In monogastric animals, the range of ileal bacterial nitrogen most commonly reported by researchers appears to be between 36%–55%, therefore a value of 2.5% reported by Kluess *et al.* (2010) is very unusual. The substantial use of dietary nitrogen by commensal bacteria for the synthesis of microbial protein has been determined in recent studies (Bartelt *et al.*, 1999; Libao-Mercado *et al.*, 2007)

VIII.4 Urea and Ammonia

Urea and ammonia are major components of the non-protein nitrogen fraction of ileal digesta. The maintenance of a correct balance of body N is essential for life. In mammals, the ultimate sources of nitrogen are the dietary amino acids and peptides derived from ingested proteins. Urea is formed from the catabolism of amino acids in the liver as part of the ornithine–urea cycle. Mammals do not possess urease which is necessary to hydrolyse urea, and the majority of this simple waste product is excreted in the urine. However, some of the urea can pass into the GIT, where bacteria can hydrolyse the urea to form ammonia and carbon dioxide. Bacteria utilise the ammonia in the *de novo* synthesis of amino acids and peptides necessary for their own growth.

However, with the lysis of bacterial cells inhabiting the GIT such amino acids and peptides can be reabsorbed and utilised by the mammalian host. This entire process is known as ‘urea nitrogen salvaging’ (Meakins and Jackson, 1996). Traditionally it was believed that as the majority of nutrient absorption occurs in the small intestine and the majority of intestinal bacteria inhabit the caecum and colon, then urea nitrogen salvage had little, if any, role in non-ruminant animals and man (Stewart and Smith, 2005).

The secretion of urea occurs throughout the digestive tract, including saliva, gastric juice, bile and pancreatic juice together with significant quantities directly from the bloodstream (Mosenthin *et al.*, 1992). However, the secretion of urea is more active in the small intestine than in the stomach or large intestine (Bergner *et al.*, 1986; Mosenthin *et al.*, 1992). Urea entering the GIT in pancreatic juice and/or bile (2.4g/day in the growing pig) appears mostly in the proximal small intestine. In vertebrates, urea represents a metabolic cul-de-sac as they do not possess urease, the enzyme necessary to hydrolyse urea.

Therefore the majority of urea utilization in the intestinal lumen is thought to be via commensal bacteria in the small intestine (Fuller and Reeds, 1998). However, there is little consensus as to the fate of urea in the lumen. Although 20%-30% of the urea is hydrolysed (Fouillet *et al.*, 2008; Jackson, 1995; Long *et al.*, 1978) there is little agreement as to the final outcome of the nitrogen released. Whereas Jackson *et al.* (1984) suggest that approximately 80% is retained as amino acids within the body, Long *et al.* (1978) suggest that more than 75% returns rapidly to the urea pool. Uncertainty also surrounds whether control of the body nitrogen balance is achieved via changes in urea production in parallel with protein intake (Young *et al.*, 2000) or by the regulation of urea hydrolysis (Jackson, 1999).

Mosenthin *et al.* (1992) suggested that urea recycling is affected primarily by plasma levels which are concomitantly affected by both the intake and quality of dietary protein. However the mechanisms of urea nitrogen salvage are not fully understood, although it is evident that such processes may be regulated by nutritional demands (Stewart and Smith, 2005). Although little data are available regarding the influence of dietary protein and peptides on urea kinetics (Luiking *et al.*, 2005), they play an important role in nitrogen homeostasis. A study by Fouillet *et al.* (2008) found that although urinary urea excretion was not influenced by the protein source it was influenced by the level of protein in the diet, a conclusion that is in accord with others (Mosenthin *et al.*, 1992; Stewart and Smith, 2005). Likewise, when Columbus *et al.* (2010) calculated urea and ammonia flux, based on isotope dilution, lowering the quantity of dietary protein resulted in a reduction in urea flux and urea recycling ($P < 0.0001$) but there was no effect on ammonia flux ($P > 0.10$).

In conditions of decreased dietary protein, such as with a synthetic amino acid based diet, the amount of urea excreted by the kidney is reduced, circumstances in which Stewart and Smith (2005) suggest urea nitrogen salvage from the gut is maximised, and represents a regulated compensation mechanism within the urea nitrogen salvage process that is sensitive to host nitrogen balance. Such a process would regulate urea secretions, via facilitative urea transporters, from the host into the gut and/or regulate the movement of the products of microbial urea hydrolysis, specifically ammonia, amino acids and peptides, from enteral bacteria back to the host (Stewart and Smith, 2005). In cattle it has been shown that the incorporation of urea into bacterial protein is

inversely proportional to protein intake (Bunting *et al.*, 1989). However, Stewart and Smith (2005) point out that it is still unclear whether this is because less urea enters the gut, or that the amount of urea-nitrogen is being 'diluted' by increased levels of ammonia (derived from dietary nitrogen), and/or that bacteria preferentially use amino acid-nitrogen.

The release of ammonia into the intestinal lumen represents the catabolism and oxidation of amino acids by enterocytes and/or bacteria. Using low protein amino acid supplemented diets in weaned piglets Nyachoti *et al.* (2006) determined that the digesta ammonia concentration decreased as the amount of protein in the diet was reduced and suggested that this represented a reduction in bacterial hydrolysis of nitrogenous dietary components. However bacterial urease is affected by non-competitive product inhibition as many researchers have reported that the enzyme activity is inversely proportional to ammonia concentration (Fidaleo and Lavecchia, 2003; Hoare and Laidler, 1950; Lal *et al.*, 1993). In view of the research by Fouillet *et al.* (2008), if both the numbers of bacteria in the GIT and the percentage nitrogen in the diet are relatively constant, it may be postulated that the concentration of both urea and ammonia in ileal digesta of both monogastric animals and humans would remain the same due to alterations in the quantity of urea secreted into the GIT; an action of the urea nitrogen salvage system.

Moughan and Schuttert (1991) estimated that urea and ammonia in the ileal digesta, of pigs fed a protein-free diet, was 2.9% and 1.1% of the total digesta nitrogen respectively. In ileal digesta of rats fed a protein-free diet, Butts *et al.* (1992) determined the mean proportions of nitrogen contained in urea and ammonium to be 1.8% and 1.5% respectively; a result in line with that of Moughan and Schuttert (1991). In a later study, Butts *et al.* (1993) determined the urinary urea excretion of growing pigs fed two isonitrogenous diets, a synthetic amino acid diet and a protein (zein) diet. They found that protein alimentation increased urea excretion by 63%. Nyachoti *et al.* (2006), and determined that for weaned piglets, plasma urea nitrogen levels were proportional to the percentage protein in the diets. However, in both of the latter studies the proportion of urea and ammonia in the ileal digesta was not determined. The proportions of urea and ammonia in the terminal ileal digesta of healthy humans have not been determined under conditions of protein alimentation.

VIII.5. Total protein

It is useful to be able to determine the total protein content of digesta directly. Many methods for the determination of protein are subject to interference by a number of chemical agents. With the Lowry procedure (Lowry *et al.*, 1951), for example, there is interference from:

- Potassium ions (Vallejo and Lagunas, 1970).
- Magnesium ion (Kuno and Kihara, 1967).
- EDTA (Neurath, 1966).
- Tris (Kuno and Kihara, 1967).
- Thiol reagents (Vallejo and Lagunas, 1970).
- Carbohydrates (Lo and Stelson, 1972).

For the Biuret reaction (Mokrasch and McGilvery, 1956) there is interference from:

- Tris (Robson *et al.*, 1968).
- Ammonia (Gornall *et al.*, 1949).
- Glycerol (Zishka and Nishimur, 1970).

Even the modified Lowry and Biuret assays (Bennett, 1967; Grassmann and Hannig, 1950) present problems (Bradford, 1976). The Orange G dye binding techniques are largely insensitive assays (Ashworth, 1971; Colenbrander and Martin, 1971), and although the Amidoschwarz 10-B binding assay (Schaffner and Weissman, 1973) is effective, the procedure involves both a precipitation step (trichloroacetic acid) and a filtration step.

The Bradford protein assay (Bradford, 1976) eliminates most of the problems listed above, and is easily adapted for the analysis of large numbers of samples. The assay (cited more than 137 000 times) utilises Coomassie Brilliant Blue G-250 which exists in two colour forms, the red form is converted to the blue form as the dye binds to protein (Reisner *et al.*, 1975). The protein-dye complex forms within two minutes, and

is stable in solution for up to an hour. The extinction coefficient is high making the assay sensitive to small quantities of protein.

IX. Conclusion

The primary purpose of the GIT is to undertake the controlled digestion and absorption of essential nutrients from ingested dietary material. Amino acids are essential for nutritional homeostasis in humans and although holistically the net flux of the GIT is absorption, a dynamic equilibrium exists between the secretion of endogenous protein into the gut and the concomitant absorption of both exogenous and endogenous material from the intestinal lumen. A cascade of neural, hormonal, chemical and mechanical stimuli cause salivary, gastric, hepatic, pancreatic and intestinal secretory cells to release endogenous proteinaceous materials into the lumen of the gut. These are vital for the chyme to attain the characteristics necessary for digestion and absorption.

The quantity of endogenous proteinaceous secretions into the gastrointestinal lumen has been estimated to be of equal mass to that of ingested protein. Much of the proteinaceous secreta, together with protein from desquamated cells of the mucosa and commensal bacteria inhabiting the intestinal lumen are digested and reabsorbed before reaching the terminal ileum. What endogenous and exogenous material remains and enters the colon is deemed to be of little nutritional benefit to the host. It is therefore fundamentally important to accurately quantify the endogenous proteins, amino acids and nitrogen that remain at the terminal ileum.

Although the literature is replete with studies that quantify the amino acid and nitrogenous fractions of ileal digesta there is a paucity of information regarding its complete composition. Therefore the aim of this study was to identify and then quantify the nitrogenous component fractions present in digesta collected from the terminal ileum of humans and the pig as an animal model for man.

Based upon the information collated here the method of choice for the collection of digesta in normal human subjects would be via a naso-ileal intubation. However, before any invasive techniques for the collection of digesta from human subjects is undertaken it is proposed that the experimental parameters should first be undertaken using the pig as a model for the human subject. As the naso-ileal intubation of the pig is impractical, ileal digesta would be collected using post-valve T-cecum cannulation, as this method allows the almost complete collection of digesta with only minimal changes to the nutritional metabolism of the pig.

Lactic casein is the protein of choice for the determination of endogenous nitrogen losses as this protein is easily digested and absorbed. Although small amounts of dietary peptides and amino acids may remain unabsorbed at the terminal ileum when casein is fed to an experimental subject/animal, these would have no bearing on the estimation of the endogenous nitrogen-containing compounds, which can be determined directly.

Based upon a critical review of the literature there are considerable gaps in the knowledge concerning gut endogenous protein. In particular:

- What are the principal forms of endogenous protein present in ileal digesta collected from the terminal ileum of an experimental subject/animal, given a diet of protein/peptide diet?
- What are the principal forms of non-protein nitrogen found in the digesta from the terminal ileum?
- Are the proportions of protein and non-protein nitrogen present in terminal ileal digesta affected by the form of dietary nitrogen?
- What proportion of ileal effluent originates from bacteria?
- Is the proportion of bacterial protein present in terminal ileal digesta affected by the form of dietary nitrogen?
- What proportion of bacterial protein in digesta from the terminal ileum, originates from lysed bacterial cells?
- What proportion of endogenous protein originates from intestinal mucins?

- Is the quantity of mucin present in terminal ileal digesta affected by the form of dietary nitrogen?

Before being able to quantify some of these components it is necessary evaluate the various methodologies reported in the literature to ascertain the most appropriate techniques to determine the nitrogenous composition of ileal digesta. Therefore the objectives of this study reported in this dissertation were to:

- a) Determine the best methods to quantify the major nitrogenous compounds collected from ileal digesta.
- b) Identify the major nitrogenous compounds present in digesta collected from the terminal ileum of the pig given a lactic casein diet.
- c) Quantify the principal nitrogenous compounds in terminal ileal digesta collected from adult humans given the purified protein, lactic casein.
- d) Quantify any changes in the composition of endogenous nitrogen containing compounds in the ileal digesta of humans fed a casein-, enzyme hydrolysed casein-, or a synthetic amino acid-based experimental diet (with the same amino acid composition as casein).

X. Literature cited

- Adibi, S. A. (1971). Intestinal transport of dipeptides in man - relative importance of hydrolysis and intact absorption. *Journal of Clinical Investigation*, 50(11), 2266-2275.
- Adibi, S. A. (1997). The oligopeptide transporter (Pept-1) in human intestine: Biology and function. *Gastroenterology*, 113(1), 332-340.
- Adibi, S. A., and Morse, E. L. (1977). Number of glycine residues which limits intact absorption of glycine oligopeptides in human jejunum *Journal of Clinical Investigation*, 60(5), 1008-1016.
- Alderberth, I., Hanson, L. A., and Wold, A. E. (1999). Ontogeny of the intestinal flora. In R. Ogra, J. Mestecky and J. E. A. McGhee (Eds.), *Handbook of Mucosal Immunology* (2nd ed., pp. 279-292). San Diego: Academic Press.
- Allen, A., and Flemstrom, G. (2005). Gastroduodenal mucus bicarbonate barrier: Protection against acid and pepsin. *American Journal of Physiology-Cell Physiology*, 288(1), C1-C19.
- Allescher, H. D., Storr, M., Brechmann, C., Hahn, A., and Schusdziarra, V. (2000). Modulatory effect of endogenous and exogenous opioids on the excitatory reflex pathway of the rat ileum. *Neuropeptides*, 34(1), 62-68.
- Alverdy, J. C. (1990). Effects of glutamine-supplemented diets on immunology of the gut. *Journal of Parenteral and Enteral Nutrition*, 14(4), S109-S113.
- Amann, R. I., Ludwig, W., and Schleifer, K. H. (1995). Phylogenetic identification and in-situ detection of individual microbial-cells without cultivation. *Microbiological Reviews*, 59(1), 143-169.
- Amerongen, A. V. N., Bolscher, J. G. M., Bloemena, E., and Veerman, E. C. I. (1998). Sulfomucins in the human body. *Biological Chemistry*, 379(1), 1-18.
- Aminoff, D. (1961). Methods for Quantitative Estimation of N-Acetylneuraminic Acid and Their Application to Hydrolysates of Sialomucoids. *Biochemical Journal*, 81(2), 384-392.
- Anumula, K. R. (2006). Advances in fluorescence derivatization methods for high-performance liquid chromatographic analysis of glycoprotein carbohydrates. *Analytical Biochemistry*, 350(1), 1-23.

- Asche, G. L., Lewis, A. J., and Peo, E. R. (1989). Protein digestion in weanling pigs - effect of feeding regimen and endogenous protein secretion. *Journal of Nutrition*, 119(8), 1083-1092.
- Ashworth, U. S. (1971). Proteins in meat and egg products determined by dye binding. *Journal of Food Science*, 36(3), 509-510.
- Autenrieth, I. B. (1998). Physiology and pathophysiology of intestinal flora. *Wiener Klinische Wochenschrift*, 110, 16-20.
- Azuumi, Y., Ichikawa, T., Ishihara, K., and Hotta, K. (1993). The Validity of the Ethanol Precipitation Method for the Measurement of Mucin Content in Human Gastric Juices and Its Possible Relationship to Gastroduodenal Diseases. *Clinica Chimica Acta*, 221(1-2), 219-225.
- Badawy, A. M. (1964). Changes in the protein and non-protein nitrogen in the digesta of the sheep. In H. N. Munro (Ed.), *The Role of the Gastrointestinal Tract in Protein Metabolism*. (Vol. 175-185). Oxford: Blackwell.
- Badawy, A. M., Campbell, R. M., Cuthbertson, D. P., and Fell, B. F. (1957). Changes in the intestinal mucosa of the sheep following death by humane killer. *Nature*, 180, 756-757.
- Badawy, A. M., Campbell, R. M., Cuthbertson, D. P., Fell, B. F., and Mackie, W. S. (1958). Further studies on changing composition of the digesta along the alimentary tract of the sheep. Total and nonprotein nitrogen. *British Journal of Nutrition*, 12, 367-383.
- Bajaj-Elliott, M., and Sanderson, I. R. (2004). Structure and function of the gastrointestinal mucosa. In O. Morteau (Ed.), *Oral Tolerance: The Responce of the Intestinal Mucosa to Dietary Antigens* (pp. 1-13). New York: Kluwer Academic / Plenum Publishers.
- Bakalkin, G. Y., Demuth, H. U., and Nyberg, F. (1992). Relationship between Primary Structure and Activity in Exorphins and Endogenous Opioid-Peptides. *Febs Letters*, 310(1), 13-16.
- Baldwin, R. L. (1995). *Modeling ruminant digestion and metabolism*. London: Chapman and Hall.
- Bannink, A., Dijkstra, J., Koopmans, S. J., and Mroz, Z. (2006). Physiology, regulation and multifunctional activity of the gut wall: A rationale for multicompartamental modelling. *Nutrition Research Reviews*, 19(2), 227-253.

- Bansil, R., Cao, X. X., Bhaskar, K. R., and LaMont, J. T. (1996). Gelation and aggregation of mucin in relation to the stomach's protective barrier. *Macromolecular Symposia*, 109, 105-113.
- Bansil, R., Stanley, E., and Lamont, J. T. (1995). Mucin biophysics. *Annual Review of Physiology*, 57, 635-657.
- Barcelo, A., Claustre, J., Moro, F., Chayvialle, J. A., Cuber, J. C., and Plaisancie, P. (2000). Mucin secretion is modulated by luminal factors in the isolated vascularly perfused rat colon. *Gut*, 46(2), 218-224.
- Bartelt, J., Drochner, W., Gotz, K. P., Szakacz, J., Ceresnakova, Z., and Sommer, A. (1999). Determination of endogenous nitrogen associated with bacteria in ileal digesta of pigs receiving cereal-based diets with or without fish meal and various fibre supplements by using a simple N-15-dilution technique. *Journal of Animal and Feed Sciences*, 8(3), 425-440.
- Bazin, H., Andre, C., and Heremans, J. F. (1973). Immunological responses to orally ingested antigens. *Annales D Immunologie*, C124(2), 253-272.
- Beach, R. C., Menzies, I. S., Clayden, G. S., & Scopes, J. W. (1982). Gastrointestinal permeability changes in the preterm neonate. *Archives of Disease in Childhood*, 57(2), 141-145.
- Bedecs, K., Berthold, M., and Bartfai, T. (1995). Galanin-10 years with a neuroendocrine peptide. *International Journal of Biochemistry and Cell Biology*, 27, 337-349.
- Beeley, J. G. (1985). *Glycoprotein and Proteoglycan Techniques* (Vol. 16). Amsterdam: Elsevier Science Publishers B. V.
- Belanche, A., Erroa, I. R., Balcells, J., and Calleja, L. (2010). Use of quantitative real-time PCR to assess the in vitro survival of specific DNA gene sequences of rumen microbes under simulated abomasal conditions. *Journal of Animal Physiology and Animal Nutrition*, 94(2), 204-211.
- Bennett, T. P. (1967). Membrane filtration for determining protein in presence of interfering substances. *Nature*, 213(5081), 1131-1132.
- Bergen, W. G., and Wu, G. Y. (2009). Intestinal nitrogen recycling and utilization in health and disease. *Journal of Nutrition*, 139(5), 821-825.
- Bergner, H., Simon, O., Zebrowska, T., and Munchmeyer, R. (1986). Studies on the secretion of amino-acids and of urea into the gastrointestinal-tract of pigs .3.

- Secretion of urea determined by continuous intravenous-infusion of N-15-urea. *Archiv Fur Tierernahrung-Archives of Animal Nutrition*, 36(6), 479-490.
- Berrocal, R., Chanton, S., Juillerat, M. A., Pavillard, B., Scherz, J. C., & Jost, R. (1989). Tryptic Phosphopeptides from Whole Casein .2. Physicochemical Properties Related to the Solubilization of Calcium. *Journal of Dairy Research*, 56(3), 335-341.
- Beucher, S., Levenez, F., Yvon, M., and Corring, T. (1994). effects of gastric digestive products from casein on cck release by intestinal-cells in rat. *Journal of Nutritional Biochemistry*, 5(12), 578-584.
- Bevins, C. L., Martin-Porter, E., and Ganz, T. (1999). Defensins and innate host defence of the gastrointestinal tract. *Gut*, 271, 14038-14045.
- Bhalla, D. K., and Owen, R. L. (1983). Migration of lymphocyte-B and lymphocyte-a to M-cells in peyers patch follicle epithelium - an autoradiographic and immunocytochemical study in mice. *Cellular Immunology*, 81(1), 105-117.
- Biolo, G., Tessari, P., Inchiostro, S., Bruttomesso, D., Fongher, C., Sabadin, L., Fratton, M.G., Valerio, A., and Tiengo, A. (1992). Leucine and phenylalanine kinetics during mixed meal ingestion - a multiple tracer approach. *American Journal of Physiology*, 262(4), E455-E463.
- Blachier, F., Lancha, A. H., Jr., Boutry, C., and Tome, D. (2010). Alimentary proteins, amino acids and cholesterolaemia. *Amino Acids*, 38(1), 15-22.
- Blachier, F., Mariotti, F., Huneau, J. F., and Tome, D. (2007). Effects of amino acid-derived luminal metabolites on the colonic epithelium and physiopathological consequences. *Amino Acids*, 33(4), 547-562.
- Blakeney, A. B., Harris, P. J., Henry, R. J., and Stone, B. A. (1983). A Simple and Rapid Preparation of Alditol Acetates for Monosaccharide Analysis. *Carbohydrate Research*, 113(2), 291-299.
- Boas, N. F. (1953). Method for the Determination of Hexosamines in Tissues. *Journal of Biological Chemistry*, 204(2), 553-563.
- Boisen, S. (2007). In vitro analyses for predicting standardised ileal digestibility of protein and amino acids in actual batches of feedstuffs and diets for pigs. *Livestock Science*, 109(1-3), 182-185.
- Boisen, S., and Moughan, P. J. (1996). Dietary influences on endogenous ileal protein and amino acid loss in the pig - A review. *Acta Agriculturae Scandinavica Section a-Animal Science*, 46(3), 154-164.

- Bolscher, J., Veerman, E., Amerongen, A. V., Tulp, A., and Verwoerd, D. (1995). Distinct populations of high-M(r) mucins secreted by different human salivary-glands discriminated by density-gradient electrophoresis. *Biochemical Journal*, 309, 801-806.
- Bos, C., Airinei, G., Mariotti, F., Benamouzig, R., Berot, S., Evrard, J., Fenart, E., Tome, D., and Gaudichon, C. (2007). The poor digestibility of rapeseed protein is balanced by its very high metabolic utilization in humans. *Journal of Nutrition*, 137(3), 594-600.
- Bouteloup-Demange, C., Boirie, Y., Dechelotte, P., Gachon, P., and Beaufrere, B. (1998). Gut mucosal protein synthesis in fed and fasted humans. *American Journal of Physiology-Endocrinology and Metabolism*, 274(3), E541-E546.
- Bowen, R. (2005). Control of digestive system function. *The digestive system*. Retrieved 5th November, 2009 from: <http://www.vivo.colostate.edu/hbooks/pathphys/digestion/basics/control.html>
- Bradford, M. M. (1976). Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Analytical Biochemistry*, 72(1-2), 248-254.
- Brandsch, M., and Brandsch, C. (2003). Intestinal transport of amino acids, peptides and proteins. In W. B. Souffrant and C. C. Metges (Eds.), *Progress in Research on Energy and Protein Metabolism* (pp. 667-680).
- Brandsch, M., Brust, P., Neubert, K., and Ermisch, A. (1994). Evidence for a functional significance of natural α -casomorphins. β -casomorphins: Chemical signals of intestinal transport systems. In V. Brantl and H. Teschemacher (Eds.), *β -Casomorphins and Related Peptides: Recent Developments*. (pp. 207-219): VCH-Weinheim,.
- Brantl, V., Teschemacher, H., Henschen, A., and Lottspeich, F. (1979). Novel opioid peptides derived from casein (beta-casomorphins). 1. Isolation from bovine casein peptone. *Hoppe-Seylers Zeitschrift Fur Physiologische Chemie*, 360(9), 1211-1216.
- Braude, R., Buttle, H. L., Horszcza, F., and Myres, A. W. (1969). Preliminary communication on studies of digestive processes in pigs with intestinal cannulas. *Proceedings of the Nutrition Society*, 28(2), A45.
- Bray, G. A. (2000). Afferent signals regulating food intake. *Proceedings of the Nutrition Society*, 59(4), 676-677.

- Bremer, H., and Dennis, P. P. (1996). Modulation of chemical composition and other parameters of the cell growth rate. In F. C. Neidhardt (Ed.), *Escherichia coli and Salmonella: cellular and molecular biology* (2nd ed., pp. 1553-1568). Washington DC: American Society for Microbiology.
- Britton, R., and Krehbiel, C. (1993). Nutrient metabolism by gut tissues. *Journal of Dairy Science*, 76(7), 2125-2131.
- Broderick, G. A., and Merchen, N. R. (1992). Markers for quantifying microbial protein-synthesis in the rumen. *Journal of Dairy Science*, 75(9), 2618-2632.
- Broudiscou, L., and Jouany, J. P. (1995). Reassessing the manipulation of protein-synthesis by rumen microbes. *Reproduction Nutrition Development*, 35(5), 517-535.
- Bunting, L. D., Boling, J. A., and Mackown, C. T. (1989). Effect of dietary-protein level on nitrogen-metabolism in the growing bovine .1. nitrogen recycling and Intestinal Protein Supply in Calves. *Journal of Animal Science*, 67(3), 810-819.
- Buraczewska, L. (1979). Secretion of nitrogenous compounds into the small intestine of pigs. In A. G. Low and I. G. Partridge (Eds.), *Current Concepts of Digestion and Absorption in Pigs* (pp. 151-156). Ayr: The National Institute for Research in Dairying and Hannah Research Institute.
- Buraczewska, L., Buraczewski, S., and Zebrowska, T. (1975). Digestion and absorption in the small intestine of pigs. 2. Amino acid content in digesta and their absorption. *Roczniki Nauk Rolniczych: Zootech.*, 97B, 103-115.
- Buraczewski, S., Porter, J. W. G., Rolls, B. A., and Zebrowska, T. (1971). The course of digestion of different food proteins in the rat. 2. The effect of feeding carbohydrates with proteins. *British Journal of Nutrition*, 25, 299-306.
- Burks, T. F., Hirning, L. D., Galligan, J. J., and Davis, T. P. (1982). Motility effects of opioid-peptides in dog intestine. *Life Sciences*, 31(20-2), 2237-2240.
- Burrin, D. G., Stoll, B., Jiang, R. H., Chang, X. Y., Hartmann, B., Holst, J. J., Greeley, G. H., and Reeds, P. J. (2000). Minimal enteral nutrient requirements for intestinal growth in neonatal piglets: How much is enough? *American Journal of Clinical Nutrition*, 71(6), 1603-1610.
- Burston, D., Addison, J. M., and Matthews, D. M. (1972). Uptake of dipeptides containing basic and acidic amino-acids by rat small-intestine in-vitro. *Clinical Science*, 43(6), 823-837.

- Buttery, P. J., and Cole, D. J. A. (1977). Chemical-analysis - sources of error. *Proceedings of the Nutrition Society*, 36(2), 211-218.
- Butts, C. A., Moughan, P. J., and Smith, W. C. (1991). Endogenous amino-acid flow at the terminal ileum of the rat determined under conditions of peptide alimentation. *Journal of the Science of Food and Agriculture*, 55(2), 175-187.
- Butts, C. A., Moughan, P. J., and Smith, W. C. (1992). Protein nitrogen, peptide nitrogen and free amino-acid nitrogen in endogenous digesta nitrogen at the terminal ileum of the rat. *Journal of the Science of Food and Agriculture*, 59(3), 291-298.
- Butts, C. A., Moughan, P. J., Smith, W. C., and Carr, D. H. (1993). Endogenous lysine and other amino-acid flows at the terminal ileum of the growing pig (20 Kg bodyweight) - the effect of protein-free, synthetic amino-acid, peptide and protein alimentation. *Journal of the Science of Food and Agriculture*, 61(1), 31-40.
- Butts, C. A., Moughan, P. J., Smith, W. C., Reynolds, G. W., and Garrick, D. J. (1993). The effect of food dry-matter intake on endogenous ileal amino-acid excretion determined under peptide alimentation in the 50 kg liveweight pig. *Journal of the Science of Food and Agriculture*, 62(3), 235-243.
- Caine, W. R., Tamminga, S., Sauer, W. C., Verstegen, M. W. A., and Schulze, H. (1999). Bacterial contributions to total and endogenous recoveries of nitrogen and amino acids in ileal digests of newly weaned piglets fed protease-treated soybean meal. *Livestock Production Science*, 57(2), 147-157.
- Carlson, K. H., and Bayley, H. S. (1970). Nitrogen and amino acids in faeces of young pigs receiving a protein-free diet and diets containing graded levels of soybean oil meal or casein. *Journal of Nutrition*, 100(11), 1353-1362.
- Carlstedt, I., Herrmann, A., Hovenberg, H., Lindell, G., Nordman, H., Wickstrom, C., et al. (1995). 'Soluble' and 'insoluble' mucins - Identification of distinct populations. *Biochemical Society Transactions*, 23(4), 845-851.
- Carlstedt, I., Lindgren, H., Sheehan, J. K., Ulmsten, U., and Wingerup, L. (1983). Isolation and characterization of human cervical-mucus glycoproteins. *Biochemical Journal*, 211(1), 13-22.
- Carmen Collado, M., and Sanz, Y. (2007). Quantification of mucosa-adhered microbiota of lambs and calves by the use of culture methods and fluorescent

- in situ hybridization coupled with flow cytometry techniques. *Veterinary Microbiology*, 121(3-4), 299-306.
- Castagna, M., Shayakul, C., Trotti, D., Sacchi, V. F., Harvey, W. R., and Hediger, M. A. (1997). Molecular characteristics of mammalian and insect amino acid transporters: Implications for amino acid homeostasis. *Journal of Experimental Biology*, 200(2), 269-286.
- Cayol, M., Boirie, Y., Rambourdin, F., Prugnaud, J., Gachon, P., Beaufrere, B., and Obled, C. (1997). Influence of protein intake on whole body and splanchnic leucine kinetics in humans. *American Journal of Physiology-Endocrinology and Metabolism*, 272(4), E584-E591.
- Cecava, M. J., Merchen, N. R., Gay, L. C., and Berger, L. L. (1990). Composition of ruminal bacteria harvested from steers as influenced by dietary energy-level, feeding frequency, and isolation techniques. *Journal of Dairy Science*, 73(9), 2480-2488.
- Chabance, B., Jolles, P., Izquierdo, C., Mazoyer, E., Francoual, C., Drouet, L., et al. (1995). Characterization of an antithrombotic peptide from kappa-casein in newborn plasma after milk ingestion. *British Journal of Nutrition*, 73(4), 583-590.
- Chabance, B., Marteau, P., Rambaud, J. C., Migliore-Samour, D., Boynard, M., Perrotin, P., Guillet, R., Jolles, P., and Fiat, A. M. (1998). Casein peptide release and passage to the blood in humans during digestion of milk or yogurt. *Biochimie*, 80(2), 155-165.
- Chacko, A., and Cummings, J. H. (1988). Nitrogen losses from the human small bowel: Obligatory losses and the effect of physical form of food. *Gut*, 29, 809-815.
- Champ, M. M. J., Molis, C., Flourie, B., Bornet, F., Pellier, P., Colonna, P., Galmiche, J. P., and Rambaud, J. C. (1998). Small-intestinal digestion of partially resistant cornstarch in healthy subjects. *American Journal of Clinical Nutrition*, 68(3), 705-710.
- Chandrasekharaiah, M., Thulasi, A., and Sampath, K. T. (2010). Estimation of microbial protein synthesis efficiency in sheep using purine nitrogen index and nitrogen capture efficiency. *Indian Journal of Animal Sciences*, 80(8), 758-763.

- Chang, E. B., and Rao, M. C. (Eds.). (1994). Intestinal water and electrolyte transport. *Mechanisms of physiological and adaptive responses*. (3rd ed.). New York: Raven Press Ltd.
- Chang, K. J., Cuatrecasas, P., Wei, E. T., & Chang, J. K. (1982). Analgesic Activity of Intracerebroventricular Administration of Morphiceptin and Beta-Casomorphins - Correlation with the Morphine (Mu) Receptor-Binding Affinity. *Life Sciences*, 30(18), 1547-1551.
- Chang, K. J., Killian, A., Hazum, E., Cuatrecasas, P., and Chang, J. K. (1981). Morphiceptin (NH₄-Tyr-Pro-Phe-Pro-ConH₂) - a potent and specific agonist for morphine (Mu) receptors. *Science*, 212(4490), 75-77.
- Chen, L. X., Li, P., Wang, J. J., Li, X. L., Gao, H. J., Yin, Y. L., Hou, Y. Q., and Wu, G. Y. (2009). Catabolism of nutritionally essential amino acids in developing porcine enterocytes. *Amino Acids*, 37(1), 143-152.
- Chen, L. X., Yin, Y. L., Jobgen, W. J. S., Jobgen, S. C., Knabe, D. A., Hu, W. X., and Wu, G. Y. (2007). In vitro oxidation of essential amino acids by jejunal mucosal cells of growing pigs. *Livestock Science*, 109(1-3), 19-23.
- Cheng, B., Navab, F., Lis, M. T., Miller, T. N., and Matthews, D. M. (1971). Mechanisms of dipeptide uptake by rat small intestine in-vitro. *Clinical Science*, 40(3), 247-259.
- Cheng, F. Y., Liu, Y. T., Wan, T. C., Lin, L. C., and Sakata, R. (2008). The development of angiotensin I-converting enzyme inhibitor derived from chicken bone protein. *Animal Science Journal*, 79(1), 122-128.
- Cheng, H., and Leblond, C. (1974). Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. *American Journal of Anatomy*, 141, 461-479.
- Cho, C. Y., Horney, F. D., and Bayley, H. S. (1971). Examination of amino acids in ileal digesta and faeces of cannulated pigs. *Federation Proceedings*, 30(2), A235.
- Christl, S. U., and Scheppach, W. (1997). Metabolic consequences of total colectomy. *Scandinavian Journal of Gastroenterology*, 32, 20-24.
- Clamp, J. R., Allen, A., Gibbons, R. A., and Roberts, G. P. (1978). Chemical Aspects of Mucus. *British Medical Bulletin*, 34(1), 25-41.
- Clamp, J. R., and Gough, M. (1991). The glycoconjugate content of human faeces and ileostomy effluents. *Clinica Chimica Acta*, 203(2-3), 243-248.

- Clare, D. A., Catignani, G. L., and Swaisgood, H. E. (2003). Biodefense properties of milk: The role of antimicrobial proteins and peptides. *Current Pharmaceutical Design*, 9(16), 1239-1255.
- Clare, D. A., and Swaisgood, H. E. (2000). Bioactive milk peptides: A prospectus. *Journal of Dairy Science*, 83(6), 1187-1195.
- Claustre, J., Toumi, F., Trompette, A., Jourdan, G., Guignard, H., Chayvialle, J. A., and Plaisancie, P. (2002). Effects of peptides derived from dietary proteins on mucus secretion in rat jejunum. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 283(3), G521-G528.
- Colenbrander, V. F., and Martin, T. G. (1971). Orange-G dye binding for determination of protein of sows milk. *Journal of Dairy Science*, 54(4), 531-533.
- Columbus, D., Cant, J. P., and de Lange, C. F. M. (2010). Estimating fermentative amino acid losses in the upper gut of pigs. *Livestock Science*, 133(1-3), 124-127.
- Combe, E., Patureaumirand, P., Bayle, G., and Pion, R. (1980). Effect of Diet and Microflora on Amino-Sugars in the Digestive Contents and Feces of Rats, Lambs and Preruminant Calves. *Reproduction Nutrition Development*, 20(5B), 1707-1715.
- Cook, L. J., Musa, O. A., and Case, R. M. (1996). Intracellular transport of pancreatic enzymes. *Scandinavian Journal of Gastroenterology*, 31, 1-5.
- Cooke, H. J. (1994). Neuroimmune signaling in regulation of intestinal ion transport. *American Journal of Physiology*, 266, G167-G178.
- Cooke, H. J. (2000). Neurotransmitters in neuronal reflexes regulating intestinal secretion. *Annals of New York Academy of Science*, 915, 77-80.
- Corfield, A. P., Carroll, D., Myerscough, N., and Probert, C. S. J. (2001). Mucins in the gastrointestinal tract in health and disease. *Frontiers in Bioscience*, 6, D1321-D1357.
- Corring, T., and Saucier, R. (1972). Pancreatic-secretion of fistulated pig - adaptation to protein content in diet. *Annales De Biologie Animale Biochimie Biophysique*, 12(2), 233-241.
- Cowieson, A. J., and Ravindran, V. (2007). Effect of phytic acid and microbial phytase on the flow and amino acid composition of endogenous protein at the terminal ileum of growing broiler chickens. *British Journal of Nutrition*, 98(4), 745-752.

- Craft, I. L., Geddes, D., Hyde, C. W., Wise, I. J., and Matthews, D. M. (1968). Absorption and malabsorption of glycine and glycine peptides in man. *Gut*, 9(4), 425-437.
- Crampton, E. W., and Bell, J. M. (1946). The effect of fineness of grinding on the utilisation of oats by market hogs. *Journal of Animal Science*, 5, 200-210.
- Csapo, J., Csapo-Kiss, Z., Schmidt, J., and Martin, T. G. (2001a). Quantitative determination of protein of bacterial origin. *Trac-Trends in Analytical Chemistry*, 20(1), 42-48.
- Csapo, J., Schmidt, J., Csapo-Kiss, Z., Hollo, G., Hollo, I., Wagner, L., et al. (2001b). A new method for the quantitative determination of protein of bacterial origin on the basis of D-aspartic acid and D-glutamic acid content. *Acta Alimentaria*, 30(1), 37-52.
- Csapo, J., Schmidt, J., Csapo-Kiss, Z., Varga-Visi, E., Pohn, G., and Csokona, E. (2002). Quantitative determination of protein of bacterial origin on the basis of D-aspartic acid and D-glutamic acid content. *Chromatographia*, 56, S169-S171.
- Cuff, M. A., Lambert, D. W., and Shirazi-Beechey, S. P. (2002). Substrate-induced regulation of the human colonic monocarboxylate transporter, MCT1. *Journal of Physiology-London*, 539(2), 361-371.
- Cunningham-Rundles, C. (1987). Failure of antigen exclusion. In J. Brostoff and C. J. Chalcombe (Eds.), *Food Allergy and Intolerance*. (pp. 223-236). London: Bailliere Tindall.
- Cunningham, H. M., Friend, D. W., and Nicholson, J. W. G. (1962). Note on a re-entrant fistula for digestion studies with pigs. *Canadian Journal of Animal Science*, 42, 112-113.
- Cunningham, H. M., Friend, D. W., and Nicholson, J. W. G. (1963). Observations on digestion in the pig using a re-entrant intestinal fistula. *Canadian Journal of Animal Science*, 43, 215-225.
- Cuthbert, A. W. (2001). Kinins and epithelial ion transport in the alimentary tract. *Biological Chemistry*, 382, 57-60.
- Czerkawski, J. W. (1974). Methods for determining 2-6-diaminopimelic acid and 2-aminoethylphosphonic acid in gut contents. *Journal of the Science of Food and Agriculture*, 25(1), 45-55.

- Dai, Z. L., Wu, G. Y., and Zhu, W. Y. (2011). Amino acid metabolism in intestinal bacteria: Links between gut ecology and host health. *Frontiers in Bioscience-Landmark*, 16, 1768-1786.
- Dai, Z. L., Zhang, J., Wu, G. Y., and Zhu, W. Y. (2010). Utilization of amino acids by bacteria from the pig small intestine. *Amino Acids*, 39(5), 1201-1215.
- Dalmasso, G., Charrier-Hisamuddin, L., Nguyen, H. T. T., Yan, Y., Sitaraman, S., and Merlin, D. (2008). PepT1-mediated tripeptide KPV uptake reduces intestinal inflammation. *Gastroenterology*, 134(1), 166-178.
- Dangin, M., Boirie, Y., Garcia-Rodenas, C., Gachon, P., Fauquant, J., Callier, P., Balleve, O., and Beaufrere, B. (2001). The digestion rate of protein is an independent regulating factor of postprandial protein retention. *American Journal of Physiology-Endocrinology and Metabolism*, 280(2), E340-E348.
- Daniel, H. (2004). Molecular and integrative physiology of intestinal peptide transport. *Annual Review of Physiology*, 66, 361-384.
- Daniel, H., and Hahn, A. (1990). Beta-casomorphins opioid-peptides derived from milk-proteins. *Ernahrungs-Umschau*, 37(3), 95-101.
- Daniel, H., Vohwinkel, M., and Rehner, G. (1990a). Effect of casein and beta-casomorphins on gastrointestinal motility in rats. *Journal of Nutrition*, 120(3), 252-257.
- Daniel, H., Wessendorf, A., Vohwinkel, M., and Brantl, V. (1990b). Effect of D-Ala 2,4,Tyr5-beta-casomorphin-5-amide on gastrointestinal functions. In . In F. Nyberg and V. Brantl (Eds.), *Beta-Casomorphins and Related Peptides* (pp. 95-104). Uppsala: Fyris-Tryck AB.
- Darcy-Vrillon, B., and Laplace, J. P. (1985). Ileal amino acid digestibility measurement in pigs fed high-fibre diet: Ileo-rectal anastomosis versus ileo-colic post-valve fistulation. In A. Just, H. Jørgensen and L. A. Fernandez (Eds.), *Digestive Physiology in the Pig* (Vol. Report no. 580, pp. 184-187). Copenhagen, Denmark: National Institute of Animal Science.
- Darcy-Vrillon, B., and Laplace, J. P. (1990). Digesta collection procedure may affect ileal digestibility in pigs fed diets based on wheat bran or beet pulp. *Animal Feed Science and Technology*, 27, 307-316.
- Darcy, B., Laplace, J. P., and Villiers, P. A. (1980a). Digestion in the pig small-intestine .2. comparative kinetics of the passage of digesta according to the

- mode of fistulation, ileocecal or post-valvular ileocolic, in various feeding Conditions. *Annales De Zootechnie*, 29(2), 147-177.
- Darcy, B., Laplace, J. P., and Villiers, P. A. (1980b). Collection of digesta flowing into the large-intestine after post-ileocolic valve fistulation - preliminary-results. *Reproduction Nutrition Development*, 20(4B), 1197-1202.
- Darragh, A. J., Cranwell, P. D., and Moughan, P. J. (1994). Absorption of lysine and methionine from the proximal colon of the piglet. *British Journal of Nutrition*, 71(5), 739-752.
- Darragh, A. J., and Hodgkinson, S. M. (2000). Quantifying the digestibility of dietary protein. *Journal of Nutrition*, 130(7), 1850S-1856S.
- Darragh, A. J., and Moughan, P. J. (1995). The 3-week-old piglet as a model animal for studying protein digestion in human infants. *Journal of Pediatric Gastroenterology and Nutrition*, 21(4), 387-393.
- Darragh, A. J., Moughan, P. J., and Smith, W. C. (1990). The effect of amino-acid and peptide alimentation on the determination of endogenous amino-acid flow at the terminal ileum of the rat. *Journal of the Science of Food and Agriculture*, 51(1), 47-56.
- Das, M., and Radhakri, A. (1974). Comparative study of distribution of soluble and particulate glycyl-l-leucine hydrolase in small-intestine. *Clinical Science and Molecular Medicine*, 46(4), 501-510.
- Davenport, H. W. (1966). *Physiology of the Digestive Tract Year Book* (2nd ed.). Chicago, USA: Medical Publishers Inc.
- De Noni, I. (2008). Release of beta-casomorphins 5 and 7 during simulated gastro-intestinal digestion of bovine beta-casein variants and milk-based infant formulas. *Food Chemistry*, 110(4), 897-903.
- De Noni, I., and Cattaneo, S. (2010). Occurrence of beta-casomorphins 5 and 7 in commercial dairy products and in their digests following in vitro simulated gastro-intestinal digestion. *Food Chemistry*, 119(2), 560-566.
- de Vrese, M., Frik, R., Roos, N., and Hagemester, H. (2000). Proteinbound D-amino acids, and to a lesser extent lysinoalanine, decrease true ileal protein digestibility in minipigs as determined with ¹⁵N-labelling. *Journal of Nutrition*, 130, 2026-2031.
- Deglaire, A. (2008). *Gut endogenous protein flows and post prandial metabolic utilization of dietary amino acids in simple stomached animals and humans.*

- Doctor of Philosophy Thesis, Massey University, Palmerston North. New Zealand.
- Deglaire, A., Bos, C., Airinei, G., Luengo, C., Benamouzig, R., Fauquant, J., Gaudichon, C., Moughan, P. J., and Tome, D. (2007). Postprandial metabolic utilization of intact and hydrolyzed casein in humans. *Faseb Journal*, 21(5), A333-A333.
- Deglaire, A., Bos, C., Tomé, D., and Moughan, P. J. (2009). Ileal digestibility of dietary protein in the growing pig and adult human. *British Journal of Nutrition*, 102(12), 1752-1759.
- Deglaire, A., Moughan, P. J., Bos, C., Petzke, K., Rutherfurd, S. M., and Tomé, D. (2008). A casein hydrolysate does not enhance gut endogenous protein flows compared with intact casein when fed to growing rats. *Journal of Nutrition*, 138(3), 556-561.
- Dekanski, J. B., Macdonald, A., Sacra, P., and Parke, D. V. (1975). Effects of Fasting, Stress and Drugs on Gastric Glycoprotein Synthesis in Rat. *British Journal of Pharmacology*, 55(3), 387-392.
- Dekker, J., Aelmans, P. H., and Strous, G. J. (1991). The oligomeric structure of rat and human gastric mucins. *Biochemical Journal*, 277, 423-427.
- Del Valle, J., and Gantz, I. (1997). Novel insights into histamine H2 receptor biology. *American Journal of Physiology*, 273, G987-G996.
- De Lange, C. F. M., Sauer, W. C., Mosenthin, R., and Souffrant, W. B. (1989). The effect of feeding different protein-free diets on the recovery and amino-acid composition of endogenous protein collected from the distal ileum and faeces in pigs. *Journal of Animal Science*, 67(3), 746-754.
- De Lange, C. F. M., Sauer, W. C., Souffrant, W. B., and Lien, K. A. (1992). N-15 leucine and N-15-isoleucine isotope-dilution techniques versus the N-15-isotope dilution technique for determining the recovery of endogenous protein and amino-acids in digesta collected from the distal ileum in pigs. *Journal of Animal Science*, 70(6), 1848-1856.
- De Lange, C. F. M., Souffrant, W. B., and Sauer, W. C. (1990). Real ileal protein and amino-acid digestibilities in feedstuffs for growing pigs as determined with the N-15-isotope dilution technique. *Journal of Animal Science*, 68(2), 409-418.
- Dierick, N. A., Vervaeke, I. J., Decuyper, J. A., van der Heyde, H., and Henderickx, H. K. (1988). Correlation of ileal and fecal digested protein and organic matter

- to production performance in growing pigs. In W. Z. (Ed.) (Vol. 37, pp. 50-51). Rostock, N-Reihe: WPU.
- Dodgson, K. S. (1961). Determination of Inorganic Sulphate in Studies on Enzymic and Non-Enzymic Hydrolysis of Carbohydrate and Other Sulphate Esters. *Biochemical Journal*, 78(2), 312-and.
- Dodgson, K. S., and Price, R. G. (1962). A Note on Determination of Ester Sulphate Content of Sulphated Polysaccharides. *Biochemical Journal*, 84(1), 106-and.
- Donkoh, A., Moughan, P. J., and Morel, P. C. H. (1995). Comparison of methods to determine the endogenous amino-acid flow at the terminal ileum of the growing rat. *Journal of the Science of Food and Agriculture*, 67(3), 359-366.
- Donkoh, A., Moughan, P. J., and Smith, W. C. (1994). Comparison of the slaughter method and simple t-piece cannulation of the terminal ileum for determining ileal amino-acid digestibility in meat and bone meal for the growing pig. *Animal Feed Science and Technology*, 49(1-2), 43-56.
- Drochner, W. (1984). Einfluß wechselnder Rohfaser- und Pektinabgehalte im Futter auf einige präcaecale und postileale Verdauungsvorgänge beim wachsenden Schwein. *Fortschr. Tierphysiol. Tierernähr.*, Heft 14, 1-125.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, 28(3), 350-356.
- Dubois, V., Nedjar-Arroume, N., and Guillochon, D. (2005). Influence of pH on the appearance of active peptides in the course of peptic hydrolysis of bovine haemoglobin. *Preparative Biochemistry and Biotechnology*, 35(2), 85-102.
- Dufva, G. S., Bartley, E. E., Arambel, M. J., Nagaraja, T. G., Dennis, S. M., Galitzer, S. J., et al. (1982). Diaminopimelic Acid Content of Feeds and Rumen Bacteria and Its Usefulness as a Rumen Bacterial Marker. *Journal of Dairy Science*, 65(9), 1754-1759.
- Dugan, M. E. R., Knabe, D. A., and Wu, G. (1994). Glutamine and glucose-metabolism in intraepithelial lymphocytes from pre-weaning and post-weaning pigs. *Comparative Biochemistry and Physiology B-Biochemistry and Molecular Biology*, 109(4), 675-681.
- Easter, R. A., Driedger, A., and Tanksley, T. D. (1972). Technique for reentrant ileocecal cannulation of swine. *Journal of Animal Science*, 35(1), 215-216.

- Easter, R. A., and Tanksley, T. D. (1973). Technique for reentrant ileocecal cannulation of swine. *Journal of Animal Science*, 36(6), 1099-1103.
- Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E., and Relman, D. A. (2005). Diversity of the human intestinal microbial flora. *Science*, 308(5728), 1635-1638.
- Einarsson, S., Folestad, S., and Josefsson, B. (1987). Separation of Amino-Acid Enantiomers Using Precolumn Derivatization with Ortho-Phthalaldehyde and 2,3,4,6-Tetra-O-Acetyl-1-Thio-Beta-Glucopyranoside. *Journal of Liquid Chromatography*, 10(8-9), 1589-1601.
- Ekblad, E., and Sundler, F. (1997). Distinct receptors mediate pituitary adnylate cyclase-activating peptide- and vasoactive intestinal peptide-induced relaxation of rat ileal longitudinal muscle. *European Journal of Pharmacology*, 334, 61–66.
- Elson, L. A., & Morgan, W. T. J. (1933). A colorimetric method for the determination of glucosamine and chondrosamine. *Biochemical Journal*, 27, 1824-1828.
- Encheva, V., Gharbia, S. E., Wait, R., Begum, S., and Shah, H. N. (2006). Comparison of extraction procedures for proteome analysis of *Streptococcus pneumoniae* and a basic reference map. *Proteomics*, 6(11), 3306-3317.
- Erdmann, K., Cheung, B. W. Y., and Schroder, H. (2008). The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *Journal of Nutritional Biochemistry*, 19(10), 643-654.
- Escudero, E., Sentandreu, M. A., Arihara, K., and Toldra, F. (2010). Angiotensin I-converting enzyme inhibitory peptides generated from in vitro gastrointestinal digestion of pork meat. *Journal of Agricultural and Food Chemistry*, 58(5), 2895-2901.
- Esteller, A. (2008). Physiology of bile secretion. *World Journal of Gastroenterology*, 14(37), 5641-5649.
- Exley, C. (1998). The precipitation of mucin by aluminium. *Journal of Inorganic Biochemistry*, 70(3-4), 195-206.
- Fan, M. Z., Sauer, W. C., and McBurney, M. I. (1995). Estimation by regression-analysis of endogenous amino-acid levels in digesta collected from the distal ileum of pigs. *Journal of Animal Science*, 73(8), 2319-2328.
- Fanciulli, G., Dettori, A., Demontis, M. P., Tomasi, P. A., Anania, V., and Delitala, G. (2005). Gluten exorphin B5 stimulates prolactin secretion through opioid

- receptors located outside the blood-brain barrier. *Life Sciences*, 76(15), 1713-1719.
- FAO. (1991). Protein quality evaluation in human diets. Report of a joint FAO/WHO expert consultation. *Food and Agriculture Organization Food Nutrition Paper*(51).
- Farthing, M. J. G. (2000). Enterotoxins and the enteric nervous system: A fatal attraction. *International Journal of Microbiology*, 290, 491-496.
- Fauconneau, G., and Michel, M. C. (1970). The role of the gastrointestinal tract in the regulation of protein metabolism. In J. N. Munro and J. B. Allison (Eds.), *Mammalian protein metabolism*. (pp. 481–522). New York: Academic Press.
- Franklin, R. E., and Gosling, R. G. (1953). Molecular configuration in sodium thyminucleate. *Nature*, 171(4356), 740-741.
- Fell, B. F. (1961). Cell shedding in the epithelium of the intestinal mucosa: Fact and artefact. *Journal of Pathology and Bacteriology*, 81, 251-254.
- Felske, A., Akkermans, A. D. L., and De Vos, W. M. (1998). Quantification of 16S rRNAs in complex bacterial communities by multiple competitive reverse transcription PCR in temperature gradient gel electrophoresis fingerprints. *Applied and Environmental Microbiology*, 64(11), 4581-4587.
- Fiat, A. M., Miglioresamour, D., Jolles, P., Drouet, L., Sollier, C. B. D., and Caen, J. (1993). Biologically-active peptides from milk-proteins with emphasis on 2 examples concerning antithrombotic and immunomodulating activities. *Journal of Dairy Science*, 76(1), 301-310.
- Fidaleo, M., and Lavecchia, R. (2003). Kinetic study of enzymatic urea hydrolysis in the pH range 4-9. *Chemical and Biochemical Engineering Quarterly*, 17(4), 311-318.
- Field, M., and Frizzel, R. A. (1991). Intestinal absorption and secretion. In S. G. Schultz (Ed.), *The Gastrointestinal System* (Vol. 4). Bethesda: American Physiology Society.
- Firkins, J. L., Weiss, W. P., and Piwonka, E. J. (1992). Quantification of intraruminal recycling of microbial nitrogen using N-15. *Journal of Animal Science*, 70(10), 3223-3233.
- Fitzgerald, R. J., and Murray, B. A. (2006). Bioactive peptides and lactic fermentations. *International Journal of Dairy Technology*, 59(2), 118-125.

- Fitzgerald, R. J., Murray, B. A., and Walsh, D. J. (2004). Hypotensive peptides from milk proteins. *Journal of Nutrition*, 134(4), 980S-988S.
- Flamand, M. C., Pelsser, S., Ewbank, E., and Maraite, H. (1996). Production of syringotoxin and other bioactive peptides by *Pseudomonas fuscovaginae*. *Physiological and Molecular Plant Pathology*, 48(4), 217-231.
- Fleming, S. E., and Wasilewski, M. M. (1984). Using the pig as a tool for studying fermentation in the human gut. *Nutrition Reports International*, 30(4), 825-834.
- Florey, H. W., Wright, R. D., and Jennings, M. A. (1941). The secretions of the intestine. *Physiological Reviews*, 21(1), 36-69.
- Foltz, M., Meynen, E. E., Bianco, V., van Platerink, C., Koning, T., and Kloek, J. (2007). Angiotensin I-converting enzyme inhibitory peptides from a lactotripeptide-enriched milk beverage are absorbed intact into the circulation. *Journal of Nutrition*, 137(4), 953-958.
- Fone, D. R., Horowitz, M., Heddle, R., Maddox, A. F., Collins, P. J., Read, N. W., and Dent, J. (1991). Comparative effects of duodenal and ileal intubation on gastric-emptying and postprandial antral, pyloric, and duodenal motility. *Scandinavian Journal of Gastroenterology*, 26(1), 16-22.
- Ford, D., Howard, A., and Hirst, B. H. (2003). Expression of the peptide transporter hPepT1 in human colon: A potential route for colonic protein nitrogen and drug absorption. *Histochemistry and Cell Biology*, 119(1), 37-43.
- Forsberg, C. W., and Lam, K. (1977). Use of adenosine 5'-triphosphate as an indicator of microbiota biomass in rumen contents. *Applied and Environmental Microbiology*, 33(3), 528-537.
- Forstner, J. F., and Forstner, G. G. (1994). Gastrointestinal mucus In L. R. Johnson (Ed.), *Physiology of the Gastrointestinal Tract* (3rd ed., pp. 1256-1283). New York: Raven Press.
- Forte, L. R., and Hamra, F. K. (1996). Guanylin and uroguanylin: Intestinal peptide hormones that regulate epithelial transport. *News in Physiological Sciences*, 11, 17-24.
- Fouillet, H., Gaudichon, C., Mariotti, F., Bos, C., Huneau, J. F., and Tome, D. (2001). Energy nutrients modulate the splanchnic sequestration of dietary nitrogen in humans: A compartmental analysis. *American Journal of Physiology-Endocrinology and Metabolism*, 281(2), E248-E260.

- Fouillet, H., Juillet, B., Bos, C., Mariotti, F., Gaudichon, C., Benamouzig, R., *et al.* (2008). Urea-nitrogen production and salvage are modulated by protein intake in fed humans: results of an oral stable-isotope-tracer protocol and compartmental modeling. *American Journal of Clinical Nutrition*, 87(6), 1702-1714.
- Franks, A. H., Harmsen, H. J. M., Raangs, G. C., Jansen, G. J., Schut, F., and Welling, G. W. (1998). Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-Targeted oligonucleotide probes. *Applied and Environmental Microbiology*, 64(9), 3336-3345.
- Froetschel, M. A. (1996). Bioactive peptides in digesta that regulate gastrointestinal function and intake. *Journal of Animal Science*, 74(10), 2500-2508.
- Fuchs, B. M., Wallner, G., Beisker, W., Schwippl, I., Ludwig, W., and Amann, R. (1998). Flow cytometric analysis of the in situ accessibility of Escherichia coli 16S rRNA for fluorescently labeled oligonucleotide probes. *Applied and Environmental Microbiology*, 64(12), 4973-4982.
- Fujita, H., and Yoshikawa, M. (1999). LKPNM: A prodrug-type ACE-inhibitory peptide derived from fish protein. *Immunopharmacology*, 44(1-2), 123-127.
- Fukudome, S., and Yoshikawa, M. (1992). Opioid-peptides derived from wheat gluten - their isolation and characterization. *Febs Letters*, 296(1), 107-111.
- Fuller, M. F. (1988). Methods of protein evaluation for non-ruminants. In E. R. Orskov (Ed.), *World Animal Science B4. Feed Science*. (pp. 81-101). Amsterdam: Elsevier.
- Fuller, M. F. (1991). Methodologies for the measurement of digestion. In M. W. A. Verstegen, J. Huisman and L. A. den Hartog (Eds.), *Vth International Symposium on Digestive Physiology in Pigs*. (pp. 273-288). Wageningen: Pudoc.
- Fuller, M. F., Darcy-Vrillon, B., Laplace, J. P., Picard, M., Cadenhead, A., Jung, J., Brown, D., and Franklin, M. F. (1994a). The measurement of dietary amino-acid digestibility in pigs, rats and chickens - a comparison of methodologies. *Animal Feed Science and Technology*, 48(3-4), 305-324.
- Fuller, M. F., and Livingstone, R. M. (1982). Effect of ileorectal anastomosis on intestinal propulsion. *Annual report of studies in animal nutrition and allied sciences. Rowett Research Institute*, 38, 39.

- Fuller, M. F., Milne, A., Harris, C. I., Reid, T. M. S., and Keenan, R. (1994). Amino-acid losses in ileostomy fluid on a protein-free diet. *American Journal of Clinical Nutrition*, 59(1), 70-73.
- Fuller, M. F., and Reeds, P. J. (1998). Nitrogen cycling in the gut. *Annual Review of Nutrition*, 18, 385-411.
- Furrie, E. (2006). A molecular revolution in the study of intestinal microflora. *Gut*, 55(2), 141-143.
- Furuya, S., and Kaji, Y. (1989). Estimation of the true ileal digestibility of amino-acids and nitrogen from their apparent values for growing-pigs. *Animal Feed Science and Technology*, 26(3-4), 271-285.
- Furuya, S., and Kaji, Y. (1986). True ileal digestibility of crude protein and amino acids in protein sources as determined by a regression method for growing pigs. *Japanese Journal of Zootechnical Science*, 57, 859-875.
- Gabert, V. M., Canibe, N., Jorgensen, H., Eggum, B. O., and Sauer, W. C. (1997). Use of N-15-amino acid isotope dilution techniques to determine endogenous amino acids in ileal digesta in growing pigs. *Acta Agriculturae Scandinavica Section a-Animal Science*, 47(3), 168-177.
- Galligan, J. J., and Burks, T. F. (1982). Opioid-peptides inhibit intestinal transit in the rat by a central mechanism. *European Journal of Pharmacology*, 85(1), 61-68.
- Gandolfi, I., Palla, G., Marchelli, R., Dossena, A., Puelli, S., and Salvadori, C. (1994). D-alanine in fruit juices - a molecular marker of bacterial-activity, heat-treatments and shelf-life. *Journal of Food Science*, 59(1), 152-154.
- Gardner, M. L. G. (1984). Intestinal assimilation of intact peptides and proteins from the diet - a neglected field. *Biological Reviews of the Cambridge Philosophical Society*, 59(3), 289-331.
- Gardner, M. L. G. (1988). Gastrointestinal absorption of intact proteins. *Annual Review of Nutrition*, 8, 329-350.
- Gardner, M. L. G. (1998). Transmucosal passage of intact peptides. In G. K. Grimble and F. R. C. Backwell (Eds.), *Peptides in Mammalian Protein Metabolism - Tissue Utilization and Clinical Targeting* (Vol. 11, pp. 11-29).
- Gargallo, J., and Zimmerman, D. (1981). Effect of casein and starch infusion in the large-intestine on nitrogen-metabolism of growing swine. *Journal of Nutrition*, 111(8), 1390-1396.

- Garrett, J. E., Goodrich, R. D., and Meiske, J. C. (1987). Measurement and use of D-alanine as a bacterial marker. *Canadian Journal of Animal Science*, 67(3), 735-743.
- Gaskins, H. R. (2003). *The commensal microbiota and development of mucosal defense in the mammalian intestine*. Paper presented at the 9th International Symposium on Digestive Physiology in Pigs. University of Alberta, Banff, Canada
- Gaudichon, C., Bos, C., Morens, C., Petzke, K. J., Mariotti, F., Everwand, J., Benamouzig, R., Dare, S., Tome, D., and Metges, C. C. (2002). Ileal losses of nitrogen and amino acids in humans and their importance to the assessment of amino acid requirements. *Gastroenterology*, 123(1), 50-59.
- Gaudichon, C., Laurent, C., Mahe, S., Marks, L., Tome, D., and Krempf, M. (1994). Rate of N-15 leucine incorporation and determination of nitrogenous fractions from gastro-jejunal secretion in fasting humans. *Reproduction Nutrition Development*, 34(4), 349-359.
- Gaudichon, C., Mahe, S., Luengo, C., Laurent, C., Meugeais, P., Krempf, M., and Tome, D. (1996). A N-15-leucine-dilution method to measure endogenous contribution to Luminal nitrogen in the human upper jejunum. *European Journal of Clinical Nutrition*, 50(4), 261-268.
- Gaussère, B., and Fauconne, G. (1965). Evaluation quantitative a laide de sa teneur en acides nucleiques de la population microbienne du tube digestif des ruminants. 1. Methode et application aux contenus de rumen de bovins recevant differents regimes. *Annales De Biologie Animale Biochimie Biophysique*, 5(1), 5-and.
- Gebhardt, G., Kohler, R., and Zebrowska, T. (1978). Investigations into nitrogen and amino-acid absorption of swine .2. Course of N-15-labelling in chyme, urine and blood. *Archiv Fur Tierernahrung-Archives of Animal Nutrition*, 28(1), 11-20.
- Ghoos, Y., and Vantrappen, G. (1971). The cytochemical localisation of lysozyme in Paneth cell granules. *Histochemistry Journal*, 3, 175-178.
- Gibbs, B. F., Zougman, A., Masse, R., and Mulligan, C. (2004). Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented food. *Food Research International*, 37(2), 123-131.

- Gilbert, E. R., Wong, E. A., and Webb, K. E. (2008). Board-invited review: Peptide absorption and utilization: Implications for animal nutrition and health. *Journal of Animal Science*, 86(9), 2135-2155.
- Gill, H. S., Doull, F., Rutherford, K. J., and Cross, M. L. (2000). Immunoregulatory peptides in bovine milk. *British Journal of Nutrition*, 84, S111-S117.
- Gill, S. R., Pop, M., DeBoy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., Gordon, J. I., Relman, D. A., Fraser-Liggett, C. M., and Nelson, K. E. (2006). Metagenomic analysis of the human distal gut microbiome. *Science*, 312(5778), 1355-1359.
- Gobbetti, M., Minervini, F., and Rizzello, C. G. (2004). Angiotensin I-converting-enzyme-inhibitory and antimicrobial bioactive peptides. *International Journal of Dairy Technology*, 57(2-3), 173-188.
- Goddeeris, B. M., Boersma, W. J. A., Cox, E., Stede, Y. v. d., Koenen, M. E., and Vancaeneghem, S. (2002). The porcine and avian intestinal immune system and its nutritional modulation. In M. C. Blok, H. A. Vahl, L. d. Lange, A. E. v. d. Braak, G. Hemke and M. Hessing (Eds.), *Nutrition and health of the gastrointestinal tract* (pp. 97-134).
- Goodacre, R. (2007). Metabolomics of a superorganism. *Journal of Nutrition*, 137(1), 259S-266S.
- Gordon, J. I., Hooper, L. V., McNevin, M. S., Wong, M., and Bry, L. (1997). Epithelial cell growth and differentiation. III. Promoting diversity in the intestine: Conversations between the microflora, epithelium, and diffuse GALT. *American Journal of Physiology*, 273, G565-G570.
- Gornall, A. G., Bardawill, C. J., and David, M. M. (1949). Determination of serum proteins by means of the Biuret reaction. *Journal of Biological Chemistry*, 177(2), 751-766.
- Graf, S., and Sarna, S. K. (1997). 5-HT-induced colonic contractions: Enteric locus of action and receptor subtypes. *American Journal of Physiology*, 273, G68-G74.
- Grala, W., Verstegen, M. W. A., Jansman, A. J. M., Huisman, J., and Wasilewko, J. (1998). Nitrogen utilization in pigs fed diets with soybean and rapeseed products leading to different ileal endogenous nitrogen losses. *Journal of Animal Science*, 76(2), 569-577.

- Grassmann, W., and Hannig, K. (1950). Ein einfaches verfahren zur analyse der serumproteine und anderer proteingemische. *Naturwissenschaften*, 37(21), 496-497.
- Gregersen, H., and Kassab, G. (1996). Biomechanics of the gastrointestinal tract. *Neurogastroenterology and Motility*, 8(4), 277-297.
- Grishina, O., Volant, K., Marquet, F., Pansu, D., and Descroix-Vagne, M. (1998). Effect of serotonin on unidirectional fluxes in rat intestine in vivo. *Gasastroenterologie Clinique et Biologique*, 22, 792-800.
- Guilloteau, P., Toullec, R., Grongnet, J. F., Patureaumirand, P., Prugnaud, J., and Sauvant, D. (1986). Digestion of Milk, Fish and Soybean Protein in the Preruminant Calf - Flow of Digesta, Apparent Digestibility at the End of the Ileum and Amino-Acid-Composition of Ileal Digesta. *British Journal of Nutrition*, 55(3), 571-592.
- Hagemeister, H., and Erbersdobler, H. (1985). Chemical labeling of dietary-protein by transformation of lysine to homoarginine - a new technique to follow intestinal digestion and absorption. *Proceedings of the Nutrition Society*, 44(3), A133-A133.
- Hall, R. L., Miller, R. J., and Peatfield, A. C. (1980). A colorimetric assay for mucous glycoproteins using alcian blue. *Biochemical Society Transactions*, 8, 72.
- Han, K. S., Deglaire, A., Sengupta, R., and Moughan, P. J. (2008). Hydrolyzed casein influences intestinal mucin gene expression in the rat. *Journal of Agricultural and Food Chemistry*, 56(14), 5572-5576.
- Hang, T. T. N., Dalmasso, G., Powell, K. R., Yan, Y. T., Bhatt, S., Kalman, D., Sitaraman, S. V., and Merlin, D. (2009). Pathogenic bacteria induce colonic PepT1 expression: An implication in host defense response. *Gastroenterology*, 137(4), 1435-1447.
- Haque, E., Chand, R., and Kapila, S. (2009). Biofunctional properties of bioactive peptides of milk origin. *Food Reviews International*, 25(1), 28-43.
- Hardcastle, J., and Hardcastle, P. T. (1987). The secretory actions of histamine in rat small intestine. *Journal of Physiology*, 388, 521-532.
- Harmsen, H. J., Raangs, G. C., He, T., Degener, J. E., and Welling, G. W. (2002). Extensive set of 16S rRNA-based probes for detection of bacteria in human feces. *Applied and Environmental Microbiology*, 68, 2982-2990.

- Harmsen, H. J. M., Wildeboer-Veloo, A. C. M., Grijpstra, J., Knol, J., Degener, J. E., and Welling, G. W. (2000). Development of 16S rRNA-based probes for the Coriobacterium group and the Atopobium cluster and their application for enumeration of Coriobacteriaceae in human feces from volunteers of different age groups. *Applied and Environmental Microbiology*, 66(10), 4523-4527.
- Hedner, J., and Hedner, T. (1987). Beta-casomorphins induce apnea and irregular breathing in adult-rats and newborn rabbits. *Life Sciences*, 41(20), 2303-2312.
- Heine, W., Wutzke, K. D., Richter, I., Walther, F., and Plath, C. (1987). Evidence for colonic absorption of protein nitrogen in infants. *Acta Paediatrica Scandinavica*, 76(5), 741-744.
- Henare, S. J., Rutherford, S. M., Drummond, L. N., Borges, V., Boland, M. J., and Moughan, P. J. (2012). Digestible nutrients and available (ATP) energy contents of two varieties of kiwifruit (*Actinidia deliciosa* and *Actinidia chinensis*). *Food Chemistry*, 130(1), 67-72.
- Henderickx, H. K., Teague, H. S., Redman, D. R., and Grifo, A. P. (1964). Absorption of vitamin B12 from colon of pig. *Journal of Animal Science*, 23(4), 1036-1038.
- Hendriks, W. H., Moughan, P. J., and Tarttelin, M. F. (1996). Gut endogenous nitrogen and amino acid excretions in adult domestic cats fed a protein-free diet or an enzymatically hydrolyzed casein-based diet. *Journal of Nutrition*, 126(4), 955-962.
- Hendriks, W. H., Sritharan, K., and Hodgkinson, S. M. (2002). Comparison of the endogenous ileal and faecal amino acid excretion in the dog (*Canis familiaris*) and the rat (*Rattus rattus*) determined under protein-free feeding and peptide alimentation. *Journal of Animal Physiology and Animal Nutrition*, 86(9-10), 333-341.
- Hendrix, T. R., and Bayless, T. M. (1970). Digestion: Intestinal secretion. *Annual Review of Physiology*, 32, 139-164.
- Hoare, J. P., and Laidler, K. J. (1950). The Molecular Kinetics of the Urea-Urease System .2. The Inhibition by Products. *Journal of the American Chemical Society*, 72(6), 2487-2489.
- Hodgkinson, S. M., and Moughan, P. J. (2000). *Amino acids - The collection of ileal digesta and characterisation of the endogenous component*. Wageningen: Wageningen Pers, Stichting.

- Hodgkinson, S. M., and Moughan, P. J. (2001). Effectiveness of an ultrafiltration device for use with the enzyme-hydrolysed protein method for determining endogenous ileal nitrogen and amino acid excretion in the pig. *Journal of the Science of Food and Agriculture*, 81(15), 1592-1596.
- Hodgkinson, S. M., and Moughan, P. J. (2003). The enzyme hydrolysed protein method for the determination of endogenous ileal nitrogen and amino acid flows - a modification. *Animal Feed Science and Technology*, 108(1-4), 207-214.
- Hodgkinson, S. M., Moughan, P. J., and Reynolds, G. W. (2000a). Effect of the duration of feeding of a protein-free diet on endogenous ileal nitrogen and amino acid loss in the growing pig. *Journal of the Science of Food and Agriculture*, 80(9), 1407-1412.
- Hodgkinson, S. M., Moughan, P. J., Reynolds, G. W., and James, K. A. C. (2000b). The effect of dietary peptide concentration on endogenous ileal amino acid loss in the growing pig. *British Journal of Nutrition*, 83(4), 421-430.
- Hoerr, R. A., Matthews, D. E., Bier, D. M., and Young, V. R. (1991). Leucine kinetics from H-2(3) - and C-13 leucine infused simultaneously by gut and vein. *American Journal of Physiology*, 260(1), E111-E117.
- Hoerr, R. A., Matthews, D. E., Bier, D. M., and Young, V. R. (1993). Effects of protein restriction and acute refeeding on leucine and lysine kinetics in young men. *American Journal of Physiology*, 264(4), E567-E575.
- Höllt, V. (1986). Opioid peptide processing and receptor selectivity. *Annual Review of Pharmacology and Toxicology*, 26, 59-77.
- Hopkins, M. J., and Macfarlane, G. T. (2000). Evaluation of 16s rRNA and cellular fatty acid profiles as markers of human intestinal bacterial growth in the chemostat. *Journal of Applied Microbiology*, 89(4), 668-677.
- Horiguchi, M., and Kandatsu, M. (1959). Isolation of 2-aminoethane phosphonic acid from rumen protozoa. *Nature*, 184(4690), 901-902.
- Hovgaard, L., and Brondsted, H. (1996). Current applications of polysaccharides in colon targeting. *Critical Reviews in Therapeutic Drug Carrier Systems*, 13(3-4), 185-223.
- Huang, S. X., Sauer, W. C., and Marty, B. (2001). Ileal digestibilities of neutral detergent fiber, crude protein, and amino acids associated with neutral

- detergent fiber in wheat shorts for growing pigs. *Journal of Animal Science*, 79(9), 2388-2396.
- Huisman, J., Heinz, T., Vanderpoel, A. F. B., Vanleeuwen, P., Souffrant, W. B., and Verstegen, M. W. A. (1992). True protein digestibility and amounts of endogenous protein measured with the 15N-dilution technique in piglets fed on peas (*pisum-sativum*) and common beans (*phaseolus-vulgaris*). *British Journal of Nutrition*, 68(1), 101-110.
- Hurrell, R. F., and Finot, P. A. (1985). Effects of food processing on protein digestibility and amino acid availability. In J. W. Finley and D. T. Hopkins (Eds.), *Digestibility and Amino Acid Availability in Cereals and Oilseeds*. (pp. 233-246). MN: American Association of Cereal Chemists.
- Husby, S., Foged, N., Host, A., and Svehag, S. E. (1987). Passage of dietary antigens into the blood of children with celiac-disease - quantification and size distribution of absorbed antigens. *Gut*, 28(9), 1062-1072.
- Ibrahim, E. A., and Ingalls, J. R. (1972). Microbial Protein-Biosynthesis in Rumen. *Journal of Dairy Science*, 55(7), 971-978.
- Ichikawa, T., Ishihara, K., Saigenji, K., and Hotta, K. (1993). Stimulation of mucus glycoprotein biosynthesis in rat gastric mucosa by gastrin. *Biochemical Pharmacology*, 46, 1551-1557.
- Irie, M., Terada, T., Okuda, M., and Inui, K. (2004). Efflux properties of basolateral peptide transporter in human intestinal cell line Caco-2. *Pflugers Archiv-European Journal of Physiology*, 449(2), 186-194.
- Ivanov, V. T., Karelin, A. A., Philippova, M. M., Nazimov, I. V., and Pletnev, V. Z. (1997). Haemoglobin as a source of endogenous bioactive peptides: The concept of tissue-specific peptide pool. *Biopolymers*, 43(2), 171-188.
- Jabbal, I., Forstner, G., Forstner, J., and Kells, D. I. C. (1975). Sedimentation velocity studies on microgram quantities of rat intestinal goblet cell mucin. *Analytical Biochemistry*, 69(2), 558-571.
- Jackson, A. A., Picou, D., and Landman, J. (1984). The non-invasive measurement of urea kinetics in normal man by a constant infusion of N-15 N-15 urea. *Human Nutrition-Clinical Nutrition*, 38C(5), 339-354.
- Jackson, A. A. (1995). Salvage of urea-nitrogen and protein-requirements. *Proceedings of the Nutrition Society*, 54(2), 535-547.

- Jackson, A. A. (1999). Limits of adaptation to high dietary protein intakes. *European Journal of Clinical Nutrition*, 53, S44-S52.
- Jakobsson, I., Lindberg, T., Lothe, L., Axelsson, I., and Benediktsson, B. (1986). Human alpha-lactalbumin as a marker of macromolecular absorption. *Gut*, 27(9), 1029-1034.
- James, P. S., and Smith, M. W. (1976). Methionine transport by pig colonic mucosa measured during early postnatal-development. *Journal of Physiology-London*, 262(1), 151-168.
- Jansman, A. J. M., Schulze, H., van Leeuwen, P., and Verstegen, M. W. A. (1994). Effects of protease inhibitors and lectins from soya on the true digestibility and the endogenous excretion of CP in piglets. *Forschungsinstitut für die Biologie landwirtschaftlicher Nutztiere*. In W. B. Souffrant and H. Hagemester (Eds.), *Proceedings of the VIth International Symposium on Digestive Physiology in Pigs*. (pp. 322–324). Bad Doberan: EAAP Publication No. 80.
- Jansman, A. J. M., Smink, W., van Leeuwen, P., and Rademacher, M. (2002). Evaluation through literature data of the amount and amino acid composition of basal endogenous crude protein at the terminal ileum of pigs. *Animal Feed Science and Technology*, 98(1-2), 49-60.
- Jansman, A. J. M., Verstegen, M. W. A., Huisman, J., and Vandenberg, J. W. O. (1995). Effects of hulls of faba beans (viola-faba l) with a low or high content of condensed tannins on the apparent ileal and fecal digestibility of nutrients and the excretion of endogenous protein in ileal-digesta and faeces of pigs. *Journal of Animal Science*, 73(1), 118-127.
- Jensen, C., Weisbjerg, M. R., and Hvelplund, T. (2006). Evaluation of methods for estimating the amino acid supply to the duodenum of microbial, endogenous and undegraded feed protein on maize silage diets fed to dairy cows. *Animal Feed Science and Technology*, 131(1-2), 1-24.
- Jin, D. R., Miyahara, T., Oe, T., and Toyo'oka, T. (1999). Determination of D-amino acids labeled with fluorescent chiral reagents, R(-)- and S(+)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(N,N-dimethylaminosulfonyl)-2,1,3-benzoxadiazoles, in biological and food samples by liquid chromatography. *Analytical Biochemistry*, 269(1), 124-132.
- Johnson, G., Wilks, M., Warwick, S., Millar, M. R., and Fan, S. L. S. (2006). Comparative study of diagnosis of PD peritonitis by quantitative polymerase

- chain reaction for bacterial DNA vs culture methods. *Journal of Nephrology*, 19(1), 45-49.
- Jolles, P., Levytoledano, S., Fiat, A. M., Soria, C., Gillessen, D., Thomaidis, A., et al. (1986). Analogy between fibrinogen and casein - effect of an undecapeptide isolated from kappa-casein on platelet-function. *European Journal of Biochemistry*, 158(2), 379-382.
- Joly, F., Mayeur, C., Messing, B., Lavergne-Slove, A., Cazals-Hatem, D., Noordine, M. L., Cherbuy, C., Duce, P., and Thomas, M. (2009). Morphological adaptation with preserved proliferation/transporter content in the colon of patients with short bowel syndrome. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 297(1), G116-G123.
- Jones, D. E., and Bevins, C. L. (1992). Paneth cells of the human small intestine express an antimicrobial peptide gene. *Journal of Biological Chemistry*, 267, 2316-2322.
- Juntunen, M., Kirjavainen, P. V., Ouwehand, A. C., Salminen, S. J., and Isolauri, E. (2001). Adherence of probiotic bacteria to human intestinal mucus in healthy infants and during rotavirus infection. *Clinical and Diagnostic Laboratory Immunology*, 8(2), 293-296.
- Kaplan, R., and Apirion, D. (1975). Decay of ribosomal ribonucleic-acid in escherichia-coli-cells starved for various nutrients. *Journal of Biological Chemistry*, 250(8), 3174-3178.
- Karras, P. J., and North, R. A. (1981). Acute and chronic effects of opiates on single neurons of the myenteric-plexus. *Journal of Pharmacology and Experimental Therapeutics*, 217(1), 70-80.
- Karr-Lilienthal, L. K., Grieshop, C. M., Spears, J. K., Patil, A. R., Czarnecki-Maulden, G. L., Merchen, N. R., et al. (2004). Estimation of the proportion of bacterial nitrogen in canine feces using diaminopimelic acid as an internal bacterial marker. *Journal of Animal Science*, 82(6), 1707-1712.
- Kayser, H., & Meisel, H. (1996). Stimulation of human peripheral blood lymphocytes by bioactive peptides derived from bovine milk proteins. *Febs Letters*, 383(1-2), 18-20.
- Kellow, J. E., and Malcolm, A. (1996). Motility. *Current Opinion in Gastroenterology*, 12(2), 134-140.

- Kelly, D., and Coutts, A. P. G. (1997). Biologically active peptides in colostrum and milk. In J. P. Laplace, C. Fevrier and A. Barbeau (Eds.), *Digestive Physiology in Pigs. Proceedings of the VII International Symposium*. (Vol. 88). Saint Malo, France: EAPP.
- Kenrick, K. G. (1970). Immunoglobulins and dietary protein antibodies in childhood coeliac disease. *Gut*, 11(8), 635-640.
- Kerneis, S., Bogdanova, A., ColucciGuyon, E., Kraehenbuhl, J. P., and Pringault, E. (1996). Cytosolic distribution of villin in M cells from mouse Peyer's patches correlates with the absence of a brush border. *Gastroenterology*, 110(2), 515-521.
- Kies, A. K., Moughan, P. J., and Smith, W. C. (1986). The apparent and true Ileal digestibility of nitrogen and amino-acids in lactic casein for the growing-pig. *Animal Feed Science and Technology*, 16(3), 169-178.
- Kilberg, M. S., Stevens, B. R., and Novak, D. A. (1993). Recent advances in mammalian amino-acid-transport. *Annual Review of Nutrition*, 13, 137-165.
- Kimball, J. W. (1978). *Biology* (4th ed.). Reading, Mass: Addison-Wesley Pub. Co.
- Kitts, D. D., and Weiler, K. (2003). Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Current Pharmaceutical Design*, 9(16), 1309-1323.
- Kitts, D. D., and Yuan, Y. V. (1992). Caseinophosphopeptides and calcium bioavailability. *Trends in Food Science and Technology*, 3, 31-35.
- Klee, W. A., Zioudrou, C., and Streaty, R. A. (1978). *Endorphins in Mental Health Research*. New York: Macmillan.
- Kluess, J., Schoenhusen, U., Souffrant, W. B., Jones, P. H., and Miller, B. G. (2010). Impact of diet composition on ileal digestibility and small intestinal morphology in early-weaned pigs fitted with a T-cannula. *Animal*, 4(4), 586-594.
- Köhler, R., Zebrowska, T., and Gebhardt, G. (1978). Investigations into nitrogen and amino-acid absorption of swine .3. Endogenous secretion and absorption of nitrogen. *Archiv Fur Tierernahrung-Archives of Animal Nutrition*, 28(5), 317-327.
- Köhler, T., Denhartog, L. A., Huisman, J., Mosenthin, R., and Verstegen, M. W. A. (1991). Digestibility measurements in pigs by using post-valve t-cecum cannulation or end-to-side ileorectal anastomosis. *Journal of Animal*

*Physiology and Animal Nutrition-Zeitschrift Fur Tierphysiologie
Tierernahrung Und Futtermittelkunde*, 66(5), 278-286.

- Köhler, T., Huisman, J., Denhartog, L. A., and Mosenthin, R. (1990). Comparison of different digesta collection methods to determine the apparent digestibilities of the nutrients at the terminal ileum in pigs. *Journal of the Science of Food and Agriculture*, 53(4), 465-475.
- Köhler, T., Mosenthin, R., Verstegen, M. W. A., Huisman, J., den Hartog, L. A., and Ahrens, F. (1992a). Effect of ileo-rectal anastomosis and post-valve T-caecum cannulation on growing pigs. 1) Growth performances, N-balance, and intestinal adaptation. *British Journal of Nutrition*, 68, 293-303.
- Köhler, T., Mosenthin, R., Verstegen, M. W. A., Huisman, J., den Hartog, L. A., and Wensing, T. (1992b). Effect of ileo-rectal anastomosis and post-valve T-caecum cannulation on growing pigs. 2) Blood variables and mineral balances. *British Journal of Nutrition*, 68, 305-315.
- Korhonen, H., and Pihlanto, A. (2003). Food-derived bioactive peptides - Opportunities for designing future foods. *Current Pharmaceutical Design*, 9(16), 1297-1308.
- Korhonen, H., and Pihlanto, A. (2006). Bioactive peptides: Production and functionality. *International Dairy Journal*, 16(9), 945-960.
- Kotwal, S. M., Gote, M. M., Khan, M. I., and Khire, J. M. (1999). Production, purification and characterization of a constitutive intracellular alpha-galactosidase from the thermophilic fungus *Humicola* sp. *Journal of Industrial Microbiology and Biotechnology*, 23(1), 661-667.
- Kraehenbuhl, J. P., and Neutra, M. R. (2000). Epithelial M cells: Differentiation and function. *Annual Review of Cell and Developmental Biology*, 16, 301-332.
- Kraus, R. J., Shinnick, F. L., and Marlett, J. A. (1990). Simultaneous Determination of Neutral and Amino-Sugars in Biological-Materials. *Journal of Chromatography*, 513, 71-81.
- Krause, W. J. (2000). Brunner's glands: A structural, histochemical and pathological profile. *Progress in Histochemistry and Cytochemistry*, 35(4), 255-+.
- Krawielitzki, K., Kreienbring, F., Zebrowska, T., Schadereit, R., and Kowalczyk, J. (1994). Estimation of absorption, secretion and reabsorption in different intestinal sections of growing pigs using the N-15 isotope dilution method. In

- W. B. Souffrant and H. Hagemester (Eds.), *Vith International Symposium on Digestive Physiology in Pigs, Proceedings, Vols 1 and 2* (pp. 79-82).
- Krawielitzki, K., Zebrowska, T., Kreienbring, F., Schadereit, R., and Kowalczyk, J. (1996). Absorption and secretion of exogenous and endogenous N along the digestive tract and kinetic parameters of protein metabolism in growing pigs .1. Estimation by digesta exchange between N-15-labelled and unlabelled pigs. *Journal of Animal Physiology and Animal Nutrition*, 76(1), 46-56.
- Krawielitzki, K., Zebrowska, T., Schadereit, R., Kowalczyk, K., Hennig, U., Wünsche, J., and Herrmann, U. (1990). Determining of nitrogen absorption and nitrogen excretion in different sections of the pig's intestine by digesta exchange between 15N labelled and unlabelled animals. *Archives of Animal Nutrition*, 40, 25-37.
- Kreil, G., Umbach, M., Brantl, V., and Teschemacher, H. (1983). Studies on the enzymatic degradation of beta-casomorphins. *Life Sciences*, 33, 137-140.
- Kuiken, K. A., and Lyman, C. M. (1948). Availability of amino acids in some foods. *Journal of Nutrition*, 36, 359-368.
- Kuno, H., and Kihara, H. K. (1967). Simple microassay of protein with membrane filter. *Nature*, 215(5104), 974-975.
- Kurek, M., Rueff, F., CzerwionkaSzaflarska, M., Doroszewska, G., and Przybilla, B. (1996). Exorphins derived from cow's milk casein elicit pseudo-allergic wheal-and-flare reactions in healthy children. *Revue Francaise D Allergologie Et D Immunologie Clinique*, 36(2), 191-196.
- Lacomba, R., Salcedo, J., Alegria, A., Lagarda, M. J., Barbera, R., and Matencio, E. (2010). Determination of sialic acid and gangliosides in biological samples and dairy products: A review. *Journal of Pharmaceutical and Biomedical Analysis*, 51(2), 346-357.
- Lahov, E., & Regelson, W. (1996). Antibacterial and immunostimulating casein-derived substances from milk: Casecidin, isracidin peptides. *Food and Chemical Toxicology*, 34(1), 131-145.
- Lai, L. S., and Liang, H. Y. (2012). Chemical compositions and some physical properties of the water and alkali-extracted mucilage from the young fronds of *Asplenium australasicum* (J. Sm.) Hook. *Food Hydrocolloids*, 26(2), 344-349.

- Lal, R. B., Kissel, D. E., Cabrera, M. L., and Schwab, A. P. (1993). Kinetics of Urea Hydrolysis in Wheat Residue. *Soil Biology and Biochemistry*, 25(8), 1033-1036.
- Lamont, J. T. (1992). Mucus - the front-line of intestinal mucosal defence. In R. H. Stead, M. H. Perdue, H. Cooke, D. W. Powell and K. E. Barrett (Eds.), *Neuro-Immuno-Physiology of the Gastrointestinal Mucosa: Implications for Inflammatory Diseases* (Vol. 664, pp. 190-201). New York: New York Acad Sciences.
- Langendijk, P. S., Schut, F., Jansen, G. J., Raangs, G. C., Kamphuis, G. R., Wilkinson, M. H. F., *et al.* (1995). Quantitative fluorescence in-situ hybridization of bifidobacterium spp with genus-specific 16s ribosomal-RNA-targeted probes and its application in fecal samples. *Applied and Environmental Microbiology*, 61(8), 3069-3075.
- Langkilde AM, A. H., Faisant N and Champ M. (1994). *A comparison between the intubation technique and the ileostomy model for in vivo measurement of RS.* Paper presented at the Proceedings of the Concluding Plenary Meeting of EURESTA Den Haag, The Netherlands.
- Laplace, J. P., Darcyvrillon, B., Duvaliflah, Y., and Raibaud, P. (1985). Proteins in the Digesta of the Pig - Amino-Acid-Composition of Endogenous, Bacterial and Fecal Fractions. *Reproduction Nutrition Development*, 25(6), 1083-1099.
- Laplace, J. P., Darcy-Vrillon, B., Perez, J. M., Henry, Y., Giger, S., and Sauvant, D. (1989). Associative effects between two fibre sources on ileal and faecal digestibility of amino acids, energy and cell-wall components in growing pigs. *British Journal of Nutrition*, 61, 75-87.
- Laplace, J. P., Darcy-Vrillon, B., and Picard, M. (1985a). Evaluation de la disponibilité des acides aminés: Choix raisonné d'une méthode. *Journal Rech Porcine. France*, 17, 353-370.
- Laplace, J. P., Souffrant, W. B., Hennig, U., Chabeauti, E., and Fevrier, C. (1994). Measurement of precaecal dietary protein and plant cell digestion in pigs; comparison of four surgical procedures for ileo-rectal anastomosis. *Livestock Production Science*, 40, 313-328.
- Leibholz, J. (1982). The flow of endogenous nitrogen in the digestive-tract of young-pigs. *British Journal of Nutrition*, 48(3), 509-517.

- Leterme, P., Froidmont, E., Rossi, F., and Thewis, A. (1998). The high water-holding capacity of pea inner fibers affects the ileal flow of endogenous amino acids in pigs. *Journal of Agricultural and Food Chemistry*, 46(5), 1927-1934.
- Leterme, P., Monmart, T., Thewis, A., and Morandi, P. (1996a). Effect of oral and parenteral N nutrition vs N-free nutrition on the endogenous amino acid flow at the ileum of the pig. *Journal of the Science of Food and Agriculture*, 71(2), 265-271.
- Leterme, P., and Thewis, A. (2004). Effect of pig bodyweight on ileal amino acid endogenous losses after ingestion of a protein-free diet enriched in pea inner fibre isolates. *Reproduction Nutrition Development*, 44(5), 407-417.
- Leterme, P., Thewis, A., Beckers, Y., and Baudart, E. (1990). Apparent and true ileal digestibility of amino-acids and nitrogen-balance measured in pigs with ileorectal anastomosis or T-cannulas, given a diet containing peas. *Journal of the Science of Food and Agriculture*, 52(4), 485-497.
- Leterme, P., Thewis, A., Francois, E., Van Leeuwen, P., Wathelet, B., and Huisman, J. (1996b). The use of ¹⁵N-labelled dietary proteins for determining true ileal amino acid digestibilities is limited by their rapid recycling in the endogenous secretions of pigs. *Journal of Nutrition*, 126, 2188-2198.
- Ley, R. E., Turnbaugh, P. J., Klein, S., and Gordon, J. I. (2006). Microbial ecology - Human gut microbes associated with obesity. *Nature*, 444(7122), 1022-1023.
- Li, G. H., Le, G. W., Shi, Y. H., and Shrestha, S. (2004). Angiotensin I-converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects. *Nutrition Research*, 24(7), 469-486.
- Libao-Mercado, A. J., Zhu, C. L., Cant, J. P., Lapierre, H. N., Thibault, J. N., Seve, B., Fuller, M. F., and de Lange, C. F. M. (2007). *Sources of nitrogen for pig gut microbes: effect of feeding fermentable fiber*. (Vol. 124). Wageningen (The Netherlands): Academic Publishers.
- Libao-Mercado, A. J. O., Zhu, C. L., Cant, J. P., Lapierre, H., Thibault, J. N., Seve, B., Fuller, M. F., and de Lange, C. F. M. (2009). Dietary and endogenous amino acids are the main contributors to microbial protein in the upper gut of normally nourished pigs. *Journal of Nutrition*, 139(6), 1088-1094.
- Liddle, R. A. (1995). Regulation of cholecystokinin secretion by intraluminal releasing factors. *American Journal of Physiology*, 269, G319-G327.

- Lien, K. A. (1995). *Endogenous protein in ileal digesta of pigs: Evaluation of the 15N-isotope dilution technique and an estimation of the mucin content*. Ph.D. Dissertation, University of Alberta, Canada., Edmonton.
- Lien, K. A., McBurney, M. I., Beyde, B. I., Thomson, A. B. R., and Sauer, W. C. (1996). Ileal recovery of nutrients and mucin in humans fed total enteral formulas supplemented with soy fiber. *American Journal of Clinical Nutrition*, 63(4), 584-595.
- Lien, K. A., Sauer, W. C., and Dugan, M. E. R. (1997a). Evaluation of the N-15-isotope dilution technique for determining the recovery of endogenous protein in ileal digesta of pigs: Effect of the pattern of blood sampling, precursor pools, and isotope dilution technique. *Journal of Animal Science*, 75(1), 159-169.
- Lien, K. A., Sauer, W. C., and Fenton, M. (1997b). Mucin output in ileal digesta of pigs fed a protein-free diet. *Zeitschrift Fur Ernährungswissenschaft*, 36(2), 182-190.
- Lien, K. A., Sauer, W. C., and He, J. M. (2001). Dietary influences on the secretion into and degradation of mucin in the digestive tract of monogastric animals and humans. *Journal of Animal and Feed Sciences*, 10(2), 223-245.
- Lieverse, R. J., Masclee, A. A., Jansen, J. B., Rovati, L. C., and Lamers, C., B. (1995). Satiety effects a type A CCK receptor antagonist loxiglumide in lean and obese women. *Biological Psychiatry*, 37, 331-335.
- Lim, R. K. S., Ivy, A. C., and McCarthy, J. E. (1925). Contributions to the physiology of gastric secretion I Gastric secretion by local (mechanical and chemical) stimulation. *Quarterly Journal of Experimental Physiology*, 15, 13-53.
- Lin, L., Umahara, M., York, D. A., and Bray, G. A. (1998). beta-casomorphins stimulate and enterostatin inhibits the intake of dietary fat in rats. *Peptides*, 19(2), 325-331.
- Lin, S. Y., Wu, C. W., Li, M. J., and Liang, R. C. (1997). Infra-red (ATR/FT) spectroscopic study on the conformational structure of the isolated human gastric mucus pretreated with ethanol. *Journal of Gastroenterology and Hepatology*, 12(11), 707-712.
- Ling, J. R. (1990). Digestion of bacterial cell walls in the rumen. In S. Hoshino, R. Onodera & H. Minato (Eds.), *The rumen ecosystem: the microbial metabolism and its regulation*. (pp. 83-90). New York: Springer-Verlag.

- Ling, J. R., and Buttery, P. J. (1978). Simultaneous Use of Ribonucleic-Acid, S-35, 2-6-Diaminopimelic Acid and 2-Aminoethylphosphonic Acid as Markers of Microbial Nitrogen Entering Duodenum of Sheep. *British Journal of Nutrition*, 39(1), 165-179.
- Livingstone, R. M., Fowler, V. R., Atkinson, T., and Baird, B. (1977a). Diurnal patterns of digestion in pigs fitted with annealed glass cannulae. *Animal Production*, 24(FEB), 156-156.
- Livingstone, R. M., Fowler, V. R., White, F., and Wenham, G. (1977b). Annealed Glass Cannulae for Use in Digestion Studies with Pigs. *Veterinary Record*, 101(18), 368-368.
- Lo, C. H., and Stelson, H. (1972). Interference by polysucrose in protein determination by Lowry method. *Analytical Biochemistry*, 45(1), 331-336.
- Lo, W. M. Y., and Li-Chan, E. C. Y. (2005). Angiotensin I-converting enzyme inhibitory peptides from in vitro pepsin-pancreatin digestion of soy protein. *Journal of Agricultural and Food Chemistry*, 53(9), 3369-3376.
- Lobley, G. E., Connell, A., Revell, D. K., Bequette, B. J., Brown, D. S., and Calder, A. G. (1996). Splanchnic-bed transfers of amino acids in sheep blood and plasma, as monitored through use of a multiple U-C-13-labelled amino acid mixture. *British Journal of Nutrition*, 75(2), 217-235.
- Long, C. L., Jeevanandam, M., and Kinney, J. M. (1978). Metabolism and recycling of urea in man. *American Journal of Clinical Nutrition*, 31(8), 1367-1382.
- Lonnerdal, B. (2003). Nutritional and physiologic significance of human milk proteins. *American Journal of Clinical Nutrition*, 77(6), 1537S-1543S.
- Loukas, S., Varoucha, D., Zioudrou, C., Streaty, R. A., and Klee, W. A. (1983). Opioid activities and structures of alpha-casein-derived exorphins. *Biochemistry*, 22(19), 4567-4573.
- Low, A. G. (1980). Nutrient absorption in pigs. *Journal of the Science of Food and Agriculture*, 31(11), 1087-1130.
- Low, A. G., and Zebrowska, T. (1989). Digestion in pigs. In H. D. Bock, B. O. Eggum, A. G. Low, O. Simon and T. Zebrowska (Eds.), *Protein Metabolism in Farm Animals* (pp. 53–121). Oxford, UK.: Oxford University Press.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, 193, 265-275.

- Ludowieg, J. J., and Benmaman, J. D. (1968). A Method for Analysis of Amino Sugars - Specificity and Mechanism of Reaction. *Carbohydrate Research*, 8(2), 185- and.
- Luiking, Y. C., Deutz, N. E. P., Jakel, M., and Soeters, P. B. (2005). Casein and soy protein meals differentially affect whole-body and splanchnic protein metabolism in healthy humans. *Journal of Nutrition*, 135(5), 1080-1087.
- MacDonald, T. T., & Monteleone, G. (2005). Immunity, inflammation, and allergy in the gut. *Science*, 307(5717), 1920-1925.
- MacRae, J. C., Smith, J. S., and White, F. (1982). Effects of gastrointestinal cannulation and jugular vein catheterization on the metabolism in sheep. *British Journal of Nutrition*, 47, 637-644.
- Madara, J. L., and Pappenheimer, J. R. (1987). Structural basis for physiologic regulation of paracellular pathways in intestinal epithelia. *Journal of Membrane Biology*, 100, 149-164.
- Madara, J. L., and Trier, J. S. (1994). The functional morphology of the mucosa of the small intestine. In L. R. Johnson (Ed.), *The Physiology of the Gastrointestinal Tract* (3rd ed.). New York: Raven Press.
- Maga, J. A. (1981). Measurement of available lysine using the guanidination reaction. *Journal of Food Science*, 46(1), 132-134.
- Mahe, S., Huneau, J. F., Marteau, P., Thuillier, F., and Tome, D. (1992). Gastroileal nitrogen and electrolyte movements after bovine-milk ingestion in humans. *American Journal of Clinical Nutrition*, 56(2), 410-416.
- Mahe, S., Roos, N., Benamouzig, R., Sick, H., Baglieri, A., Huneau, J. F., and Tome, D. (1994). True exogenous and endogenous nitrogen fractions in the human jejunum after ingestion of small amounts of N-15-labeled casein. *Journal of Nutrition*, 124(4), 548-555.
- Mahe, S., Tome, D., Dumontier, A. M., and Desjeux, J. F. (1989). Absorption of intact morphiceptin by diisopropylfluorophosphate-treated rabbit ileum. *Peptides*, 10(1), 45-52.
- Malinen, E., Matto, J., Salmite, M., Alander, M., Saarela, M., & Palva, A. (2002). PCR-ELISA - II: Analysis of *Bifidobacterium* populations in human faecal samples from a consumption trial with *Bifidobacterium lactis* Bb-12 and a galacto-oligosaccharide preparation. *Systematic and Applied Microbiology*, 25(2), 249-258.

- Mansour, A., and Watson, S. J. (1993). *Handbook of Experimental Pharmacology*. (Vol. 104/ I). Berlin: Springer.
- Mantle, M., and Allen, A. (1978). A colorimetric assay for glycoproteins based on the Periodic Acid/Schiff stain. *Biochemical Society Transactions*, 6, 607-609.
- Mantle, M., and Allen, A. (1981). Isolation and Characterization of the Native Glycoprotein from Pig Small-Intestinal Mucus. *Biochemical Journal*, 195(1), 267-275.
- Mantle, M., and Allen, A. (1989). Gastrointestinal mucus. In J. S. Davison (Ed.), *Gastrointestinal Secretions* (pp. 202-229). London: Butterworth and Co.
- Manzi, A. E., Diaz, S., and Varki, A. (1990). High-pressure liquid-chromatography of sialic acids on a pellicular resin anion-exchange column with pulsed amperometric detection - a comparison with 6 other systems. *Analytical Biochemistry*, 188(1), 20-32.
- Marini, J. C., Fox, D. G., and Murphy, M. R. (2008). Nitrogen transactions along the gastrointestinal tract of cattle: A meta-analytical approach. *Journal of Animal Science*, 86(3), 660-679.
- Mariotti, F., Mahe, S., Benamouzig, R., Luengo, C., Dare, S., Gaudichon, C., and Tome, D. (1999). Nutritional value of N-15 -soy protein isolate assessed from ileal digestibility and postprandial protein utilization in humans. *Journal of Nutrition*, 129(11), 1992-1997.
- Mariotti, F., Mahe, S., Luengo, C., Benamouzig, R., and Tome, D. (2000). Postprandial modulation of dietary and whole-body nitrogen utilization by carbohydrates in humans. *American Journal of Clinical Nutrition*, 72(4), 954-962.
- Mariscal-Landin, G., and de Souza, T. C. R. (2006). Endogenous ileal losses of nitrogen and amino acids in pigs and piglets fed graded levels of casein. *Archives of Animal Nutrition*, 60(6), 454-466.
- Maruyama, S., Suzuki, H., & Tomizuka, N. (1985). Angiotensin I-converting enzyme-inhibitor derived from an enzymatic hydrolysate of casein .3. Effects of zinc ion on inhibition by angiotensin i-converting enzyme-inhibitor derived from casein. *Agricultural and Biological Chemistry*, 49(9), 2809-2810.
- Mason, V. C. (1969). Some observations on distribution and origin of nitrogen in sheep faeces. *Journal of Agricultural Science*, 73, 99-and.

- Mason, V. C. (1984). Metabolism of Nitrogenous Compounds in the Large Gut. *Proceedings of the Nutrition Society*, 43(1), 45-53.
- Masson, H. A., Denholm, A. M., and Ling, J. R. (1991). In vivo metabolism of 2,2'-diaminopimelic acid from gram-positive and gram-negative bacterial-cells by ruminal microorganisms and ruminants and its use as a marker of bacterial biomass. *Applied and Environmental Microbiology*, 57(6), 1714-1720.
- Masuda, O., Nakamura, Y., and Takano, T. (1996). Antihypertensive peptides are present in aorta after oral administration of sour milk containing these peptides to spontaneously hypertensive rats. *Journal of Nutrition*, 126(12), 3063-3068.
- Matsufuji, H., Matsui, T., Seki, E., Osajima, K., Nakashima, M., and Osajima, Y. (1994). Angiotensin I-converting enzyme-inhibitory peptides in an alkaline protease hydrolyzate derived from sardine muscle. *Bioscience Biotechnology and Biochemistry*, 58(12), 2244-2245.
- Matthews, D. M., Craft, I. L., Geddes, D. M., Wise, I. J., and Hyde, C. W. (1968). Absorption of glycine and glycine peptides from small intestine of rat. *Clinical Science*, 35(3), 415-424.
- Matthews, D. M., Lis, M. T., Cheng, B., and Crampton, R. F. (1969). Observations on intestinal absorption of some oligopeptides of methionine and glycine in rat. *Clinical Science*, 37(3), 751-764.
- Matthews, D. E., Marano, M. A., and Campbell, R. G. (1993). Splanchnic bed utilization of glutamine and glutamic-acid in humans. *American Journal of Physiology*, 264(6), E848-E854.
- Matthies, H., Stark, H., Hartrodt, B., Ruethrich, H. L., Spieler, H. T., Barth, A., and Neubert, K. (1984). Derivatives of beta-casomorphins with high analgesic potency. *Peptides*, 5, 463-470.
- Mazoyer, E., Bal Dit Sollier, C., Drouet, L., Fiat, A. M., Jollet, P., and Caen, J. (1992). Active peptides from human and cow's milk proteins effects on platelets function and vessel wall. In M. Paubert-Braquet, C. Dupont & R. Paoletti (Eds.), *Foods, Nutrition, and Immunity*. (Vol. 1, pp. 88-95). Basel: Dyn. Nutrition Research.
- McFadden, D., Zinner, M. J., and Jaffe, B. M. (1986). Substance P-induced intestinal secretion of water and electrolytes. *Gut*, 27, 267-272.
- McLean, E., and Ash, R. (1986). The time-course of appearance and net accumulation of horseradish-peroxidase (HRP) presented orally to juvenile carp cyprinus-

- carpio (l). *Comparative Biochemistry and Physiology a-Physiology*, 84(4), 687-690.
- McLean, E., and Ash, R. (1987). The time-course of appearance and net accumulation of horseradish-peroxidase (HRP) presented orally to rainbow-trout *salmo-gairdneri* (richardson). *Comparative Biochemistry and Physiology a-Physiology*, 88(3), 507-510.
- McNurlan, M. A., and Garlick, P. J. (1980). Contribution of rat-liver and gastrointestinal-tract to whole-body protein-synthesis in the rat. *Biochemical Journal*, 186(1), 381-383.
- Meakins, T. S., and Jackson, A. A. (1996). Salvage of exogenous urea nitrogen enhances nitrogen balance in normal men consuming marginally inadequate protein diets. *Clinical Science*, 90(3), 215-225.
- Medhus, A. W., Sandstad, O., Bredesen, J., and Husebye, E. (1999). Delay of gastric emptying by duodenal intubation: Sensitive measurement of gastric emptying by the paracetamol absorption test. *Alimentary Pharmacology and Therapeutics*, 13(5), 609-620.
- Meisel, H. (1997a). Biochemical properties of bioactive peptides derived from milk proteins: Potential nutraceuticals for food and pharmaceutical applications. *Livestock Production Science*, 50(1-2), 125-138.
- Meisel, H. (1997b). Biochemical properties of regulatory peptides derived from milk proteins. *Biopolymers*, 43(2), 119-128.
- Meisel, H. (2004). Multifunctional peptides encrypted in milk proteins. *Biofactors*, 21(1-4), 55-61.
- Meisel, H. (2005). Biochemical properties of peptides encrypted in bovine milk proteins. *Current Medicinal Chemistry*, 12(16), 1905-1919.
- Meisel, H., and Fitzgerald, R. J. (2000). Opioid peptides encrypted in intact milk protein sequences. *British Journal of Nutrition*, 84, S27-S31.
- Meisel, H., and Fitzgerald, R. J. (2003). Biofunctional peptides from milk proteins: Mineral binding and cytomodulatory effects. *Current Pharmaceutical Design*, 9(16), 1289-1295.
- Meisel, H., and Schlimme, E. (1990). Milk Proteins: Precursors of bioactive peptides. *Trends in Food Science and Technology*, 1, 41-43.
- Meredith, D., & Boyd, C. A. R. (2000). Structure and function of eukaryotic peptide transporters. *Cellular and Molecular Life Sciences*, 57(5), 754-778.

- Metges, C. C. (2000). Contribution of microbial amino acids to amino acid homeostasis of the host. *Journal of Nutrition*, 130(7), 1857S-1864S.
- Metges, C. C., Eberhard, M., and Petzke, K. J. (2006). Synthesis and absorption of intestinal microbial lysine in humans and non-ruminant animals and impact on human estimated average requirements of dietary lysine. *Current Opinion in Clinical Nutrition and Metabolic Care*, 9(1), 37-41.
- Metges, C. C., El-Khoury, A. E., Henneman, L., Petzke, K. J., Grant, I., Bedri, S., Pereira, P. P., Ajami, A. M., Fuller, M. F., and Young, V. R. (1999a). Availability of intestinal microbial lysine for whole body lysine homeostasis in human subjects. *American Journal of Physiology-Endocrinology and Metabolism*, 277(4), E597-E607.
- Metges, C. C., Petzke, K. J., Bedri, S., Fuller, M. F., and Young, V. R. (1997). The quantitative contribution of microbial lysine to lysine flux in healthy male subjects. *Journal of the Federation of American Societies for Experimental Biology.*, 11, A149.
- Metges, C. C., Petzke, K. J., El-Khoury, A. E., Henneman, L., Grant, I., Bedri, S., Regan M. M., Fuller, M. F., and Young, V. R. (1999b). Incorporation of urea and ammonia nitrogen into ileal and fecal microbial proteins and plasma free amino acids in normal men and ileostomates. *American Journal of Clinical Nutrition*, 70(6), 1046-1058.
- Metges, C. C., Petzke, K. J., and Hennig, U. (1996). Gas chromatography combustion isotope ratio mass spectrometric comparison of N-acetyl- and N-pivaloyl amino acid esters to measure N-15 isotopic abundances in physiological samples: A pilot study on amino acid synthesis in the upper gastro-intestinal tract of minipigs. *Journal of Mass Spectrometry*, 31(4), 367-376.
- Metzner, L., Natho, K., Zebisch, K., Dorn, M., Bosse-Doenecke, E., Ganapathy, V., and Brandsch, M. (2008). Mutational analysis of histidine residues in the human proton-coupled amino acid transporter PAT1. *Biochimica Et Biophysica Acta-Biomembranes*, 1778(4), 1042-1050.
- Mierke, D. F., Nossner, G., Schiller, P. W., and Goodman, M. (1990). Morphiceptin analogs containing 2-aminocyclopentane carboxylic-acid as a peptidomimetic for proline. *International Journal of Peptide and Protein Research*, 35(1), 35-45.

- Migliore-Samour, D., Floc'h, F., & Jollès, P. (1989). Biologically-active casein peptides implicated in immunomodulation. *Journal of Dairy Research*, 56(3), 357-362.
- Miguel, M., Muguerza, B., Sanchez, E., Delgado, M. A., Recio, I., Ramos, M., and Aleixandre, M. A. (2005). Changes in arterial blood pressure in hypertensive rats caused by long-term intake of milk fermented by *Enterococcus faecalis* CECT 5728. *British Journal of Nutrition*, 94(1), 36-43.
- Mihatsch, W. A., Hogel, J., & Pohlandt, F. (2001). Hydrolysed protein accelerates the gastrointestinal transport of formula in preterm infants. *Acta Paediatrica*, 90(2), 196-198.
- Miller, E. R., and Ullrey, D. E. (1987). The pig as a model for human-nutrition. *Annual Review of Nutrition*, 7, 361-382.
- Millipore. (2001). Amicon ultra: Centrifugal filter devices for the concentration and purification of biological samples. Retrieved 1st October, 2009, from http://teachline.ls.huji.ac.il/72682/Booklets/AMICON_ultracentrifuge_devices.pdf
- Millipore. (2009). Centriprep ® Centrifugal Filter Devices User Guide. Retrieved 9th June, 2009, from [http://www.millipore.com/userguides.nsf/a73664f9f981af8c852569b9005b4eee/c02dc7dfe67e983a852575c900506a7b/\\$FILE/PR03222.pdf](http://www.millipore.com/userguides.nsf/a73664f9f981af8c852569b9005b4eee/c02dc7dfe67e983a852575c900506a7b/$FILE/PR03222.pdf)
- Millward, D. J., Garlick, P. J., James, W. P. T., Sender, P. M., and Waterlow, J. C. (1976). Protein turnover. In D. J. A. Cole, K. N. Boorman, P. J. Buttery, D. Lewis, R. J. Neale and H. Swan (Eds.), *Protein Metabolism and Nutrition*. (Vol. 16, pp. 49-69.). London: European Association of Animal Production, Butterworths.
- Mizuno, S., Matsuura, K., Gotou, T., Nishimura, S., Kajimoto, O., Yabune, M., Kajimoto, Y., and Yamamoto, N. Antihypertensive effect of casein hydrolysate in a placebo-controlled study in subjects with high-normal blood pressure and mild hypertension. *British Journal of Nutrition*, 94(1), 84-91.
- Modigliani, R., Rambaud, J. C., and Bernier, J. J. (1973). Method of intraluminal perfusion of human small-intestine .1. Principle and technique. *Digestion*, 9(2), 176-192.
- Mokrasch, L. C., and McGilvery, R. W. (1956). Purification and Properties of Fructose-1,6-Diphosphatase. *Journal of Biological Chemistry*, 221(2), 909-917.

- Mollereau, C., Parmentier, M., Mailleux, P., Butour, J. L., Moisand, C., Chalon, P., Caput, D., Vassart, G. and Meunier, J. C. (1994). Or11, a novel member of the opioid receptor family - cloning, functional expression and localization. *Febs Letters*, 341(1), 33-38.
- Molmenti, E. P., Perlmutter, D. H., and Rubin, D. C. (1993). Cell specific expression of anti-trypsin in human intestinal epithelium. *Journal of Clinical Investigation*, 92, 2022-2034.
- Montagne, L., Piel, C., and Lalles, J. P. (2004). Effect of diet on mucin kinetics and composition: Nutrition and health implications. *Nutrition Reviews*, 62(3), 105-114.
- Montagne, L., Toullec, R., Formal, M., and Lalles, J. P. (2000). Influence of dietary protein level and origin on the flow of mucin along the small intestine of the preruminant calf. *Journal of Dairy Science*, 83(12), 2820-2828.
- Moon, H. W., Whipp, S. C., and Baetz, A. L. (1971). Comparative effects of enterotoxins from E.coli and V.cholera on rabbit and swine small intestine. *Laboratory Investigations*, 25, 133-140.
- Moore, W. E. C., and Holdeman, L. V. (1974). Human fecal flora: the normal flora of 20 Japanese-Hawaiians. *Applied Microbiology*, 27, 961- 979.
- Moosker, M. S. (1985). Organisation, chemistry and assembly of the cytoskeletal apparatus of the intestinal brush border. *Annual Review of Cell Division Biology*, 1, 209-241.
- Morley, J. E. (1982). Food Peptides - a New Class of Hormones. *Jama-Journal of the American Medical Association*, 247(17), 2379-2380.
- Mosenthin, R., Sauer, W. C., Blank, R., Huisman, J., and Fan, M. Z. (2000). The concept of digestible amino acids in diet formulation for pigs. *Livestock Production Science*, 64(2-3), 265-280.
- Mosenthin, R., Sauer, W. C., and De Lange, C. F. M. (1992). Tracer Studies of Urea Kinetics in Growing Pigs .1. The Effect of Intravenous-Infusion of Urea on Urea Recycling and the Site of Urea Secretion into the Gastrointestinal-Tract. *Journal of Animal Science*, 70(11), 3458-3466.
- Moughan, P. J. (2005). Dietary protein quality in humans - An overview. *Journal of Aoac International*, 88(3), 874-876.
- Moughan, P. J. (2003). Amino acid availability: Aspects of chemical analysis and bioassay methodology. *Nutrition Research Reviews*, 16(2), 127-141.

- Moughan, P. J., Birtles, M. J., Cranwell, P. D., Smith, W. C., and Pedraza, M. (1992a). The piglet as a model animal for studying aspects of digestion and absorption in milk-fed human infants. *World review of nutrition and dietetics* 67, 40-113.
- Moughan, P. J., BATTERY, P. J., Essex, C. P., and Soar, J. B. (1992b). Evaluation of the isotope-dilution technique for determining ileal endogenous nitrogen-excretion in the rat. *Journal of the Science of Food and Agriculture*, 58(2), 165-172.
- Moughan, P. J., Butts, C. A., Rowan, A. M., and Deglaire, A. (2005b). Dietary peptides increase endogenous amino acid losses from the gut in adults. *American Journal of Clinical Nutrition*, 81(6), 1359-1365.
- Moughan, P. J., Butts, C. A., van Wijk, H., Rowan, A. M., and Reynolds, G. W. (2005a). An acute ileal amino acid digestibility assay is a valid procedure for use in human ileostomates. *Journal of Nutrition*, 135(3), 404-409.
- Moughan, P. J., Cranwell, P. D., Darragh, A. J., and Rowan, A. M. (1994). The domestic pig as a model animal for studying digestion in humans In W. B. Souffrant and H. Hagemester (Eds.), *Vith International Symposium on Digestive Physiology in Pigs, Proceedings, Vols 1 and 2* (pp. 389-396).
- Moughan, P. J., Darragh, A. J., Smith, W. C., and Butts, C. A. (1990a). Perchloric and trichloroacetic acids as precipitants of protein in endogenous ileal digesta from the rat. *Journal of the Science of Food and Agriculture*, 52(1), 13-21.
- Moughan, P. J., Gall, M. P. J., and Rutherford, S. M. (1996). Absorption of lysine and deoxyketosyllysine in an early-Maillard browned casein by the growing pig. *Journal of Agricultural and Food Chemistry*, 44(6), 1520-1525.
- Moughan, P. J., and Rutherford, S. M. (1990b). Endogenous flow of total lysine and other amino-acids at the distal ileum of the protein-fed or peptide-fed rat - the chemical labeling of gelatin protein by transformation of lysine to homoarginine. *Journal of the Science of Food and Agriculture*, 52(2), 179-192.
- Moughan, P. J., and Schuttert, G. (1991). Composition of nitrogen-containing fractions in digesta from the distal ileum of pigs fed a protein-free diet. *Journal of Nutrition*, 121(10), 1570-1574.
- Moughan, P. J., Schuttert, G., and Leenaars, M. (1992). Endogenous amino-acid flow in the stomach and small-intestine of the young growing pig. *Journal of the Science of Food and Agriculture*, 60(4), 437-442.

- Moughan, P. J., and Smith, W. C. (1984). Assessment of a balance of dietary amino-acids required to maximize protein-utilization in the growing-pig (20-80 Kg Liveweight). *New Zealand Journal of Agricultural Research*, 27(3), 341-347.
- Moughan, P. J., and Smith, W. C. (1985). Determination and assessment of apparent ileal amino-acid digestibility coefficients for the growing-pig. *New Zealand Journal of Agricultural Research*, 28(3), 365-370.
- Moughan, P. J., Souffrant, W. B., and Hodgkinson, S. M. (1998). Physiological approaches to determining gut endogenous amino acid flows in the mammal. *Archives of Animal Nutrition-Archiv Fur Tierernahrung*, 51(2-3), 237-252.
- Mroz, Z., Bakker, G. C. M., Dekker, J., Jongbloed, A. W., and Jongbloed, R. (1994). *Application of the steered ileo-cecal valve cannulation (SICV) technique to measure digesta kinetics and ileal digestibility in pigs fed high fibrous diets.* Paper presented at the VIth International Symposium on Digestive Physiology in Pigs, Dummerdorf, Germany.
- Mroz, Z., Bakker, G. C. M., Jongbloed, A. W., Dekker, R. A., Jongbloed, R., and vanBeers, A. (1996). Apparent digestibility of nutrients in diets with different energy density, as estimated by direct and marker methods for pigs with or without ileo-cecal cannulas. *Journal of Animal Science*, 74(2), 403-412.
- Muguerza, B., Ramos, M., Sanchez, E., Manso, M. A., Miguel, M., Aleixandre, A., Delgado, M. A., and Recio, I. (2006). Antihypertensive activity of milk fermented by *Enterococcus faecalis* strains isolated from raw milk. *International Dairy Journal*, 16(1), 61-69.
- Mulherkar, R., Rao, R. S., and Wagle, A. S., Patki, V., and Deo, M. G. (1993). Enhancing factor, a Paneth cell specific protein from mouse small intestine: Predicted amino acid sequences from RT-PCR amplified cDNA and its expression. *Biochemical and Biophysical Research Communications*, 195, 1254-1263.
- Mullally, M. M., Meisel, H., & Fitzgerald, R. J. (1996). Synthetic peptides corresponding to alpha-lactalbumin and beta-lactoglobulin sequences with angiotensin-I-converting enzyme inhibitory activity. *Biological Chemistry Hoppe-Seyler*, 377(4), 259-260.
- Munck, B. G., and Munck, L. K. (1994). Phenylalanine transport in rabbit small-intestine *Journal of Physiology-London*, 480, 99-107.

- Munro, H. N. and Goldberg, D. M. (1964): The effect of protein intake on the protein and nucleic acid metabolism of the intestinal mucosal cell. In: H. N. Munro (Ed.), *The Role of the Gastrointestinal Tract in Protein Metabolism*. Blackwell Scientific Publications, Oxford, England, pp. 189-98.
- Muramatsu, T. (1990). Nutrition and whole-body protein turnover in the chicken in relation to mammalian species. *Nutrition Research Reviews*, 3, 211-228.
- Murphy, J. M., Murch, S. J., and Ball, R. O. (1996). Proline is synthesized from glutamate during intragastric infusion but not during intravenous infusion in neonatal piglets. *Journal of Nutrition*, 126(4), 878-886.
- Nakamura, Y., Yamamoto, N., Sakai, K., and Takano, T. (1995). Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors to angiotensin I-converting enzyme. *Journal of Dairy Science*, 78(6), 1253-1257.
- Nasset, E. S. (1957). Role of the digestive tract in the utilisation of protein and amino acids. *Journal of the American Medical Association*, 164, 172-178.
- Nasset, E. S., and Ju, J. S. (1961). Mixture of endogenous and exogenous protein in the alimentary tract. *Journal of Nutrition*, 74, 461-465.
- Neurath, A. R. (1966). Interference of sodium ethylenediaminetetraacetate in determination of proteins and its elimination. *Experientia*, 22(5), 290.
- Neutra, M. R., Phillips, T. L., Mayer, E. L., and Fishkind, D. J. (1987). Transport of membrane-bound macromolecules by M-cells in follicle-associated epithelium of rabbit peyer patch. *Cell and Tissue Research*, 247(3), 537-546.
- Neutra, M. R., Pringault, E., and Kraehenbuhl, J. P. (1996). Antigen sampling across epithelial barriers and induction of mucosal immune responses. *Annual Review of Immunology*, 14, 275-300.
- Neutra, M. R., Wilson, J. M., Weltzin, R. A., and Kraehenbuhl, J. P. (1988). Membrane domains and macromolecular transport in intestinal epithelial-cells. *American Review of Respiratory Disease*, 138(6), S10-S16.
- Newey, H., and Smyth, D. H. (1959). The intestinal absorption of some dipeptides. *Journal of Physiology-London*, 145(1), 48-56.
- Nishimura, E., Buchan, A. M. J., and McIntosh, C. H. S. (1986). Autoradiographic localization of mu-type and delta-type opioid receptors in the gastrointestinal-tract of the rat and guinea-pig. *Gastroenterology*, 91(5), 1084-1094.

- Noah, L., Guillon, F., Bouchet, B., Buleon, A., Molis, C., Gratas, M., and Champ, M. (1998). Digestion of carbohydrate from white beans (*Phaseolus vulgaris* L.) in healthy humans. *Journal of Nutrition*, 128(6), 977-985.
- Nock, B., Giordano, A. L., Moore, B. W., and Cicero, T. J. (1993). Properties of the putative epsilon opioid receptor - identification in rat, guinea-pig, cow, pig and chicken brain. *Journal of Pharmacology and Experimental Therapeutics*, 264(1), 349-359.
- Nyachoti, C. M., de Lange, C. F. M., and Schulze, H. (1997a). *The homoarginine method for determining true ileal lysine digestibilities in casein, barley and canola meal fed to growing pigs*. Paper presented at the Proceedings of the VIIIth International Symposium on Digestive Physiology in Pigs, Saint Malo, France.
- Nyachoti, C. M., de Lange, C. F. M., and Schulze, H. (1997b). Estimating endogenous amino acid flows at the terminal ileum and true ileal amino acid digestibilities in feedstuffs for growing pigs using the homoarginine method. *Journal of Animal Science*, 75(12), 3206-3213.
- Nyachoti, C. M., Omogbenigun, F. O., Rademacher, M., and Blank, G. (2006). Performance responses and indicators of gastrointestinal health in early-weaned pigs fed low-protein amino acid-supplemented diets. *Journal of Animal Science*, 84(1), 125-134.
- Nyberg, F., Sanderson, K., and Glamsta, E. L. (1997). The haemorphins: A new class of opioid peptides derived from the blood protein haemoglobin. *Biopolymers*, 43(2), 147-156.
- O'Keefe, B. R., Beutler, J. A., Cardellina, J. H., Gulakowski, R. J., Krepps, B. L., McMahon, J. B., *et al.* (1997). Isolation and characterization of niphatevirin, a human-immunodeficiency-virus-inhibitory glycoprotein from the marine sponge *Niphates erecta*. *European Journal of Biochemistry*, 245(1), 47-53.
- Ohara, S., Watanabe, T., and Hotta, K. (1997). Comparative Study of Carbohydrate Portion of Gastrointestinal Mucins Using Enzyme-linked Lectin-Binding Assay (ELLA). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 116(2), 167-172.
- Ouellette, A. J. (1999). Mucosal immunity and inflammation. IV. Paneth cell antimicrobial peptides and the biology of the mucosal barrier. *American Journal of Physiology*, 40, G257-G261.

- Paganelli, R., and Levinsky, R. J. (1980). Solid-phase radioimmunoassay for detection of circulating food protein antigens in human-serum. *Journal of Immunological Methods*, 37(3-4), 333-341.
- Paintal, A. S. (1957). Responses from mucosal mechanoreceptors in the small intestine of the cat. *Journal of Physiology-London*, 139(3), 353-368.
- Paroli, E. (1988). Opioid peptides from food (the exorphins). *World Review of Nutrition and Dietetics*, 55, 58-97.
- Partanen, K., Jalava, T., & Valaja, I. (2007). Effects of a dietary organic acid mixture and of dietary fibre levels on ileal and faecal nutrient apparent digestibility, bacterial nitrogen flow, microbial metabolite concentrations and rate of passage in the digestive tract of pigs. *Animal*, 1(3), 389-401.
- Paterson, S. J., Robson, L. E., and Kosterlitz, H. W. (1983). Classification of opioid receptors. *British Medical Bulletin*, 39(1), 31-36.
- Patterson, B. W., Carraro, F., Klein, S., and Wolfe, R. R. (1995). Quantification of incorporation of N-15 ammonia into plasma amino-acids and urea. *American Journal of Physiology-Endocrinology and Metabolism*, 269(3), E508-E515.
- Pauling, L., and Corey, R. B. (1951). Configuration of polypeptide chains. *Nature*, 168(4274), 550-551.
- Pauling, L., Corey, R. B., and Branson, H. R. (1951). The structure of proteins - 2 hydrogen-bonded helical configurations of the polypeptide chain. *Proceedings of the National Academy of Sciences of the United States of America*, 37(4), 205-211.
- Payne, W. L., Combs, G. F., Kifer, R. R., and Snyder, D. G. (1968). Investigation of protein quality - ileal recovery of amino acids. *Federation Proceedings*, 27, 199-203.
- Pedersen, C., Boisen, S., and Fernandez, J. A. (2002). Studies on the effect of dietary crude protein supply on the composition of ileal endogenous crude protein loss in growing pigs. *Acta Agriculturae Scandinavica Section a-Animal Science*, 52(3), 141-149.
- Pellegrino, J. (2000). Filtration and ultrafiltration equipment and techniques. *Separation and Purification Methods*, 29(1), 91-118.
- Perez-Vilar, J., and Hill, R. L. (1999). The structure and assembly of secreted mucins. *Journal of Biological Chemistry*, 274(45), 31751-31754.

- Pert, C. B., and Snyder, S. H. (1973). Opiate Receptor - Demonstration in Nervous-Tissue. *Science*, 179(4077), 1011-1014.
- Petrilli, P., Picone, D., Caporale, C., Addeo, F., Auricchio, S., and Marino, G. (1984). Does casomorphin have a functional-role. *Febs Letters*, 169(1), 53-56.
- Phillips, R. J., and Powley, T. L. (2000). Tension and stretch receptors in gastrointestinal smooth muscle: Re-evaluating vagal mechanoreceptor electrophysiology. *Brain Research Reviews*, 34(1-2), 1-26.
- Phillipson, A. T. (1952). The Passage of digesta from the abomasum of sheep. *Journal of Physiology-London*, 116(1), 84-97.
- Picard, M., Bertrand, S., Genin, F., and Maillard, R. A. (1984). Digestibilité des acides aminés: Intérêt de la technique du shunt iléorectal chez le porc. *Journal Rech Porcine France*, 16, 355-360.
- Picariello, G., Ferranti, P., Fierro, O., Mamone, G., Caira, S., Di Luccia, A., Monica, S., and Addeo, F. (2010). Peptides surviving the simulated gastrointestinal digestion of milk proteins: Biological and toxicological implications. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, 878(3-4), 295-308.
- Piel, C., Montagne, L., Salgado, P., and Lalles, J. P. (2004). Estimation of ileal output of gastro-intestinal glycoprotein in weaned piglets using three different methods. *Reproduction Nutrition Development*, 44(5), 419-435.
- Pihlanto-Leppala, A. (2000). Bioactive peptides derived from bovine whey proteins: Opioid and ace-inhibitory peptides. *Trends in Food Science and Technology*, 11(9-10), 347-356.
- Poppe, S., and Meier, H. (1977). Assessment of true digestibility of amino acids and its value in swine. *Protein Metabolism and Nutrition. Proceedings of the 2nd International Symposium on Protein Metabolism and Nutrition, The Netherlands, May 1977.* (Vol. 22, pp. 79-81): E.A.A.P Publication. Publishing Centre for Agricultural Publishing and Documentation.
- Potkins, Z. V., Lawrence, T. L. J., & Thomlinson, J. R. (1991a). Effects of structural and nonstructural polysaccharides in the diet of the growing pig on gastric-emptying rate and rate of passage of digesta to the terminal ileum and through the total gastrointestinal-tract. *British Journal of Nutrition*, 65(3), 391-413.
- Potkins, Z. V., Lawrence, T. L. J., and Thomlinson, J. R. (1991b). Effects on ileal apparent digestibility in the growing pig of replacing barley with bran, oatmeal

- by-product, guar gum and pectin. *Animal Feed Science and Technology*, 35(1-2), 171-179.
- Powell, D. W. (1986). Ion and water transport in the intestine. In Andreoli, Hoffman, Fanestil and W. H. Schultz (Eds.), *Physiology of Membrane Disorders*. (2nd ed., pp. 559-596). New York: Plenum Incorporated.
- Prinz, C., Tegenfeldt, J. O., Austin, R. H., Cox, E. C., and Sturm, J. C. (2002). Bacterial chromosome extraction and isolation. *Lab on a Chip*, 2(4), 207-212.
- Purser, D. B., and Buechler, S. M. (1966). Amino acid composition of rumen organisms. *Journal of Dairy Science*, 49(1), 81-and.
- Quigley, J. D., and Schwab, C. G. (1988). Comparison of D-alanine and diaminopimelic acid as bacterial markers in young calves. *Journal of Animal Science*, 66(3), 758-763.
- Radcliffe, J. S., Rice, J. P., Pleasant, R. S., and Apgar, G. A. (2005). Technical Note: Improved technique for fitting pigs with steered ileocecal valve cannulas. *Journal of Animal Science*, 83(7), 1563-1567.
- Rambaud, J. C., Duprey, F., Nouel, O., Hostein, J., Delpech, B., and Bernier, J. J. (1981). Assessment of the accuracy of segmental perfusion under an occluding balloon for measuring the intra-jejunal secretion of albumin and immunoglobulin-A. *Gut*, 22(5), 371-375.
- Ravindran, V., and Hendriks, W. H. (2004). Endogenous amino acid flows at the terminal ileum of broilers, layers and adult roosters. *Animal Science*, 79, 265-271.
- Ravindran, V., Imbeah, M., Angkanaporn, K., and Bryden, W. L. (1996). Guanidination of lysine in cottonseed protein. *Journal of Agricultural and Food Chemistry*, 44(7), 1812-1815.
- Ravindran, V., Morel, P. C. H., Rutherford, S. M., and Thomas, D. V. (2009). Endogenous flow of amino acids in the avian ileum as influenced by increasing dietary peptide concentrations. *British Journal of Nutrition*, 101(6), 822-828.
- Read, L. C., Lord, A. P. D., Brantl, V., and Koch, G. (1990). Absorption of beta-casomorphins from autoperfused lamb and piglet small-intestine. *American Journal of Physiology*, 259(3), G443-G452.
- Read, N. W., Aljanabi, M. N., Bates, T. E., and Barber, D. C. (1983). Effect of gastrointestinal intubation on the passage of a solid meal through the stomach and small-intestine in humans. *Gastroenterology*, 84(6), 1568-1572.

- Redman, D. R., King, N. B., Henderickx, H. K., and Teague, H. S. (1964). Cecal fistulation of pig using 2 forms of indwelling cannulas. *Journal of Animal Science*, 23(4), 1032-1035.
- Reeds, P. J., and Burrin, D. G. (2000). The gut and amino acid homeostasis. *Nutrition*, 16(7-8), 666-668.
- Reeds, P. J., Burrin, D. G., Stoll, B., Jahoor, F., Wykes, L., Henry, J., and Frazer, M. E. (1997). Enteral glutamate is the preferential source for mucosal glutathione synthesis in fed piglets. *American Journal of Physiology-Endocrinology and Metabolism*, 273(2), E408-E415.
- Reeds, P. J., Burrin, D. G., Stoll, B., and van Goudoever, J. B. (1999). Consequences and regulation of gut metabolism. In G. E. Lobley, A. White and J. C. MacRae (Eds.), *Protein Metabolism and Nutrition* (pp. 127-153).
- Reisner, A. H., Nemes, P., and Bucholtz, C. (1975). Use of Coomassie brilliant blue G250 perchloric-acid solution for staining in electrophoresis and isoelectric focusing on polyacrylamide gels. *Analytical Biochemistry*, 64(2), 509-516.
- Reynal, S. M., and Broderick, G. A. (2009). Technical note: A new high-performance liquid chromatography purine assay for quantifying microbial flow. *Journal of Dairy Science*, 92(3), 1177-1181.
- Reynal, S. M., Broderick, G. A., and Bearzi, C. (2005). Comparison of four markers for quantifying microbial protein flow from the rumen of lactating dairy cows. *Journal of Dairy Science*, 88(11), 4065-4082.
- Ririe, D. G., Roberts, P. R., Shouse, M. N., and Zaloga, G. P. (2000). Vasodilatory actions of the dietary peptide carnosine. *Nutrition*, 16(3), 168-172.
- Robert, M. C., Razaname, A., Mutter, M., and Juillerat, M. A. (2004). Peptides derived from sodium caseinate hydrolysates produced by *Lactobacillus helveticus* NCC 2765. *Journal of Agricultural and Food Chemistry*, 52(23), 6923-6931.
- Roberton, D. M., Paganelli, R., Dinwiddie, R., and Levinsky, R. J. (1982). Milk antigen absorption in the preterm and term neonate. *Archives of Disease in Childhood*, 57(5), 369-372.
- Roberts, P. R., Burney, J. D., Black, K. W., and Zaloga, G. P. (1999). Effect of chain length on absorption of biologically active peptides from the gastrointestinal tract. *Digestion*, 60(4), 332-337.
- Robinson, P. H., Fadel, J. G., and Ivan, M. (1996). Critical evaluation of diaminopimelic acid and ribonucleic acid as markers to estimate rumen pools

- and duodenal flows of bacterial and protozoal nitrogen. *Canadian Journal of Animal Science*, 76(4), 587-597.
- Robson, R. M., Goll, D. E., and Temple, M. J. (1968). Determination of proteins in tris buffer by Biuret reaction. *Analytical Biochemistry*, 24(2), 339-341.
- Roden, L., and Horowitz, M. I. (1978). Structure and biosynthesis of connective tissue proteoglycans. In M. I. Horowitz and W. Pigman (Eds.), *The glycoconjugates*. (Vol. II, pp. 3-71). New York: Academic Press.
- Rodriguez, J. V., Dubin, M., Carrillo, M. C., and Garay, E. A. R. (1982). Influence of a protein-free diet on bile secretion in the rat. *Nutrition Reports International*, 26(1), 147-155.
- Rouanet, J. M., Infante, J. L. Z., Caporiccio, B., and Pejoan, C. (1990). Nutritional-value and intestinal effects of dipeptides and tripeptides - comparison with their issuing bovine plasma-protein in rats. *Annals of Nutrition and Metabolism*, 34(3), 175-182.
- Roussel, P., and Delmotte, P. (2004). The diversity of epithelial secreted mucins. *Current Organic Chemistry*, 8(5), 413-437.
- Rowan, A. M., Moughan, P. J., and Wilson, M. N. (1992). The flows of deoxyribonucleic-acid and diaminopimelic acid and the digestibility of dietary fiber components at the terminal ileum, as indicators of microbial activity in the upper digestive-tract of ileostomized pigs. *Animal Feed Science and Technology*, 36(1-2), 129-141.
- Rowan, A. M., Moughan, P. J., Wilson, M. N., Maher, K., and Tasman-Jones, C. (1994). Comparison of the ileal and fecal digestibility of dietary amino-acids in adult humans and evaluation of the pig as a model animal for digestion studies in man. *British Journal of Nutrition*, 71(1), 29-42.
- Rubio, L. A. (2003). Determination of diaminopimelic acid in rat feces by high-performance liquid chromatography using the Pico Tag method. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, 784(1), 125-129.
- Rutherford-Markwick, K. J., and Moughan, P. J. (2005). Bioactive peptides derived from food. *Journal of Aoac International*, 88(3), 955-966.
- Rutherford, S. M., & Moughan, P. J. (1990). Guanidination of lysine in selected dietary proteins. *Journal of Agricultural and Food Chemistry*, 38(1), 209-211.

- Rutherford, S. M., and Moughan, P. J. (1998). The digestible amino acid composition of several milk proteins: Application of a new bioassay. *Journal of Dairy Science*, 81(4), 909-917.
- Sambrook, I. E. (1981). Studies on the flow and composition of bile in growing pigs. *Journal of the Science of Food and Agriculture*, 32(8), 781-791.
- Sanderson, I. R., and Walker, W. A. (1993). Uptake and transport of macromolecules by the intestine: Possible role in clinical disorders. *Gastroenterology*, 104, 622-639.
- Sanderson, I. R., and Walker, W. A. (1999). In R. Ogra and J. e. a. Mestecky (Eds.), *Handbook of Mucosal Immunology* (2nd ed., pp. 5-17). San Diego: Academic Press.
- Satake, M., Enjoh, M., Nakamura, Y., Takano, T., Kawamura, Y., Arai, S., and Shimizu, M. (2002). Transepithelial transport of the bioactive tripeptide, Val-Pro-Pro, in human intestinal Caco-2 cell monolayers. *Bioscience Biotechnology and Biochemistry*, 66(2), 378-384.
- Satchithanandam, S., Klurfeld, D. M., Calvert, R. J., and Cassidy, M. M. (1996). Effects of dietary fibers on gastrointestinal mucin in rats. *Nutrition Research*, 16(7), 1163-1177.
- Satoh, M., and Minami, M. (1995). Molecular pharmacology of the opioid receptors. *Pharmacology and Therapeutics*, 68(3), 343-364.
- Sauer, W., and Ozimek, L. (1986). Digestibility of amino acids in swine: Results and their practical application. A review. *Livestock Production Science*, 15, 367-369.
- Sauer, W. C., Dugan, M. E. R., de Lange, C. F. M., Imbeah, M., and Mosenthin, R. (1989). Considerations in methodology for the determination of amino acid digestibilities in feedstuffs for pigs. In M. Friedmann (Ed.), *Absorption and Utilization of Amino Acids* (Vol. III, pp. 217-230). Boca Raton, Florida.: CRC Press Inc.
- Schaafsma, G. (2009). Safety of protein hydrolysates, fractions thereof and bioactive peptides in human nutrition. *European Journal of Clinical Nutrition*, 63(10), 1161-1168.
- Schafer, J. A., and Andreoli, T. E. (1986). Principles of water and non-electrolyte transport across membranes. In T. E. Andreoli, J. F. Hoffman, D. D. Fanestil

- and S. G. Schultz (Eds.), *Physiology of Membrane Disorders* (2nd ed., pp. 177-190). New York: Plenum Inc.
- Schaffner, W., and Weissman, C. (1973). Rapid, sensitive, and specific method for determination of protein in dilute-solution. *Analytical Biochemistry*, 56(2), 502-514.
- Schaible, U. E., and Kaufmann, S. H. E. (2005). A nutritive view on the host-pathogen interplay. *Trends in Microbiology*, 13(8), 373-380.
- Schauer, R. (1982). Chemistry, metabolism, and biological functions of sialic acids. *Advances in Carbohydrate Chemistry and Biochemistry*, 40, 131-234.
- Schedl, H. P., and Clifton, J. A. (1961). Kinetics of intestinal absorption in man - normal subjects and patients with sprue. *Journal of Clinical Investigation*, 40(6), 1079-1085.
- Schieber, A., Bruckner, H., and Ling, J. R. (1999). GC-MS analysis of diaminopimelic acid stereoisomers and amino acid enantiomers in rumen bacteria. *Biomedical Chromatography*, 13(1), 46-50.
- Schleife, K. H., and Kandler, O. (1967). Chemical composition of cell wall of *streptococci*. 1. Amino acid sequence of mureins of *Str thermophilus* and *Str faecalis*. *Archiv Fur Mikrobiologie*, 57(4), 335-364.
- Schlimme, E., & Meisel, H. (1995). Bioactive peptides derived from milk-proteins - structural, physiological and analytical aspects. *Nahrung-Food*, 39(1), 1-20.
- Schloss, B. (1951). Colorimetric determination of glucosamine. *Analytical Chemistry*, 23(9), 1321-1325.
- Schmelzer, C. E. H., Schops, R., Reynell, L., Ulbrich-Hofmann, R., Neubert, R. H. H., and Raith, K. (2007). Peptic digestion of beta-casein. Time course and fate of possible bioactive peptides. *Journal of Chromatography A*, 1166(1-2), 108-115.
- Schmitz, M., Hagemeister, H., and Erbersdobler, H. F. (1991). Homoarginine labelling is suitable for determination of protein-absorption in miniature pigs. *Journal of Nutrition*, 121(10), 1575-1580.
- Schneeman, B. O. (1982). Digestive enzyme activities from the pancreas in response to diet. In J. P. Laplace, T. Corring and A. Rerat (Eds.), *Physiologie Digestive Chez le Porc* (pp. 125-131). Paris: Institut National de la Recherche Agronomique.

- Schoenhusen, U., Voigt, J., Hennig, U., Kuhla, S., Zitnan, R., and Souffrant, W. B. (2008). Bacterial D-alanine concentrations as a marker of bacterial nitrogen in the gastrointestinal tract of pigs and cows. *Veterinarni Medicina*, 53(4), 184-192.
- Schonhusen, U., Voigt, J., Freienbring, F., & Teuscher, F. (1995). Evaluation of different markers for determination of the microbial nitrogen flow into the duodenum of dairy-cows. *Archives of Animal Nutrition-Archiv Fur Tierernahrung*, 48(1-2), 147-158.
- Schrezenmeir, J., Korhonen, H., Williams, C., Gill, H. S., and Shah, N. (2000). Beneficial natural bioactive substances in milk and colostrum - Occurrence, biochemical and technological characteristics of bioactive substances - Physiological effects and potential health benefits - Foreword. *British Journal of Nutrition*, 84, S1-S1.
- Schröder, H. (1988). Investigations on the apparent digestibility of N-compounds in various sections of the intestinal tract in growing pigs. Doctor of Philosophy Thesis, Kiel University. Kiel, Germany.
- Schulte-Frohlinde, E., Schimid, R., Brantl, V., and Schusdziarra, V. (1994). Effect of bovine β -casomorphins-4-amide on gastrointestinal transit and pancreatic endocrine function in man. In V. Brantl and H. Teschemacher (Eds.), *β -Casomorphins and Related Peptides: Recent Developments*. (pp. 155–160): VCH-Weinheim, Germany.
- Schulz, R., Wuster, M., Krenss, H., and Herz, A. (1979). Selective receptors for beta-endorphin on the rat vas deferens. *Life Sciences*, 24, 843-850.
- Schulze, H., Saini, H. S., Huisman, J., Helsing, M., Vandenberg, W., and Verstegen, M. W. A. (1995). Increased nitrogen secretion by inclusion of soya lectin in the diets of pigs. *Journal of the Science of Food and Agriculture*, 69(4), 501-510.
- Schulze, H., Vanleeuwen, P., Verstegen, M. W. A., Huisman, J., Souffrant, W. B., and Ahrens, F. (1994). Effect of level of dietary neutral detergent fiber on ileal apparent digestibility and ileal nitrogen losses in pigs. *Journal of Animal Science*, 72(9), 2362-2368.
- Schulze, H., Vanleeuwen, P., Verstegen, M. W. A., and Vandenberg, J. W. O. (1995). Dietary level and source of neutral detergent fiber and ileal endogenous nitrogen flow in pigs. *Journal of Animal Science*, 73(2), 441-448.

- Schusdziarra, V., Henrichs, I., Holland, A., Klier, M., and Pfeiffer, E. F. (1981). Evidence for an effect of exorphins on plasma-insulin and glucagon-levels in dogs. *Diabetes*, 30(4), 362-364.
- Schusdziarra, V., Rewes, B., Lenz, N., Maier, V., and Pfeiffer, E. F. (1983a). Evidence for a role of endogenous opiates in postprandial somatostatin release. *Regulatory Peptides*, 6(4), 355-361.
- Schusdziarra, V., Schick, A., Delafuente, A., Specht, J., Klier, M., Brantl, V., and Pfeiffer, E. F. (1983b). Effect of beta-casomorphins and analogs on insulin release in dogs. *Endocrinology*, 112(3), 885-889.
- Schusdziarra, V., Schick, R., Delafuente, A., Holland, A., Brantl, V., and Pfeiffer, E. F. (1983c). Effect of beta-casomorphins on somatostatin release in dogs. *Endocrinology*, 112(6), 1948-1951.
- Seifter, S., Dayton, S., Novic, B., and Muntwyler, E. (1950). The Estimation of Glycogen with the Anthrone Reagent. *Archives of Biochemistry*, 25(1), 191-200.
- Sepulveda, F. V., and Smith, M. W. (1979). Different mechanisms for neutral amino-acid uptake by newborn pig colon. *Journal of Physiology-London*, 286(JAN), 479-490.
- Severin, S., and Xia, W. S. (2005). Milk biologically active components as nutraceuticals: Review. *Critical Reviews in Food Science and Nutrition*, 45(7-8), 645-656.
- Sghir, A., Gramet, G., Suau, A., Rochet, V., Pochart, P., and Dore, J. (2000). Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. *Applied and Environmental Microbiology*, 66(5), 2263-2266.
- Shah, N. P. (2000). Effects of milk-derived bioactives: An overview. *British Journal of Nutrition*, 84, S3-S10.
- Shepherd, E. J., Lister, N., Affleck, J. A., Bronk, J. R., Kellett, G. L., Collier, I. D., Bailey, P. D., and Boyd, C. A. R. (2002). Identification of a candidate membrane protein for the basolateral peptide transporter of rat small intestine. *Biochemical and Biophysical Research Communications*, 296(4), 918-922.
- Shi, B., Song, D. S., Xue, H., Li, J. H., Li, N., and Li, J. S. (2006). Abnormal expression of the peptide transporter PepT1 in the colon of massive bowel

- resection rat: A potential route for colonic mucosa damage by transport of fMLP. *Digestive Diseases and Sciences*, 51(11), 2087-2093.
- Shier J, Butler J, Lewis R. Hole's Human Anatomy and Physiology. 8th ed. New York: WCB McGraw-Hill; 1999.
- Shook, J. E., Lemcke, P. K., and Burks, T. F. (1986). Mu-opioid and kappa-opioid receptors regulate CCK actions in the guinea-pig ileum (Gpi). *Gastroenterology*, 91(4), 1068-1068.
- Siddons, R. C., Nolan, J. V., Beever, D. E., and Macrae, J. C. (1985). Nitrogen digestion and metabolism in sheep consuming diets containing contrasting forms and levels of N. *British Journal of Nutrition*, 54(1), 175-187.
- Silva, S. V., and Malcata, F. X. (2005). Caseins as source of bioactive peptides. *International Dairy Journal*, 15(1), 1-15.
- Simon, E. J., Hiller, J. M., and Edelman, I. (1973). Stereospecific binding of potent narcotic analgesic h-3)etorphine to rat-brain homogenate. *Proceedings of the National Academy of Sciences of the United States of America*, 70(7), 1947-1949.
- Simon, N., Lebot, N., Marie, D., Partensky, F., and Vaultot, D. (1995). Fluorescent in-situ hybridization with ribosomal-RNA-targeted oligonucleotide probes to identify small phytoplankton by flow-cytometry. *Applied and Environmental Microbiology*, 61(7), 2506-2513.
- Simon, O. (1989): Metabolism of proteins and amino acids. In: H.-D. Bock, B. O. Eggum, A. G. Low, O. Simon and T. Zebrowska (Eds.). Protein Metabolism in Farm Animals, pp. 273-366. Oxford University Press, Oxford, England.
- Skilton, G. A., Moughan, P. J., and Smith, W. C. (1988). Determination of endogenous amino-acid flow at the terminal ileum of the rat. *Journal of the Science of Food and Agriculture*, 44(3), 227-235.
- Sladen, G. E., and Dawson, A. M. (1969). Effects of flow rate on absorption of glucose in a steady state perfusion system in man. *Clinical Science*, 36(1), 133-and.
- Smith, K., Diggle, M. A., and Clarke, S. C. (2003). Comparison of commercial DNA extraction kits for extraction of bacterial genomic DNA from whole-blood samples. *Journal of Clinical Microbiology*, 41(6), 2440-2443.
- Smith, M. W., and James, P. S. (1976). Amino-acid transport by helicoidal colon of newborn pig. *Biochimica Et Biophysica Acta*, 419(2), 391-394.

- Smith, R. H., and McAllan, A. B. (1970). Nucleic acid metabolism in ruminant .2. Formation of microbial nucleic acids in rumen in relation to digestion of food nitrogen, and fate of dietary nucleic acids. *British Journal of Nutrition*, 24(2), 545.
- Snary, D., Allen, A., and Pain, R. H. (1974). Conformational-changes in gastric mucoproteins induced by cesium-chloride and guanidinium chloride. *Biochemical Journal*, 141(3), 641-646.
- Snook, J. T., and Meyer, J. H. (1964). Response of digestive enzymes to dietary protein. *Journal of Nutrition*, 82(4), 409-414.
- Souffrant, W. (1991). Endogenous nitrogen losses during digestion in pigs. In M. W. A. Verstegen, J. Huisman and L. A. den Hartog (Eds.), *Digestive Physiology in Pigs*. (pp. 147). Wageningen, The Netherlands.: EAAP Publication No. 54 PUDOC.
- Souffrant, W. B., Rerat, A., Laplace, J. P., Darcyvrillon, B., Kohler, R., Corring, T., and Gebhardt, G. (1993). Exogenous and endogenous contributions to nitrogen fluxes in the digestive-tract of pigs fed a casein diet .3. recycling of endogenous nitrogen. *Reproduction Nutrition Development*, 33(4), 373-382.
- Souffrant, W. B., Schumann, B., Matkowitz, R., and Gebhardt, G. (1985). Untersuchungen zur stickstoff- und aminosäurenresorption im dünn darm von wachsenden schweinen. I. mitteilung. *Archiv Fur Tierernahrung-Archives of Animal Nutrition*, 11, 781-789.
- Spichtig, V., Michaud, J., and Austin, S. (2010). Determination of sialic acids in milks and milk-based products. *Analytical Biochemistry*, 405(1), 28-40.
- Spiller, R. C. (2001). Effects of serotonin on intestinal secretion and motility. *Current Opinion in Gastroenterology*., 17, 99-103.
- Stanley, R. A., Lee, S. P., and Robertson, A. M. (1983). Heterogeneity in Gastrointestinal Mucins. *Biochimica Et Biophysica Acta*, 760(2), 262-269.
- Stanley, R. A., Ram, S. P., Wilkinson, R. K., and Robertson, A. M. (1986). Degradation of pig gastric and colonic mucins by bacteria isolated from the pig colon. *Applied and Environmental Microbiology*, 51(5), 1104-1109.
- Starkey, B. J., Snary, D., and Allen, A. (1974). Characterization of Gastric Mucoproteins Isolated by Equilibrium Density-Gradient Centrifugation in Cesium-Chloride. *Biochemical Journal*, 141(3), 633-639.

- Steadman, C. J., Talley, N. J., Phillips, S. F., and Zinsmeister, A. R. (1992). Selective 5-hydroxytryptamine type 3 receptor antagonism with ondansetron as treatment for diarrhea-predominant irritable bowel syndrome: A pilot study. *Mayo Clinic Proceedings*, 67, 732–738.
- Steendam, C. A., Verstegen, M. W. A., Tamminga, S., Boer, H., van 't End, M., Verstappen, B., Caine, W. R., and Visser, G. H. (2004). Route of tracer administration does not affect real endogenous nitrogen recovery measured with the N-15-isotope dilution technique in pigs fed rapidly digestible diets. *Journal of Nutrition*, 134(11), 3068-3075.
- Stepnik, M., and Kurek, M. (2002). The influence of bovine casein-derived exorphins on mast cells in rodents. *Revue Francaise D Allergologie Et D Immunologie Clinique*, 42(5), 447-453.
- Stern, J., Bruwer, M., Huber, F. X., and Decker-Baumann, C. (1999). Metabolic consequences after ileostomy. *Chirurg*, 70(6), 627-634.
- Stewart, G. S., and Smith, C. P. (2005). Urea nitrogen salvage mechanisms and their relevance to ruminants, non-ruminants and man. *Nutrition Research Reviews*, 18(1), 49-62.
- Stevens, B. R., Ross, H. J., and Wright, E. M. (1982). Multiple transport pathways for neutral amino-acids in rabbit jejunal brush-border vesicles. *Journal of Membrane Biology*, 66(3), 213-225.
- Stoll, B., Burrin, D. G., Henry, J., Jahoor, F., and Reeds, P. J. (1997). Phenylalanine utilization by the gut and liver measured with intravenous and intragastric tracers in pigs. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 273(6), G1208-G1217.
- Stoll, B., Burrin, D. G., Henry, J., Yu, H., Jahoor, F., and Reeds, P. J. (1999). Substrate oxidation by the portal drained viscera of fed piglets. *American Journal of Physiology-Endocrinology and Metabolism*, 277(1), E168-E175.
- Stoll, B., Henry, J., Reeds, P. J., Yu, H., Jahoor, F., and Burrin, D. G. (1998). Catabolism dominates the first-pass intestinal metabolism of dietary essential amino acids in milk protein-fed piglets. *Journal of Nutrition*, 128(3), 606-614.
- Sugimoto, K., Ichikawa, Y., and Nakamura, I. (1981). Absorption of protein molecules by the small-intestine of the bullfrog tadpole, *rana-catesbeiana*. *Journal of Experimental Zoology*, 215(1), 53-62.

- Summers, M., McBride, B. W., and Milligan, L. P. (Eds.). (1988). *Components of basal energy expenditure. In Aspects of Digestive Physiology in Ruminants*. Ithaca, New York: Comstock Publishing Associates.
- Svedberg, J., Dehaas, J., Leimenstoll, G., Paul, F., and Teschemacher, H. (1985). Demonstration of beta-casomorphin immunoreactive materials in invitro digests of bovine-milk and in small-intestine contents after bovine-milk ingestion in adult humans. *Peptides*, 6(5), 825-830.
- Sweeley, C. C., Bentley, R., Makita, M., and Wells, W. W. (1963). Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. *Journal of the American Chemical Society*, 85(16), 2497-2507.
- Sylvester, J. T., Karnati, S. K. R., Yu, Z. T., Morrison, M., and Firkins, J. L. (2004). Development of an assay to quantify rumen ciliate protozoal biomass in cows using real-time PCR. *Journal of Nutrition*, 134(12), 3378-3384.
- Synge, R. L. M. (1953). Note on the occurrence of diaminopimelic acid in some intestinal micro-organisms from farm animals. *Journal of General Microbiology*, 9(3), 407-409.
- Tabuchi, Y., Sugiyama, N., Horiuchi, T., Furuhashi, K., and Furusawa, M. (1997). Insulin stimulates production of glycoconjugate layers on the cell surface of gastric surface mucous cell line GSM06. *Digestion*, 58(1), 28-33.
- Taira, T., Hilakivi, L. A., Aalto, J., and Hilakivi, I. (1990). Effect of beta-casomorphin on neonatal sleep in rats. *Peptides*, 11(1), 1-4.
- Tamminga, S., Schulze, H., Vanbruchem, J., and Huisman, J. (1995). Nutritional significance of endogenous n-losses along the gastrointestinal-tract of farm-animals. *Archives of Animal Nutrition-Archiv Fur Tierernahrung*, 48(1-2), 9-22.
- Tani, S., Okuda, M., Morishige, R., and Tanaka, T. (1997). Gastric mucin secretion from cultured rat epithelial cells. *Biological and Pharmaceutical Bulletin*, 20(5), 482-485.
- Tenovuo, J. (2002). Antimicrobial agents in saliva - Protection for the whole body. *Journal of Dental Research*, 81(12), 807-809.
- Terada, T., and Inui, K. (2004). Peptide transporters: Structure, function, regulation and application for drug delivery. *Current Drug Metabolism*, 5(1), 85-94.
- Terada, T., Sawada, K., Saito, H., Hashimoto, Y., and Inui, K. I. (1999). Functional characteristics of basolateral peptide transporter in the human intestinal cell

- line Caco-2. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 276(6), G1435-G1441.
- Terada, T., Shimada, Y., Pan, X. Y., Kishimoto, K., Sakurai, T., Doi, R., Onodera, H., Katsura, T., Imamura, M. and Inui, K. I. (2005). Expression profiles of various transporters for oligopeptides, amino acids and organic ions along the human digestive tract. *Biochemical Pharmacology*, 70(12), 1756-1763.
- Teran-Ventura, E., Roca, M., Teresa Martin, M., Lourdes Abarca, M., Martinez, V., and Vergara, P. (2010). Characterization of Housing-Related Spontaneous Variations of Gut Microbiota and Expression of Toll-Like Receptors 2 and 4 in Rats. *Microbial Ecology*, 60(3), 691-702.
- Terenius, L. (1973). Characteristics of receptor for narcotic analgesics in synaptic plasma-membrane fraction from rat-brain. *Acta Pharmacologica Et Toxicologica*, 33(5-6), 377-384.
- Teschemacher, H. (2003). Opioid receptor ligands derived from food proteins. *Current Pharmaceutical Design*, 9(16), 1331-1344.
- Teschemacher, H., Koch, G., and Brantl, V. (1994). Milk protein derived atypical peptides and related compounds with opioid antagonist activity (review). In V. Brantl and H. Teschemacher (Eds.), *β -Casomorphins and Related Peptides: Recent Developments*. (pp. 3-17): VCH-Weinheim, Germany.
- Teschemacher, H., Koch, G., and Brantl, V. (1997). Milk protein-derived opioid receptor ligands. *Biopolymers*, 43(2), 99-117.
- Teschemacher, H., Umbach, M., Hamel, U., Praetorius, K., Ahnerthilger, G., Brantl, V., Lottspeich, F., and Henschen, A. (1986). No evidence for the presence of beta-casomorphins in human-plasma after ingestion of cows milk or milk-products. *Journal of Dairy Research*, 53(1), 135-138.
- Tettamanti, G. and Pigman, W. (1968). Purification and Characterization of Bovine and Ovine Submaxillary Mucins. *Archives of Biochemistry and Biophysics*, 124(1-3), 41-50.
- Thanantong, N., Edwards, S., and Sparagano, O. A. E. (2006). Characterization of lactic acid bacteria and other gut bacteria in pigs by a macroarraying method. In E. F. Blouin and J. C. Maillard (Eds.), *Impact of Emerging Zoonotic Diseases on Animal Health* (Vol. 1081, pp. 276-279).

- Thornton, D. J., Carlstedt, I., Howard, M., Devine, P. L., Price, M. R., and Sheehan, J. K. (1996). Respiratory mucins: Identification of core proteins and glycoforms. *Biochemical Journal*, 316, 967-975.
- Thornton, D. J., Howard, M., Devine, P. L., and Sheehan, J. K. (1995). Methods for separation and deglycosylation of mucin subunits. *Analytical Biochemistry*, 227(1), 162-167.
- Thorpe, E., and Thomlinson, J. R. (1967). Autolysis and post-mortem bacteriological changes in the alimentary tract of the pig. *Journal of Pathology and Bacteriology*, 93, 600-610.
- Thwaites, D. T., and Anderson, C. M. H. (2007). Deciphering the mechanisms of intestinal imino (and amino) acid transport: The redemption of SLC36A1. *Biochimica Et Biophysica Acta-Biomembranes*, 1768(2), 179-197.
- Tirupathi, C., Miyamoto, Y., Ganapathy, V., and Leibach, F. H. (1993). Genetic-evidence for role of DPP-IV in intestinal hydrolysis and assimilation of prolyl peptides. *American Journal of Physiology*, 265(1), G81-G89.
- Tomasi, T. B. (1972). Secretory immunoglobulins. *New England Journal of Medicine*, 287(10), 500-506.
- Tome, D., Ben Mansour, A., Hautefeville, M., and Desjeux, J. F. (1987). Opiate activity and transepithelial passage of intact β -casomorphins in rabbit ileum. *American Journal of Physiology*, 253, G737-G744.
- Tome, D., Benmansour, A., Hautefeuille, M., Dumontier, A. M., and Desjeux, J. F. (1988). Neuromediated action of beta-casomorphins on ion-transport in rabbit ileum. *Reproduction Nutrition Development*, 28(4A), 909-918.
- Tome, D., and Debabbi, H. (1998). Physiological effects of milk protein components. *International Dairy Journal*, 8(5-6), 383-392.
- Torrallardona, D., Harris, C. I., Coates, M. E., and Fuller, M. F. (1996). Microbial amino acid synthesis and utilization in rats: Incorporation of N-15 from (NH₄Cl)-N-15 into lysine in the tissues of germ-free and conventional rats. *British Journal of Nutrition*, 76(5), 689-700.
- Torrallardona, D., Harris, C. I., and Fuller, M. F. (2003a). Lysine synthesized by the gastrointestinal microflora of pigs is absorbed, mostly in the small intestine. *American Journal of Physiology-Endocrinology and Metabolism*, 284(6), E1177-E1180.

- Torrallardona, D., Harris, C. I., and Fuller, M. F. (2003b). Pigs' gastrointestinal microflora provide them with essential amino acids. *Journal of Nutrition*, 133(4), 1127-1131.
- Torrallardona, D., Harris, C. I., Milne, E., and Fuller, M. F. (1994). *The contribution of intestinal microflora to amino acid requirements in pigs*. Paper presented at the VIth International Symposium on Digestive Physiology in Pigs, Proceedings., Dummerstorf, Germany.
- Trompette, A., Claustre, J., Caillon, F., Jourdan, G., Chayvialle, J. A., and Plaisancie, P. (2003). Milk bioactive peptides and beta-casomorphins induce mucus release in rat jejunum. *Journal of Nutrition*, 133(11), 3499-3503.
- Turroni, F., Ribbera, A., Foroni, E., van Sinderen, D., and Ventura, M. (2008). Human gut microbiota and bifidobacteria: From composition to functionality. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology*, 94(1), 35-50.
- Tytgat, K., vanderWal, J. W. G., Einerhand, A. W. C., Buller, H. A., and Dekker, J. (1996). Quantitative analysis of MUC2 synthesis in ulcerative colitis. *Biochemical and Biophysical Research Communications*, 224(2), 397-405.
- Ueda, K., Morgan, S. L., Fox, A., Gilbert, J., Sonesson, A., Larsson, L., et al. (1989). D-alanine as a chemical marker for the determination of streptococcal cell-wall levels in mammalian-tissues by gas-chromatography negative-ion chemical ionization mass-spectrometry. *Analytical Chemistry*, 61(3), 265-270.
- Ugawa, S., Sunouchi, Y., Ueda, T., Takahashi, E., Saishin, Y., and Shimada, S. (2001). Characterization of a mouse colonic system B0+ amino acid transporter related to amino acid absorption in colon. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 281(2), G365-G370.
- Ushida, K., Lassalas, B., and Jouany, J. P. (1985). Determination of assay parameters for rna analysis in bacterial and duodenal samples by spectrophotometry - influence of sample treatment and preservation. *Reproduction Nutrition Development*, 25(6), 1037-1046.
- Vaahrovuo, J., Korkeamaki, M., Munukka, E., Hameenoja, P., and Vuorenmaa, J. (2007). Microbial balance index - A view on the intestinal microbiota. *Livestock Science*, 109(1-3), 174-178.

- Vallejo, C. G., and Lagunas, R. (1970). Interferences by sulfhydryl, disulfide reagents and potassium ions on protein determination by Lowry's method. *Analytical Biochemistry*, 36(1), 207-212.
- van Barneveld, R. J., Batterham, E. S., and Norton, B. W. (1991). A comparison of ileal digestibility using cannulation and dissection techniques. In D. J. Farrell (Ed.), *Recent Advances in Animal Nutrition in Australia*. (pp. 22A). Armidale: University of New England.
- van der Pijl, P. C., Kies, A. K., Ten Have, G. A. M., Duchateau, G., and Deutz, N. E. P. (2008). Pharmacokinetics of proline-rich tripeptides in the pig. *Peptides*, 29(12), 2196-2202.
- van Goudoever, J. B., Stoll, B., Henry, J. F., Burrin, D. G., and Reeds, P. J. (2000). Adaptive regulation of intestinal lysine metabolism. *Proceedings of the National Academy of Sciences of the United States of America*, 97(21), 11620-11625.
- van Klinken, B. J. W., Dekker, J., van Gool, S. A., van Marle, J., Buller, H. A., and Einerhand, A. W. C. (1998). MUC5B is the prominent mucin in human gallbladder and is also expressed in a subset of colonic goblet cells. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 274(5), G871-G878.
- van Klinken, B. J. W., Einerhand, A. W. C., Buller, H. A., and Dekker, J. (1998a). The oligomerization of a family of four genetically clustered human gastrointestinal mucins. *Glycobiology*, 8(1), 67-75.
- van Klinken, B. J. W., Einerhand, A. W. C., Buller, H. A., and Dekker, J. (1998b). Strategic biochemical analysis of mucins. *Analytical Biochemistry*, 265(1), 103-116.
- van Leeuwen, P., Huisman, J., Verstegen, M. W. A., Baak, M. J., van Kleef, D. J., Van and Weerden, E. J. (1988). *A new technique for collection of ileal chyme in pigs*. Paper presented at the Proceedings of Symposium on Digestive Physiology in the Pig, Warsaw.
- van Leeuwen, P., van Kleef, D. J., van Kempen, G. J. M., Huisman, J., and Verstegen, M. W. A. (1991). The post valve T-caecum cannulation technique in pigs applicated to determine the digestibility of amino-acid in maize, groundnut and sunflower meal. *Journal of Animal Physiology and Animal Nutrition-Zeitschrift Fur Tierphysiologie Tierernahrung Und Futtermittelkunde*, 65(3-4), 183-193.

- van Nevel, C. J., and Demeyer, D. I. (1977). Determination of rumen microbial-growth invitro from P-32-labeled phosphate incorporation. *British Journal of Nutrition*, 38(1), 101-114.
- van Platerink, C. J., Janssen, H. G. M., and Haverkamp, J. (2007). Development of an at-line method for the identification of angiotensin-I inhibiting peptides in protein hydrolysates. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, 846(1-2), 147-154.
- van Winkle, L. J. (1993). Endogenous amino-acid-transport systems and expression of mammalian amino-acid-transport proteins in xenopus-oocytes. *Biochimica Et Biophysica Acta*, 1154(2), 157-172.
- Variyam, E. P., and Hoskins, L. C. (1981). Mucin degradation in human-colon ecosystems - degradation of hog gastric mucin by fecal extracts and fecal cultures. *Gastroenterology*, 81(4), 751-758.
- Vasilevskaya, L. S., Stan, E. Y., Chernikov, M. P., and Shlygin, G. K. (1977). Inhibitory action of glycomacropptide produced on the gastric secretion by various humoral stimulants. *Voprosy Pitaniya*, 21-24.
- Vegarud, G. E., Langsrud, T., & Svenning, C. (2000). Mineral-binding milk proteins and peptides; occurrence, biochemical and technological characteristics. *British Journal of Nutrition*, 84, S91-S98.
- Vermeirssen, V., Van Camp, J., and Verstraete, W. (2004). Bioavailability of angiotensin I-converting enzyme inhibitory peptides. *British Journal of Nutrition*, 92(3), 357-366.
- Viljoen, J., Coetzee, S. E., Fick, J. C., Siebrits, F. K., and Hayes, J. P. (2000). Evaluation of in vivo techniques for the determination of apparent ileal amino acid digestibilities in feedstuffs for piglets. *South African Journal of Animal Science*, 30(1), 7-15.
- Vince, A., Ogrady, F., and Dawson, A. M. (1973). Development of ileostomy flora. *Journal of Infectious Diseases*, 128(5), 638-641.
- Voet, D. (1995). Three-dimensional structures of proteins. In J. G. Voet (Ed.), *Biochemistry* (2nd ed., pp. 141-190). New York: John Wiley and Sons.
- Voss, K., and Galensa, R. (2000). Determination of L- and D-amino acids in foodstuffs by coupling of high-performance liquid chromatography with enzyme reactors. *Amino Acids*, 18(4), 339-352.

- Walker, A. W., Duncan, S. H., Harmsen, H. J. M., Holtrop, G., Welling, G. W., and Flint, H. J. (2008). The species composition of the human intestinal microbiota differs between particle-associated and liquid phase communities. *Environmental Microbiology*, *10*(12), 3275-3283.
- Walker, W. A., Abel, S. N., Wu, M., and Bloch, K. J. (1976). Intestinal uptake of macromolecules 5. Comparison of invitro uptake by rat small-intestine of antigen-antibody complexes prepared in antibody or antigen excess. *Journal of Immunology*, *117*(3), 1028-1032.
- Walker, W. A., Wu, M., Isselbacher, K. J., and Bloch, K. J. (1975). Intestinal uptake of macromolecules 4. Effect of pancreatic duct ligation on breakdown of antigen and antigen-antibody complexes on intestinal surface. *Gastroenterology*, *69*(6), 1223-1229.
- Wallace, R. J., and West, A. A. (1982). Adenosine 5'-triphosphate and adenylate energy-charge in sheep digesta. *Journal of Agricultural Science*, *98*, 523-528.
- Wallner, G., Erhart, R., and Amann, R. (1995). Flow cytometric analysis of activated-sludge with ribosomal-RNA-targeted probes. *Applied and Environmental Microbiology*, *61*(5), 1859-1866.
- Wallner, G., Fuchs, B., Spring, S., Beisker, W., and Amann, R. (1997). Flow sorting of microorganisms for molecular analysis. *Applied and Environmental Microbiology*, *63*, 4223- 4231.
- Wang, W. W., Qiao, S. Y., and Li, D. F. (2009). Amino acids and gut function. *Amino Acids*, *37*(1), 105-110.
- Wapnir, R. A., and Teichberg, S. (2002). Regulation mechanisms of intestinal secretion: Implications in nutrient absorption. *Journal of Nutritional Biochemistry*, *13*(4), 190-199.
- Warren, L. (1959). Thiobarbituric Acid Assay of Sialic Acids. *Journal of Biological Chemistry*, *234*(8), 1971-1975.
- Watanabe, K., Tsuge, Y., and Shimoyamada, M. (1998). Binding activities of pronase-treated fragments from egg white ovomucin with anti-ovomucin antibodies and Newcastle disease virus. *Journal of Agricultural and Food Chemistry*, *46*(11), 4501-4506.
- Watson, J. D., and Crick, F. H. C. (1953). Molecular structure of nucleic acids - a structure for deoxyribose nucleic acid. *Nature*, *171*(4356), 737-738.

- Webb, K. E. (1990). Intestinal-absorption of protein hydrolysis products - a review. *Journal of Animal Science*, 68(9), 3011-3022.
- Webb, K. E., and Bergman, E. N. (1991). *Amino acid and peptide absorption and transport across the intestine*. San Diego: Academic Press Inc.
- Weber, E., and Ehrlein, H. J. (1998). Relationships between gastric emptying and intestinal absorption of nutrients and energy in mini pigs. *Digestive Diseases and Sciences*, 43(6), 1141-1153.
- Weimer, H. E., and Moshin, J. R. (1953). Serum Glycoprotein Concentrations in Experimental Tuberculosis of Guinea Pigs. *American Review of Tuberculosis*, 68(4), 594-602.
- Weller, R. A., Gray, F. V., and Pilgrim, A. F. (1958). The Conversion of Plant Nitrogen to Microbial Nitrogen in the Rumen of the Sheep. *British Journal of Nutrition*, 12(4), 421-429.
- Weltzien, F. A., Norberg, B., and Swanson, P. (2003). Isolation and characterization of FSH and LH from pituitary glands of Atlantic halibut (*Hippoglossus hippoglossus* L.). *General and Comparative Endocrinology*, 131(2), 97-105.
- Whitelaw, F. G., Eadie, J. M., Bruce, L. A., and Shand, W. J. (1984). Microbial protein-synthesis in cattle given roughage-concentrate and all-concentrate diets - the use of 2,6-diaminopimelic acid, 2-aminoethylphosphonic acid and S-35 as markers. *British Journal of Nutrition*, 52(2), 249-260.
- Wijey, C., Crawford, C. G., Tu, S. I., and Paoli, G. C. (2004). Effect of bacterial protein extraction reagent combined with sodium azide on the detection of *Escherichia coli* O157 : H7 using immunomagnetic-electrochemiluminescence. *Journal of Rapid Methods and Automation in Microbiology*, 12(2), 115-126.
- Wilkinson, P. C. (1974). Surface and cell-membrane activities of leukocyte chemotactic factors. *Nature*, 251(5470), 58-60.
- Windmueller, H. G., and Spaeth, A. E. (1980). Respiratory fuels and nitrogen-metabolism invivo in small-intestine of fed rats - quantitative importance of glutamine, glutamate, and aspartate. *Journal of Biological Chemistry*, 255(1), 107-112.
- Wolf, J. L., Rubin, D. H., Finberg, R., Kauffman, R. S., Sharpe, A. H., Trier, J. S., and Fields B. N. (1981). Intestinal M-cells - a pathway for entry of reovirus into the host. *Science*, 212(4493), 471-472.

- Wood, J. D., Alpers, D. H., and Andrews, P. L. R. (1999). Fundamentals of neurogastroenterology. *Gut*, 45, 6-16.
- Work, E. (1950). A new naturally occurring amino-acid. *Nature*, 165(4185), 74-75.
- Wu, G. Y. (1998). Intestinal mucosal amino acid catabolism. *Journal of Nutrition*, 128(8), 1249-1252.
- Wunsche, J., Bock, H. D., Hennig, U., Kreienbring, F., and Borgmann, E. (1979). Untersuchungen über die Proteinverdaulichkeit und Aminosäurenresorption in verschiedenen Abschnitten des Verdauungstraktes beim Schwein. *Archives of Animal Nutrition*, 29, 221-234.
- Wunsche, J., Volker, T., Souffrant, W. B., and Borgmann, E. (1991). Assessment of the bacteria-N proportions in feces and differently obtained ileal digesta of pigs. *Archiv Fur Tierernahrung-Archives of Animal Nutrition*, 41(7-8), 703-716.
- Yamamoto, N., Ejiri, M., and Mizuno, S. (2003). Biogenic peptides and their potential use. *Current Pharmaceutical Design*, 9(16), 1345-1355.
- Yang, C. Y., Dantzig, A. H., and Pidgeon, C. (1999). Intestinal peptide transport systems and oral drug availability. *Pharmaceutical Research*, 16(9), 1331-1343.
- Yang, S. Z., Yunden, J., Sonoda, S., Doyama, N., Lipkowski, A. W., Kawamura, Y., and Yoshikawa, M. (2001). Rubiscolin, a delta selective opioid peptide derived from plant Rubisco. *Febs Letters*, 509(2), 213-217.
- Yen, S. S. C., Quigley, M. E., Reid, R. L., Ropert, J. F., & Cetel, N. S. (1985). Neuroendocrinology of opioid-peptides and their role in the control of gonadotropin and prolactin secretion. *American Journal of Obstetrics and Gynecology*, 152(4), 485-493.
- Yin, L. Y., and McCracken, K. J. (1996). Methodological aspects of in vivo measurement of ileal amino acid digestibility in pigs- a review. *Asian-Australasian Journal of Animal Sciences*, 9, 495-502.
- Yin, Y. L., Huang, R. L., Libao-Mercado, A. J., Jeurond, E. A., de Lange, C. F. M., and Rademacher, M. (2004). Effect of including purified jack bean lectin in casein or hydrolysed casein-based diets on apparent and true ileal amino acid digestibility in the growing pig. *Animal Science*, 79, 283-291.
- Yin, Y. L., McEvoy, D. G., McEvoy, J. D. G., Schulze, H., and McCracken, K. J. (2000a). Studies on cannulation method and alternative indigestible markers

- and the effects of food enzyme supplementation in barley-based diets on ileal and overall apparent digestibility in growing pigs. *Animal Science*, 70, 63-72.
- Yin, Y. L., McEvoy, J. D. G., Schulze, H., Hennig, U., Souffrant, W. B., and McCracken, K. J. (2000b). Apparent digestibility (ileal and overall) of nutrients as evaluated with PVTC-cannulated or ileo-rectal anastomised pigs fed diets containing two indigestible markers. *Livestock Production Science*, 62(2), 133-141.
- Yoshikawa, M., Tani, F., Ashikaga, T., Yoshimura, T., and Chiba, H. (1986). Purification and characterization of an opioid antagonist from a peptic digest of bovine kappa-casein. *Agricultural and Biological Chemistry*, 50(11), 2951-2954.
- Yoshikawa, M., Tani, F., Shiota, H., Suganuma, H., Usui, H., Kurahashi, K., and Chiba, H. (1994). Casoxin D, an opioid antagonist ileum-contracting/vasorelaxing peptide derived from human alpha-s1-casein. In V. Brantl and H. Teschemacher (Eds.), *β -Casomorphins and Related Peptides: Recent Developments*. (pp. 43-48). Weinheim, Germany.: VCH.
- Young, V. R., El-Khoury, A. E., Raguso, C. A., Forslund, A. H., and Hambraeus, L. (2000). Rates of urea production and hydrolysis and leucine oxidation change linearly over widely varying protein intakes in healthy adults. *Journal of Nutrition*, 130(4), 761-766.
- Yu, Y. M., Burke, J. F., Vogt, J. A., Chambers, L., and Young, V. R. (1992). Splanchnic and whole-body L- 1-C-13,N-15 leucine kinetics in relation to enteral and parenteral amino-acid supply. *American Journal of Physiology*, 262(5), E687-E694.
- Zahradníčková, H., Husek, P., Simek, P., Hartvich, P., Marsalek, B., and Holoubek, I. (2007). Determination of D- and L-amino acids produced by cyanobacteria using gas chromatography on Chirasil-Val after derivatization with pentafluoropropyl chloroformate. *Analytical and Bioanalytical Chemistry*, 388(8), 1815-1822.
- Zaloga, G. P., Roberts, P. R., Black, K. W., Lin, M., Zapata-Sudo, G., Sudoh, R. T., and Nelson, T. E. (1997). Carnosine is a novel peptide modulator of intracellular calcium and contractility in cardiac cells. *American Journal of Physiology*, 272, H462-H468.

- Zaloga, G. P., and Siddiqui, R. A. (2004). Biologically active dietary peptides. *Mini-Reviews in Medicinal Chemistry*, 4(8), 815-821.
- Zebrowska, T. (1973a). Digestion and absorption of nitrogenous compounds in the large intestine of pigs., *95b*, 85-90.
- Zebrowska, T. (1973b). Digestion and absorption of nitrogenous compounds in the large intestine of pigs. *Roczniki Nauk Rolniczych: Zootech.*, *95b*, 85-90.
- Zebrowska, T. (1975). The apparent digestibility of nitrogen and individual amino acids in the large intestine of pigs. *Roczniki Nauk Rolniczych: Zootech.*, *97B*, 117-123.
- Zebrowska, T., Low, A. G., and Zebrowska, H. (1983). Studies on gastric digestion of protein and carbohydrate, gastric-secretion and exocrine pancreatic-secretion in the growing-pig. *British Journal of Nutrition*, 49(3), 401-410.
- Zhao, Q., Garreau, I., Sannier, F., and Piot, J. M. (1997a). Opioid peptides derived from haemoglobin: Haemorphins. *Biopolymers*, 43, 75-98.
- Zhao, X. T., McCamish, M. A., Miller, R. H., Wang, L. J., and Lin, H. C. (1997b). Intestinal transit and absorption of soy protein in dogs depend on load and degree of protein hydrolysis. *Journal of Nutrition*, 127(12), 2350-2356.
- Ziegler, T. R., Fernandez-Estivariz, C., Gu, L. H., Bazargan, N., Umeakunne, K., Wallace, T. M., Diaz, E. E., Rosado, K. E., Pascal, R. R., Galloway, J. R., Wilcox, J. N., and Leader, L. M. (2002). Distribution of the H⁺/peptide transporter PepT1 in human intestine: Up-regulated expression in the colonic mucosa of patients with short-bowel syndrome. *American Journal of Clinical Nutrition*, 75(5), 922-930.
- Zinn, R. A., and Owens, F. N. (1986). A rapid procedure for purine measurement and its use for estimating net ruminal protein-synthesis. *Canadian Journal of Animal Science*, 66(1), 157-166.
- Zioudrou, C., Streaty, R. A., and Klee, W. A. (1979). Opioid peptides derived from food proteins - exorphins. *Journal of Biological Chemistry*, 254(7), 2446-2449.
- Zishka, M. K., and Nishimur.Js. (1970). Effect of glycerol on Lowry and Biuret methods of protein determination. *Analytical Biochemistry*, 34(1), 291-297.
- Zucht, H. D., Shan, Y., Schreeb, R., Adermann, K., & Forssmann, W. G. (1996). Antimicrobial properties of casocidin-I: A peptide processed from casein-alpha(s2). *Faseb Journal*, 10(3), 4599-4599.

Chapter II

Methods for mucin analysis - a comparative study

The objective of the present study was to evaluate five techniques commonly used for the quantification of mucin in the digesta collected from the terminal ileum of pigs fed a casein-based diet.

Data published in Miner-Williams, W., Moughan, P. J., and Fuller, M. F. (2009). Methods for mucin analysis - a comparative study. *Journal of Agricultural and Food Chemistry*, 57, 6029-6035.

Abstract

The aim was to compare five of the techniques commonly used to quantify mucin concentrations in ileal digesta collected from three growing pigs that had been fed a diet in which the sole protein was casein. Ileal mucin output was estimated by the periodic acid-Schiff, ethanol precipitation and phenol-sulphuric acid methods as 25.1, 19.3 and 20.7 g·kg⁻¹ dry matter intake (DMI), respectively. The mucin concentration estimated from sialic acid was only 5.9 g·kg⁻¹ DMI. Based on the concentrations of the hexosamines N-acetylglucosamine and N-acetylgalactosamine mucin output was estimated as 44.9 g·kg⁻¹ DMI. Of the five assays studied, the ethanol precipitation, the periodic acid-Schiff, the phenol-sulphuric acid and the sialic acid method may considerably underestimate mucin in the digesta which calls into question the accuracy of all these approaches. In contrast, the gas chromatography method for the determination of hexosamines gave more information on the type and state of the mucin present.

Introduction

Given the nature of the alimentary canal, the gastrointestinal mucosal epithelia are exposed to the external environment. Bile salts, acids, digestive enzymes and pathogenic bacteria all contribute to make the intestinal lumen a particularly noxious environment (Bevins *et al.*, 1999; Sanderson and Walker, 1999). To protect itself, the gastrointestinal mucosa is covered with a layer of mucus, characterised by aggregated glycoprotein molecules (mucins) secreted by specialist cells of the underlying mucosa. Mucins are a family of polydisperse molecules with high molecular mass and a high proportion of covalently-bound oligosaccharide side chains (Corfield *et al.*, 2001) which afford high resistance to the effects of acid and digestive enzymes. They may be characterised as secreted or membrane-bound.

Secreted mucins, up to 0.5 to 20 x 10⁶ Da (Bansil and Turner, 2006; Montagne *et al.*, 2004) contain a central polypeptide core, of 1500 to 4500 amino acids in length, with

100-200 oligosaccharide side chains that contain 1 to 20 or more monosaccharides; such oligosaccharides may account for 50-80% of the molecule's mass (Roussel and Delmotte, 2004). In mammals, mucins typically contain fucose, galactose, N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc) and sialic acid, together with small amounts of sulphate and mannose. The carbohydrate chains are bound to the polypeptide by O-glycosidic linkages between N-acetylgalactosamine and the hydroxylated amino acids serine and threonine (Roussel and Delmotte, 2004). Highly glycosylated regions of the polypeptide, rich in threonine, serine and proline, may account for 70-80% of the molecule, in a structure reminiscent of a bottle brush. The poorly glycosylated regions of the mucin molecule contain less serine and threonine but are rich in cysteine, which allows the formation of disulphide bridges between mucin molecules to form very high molecular mass mucous polymers (Krause, 2000). The unique capacity of the secreted mucins to protect the delicate epithelial surfaces of the mucosa is primarily due to the polymerisation of mucin monomers to form viscoelastic gels (Krause, 2000).

Membrane-bound mucins share many of the structural properties of secreted mucins but remain monomeric, do not form gels, and stretch out from the epithelial surface to form the cell-surface membrane glycocalyx. The mucin layer also contains a number of other compounds including bicarbonate ions, epidermal growth factor, trefoil peptides, bactericidal factors, protease inhibitors, and surface-active lipids (Krause, 2000). Such compounds, when incorporated into the mucus layer, guard against its degradation and protect the underlying mucosa from gastric acid and pancreatic enzymes.

Within the gastrointestinal tract (GIT) a dynamic equilibrium exists between the rate at which mucin is synthesised and secreted and the rate at which it is degraded, by both proteolysis and physical erosion. As a result of these degradative processes, mucins constitute a significant proportion of the endogenous protein that reaches the terminal ileum. It has been estimated that between 50 and 65% of the endogenous protein secreted into the gut is mucin (Montagne et al., 2004). Lien *et al.* (1997) estimated that mucin accounted for 5 to 11% of the endogenous protein leaving the ileum of pigs fed a protein-free diet (with amino acid supplementation) whilst, in the calf, protein derived from mucin was estimated to account for 19% of the total basal endogenous

losses at the terminal ileum (Montagne *et al.*, 2004). This equates to a mass of mucin at the terminal ileum of pre-ruminant calves and of pigs amounting to 7.5 and 3.9 g•kg⁻¹ of dry matter intake, respectively, with 25% of this originating from the upper GI tract (Lien *et al.*, 1997; Montagne *et al.*, 2000).

Several studies have shown that the loss of intestinal mucin may be affected by certain dietary components such as fibre, peptides and anti-nutritional factors, Claustre *et al.*, 2002; Lien *et al.*, 1996; Montagne *et al.*, 2004; Satchithanandam *et al.*, 1996). As mucin is so important in the protection of the mucosa of the GI tract any mechanism that alters this defensive barrier has important physiological implications, especially in the control and management of inflammatory diseases of the bowel (Barcelo *et al.*, 2000). There are few studies that detail the quantification of gastrointestinal mucin because it is particularly difficult to assay (Barcelo *et al.*, 2000).

Because ileal digesta are a complex mixture of endogenous and exogenous substances the isolation and quantification of mucin in ileal digesta is often undertaken using carbohydrate markers specific to glycoproteins, such as amino sugars and sialic acid. The aim of this study was to compare some of the common techniques used to quantify mucin concentrations in mammalian ileal digesta.

Materials and methods

Animals and diets.

Digesta samples were collected from three Large White x Duroc pigs of mean body weight 79 (\pm 4.8) kg (\pm SEM), housed individually in steel metabolism crates, in a room maintained at 24 \pm 1°C, at the Small Animal Production Unit, Massey University, Palmerston North, New Zealand. Approval for the study was granted by the Massey University Animal Ethics Committee (protocol 05/29). After one week's acclimation each pig was fitted with a post-valve T caecum cannula, as described by van Leeuwen *et al.* (1991). Following surgery, food was progressively reintroduced within one week up to a daily level of 0.08 metabolic body weight ($W^{0.75}$) kg•d⁻¹ and this level of food intake was maintained for the remainder of the trial. Collection of

digesta took place 8 weeks following surgery. The pigs were fed a lactic casein-based basal diet (**Table 1**) mixed with water (1:1, w/w), 3 times daily (0800, 1200 and 1600 h), in equal portions. Water was available *ad libitum*.

Table 1: Ingredient compositions of the basal diet.

Ingredient	g/kg air dry weight	Ingredient	g/kg air dry weight
Cooked Wheat ¹	485.8	Vitamin / mineral mix ³	2.5
Sucrose	168	Sodium chloride	1.6
Lactic casein ²	159	Synthetic methionine	0.7
Skim milk powder	80	Calcium carbonate	0.2
Soybean oil	80	Antioxidant ⁴	0.2
Dicalcium phosphate	22		

¹ Weet-bix, Sanitarium, Auckland, New Zealand.

² NZMP, Palmerston North, New Zealand.

³ Vitalean, Vitac Nutrition Ltd, Auckland, New Zealand. Vitamins provided: (g•kg⁻¹ of diet) vitamin A 3.0; (mg•kg⁻¹ of diet) cholecalciferol 500.0, choline 83.3; niacin 12.5, panthotenic acid 8.3, riboflavin 2.1, vitamin B6 1.7, vitamin E 41.7, vitamin K 1.7; (µg•kg⁻¹ of diet) biotin 8.3, folic acid 417.0, thiamin 833.0, vitamin B12 8.3, Minerals provided: (mg•kg⁻¹ of diet) Cu 104.0, Fe 83.0, Mn 38.0, Zn 100.0; (µg•kg⁻¹ of diet) I 833.0, Co 417.0, Se 250.0.

⁴ Ethoxyquin, Kemin Industries Ltd, Auckland, New Zealand.

On the day of the digesta collection at 0800 h the pigs were fed one-third of the daily intake of an experimental diet (**Table 2**). Digesta were then collected into polythene bags for a period of ten hours after the morning meal. Each specimen was weighed before sodium benzoate (10 g•kg⁻¹ of digesta), as a bactericide and phenylmethylsulphonyl fluoride (0.37 g•kg⁻¹ of digesta) as an anti-protease were added according to the protocol of Salgado *et al.* (2001). The digesta samples for each pig were then pooled and frozen at -20 °C until chemical analysis. The pigs received no food during digesta collection.

Table 2 Composition of the experimental diet.

Ingredient	g/kg air dry weight¹
Maltodextrin	453
Sucrose	161
Soybean oil	154
Sodium hydrogen carbonate	18
Titanium dioxide	3
Lactic casein	211
Nitrogen content of the test diet	31.9

¹No vitamins, minerals or fibre were added to this diet as the same diet was used in a separate acute feeding study with human subjects (Deglaire *et al.*, 2007).

Chemical analysis.

All analyses were undertaken in triplicate. Dry matter was determined by drying the material to a constant mass in a forced air oven at 95 °C. Total Nitrogen was determined by the Leco total combustion method (AOAC, 2000), a variation of the Dumas method. Duplicate samples were combusted at 1050 °C in oxygen gas; the nitrogen was then reduced to N₂ by a copper catalyst at 750°C and this was measured by a thermal conductivity cell in a Leco FP2000 analyser (Leco Corporation, St Joseph, Michigan, USA). Titanium dioxide was determined by the method of Short *et al.* (Short *et al.*, 1996). Amino acid concentrations were measured using a Waters ion exchange HPLC system (Waters, Millipore, Milford, MA) calibrated against a reference amino acid mixture. Norleucine and lysozyme were used as internal and external standards, respectively. Methionine and cysteine were measured as methionine sulphone and cysteic acid respectively, after hydrolysis of samples that had been oxidized using performic acid. Tryptophan was not determined. Amino acid concentrations are given as a percentage of total determined amino acid concentration.

Samples of digesta were fractionated by differential centrifugation using the method of Metges *et al.* (1999), first at 250 x g for 15 minutes at 4°C to separate food particles

and porcine cells, then at 14500 x g for 30 minutes at 4°C to separate microbial cells and porcine cellular detritus. The soluble mucins were assumed to be quantitatively recovered in the supernatant, together with proteins, peptides, free amino acids, neutral sugars, urea, creatinine and ammonia.

The determination of mucin and mucin markers

An attempt was made to prepare a standard mucin solution by redissolving pure mucin obtained from Sigma (Sigma-Aldrich Corp. St. Louis, MO) This proved difficult however particularly because many surfactants interfered with the colorimetric methods. To overcome this, for the colorimetric assays, a standard solution was obtained using mucin precipitated from porcine ileal digesta by ethanol. The standard curve obtained correlated with that of Mantle and Allen (1978) ($\rho = 0.99$; $P < 0.001$)

Quantification of mucin by ethanol precipitation.

Following the method of Piel *et al* (2004), the digesta were treated in the following manner: Three ml of each fractionated digesta, was added to 25 ml of a 0.15M sodium chloride solution and mixed by vortex. Each of the diluted digesta samples was centrifuged at 12 000 x g for 30 minutes at 4 °C); 15 ml of each supernatant was added to 22 ml ethanol at 0°C, mixed then kept overnight at -20 °C. Each supernatant/precipitate mixture was then centrifuged at 1400 x g for 10 minutes at 4 °C). The precipitate from each mixture was recovered and redissolved in 15 ml of 0.15 M sodium chloride solution, before being precipitated again using the same procedure. Each precipitate was then be re-solubilised in 10 ml distilled water, frozen, freeze-dried and weighed.

Colorimetric analysis of mucin based on the periodic acid/Schiff histological stain method

The determination of digesta mucin using Periodic Acid Schiff (PAS) followed a method taken from Mantle and Allen (1978). Both the Schiff reagent and 50% periodic acid solution were purchased from BDH Chemicals (Merck, Darmstadt, Germany). A periodic acid solution was prepared by adding 10 µl of periodic acid (50% solution from BDH Chemicals) to 10 ml of a 7% ethanoic acid solution. Samples and standards,

containing between 5 to 100 μg of mucin and a blank dissolved in 2 ml of water, were incubated for 2 hours at 37 °C with 200 μl of freshly made periodic acid solution. After periodic acid oxidation, 200 μl of the Schiff solution was added to the glycoprotein solutions. The resultant solutions were then incubated for 30 min at room temperature for maximum colour development. Absorbances were read at 555 nm.

Colorimetric analysis of mucin by the Phenol-Sulphuric Acid assay for neutral sugars.

The method used for the detection of neutral sugars using Phenol-Sulphuric acid (PSA) was adapted from that of Beeley (1985); a scaled down version of that used by Dubois *et al* (1956). Concentrated sulphuric acid (specific gravity 1.84) D-mannose and phenol were purchased from BDH Chemicals (Merck, Darmstadt, Germany). A standard aqueous solution of D-mannose (40 μml) was pre-prepared and stored at -18 °C. In test tubes (14mm i.d.), 0.5 ml of an aqueous test, blank or standard solution was added to 300 μl of a phenol reagent (5% w/v aqueous solution) and then mixed. Two ml of concentrated sulphuric acid was then added rapidly from a fast flowing pipette and mixed immediately. A blank (water) and standards of 5-20 μg mannose were included in each assay. The test, blank and standard solutions were left for 30 min for maximum colour development and for the tubes to cool before reading absorbance at 484 nm.

Total, bound and free sialic acids

Sialic acids were determined following Beeley (1985), who adapted a periodate-thiobarbituric acid assay developed by Aminoff (1961). The following reagents were prepared several hours before they were required: Periodate, 0.025 M periodic acid in 0.0625 M sulphuric acid, pH 1.2; Sodium arsenite, 2% wt/v sodium arsenite in 0.5 M HCl; 2-Thiobarbituric acid, 0.1 M 2-thiobarbituric acid in water adjusted to pH 9.0 by adding NaOH; Acid butan-1-ol, butan-1-ol containing 5% (v/v) of 12 M HCl; an aqueous standard of N-Acetylneuraminic acid, in a range of 10-80 $\mu\text{g/ml}$. All chemicals were purchased from BDH Chemicals (Merck, Darmstadt, Germany). The procedure for the analysis of free sialic acid was as follows: Five hundred μl of sample, blank or standard were added to 0.25 ml of the periodic acid reagent and then

mixed by vortex. The tubes were incubated for 30 min at 37 °C before 1ml of the sodium arsenite was added to reduce the excess periodic acid and vortexed. The tubes were left for 1-2 minutes for the yellow colour of the liberated iodine to disappear. Two ml of the thiobarbituric acid reagent were added before vortexing once again. The samples were then heated in a boiling water bath for 7.5 minutes before being cooled on ice. Once cool 5 ml of the acid-butanol was added and then mixed once more by vortex. The tubes were centrifuged briefly to separate the two phases before the butanol layer was collected and the absorbance measured at 549 nm.

The procedure for analysis of total sialic acid was as follows: One hundred µl 0.5M HCl were added to 0.4 ml of digesta and the tubes incubated at 80 °C for 1 hour. After allowing the hydrolysates to cool the procedure for analysis of free sialic acid as described above, was repeated.

Hexosamines by gas chromatography.

The hexosamines, GlcNAc and GalNAc, used as mucin markers, were estimated as the alditol acetates by the gas-liquid chromatography method described by Lien *et al.* (1997) from the procedures of Blakeney *et al* (1983) and Kraus *et al* (1990), using a Shimadzu 2010 (Shimadzu Scientific Instruments Inc, Columbia, MD) chromatograph with a DB-17 fused silica capillary column (J and W Scientific, Folsom, CA; 0.25 mm internal diameter x 30 metres) and using helium (1.5 ml/minute) as the carrier. For the extraction, hydrolysis and acetylation of hexosamine sugars all chemicals were purchased from BDH Chemicals (Merck, Darmstadt, Germany) and the method was as follows: To approximately 50 mg of freeze dried digesta 1.5ml of 12M sulphuric acid was added and left for 1 hour at room temperature. The solutions were then diluted to 3M by adding 4.5 ml of water before being hydrolysed at 110 °C for a further hour. Following hydrolysis 200 µl of an internal standard, N-methylglucamine, was added (10 mg/ml in distilled water). One ml aliquots of the acid hydrolysates were then cooled on ice before being made basic by adding 0.7 ml of concentrated ammonium hydroxide. One hundred µl of this basic solution was reduced by adding 1 ml of sodium borohydride (30 mg/ml in anhydrous dimethyl sulphoxide) and incubating at 40 °C for 90 min. Excess sodium borohydride was then decomposed by adding 200 µl of glacial acetic acid. Acetylation was achieved by the addition of 0.2

ml of 1-methylimidazole and 2ml of acetic anhydride and then leaving for 10-15 min at room temperature. Following acetylation, the acetic anhydride was decomposed by the adding 5 ml of water and allowing the solutions to cool to room temperature. The alditol acetates were extracted into 4 ml of dichloromethane by vortexing. Following a brief centrifugation the aqueous layer was discarded. The dichloromethane solutions were then washed twice with another 4 ml of water before the final dichloromethane layer was evaporated to dryness under a stream of nitrogen. Before analysis by gas-liquid chromatography the alditol residues were redissolved in 1 ml dichloromethane.

Five μ l of the redissolved alditol acetate solutions were injected onto a DB-17 fused silica capillary column (J and W Scientific, Folsom, CA; 0.25 mm internal diameter x 30 metre). The gas chromatograph, a Shimadzu 2010 (Shimadzu Scientific Instruments Inc, Columbia, MD), was set up using the following conditions: Carrier gas, Helium, at a rate of 1.5 ml/minute, and the injector temperature, 270 °C. The oven temperature was raised from 50 °C to 190 °C at 30 °C/minute and maintained for 3 minutes, then increased by 5 °C/minute to 270 °C and maintained for 10 minutes. The detector temperature was set at 270 °C. Peak area integration was analysed using the Shimadzu GC solutions V2-30su6 data system (Shimadzu Scientific Instruments Inc, Columbia, MD).

The ratio GlcNAc:GalNAc in mucins varies according to the origins of the mucin molecules (Lien *et al.*, 1997). Utilising this difference Lien *et al.* (1997) estimated mucin output using regression equations derived from the ratios of these hexosamines in purified gastric and intestinal mucins. These equations have been used in this study and are as follows:

$$\text{Mucin output } \text{g}\cdot\text{day}^{-1} = \text{GalNAc } \text{g}\cdot\text{day}^{-1} / \% \text{ GalNAc.}$$

$$\% \text{ GalNAc} = 32.30 - 22.74x + 8.83x^2 - 1.37x^3 \text{ (equation 1)}$$

Where x = GlcNAc:GalNAc ratio.

The contribution (%) of gastric mucin was determined using the following equation:

$$\% \text{ gastric mucin} = -80.23 + 183.26x - 71.19x^2 + 11.05x^3 \text{ (equation 2)}$$

The recovery of an indigestible marker (TiO_2) was used to correct the concentrations of mucin and mucin markers for each of the methods using the following equation:

$$\begin{aligned} \text{Corrected concentration of mucin or mucin marker (g}\cdot\text{kg}^{-1} \text{ dry matter intake (DMI))} = \\ \text{Concentration of mucin or mucin marker (g}\cdot\text{kg}^{-1} \text{ DMI)} \times \text{Concentration of TiO}_2 \text{ in the} \\ \text{diet (g}\cdot\text{kg}^{-1} \text{ DMI)} / \text{Concentration of TiO}_2 \text{ in the ileal digesta (g}\cdot\text{kg}^{-1} \text{ DMI)} \end{aligned}$$

Where DMI = dry matter intake

Having obtained values for the concentrations of mucin markers by the PAS, PSA and sialic acid assays the final concentration of mucin was calculated using multiples derived from the composition data published by Mantle and Allen (1989)

Results

The amino acid composition of the total ileal digesta is presented in **Table 3**. In diminishing order of concentration the amino acids glutamic acid, glycine, threonine, aspartic acid, proline and serine were predominant. Glutamic acid was particularly high being nearly 15% of the total amino acid content of the digesta. The concentration of tryptophan was not determined in this study.

Table 4 gives the concentrations of the mucin markers in the ileal digesta of pigs fed a casein based diet determined using the various methods studied. It may be noted that the concentration of sialic acid found in the digesta is low in comparison to the other mucin markers. Nearly 74% of the total sialic acid is unbound with only 26% remaining bound to mucin subunits. These data are only indirectly representative of the mucin present in the ileal digesta.

Table 3. Amino acid composition of the terminal ileal digesta of pigs fed a casein-based diet.

	Amino acid concentration¹.	SEM².
Indispensable amino acids		
Arginine	2.6	0.23
Cysteine	2.2	0.40
Histidine	2.5	0.23
Isoleucine	3.8	0.48
Leucine	5.8	0.68
Lysine	3.4	0.42
Methionine	1.1	0.09
Phenylalanine	2.7	0.36
Threonine*	9.4	1.67
Valine	6.5	0.86
Dispensable amino acids		
Alanine	7.7	0.96
Aspartic Acid	9.3	0.65
Glutamic Acid	14.8	0.90
Glycine	9.6	1.34
Proline*	8.8	0.73
Serine*	7.8	0.78
Tyrosine	2.0	0.24

¹ Amino acid concentration as a percentage of total determined amino acid concentration.

² SEM = Standard error of mean.

Tryptophan not assayed. * Predominant amino acids in mucin.

Table 4. Mean (\pm SEM) concentrations of mucin markers in the ileal effluent of pigs given a casein-based diet.

Method	Reference	Mucin marker	Mucin marker concentration g • kg ⁻¹ DMI	SEM
Periodic Acid Schiff	(1)	Polysaccharide	20.7	0.77
Phenol Sulphuric Acid	(2)	Neutral Sugars	17.1	1.43
Aminoff	(3)	Sialic Acid	1. Total. 0.17 2. Free. 0.12 3. Bound. 0.05	0.006 0.004 0.002
Hexosamines by gas chromatography	(4)	GalNAc ¹ GluNAc ¹	6.5 8.6	0.33 0.28

¹ GalNAc = N-acetylgalactosamine and GluNAc = N-acetylglucosamine.

1. Mantle and Allen 1978
2. Dubois *et al.*, 1956
3. Aminoff 1961
4. Lien *et al.*, 1997

The predicted concentrations of mucin based on the markers are presented in **Table 5**. These concentrations were calculated using compositional data from the study of Mantle and Allen (1989). The mucin concentration determined by the hexosamine method was 44.9 g•kg⁻¹ and considerably higher than the other methods. In comparison the PAS, PSA, and ethanol precipitation methods were 56%, 46% and 43% of the concentration on mucin determined by the hexosamine method. The mucin concentration determined by the Aminoff method was only 5.9 g • kg⁻¹ a value only 13% of that determined using the hexosamine method.

Pearson correlation coefficients describing the degree of relationship between the respective mucin analysis methods are presented in **Table 6**. The relationship between

the ethanol precipitation analysis and the two colorimetric methods of PAS and the PSA gave particularly high Pearson correlation coefficients of 0.953 and 0.944.

Table 5. Mean (\pm SEM) concentrations of mucin ¹ in the ileal effluent of pigs given a casein based diet.

Method	Reference	Mucin concentration g•kg ⁻¹ DMI	SEM
Periodic Acid Schiff	(1)	25.1 ¹ .	0.95
Phenol Sulphuric Acid	(2)	20.7 ¹ .	1.76
Aminoff	(3)	5.9 ¹ .	0.13
Ethanol Precipitation	(4)	19.3	1.50
Hexosamines by gas chromatography	(5)	44.9 ² .	1.48

¹ Calculated using data from Mantle and Allen (1989).

² Calculated using regression equations from Lien *et al.* (1997).

1. Mantle and Allen 1978

2. Dubois *et al.*, 1956

3. Aminoff 1961

4. Piel *et al.*, 2004

4. Lien *et al.*, 1997

Table 6 Pearson correlation coefficients for relationships between chosen mucin analysis methods.

Mucin marker	Hexosamine	Periodic acid Schiff	Phenol/sulphuric acid	Ethanol precipitation
Periodic acid Schiff	0.687			
Phenol/sulphuric acid	0.623	0.949		
Ethanol precipitation	0.634	0.953	0.944	
Sialic acid	0.777	0.761	0.781	0.770

All correlations were significant $p < 0.001$

The correlation between the two acid colorimetric methods was also high (0.949). The correlation between the hexosamine method and the other methods was considerably lower, only 0.687, 0.623, 0.634, and 0.777 for the PAS, PSA, ethanol precipitation and Aminoff methods respectively. Interestingly the correlation between the Aminoff method and the other methods remained fairly constant, being 0.777, 0.761, 0.781, and 0.770 for the hexosamine, PAS, PSA, and ethanol precipitation methods respectively.

Discussion

The aim of this study was to evaluate five of the techniques used to quantify mucin concentrations in ileal digesta. The results of this study that the hexosamine assay, using a gas liquid chromatography method was judged to give the best estimate of the mucin concentration present in ileal digesta at the terminal ileum.

The proportion of protein in purified adult porcine small intestinal glycoprotein has been reported as 23 % (Piel et al., 2004; Snary and Allen, 1971) and with the sum of serine, proline and threonine in the protein core of the glycoprotein being as much as 52% (by weight) according to Mantle and Allen (1989). In the present study these three amino acids represented only 23% (by weight) of the total amino acids present in the digesta, a result which reflects the presence of other sources of protein in the digesta. In earlier research (Miner-Williams *et al.*, 2009) it was demonstrated that only 14% of the total protein present in the ileal digesta originated from mucin. Although the six most abundant amino acids in the digesta are those associated with mucin they are diluted by the high proportion of amino acids originating from bacterial sources, nearly 61% of total protein found in the ileal digesta of animals fed a casein based diet (Miner-Williams et al., 2009).

Ileal digesta may contain several different mucin types, originating from a range of secretory organs in different parts of the GI tract. The chemical composition of these mucins is known to vary with both their origin and with the animal species (**Table 7**) (Corfield et al., 2001; Mantle and Allen, 1989). However, it would be expected that, in ileal digesta, mucins originating from the stomach and the small intestine would predominate.

The value of GalNAc as a mucin marker lies in its limited occurrence in dietary constituents (Lien et al., 1997). GluNAc is less specific as it is found in some dietary proteoglycans (Roden and Horowitz, 1978). However no GalNAc or GluNAc were found in the dietary material in this study.

The GluNAc:GalNAc ratio differs considerably between gastric and intestinal mucin (Lien et al., 1997; Mantle and Allen, 1981; Stanley *et al.* 1983), as gastric mucin contains approximately 30% GlcNAc and 13 % GalNAc, whereas intestinal mucin contains approximately 20% and 40% respectively. Given this difference in the concentrations of these two amino sugars, the relative proportions of gastric and intestinal mucins in the ileal digesta can be estimated.

Table 7. Composition of mucus glycoproteins from the mammalian gastrointestinal tract, [after Mantle and Allen (1989)].

Mucin	Percentage by weight		
	Sulphate	Carbohydrate ¹	Protein
Salivary Mucins			
a) Pig	0.0	66	34.0
Gastric Mucins			
a) Human	7.0	76	17.0
b) Pig	3.1	83.9	13.0
Small Intestinal Mucins			
a) Human	1.6	80.4	18.0
b) Pig	2.6	79.4	18.0
Colonic Mucins			
a) Human	2.0	66.9	31.1
b) Pig	3.0	83.7	13.3

¹. Data from Mantle and Allen (1989). The percentage of carbohydrate was calculated as the difference between the percentages given for sulphate and protein.

The concentrations of GalNAc and GluNAc in the ileal digesta were estimated to be 6.5 and 8.6 g•kg⁻¹DMI (Table 4) respectively.

Using regression equations derived by Lien *et al.* (1997) the mucin output was then calculated to be 44.9 g•kg⁻¹DMI (Table 5) a value much higher than the estimates reported by Lien *et al.* (1997) for pigs fed a protein free diet. This discrepancy may be due to the influence of dietary protein per se (Claustre *et al.*, 2002).

GluNAc:GalNAc ratios in purified pig gastric and intestinal mucins of 2.8 and 0.6, respectively were reported by Mantle and Allen (1989). The ratio observed in this study for the mixed proteins in the ileal digesta was 1.36, which suggests that there was proportionately more gastric mucin than intestinal mucin in the ileal effluent. This

is corroborated by the ratio of threonine:serine. Ratios for threonine:serine of 1.15 and 2.55 in purified pig gastric and intestinal mucins, respectively, were reported by Mantle and Allen (1989). In the present study the threonine:serine ratio was 1.32, similar to a mean value of 1.29 reported by Lien *et al.* (1997) for adult pigs given a protein-free diet. Correlation analysis of the concentrations of the two hexosamines over the three pigs yielded a Pearson correlation coefficient (ρ) of 0.995 ($P < 0.001$) indicating a constancy in the ratio of gastric and small intestinal mucins between animals given the casein based diet.

Degradation of the oligosaccharide side chains may reduce the concentration of some mucin markers found in the ileal digesta. However as every oligosaccharide side chain begins with one GalNAc residue these would be conserved more than any other. As GluNAc is found along the length of the oligosaccharide side chains the ileal concentration of this hexosamine may be subject to greater variation than GalNAc when the side chains are degraded. However, as the ratio of GluNAc and GalNAc appears to be relatively constant (as evident from the high correlation coefficient) it suggests that GalNAc and GluNAc are reliable mucin markers.

The precipitation of glycoproteins using high concentrations of ethanol is considered by many researchers to be non-specific (e.g. Piel *et al.*, 2004). This is further illustrated by the variety of different compounds ethanol has been used to precipitate (e.g., α -galactosidase from a fungus (Kotwal *et al.*, 1999); human-immunodeficiency-virus-inhibitory glycoprotein from aqueous extracts of a Caribbean sponge (O'Keefe *et al.*, 1997); and the two gonadotropins, follicle-stimulating hormone and luteinising hormone, from the pituitary gland of halibut (Weltzien *et al.*, 2003). Non-covalently bound proteins associated with mucin precipitated by ethanol have been noted by Leterme *et al.* (1996), and are associated with the polymerisation of mucin subunits. Although the validity of this assay for the determination of mucin in human gastric juices has been accepted by some workers (e.g., Azuumi *et al.*, 1993), its effectiveness has been questioned by others (O'Keefe *et al.*, 1997; Piel *et al.*, 2004). In the present study both the precipitate and the ethanolic supernatant were assayed for carbohydrates using the phenol/sulphuric acid technique in an effort to ascertain if the precipitation of mucins using ethanol was effective. The final concentration of ethanol in the precipitation of glycoprotein from the digesta seems to be critical, as once the resulting

precipitate was redissolved, its carbohydrate content varied from almost zero to 124 $\mu\text{g}\cdot\text{ml}^{-1}$ of ileal digesta. When the protein content of each fraction was determined, using Bradford reagent, there was often twice as much protein in the supernatant as there was in the precipitate. When Piel *et al.* (2004) reported that their ELISA showed no immunoreactivity in the precipitates, they concluded that this may have been because high concentrations of ethanol are known to denature human gastric mucin (Lin *et al.*, 1997). However, the unreliability of the assay found during this study suggests that ineffective precipitation may also be a factor and one that requires further investigation.

The estimate of intestinal mucin output obtained by ethanol precipitation was less than half that estimated by the hexosamine assay (Table 5). A similar discrepancy was reported by Piel *et al.* (2004). In their assay the ethanol precipitate value was 78% of that determined by the hexosamine assay. Björling (1976) reported that highly glycosylated glycoproteins remain soluble in relatively high concentrations of ethanol. Ineffective precipitation may well be the reason for such inconsistent results. In the light of the results from this study and of others, the reliability of ethanol precipitation for quantitative and specific estimation of digesta glycoproteins must be queried. From our own observations and supported by other published results (Björling, 1976; O'Keefe *et al.*, 1997; Piel *et al.*, 2004), it would appear that the ethanol precipitation method considerably underestimates mucin in digesta. Moreover, given the sialic acid, Periodic Acid-Schiff (PAS) and phenol sulphuric acid (PSA) methods gave similar or lower values for mucin compared to ethanol precipitation (Table 4), this calls into question the accuracy of all these approaches.

Although the PAS staining of electrophoresis gels or histological specimens containing glycoproteins is a sensitive and widely used procedure, its use for the analysis of mucins in biological fluids does not appear to be common. Developed by Mantle and Allen (1978), the assay is quick and sensitive for the analysis of polysaccharides that are oxidised by periodate. However, there is considerable variation in the oligosaccharide side chains of the various mucins found within the gastrointestinal tract and between different species (**Table 8**). Also pig gastric mucin contains many sugar residues that are 1-3 linked and therefore are not oxidised by periodic acid (Clamp *et al.* 1978).

Table 8. Carbohydrate concentration of mucin expressed as a molar ratio to GalNAc, after Mantle and Allen (1989).

Mucin	Carbohydrate Composition*				
	GalNAc ¹	GlcNAc ²	Galactose	Fucose	Sialic Acid
Salivary Mucins					
a) Pig	1	0.0	0.5	0.4	0.4
Gastric Mucins					
a) Human	1	1.8	2.3	1.6	0.9
b) Pig	1	2.8	2.9	1.9	0.2
Small Intestinal Mucins					
a) Human	1	1.1	1.0	1.0	0.4
b) Pig	1	0.6	0.6	0.3	0.6
Colonic Mucins					
a) Human	1	0.9	1.2	0.3	0.2
b) Pig	1	2.9	2.5	1.5	0.2

* Carbohydrate composition expressed as the molar ratio to GalNAc.

¹ GalNAc: N-Acetylgalactosamine.

² GluNAc: N-Acetylglucosamine.

Such differences in composition affect the sensitivity of the PAS assay such that 150 µg of pig intestinal mucin gives an absorbance at 555 nm, 35% greater than a similar mass of pig gastric mucin (Mantle and Allen, 1978). With the mixtures of different mucins that are present in ileal effluent it is not possible to obtain absolute values for the mass of oxidizable sugar residues (Beeley, 1985) and the aforementioned different monosaccharide composition may account for the apparent underestimation of mucin using this method (Table 5).

The colorimetric assay for the quantitative analysis of neutral sugars, described by Dubois *et al.* (1956) is undertaken in strongly acidic conditions and can be applied to free monosaccharides or to unhydrolysed samples of glycoprotein (Beeley 1985).

Although this assay is quick and simple, like the PAS reaction, it too lacks specificity as it cannot distinguish between monosaccharides such as galactose, fucose and glucose. The extinction coefficients of these sugars also differ. Using correlation analysis of the concentrations of mucin for the three pigs as determined by the PAS and phenol sulphuric acid assays a Pearson correlation coefficient of 0.95 ($p < 0.001$; $n = 18$) (Table 6) was obtained, a value which is not surprising as the two assays measure similar monosaccharides. There was also good agreement between the ethanol precipitation method and the two colorimetric assays ($\rho = 0.95$ and 0.94 respectively).

If it is accepted that the ethanol precipitation method underestimates the mucin concentration because of incomplete precipitation (Björling, 1976; Piel et al., 2004) it is also not surprising that these three methods indicate such proportionality.

The concentrations of galactose and fucose also differ greatly between the different types of gastrointestinal mucin (Table 8). Pig gastric mucin has over five times the amount of galactose and fucose than pig intestinal mucin. As the type of mucin found in digesta from the terminal ileum varies, the different concentrations of galactose and fucose cannot be relied upon for the determination of absolute concentrations of mucin. Similarly the Periodic acid-Schiff reaction also seems more suitable for comparative analysis and cannot be relied upon for determining absolute concentrations of mucin. Therefore an apparent underestimation of mucin using this method (Table 5) may be attributable to the variance in monosaccharide composition, between the types of mucin present in the digesta, and their differential reactions to the chromophore.

Sialic acid is a collective term for the acylated derivatives of neuraminic acid. In mammalian glycoproteins sialic acids occur at the terminal end of oligosaccharide side chains of the glycoconjugate and give the mucin molecules an electronegative charge. Due to the weakness of its glycosidic linkage to the carbohydrate side chains, sialic acid units are easily cleaved from such side chains with only mild hydrolysis. Thus, the concentration of free sialic acid may be used as a measure of desialylation of the glycoconjugates (Yusuf *et al.*, 2005) and the quantification of free sialic acid in the ileal effluent can indicate the degree of degradation of glycoproteins.

The mucin concentration estimated using the sialic acid assay was $5.9 \text{ g}\cdot\text{kg}^{-1}$ DMI, a value more than seven times lower than the value obtained using the gas chromatograph hexosamine assay (Table 5). The reason for such a large difference is not known, however the degradation of sialic acid along the gastrointestinal tract and incomplete reaction of the breakdown products with the chromophore may account for the low values observed.

The concentration of free sialic acid ($0.12 \text{ g}\cdot\text{kg}^{-1}$ DMI, Table 4) was nearly 2.4 times that of the bound fraction ($0.05 \text{ g}\cdot\text{kg}^{-1}$ DMI), suggesting a substantial degree of mucin degradation. It is not therefore surprising that the sialic acid assay yielded lower, though still statistically significant, inter-assay correlations compared to the other colorimetric and ethanol precipitation assays.

With the ethanol precipitation method there may have been differential precipitation of highly glycosylated glycoproteins and of other non-mucin components. There seems no reason why this gravimetric assay produces results 2.3 times lower than the hexosamine assay, other than that it ineffectively precipitates degraded forms of mucin as indicated by the free sialic acid concentrations present in the digesta. Although the two colorimetric assays for neutral sugars are both straightforward and relatively inexpensive they can only be justifiably used for comparative studies. Given that the ethanol precipitation method clearly gave low recoveries of mucin (based upon chemical analysis of the supernatant and precipitate), this also calls into question the accuracy of the absolute values for mucin excretion determined by the periodic acid Schiff, the phenol sulphuric acid and the sialic acid methods, all of which gave values similar to or lower than the ethanol precipitation method. One reason for this may be that the mucins present have been degraded to such an extent that the other mucin markers present in the digesta may not be conserved as much as the two hexosamines. It would therefore be useful to determine the nature of the ethanol precipitate and the extent of its degradation.

One notable omission from the range of methods investigated here is the ELISA assay used by Piel *et al.* (2004). In their hands, mucin levels in digesta estimated by this means were only one third those derived from the hexosamine assay. They attribute this discrepancy to the hexosamine and ethanol precipitation assays being less specific.

They also found a fourfold greater individual variability with the ELISA technique than with the other two assays. They suggested that this may also be due to a lack of specificity. However, it seems to us that a more likely reason for these results stems from the extensive degradation of mucins during their transit of the GI tract, which is likely to diminish progressively the specificity of an antibody-based method.

Each of the five methods studied has advantages and disadvantages from a technical perspective. The present results highlight that both the hexosamine assay, using a gas liquid chromatography method and the quantification of free and bound sialic acid give information that is useful in describing the nature of the mucins present at the terminal ileum. The hexosamine assay gives a measure of the ratio of gastric to intestinal mucin, and the sialic acid assay a measure of the extent of digestive degradation, both useful in describing the composition of ileal effluent. If the quantities of gastric and small intestinal mucin are to be determined it would appear that the hexosamine assay would be the current method of choice.

Acknowledgements

Grateful thanks are given for the assistance of Dr Amélie Deglaire during the pig trial and Dr Gordon Reynolds for surgical procedures involved with installing the post-valve T caecum cannulae.

Literature cited

- Aminoff, D. (1961). Methods for Quantitative Estimation of N-Acetylneuraminic Acid and Their Application to Hydrolysates of Sialomucoids. *Biochemical Journal*, 81(2), 384-392.
- AOAC. (2000). Official Method for protein (Dumas method), 968.06. *Official methods of analysis of association of official analytical chemists international*.
- Azuumi, Y., Ichikawa, T., Ishihara, K., and Hotta, K. (1993). The Validity of the Ethanol Precipitation Method for the Measurement of Mucin Content in

- Human Gastric Juices and Its Possible Relationship to Gastroduodenal Diseases. *Clinica Chimica Acta*, 221(1-2), 219-225.
- Bansil, R., and Turner, B. S. (2006). Mucin structure, aggregation, physiological functions and biomedical applications. *Current Opinion in Colloid and Interface Science*, 11(2-3), 164-170.
- Barcelo, A., Claustre, J., Moro, F., Chayvialle, J. A., Cuber, J. C., and Plaisancie, P. (2000). Mucin secretion is modulated by luminal factors in the isolated vascularly perfused rat colon. *Gut*, 46(2), 218-224.
- Beeley, J. G. (1985). *Glycoprotein and Proteoglycan Techniques* (Vol. 16). Amsterdam: Elsevier Science Publishers B. V.
- Bevins, C. L., Martin-Porter, E., and Ganz, T. (1999). Defensins and innate host defence of the gastrointestinal tract. *Gut*, 271, 14038-14045.
- Björling, H. (1976). Cohn fractionation. In B. Blombäck and L. A. Hanson (Eds.), *Plasma Proteins* (pp. 29-37). Chichester: Wiley.
- Blakeney, A. B., Harris, P. J., Henry, R. J., and Stone, B. A. (1983). A Simple and Rapid Preparation of Alditol Acetates for Monosaccharide Analysis. *Carbohydrate Research*, 113(2), 291-299.
- Clamp, J. R., Allen, A., Gibbons, R. A., and Roberts, G. P. (1978). Chemical Aspects of Mucus. *British Medical Bulletin*, 34(1), 25-41.
- Claustre, J., Toumi, F., Trompette, A., Jourdan, G., Guignard, H., Chayvialle, J. A., *et al.* (2002). Effects of peptides derived from dietary proteins on mucus secretion in rat jejunum. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 283(3), G521-G528.
- Corfield, A. P., Carroll, D., Myerscough, N., and Probert, C. S. J. (2001). Mucins in the gastrointestinal tract in health and disease. *Frontiers in Bioscience*, 6, D1321-D1357.
- Deglaire, A., Bos, C., Airinei, G., Luengo, C., Benamouzig, R., Fauquant, J., *et al.* (2007). Postprandial metabolic utilization of intact and hydrolyzed casein in humans. *FASEB Journal*, 21(5), A333-A333.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, 28(3), 350-356.
- Hodgkinson, S. M., and Moughan, P. J. (2003). The enzyme hydrolysed protein method for the determination of endogenous ileal nitrogen and amino acid

- flows - a modification. *Animal Feed Science and Technology*, 108(1-4), 207-214.
- Kotwal, S. M., Gote, M. M., Khan, M. I., and Khire, J. M. (1999). Production, purification and characterization of a constitutive intracellular alpha-galactosidase from the thermophilic fungus *Humicola* sp. *Journal of Industrial Microbiology and Biotechnology*, 23(1), 661-667.
- Kraus, R. J., Shinnick, F. L., and Marlett, J. A. (1990). Simultaneous Determination of Neutral and Amino-Sugars in Biological-Materials. *Journal of Chromatography*, 513, 71-81.
- Krause, W. J. (2000). Brunner's glands: A structural, histochemical and pathological profile. *Progress in Histochemistry and Cytochemistry*, 35(4), 259-367.
- Leterme, P., Thewis, A., Francois, E., Van Leeuwen, P., Wathelet, B., and Huisman, J. (1996). The use of ¹⁵N-labelled dietary proteins for determining true ileal amino acid digestibilities is limited by their rapid recycling in the endogenous secretions of pigs. *Journal of Nutrition*, 126, 2188-2198.
- Lien, K. A., McBurney, M. I., Beyde, B. I., Thomson, A. B. R., and Sauer, W. C. (1996). Ileal recovery of nutrients and mucin in humans fed total enteral formulas supplemented with soy fiber. *American Journal of Clinical Nutrition*, 63(4), 584-595.
- Lien, K. A., Sauer, W. C., and Fenton, M. (1997). Mucin output in ileal digesta of pigs fed a protein-free diet. *Zeitschrift Fur Ernährungswissenschaft*, 36(2), 182-190.
- Lin, S. Y., Wu, C. W., Li, M. J., and Liang, R. C. (1997). Infra-red (ATR/FT) spectroscopic study on the conformational structure of the isolated human gastric mucus pretreated with ethanol. *Journal of Gastroenterology and Hepatology*, 12(11), 707-712.
- Mantle, M., and Allen, A. (1978). A colorimetric assay for glycoproteins based on the Periodic Acid/Schiff stain. *Biochemical Society Transactions*, 6, 607-609.
- Mantle, M., and Allen, A. (1981). Isolation and Characterization of the Native Glycoprotein from Pig Small-Intestinal Mucus. *Biochemical Journal*, 195(1), 267-275.
- Mantle, M., and Allen, A. (1989). Gastrointestinal mucus. In J. S. Davison (Ed.), *Gastrointestinal Secretions* (pp. 202-229). London: Butterworth and Co.
- Metges, C. C., Petzke, K. J., El-Khoury, A. E., Henneman, L., Grant, I., Bedri, S., et al. (1999). Incorporation of urea and ammonia nitrogen into ileal and fecal

- microbial proteins and plasma free amino acids in normal men and ileostomates. *American Journal of Clinical Nutrition*, 70(6), 1046-1058.
- Miner-Williams, W., Moughan, P. J., and Fuller, M. F. (2009). Endogenous components of digesta protein from the terminal ileum of pigs fed a casein based diet. *Journal of Agricultural and Food Chemistry* 57, 2072-2078.
- Montagne, L., Piel, C., and Lalles, J. P. (2004). Effect of diet on mucin kinetics and composition: Nutrition and health implications. *Nutrition Reviews*, 62(3), 105-114.
- Montagne, L., Toullec, R., Formal, M., and Lalles, J. P. (2000). Influence of dietary protein level and origin on the flow of mucin along the small intestine of the preruminant calf. *Journal of Dairy Science*, 83(12), 2820-2828.
- O'Keefe, B. R., Beutler, J. A., Cardellina, J. H., Gulakowski, R. J., Krepps, B. L., McMahon, J. B., *et al.* (1997). Isolation and characterization of niphatevirin, a human-immunodeficiency-virus-inhibitory glycoprotein from the marine sponge *Niphates erecta*. *European Journal of Biochemistry*, 245(1), 47-53.
- Piel, C., Montagne, L., Salgado, P., and Lalles, J. P. (2004). Estimation of ileal output of gastro-intestinal glycoprotein in weaned piglets using three different methods. *Reproduction Nutrition Development*, 44(5), 419-435.
- Roden, L., and Horowitz, M. I. (1978). Structure and biosynthesis of connective tissue proteoglycans. In M. I. Horowitz and W. Pigman (Eds.), *The glycoconjugates*. (Vol. II, pp. 3-71). New York: Academic Press.
- Roussel, P., and Delmotte, P. (2004). The diversity of epithelial secreted mucins. *Current Organic Chemistry*, 8(5), 413-437.
- Salgado, P., Lalles, J. P., Toullec, R., Mourato, M., Cabral, F., and Freire, J. P. B. (2001). Nutrient digestibility of chickpea (*Cicer arietinum* L.) seeds and effects on the small intestine of weaned piglets. *Animal Feed Science and Technology*, 91(3-4), 197-212.
- Sanderson, I. R., and Walker, W. A. (1999). In R. Ogra and J. e. a. Mestecky (Eds.), *Handbook of Mucosal Immunology* (2nd ed., pp. 5-17). San Diego: Academic Press.
- Satchithanandam, S., Klurfeld, D. M., Calvert, R. J., and Cassidy, M. M. (1996). Effects of dietary fibers on gastrointestinal mucin in rats. *Nutrition Research*, 16(7), 1163-1177.

- Short, F. J., Gorton, P., Wiseman, J., and Boorman, K. N. (1996). Determination of titanium dioxide added as an inert marker in chicken digestibility studies. *Animal Feed Science and Technology*, 59(4), 215-221.
- Snary, D., and Allen, A. (1971). Studies on Gastric Mucoproteins - Isolation and Characterization of Mucoprotein of Water-Soluble Mucus from Pig Cardiac Gastric Mucosa. *Biochemical Journal*, 123(5), 845-853.
- Stanley, R. A., Lee, S. P., and Robertson, A. M. (1983). Heterogeneity in Gastrointestinal Mucins. *Biochimica Et Biophysica Acta*, 760(2), 262-269.
- van Leeuwen, P., van Kleef, D. J., van Kempen, G. J. M., Huisman, J., and Verstegen, M. W. A. (1991). The Post Valve T-Cecum Cannulation Technique in Pigs Applied (sic) to Determine the Digestibility of Amino-Acid in Maize, Groundnut and Sunflower Meal. *Journal of Animal Physiology and Animal Nutrition-Zeitschrift Fur Tierphysiologie Tierernahrung Und Futtermittelkunde*, 65(3-4), 183-193.
- Weltzien, F. A., Norberg, B., and Swanson, P. (2003). Isolation and characterization of FSH and LH from pituitary glands of Atlantic halibut (*Hippoglossus hippoglossus* L.). *General and Comparative Endocrinology*, 131(2), 97-105.
- Yusuf, S., Nok, A. J., Ameh, D. A., Adelaiye, A. B., and Balogun, E. O. (2005). Correlation of gastric mucosal damage with sialic acid profile in rats: effect of hydrochloric acid, pepsin and hypertonic saline. *Cell Biochemistry and Function*, 23(5), 339-345.

Chapter III

Analysis of an ethanol precipitate from the soluble fraction of ileal digesta: evaluation of a method for the determination of mucin.

The objective of the present study was to evaluate the precipitation of mucins using high concentrations of ethanol used for the isolation of mucin in the digesta collected from the terminal ileum of pigs fed a casein-based diet.

Abstract

The precipitation of crude mucin using high concentrations of ethanol has been used by many researchers to quantify mucin in the gut and yet others have called into question the validity of the technique. An investigative analysis of an ethanol precipitate, produced from the soluble fraction of terminal ileal digesta, from pigs fed a casein-based diet, was undertaken using molecular weight profiling and polyacrylamide gel electrophoresis. The precipitate contained 201 mg·g⁻¹ protein, 87% of which had a molecular weight in excess of 20 KDa. Polyacrylamide gel electrophoresis stained with both Coomassie blue and periodic acid/Schiff, revealed that most of the glycoprotein had a molecular weight between 37 and 100 KDa. The molecular weight of glycoprotein in the precipitate was therefore considerably lower than that of intact mucin. These observations, together with a high proportion of free sialic acid, indicated that the glycoprotein in the ethanol precipitate was significantly degraded. The large amount of protein and carbohydrate detected in the supernatant from ethanol precipitation indicated that the precipitation of glycoprotein using high concentrations of ethanol was incomplete. As a quantitative method for determining the concentration of mucin in digesta, ethanol precipitation is demonstrated to be an unreliable technique. Although the validity of this assay for the determination of mucin in human gastric juices has been accepted by some workers its effectiveness has been questioned by others. This paper describes the first complete evaluation of the ethanol precipitation of mucin from ileal digesta as a method for the quantification of mucin.

Introduction

The epithelial tissues of the gastrointestinal tract are subject to degradative challenges from extremes in pH, enzymatic hydrolysis, attack from pathogenic bacteria and physical erosion (Bevins *et al.*, 1999; Sanderson and Walker, 1999). The gastrointestinal mucosa is protected by layers of secreted or membrane-bound mucus formed from polymeric glycoproteins, mucins, secreted by specialist cells of the underlying mucosa. Secreted mucins are high molecular mass, [2×10^6 Da (Montagne Bevins *et al.*, 2004)] polydisperse molecules that contain a central polypeptide core of 1500 to 4500 amino acids and 100-200 covalently bound oligosaccharide side chains containing 1 to 20 or more monosaccharides. Likened to the structure of a bottlebrush the oligosaccharides, arranged around the protein core make up 50 to 80% of the mucin's mass. The carbohydrate side chains are bound to the polypeptide by O-glycosidic linkages between N-acetylgalactosamine and the hydroxylated amino acids serine and threonine (Roussel and Delmotte, 2004). The glycosylated regions of the polypeptide are rich in serine, threonine and proline whilst the non-glycosylated regions are rich in cysteine which allows the formation of disulphide bridges between glycoprotein polymeric sub-units to form a cohesive viscoelastic gel across the surface of the gastrointestinal tract (Krause, 2000).

All along the gastrointestinal tract a dynamic equilibrium exists between the synthesis of mucins and their degradation by proteolysis and physical erosion. As mucins are somewhat resistant to mammalian digestive processes they represent a significant fraction of endogenous losses at the terminal ileum. In our own work (Miner-Williams *et al.*, 2009a) the mucin content of digesta, collected from the terminal ileum of pigs fed a casein-based diet, equalled nearly 16% of the overall dry matter and mucins were the single most abundant, truly endogenous component secreted into the gastrointestinal tract. As mucin is so important in the protection of the gastrointestinal mucosa any dietary or physiological factor that affects the amount of mucin secreted into the digestive tract will have important implications on both metabolism and the integrity of the defensive mechanism.

There are few studies that detail the quantification of gastrointestinal mucin because it is particularly difficult to assay (Piel *et al.*, 2004). In a previous study (Miner-Williams *et al.*, 2009b) a number of methods to determine the concentration of mucin in ileal digesta were evaluated, including the gravimetric method of precipitating mucins using high concentrations of ethanol and the determination of mucin based on the markers N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GluNAc) (Piel *et al.*, 2004). In our study, values obtained by ethanol precipitation were on average 43% lower than for the hexosamine assay, whilst Piel *et al.* (2004) found the difference was as much as 33%.

The precipitation of glycoproteins using high concentrations of ethanol is considered by many researchers to be non-specific, as Leterme *et al.* (1998) concede, because the precipitate does not only contain raw mucus but may be contaminated by non-covalently bound proteins. This is corroborated by considering the various compounds ethanol has been used to precipitate (e.g., α -galactosidase from a fungus (Kotwal *et al.*, 1999), human-immunodeficiency-virus-inhibitory glycoprotein from aqueous extracts of a Caribbean sponge (O'Keefe *et al.*, 1997), gonadotropins, follicle-stimulating hormone and luteinising hormone from halibut pituitary gland (Weltzien *et al.*, 2003). Although the validity of this assay for the determination of mucin in human gastric juices has been accepted by some workers [e.g., Azuumi *et al.* (Azuumi *et al.*, 1993)], its effectiveness has been questioned by others (Piel *et al.*, 2004; O'Keefe *et al.*, 1997).

The results from our previous study (Miner-Williams *et al.*, 2009b) and that of others (Piel *et al.*, 2004) call into question the reliability of the ethanol precipitation method for the quantification of mucin in ileal digesta. The aim of this study was to investigate ethanol precipitates from ileal digesta as the source of mucins to evaluate the ethanol precipitation of digesta as a method for the quantification of mucin.

Materials and methods

Terminal ileal digesta samples were collected during the conduct of another study (Miner-Williams *et al.*, 2009a) from 6 Large White × Duroc pigs of mean body weight 79 (\pm 4.8) kg, [\pm standard error of mean (SEM)] fitted with a post-valve T caecum cannula (van Leeuwen *et al.*, 1991). The pigs were fed a lactic casein-based diet mixed with water (**Table 1**).

Table 1 Composition of the experimental diet (g/kg air dry weight).

Ingredient	Experimental Diet ¹
Maltodextrin	453
Sucrose	161
Soyabean oil	154
Sodium Hydrogen Carbonate	18
Titanium Dioxide	3
Lactic casein	211

¹No vitamins, minerals or fibre were added to this diet as the same diet was used in an acute feeding study with human subjects.

The fresh digesta were fractionated by differential centrifugation following the method of Metges *et al.* (1999). Samples were centrifuged first at 250 RCF for 15 minutes at 4°C to separate food particles and porcine cells, then at 14,500 RCF for 30 minutes at 4°C to separate microbial cells and porcine cellular detritus. It was assumed that the mucins, which are soluble, were quantitatively recovered in the supernatant, together with other soluble materials including proteins, peptides, free amino acids, neutral sugars, urea, creatinine and ammonia.

The precipitation of crude mucin from the 14500 RCF supernatant using ethanol followed a method adapted from that of Piel *et al.* (2004). 3 mL of the supernatant were added to 25 mL of 0.15 M aqueous sodium chloride and centrifuged at 14,500 RCF for 30 minutes at 4 °C. After centrifugation, 15 mL of the supernatant were added to 22 mL of absolute ethanol at 0 °C and kept at -20 °C overnight. The tubes

were then centrifuged again at 14,500 RCF (at 4 °C) before the supernatant was removed and the precipitate dissolved in a further 15mL of 0.15 M aqueous sodium chloride. The ethanol precipitation was then repeated before the final precipitate was dissolved in 10 mL of deionised water, freeze-dried and weighed. All the ethanol supernatant was recovered, dried and redissolved in 10 mL of deionised water.

Polyacrylamide gel electrophoresis (SDS-PAGE) followed the protocol used by Piel *et al.* (2004) for analysing the purity of protein, using 7.5% and 4% polyacrylamide gels for migrating and stacking gels respectively under conditions described by Laemmli (1970). Mucins on SDS-PAGE gels were detected using periodic acid/Schiff (PAS) staining, using a glycoprotein detection kit (GLYCO-PRO, Sigma, St Louis, MO, USA), and Coomassie Blue staining, using 0.1% Coomassie Brilliant Blue R-250.

Molecular weight profiling was adapted from the method described by Swergold and Rubin (1983) using high performance liquid chromatography with a size exclusion column. Briefly, peptides and amino acids in a hydrolysate were separated according to their size using two 2 × 30 cm TSK G2000 SWXL columns in series, with a TSK SWXL guard column, with a mobile phase that contained acetonitrile to denature the peptides and to promote better separation in the 0-10,000 molecular weight range. The column flow rate was 0.5 mL/minute (isocratic) at ambient temperature with a run length of 65 minutes. Peptides were detected by monitoring the absorbance at 205 or 210 nm. A calibration standard was obtained by using nine molecular weight markers to establish a plot of log molecular weight versus retention time ($r^2 = 0.995$). The curve allowed the estimation of molecular weights corresponding to specific retention times.

Soluble protein was determined using the Bradford method (Bradford, 1976). The detection of neutral sugars, using phenol-sulphuric acid, and of sialic acid, using periodate-thiobarbituric acid, were undertaken using an adaptation of the methods described by Beeley (1985). Dry matter was determined by drying material to a constant mass in a forced-air oven at 95 °C.

Results and Discussion

The average mass of the ethanol precipitate was $69.7 \text{ mg}\cdot\text{g}^{-1}$ digesta dry matter ([DDM] coefficient of variation = 19.1%; n = 6) The molecular weight profile (**Figure 1, Table 2**) of the residue from the ethanol precipitation showed that 87% of the material consisted of protein molecules with a molecular weight greater than 20 KDa, with a further 10% of the material consisting of molecules with a molecular weight less than 1KDa.

Figure 1 Molecular weight profile (absorbance versus time) of the residue after ethanol precipitation of the 14,500 RCF digesta supernatant.

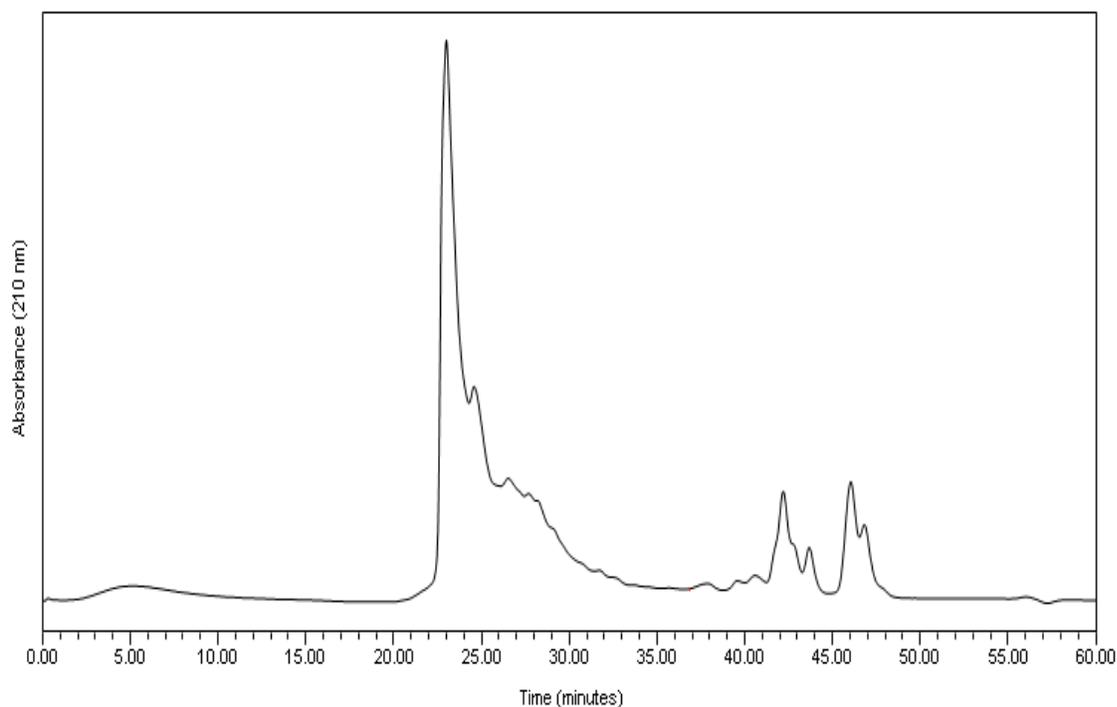


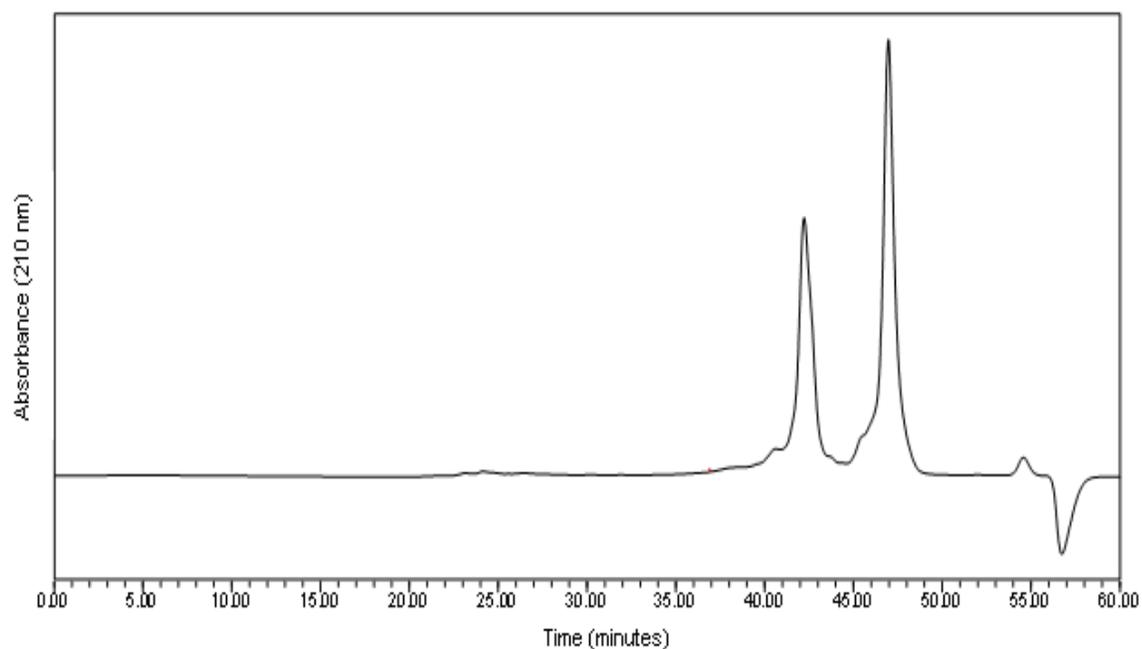
Table 2 Molecular weight distribution within the residue and supernatant following ethanol precipitation of the 14,500 RCF digesta supernatant.

Molecular weight grouping	Molecular weight (KDa)	Ethanol-precipitation fraction	
		Residue ^{1.}	Supernatant ^{1.}
1	> 20	87.2	4.0
2	5-20	2.9	0.1
3	1-5	0.01	0.1
4	< 1	9.9	95.8

^{1.} Recorded as a percentage of total mass within each fraction.

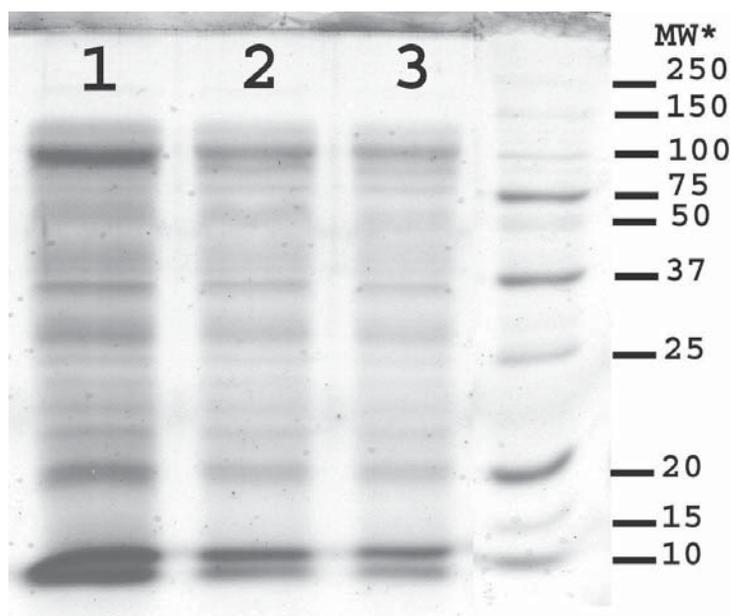
In comparison, the supernatant contained predominantly protein molecules with a molecular weight less than 1 KDa (**Figure 2**, Table 2). Only a small amount of material in the supernatant (4%) had a molecular weight greater than 20 KDa.

Figure 2 Molecular weight profile (absorbance versus time) of the supernatant after ethanol precipitation of the 14,500 RCF digesta supernatant.



SDS-PAGE analysis with Coomassie staining (**Figure 3**) revealed banding that indicated that the residue contained proteins with molecular weights ranging from less than 10 to just over one hundred KDa and confirmed what was observed with the molecular weight profiling.

Figure 3 Polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the residue from ethanol precipitation of the 14,500 RCF digesta supernatant using Coomassie Blue staining for protein.



* MW = Molecular weight (KDa)

1, 2 and 3 represent the volume of solubilised ethanol precipitate applied to the electrophoresis gel. 1 = 5 µL, 2 = 2.5 µL and 3 = 1 µL.

Bands more heavily stained than others were seen at <10, 12, 20, 31, 37, 69, and 120 KDa (**Table 3**). When the SDS-PAGE analysis was repeated, using the same conditions but using a periodic acid/Schiff stain (**Figure 4**) for sugars that are oxidizable by periodate, densely stained bands were observed equivalent to 23, 40, 63, 80 and 100 KDa (Table 3). However, there was diffuse staining in two separate bands between 21 and 28 and between 37 and 100 KDa which also matched areas of diffuse staining on the Coomassie blue plate.

Table 3 The molecular weights of observed bands within the polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the residue from ethanol precipitation of the 14,500 RCF digesta supernatant using Coomassie Blue staining for protein and periodic acid Schiff staining for sugar.

Band	Molecular weight (KDa)	
	Coomassie Blue	Periodic Acid Schiff
1	<10*	21
2	12*	23*
3	20*	28
4	22	37
5	23	40*
6	24	63*
7	27	80*
8	31*	100*
9	37*	
10	47	
11	69*	
12	80	
13	88	
14	120*	

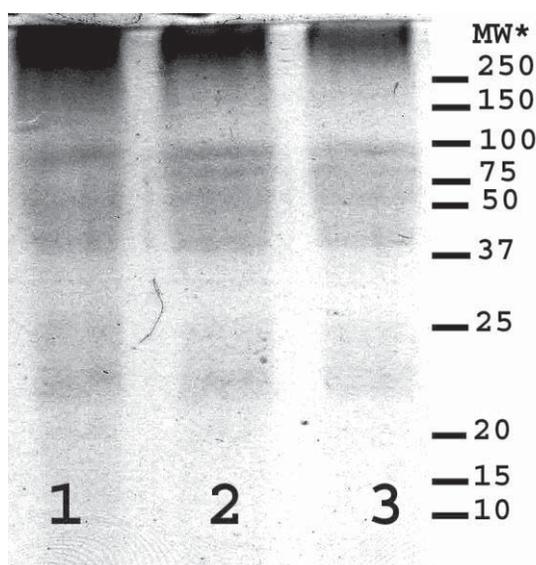
* Significantly dense stained bands.

a and b areas with diffuse bands stained by periodic acid Schiff.

In an earlier study (Miner-Williams *et al.*, 2009b) it was determined that terminal ileal digesta from pigs contains a mixture of gastric and small intestinal mucins in a ratio of nearly 2:1. Using this ratio, and data published by Allen *et al.* (1982) on the composition of both porcine gastric and small intestinal glycoproteins, the expected composition of the ethanol precipitate was calculated (**Table 4**). The ester sulphate content of the ethanol precipitate was not determined during the present study. This expected composition was then compared with the determined composition of the ethanol precipitate.

The determined chemical composition of the ethanol precipitate is given in **Table 5**. If the ester sulphate content of porcine gastric and small intestinal mucin is assumed to be 2.7% [(Allen *et al.*, 1982); Table 4], then only 1.4% of the dry matter remained unaccounted for. Analysis of the carbohydrate and protein content of the supernatant estimated the concentrations to be 25.6 and 14.1 mg·g⁻¹ DDM respectively.

Figure 4 Polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the residue from ethanol precipitation of the 14,500 RCF digesta supernatant using periodic acid Schiff staining for carbohydrate.



* MW = Molecular weight (KDa)

1, 2 and 3 represent the volume of solubilised ethanol precipitate applied to the electrophoresis gel. 1 = 5 μ L, 2 = 2.5 μ L and 3 = 1 μ L.

When the mass of the precipitate (69.7 mg·g⁻¹ DDM) was compared with the cumulative mass of the components determined individually (69.2 mg·g⁻¹ DDM) only a small (0.7%) deficit was observed. However when the concentration of carbohydrate in the ethanol supernatant is taken as the difference between that in the ethanol precipitate (65% of its mass, Table 5) and the total concentration of carbohydrate in the original 14,500 RCF supernatant, a concentration of 21.1 mg·g⁻¹ DDM should be present, 6.7% less than what was actually found.

Table 4 Composition of pure pig gastric and small intestinal glycoprotein together with the expected composition of glycoprotein in the ethanol precipitate (of the 14,500 RCF digesta supernatant fraction) found in the present study.

Constituent	Dry weight composition (%) ^{1.}		Expected composition of ethanol precipitate (%) ^{2.}	
	Gastric	Small intestinal	Dry	Wet
Carbohydrate	82.9	77.5	81.6	71.8
Protein	13.2	19.6	15.3	13.5
Ester sulphate	3.2	2.8	3.1	2.7
Water	-	-	-	12 ^{3.}
Total	99.3	99.9	100	100

^{1.} Taken from Allen et al. (1982).

^{2.} Calculated using the estimated ratio of gastric and small intestinal mucin (2.05:1) present in the digesta from data of pigs fed a casein based diet (Miner-Williams *et al.*, 2009).

^{3.} Calculated using data from Mantle and Allen (1981) and Scawen and Allen (1977)

Over 70% of the total sialic acid in the 14,500 RCF supernatant was free. Pig gastric mucin is thought to contain an average of four glycoprotein subunits, of 5×10^5 Da, joined together by covalent disulphide bridges, whereas pig small intestine has an average of eight subunits each with a molecular weight of 2×10^5 Da. During proteolysis the glycoprotein sub-units separate and some degradation of the non-glycosylated regions of the glycoprotein core occurs. Although proteases may degrade polymeric mucins into smaller glycosylated units, any further breakdown of the protein core cannot occur until the carbohydrate side chains are removed. This occurs primarily in the colon, mediated by bacterial exoenzymes (Mantle and Allen, 1989).

Table 5 Composition of the ethanol precipitate (from the 14,500 RCF digesta supernatant fraction).

<i>Constituent</i>	<i>mg·g⁻¹ DDM</i>	<i>SEM</i>	<i>% of total</i>
Carbohydrate	45.6	1.1	65.0
Protein	13.9	0.83	20.1
Ester sulphate ^{1.}	1.9 ^{1.}	-	2.7
Water	7.8	0.70	10.8
Total	69.2		98.6

^{1.} Estimate from Table 2

Sialic acid residues usually occur as the terminal non-reducing residues (Beeley 1985) of the oligosaccharide chains of the glycoconjugate and their electronegativity is known to significantly influence mucin rheology and degradation (Pasquier *et al.*, 1991; Yusuf *et al.*, 2005). As the glycosidic linkages between sialic acids and the oligosaccharide chains are easily hydrolysed, the concentration of free sialic acid present in the digesta may be used as an estimate of mucin degradation (Yusuf *et al.*, 2005). With more than 70% of the sialic acid in the 14,500 RCF supernatant found to be unbound, this degree of desialylation represents significant mucin degradation and supports the SDS PAGE data that the glycoproteins detected in the ethanol precipitate were degraded mucin subunits of lower molecular weight.

The dry weight of the precipitate was determined to be 32.5% of the dry matter present in the 14,500 RCF digesta supernatant. The concentration of protein in the ethanol precipitate and supernatant were estimated to be 13.9 and 14.1 mg·g⁻¹ DDM respectively. Less than 50% of the total protein and 64% of the total carbohydrate present in the 14,500 RCF digesta supernatant were precipitated by the ethanol. Such ineffectual precipitation might explain why, in an earlier study comparing different methods of quantifying mucin in ileal digesta (Miner-Williams 2009b), the ethanol precipitation method gave an estimated mucin concentration less than half of that determined using the mucin markers GalNAc and GluNAc.

The determined carbohydrate concentration of the ethanol precipitate (65%, Table 5), is 7% less than expected (72% estimated from the composition data of Allen *et*

al. (1982), Table 4), and the protein concentration is nearly 7 percentage units higher than expected [20% determined in this study compared to 13.5% derived from the data of Allen *et al.* (1982)]. This together with the free sialic acid data indicate that the glycoprotein present in the ethanol precipitate, and the original digesta, had been degraded and that some sugar moieties had been stripped off the bottlebrush structure by both α - and β -glycosidases. Such degradation of the glycoprotein subunits would also explain the range of smaller glycoproteins observed in the SDS PAGE analysis where the bulk of the proteins, with associated sugar residues, had molecular weights ranging from between 37 and 100 KDa.

Mucin in ileal effluent would be expected to contain predominantly a mixture of gastric and small intestinal mucins. It has been assumed that there is minimal digestion of mucin in the small intestine and that this largely takes place in the colon (Montagne *et al.*, 2004; Morel *et al.*, 2003). However the data from this study show that considerable degradation has occurred proximal to the terminal ileum. Mucin degradation by proteolysis and physical erosion is a multistep process initiated by the enzymatic proteolysis of the non-glycosylated regions of the mucin oligomers by host and microbial proteases: this process, together with the disruption of the intermolecular disulphide bridges, occurs continuously throughout the gastrointestinal tract (Deplancke and Gaskins, 2001). As a result the chyme will contain fragments of the non-glycosylated apomucin and an accumulation of the heavily glycosylated STP domains (>500 KDa) now termed mucin glycopeptides or T-domains (T = trypsin) where the oligosaccharide side chains protect the polypeptide chain from further proteolytic attack (Deplancke and Gaskins, 2001; Roussel and Delmotte, 2004).

It is therefore anticipated that although the T-domains are conserved some sugar moieties from the termini of the oligosaccharide chains will have been degraded and only small remnants of the non-glycosylated apomucin remain in the ileal effluent. The data from this study largely corroborates these conclusions.

The precipitation of crude mucin using high concentrations of ethanol is not an easily reproducible assay as a coefficient of variation of 19% found in this study suggests. As reported previously Miner-Williams *et al.* (2009) the final ethanol

concentration is critical and any deviation from the standard conditions can lead to spurious results. Ethanol precipitation is not specific to mucin (Kotwal *et al.*, 1999; O'Keefe *et al.*, 1997; Weltzien *et al.*, 2003) and its effectiveness has been called into question by a number of researchers (O'Keefe *et al.*, 1997; Piel *et al.*, 2004). Moughan *et al.* (1990), using perchloric and trichloroacetic acids as protein precipitants and indeed Björling (1976) suggested that some highly glycosylated glycoproteins remain soluble even in high concentrations of ethanol. In the light of this evidence, the high concentration of carbohydrate and protein found in the ethanol supernatant in the present study may represent either non-precipitated mucin or other non-associated sugars and proteins found in the soluble fraction of digesta.

The ineffective precipitation of degraded glycoprotein subunits present in the digesta may explain why the ethanol precipitation procedure appears to underestimate digesta mucin concentration (Miner-Williams *et al.*, 2009b; Piel *et al.*, 2004). This procedure for the quantification of mucin is likely to lead to considerable error.

If accurate estimations are to be made, any estimation using mucin markers must involve those markers that are best conserved, even though mucin degradation has been demonstrated proximal to the ileocaecal junction. In our earlier study (Miner-Williams *et al.*, 2008b) it was demonstrated that using the ileal concentrations of neutral sugars and sialic acid underestimated the concentration of mucin in ileal digesta. Data from this study confirms that such mucin markers are unreliable as mucin degradation lowers the concentration of many carbohydrate moieties and leads to the underestimation of mucin in the ileal effluent. However, as every oligosaccharide side chain begins with one GalNAc residue and that these residues are conserved more than any other, we suggest that using GalNAc as a mucin marker is justifiably more reliable.

Literature cited

- Allen, A., Bell, A., Mantle, M., and Pearson, J. P. (1982). The Structure and Physiology of Gastrointestinal Mucus. *Advances in Experimental Medicine and Biology*, 144, 115-133.
- Azuumi, Y., Ichikawa, T., Ishihara, K., and Hotta, K. (1993). The Validity of the Ethanol Precipitation Method for the Measurement of Mucin Content in Human Gastric Juices and Its Possible Relationship to Gastroduodenal Diseases. *Clinica Chimica Acta*, 221(1-2), 219-225.
- Beeley, J. G. (1985). *Glycoprotein and Proteoglycan Techniques* (Vol. 16). Amsterdam: Elsevier Science Publishers B. V.
- Bevins, C. L., Martin-Porter, E., and Ganz, T. (1999). Defensins and innate host defence of the gastrointestinal tract. *Gut*, 271, 14038-14045.
- Björling, H. (1976). In B. Blombäck and L. A. Hanson (Eds.), *Plasma Proteins* (29-37). Chichester: Wiley.
- Bradford, M. M. (1976). Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein Utilizing Principle of Protein-Dye Binding. *Analytical Biochemistry*, 72(1-2), 248-254.
- Deplancke, B., and Gaskins, H. R. (2001). Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. *American Journal of Clinical Nutrition*, 73(6), 1131S-1141S.
- Kotwal, S. M., Gote, M. M., Khan, M. I., and Khire, J. M. (1999). Production, purification and characterization of a constitutive intracellular alpha-galactosidase from the thermophilic fungus *Humicola* sp. *Journal of Industrial Microbiology and Biotechnology*, 23(1), 661-667.
- Krause, W. J. (2000). Brunner's glands: A structural, histochemical and pathological profile. *Progress in Histochemistry and Cytochemistry*, 35(4), 255-+.
- Laemmli, U. K. (1970). Cleavage of Structural Proteins During Assembly of Head of Bacteriophage-T4. *Nature*, 227(5259), 680-and.
- Leterme, P., Seve, B., and Thewis, A. (1998). The current N-15-Leucine infusion technique is not suitable for quantitative measurements of ileal endogenous amino acid flows in pigs. *Journal of Nutrition*, 128(11), 1961-1968.

- Mantle, M., and Allen, A. (1989). Gastrointestinal mucus. In J. S. Davison (Ed.), *Gastrointestinal Secretions* (pp. 202-229). London: Butterworth and Co.
- Metges, C. C., Petzke, K. J., El-Khoury, A. E., Henneman, L., Grant, I., Bedri, S., et al. (1999). Incorporation of urea and ammonia nitrogen into ileal and fecal microbial proteins and plasma free amino acids in normal men and ileostomates. *American Journal of Clinical Nutrition*, 70(6), 1046-1058.
- Miner-Williams, W., Moughan, P. J., & Fuller, M. F. (2009a). Endogenous components of digesta protein from the terminal ileum of pigs fed a casein based diet. *Journal of Agricultural and Food Chemistry* 57, 2072-2078.
- Miner-Williams, W., Moughan, P. J., and Fuller, M. F. (2009b). Methods for Mucin Analysis - a Comparative Study. *Journal of Agricultural and Food Chemistry*, 57, 6029-6035.
- Montagne, L., Piel, C., and Lalles, J. P. (2004). Effect of diet on mucin kinetics and composition: Nutrition and health implications. *Nutrition Reviews*, 62(3), 105-114.
- Morel, P. C. H., Padilla, R. M., and Ravindran, G. (2003). Effect of non-starch polysaccharides on mucin secretion and endogenous amino acid losses in pigs. *Asian-Australasian Journal of Animal Sciences*, 16(9), 1332-1338.
- Moughan, P. J., Smith, W. C., and Stevens, E. V. J. (1990). Allometric Growth of Chemical Body Components and Several Organs in the Pig (20-90 Kg Liveweight). *New Zealand Journal of Agricultural Research*, 33(1), 77-84.
- O'Keefe, B. R., Beutler, J. A., Cardellina, J. H., Gulakowski, R. J., Krepps, B. L., McMahon, J. B., et al. (1997). Isolation and characterization of niphatevirin, a human-immunodeficiency-virus-inhibitory glycoprotein from the marine sponge *Niphates erecta*. *European Journal of Biochemistry*, 245(1), 47-53.
- Pasquier, M. C., Vatier, J., Poitevin, C., Vallot, T., and Mignon, M. (1991). Assessment of Mucus Glycoprotein Erosion by Measurement of Sialic-Acid in Gastric Secretions - Pathophysiologic and Therapeutic Aspects. *Journal of Clinical Gastroenterology*, 13, S22-S31.
- Piel, C., Montagne, L., Salgado, P., and Lalles, J. P. (2004). Estimation of ileal output of gastro-intestinal glycoprotein in weaned piglets using three different methods. *Reproduction Nutrition Development*, 44(5), 419-435.
- Roussel, P., and Delmotte, P. (2004). The diversity of epithelial secreted mucins. *Current Organic Chemistry*, 8(5), 413-437.

- Sanderson, I. R., and Walker, W. A. (1999). In R. Ogra and J. e. a. Mestecky (Eds.), *Handbook of Mucosal Immunology* (2nd ed., pp. 5-17). San Diego: Academic Press.
- Swergold, G. D., and Rubin, C. S. (1983). High-Performance Gel-Permeation Chromatography of Polypeptides in a Volatile Solvent - Rapid Resolution and Molecular-Weight Estimations of Proteins and Peptides on a Column of Tsk-G3000-Pw. *Analytical Biochemistry*, 131(2), 295-300.
- van Leeuwen, P., van Kleef, D. J., van Kempen, G. J. M., Huisman, J., and Verstegen, M. W. A. (1991). The Post Valve T-Cecum Cannulation Technique in Pigs Applied (sic) to Determine the Digestibility of Amino-Acid in Maize, Groundnut and Sunflower Meal. *Journal of Animal Physiology and Animal Nutrition-Zeitschrift Fur Tierphysiologie Tierernahrung Und Futtermittelkunde*, 65(3-4), 183-193.
- Weltzien, F. A., Norberg, B., and Swanson, P. (2003). Isolation and characterization of FSH and LH from pituitary glands of Atlantic halibut (*Hippoglossus hippoglossus* L.). *General and Comparative Endocrinology*, 131(2), 97-105.
- Yusuf, S., Nok, A. J., Ameh, D. A., Adelaiye, A. B., and Balogun, E. O. (2005). Correlation of gastric mucosal damage with sialic acid profile in rats: effect of hydrochloric acid, pepsin and hypertonic saline. *Cell Biochemistry and Function*, 23(5), 339-345.

Chapter IV

Comparison of three markers for the determination of bacterial protein in terminal ileal digesta in the growing pig.

The objective was to evaluate three techniques commonly used for the quantification of bacterial protein in the digesta collected from the terminal ileum of pigs fed a casein-based diet.

Abstract

Although the upper digestive tract in humans was once thought to be virtually devoid of microbial activity it is now accepted that the entire gastrointestinal tract (GIT) is colonised by a multitude of microbial species. Such a microflora form a diverse community that profoundly influences many of the physiological processes of the host. The host and intestinal microflora are in a state of symbiotic mutualism. Given that microbial protein is likely to make a significant contribution to total ileal digesta protein, this fraction needs to be accurately determined when studying ileal digesta protein composition.

The aim of the study was to compare three methods commonly used to determine the concentrations of bacterial protein in digesta collected from ileal digesta of growing pigs that had been fed a casein-based diet. The amounts of bacterial protein in terminal ileal digesta were determined using three different markers: 2,6-diaminopimelic acid (DAPA) and the D-amino acids, D-aspartic acid (D-Asp) and D-glutamic acid (D-Glu). The effectiveness of each marker was compared against a control (based on physical centrifugation and DNA contents).

The total bacterial protein concentrations derived from the markers D-Asp and D-Glu were significantly different ($P = 0.05$) to those calculated from DAPA and the control, but there was no difference between DAPA and the control. The percentage of bacterial nitrogen ranged from 40% to 52% dependent on the marker used. Bacterial protein expressed as a percentage of the total protein, ranged from 48% to 62%, a substantial proportion of which (12% – 28%) was derived from lysed bacterial cells. Statistical correlations between the estimation methods were low. Such poor correlation between the markers may be the result of random errors such as variance in the epimerization of the two D-amino acids during protein hydrolysis. DAPA was accepted as a reliable marker for determining microbial protein in ileal digesta as the values of this assay matched more precisely those of the independent control.

Introduction

The gastrointestinal tract (GIT) of all mammals is colonised by a plethora of microbial species. In adult humans the high density of microbes in the human intestinal tract is estimated to outnumber the host cells by more than ten to one with a luminal concentration in the whole digestive tract of 10^{11} – 10^{12} microbes/mL of digesta (Palmer *et al.*, 2007). Although other types of microorganisms inhabit the GIT (e.g. protozoa and yeasts) the vast majority are bacteria. In the adult pig the number of bacteria in the GIT may be as high as 10^{14} indigenous prokaryotic and eukaryotic microbial cells (Shirkey *et al.*, 2006). Although the microbial diversity of the external environment is vast the intestine is remarkable for its exclusivity as the microbiota of the GIT is dominated by members of just two divisions of bacteria –the *Bacteroidetes* and *Firmicutes* (Ley *et al.*, 2006). While many microbes are autochthonous permanent residents, others are allochthonous, originating from ingested food and water (Ley *et al.*, 2006). Such colonising intra-luminal microbiota form a diverse community (Ley *et al.*, 2006) that profoundly influences some of the physiological processes of the animal host that include: nutrient digestion, nitrogen cycling, mucus secretion, regulation of host fat storage, stimulating the proliferation of the intestinal epithelium and protection against pathogens (MacDonald and Monteleone, 2005). Numerous diseases and conditions have been linked to dysbiosis of GIT microbial communities (Nam *et al.* 2011) including: diarrhoea, type 1 diabetes, inflammatory bowel disease, necrotising enterocolitis together with both gastric and colonic cancers.

The principal function of the GIT is the digestion of exogenous macromolecules and the absorption of the resulting nutrients. To achieve this, significant quantities of proteinaceous compounds are secreted into the lumen of the GIT. The mass of the endogenous proteinaceous secretions has been estimated to be equal to that of ingested protein (Nasset and Ju, 1961) Residual amounts of unabsorbed proteinaceous materials, both exogenous and endogenous, arriving at the terminal ileum are thought to have no further nutritional value (Wunsche *et al.*, 1979). Therefore the accurate determination of endogenous total nitrogen and amino acid flows at the terminal ileum is necessary to allow the determination of true dietary amino acid digestibility coefficients and for the factorial estimation of dietary amino acid requirements.

Although not strictly of endogenous origin, non-dietary protein from the microbiota biomass inhabiting the GIT also contributes to the endogenous protein losses. When the composition of ileal digesta is analysed the greatest proportion of nitrogen and amino acids found at the terminal ileum is derived from microbial sources; believed to be close to 45% for the pig (Caine *et al.*, 1999). Therefore the accurate determination of such a critical component of endogenous nitrogen losses is essential.

Several methods have been developed for the determination of microbial nitrogen or protein in digesta and commonly these methods fall into two categories: methods for tracing bacterial protein or those based on the analysis of marker components believed to be uniquely microbial in origin. Methods for tracing the bacterial protein include labelling of bacterial protein with ^{15}N (Firkins *et al.*, 1992), or ^{35}S (Whitelaw *et al.*, 1984) and bacterial phospholipids with ^{32}P (Van Nevel and Demeyer, 1977). Bacterial markers have included: ribonucleic acids (RNA) (Schonhusen *et al.*, 1990), adenosine triphosphate (ATP) (Forsberg and Lam, 1977), 2,6-diaminopimelic acid (DAPA) (Weller *et al.*, 1958), or D-amino acids such as D-alanine (D-Ala) (Garrett *et al.*, 1987), or D-aspartic acid (D-Asp) and D-glutamic acid (D-Glu) (Csapo *et al.*, 2001).

Although structurally diverse the cell walls of both gram-positive and gram-negative bacteria contain peptidoglycan consisting of polysaccharide strands cross-linked with short “stem” peptides. The peptides are composed of alternating D- and L-amino acid isomers with a diamino acid, most commonly D-Asp, D-Glu and DAPA. A typical stem peptide in a Gram-negative bacterial cell wall might be; L-Ala–D-Glu–DAPA–D-Ala. However, the peptidoglycan content of bacterial cell walls varies according to the cell type. Gram-positive bacteria contain about 30–60% peptidoglycan whereas gram-negative bacteria have only 10% peptidoglycan (Schoenhusen *et al.*, 2008). The amino acid composition and structure of peptidoglycans in gram-positive bacteria varies greatly, whereas that of gram-negative bacteria is more consistent. Despite this many researchers have quantified these bacterial markers to estimate the amount of bacterial nitrogen and/or protein in fluids taken from the GIT of humans and livestock (Csapo *et al.*, 2001). However, the suitability of these markers is moot. While some researchers show that D-Ala is an effective bacterial marker (Garrett *et al.*, 1987) others claim the reverse (Schoenhusen *et al.*, 2008).

Of the potential methods DAPA and the D-sugars, D-Asp and D-Glu seem to have particular utility. The aim here was to evaluate the three markers; DAPA, D-Asp and D-Glu for the estimation of bacterial protein present in ileal digesta sampled from pigs fed diets with casein as the only source of dietary nitrogen. Using the differential centrifugation method devised by Metges *et al.* (1999) total bacterial DNA and total bacterial protein in the digesta were determined and served as a control.

Materials and methods

Animals and diets

Samples of digesta were collected from the terminal ileum of six Large White x Duroc pigs of mean (\pm SEM) body weight 79 ± 4.8 kg. The pigs were kept singly in steel metabolism crates, in a room maintained at $24 \pm 1^\circ\text{C}$, at the Animal Physiology Unit, Massey University, Palmerston North, New Zealand. Approval for the study was granted by the Massey University Animal Ethics Committee (protocol 05/29). The pigs arrived in the unit one week prior to surgery. During surgery each pig was fitted with a post-valvular T caecum (PVTC) cannula, as described by van Leeuwen *et al.* (1991). The study commenced eight weeks after surgery. Following surgery, food (preliminary diet) was progressively reintroduced within one week up to a level of 0.08 metabolic body weight ($W^{0.75}$)/d and this level of food intake was maintained for the remainder of the trial. The pigs were fed a nutritionally balanced casein-based diet (experimental diet), mixed with water (1:1, w/w), 3 times daily (0800, 1200 and 1600 h), in equal portions for the remainder of the trial. Water was available ad libitum. The composition of the preliminary diet is presented in **Table 1**. On the day of digesta collection the pigs were fed one-third of the daily ration of the experimental diet at 0800h. The composition of the experimental diet, which included titanium dioxide as an indigestible marker, is presented in **Table 2**. The pigs did not receive any food during the digesta collection period. In the evening, following the ten hour digesta collection, they received the remaining two-thirds of their daily ration and water.

Table 1 Ingredient composition of the preliminary diet.

<i>Ingredient</i>	<i>g/kg air dry weight</i>
Cooked Wheat ¹	485.8
Sucrose	168
Lactic casein ²	159
Skim milk powder	80
Soyabean oil	80
Dicalcium phosphate	22
Vitamin / mineral mix ³	2.5
Sodium chloride	1.6
Synthetic methionine	0.7
Calcium carbonate	0.2
Antioxidant ⁴	0.2

¹ Weet-bix, Sanitarium, Auckland, New Zealand.

² NZMP, Palmerston North, New Zealand.

³ Vitalean, Vitec Nutrition Ltd, Auckland, New Zealand. Vitamins provided: (g/kg of diet) vitamin A 3.0; (mg/kg of diet) cholecalciferol 500.0, choline 83.3; niacin 12.5, panthotenic acid 8.3, riboflavin 2.1, vitamin B6 1.7, vitamin E 41.7, vitamin K 1.7; (μ g/kg of diet) biotin 8.3, folic acid 417.0, thiamin833.0, vitamin B12 8.3, Minerals provided: (mg/kg of diet) Cu 104.0, Fe 83.0, Mn 38.0, Zn 100.0; (μ g/kg of diet) I 833.0, Co 417.0, Se 250.0.

⁴ Ethoxyquin, Kemin Industries Ltd, Auckland, New Zealand.

Table 2 Composition of the experimental diet (g/kg air dry weight).

Ingredient	Experimental Diet ¹.
Maltodextrin	453
Sucrose	161
Soyabean oil	154
Sodium Hydrogen Carbonate	18
Titanium Dioxide ²	3
Lactic casein	211

¹ No vitamins, minerals or fibre were added to this diet as the same diet was used in a comparative acute feeding study with human subjects. The preliminary casein based diet contained a vitamin mineral premix and cellulose.

² Titanium Dioxide was included in the experimental diet as an indigestible marker necessary for the calculation of endogenous protein flows in a separate study using the same digesta samples (Miner-Williams *et al.*, 2009).

Digesta were collected into polythene bags. After weighing, sodium benzoate, (10g•kg⁻¹ of digesta), as a bactericide, and phenylmethylsulphonyl fluoride (0.37g•kg⁻¹ of digesta), as a protease inhibitor, were added according to the protocol of Salgado *et al.* (Salgado *et al.*, 2001). For each pig the digesta samples collected were pooled and frozen at -20 °C until chemical analysis.

Chemical analyses

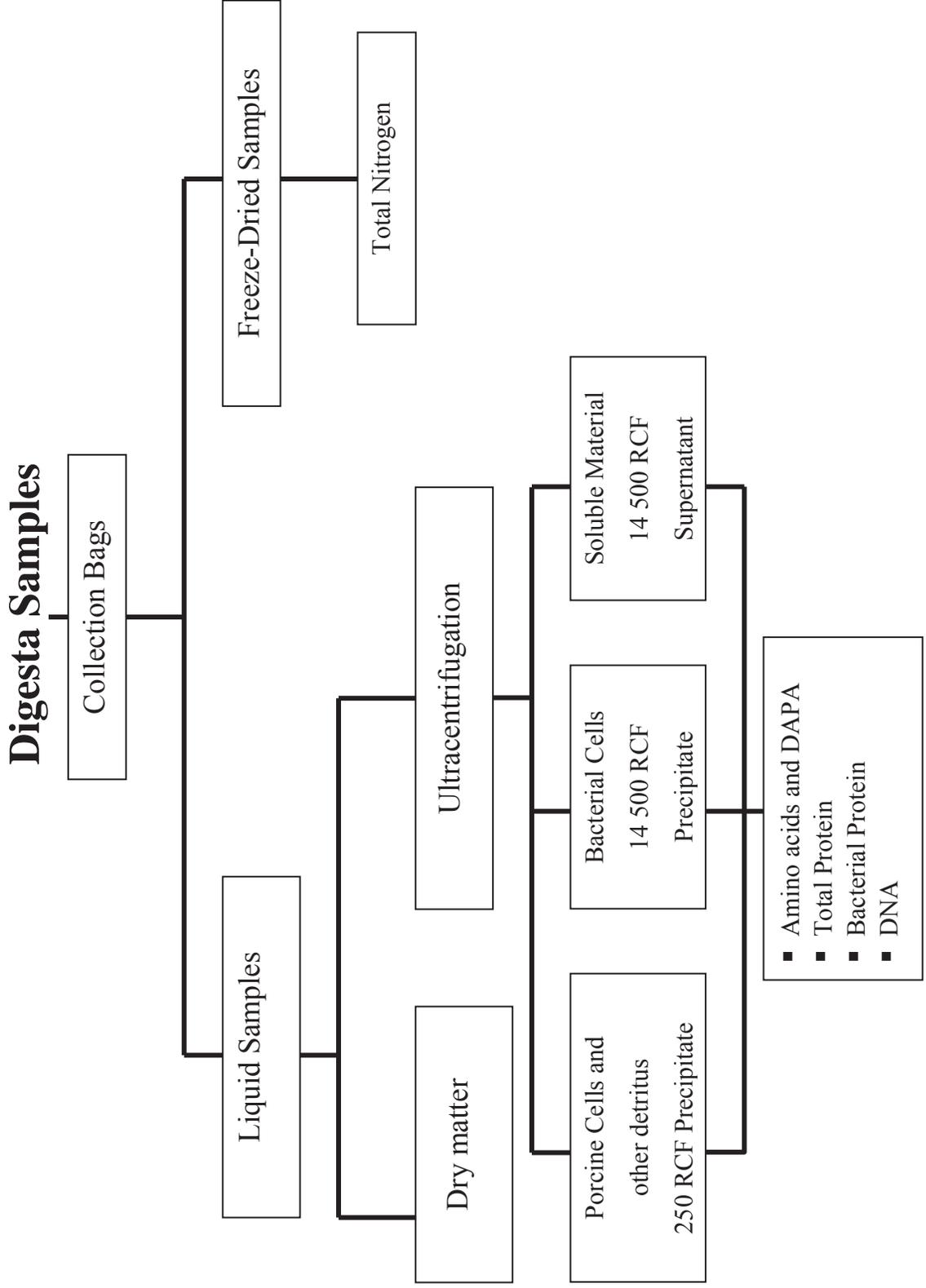
All analyses were undertaken in triplicate. Aliquots of the pooled digesta samples were freeze-dried, ground and mixed. Dry matter was measured by drying to a constant mass in a forced air oven at 95 °C. The remaining sample of digesta was fractionated by differential centrifugation using the method of Metges *et al.* (1999). Briefly the pooled digesta were centrifuged first at 250 RCF (relative centrifugal force) for 15 minutes at 4°C, giving a fraction expected to contain food particles and intact porcine cells, then at 14 500 RCF for 30 minutes at 4°C, to give a precipitate expected to contain microbial cells, and a supernatant expected to contain mainly proteins,

peptides, free amino acids, mucins, neutral sugars, urea, creatinine and ammonia. A summary of the centrifugation protocol is given in **Figure 1**.

Total Nitrogen, derived from both protein and non-protein compounds within the digesta, was determined by the Leco total combustion method (AOAC, 2000), a variation of the Dumas method. Amino acid compositions were determined using the procedure outlined by Hodgkinson and Moughan (2003). No corrections were made for potential losses of amino acids during hydrolysis. Methionine and cysteine were measured as methionine sulphone and cysteic acid, respectively, after the hydrolysis of samples that had been oxidized using performic acid. Diaminopimelic acid (DAPA) was quantified, following oxidation with performic acid, using an HPLC system with a UV detector. Tryptophan was not determined.

Soluble protein was determined by the Bradford method (1976). Bacterial protein in the 14500 RCF precipitate was determined using a Bacterial Protein Extraction Lysis Buffer (PELB) kit combined with a Non-interfering Protein Assay (NPA) kit, both obtained from G-Biosciences, St Louis, MO, USA. Following the manufacturer's instructions, total protein estimation in the 14500 RCF precipitate, comprising mainly bacterial matter, was achieved by first using the Bacterial PELB kit to lyse the bacterial cells, before using the Non-interfering Protein Assay Kit. The extracellular protein concentration was then determined for the same quantity of the 14500 RCF precipitate without the initial lysis step. The difference between the resultant concentrations was considered to be the bacterial protein concentration. As there were no porcine cells detected in the 14500 RCF precipitate all the DNA in this fraction was assumed to be of bacterial origin. The determination of a bacterial protein per unit DNA ratio from the 14500 RCF precipitate could then be used to estimate the bacterial protein concentration of the 14500 RCF supernatant and then utilised as an independent control.

Figure 1. Schematic diagram of the processing of the digesta samples



D-Asp and D-Glu were quantified using the HPLC method of Csapo *et al.*, in accordance with the method of Einarsson *et al.* (1987). Briefly, lyophilised samples, were first hydrolysed with 6M HCl for 30 minutes at 170 °C to restrict any racemisation. Derivatisation was affected by O-phthaldehyde (OPA) and 2,3,4,6-Tetra-O-Acetyl-1-Thio-B-D-Glucopyranoside (TGTA) dissolved in acetonitrile. Enantiomer separation was performed in a reverse phase analytical column (250 x 4.6 mm ID, 5mm particle size, Kromasil octyl (C8) charge). To lengthen the life of the column a safety column was used (RP8, Newguard, 25 x 3.2 ID 7 mm particle size, Brownlee), between the sample doser and the analytical column. A cleaning column (C18 36x4.5 ID, 20 mm particle size, Rsil), was also fitted between the pump and the sample doser. A gradient system, consisting of two components was then used for enantiomer separation. Solution A comprised 40% methanol in phosphate buffer (9.5 mmol, pH 7.05). Solution B was acetonitrile (CHROMASOLV[®] Plus Sigma-Aldrich, Auckland, New Zealand). The rate of flow was 1 ml per minute. Fluorescence detector excitation and emission maxima were 342 nm and 410 nm respectively.

DNA in all centrifugation fractions was determined using a QIAamp DNA Stool Mini Kit obtained from QIAGEN Inc. Valencia, CA, USA. The kit was used following the manufacturer's instructions for the isolation of DNA from stools for pathogen detection. The absorbance of the extracted samples was determined using a NanoDrop[®] ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies Wilmington, DE 19810, USA). The concentration of DNA was determined from its absorbance at A260 nm and its purity, with respect to contaminants that absorb UV, such as protein, from the ratio of the readings at A260 nm and A280 nm.

Bacterial protein in the digesta, determined using D-Asp and D-Glu as bacterial protein markers, was calculated using multiplication factors reported by Csapo *et al.* (2001). For the determination of bacterial protein using DAPA as a marker, the ratio of 26.4 mg of DAPA per g bacterial nitrogen was utilised, a value reported by Wunsche *et al.* (1991).

Results were expressed as means \pm standard error of the mean. Statistical analysis was undertaken using Minitab 15 (Minitab Pty Ltd, Sydney NSW Australia). Data were tested for homogeneity of variance using the Levene's test. Variables with equal variance were analysed using one-way ANOVA. Mucin concentration and flow data were analysed using non-parametric analyses (Kruskal-Wallis one-way Analysis of Variance) as the Levene's tests indicated that the variances in these parameters were not equal. Statistical significance was considered to be reached at $P < 0.05$. Results are means \pm standard error of the mean.

Results

The amounts of bacterial protein in the terminal ileal digesta determined using each marker, and the control values are presented in **Table 3**. The total bacterial protein concentrations derived from the markers D-Asp and D-Glu were significantly different to those calculated from DAPA and the control ($P = 0.05$).

Table 3 Digesta bacterial protein concentrations ($\text{mg}\cdot\text{g}^{-1}$ DDM) estimated using three markers compared to a control value based upon determined DNA

	DNA control	D-Asp	D-Glu	DAPA	SEM	<i>P</i>
Bacterial protein	186.8 ^b	204.2 ^a	157.7 ^a	180.4 ^b	18.1	0.05
Bacterial nitrogen as a % of total nitrogen ¹	47.3	51.7	39.9	45.7		NS
Bacterial protein as a % of total protein ²	56.6 ^b	61.9 ^a	47.8 ^a	54.7 ^b		0.05

Values are means (of 6 animals). DNA = Deoxyribose nucleic acid. DAPA = 2,6-diaminopimelic acid. D-Asp = D-aspartic acid and D-Glu = D-glutamic acid. SEM = standard error of the mean. DDM = Digesta dry matter.

Total nitrogen = $63.2 \text{ mg}\cdot\text{g}^{-1}$ DDM and total protein $329.7 \text{ mg}\cdot\text{g}^{-1}$ DDM. Mean (\pm SEM) marker concentrations were: 1.34 (± 0.16); 1.51 (± 0.15); 1.56 (± 0.16); 0.76 (± 0.06); for the DNA control, D-Asp, D-Glu and DAPA respectively. Means within a row with different superscripts were significantly different.

The percentage of bacterial nitrogen ranged from nearly 40% when estimated using D-glutamic acid (D-Glu) as the bacterial marker to nearly 52% when D-aspartic acid (D-Asp) was the chosen bacterial marker. Bacterial protein expressed as a percentage of the total protein, ranged from 48% to 62%. The correlation coefficients (r) for the relationships between the different bacterial protein estimation methods were 0.41 ($P = 0.094$) for DAPA–D-Glu, 0.11 ($P = 0.657$) for DAPA–D-Asp and 0.58 ($P = 0.012$) for D-Glu–D-Asp.

The amino acid composition of both the total protein and bacterial protein is given in **Table 4**. Within each fraction (total digesta protein, and digesta bacterial protein) the mean amino acid concentrations are given as $\text{mg}\cdot\text{g}^{-1}$ of total nitrogen and bacterial nitrogen respectively. The bacterial amino acid profile was determined from the 14500 RCF precipitate. The five most abundant amino acids (in diminishing order) were: glutamic acid; aspartic acid; proline; threonine and serine. The greatest proportion of these five was determined to be present in the endogenous protein fraction and to contribute nearly 29% of the total nitrogen. These amino acids probably originate from the soluble mucin present in this fraction (Miner-Williams *et al.*, 2009).

Threonine, serine and proline are most abundant in the glycosylated region of mucin polymers that holds the great majority of these three amino acids. Glutamic acid, glycine and aspartic acid are most common in the nonglycosylated regions of mucin.

The concentrations of total, bacterial and porcine cellular DNA (**Table 5**) were determined to be 21.2, 18.2, and $3.0 \mu\text{g}\cdot\text{g}^{-1}$ nitrogen respectively (values equivalent to 1.34, 1.15, $0.19 \text{ mg}\cdot\text{g}^{-1}$ DDM). The total bacterial DNA represented 86% of the total DNA detected in the ileal digesta with nearly 51% of that present in the 14500 RCF supernatant and 33% found in the 14500 RCF precipitate which constituted the bacterial pellet.

Table 4 Amino acid compositions of total protein and bacterial protein in ileal digesta.

Amino Acid	Total digesta amino acids ¹	SEM	Digesta bacterial amino acids ²	SEM
Glutamic Acid*	0.94	0.11	0.39	0.03
Aspartic Acid*	0.53	0.05	0.25	0.03
Proline*	0.42	0.03	0.12	0.01
Threonine*	0.41	0.03	0.14	0.02
Serine*	0.33	0.02	0.13	0.02
Valine	0.32	0.02	0.15	0.02
Leucine	0.31	0.03	0.16	0.02
Alanine	0.28	0.03	0.14	0.02
Glycine*	0.27	0.02	0.13	0.02
Lysine	0.21	0.02	0.12	0.02
Isoleucine	0.20	0.02	0.11	0.01
Histidine	0.19	0.02	0.06	0.01
Arginine	0.18	0.02	0.11	0.01
Phenylalanine	0.18	0.02	0.10	0.01
Tyrosine	0.16	0.01	0.07	0.01
Cysteine	0.09	0.01	0.05	0.01
Methionine	0.07	0.01	0.04	0.01
Nitrogen	63.2		28.3	

Values are means (of 6 animals). SEM = standard error of the mean.

¹ mg•g⁻¹ total nitrogen.

² mg•g⁻¹ bacterial nitrogen in the 14500 RCF precipitate.

Tryptophan not assayed. * Predominant amino acids in mucin

Table 5 Concentrations of DNA in terminal ileal digesta.

Centrifugation Fraction	DNA $\mu\text{g}\cdot\text{g}^{-1}$ Nitrogen		
	Total ^{1.}	Bacterial cellular	Porcine cellular ^{4.}
250 RCF ² precipitate	2.85	0.32	2.53
14500 RCF ³ precipitate	7.12	7.12	
14500 RCF ² supernatant	11.23	10.76	0.47
Sum	21.20	18.20	3.01

DNA = Deoxyribose nucleic acid. DAPA = 2.6-diaminopimelic acid.

^{1.} Concentration in digesta fraction.

^{2.} The concentration of bacterial DNA in this fraction was assumed to originate from lysed bacterial cells and gave a DNA:DAPA ratio of 1.77 in the 14500 RCF precipitate.

^{3.} It was assumed that all of the DNA detected in the 14500 precipitate was bacterial in origin.

^{4.} Porcine cellular DNA was determined as being the difference between total DNA detected and estimated bacterial DNA.

The distribution of protein across the three centrifugation fractions is presented in **Table 6**. Nearly 60% and 28% of the protein was detected in the 14500 RCF supernatant and 14500 RCF precipitate respectively. Dependent upon the marker, between 48% – 62% of total protein was bacterial in origin, with 12% – 28% detected in the 14500 RCF supernatant and 19% – 36% in the 14500 RCF precipitate.

Discussion

Casein, a protein known to be almost completely digested and absorbed (Deglaire et al., 2009; Eklund *et al.*, 2008; Kies *et al.*, 1986), was used in this study because it allowed the direct determination of the proteinaceous components in the terminal ileal digesta of pigs fed a single purified dietary protein. The true digestibility of casein has been estimated by Deglaire et al. (2009) to be in excess of 97%.

Table 6 Distribution of protein of microbial origin within the different centrifugation fractions of ileal digesta.

	Microbial protein calculated from DAPA			Microbial protein calculated from D-Asp			Microbial protein calculated from D-Glu		
	mg/g DDM	SEM	Percentage of total protein	mg/g DDM	SEM	Percentage of total protein	mg/g DDM	SEM	Percentage of total protein
Total microbial protein	180.4	17.3	54.8	204.2	21.3	61.9	157.7	17.9	47.8
250 RCF Precipitate									
Microbial protein	26.0	2.4	7.9	26.9	2.1	8.2	22.6	1.8	6.9
14500 RCF Precipitate									
Microbial protein	61.3	6.6	19.0	56.1	5.8	17.0	39.4	3.4	12.0
14500 RCF Supernatant									
Microbial protein	93.1	7.5	28.3	121.2	9.9	36.8	95.7	9.5	29.0

Values are means (of 6 animals). DNA = Deoxyribose nucleic acid. DAPA = 2,6-diaminopimelic acid. D-Asp = D-aspartic acid and D-Glu = D-glutamic acid. SEM = standard error of the mean. DDM = Digesta dry matter.

Total digesta protein was estimated to be (mean \pm SEM) 329.7 (\pm 11.7) mg•g⁻¹ DDM. The total protein in the 250 RCF, 14500 RCF precipitate and supernatant were; 43.4(\pm 3.1), 91.0 (\pm 10.9) and 195.4 (\pm 19.9) mg•g⁻¹ DDM respectively. Porcine mucosal cell protein in the 250 RCF precipitate was 17.4 (\pm 1.8) mg•g⁻¹ DDM.

Although small amounts of dietary peptides and amino acids may remain unabsorbed at the terminal ileum these were assumed to be minimal and to have no bearing on the overall results, as bacterial protein was determined directly.

Light microscopy confirmed that the 14500 RCF precipitate was largely composed of microbial cells with no contamination from porcine mucosal cells. Accepting this, all the DNA in this fraction can be assumed to be of microbial origin. Using the PELB kit the quantity of bacterial protein in this fraction was determined to be $62.7 \text{ mg}\cdot\text{g}^{-1}$ DDM. The concentration of bacterial protein per unit DNA was then determined as $139.3 \text{ mg}\cdot\text{g}^{-1}$ DDM; a value that is in accord with values calculated from data published by Obispo and Dehority (Obispo and Dehority, 1999) and Calsamiglia *et al.* (1996). The DNA:DAPA ratio for the 14500 RCF precipitate was determined to be 1.77 a figure that is in agreement with Rowan *et al.* (1992). It cannot be assumed that the DNA present in the 250 RCF precipitate and 14500 RCF supernatant originates solely from microbial material. Using the DNA:DAPA ratio of 1.77 the DNA of microbial and non-microbial origin can be calculated for both the 250 RCF precipitate and 14500 RCF supernatant. Using the microbial DNA values determined for these fractions and the bacterial protein per unit bacterial DNA from the 14500 RCF precipitate the bacterial protein content of the 14500 RCF supernatant and the 250 RCF precipitate were determined to be; $94.7 (\pm 8.4)$ and $29.4 (\pm 0.3) \text{ mg}\cdot\text{g}^{-1}$ DDM respectively. Adding these values to the direct estimate for the 14500 RCF precipitate the total bacterial protein concentration for the control was $186.8 \text{ mg}\cdot\text{g}^{-1}$ DDM. Compared to the value of the control the concentrations of bacterial protein determined from the three markers, DAPA, D-Asp, and D-Glu were; -3.4%, +9.3% and -15.6%.

Although the estimated mean concentrations of bacterial protein in the ileal digesta of pigs fed a casein-based meal calculated using the three different markers were not too dissimilar the correlations between the estimation methods were low. These results are in contrast to those of Csapo *et al.* (2001) who found a much closer correlation between the microbial markers, with r values that ranged from 0.7 for DAPA and D-Glu to 0.78 for DAPA and D-Asp in duodenal chyme taken through a fistula from 5 growing bulls. In their study the highest correlation ($r = 0.95$) between the markers of bacterial protein was between D-Asp–D-Glu, in the present study this correlation was just 0.58.

However, Csapo did not find a close correlation between the concentration of bacterial protein and all three bacterial markers (D-Asp, D-Glu and DAPA) in duodenal chyme. In their study they found that bacterial protein values estimated using DAPA as the marker for bacterial protein were 10% higher than values estimated using the two D-amino acids. In the presently reported study the bacterial protein concentration determined with DAPA was nearly 12% lower than that determined with D-Asp and 14% higher than that determined with D-Glu. Interestingly Csapo *et al.*, found the correlation between DAPA and ruminal bacteria to be the highest with an r value of 0.74. In the present study, the poor correlation between the markers may be the result of random errors such as variance in the epimerization (partial racemisation) during protein hydrolysis, which Csapo *et al.* (2002) acknowledge may lead to error in estimation. Although the protein hydrolysis procedure developed by Csapo *et al.*, to minimise epimerization was adopted in this study this source of error cannot be discounted. As the degree of epimerization cannot be quantified this is a source of error which casts doubt on the reliability of using D-Asp and D-Glu as markers of bacterial protein. Likewise the D-amino acids have been detected in foodstuffs, particularly milk and milk products, as a result of microbial activity (Csapo *et al.*, 1997) or the heat treatment of foodstuffs (Bruckner *et al.*, 2001). In recent years, the ratio of D- to L-amino acids in the environment has increased as a result of water contamination due to human activities (chemical waste, fertilizers, sweeteners, cleansers and other artificial sources containing racemic mixtures of amino acids) (Zahradnickova *et al.*, 2007). Such interference has not been found with DAPA. In spite of the potential drawbacks of using the D-amino acid markers, their application here did lead to similar mean values for the bacterial protein content of ileal digesta.

Bacterial amino acids are, strictly speaking, neither of exogenous or endogenous origin and it is pertinent to determine the amino acid composition of the bacterial component (Table 4). The amino acid profile of the 14500 RCF precipitate may be considered to be derived solely from bacterial protein. It should be noted that there were considerable quantities of bacterial protein markers present in the 14500 RCF supernatant that are likely to have originated from lysed bacterial cells. Visual analysis (using light microscopy) of the three centrifugation fractions confirmed that most intact porcine cells were present in the 250RCF precipitate; most whole bacterial cells

were present in the 14500 RCF precipitate; and the number of intact porcine or bacterial cells in the 14500 RCF supernatant was negligible.

The distribution of DNA throughout the three centrifugation fractions (Table 5) indicated that with 51% more bacterial DNA in the 14500 RCF supernatant than in the 14500 RCF precipitate it is evident that the majority of bacterial protein in the terminal ileal digesta originates from lysed bacterial cells.

The total microbial protein values (Table 6) expressed as a proportion of total protein were determined as, 48% 55% and 61% for D-Glu, DAPA, and D-Asp respectively. Bacterial protein present in the ileal effluent contributes a substantial amount to the endogenous nitrogen flows of pigs fed a diet where casein was the sole source of protein. The greatest quantity of protein from bacteria was determined to be in the 14500 RCF supernatant originating from lysed bacterial cells.

The ideal bacterial marker should: 1) be absent in the feed; 2) not be absorbed; 3) be biologically stable; 4) have a relatively straightforward assay; 5) be exclusively bacterial in origin; 6) occur in similar proportions in the different species of bacteria colonizing the GIT; 7) be in constant proportions throughout all stages of microbial cell growth; and 8) flow throughout the GIT at a similar rate in both free and bound forms.

Although detailed information of the microbiota that inhabit the mammalian GIT can be gained from the phylogenetic analysis of 16s ribosomal DNA (16s rDNA) this is not an ideal method for the quantification of bacterial protein for mixed bacterial populations. In addition to low sensitivity and inconvenience of use for large batches of samples, quantitative PCR is not straightforward. Since the DNA molecules are amplified during PCR, the initial quantity of the target DNA can only be estimated on the assumption that such amplification efficiency is reproducible. Because of the exponential nature of this process any disturbance of the amplification efficiency may result in major PCR bias (Felske *et al.*, 1998). As with other microbial markers the 16s rDNA content of bacteria varies with different species and environment (Hopkins and Macfarlane, 2000). As bacteria become more metabolically active, their demand for protein synthesis increases and ribosome production is upregulated (Hopkins and

Macfarlane, 2000). In addition, the relationship between growth rate and 16s rDNA may not always be linear (Kaplan and Apirion, 1975). Having determined the number of bacterial cells in a sample using PCR, conversion factors are required to estimate the quantity of bacterial protein. For ileal digesta samples containing a large variety of bacterial species, with bacterial cells in different stages of development such conversions are subject to much error. For these reasons a quantitative PCR assay of 16s rDNA was not undertaken.

Although some researchers suggest that molecular techniques, especially 16s rDNA sequence comparisons, have rendered many of the more conventional methods for the determination of bacterial protein superfluous, there is still a place for using DAPA as a marker for bacterial protein (Jensen *et al.*, 2006). The classic argument against the use of DAPA as a marker of bacterial protein has been that the quantity of DAPA present in the cell wall is strongly dependent on the species and size of bacteria (Dufva *et al.*, 1982). Interestingly the same can be said of the D-amino acids which also originate from the peptidoglycans present in the cell wall of bacteria. With the exception of points 6) and 7) (previous page) DAPA fulfils each of the criteria of an ideal bacterial marker. The ratios of DAPA to bacterial nitrogen (Wunsche *et al.*, 1991) and to bacterial dry matter (Czerkawski, 1974) are well known and widely used by researchers. The microbial community within the GIT of experimental animals may be diverse but it remains stable, with a population of limited species and bacterial density, for many months (Ley *et al.*, 2006). DAPA is not subject to interference from epimerization. In comparative experiments, the quantity of DAPA and its relationship to total bacterial protein does not vary and can be used satisfactorily to determine the proportion of protein of bacterial origin in the luminal contents of the GIT (Csapo *et al.*, 2001). In the present work application of the marker DAPA gave the best and an accurate estimate of the total microbial protein based upon differential centrifugation and analysis of DNA.

Acknowledgements

Grateful thanks are given for the assistance of Dr Amélie Deglaire during the pig trial and Dr Gordon Reynolds for surgical procedures involved with installing the post-valve T caecum cannulae.

Literature cited

- AOAC. (2000). Official Method for protein (Dumas method), 968.06. *Official methods of analysis of association of official analytical chemists international*.
- Bradford, M. M. (1976). Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Analytical Biochemistry*, 72(1-2), 248-254.
- Bruckner, H., Justus, J., and Kirschbaum, J. (2001). Saccharide induced racemization of amino acids in the course of the Maillard reaction. *Amino Acids*, 21(4), 429-433.
- Caine, W. R., Tamminga, S., Sauer, W. C., Verstegen, M. W. A., and Schulze, H. (1999). Bacterial contributions to total and endogenous recoveries of nitrogen and amino acids in ileal digests of newly weaned piglets fed protease-treated soybean meal. *Livestock Production Science*, 57(2), 147-157.
- Calsamiglia, S., Stern, M. D., and Firkins, J. L. (1996). Comparison of nitrogen-15 and purines as microbial markers in continuous culture. *Journal of Animal Science*, 74(6), 1375-1381.
- Csapo, J., Csapo-Kiss, Z., Csordas, E., Fox, P. F., Wagner, L., and Talos, T. (1997). Total free and free D-amino acid content of cheeses produced by different technologies Kulonbozo technologiaval keszult sajtok osszes szabad- es szabad D-aminosav tartalma. *Tejgazdasag*, 57(1), 25-30.
- Csapo, J., Csapo-Kiss, Z., Schmidt, J., and Martin, T. G. (2001). Quantitative determination of protein of bacterial origin. *Trac-Trends in Analytical Chemistry*, 20(1), 42-48.
- Csapo, J., Schmidt, J., Csapo-Kiss, Z., Hollo, G., Hollo, I., Wagner, L., *et al.* (2001). A new method for the quantitative determination of protein of bacterial origin on

- the basis of D-aspartic acid and D-glutamic acid content. *Acta Alimentaria*, 30(1), 37-52.
- Csapo, J., Schmidt, J., Csapo-Kiss, Z., Varga-Visi, E., Pohn, G., and Csokona, E. (2002). Quantitative determination of protein of bacterial origin on the basis of D-aspartic acid and D-glutamic acid content. *Chromatographia*, 56, S169-S171.
- Czerkawski, J. W. (1974). Methods for determining 2-6-diaminopimelic acid and 2-aminoethylphosphonic acid in gut contents. *Journal of the Science of Food and Agriculture*, 25(1), 45-55.
- Deglaire, A., C. Bos, et al. (2009). "Ileal digestibility of dietary protein in the growing pig and adult human." *British Journal of Nutrition* 102(12): 1752-1759.
- Dufva, G. S., Bartley, E. E., Arambel, M. J., Nagaraja, T. G., Dennis, S. M., Galitzer, S. J., et al. (1982). Diaminopimelic Acid Content of Feeds and Rumen Bacteria and Its Usefulness as a Rumen Bacterial Marker. *Journal of Dairy Science*, 65(9), 1754-1759.
- Einarsson, S., Folestad, S., and Josefsson, B. (1987). Separation of Amino-Acid Enantiomers Using Precolumn Derivatization with Ortho-Phthalaldehyde and 2,3,4,6-Tetra-O-Acetyl-1-Thio-Beta-Glucopyranoside. *Journal of Liquid Chromatography*, 10(8-9), 1589-1601.
- Eklund, M., Mosenthin, R., Piepho, H. P., and Rademacher, M. (2008). Effect of dietary crude protein level on basal ileal endogenous losses and standardized ileal digestibilities of crude protein and amino acids in newly weaned pigs. *Journal of Animal Physiology and Animal Nutrition*, 92(5), 578-590.
- Felske, A., Akkermans, A. D. L., and De Vos, W. M. (1998). Quantification of 16S rRNAs in complex bacterial communities by multiple competitive reverse transcription PCR in temperature gradient gel electrophoresis fingerprints. *Applied and Environmental Microbiology*, 64(11), 4581-4587.
- Firkins, J. L., Weiss, W. P., and Piwonka, E. J. (1992). Quantification of intraruminal recycling of microbial nitrogen using N-15. *Journal of Animal Science*, 70(10), 3223-3233.
- Forsberg, C. W., and Lam, K. (1977). Use of Adenosine 5'-Triphosphate as an Indicator of Microbiota Biomass in Rumen Contents. *Applied and Environmental Microbiology*, 33(3), 528-537.

- Garrett, J. E., Goodrich, R. D., and Meiske, J. C. (1987). Measurement and use of D-alanine as a bacterial marker. *Canadian Journal of Animal Science*, 67(3), 735-743.
- Hodgkinson, S. M., and Moughan, P. J. (2003). The enzyme hydrolysed protein method for the determination of endogenous ileal nitrogen and amino acid flows - a modification. *Animal Feed Science and Technology*, 108(1-4), 207-214.
- Hopkins, M. J., and Macfarlane, G. T. (2000). Evaluation of 16s rRNA and cellular fatty acid profiles as markers of human intestinal bacterial growth in the chemostat. *Journal of Applied Microbiology*, 89(4), 668-677.
- Jensen, C., Weisbjerg, M. R., and Hvelplund, T. (2006). Evaluation of methods for estimating the amino acid supply to the duodenum of microbial, endogenous and undegraded feed protein on maize silage diets fed to dairy cows. *Animal Feed Science and Technology*, 131(1-2), 1-24.
- Kaplan, R., and Apirion, D. (1975). Decay of ribosomal ribonucleic-acid in escherichia-coli-cells starved for various nutrients. *Journal of Biological Chemistry*, 250(8), 3174-3178.
- Kies, A. K., Moughan, P. J., and Smith, W. C. (1986). The apparent and true Ileal digestibility of nitrogen and amino-acids in lactic casein for the growing-pig. *Animal Feed Science and Technology*, 16(3), 169-178.
- Ley, R. E., Peterson, D. A., and Gordon, J. I. (2006). Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell*, 124(4), 837-848.
- Ley, R. E., Turnbaugh, P. J., Klein, S., and Gordon, J. I. (2006). Microbial ecology - Human gut microbes associated with obesity. *Nature*, 444(7122), 1022-1023.
- MacDonald, T. T., and Monteleone, G. (2005). Immunity, inflammation, and allergy in the gut. *Science*, 307(5717), 1920-1925.
- Metges, C. C., Petzke, K. J., El-Khoury, A. E., Henneman, L., Grant, I., Bedri, S., *et al.* (1999). Incorporation of urea and ammonia nitrogen into ileal and fecal microbial proteins and plasma free amino acids in normal men and ileostomates. *American Journal of Clinical Nutrition*, 70(6), 1046-1058.
- Miner-Williams, W., Moughan, P. J., and Fuller, M. F. (2009). Endogenous components of digesta protein from the terminal ileum of pigs fed a casein based diet. *Journal of Agricultural and Food Chemistry* 57, 2072-2078.

- Nam, Y. D., Jung, M. J., Roh, S. W., Kim, M. S., and Bae, J. W. (2011). Comparative Analysis of Korean Human Gut Microbiota by Barcoded Pyrosequencing. *Plos One*, 6(7).
- Nasset, E. S., and Ju, J. S. (1961). Mixture of endogenous and exogenous protein in the alimentary tract. *Journal of Nutrition*, 74, 461-465.
- Obispo, N. E., and Dehority, B. A. (1999). Feasibility of using total purines as a marker for ruminal bacteria. *Journal of Animal Science*, 77(11), 3084-3095.
- Palmer, C., Bik, E. M., DiGiulio, D. B., Relman, D. A., and Brown, P. O. (2007). Development of the human infant intestinal microbiota. *Plos Biology*, 5(7), 1556-1573.
- Rowan, A. M., Moughan, P. J., & Wilson, M. N. (1992). The flows of deoxyribonucleic-acid and diaminopimelic acid and the digestibility of dietary fiber components at the terminal ileum, as indicators of microbial activity in the upper digestive-tract of ileostomized pigs. *Animal Feed Science and Technology*, 36(1-2), 129-141.
- Salgado, P., Lalles, J. P., Toullec, R., Mourato, M., Cabral, F., and Freire, J. P. B. (2001). Nutrient digestibility of chickpea (*Cicer arietinum L.*) seeds and effects on the small intestine of weaned piglets. *Animal Feed Science and Technology*, 91(3-4), 197-212.
- Schoenhusen, U., Voigt, J., Hennig, U., Kuhla, S., Zitnan, R., and Souffrant, W. B. (2008). Bacterial D-alanine concentrations as a marker of bacterial nitrogen in the gastrointestinal tract of pigs and cows. *Veterinarni Medicina*, 53(4), 184-192.
- Schonhusen, U., Voigt, J., Piatkowski, B., and Kreienbring, F. (1990). Studies of the utilization of ribonucleic-acid as marker of the microbial protein yield in the rumen .2. The influence of the treatment of samples, the sampling time and the composition of the ration on the RNA-N ratio in the rumen microbes. *Archiv Fur Tierernahrung-Archives of Animal Nutrition*, 40(1-2), 75-84.
- Shirkey, T. W., Siggers, R. H., Goldade, B. G., Marshall, J. K., Drew, M. D., Laarveld, B., *et al.* (2006). Effects of commensal bacteria on intestinal morphology and expression of proinflammatory cytokines in the gnotobiotic pig. *Experimental Biology and Medicine*, 231(8), 1333-1345.
- van Leeuwen, P., van Kleef, D. J., van Kempen, G. J. M., Huisman, J., and Verstegen, M. W. A. (1991). The Post Valve T-Cecum Cannulation Technique in Pigs

- Applied (sic) to Determine the Digestibility of Amino-Acid in Maize, Groundnut and Sunflower Meal. *Journal of Animal Physiology and Animal Nutrition-Zeitschrift Fur Tierphysiologie Tierernahrung Und Futtermittelkunde*, 65(3-4), 183-193.
- Van Nevel, C. J., and Demeyer, D. I. (1977). Determination of rumen microbial-growth invitro from P-32-labeled phosphate incorporation. *British Journal of Nutrition*, 38(1), 101-114.
- Weller, R. A., Gray, F. V., and Pilgrim, A. F. (1958). The Conversion of Plant Nitrogen to Microbial Nitrogen in the Rumen of the Sheep. *British Journal of Nutrition*, 12(4), 421-429.
- Whitelaw, F. G., Eadie, J. M., Bruce, L. A., and Shand, W. J. (1984). Microbial protein-synthesis in cattle given roughage-concentrate and all-concentrate diets - the use of 2,6-diaminopimelic acid, 2-aminoethylphosphonic acid and S-35 as markers. *British Journal of Nutrition*, 52(2), 249-260.
- Wunsche, J., Bock, H. D., Hennig, U., Kreienbring, F., and Borgmann, E. (1979). Untersuchungen über die proteinverdaulichkeit und aminosäurenresorption in verschiedenen abschnitten des verdauungstraktesbeim schwein. *Archives of Animal Nutrition*, 29, 221-234.
- Wunsche, J., Volker, T., Souffrant, W. B., and Borgmann, E. (1991). Assessment of the bacteria-N proportions in feces and differently obtained ileal digesta of pigs. *Archiv Fur Tierernahrung-Archives of Animal Nutrition*, 41(7-8), 703-716.
- Zahradnickova, H., Husek, P., Simek, P., Hartvich, P., Marsalek, B., and Holoubek, I. (2007). Determination of D- and L-amino acids produced by cyanobacteria using gas chromatography on Chirasil-Val after derivatization with pentafluoropropyl chloroformate. *Analytical and Bioanalytical Chemistry*, 388(8), 1815-1822.

Chapter V

Endogenous components of digesta protein from the terminal ileum of pigs fed a casein-based diet.

The aim of the current study was to gain a clearer understanding of the nature and composition of endogenous nitrogen in the terminal ileal digesta collected from pigs fed a casein-based diet. The pig was used as an animal model for the adult human.

Data published in Miner-Williams, W., Moughan, P. J., and Fuller, M. F. (2009). Endogenous components of digesta protein from the terminal ileum of pigs fed a casein based diet. *Journal of Agricultural and Food Chemistry* 57, 2072-2078.

Abstract

To gain a clearer understanding of the nature and composition of endogenous nitrogen containing substances lost from the upper mammalian digestive tract, digesta were collected from the terminal ileum of six growing pigs that had been fed a casein- based diet with titanium dioxide as an indigestible marker.

Total nitrogen lost at the terminal ileum was in excess of $63 \text{ mg}\cdot\text{g}^{-1}$ digesta dry matter. Of this, nearly 73% was proteinaceous, with nearly 45% being bacterial protein, 13% from soluble free protein and 11% from mucin. Of the non-protein nitrogen 11% was as ammonia and 5% as urea. Bacterial and porcine, cellular DNA nitrogen were collectively 0.2% of the total nitrogen. Only 8.3% of the total nitrogen remained unidentified and was assumed to include free amino acids, RNAs, amines and the tetrapyrroles bilirubin and biliverdin.

Although mucin contributed just 10.4% of the nitrogen losses, it was the single most abundant truly endogenous component, comprising 13% of the total dry matter.

Bacterial nitrogen, combined with ammonia and urea nitrogen, represented nearly 61% of the total nitrogenous losses: this suggests substantial microbial activity in the stomach and small intestine of the pig. Centrifugal separation of a bacterial fraction from the digesta produced a microbial amino acid profile that, when subtracted from the overall amino acid content, provided an amino acid profile more representative of true endogenous amino acid losses.

Introduction

Although the principal functions of the gastrointestinal tract are the chemical breakdown of exogenous dietary macromolecules and the absorption of the resultant smaller and simpler products, there are significant amounts of material secreted into the gut. Salivary, gastric, hepatic, pancreatic and intestinal secretory cells all secrete endogenous proteins into the lumen of the gut (Jansman *et al.*, 2002) that are vital for digestion and absorption (Wapnir and Teichberg, 2002). It has been estimated that secreted materials originating from the pancreas might contribute 3-5g nitrogen per day (Corring and Saucier, 1972), from the gall bladder 2g nitrogen per day (Sambrook, 1981), from salivary and gastric secretions 0.3-0.6g nitrogen per day (Zebrowska *et al.*, 1983), together with sloughed epithelial cells and secretions from the duodenal mucosa amounting to 3-5g nitrogen per day (Leibholz, 1982). Although not strictly endogenous, bacterial protein is commonly included in estimations of endogenous materials (Moughan *et al.*, 2005) and may be the largest single contributor to the nitrogen of terminal ileal digesta (Caine *et al.*, 1999). The mass of the endogenous proteinaceous secretions has been estimated to be equal to that of ingested protein (Nasset and Ju, 1961). The protein content of the digesta is thus a dynamic equilibrium between dietary intake and the secretion of endogenous material into the lumen on one hand and the concomitant absorption from the gut of digested materials, both exogenous and endogenous in origin on the other.

Residual amounts of unabsorbed proteinaceous materials, both exogenous and endogenous, arriving at the terminal ileum are thought to have no further nutritional value (Wunsche *et al.*, 1979) because, although limited uptake of amino acids may occur in the large intestine, such trivial amounts are of little nutritional benefit to the host (Moughan, 2003). However, the result of microbial fermentation in the hindgut is that the composition of nitrogenous materials excreted in the faeces bears little relation to that in ileal effluent: in fact, some 80% of faecal nitrogen originates from microbial activity (Rutherford and Moughan, 1998). Because of this, the composition of gut endogenous nitrogen losses cannot be determined from faecal analysis. The nitrogenous materials leaving the ileum thus represent the net balance between secretion and reabsorption.

Classically, losses of endogenous nitrogen and amino acids in ileal effluent have been determined by giving a protein-free diet, when any proteinaceous material in the digesta is assumed to be from endogenous sources (Low, 1980). However, such a diet is physiologically unnatural (Low, 1980), as it decreases the rate of both protein synthesis by the gut tissues (Millward *et al.*, 1976) and of protein secretion into the gut, leading to the underestimation of endogenous nitrogen losses (Hodgkinson and Moughan, 2003). For this reason several alternative methods have been developed to measure ileal endogenous nitrogen and amino acid losses (Moughan, 2003).

An accurate estimation of endogenous total nitrogen and amino acid flows is necessary to allow the determination of true dietary amino acid digestibility coefficients and for the factorial estimation of dietary amino acid requirements (Moughan *et al.*, 2005). Although the literature is replete with studies that quantify the amino acid composition of ileal digesta (reviewed in Jansman *et al.*, 2002; Moughan, 2003; Pedersen and Boisen, 2002), there are, to the authors' knowledge, no systematic studies regarding the complete nitrogen and protein composition of ileal digesta.

The aim of this study was to quantify the endogenous components of terminal ileal digesta from pigs given a diet with a purified animal protein, lactic casein, known to be highly digested and absorbed. Although small amounts of dietary peptides and amino acids may remain unabsorbed when casein is fed to an animal, these were assumed to have little bearing on the results overall.

Materials and methods

Animals and diets

Digesta samples were collected from the terminal ileum of six Large White x Duroc pigs of mean (\pm SEM) body weight 79 ± 4.8 kg. The pigs were kept singly in steel metabolism crates, in a room maintained at $24 \pm 1^\circ\text{C}$, at the Animal Physiology Unit, Massey University, Palmerston North, New Zealand. Approval for the study was granted by the Massey University Animal Ethics Committee (protocol 05/29). The pigs arrived in the unit one week prior to surgery. During surgery each pig was fitted with a post-valvular T caecum (PVTC) cannula, as described by van Leeuwen *et al.* (1991). The study commenced some eight weeks after surgery. Following surgery, food was progressively reintroduced within one week up to a level of 0.08 metabolic body weight ($W^{0.75}$)/d and this level of food intake was maintained for the remainder of the trial. The pigs were fed a nutritionally balanced casein-based diet, mixed with water (1:1, w/w), 3 times daily (0800, 1200 and 1600 h), in equal portions for the remainder of the trial. Water was available *ad libitum*. On the day of digesta collection the pigs were fed one-third of the daily ration of the test diet at 0800h. The composition of the test diet, which included titanium dioxide as an indigestible marker, is presented in **Table 1**. The pigs did not receive any food during digesta collection. In the evening, following the ten hour digesta collection, they received the remaining two-thirds of their daily ration and water.

Digesta were collected into polythene bags. After weighing, sodium benzoate, ($10\text{g}\cdot\text{kg}^{-1}$ of digesta), as a bactericide, and phenylmethylsulphonyl fluoride ($0.37\text{g}\cdot\text{kg}^{-1}$ of digesta), as a protease inhibitor, were added according to the protocol of Salgado *et al.* (2001). The digesta samples for each pig were then pooled separately and frozen at -20°C until chemical analysis.

Table 1 Composition of the experimental diet (g/kg air dry weight).

Ingredient	Experimental Diet ¹ .
Maltodextrin	453
Sucrose	161
Soybean oil	154
Sodium Hydrogen Carbonate	18
Titanium Dioxide	3
Lactic casein	211

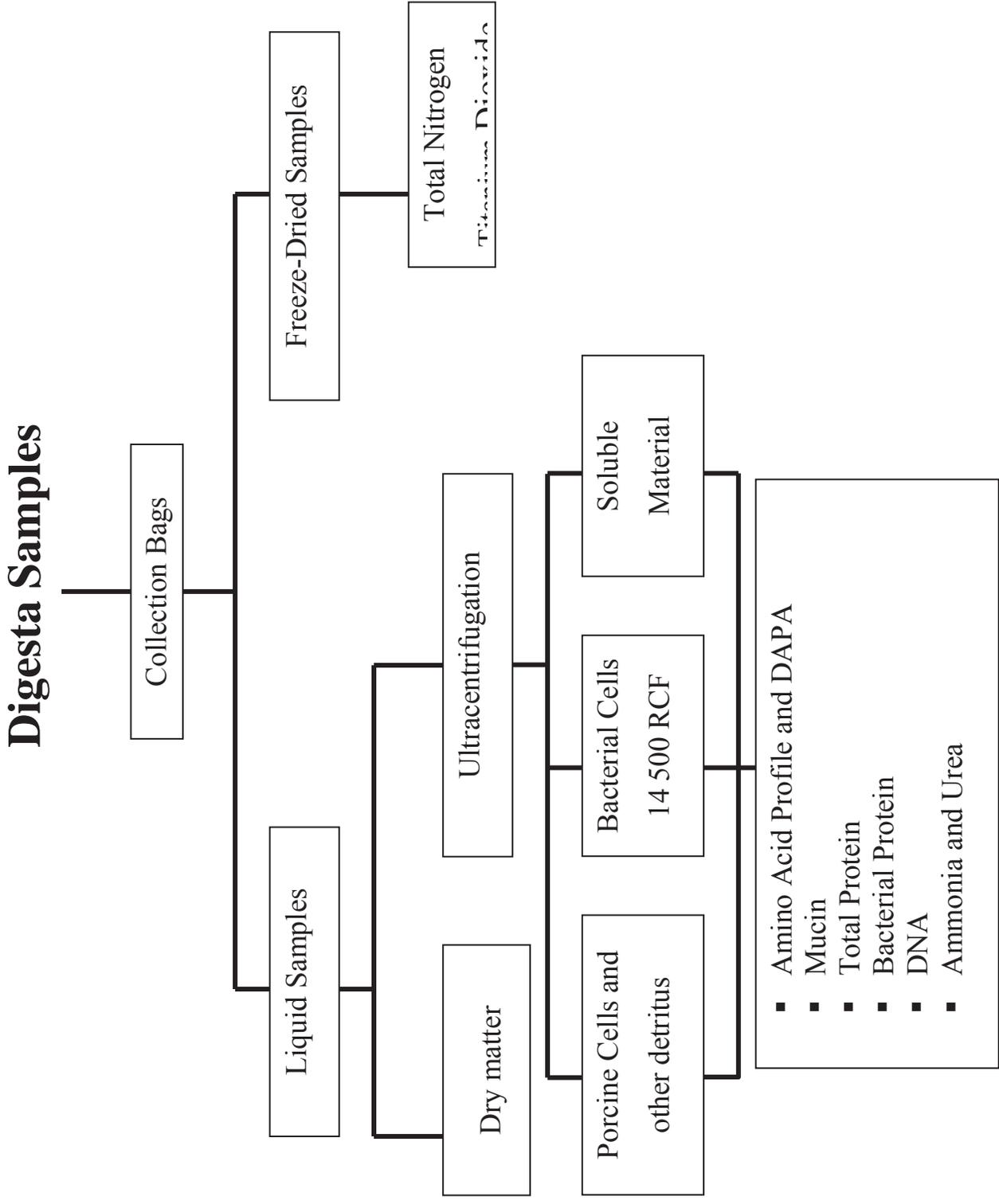
¹No vitamins, minerals or fibre were added to this diet as the same diet was used in an acute feeding study with human subjects. A preliminary casein based diet contained a vitamin mineral premix and cellulose.

Chemical analyses

All analyses were undertaken in triplicate. Dry matter was measured by drying to a constant mass in a forced air oven at 95 °C. Other samples of the digesta were fractionated by differential centrifugation using the method of Metges *et al.* (1999). Briefly the pooled digesta were centrifuged first at 250 RCF for 15 minutes at 4 °C, giving a fraction expected to contain food particles and porcine cells, then at 14 500 RCF for 30 minutes at 4 °C, to give a precipitate expected to contain microbial cells, and a supernatant expected to contain mainly proteins, peptides, free amino acids, mucins, neutral sugars, urea, creatinine and ammonia. A summary of the centrifugation protocol is given in **Figure 1**.

Total Nitrogen was determined by the Leco total combustion method (AOAC, 2000), a variation of the Dumas method. Amino acid compositions were determined using the procedure outlined by Hodgkinson and Moughan (2003). No corrections were made for potential losses of amino acids during hydrolysis. Methionine and cysteine were measured as methionine sulphone and cysteic acid, respectively, after the hydrolysis of samples that had been oxidized using performic acid.

Figure 1. Schematic diagram of the processing of the digesta samples



Diaminopimelic acid (DAPA) was quantified, following oxidation with performic acid, using an HPLC system with a UV detector. Tryptophan was not measured. Titanium Dioxide was determined by the method of Short *et al.* (1996), and soluble protein was determined by the Bradford method (1976).

Bacterial protein in the 14500 RCF precipitate was determined using a Bacterial Protein Extraction Lysis Buffer (PELB) kit combined with a Non-interfering Protein Assay Kit, both obtained from G-Biosciences, St Louis, MO, USA. Following the manufacturer's instructions, total protein estimation in the 14500 RCF precipitate, comprising mainly bacterial matter, was achieved by first using the Bacterial PELB kit to lyse the bacterial cells, before using the Non-interfering Protein Assay Kit. The extracellular protein concentration was then determined for the same quantity of the 14500 RCF precipitate without the initial lysis step. The difference between the resultant concentrations was considered to be the bacterial protein concentration.

Mucins were quantified by the determination of the amino sugars N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GlcNAc), carbohydrate markers peculiar to glycoproteins. The method used was similar to that described by Lien *et al.* (1997).

Gas-liquid chromatography was undertaken using a Shimadzu 2010 chromatograph (Shimadzu Scientific Instruments Inc, Columbia, MD), with a DB-17 fused silica capillary column (J and W Scientific, Folsom, CA; 0.25 mm internal diameter x 30 meter) with helium (1.5mL/minute) as the carrier. A Shimadzu GC solutions V2-30su6 data system (Shimadzu Scientific Instruments Inc, Columbia, MD) was used for peak area integration.

Mucin output was estimated using regression equations devised by Lien *et al.* (1997). The regression equations for mucin, assuming no digestion, were as follows:

Where GalNAc is equal to GalNAc output in g·day⁻¹

For native mucin: % GalNAc = 32.30 - 22.74x + 8.83x² - 1.37x³.

Where x = GlcNAc:GalNAc ratio.

Thus,

Mucin output = GalNAc / % GalNAc

Ammonia and Urea were determined using an adaptation of the method devised by Chaney and Marbach (1962). DNA was determined using a QIAamp DNA Stool Mini Kit obtained from QIAGEN Inc. Valencia, CA, USA. The kit was used following the manufacturer's instructions for the isolation of DNA from stools for pathogen detection. The absorbance of the extracted samples was determined using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies Wilmington, DE 19810, USA). The concentration of DNA was determined from its absorbance at A_{260} nm and its purity, with respect to contaminants that absorb UV, such as protein, from the ratio of the readings at A_{260} nm and A_{280} nm.

The recovery of an indigestible marker (TiO_2) was used to correct the concentrations of mucin and mucin markers for each of the methods using the following equation:

$$\text{Corrected concentration of mucin or mucin marker (g}\cdot\text{kg}^{-1} \text{ dry matter intake (DMI))} = \frac{\text{Concentration of mucin or mucin marker (g}\cdot\text{kg}^{-1} \text{ DMI)} \times \text{Concentration of TiO}_2 \text{ in the diet (g}\cdot\text{kg}^{-1} \text{ DMI)}}{\text{Concentration of TiO}_2 \text{ in the ileal digesta (g}\cdot\text{kg}^{-1} \text{ DMI)}}$$

Where DMI = dry matter intake

Results and Discussion.

In this study the experimental diet contained the protein, casein, which is almost completely digested and absorbed (Eklund *et al.*, 2008; Kies *et al.*, 1986); indeed because of its high digestibility casein is a preferred protein source to determine basal ileal endogenous losses (Eklund *et al.*, 2008; Mariscal-Landin and de Souza, 2006). The true digestibility of casein has been estimated by Deglaire *et al.* (2009) to be in excess of 97%. It is expected that almost all of the soluble protein found in the ileal effluent would have originated from endogenous or bacterial sources. The study allowed the direct determination of endogenous proteinaceous components in the digesta of protein fed animals

A description of the nitrogenous components of the digesta is given in **Table 2**. Total nitrogen made up 6.3% of digesta dry matter (DDM). Of the total nitrogen in the digesta, 73% was protein nitrogen and 27% non-protein nitrogen. Some 60% of the protein nitrogen was soluble, and present in the 14500 RCF supernatant, with the remaining 40% being insoluble, and found in the two centrifugation precipitates.

The differential centrifugation method used here separated the digesta into three fractions. Microscopic examination of the 250 RCF precipitate and the 14500 RCF supernatant confirmed that although there were bacterial cells in these fractions, their numbers were proportionally insignificant compared to the precipitate from the 14 500 RCF centrifugation stage.

Table 2. Nitrogen content of terminal ileal digesta for pigs given a casein based diet.

	$\text{g}\cdot\text{kg}^{-1}$ DMI ¹		$\text{mg}\cdot\text{g}^{-1}$ DDM ²		Percentage
	Mean	SEM	Mean	SEM	
Total Nitrogen	3.9	0.45	63.2	1.48	100
Protein Nitrogen	2.8	0.30	46.4	1.83	73.0
Non-protein Nitrogen	1.1	0.04	16.8	0.98	27
Soluble Protein Nitrogen	1.7	0.21	27.8	1.25	60.0 ⁴
Insoluble Protein Nitrogen	1.1	0.18	18.6	2.03	39.9 ⁴

¹ DMI = Dry matter intake.

² DDM = Digesta dry matter.

³SEM = Standard error of Mean.

⁴ Percentage values of Total Protein Nitrogen. n = 18.

The amino acid composition of the digesta is presented in **Table 3**, and the distribution of amino acids in the three centrifugation fractions is shown graphically in **Figure 2**.

Table 3. Amino acid composition of terminal ileal digesta.

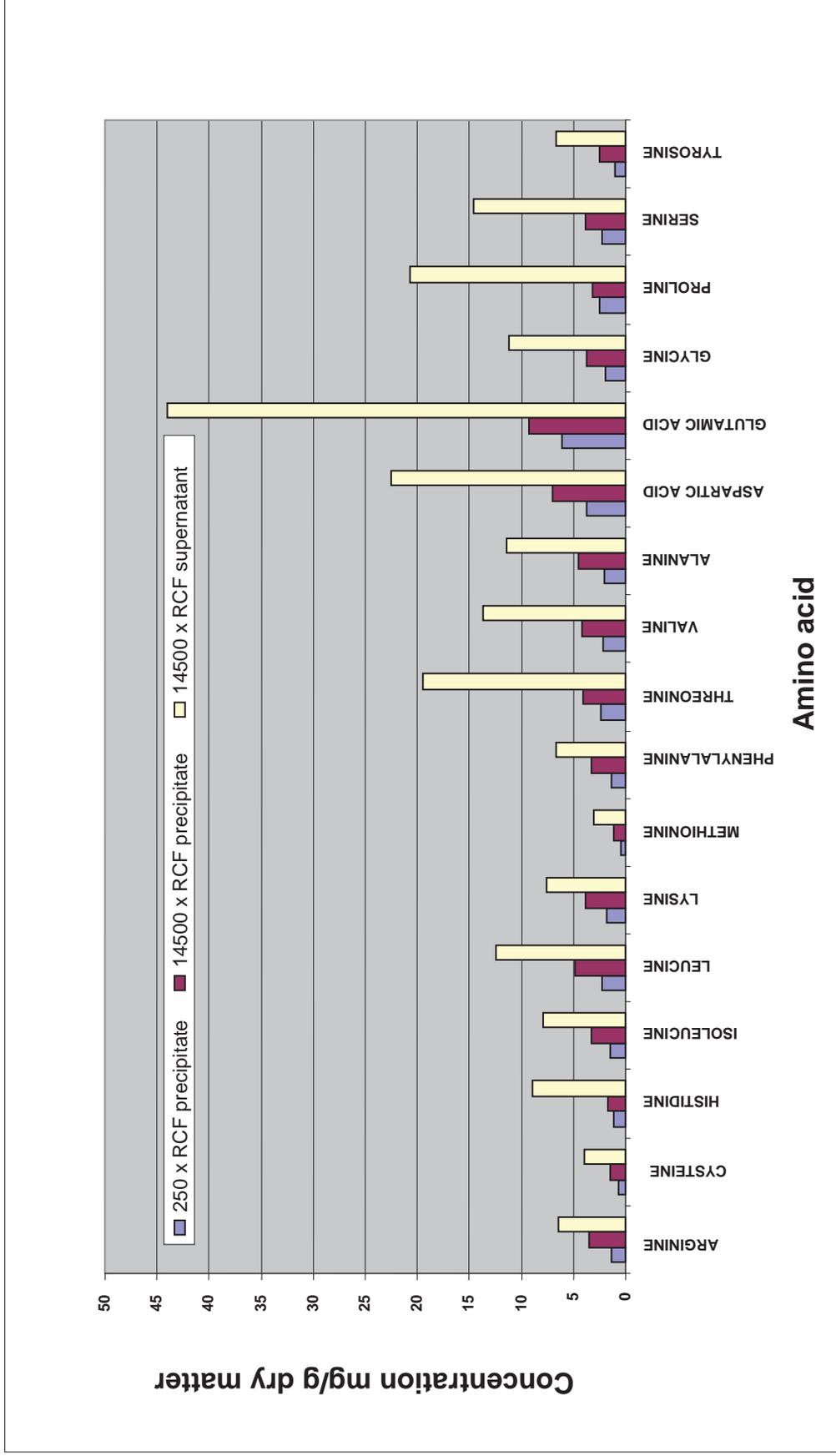
Amino acid	mol/100mol	SEM ¹	mg•g ⁻¹ DDM ²	SEM ¹
Indispensable amino acids				
Arginine	2.5	0.19	11.4	1.09
Cysteine	2.0	0.28	5.9	0.69
Histidine	3.0	0.21	11.8	0.98
Isoleucine	3.8	0.30	12.7	0.96
Leucine	5.8	0.42	19.7	1.58
Lysine	3.5	0.24	13.3	1.29
Methionine	1.1	0.06	4.4	0.37
Phenylalanine	2.2	0.23	11.4	1.16
Threonine*	8.8	1.00	25.8	2.13
Valine	6.7	0.50	20.0	1.47
Dispensable amino acids				
Alanine	7.7	0.60	17.9	1.67
Aspartic Acid	9.5	0.68	33.3	2.91
Glutamic Acid	15.1	1.06	59.4	6.67
Glycine	9.1	0.86	16.9	0.97
Proline*	8.9	0.64	26.4	2.21
Serine*	7.8	0.64	20.7	1.36
Tyrosine	2.1	0.13	10.0	0.87

¹ SEM = Standard error of mean.

² DDM = Digesta dry matter.

Tryptophan not determined. * Predominant amino acids in mucin. n = 18.

Figure 2 Amino acid concentrations for the different centrifugation fractions of ileal digesta.



The six most abundant amino acids detected in the digesta, in decreasing order of abundance ($\text{mg}\cdot\text{g}^{-1}$ DDM), were: glutamic acid, aspartic acid, proline, threonine, serine, and glycine. These six amino acids are present in the highest proportions in the protein core of glycoproteins from the gastrointestinal tract. Of these six, threonine, serine and proline are most abundant in the glycosylated region of mucin polymers, a region that holds almost 90% of these three amino acids (Montagne et al., 2000). The other three amino acids, glutamic acid, glycine and aspartic acid, are predominant in the non-glycosylated regions of mucin. The high proportions (nearly 52% of the total by mass of amino acids) of these six mucin-associated amino acids, is consistent with a high rate of secretion of mucins into the gastrointestinal tract and a relatively poor digestion and absorption of their amino acids from the small intestine. These mucin-associated amino acids predominate in the 14500 RCF supernatant (Figure 2), from which it may be inferred that a large proportion of soluble mucin is present in this fraction.

The concentrations of DAPA and DAPA nitrogen were estimated to be 0.62 and 0.09 $\text{mg}\cdot\text{g}^{-1}$ digesta dry matter respectively, values that are in accord with Rowan et al. (Rowan, Moughan, & Wilson, 1992). Using the ratio of 2.88 mg of DAPA per gram of bacterial dry matter (Czerkawski, 1974), it was calculated that the mass of bacterial dry matter was 216 $\text{mg}\cdot\text{g}^{-1}$, a value also in accord with Rowan et al. (1992), and represents 21% of the total dry matter of the digesta.

Diaminopimelic acid (DAPA) is a component of peptidoglycans found in bacterial cell walls. Although traces of DAPA may be found in protozoa, it is almost unique to bacteria and consequently has been used as a marker of bacterial protein (Weller et al., 1958). The accuracy of this approach however has been called into question by a number of researchers because the concentration of DAPA varies with bacterial size and species (Ling, 1990). Although alternative markers have been proposed (Csapo et al., 2002), the degree of error associated with bacterial nitrogen estimations using DAPA is not known (Caine et al., 1999). Whilst DAPA was judged by Robinson et al. (1996) to underestimate the microbial nitrogen pool, Csapo et al. (2002) suggested that it overestimated it by some 10% in comparison with D-aspartic acid and D-glutamic acid, used as alternative bacterial protein markers. Although there may be limitations in the use of DAPA it was adopted in this study as a useful comparative method in line

with the work of other researchers. As bacterial amino acids are not of direct dietary origin, it is pertinent to determine the amino acid composition of endogenous ileal digesta adjusted for the bacterial component. The amino acid profile of the 14500 RCF precipitate, the bacterial pellet (**Table 4**) can be considered, with some certainty, to be derived solely from bacterial protein. The flows of amino acids related to this fraction of the digesta were thus subtracted from the total endogenous flow to give an estimate (Table 4) of the true endogenous ileal amino acid flows. However, it should be noted that there will be quantities of soluble bacterial protein present in the 14500 RCF supernatant that have originated from lysed bacterial cells: these are not accounted for in this correction.

The lysine derived from the bacterial pellet, present in the 14500 RCF precipitate (see Table 4), represented nearly 25% of the total amount of lysine present in the ileal digesta. There is a marked difference between the lysine concentrations of microbial protein and of porcine intestinal tissue. The concentration of lysine in the 14500 RCF precipitate, which represents the microbial fraction of the digesta, was 3.6 g/16 g of bacterial nitrogen, a figure that is in accord with that of Metges *et al.* (2000) and very different from the accepted lysine concentration of porcine intestinal tissue (7.8 g/16 g nitrogen (Munro and Fleck, 1970). The concentration of lysine in the other fractions, the 250 RCF precipitate and the supernatant from the high speed centrifugation, which together are considered to be non-microbial, was found to be 8.3 g/16 g nitrogen, a value that is more in line with the value for cellular protein determined by Munro and Fleck (1970).

The concentrations of deoxyribonucleic acid (DNA) and DAPA in the different centrifugation fractions are presented in **Table 5**. The mean value for digesta DNA was 1.34 mg·g⁻¹ of dry matter, a value that compares well with 1.1 mg·g⁻¹ of digesta dry matter reported by Rowan *et al.* (1992). It was assumed that the DNA concentration of 0.45 mg·g⁻¹ of digesta dry matter (DDM) detected in the 14500 RCF precipitate was solely of bacterial origin. Using this concentration of DNA together with the estimate of DAPA in the same centrifugation fraction (0.26 mg·g⁻¹ of DDM), a DNA:DAPA ratio of 1.73 was determined, a figure also in agreement with that obtained by Rowan *et al.* (1992). The latter ratio was then used to determine the amounts of bacterial DNA in the other centrifugation fractions.

Table 4. Amino acid composition of total and endogenous (corrected for bacterial amino acids) ileal digesta flows.

Amino acid	Total amino acids	Bacterial amino acids ²	Endogenous amino acids ³
	mg•g ⁻¹ DDM ¹	mg•g ⁻¹ DDM	mg•g ⁻¹ DDM
Indispensable amino acids			
Arginine	11.4	3.1	8.2
Cysteine	5.9	1.4	4.6
Histidine	11.8	1.7	10.7
Isoleucine	12.7	3.0	9.8
Leucine	19.7	4.4	15.3
Lysine	13.3	3.3	10.0
Methionine	4.4	1.1	3.3
Phenylalanine	11.4	2.9	8.5
Threonine*	25.8	4.0	21.8
Valine	20.0	4.0	16.0
Dispensable amino acids			
Alanine	17.9	4.0	13.9
Aspartic Acid	33.3	7.0	26.3
Glutamic Acid	59.4	10.9	48.5
Glycine	16.9	3.6	13.3
Proline*	26.4	3.5	22.9
Serine*	20.7	3.6	17.0
Tyrosine	10.0	2.1	7.9

¹ DDM = Digesta dry matter.

² 14500 RCF precipitate.

³ Total amino acid flow corrected for bacterial amino acid flow.

Tryptophan not assayed.* Predominant amino acids in mucin. n = 18.

Table 5 Concentrations of DNA and DAPA in terminal ileal digesta.

Centrifugation Fraction	DAPA mg/g DDM ¹ .	DNA mg/g DDM		
	Total DAPA	Total DNA	Bacterial cellular	Porcine cellular ⁴ .
250 RCF precipitate	0.01	0.18	0.02	0.16
14500 RCF precipitate	0.26	0.45	0.45 ²	
14500 RCF supernatant	0.36	0.71	0.68 ³	0.03
Totals	0.62	1.34	1.15	0.19

¹. DDM = Digesta dry matter. DNA = Deoxyribose nucleic acid. DAPA = 2,6-diaminopimelic acid.

². It was assumed that all of the DNA detected in the 14500 precipitate was bacterial in origin. A DNA:DAPA ratio of 1.77 was calculated (when expressed as mg·g⁻¹DDM), and used to estimate the bacterial DNA in the other two centrifugation fractions.

³. The concentration of bacterial DNA in this fraction was assumed to originate from lysed bacterial cells and was determined from the DNA:DAPA ratio of 1.77 (see ². above).

⁴. Cellular DNA was determined as being the difference between total DNA detected and estimated bacterial DNA. n = 18.

DNA in the 14500 RCF supernatant was assumed to have originated from lysed bacterial and porcine cells (inspection using light microscopy revealed that the number of intact cells in this fraction was negligible). Porcine cellular DNA material was determined as the difference between total DNA detected and the estimated quantity of bacterial DNA. The higher proportion of porcine cellular DNA in the 250 RCF centrifugation fraction supports the effectiveness of the separation, by centrifugation, of the porcine cellular material and bacterial material from the soluble components of the digesta. Using the QIAamp DNA Stool Mini Kit, the DNA extraction gave a particularly pure sample, such that protein interference was minimal.

An average DAPA:bacterial nitrogen ratio of 26.42 was determined, a value very close to that of 26.4 reported by Wünsche *et al.* (1991). Utilizing this value, the proportions of the protein from microbial and non-microbial sources can be calculated: they are presented in **Table 6**. It is evident that a substantial proportion of protein of microbial origin is present in the digesta, nearly 54%, with over half of this being present in the 14500 RCF supernatant.

The concentration of both amino sugars and the calculated mucin output are presented in **Table 7**. Although the concentrations of both GalNAc and GluNAc are in accord with those reported by Piel *et al.* (2004), the total mucin output determined in this study is much higher. This may be because of differences between the control diet used by Piel *et al.* (2004) and the casein diet used in this study or, more likely, as noted by Piel *et al.* (2004), due to differences in the composition of mucins in weaned piglets and older pigs.

Different types of mucin are secreted by different regions of the gastrointestinal tract and the composition of such mucins is known to vary between different animal species and the various sites of secretion (Montagne *et al.*, 2000). However, mucins in digesta taken from the terminal ileum would be expected to originate mostly from the stomach and the small intestine.

Table 6 Distribution of protein of microbial or non-microbial origin within the different centrifugation fractions of ileal digesta.

	g/kg DMI ¹	SEM	mg/g DDM	SEM	% ⁴
Total digesta protein	20.5	2.25	329.7	11.69	100
Total microbial protein ²	11.1	0.83	180.4	17.3	54.1
Total non-microbial protein	9.4	0.86	149.3	15.3	45.9
250 RCF Precipitate					
Protein	2.1	0.25	43.4	3.08	10.2
Microbial protein	1.0	0.18	24.3	2.87	4.9
Porcine cellular protein	1.1	0.19	19.0	2.07	5.4
14500 RCF Precipitate					
Protein ³	6.7	0.73	91.0	10.91	32.7
Microbial protein	3.5	0.31	62.7	7.65	17.1
non-microbial Protein	3.2	0.37	28.2	3.52	15.6
14500 RCF Supernatant					
Protein	11.7	1.28	195.4	10.92	57.1
Microbial protein	6.6	0.79	93.3	7.51	32.2
Non-microbial protein	5.1	0.58	102.0	9.63	24.9

¹ Values are the means \pm standard error of mean.

² Microbial Protein was determined using the value of 26.42 mg DAPA/ g of bacterial nitrogen.

³ Protein in this fraction was estimated using the Bacterial Protein Extraction Lysis Buffer (PELB), kit combined with a Non-interfering protein assay kit.

⁴ Percentages of total protein calculated from protein values expressed in $\text{g}\cdot\text{kg}^{-1}$ DMI. n = 18.

The amino sugars N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GluNAc) are useful mucin markers, and once the ratio of the two is known, the relative proportions of gastric and intestinal mucin can be estimated. This is possible because the GluNAc:GalNAc ratio differs considerably between the two types of mucin, with gastric mucin containing approximately 30% GlcNAc and 13% GalNAc, whereas intestinal mucin contains approximately 20% and 40% (Lien et al., 1997; Montagne et al., 2000; Stanley *et al.*, 1983).

Table 7 Mean concentrations of the amino sugars N-Acetylgalactosamine and N-Acetylglucosamine, and the concentration of mucin in terminal ileal digesta.

		Mean	SEM
N-Acetylgalactosamine	Diet	ND ^a	.
	Ileal digesta g•100 g ⁻¹ DDM ^c	2.0	0.28 ^b
	Ileal digesta g•kg ⁻¹ DMI ^d	5.35	0.32
N-Acetylglucosamine	Diet	ND	
	Ileal digesta g•100 g ⁻¹ DDM	2.6	0.22
	Ileal digesta g•kg ⁻¹ DMI	7.0	0.49
GluNAc: GalNAc ratio ^e		1.3	0.06
Mucin Output	g•kg ⁻¹ DMI	36.9	2.22
	mg•g ⁻¹ DDM	131.0	10.69
Mucin Nitrogen	g•100g ⁻¹ Digesta total nitrogen	9.4	0.68

^a ND = Not detected.

^b SEM = standard error of mean.

^c DDM = Digesta dry matter.

^d DMI = Dry matter intake.

^e When expressed as g•100g⁻¹ DDM.

The estimated proportions of gastric and intestinal mucins in the digesta were 64% and 36%, respectively. n = 18.

Regression equations developed by Lien *et al.* (1997) can be used to estimate the proportions of gastric and intestinal mucin. Of several methods that may be used to detect mucin (Miner-Williams *et al.*, 2009), the assay used here provides the most accurate information as to the type of mucin present in digesta.

In their studies of gastrointestinal mucus, Mantle and Allen (1989) reported GalNAc:GluNAc ratios of 2.8 and 0.6 for purified pig gastric and intestinal mucin respectively. In this study a value of 1.3 for total mucin was determined: using the regression equations developed by Lien *et al.* (1997). The proportions of gastric and intestinal mucin present in the digesta were estimated to be 64% and 36%, respectively.

It is known that the presence of dietary proteins in the abomasum of the calf increases the secretion of pepsin and chymosin into the stomach (Sissons, 1981) and that these enzymes can hydrolyse gastric mucus to release mucins into the abomasal digesta, which in turn flow into the duodenum. The high proportion of gastric mucin found in the ileal digesta in the present study supports the thesis that dietary casein stimulates the secretion of gastric proteases and acid which in turn erode the gastric mucus layer and release gastric mucins into the chyme, which are not fully digested before arriving at the terminal ileum.

When the threonine:serine ratios of the different centrifugation fractions were determined (see Table 4, the ratio for the supernatant was nearly 30% higher than that of the 250 RCF precipitate and nearly 34% higher than that in the 14500 RCF precipitate. Once again this indicates that a large proportion of the mucin in the digesta is soluble and is in the supernatant, with a much smaller amount of insoluble mucin in the 250 RCF centrifugation fraction. At 13% of the digesta dry matter, mucin is the single most abundant truly endogenous component secreted into the gastrointestinal tract.

Concentrations of ammonia, urea and creatinine are shown in **Table 8**. When expressed in terms of nitrogen these three components accounted for nearly 65% of the non-protein nitrogen in the digesta. Although accounting for only 1.6% of the digesta dry matter, urea and ammonia contribute a disproportionate amount of nitrogen.

Table 8 Determined concentrations of urea, ammonia and creatinine in terminal ileal digesta.

	g•kg⁻¹DMI	SEM	mg•g⁻¹DDM	SEM
Urea	1.9	0.34	7.1	0.84
Ammonia	1.8	0.29	8.4	1.43
Creatinine	0.12	0.03	3.1	0.73

Values are means, ± standard error of mean. n = 18.

Ammonia, urea and the metabolic activity of intestinal bacteria are all linked to the nitrogen cycling systems of the gut in simple-stomached animals, including humans. The release of ammonia into the intestinal lumen may result from the catabolism and oxidation of amino acids by both enterocytes and bacteria, as well as the enzymatic breakdown of urea by microbial flora. The enteric metabolism of amino acids has important implications for the apparent digestibilities of proteins and amino acids, becoming, as Stoll *et al.* suggest, a source of nutritional inefficiency (Stoll *et al.*, 1998). In this study the combined contributions of bacteria, urea, and ammonia account for nearly 55% of the total nitrogen in the digesta at the terminal ileum and may reflect nutritional inefficiency noted by Stoll, *et al.* (1998). It is interesting to note that the nitrogenous composition of the digesta of pigs fed a casein-based diet are in accord with the results of Chacko and Cummings (1988), who suggested that 80-85% of nitrogen lost from the small bowel was from proteins and peptides.

In summary the sources of nitrogen determined in the ileal digesta of pigs fed a casein-based diet is given in **Table 9**. The estimate of bacterial protein nitrogen, nearly 45% of total digesta nitrogen is suggestive of large populations of bacteria in the small intestine, contrary to the earlier view that the upper digestive tract is virtually devoid of microbial activity (Hill, 1982). Although the number of bacteria in the small intestine may have been influenced by the presence of the PVTC cannula its effect on the proportion of microbial protein relative to total protein is considered to be much less than found with other collection techniques such as ileo-rectal anastomosis (Köhler *et al.*, 1992).

Table 9 Summary of the sources of nitrogen in terminal ileal digesta of pigs given a casein-based diet.

Source of Nitrogen	mg/g DDM ¹ .	SEM	Percentage of total protein	Percentage of non-bacterial protein	Percentage of total nitrogen
Bacterial	28.3 ² .	3.80	60.9		44.7
Porcine Cellular ³ .	3.1	0.69	6.7	17.2	4.9
Soluble Free Protein ⁴ .	8.5	0.80	18.2	46.7	13.4
Mucin	6.6	0.48	14.1	36.1	10.4
DNA	0.12	0.02			0.2
Urea	3.3	0.40			5.2
Ammonia	6.9	0.98			11.0
Creatinine	1.2	0.20			1.8
Non-specific ⁵ .	5.2	0.71			8.3
Sum	63.2	2.31			100.0

¹. Values are means \pm standard errors. DNA = Deoxyribose nucleic acid. DAPA = 2,6-diaminopimelic acid.

² Equivalent to 26.4 mg DAPA/g Bacterial Nitrogen. Bacterial nitrogen was principally determined from the 14500 RCF precipitate with corrections for DAPA found in other centrifugation fractions.

³ Porcine cellular nitrogen was calculated from the 250 RCF centrifugation precipitate with corrections for the DAPA found in that fraction. This fraction represents intact porcine cells and does not include cellular debris.

⁴ Soluble free protein was determined from the 14500 RCF supernatant using the Bradford reagent, may include: albumin, immunoglobulins, digestive enzymes and small peptides such as digestion resistant bioactive peptides and soluble cell debris.

⁵ This fraction contains non-identified nitrogenous material that may include non-protein compounds such as: free amino acids, RNA's, amines and the tetrapyrroles, bilirubin and biliverdin.

Present in the 14500 RCF supernatant was a protein fraction that was determined using the Bradford reagent. This fraction was estimated to contain nearly 9 mg of soluble free protein per gram of dry matter (see Table 9), and at 13% represents a not insignificant proportion of total nitrogen present in the digesta dry matter. This fraction would be expected to contain immunoglobulins, digestive enzymes and small protease-resistant peptides.

Acknowledgements

Grateful thanks are offered to Dr Amélie Deglaire for her assistance with the pig trial and Dr Gordon Reynolds for undertaking the surgery to install the PVTC's

Literature cited

- AOAC. (2000). Official Method for protein (Dumas method), 968.06. *Official methods of analysis of association of official analytical chemists international*.
- Bradford, M. M. (1976). Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Analytical Biochemistry*, 72(1-2), 248-254.
- Caine, W. R., Tamminga, S., Sauer, W. C., Verstegen, M. W. A., and Schulze, H. (1999). Bacterial contributions to total and endogenous recoveries of nitrogen and amino acids in ileal digests of newly weaned piglets fed protease-treated soybean meal. *Livestock Production Science*, 57(2), 147-157.
- Chaney, A. L., and Marbach, E. P. (1962). Modified reagents for determination of urea and ammonia. *Clinical Chemistry*, 8(2), 130-and.
- Corring, T., and Saucier, R. (1972). Pancreatic-secretion of fistulated pig - adaptation to protein content in diet. *Annales De Biologie Animale Biochimie Biophysique*, 12(2), 233-241.
- Csapo, J., Schmidt, J., Csapo-Kiss, Z., Varga-Visi, E., Pohn, G., and Csokona, E. (2002). Quantitative determination of protein of bacterial origin on the basis of D-aspartic acid and D-glutamic acid content. *Chromatographia*, 56, S169-S171.

- Czerkawski, J. W. (1974). Methods for determining 2-6-diaminopimelic acid and 2-aminoethylphosphonic acid in gut contents. *Journal of the Science of Food and Agriculture*, 25(1), 45-55.
- Deglaire, A., C. Bos, *et al.* (2009). "Ileal digestibility of dietary protein in the growing pig and adult human." *British Journal of Nutrition* 102(12): 1752-1759.
- Eklund, M., Mosenthin, R., Piepho, H. P., and Rademacher, M. (2008). Effect of dietary crude protein level on basal ileal endogenous losses and standardized ileal digestibilities of crude protein and amino acids in newly weaned pigs. *Journal of Animal Physiology and Animal Nutrition*, 92(5), 578-590.
- Hill, M. J. (1982). Gut flora associated diseases in man. *Advances in Veterinary Medicine* 22: 32-38.
- Hodgkinson, S. M., and Moughan, P. J. (2003). The enzyme hydrolysed protein method for the determination of endogenous ileal nitrogen and amino acid flows - a modification. *Animal Feed Science and Technology*, 108(1-4), 207-214.
- Jansman, A. J. M., Smink, W., van Leeuwen, P., and Rademacher, M. (2002). Evaluation through literature data of the amount and amino acid composition of basal endogenous crude protein at the terminal ileum of pigs. *Animal Feed Science and Technology*, 98(1-2), 49-60.
- Kies, A. K., Moughan, P. J., and Smith, W. C. (1986). The apparent and true Ileal digestibility of nitrogen and amino-acids in lactic casein for the growing-pig. *Animal Feed Science and Technology*, 16(3), 169-178.
- Köhler, T., R. Mosenthin, *et al.* (1992). "Effect of ileo-rectal anastomosis and post-valve T-caecum cannulation on growing pigs. 1) Growth performances, N-balance, and intestinal adaptation." *British Journal of Nutrition* 68: 293-303.
- Leibholz, J. (1982). The flow of endogenous nitrogen in the digestive-tract of young-pigs. *British Journal of Nutrition*, 48(3), 509-517.
- Lien, K. A., Sauer, W. C., and Fenton, M. (1997). Mucin output in ileal digesta of pigs fed a protein-free diet. *Zeitschrift Fur Ernährungswissenschaft*, 36(2), 182-190.
- Ling, J. R. (1990). *The Rumen Ecosystem. The Microbial Metabolism and its Regulation*. New York: Spinger-Verlag.
- Low, A. G. (1980). Nutrient absorption in pigs. *Journal of the Science of Food and Agriculture*, 31(11), 1087-1130.

- Mantle, M., and Allen, A. (1989). Gastrointestinal mucus. In J. S. Davison (Ed.), *Gastrointestinal Secretions* (pp. 202-229). London: Butterworth and Co.
- Mariscal-Landin, G., and de Souza, T. C. R. (2006). Endogenous ileal losses of nitrogen and amino acids in pigs and piglets fed graded levels of casein. *Archives of Animal Nutrition*, 60(6), 454-466.
- Metges, C. C. (2000). Contribution of microbial amino acids to amino acid homeostasis of the host. *Journal of Nutrition*, 130(7), 1857S-1864S.
- Metges, C. C., Petzke, K. J., El-Khoury, A. E., Henneman, L., Grant, I., Bedri, S., *et al.* (1999). Incorporation of urea and ammonia nitrogen into ileal and fecal microbial proteins and plasma free amino acids in normal men and ileostomates. *American Journal of Clinical Nutrition*, 70(6), 1046-1058.
- Millward, D. J., Garlick, P. J., James, W. P. T., Sender, P. M., and Waterlow, J. C. (1976). Protein turnover. In D. J. A. Cole, K. N. Boorman, P. J. Buttery, D. Lewis, R. J. Neale and H. Swan (Eds.), *Protein Metabolism and Nutrition*. (Vol. 16, pp. 49-69.). London: European Association of Animal Production, Butterworths.
- Miner-Williams, W., Moughan, P. J., and Fuller, M. F. (2009). Methods for mucin analysis - a comparative study. *Journal of Agricultural and Food Chemistry*, 57, 6029-6035.
- Montagne, L., Toullec, R., Formal, M., and Lalles, J. P. (2000). Influence of dietary protein level and origin on the flow of mucin along the small intestine of the preruminant calf. *Journal of Dairy Science*, 83(12), 2820-2828.
- Moughan, P. J. (2003). Amino acid availability: aspects of chemical analysis and bioassay methodology. *Nutrition Research Reviews*, 16(2), 127-141.
- Moughan, P. J., Butts, C. A., Rowan, A. M., and Deglaire, A. (2005). Dietary peptides increase endogenous amino acid losses from the gut in adults. *American Journal of Clinical Nutrition*, 81(6), 1359-1365.
- Munro, H. N., and Fleck, A. (1970). Analysis of tissues and body fluids for nitrogenous constituents. In H. N. Munro (Ed.), *Mammalian Protein Metabolism* (Vol. III, pp. 423-525). New York: Academic.
- Nasset, E. S., and Ju, J. S. (1961). Mixture of endogenous and exogenous protein in the alimentary tract. *Journal of Nutrition*, 74, 461-465.
- Pedersen, C., and Boisen, S. (2002). Establishment of tabulated values for standardized ileal digestibility of crude protein and essential amino acids in common

- feedstuffs for pigs. *Acta Agriculturae Scandinavica Section a-Animal Science*, 52(3), 121-140.
- Piel, C., Montagne, L., Salgado, P., and Lalles, J. P. (2004). Estimation of ileal output of gastro-intestinal glycoprotein in weaned piglets using three different methods. *Reproduction Nutrition Development*, 44(5), 419-435.
- Robinson, P. H., Fadel, J. G., and Ivan, M. (1996). Critical evaluation of diaminopimelic acid and ribonucleic acid as markers to estimate rumen pools and duodenal flows of bacterial and protozoal nitrogen. *Canadian Journal of Animal Science*, 76(4), 587-597.
- Rowan, A. M., Moughan, P. J., and Wilson, M. N. (1992). The flows of deoxyribonucleic-acid and diaminopimelic acid and the digestibility of dietary fiber components at the terminal ileum, as indicators of microbial activity in the upper digestive-tract of ileostomized pigs. *Animal Feed Science and Technology*, 36(1-2), 129-141.
- Rutherford, S. M., and Moughan, P. J. (1998). The digestible amino acid composition of several milk proteins: Application of a new bioassay. *Journal of Dairy Science*, 81(4), 909-917.
- Salgado, P., Lalles, J. P., Toullec, R., Mourato, M., Cabral, F., and Freire, J. P. B. (2001). Nutrient digestibility of chickpea (*Cicer arietinum* L.) seeds and effects on the small intestine of weaned piglets. *Animal Feed Science and Technology*, 91(3-4), 197-212.
- Sambrook, I. E. (1981). Studies on the flow and composition of bile in growing pigs. *Journal of the Science of Food and Agriculture*, 32(8), 781-791.
- Short, F. J., Gorton, P., Wiseman, J., and Boorman, K. N. (1996). Determination of titanium dioxide added as an inert marker in chicken digestibility studies. *Animal Feed Science and Technology*, 59(4), 215-221.
- Sissons, J. W. (1981). Digestive Enzymes of Cattle. *Journal of the Science of Food and Agriculture*, 32(2), 105-114.
- Stanley, R. A., Lee, S. P., and Roberton, A. M. (1983). Heterogeneity in Gastrointestinal Mucins. *Biochimica Et Biophysica Acta*, 760(2), 262-269.
- Stoll, B., Henry, J., Reeds, P. J., Yu, H., Jahoor, F., and Burrin, D. G. (1998). Catabolism dominates the first-pass intestinal metabolism of dietary essential amino acids in milk protein-fed piglets. *Journal of Nutrition*, 128(3), 606-614.

- van Leeuwen, P., van Kleef, D. J., van Kempen, G. J. M., Huisman, J., and Verstegen, M. W. A. (1991). The Post Valve T-Cecum Cannulation Technique in Pigs Applied (sic) to Determine the Digestibility of Amino-Acid in Maize, Groundnut and Sunflower Meal. *Journal of Animal Physiology and Animal Nutrition-Zeitschrift Fur Tierphysiologie Tierernahrung Und Futtermittelkunde*, 65(3-4), 183-193.
- Wapnir, R. A., and Teichberg, S. (2002). Regulation mechanisms of intestinal secretion: implications in nutrient absorption. *Journal of Nutritional Biochemistry*, 13(4), 190-199.
- Weller, R. A., Gray, F. V., and Pilgrim, A. F. (1958). The Conversion of Plant Nitrogen to Microbial Nitrogen in the Rumen of the Sheep. *British Journal of Nutrition*, 12(4), 421-429.
- Wunsche, J., Bock, H. D., Hennig, U., Kreienbring, F., and Borgmann, E. (1979). Untersuchungen über die proteinverdaulichkeit und aminosäurenresorption in verschiedenen abschnitten des verdauungstraktes beim schwein. *Archives of Animal Nutrition*, 29, 221-234.
- Wunsche, J., Volker, T., Souffrant, W. B., and Borgmann, E. (1991). Assessment of the bacteria-N proportions in feces and differently obtained ileal digesta of pigs. *Archiv Fur Tierernahrung-Archives of Animal Nutrition*, 41(7-8), 703-716.
- Zebrowska, T., Low, A. G., and Zebrowska, H. (1983). Studies on gastric digestion of protein and carbohydrate, gastric-secretion and exocrine pancreatic-secretion in the growing-pig. *British Journal of Nutrition*, 49(3), 401-410.

Chapter VI

Endogenous components of digesta protein from the terminal ileum of adult subjects fed a casein-based diet

The aim of the present study was to determine the nature and composition of endogenous nitrogen in the terminal ileal digesta collected from adult humans fed a casein-based diet.

Abstract

Although there are several published estimates of the amino acid composition of human ileal digesta there are no systematic studies of the protein of ileal digesta in humans. To obtain a better understanding of the nature and composition of endogenous substances containing nitrogen lost from the upper digestive tract of humans. Digesta were collected from the terminal ileum of six adult subjects using a naso-ileal tube and given a diet that contained casein as the only source of nitrogen.

The total nitrogen passing the terminal ileum was $39.3 \text{ mg}\cdot\text{g}^{-1}$ native digesta dry matter. Of this 86% was proteinaceous, nearly 60% was bacterial protein, 6% soluble free protein, and nearly 15% derived from mucin. Of the non-protein nitrogen, nearly 5% was as ammonia and 4% as urea. Bacterial and human mucosal cellular DNA nitrogen were collectively nearly 0.5% of the total nitrogen. Only 4.0% of the total nitrogen remained unidentified: this was assumed to include free amino acids, RNAs, amines, and the tetrapyrroles bilirubin and biliverdin. Mucin contributed 14.6% of the nitrogen losses and was the most abundant, truly endogenous component within the terminal ileal digesta, and comprised almost 13% of the total dry matter. Bacterial nitrogen, combined with ammonia and urea nitrogen, represented over 68% of the total nitrogenous losses, indicating substantial microbial activity within the human small intestine.

This is the first study detailing the proteinaceous composition of ileal digesta, quantifying the endogenous nitrogen containing compounds leaving the terminal ileum.

Introduction

Proteinaceous compounds are secreted into the lumen of the gastrointestinal tract (GIT) to assist the chemical breakdown of complex food molecules. Whether for the purpose of defence or for active digestion, such proteinaceous materials are secreted by specialist cells within salivary, gastric, hepatic and pancreatic glandular tissues and are termed endogenous proteins. Such proteins are often somewhat resistant to the digestive processes and may partly remain in the digesta leaving the ileum (Jansman *et al.*, 2002; Wapnir and Teichberg, 2002). Estimates of the amount of these secretions from the various glands along the GIT vary greatly though, on average, in the pig (an often used human analogue), approximately 8.3–12.6g nitrogen/day are secreted into the gut (Corring and Saucier, 1972; Krawielitzki *et al.*, 1994; Krawielitzki *et al.*, 1990; Leibholz, 1982; Sambrook, 1981; Zebrowska *et al.*, 1983). The total mass of endogenous material secreted into the GIT has been estimated to be approximately equal to the exogenous protein ingested (Nasset and Ju, 1961). Protein of the microbiota biomass that normally inhabits the GIT, although not strictly of endogenous origin, also contributes to the endogenous protein losses and may constitute the largest proportion of nitrogen and amino acids found at the terminal ileum (Caine *et al.*, 1999). Residual amounts of exogenous and endogenous proteinaceous material that remain unabsorbed at the terminal ileum are considered to have little nutritional value (Wunsche *et al.*, 1979) as amino acid absorption in the large intestine is thought to be minimal (Gaudichon *et al.*, 2002; Moughan and Rutherfurd, 2011; Moughan, 2003).

A dynamic equilibrium exists in the GIT between dietary protein intake and the secretion of endogenous proteinaceous material into the lumen with the concomitant absorption from the gut of digested materials, both exogenous and endogenous in origin. It has long been realised that the mass of nitrogenous materials leaving the ileum represents the net balance between nitrogen intake and secretion minus the absorption of dietary nitrogen and reabsorption of endogenous nitrogen (Moughan, 2003). Previously, methods to distinguish between endogenous nitrogen losses at the terminal ileum and undigested exogenous dietary nitrogen have involved the administration of a protein-free diet (Low, 1980). However, this has been shown to be physiologically unnatural and leads to an underestimation of endogenous nitrogen

losses due to a decrease in protein synthesis by glandular tissues of the GIT (Millward *et al.*, 1976) and the diminution of proteinaceous secretions into the gut lumen (Hodgkinson and Moughan, 2003). As a result alternative methods to measure endogenous nitrogen and amino acid losses at the terminal ileum, which involve feeding protein-containing diets, have been developed (Moughan, 2003; Moughan *et al.*, 1998; Stein *et al.*, 2007; White and Ashes, 1999). Accurate determinations of endogenous total nitrogen and amino acid flows are necessary to allow the determination of true dietary amino acid digestibility coefficients and for the factorial estimation of dietary amino acid requirements (Moughan *et al.*, 2005). Although there are several published estimates of the amino acid composition of human ileal digesta (Moughan and Rutherford, 2011) there are no systematic studies of the protein composition (i.e. sources of protein) of ileal digesta in humans. Such a study has been recently undertaken in the growing pig (Miner-Williams *et al.*, 2009b).

The aim of this study was to quantify the endogenous protein components of terminal ileal digesta collected from humans given a diet containing casein, a protein known to be highly digested and absorbed. Although small amounts of dietary peptides and amino acids may remain unabsorbed when casein is fed to an experimental subject, these were assumed to be minimal and to have no bearing on the overall results, as the respective constituent proteins were determined directly.

Materials and Methods

Subjects and Diets.

Six adult subjects (four female and two male) were involved in the study. The mean age of the subjects was 35.7 (\pm 6.6) years, with a mean bodyweight of 62.5 (\pm 12.6) kilograms and a mean body mass index being 22.1 (\pm 4). Their inclusion in the study followed a thorough medical examination that included routine blood tests. All of the subjects received detailed information of the experimental protocol and consequently gave their informed consent to participate in the study. The study was conducted at the Avicenne Hospital, Bobigny, France and the protocol was approved by the

Institutional Review Board of St- Germain-en-Laye Hospital, France. The volunteers were admitted to the hospital in the morning of day 1, when a 3 m polyvinyl chloride (PVC) triple-lumen tube was inserted via the nose under local anaesthesia. At the distal end of the triple lumen tube a 2mm diameter tube was pierced with 2 mm holes for a length of 10 cm and utilized for the collection of digesta. A second radio-opaque tube terminated 20cm from the digesta collection zone and was used to perfuse a flow rate marker. A final inner tube was then utilized to inflate a balloon at the terminal end of the tube 5 cm below the digesta collection zone. Once past the pyloric sphincter a terminal balloon was inflated to stimulate peristalsis and hasten the progression of the tube through the gut. Once it had reached the terminal ileum the tube was restrained from further movement. The subjects were then given a standard easily digested hospital meal and received a second meal at 19.00 hours before being fasted overnight. The experimental protocol commenced at 09.30 hours on Day 2. To enable base-line data to be obtained digesta were first collected 30 minutes before the ingestion of the test meal. A saline solution, containing polyethylene glycol PEG-4000 (20 g/l) as a liquid-phase marker, was then infused continuously through one of the tube's lumens at a constant rate of 1 ml/min for the duration of the test. The composition of the test meal (g/kg air dry weight) was: 220 g casein; 470 g maltodextrin; 157 g sucrose and 153.3 soya bean oil. No vitamins, minerals or fibre were added to this diet, which was fed acutely, as the same diet was used in a previous pig trial (Miner-Williams *et al.*, 2009a). The test meal was given at 10.00 hours and consisted of 150 g of the test diet (containing 33 g of casein), which was then made up to a final volume of 550 mL with water. The total metabolizable energy content of each test meal was 700 kcal (2.93 MJ).

The collection of digesta was undertaken while the subjects were resting in a semi-recumbent position and no further food was allowed until the end of the collection period, which lasted for the 8 hours following the ingestion of the meal. Water was given to the subject hourly. Digesta were collected on ice and pooled over 30 minute periods; following the addition of the antiprotease di-isopropylfluorophosphate (1 mmol/l) and sodium benzoate (10g•kg⁻¹ of digesta) as a bactericide, they were frozen immediately. Later each 30 minute digesta sample was freeze-dried, ground and mixed.

Chemical Analysis.

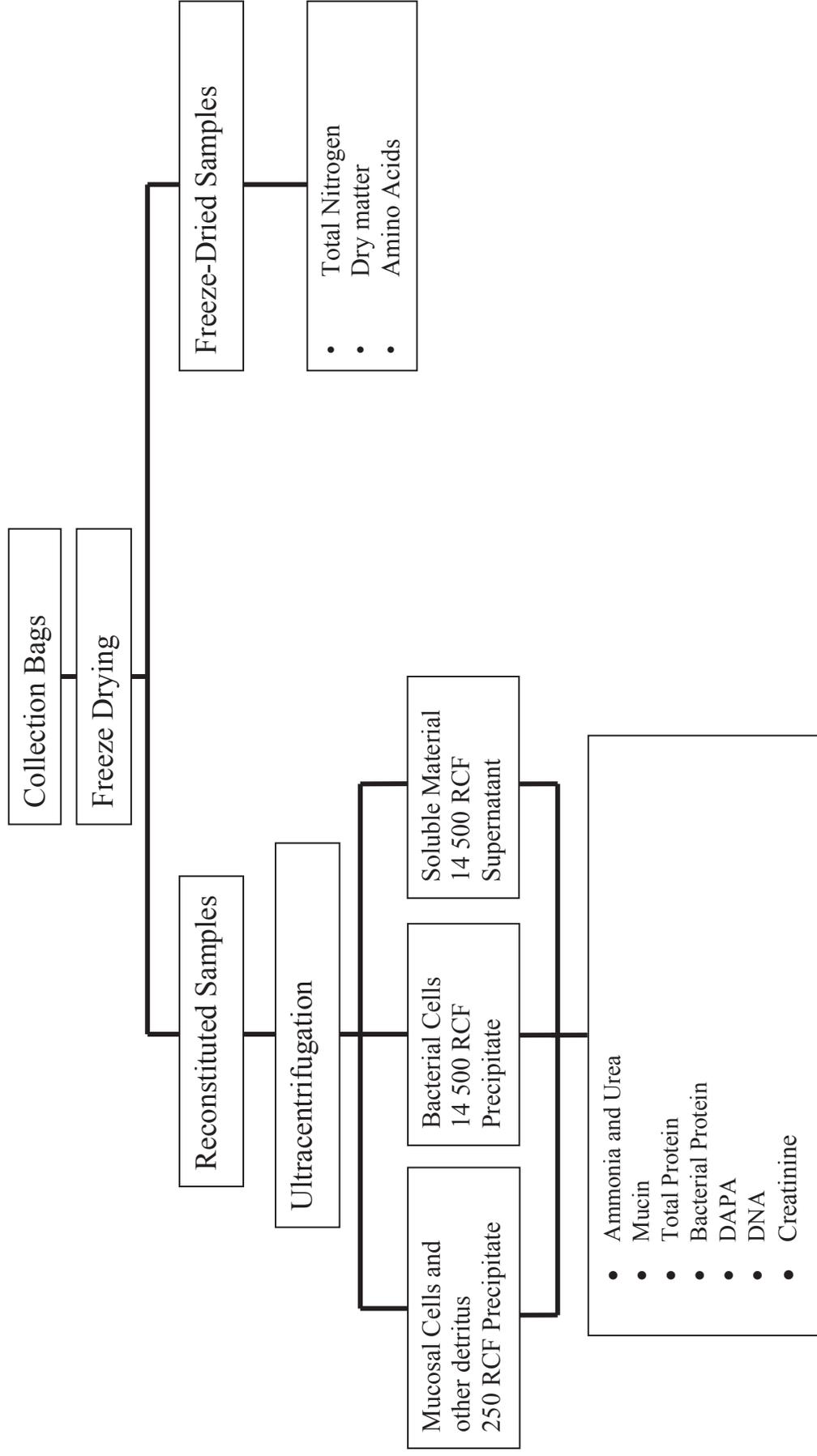
All analyses were undertaken in triplicate. Amino acids were determined on the freeze dried digesta samples (Deglaire *et al.*, 2009) after acid hydrolysis for 24 hours with hydrochloric acid (6 mol/l containing 0.1% phenol) using a Waters ion exchange HPLC (AOAC, 2003). Tryptophan and cysteine were not determined. Other nitrogen containing components of the ileal digesta were determined as described previously (Miner-Williams *et al.*, 2009b). Briefly samples of freeze dried digesta were reconstituted in normal saline and fractionated by differential centrifugation using the method of Metges *et al.* (1999) (**Figure 1**). Digesta were first centrifuged at 250 RCF for 15 minutes at 4°C, then at 14500 RCF for 30 minutes at 4°C. The first precipitate at 250 RCF was expected to contain food particles and mucosal cells. The second precipitate at 14500 RCF was expected to contain microbial cells with the supernatant expected to contain soluble nitrogenous components such as: proteins, peptides, free amino acids, mucins, neutral sugars, urea, creatinine, and ammonia.

Total nitrogen was determined by the Leco total combustion method a variation of the Dumas method (AOAC, 2000). Diaminopimelic acid (DAPA) was quantified, following oxidation with performic acid, using an HPLC system with a UV detector. Soluble protein was determined by the Bradford method (1976). Bacterial protein in the 14500 RCF precipitate was determined using a bacterial protein extraction lysis buffer (PELB) kit combined with a Noninterfering Protein Assay Kit (NPA), both obtained from G-Biosciences, St Louis, MO. Following the manufacturer's instructions total protein in the 14500 RCF precipitate was determined using the PELB kit to lyse the bacterial cells before using an NPA kit. Extracellular protein was then determined for the same quantity of precipitate without the initial lysis step. The difference between the resultant concentrations was considered to be the bacterial protein concentration.

Mucins were quantified by the determination of the amino sugars N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GlcNAc), carbohydrate markers peculiar to glycoproteins.

Figure 1 Schematic diagram of the processing of the digesta samples.

Digesta Samples



Ammonia and urea were determined using an adaptation of the method devised by Chaney and Marbach (1962). DNA was determined using a QIAamp DNA Stool Mini Kit obtained from QIAGEN Inc., Valencia, CA according to the manufacturer's instructions. The absorbance of the extracted samples was determined using a NanoDrop ND-1000 UV-vis Spectrophotometer (NanoDrop Technologies Wilmington, DE 19810). The DNA concentration was determined from its absorbance at A260 nm and its purity from the ratio of the readings at A260 nm and A280 nm.

Data Analysis.

The total ileal component flows were determined in the timed (30 minute) samples or pooled freeze-dried ileal digesta using the following equation:

$$\text{TICF} = C_{\text{digesta}(T)} \times \text{DM}_{\text{s}(T)} \times F_{\text{tot}(T)}$$

where TICF represents the total ileal component flow (mg); $C_{\text{digesta}(T)}$ represents the component concentration in the terminal ileal digesta for the given time period, (mg/g DM); $\text{DM}_{\text{s}(T)}$ represents the dry matter concentration in the sample for time T (mg/g digesta) and $F_{\text{tot}(T)}$ represents the total ileal digesta flow over time T (mL).

$F_{\text{tot}(T)}$ was determined using the following equation:

$$F_{\text{tot}(T)} = (\text{PEG}_i / \text{PEG}_{\text{s}(T)}) \times F_i \times T$$

where PEG_i represents the concentration of the indigestible marker (PEG) in the solution perfused and $\text{PEG}_{\text{s}(T)}$ is the PEG concentration in the sample of ileal digesta (g/L); F_i represents the PEG infusion rate (mL/minute) and T represents the sampling time period (minutes).

Native digesta dry matter (NDDM) was determined using the following equation:

$$\text{NDDM} = \text{DM}_{s-(T)} \times F_{\text{tot}-(T)} - \text{PEG}_i \times F_i \times T$$

where NDDM represents the dry matter after excluding the DM of the PEG infused in the time period T (g).

Results were expressed as means \pm standard error of the mean. Statistical analysis was undertaken using Minitab 15 (Minitab Pty Ltd, Sydney NSW Australia).

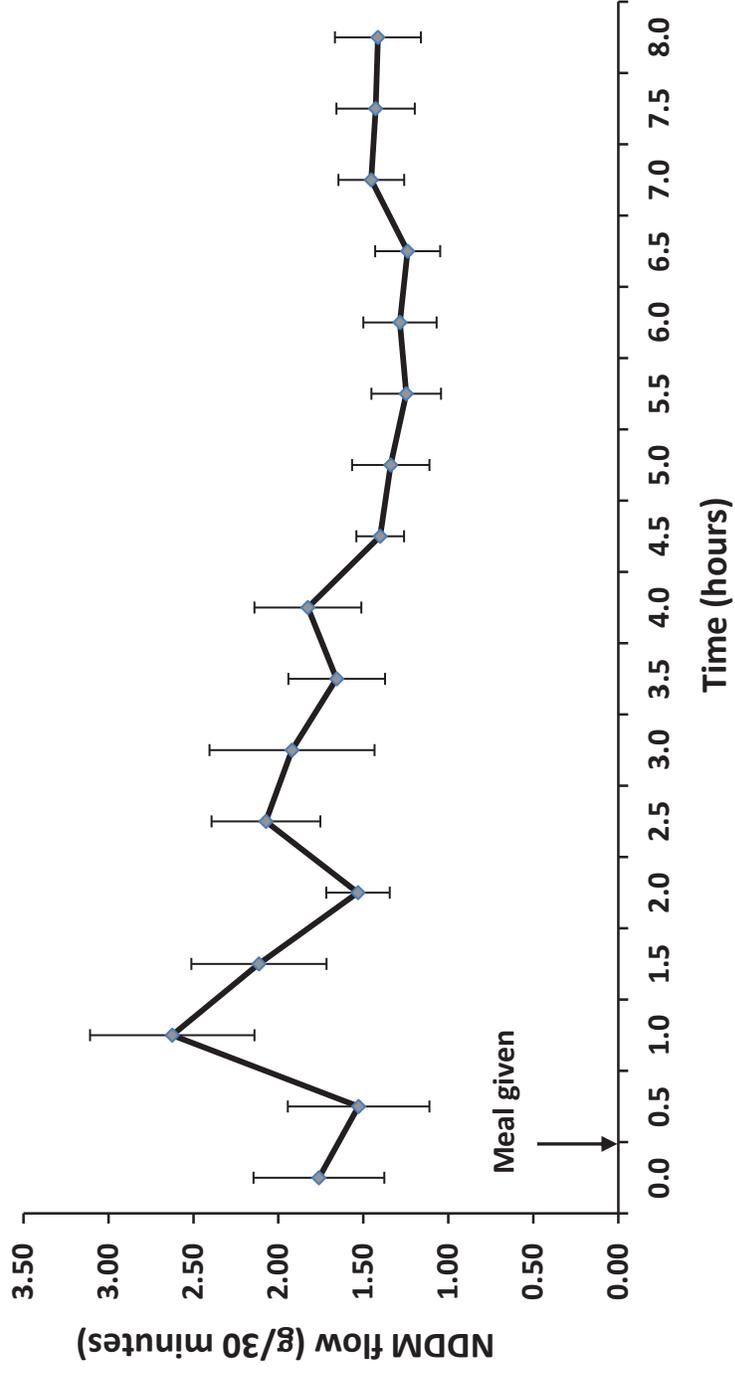
Results

The flow of native digesta dry matter (NDDM) is presented in **Figure 2** and shows that following a peak flow of 2.6 g/30 min during the period between 30-60 minutes the flow declines to a more constant and surprisingly low rate of \sim 1.3g/30 min. The mean NDDM flow in the ileal effluent over the 8 hour collection period for the 6 subjects was 1.73g/min (\pm 0.13 SEM). The total NDDM concentration was 29.3g/L (\pm 2.5 SEM).

The nitrogen composition of the digesta is given in **Table 1**. Total nitrogen was 3.9% of NDDM. Proteinaceous nitrogen was 86% of the total nitrogen, and non-protein nitrogen the remaining 14%. Most (77%) of the protein nitrogen was soluble and present in the 14500 RCF supernatant leaving the remaining 23% insoluble protein nitrogen being detected in the 250 RCF and 14500 RCF precipitates.

The amino acid profile of the human ileal digesta is presented in **Table 2** as $\text{mg} \cdot \text{g}^{-1}$ NDDM and $\text{mg} \cdot \text{g}^{-1}$ total nitrogen. The concentrations of both DAPA and DNA in the three centrifugation fractions were used to determine the proportion and distribution of bacterial and human protein, these data are given in **Table 3**. The distribution of protein across the different centrifuge fractions is given in **Table 4**. Just over 69% of the protein detected in the ileal digesta was of bacterial origin.

Figure 2 Changes in the mean flow of NDDM ($\text{g} \cdot 30 \text{ minutes}^{-1}$) over the 8 hour collection period with human subjects.



— Plot of mean NDDM values, error bars are \pm standard error of the mean.
Abbreviations: NDDM native digesta dry matter.

Table 1 Digesta nitrogen content of terminal ileal digesta for human subjects given a casein-based diet.

	$\text{mg}\cdot\text{g}^{-1}$ NDDM ^a		Percentage
	Mean	SEM ^b	
Total Digesta Nitrogen	39.3	2.05	100
Protein Nitrogen	34.0	3.21	86.4
Non-protein Nitrogen	5.3	0.55	13.6
Soluble Protein Nitrogen	26.2	1.60	77.0 ^c .
Insoluble Protein Nitrogen	7.8	0.77	23.0 ^c .

^a NDDM = native digesta dry matter. ^b SEM = standard error of the mean.

^c Percentage values of Total Protein Nitrogen.

The greatest concentration of protein (53% of total protein) was detected in the 14500 RCF supernatant, nearly 70% of which was bacterial.

The mucin output was determined as being $127 \text{ mg}\cdot\text{g}^{-1}$ NDDM (**Table 5**) and nearly 13% of the digesta dry matter. The change in mucin concentration in the digesta throughout the collection period ($\text{mg}\cdot\text{g}^{-1}$ NDDM every 30 minutes) is presented in **Figure 3**. A summary of the sources and the concentrations of nitrogen in terminal ileal digesta of humans given a casein based diet are presented in **Table 6**. Nearly 60% of the nitrogen in the ileal effluent was determined to be of bacterial origin with mucin contributing nearly 15% of the nitrogen and was the single most abundant truly endogenous component found in the GIT contents.

Table 2 Amino Acid Flow Composition of Terminal Ileal Digesta^a

Amino Acid	mg•g ⁻¹ Nitrogen	SEM ^b	mg•g ⁻¹ NDDM ^c	SEM ^b
Aspartic acid ^d	460.0	24.13	17.3	1.93
Threonine ^d	265.0	9.36	10.0	1.06
Serine ^d	356.9	18.55	13.4	1.50
Glutamic acid ^d	642.0	43.98	24.2	3.03
Proline ^d	274.6	10.41	10.3	1.03
Glycine ^d	522.5	52.34	19.5	2.56
Alanine	177.0	7.88	6.6	0.72
Valine	267.8	12.50	9.9	1.04
Methionine	54.4	2.51	2.0	0.24
Isoleucine	217.4	9.18	8.1	0.82
Leucine	256.9	10.46	9.6	1.00
Tyrosine	175.2	7.79	6.6	0.71
Phenylalanine	157.1	7.60	5.9	0.63
Histidine	152.3	3.84	5.7	0.57
Lysine	170.4	13.57	6.4	0.81
Arginine	149.6	10.76	5.6	0.69

These data were previously published in the study by Deglaire *et al.* (2009) as total amino acid flows over the entire 8 hour collection period. ^aTryptophan was not determined. ^bSEM = standard error of mean. ^cNDDM = native digesta dry matter. ^dpredominant amino acids in mucin.

Table 3 Concentrations of DAPA and DNA in the terminal ileal digesta.

	DAPA ^a		DNA ^c mg·g ⁻¹ NDDM		
	mg·g ⁻¹ NDDM ^b		total	bacterial cellular	human cellular ^g
Centrifugation fraction					
250 RCF precipitate	0.03		0.35	0.07	0.28
14500 RCF precipitate	0.26		0.59	0.59 ^e	
14500 RCF supernatant	0.33		0.82	0.76 ^f	0.06
Total	0.62 ^d		1.35 ^d	1.42	0.34

^a DAPA Diaminopimelic acid. ^b NDDM native digesta dry matter. ^c DNA deoxyribonucleic acid.

^d These totals represent the DAPA and DNA detected in the ileal digesta.

^e It was assumed that all of the DNA detected in the 14500 precipitate was bacterial in origin. A DNA/DAPA ratio of 2.27 was calculated (when expressed as mg·g⁻¹ DDM), and used to estimate the bacterial DNA in the other two centrifugation fractions.

^f The concentration of bacterial DNA in this fraction was assumed to originate from lysed bacterial cells and was determined from the DNA/DAPA ratio of 2.27 (see ^b above).

^g Cellular DNA was determined as being the difference between the total DNA detected and estimated bacterial DNA.

Discussion

The method of collecting ileal digesta from fit healthy subjects, using a naso-ileal tube, was chosen after a meta-analysis study confirmed that this method allowed precise and accurate sampling (Deglaire, 2008). Although total digesta collection is not possible with naso-ileal intubation determining the recovery of a perfused non-digestible marker (polyethylene glycol 4000) allows the precise and accurate sampling of digesta.

Table 4 Distribution of microbial and non-microbial protein within the different centrifugation fractions of ileal digesta.

	mg·g ⁻¹ NDDM ^a	SEM ^b	% of total protein ^e
total protein	306.7	11.42	100.0
total microbial protein ^c	212.6	10.31	69.3
total non-microbial protein	94.1	4.26	30.7
250 RCF Precipitate			
protein	31.3	2.65	10.2
microbial protein	9.9	4.85	3.2
human protein	21.4	1.69	7.0
14500 RCF Precipitate			
Protein ^d	112.9	5.37	36.8
microbial protein	89.0	4.51	29.0
non-microbial protein	23.9	1.66	7.8
14500 RCF Supernatant			
protein	162.6	7.42	53.0
microbial protein	113.7	5.38	37.1
non-microbial protein	48.9	1.67	15.9

^a NDDM= native digesta dry matter. ^b SEM = standard error of the mean. ^c Microbial protein was determined using the value of 26.4 mg DAPA/g of bacterial nitrogen. ^d Protein in this fraction was estimated using the bacterial protein extraction lysis buffer (PELB), kit combined with a non-interfering protein assay kit, obtained from G-Biosciences. ^e Percentages of total protein calculated from protein values expressed in mg·g⁻¹ NDDM.

Table 5 Mean concentrations of the amino sugars N-Acetylgalactosamine and N-Acetylglucosamine and the concentration of mucin in terminal ileal digesta.

		Mean	SEM ^a
N-Acetylgalactosamine	mg•g ⁻¹ NDDM ^b	10.5	1.36
N-Acetylglucosamine	mg•g ⁻¹ NDDM	25.5	3.42
Mucin Outputc	mg•g ⁻¹ NDDM	127.0	11.08
GluNAc/GalNAc ratio		2.4	
Mucin Nitrogen	g•100g ⁻¹ digesta total nitrogen	22.5	1.86

^a SEM = standard error of the mean. ^b NDDM = native digesta dry matter.

Collecting digesta from ileostomy effluent was rejected because normal digestive function may be altered by the primary pathological condition that resulted in the patient's colectomy, and it is known that colectomy may induce adaptations in a number of physiological and metabolic processes (Christl and Scheppach, 1997; Stern *et al.*, 1999). In addition, given the elevated population of ileal microorganisms associated with ileostomy, the digesta collected from ileostomates may have a composition very different from that of subjects with an intact digestive system (Christl and Scheppach, 1997; Fuller *et al.*, 1994).

The PEG recovered in the digesta collected over the 8-hour period was 55.6 % of that infused (Deglaire, 2008), indicating that a relatively large part of the total ileal digesta had been collected, thus ensuring that the samples were representative of the total digesta leaving the ileum.

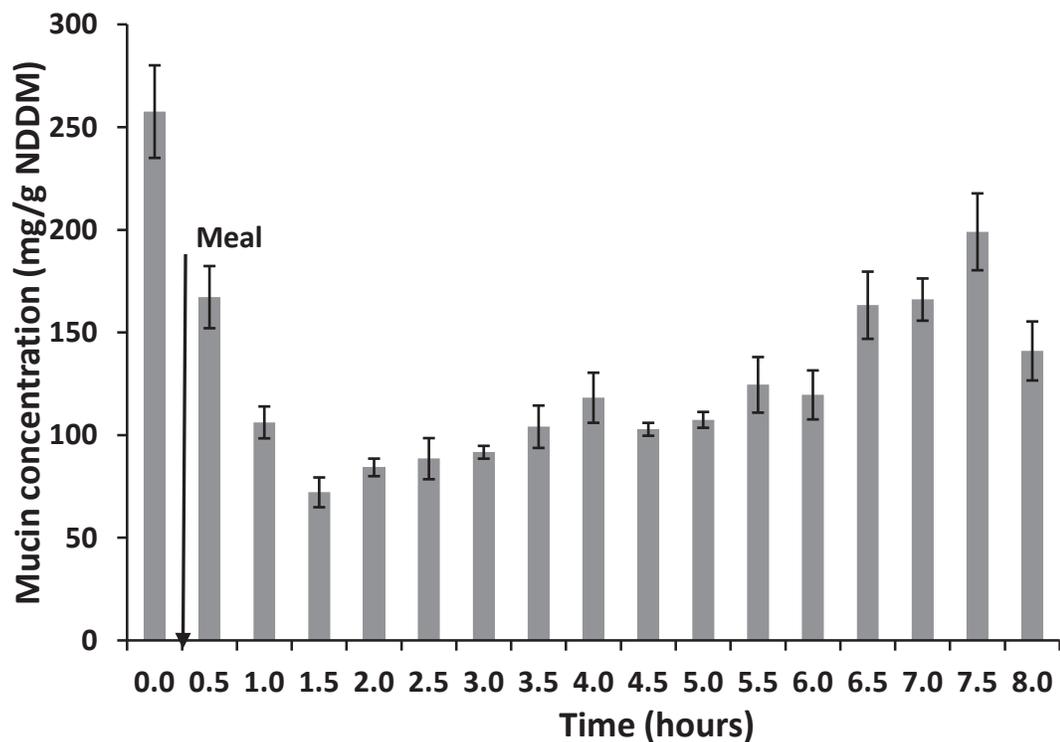
Casein, a protein known to be almost completely digested and absorbed (Eklund *et al.*, 2008; Kies *et al.*, 1986), was used in this experiment so that most of the soluble protein found in the ileal digesta should have been of non-dietary (endogenous or bacterial) origin. The true digestibility of casein has been estimated by Deglaire *et al.* (2009) to

be in excess of 97%. The study allowed the direct determination of the proteinaceous components in the terminal ileal digesta of subjects fed a single purified dietary protein.

When the nitrogen composition of the digesta (Table 1) is compared to the results of an earlier pig study, in which the animals were fed the same-semi-purified casein-based diet (Miner-Williams *et al.*, 2009a) the total nitrogen content of the digesta was higher in the pig than the value found here for humans (6.4% and 3.9% respectively).

However, when the percentage composition is compared the results are more similar, the protein nitrogen as a percentage of total nitrogen being 74% in the pig and nearly 86% in the human.

Figure 3 Changes in the mean digesta mucin concentration ($\text{g} \cdot 30 \text{ minutes}^{-1}$) over the 8 hour collection period with human subjects.



Plot of mean mucin values, error bars are \pm standard error of the mean

Abbreviations: NDDM, native digesta dry matter; min, minutes.

Table 6 Summary of the sources of nitrogen in terminal ileal digesta of humans given a casein based diet.

Source of Nitrogen	mg•g ⁻¹ NDDM ^a	SEM ^b	Percentage of total protein nitrogen	Percentage of nonbacterial protein nitrogen	Percentage of total nitrogen
Protein					
Bacteria	23.5 ^{c,d}	1.19	69.2		59.9
Mucin	5.8	0.20	16.9	55.0	14.6
Soluble Free Protein ^e	2.6	0.40	7.6	24.9	6.6
Mucosal Cells	2.1	0.07	6.2	20.2	5.4
Non-protein					
DNA	0.2	0.03			0.5
Urea	1.6	0.13			3.9
Ammonia	1.9	0.16			4.7
Creatinine	0.12	0.01			0.3
Non-specific ^f	1.6	0.04			4.1
Total N	39.3	2.07			100

^a NDDM native digesta dry matter.

^b SEM standard error of the mean.

^c Equivalent to 26.4 mg DAPA/g bacterial nitrogen.

^d Bacterial nitrogen was principally determined from the 14500 RCF with corrections for DAPA found in other centrifugation fractions.

^e Soluble free protein was determined from the 14500 RCF supernatant using the Bradford reagent and may include albumin, immunoglobulins, digestive enzymes, and small peptides such as digestion resistant bioactive peptides and soluble cell debris.

^f This fraction contains nonidentified nitrogenous material that may include nonprotein compounds such as free amino acids, RNAs, amines, and the tetrapyrroles, bilirubin and biliverdin.

The soluble protein nitrogen, expressed as a percentage of total nitrogen, was also similar, 61% in the pig and 66% in the human. As the dietary nitrogen, derived from casein, was similar for both the pig and the human (211 and 220g/kg air dry weight respectively) such a difference in total nitrogen is surprising. However, the reason for this difference may be related to the human subjects receiving a considerably lower absolute amount of casein. The amount of casein given to the human subjects in the test meal was a quarter of that in the test meal given to the pig (33g and 121g for the human and pig respectively). This may have resulted in less undigested dietary protein being present in the terminal ileal digesta collected from the human subjects.

In the present study the six most abundant amino acids detected in the digesta, Table 2 (in decreasing order of abundance, $\text{mg}\cdot\text{g}^{-1}$ NDDM) were: glutamic acid; glycine; aspartic acid; serine; proline and threonine. This results compare well with a previous study showing in human ileal effluents high concentrations for glycine, proline, aspartate/asparagine, threonine, serine, and alanine (Gaudichon *et al.*, 2002; Moughan and Rutherford, 2011; Moughan, 2003).

It also agrees with an earlier pig study, when a similar amount of purified casein was included in the test meal (Miner-Williams *et al.*, 2009b). Threonine, serine and proline are most abundant in the glycosylated region of mucin polymers that holds the great majority of these three amino acids. Glutamic acid, glycine and aspartic acid are most common in the nonglycosylated regions of mucin. In our earlier pig study these six amino acids accounted for almost 52% of the total mass of amino acids, and in this study the proportion was somewhat higher, almost 59%. The high proportion of these six amino acids is consistent with the high rates of mucin secretion throughout the GIT and, the minimal rate of degradation of these macro-molecules. A high concentration of the mucin associated amino acids together with the high proportion of protein nitrogen found in the 14500 RCF supernatant in this study (77%), would indicate that a high proportion of soluble mucin is present in this fraction; an observation consistent with our earlier findings. It is interesting to note that the mean concentration of lysine in the human ileal digesta collected over 8 hours was $6.38 \text{ mg}\cdot\text{g}^{-1}$ NDDM which equates to nearly 4 % of the total amino acids present, a figure consistent with that (4.75%) reported by Metges *et al.* (1999) for ileal microbial protein collected from human ileostomates. However, it is only half that of endogenous cellular protein

determined by Munro and Fleck (1970) suggesting that there was a high proportion of bacterial and other non-mucosal cell proteins in the ileal digesta.

The method of differential centrifugation used in this study allowed us to expect that most of the whole bacterial cells would be present in the 14500 RCF precipitate. Microscopic investigation of the three centrifugation fractions confirmed this and although some bacterial cells were present in the 250 RCF precipitate and the 14500 RCF supernatant the great majority of whole bacterial cells was found in the 14500 RCF precipitate. The concentration of DAPA in the respective fractions (**Table 3**) did not entirely concur with these observations as a relatively high concentration of DAPA was found in the 14500 RCF supernatant. However, as many fewer intact bacterial cells were observed visually in the 14500 RCF supernatant it was assumed that this was free DAPA from lysed bacterial cells. The total concentrations of DAPA and DAPA nitrogen were determined to be 0.62 and 0.09 mg·g⁻¹ NDDM respectively, values similar to those found in our previous work with pigs (0.63 and 0.09 respectively). The mass of bacterial dry matter was calculated assuming 2.88 mg of DAPA per gram of bacterial dry matter (Czerkawski, 1974) and determined as 215 mg·g⁻¹ NDDM, a figure that represents almost 22% of the total digesta dry matter, a value similar to 208 mg·g⁻¹ determined by Rowan *et al.* (1992).

The DNA found in the 14500 RCF supernatant was assumed to be derived from lysed bacterial and human cells as inspection using light microscopy confirmed that the number of intact cells in this fraction was negligible. Protein interference was found to be minimal when using the QIAmp DNA Stool Mini Kit as the extraction of DNA was particularly pure.

The concentrations of DNA in each of the centrifugation fractions are also given in Table 3. The total amount of DNA detected in the human ileal digesta was 1.76 mg·g⁻¹ NDDM, a result consistent with the pig studies of Rowan *et al.* (1992) and Miner-Williams *et al.* (2009b) If the concentration of DNA present in the 14500 RCF precipitate (0.59 mg·g⁻¹ NDDM), is assumed to be entirely of bacterial origin and the DAPA concentration for this fraction was 0.26 mg·g⁻¹ NDDM then the DNA/DAPA ratio can be calculated to be 2.27 and similar to that (2.86) reported by Rowan *et al.* (1994) in humans. This ratio can then be used to determine the bacterial DNA in the

remaining two centrifugation fractions (0.07 and 0.76 mg bacterial DNA/g⁻¹ NDDM for the 250 RCF precipitate and 14500 RCF supernatant respectively). Human cellular material was thus determined as the difference between the total DNA and the estimated bacterial DNA. As in our earlier pig study (Miner-Williams *et al.*, 2009b) the higher proportion of human DNA in the 250RCF precipitate supports the effectiveness of using differential centrifugation for the separation of human and bacterial cellular material from soluble components in the ileal digesta (Miner-Williams *et al.*, 2009a).

The proportions of microbial and nonmicrobial protein were calculated using the DAPA/bacterial nitrogen ratio of 26.4 reported by Wünsche *et al.* (Wunsche *et al.*, 1991). The proportions of microbial and nonmicrobial protein are presented in **Table 4**. The percentage of microbial protein (69%) seems to be relatively high when compared to that detected in the ileal digesta taken from pigs (55%), fed a similar diet containing casein as the sole dietary protein, reported in our earlier study (Miner-Williams *et al.*, 2009b). The reason for this is not clear. A greater amount of the microbial protein was found in the 14500 RCF supernatant (~37% as opposed to 29% in the 14500 RCF precipitate), suggesting that substantial degradation of bacterial cells takes place in the small intestine.

The concentration of mucin in the ileal effluent was calculated using the mucin markers N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GluNAc). The concentration of these two amino sugars together with the GluNAc/GalNAc ratio, and the calculated mean mucin concentration are presented in **Table 5**. The predominant types of mucin found in the ileal effluent are gastric and small intestinal mucin. Knowing the ratio of these two amino sugars in gastric and intestinal secretions, Lien *et al.* (1997) used regression equations to estimate the proportions of these two types of mucin in the ileal effluent of pigs fed a protein free diet. However, the proportions of the two amino sugars are different in the human and with a GluNAc/GalNAc ratio of ~2.4 detected in the human ileal digesta in this study, it was not possible to calculate the proportions of the two types of mucin as we did in our earlier work (Miner-Williams *et al.*, 2009a). The concentration of digesta mucin detected here lies within the range of other published values (Lien *et al.*, 1996; Montagne *et al.*, 2004). Mucin present in the human ileal digesta represents nearly 13% of the digesta dry matter and

is thus the single most abundant truly endogenous component found in the GIT contents and a figure almost identical to that found in our earlier study with the pig.

It is known that the insertion of a naso-ileal tube affects gastric emptying (Fone *et al.*, 1991; Medhus *et al.*, 1999) increases intestinal transit time (Read *et al.*, 1983) and can stimulate intestinal secretions (Hendrix and Bayless, 1970; Read *et al.*, 1983).

However, as the naso-ileal tube had been inserted into the gut of the experimental subjects approximately 24 hours previous to the commencement of the test it was assumed that any mechanical stimulation of the gut caused by the insertion of the tube would have diminished and that the luminal conditions of the GIT had once again reached a normal equilibrium. However, it is interesting to note that the digesta mucin concentration fell by nearly 57% in the 90 minutes of collection after the test meal was administered. Such a high mucin concentration in the 30 minutes before the meal was given may be due to the infusion of the saline solution containing polyethylene glycol, and then as the GIT recovers the mucin concentration continues to fall until it begins to rise after 90 minutes. Such a high level of mucin at the start of the study period may affect the measurements of the other parameters such as protein and nitrogen. The gradual rise in mucin concentration at the terminal ileum between 1.5-8 hours likely reflects the passage of the meal and its degradation products together with the mucins secreted by the stomach and the small intestine, that are stimulated by its passage along the GIT, until the spike at 7.5 hours when the digesta bolus reaches the terminal ileum and the mucin level then begins to fall between 7.5 hours and the end of the collection period. Had the resulting mucin pattern been known prior to the trial the collection period should have been extended by one hour to ensure that the mucin levels were returning to a normal level.

The mean concentrations (\pm standard error of the mean) of ammonia, urea and creatinine in the digesta were 2.27 (0.31), 3.31 (0.30), and 0.31 (0.01) $\text{mg}\cdot\text{g}^{-1}$ NDDM respectively. These three compounds made up 66% of the non-protein nitrogen (**Table 6**). Although urea and ammonia made up only ~0.4% of the digesta dry matter these components of the digesta contribute over 9% of the total digesta nitrogen, a disproportionate amount of nitrogen. Urea, ammonia and the metabolic activity of intestinal bacteria are linked to nitrogen cycling systems in the gut. Both the pig and human small intestine, once thought to be largely devoid of microbial activity (Hill,

1982), are now known to be colonised with large populations of bacteria (Leser *et al.*, 2002; Turrone *et al.*, 2008). Indeed antimicrobial peptides secreted by the paneth cells within the crypts of the small intestine protect the underlying mucosa, while still allowing the presence of an enteric microbiota (Meyer-Hoffert *et al.*, 2008). Although the microbial populations of the large intestine are greater than that of the small intestine, Ahmed *et al.* (2007) revealed that bacterial population densities in the small intestine were highest in the terminal ileum. The microbiota of the human gut represents a complex microbial community which is believed to have a significant impact on human physiology (Turrone *et al.*, 2008).

In summary the loss of proteinaceous nitrogen from both microbial and endogenous sources was over 86% of total ileal nitrogen losses (Table 1), a value in accord with the estimates by Chacko and Cummings (1988) who gave a range of 80-85%. The fraction of non-specific protein present in the 14500 RCF supernatant was 2.6 mg·g⁻¹ NDDM an amount that contributed 7.6% of the total nitrogen at the terminal ileum (see **Table 7**), a not insignificant amount that would be expected to contain immunoglobulins, digestive enzymes, serum albumin, small protease-resistant peptides and undigested dietary protein fractions.

Acknowledgements

I would like to offer my thanks to Dr Amélie Deglaire for her assistance with the acquisition of samples from the human subjects and Dr Robert Benamouzig for the installation of the naso-ileal tubes at the start of the human trial.

Literature cited

- Ahmed, S., Macfarlane, G. T., Fite, A., McBain, A. J., Gilbert, P., and Macfarlane, S. (2007). Mucosa-associated bacterial diversity in relation to human terminal ileum and colonic biopsy samples. *Applied and Environmental Microbiology*, 73(22), 7435-7442.
- AOAC. (2000). Official Method for protein (Dumas method), 968.06. *Official methods of analysis of association of official analytical chemists international*.
- AOAC. (2003). Official Method of Analysis for Amino Acids *Official methods of analysis of association of official analytical chemists international* (Vol. 17th Edition). Gaithersburg MD: Association of Official Analytical Chemists International.
- Bradford, M. M. (1976). Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Analytical Biochemistry*, 72(1-2), 248-254.
- Caine, W. R., Tamminga, S., Sauer, W. C., Verstegen, M. W. A., and Schulze, H. (1999). Bacterial contributions to total and endogenous recoveries of nitrogen and amino acids in ileal digests of newly weaned piglets fed protease-treated soybean meal. *Livestock Production Science*, 57(2), 147-157.
- Chacko, A., and Cummings, J. H. (1988). Nitrogen losses from the human small bowel: obligatory losses and the effect of physical form of food. *Gut*, 29, 809–815.
- Chaney, A. L., and Marbach, E. P. (1962). Modified reagents for determination of urea and ammonia. *Clinical Chemistry*, 8(2), 130-and.
- Christl, S. U., and Scheppach, W. (1997). Metabolic consequences of total colectomy. *Scandinavian Journal of Gastroenterology*, 32, 20-24.
- Corring, T., and Saucier, R. (1972). Pancreatic-secretion of fistulated pig - adaptation to protein content in diet. *Annales De Biologie Animale Biochimie Biophysique*, 12(2), 233-241.
- Czerkawski, J. W. (1974). Methods for determining 2-6-diaminopimelic acid and 2-aminoethylphosphonic acid in gut contents. *Journal of the Science of Food and Agriculture*, 25(1), 45-55.

- Deglaire, A. (2008). *Gut endogenous protein flows and post prandial metabolic utilization of dietary amino acids in simple stomached animals and humans*. Doctor of Philosophy Doctor of Philosophy, PhD Thesis, Massey University, Palmerston North.
- Deglaire, A., Bos, C., Tomé, D., and Moughan, P. J. (2009). Ileal digestibility of dietary protein in the growing pig and adult human. *British Journal of Nutrition*, 102(12), 1752-1759.
- Eklund, M., Mosenthin, R., Piepho, H. P., and Rademacher, M. (2008). Effect of dietary crude protein level on basal ileal endogenous losses and standardized ileal digestibilities of crude protein and amino acids in newly weaned pigs. *Journal of Animal Physiology and Animal Nutrition*, 92(5), 578-590.
- Fone, D. R., Horowitz, M., Heddle, R., Maddox, A. F., Collins, P. J., Read, N. W., et al. (1991). Comparative effects of duodenal and ileal intubation on gastric-emptying and postprandial antral, pyloric, and duodenal motility. *Scandinavian Journal of Gastroenterology*, 26(1), 16-22.
- Fuller, M. F., Milne, A., Harris, C. I., Reid, T. M. S., and Keenan, R. (1994). Amino-acid losses in ileostomy fluid on a protein-free diet. *American Journal of Clinical Nutrition*, 59(1), 70-73.
- Gaudichon, C., Bos, C., Morens, C., Petzke, K. J., Mariotti, F., Everwand, J., et al. (2002). Ileal losses of nitrogen and amino acids in humans and their importance to the assessment of amino acid requirements. *Gastroenterology*, 123(1), 50-59.
- Hendrix, T. R., and Bayless, T. M. (1970). Digestion: intestinal secretion. *Annual Review of Physiology*, 32, 139-164.
- Hill, M. J. (1982). Gut flora associated diseases in man. *Advances in Veterinary Medicine*, 22, 32-38.
- Hodgkinson, S. M., and Moughan, P. J. (2003). The enzyme hydrolysed protein method for the determination of endogenous ileal nitrogen and amino acid flows - a modification. *Animal Feed Science and Technology*, 108(1-4), 207-214.
- Jansman, A. J. M., Smink, W., van Leeuwen, P., and Rademacher, M. (2002). Evaluation through literature data of the amount and amino acid composition of basal endogenous crude protein at the terminal ileum of pigs. *Animal Feed Science and Technology*, 98(1-2), 49-60.

- Kies, A. K., Moughan, P. J., and Smith, W. C. (1986). The apparent and true ileal digestibility of nitrogen and amino-acids in lactic casein for the growing-pig. *Animal Feed Science and Technology*, 16(3), 169-178.
- Krawielitzki, K., Kreienbring, F., Zebrowska, T., Schadereit, R., and Kowalczyk, J. (1994). Estimation of N absorption, secretion and reabsorption in different intestinal sections of growing pigs using the N-15 isotope dilution method. In W. B. Souffrant and H. Hagemester (Eds.), *VIth International Symposium on Digestive Physiology in Pigs, Proceedings, Vols 1 and 2* (pp. 79-82).
- Krawielitzki, K., Zebrowska, T., Schadereit, R., Kowalczyk, J., Hennig, U., Wunsche, J., et al. (1990). Determining of nitrogen absorption and nitrogen secretion in different sections of the pigs intestine by digesta exchange between N-15 labeled and unlabeled animals. *Archiv Fur Tierernahrung-Archives of Animal Nutrition*, 40(1-2), 25-37.
- Leibholz, J. (1982). The flow of endogenous nitrogen in the digestive-tract of young-pigs. *British Journal of Nutrition*, 48(3), 509-517.
- Leser, T. D., Amenuvor, J. Z., Jensen, T. K., Lindecrona, R. H., Boye, M., and Moller, K. (2002). Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. *Applied and Environmental Microbiology*, 68(2), 673-690.
- Lien, K. A., McBurney, M. I., Beyde, B. I., Thomson, A. B. R., and Sauer, W. C. (1996). Ileal recovery of nutrients and mucin in humans fed total enteral formulas supplemented with soy fiber. *American Journal of Clinical Nutrition*, 63(4), 584-595.
- Lien, K. A., Sauer, W. C., and Fenton, M. (1997). Mucin output in ileal digesta of pigs fed a protein-free diet. *Zeitschrift Fur Ernährungswissenschaft*, 36(2), 182-190.
- Low, A. G. (1980). Nutrient absorption in pigs. *Journal of the Science of Food and Agriculture*, 31(11), 1087-1130.
- Medhus, A. W., Sandstad, O., Bredesen, J., and Husebye, E. (1999). Delay of gastric emptying by duodenal intubation: sensitive measurement of gastric emptying by the paracetamol absorption test. *Alimentary Pharmacology and Therapeutics*, 13(5), 609-620.
- Metges, C. C., Petzke, K. J., El-Khoury, A. E., Henneman, L., Grant, I., Bedri, S., et al. (1999). Incorporation of urea and ammonia nitrogen into ileal and fecal

- microbial proteins and plasma free amino acids in normal men and ileostomates. *American Journal of Clinical Nutrition*, 70(6), 1046-1058.
- Meyer-Hoffert, U., Hornef, M. W., Henriques-Normark, B., Axelsson, L. G., Midtvedt, T., Putsep, K., *et al.* (2008). Secreted enteric antimicrobial activity localises to the mucus surface layer. *Gut*, 57(6), 764-771.
- Millward, D. J., Garlick, P. J., James, W. P. T., Sender, P. M., and Waterlow, J. C. (1976). Protein turnover. In D. J. A. Cole, K. N. Boorman, P. J. Buttery, D. Lewis, R. J. Neale and H. Swan (Eds.), *Protein Metabolism and Nutrition*. (Vol. 16, pp. 49-69.). London: European Association of Animal Production, Butterworths.
- Miner-Williams, W., Moughan, P. J., and Fuller, M. F. (2009a). Endogenous Components of Digesta Protein from the Terminal Ileum of Pigs Fed a Casein-Based Diet. *Journal of Agricultural and Food Chemistry*, 57(5), 2072-2078.
- Miner-Williams, W., Moughan, P. J., and Fuller, M. F. (2009b). Endogenous components of digesta protein from the terminal ileum of pigs fed a casein based diet. *Journal of Agricultural and Food Chemistry* 57, 2072-2078.
- Montagne, L., Piel, C., and Lalles, J. P. (2004). Effect of diet on mucin kinetics and composition: Nutrition and health implications. *Nutrition Reviews*, 62(3), 105-114.
- Moughan, J. P., and Rutherfurd, S. M. (2011). Gut luminal endogenous protein: Implications for the determination of ileal amino acid digestibility in humans. *British Journal of Nutrition (In Press)*.
- Moughan, P. J., C. A. Butts, *et al.* (2005). Dietary peptides increase endogenous amino acid losses from the gut in adults. *American Journal of Clinical Nutrition* 81(6): 1359-1365.
- Moughan, P. J. (2003). Amino acid availability: aspects of chemical analysis and bioassay methodology. *Nutrition Research Reviews*, 16(2), 127-141.
- Moughan, P. J., Souffrant, W. B., and Hodgkinson, S. M. (1998). Physiological approaches to determining gut endogenous amino acid flows in the mammal. *Archives of Animal Nutrition-Archiv Fur Tierernahrung*, 51(2-3), 237-252.
- Munro, H. N., and Fleck, A. (1970). Analysis of tissues and body fluids for nitrogenous constituents. In H. N. Munro (Ed.), *Mammalian Protein Metabolism* (Vol. III, pp. 423-525). New York: Academic.

- Nasset, E. S., and Ju, J. S. (1961). Mixture of endogenous and exogenous protein in the alimentary tract. *Journal of Nutrition*, 74, 461-465.
- Read, N. W., Aljanabi, M. N., Bates, T. E., and Barber, D. C. (1983). Effect of gastrointestinal intubation on the passage of a solid meal through the stomach and small-intestine in humans. *Gastroenterology*, 84(6), 1568-1572.
- Rowan, A. M., Moughan, P. J., and Wilson, M. N. (1992). The flows of deoxyribonucleic-acid and diaminopimelic acid and the digestibility of dietary fiber components at the terminal ileum, as indicators of microbial activity in the upper digestive-tract of ileostomized pigs. *Animal Feed Science and Technology*, 36(1-2), 129-141.
- Rowan, A. M., Moughan, P. J., Wilson, M. N., Maher, K., and Tasmanjones, C. (1994). Comparison of the ileal and fecal digestibility of dietary amino-acids in adult humans and evaluation of the pig as a model animal for digestion studies in man. *British Journal of Nutrition*, 71(1), 29-42.
- Sambrook, I. E. (1981). Studies on the flow and composition of bile in growing pigs. *Journal of the Science of Food and Agriculture*, 32(8), 781-791.
- Stein, H. H., Seve, B., Fuller, M. F., Moughan, P. J., and de Lange, C. F. M. (2007). Invited review: Amino acid bioavailability and digestibility in pig feed ingredients: Terminology and application. *Journal of Animal Science*, 85(1), 172-180.
- Stern, J., Bruwer, M., Huber, F. X., and Decker-Baumann, C. (1999). Metabolic consequences after ileostomy. *Chirurg*, 70(6), 627-634.
- Turrioni, F., Ribbera, A., Foroni, E., van Sinderen, D., and Ventura, M. (2008). Human gut microbiota and bifidobacteria: from composition to functionality. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology*, 94(1), 35-50.
- Wapnir, R. A., and Teichberg, S. (2002). Regulation mechanisms of intestinal secretion: implications in nutrient absorption. *Journal of Nutritional Biochemistry*, 13(4), 190-199.
- White, C. L., and Ashes, J. R. (1999). A review of methods for assessing the protein value of grain fed to ruminants. *Australian Journal of Agricultural Research*, 50(5), 855-869.
- Wunsche, J., Bock, H. D., Hennig, U., Kreienbring, F., and Borgmann, E. (1979). Untersuchungen über die proteinverdaulichkeit und aminosäurenresorption in

verschiedenen abschnitten des verdauungstraktesbeim schwein. *Archives of Animal Nutrition*, 29, 221-234.

Wunsche, J., Volker, T., Souffrant, W. B., and Borgmann, E. (1991). Assessment of the bacteria-N proportions in feces and differently obtained ileal digesta of pigs. *Archiv Fur Tierernahrung-Archives of Animal Nutrition*, 41(7-8), 703-716.

Zebrowska, T., Low, A. G., and Zebrowska, H. (1983). Studies on gastric digestion of protein and carbohydrate, gastric-secretion and exocrine pancreatic-secretion in the growing-pig. *British Journal of Nutrition*, 49(3), 401-410.

Chapter VII

Endogenous proteins in terminal ileal digesta sampled from adult subjects fed casein, enzyme hydrolysed casein, or crystalline amino acid-based diets

The present study aimed to complement the study reported in the previous chapter and gain a clearer understanding of the nature and composition of endogenous nitrogen in the terminal ileal digesta collected from humans fed different forms of nitrogen: a casein-based diet, an enzyme hydrolysed casein-based diet and a crystalline amino acid-based diet.

Abstract

The aim was to ascertain if the form of dietary nitrogen (free amino acids, peptides, intact protein) affects the nature and composition of nitrogen-containing endogenous substances lost from the upper digestive tract of humans. Digesta were collected from the terminal ileum of sixteen human subjects given a diet that contained casein (CA), hydrolysed casein (HC) or crystalline amino acids (AA) as the sole source of nitrogen. The total nitrogen passing the terminal ileum ranged from 33–42 mg·g⁻¹ native digesta dry matter (NDDM). Of this ~86% was proteinaceous, 59%–62% was bacterial protein, 6%–8% soluble free protein, and 12%–15% derived from mucin. Of the non-protein nitrogen, nearly 5% was derived from ammonia and 4% from urea. Bacterial and human mucosal cellular DNA nitrogen were collectively nearly 0.5% of the total nitrogen. Mucin contributed 12%–15% of the nitrogen losses and was the most abundant, truly endogenous component within the terminal ileal digesta, and ranged between 9%–13% of the total dry matter. Bacterial nitrogen, combined with ammonia and urea nitrogen, represented 68%–72% of the total nitrogenous losses, indicating substantial microbial activity within the human small intestine. A fraction of non-specific protein was between 2.1–3.4 mg·g⁻¹ NDDM an amount that contributed between 6.3%–9.4% of the total nitrogen at the terminal ileum. Only a relatively small fraction of the non-protein nitrogen remained unidentified (2.6%–4.1% of the total nitrogen), this was assumed to include free amino acids, RNAs, amines, and the tetrapyrroles bilirubin and biliverdin.

The form of dietary nitrogen (protein, short peptides and amino acids) had a significant effect ($P < 0.01$) upon the amount of protein found in the small intestinal digesta (39.3, 42.6, and 33.4 mg·g⁻¹ of nitrogen determined in the digesta of subjects fed the CA-, HC- and AA-based diets respectively). The mucin concentration in digesta collected from subjects fed the CA and HC diet were both significantly greater (41% and 24% respectively) than that detected in the digesta collected from subjects fed the AA diet. Both the concentrations and flows of two of the amino acids common to gastrointestinal mucins (threonine and serine) were significantly higher in digesta collected from subjects fed the CA- and HC-based diets than those determined for the AA-based diet. Interestingly the quantity of microbial protein originating from lysed

cells detected in the 14500 RCF supernatant was significantly greater in digesta collected from subjects fed the CA- and HC-based diets than that collected from the subjects fed the AA-based diet. However, the form of dietary nitrogen had no significant effect upon total bacterial protein, ammonia or urea which constituted between 69% – 72% of the total digesta nitrogen.

Introduction

Proteinaceous compounds are secreted by specialist cells into the lumen of the gastrointestinal tract (GIT) for either the purpose of digestion or defence. Such endogenous proteins may be partly resistant to the digestive processes within the GIT and may remain in ileal effluent, entering the large bowel where they are largely catabolised (Wapnir and Teichberg, 2002). Also, cells of the mucosa are regularly sloughed off into the gut lumen. Although estimates of these inputs vary greatly, in the pig, an often used human analogue (Moughan *et al.*, 1994), approximately 8.3-12.6g of nitrogen/day enter the gut from the body (Krawielitzki *et al.*, 1994). The total mass of endogenous material entering into the GIT has been estimated to approximately equal that of the dietary exogenous protein ingested (Nasset and Ju, 1961). Although not strictly endogenous (but non-dietary) in origin, protein from the microbiota that normally inhabit the GIT also contribute to the endogenous nitrogen loss component and may amount to the largest proportion of nitrogen and amino acids found at the terminal ileum (Caine *et al.*, 1999). Both exogenous and endogenous proteinaceous materials that exit the terminal ileum unabsorbed are considered to have little nutritional value as absorption of such material, as amino acids *per se*, in the large intestine is thought to be minimal (Moughan and Rutherfurd, 2011).

Many methods have been used to quantitate the endogenous ileal nitrogen losses and these have been reviewed extensively (Fuller and Tome, 2005; Moughan, 2003). The administration of a protein-free diet to distinguish between endogenous nitrogen losses at the terminal ileum and undigested exogenous dietary nitrogen has been shown to be an unphysiological approach and to lead to an underestimation of endogenous nitrogen losses due to a decrease in protein synthesis by glandular tissues of the GIT (Millward

et al., 1976) and the diminution of proteinaceous secretions into the gut lumen (Schneeman, 1982; Snook and Meyer, 1964). To obviate this, alternative methods to determine endogenous nitrogen and amino acid losses at the terminal ileum, which involve feeding protein-containing diets, have been developed (Stein *et al.*, 2007). A dynamic equilibrium exists in the GIT between dietary protein intake and the secretion of endogenous proteinaceous material into the lumen with the concomitant absorption of both exogenous and endogenous digested materials. Such equilibria are known to be influenced by the composition of the diet (Pedersen *et al.*, 2002). Indeed the influence of the form of dietary nitrogen (free amino acids, peptides, protein) has been studied and found to affect gastric secretion (Calbet and Holst, 2004), increase endogenous amino acid losses from the gut (Butts *et al.*, 1993; Moughan *et al.*, 2005; Skilton *et al.*, 1988) and the absorption of amino acids and their appearance in the portal blood (Rerat, 1995). Information on the composition of ileal digesta is of fundamental interest, and accurate estimates of endogenous total nitrogen and amino acid flows are necessary to allow the determination of true ileal dietary amino acid digestibility coefficients and for the factorial estimation of dietary amino acid requirements (Moughan *et al.*, 2005). Although there are several published estimates of the amino acid composition of human ileal digesta (Deglaire *et al.*, 2009b; Moughan and Rutherford, 2011) and some published data on the sources of ileal endogenous protein (Miner-Williams *et al.*, 2009) and refer to Chapter V page 265) there have been no systematic studies reported as to the effect of the form of dietary nitrogen (intact protein, peptides and free amino acids) on the protein composition of ileal digesta in humans.

The aim was to quantify the endogenous protein components of terminal ileal digesta collected from humans given three different diets. The three semi-synthetic starch based diets used were based on casein, a protein known to be highly digested and absorbed. The diets contained intact casein, enzyme-hydrolysed casein or free amino acids mimicking the pattern of amino acids in the casein and casein hydrolysate. The diets were iso-nitrogenous and iso-caloric. Although small amounts of dietary peptides and amino acids may remain unabsorbed when such casein based diets are given to experimental subjects these were assumed to be minimal and to have no bearing on the overall results, as the respective constituent endogenous proteins were determined directly.

Materials and Methods

Subjects

Sixteen adult subjects (seven female and nine male) were involved in the study. Their age, body weight and body mass index are presented in **Table 1**. All of the subjects gave their informed consent to participate in the study after receiving detailed information of the experimental protocol. Their inclusion in the study followed a thorough medical examination and routine blood tests. The subjects were randomly allocated to the test diets. The study was conducted according to the ethical guidelines laid down in the Declaration of Helsinki; at the Avicenne Hospital, Bobigny, France and the experimental protocol was approved by the Institutional Review Board of the St- Germain-en-Laye Hospital, France.

Table 1 Subject characteristics.

Characteristic	Experimental Diet		
	Casein	Hydrolysed Casein	Amino Acid
Number of subjects	^a 4F/2M ^b	2F/3M*	1F/4M*
Age in years	35.7 ± 6.6	28.4 ± 8.5	24.6 ± 8.1
Body weight (kg)	62.5 ± 12.6	64.2 ± 7.2	67.2 ± 9.2
BMI ^c (kg/m ²)	22.1 ± 4	21.8 ± 4.2	22.2 ± 4.3

Data are Means ± standard deviation. ^aF: female, ^bM: male. ^c Body mass index

* Initially eighteen subjects were recruited for the study. In two subjects the naso-ileal tube failed to reach the ileo-caecal junction and therefore those subjects were withdrawn from the ileal study.

Test Meals

The ingredient compositions of the test diets are outlined in **Table 2**. The test meals consisted of 150g of each test diet and each meal was formulated to have equal amounts of nitrogen and amino acids (320 mmol of nitrogen in the CA- and HC-based diets and 306 mmol of nitrogen in the AA-based diet). The meal was made up to a final volume of 550 mL with water. The metabolizable energy content of each test meal was 700 kcal (2.93 MJ). The hydrolysed casein was obtained by the enzymatic hydrolysis of the parent intact micellar casein following the method described in detail by Deglaire *et al.* (2009b) and was found to contain 21% of peptides between 1-5kDa

and 79% < 1kDa. All processes were carried out at the National Institute of Agronomic Research (Rennes, France). The crystalline L-isomer amino acids were obtained from Ajinomoto Co. Inc., Kawazaki Japan.

Table 2. Composition of the experimental diets (g/kg air dry weight)

Ingredient	Form of Dietary Nitrogen ^a		
	Casein	Hydrolysed Casein	Crystalline Amino Acid
Lactic Casein	220.0		
Hydrolysed casein		226.7	
Crystalline amino acids			206.7
Maltodextrin	470.0	465.0	480.0
Sucrose	156.7	155.0	160.0
Soya bean oil	153.3	153.3	153.3

^a No vitamins, minerals or fibre were added to this diet as the diet was given acutely.

^b The hydrolysed casein was an enzyme hydrolysate of the parent intact casein and the crystalline amino acid mixture was formulated to meet the determined amino acid composition of the casein.

Experimental protocol

The volunteers were admitted to the hospital on the morning of day 1 of the study, when a 3 m polyvinyl chloride (PVC) triple-lumen tube was inserted via the nose under local anaesthesia. At the distal end of the triple lumen tube a 2 mm diameter tube, perforated with 2 mm holes for a length of 10 cm, was utilized for digesta collection. A second radio-opaque tube terminated 20cm from the digesta collection zone and was used to perfuse polyethylene glycol (PEG) as a flow rate marker. A third inner tube was utilized to inflate a balloon at the terminal end of the tube, 5 cm below the digesta collection zone. Having passed the pyloric sphincter, the terminal balloon was inflated to stimulate peristalsis and hasten the tube's progression through the gut. Once the tube had reached the terminal ileum its position was confirmed by X-ray and then restrained from further movement by the inflation of the terminal balloon proximal to the ileo-caecal junction. The subjects were given a standard hospital meal and received a second meal at 19.00 hours before being fasted overnight.

The experimental protocol commenced at 09.30 h on Day 2 when a saline solution, containing polyethylene glycol (PEG-4000, 20 g/l) as a liquid-phase marker, was infused continuously at a constant rate of 1 ml/min for the duration of the test. To enable base-line data to be obtained digesta were first collected 30 minutes before the ingestion of the test meal.

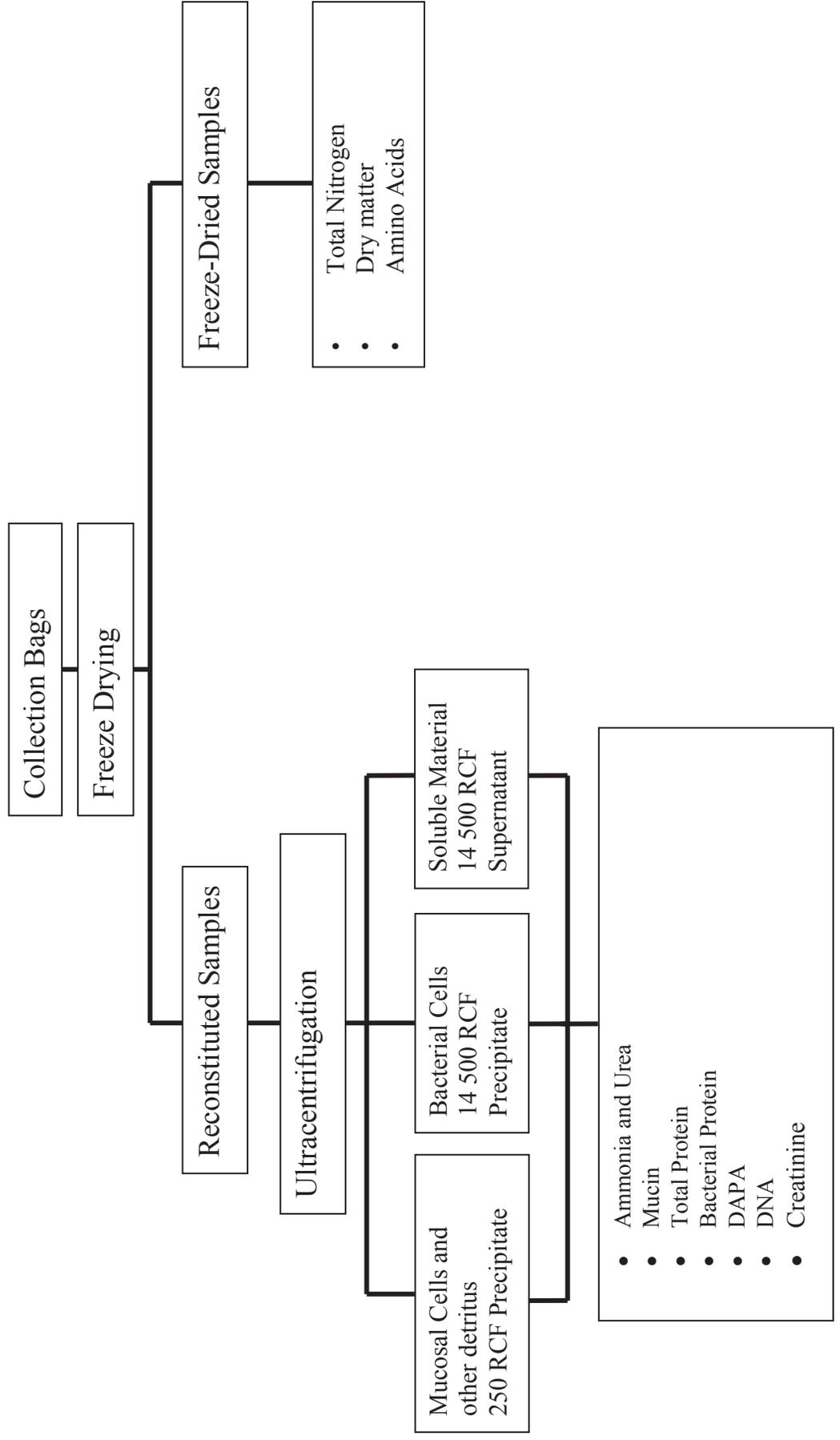
At 10.00 h the test meal was given to the subjects. Digesta were collected while the subjects were resting in a semi-recumbent position and subjects did not receive any further food until the end of the collection period, which lasted for the 8 hours following the ingestion of the test meal. Water was given to the subjects hourly. Digesta were collected on ice and pooled over 30 minute periods. Following the addition of the antiprotease di-isopropylfluorophosphate (1 mmol/L) and sodium benzoate ($10\text{g}\cdot\text{kg}^{-1}$ of digesta) as a bactericide, they were frozen (-20°C) immediately. Later each 30 minute digesta sample was freeze dried, ground and mixed.

Chemical Analysis.

All analyses were undertaken in triplicate. Amino acids were determined on the freeze dried digesta samples after hydrolysis for 24 hours with hydrochloric acid (6 mol/l containing 0.1% phenol) using a Waters ion exchange HPLC (AOAC, 2003; Deglaire *et al.*, 2009a). Tryptophan and cysteine were not determined. Other nitrogen containing components of the ileal digesta were determined as described previously (Miner-Williams *et al.*, 2009). Briefly, samples of freeze dried digesta were reconstituted in normal saline and fractionated by differential centrifugation using the method of Metges *et al.* (1999) (**Figure 1**). Digesta were first centrifuged at 250 RCF for 15 minutes at 4°C , then at 14500 RCF for 30 minutes at 4°C . The first precipitate, at 250 RCF, was expected to contain mainly food particles and mucosal cells. The second precipitate, at 14500 RCF, was expected to contain mainly microbial cells with the supernatant expected to mainly contain soluble nitrogenous components such as: proteins, peptides, free amino acids, mucins, neutral sugars, urea, creatinine, and ammonia. Total nitrogen was determined by the Leco total combustion method, a variation of the Dumas method (AOAC, 2000). Diaminopimelic acid (DAPA) was quantified, following oxidation with performic acid, using an HPLC system with a UV detector. Soluble protein was determined by the Bradford method (Bradford, 1976).

Figure 1 Schematic diagram of the processing of the digesta samples.

Digesta Samples



Mucins were quantified by the determination of the amino sugars N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GlcNAc), carbohydrate markers peculiar to glycoproteins. Ammonia and urea were determined using an adaptation of the method devised by Chaney and Marbach (1962). DNA was determined using a QIAamp DNA Stool Mini Kit obtained from QIAGEN Inc., Valencia, CA according to the manufacturer's instructions. The absorbance of the extracted samples was determined using a NanoDrop ND-1000 UV-vis Spectrophotometer (NanoDrop Technologies Wilmington, DE 19810). The DNA concentration was determined from its absorbance at A260 nm and its purity from the ratio of the readings at A260 nm and A280 nm.

Data Analysis.

The total ileal component flows were determined in the timed (30 minute) samples or pooled freeze-dried ileal digesta using the following equation:

$$\text{TICF} = C_{\text{digesta}(T)} \times \text{DM}_{\text{s}(T)} \times F_{\text{tot}(T)}$$

where TICF represents the total ileal component flow (mg); $C_{\text{digesta}(T)}$ represents the component concentration in the terminal ileal digesta for the given time period, (mg/g DM); $\text{DM}_{\text{s}(T)}$ represents the dry matter concentration in the sample for time T (mg/g digesta) and $F_{\text{tot}(T)}$ represents the total ileal digesta flow over time T (mL).

$F_{\text{tot}(T)}$ was determined using the following equation:

$$F_{\text{tot}(T)} = (\text{PEG}_i / \text{PEG}_{\text{s}(T)}) \times F_i \times T$$

where PEG_i represents the concentration of the indigestible marker (PEG) in the solution perfused and $\text{PEG}_{\text{s}(T)}$ is the PEG concentration in the sample of ileal digesta (g/L); F_i represents the PEG infusion rate (mL/minute) and T represents the sampling time period (minutes).

Native digesta dry matter (NDDM) was determined using the following equation:

$$\text{NDDM} = \text{DM}_{s-(T)} \times F_{\text{tot-(T)}} - \text{PEG}_i \times F_i \times T$$

where NDDM represents native digesta dry matter (i.e., excluding the DM of the PEG infused) in the time period T (g).

Results were expressed as means \pm standard error of the mean. Statistical analysis was undertaken using Minitab 15 (Minitab Pty Ltd, Sydney NSW Australia). Data were tested for homogeneity of variance using the Levene's test. Variables with equal variance were analysed using one-way ANOVA. Mucin concentration and flow data were analysed using non-parametric analyses (Kruskal-Wallis one-way Analysis of Variance) as the Levene's tests indicated that the variances in these parameters were not equal. Statistical significance was considered to be reached at $P < 0.05$. Results are means \pm standard error of the mean.

Results and Discussion

Some earlier studies have used ileostomy patients for this type of work because of the ease of collecting total digesta from the terminal ileum (Moughan *et al.*, 2005).

However, this approach was rejected in this study for two reasons: (1) normal digestive function may be altered by the primary pathological condition that resulted in the patient's colectomy, and it is known that colectomy may induce adaptations in a number of physiological and metabolic processes (Stern *et al.*, 1999); (2) given the elevated population of ileal microorganisms associated with ileostomy, the digesta collected from ileostomates may have a composition different from that of subjects with an intact digestive system (Fuller *et al.*, 1994). The method of sampling digesta via naso-ileal intubation has been developed (Modigliani *et al.*, 1973) and widely accepted as a valid procedure for collecting digesta from healthy human adults (Deglaire *et al.*, 2007). The method of collecting ileal digesta from fit healthy subjects, using this technique, was chosen after a meta-analysis study confirmed that this method allows precise and accurate sampling of ileal digesta (Deglaire, 2008).

Although total digesta collection is not possible with naso-ileal intubation, determining the recovery of a perfused non-digestible marker (polyethylene glycol 4000) allows the precise and accurate sampling of digesta. The PEG recovered in the digesta collected over the 8-hour period in the present study was 56 % of that infused (as reported

elsewhere, 32), indicating that a relatively large proportion of the total ileal digesta had been collected.

The three iso-nitrogenous and iso-caloric meals were given at the same dry matter intakes and the only difference between them was the form of nitrogen administered. Casein was used in this experiment because it is a well characterised protein which is known to be almost completely digested and absorbed (Eklund *et al.*, 2008). Its amino acid composition is well documented and using enzyme hydrolysis it is easily broken up into smaller peptides. This made casein an ideal parent protein to study the effect of the physical form of the dietary nitrogen on the composition of terminal ileal digesta. Using casein, a casein hydrolysate or crystalline amino acids ensured that most of the soluble protein found in the ileal digesta would be of non-dietary (endogenous or bacterial) origin and that any unabsorbed dietary material would likely be small peptides and amino acids, thus allowing an unambiguous direct determination of the endogenous proteinaceous components of the terminal ileal digesta.

The total nitrogen composition of the digesta is given in **Table 3**. Mean total nitrogen for the 8 hour collection period was determined as 39, 43, and 33 mg·g⁻¹ NDDM for the CA, HC, and AA diets respectively and although the results were not statistically significantly different between the CA and HC diets there was a significant ($P<0.01$) difference between these two diets and the AA diet. There was also a significant difference ($P<0.01$) between the AA diet and the CA and HC diets, respectively (29, 34, and 36 mg·g⁻¹ NDDM) for the proteinaceous nitrogen content. However, when proteinaceous nitrogen was expressed as a percentage of the total nitrogen there were no statistically significant differences between the three diets (86%, 85% and 87% for the CA, HC and AA diets respectively). The statistically significant differences in protein nitrogen concentrations related to similar differences ($P < 0.01$) in the soluble protein nitrogen fraction of the digesta (26, 27, and 20 mg·g⁻¹ NDDM for the CA, HC and AA diets respectively). When the total nitrogen and protein nitrogen flows (per unit food dry matter intake) were determined (data not given) there were statistically significant differences between the mean data for the CA and HC diets and the AA diet, with the subjects receiving the free amino acids having lower flows. This result is consistent with that of other researchers (Butts *et al.*, 1993; Claustre *et al.*, 2002; Darragh *et al.*, 1990; Deglaire *et al.*, 2009b; Deglaire *et al.*, 2007).

Table 3 Nitrogen content of terminal ileal digesta of human subjects given the three experimental diets.

	Form of Dietary Nitrogen												
	Casein			Hydrolysed casein			Crystalline amino acid			P			
	mg•g ⁻¹ NDDM	Mean	SEM	%	Mean	SEM	%	Mean	SEM	%	Mean	SEM	%
Total Digesta Nitrogen	39.3 ^a	1.04	1.04	100.0	42.6 ^a	1.19	100.0	33.4 ^b	1.07	100.0	33.4 ^b	1.07	<0.01
Protein Nitrogen	34.0 ^a	1.73	1.73	86.5 ¹	36.2 ^a	0.96	85.0 ¹	28.9 ^b	1.85	86.6 ¹	28.9 ^b	1.85	<0.01
Non-protein Nitrogen	5.3	0.55	0.55	13.5 ¹	6.4	0.37	15.0 ¹	4.5	0.38	13.4 ¹	4.5	0.38	NS
Soluble Protein Nitrogen	26.2 ^a	1.58	1.58	76.9 ²	28.1 ^a	2.23	77.5 ²	21.0 ^b	1.88	72.6 ²	21.0 ^b	1.88	<0.01
Insoluble Protein Nitrogen	7.8	0.77	0.77	23.0 ²	8.1	0.91	22.5 ²	7.9	0.68	27.4 ²	7.9	0.68	NS

Abbreviations: NDDM, native digesta dry matter. SEM, standard error of the mean. NS, not significant.

¹ Percentage values of total nitrogen. ² Percentage values of total protein nitrogen. Statistical probability shown for the concentration data (mg•g⁻¹ NDDM).

Means within a row with different superscripts were significantly different.

There was no statistically significant difference between the non-protein nitrogen values (5.3 , 6.4 and $4.5 \text{ mg}\cdot\text{g}^{-1}$ NDDM for the CA, HC, and AA diets respectively) or when these were expressed as a percentage of total nitrogen (13.6% , 15.4% and 13.8% for the CA, HC, and AA diets respectively). There was also no statistically significant difference for insoluble protein nitrogen content (7.8 , 7.8 and $7.5 \text{ mg}\cdot\text{g}^{-1}$ NDDM for the CA, HC, and AA diets respectively). For all three diets most of the protein nitrogen (77% , 78% and 73% for the CA, HC, and AA diets respectively) was soluble and present in the 14500 RCF supernatant leaving the remaining insoluble protein nitrogen (23% , 22% and 27% for the CA, HC, and AA diets respectively) being detected in the 250 RCF and 14500 RCF precipitates. It appears that protein and/or peptide alimantation stimulates proteinaceous nitrogen inputs into the GIT (23% and 26% more than the AA diet for the CA and HC diets respectively). This increase in proteinaceous nitrogen produced a proportional increase in digesta soluble protein concentration (25% and 34% greater for the CA and HC diets respectively than for the soluble protein concentration of the AA diet), results that are in keeping with other researchers working with pigs and rats (Butts *et al.*, 1993; Moughan *et al.*, 2005; Pedersen *et al.*, 2002; Skilton *et al.*, 1988). Dietary protein/peptide secretagogues may thus stimulate the secretions of proteinaceous compounds such as digestive enzymes and protective mucins, which are at least partly resistant to digestion and remain unabsorbed at the terminal ileum. Such differences in secretions are not due to differences in the supply of dietary amino acids to the enterocytes, as all of the diets supplied amino acids equally.

The amino acid profile of the human ileal digesta for the three diets is given in **Table 4** as $\text{mg}\cdot\text{g}^{-1}$ NDDM and $\text{mg}\cdot\text{g}^{-1}$ total nitrogen. The six most abundant amino acids detected in the ileal digesta for the CA and HC diets are those most commonly found in mucin. Similar to our earlier pig study (Miner-Williams *et al.*, 2009), where a similar amount of purified casein was included in the test meal, the six most abundant amino acids detected in the digesta (in decreasing order of abundance, $\text{mg}\cdot\text{g}^{-1}$ NDDM) were: glutamic acid; glycine; aspartic acid; serine; proline and threonine. Threonine, serine and proline are abundant in the glycosylated region of mucin polymers. Glutamic acid, glycine and aspartic acid are most common in the nonglycosylated regions of mucin. Surprisingly the same is not true for the AA diet, as serine was only the thirteenth most abundant amino acid.

Table 4 Amino acid composition of terminal ileal digesta of human subjects given the three experimental diets

	Form of Dietary Nitrogen				Hydrolysed casein				Crystalline amino acid				
	Casein mg·g ⁻¹ Nitrogen	SEM	mg·g ⁻¹ NDDM	SEM	mg·g ⁻¹ Nitrogen	SEM	mg·g ⁻¹ NDDM	SEM	mg·g ⁻¹ Nitrogen	SEM	mg·g ⁻¹ NDDM	SEM	P
Aspartic acid ¹	664.7	34.9	17.0	1.5	701.0	45.3	19.0	1.4	818.0	93.7	18.4	2.1	NS
Threonine ¹	383.0	13.5	9.8 ^a	0.9	394.3	21.8	10.6 ^a	0.6	357.5	13.0	8.0 ^b	0.5	0.05
Serine ¹	515.8	26.8	13.2 ^a	1.3	572.8	32.5	15.5 ^a	1.0	259.1	23.1	5.8 ^b	0.6	<0.001
Glutamic acid ¹	927.8	63.6	23.9	2.5	1096.7	77.1	29.7	2.7	1087.7	216.2	24.3	4.6	NS
Proline ¹	396.8	15.0	10.2 ^a	0.9	410.8	25.2	11.1 ^a	0.8	597.1	73.7	13.4 ^b	1.6	<0.01
Glycine ¹	755.2	75.6	19.3	2.3	579.2	68.7	15.4	1.4	700.7	72.6	15.5	1.2	NS
Alanine	255.8	11.4	6.6	0.6	270.4	17.2	7.4	0.6	305.2	35.0	6.8	0.8	NS
Valine	387.0	18.1	9.9 ^a	0.9	406.7	27.3	11.0 ^a	0.8	314.7	24.8	7.1 ^b	0.6	0.05
Methionine	78.6	3.6	2.0 ^a	0.2	110.7	8.0	3.0	0.3	84.7	11.3	1.9 ^b	0.3	0.03
Isoleucine	314.2	13.3	8.0 ^a	0.7	331.7	19.8	9.0 ^a	0.7	190.9	11.0	4.3 ^b	0.3	<0.01
Leucine	371.3	15.1	9.5 ^a	0.8	363.3	23.8	9.8 ^a	0.8	346.2	24.6	7.8 ^b	0.7	0.05
Tyrosine	253.2	11.3	6.5 ^a	0.6	241.0	20.1	6.5 ^a	0.6	341.0	44.8	7.7 ^b	1.2	0.01
Phenylalanine	227.1	11.0	5.8	0.5	219.0	17.7	5.9	0.6	263.8	28.0	5.9	0.7	NS
Histidine	220.2	5.5	5.6 ^b	0.4	248.6	12.5	6.7 ^a	0.2	263.7	18.1	5.9 ^b	0.4	0.03
Lysine	246.2	19.6	6.3	0.7	236.2	27.0	6.4	0.8	286.8	52.2	6.4	1.2	NS
Arginine	216.2	15.5	5.6	0.6	203.3	21.5	5.5	0.6	182.9	14.3	4.1	0.4	NS

Tryptophan was not determined. Statistical probability shown for the concentration data (mg·g⁻¹ NDDM).¹ Predominant amino acids in mucin. Means written within a row and diet type with different superscripts were significantly different. Abbreviations: SEM, standard error of the mean. NDDM, native digesta dry matter. NS, not significant. Means within a row and type of measure (mg·g⁻¹ NDDM) with different superscripts were significantly different.

Why the concentration of this amino acid was so low ($P < 0.001$) in digesta taken from subjects given the AA diet is unclear. In the rat, Darragh *et al.* (1990) also found that the flow of serine was much lower for animals fed a crystalline amino acid based diet compared to those given an HC diet ($254\mu\text{g}\cdot\text{g}^{-1}$ and $795\mu\text{g}\cdot\text{g}^{-1}$ respectively). A plausible explanation may be that the concentration of serine was lower because the mucin concentration was lower in the digesta from subjects given the AA diet. In the presently reported study the proportion of these six amino acids (with the exception of serine in the AA diet) was common to all three diets and ranged from 58.6%–59.5%. The high proportion of these six amino acids is consistent with the high rates of mucin secretion throughout the GIT and the minimal rate of degradation of these macromolecules; this together with the high proportion of protein nitrogen found in the 14500 RCF supernatant in this study (73–77%), would indicate that a high proportion of soluble mucin was present in this fraction. The mean concentration of lysine found in the ileal digesta collected over 8 hours was virtually the same for all three diets ranging between $6.34\text{--}6.45\text{ mg}\cdot\text{g}^{-1}$ NDDM. Such lysine concentrations equate to nearly 3.7%–4.5% of the total amino acids present, figures not too different from the value for lysine content (4.75%) reported by Metges *et al.* (1999) for ileal microbial protein collected from human ileostomates. Interestingly, Moughan *et al.* (2005) found that dietary peptides exert a positive influence on endogenous ileal amino acid loss in humans. In their study the endogenous total ileal flows of all amino acids (with the exception of glycine, phenylalanine, tyrosine and cysteine) were increased substantially in subjects given an HC diet when compared to subjects given a protein free diet. As other studies have shown that the endogenous ileal flows of amino acids in animals given an AA diet are comparable to those given a protein free diet (Darragh *et al.*, 1990; Skilton *et al.*, 1988) then it is surprising that in the present study there was so little difference between the amino acid concentrations (and flows, data not shown) determined in the ileal digesta of human subjects given the CA and HC diets when compared to those given the AA diet. Only five of the amino acids (threonine, serine, valine, isoleucine and leucine) were significantly and quantitatively lower in the digesta of subjects given the AA diet than those given the CA and HC diets. One possible explanation may be that the secretion of proteinaceous materials in subjects given the AA diet was influenced by the presence of the naso-ileal tube and/or the effect of the PEG marker stimulating the increased secretion of proteinaceous materials, such as mucin. Although an additional four amino acid concentrations were

statistically significantly different, two were highest in digesta from subjects given the AA diet (proline and tyrosine) and two were highest in the digesta from those given the HC diet (methionine and histidine). The reason for these differences is unclear.

Table 5 shows correlation coefficients for the threonine, serine and proline digesta concentrations calculated within each of the three diets. There was a close relationship between these three amino acids in the digesta of subjects given the CA diet but a low correlation was observed between the same three amino acids in the digesta from subjects given the AA diet. Threonine, serine and proline are most common in the glycosylated regions of mucins secreted by the mucosa of the GIT (Allen and Pearson, 1993). In mucin polymers the glycosylated regions are more resistant to digestion than the non-glycosylated protein regions. As the carbohydrate chains of the glycosylated regions of gastric mucins are longer than those in small intestinal mucins, gastric mucins are more resistant to hydrolytic degradation than small intestinal mucins and as a result would be conserved in greater concentrations at the terminal ileum.

Table 5 Correlation coefficients (*r*) between threonine, serine and proline concentrations ($\text{mg}\cdot\text{g}^{-1}$ NDDM) in the terminal ileal digesta of subjects receiving the three experimental diets.

Form of Dietary Nitrogen		Proline	<i>P</i>
Casein	Threonine	0.96	(0.002)
	Serine	0.88	(0.002)
Enzyme Hydrolysed Casein	Threonine	0.90	(0.012)
	Serine	0.79	(0.064)
Free Amino Acid	Threonine	0.40	(0.506)
	Serine	-0.48	(0.414)

The values are means together with the *P* values in brackets.

This would support the hypothesis that native protein polymers such as casein in the CA diet stimulate the secretion of more gastric mucin than do the AA diet.

The method of differential centrifugation used in this study allowed the separation of the whole bacterial cells from human mucosal cells and food particles present in the digesta. Microscopic investigation of stained samples of all three centrifugation fractions confirmed that most of the intact human mucosal cells were in the 250 RCF precipitate. Although some bacterial cells were present in the 250 RCF precipitate and the 14500 RCF supernatant the great majority of whole bacterial cells were found in the 14500 RCF precipitate. A relatively high concentration of DAPA was found in the 14500 RCF supernatant (**Table 6**) of digesta taken from subjects given all three diets (and few intact bacterial cells were observed visually in this fraction), and it was assumed that this was the result of free DAPA from lysed bacterial cells. The mean total concentrations of DAPA determined in the digesta from subjects given the three diets did not differ significantly and ranged from 0.55–0.66 mg·g⁻¹ NDDM, values similar to those found in a previous pilot study (W. M. Miner-Williams and P.J. Moughan, unpublished data) with pigs (0.60–0.68 mg·g⁻¹ NDDM). The mass of total bacterial dry matter was calculated assuming that there was 2.88 mg of DAPA per gram of bacterial dry matter (Czerkawski, 1974) and ranged from 191–229 mg·g⁻¹ NDDM, which represents 19%–23% of the total digesta dry matter, values similar to those determined by Rowan *et al.* (1992).

The proportions of total, microbial and non-microbial protein are presented in **Table 7**. The total protein in the ileal digesta for subjects given the AA diet was significantly lower than that in the digesta collected from subjects given the CA and HC meals (212, 226 and 181 mg·g⁻¹ NDDM for the AA, CA and HC diets respectively). However, only the total nonmicrobial protein flows and the nonmicrobial protein flows in the 14500 RCF precipitate were determined to be significantly lower for subjects given the AA diet when compared to subjects given the CA and HC diets (data not shown).

Table 6 Mean (\pm SEM) concentrations ($\text{mg}\cdot\text{g}^{-1}$ NDDM) of DAPA and DNA in the terminal ileal digesta of subjects given the three experimental diets.

Centrifugation Fraction	Form of Dietary Nitrogen												P ⁵
	Casein			Hydrolysed Casein			Crystalline amino acid			Total			
	DAPA $\text{mg}\cdot\text{g}^{-1}$ NDDM	DNA $\text{mg}\cdot\text{g}^{-1}$ NDDM	Bacterial cellular ⁴	DAPA $\text{mg}\cdot\text{g}^{-1}$ NDDM	DNA $\text{mg}\cdot\text{g}^{-1}$ NDDM	Bacterial cellular ⁴	DAPA $\text{mg}\cdot\text{g}^{-1}$ NDDM	DNA $\text{mg}\cdot\text{g}^{-1}$ NDDM	Bacterial cellular ⁴	DAPA $\text{mg}\cdot\text{g}^{-1}$ NDDM	DNA $\text{mg}\cdot\text{g}^{-1}$ NDDM	Bacterial cellular ⁴	
250 RCF precipitate	0.03 (± 0.005)	0.06 (± 0.005)	0.20 (± 0.01)	0.26 (± 0.04)	0.03 (± 0.004)	0.06 (± 0.005)	0.24 (± 0.03)	0.30	0.03 (± 0.004)	0.06 (± 0.005)	0.23 (± 0.025)	0.29 (± 0.04)	NS
14500 RCF Precipitate²	0.26 (± 0.03)	0.45 (± 0.05)	0.45 (± 0.05)	0.45 (± 0.05)	0.26 (± 0.18)	0.50 (± 0.05)	0.50	0.50 (± 0.05)	0.24 (± 0.02)	0.37 (± 0.04)	0.37 (± 0.04)	0.37 (± 0.04)	NS
14500 RCF Supernatant³	0.33 ^a (± 0.04)	0.58 ^a (± 0.05)	0.06 (± 0.005)	0.62 ^a (± 0.05)	0.38 ^a (± 0.05)	0.72 ^a (± 0.06)	0.04 (± 0.004)	0.78 ^a (± 0.07)	0.28 ^b (± 0.03)	0.45 ^b (± 0.05)	0.05 (± 0.004)	0.49 ^b (± 0.05)	< 0.01
Sum¹	0.62 (± 0.07)	1.07 (± 0.14)	0.26 (± 0.03)	1.33 (± 0.16)	0.66 (± 0.62)	1.28 (± 0.15)	0.28	1.58 (± 0.13)	0.55 (± 0.05)	0.85 (± 0.07)	0.28 (± 0.04)	1.14 (± 0.19)	NS

Abbreviations: DAPA, Diaminopimelic acid. NDDM, native digesta dry matter. DNA, deoxyribonucleic acid. NS, not significant. Values are means \pm standard error of the mean

¹. These totals represent the DAPA and DNA detected in the ileal digesta. ² It was assumed that all the DNA detected in the 14500 precipitate was bacterial in origin. A DNA/DAPA ratio was calculated based upon the 14500 RCF precipitate (each component expressed as $\text{mg}\cdot\text{g}^{-1}$ DDM), and used to estimate the bacterial DNA in the other two centrifugation fractions. ³ The bacterial DNA in this fraction was assumed to originate from lysed bacterial cells and was determined from the DNA/DAPA ratio of 1.77. ⁴ Cellular DNA was determined as being the difference between the total DNA detected and the estimated bacterial DNA. ⁵ Statistical probability given for the concentrations ($\text{mg}\cdot\text{g}^{-1}$ NDDM). Means within a row and measure (DNA or DAPA) with different superscripts were significantly different.

Table 7 Microbial and non-microbial protein within the different centrifugation fractions of ileal digesta of subjects receiving the three experimental diets.

	Form of Dietary Nitrogen						P¹
	Casein		Hydrolysed casein		Crystalline amino acid		
	mg·g⁻¹ NDDM	SEM	mg·g⁻¹ NDDM	SEM	mg·g⁻¹ NDDM	SEM	
Total protein	212.3 ^a	20.49	226.4 ^a	19.99	180.7 ^b	18.01	<0.01
Total microbial protein	147.1	10.26	157.0	16.02	130.4	14.82	NS
Total non-microbial protein	65.4 ^a	4.19	69.3 ^a	7.23	50.3 ^b	4.19	<0.01
250 RCF Precipitate							
Protein	20.3 ^a	2.48	22.2 ^a	2.51	16.4 ^b	1.54	<0.01
Microbial protein	7.1	1.08	6.98	0.80	7.1	0.74	NS
Mucosal cell protein	13.2 ^a	1.47	15.2 ^a	1.41	9.3 ^b	0.82	<0.01
14500 RCF Precipitate							
Protein²	78.1 ^a	5.42	78.8 ^a	6.13	65.4 ^b	6.37	<0.02
Microbial protein	61.6	4.49	61.1	4.28	56.7	4.87	NS
Non-microbial protein	16.5 ^a	1.65	17.7 ^a	1.51	8.7 ^b	0.81	<0.01
14500 RCF Supernatant							
Protein	114.1 ^a	7.52	125.4 ^a	8.47	98.4 ^b	7.27	<0.01
Microbial protein	78.5 ^a	5.42	89.0 ^a	6.59	66.2 ^b	5.23	<0.01
Non-microbial protein	35.6	1.82	36.4	2.74	32.2	1.91	NS

Abbreviations: NDDM, native digesta dry matter. SEM, standard error of the mean. NS, not significant.

Microbial protein was determined using the value of 26.4 mg DAPA/g of bacterial nitrogen. ¹ Statistical probability shown for the concentration data (mg·g⁻¹ NDDM). ² Protein in this fraction was determined using the bacterial protein extraction lysis buffer (PELB), kit combined with a non-interfering protein assay kit, obtained from G-Biosciences. Means written within a row with different superscripts were significantly different.

These results support the observation that the form of dietary nitrogen has an important effect upon the secretion and/or reabsorption of protein into the lumen of the small intestine of humans. The proportions of microbial and nonmicrobial protein were calculated using the DAPA/bacterial nitrogen ratio of 26.4 reported by Wünsche *et al.* (1991). The percentage of microbial protein ranged between 69%–76%. A greater amount of the microbial protein was found in the 14500 RCF supernatant (37%–41% as opposed to 28%–33%) in comparison with the 14500 RCF precipitate, suggesting that substantial degradation of bacterial cells takes place in the small intestine. As the concentration of DAPA and thus the level of microbial protein, was similar for all of the human subjects in this study it can be concluded that bacterial protein levels (and thus the microbial population) were largely unaffected by diet, a result in accord with other researchers (Robinson *et al.*, 1996).

The concentrations of DNA in each of the centrifugation fractions are also given in Table 6. The total amounts of DNA detected in the human ileal digesta ranged from 1.1–1.8 mg·g⁻¹ NDDM, values consistent with the pig studies of Rowan *et al.* (1992) and Miner-Williams *et al.* (2009). If the concentrations of DNA present in the 14500 RCF precipitates are assumed to be entirely of bacterial origin then the DNA/DAPA ratio can be calculated. This ratio ranged from 1.5–2.3 and was used to determine the bacterial DNA in the remaining two centrifugation fractions (these ranged from 0.05–0.07 and 0.42–0.76 mg bacterial DNA·g⁻¹ NDDM for the 250 RCF precipitates and 14500 RCF supernatants respectively). Human cellular material was thus determined as the difference between the total DNA and the estimated bacterial DNA (these values ranged from 0.23–0.29 and 0.04–0.06 mg bacterial DNA·g⁻¹ NDDM for the 250 RCF precipitates and 14500 RCF supernatants respectively). As in our earlier pig study (Miner-Williams *et al.*, 2009) the higher proportion of human DNA in the 250RCF precipitate supports the effectiveness of using differential centrifugation for the separation of human and bacterial cellular material from soluble components in the ileal digesta (Miner-Williams *et al.*, 2009). Inspection using light microscopy confirmed that the number of intact cells in the 14500 RCF supernatants was negligible and the DNA found in these fractions was assumed to be derived from lysed bacterial cells. Protein interference was minimal when using the QIAmp DNA Stool Mini Kit as the extraction of DNA was particularly pure. There were no statistically

significant differences in the ileal digesta concentrations of DNA between all three diets.

The concentration of mucin in the ileal effluent was calculated using the mucin markers N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GluNAc). The concentrations of these two amino sugars together with the GluNAc/GalNAc ratio, and the calculated mean mucin concentrations are presented in **Table 8**. Knowing the ratio of these two amino sugars in gastric and intestinal secretions, Lien *et al.* (1997) used regression equations to estimate the proportions of these two types of mucin in the ileal effluent of pigs fed a protein free diet. However, the proportions of the two amino sugars are different in the human and therefore it was not possible to calculate the proportions of these two types of mucin as we did in our earlier work (Miner-Williams *et al.*, 2009). The mucin concentrations in the ileal effluent were 91, 112 and 127 mg·g⁻¹ NDDM for the AA, HC and CA diets respectively and these values lie within the range of other published values (Montagne *et al.*, 2004). The total mucin flows over the collection period were: 3.3, 2.6 and 2.2 g·8 hours⁻¹ for the CA, HC and AA diets respectively, with the flow for the AA diet being significantly lower ($P < 0.05$) than the flows for the CA and HC diets. The digesta concentrations of mucin ranged from 9% to 13% of the digesta dry matter and mucin is the single most abundant truly endogenous component found in the digesta. Such values for digesta mucin content agree well with values found in our earlier study with the pig (Miner-Williams *et al.*, 2009).

Based on the observed differences in digesta mucin concentrations and flows it would appear that the form of dietary nitrogen has a direct effect upon the quantity of mucin secreted into the GIT (or reabsorbed from the GIT) and thus detected at the terminal ileum. The mean mucin concentrations in digesta for the subjects given the HC and CA diet were (24% and 41% respectively) greater than that in the digesta for subjects given the AA diet. The results concur with those of Sissons (1981) and indicate that casein stimulates the secretion of gastric mucin as a response to the increased secretion of pepsin, chymosin and hydrochloric acid once protein enters the stomach.

Table 8 Mean concentrations of the amino sugars N-Acetylgalactosamine and N-Acetylglucosamine and the concentration of mucin in terminal ileal digesta of subjects receiving the three experimental diets.

	Form of Dietary Nitrogen							
		Casein		Hydrolysed casein		Crystalline amino acid		
		Mean	SEM	Mean	SEM	Mean	SEM	P
N-Acetylgalactosamine	mg·g ⁻¹ NDDM	10.5 ^a	1.36	9.3 ^a	1.56	7.7 ^b	0.97	0.01
N-Acetylglucosamine	mg·g ⁻¹ NDDM	25.5 ^a	1.04	22.5 ^a	1.78	18.2 ^b	1.75	0.01
GluNAc/GalNAc ratio		2.42		2.41		2.38		
Mucin	mg·g ⁻¹ NDDM	127.0 ^a	4.47	112.0 ^a	8.85	90.6 ^b	5.95	0.01
Mucin Nitrogen	g·100g ⁻¹ digesta total nitrogen	14.6	1.26	11.9	1.27	12.3	1.18	NS

Abbreviations: SEM, standard error of the mean. NDDM, native digesta dry matter. NS, not significant. GalNAc, N-Acetylgalactosamine. GluNAc, N-Acetylglucosamine. Statistical probability shown for the concentration data (mg·g⁻¹ NDDM). Means within a row with different superscripts were significantly different.

Keogh *et al.* (1997) also suggested that the erosion and consequent solubilisation of the adherent gastric mucus gel observed in their studies may have arisen from stimulation of acid/pepsin secretion. The synthesis and secretion of mucin, stimulated by dietary whole protein secretagogues has been noted by a number of other researchers; for example, it is known that the presence of whole, dietary protein secretagogues, such as bovine α -lactalbumin, a major milk (whey) protein of 14.2 kDa, stimulates the synthesis and secretion of mucin in the gastric mucosa of the rat (Ushida *et al.*, 2007). In an earlier study Ushida *et al.* (2003) demonstrated that α -lactalbumin stimulated an increase in the amount of both soluble and adherent gel forming gastric mucin. Whether such stimulation of the mucosal mucin producing cells is via a gastrin mediated process that leads to the independent secretion of gastric acid is unknown and requires further study. In the pig, Butts *et al.* (1993) reported that dietary protein and peptides or their digestion products increase amino acid excretion at the terminal ileum when compared to an AA diet. They suggest that the presence of protein, peptides or both in the gut lumen stimulates the secretion of protein into the GIT. An *in vitro* study by Claustre *et al.* (2002) suggested that bioactive peptides, such as the opioid peptide β -casomorphin-7, released during the hydrolysis of casein might be the cause of increased mucin secretion when protein and/or peptides are present in the lumen of the GIT. Han *et al.* (Han *et al.*, 2008) found that in rats an HC diet up-regulates the *in vivo* expression of some individual mucin genes (Muc3 in the small intestine and Muc4 in the colon) when compared to an AA diet.

The concentrations of ammonia, urea and creatinine in the digesta are presented in **Table 9**. These three compounds make up between 66%–77% of the non-protein nitrogen in digesta (**Table 10**). Although urea and ammonia make up only about 4% of the digesta dry matter these components of the digesta contribute between 8.7%–10% of the total digesta nitrogen, a disproportionate amount of nitrogen. Similar to the concentrations of DAPA and microbial protein the concentrations of ammonia and urea were not significantly affected by the form of dietary nitrogen given to the human subjects in this study. Urea, ammonia and the metabolic activity of intestinal bacteria are linked to nitrogen cycling systems in the gut. Both the pig and human small intestine, once thought to be largely devoid of microbial activity (Hill, 1982), are now known to be colonised with large populations of bacteria (Turrone *et al.*, 2008).

Table 9 Concentrations of ammonia, urea and creatinine in the terminal ileal digesta of subjects receiving the three experimental diets.

	Casein		Hydrolysed casein		Crystalline amino acid		P
	mg·g ⁻¹	SEM	mg·g ⁻¹	SEM	mg·g ⁻¹	SEM	
	NDDM		NDDM		NDDM		
Ammonia	2.3	0.33	3.0	0.19	2.3	0.24	NS
Urea	3.3	0.30	3.9	0.28	3.0	0.34	NS
Creatinine	0.3	0.01	0.3	0.03	0.5	0.07	NS
Sum	5.9	0.62	7.2	0.49	5.8	0.42	NS

Abbreviations: NDDM, native digesta dry matter. SEM, standard error of the mean. NS, not significant. Statistical probability shown for the concentration data (mg·g⁻¹ NDDM).

Indeed antimicrobial peptides secreted by the paneth cells within the crypts of the small intestine protect the underlying mucosa, while still allowing the presence of an enteric microbiota (Meyer-Hoffert *et al.*, 2008). Although the microbial populations of the large intestine are greater than that of the small intestine, Ahmed *et al.* (2007) revealed that bacterial population densities in the small intestine were highest at the terminal ileum. The microbiota of the human gut represents a complex microbial community which is believed to have a significant impact on human physiology (Turroni *et al.*, 2008).

The loss of proteinaceous nitrogen from both microbial and truly endogenous sources, was between 85%–86% of total ileal nitrogen losses' (Table 3), a value in accord with the estimates by Chacko and Cummings (1988) who gave a range of 80-85%. The fraction of non-specific protein present in the 14500 RCF supernatant was between 2.1–3.4 mg·g⁻¹ NDDM, an amount that contributed between 6.3%–9.4% of the total nitrogen at the terminal ileum (see Table 10), a not insignificant amount that would be expected to contain compounds such as immunoglobulins, digestive enzymes, serum albumin, small protease-resistant peptides and undigested dietary protein fractions.

Table 10 Summary of the effect of the form of dietary nitrogen on the sources of nitrogen in terminal ileal digesta.

Source of Nitrogen	Casein		Hydrolysed casein		Crystalline amino acid		P
	Nitrogen (mg•g ⁻¹ NDDM)	SEM	Nitrogen (mg•g ⁻¹ NDDM)	SEM	Nitrogen (mg•g ⁻¹ NDDM)	SEM	
Bacterial ¹	23.5	1.19	25.3	0.22	20.9	1.34	NS
Mucin	5.8 ^a	0.20	5.1 ^a	0.43	4.1 ^b	0.27	0.05
Soluble Free Protein ²	2.6 ^a	0.40	3.4 ^a	0.25	2.1 ^b	0.13	0.02
Mucosal Cellular	2.1	0.12	2.4	0.07	1.9	0.44	NS
Protein							
Non-protein							
DNA ³	0.2	0.03	0.2	0.10	0.7	0.01	NS
Urea	1.5	0.13	1.8	0.22	1.4	0.16	NS
Ammonia	1.9	0.16	2.5	0.30	1.9	0.20	NS
Creatinine	0.1	<0.01	0.12	<0.01	0.2	0.03	NS
Non-specific ⁴	1.6 ^a	0.12	1.8 ^a	0.13	0.9 ^b	0.11	0.01
Sum	39.3	0.20	42.6	1.19	33.4	1.07	<0.01

Abbreviations: NDDM, native digesta dry matter. SEM, standard error of the mean. NS, not significant.

¹. Bacterial protein in the 14500 RCF precipitate was determined using the bacterial protein extraction lysis buffer (PELB), kit combined with a non-interfering protein assay kit, protein in the remaining fractions was calculated using the ratio of 26.4 mg DAPA/g bacterial nitrogen. ² Soluble free protein was determined from the 14500 RCF supernatant using the Bradford reagent and may include compounds such as albumin, immunoglobulins, digestive enzymes, and small peptides such as digestion resistant bioactive peptides and soluble cell debris. ³ A DNA/DAPA ratio was calculated for the 14500 RCF precipitate (each component expressed as mg•g⁻¹ DDM), and used to estimate the quantity of bacterial DNA in the other two centrifugation fractions. ⁴ This fraction contains non-identified nitrogenous material that may include non-protein compounds such as free amino acids, RNAs, amines, and the tetrapyrroles, bilirubin and biliverdin. Statistical probability shown for the concentration data (mg•g⁻¹ NDDM). Means within a row with different superscripts were significantly different.

The remaining fraction of unidentified non-protein nitrogenous material may include free amino acids, RNAs, amines, and the tetrapyrroles, bilirubin and biliverdin.

The key findings of this study were that the form of dietary nitrogen (protein, small peptides or free amino acids) has an effect upon the amount and composition of endogenous protein at the end of the ileum; either the secretion of protein into the small intestine or reabsorption of protein from the small intestine. It would appear that a significant proportion of the truly endogenous protein secretions that remain unabsorbed at the end of the ileum are mucins. Dietary casein stimulated the secretion of more mucins in comparison to a free amino acid based diet. The form of dietary nitrogen largely had no effect upon bacterial protein, ammonia and urea concentrations which collectively contributed around 70% of the total digesta nitrogen.

Acknowledgements

I would like to offer my thanks to Dr Amélie Deglaire for her assistance with the acquisition of samples from the human subjects and Dr Robert Benamouzig for the installation of the naso-ileal tubes at the start of the human trial.

Literature cited

Ahmed, S., Macfarlane, G. T., Fite, A., McBain, A. J., Gilbert, P., and Macfarlane, S. (2007). Mucosa-associated bacterial diversity in relation to human terminal ileum and colonic biopsy samples. *Applied and Environmental Microbiology*, 73(22), 7435-7442.

Allen, A., and Pearson, J. P. (1993). Mucus glycoproteins of the normal gastrointestinal-tract. *European Journal of Gastroenterology & Hepatology*, 5(4), 193-199.

AOAC. (2000). Official Method for protein (Dumas method), 968.06. *Official methods of analysis of association of official analytical chemists international*.

AOAC. (2003). Official Method of Analysis for Amino Acids *Official methods of analysis of association of official analytical chemists international* (Vol. 17th

- Edition). Gaithersburg MD: Association of Official Analytical Chemists Internationa.
- Bradford, M. M. (1976). Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Analytical Biochemistry*, 72(1-2), 248-254.
- Butts, C. A., Moughan, P. J., Smith, W. C., and Carr, D. H. (1993). Endogenous Lysine and Other Amino-Acid Flows at the Terminal Ileum of the Growing Pig (20 Kg Bodyweight) - the Effect of Protein-Free, Synthetic Amino-Acid, Peptide and Protein Alimentation. *Journal of the Science of Food and Agriculture*, 61(1), 31-40.
- Caine, W. R., Tamminga, S., Sauer, W. C., Verstegen, M. W. A., and Schulze, H. (1999). Bacterial contributions to total and endogenous recoveries of nitrogen and amino acids in ileal digests of newly weaned piglets fed protease-treated soybean meal. *Livestock Production Science*, 57(2), 147-157.
- Calbet, J. A. L., and Holst, J. J. (2004). Gastric emptying, gastric secretion and enterogastrone response after administration of milk proteins or their peptide hydrolysates in humans. *European Journal of Nutrition*, 43(3), 127-139.
- Chacko, A., and Cummings, J. H. (1988). Nitrogen losses from the human small bowel: obligatory losses and the effect of physical form of food. *Gut*, 29, 809-815.
- Chaney, A. L., and Marbach, E. P. (1962). Modified reagents for determination of urea and ammonia. *Clinical Chemistry*, 8(2), 130-and.
- Claustre, J., Toumi, F., Trompette, A., Jourdan, G., Guignard, H., Chayvialle, J. A., et al. (2002). Effects of peptides derived from dietary proteins on mucus secretion in rat jejunum. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 283(3), G521-G528.
- Czerkawski, J. W. (1974). Methods for determining 2-6-diaminopimelic acid and 2-aminoethylphosphonic acid in gut contents. *Journal of the Science of Food and Agriculture*, 25(1), 45-55.
- Darragh, A. J., Moughan, P. J., and Smith, W. C. (1990). The Effect of Amino-Acid and Peptide Alimentation on the Determination of Endogenous Amino-Acid Flow at the Terminal Ileum of the Rat. *Journal of the Science of Food and Agriculture*, 51(1), 47-56.

- Deglaire, A. (2008). *Gut endogenous protein flows and post prandial metabolic utilization of dietary amino acids in simple stomached animals and humans*. Doctor of Philosophy Doctor of Philosophy, PhD Thesis, Massey University, Palmerston North.
- Deglaire, A., Bos, C., Airinei, G., Luengo, C., Benamouzig, R., Fauquant, J., *et al.* (2007). Postprandial metabolic utilization of intact and hydrolyzed casein in humans. *FASEB Journal*, 21(5), A333-A333.
- Deglaire, A., Bos, C., Tomé, D., and Moughan, P. J. (2009a). Ileal digestibility of dietary protein in the growing pig and adult human. *British Journal of Nutrition*, 102(12), 1752-1759.
- Deglaire, A., Fromentin, C., Fouillet, H., Airinei, G., Gaudichon, C., Boutry, C., *et al.* (2009b). Hydrolyzed dietary casein as compared with the intact protein reduces postprandial peripheral, but not whole-body, uptake of nitrogen in humans. *American Journal of Clinical Nutrition*, 90(4), 1011-1022.
- Deglaire, A., Moughan, P. J., Rutherford, S. M., Bos, C., and Tomé, D. (2007). Feeding dietary peptides to growing rats enhances gut endogenous protein flows compared with feeding protein-free or free amino acid-based diets. *Journal of Nutrition*, 137(11), 2431-2436.
- Eklund, M., Mosenthin, R., Piepho, H. P., and Rademacher, M. (2008). Effect of dietary crude protein level on basal ileal endogenous losses and standardized ileal digestibilities of crude protein and amino acids in newly weaned pigs. *Journal of Animal Physiology and Animal Nutrition*, 92(5), 578-590.
- Fuller, M. F., Milne, A., Harris, C. I., Reid, T. M. S., and Keenan, R. (1994). Amino-acid losses in ileostomy fluid on a protein-free diet. *American Journal of Clinical Nutrition*, 59(1), 70-73.
- Fuller, M. F., and Tome, D. (2005). In vivo determination of amino acid bioavailability in humans and model animals. *Journal of AOAC International*, 88(3), 923-934.
- Han, K. S., Deglaire, A., Sengupta, R., and Moughan, P. J. (2008). Hydrolyzed casein influences intestinal mucin gene expression in the rat. *Journal of Agricultural and Food Chemistry*, 56(14), 5572-5576.
- Hill, M. J. (1982). Gut flora associated diseases in man. *Advances in Veterinary Medicine*, 22, 32-38.
- Keogh, J. P., Allen, A., and Garner, A. (1997). Relationship between gastric mucus synthesis, secretion and surface gel erosion measured in amphibian stomach in

- vitro. *Clinical and Experimental Pharmacology and Physiology*, 24(11), 844-849.
- Krawielitzki, K., Kreienbring, F., Zebrowska, T., Schadereit, R., and Kowalczyk, J. (1994). Estimation of N absorption, secretion and reabsorption in different intestinal sections of growing pigs using the N-15 isotope dilution method. In W. B. Souffrant and H. Hagemester (Eds.), *VIth International Symposium on Digestive Physiology in Pigs, Proceedings, Vols 1 and 2* (pp. 79-82).
- Lien, K. A., Sauer, W. C., and Fenton, M. (1997). Mucin output in ileal digesta of pigs fed a protein-free diet. *Zeitschrift Fur Ernährungswissenschaft*, 36(2), 182-190.
- Metges, C. C., Petzke, K. J., El-Khoury, A. E., Henneman, L., Grant, I., Bedri, S., *et al.* (1999). Incorporation of urea and ammonia nitrogen into ileal and fecal microbial proteins and plasma free amino acids in normal men and ileostomates. *American Journal of Clinical Nutrition*, 70(6), 1046-1058.
- Meyer-Hoffert, U., Hornef, M. W., Henriques-Normark, B., Axelsson, L. G., Midtvedt, T., Putsep, K., *et al.* (2008). Secreted enteric antimicrobial activity localises to the mucus surface layer. *Gut*, 57(6), 764-771.
- Millward, D. J., Garlick, P. J., James, W. P. T., Sender, P. M., and Waterlow, J. C. (1976). Protein turnover. In D. J. A. Cole, K. N. Boorman, P. J. Buttery, D. Lewis, R. J. Neale and H. Swan (Eds.), *Protein Metabolism and Nutrition*. (Vol. 16, pp. 49-69.). London: European Association of Animal Production, Butterworths.
- Miner-Williams, W., Moughan, P. J., and Fuller, M. F. (2009). Endogenous components of digesta protein from the terminal ileum of pigs fed a casein based diet. *Journal of Agricultural and Food Chemistry* 57, 2072-2078.
- Modigliani, R., Rambaud, J. C., and Bernier, J. J. (1973). Method of intraluminal perfusion of human small-intestine. 1. Principle and technique. *Digestion*, 9(2), 176-192.
- Montagne, L., Piel, C., and Lalles, J. P. (2004). Effect of diet on mucin kinetics and composition: Nutrition and health implications. *Nutrition Reviews*, 62(3), 105-114.
- Moughan, J. P., and Rutherfurd, S. M. (2011). Gut luminal endogenous protein: Implications for the determination of ileal amino acid digestibility in humans. *British Journal of Nutrition (In Press)*.

- Moughan, P. J. (2003). Amino acid availability: aspects of chemical analysis and bioassay methodology. *Nutrition Research Reviews*, 16(2), 127-141.
- Moughan, P. J., Butts, C. A., Rowan, A. M., and Deglaire, A. (2005). Dietary peptides increase endogenous amino acid losses from the gut in adults. *American Journal of Clinical Nutrition*, 81(6), 1359-1365.
- Moughan, P. J., Butts, C. A., van Wijk, H., Rowan, A. M., and Reynolds, G. W. (2005). An acute ileal amino acid digestibility assay is a valid procedure for use in human ileostomates. *Journal of Nutrition*, 135(3), 404-409.
- Moughan, P. J., Cranwell, P. D., Darragh, A. J., and Rowan, A. M. (1994). The domestic pig as a model animal for studying digestion in humans. In W. B. Souffrant and H. Hagemester (Eds.), *Vith International Symposium on Digestive Physiology in Pigs, Proceedings, Vols 1 and 2* (pp. 389-396).
- Nasset, E. S., and Ju, J. S. (1961). Mixture of endogenous and exogenous protein in the alimentary tract. *Journal of Nutrition*, 74, 461-465.
- Pedersen, C., Boisen, S., and Fernandez, J. A. (2002). Studies on the effect of dietary crude protein supply on the composition of ileal endogenous crude protein loss in growing pigs. *Acta Agriculturae Scandinavica Section a-Animal Science*, 52(3), 141-149.
- Rerat, A. (1995). Nutritional-value of protein hydrolysis products (oligopeptides and free amino-acids) as a consequence of absorption and metabolism kinetics. *Archives of Animal Nutrition-Archiv Fur Tierernahrung*, 48(1-2), 23-36.
- Robinson, P. H., Fadel, J. G., and Ivan, M. (1996). Critical evaluation of diaminopimelic acid and ribonucleic acid as markers to estimate rumen pools and duodenal flows of bacterial and protozoal nitrogen. *Canadian Journal of Animal Science*, 76(4), 587-597.
- Rowan, A. M., Moughan, P. J., and Wilson, M. N. (1992). The flows of deoxyribonucleic-acid and diaminopimelic acid and the digestibility of dietary fiber components at the terminal ileum, as indicators of microbial activity in the upper digestive-tract of ileostomized pigs. *Animal Feed Science and Technology*, 36(1-2), 129-141.
- Schneeman, B. O. (1982). Digestive enzyme activities from the pancreas in response to diet. In J. P. Laplace, T. Corring and A. Rerat (Eds.), *Physiologie Digestive Chez le Porc* (pp. 125-131). Paris: Institut National de la Recherche Agronomique.

- Sissons, J. W. (1981). Digestive Enzymes of Cattle. *Journal of the Science of Food and Agriculture*, 32(2), 105-114.
- Skilton, G. A., Moughan, P. J., and Smith, W. C. (1988). Determination of endogenous amino-acid flow at the terminal ileum of the rat. *Journal of the Science of Food and Agriculture*, 44(3), 227-235.
- Snook, J. T., and Meyer, J. H. (1964). Response of Digestive Enzymes to Dietary Protein. *Journal of Nutrition*, 82(4), 409-414.
- Stein, H. H., Seve, B., Fuller, M. F., Moughan, P. J., and de Lange, C. F. M. (2007). Invited review: Amino acid bioavailability and digestibility in pig feed ingredients: Terminology and application. *Journal of Animal Science*, 85(1), 172-180.
- Stern, J., Bruwer, M., Huber, F. X., and Decker-Baumann, C. (1999). Metabolic consequences after ileostomy. *Chirurg*, 70(6), 627-634.
- Turroni, F., Ribbera, A., Foroni, E., van Sinderen, D., and Ventura, M. (2008). Human gut microbiota and bifidobacteria: from composition to functionality. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology*, 94(1), 35-50.
- Ushida, Y., Shimokawa, Y., Matsumoto, H., Toida, T., and Hayasawa, H. (2003). Effects of bovine alpha-lactalbumin on gastric defense mechanisms in naïve rats. *Bioscience Biotechnology and Biochemistry*, 67(3), 577-583.
- Ushida, Y., Shimokawa, Y., Toida, T., Matsui, H., and Takase, M. (2007). Bovine alpha-lactalbumin stimulates mucus metabolism in gastric mucosa. *Journal of Dairy Science*, 90(2), 541-546.
- Wapnir, R. A., and Teichberg, S. (2002). Regulation mechanisms of intestinal secretion: implications in nutrient absorption. *Journal of Nutritional Biochemistry*, 13(4), 190-199.
- Wunsche, J., Volker, T., Souffrant, W. B., and Borgmann, E. (1991). Assessment of the bacteria-N proportions in feces and differently obtained ileal digesta of pigs. *Archiv Fur Tierernahrung-Archives of Animal Nutrition*, 41(7-8), 703-716.

Chapter VIII

General Discussion

Separating the often noxious external environment from the internal environment of multicellular organisms is a thin layer of epithelial cells, whose principal function is to keep the two environments separate and to assist in maintaining a stable internal environment, despite the ever changing conditions of the external environment in which we live. One of the essential characteristics of life is digestion – the mechanical and chemical partitioning of food macromolecules into smaller and simpler compounds that can be absorbed through the same epithelia that separates the outside from the inside. During digestion there is a significant amount of proteinaceous material secreted into the gut which is a necessary requirement of the digestive process and for the defence of the mucosa from the noxious luminal environment that persists along the GIT. Whether for the purpose of defence or for active digestion, such proteinaceous materials are secreted by specialist cells within salivary, gastric, hepatic and pancreatic glandular tissues and are termed endogenous proteins (Jansman *et al.*, 2002; Wapnir and Teichberg, 2002). The total mass of the endogenous proteinaceous secretions has been estimated to be equal to that of ingested protein (Nasset and Ju, 1961). Although not strictly endogenous, bacterial protein is commonly included in estimations of endogenous materials (Moughan, 2005) and may be the largest single contributor to the nitrogen pool of terminal ileal digesta (Caine *et al.*, 1999). A dynamic equilibrium thus exists in the GIT between dietary protein intake and the secretion of endogenous proteinaceous material into the lumen with the concomitant absorption from the gut of digested materials, both exogenous and endogenous in origin. It has long been realised that the mass of nitrogenous materials leaving the ileum represents the net balance between nitrogen intake and secretion minus the absorption of dietary nitrogen and reabsorption of endogenous nitrogen (Moughan, 2003) and represents a significant metabolic cost. Although the secretion and reabsorption of specific endogenous proteins is of fundamental interest there have been no systematic studies of the protein composition (i.e. sources of protein) of ileal digesta in humans.

In this dissertation studies are presented, the aim of which was to quantify the endogenous protein components of ileal digesta collected from the terminal ileum of both humans and pigs. The growing pig was used as an animal model for humans.

Before embarking on the determination of the endogenous proteins, preliminary work needed to be undertaken investigating suitable analytical methodologies. The first study (Chapter II) aimed at comparing five of the common techniques used to quantify mucin concentrations in mammalian ileal digesta. Mucins are a family of polydisperse molecules with high molecular mass and a high proportion of covalently-bound oligosaccharide side chains (Corfield *et al.*, 2001) which afford high resistance to acid and digestive enzymes. The unique capacity of the secreted mucins to protect the delicate epithelial surfaces of the mucosa is primarily due to the polymerisation of mucin monomers to form viscoelastic gels (Krause, 2000). Secreted mucins, 0.5 – 20 x 10⁶ Da (Bansil and Turner, 2006; Montagne *et al.*, 2004) contain a central polypeptide core of 1500 – 4500 amino acids in length, with 100-200 oligosaccharide side chains that contain 1 – 20 or more monosaccharides; such oligosaccharides may account for 50-80% of the molecule's mass (Roussel and Delmotte, 2004). In mammals, the oligosaccharides typically contain fucose, galactose, N-acetylglucosamine (GluNAc), N-acetylgalactosamine (GalNAc) and sialic acid, together with small amounts of sulphate and mannose. The isolation and quantification of mucin in ileal digesta are often undertaken using one or more of the carbohydrate markers specific to glycoproteins, such as amino sugars and sialic acid.

From a technical perspective, each of the five methods studied had both advantages and disadvantages. Of the five assays studied, the ethanol precipitation method and the two colorimetric assays (periodic acid-Schiff and phenol-sulphuric acid) appear to considerably underestimate mucin in the digesta, which calls into question the accuracy of these approaches. The hexosamine assay, using a gas liquid chromatography method, and the quantification of free and bound sialic acid together, gave information that was most useful in describing the nature of the mucins present at the terminal ileum. Following the method of Lien *et al.* (1997) mucin output in the pig was estimated using regression equations derived from the ratios of GluNAc and GalNAc in purified gastric and intestinal mucins. Using these equations gave a measure of the flow of both gastric and intestinal mucin. Coupled with the sialic acid assay an estimation of the extent of digestive degradation of the mucin could be obtained. The value of GalNAc as a mucin marker lies in its limited occurrence in dietary constituents (Lien, Sauer, and Fenton, 1997). Found in some dietary proteoglycans, GluNAc is less specific than GalNAc (Roden and Horowitz, 1978).

However neither GalNAc or GluNAc were found in the dietary material used in this study. One notable omission from the range of methods investigated was the ELISA assay used by Piel *et al.* (2004). They found that ileal digesta mucin concentrations obtained using this method were only one third of those derived from the hexosamine assay. In view of the extensive degradation of mucins passing along the GIT, as determined by the sialic acid assay, it was decided that such degradation was likely to diminish the specificity of an antibody-based method.

The mucin values obtained by ethanol precipitation were on average 43% lower than for the hexosamine assay, whilst Piel *et al.* (2004) found the difference was 33%. It is hypothesised that this stemmed from the amount of mucin degradation, causing ineffectual precipitation of the more degraded mucin fragments. In view of the results from the mucin methodology study an investigation was undertaken (reported in Chapter III) to evaluate the effectiveness of the ethanol precipitation method to quantify mucins contained in ileal digesta. The precipitation of crude mucin using high concentrations of ethanol was a poorly reproducible assay as a determined coefficient of variation of 19% suggests. As determined in the mucin methodology study the final ethanol concentration is critical and any deviation from the standard conditions leads to spurious inconsistent results. Ethanol precipitation is not specific to mucin, (Kotwal *et al.*, 1999; O'Keefe *et al.*, 1997; Weltzien *et al.*, 2003) and although the validity of this assay for the determination of mucin in human gastric juices has been accepted by some workers (Azuumi *et al.*, 1993), its effectiveness has been questioned by others (O'Keefe *et al.*, 1997; Piel *et al.*, 2004). Both Moughan *et al.* (1990) and Björling (1976) suggested that some highly glycosylated glycoproteins may remain soluble even in high concentrations of ethanol. High concentrations of both carbohydrate and protein found in the supernatant following ethanol precipitation may represent non-precipitated mucin, or other sugars and proteins not associated with mucin.

In the presently described work the precipitate (after ethanol treatment) contained 201 mg•g⁻¹ protein, 87% of which had a molecular weight in excess of 20 KDa.

Polyacrylamide gel electrophoresis stained with both Coomassie blue and periodic acid/Schiff, revealed that most of the glycoprotein had a molecular weight between 37 and 100 KDa. The molecular weight of glycoprotein in the precipitate was therefore considerably lower than that of intact mucin. Less than 50% of the total protein and

64% of the total carbohydrate present in the 14,500 RCF digesta supernatant were precipitated by the ethanol. These observations, together with an observed high proportion of free sialic acid, indicated that the glycoprotein in the ethanol precipitate was significantly degraded. The large amounts of protein and carbohydrate detected in the supernatant after ethanol precipitation indicated that the precipitation of glycoprotein using high concentrations of ethanol was incomplete. The ineffective precipitation of degraded glycoprotein subunits present in the digesta may explain why the ethanol precipitation procedure appears to underestimate digesta mucin concentration, (Piel et al., 2004). This procedure for the quantification of mucin is likely to lead to considerable error.

The GIT of all mammals is colonised by a plethora of microbial species which in adult humans is estimated to give a luminal concentration of typically 10^{11} – 10^{12} microbes/mL of digesta (Palmer *et al.*, 2007; Savage, 1977). In adult pigs the concentration may be in the order of 10^{14} indigenous prokaryotic and eukaryotic microbial cells (Shirkey *et al.*, 2006). Although the microbial diversity of the external environment is vast, the mammalian intestine is remarkable for its exclusivity, with the microbiota within the GIT being dominated by members of just two divisions of bacteria –the *Bacteroidetes* and *Firmicutes* (Ley *et al.*, 2006). Microbiota of the GIT form a diverse community (Hill *et al.*, 2002; Leser *et al.*, 2002) that profoundly influence some of the physiological processes of their animal host, (Turroni *et al.*, 2008) including processes that affect endogenous nitrogen losses such as nutrient digestion (Turnbaugh *et al.*, 2006), nitrogen cycling (Fuller and Reeds, 1998), mucus secretion (Meyer-Hoffert *et al.*, 2008), regulation of host fat storage (Backhed *et al.*, 2004), stimulating the proliferation of the intestinal epithelium (Mazmanian *et al.*, 2005) and protection against pathogens (Guarner and Malagelada, 2003; MacDonald and Monteleone, 2005). Although not strictly endogenous in origin, protein from the microbiota biomass inhabiting the GIT also contributes to the measured endogenous protein losses. Approximately 50% of the dry mass of a bacterial cell is protein (Bremer and Dennis, 1996) As the proportion of nitrogen and amino acids found at the terminal ileum, derived from microbial sources is high (nearly 45% for the pig, Caine et al., 1999), the accurate determination of protein and amino acid flows associated with the microbiota is important.

Many methods have been developed for the determination of microbial nitrogen and/or protein. Most commonly these methods fall into two categories: 1) methods for tracing bacterial protein or 2) those based on the analysis of marker components believed to be uniquely microbial in origin. In recent times D-amino acids, components of the peptidoglycan content of bacterial cell walls, have been proposed as suitably unique markers of bacterial protein, suitable markers could be D-alanine (D-Ala) (Garrett *et al.*, 1987; Ueda *et al.*, 1989), or D-aspartic acid (D-Asp) and D-glutamic acid (D-Glu) (Csapo *et al.*, 2001; Zahradnickova *et al.*, 2007 and 2005). However, the effectiveness of these markers is moot, as few researchers can agree on which of the markers is subject to the least error. For example while some researchers show that D-Ala is an effective bacterial marker, (Garrett *et al.*, 1987; Ueda *et al.*, 1989) others claim the reverse, (Quigley and Schwab, 1988; Schoenhusen *et al.*, 2008).

The aim of the work described in Chapter IV, therefore, was to compare three markers for the determination of bacterial protein in terminal ileal digesta. Firstly, collected digesta were fractionated by differential centrifugation using the method of Metges *et al.*, (1999) The three centrifugation fractions were: at 250 RCF a fraction expected to contain food particles and porcine cells; at 14500 RCF, a precipitate that was expected to contain microbial cells; and at 14500 RCF a supernatant expected to contain mainly proteins, peptides, free amino acids, soluble mucins, neutral sugars, urea, creatinine and ammonia and lysed microbial cell content. Microscopic examination of the 250 RCF precipitate and the 14500 RCF supernatant confirmed that although there were some intact bacterial cells in these fractions, their numbers were proportionally insignificant compared to the precipitate from the 14500 RCF centrifugation stage.

The three markers compared in this study were 2,6-diaminopimelic acid (DAPA), a component of the peptidoglycan of bacterial cell walls and a classic marker of microbial protein used for many years (Czerkawski, 1974; Masson *et al.*, 1991; Purser and Buechler, 1966; Weller *et al.*, 1958); D-Asp and D-Glu. As a marker of bacterial protein, DAPA has been criticised because the quantity of DAPA present in the bacterial cell wall is strongly dependent on the species and size of bacteria (Dufva *et al.*, 1982; Ling and Buttery, 1978), however, the degree of error associated with bacterial nitrogen estimations using DAPA is not known (Caine *et al.*, 1999). Whilst DAPA was judged by Robinson *et al.*, (1996) to underestimate the microbial nitrogen

pool, Csapo *et al.*, (2002) suggested that it overestimated it by some 10%. For the same reasons the validity of bacterial protein assays determined using DAPA have been called into question. However, the same can be said of the D-amino acids which also originate from the peptidoglycans present in the cell wall of bacteria.

Although the standard error of the means for bacterial protein determined using DAPA, D-Asp and D-Glu as markers were 9.6%, 10.3% and 11.3% respectively, surprisingly there was little correlation between the three markers (Pearson correlation coefficients were 0.58, -0.26 and 0.15 respectively). Such a lack of correlation between them may be the result of a systematic error such as variance in the degree of epimerization (partial racemisation) occurring during protein hydrolysis, which Csapo *et al.*, (2002) acknowledged may falsify the data obtained. Even though the protein hydrolysis procedure developed by Csapo *et al.*, (1997) to minimise epimerization was adopted, this source of error cannot be discounted. As the degree of epimerization cannot be quantified this source of error casts doubt on the reliability of using D-Asp and D-Glu as markers of bacterial protein. D-amino acids have also been detected in foodstuffs, particularly milk and milk products, as a result of microbial activity (Csapo *et al.*, 1997; Csapo *et al.*, 1995) or the heat treatment of foodstuffs (Bruckner *et al.*, 2001; Csapo *et al.*, 2007; Erbe and Bruckner, 2000). Moreover in recent years, the ratio of D- to L-amino acids in the background environment has increased as a result of water contamination due to human activities [chemical waste, fertilizers, sweeteners, cleansers and other artificial sources containing racemic mixtures of amino acids (Zahradnickova *et al.*, 2007)]. Having said this, the three markers gave broadly similar mean values for the microbial protein content of digesta. Compared to the value of a control (based on physical centrifugation and DNA contents) the mean concentrations of bacterial protein determined from the three markers (DAPA, D-Asp, and D-Glu) were; -3.4%, +9.3% and -15.6% respectively. DAPA, however, is not subject to interference from epimerization and was not detected in the dietary materials. The ratios of DAPA to bacterial nitrogen (Wunsche *et al.*, 1991) and to bacterial dry matter (Czerkawski, 1974) are well known and widely used by researchers. In comparative experiments, the quantity of DAPA and its relationship to total bacterial protein does not vary and can be used satisfactorily to determine the proportion of protein of bacterial origin in the luminal contents of the GIT (Csapo *et al.*, 2001). Taking into

account the results of this study, and that DAPA is a moiety readily assayed, it was the method of choice in the digesta composition studies to be undertaken.

Having devised suitable analytical methods the aim of the work reported in Chapter V was to quantify the endogenous components of terminal ileal digesta from growing pigs (as an animal model for humans) given a diet containing purified lactic casein.

It was found that total nitrogen made up 6.3% of digesta dry matter (DDM). Of the total nitrogen in the digesta, 73% was protein nitrogen, with 60% being soluble and present in the 14500 RCF supernatant, the remaining 40% was insoluble and found in the two centrifugation precipitates. It was found that bacterial protein nitrogen was nearly 45% of the total digesta nitrogen and suggestive of large populations of bacteria in the small intestine which is contrary to the earlier held view that the upper digestive tract is virtually devoid of microbial activity (Hill, 1982). At over 13% of the total nitrogen, mucin was determined to be the single most abundant truly endogenous component in the ileal digesta. Most of the mucin was soluble and present in the 14500 RCF supernatant, with much less insoluble mucin in the 250 RCF precipitate. The proportions of gastric and intestinal mucin were estimated to be 64% and 36%, respectively. The high proportion of gastric mucin found in the ileal digesta in this study supports the hypothesis that dietary casein stimulates the secretion of gastric proteases and acid which in turn erode the gastric mucus layer and release gastric mucins into the chyme, which are not fully digested before arriving at the terminal ileum. Nitrogen originating from sloughed porcine mucosa (intact cells) was determined as nearly 5% of the total nitrogen. The final fraction of protein nitrogen was soluble and determined to be 13% of the total nitrogen present in the digesta and would be expected to contain compounds such as immunoglobulins, digestive enzymes and small protease-resistant peptides. The non-protein nitrogen originating from DNA, urea, ammonia, and creatinine was determined to be 0.2%, 5.2%, 11.0%, and 1.8% of the total nitrogen respectively. The remaining fraction of non-protein nitrogen, containing non-identified nitrogenous material, was 8.3% and may include non-protein compounds such as: free amino acids, RNA's, amines and the tetrapyrroles, bilirubin and biliverdin.

The study in Chapter VI was designed to parallel that of the pig study and aimed to quantify the endogenous protein components of terminal ileal digesta collected from humans given a diet containing casein. Although the diet given to the human subjects was similar to that given to the pig, the collection of digesta from fit healthy subjects was via a naso-ileal tube.

The mean total nitrogen content of the digesta for the human subjects was lower than that in the pig, (3.9% and 6.4% for human subjects respectively). The reason for this difference may be related to the human subjects receiving a test meal only a quarter of that given to the pig (33g and 121g for the human and pig respectively). This may have resulted in less undigested dietary protein being present in the terminal ileal digesta collected from the human subjects. However, as a percentage of total nitrogen, the values for protein nitrogen were more similar, being 74% and 86% in the pig and human respectively. Soluble protein nitrogen, expressed as a percentage of total nitrogen, was also similar, 61% in the pig and 66% in the human.

The six most abundant amino acids detected in the digesta, in decreasing order of abundance ($\text{mg} \cdot \text{g}^{-1}$ native digesta dry matter NDDM) were: glutamic acid; glycine; aspartic acid; serine; proline and threonine. These six amino acids are present in the highest proportions of the protein core of glycoproteins from the GIT. Of these six, threonine, serine and proline are most abundant in the glycosylated region of mucin polymers, and glutamic acid, glycine and aspartic acid, are predominant in the non-glycosylated regions of mucin. (Mantle and Allen, 1989). In the pig study these six amino acids accounted for almost 52% of the total mass of amino acids although with the human subjects the proportion was somewhat higher, (almost 59%).

The proportions of microbial and nonmicrobial protein were determined to be 69% and 31% respectively. The percentage of microbial protein was relatively high when compared to that (55%) detected in the ileal digesta taken from pigs. The amount of microbial protein in the 14500 RCF supernatant was ~37%, with 29% in the 14500 RCF precipitate, and suggests that substantial degradation of bacterial cells takes place in the small intestine releasing DAPA into the soluble fraction of digesta. Bacterial dry matter was determined to be $215 \text{ mg} \cdot \text{g}^{-1}$ NDDM, a figure that represents almost 22%

of the total digesta dry matter, a value similar to $208 \text{ mg}\cdot\text{g}^{-1}$ determined by Rowan *et al.*, (1992).

The mean mucin concentration determined in the human ileal digesta was $127 \text{ mg}\cdot\text{g}^{-1}$ NDDM, a concentration that lies within the range of other published values (Lien *et al.*, 1996; Montagne *et al.*, 2004). Mucin present in the human ileal digesta represents nearly 13% of the digesta dry matter a figure almost identical to that found in the pig. Once again mucin was the single most abundant truly endogenous component found in the GIT.

The loss of proteinaceous nitrogen from both microbial and true endogenous sources was over 86% of the total ileal nitrogen losses, a value in accord with the estimates by Chacko and Cummings (1988) who gave a range of 80-85%. Bacterial nitrogen represented nearly 60% of the mean total nitrogen present in the digesta. Other proteinaceous nitrogen was determined as: mucin nearly 15%, soluble free protein (presumably from immunoglobulins, digestive enzymes, small protease-resistant peptides and cell contents) nearly 7% and that derived from intact human mucosal cells as being just over 5%. The non-protein nitrogen originating from DNA, urea, ammonia, and creatinine were determined to be 0.5%, 3.9%, 4.7%, and 0.3%, respectively. The remaining fraction of nitrogen was nearly 4% of the total nitrogen contained in the ileal digesta.

The final study reported in Chapter VII compared the composition of digesta from the terminal ileum of human subjects fed isonitrogenous diets that contained casein, enzyme hydrolysed casein, or synthetic amino acids as the sole source of dietary protein. The aim was to ascertain if the quantity and composition of nitrogen containing endogenous substances lost from the upper digestive tract of humans is affected by the nature of dietary nitrogen, (specifically intact protein, peptides or free amino acids).

The flow of native digesta dry matter (NDDM) for all diets gave a peak flow between 0.5-2.0 hours, with a gradual decrease to a fairly constant and surprisingly low rate of flow of 1-1.6 g/30 min during the final four hours of the digesta collection period. Total digesta nitrogen concentrations in humans, irrespective of diet, ranged between

32.0–41.2 mg·g⁻¹ NDDM and although there was no statistically significant difference between the CAS and EHC diets there was a significant difference between these two diets and the SAA diet for the digesta concentrations. There were also significant differences between the SAA diet and the CAS and EHC diets for the proteinaceous nitrogen and soluble protein nitrogen concentrations. There were no significant differences between the values for digesta non-protein nitrogen.

The amino acid profile of the digesta from subjects fed the CAS and EHC diets indicated that the six most abundant amino acids were those most commonly found in mucin; (in decreasing order of abundance, mg·g⁻¹ NDDM glutamic acid, glycine, aspartic acid, serine, proline and threonine). However, the digesta serine concentration for subjects receiving the SAA diet was particularly low in comparison to the CAS and EHC diets. A plausible explanation for this may be that the concentration of serine is much higher in the digesta taken from subjects given the CAS and EHC diets because of higher concentrations of mucin in those digesta. The high proportions of these six amino acids is consistent with high rates of mucin secretion throughout the GIT and the minimal rate of degradation of these macro-molecules. The high proportion of protein nitrogen found in the 14500 RCF supernatant of this study (73–77%), would indicate that a high proportion of soluble mucin was present in this fraction.

The concentrations of DAPA determined in the digesta from subjects given the three diets ranged from 0.55–0.66 mg·g⁻¹ NDDM. The mass of bacterial dry matter was calculated assuming 2.88 mg of DAPA per gram of bacterial dry matter, (Czerkawski, 1974) and ranged from 191 to 229 mg·g⁻¹ NDDM, representing 19%–23% of the total digesta dry matter, values similar to those determined by Rowan *et al.*, (1992).

There were significant differences for total protein, and total non-microbial protein between subjects given the SAA diet and those given the CAS and EHC diet. The percentage of microbial protein ranged between 69%–76%. A greater amount of the microbial protein was found in the 14500 RCF supernatants than in the 14500 RCF precipitates suggests that substantial degradation of bacterial cells takes place in the small intestine. Although the total concentration of bacterial protein is high, diet had no statistically significant effect on the quantity of bacterial protein present in the ileal digesta.

Mucin concentrations determined in the digesta collected from subjects given each diet were statistically significantly different. It would appear that the form of dietary protein has a direct effect upon the quantity of mucin secreted into the GIT and thus detected at the terminal ileum. The level of mucin determined in this study ranged from 9%–13% of the digesta dry matter and is the single most abundant truly endogenous component found in the digesta. It is interesting to note that the mucin concentration was high before the test meal was given, in all subjects irrespective of diet; and then reduced dramatically over the following 90 minutes. This may be due to the infusion of the saline solution containing polyethylene glycol. Such a high level of mucin at the start of the study period may affect the measurements of the other parameters such as protein and nitrogen. The pattern of a gradual rise in the mucin concentration between 2-8 hours was common to all three diets and most likely reflects the passage of the meal and its degradation products, together with the mucins secreted by the stomach and the small intestine until the spike between 7.0–7.5 hours when the digesta bolus reaches the terminal ileum.

Ammonia, urea and creatinine made up between 66%–77% of the non-protein nitrogen, and although urea and ammonia make up between ~4% of the digesta dry matter they contribute between 8.7%–10% of the total digesta nitrogen. The loss of proteinaceous nitrogen from both microbial and endogenous sources was between 85%–86% of total ileal nitrogen losses, a value in accord with the estimates by Chacko and Cummings (1988) who gave a range of 80-85%.

In summary and based on the overall study the following conclusions were made:

- For the determination of mucins in digesta from the terminal ileum the method of choice quantifies the amino sugars N-acetylglucosamine and N-acetylgalactosamine using gas liquid chromatography, coupled with the quantification of free and bound sialic acid. The hexosamine assay gives a measure of the ratio of gastric to intestinal mucin in pigs, and the sialic acid assay a measure of the extent of digestive degradation.
- The precipitation of crude mucin from ileal digesta using high concentrations of ethanol is ineffective, as the precipitation of degraded glycoprotein subunits present in the digesta is incomplete. This procedure for the quantification of

mucin is likely to underestimate the digesta mucin concentration and lead to considerable error.

- Although not an ideal marker of bacterial protein, the quantification of 2,6-diaminopimelic acid (DAPA) is not subject to many of the errors implicit in the determination of the D-amino acids and appears to provide accurate data. In comparative experiments, the quantity of DAPA and its relationship to total bacterial protein can be used satisfactorily to determine the proportion of protein that is of bacterial origin in the luminal contents of the GIT.
- Of the total nitrogen lost at the terminal ileum of pigs fed a casein-based diet nearly 73% was proteinaceous. Nearly 45% of the total nitrogen originated from bacterial protein, 13% from soluble free protein and 11% from mucin. Of the non-protein nitrogen in the digesta 11% was from ammonia and 5% from urea. Only 8.3% of the total nitrogen remained unidentified. Mucin was the single most abundant truly endogenous component, comprising 13% of the total dry matter.
- Of the total nitrogen lost at the terminal ileum of humans fed a casein-based diet 86% was proteinaceous. Nearly 60% of the total nitrogen originated from bacterial protein, with 15% from mucin and 6% from soluble free protein. Of the non-protein nitrogen in the digesta 5% was from ammonia and 4% from urea. Only 4% of the total nitrogen remained unidentified. Once again mucin was the single most abundant truly endogenous component, comprising 13% of the total dry matter.
- In comparison with a synthetic amino acid-based diet, casein increased endogenous nitrogen losses in both the pig and human. When peptides from dietary casein or enzyme hydrolysed casein are released into the small intestine they appear to stimulate mucin secretions from the small intestine. Changes in the form of dietary protein did not affect the proportion of bacterial protein in the ileal effluent.
- In comparison with a synthetic amino acid-based diet, both casein and hydrolysed casein affected endogenous nitrogen losses in humans.

Based upon the results presented here the following recommendations for future research are proposed:

- To confirm the effect of the form of dietary nitrogen on digesta mucin concentration.
- To investigate the effect of specific dietary bioactive peptides, such as β -casomorphin-7, on digesta mucin concentration in the pig.
- To develop methods to determine the different types of mucin in ileal digesta.
- To investigate the up-regulation of mucin genes in response to changes in the form of dietary protein.
- To investigate the absorption of bioactive peptides in the gut of humans and pigs and their effect on endogenous nitrogen losses and host physiology.

Literature cited

- Azuumi, Y., Ichikawa, T., Ishihara, K., and Hotta, K. (1993). The Validity of the Ethanol Precipitation Method for the Measurement of Mucin Content in Human Gastric Juices and Its Possible Relationship to Gastroduodenal Diseases. *Clinica Chimica Acta*, 221(1-2), 219-225.
- Backhed, F., Ding, H., Wang, T., Hooper, L. V., Koh, G. Y., Nagy, A., *et al.* (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences of the United States of America*, 101(44), 15718-15723.
- Bansil, R., and Turner, B. S. (2006). Mucin structure, aggregation, physiological functions and biomedical applications. *Current Opinion in Colloid and Interface Science*, 11(2-3), 164-170.
- Björling, H. (1976). Cohn fractionation. In B. Blombäck and L. A. Hanson (Eds.), *Plasma Proteins* (pp. 29-37). Chichester: Wiley.
- Bremer, H., and Dennis, P. P. (1996). Modulation of chemical composition and other parameters of the cell growth rate. In F. C. Neidhardt (Ed.), *Escherichia coli and Salmonella: cellular and molecular biology* (2nd ed., pp. 1553-1568). Washington DC: American Society for Microbiology.
- Bruckner, H., Justus, J., and Kirschbaum, J. (2001). Saccharide induced racemization of amino acids in the course of the Maillard reaction. *Amino Acids*, 21(4), 429-433.
- Caine, W. R., Tamminga, S., Sauer, W. C., Verstegen, M. W. A., and Schulze, H. (1999). Bacterial contributions to total and endogenous recoveries of nitrogen and amino acids in ileal digests of newly weaned piglets fed protease-treated soybean meal. *Livestock Production Science*, 57(2), 147-157.
- Chacko, A., and Cummings, J. H. (1988). Nitrogen losses from the human small bowel: obligatory losses and the effect of physical form of food. *Gut*, 29, 809-815.
- Corfield, A. P., Carroll, D., Myerscough, N., and Probert, C. S. J. (2001). Mucins in the gastrointestinal tract in health and disease. *Frontiers in Bioscience*, 6, D1321-D1357.

- Csapo, J., Csapo-Kiss, Z., Csordas, E., Fox, P. F., Wagner, L., and Talos, T. (1997). Total free and free D-amino acid content of cheeses produced by different technologies Kulonbozo technologiaval készült sajtok összes szabad- és szabad D-aminosav tartalma. *Tejgazdaság*, 57(1), 25-30.
- Csapo, J., Csapo-Kiss, Z., Stefler, J., Martin, T. G., and Nemethy, S. (1995). Influence of mastitis on D-amino acid content of milk. *Journal of Dairy Science*, 78(11), 2375-2381.
- Csapo, J., Csapo-Kiss, Z., Wagner, L., Talos, T., Martin, T. G., Folestad, S., *et al.* (1997). Hydrolysis of proteins performed at high temperatures and for short times with reduced racemization, in order to determine the enantiomers of D- and L-amino acids. *Analytica Chimica Acta*, 339(1-2), 99-107.
- Csapo, J., Schmidt, J., Csapo-Kiss, Z., Hollo, G., Hollo, I., Wagner, L., *et al.* (2001). A new method for the quantitative determination of protein of bacterial origin on the basis of D-aspartic acid and D-glutamic acid content. *Acta Alimentaria*, 30(1), 37-52.
- Csapo, J., Schmidt, J., Csapo-Kiss, Z., Varga-Visi, E., Pohn, G., and Csokona, E. (2002). Quantitative determination of protein of bacterial origin on the basis of D-aspartic acid and D-glutamic acid content. *Chromatographia*, 56, S169-S171.
- Csapo, J., Varga-Visi, E., Loki, K., and Albert, C. (2007). The influence of manufacture on the free D-amino acid content of Cheddar cheese. *Amino Acids*, 32(1), 39-43.
- Czerkawski, J. W. (1974). Methods for determining 2-6-diaminopimelic acid and 2-aminoethylphosphonic acid in gut contents. *Journal of the Science of Food and Agriculture*, 25(1), 45-55.
- Dufva, G. S., Bartley, E. E., Arambel, M. J., Nagaraja, T. G., Dennis, S. M., Galitzer, S. J., *et al.* (1982). Diaminopimelic Acid Content of Feeds and Rumen Bacteria and Its Usefulness as a Rumen Bacterial Marker. *Journal of Dairy Science*, 65(9), 1754-1759.
- Erbe, T., and Bruckner, H. (2000). Studies on the optical isomerization of dietary amino acids in vinegar and aqueous acetic acid. *European Food Research and Technology*, 211(1), 6-12.
- Fuller, M. F., and Reeds, P. J. (1998). Nitrogen cycling in the gut. *Annual Review of Nutrition*, 18, 385-411.

- Garrett, J. E., Goodrich, R. D., and Meiske, J. C. (1987). Measurement and use of D-alanine as a bacterial marker. *Canadian Journal of Animal Science*, 67(3), 735-743.
- Guarner, F., and Malagelada, J. R. (2003). Gut flora in health and disease. *Lancet*, 361(9356), 512-519.
- Hill, J. E., Seipp, R. P., Betts, M., Hawkins, L., Van Kessel, A. G., Crosby, W. L., et al. (2002). Extensive profiling of a complex microbial community by high-throughput sequencing. *Applied and Environmental Microbiology*, 68(6), 3055-3066.
- Hill, M. J. (1982). Gut flora associated diseases in man. *Advances in Veterinary Medicine*, 22, 32-38.
- Jansman, A. J. M., Smink, W., van Leeuwen, P., and Rademacher, M. (2002). Evaluation through literature data of the amount and amino acid composition of basal endogenous crude protein at the terminal ileum of pigs. *Animal Feed Science and Technology*, 98(1-2), 49-60.
- Kotwal, S. M., Gote, M. M., Khan, M. I., and Khire, J. M. (1999). Production, purification and characterization of a constitutive intracellular alpha-galactosidase from the thermophilic fungus *Humicola* sp. *Journal of Industrial Microbiology and Biotechnology*, 23(1), 661-667.
- Krause, W. J. (2000). Brunner's glands: A structural, histochemical and pathological profile. *Progress in Histochemistry and Cytochemistry*, 35(4), 259-367.
- Leser, T. D., Amenuvor, J. Z., Jensen, T. K., Lindecrona, R. H., Boye, M., and Moller, K. (2002). Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. *Applied and Environmental Microbiology*, 68(2), 673-690.
- Ley, R. E., Peterson, D. A., and Gordon, J. I. (2006). Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell*, 124(4), 837-848.
- Lien, K. A., McBurney, M. I., Beyde, B. I., Thomson, A. B. R., and Sauer, W. C. (1996). Ileal recovery of nutrients and mucin in humans fed total enteral formulas supplemented with soy fiber. *American Journal of Clinical Nutrition*, 63(4), 584-595.
- Lien, K. A., Sauer, W. C., and Fenton, M. (1997). Mucin output in ileal digesta of pigs fed a protein-free diet. *Zeitschrift Fur Ernährungswissenschaft*, 36(2), 182-190.

- Ling, J. R., and Buttery, P. J. (1978). Simultaneous Use of Ribonucleic-Acid, S-35, 2-6-Diaminopimelic Acid and 2-Aminoethylphosphonic Acid as Markers of Microbial Nitrogen Entering Duodenum of Sheep. *British Journal of Nutrition*, 39(1), 165-179.
- MacDonald, T. T., and Monteleone, G. (2005). Immunity, inflammation, and allergy in the gut. *Science*, 307(5717), 1920-1925.
- Mantle, M., and Allen, A. (1989). Gastrointestinal mucus. In J. S. Davison (Ed.), *Gastrointestinal Secretions* (pp. 202-229). London: Butterworth and Co.
- Masson, H. A., Denholm, A. M., and Ling, J. R. (1991). In vivo metabolism of 2,2'-diaminopimelic acid from gram-positive and gram-negative bacterial-cells by ruminal microorganisms and ruminants and its use as a marker of bacterial biomass. *Applied and Environmental Microbiology*, 57(6), 1714-1720.
- Mazmanian, S. K., Liu, C. H., Tzianabos, A. O., and Kasper, D. L. (2005). An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell*, 122(1), 107-118.
- Metges, C. C., Petzke, K. J., El-Khoury, A. E., Henneman, L., Grant, I., Bedri, S., *et al.* (1999). Incorporation of urea and ammonia nitrogen into ileal and fecal microbial proteins and plasma free amino acids in normal men and ileostomates. *American Journal of Clinical Nutrition*, 70(6), 1046-1058.
- Meyer-Hoffert, U., Hornef, M. W., Henriques-Normark, B., Axelsson, L. G., Midtvedt, T., Putsep, K., *et al.* (2008). Secreted enteric antimicrobial activity localises to the mucus surface layer. *Gut*, 57(6), 764-771.
- Montagne, L., Piel, C., and Lalles, J. P. (2004). Effect of diet on mucin kinetics and composition: Nutrition and health implications. *Nutrition Reviews*, 62(3), 105-114.
- Moughan, P. J. (2003). Amino acid availability: aspects of chemical analysis and bioassay methodology. *Nutrition Research Reviews*, 16(2), 127-141.
- Moughan, P. J. (2005). Dietary protein quality in humans - An overview. *Journal of Aoac International*, 88(3), 874-876.
- Moughan, P. J., Smith, W. C., and Stevens, E. V. J. (1990). Allometric Growth of Chemical Body Components and Several Organs in the Pig (20-90 Kg Liveweight). *New Zealand Journal of Agricultural Research*, 33(1), 77-84.
- Nasset, E. S., and Ju, J. S. (1961). Mixture of endogenous and exogenous protein in the alimentary tract. *Journal of Nutrition*, 74, 461-465.

- O'Keefe, B. R., Beutler, J. A., Cardellina, J. H., Gulakowski, R. J., Krepps, B. L., McMahon, J. B., *et al.* (1997). Isolation and characterization of niphatevirin, a human-immunodeficiency-virus-inhibitory glycoprotein from the marine sponge *Niphates erecta*. *European Journal of Biochemistry*, 245(1), 47-53.
- Palmer, C., Bik, E. M., DiGiulio, D. B., Relman, D. A., and Brown, P. O. (2007). Development of the human infant intestinal microbiota. *Plos Biology*, 5(7), 1556-1573.
- Piel, C., Montagne, L., Salgado, P., and Lalles, J. P. (2004). Estimation of ileal output of gastro-intestinal glycoprotein in weaned piglets using three different methods. *Reproduction Nutrition Development*, 44(5), 419-435.
- Purser, D. B., and Buechler, S. M. (1966). Amino acid composition of rumen organisms. *Journal of Dairy Science*, 49(1), 81-and.
- Quigley, J. D., and Schwab, C. G. (1988). Comparison of D-alanine and diaminopimelic acid as bacterial markers in young calves. *Journal of Animal Science*, 66(3), 758-763.
- Robinson, P. H., Fadel, J. G., and Ivan, M. (1996). Critical evaluation of diaminopimelic acid and ribonucleic acid as markers to estimate rumen pools and duodenal flows of bacterial and protozoal nitrogen. *Canadian Journal of Animal Science*, 76(4), 587-597.
- Roden, L., and Horowitz, M. I. (1978). Structure and biosynthesis of connective tissue proteoglycans. In M. I. Horowitz and W. Pigman (Eds.), *The glycoconjugates*. (Vol. II, pp. 3-71). New York: Academic Press.
- Roussel, P., and Delmotte, P. (2004). The diversity of epithelial secreted mucins. *Current Organic Chemistry*, 8(5), 413-437.
- Rowan, A. M., Moughan, P. J., and Wilson, M. N. (1992). The flows of deoxyribonucleic-acid and diaminopimelic acid and the digestibility of dietary fiber components at the terminal ileum, as indicators of microbial activity in the upper digestive-tract of ileostomized pigs. *Animal Feed Science and Technology*, 36(1-2), 129-141.
- Savage, D. C. (1977). Microbial ecology of gastrointestinal-tract. *Annual Review of Microbiology*, 31, 107-133.
- Schoenhusen, U., Voigt, J., Hennig, U., Kuhla, S., Zitnan, R., and Souffrant, W. B. (2008). Bacterial D-alanine concentrations as a marker of bacterial nitrogen in

- the gastrointestinal tract of pigs and cows. *Veterinarni Medicina*, 53(4), 184-192.
- Shirkey, T. W., Siggers, R. H., Goldade, B. G., Marshall, J. K., Drew, M. D., Laarveld, B., *et al.* (2006). Effects of commensal bacteria on intestinal morphology and expression of proinflammatory cytokines in the gnotobiotic pig. *Experimental Biology and Medicine*, 231(8), 1333-1345.
- Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., and Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature*, 444(7122), 1027-1031.
- Turroni, F., Ribbera, A., Foroni, E., van Sinderen, D., and Ventura, M. (2008). Human gut microbiota and bifidobacteria: from composition to functionality. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology*, 94(1), 35-50.
- Ueda, K., Morgan, S. L., Fox, A., Gilbert, J., Sonesson, A., Larsson, L., *et al.* (1989). D-alanine as a chemical marker for the determination of streptococcal cell-wall levels in mammalian-tissues by gas-chromatography negative-ion chemical ionization mass-spectrometry. *Analytical Chemistry*, 61(3), 265-270.
- Wapnir, R. A., and Teichberg, S. (2002). Regulation mechanisms of intestinal secretion: implications in nutrient absorption. *Journal of Nutritional Biochemistry*, 13(4), 190-199.
- Weller, R. A., Gray, F. V., and Pilgrim, A. F. (1958). The Conversion of Plant Nitrogen to Microbial Nitrogen in the Rumen of the Sheep. *British Journal of Nutrition*, 12(4), 421-429.
- Weltzien, F. A., Norberg, B., and Swanson, P. (2003). Isolation and characterization of FSH and LH from pituitary glands of Atlantic halibut (*Hippoglossus hippoglossus* L.). *General and Comparative Endocrinology*, 131(2), 97-105.
- Wunsche, J., Volker, T., Souffrant, W. B., and Borgmann, E. (1991). Assessment of the bacteria-N proportions in feces and differently obtained ileal digesta of pigs. *Archiv Fur Tierernahrung-Archives of Animal Nutrition*, 41(7-8), 703-716.
- Zahradnickova, H., Husek, P., Simek, P., Hartvich, P., Marsalek, B., and Holoubek, I. (2007). Determination of D- and L-amino acids produced by cyanobacteria using gas chromatography on Chirasil-Val after derivatization with

pentafluoropropyl chloroformate. *Analytical and Bioanalytical Chemistry*, 388(8), 1815-1822.

Zahradnickvia, H., Hartvich, P., and Holoubek, I. (2005). History and significance of chiral analysis of amino acids in biological matrices and environment. *Chemicke Listy*, 99(10), 703-710.