Nutritional Influences on Gut Physiology and Microflora in the Post-Weaned Piglet

By

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DEDICATED TO MY PARENTS

John and Peggy

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CHAPTER 5B: THE EFFECTS OF THE DIETARY ADDITION OF A YEAST-BASED NUCLEOTIDE SOURCE ON POST-WEANING PIGLETS

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ABSTRACT

In piglets, the post-weaning growth check is commonly associated with the weaning process that occurs within the European Union at approximately 3 – 4 weeks of age. The aims of the studies reported here were to investigate the contribution and importance of nutritional influences on the complex and multi-factorial problem of the post-weaning growth check in the piglet. Multi-disciplinary investigations focussed upon the relationship between post-weaning nutrition and the gut ecosystem with specific emphasis on gut physiology, immunity and microflora.

The influence of dietary acid binding-capacity (ABC) on gut morphology characteristics was investigated in 20 newly-weaned piglets up to 14 days post-weaning. Piglets offered the low ABC diet displayed more rapid recovery of villus height (after a degree of villus atrophy) than control animals ($P<0.001$). Prior to the initial villus atrophy, villus height increased significantly throughout the experimental period for both dietary treatment groups ($P<0.001$; <0.001 (L); <0.001 (Q)). In control animals, villus width was greater ($P=0.006$) compared with treatment animals, and villus width increased over time for both groups ($P<0.001$; <0.001 (L); 0.014 (Q)). Crypt depth also increased temporally ($P<0.001$; <0.001 (L)) for both dietary groups, with treatment animals exhibiting the greatest overall dietary mean ($P=0.009$). No significant differences between ileal digesta pH and feed intake levels were determined. Despite the improvements in intestinal structure post-weaning, these effects were not manifested in increased performance, *i.e.* DLWG. The improvements in intestinal structure may not have been of significant magnitude to influence performance parameters.

Dietary zinc oxide (ZnO) and avilamycin supplementation was found to exert a beneficial (although non-significant: $P>0.05$) effect on gut morphology; villus atrophy occurred over the initial 2 days post-weaning for animals fed ZnO, avilamycin or ZnO plus avilamycin (diets 2 – 4 respectively), compared to 4 days for control animals. No significant differences between intestinal coliform and lactobacilli load were established with respect to dietary treatment. Any differences observed in microflora load are most likely to be age-dependent. A positive relationship was established between dietary treatment (ZnO,

avilamycin and ZnO plus avilamycin) on daily live weight gain post-weaning (P<0.001). Although not significant (P>0.05), a positive influence of dietary ZnO supplementation on feed intake levels was apparent, which may account, in part, for the enhanced growth performance. This finding was not however manifested through modifications of intestinal morphology or the lactobacilli and coliform populations studied. This further suggests that dietary ZnO may exert an effect either luminally or systemically. Further research is required to determine the mechanism responsible for the enhanced feed intake and DLWG response.

The effects of feeding a yeast-based nucleotide source pre- and post-weaning revealed no significant differences with respect to villus height and width. Crypt depth was significantly greater in animals fed the treatment diet post-weaning (P<0.001). Post-weaning nucleotide-supplemented diets were found to significantly reduce intestinal coliform load (P=0.033). Such an effect was not evident in animals fed the diets pre-weaning, suggesting that the gut microflora may have adapted to the dietary regimen throughout the pre-weaning period.

Lymphocyte blastogenesis assays revealed that piglets fed a yeast-based nucleotide source post-weaning might be immunosuppressed at the time weaning. Conversely, when the same diets (in terms of composition) were fed from 14 days pre-weaning (study 4), no indication of immunosuppression was evident. Since no dietary effects were apparent in either study, it is postulated that this could be a general effect of the diet *per se* and not the actual dietary composition. It is however also possible that the animals involved in study 4 were experiencing hypersensitivity reactions to the pre-weaning dietary antigens. These animals were also combating an *E. coli* infection. Additional studies are however required to identify conclusively a cause and effect relationship, and elucidate the complicated interactions between nutrition or feed intake and immunobiology in the post-weaned piglet. Implementation of dietary nucleotide-supplementation from 7 days pre-weaning through to 25 days post-weaning within a commercial environment was found to enhance significantly DLWG (P<0.001).

In summary, the current work extends current knowledge and offers a greater understanding of the factors and complex process that influence the gut ecosystem and physiology in the post-weaned piglet. This thesis confirms the crucial role feed intake or, more specifically luminal nutrition, in post-weaned piglets and has highlighted key areas for future investigation.

PUBLICATIONS

Pickard, J. A., and Wiseman J. (2002). Nutritional influences on gut microflora post-weaning. *Perspectives in Pig Science* (Eds. J. Wiseman, M. A. Varley & B. Kemp). In press.

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ABBREVIATIONS AND GLOSSARY

α	alpha
AAS	atomic absorption spectrophotometry
ABC	acid binding-capacity
AGP	antimicrobial growth promoter
Avil	avilamycin
β	beta
BMC	blood mononuclear cells
bp	base pair
BPT	biochemical phenotype
BUN	blood urea nitrogen
BVR	baseline versus the rest
Bwt	body weight
Ca^{2+}	calcium
CCK	cholecystokinin
CD	crypt depth
cfu	colony forming unit
Ci	curie
Con A	concanavalin A
CONV	conventional
CP	crude Protein
CTC	chlortetracycline
d	day
δ	delta
dATP	deoxy-adenosine triphosphate

dCTP	deoxy-cytidine triphosphate
DE	digestible energy
Dendrogram	diagram showing the inter-relatedness of DNA fingerprints
DLWG	daily live-weight gain
DNA	deoxyribonucleic acid
DNA Fingerprint	DNA fragments generated by PCR reaction
DM	dry matter
DTH	delayed-type hypersensitivity
dGTP	deoxy-guanosine triphosphate
dTTP	deoxy-thymidine triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylamine diamine tetra acetic acid
Eh	oxidation / reduction potential
ETEC	enterotoxigenic *Escherichia coli*
ERIC-PCR	enterobacterial repetitive intergenic consensus sequence
EU	European Union
FCR	feed conversion ratio
g	gram
g	G force
GE	gross energy
GF	germ-free
GIT	gastro-intestinal tract
HCl	hydrochloric acid
IEL	intraepithelial-lymphocyte
IGF-1	insulin-like growth factor-1
IMS	industrial methylated spirits

IU	international unit
kg	kilogramme
kJ	kilo-joule
L	linear
LAB	lactic acid bacteria
ME	metabolisable energy
ME_m	ME maintenance
meq	miliequimolar
$MgCl_2$	magnesium chloride
mg	miligramme
ml	millilitre
mmol	milimolar
MT	metallothioncin
$NaHCO_3$	sodium bicarbonate
NSP	non-starch polysaccharide
NH_4^+	ammonium ion
P	probability
PBL	peripheral blood lymphocytes
PCR	polymerase chain reaction
PHA	phytohaemaglutinin
pM	pico-molar
POL	polynomial function
PWD	post-weaning diarrhoea
PWM	pokeweed mitogen
SCFA	short-chain fatty acids
s.e.d	standard error of the difference

S_D	dice similarity co-efficient
SI	small intestine
S1	duodenal sample
S2	jejunal sample
S3	ileal sample
S4	caecal sample
S5	colonic sample
SPF	specific pathogen free
t	tonne
TE	tris-EDTA
µm	micron
µl	microlitre
UPGMA	unweighted pair group method arithmetic average
V	volt
v/v	volume by volume
VFA	volatile fatty acid
VH	villus height
VH:CD	villus height : crypt depth ratio
VIR	virginamycin
VW	villus width
VRB	violet red bile
w/v	water by volume
Zn	zinc
ZnO	zinc oxide
$ZnSO_4$	zinc sulfate
Q	quadratic

CHAPTER 1:

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Under natural conditions weaning is a gradual process, but in modern pig production it is now common practice to wean abruptly (Fowler, 1985) and both animal and microflora require rapid adjustment to a radically different diet. At weaning, the young piglet is subject to a myriad of abrupt and simultaneous changes such as mixing, moving to a new environment, separation from the sow (and possibly littermates) and most importantly, change in food type and form associated with a move from a liquid to solid diets (which may also result in reduced water intake and decreased digestive enzyme activities). This frequently results in perturbation of the gut ecosystem. Collectively these factors often contribute to the post-weaning 'growth check', which is characterised by low voluntary food intake, poor growth performance, suppressed immunity, expression of enteric diseases and in some cases mortality. Even mild growth checks at this stage may compromise the overall growing/finishing performance (in terms of daily live-weight gain and days to slaughter). The interactions between pre- and post-weaning nutrition (including level of feed intake), gut physiology, microflora and immunology together with their relevance to health, welfare and performance are fundamental to solving the problems associated with weaning.

A major challenge for the pig industry is to formulate economically viable growth-promoting diets to ease the transition from sow's milk to weaner diets (Thacker, 1999). Presently it is not sufficient for such diets merely to satisfy the nutritional requirements of weaned pigs, but they must also modulate microbial succession, stabilise the balance of commensal microbiota, improve immune function and thus enhance disease resistance (Kelly and King, 2001a). Furthermore, although the importance of the commensal / resident bacteria for health and disease is well documented, relatively little is known of the interaction between the diet, the microflora, the host and the implications for health and disease (Bach Knudsen, 2001).

1.2 GASTRO-INTESTINAL SYSTEM IN THE PRE-WEANING PIG

1.2.1 Introduction

The maintenance of body functions requires animals to obtain energy and nutrients from food in order to carry out several body processes. Food is broken down into small particles by physical and chemical means and these structural units enter the alimentary tract. The process of food degradation within the animal is digestion. Smaller chemical compounds cross the intestinal epithelium and enter the bloodstream, in a process termed absorption. Pigs are classified as omnivorous animals with a relatively long small intestine for digestion and absorption.

The diet exerts a major influence on intestinal epithelial differentiation and growth and this is most pronounced at periods of dietary change such as birth and weaning. During neonatal life extensive changes in gut morphology, transiently elevated protein transcytosis, sustained increases in *de novo* protein synthesis and both age-related and diet induced changes in brush-border membrane digestive and transporter functions occur (Kelly and King, 2001a). Furthermore, the mucosal surface becomes colonised by successions of gut bacterial groups.

1.2.2 Morphological development

1.2.2.1 Digestive tract

The digestive tract has a tubelike structure, extends from the mouth to the anus and is divided into several components: mouth, pharynx, oesophagus, stomach, small intestine and large intestine (Figure 1.1). In the buccal cavity food is ground slightly, into small particles (assisted by the action of the teeth and tongue) to increase the surface area that aids chemical and microbiological degradation. Salivary secretions comprise primarily of water (0.99 as a proportion), with the remainder consisting of mucin, inorganic salts and some α-amylase capable of digesting starch. These provide lubrication and, due to the watery nature, facilitate mastication and deglutition. However, it is important to emphasise that mastication in the pig is not of major significance. Corring (1980) reported that in 45 kg pigs, an increase in the feed intake of a cereal-based diet resulted in both enhanced saliva volume secreted and total amylase output. Furthermore, when the same diets were fed wet, the saliva volume decreased while α-amylase output remained constant.

The pharynx is the common pathway for both food and air, whereas the oesophagus is the muscular tube that extends to the stomach, receives food for storage and is the site of digestion initiation (other than initial amylolysis through salivary amylase). The stomach is subdivided into four parts, which are continuous from one another, although they function separately: oesophageal, cardic gland, fundic gland and finally pyloric gland. The oesophageal region is the area around the cardiac region containing no glands and having the same epithelium as the oesophagus. All of the remaining three regions secrete mucus from their associated glands. In addition to mucus, which is usually secreted throughout the digestive tract, the stomach secretes hydrochloric acid (HCl) and pepsinogen (gastric glands), whereas the hormone gastrin is produced in the pyloric glands. Gastrin is synthesised in response to the amount of protein present in the stomach, and stimulates the secretion of additional HCl. Pepsinogen and HCl are secreted into the lumen of the stomach where they initiate protein digestion, and gastrin is secreted into the blood. Pepsinogen is a precursor of pepsin, a proteolytic enzyme with autocatalytic properties, and conversion occurs in the lumen under the influence of HCl, and begins at about pH 5. Optimal pepsin activity occurs at pH 1.8 to 3.5 and initiates gastric digestion by degrading protein to peptides.

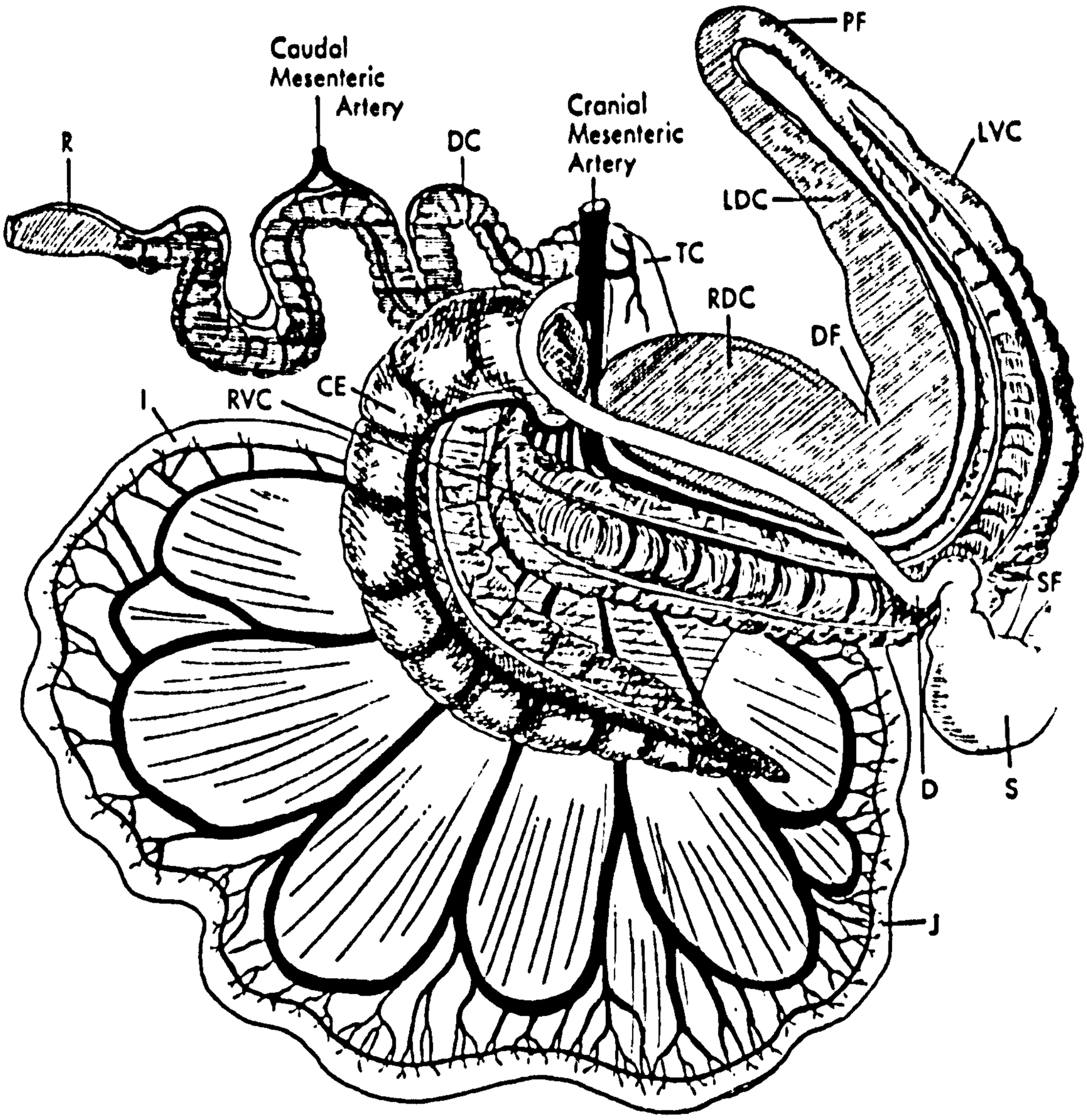


Figure 1.1: Gastro-intestinal tract of the pig. S=stomach; D=duodenum; J=jejunum; I=ileum; C=caecum; CC=coiled colon; TC=transverse colon; DC=descending colon; R=rectum (after Frandson and Spurgeon, 1992).

1.2.2.2 Anatomy and function of the small intestine

The intestinal tract provides a complex interface between the animal and its environment and has two main functions: the absorption of nutrients, enabled by a single-layered epithelium, and the barrier mechanism against microbial and nutritional antigens by cells of the immune system (Kelly and King, 2001a).

The small intestine is divided into three components based on anatomical, histological and functional characteristics: duodenum, jejunum and ileum, and can be up to 20 m in length and account for one third of the total volume capacity of the whole GIT (Moran, 1982). Stomach contents enter the duodenum, which is closely associated with the pancreas. Secretions such as pancreatic juice and enzymes enter through the pancreatic

duct, and are mixed with chyme, and bile that is formed in the liver and enters through the common bile duct. The movements of the small intestine facilitate mixing of the aforementioned enzymes and secretions. This also allows luminal digestion of carbohydrates, fats and proteins to occur, whilst maximising the exposure of the digested nutrient surface to the mucosa of the small intestine. Small intestinal activity can be either increased or decreased via the action of parasympathetic or sympathetic stimulation respectively.

1.2.2.3 Secretions of the small intestine

The intestinal juice is derived from glands located in the *crypts of Lieberkükn* called *Brunner's or duodenal glands*. These glands are stimulated by the presence of food within the small intestine to produce mucus. In addition to water, salt and mucus, intestinal cells also produce a number of enzymes and hormones. For example, when the stomach contents enter the intestine, the hormones secretin and cholecystokinin (CCK) are secreted by the mucosal cells in the dudodenum. Secretin inhibits, whilst CCK stimulates, small intestinal motility. Both are secreted into the blood and are carried to the stomach where they reduce the motility of the stomach and inhibit the release of gastrin and HCl. Secretin release is stimulated by acid perfusion of the duodenum and causes the pancreas to secrete $NaHCO_3$. The hormone CCK is secreted in response to the presence of protein and fat in the duodenum, and causes the pancreas to secrete both enzymes and pro-enzymes.

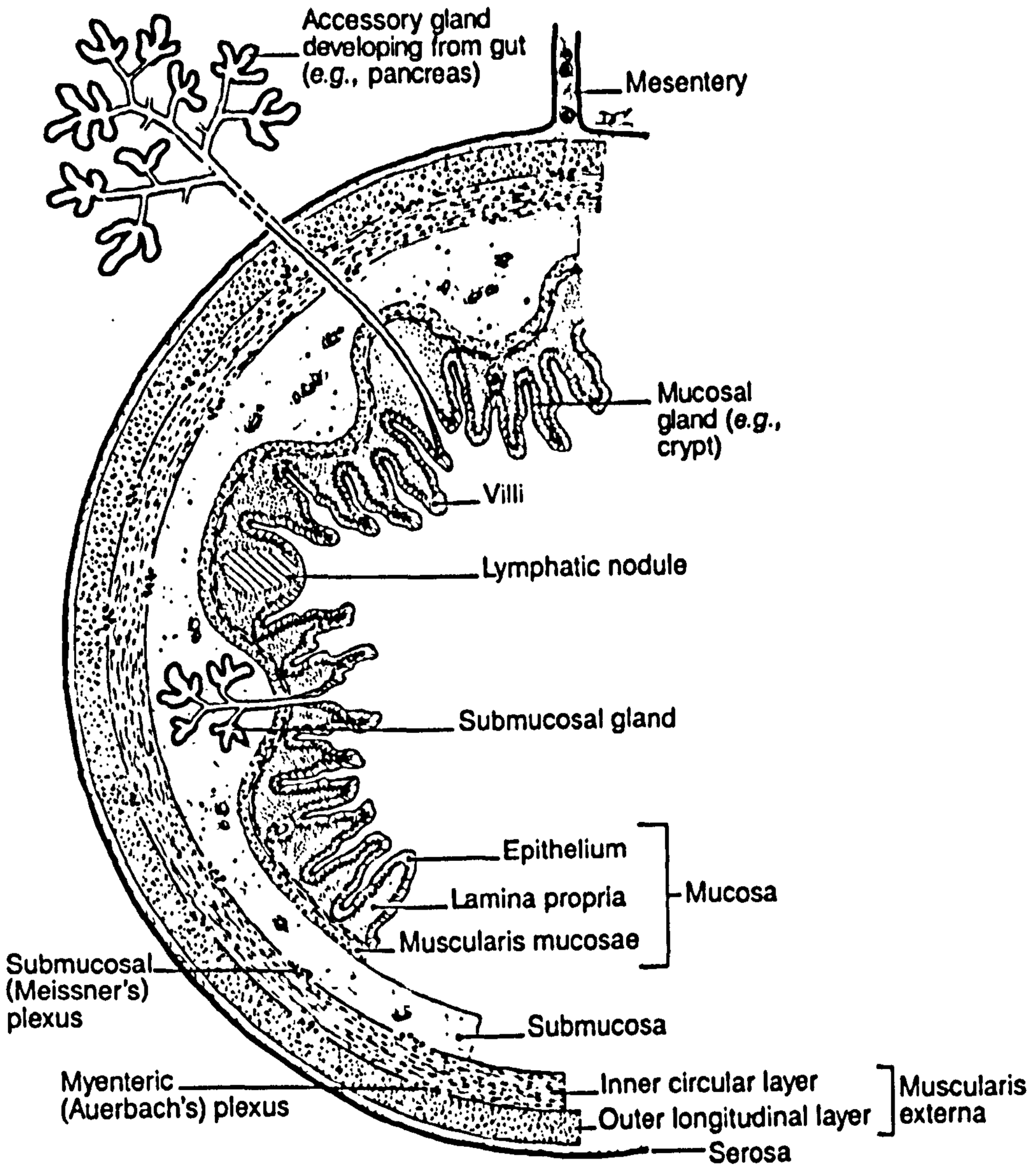


Figure 1.2: Diagrammatic representation of the wall structure of the digestive tract seen in the small and large intestine (after Cormack, 1987).

1.2.2.4 Structure of the small intestine

The small intestine possesses the same structure in all three sections (duodenum, jejunum and ileum) and consists of four principal components: serosa, muscle layer, connective tissue layer and submucosa (Figure 1.2). The serosa covers the intestine and is continuous with the mesentery, the muscle layer is comprised of two sub-layers (circular smooth and longitudinal smooth muscle), the submucosa consists of connective tissue innervated with blood vessels, lymph vessels and nerve fibres and finally the mucosa which is an inert layer full of epithelial cells. The epithelial surface area is significantly increased by the mucosa, with the *villi*, (covered with columnar cells referred to as enterocytes), and *microvilli* increasing the surface area ten-fold and thirty- to sixty-fold respectively (Caspary, 1987; Smith, 1992) thus rendering small intestinal digestion and absorption

more efficient. Furthermore, numerous circular or spiral folds of the *submucosa* of the gut increase the small intestinal surface area by a factor of 3 (Caspary, 1987). Buddle and Bolton (1992) stated that the small intestine of a 3 kg piglet at 10 days of age has a total absorptive area of 114 m^2. Between the *villi*, smaller tubular depressions are found, called *crypts of Lieberkükn* (Figure 1.3), which secrete enterokinase, (which activates trypsinogen), and a limited amount of amylase. Intestinal epithelium cells are renewed through the process of mitosis from the crypt cells.

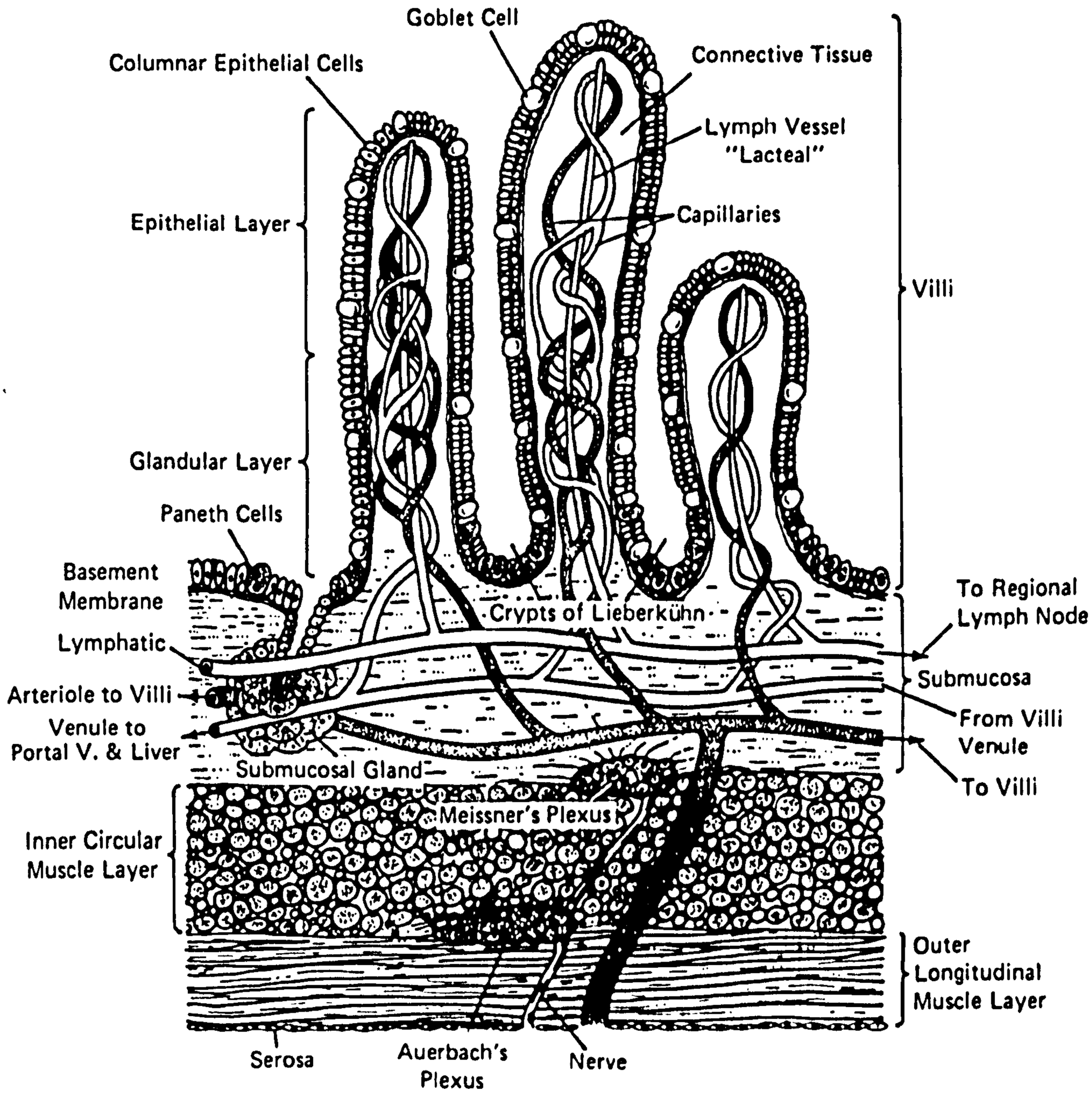


Figure 1.3: Schematic longitudinal section of small intestinal villi and its associated crypts (after Bone, 1988).

The anatomy and morphology of the small intestinal epithelium has been described previously by Kelly *et al.* (1992) and Cranwell (1995). Intestinal epithelium functionality is determined by the level of maturity and state of differentiation of the enterocytes in addition to the number of functional cells present on the intestinal mucosa (Kelly *et al.*, 1992).

1.2.3 Effect of maternal factors on intestinal structure and function

The changing dietary input during ontogeny (e.g. amniotic fluid, maternal milk, weaning, post-weaning diet) imposes different demands on the intestine and influences its morphology, enzyme equipment and transport (Buddington, 1994). Colostrum and milk feeding have been shown to promote the maturation and growth of the developing intestinal epithelium (Kelly *et al.*, 1991a; Burrin *et al.*, 1992; Kelly *et al.*, 1993; Wang and Xu, 1996) and is supported by a high rate of protein synthesis (Burrin *et al.*, 1992). The ingestion of colostrum stimulates crypt cell proliferation (Zhang *et al.*, 1997). The non-nutritive colostral factors that elicit proliferation and maturation of the gut include both immunoglobulins and biologically active substances, including insulin-like growth factor-I (IGF-I) (Xu, 1996; Zhang *et al.*, 1998). Similarly, maternal milk and colostrums (Grosvenor *et al.*, 1993; Odle *et al.*, 1996) or amniotic fluid contain many biologically active substances (Weaver *et al.*, 1988) that stimulate both intestinal growth and development (Xu, 1996), and this stimulatory effect can also be detected in various intestinal cell lines (Cera *et al.*, 1987; Ichiba *et al.*, 1992). It can therefore be postulated that some macronutrients (such as hormones and growth factors) play an important role in the growth and development of neonates (Pacha, 2000).

Specifically, in the pig, colostrums accelerate enterocyte proliferation and maturation of the intestine (Jaeger *et al.*, 1987; Koldovsky *et al.*, 1992), increase transport of electrolytes and nutrients (Bird *et al.* 1994), stimulate secretion of glucoregulatory hormones (Lepine *et al.*, 1989; Burrin *et al.*, 1992) and influence later development of the intestine (Kelly *et al.*, 1993). It is thought that these influences are mediated by the high concentrations of growth factors present in colostrum. However, nutrients in colostrums may also be influential, either directly or indirectly by stimulating secretions (Zhang *et al.*, 1997). Furthermore, variations in milk composition during lactation are associated with alterations in the synthesis and intracellular processing of brush-border hydrolases and increased transport of nutrients and electrolytes in the suckled animal (Kelly and King, 2001a).

The transfer of macromolecules across the intestinal wall represents an important transport mode that facilitates the uptake of a number of protein molecules such as immunoglobulins, growth factors, and many antigens including microorganisms (Pacha, 2000). The specific transport of macromolecules is generally achieved by binding of luminal factors to specific receptors that shuttle them across the intestinal mucosa without intracellular hydrolysis (Pacha, 2000). This macromolecular uptake is non-selective and several colostral components, including immunoglobulins and trophic factors may be rapidly absorbed through the epithelium and transported into the circulation (Gaskins, 1998). The transport of macromolecules is particularly important physiologically during the postnatal period, as it facilitates the absorption of growth factors and immunoglobulin (Ig) G from maternal colostrums and milk (Pacha, 2000). Immunoglobulins comprise the majority of the total protein fraction of colostrums, with milk-specific proteins such as casein, β-lactoglobulin and α-lactalbumin constituting the remainder. The levels of these maternally derived antibodies (immunoglobulins) are highest at day 1 post-farrowing and then decline to very low levels by the time the pig reaches 3 weeks of age. This is crucial for piglets and other ungulates such as calves that are born almost agammaglobulinemic but are capable of transferring intact immunoglobulins from ingested colostrums to the circulation during the initial postnatal period (Westrom *et al.*, 1984). In pigs, transport and macromolecular uptake declines rapidly within the first postnatal days (often referred to as 'gut closure') and although not fully understood, depends on factors such as epithelial maturation or increased intraluminal proteolysis (Telemo *et al.*, 1987; Martin *et al.*, 1993; 1997). Furthermore, Sangild *et al.* (1999) provided evidence that factors responsible for mediating closure are present in the whey fraction of colostrum. Ekstrom and Westrom, (1991) stated that intestinal closure is thought to result from a decreased transfer of the intestinal macromolecules into the blood. Additionally, several authors (for example, Leece *et al.*, 1962; Leece, 1973) have also reported that the ability of the gut to absorb macromolecules, in the case of immunoglobulins, is decreased as the total food intake increases. Moreover, since administration of immunoglobulin-free food can totally remove the ability of the gut to absorb immunoglobulins, the trigger for 'gut-closure' to immunoglobulins is not dependent on the quantity of ingested food (Leece, 1973). Once gut closure has occurred, the animals must then rely for 1-2 weeks on the persistence of circulating passively acquired immunoglobulins (Kelly, 1994).

Rapid increases in intestinal dimensions occur during the early postnatal period, particularly the first 6 h of suckling (Zhang *et al.*, 1997). Compared with unsuckled newborns, the intestines of pigs 24 h old were 29% longer and 86% heavier, with 130% more mucosa (Buddington *et al.*, 2001). Adult-type enterocytes replace the fetal-like cells over the initial 2 weeks of life, and exhibit much lower endocytic activity (Smith and Jarvis, 1977; Smith and Peacock, 1980). The cell replacement proceeds in a proximal-distal direction along the intestine, being complete in the proximal section by the time the pig is 6 days of age (Ekstrom and Westrom, 1991). Distally there is still uptake of macromolecules, which terminates by the time the pigs are 4-8 weeks of age and is concomitant with the complete replacement of the fetal-type cells.

1.2.4 Microbial colonisation of the GIT

1.2.4.1 Introduction

The gut is a dynamic ecosystem of major complexity, and gastrointestinal health can be defined as the ability to maintain a balance within this system (Melin *et al.*, 2001). Furthermore, it is important to note that the health of the gut has a direct bearing on the growth and productivity of livestock animals, since the gut comprises the body's largest organ and its activity represents a considerable proportion of the animal's protein and energy requirements (Edmunds *et al.*, 1980; Muramatsu *et al.*, 1987).

Dietary components and the gut microflora interact to exhibit a significant impact on the growth, development and intrinsic differentiation of the digestive tract in neonatal pigs. Extensive nutrient-gene interactions underpin the successful adaptation of neonates to non-liquid, lower fat, higher carbohydrate weaner diets (Kelly and King, 2001a). These changes in gene expression and the processing of gene products are entrained through diet-induced events that begin very early in postnatal life (Kelly and King, 2001a). Furthermore, Kelly and King (2001a) reasoned that due to the vast ecological diversity of the microflora, it is logical to assume that there is intimate contact and interaction between gut bacteria with subsequent homeostatic effects. The influences of the gastrointestinal microflora include: a) the growth and development of the host, b) nutritional requirements, c) morphogenesis of the GIT, d) modification (by metabolic activity) of endogenous and exogenous substances introduced into the gastrointestinal lumen and e) possession of an active role in preventing foreign microorganisms from becoming established (Visek, 1978).

Colonising microorganisms (both benign and pathogenic) induce varied host responses extensively influencing digestive physiology and gut development. Microbial colonisation stimulates the intestinal immune system producing a constitutive, low-level inflammation and epithelial changes that can have both negative and positive effects on nutrient and energy absorption in the young (Gaskins, 1997; Anderson *et al.*, 2000). Mostly non-pathogenic, anaerobic microorganisms from maternal and environmental origins continually colonise the intestinal tract and other mucous membranes (Finegold *et al.*, 1983; Conway, 1997). Generally, the dynamic balance between host physiology, diet and the gastrointestinal microbiota (involving synergic and antagonistic interactions) (Linton and Hinton, 1990) leads to the establishment of a stable microbial ecology, and the resultant bacterial population is commonly referred to as the natural, indigenous, resident, normal or commensal microflora that exert a positive influence in maintaining and establishing a healthy gut immune system (Kelly and King, 2001a).

It has been estimated that several hundred microbial species inhabit the mammalian GIT within the first few days of life (Kenworthy and Crabb, 1963; Finegold *et al.*, 1983; Drasar and Barrow, 1985), and that almost 99% of them are obligate anaerobes (Drasar and Hill, 1974). In pigs, concentrations of up to 10^9 and 10^{11} bacteria / g occur in the small intestine and colon/caecum, respectively (Smith and Jones, 1963; Leece, 1973) and much smaller numbers of microorganisms are present in the stomach. The pattern of colonisation is similar for most animals, with lactic acid bacteria, enterobacteria and streptococcci appearing first, followed by obligate anerobes (Conway, 1997). Facultatively anaerobic strains such as *Escherichia coli* and streptococci initially exist in highest numbers (Rotimi and Duerden, 1981; Stark and Lee, 1982). These bacteria may subsequently create a highly reduced environment that allows the growth of strictly anaerobic species. A preferred microbiota is that in which the so-called beneficial strains predominate over the potentially pathogenic species. Generally, those species classified as beneficial are bifidobacteria and lactobacilli, which possess various health promoting properties such as production of SCFA (which acidify gut contents), immuno-potentiation and inhibitory effects on the growth of harmful bacteria (Gibson and Roberfroid, 1995).

The bacterial population in the porcine GIT (viable counts / g fresh material) increases from 10^7-10^9 in the stomach to 10^9 in the distal small intestine, and further to 10^{10}- 10^{11} in the colon, and with increasing proximal to distal abundance of obligate anaerobes (Bach

Knudsen *et al.*, 1993; Jensen and Jorgensen, 1994). The majority of the culturable bacteria in the colon are Gram-positive strictly anaerobic streptococci, lactobacilli, eubacteria, clostridia and peptostreptococci, while Gram-negative organisms comprise about 10% of the total culturable flora (Bach Knudsen, 2001). Dietary composition exerts a huge impact on the population, composition and activity of the commensal microflora. In addition to this horizontal variation in bacterial composition, there is also a vertical gradient of species distribution (Kelly *et al.*, 1994). The mucosal environment differs both physically and chemically from the environment within the lumen. Additionally, the bacteria associated with the mucosa are likely to have a greater potential to influence the host than those present in the lumen (Kelly *et al.*, 1994).

Once established, this normal gut flora is believed to play an important role in the health of the host by competitively inhibiting colonisation by pathogenic forms by 'microbial interference or exclusion' (Tannock, 1988; Schoeni and Wong, 1994). Several microhabitats exist within the intestines that exert a selective influence on the local composition and metabolic activity of the microflora. These microniches are found in the proximal and distal intestine and are associated with the villus surface, crypts, epithelial-associated mucins and luminal mucus (Kelly and King, 2001a). Variables that contribute to the regional compositional diversity include immune reactivity, the presence of gut receptors, nutrient availability and composition, the flow of digesta, pH and Eh (oxidation/reduction potential) and available molecular oxygen (Stewart *et al.*, 1993). Furthermore, the normal microbiota in a single community may also differ from that of another of the same species due to differences in diet, husbandry or climatic conditions. The bacterial composition of the intestine of mammalian species has been reviewed by a number of authors, for example, Moughan *et al.* (1992) and Maxwell and Stewart (1994).

For growth, bacteria require energy sources and nutrients, derived either exogenously from the host diet or endogenously from sloughed-off epithelial cells, and cell secretions from the mucous blanket that coats much of the inner surface of the gut (Stewart *et al.*, 1993). Competition for available substrates and adhesion sites, on both food particles and gut mucosa, are major factors determining the composition of the intestinal microbial population. Carbohydrates and proteins are degraded by the microbiota via fermentation and the end products provide a source of energy for the host. It is important to maintain homeostasis within the gut ecosystem, as invasion by non-commensal or pathogenic flora

can result in diarrhoea (by damaging the gut wall) (Gibson and Wang, 1994). As such, the host has evolved defence mechanisms to prevent the establishment of such microbes. These include gastric acidity, rapid peristaltic movements and physical barriers such as mucin production. However, some gut species such as bacteroides, bifidobacteria, ruminococci are able to utilise mucin as a source of carbon and energy therefore reducing its protective effects (Gibson and Wang, 1994). As such a dynamic balance exists between mucin secretion by the host and its utilisation by the microbiota. A further defence mechanism is the normal indigenous flora itself, which may prevent enteric colonisation by opportunistic pathogens. This may be a consequence of competitive exclusion of pathogens by the indigenous flora. Alternatively, the indigenous flora may synthesize molecules that non-specifically stimulate the immune system or chemically interfere with the interaction between pathogens and intestinal epithelia (Kelly *et al.*, 1994).

Studies comparing germ-free (GF) and conventional (CONV) rodents suggest that normal gut flora also stimulates, or plays a regulatory role, in the development of the immune system (Thorbecke, 1959; Berg, 1983). For example, MacDonald and Carter (1979) and Woolverton *et al.* (1992) showed that the poor cell-mediated responses of GF mice could be restored by bacterial colonisation.

1.2.4.2 Bifidobacteria; beneficial effects on host health

Bifidobacterium is a major group of saccharolytic bacteria in the colon, and constitutes up to 25% of the total population in the gut of adult and 95% in newborn humans (Kawase, 1982). Bifidobacteria have been shown to exert a number of beneficial effects on the host, which are summarised in Figure 1.4

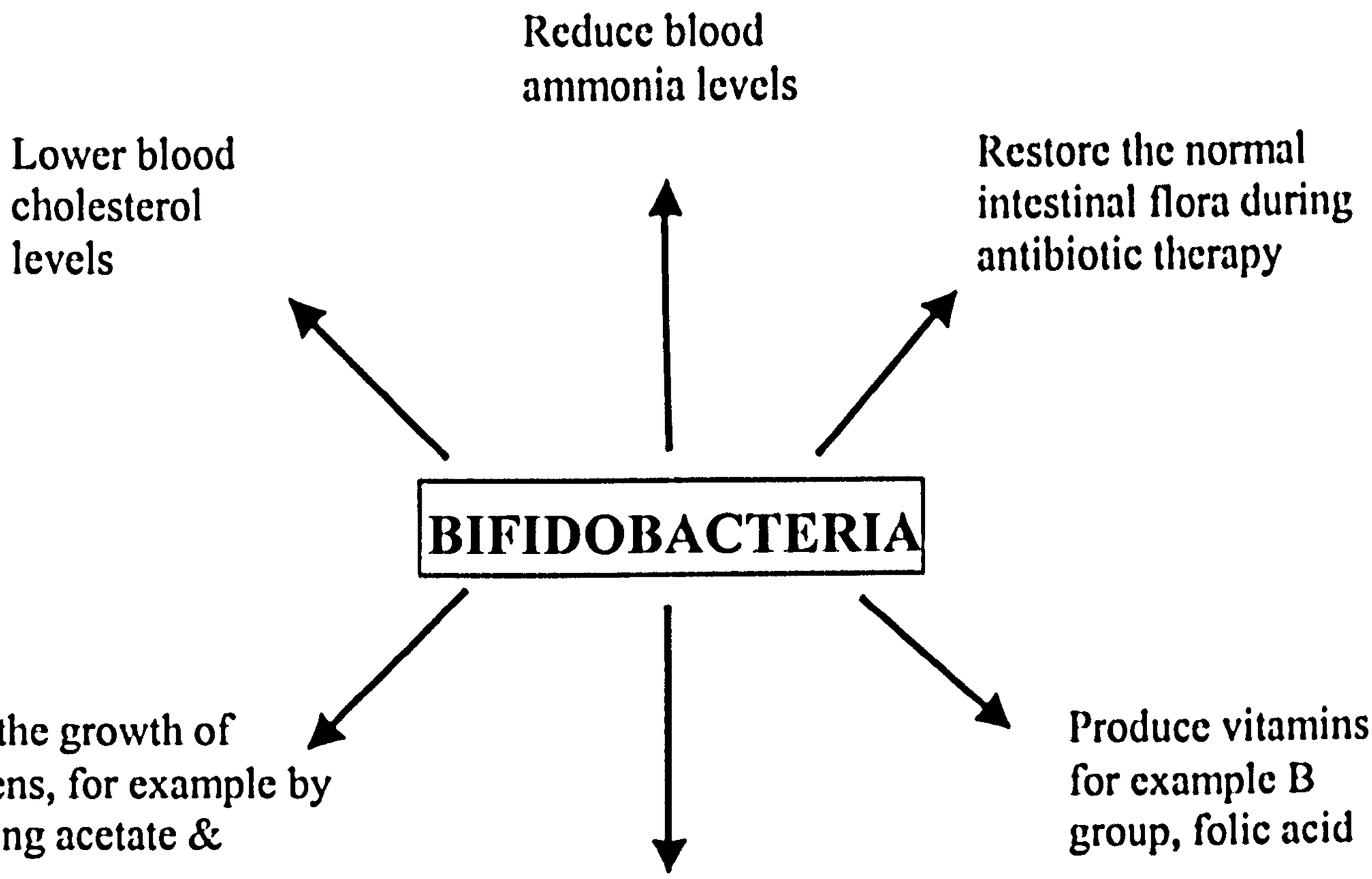


Figure 1.4: Properties of bifidobacteria that are thought to be beneficial to host health (adapted from Gibson and Roberfroid, 1995).

1. Bifidobacteria produce metabolic end products (acetate, lactate), which are strong acids that reduce the pH of the surrounding medium. This may therefore exert an antibacterial effect by inhibiting acid intolerant bacteria (Kawase, 1982; Rasic, 1983). 2. This acid production results in the protonation of ammonia and amines which are potentially toxic, to produce NH_4^+ which is non-diffusable and therefore reduces blood ammonia levels (Hansen, 1985). Furthermore bifidobacteria do not form aliphatic amines, hydrogen sulfide or nitrites (Bezkorovainy and Miller-Catchpole, 1989; Miller-Catchpole, 1989). 3. Bifidobacteria produce vitamins, principally B (Nishizawa, 1960; Liescher, 1961) as cited by Gibson and Roberfroid (1995), in addition to digestive enzymes e.g. casein

phosphatase and lysozyme (Mingawa, 1970; Kawase, 1982). 4. Specific cellular components of bifidobacteria act as immunomodulators and these bacteria have successfully restored normal intestinal flora during antibiotic therapy (Korshunov *et al.*, 1985 as cited Gibson and Roberfroid, 1995).

Although the gut can tolerate and indeed benefit from a threshold level of indigenous adhering bacteria (Fuller, 1992) it would be wrong to assume that the indigenous flora is always benign. Under conditions of nutritional or other physiological stress the indigenous population may exceed a safe threshold and constitute a pathological burden (King *et al.*, 1993; Spitz *et al.*, 1994).

Malnutrition and long term parenteral nutrition have been shown to alter intestinal morphology, impair immune function and induce bacterial overgrowth. In the extreme, these changes have been implicated in the pathophysiology of translocation of bacteria leading to sepsis (Langkamp-Henken *et al.*, 1992; Deitch, 1994; Silk and Grimble, 1994; Spitz *et al.*, 1994). Surface proteins (invasins, internalins) implicated in the process of cellular invasion and bacterial translocation are expressed by several enteric bacteria including *Shigella, Yersinia* and *Salmonella* (Wick *et al.*, 1991). Furthermore, it has recently been demonstrated that ETEC which normally colonise the mucosal surface possess specialised features which facilitate cellular invasion (Elsinghorst and Kopecko, 1992). It has been suggested that under conditions of environmental stress such invasive gene products are expressed in a wide variety of normally non-invasive organisms. An important research goal of the future is to elucidate the mechanisms by which both adherent and non-adherent bacteria become invasive (Boedeker, 1994). The invasion processes are complex and involve the interaction of bacteria and extracellular matrix components and cytoskeletal elements and plagiarisation of normal signal transduction mechanisms of the intestinal epithelium (Wick *et al.*, 1991). Dietary management is important for the prevention of colonisation of the gut by enteropathogens but also has a protective role in prevention of transepithelial translocation of bacteria.

1.2.5 Digestive enzyme activities

At weaning (3–5 weeks of age), the digestive system of the piglet is extremely immature and under-developed. Dietary carbohydrates undergo the process of enzymatic breakdown by intestinal disaccharidase activity and are an important component of the overall digestive process in young piglets. It has been suggested that in early-weaned

pigs, low feed consumption, growth check and diarrhoea in the immediate post-weaning period are related to the limited capacity of the gastrointestinal tract to digest and effectively utilise weaner diets (Hampson and Kidder, 1984; Hampson, 1994). Marked alterations in the intestinal mucosa have also been reported to reduce the ability of villi to transport amino acids (Smith, 1984), but food intake tends to be positively correlated with enzymic activity (McCracken and Kelly, 1984). Peak carbohydrase activity is known to occur between the first 0.2 and 0.3 (as a proportion) of the small intestine (Manners and Stevens, 1972; Kelly *et al.*, 1991a).

Lactase activity normally declines with age, and weaning has been reported to influence this. Lactase is present at birth in large amounts and its activity reaches its maximum during the first week of life (Aumaitre and Corring, 1978). Furthermore, Miller *et al.* (1986) reported a significant decrease in lactase activity in the intestine 5 days after weaning. In a more recent study, Kelly *et al.* (1990) reported that weaned pigs which had received creep feed pre-weaning tended to have higher lactase activity during the immediate post-weaning period compared to those which received no creep feed, however this effect was not significant (80.2 compared to 67.2 µmol min / g protein for creep and non-creep fed respectively). When uncooked cereals (320 g / kg) were given to weaned pigs lactase activity was slightly decreased (9.4 vs. 11.4 µmol min / g mucosa) in comparison to those fed a dried skim milk diet (McCracken and Kelly, 1984).

Aumaitre and Corring (1978) were unable to detect any sucrase activity at birth, or during the first week of life but it increased gradually with age; sucrase activity in the duodenum was recorded at only 6 weeks of age while it appeared in the jejunum and ileum after the first week. Although the provision of creep feed pre-weaning had no effect on the development of sucrase activity (Kelly *et al.*, 1990), it was reported to be approximately 50% higher in the high creep feed groups than zero and no creep feed in unweaned piglets.

Maltase, which is also found in the pancreatic juice, hydrolyses each maltose molecule into two glucose molecules. Activity of maltase is low at birth and increases gradually with age. Aumaitre and Corring (1978) reported that maltase activity increased up to the eighth week of life several fold (x 143). Furthermore, maltase II activity has been reported to be significantly higher during the post-weaning period for pigs fed uncooked cereal based diets (McCracken and Kelly, 1984). This is supported by findings of Miller *et al.* (1986) who weaned pigs at 6 weeks of age. Kelly *et al.* (1990) stated that creep

feeding of piglets prior to weaning was not associated with the post-weaning increase in the activity of maltase enzymes. Kelly *et al.* (1991a) later reported that total activity steadily increased during the post-weaning period and after 7 days was twice that found 3 days after weaning. Furthermore, in a study conducted by Kelly *et al.* (1991b) where gastric intubation was employed to compare continuous and restricted nutrient supply on the digestive development of pigs weaned at 14 days of age, during the first 5 days post-weaning, it was also demonstrated that total maltase activity was significantly higher for the continuously fed pigs.

1.2.6 Enzyme secretion of the pancreas

The pancreas is located near the first part of the duodenum and possesses both endocrine (produces hormones) and exocrine functions (produces sodium bicarbonate ($NaHCO_3$) and digestive enzymes). The pancreatic duct enters the initial part of the duodenum close to the common bile duct. The endocrine components of the pancreas (*islets of Langerhans*) are isolated groups of cells scattered throughout the connective tissue of the gland. The alpha (α) cells produce glucagons, the beta (β) cells produce insulin and the delta (δ) cells somatostatin. Glucagon and insulin pass directly to the blood, while somatostatin inhibits the release of insulin and glucagons from the pancreas and inhibits the release of growth hormone, thyrotropin and corticotropin from the adenohypophysis.

Only the exocrine secretions ($NaHCO_3$ and digestive enzymes or their precursors) of the pancreas are involved in the digestive process. The secretion of $NaHCO_3$ functions to neutralise or raise the pH of the acid component (HCl) of the chyme received from the stomach which then enters the duodenum. A further role is the neutralisation of the acids produced from the fermentation process in the large intestine, so that the degradation products can be absorbed. The pancreas secretes all the enzymes and their precursors (pro-enzymes) necessary for hydrolysing the proteins, fats and carbohydrates. The proteases are secreted in pro-enzyme form and include trypsinogen, chymotrypsinogen, elastase and carboxypeptidases A and B. Trypsinogen is activated by enterokinase which is present in the intestinal epithelium, to form trypsin only after it reaches the brush border of the intestinal lumen. Trypsin then becomes the activator for the other pro-enzymes. Spontaneous conversion of trypsinogen to trypsin is prevented in the pancreas by the presence of trypsin inhibitors. Control of pancreatic exocrine secretion is also dependent on the relative stimulation of the vagal autonomic nerves that innervate the

pancreas and also control the degree of secretion of three other hormones; secretin, cholecystokinin and gastrin.

Changes in enzyme secretion of the pancreas during the initial weeks of life in piglets have been examined by several workers such as Corring *at al.* (1978); Efird *et al.* (1982); Lindeman *et al.* (1986); Owsley *et al.* (1986); Makkink *et al.* (1994). For example, Corring *et al.* (1978) stated that the activities of lipase, amylase, trypsin and chymotrypsin undergo a dual-stage development; rapid growth in the first week of life and again from the 4th to the 8th week of age. The authors also stated that there is a high correlation between pancreas to body weight ratio, which increases in the first week, and from the 4th week of age growth is again more rapid (Corring *et al.*, 1978). Similarly, Efird *et al.* (1982) observed dramatic changes in the development of the digestive system from birth to the 3rd week of age and at weaning. Later, Lindemann *et al.* (1986) concluded that enzyme activity increases with age and that these were due to increases in both tissue weight and enzyme activity per gram of tissue.

Pancreatic lipase hydrolyses fats (dietary triglycerides) into products (fatty acids and glycerol), which can then be absorbed. An optimum pH for the action of trypsin in protein hydrolysis and lipase in fat hydrolysis is approximately 8. From birth to 6 weeks of age, specific activity (expressed in relation to total tissue proteins, mg RNA, or mg DNA) increased several fold (x 4.6) but after 2 weeks it was significantly lower (Corring *et al.*, 1978). In a further study, lipase activity was found to increase from birth until 4 weeks of age, when maximum levels were obtained, but post-weaning the increase in total activity was due to increases in pancreas weight (Lindemann *et al.*, 1986).

Pancreatic amylase is secreted in its active form. This carbohydrase enzyme hydrolyses starch to maltose, a disaccharide. Amylase activity has been reported to increase from 5 to 8 weeks of age (Corring *et al.*, 1978; Lindeman *et al.*, 1986; Owsley *et al.*, 1986), and this increase is most likely related to the requirement to digest dietary starch. Corring *et al.* (1978) reported that, at 3 weeks of age, specific activity was higher than at birth (x 2.8) and thereafter increased more rapidly until the 6th week (x 27.5 between 0 and 42 days of age). The same pattern of increasing activity, as for lipase and amylase, is also observed for trypsin and chymotrypsin. From birth to 3-4 weeks of age, total activities per pancreatic gland increased by 2 and 3 fold for trypsin and chymotrypsin respectively, whereas until the 8th week the increase was more rapid (by 94 and 10 fold respectively; Corring *et al.*, 1978). Moreover, this is in agreement with the findings of Owsley *et al.*

(1986), who measured activities in both the pancreas and small intestine, and reported that the total activity of amylase, trypsin and chymotrypsin increased significantly until 56 days (8 weeks) of age. Similar results for total trypsin and chymotrypsin activities were also reported by Efird *et al.* (1982) who observed a significant linear effect with age, from birth to 22 days of age; the higher proportional contribution for both enzymes was mainly attributable to the pancreas. Furthermore, Lindemann *et al.* (1986), reported a general depression in pancreatic enzyme activities, but not in gastric proteolytic activities, during the 1st week post-weaning. More recently, Kelly *et al.* (1991a) observed that anatomy, morphology and function of the gut in weaned pigs were affected by a continuous nutrient supply.

1.3 GASTRO-INTESTINAL SYSTEM IN THE WEANED PIG

1.3.1 Introduction

Over the past 50 years, the weaning age of piglets has been decreased from 10-12 weeks to current ages of 3-5 weeks (Nabuurs, 1998). At weaning, the piglet must rapidly adapt to major changes in both nutrition and environment, which is extremely stressful, both physically and psychologically. As such, the weaning transition is commonly accompanied by adverse changes in intestinal morphology, including villus atrophy, crypt hyperplasia, increased villus width, and reduced absorptive and digestive capacity and reduced brush-border enzyme activity in the small intestine (McCracken *et al.*, 1999). Specifically, factors such as the withdrawal of immunoglobulins and growth factors (present in sows' milk) at weaning, the presence of antigenic feed components and the proliferation of certain bacteria in the gut, are known to influence these morphological changes. These modifications may render the host vulnerable to infection by opportunistic and other pathogens.

1.3.2 Structure and function of the intestine

Several factors may be implicated in weaning-associated morphological changes in the pig intestine. However, the precise aetiology of changes in gut structure and function after weaning remains to be fully elucidated. The dynamic process of small intestinal cell turnover is a function of the rates of crypt cell proliferation, migration along the crypt-villus axis and cell extrusion from the villus apex via apoptosis and sloughing (Ziegler *et al.*, 1999). An increased rate of cell loss or a reduced rate of cell renewal could be responsible for villus atrophy post-weaning. If an increased rate of cell loss were apparent, then this would be associated with greater crypt-cell production and generally

increased crypt depth. However, villus atrophy may be a consequence of reduced cell division in the crypts, such as that which occurs as a result of fasting (Altmann, 1972; Goodlad and Wright, 1984; Goodlad *et al.*, 1988). Although both of these factors are likely to occur during the post-weaning period, resulting in lowering the villus height to crypt depth ratio, a reduced rate of cell renewal will have the most profound effect on gut structure.

Hall and Byrne (1989) also reported a reduction in crypt cell production rate associated with villus atrophy, a phenomenon attributed to sub-optimal intakes (refer to section 1.6.5) of energy and protein. As crypt depth declined at 3 days, Hall and Byrne (1989) suggested that villus atrophy was due to a slowed production of new cells and not an increased rate of loss of mature enterocytes from the surface of the villi. Hampson (1986) stated that the number of cells in the crypts did not increase 2 days post-weaning, but steadily increased thereafter until day 11. Whilst crypt elongation also occurred in unweaned pigs, the extent of the increase was greater in weaned animals. Therefore, as a consequence of these changes, the villus height:crypt depth ratio in weaned pigs is decreased compared to unweaned animals. Hampson (1986) suggested that this represents a balance of cell production in the crypts and cell loss from the villi that began on day 5 post-weaning and continued for at least 5 weeks. This is evident in the change from longer, finger-like villi (newborn and suckling pigs) to wider leaf-like or tongue-like villi post-weaning. However, this only occurs post-weaning with continuous absence from the sow (Hampson, 1986).

a)

b)

Figure 1.5: Scanning electron micrograph showing typical appearance of a) long fingerlike intestinal villi in a newborn pig and b) shorter leaf-shaped intestinal villi in a newly weaned pig (micrographs courtesy of D. Kelly).

1.4 IMMUNE SYSTEM OF THE PIGLET

1.4.1 Introduction

At birth, piglets are immunocompetent, however they are also immunologically naïve, having been protected within an antigen-free environment throughout gestation, and as such, antigen exposure is necessary for the development of the immune system. The antibodies present in colostrum therefore protect the neonate while it mounts an immune response to the organisms in the environment. Furthermore, the response to antigen

primes and establishes the functional immune response early in ontogeny (Zanetti, 1992). It is well established that germ-free animals that are exposed to dietary but not bacterial antigens possess only a rudimentary immune system (Kelly and Coutts, 2000b). At weaning, protective maternal antibodies are removed and the weaned pig must rely on active rather than passive immune responses to protect against environmental microorganisms. Hence weaning may render some piglets immunocompromised, which creates a window of susceptibility to infections.

The main sites of entry for microbes into the body are through the epithelial surfaces containing mucosal epithelial cells and 50% of the lymphoid mass is associated with these surfaces. These are collectively called the mucosa-associated lymphoid tissues (MALT) and include NALT (nasal-associated lymphoid tissue), BALT (bronchus-associated lymphoid tissue), GALT (gut-associated lymmphoid tissue) and lymphoid tissue associated with the genito-urinary system.

1.4.2 General overview of the immune system

The primary function of the immune system is the elimination of infectious agents and minimisation of any damage. Broadly, the immune system can be separated into two categories: innate and acquired, the main difference between the two being that acquired is highly specific for a particular pathogen. The primary immunological organs consist of the bone marrow and the thymus, and the secondary organs include spleen, mesenteric lymph nodes and Peyer's patches (Kelly and King, 2001c). Immune responses are produced primarily by leucocytes, which can be grouped into two major categories; lymphocytes and phagocytes. The latter group includes monocytes, macrophages and polymorphonuclear neutrophils and bind to the invading microorganisms, internalise and destroy them. These cells use primitive non-specific recognition systems enabling them to bind to a host of microbial products, hence they mediate innate immune responses. The lymphocytes are central and exclusive to the specific immune recognition of foreign antigens, both inside host cells and outside in the tissue fluids or blood. The lymphoid progenitor cell has the capacity to differentiate into T-cells (t-helper (Th) 1 and 2) or B-cells, and this is principally determined by the microenvironment to which they migrate (Kelly and Coutts, 2000b). T-cells develop from precursors in the thymus, whereas mammalian B-cells differentiate in the fetal liver and postnatal bone marrow.

B-cells combat extracellular pathogens and their products by releasing antibody, which binds to the antigen. In the gut, this antibody is mainly an immunoglobulin (Ig) isotype. The antigen could be a molecule on the surface of a pathogen or a toxin, which it produces. T-lymphocytes posses a broader range of activities and are primarily involved in antigen recognition and clearance, and in co-ordinating the response of B-cells. For example, some are involved in the control of B-lymphocyte development and antibody production. A further group interacts with phagocytic cells to assist in the destruction of the pathogen that they have taken up. A final set of T-lymphocytes recognises cells infected by virus and destroys them. Interactions between T- and B-cells, as well as antigen presenting cells, are critical to the development of specific immunity. Phagocytic cells remove particulate antigen by phagocytosis and are derived from myeloid progenitor cells. Macrophages, which are mononuclear phagocytes, are also involved in antigen presentation to T-cells (Kelly and Coutts, 2000b).

1.4.3 Cytokines

Cytokines are small protein, signalling molecules, secreted by cells in response to a stimulus and can act in an autocrine or a paracrine manner. One of the most important roles of cytokines is in controlling the activity of lymphocytes. For example, intestinal epithelial-derived cytokines also play key roles in the cellular and functional development of the IEL (intra-epithelial T-lymphocytes) and lamina propria compartments; however, the specific cytokines involved and modes of action have yet to be identified. Other functions include regulation of the amplitude and duration of immune responses and the activation of immune cells and the extent of their cytostatic, cytotoxic and antiviral activities (Reeves and Todd, 1996). There is a wide range of different cytokines that exert their effects in the immune system. These include interleukins (IL-1 etc.), growth factors and necrosis factors and there are more than 100 in total. Cytokines are highly multifunctional and as such different cytokines have overlapping functions, which can yield them redundant. However, some cytokines can in combination function in a synergistic manner. For the pig, published reports on intestinal epithelial cell cytokines have not yet been identified.

1.4.4 Immune mechanisms operative at mucosal surfaces

The intestinal immune system is organised anatomically to provide several layers of defence with final protection being effected through complex interactions among the various cell types that reside in distinct locations. Cellular immune components of the

intestinal immune system include dispersed or non-organised cells found throughout the epithelium (lamina propria leukocytes and IEL), as well as highly organised lymphoid follicles such as Peyer's patches and lymph nodes. In addition to providing a protective physical barrier, the intestinal epithelium actively contributes to intestinal immunity by transducing information regarding the relative state of intestinal health to resident or transient immune cells in the underlying lamina propria. Those functions are mediated through the ability of intestinal epithelial cells to present foreign antigens and to synthesise, secrete, and respond to numerous cytokines (Gaskins, 1997).

1.4.5 Immunologic functions of the intestinal epithelium

Throughout the intestine, the outermost epithelial cell monolayer is organised into two morphologically and functionally distinct compartments, the crypt regions containing stem cells and Paneth cells, and the villi (small intestine) or epithelial cuffs (large intestine) containing one of several terminally differentiated epithelial cell types (Gaskins, 1997). The formation of tight junctions between adjacent epithelial cells in mature epithelia provides a key innate component of mucosal defence by serving simply as a physical barrier to the external environment (Gordon, 1989; Potten and Loefler, 1990). Crypt stem cells move in a polarised fashion at a rate of one to two cell positions per hour, up the villus base towards its tip (Potten and Loefler, 1990). During the translocation process, epithelial precursors differentiate into enterocytes, goblet cells, or enteroendocrine cells (Gordon, 1989). Upon reaching the apical area of each villus, the resulting differentiated cells are extruded into the intestinal lumen. The process of continual desquamation and renewal of the gut epithelium plays a key defensive role by limiting opportunities for pathogens to colonise epithelial cells. Two epithelial cell types that also contribute important innate defence functions are the Paneth cells which synthesise and secrete anti-bacterial peptides (Lehrer *et al.*, 1993) and the mucin-producing goblet cells (Neutra and Forstner, 1987). Although certainly key protective components of the neonatal intestine, the developmental biology of both Paneth and goblet cells has not yet been described for the pig.

Being the first site of intimate contact with the external environment, the epithelium is in direct contact with IEL and the numerous immune effector cells located in the laminar propria. Indeed, complex communication networks between epithelial and submucosal cells, via a variety of bioactive molecules, appear to underlie concerted inflammatory responses to luminal insults in the intestine (Gaskins, 1997). For example, in response to

bacterial toxins or food antigens, activated lamina propria cells secrete cytokines and bioactive lipids which collectively increase motility and blood flow to the intestine, while inhibiting absorption and stimulating water and ion secretion by intestinal epithelial cells. When integrated, these physiological responses culminate in secretory diarrhoea, the common and major symptom of intestinal inflammation, regardless of precipitating insult (Hinterleitner and Powell, 1991).

Intestinal epithelial-derived cytokines also play key roles in the cellular and functional development of the IEL and lamina propria compartments; however, the specific cytokines involved and modes of action have yet to be identified.

1.4.6 Influence of microflora

Although bacteria play an important role in the development of the intestinal immune system (Gaskins, 1997; Gaskins, 1998; MacDonald and Pettersson, 2000), some may actually also help to reduce maintenance costs of the gastrointestinal system (Kelly and King, 2001a). Commensal and pathogenic bacteria have evolved a diverse range of mechanisms that promote their survival within the gut ecosystem. Bacteria can produce a vast array of cytokine-inducing or cytokine-modulating molecules that will regulate or direct the host response. Certain of these factors may promote the virulence and pathogenic potential of bacteria but others, paradoxically, may facilitate the maintenance of the indigenous microflora by beneficially regulating the immuno-inflammatory status of the gut (Kelly and King, 2001a). Furthermore, commensal microorganisms colonising the intestinal mucosa provide a barrier effect against pathogens by using a variety of mechanisms, such as occupation of niches, competition for nutrients and production of antimicrobial substances. Moreover, evidence is accumulating that these symbiotic microorganisms, including certain lactic acid bacteria (LAB) can modulate the homeostasis of the host's defence mechanisms, both innate and adaptive immune functions. The molecular basis and importance of crosstalk between the luminal microorganisms and the intestinal epithelium and immune cells is beginning to emerge (Kelly and King, 2001a).

1.5 GROWTH PROMOTION POST-WEANING

1.5.1 Introduction

Antibiotics are well established in animal production where they have been historically used as a prophylactic against bacterial infection, therapy and as antimicrobial growth promoters (AGP). The use of antimicrobial agents for food animals may cause problems in the therapy of infections by selecting for resistance among bacteria pathogenic for animals or humans (Aarestrup, 1999). The emergence of resistant bacteria and resistance genes following the use of antimicrobial agents is relatively well documented and it seems evident that the use of all antimicrobial agents will encourage selection for resistance.

1.5.2 Antibiotic addition

AGPs have been utilised within the agricultural industry for some 30 years even though their exact mode of action has not yet been fully elucidated. Concern regarding the predicted emergence of resistant strains of pathogenic species which may then be transferred to other bacteria, thus rendering them resistant (Aarestrup, 1999), has however, provoked an increasing public rejection of this farming practice. As a result of the risk of transmission of antibiotic resistance from food animals to man, the EU banned the use of virginamycin, tylosin phosphate, spiramycin and zinc bacitracin in animal feedstuffs from 1 July 1999 (Wegener *et al.*, 1999). Furthermore, the EU has recently produced legislation to ban the 4 remaining products (flavophospholipol, monensin sodium, salinomycin sodium and avilamycin) by 2006. There will be minimal, if any, benefit in withdrawing antibiotic growth promoters if this is accompanied by an escalation in the use of such products for clinical purposes. Hence, this has placed new emphasis on utilising the diet to control enteric bacterial infections, promote performance and the role and importance of gut health in achieving this in pigs. Moreover, alternative strategies to ensure a smooth and economic transition from the suckling to post-weaning period are required.

Although the precise mechanisms underlying the beneficial effects of antibiotics remains unclear, several mechanisms have been proposed. Bach Knudsen (2001) outlined a number of mechanisms, which may be responsible; 1. nutrients are more efficiently absorbed and fewer are utilised by the gut wall due to a thinner epithelium; 2. more nutrients are available to the host because of a reduced flora with subsequent alterations in epithelial functions within the porcine small intestine; 3. there is a reduction in harmful

gut bacteria which may reduce performance (due to low-level inflammation) and cause subclinical infection; 4. production of growth-suppressing toxins or metabolites is reduced; 5. microbial deconjugation of bile acids is reduced. These are in agreement with hypotheses of Visek (1978) and Andersen *et al.* (1999). Moreover, the response to dietary antibiotic supplementation is often greater in younger, unthrifty or stressed animals maintained under adverse management and environmental conditions. Basically, antibiotics are thought to exert their effects through the increased utilisation of nutrients and a reduction in the maintenance costs of the gastrointestinal system.

1.5.3 Alternatives feed strategies

Gut microflora can play a crucial role in host health, hence manipulation of the gut ecosystem seems a possible method in promoting health. There are numerous alternative strategies including dietary supplementation with enzymes, copper sulfate, zinc oxide, acidification, pro- and pre-biotics, herbal extracts and spices, or immuno-potentiators. At present 47 enzyme-products are provisionally authorised within the EU. The latter supplements are perceived as being more 'natural' in concept and are therefore likely to be more readily accepted by the consumer.

Probiotics, are often defined as 'a live microbial food supplement that beneficially affects the host animal by improving its intestinal microbial balance' (Fuller, 1989; Fuller, 1992; Fuller and Gibson, 1997). Probiotics are usually based on lactic acid producers such as lactobacilli and bifidobacteria and have been used in animals and humans to change the composition or balance of the gut flora. Postulated health advantages associated with probiotic intake include 1) alleviation of symptoms of lactase deficiency, 2) enhanced natural resistance to infectious diseases of the GIT, 3) improved digestion, and 4) stimulation of gastrointestinal immunity (Fuller, 1989; Fuller, 1992; Gibson and Roberfroid, 1995). However, any changes in colonic microbiota are often transient and the colonisation of exogenous bacteria therefore becomes limited (*i.e.* the necessity to feed probiotics on a daily basis to replace the bacteria which are washed out of the colon).

A prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon (Gibson and Roberfroid, 1995). Intake of prebiotics can significantly modulate the colonic microbiota by enhancing the numbers of specific, beneficial bacteria. Such compounds include nondigestible carbohydrates (such as oligo-

and polysaccharides, resistant starch), some proteins and peptides and certain lipids such as ethers and esters. For example, evidence suggests that fructooligosaccharides are selectively fermented by most strains of bifidobacteria (Wang and Gibson, 1993).

Synbiotics are 'a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the GIT, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare' (Gibson and Roberfroid, 1995). This combination of pre- and probiotics could improve the survival of the probiotic organism, because its specific substrate is readily available for its fermentation, and the resultant advantages include a combination of the benefits of live microorganisms and prebiotics to the host (Collins and Gibson, 1999). This could produce a number of benefits including improved survival of live bacteria in food products; enhanced number of ingested viable bacteria reaching the colon; stimulation of the growth and implantation of both exogenous and endogenous bacteria in the colon; and activation of the metabolism of these bacteria (only metabolically active bacteria can promote health).

1.6 PROBLEMS ASSOCIATED WITH THE POST-WEANING PERIOD

1.6.1 Introduction

Numerous micro-ecosystems exist within the GIT, and a high diversity of the bacterial flora within these micro-ecosystems enhances the stability of the microbial population and contributes to a high colonisation resistance against invading pathogens (Hentges, 1983; Kuhn *et al.*, 1993). Changes/fluctuations in the stability of this flora may result in overgrowth of indigenous and/or exogenous pathogenic bacteria possibly resulting in disease particularly diarrhoea.

Enterotoxigenic *E. coli* and rotaviral infections are common causes or contributing factors to scours, or diarrhoea, in suckled and weaned pigs. Possibly the most important predisposing factor in susceptibility of suckling pigs to infections is insufficient uptake of colostrum in the first hours of suckling which provides protective levels of IgA and other beneficial factors. A second important predisposing factor to enteric infections is the prevalence and expression of membrane and mucin glycoconjugates that serve as binding sites for enteropathogens (Kelly *et al.*, 1994; Thacker, 1999; Kelly and Coutts, 2000a; Kelly and King, 2001a). The chemistry and distribution of bacterial and viral binding

sites on gut mucosal surfaces play important roles in determining host and tissue susceptibility and in triggering host responses. This is particularly noticeable in neonates, where both beneficial and harmful swings in microbial balance can accompany epithelial differentiation (Kelly *et al.*, 1992; Stewart *et al.*, 1993). Other predisposing factors in such infections include: inadequate feed and water intake; inadequate gastric acid secretion and unstable/fluctuating microbiota (Kelly *et al.*, 1994; Thacker, 1999; Kelly and Coutts, 2000a; Kelly and King, 2001a).

1.6.2 Voluntary feed intake / post-weaning anorexia

Evidence suggests that voluntary feed intake immediately post-weaning has a fundamental role in the subsequent health and performance of the animal. For example, malnutrition studies have provided evidence that diet influences intestinal transport. Moreover, malnutrition and fasting have been shown to result in dramatic changes in mucosal barrier functions, and also modulate intestinal transport of nutrients and ions. Specifically, the permeability of the intestinal epithelium for macromolecules is increased (Rothman *et al.*, 1985; Carey *et al.*, 1994) and the content of mucosal IgA and the secretory component is markedly diminished (Ha and Woodward, 1997). Post-weaning malnutrition predisposes to infection by compromising the barrier and immune functions of the gut. At the same time, the infections adversely influence dietary intake and absorption and cause loss of endogenous nutrients (Calder and Jackson, 2000).

1.6.2.1 'Luminal nutrition' and gut structure and function

The weaning transition period usually results in a critical period of underfeeding during which the piglet needs to eat and adapt to digest the solid feed. Feed intake of piglets post-weaning is very low and both the extent and duration of underfeeding are extremely variable (Figure 1.6). This has both immediate and mid-term and marked effects on piglet growth, energy metabolism, and on metabolic changes associated with endocrine adjustments. Le Dividich and Seve, (2000) observed that the level of metabolisable energy (ME) intake achieved at the end of the first week post-weaning ranged between 700 and 800 kJ ME/$kg^{0.75}$, irrespective of weaning age, and that this was only 60-70% of the pre-weaning milk ME intake; it was also stated that the pre-weaning ME intake is not achieved until approximately 2 weeks post-weaning. A reduction in feed intake immediately post-weaning (up to day 7) has also been shown to result in an increased critical body temperature (Le Dividich *et al.*, 1979 as cited by Le Dividich, 1981); this is further supported by later studies of Le Dividich *et al.* (1980).

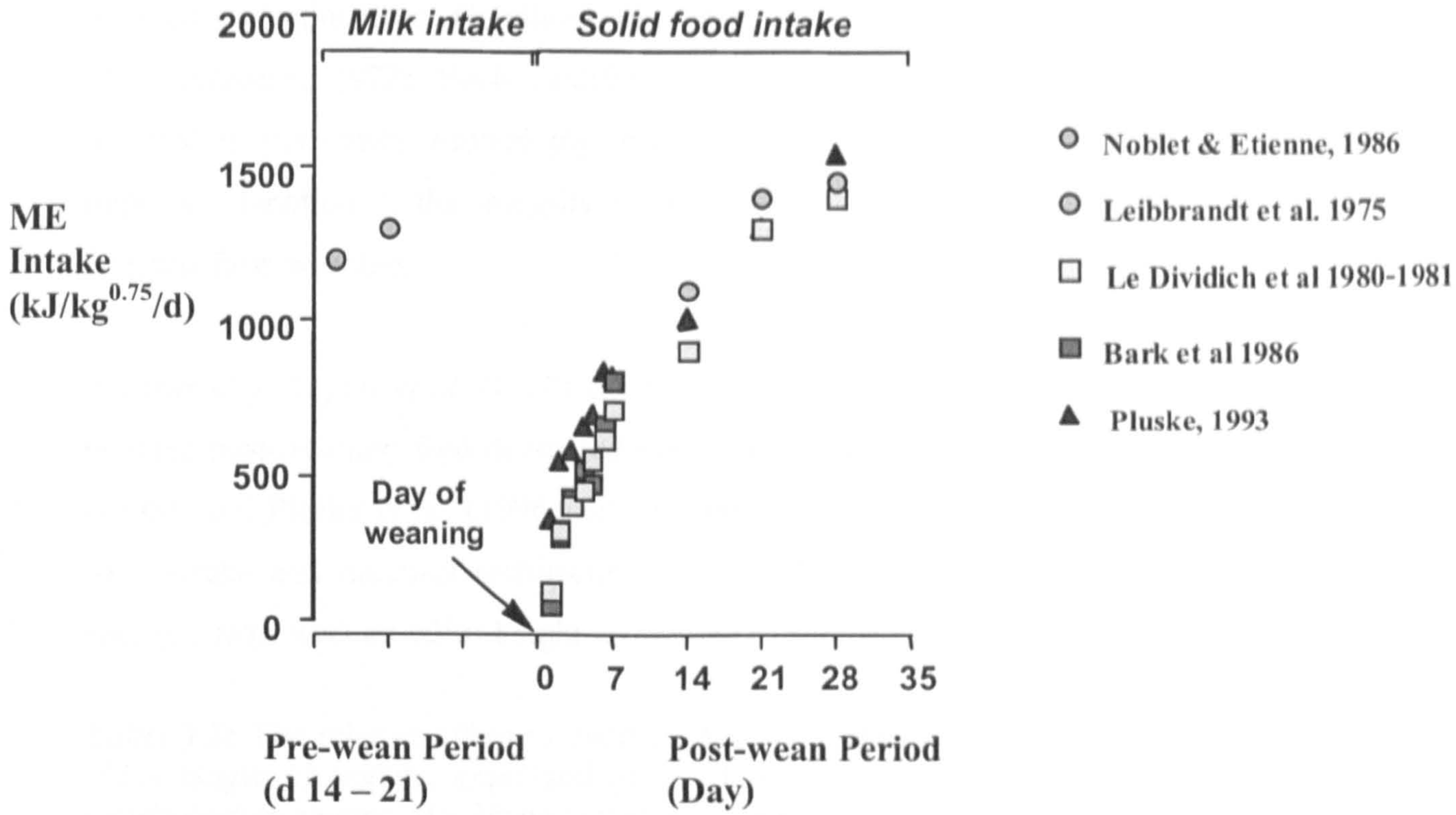


Figure 1.6: The voluntary feed intake of pigs pre- and post-weaning (expressed as metabolisable energy (ME) per kg metabolic live weight) (adapted from Le Dividich and Seve, 2000)

It is well established that feed intake and subsequent nutrient flow along the gut is essential for its structural and functional maintenance (Diamond and Karasov, 1983; Kelly *et al.,* 1992). The consequences of this lack of nutrients to the small intestinal lumen immediately post-weaning include detrimental effects on the rate of cell differentiation and cell turnover. This presence of food within the GIT has both direct and indirect effects on epithelial cell proliferation (Johnson, 1987). For example, Burrin *et al.* (2000) performed a study in which suckling piglets were weaned and fed enterally in order to investigate the minimum amount of enteral nutrition necessary for the stimulation and maintenance of intestinal growth. The authors reported that 40% of total nutrient intake was required to increase mass and protein content of the intestine and 60% of total nutrient intake was necessary to stimulate proliferative activity (as determined from analysis of DNA content, crypt depth and 5-bromodeoxyuridine incorporation into crypt cell nuclei).

Furthermore, villus atrophy and reduced crypt-cell production rates have all been demonstrated to be consequences of either dietary restriction (e.g. Nunez *et al.*, 1995), intravenous feeding (e.g., Goodlad *et al.*, 1992) or starvation (e.g., McNeill and Hamilton, 1971; Altmann, 1972). Such modifications in small intestinal morphology have been reported in the newly weaned pig, hence it seems likely that luminal nutrition is an important function in the integrity of the growth, structure and function of the small intestine post-weaning.

Additionally, Deprez *et al.* (1987) and Cera *et al.* (1988) demonstrated that feeding a dry pelleted post-weaning feed decreased villus height. More specifically, McCracken *et al.* (1995), and Pluske *et al.* (1996a; b) illustrated the interdependence between voluntary food intake and mucosal architecture. Table 1.1 provides the effect of post-weaning energy intake level on villus height, expressed as percentage of villus height at weaning.

Table 1.1: The relative effect of energy intake post-weaning on average small intestinal villus height of piglets, expressed as % of that at weaning (GE: gross energy; ME: metabolisable energy; MJ: Mega joules; BW: body weight (Adapted from Bruininx *et al.*, 2001).

Reference	Energy Source	Energy Intake		Age (days)		Villus height (%)
		MJ day^{-1}	Unit	Weaning	Slaughter	
Pluske *et al.*,						
(1996a)	Starter diet	5.7	GE	28	33	-30
	Ewe milk	7.4	GE	28	33	-2
(1996b)	Cow milk	2.3	GE	29	34	-27
	Starter diet	5.1	GE	29	34	-18
	Cow milk	5.2	GE	29	34	-4
	Cow milk	8.9	GE	29	34	.+11
(1996c)	Cow milk	5.5	GE	28	33	-4.8
Kelly *et al.*,						
(1991b)	Starter diet	2.9	GE	14	20	-55
van Beers-Schreurs						
(1996)	Starter diet	0.53	ME BW$^{-0.75}$	28	32	-40
	Sow milk	0.48	ME BW$^{-0.75}$	28	32	-35
	Sow milk	1.4	ME BW$^{-0.75}$	28	32	-11

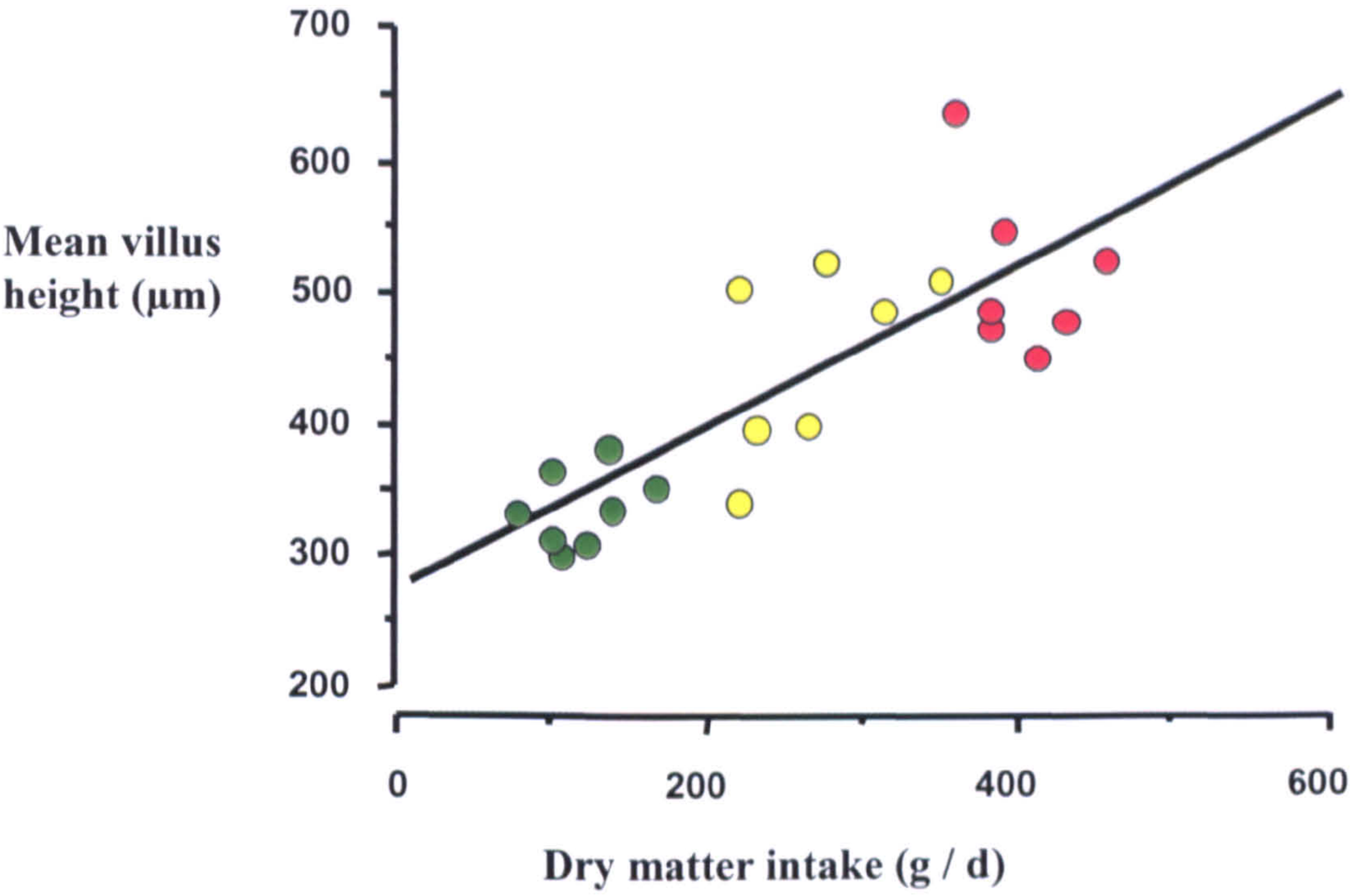
Many authors have tried to reduce both the extent and duration of post-weaning anorexia. For example, Dunshea *et al.* (1997) provided piglets (weaned at 21 d) with supplemental skim milk during the suckling period (from 10 to 20 d) and reported increased feed intake immediately post-weaning (initial 2 d), 257 vs 30 g DM / d compared to piglets offered a

pelleted diet. Additionally, Azain (1997) weaned piglets at 7-10 d and provided liquid artificial milk and recorded feed intake levels double (300 vs 130 gDM / d) than littermates fed dry feed during the first week post-weaning. Moreover, Pluske *et al.* (1996b; c) illustrated that, in comparison to unweaned piglets, feeding a milk liquid diet (ewes' or cows' fresh milk at 2 hourly intervals) immediately after weaning maintained both villus height and crypt depth.

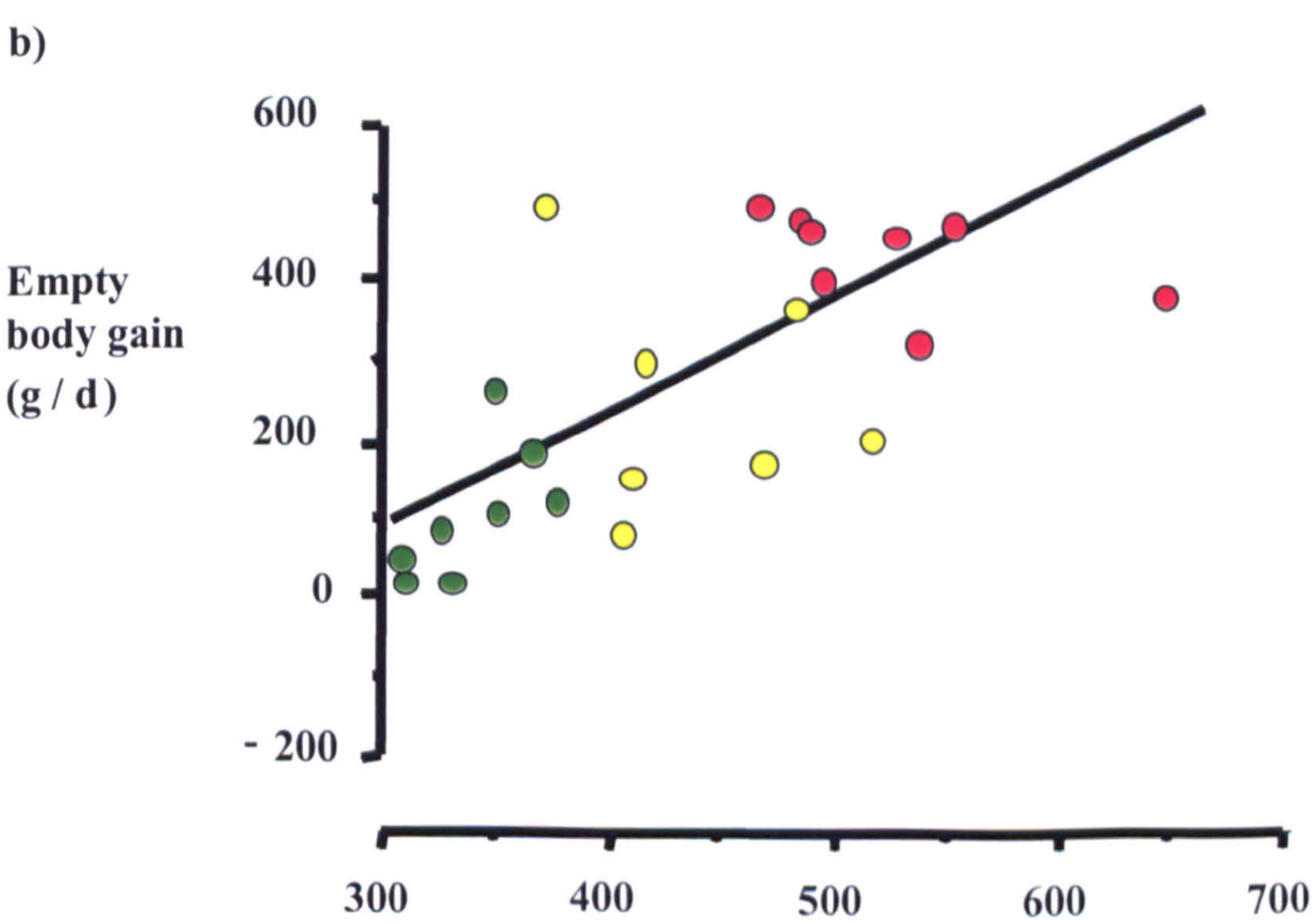
Pluske *et al.* (1996c) also illustrated a linear relationship between total dry matter intake and mean villus height along the length of the small intestine (Figure 1.7a). In turn, mean villus height explained 47% of the total variation in empty body-weight gain in the first five days after weaning (Figure 1.7b). This observation is in agreement with similar relationships reported in other studies (Li *et al.*, 1991). It was concluded that as villus height can be correlated to the number of enterocytes on the villus column in 'normal' villi (Wright, 1982; Hampson and Smith, 1986), maintenance of food intake after weaning must have preserved enterocyte number commensurate with a maintenance, or even enhancement (e.g., James *et al.*, 1988), of digestive and absorptive capacity of digestive and absorptive capacity of each enterocyte (Pluske, 2000). Furthermore, in the study of Pluske *et al.* (1996c), mean villus height was highly correlated ($r = 0.79$; $P < 0.05$) to empty body-weight gain (Figure 1.7c). Whilst the authors were unable to state definitively that cause and effect relationships exist between gut structure and weight gain, these data illustrate the apparent interdependence between voluntary food intake and mucosal structure in determining the post-weaning performance of piglets (Pluske *et al.*, 1996b; c). Additionally, in the study of Pluske *et al.* (1996c), pigs fed *ad libitum* exhibited growth rates in excess of 500 g / d indicating that digestive and absorptive capacity was not restricted. These data highlight the importance of stimulating food intake after weaning, whilst also emphasising the interdependence between absorbed nutrients, intestinal structure and growth rate in the immediate post-weaning period.

Goodlad *et al.* (1988) proposed an alternative hypothesis for the preservation of villus height, in that villus cell population might increase by way of cell migration from the crypts without an accompanying increase in crypt-cell production rate. However, this is unlikely to explain the maintenance of villus height reported by Pluske *et al.* (1996b; c) as this has only been observed in rats re-fed after a period of short-term starvation (Altmann,1972; Clarke, 1975).

a)


Mean villus height (µm)
700
600
500
400
300
200
0
200
400
600
Dry matter intake (g / d)

b)


Empty body gain (g / d)
600
400
200
0
- 200
300
400
500
600
700
Mean villus height (µm)

c)

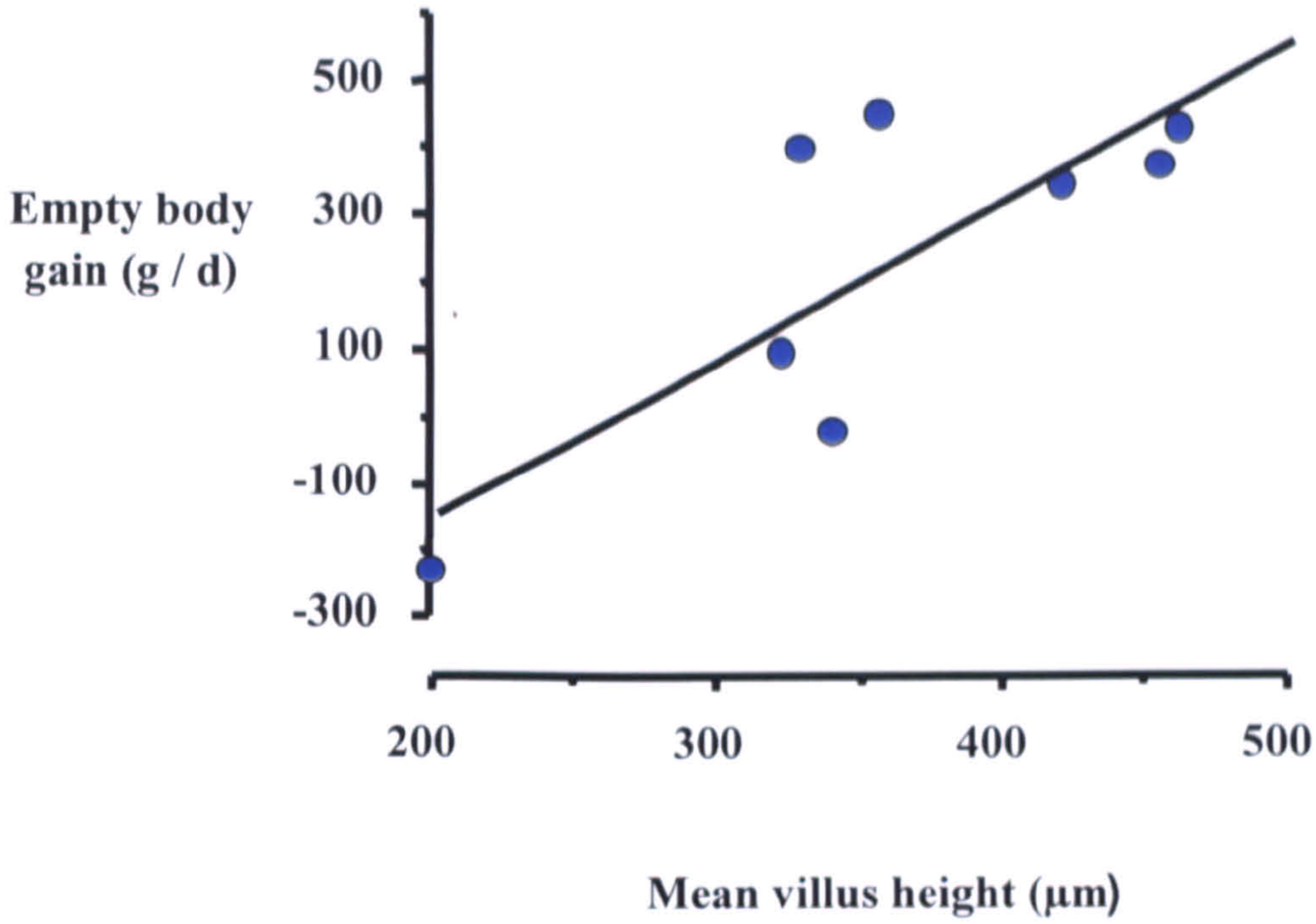


Figure 1.7: Relationship between

a) dry matter intake and mean villus height along the small intestine [y = 286.10 + 0.54x, R^2 = 0.68 (RSD = 56.91); P < 0.00];

b) mean villus height along the small intestine and empty body-weight gain [y = -325.69 + 1.39x, R^2 = 0.48 (RSD = 127.10); P = 0.002], in pigs offered cow's liquid milk at maintenance ( ), 2.5 times maintenance ( ), or *ad libitum,* ( ) energy intake for 5 days after weaning (8 pigs per treatment) and

c) [y = -612.0 + 2.33x, R = 0.79; P = 0.020] in pigs offered a pelleted starter ( ) diet

1.7 IMMUNO-POTENTIATION

1.7.1 Introduction

The mammalian immune system possesses mechanisms to detect chemical structures or molecules that are potentially dangerous, and utilise them as alarm signals to switch on the defence against infections. As a result, the immune system will respond to immuno-potentiators as if challenged by a pathogenic microbe (Sohn *et al.*, 2000).

The ability to control an immune response for the benefit of both the animal and production efficiency is the objective of immunomo-potentiation in food-producing animals, and substances that exert this control are called immuno-potentiators or immunomodulators (Blecha, 2001). Broad categories of immuno-potentiators include cytokines, pharmaceuticals, microbial products, neutraceuticals, and traditional medicinal plants. Immuno-potentiators are chemcials, drugs, stressors or actions that elevate the

non-specific defence mechanism or the specific immune response (Anderson, 1992), and hence render animals more resistant to infections by viruses, bacteria, fungi and parasites. This definition also emphasises the complexity of the interaction between the immune system and its environment. If the non-specific defence mechanism is enhanced, there is no memory element and so the response is likely to be of short duration. This explains why supplements such as nucleotides are administered over a relatively short time period and on a daily basis.

Although the mode of action at a molecular level is known only for a few immuno-potentiators (Sohn *et al.*, 2000), a number have been evaluated in a range of species including laboratory animals, ducks and fish, such as structural elements of bacteria (lipopolysaccharides, lipopeptides, glycoproteins), various β-1,3-glucan products from bacteria and mycelial fungi, β-1,3/1,6-glucans from the cell wall of baker's yeast, complex carbohydrate structures (glycans) from various biological sources including seaweed, peptides in extracts of certain animals and made by enzymatic hydrolysis of fish protein, blood plasma, vitamins, minerals, and synthetic products. The various immuno-potentiators of bacterial origin interact directly with phagocytic leukocytes as well as with the leukocytes involved in antibody production (refer to Sakai, (1999) for a review in fish). Immno-potentiators of bacterial origin therefore seem to cause an enhancement of many different immune reactions within the body and are also antigenic in nature, inducing antibody production against the immuno-potentiator itself. However, bacterial immuno-potentiators, used as adjuvants in vaccines (large-scale utilisation in aquaculture) have the disadvantage that much of the antibody producing capability of the body is wasted on the adjuvant. Macrophage activation by β-glucans has been shown to enhance the capacity of the non-specific component of the immune system to resist bacterial infections (Burrells *et al.*, 2001). Administration of an immuno-potentiator in early life should boost the immune system (thus increasing the animal's immunocompetence) throughout the post-weaning period when the piglet is most susceptible to infection and disease leading to reduced performance. Uses of immuno-potentiators such as those described above provide an effective means of increasing the immunocompetency and disease resistance of animals.

1.7.2 Nucleotides

Nucleotides consist of a nitrogenous base, a pentose sugar and one or more phosphate groups. The nitrogenous base is either a purine or pyrimidine, the atoms of which are

primarily derived from amino acids. Pyrimidine bases are six-membered rings, and include uracil (U), cytosine (C) and thymidine (T). Purine bases have a second five-membered ring, and include adenine (A), guanine (G), hypoxanthine and xanthine. A purine or pyrimidine base linked to a pentose molecule constitutes a nucleoside. Nucleotides (low molecular weight biological compounds that are involved in almost all biochemical processes) are the building blocks of DNA and RNA and play essential roles in structural, metabolic, energetic, and regulatory functions particularly as components of adenosine triphosphate and other nucleotide triphosphates and in many co-enzymes (Adjei *et al.*, 1995).

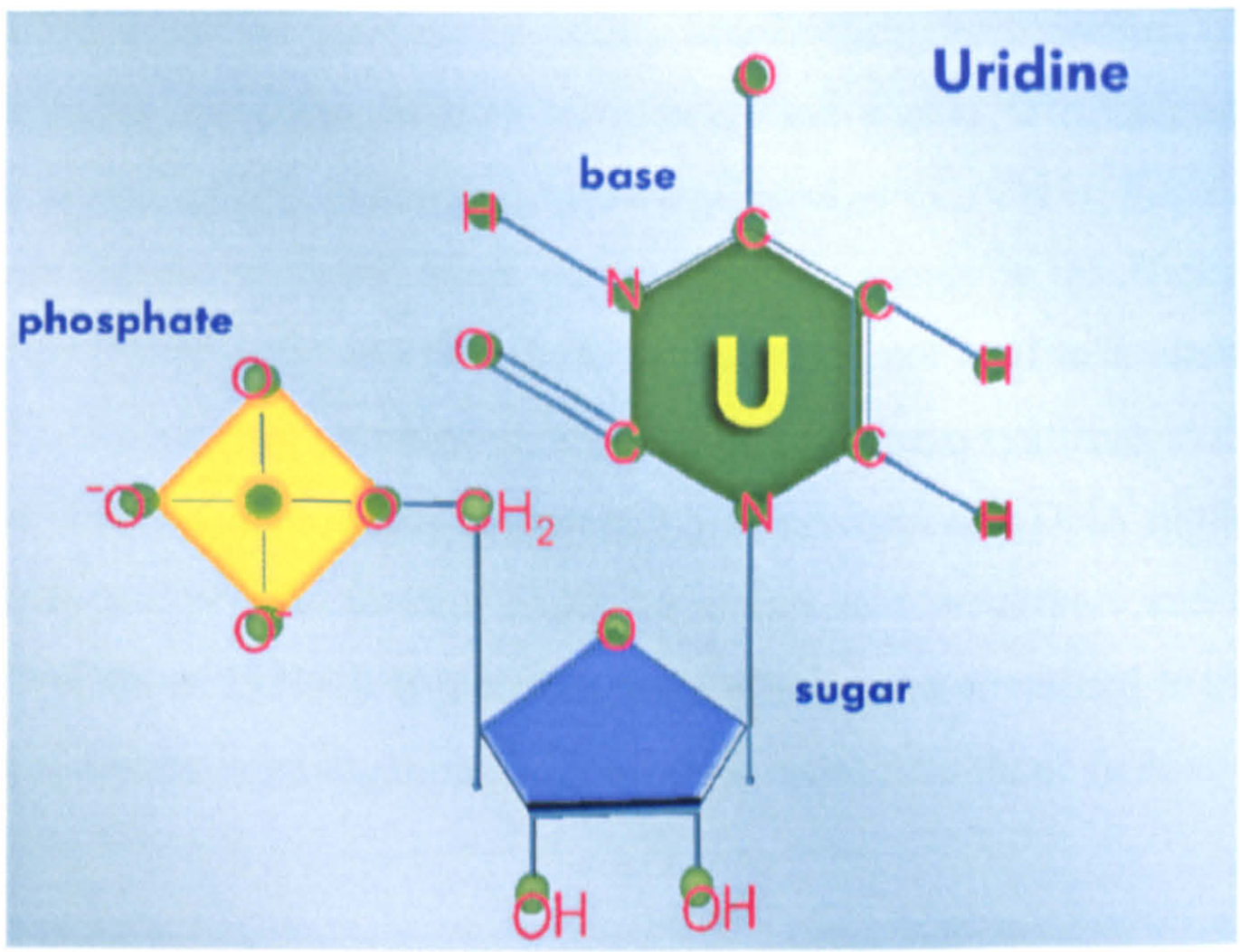


Figure 1.8: Diagrammatic representation of a nucleotide comprising a nitrogen-containing ring compound (the base; uracil), linked to a 5-carbon sugar (ribose or deoxyribose) that carries a phosphate group.

Nucleotides are contained within milk (in the form of nucleic acids, nucleosides, nucleotides and related metabolic products (Carver, 1999) and are thought to contribute to the enhanced development of the immune and gastrointestinal systems of milk-fed young (Jyonouchi, 1994). The nucleotide content of human milk is significantly higher than most cow's milk-based infant formulae. Evidence suggests that dietary nucleotides enhance the gastrointestinal and immune systems of formula-fed infants. For example, infants fed nucleotide-supplemented versus non-supplemented formula have a lower incidence of diarrhoea, and higher natural killer cell activity (Carver, 1999). These data

suggest that human milk nucleotide may contribute to the superior clinical performance of the breast-fed infant.

Although most tissues can synthesise nucleotides *de novo*, other cells such as immune cells and cells in the intestine (*i.e.* erythrocytes, polymorphonuclear leukocytes, intestinal mucosa, bone marrow, hematopoietic cells, brain cells) are lacking in this capacity and must depend on pre-formed nucleotides (Carver *et al.*, 1991; Quan, 1991). Mammalian requirements for exogenous nucleotides can vary considerably and can increase dramatically at times of rapid growth (*i.e.* the young, immature animal) or physiological stress (*i.e.* weaning) (Carver, 1994; Jyonouchi, 1994).

Since nucleotides can be synthesised endogenously they are therefore not considered essential nutrients. However, studies have suggested a role of nucleotides as 'semi-essential' or 'conditionally essential' nutrients (Carver *et al.*, 1991). For example, these nutrients may become essential when the endogenous supply is insufficient for normal function, even though their absence from the diet does not lead to a classic deficiency syndrome. Nucleotides are provided endogenously by *de novo* synthesis in tissues such as the liver. At least 10^9 nucleotides are necessary to perform one DNA replication; hence this is an energy-expensive process requiring amino acid precursors and energy in the form of ATP (Carver, 1999). It is therefore more efficient for an animal to use pre-formed nucleotides of dietary or endogenous origin than to synthesise them *de novo* (Roux, 1973; Uauy, 1994).

Most dietary nucleotides are rapidly catabolised and excreted. However, some are incorporated into tissues, particularly at younger ages (during periods of rapid growth) and with fasting. Under conditions of limited nucleotide intake, rapid growth or certain disease challenge, dietary nucleotide may spare the cost of *de novo* nucleotide synthesis and optimise the function of rapidly dividing tissues such as those of the gastrointestinal and immune systems. Animals fed nucleotide-supplemented versus non-nucleotide-supplemented diets exhibit enhanced gastrointestinal growth and maturation (Iijima *et al.*, 1993), and improved recovery following small and large bowel injury. Indices of humoral and cellular immunity are enhanced, and survival rates are higher following pathogenic infection (Carver, 1999).

Despite the considerable research effort, the role of dietary nucleotides in the functioning of the immune system has not yet been fully elucidated. However, evidence suggests that immature lymphocytes are not capable of synthesising purine and pyrimidine bases required to produce nucleotides and must therefore rely on the *de novo* synthesis of nucleotides by other organs such as the liver (Rudolph *et al.*, 1990) or an exogenous source such as the diet.

1.7.2.1 Immunological effects

Feeding nucleotide-supplemented compared with a nucleotide-free diet to rodents has been associated with increases in the following immune responses;

1. graft-versus host disease mortality (Kulkarni *et al.*, 1994a);
2. rejection of allogenic grafts (Kulkarni *et al.*, 1994a);
3. delayed cutaneous hypersensitivity (Kulkarni *et al.*, 1994a; Yamauchi *et al.*, 1996);
4. alloantigen- and mitogen-induced hyperproliferation (Kulkarni *et al.*, 1994a; Yamauchi *et al.*, 1996);
5. reversal of malnutrition and starvation-induced immunosuppression (Kulkarni *et al.*, 1994a);
6. natural killer cell activity and macrophage activation (Carver *et al.*, 1990);
7. resistance to microbial challenge (Kulkarni *et al.*, 1994a);
8. macrophage phagocytic capacity (Kulkarni *et al.*, 1994a);
9. spleen cell production of interleukin-2 (IL-2) and expression of IL-2 receptors and lyt-1 surface markers (Kulkarni *et al.*, 1994a);
10. the number of antibody-secreting spleen cells produced in culture (Jyonouchi and Sun, 1996; Navarro *et al.*, 1996);
11. popliteal lymph-node cytokine secretion (Yamauchi *et al.*, 1996);
12. peripheral blood total leukocyte counts and neutrophil numbers following infection (Matsumoto *et al.*, 1995).

Many studies have investigated the role of dietary nucleosides and nucleotides in the maintenance of the celluar immune system. For example, the addition of dietary nucleosides and nucleotides to diets has been shown to increase DTH (delayed-type hypersensitivity) responses (Kulkarni *et al.*, 1987), enhance host resistance to infection (Kulkarni *et al.* (1986), Fanslow *et al.* (1988), Adjei *et al.* (1993)), affect the number and

function of T-helper cells (Van Buren *et al.*, 1985; Kulkarni *et al.*, 1989; Rudolph *et al.*, 1990; Jyonouchi *et al.*, 1994) and enhance the cytotoxic function of natural killer cells in BALB/c mice (Carver *et al.*, 1990; Kulkarni *et al.*, 1994a).

Van Buren *et al.* (1985) proposed that dietary nucleotides exert effects on immune responsiveness by acting on the T-helper/inducer population, with the predominant effect on the initial phase of antigen processing and lymphocyte proliferation. The presumed mechanism is suppression of uncommitted T-lymphocyte responses, as demonstrated by higher levels of specific intracellular marker for undifferentiated lymphocytes in primary lymphoid organs in mice fed a nucleotide-free diet (Rudolph *et al.*, 1983). A regulatory role of dietary nucleotides in immunohematopoiesis has also been proposed (Kulkarni *et al.*, 1992). Rudolph *et al.* (1990) suggested that dietary nucleotide effects on immunity were not previously observed, since they are only evident under conditions of stress, such as immune challenge. Van Buren *et al.* (1983) demonstrated that dietary nucleotides enhanced *in vitro* and *in vivo* cell-mediated immunity to alloantigens and cardiac allografts. Kulkarni *et al.* (1989) showed that mice fed nucleotide-free diet containing 21% casein showed no recovery of the immune response, and that the host response against bacterial and fungal pathogens was decreased (Kulkarni, 1986 and Fanslow, 1988). These findings suggest that exogenous supply of nucleic acids and/or their component from dietary sources may be beneficial in maintaining normal body functions.

1.8 CONCLUSIONS

The development of the GIT and its associated immune system, under the protection afforded by maternal passive immunity, occurs in a precise and highly regulated manner and results in an optimally primed system capable of providing nutrients for normal bodily functions and growth and also for immune protection, all essential for the survival of the young animal (Kelly and King, 2001a). However, current high-health farming systems with increasing production costs require a high throughput of animals, and the use of antibiotics to promote growth and combat infection is common practice. This is now threatened by legislation that prevents the use of those antibiotics to reduce any perceived health risks, and to combat the potential emergence of antibiotic resistance. The remaining 4 growth promoting antibiotics are due to be banned in the European Union from 2006. Furthermore legislation has recently been proposed to increase the weaning age of piglets to a minimum of 28 days. It is postulated that this will have a significant

impact on the weaned pig, which will be more mature and better able to cope with, and adapt to, the weaning process.

The compromise between production levels, and the health and welfare of both animals and humans is complex. Alternative strategies must be developed which are in line with health and welfare requirements, whilst also matching demand and market requirements. The modern consumer is increasingly demanding more stringent controls regarding the way in which food-producing animals are reared. As such new food production strategies, which are in-line with current legislation and consumer demands, are imperative. Information on the 'signals and inputs' required in early life to promote optimum immune function and disease resistance, and the energetics of these processes, may enable the manipulation of the nutrition and the environment (microbial exposure) of the young animal to satisfy both welfare, environmental and production criteria (Kelly and King, 2001a).

1.9 OVERALL HYPOTHESES

It was postulated that dietary manipulation would exert a beneficial effect on piglet post-weaning performance. Such beneficial effects would be monitored in terms of gut health *i.e.* gut morphology characteristics, identification and enumeration of a limited number of microbiological species, growth performance and in some later studies, peripheral lymphocyte blastogenesis assays. Such measurements would provide an indication of intestinal structure, microfloral load and immune status, which all impact on post-weaning growth performance. Hypotheses for individual studies are presented in the appropriate sections.

CHAPTER 2:

GENERAL MATERIALS AND METHODS

2.1 INTRODUCTION

Studies were undertaken to investigate the digestive physiology of the post-weaned pig with specific emphasis upon nutritional influences on gut microflora and health throughout the initial 2 weeks post-weaning. The same basal conventional starter diet (Provimi Ltd, Yorkshire, UK) was used throughout all studies, with treatment diets being altered in some manner. Certain details concerning housing, management, and parameters measured along with analytical procedures that were followed were similar for all studies and details of these are given below. Any departures from the general description, which occurred, are given in the appropriate sections. No procedures requiring a Home Office Project Licence were undertaken in any of the studies.

2.2 EXPERIMENTAL ANIMALS

All experiments were performed under the same basic principles. Entire male, post-weaned piglets were employed for each study (except studies 1 and 5) of approximately 5 to 8 kg initial live-weight (studies 2–4 n = 36). In study 1, 20 male piglets were employed and in study 5, 96 mixed male and female piglets were employed. All animals were sourced from a local multiplier herd (commercial white hybrid) and litters were weaned at 28 days of age. Upon arrival at the site, the piglets were individually weighed and transferred to an environmentally controlled (temperature 28 – 32 ^{0}C, humidity 45%, and lighting regime 16 h light and 8 h dark) room in the Pig Metabolism Building (experimental unit).

2.2.1 Housing and management

Upon arrival at the experimental unit, piglets were randomly allocated to one experimental diet and were individually housed in pens with 1 m^2 slatted floor space/pen (study 2 - 4) or group housed (study 1), with 2 nipple drinkers and a feed trough. Each pen had partitions allowing visual, olfactory and limited tactile contact between adjacent pens (conforming to appropriate DEFRA (formerly MAFF) guidelines). Water was available *ad libitum* from

nipple drinkers located in each pen at all times. Feed was also available *ad libitum*, refusals were removed, weighed and discarded twice daily.

2.3 DIETS

Two experimental diets were evaluated in all trials except trial 2 which employed 4 diets. All the ingredients were conventional raw feed materials and diets were designed to be general starter diets. All diets were isoenergetic and isonitrogenous (15.4 MJ digestible energy (DE) / kg and 230g crude protein (CP) / kg as fed) and were based on cooked cereals, wheatfeed, soya products, fishmeal, milk products, soya oil and a mineral / vitamin supplement. The control diet was the same for all five studies. All diets were produced by Provimi Ltd, Dalton Mill, Thirsk, North Yorkshire, England, and were bagged in 25 kg lots. The chemical composition of the diets used is presented in table 2.1. Although the diets were formulated to be identical in energy and nutrient specification, both seasonal variations together with variation in batch manufacture resulted in slight differences between diets used in different studies. As such, specific details concerning the diet specification for each study are presented in relevant sections (sections 3.1; 4.1 and 5A.1).

Table 2.1 Chemical composition of the diets used in studies 1 to 5[a]

Nutrient Specification * g/kg (as fed)	Trial 1	Trial 2	Trials 3, 4 & 5
Moisture (g/kg)	120	110	100
Oil A (Ether Extract)	70	75	90
Protein	230	220	230
Crude Fibre	30	25	25
Ash	60	54	60
DE (MJ/kg)	15.5	16.0	16.0
Total Lysine	15	16	16
Vitamin A (iu/kg)	16,000	16,000	12,500
Vitamin D_3 (iu/kg)	2000	2000	2000
Vitamin E (iu/kg)	150	150	250
Copper (mg/kg)	165	165	165
Zinc (mg/kg)	140	140	140

[a] Calculated values from formulation programme

2.4 EXPERIMENTAL PROCEDURE

All piglets were weighed on days 0 (day of weaning and delivery to the experimental unit), 2, 4, 6, 9, 12 and 14. The time of weighing remained the same throughout the trial period (07.30 h) in order to minimise any variability due to the influence of gut contents.

The first 4 piglets were slaughtered on day 0 (and shall now be referred to as baseline animals). This group of animals was expected to yield the same results as if they had been slaughtered pre-weaning as animals were slaughtered within 1 hour of weaning; it is assumed

that the gut morphology will be the same as a pre-weaned animal. The piglets were serially slaughtered as follows:

Table 2.2: Slaughter timetable

Group	Day
1	0
2	+2
3	+4
4	+6
5	+14

Eight piglets were slaughtered on each of the above 5 days; specific details of the number of animals slaughtered for each treatment group are described at appropriate points (sections 3.3; 4.3; 5A.3; 5B.3 and 5C.3).

2.5. SLAUGHTER PROCEDURE

Food was withdrawn from the piglets for 1 hour prior to slaughter to standardise any impact that gut contents may have. Animals were slaughtered by electrical stunning requiring a minimum of 1.3 Amps, with the maximum stun-stick interval being 30 seconds. The main jugular vein was severed, resulting in exsanguination (3 to 4 minutes were allowed to bleed out completely). The small intestine was then removed and the remaining parts discarded. The jejunum, duodenum, ileum, colon and rectal contents (studies 3 and 4; study 2 ileal samples alone) were removed immediately and the pH measured directly using a digital pH meter (Mettle Toledo 320; Fisher Scientific UK). The pH meter was calibrated prior to pH measurement using pH 4.0, 7.0 and 9.2 buffering solutions (Fisher Scientific UK).

2.6 HISTOLOGY

2.6.1 Fixing and embedding tissue samples

2.6.1.1 Introduction

A standard operating procedure, which describes the basic steps for fixing and embedding tissue samples, was followed. Preliminary studies performed in this laboratory have shown that Bouin's fixative (Zarkadas, 1999) adequately prepares piglet intestinal tissue for subsequent morphological measurements and was therefore used for all samples.

2.6.1.2 Fixing tissue samples

Pieces of intestinal tissue (6 to 8 cm in length and 1 to 2 cm in diameter), were ligated at distances of proportionately 0.25 (proximal), 0.50 (medial) and 0.75 (distal) along the whole length of the small intestine, from the gastric pylorus to the ileo-caecal valve. At each side, both ends of the tissue were doubly tied and the loops filled by injecting Bouin's fixative (consisting of Picric acid, saturated aqueous 714.3 ml l^{-1} (Sigma-Aldrich Co), formaldehyde (formalin) concentration 238.1 ml l^{-1} (Sigma-Aldrich Co), and glacial acetic acid 47.6 ml l^{-1} (Fisher Scientific UK) into the lumen, then submersing the tissue in Bouin's fixative for a minimum period of 15 to 20 minutes. The lengths of fixed intestine were then cut off, rinsed with normal saline (0.9% w/v) to flush out the intestinal contents, and the tissue returned to fresh Bouin's fixative for storage. The period of time required for complete fixation depends on the volume / density of tissue present and the rate of penetration of the fixative. Previous work within this laboratory has shown 24 hours to allow full tissue penetration by the fixative (Zarkadas, 1999).

After approximately 24 hours, the tissue was removed from the fixative and transferred to 70% (v/v) Industrial Methylated Spirit (I.M.S.; Fisher Scientific UK), with the solution being changed a number of times in order to remove excess fixative from the tissue, and stored in 70% I.M.S. until embedding.

A 1 cm ring-shaped length of the tissue was then dehydrated by immersion in an ascending series of aqueous alcohol solutions (70, 90 and 100% IMS) using a histokinette (Model TP1020 Leica, Germany). Following dehydration the tissue was cleared with toluene (Fisher Scientific UK), a substance that is miscible with both alcohol and paraffin wax thus allowing

molten wax to penetrate the tissue. The duration of each step is dependent upon the size and thickness of the tissue sample. The schedule used for piglet gut samples is shown below in table 2.3.

Table 2.3: Schedule of paraffin wax embedding of intestinal tissue.

Step	Chemical	Duration
1	70% IMS 1	Overnight
2	70% IMS 2	1 hour
3	90% IMS 1	1 hour
4	90% IMS 2	1 hour
5	100% IMS 1	1 hour
6	100% IMS 2	1 hour
7	Toluene 1	1 hour
8	Toluene 2	1 hour
9	Wax 1	1 hour
10	Wax 2	1 hour

Once the tissue had been infiltrated with wax, it was transferred as rapidly as possible into a pre-warmed embedding mould filled with molten wax and orientated as desired. The mould was cooled immediately in water at 15^{0}C to prevent fracturing. Sample blocks were stored at 4^{0}C to prevent softening of the wax and for ease of subsequent sectioning.

2.6.1.3 Sectioning and staining tissue samples

After embedding, the tissue was subsequently sectioned using a rotary microtome (HM 355; Microm Laborgeräte GmbH, Walldorf, Germany) with the specimen feed being set at 8 microns (μm). Previous work within this laboratory has shown using serial sections, that villi in piglets of this age are less than 150 microns in diameter (Zarkadas, 1999). Therefore selecting every 28th section ensures that a new villus is studied on each section. For each sample, 28, 8 μm thick, transverse sections were cut, placed on slides, stretched flat using 1% glycerin albumin (Raymond .A. Lamb Ltd) on a hot plate and baked overnight at 35 ^{0}C.

The slides were run down a series of alcohols to rehydrate them prior to staining using a modified Milligan trichrome staining protocol in which a regressive haematoxylin stain replaces the acid fuchsin stage. The procedure for staining the tissue slides is outlined below (all chemicals and reagents were obtained from Fisher Scientific UK, unless otherwise stated).

1. Xylene 1: 2 min
2. Xylene 2: 2 min
3. Absolute I.M.S. 1: 2 min
4. Absolute I.M.S. 2: 2 min
5. 95% (v/v) I.M.S.1: 3 min
6. 95% (v/v) I.M.S.2: 3 min
7. Running water: 3 min
8. Harris haematoxylin (BDH): 15 min
9. Wash in running water: 3 min
10. Destain in 0.5% HCl in 70% (v/v) I.M.S. for a few seconds, (sections will turn pink). Transfer to ammoniated 70% (v/v) I.M.S. until sections turn blue. Examine under microscope; if nuclei are too dark, repeat above procedure. When nuclei stand out as a sharp blue against a colourless background, rinse slides thoroughly in water: 3 min
11. Fix stain in 1% (w/v) phosphomolybdic acid solution: 2.5 min
    (Sigma-Aldrich Co.)
12. Stain in orange G (Raymond .A. Lamb Ltd): 5 min
13. Rinse in distilled water
14. Treat with 1% (w/v) acetic acid: 2 min
15. Stain in fast green (Fisons Scientific): 5 min
16. Treat with 1% (w/v) acetic acid: 3 min
17. Rinse in 95% (v/v) I.M.S., transfer to second 95% I.M.S.: 5 min
18. Absolute I.M.S. 3: 3 min
19. Absolute I.M.S. 4: 3 min
20. Xylene 3: 3 min
21. Xylene 4: 3 min

22. Mount slides: using metal forceps remove one slide at a time from the xylene, quickly dry the back of the slide with paper towel and place on the bench. Using a glass Pasteur pipette, apply DPX Mounting Medium (Raymond .A. Lamb Ltd) to the slide. Add a cover slip. Using a mounted needle or similar, carefully remove any bubbles by applying gentle pressure to the coverslip.

2.7 HISTOLOGICAL MEASUREMENTS

Slide mounted sections were measured using an Olympus system microscope (Model BHS; Olympus Japan), with an eyepiece ocular graticule and stage micrometer (Raymond .A. Lamb Ltd). The stage micrometer has a 2mm scale divided into 0.01 mm divisions. The eyepiece ocular graticule has 100 divisions, which are arbitrary and therefore require calibrating at each magnification used. The calibration is shown in below table 2.4. For study 1, sections were measured manually. However, for studies 2-4, sections were measured using a computerised image analysis system. This consisted of a Leitz system microscope (Model Diaplan; Leitz Wetzlar Germany) fitted with a Panasonic system camera (Model WV-F15E; Panasonic Japan). The camera projected live images to a computer that utilised an AGP video frame grabber (Model Flashpoint 3D Lite; Integral Technologies Inc). The villus height, width and crypt depth where then measured using Scion Image for Windows (version Beta 4.02 Scion Corporation). This software was calibrated using the stage micrometer as described above.

Table 2.4: Graticule calibration

Magnification	mm*	µm*
x 40	0.0250	25.0
x 100	0.0100	10.0
x 200	0.0050	5.0
x 400	0.0025	2.5
x 1000	0.0010	1.0

*calibration for one section on the graticule.

Villi were selected for measurement from each section by the following criteria: the villus must be complete from tip to base (shown by a single layer of epithelial cells), have a clearly visible capillary network running down the centre of the villus, and include obvious crypt cells. Measurements were only taken from sections where the plane of section ran vertically from the tip of a villus to the base of an adjacent crypt. Ten of the tallest well-orientated villi (complete distance from muscle layer to tip), 10 associated crypts (taken as the distance between the villus base and the muscularis layer), and the villus width (one third down from the tip of the villus) were measured. All villi measurements were performed 'blind', as such piglet identification and dietary treatment where unknown at the time of measurement. A random selection of slides was given to an independent person to verify the measurements that had been taken.

2.8 MICROBIOLOGY

2.8.1 Introduction

No microbiological analyses were undertaken for studies 1 and 5. Studies 2 - 4 employed microbial enumeration of digesta and faecal samples. However, study 4 employed a limited sampling procedure (Section 5B3.4).

2.8.2 Bacterial enumeration

Bacterial enumeration expressed as colony forming units per ml or per g (cfu ml^{-1} or cfu g^{-1}) was calculated using a serial dilution method. The sample (approx. 1g for intestinal samples and 1 swab for faecal samples) to be quantified was diluted 10-fold in maximum recovery diluent (MRD; Oxoid Ltd; Appendix 1) (9 ml for intestinal and 10 ml for faecal samples) and further 10 fold dilutions prepared as necessary. An aliquot (100 microlitres (µl) for lactobacilli and bifidobacteria, and 1 ml for coliforms and *E. coli*) of each dilution was spread plated on to the surface of an appropriate agar plate. The plates were allowed to dry and the agar plates incubated under the appropriate conditions.

2.8.3 Faecal sampling procedure

Faecal swabs were collected from all pigs upon arrival at the experimental unit and alternate days thereafter (*i.e.* days 0, 1, 3, 5, 7, 9, 11, 13 and 14) for studies 1-3. If collected and analysed correctly, faecal flora can be representative of colonic flora in terms of composition

and function (Roberfroid *et al.*, 1995). However, faecal samples are an imprecise measurement of the actual status of the even the colon, and provide no information on the microflora of the ileum or caecum. The faecal swab was collected into 10 ml MRD (Oxoid Ltd) and placed on ice. All samples were then blended using a vortex mixer (VM20 Chiltern Scientific) for a period of 30 seconds. This blended sample constituted the 1:10 dilution for lactobacilli, bifidobacteria, *E. coli* and coliform determinations where relevant.

2.8.4 Digesta and rectal sampling procedure

Changes in the microflora of the small and large intestines can provide an indication of gut health and can be related to differences in growth performance. However, it should be noted that digesta samples provide information on the status of the gut microflora at a single point in time, although this technique has been employed successfully in a number of pig studies. The digesta and rectal samples were obtained from each piglet at slaughter (refer to section 2.5). Approximately 1g of each of the 4 intestinal and 1 rectal sample obtained from each piglet were individually weighed into a separate sterile stomacher bag (Model 400, Seward Ltd), and 9 ml MRD (Oxoid Ltd) added to each bag (*i.e.* 5 stomacher bags were prepared for each pig). The stomacher bag contents were then blended in a stomacher (Model BA 6021; Seward Ltd) for a period of 30 seconds. This blended sample constituted the 1:10 dilution for lactobacilli, bifidobacteria and *E. coli* and coliform determination as stated above in section 2.8.2.

2.8.5 Culture media and enumeration of bacteria

2.8.5.1 Lactobacilli and bifidobacteria enumeration

The agar used for the lactobacilli enumeration was Rogosa agar (Oxoid Ltd), which is designed for lactobacilli specifically (Appendix 3). A modified Columbia blood agar base (Oxoid Ltd) was used to determine bifidobacteria counts using the method of Beerens (1990) (Appendix 3).

The blended samples (faecal, digesta and rectal) were serially diluted using sterile MRD (Oxoid Ltd) and appropriate dilutions were plated on duplicate plates as described in section 2.8.2. Columbia agar plates were stacked in crates with semi-slatted sides, placed in a black plastic bag, gassed with CO_2 to create capnophilic conditions and sealed. All plates were then incubated at 37^0 C for 72 hours. Following incubation, all colonies grown on Rogosa and

Columbia agar were initially identified by Gram stain and catalase tests (refer to section 2.8.5.3). The numbers of colonies were then counted on both plates and an average value taken. All plates were incubated with Quality Control plates; controls for Rogosa agar were *Lactobacillus acidophilus* (positive) and *Staphylococcus aureus* (negative), and for Columbia agar, *Staphylococcus aureus* (positive) and an uninoculated plate (negative) (Beerens, 1990).

2.8.5.2 *Escherichia coli* and coliform enumeration

Aliquots of one millilitre (ml) of each dilution of piglet faecal, intestinal and rectal samples (refer to sections 2.8.2 & 2.8.3) were spread onto coliform Petri film (3M) plates in duplicate and were incubated at 37 ^{0}C for 24 hours. Both plates were counted and an average value taken. Red colonies were classified as coliforms, and blue colonies associated with gas were characterised as *E. coli* colonies.

2.8.5.3 Biochemical Tests

Catalase: A colony under test was placed into a sterile bijoux bottle and hydrogen peroxide (1 ml, 5-10%v/v; Fisher Scientific, UK) was added. The evolution of gas indicates catalase activity.

Gram stain: A small colony was emulsified in diluent, smeared onto a glass slide and allowed to air dry. The sample was heat fixed by passing it through a blue Bunsen flame. The Gram staining was carried out by immersing the slides in a series of dyes that were washed off in water before the application of the next reagent (Collins and Lyne, 1995). This comprised methyl violet (0.5% w/v; Raymond .A. Lamb Ltd) for 1 minute; Lugol's iodine solution (Raymond .A. Lamb Ltd) for 30 seconds; alcohol (95% ethanol; Fisher Scientific UK) for 1 min and carbol fuschin (1% w/v; Raymond .A. Lamb Ltd) for 30 seconds. Finally, the colony was observed microscopically at x100 magnification under oil immersion.

2.9 IMMUNOLOGY

2.9.1 Isolation of porcine peripheral blood lymphocytes (PBL)

Studies 3 and 4 employed a blood collection procedure outlined below for the isolation of peripheral blood lymphocytes. During the exsanguination period (refer to section 2.5) when the main jugular vein had been severed with a sterile knife, 20 ml and 200 ml of blood for studies 3 and 4 respectively, were collected into heparinised (1000 iu / ml; C.P.

Pharmaceuticals) centrifuge tubes. The samples were allowed to stand for 20-30 minutes to allow rouleaux formation of erythrocytes to occur. The supernatant plasma was then removed and layered over 6 ml Ficoll (Pharmacia Biotech) in a sterile 10 ml V-bottomed centrifuge tube. This was then centrifuged at 1400 rpm (475g) for 30 minutes. The lymphocytes were then harvested at the interface between the medium and the Ficoll and made up to 50 mls with Hanks Balanced Salt Solution (HBSS; Sigma-Aldrich Co.). This was centrifuged at 1400 rpm for 15 minutes to 'pellet' the lymphocytes (*i.e.* precipitate the lymphocytes out of solution at the base of the V-bottomed centrifuge tube). These were then washed in 50 ml HBSS by centrifugation at 1000 rpm for 10 minutes. This was repeated twice to ensure removal of the platelets. The cells were then counted (using a haemocytometer) by 0.1% Trypan blue dye exclusion to determine the number of viable cells (Hudson and Hay, 1976) and re-suspended in a solution of 10% Fetal Calf Serum (FCS; Sigma-Aldrich Co.) and RPMI 1640 (Sigma-Aldrich Co.) to a concentration of 2×10^6 cell / ml.

2.9.2 Cryopreservation of porcine peripheral blood lymphocytes

For study 5A all leucocytes were frozen upon isolation and the proliferation experiments carried out at a later date. In study 5B all proliferation experiments were performed with fresh lymphocytes and a percentage frozen should any subsequent analysis be deemed necessary. The concentration at which the cells needed to be frozen (anticipating a 60% yield on thawing) was determined to achieve a final viable concentration of 2×10^6 cell / ml. The dilution or concentration factor was also calculated. The lymphocytes were then centrifuged at 1000 rpm (275g) for 7-10 minutes at room temperature and the supernatant decanted, the required volume of freezing medium (10% DMSO (Sigma-Aldrich Co.) in heat inactivated FCS (Sigma-Aldrich Co.)) added and gently re-suspended. Aliquots of 1 ml were added into each cryovial, which were then placed in a cryo 1^0C freezing container (Nalgene) and transferred immediately to a -70^0C freezer. The following day, the cryovials were placed into liquid nitrogen for long-term storage.

2.9.3 Mitogen proliferation of peripheral blood lymphocytes – lymphocyte blastogenesis assay

In study 5A, the lymphocytes were thawed, and the number of cells counted using a haemocytometer. The dilution of cells required to obtain a final concentration of 2×10^6 cell /

ml was determined. The 2 mitogens (Concanavalin A (Con A; Sigma-Aldrich Co.) and Phytohaemagglutinin (PHA; Sigma-Aldrich Co.)), were diluted to provide a working concentration of 20 μg ml^{-1}. This working solution was further diluted (with 20% RPMI-1640 containing FCS (Sigma-Aldrich Co.), penstrep (Sigma-Aldrich Co.), L-glutamine (Sigma-Aldrich Co.) and 2-Mercaptoethanol (0.01 mM; BDH)) to achieve 3 final concentrations for each mitogen of 10, 3.3 and 1.1 μg / ml. These concentrations of mitogens were shown to be optimum by dose titration assays.

For each mitogen, 100 μl of each concentration was then added in triplicate to a 96 U-bottomed well plate. The plate was then placed in the incubator at 37 ^{0}C in a humidified 5% CO_2 atmosphere to allow the CO_2 to penetrate the mitogens prior to lymphocyte addition. Aliquots of 100 μl of lymphocyte cells were then added to each well containing the mitogens, and 3 blank wells containing 20% RPMI-1640 alone (unstimulated wells; control) for each mitogen concentration were prepared. Preliminary validation assays using Con A, PHA and PWM at concentrations of 0.5, 1.0, 2.5, 5, 10 and 20 μg / ml were conducted to assess the optimum lymphocyte stimulation level within our experimental conditions and genetic background of the pigs. The plate was then incubated at 37 ^{0}C in a humidified 5% CO_2 atmosphere for 48 h. Each well containing the cells and mitogens were then pulsed with 0.5 μCi of methyl-[^{3}H]-thymidine (Amersham Pharmacia). The plate was then incubated for a further 18 h at 37 ^{0}C in a humidified 5% CO_2 atmosphere. Well contents were harvested onto fiber filters with a semi-automatic cell harvester (Model 11019 Skatron Instruments Ltd). The discs were left to dry for 40 minutes before being punched out into scintillation vials and 1 ml of scintillate (Ultima Gold; Packard Biosciences) was added. The vials were left to stand for 1 hour to allow the scintillate to penetrate the discs fully. The incorporated radioactivity was then measured using a Beta counter (Model Tri-card 2100 TR, Packard Bioscience).

2.10 ANALYTICAL PROCEDURES

2.10.1 Analysis of dietary pH

Dietary pH was determined by adding 25 ml distilled water to 5 g of feed that was then mixed thoroughly. The pH was measured directly using a digital pH meter (Mettle Toledo 320; Fisher Scientific UK). The pH meter was calibrated prior to pH measurement using pH 4.0, 7.0 and 9.2 buffering solutions (Fisher Scientific UK).

2.11 CALCULATION OF RESULTS

2.11.1 Growth performance measurements

2.11.1.1 Daily live weight gain

Determination of the average daily live-weight gain (DLWG) was performed by linear regression of live-weight, with the slope of the linear response being DLWG. This analysis is more accurate than start and final weights, and also permits a precise calculation of the number of days that an animal takes to grow to a specified weight. It also allows any differences due to dietary treatment to be determined.

Example:

The live-weights for a piglet were as follows:

Day	Live weight (kg)
0	6.4
2	6.8
6	7.9
12	11.5
14	14.4

The live-weights were plotted against days on which pigs were weighed. From the regression analysis (refer to appendix 3 for programme) the equation generated was as follows:

$$y = a\,x + b \qquad \text{where}$$

a = live-weight gain

y = live-weight

b = intercept

$y = 0.467x + 6.4$

$R^2 = 0.959$ (the limit for R^2 was 0.750)

This specific piglet weighed 6.4 kg on the first day of the trial period, and had an average daily gain of 0.467 g / day (Figure 2.1).

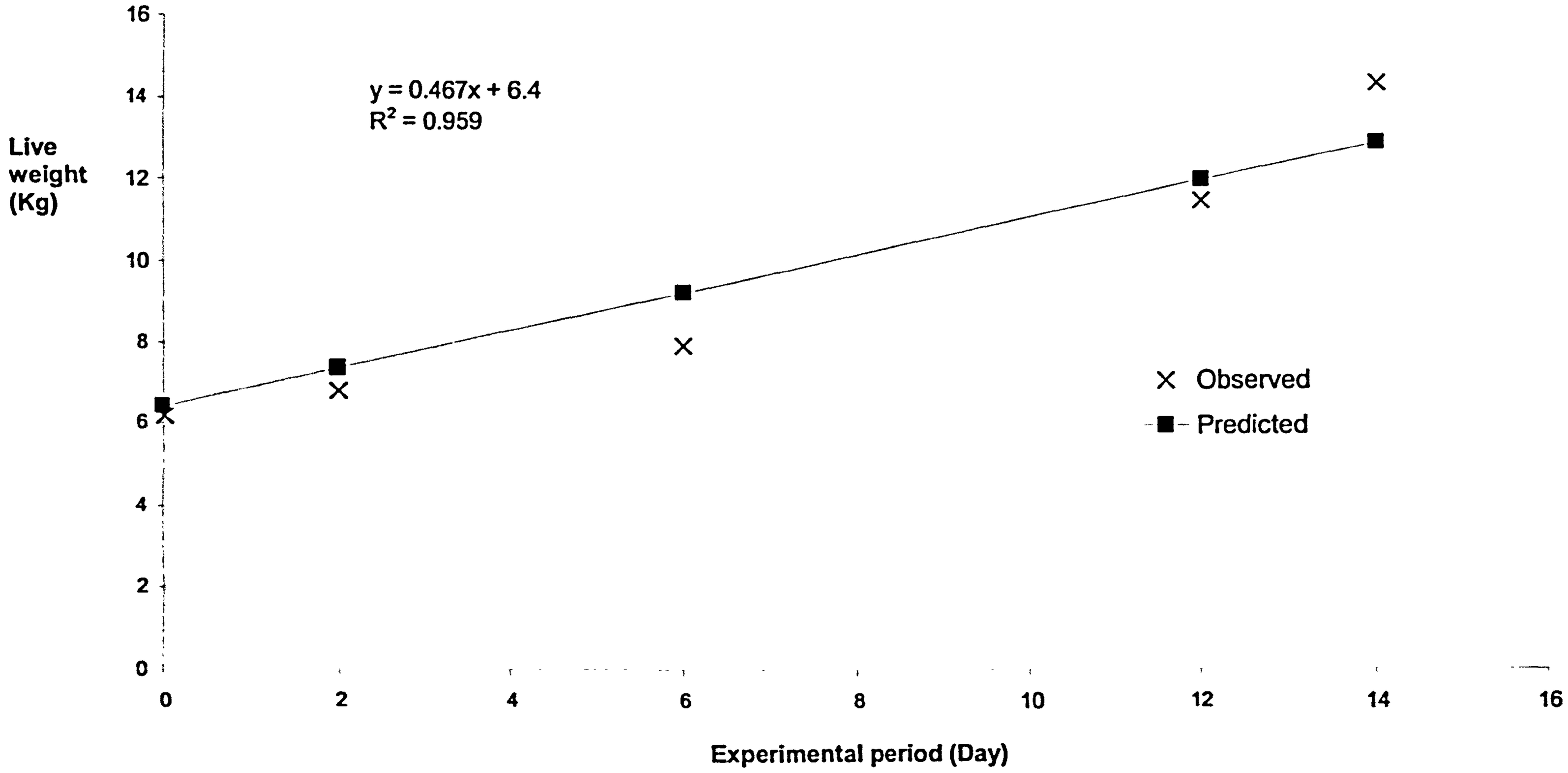


Figure 2.1: Regression analysis of live-weight

2.11.1.2 Daily feed intake

Throughout the whole experimental period, individual total dry matter feed intake (food offered less any refusals) was recorded on a daily basis (except studies 1and 5 where animals were group housed and therefore feed intake recorded on a group basis).

2.11.1.3 Feed conversion ratio

This is defined as the ratio of total feed intake (kg dry matter basis) per total weight gain (kg) over the experimental period.

2.11.2 Microbiological enumeration

cfu / swab = a x b x (c x d) where

a = mean plate count

b = dilution factor

c = plating volume

d = diluent volume

Example faecal samples:

Plate 1 lactobacilli count = 43

Plate 2 lactobacilli count = 34

Dilution = 10^{-2}

Mean plate count (a) = (43 + 34)/2 = 38.5

cfu / swab = 38.5 x 100 x 0.1 x 10

= 38.5 x 100 x [10 x 10]

= 385000

= 3.85 x 10^5 cfu / swab lactobacilli

Example digesta samples:

As above but the weight of the sample must also be incorporated

$$\text{cfu / g} = [a \times b \times (c \times d)] / e \quad \text{where}$$

e = sample weight

mean plate count = 136 cfu/plate lactobacilli

e = 2.3g digesta sample

cfu / sample = 136 x 100000 x 10 x 9

= 1.2×10^9 cfu / 9 ml

cfu / g = $[1.2 \times 10^9] / 2.3$

= 5.3×10^8 cfu / g

All microbiological results were log transformed (base 10) prior to statistical analysis.

2.12 STATISTICAL ANALYSES

All statistical analyses were undertaken using Genstat 5 (Lawes, 1997) and all graphs presented were compiled using Excel (Microsoft, 1997). The statistical analysis was undertaken using General Analysis of Variance and proceeded through establishing linear and non-linear contrasts with time and other sources of variation, and was performed in two parts. For example, the sources of variation utilised in gut morphological analyses were diet, slaughter day (main-plot factor), gut region (sub-plot factor), and all two- and three-way interactions. The between treatment variation was first partitioned into Baseline versus the Rest (BVR) which is comparing the baseline animals (*i.e.* those slaughtered on day 0; $n = 4$) with the rest of the experimental animals (study 1 $n = 16$; studies 2, 3, and 4 $n = 32$). The baseline animals were classified as receiving diet 0 which corresponds to sow milk in all studies. Secondly, the dietary effects were analysed by removing baseline values and comparing treatments. Latency period to first feed and water, and also final slaughter weight were included within the analysis as co-variates where appropriate. A summary of the sources of variation employed for the analyses of each study is presented below in table 2.5.

Table 2.5 Summary of the number of experimental animals and sources of variation employed in studies 1-5

Study	Number of Animals	Number of diets	Region	Sample Site	Faecal swabs	Lymphocytes
1	20	2	3	0	N	N
2	36	4	3	5	Y	N
3	36	2	3	5	Y	Y
4	36	2	3	5	Y	Y
5	240	2	0	0	N	N

All two-way and three-way interactions, and means of all measurements for each individual pig were determined. Table 2.6 illustrates the actual treatment structures employed (common to a number of studies) for each parameter under investigation: where day was employed, linear (L) and non-linear (Q) contrasts were established using a POLYANOVA (POL) routine.

Table 2.6 Summary of actual treatment structure used in Analysis of Variance

	Dietary Effects	BVR
Gut Morphology	Diet*POL(Slaughter Day;3)*Gut Region	BVR / (Diet*Slaughter Day*Gut Region)
Digesta Samples	Diet*POL(Slaughter Day;3)*Sample Site	BVR / (Diet*Slaughter Day*Sample Site)
Lymphocyte Proliferations	Diet*POL(Slaughter Day;3)*Mit*Mit Conc	BVR / (Diet*Slaughter Day*Mit*Mit Conc)
Faecal Samples	Diet*POL(Swab Day;3)	BVR / (Diet*Swab Day)
Feed Intake	Diet*POL(Experimental Day;3)	N/A

Gut region = The intestinal region proportionately 0.25, 0.50 and 0.75 from the gastric pylorus to the ileo-caecal valve in the small intestine

POL = Polynomial function

Sample site = The 5 intestinal sampling sites: duodenum, jejunum, ileum, caecum and colon

Mit = Mitogen; Concanavalin A (Con A); Phytohaemagglutinin (PHA) or Pokeweed mitogen (PWM)

Mit Conc = Mitogen concentration (10, 3.3 & 1.1 μg/ml for Con A and PHA and 10, 5 and 2.5 μg/ml for PWM

BVR = Baseline versus the rest

CHAPTER 3:

INFLUENCE OF DIET ACID BINDING CAPACITY (ABC) ON GUT MORPHOLOGY AND ILEAL pH IN PIGLETS

3.1 INTRODUCTION

In an attempt to overcome the post-weaning growth check and its associated problems, newly weaned piglet diets are normally highly digestible (possessing a high dietary energy and nutrient concentration), and are therefore composed of ingredients containing large concentrations of protein and minerals (for example, dried skim milk, fishmeal, and soya-bean meal), which often possess high acid binding-capacities (ABC) which bind hydrochloric acid (HCl) in the stomach. This can result in a high gastric pH, which is detrimental to the pig because it allows the proliferation of deleterious microorganisms (*i.e. E. coli* which require a pH of 6-8 for *maximum* rate of reproduction), and inhibits protein digestion. Consequently, reduced stomach efficiency may result in increased feed retention with subsequent reduced intake. The ABC of a compound feed may be defined as the quantity of mmol HCl required per 100 grammes of diet in order to reduce the pH to 4.0 (Spencer and Tilley, 1994). A number of factors have been shown to affect the ABC of diets including feed composition, principally; crude protein, fibre and mineral content. However, Jasaitis *et al.* (1987) and Hardy (1992) have suggested that the most active element affecting the phenomenon of ABC is calcium.

Evidence suggests that high calcium levels in pig starter diets can significantly reduce post-weaning growth performance (Hardy, 1992). Furthermore, restricting dietary calcium content has also been shown to prevent the hyper-proliferation of the stomach and gastrointestinal flora thus reducing the incidence of diarrhoea (Hardy, 1992). A further benefit of feeding low ABC diets is that a higher voluntary feed intake can be achieved as the stomach pH is maintained at or below 4 (Tilley, 1990). Once pH 4 and above has been attained in the stomach, the rates of proteolysis and digestion are reduced, which in turns delays gastric emptying and reduces feed intake.

Spencer and Tilley (1994) reported that the stomach of a 6.5-7.0 kg pig weaned at 28 days of age produces the equivalent of 20 ml molar HCl per day. If the feed offered requires 35 ml per 100g to achieve a pH of 4.0, the piglet can only cope with approximately 57g of that feed on the first day of feeding (Spencer and Tilley 1994). The authors further

estimated that if the pig were to consume 100g (of a good quality, highly palatable) post-weaning diet, this would result in an overload of 43g of feed causing a subsequent increase in stomach pH to well above the target of 4.0. This will have significant implications on digestion and absorption, and on gut micoflora load and composition.

During the suckling period, lactobacilli proliferate, due to the high lactose content of milk, and produce high levels of lactic acid. This maintains a low stomach pH but suppresses HCl secretion. At weaning it is therefore difficult for the weaned pig to maintain a low stomach pH as lactobacilli numbers decline and it takes time for both the hindgut and the secretion of HCl to develop (Cranwell, 1995). In addition to lactobacilli, the post-weaning feed (pre-pelleted) also contains 10^3 - 10^5 / g of various bacteria and fungi that can compromise the environment of the upper tract leading to both physiological and pathological problems. Furthermore, pepsin is required to initiate proteolysis of the "novel" proteins present in the feed, and to ensure their maximum digestion in the jejunum. In order to perform this effectively, the weaner pig requires abundant HCl production in the stomach. Schnabel (1983) as cited by Bolduan (1988), reported that HCl secretion reaches 50% of the whole stomach acidity or 3-8 mmol/100 g digesta only at 3 - 4 weeks post-weaning. This lack of acidity is accompanied by a pH increase post-weaning up to pH 4-5. Hence, the consequence of insufficient HCl production is that the consumed feed is insufficiently acidified in the stomach.

As a result, microbes are not sufficiently activated and protein digestion is reduced. It is known that the enzyme pepsin, which breaks down proteins into peptides, has an optimum pH for activity of around 2. Following a meal, the feed determines the pH value in the stomach for several hours and it has been suggested that such a pH is not attained in piglets, even 6 hours after *ad libitum* feeding (Kamphues, 1987 as cited by Bolduan 1988). The reduced protein digestion means that potentially pathogenic bacteria receive large quantities of undigested, readily available protein and starch as substrates that encourage their proliferation. The consequence is that there is a shift in the microorganisms colonising the gastrointestinal tract in favour of Gram-negative and often pathogenic bacteria (e.g. *E. coli*, *Streptococci, Clostridia* and *Salmonella*), which require a pH of between 6 and 8. The gut microflora is thus extremely unstable after weaning and it is important to consider feed as both a nutrient / energy source for the host and as a bacterial substrate. Bolduan (1988) illustrated that the ABC of a diet can be reduced by the addition of organic acids. However, there are two main disadvantages of this course of

action. Firstly, the amount of any acid is limited to less than 1% of the mass of the weaner diet, since acid addition in excess of this reduces palatability and depresses feed intake. Secondly, adding small quantities of acid to feed of high ABC has a very limited and minimal effect on stomach pH. Furthermore, the addition of organic acids may also alter the overall nutritional composition of the diet. Under the experimental conditions imposed in the current study, it was necessary for the 2 diets to be equal in all components except for ABC in order for a valid comparison between the control and treatment diets to be made.

Therefore the aim is to provide suitable post-weaning diets so that the piglet can effectively digest the protein fraction in the upper and middle-gut, and the material entering the lower gut (*i.e.* where the majority of digestive tract microorganisms reside) will be of relatively low protein / appropriate energy concentration residue, ideal for the fermentation process which occurs there, necessary to encourage a microbial load with positive benefit for the animal. In this way the microorganisms rapidly achieve what is essentially a symbiotic relationship with the piglet. This is the "stable microflora" concept of the healthy piglet.

Numerous workers have investigated the influence of dietary anion-cation balance on parameters such as acid-base status in a range of species, such as chickens (Mellière and Forbes, 1966; Mongin, 1981), cattle (Ogilvie *et al.* 1983), fish (Chiu *et al.* 1984), pigs (Yen *et al.* 1981), rabbits (Thacker, 1959) and rats (Feldman and Charney, 1980). There would appear to have been no research studying the effects of dietary ABC on gut morphological parameters in piglets.

3.2 AIMS

The exact mechanism by which high dietary ABC retards growth performance is largely unknown. However, alterations in the small intestinal morphology post-weaning are thought to contribute to the post-weaning growth check. Accordingly, a preliminary 2 x 2 factorial experiment was designed to examine the effects of diet ABC on gut morphological characteristics such as villus height, width and crypt depth. Differences between regions of the small intestine were also analysed. Additionally, ileal digesta pH was measured which is an independent factor influencing microflora colonisation. It was postulated that a low ABC diet would result in more rapid recovery of small intestinal

structure post-weaning, with subsequent positive impacts on post-weaning growth performance.

3.3 METHODS

3.3.1 Animals and housing

The experiment involved 20 newly weaned (commercial white hybrid) piglets (10 male and 10 female) of approximately 7 to 9 kg initial live-weight, the criterion for selection being animals of similar weight, and good health status. Litters were weaned at 28 d ± 1d of age and delivered to the University on the day of weaning.

Upon arrival on the experimental unit, piglets were individually weighed and randomly allocated to one of two groups (except for the 4 baseline animals, see section 3.3.3; each subsequent group containing 4 male and 4 female piglets). The piglets were group housed in an environmentally controlled (27 ^{0}C) room in the Pig Metabolism Building (experimental unit).

The piglets were group housed in 2 pens each with slatted floors, 2 feed and 1 water trough. Water was available *ad libitum* from the water trough, which was present in the pens at all times (changed 3 times/d at 08.00, 13.30 and 17.00 h). Feed was also available on an *ad libitum* basis with refusals being removed, weighed and discarded after each meal. Piglets were fed twice daily, at 08.00 h and 17.00 h with quantities offered being based on initial live-weight and calculated to exceed voluntary feed intake (Appendix 1).

3.3.2 Diets

A single-stage trial diet programme was used based on Provimi Ltd 'Multiwean' diet. Both diets were isoenergetic and isonitrogenous (15.4 MJ digestible energy (DE) / kg and 230g crude protein (CP) / kg) and were based on (g / kg) cooked cereals 488.5, wheatfeed 27.2, soya products 232, fishmeal 82.7, milk products 123, soya oil 10 and a mineral/vitamin supplement 26. Dietary ABC was altered by the removal of limestone flour. The control diet contained 14.8g limestone flour / kg yielding an ABC of 77 meq / kg, whilst the experimental diet contained 5g limestone flour / kg with an ABC of 62 meq / kg. This difference in ABC was the maximum that was achievable with no significant alterations to the basal formulation. This reduced ABC was deemed to be large enough to exert a significant impact on the gut ecosystem (Varley, personal communication). The

chemical composition has been described previously in section 2.3. The two diets were as follows:

Diet 1 - High ABC (Conventional starter diet)

Diet 2 - Low ABC (Treatment diet)

Table 3.1: Diet specification (refer to Table 2.1 for chemical composition)

Ingredient	Inclusion (g/kg) Diet 1 Control	Diet 2 Treatment
Cooked Cereals	528	536
Soya-bean meals	232	
Whey Products	120	
Fish Products	84	
Soya Oil	8	12
Limestone	16	4
Vitamins & Minerals	10	
Amino Acids	2	

3.3.3 Experimental procedure

The experimental and slaughter procedure has been described previously in sections 2.4 and 2.5. The first 4 piglets (baseline) were slaughtered on day 0, which corresponds to both the weaning and arrival date of the piglets at the experimental unit. A total of 4 piglets were subsequently slaughtered on each of days 2, 4, 6 and 14; 2 piglets having been fed diet 1 (high ABC; conventional starter diet) and 2 diet 2 (low ABC). At slaughter, the ileal digesta contents were removed immediately and the pH measured directly using a digital pH meter (Mettle Toledo 320; Fisher Scientific UK, Loughborough, Leics.). The pH meter was calibrated prior to pH measurement using pH 4.0, 7.0 and 9.2 buffering solutions (Fisher Scientific UK; Loughborough, Leics.).

3.3.4 Histology

The histological protocol has been described previously in section 2.6. No modifications to this procedure were made.

3.4 STATISTICAL ANALYSES

The statistical analyses have been described previously in section 2.12. This preliminary study utilised 20 piglets in total comprising 10 male and 10 female. Baseline animals (n = 4) were slaughtered upon arrival at the experimental unit, 4 piglets of each sex were subsequently offered 1 of each of the diets 1 and 2. This essentially formed 2 groups of animals each comprising 4 male and 4 female animals; group 1 were offered diet 1 (control), and group 2 were offered diet 2 (treatment). This experimental design is illustrated below:

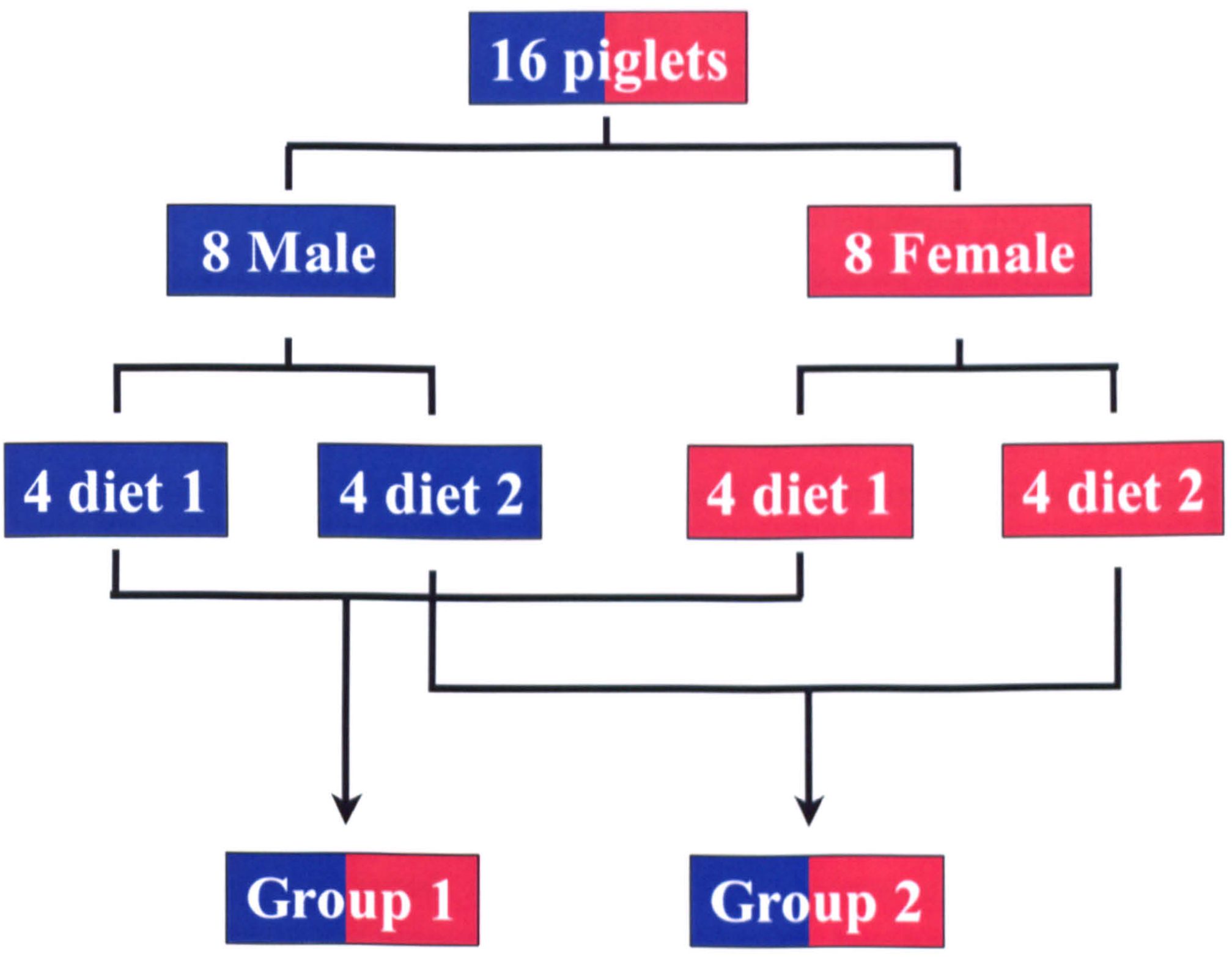


Figure 3.1: Diagrammatic representation of the experimental design utilised in this study

As such in this preliminary study there was no strict dietary replication, since one group of animals was a replicate. It must, however, be emphasised that this was a preliminary study and all subsequent studies utilised animals housed on an individual basis. It was nevertheless considered worthwhile to employ individual animals as replicates to provide an initial assessment of diet.

3.5 RESULTS

A significant relationship between day and diet (P=<0.001: <0.001 (Q)) was evident with respect to villus height (Tables 3.2 and 3.3). Piglets offered the low ABC diet displayed more rapid recovery of villus height in the post-weaning period (P<0.001; Figure 3.2), and also maintained ileal pH at a more acid level. Moreover, there was a significant difference between intestinal region, with villi at the distal region being the shortest for both control and treatment animals (P<0.001). Villus height increased (after an initial degree of villus atrophy) significantly between day 0 and day 14 (P<0.001; <0.001 (L); <0.001 (Q)), and there was a significant correlation between BVR and day (P=0.029), but no significant difference between the baseline and the remainder of the animals was found (BVR). Although not significant (P=0.101), the relationship between BVR and diet is nonetheless interesting.

Villus width followed a similar pattern; a significant relationship between day and diet was observed (P<0.001; 0.090 (L); Tables 3.4 and 3.5). Again there was a significant difference in relation to dietary treatment (P=0.006), with those animals offered the control diet (diet 1; high ABC) displaying the greatest overall average width. Villus width increased significantly throughout the experimental period (P<0.001; <0.001 (L); 0.014 (Q)), with the proximal villi possessing the greatest width (P<0.001). A significant correlation between BVR and day (P=0.033) was evident, but no significant differences due to BVR, or relationship between BVR and dietary treatment were determined.

Analysis revealed a significant non-linear relationship (P<0.001; P<0.001 (Q)) between day and diet with respect to crypt depth (Tables 3.6 and 3.7). Crypt depth also increased temporally (P<0.001; <0.001 (L)), and the overall mean crypt depth was significantly greater for the treatment animals (low ABC; P=0.009). Furthermore, no significant differences were determined between intestinal region or BVR. Significant relationships between BVR and day, or BVR and diet were not evident.

Villus height : crypt depth ratios were significantly different between intestinal region (P<0.001), and also between slaughter day (P=0.037; 0.088(Q); Tables 3.8 and 3.9). A significant relationship between day and diet (P=0.019), and BVR and region (P<0.001), were also found. Strong trends towards significant differences between BVR (P=0.087) and also a significant correlation between BVR and diet (P=0.066) were apparent.

No significant differences between ileal digesta pH with respect to dietary treatment were evident (Tables 3.10 and 3.11). However, a strong trend was observed towards significant differences between day (P=0.095 (Q)) and BVR (P=0.078). Diet pH values were 5.94 and 5.94 for diets 1 (control; high ABC) and 2 (treatment; low ABC) respectively.

Feed intake levels were similar for both dietary treatments, and there was no significant effect of dietary treatment on piglet daily live-weight gain (mean values = 0.121 and 0.170 for control (high ABC) and treatment (low ABC) diets respectively; P = 0.437; s.e.d = 0.06).

Table 3.2: Effect of dietary treatment on villus height (μm; baseline = animals slaughtered on day zero (weaning) $n = 4$; $n = 8$ per diet)

	Baseline				Diet 1				Diet 2			
	Proportion of Intestine				Proportion of Intestine				Proportion of Intestine			
Day	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean
0	607	378	409	**465**								
2					405	428	392	**408**	451	499	503	**484**
4					427	354	285	**355**	298	328	357	**328**
6					391	338	315	**348**	499	492	477	**489**
14					504	510	400	**471**	538	523	430	**497**
mean	**607**	**378**	**409**	***465***	**432**	**407**	**348**	***396***	**446**	**460**	**442**	***450***

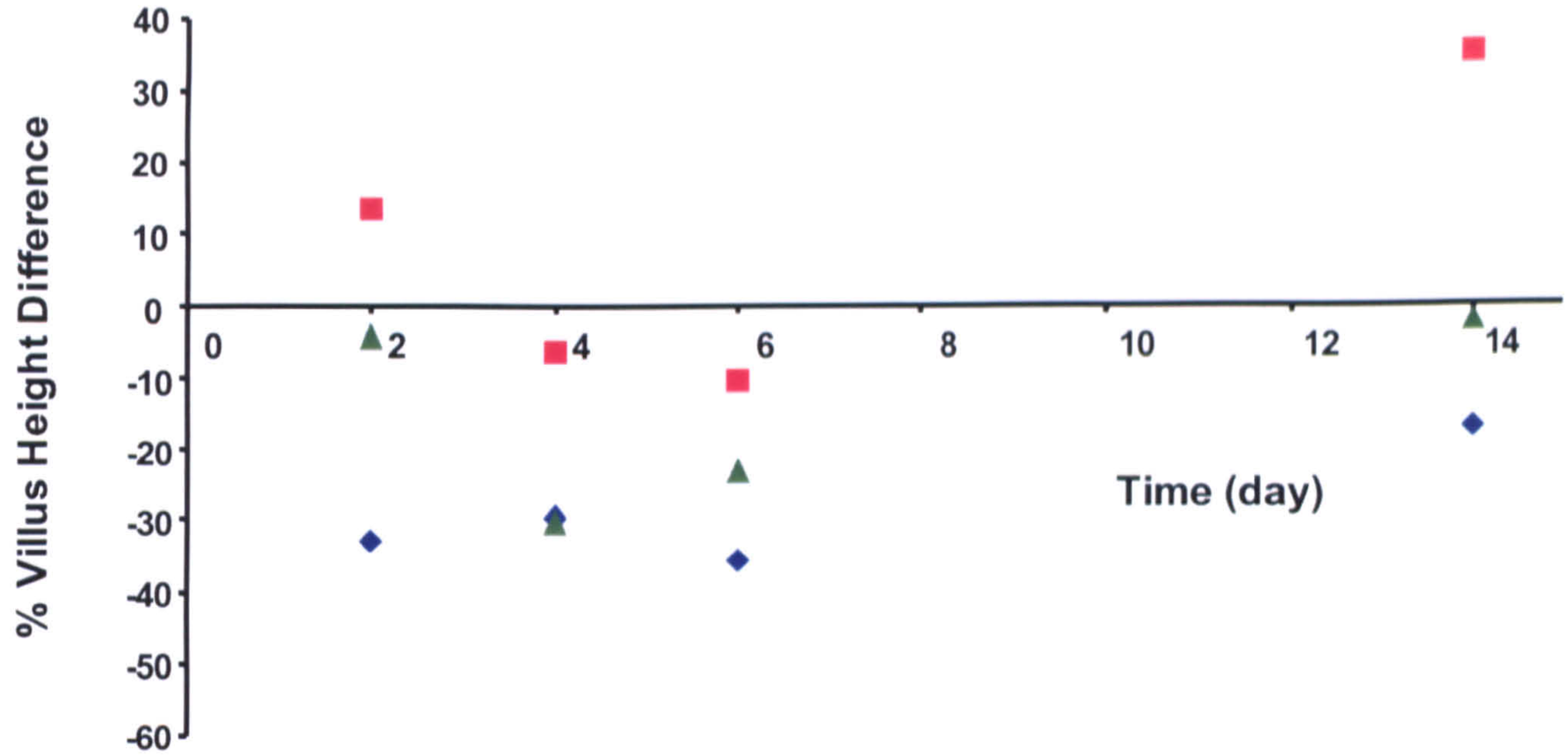
Baseline = animals slaughtered on day 0 (weaning); diet 1 = control (high ABC); diet 2 = treatment (low ABC)

Table 3.3: A summary of the statistical analysis for villus height

Diet		Day		Diet*Day		Region		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
5.3	<0.001	7.5	<0.001 <0.001 (L) <0.001 (Q)	10.6	<0.001 0.122 (L) <0.001 (Q)	6.5	<0.001	32.8	0.245	41.4	0.029	35.9	0.101

BVR = Baseline versus the rest; day refers to slaughter day

a)



b)

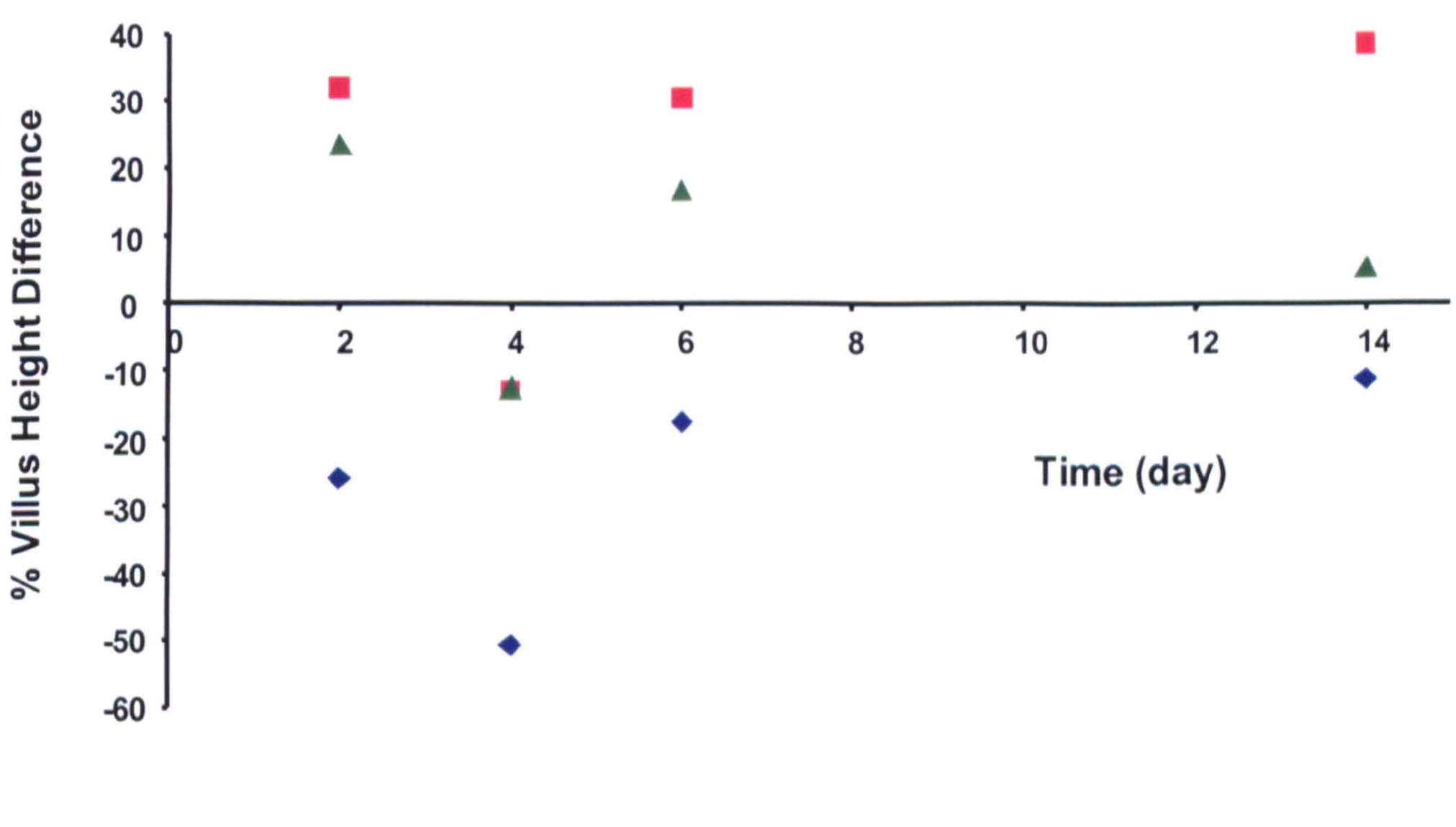


Figure 3.2: Percentage difference in villus height between baseline values and those obtained from animals slaughtered on days 2, 4, 6 or 14 fed a) diet 1 (control) or b) diet 2 (treatment) at regions proportionately 0.25 (◆), 0.50 (■) and 0.75 (▲) along the length of the intestine from the gastric pylorus to the ileo-caecal valve.

Table 3.4: Effect of dietary treatment on villus width (μm; baseline = animals slaughtered on day zero (weaning) $n = 4$; $n = 8$ per diet)

	Baseline				Diet 1				Diet 2			
	Proportion of Intestine				Proportion of Intestine				Proportion of Intestine			
Day	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean
0	110	85	94	**96**								
2					108	82	95	**95**	107	89	96	**97**
4					103	103	99	**102**	78	85	83	**82**
6					96	90	87	**91**	105	105	94	**101**
14					123	108	113	**115**	114	102	94	**103**
mean	**110**	**85**	**94**	***96***	**108**	**96**	**98**	**101**	**101**	**95**	**92**	**96**

Baseline = animals slaughtered on day 0 (weaning); diet 1 = control (high ABC); diet 2 = treatment (low ABC)

Table 3.5: A summary of the statistical analysis for villus width

Diet		Day		Diet*Day		Region		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
1.7	0.006	2.4	<0.001 <0.001 (L) 0.014 (Q)	3.4	<0.001 0.090 (L) 0.116 (Q)	2.1	<0.001	4.0	0.726	5.1	0.033	4.4	0.385

BVR = Baseline versus the rest; day refers to slaughter day

Table 3.6: Effect of dietary treatment on crypt depth (μm; baseline = animals slaughtered on day zero (weaning) $n = 4$; $n = 8$ per diet)

	Baseline				Diet 1				Diet 2			
	Proportion of Intestine				Proportion of Intestine				Proportion of Intestine			
Day	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean
0	237	213	219	**223**								
2					166	208	204	**193**	208	259	264	**244**
4					238	230	223	**231**	220	219	244	**228**
6					275	249	239	**254**	224	223	202	**216**
14					277	244	238	**253**	306	283	284	**291**
mean	**237**	**213**	**219**	***223***	**239**	**233**	**226**	***233***	**239**	**246**	**248**	***245***

Baseline = animals slaughtered on day 0 (weaning); diet 1 = control (high ABC); diet 2 = treatment (low ABC)

Table 3.7: A summary of the statistical analysis for crypt depth

Diet		Day		Diet*Day		Region		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
4.5	0.009	6.4	<0.001 <0.001 (L) 0.954 (Q)	9.1	<0.001 0.327 (L) <0.001 (Q)	5.6	0.899	17.1	0.398	21.7	0.130	18.8	0.303

BVR = Baseline versus the rest; day refers to slaughter day

Table 3.8: Effect of dietary treatment on villus height to crypt depth ratio (μm; baseline = animals slaughtered on day zero (weaning) $n = 4$; $n = 8$ per diet)

	Baseline				Diet 1				Diet 2			
	Proportion of Intestine				Proportion of Intestine				Proportion of Intestine			
Day	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean
0	2.7	1.8	1.9	2.2								
2					2.7	2.1	2.2	2.4	2.2	1.7	2.0	2.0
4					1.9	1.6	1.3	1.6	1.4	1.5	1.6	1.5
6					1.5	1.4	1.4	1.4	2.4	2.3	2.4	2.4
14					1.9	2.2	1.7	1.9	1.8	1.9	1.6	1.8
mean	2.7	1.8	1.9	2.2	2.0	1.8	1.7	1.8	1.9	1.9	1.9	1.9

Baseline = animals slaughtered on day 0 (weaning); diet 1 = control (high ABC); diet 2 = treatment (low ABC)

Table 3.9: A summary of the statistical analysis for villus height to crypt depth ratio

Diet		Day		Diet*Day		Region		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.11	0.585	0.16	0.037 0.492 (L) 0.088 (Q)	0.23	0.019 0.836 (L) 0.005 (Q)	0.05	<0.001	0.16	0.087	0.20	0.066	0.17	0.644

BVR = Baseline versus the rest; day refers to slaughter day

Table 3.10: Mean ileal digesta pH values (µm; baseline = animals slaughtered on day zero (weaning) $n = 4$; $n = 8$ per diet)

Day	Baseline	Diet 1	Diet 2	mean
0	7.0			
2		8.1	7.6	7.9
4		7.3	7.4	7.4
6		7.3	7.2	7.2
14		7.3	7.5	7.4
mean	7.0	7.5	7.4	7.5

Baseline = animals slaughtered on day 0 (weaning); diet 1 = control (high ABC); diet 2 = treatment (low ABC)

Table 3.11: A summary of the statistical analysis for ileal digesta pH

Diet		Day		Diet*Day		BVR		BVR*Diet		BVR*Day	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.22	0.733	0.31	0.274 0.376 (L) 0.095 (Q)	0.43	0.774 0.495 (L) 0.585 (Q)	0.23	0.078	0.26	0.722	0.30	0.232

BVR = Baseline versus the rest; day refers to slaughter day

3.6. EXECUTIVE SUMMARY OF RESULTS

Factors common to all studies contained within this thesis are discussed in detail later in Chapter 6.

The use of a low ABC diet for 14 days immediately post-weaning did improve gut morphology characteristics. The recovery of villus height occurred primarily between days 4 and 6 post-weaning, suggesting that the first 6 days post-weaning are the most crucial for the piglet in terms of intestinal recovery and development. There were no differences in feed intake post-weaning therefore this cannot explain this improved recovery. This is in contrast to work reported by Kelly *et al.* (1984) and McCracken and Kelly (1984) who have suggested that low feed intake immediately post-weaning may be responsible for the changes in gut structure and function in the pig (such as villus atrophy, crypt hyperplasia, changes in specific enzyme activity relating to the digestive and absorptive function of the gut). Specifically, several authors such as, Hampson and Smith (1986) and Kelly *et al.* (1990) have proposed that villus atrophy and crypt hyperplasia may be related to the withdrawal of ' intrinsic factors ', such as IgA, in sows' milk (and indeed other mammalian milks), which may also contain an array of biologically active peptides (e.g. epidermal growth factor) and compounds (e.g. polyamines) that may stimulate protein and DNA synthesis and have functional roles in intestinal differentiation. This is supported by work conducted by Pluske *et al.* (1996b; c) where villus height and crypt depth could be maintained post-weaning by feeding a milk liquid diet (ewes' or cows' fresh milk) immediately after weaning, at two-hourly intervals in comparison to unweaned controls. It was suggested that the milk may have promoted the activity of enzymes, peptides and growth factors, and hence contributed to the integrity of the small intestine.

The current data therefore suggest that the structure of the small intestine can recover more rapidly when a low ABC diet is given after weaning; this will increase the absorptive surface area in the gut and could lead to reductions in the growth check that is often observed after weaning. However, in the current study the improvements in gut morphology observed in animals fed diet 2 (low ABC) did not result in any positive effects on subsequent growth rate. This is in contrast to that reported by Spencer and Tilley (1994), where piglets fed a low ABC diet (as determined by the acid binding factor (ABF) of 17.3) displayed significantly reduced morbidity and mortality, and enhanced daily gains, compared to those fed the conventional (high ABF of 24) post-weaning diet.

The post-weaning changes in digesta pH, albeit transitory and comparatively modest (and non-significant between diets), may have important implications for gut microflora load by inhibiting the proliferation of deleterious microorganisms which typically require a less acid pH (*i.e. E. coli*, *Streptococci*, *Clostridia* and *Salmonella*).

Furthermore, dietary ABC is an important parameter to control as it is likely to impact on the acid-base balance of biological fluids (Stewart, 1978). In addition, Kronfeld (1979) stated that acid-base balance has a higher physiological priority than growth, lactation or reproduction; hence any changes in acid-base balance are likely to affect animal performance.

Diets formulated to possess a low ABC require a relatively low HCl secretion by the piglet's immature stomach in order to maintain a low pH in the upper GIT. This subsequently results in adequate pepsin production/secretion, enhanced protein digestion and utilisation, and an unfavourable environment for the proliferation of pathogens. Indeed, Spencer and Tilley (1994) also reported that where such low ABC diets were utilised commercially, a marked reduction in the quantity of medication required to treat these pigs was reported by the veterinary surgeon.

In summary, this preliminary study demonstrates that villus height can recover more rapidly if a low ABC diet is fed immediately post-weaning. In contrast, piglets offered a conventional, high ABC starter diet displayed villus atrophy and crypt hyperplasia for a prolonged period of time. The potential contribution of feeding low ABC diets to preserve gut structure and function and hence reduce post-weaning growth check seems plausible, although further research in this area is required. Aspects of this preliminary study were incorporated and improved upon to form the basis of work in subsequent Chapters (4 and 5).

CHAPTER 4:

EFFECTS OF DIETARY ZINC AND ANTIBIOTIC ADDITION ON PERFORMANCE AND GUT HEALTH CHARACTERISTICS OF POST-WEANED PIGLETS

4.1 INTRODUCTION

Zinc oxide (ZnO) has been included in post-weaning piglet diets commercially, in an attempt to reduce the incidence of post-weaning diarrhoea associated with *Escherichia coli*, and to promote growth. Evidence suggests that weanling pigs exhibit increased growth performance when fed pharmacological concentrations of Zn as ZnO (Hahn and Baker (1993) Hill *et al.* (1996), Smith *et al.* (1997), Carlson *et al.* (1999)), although there are some reports in which no growth-promoting benefits were observed (Fryer *et al.* (1992), Tokach *et al.* (1992), Schell and Kornegay (1996)). A possible explanation for this lack of response is that young, immature pigs may have a reduced capacity to clear excess Zn from the body, possibly resulting in high levels of Zn in the blood, thus preventing a ZnO-induced response in weight gain (Hahn and Baker, 1993). It should be noted however, that the direct comparison of different studies is hampered by the fact that, in many reports in the literature, the diets also contain feed grade antibiotics. Furthermore, Hahn and Baker (1993) reported improved feed intake and weight gain from feeding Zn from a ZnO source, but not from $ZnSO_4$ or Zn-Met sources. Hahn and Baker (1993) relate this to the fact that the 'coefficient of bioavailability' of Zn in ZnO is lower than that for other Zn sources such as $ZnSO_4$ (Wedekind and Baker, 1990; Wedekind *et al.*, 1992; Wedekind and Lewis, 1993). For example, Poulsen and Larsen (1994) estimated the 'bioavailability' of Zn from ZnO to be 0.2 in basic pig diets comprising barley (467 g/kg), wheat (200 g/kg) and soybean meal (270 g/kg). Furthermore, the bioavailability of zinc obtained from the same source may differ due to the presence of contaminating materials such as lead, iron or calcium oxide (Poulsen, 1995). A further factor affecting the bioavailability among ZnO sources are the processing methods and the resultant temperatures to which ZnO is exposed. For example, the Waelz process reaches 1,200^{0}C (Edwards and Baker, 1999); any temperatures either below or above this will affect bioavailability. Moreover, in a study conducted by Mavromichalis *et al.* (2000) where only relatively small ZnO responses were observed, and the responses between 2 different ZnO sources were the same, it was suggested that this may be attributed

to the variability inherent in trials initiated immediately after weaning. Indeed, Carlson *et al.* (1999) indicated that pigs need to be fed ZnO-supplemented diets for at least 2 weeks post-weaning for any growth-promoting effects to become apparent, further implying that at least a 1 week priming (or preloading) period may be required for clear ZnO responses to become manifest (Mavromichalis *et al.*, 2000).

The mode of action for the enhanced growth observed when weanling piglets are fed pharmacological concentrations of Zn has not yet been fully elucidated. One proposed mechanism concerns the protein metallothionein (MT), which is involved in maintaining Zn homeostasis (Richards and Cousins, 1975; Menard *et al.*, 1981), and alteration of intestinal morphology (Hampson, 1986) that may enhance nutrient absorption. MT regulates dietary Zn absorption by binding dietary Zn in the intestine. Excess Zn-MT complex is not absorbed and is later sloughed off in the epithelial cells (Cousins, 1985). Zn transported into the plasma is deposited in the liver, where MT functions in its release and storage (Carlson *et al.*, 1999). MT may operate in conjunction with zinc transporters but, at present, there is no evidence to support this. Furthermore, hepatic and intestinal MT synthesis is stimulated by dietary zinc supplementation (Krebs, 2000). Conversely, Evans *et al.* (1979) concluded that Zn homeostasis is maintained by the secretion of Zn into the intestinal lumen rather than by the control of Zn absorption. MT was not considered an important regulator of the absorption process. However, Starcher *et al.* (1980) suggested that although conflicting data exists concerning the role of MT in Zn metabolism, MT does protect against heavy metal toxicity.

Zinc is known to possess antimicrobial properties. ZnO-based ointments are routinely used in human medicine and ZnO has been shown to enhance wound healing (Prasad, 1983), for example to treat and prevent footrot in sheep. In pigs, high dietary ZnO supplementation has been associated with reduced bacterial translocation from the small intestine to the ileal mesenteric lymph node (Huang *et al.*, 1999), and with improved homogeneity and stability in intestinal coliform populations (Katouli *et al.*, 1999). Furthermore, Gram-positive bacteria are most susceptible to ZnO, whereas Gram-negative bacteria and streptococci are not usually inhibited by high ZnO concentrations (Mavromichalis *et al.*, 2000).

Katouli *et al.* (1999) demonstrated that individual pigs carry a number of transient and resident coliforms. Furthermore, some of the resident phenotypes within an animal appeared

during the first week of life, while others appeared at later stages of the suckling period. The majority of the resident coliforms were present in several pigs, suggesting that they possessed the ability to colonise the gut, and persist for a prolonged period within the intestine. Katouli *et al.* (1999), also demonstrated that coliform colonisation of pigs is both strain- and host-specific as very few pigs were colonised with the same resident strain. Additionally, Katouli *et al.* (1999) reported that the majority of resident coliforms in both control and ZnO-supplemented groups were not isolated in samples obtained immediately post-weaning (*i.e.* 2 days post-weaning), possibly because their numbers in the faecal samples were below detectable levels. This is supported by the fact that most of them appeared again in succeeding samples.

The growth-promoting effect of dietary antibiotic supplementation is well documented (e.g. Visek, 1978). There is also substantial evidence within the literature implicating an involvement of the intestinal bacterial population in this growth promotion, and proposed mechanisms associated with antibiotic supplementation include; a) that pathogens responsible for mild and subclinical infections are suppressed, b) the bacterial production of growth-depressing toxins is reduced, c) that bacterial destruction and utilisation of essential nutrients is reduced (Francois, 1962; Visek, 1978) and d) a beneficial shift in intestinal bacteria metabolism is created (Visek, 1978; Vervaeke *et al.*, 1979). Specifically, this beneficial shift is explained by the detoxification and / or excretion of metabolites. For example, the anaerobic bacterial degradation of tyrosine and tryptophan results in the production of several volatile phenolic and aromatic metabolites (Scheline, 1968; Bakke, 1969; Yokoyama and Carlson, 1974; Yokoyama and Carlson, 1979) such as 4-methylphenol (*p*-cresol). Based on the excreted form of *p*-cresol (Spoelstra, 1978; Kao *et al.*, 1979 as cited by Yokoyama *et al.*, 1982), it is thought that the pig utilises uridine diphosphate-glucuronic acid (derived from glucose in the uronic acid pathway), to form the glucuronide conjugate of phenolics before their excretion. Therefore, a decrease in intestinal *p*-cresol production by antibiotics would decrease the glucose and ATP necessary for the detoxification and excretion of this bacterial metabolite, thus allowing more energy to be directed towards growth by the host animal.

4.2 AIMS

To determine the effects of feeding 4 diets to piglets on gut physiology and microflora characteristics (numbers and ratios of specific species (including coliforms, and lactobacilli)), mineral status and performance characteristics between 7.5 and 20 kg live-weight; to evaluate the potential interactive or additive effects of growth-promotional levels of Zn and avilamycin on weanling pig performance. It is hypothesised that dietary Zn and/or antibiotic addition will beneficially effect small intestinal structure post-weaning, and enhance the population of lactobacilli. Such effects will result in enhanced growth performance post-weaning.

4.3 METHODS

4.3.1 Animals and housing

Thirty-six entire male piglets (commercial hybrid white genotype) obtained from a local multiplier herd, weaned at 28 ± 1 days of age (7.5 kg live-weight) were selected. The piglets were individually housed in pens with 1 m^2 slatted floor space/pen and partitions allowing visual, olfactory and limited tactile contact between adjacent pens. Four piglets were slaughtered on day 0 (weaning day), which corresponds to the start of the trial, and provide baseline data to compare with the remaining animals.

4.3.2 Diets

A single-stage trial diet programme was used based on Provimi Ltd 'Multiwean' diet. Diets are based upon identical formulations, but diets 2, 3 and 4 contained the following supplements:

Diet 1	No supplement; Conventional Starter Diet
Diet 2	+ ZnO (2500 ppm)
Diet 3	+ Avilamycin (40 ppm)
Diet 4	+ ZnO and Avilamycin (2500 and 40 ppm respectively)

The basal diet contained 140 ppm Zn that meets the requirements for a pig of this age. Additionally, the above dietary supplements were not added to the basal ration at the expense of another constituent, but as a supplement to the basal ration. One of the four diets was fed

to 8 piglets over an experimental period of 14 days. All 4 diets comprised the same raw ingredients as shown in Table 4.1.

Table 4.1: Diet specification (refer to table 2.1 for chemical composition)

Ingredient	Inclusion (g/kg)
Cooked cereals	497.6
Oilseeds	173.0
Fish Products	87.0
Whey Powders	215.5
Oils & Fats	12.0
Vitamins & Minerals	15.0
Amino Acids	2.9

4.3.3 Post-mortem procedure

On the day of weaning (day 0; $n = 4$), and on days 2, 4, 6 and 14 ($n = 8$; 2 piglets from each of the 4 experimental diets), piglets were slaughtered and samples of the small intestine at sites proportionately 0.25, 0.50 and 0.75 along the length of the small intestine were taken for subsequent histological examination. The slaughter, post-mortem procedure and preparation of small intestinal samples for measurement of villus height, width and crypt depth have been described previously in section 2.4.

In addition, a peripheral blood sample was taken and frozen at -80 ^{0}C for subsequent blood urea nitrogen (BUN) analysis. The liver and kidney were also removed, weighed and frozen at -80 ^{0}C for subsequent mineral analysis.

Digesta samples from the duodenum, jejunum, ileum, colon and rectum were immediately removed for microbiological enumeration and pH analysis of ileal digesta samples were untaken as described in sections 2.5 and 2.8 respectively.

4.4 MICROBIOLOGICAL ANALYSIS

Faecal swabs were collected from all pigs every second day throughout the experimental period. Coliform and lactobacilli counts were determined. Serial dilutions of digesta samples were plated on selective media as given in section 2.8.5 and incubated appropriately. Following incubation, the colonies were identified and counted as described in section 2.8.

4.5 MINERAL ANALYSIS

Tissue samples were analysed for zinc concentration by flame atomic absorption spectrophotometry (AAS). Blood samples underwent analysis for urea nitrogen content (BUN) by use of a Sigma diagnostic kit combined with spectrophotometry (Appendix 3).

4.6 STATISTICAL ANALYSES

Statistical analyses were undertaken as described previously in section 2.12. Tissue analyses for zinc concentration were also assessed using a 2 factor completely randomised split-plot analysis of variance, with slaughter day as the main-plot factor and sample as the sub-plot factor. The following treatment structures were employed (where sample refers either to the liver or kidney sample):

Zn*Ab*POL(Slaughter Day; 2)*Sample

BVR / (Zn*Ab*Slaughter Day*Sample)

BVR = Baseline versus the rest

POL = Polynomial function

4.7. RESULTS

4.7.1 Gut morphology

Gut morphology data obtained for baseline animals (animals slaughtered on day zero) are presented in table 4.2. Villus height (VH; $P<0.001$; s.e.d = 36.76), villus width (VW; $P=0.006$; s.e.d = 4.07) and crypt depth (CD; $P=0.008$; s.e.d 15.92) increased significantly with advancing distance along the small intestine from proportionately 0.25 to 0.50 from the gastric pylorus to the ileo-caecal valve. Measurements taken at 0.75 along the intestine were lower than those taken at 0.25 along the small intestine. A similar trend was also observed for days 2, 4, 6 and 14 across all dietary treatments.

Analysis of variance established that ileal VH increased significantly over time for all dietary treatments; a significant linear effect was established even after the effect of slaughter weight was taken into account (P=0.184; 0.045 (L); Tables 4.3 and 4.4). No significant differences due to dietary antibiotic (avilamycin) or zinc were established. Mean VH at day 2 was lower than the mean baseline values (day 0 = weaning) for all dietary treatments indicating that a degree of villus atrophy occurred between days 0 and 2. Although not significant, this villus atrophy continued until day 4 for those animals fed the control diet. Significant differences with respect to BVR (P=0.015) and region (P=<0.001) were also evident with VH generally declining with increasing distance along the small intestine from the gastric pylorus to the ileo-caecal valve.

Although not significant, VW tended to increase over time for all dietary groups (P=0.115 (Q); Tables 4.5 and 4.6). Analysis revealed no significant differences in relation to dietary treatment, intestinal region, or BVR. No significant relationships between diet and day, BVR and day, or BVR and diet were found.

There was a strong trend (P=0.088) for CD to increase throughout the experimental period (Tables 4.7 and 4.8). The mean CD was similar for all 4 dietary treatments and no significant differences with respect to BVR were determined. No significant correlations between diet and day, or BVR and diet were observed. CD at a distance of 0.50 along the length of the intestine tended to be greater than those at 0.25 or 0.75 (P=0.019), indicating a significant quadratic effect over time (P=0.047). There was also a strong trend towards a correlation between BVR and day (P=0.077), with CD increasing over time in comparison to the mean baseline measurement of 193µm.

Analysis determined no significant differences between dietary treatment, or day, and no significant relationships between diet and day, BVR and day or BVR and diet with respect to villus height to crypt depth (VH:CD) ratio (Tables 4.9 and 4.10). A significant difference between intestinal region (P<0.001) was found, with the VH:CD ratio at the proximal region of the small intestine (SI) being greatest (in terms of overall mean). Baseline animals also displayed VH:CD ratio values that were significantly different to the remainder of the experimental animals (P=0.045).

Table 4.2: Gut morphological parameters for baseline animals (slaughtered on day zero; $n = 4$)

Parameter	Proportion of intestine 0.25	0.50	0.75	mean	BVR s.e.d	P	BVR*Zn s.e.d	P	BVR*Ab s.e.d	P	BVR*Day s.e.d	P
Villus Height (VH) (µm)	438	536	310	428	48.5	0.015	44.9	0.613	44.6	0.396	67.6	0.128
Villus Width (VW) (µm)	73	82	67	74	3.9	0.204	3.6	0.720	3.6	0.473	5.5	0.122
Crypt Depth (CD) (µm)	197	202	180	193	19.9	0.180	18.4	0.634	18.3	0.366	27.7	0.077
VH to CD ratio	2.44	2.81	1.78	2.35	0.4	0.045	0.4	0.986	0.4	0.322	0.6	0.804

BVR = Baseline versus the rest; Zn = Zinc dietary treatment; Ab = Antibiotic (avilamycin) dietary treatment; day refers to slaughter day

Table 4.3: Effect of dietary treatment on villus height (μm; $n = 8$ per diet)

	Diet 1				Diet 2				Diet 3				Diet 4			
	Proportion of Intestine				Proportion of Intestine				Proportion of Intestine				Proportion of Intestine			
Day	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean
2	446	373	346	388	389	338	281	336	382	305	292	326	320	354	358	344
4	441	357	296	365	379	389	309	359	450	459	346	418	491	449	324	421
6	339	344	479	387	469	524	408	467	497	445	458	467	395	434	393	407
14	539	575	498	537	575	571	415	520	438	417	366	407	582	632	593	602
mean	441	412	405	*419*	453	456	353	*421*	442	407	365	*404*	447	467	417	*444*

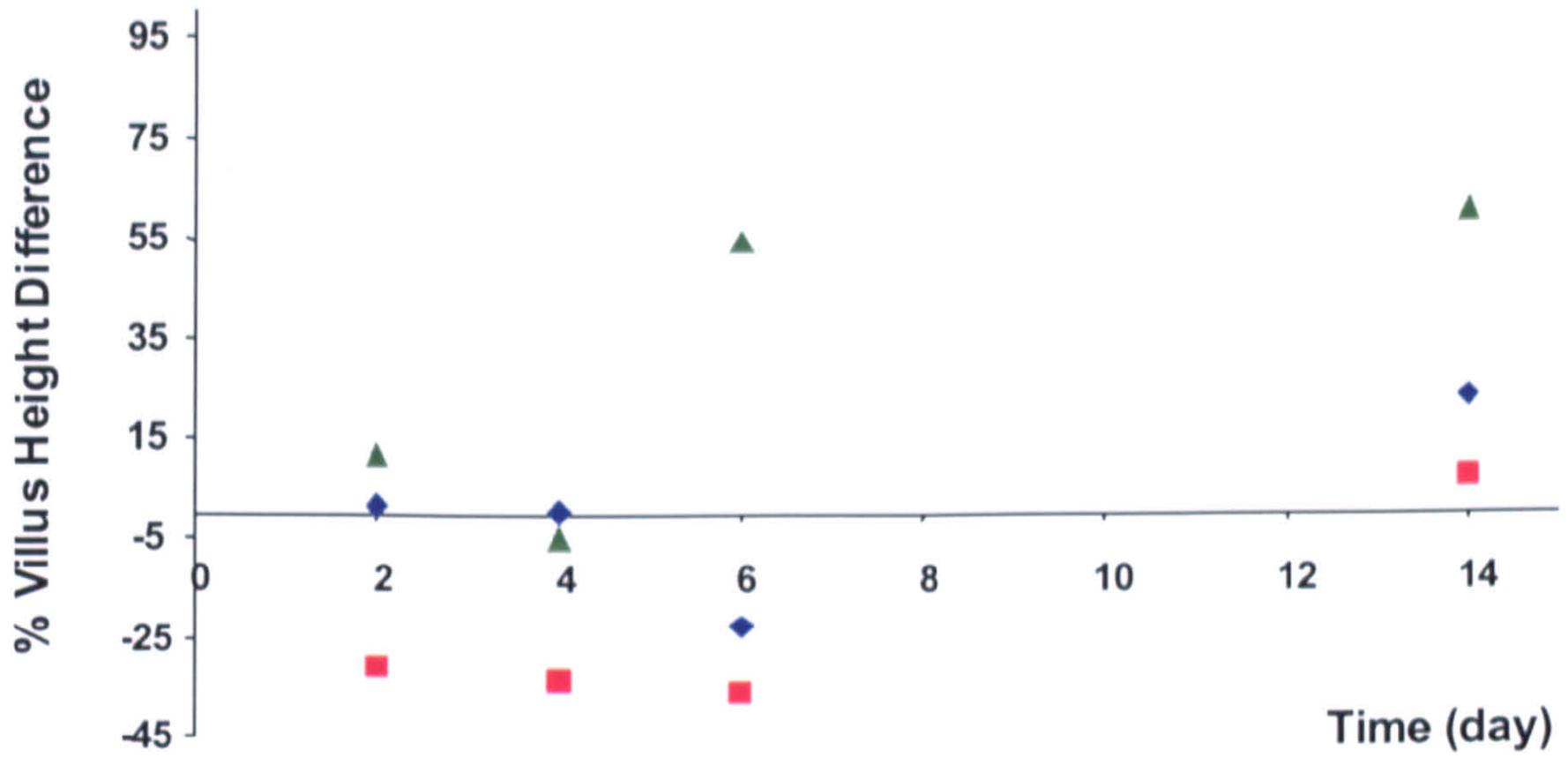
(Mean baseline values = 438, 536, 310 & 428 μm for distances proportionately 0.25, 0.50, 0.75 along the length of the SI & the overall mean respectively (table 4.2); diet 1 = Control; diet 2 = ZnO; diet 3 = Avilamycin; diet 4 = ZnO + Avilamycin)

Table 4.4: A summary of the statistical analysis for villus height

Zn		Ab		Day		Zn*Day		Ab*Day		Region	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
27.6	0.635	26.2	0.428	92.1	0.198 0.051 (L) 0.395 (Q)	95.4	0.560 0.622 (L) 0.369 (Q)	94.4	0.533 0.153 (L) 0.906 (Q)	4.8	<0.001

Zn = Zinc dietary treatment; Ab = Antibiotic (avilamycin) dietary treatment; day refers to slaughter day

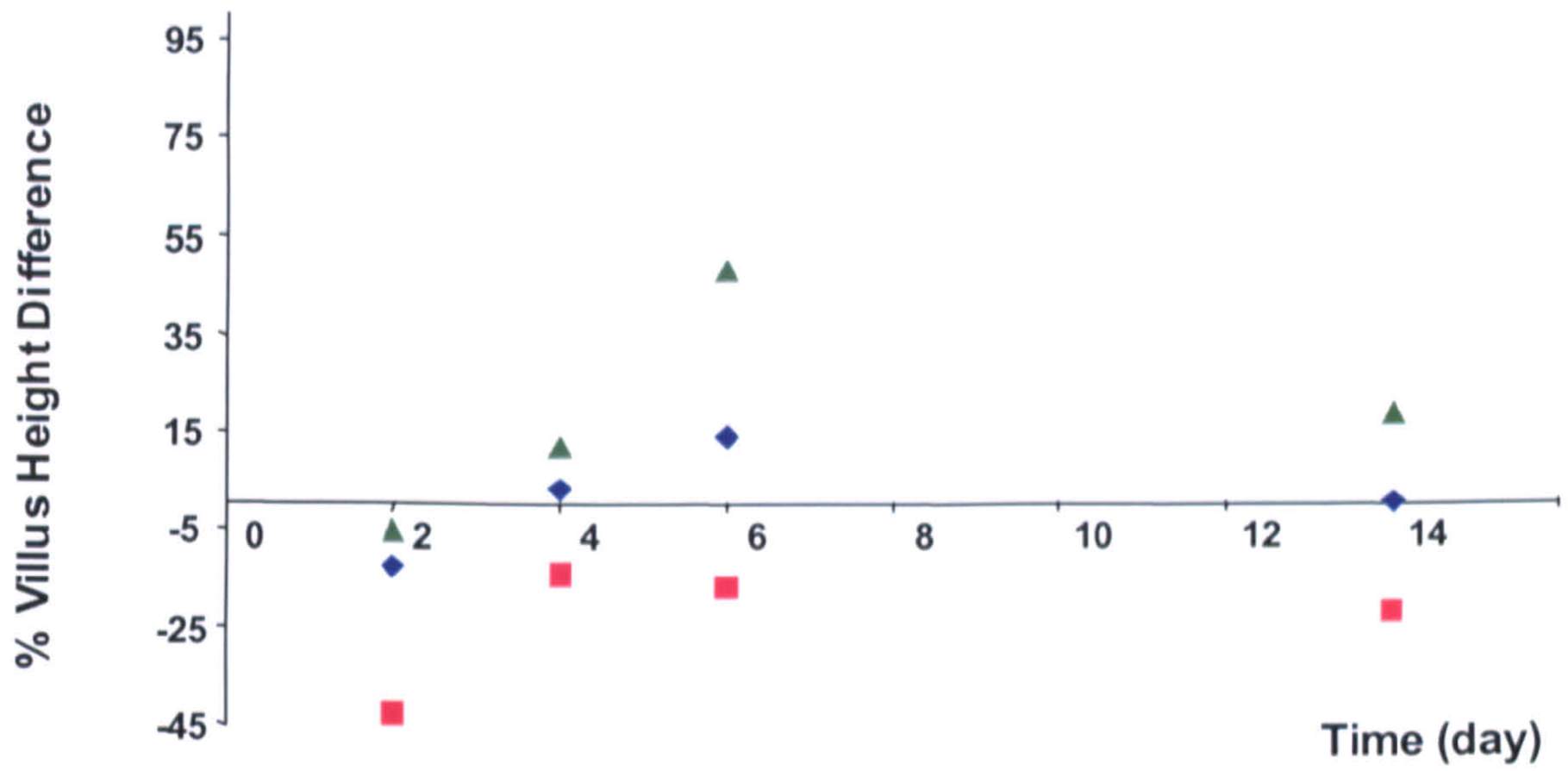
a)


% Villus Height Difference
95
75
55
35
15
-5
-25
-45
0
2
4
6
8
10
12
14
Time (day)

b)

% Villus Height Difference
95
75
55
35
15
-5
-25
-45
0
2
4
6
8
10
12
14
Time (day)

c)



d)

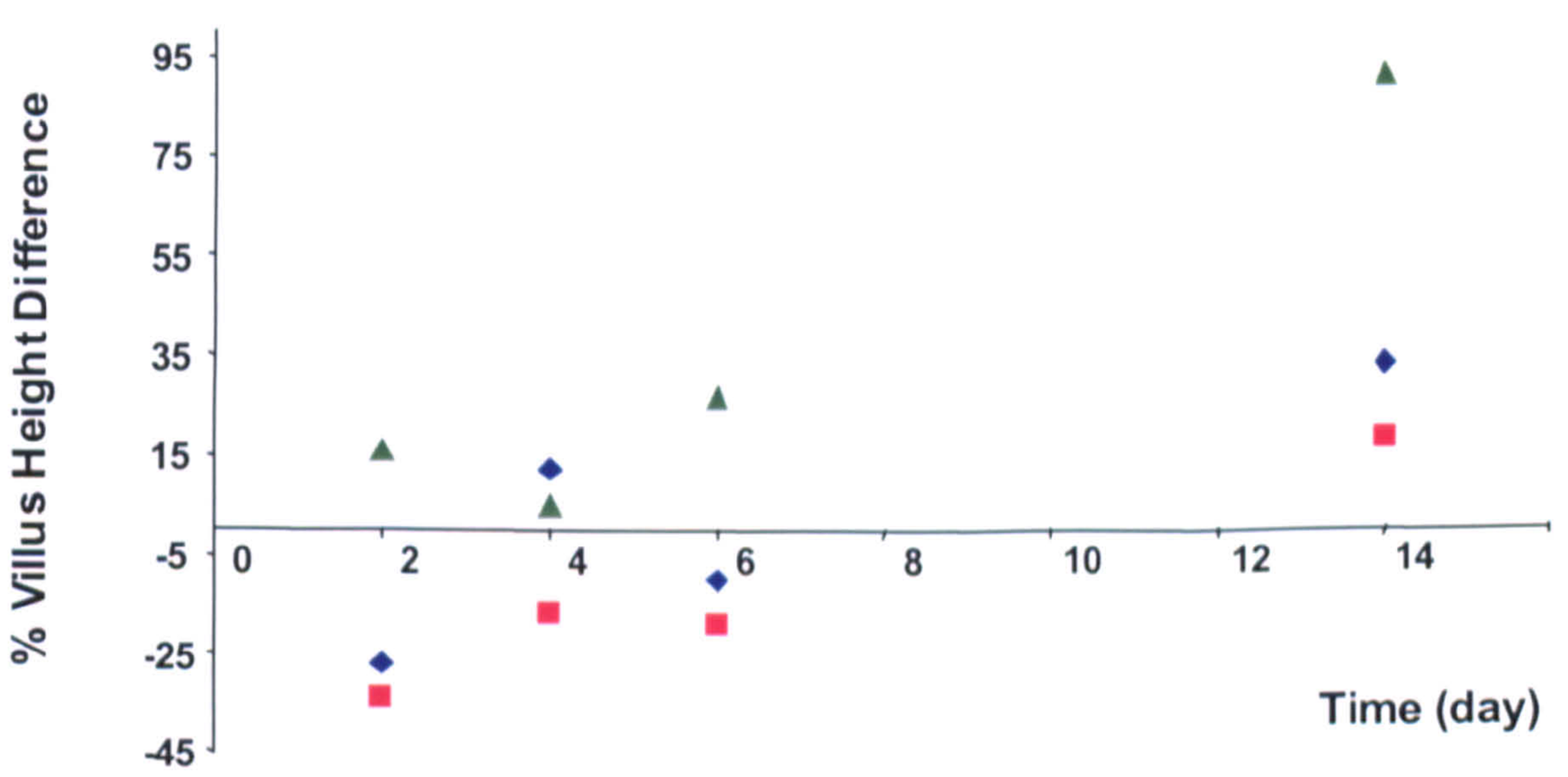


Figure 4.1: Percentage difference in villus height between baseline values and those obtained from animals slaughtered on days 2, 4, 6 or 14 fed a) diet 1 (control); b) diet 2 (ZnO); c) diet 3 (Ab) or d) diet 4 (ZnO + Ab) at regions proportionately 0.25 (◆), 0.50 (■) and 0.75 (▲) along the length of the intestine from the gastric pylorus to the ileo-caecal valve.

Table 4.5: Effect of dietary treatment on villus width (μm; $n = 8$ per diet)

	Diet 1				Diet 2				Diet 3				Diet 4			
	Proportion of Intestine				Proportion of Intestine				Proportion of Intestine				Proportion of Intestine			
Day	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean
2	86	78	81	82	82	80	79	80	89	82	79	83	81	81	81	81
4	74	64	71	69	77	74	78	76	74	79	65	72	84	86	77	82
6	73	76	86	78	82	85	76	81	83	75	80	79	81	73	76	77
14	87	116	97	100	83	92	91	88	81	87	84	84	94	99	89	94
mean	80	84	84	*82*	81	83	81	*81*	82	81	77	*80*	85	85	81	*84*

(Mean baseline values = 73, 82, 67 & 74 μm for distances proportionately 0.25, 0.50, 0.75 along the length of the SI & the overall mean respectively (table 4.2); diet 1 = Control; diet 2 = ZnO; diet 3 = Avilamycin; diet 4 = ZnO + Avilamycin)

Table 4.6: A summary of the statistical analysis for villus width

Zn		Ab		Day		Zn*Day		Ab*Day		Region	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
2.3	0.740	4.2	0.509	7.6	0.183 0.884 (L) 0.115 (Q)	7.8	0.746 0.434 (L) 0.785 (Q)	7.7	0.283 0.419 (L) 0.422 (Q)	1.5	0.305

Zn = Zinc dietary treatment; Ab = Antibiotic (avilamycin) dietary treatment; day refers to slaughter day

Table 4.7: Effect of dietary treatment on crypt depth (μm; $n = 8$ per diet)

	Diet 1				Diet 2				Diet 3				Diet 4			
	Proportion of Intestine				Proportion of Intestine				Proportion of Intestine				Proportion of Intestine			
Day	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean
2	210	228	186	208	171	200	154	175	153	186	197	179	181	179	177	179
4	184	173	199	185	220	250	217	229	221	242	180	214	215	234	231	227
6	232	239	264	245	184	182	188	185	239	235	262	246	227	219	228	225
14	222	224	218	221	219	197	243	219	239	236	197	224	183	228	209	207
mean	212	216	217	*215*	199	207	201	*202*	213	225	209	*216*	202	215	211	*209*

(Mean baseline values = 197, 202, 180 & 193 μm for distances proportionately 0.25, 0.50, 0.75 along the length of the SI & the overall mean respectively (table 4.2); diet 1 = Control; diet 2 = ZnO; diet 3 = Avilamycin; diet 4 = ZnO + Avilamycin)

Table 4.8: A summary of the statistical analysis for crypt depth

Zn		Ab		Day		Zn*Day		Ab*Day		Region	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
10.7	0.645	10.2	0.374	35.8	0.089 0.107 (L) 0.045 (Q)	37.1	0.658 0.992 (L) 0.228 (Q)	36.7	0.165 0.730 (L) 0.632 (Q)	3.5	0.019

Zn = Zinc dietary treatment; Ab = Antibiotic (avilamycin) dietary treatment; day refers to slaughter day

Table 4.9: Effect of dietary treatment on villus height to crypt depth ratio ($n = 8$ per diet)

	Diet 1				Diet 2				Diet 3				Diet 4			
	Proportion of Intestine				Proportion of Intestine				Proportion of Intestine				Proportion of Intestine			
Day	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean
2	2.2	1.7	1.9	1.9	2.5	1.8	2.1	2.1	2.7	3.1	1.8	2.0	1.9	2.1	2.2	2.1
4	2.7	2.3	1.6	2.2	1.8	1.6	1.5	1.6	2.2	2.0	2.1	2.1	2.4	2.0	1.5	2.0
6	1.6	1.5	1.9	1.6	2.7	3.1	2.3	2.7	2.1	1.9	1.8	1.9	1.8	2.1	1.8	1.9
14	2.7	3.1	2.6	2.8	3.0	3.1	1.8	2.6	1.9	1.8	2.0	1.9	3.6	3.1	3.2	3.3
mean	2.3	2.1	2.0	2.1	2.5	2.4	1.9	2.3	2.2	1.9	1.9	2.0	2.4	2.3	2.2	2.3

(Mean baseline values = 2.44, 2.81, 1.78 & 2.35 for distances proportionately 0.25, 0.50, 0.75 along the length of the SI & the overall mean respectively (table 4.2); diet 1 = Control; diet 2 = ZnO; diet 3 = Avilamycin; diet 4 = ZnO + Avilamycin)

Table 4.10: A summary of statistical analysis for villus height to crypt depth ratio (VH:CD)

Zn		Ab		Day		Zn*Day		Ab*Day		Region	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.25	0.991	0.24	0.351	0.83	0.900 0.626 (L) 0.585 (Q)	0.85	0.931 0.739 (L) 0.810 (Q)	0.85	0.86 0.291 (Q) 0.919 (Q)	0.05	<0.001

Zn = Zinc dietary treatment; Ab = Antibiotic (avilamycin) dietary treatment; day refers to slaughter day

4.7.2 Microbiological analyses

Total mean intestinal coliform counts (log cfu / g) declined significantly (P=0.004) throughout the experimental period, and also increased significantly (P= 0.002) along the length of the intestine (*i.e.* from the duodenum to the rectum) (Tables 4.12 and 4.13). The mean baseline value of 8.5 cfu / g was higher than those obtained from the other experimental animals on most days (mean values; BVR*day; P=0.003). No significant differences due to dietary treatment, or significant relationships between diet and day, or BVR and diet were evident.

Conversely, mean intestinal lactobacilli counts increased significantly (P=0.002) throughout the experimental period (Tables 4.14 and 4.15). No significant differences due to diet, or significant diet and day interactions were determined. As observed with coliforms, the mean number of lactobacilli also increased significantly (P=<0.001) along the length of the intestine. The lactobacilli count for the baseline animals was higher (mean; 9.3 cfu / g; P=0.002) than those obtained from animals on day 2. A significant relationship between BVR and diet (P=0.001), but none between BVR and day was apparent.

Intestinal lactobacilli to coliform ratio increased throughout the experimental period for all treatment groups (P = 0.026; 0.013 (L); 0.062 (Q); Tables 4.16 and 4.17). This ratio represents the increased lactobacilli and declining coliform counts throughout the 14-day experimental period. No significant differences due to diet, and sample site or their interactions were evident. There was however, a significant correlation between BVR and day (P = 0.011).

As expected, faecal coliform counts followed a similar pattern to intestinal coliform numbers, and declined significantly throughout the experimental period (P=<0.001; Tables 4.18 and 4.19). The baseline value of 6.2 log cfu / swab was the highest number of coliforms obtained throughout the experiment (P=<0.001). Significant diet and day (P=0.019), and BVR and day (P=<0.001) relationships, but no significant BVR and diet correlations were found.

Faecal lactobacilli counts displayed minimal variation throughout the experimental period (P=0.272; Tables 4.20 and 4.21). Significant differences in relation to dietary treatment (P=

0.029) were observed; those animals offered diet 2 (ZnO) achieved the lowest average count of 5.0 and the control diet (diet 1) the highest of 5.8 log cfu / swab. No significant diet and day, or BVR and day relationships were found. Baseline counts were significantly different (P=0.016) from the rest of the animals, and a significant correlation between BVR and diet (P=0.042) was also determined.

Faecal lactobacilli to coliform ratios increased significantly (P=<0.001; <0.001 (L); 0.025 (Q)) over time (Tables 4.22 and 4.23). A significant relationship between antibiotic and day (P = <0.001; <0.001 (L)) was evident together with significant differences between BVR (P=0.003), and correlations between BVR and antibiotic (P=0.012), and BVR and day (P=<0.001).

Ileal pH remained relatively constant throughout the experimental period with the range being pH 6.2 to 7.0 (Tables 4.24 and 4.25). No significant differences due to dietary treatment or BVR, or relationship between diet and day, BVR and day, or BVR and diet were determined. The mean dietary pH was 6.11 ± 0.09.

Table 4.11: Mean log coliform and lactobacilli counts (log cfu / g) obtained from digesta samples (S1-S5*) along the intestine for baseline animals (slaughtered on day zero; $n = 4$)

Species	Sample Site					mean
	S1	S2	S3	S4	S5	
Coliforms	6.6	8.0	9.4	8.4	9.8	8.5
Lactobacilli	9.5	9.8	9.0	8.9	9.1	9.3
Lactobacilli:Coliform Ratio	1.5	1.3	1.1	1.1	1.0	1.2

*S1 – S5 = duodenum, jejunum, ileum, colon and rectum respectively

Table 4.12: Mean log coliform counts (log cfu / g) obtained from digesta samples (S1-S5*) along the intestine ($n = 8$ per diet)

	Diet 1						Diet 2						Diet 3						Diet 4					
	Sample site						Sample site						Sample site						Sample site					
Day	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean
2	5.8	4.9	6.6	8.6	8.6	6.9	4.8	6.9	9.0	8.1	8.7	7.5	4.4	4.6	5.5	5.8	9.3	5.9	5.2	6.0	9.2	7.4	9.2	7.4
4	8.7	8.0	9.3	8.7	8.4	8.7	5.6	8.4	8.5	7.0	6.8	7.3	6.5	5.9	6.8	5.4	4.8	5.9	5.0	4.2	7.8	6.6	8.2	6.4
6	5.4	4.4	3.4	3.9	4.3	4.3	4.9	5.6	6.1	6.8	6.0	5.9	6.3	5.9	5.8	8.6	6.1	6.5	5.7	5.0	4.1	6.0	5.3	5.2
14	5.5	3.5	6.3	5.7	5.6	5.3	4.5	4.6	4.4	5.6	5.2	4.8	5.0	4.4	6.6	4.5	5.8	5.3	4.1	6.2	4.1	4.3	4.5	4.6
Mean	6.4	5.2	6.4	6.7	6.7	6.3	4.9	6.3	7.0	6.9	6.7	6.4	5.5	5.2	6.2	6.1	6.5	5.9	5.0	5.4	6.3	6.1	6.8	5.9

*S1 – S5 = duodenum, jejunum, ileum, colon and rectum respectively; mean baseline values = 6.6, 8.0, 9.4, 8.4, 9.8 & 8.5 cfu / g for samples obtained at locations S1-S5 & the overall mean respectively (table 4.11); diet 1 = Control; diet 2 = ZnO; diet 3 = Avilamycin; diet 4 = ZnO + Avilamycin

Table 4.13: A summary of the statistical analysis for intestinal coliforms

Zn		Ab		Day		Zn*Day		Ab*Day		Sample Site	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.40	0.990	0.40	0.258	0.57	0.005 0.001 (L) 0.290 (Q)	0.80	0.503 0.316 (L) 0.450 (Q)	0.80	0.216 0.437 (L) 0.562 (Q)	0.36	0.001

BVR		BVR*Zn		BVR*Ab		BVR*Day	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.58	<0.001	0.61	0.989	0.61	0.237	0.67	0.003

BVR = Baseline versus the rest; Zn = Zinc dietary treatment; Ab = Antibiotic (avilamycin) dietary treatment; day refers to slaughter day

Table 4.14: Mean log lactobacilli counts (log cfu/g) obtained from digesta samples (S1-S5*) along the intestine ($n = 8$ per diet)

	Diet 1 Sample site						Diet 2 Sample site						Diet 3 Sample site						Diet 4 Sample site					
Day	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean
2	6.8	4.9	5.7	8.2	8.2	6.8	5.8	5.8	5.6	7.4	7.9	6.5	5.4	5.0	4.9	6.7	9.2	6.2	4.0	5.1	4.9	7.0	7.6	5.7
4	7.0	8.9	8.8	7.2	7.9	8.0	8.4	6.5	7.3	9.3	4.8	7.2	7.6	7.5	7.9	8.3	7.7	7.8	8.1	7.2	8.7	8.2	7.5	7.9
6	7.5	7.3	8.9	10.0	9.2	8.6	6.5	6.3	6.6	7.8	8.4	7.1	7.4	7.7	8.1	4.2	7.9	7.1	7.8	8.1	8.4	9.7	10.0	8.8
14	8.7	8.5	9.0	10.2	10.6	9.4	7.1	7.1	7.7	8.8	8.1	7.8	8.2	8.8	9.4	9.8	8.8	9.0	6.5	6.8	7.0	8.4	7.7	7.3
mean	7.5	7.4	8.1	8.9	9.0	8.2	7.0	6.4	6.8	8.3	7.3	7.2	7.1	7.2	7.6	7.2	8.4	7.5	6.6	6.8	7.3	8.3	8.2	7.4

*S1 – S5 = duodenum, jejunum, ileum, colon and rectum respectively; mean baseline values = 9.5, 9.8, 9.0, 8.9, 9.1 & 9.3 cfu / g for samples obtained at locations S1-S5 & the overall mean respectively (table 4.11); diet 1 = Control; diet 2 = ZnO; diet 3 = Avilamycin; diet 4 = ZnO + Avilamycin

Table 4.15: A summary of the statistical analysis for intestinal lactobacilli counts

Zn		Ab		Day		Zn*Day		Ab*Day		Sample Site	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.32	0.111	0.32	0.581	0.46	0.002 0.002 (L) 0.019 (Q)	0.65	0.257 0.104 (L) 0.262 (Q)	0.65	0.729 0.847 (L) 0.342 (Q)	0.31	<0.001

BVR		BVR*Zn		BVR*Ab		BVR*Day	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.46	0.002	0.49	0.093	0.49	0.561	0.44	0.001

BVR = Baseline versus the rest; Zn = Zinc dietary treatment; Ab = Antibiotic (avilamycin) dietary treatment; day refers to slaughter day

Table 4.16: Mean log lactobacilli to coliform ratio obtained from digesta samples (S1-S5*) along the intestine ($n = 8$ per diet)

	Diet 1						Diet 2						Diet 3						Diet 4					
	Sample site						Sample site						Sample site						Sample site					
Day	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean
2	1.2	1.0	0.9	0.9	1.0	1.0	1.2	0.9	0.6	0.9	0.9	0.9	1.2	1.1	0.9	1.2	1.0	1.1	0.8	1.2	0.5	1.0	0.8	0.9
4	0.8	1.1	0.9	0.8	0.9	0.9	1.9	0.8	0.9	1.4	0.6	1.1	1.4	1.6	1.2	1.6	1.6	1.5	1.6	1.8	1.2	1.3	0.9	1.4
6	2.2	1.8	3.0	3.2	2.8	2.6	1.5	1.2	1.1	1.2	1.4	1.3	1.2	1.4	2.0	0.7	1.3	1.3	1.4	1.8	2.5	1.7	1.9	1.9
14	1.6	2.5	1.5	1.9	1.9	1.8	1.6	1.6	2.0	1.6	1.4	1.6	1.8	2.0	1.4	2.3	1.5	1.8	2.0	1.1	1.8	2.2	1.7	1.8
mean	1.4	1.6	1.6	1.7	1.6	1.6	1.6	1.1	1.1	1.3	1.1	1.2	1.4	1.5	1.4	1.4	1.4	1.4	1.4	1.5	1.5	1.5	1.3	1.5

*S1 – S5 = duodenum, jejunum, ileum, colon and rectum respectively; mean baseline values = 1.5, 1.3, 1.1, 1.1, 1.0 & 1.2 cfu / g for samples obtained at locations S1-S5 & the overall mean respectively (table 4.11); diet 1 = Control; diet 2 = ZnO; diet 3 = Avilamycin; diet 4 = ZnO + Avilamycin

Table 4.17: A summary of the statistical analysis for intestinal lactobacilli to coliform ratio

Zn		Ab		Day		Zn*Day		Ab*Day		Sample Site	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.19	0.524	0.19	0.876	0.27	0.026 0.013 (L) 0.062 (Q)	0.38	0.872 0.885 (L) 0.841 (Q)	0.38	0.750 0.895 (L) 0.684 (Q)	0.12	0.853

BVR		BVR*Zn		BVR*Ab		BVR*Day	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.26	0.416	0.28	0.422	0.28	0.765	0.3	0.011

BVR = Baseline versus the rest; Zn = Zinc dietary treatment; Ab = Antibiotic (avilamycin) dietary treatment; day refers to slaughter day

Table 4.18: Mean log coliform counts (log cfu / swab) obtained from faecal samples ($n = 4$ baseline; $n = 8$ per diet)

Day	Baseline	Diet 1	2	3	4	mean
0	6.2					6.2
1		5.5	6.0	5.9	5.3	5.7
3		6.0	5.3	5.6	5.5	5.6
5		6.8	5.0	5.5	5.8	5.8
7		4.6	6.2	5.7	4.8	5.3
9		3.6	5.8	4.6	5.1	4.8
11		4.5	6.8	4.7	4.4	5.1
13		4.1	4.5	4.4	4.0	4.3
mean	6.2	5.4	5.6	5.4	5.2	5.4

Baseline=animals slaughtered on day 0 (weaning); diet 1 = Control; diet 2 = ZnO; diet 3 = Avilamycin; diet 4 = ZnO + Avilamycin

Table 4.19: A summary of the statistical analysis for faecal coliforms

Zn		Ab		Day		Zn*Day		Ab*Day	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.19	0.064	0.19	0.851	0.24	<0.001	0.37	0.103	0.37	0.025
					<0.001 (L)		0.091 (L)		0.254
					0.036 (Q)		0.824 (Q)		0.383

BVR		BVR*Zn		BVR*Ab		BVR*Day	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.12	<0.001	0.15	0.028	0.15	0.828	0.2	<0.001

BVR = Baseline versus the rest; Zn = Zinc dietary treatment; Ab = Antibiotic (avilamycin) dietary treatment; day refers to slaughter day

Table 4.20: Mean log lactobacilli counts (log cfu / swab) obtained from faecal samples ($n = 8$ per diet; baseline $n = 4$)

Day	Baseline	Diet 1	2	3	4	mean
0	5.9					5.9
1		5.8	6.5	5.3	5.9	5.6
3		5.5	4.5	5.2	5.6	5.2
5		5.5	4.9	5.3	5.2	5.2
7		6.3	5.1	5.0	5.4	5.4
9		5.4	5.0	5.9	4.3	5.2
11		6.6	5.4	6.7	5.5	6.1
13		6.2	4.4	5.3	4.9	5.2
mean	5.9	5.8	5.0	5.4	5.4	5.5

Baseline=animals slaughtered on day 0 (weaning); diet 1 = Control; diet 2 = ZnO; diet 3 = Avilamycin; diet 4 = ZnO + Avilamycin

Table 4.21: A summary of the statistical analysis for faecal lactobacilli counts

Zn		Ab		Day		Zn*Day		Ab*Day	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.28	0.837	0.28	0.096	0.24	0.002 0.724 (L) 0.283 (Q)	61.38	0.605 0.416 (L) 0.764 (Q)	61.38	0.138 0.005 (L) 0.811 (Q)

BVR		BVR*Zn		BVR*Ab		BVR*Day	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.16	0.001	0.19	0.792	0.19	0.031	0.22	0.002

BVR = Baseline versus the rest; Zn = Zinc dietary treatment; Ab = Antibiotic (avilamycin) dietary treatment; day refers to slaughter day

Table 4.22: Mean log lactobacilli to coliform ratio obtained from faecal samples ($n = 8$ per diet; baseline $n = 4$)

Day	Baseline	Diet 1	2	3	4	mean
0	1.0					
1		1.1	0.9	0.9	1.1	1.0
3		0.9	0.8	0.9	0.9	0.9
5		0.8	1.0	1.0	1.0	1.0
7		1.4	0.8	0.9	1.0	1.0
9		1.5	1.9	1.3	1.1	1.1
11		1.5	1.8	1.4	1.2	1.2
13		1.5	1.0	1.2	1.3	1.3
mean	1.0	1.1	0.9	1.0	1.1	1.0

Baseline=animals slaughtered on day 0 (weaning); diet 1 = Control; diet 2 = ZnO; diet 3 = Avilamycin; diet 4 = ZnO + Avilamycin

Table 4.23: A summary of the statistical analysis for faecal lactobacilli to coliform ratio

Zn		Ab		Day		Zn*Day		Ab*Day	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.06	0.467	0.06	0.054	0.06	<0.001 <0.001 (L) 0.025 (Q)	0.10	0.132 0.757 (L) 0.310 (Q)	0.10	<0.001 <0.001 (L) 0.327 (Q)

BVR		BVR*Zn		BVR*Ab		BVR*Day	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.03	0.003	0.04	0.351	0.04	0.012	0.05	<0.001

BVR = Baseline versus the rest; Zn = Zinc dietary treatment; Ab = Antibiotic (avilamycin) dietary treatment; day refers to slaughter day

Table 4.24: Mean ileal digesta pH ($n = 8$ per diet; baseline $n = 4$)

Day	Baseline	Diet 1	2	3	4	mean
0	6.7					
2		6.8	6.7	6.2	6.8	6.6
4		6.5	6.7	6.8	6.7	6.7
6		6.6	6.7	6.7	6.7	6.7
14		6.3	6.5	6.3	7.0	6.5
mean		6.6	6.7	6.5	6.8	6.6

Baseline=animals slaughtered on day 0 (weaning); diet 1 = Control; diet 2 = ZnO; diet 3 = Avilamycin; diet 4 = ZnO + Avilamycin

Table 4.25: A summary of the statistical analysis for ileal digesta pH

Zn		Ab		Day		Zn*Day		Ab*Day	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.23	0.850	0.23	0.21	0.24	0.887 0.561 (L) 0.613 (Q)	0.31	0.785 0.461 (L) 0.502 (Q)	0.31	0.698 0.385 (L) 0.648 (Q)

BVR		BVR*Zn		BVR*Ab		BVR*Day	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.24	0.665	0.32	0.431	0.32	0.594	0.24	0.794

BVR = Baseline versus the rest; day refers to slaughter day

4.7.3 Performance parameters

A highly significant difference due to the inclusion of either avilamycin or ZnO (P=<0.001) on daily live-weight gain (DLWG; Table 4. 26), with mean DLWG being 0.11, 0.16, 0.19 and 0.18 kg/d for diets 1-4 respectively, was evident. Although highly variable, as expected, feed intake increased significantly throughout the experimental period (P=<0.001), and there was a significant relationship between diet and day (P=0.007 and 0.088 for avilamycin and ZnO respectively) in relation to DLWG. Feed conversion ratio (FCR; Table 4.28) was also variable throughout the experimental period, and was due in part, to the extremely low and often zero feed intakes immediately post-weaning. The times taken for each piglet to take food and water after weaning were also recorded (data not shown). There was huge variation between the 32 piglets, with time to first feed varying between 35 min to greater than 6 hours (2 piglets), and time to first water varying between 3h 15 min to greater than 6 hours (1 piglet).

4.7.4 Mineral analyses

A significant temporal difference (P=0.005) with respect to tissue zinc concentrations was determined, with the concentration tending to increase over time (Tables 4.29 and 4.30). There was also a highly significant difference (P<0.001) between dietary treatment on Zn tissue concentration with diets 2 (ZnO) and 4 (ZnO and avilamycin) having the highest overall mean values of 311.2 and 247.9 µg / g for liver tissue respectively, compared to an average value of approximately 5.8 for diets 1 (control) and 3 (avilamycin). However, this response was not observed for the kidney. Although kidney Zn concentration did tend to increase over time, diet 1 had the highest values of 43.2 µg / g and diet 3 the lowest of 19.2 µg / g. Significant relationships between day and Zn (P=<0.001), BVR and day (P=0.002), and BVR and Zn (P=<0.001) were also determined with respect to tissue Zn concentration. Liver Zn concentrations were 9 and 8 fold greater than the baseline values for diets 2 (ZnO) and 4 (ZnO + avil) respectively on day 14.

Blood urea nitrogen (BUN) data are shown in tables 4.31 and 4.32. There were no significant differences due to day, diet (Zn or avilamycin), BVR, or significant relationships between day and diet (Zn or avilamycin), BVR and day, and BVR and diet on BUN values.

Table 4.26: DLWG for individual animals according to day of slaughter and dietary treatment (n = 8 per diet; kg/d)

Slaughter day	Diet 1	2	3	4	Zn s.e.d	Zn P	Ab s.e.d	Ab P	Day s.e.d	Day P	Zn*Ab s.e.d	Zn*Ab P
2	-0.05	0.10	-0.15	-0.10	0.188	0.816	0.188	<0.001	0.420	<0.001	0.265	0.049
2	-0.10	-0.40	0.05	-0.10						<0.001 (L)		
4	-0.27	0.00	0.13	0.10						<0.001 (Q)		
4	0.13	-0.07	0.23	0.07								
6	0.20	0.25	0.23	0.30								
6	-0.03	0.32	0.25	0.18								
14	0.47	0.60	0.40	0.43								
14	0.52	0.49	0.35	0.52								
mean	**0.11**	**0.16**	**0.19**	**0.18**								

Diet 1 = Control; diet 2 = ZnO; diet 3 = Avilamycin; diet 4 = ZnO + Avilamycin

Table 4.27: Effect of dietary treatment on daily feed intake (g; n = 8 per diet)

Day	Diet 1	2	3	4	mean	Zn s.e.d	Zn P	Ab s.e.d	Ab P	Day s.e.d	Day P	Zn * Day s.e.d	Zn * Day P	Ab * Day s.e.d	Ab * Day P
2	129	88	84	28	82	19.4	0.672	19.4	0.702	27.4	<0.001	38.8	0.088	38.8	0.007
4	162	126	321	217	207						<0.001 (L)		0.163 (L)		0.055 (L)
6	280	390	311	331	328						0.009 (Q)		0.157 (Q)		0.087 (Q)
14	637	662	553	569	606										
mean	**302**	**316**	**317**	**286**	*306*										

Diet 1 = Control; diet 2 = ZnO; diet 3 = Avilamycin; diet 4 = ZnO + Avilamycin; refer to appendix 2 for actual feed intake data; day refers to slaughter day

Table 4.28: Effect of dietary treatment on FCR ($n = 8$ per diet)

	Diet					Zn		Ab		Day		Zn * Day		Ab * Day	
Day	1	2	3	4	mean	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
2	-2.1	0.7	0.8	-0.3	-0.2	0.64	0.576	0.64	0.030	0.91	0.337	1.28	0.371	1.28	0.384
4	0.9	-1.9	2.5	3.0	1.1						0.183 (L)		0.767 (L)		0.306 (L)
6	-2.4	1.4	1.3	1.5	0.4						0.664 (Q)		0.607 (Q)		0.321 (Q)
14	1.4	1.3	1.6	1.3	1.4										
mean	-0.5	0.4	1.5	1.4	*0.7*										

Diet 1 = Control; diet 2 = ZnO; diet 3 = Avilamycin; diet 4 = ZnO + Avilamycin; day refers to slaughter day

Table 4.29: Tissue zinc concentrations (µg / g; $n = 8$ per diet)

	Liver						Kidney					
		Diet						Diet				
Day	Baseline	1	2	3	4	mean	Baseline	1	2	3	4	mean
0	72.8						13.3					
2		29.7	50.1	51.0	42.0	**43.2**		14.3	9.3	10.2	0.3	**8.3**
4		55.5	145.3	44.1	158.1	**100.8**		12.2	3.9	5.7	13.1	**8.7**
6		68.1	369.8	51.8	195.5	**171.3**		35.3	35.8	19.00	28.9	**29.7**
14		80.4	679.5	87.9	595.8	**360.9**		43.20	114.7	41.7	75.8	**85.8**
mean	**7.28**	**58.4**	**311.2**	**58.7**	**247.9**	***169.0***	**13.3**	**26.3**	**40.7**	**19.20**	**29.5**	***33.1***

Baseline=animals slaughtered on day 0 (weaning); diet 1 = Control; diet 2 = ZnO; diet 3 = Avilamycin; diet 4 = ZnO + Avilamycin

Table 4.30: A summary of the statistical analysis for tissue zinc concentration

Zn		Ab		Day		Zn*Day		Ab*Day	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.01	<0.001	0.01	0.237	0.04	0.005 0.003 (L) 0.025 (Q)	0.04	<0.001 <0.001 (L) 0.490 (Q)	0.04	0.144 0.880 (L) 0.033 (Q)

BVR		BVR*Zn		BVR*Ab		BVR*Day	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.02	0.696	0.02	<0.001	0.02	0.106	0.03	0.002

BVR = Baseline versus the rest; Zn = Zinc dietary treatment; Ab = Antibiotic (avilamycin) dietary treatment; day refers to slaughter day

Table 4.31: Serum blood urea nitrogen (BUN) data (mg / dL; $n = 8$ per diet)

Day	Baseline	Diet 1	2	3	4	mean
0	11.4					
2		9.3	10.1	9.5	14.1	10.7
4		17.4	26.0	5.4	12.0	15.2
6		14.6	20.3	12.5	35.5	20.7
14		15.0	15.6	91.3	16.9	34.7
mean	11.4	14.1	18.0	29.7	19.6	20.3

Baseline=animals slaughtered on day 0 (weaning); diet 1 = Control; diet 2 = ZnO; diet 3 = Avilamycin; diet 4 = ZnO + Avilamycin

Table 4.32: A summary of the statistical analysis for BUN

Zn		Ab		Day		Zn*Day		Ab*Day	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
10.59	0.478	10.49	0.478	14.98	0.433 0.109 (L) 0.889 (Q)	21.18	0.347 0.122 (L) 0.366 (Q)	21.18	0.395 0.127 (L) 0.660 (Q)

BVR		BVR*Zn		BVR*Ab		BVR*Day	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
15.43	0.753	10.29	0.917	10.29	0.713	11.39	0.854

BVR = Baseline versus the rest; day refers to slaughter day

4.8 EXECUTIVE SUMMARY OF RESULTS

The results illustrate that, although villus atrophy occurred immediately post-weaning, generally this was of a relatively short duration (initial 2 days for animals fed diets 2, 3 and 4, and initial 4 days for those fed diet 1 (positive control) before recovery was observed. Although there was no significant difference between the 4 dietary treatments, such a limited duration of villus atrophy is nonetheless interesting. Evidence suggests that villus atrophy commonly occurs for longer periods. For example, Hampson (1986) observed that villus height decreased to 75% pre-weaning values within 24 hours of weaning at 21 days. Subsequent reductions in villus height were smaller but continued to decline until the fifth day post-weaning, at which point villus height at most sites along the gut was 50% of the initial weaning value. In the present study the maximum decline in villus height was 43% (in comparison to baseline values) and had occurred by day 2 post-weaning. Hampson (1986) reported that this loss of villus height was most pronounced proximally, whilst enhanced crypt depth occurred greatest distally. This pattern was not replicated in the present study, since the greatest villus atrophy occurred medially, crypt deepening (after day 2) occurred principally in both the medial and distal regions (P=0.019). Furthermore, in the current study there was no significant effect of dietary treatment on crypt depth, although those fed diets 2, 3, and 4 displayed a similar pattern of a reduction in crypt depth between day 0 (baseline values) and day 2, followed by an increase in (deepening) crypt depth between days 2 and 4. This is supported by findings of Mavromichalis *et al.* (2000), who reported no consistent positive effect of dietary ZnO supplementation on villus height, width or crypt depth. Moreover, Mavromichalis *et al.* (2000) also concluded that ZnO supplementation does not appear to ameliorate the negative effects of post-weaning dry diets on intestinal structure. Carlson *et al.* (1999), suggested that the induction of metallothionein (MT) in erythrocytes might be responsible for the enhanced growth performance due to pharmacological concentrations of Zn. As MT is involved in zinc homeostasis at both the intestinal and systemic level, its expression is directly affected by dietary Zn concentration (Menard *et al.*, 1981; Cousins, 1985). As such, Carlson *et al.* (1999) proposed that since Zn has a role in cell RNA and DNA proliferation, constantly elevated levels of Zn in the enterocyte as a consequence of up-regulation of MT (Starcher *et al.*, 1980) may improve intestinal health. However, such beneficial effects were not observed in the present study, nor in that of Mavromichalis *et al.* (2000), suggesting that the ingestion of elevated dietary ZnO concentrations may therefore exert either luminal or systemic effects (Mavromichalis *et al.*,

2000). A further benefit proposed by Prasad (1983) from the ingestion of elevated dietary zinc concentrations is the prevention of liver damage by free radicals.

Intestinal coliform numbers declined over time, but increased along the length of the small intestine from the duodenum through to the colon. As expected, this pattern was also displayed in the faecal swab enumerations. This is in agreement with findings of Katouli *et al.* (1999) who reported a high number of coliforms at the early stage of the suckling period (coliforms are among the earliest groups of bacteria colonising the gut after birth (Drasar and Barrow 1985)), but decreased as the animals aged. Furthermore, Katouli *et al.* (1999) also reported that dietary ZnO supplementation helped to maintain the stability of the intestinal microflora and the diversity of coliforms during the first two weeks post-weaning, however no effect on the coliform population size in post-weaned pigs was reported. A highly diverse bacterial flora is thought to be a major factor influencing the stability of the intestinal micro-ecosystem (Kuhn *et al.*, 1993; Kuhn *et al.*, 1996). Therefore, Katouli *et al.* (1999) concluded that ZnO supplementation helped to maintain the stability of the intestinal flora in weaned pigs, hence preserving the protective ability of the flora which would otherwise be lost at weaning. Furthermore, maintaining the high diversity of coliforms may enhance competition for colonisation sites with diarrhoegenic or pathogenic strains (Barrow, 1980; Hentges, 1983; Kuhn *et al.*, 1993; Katouli *et al.*, 1994).

Melin *et al.* (2001) conducted a study where piglets were weaned at 35 days of age and fed either a conventional or ZnO (2500 ppm) supplemented diet for 28 days post-weaning, and reported no differences between the groups in the number of coliforms, enterococci or *Clostridium perfringens* excreted / g faeces. In contrast, the decrease in diversity of the faecal flora post-weaning was more pronounced and of longer duration in the control animals compared with the ZnO treated pigs. This reduction in faecal coliform diversity displayed by the ZnO treated pigs indicates a positive effect on the stability of the enteric flora by supplementation with 2500 ppm ZnO from weaning. However, the authors reported that this effect was limited to the initial 2 weeks post-weaning, and there is also evidence of toxicity after oral exposure of high concentrations of ZnO (Jensen-Waern *et al.*, 1998), which again limits the period for which ZnO supplemented diets should be fed).

Conversely, lactobacilli numbers increased throughout the experimental period, and again there was no significant dietary effect, although numbers increased along the length of the intestine. Interestingly, faecal swabs enumerated for lactobacilli displayed no significant temporal effect, whilst there was a significant dietary effect with diet 2 (ZnO) achieving the lowest average lactobacilli count. Although faecal swab determinations are useful indicators of enteric microbial load, this is a somewhat retrospective technique, and conditions in the rectum are vastly different in comparison to other sectors of the intestine. Plausible explanations for this discrepancy between faecal and intestinal flora include the divergence of the ingesta and density of bacteria in different parts (and microenvironments) of the intestine. This is in contrast to that reported by Zoric *et al.* (2001) who compared coliform populations collected from different sites of the intestinal tract and concluded that fingerprinting of the rectal flora is a relevant method to mirror enteric microflora since microbial diversity was similar in all intestinal segments investigated. This is also supported by Roberfroid *et al.* (1995). However, it should also be noted that the animals utilised in the study of Zoric *et al.* (2001), were not weaned until 35 days of age and samples were collected and analysed at day 63 (28 days post-weaning). Hence, these animals were much older than those of the present study, and as such are more likely to contain a stable intestinal microflora. Furthermore, a study conducted by Hogberg *et al.* (2001), employing grower pigs, demonstrated a high correlation between the rectal flora and the flora of the intestine, when a standard diet was fed. However, in the same study, Hogberg *et al.* (2001) reported that dietary manipulation (alteration of NSP content) rendered the diversity of enteric coliform flora in the rectum different from the flora at the ileo-caecal sampling site. This suggests limitations in the general use of rectal flora to describe the flora in the intestine close to modifications in dietary composition (Hogberg *et al.*, 2001). It is important to note that no correlation between bacterial counts and intestinal pH was evident. One may expect to find lower coliform numbers at lower pH values, whereas lactic acid bacteria being more pH tolerant would be less affected.

Treatment differences for DLWG were not significant, and as expected, the highest DLWGs were achieved on day 14. Several titration studies have indicated that pharmacological doses of ZnO stimulate growth rates in pigs. For example, Smith *et al.* (1995b), fed up to 4,000 mg Zn/kg from ZnO from d 0 to 14 post-weaning, and observed a linear increase in weight gain. A positive growth response was also reported by Le Mieux *et al.* (1995) by feeding 3,000 to

6,000 mg Zn/kg from ZnO. However, other researchers have reported maximum growth responses at inclusion levels below these. For example, Hill *et al.* (1999), performed a large-scale 28 d study comprising 1069 weaned pigs (11 to 25d of age) and reported a maximal growth performance response at 2,000 mg Zn/kg from ZnO. In the present study, the ZnO supplemented group also displayed the highest level of feed intake on day 14. However, this was not significant, so this enhanced feed intake cannot explain the increased DLWG. There is also evidence of a small (although non-significant) additive effect of ZnO and avilamycin on DLWG. Although the literature suggests that weight gain responses to ZnO supplementation are usually positive, feed intake and FCR responses have been variable. For example, Mavromichalis *et al.* (2000), observed a stimulated feed intake and growth rate, but not FCR response to dietary ZnO supplementation, a finding that is in agreement with some studies (Hahn and Baker, 1993; Hill *et al.*, 1999), but not with others (Carlson, 1995; Poulsen, 1995; Smith *et al.*, 1997; Carlson *et al.*, 1999).

Growth promoting properties of Zn could be explained in part, by studies that concentrate on the impact of dietary Zn deficiency. The principal symptom of zinc deficiency is an inhibition of growth, which is manifested by reduced food intake (MacDonald, 2000). In a study with rats, Giugliano and Millward (1984) reported that reduced food intake and diminished growth occurs within 4 - 5 days of feeding a Zn-inadequate diet. This impaired growth could be explained by an energy deficit associated with poor feed intakes. However, a reduced energy intake is unlikely to be the factor which limits growth, as force-feeding a Zn-depleted diet to rats failed to improved growth and exacerbated the clinical symptoms of the deficiency (Park *et al.*, 1986). Hence, food intake and growth appear to be regulated by Zn through independent, although well coordinated, mechanisms. When Zn is inadequate to maintain growth or cellular metabolism, reduced food intake may be a protective mechanism to allow survival. The failure to correct growth with increased food intake suggests that growth is the first limiting role for Zn. Thus, it appears logical to propose that supplementing the diet with Zn will promote growth.

Evidence concerning the additive effects of mineral and antibiotic addition are equivocal. For example, Stahly *et al.* (1980), demonstrated that single additions of copper, chlortetracycline (CTC) or virginamycin (VIR) improved ($P<0.05$) daily gains by 22, 22 and 17% respectively, and FCRs by 5.1, 8.9 and 8.2% respectively compared to animals fed the unsupplemented,

control diets throughout the post-weaning period. Moreover, dietary inclusion of both copper and an antibiotic (CTC or VIR) further improved daily gains ($P<0.05$) by 10 to 11%, and FCRs by 2 to 5% compared with the single inclusion of each antimicrobial agent. This identifies an additive effect between copper and these antimicrobial agents. This work further signifies that the growth response observed with CTC and VIR-supplemented diets was a result of both an elevated feed intake, and improved efficiency of feed conversion (Stahly *et al.*, 1980). In contrast, the enhanced growth observed with copper supplemented diets alone appears to be merely a function of increased feed intake. The fact that these responses appeared to be additive regardless of whether a broad-spectrum antibiotic (CTC) or a Gram-positive antibiotic (VIR) is used suggests that:

1. Copper has a different bacterial spectrum from CTC or VIR or
2. Copper possesses a different mode of action than do the antibiotics used in this study (Stahly *et al.*, 1980). Since no significant additive response was observed in the current study, it could be hypothesised that zinc and avilamycin possess similar modes of action.

In the present study, average growth performance was enhanced by 31, 42 and 39% for diets 2, 3, and 4 respectively when compared to the control animals. FCR data should be interpreted with caution, due to the high variability in feed intake levels observed among individuals, the initial 2 days post-weaning are often accompanied by low or even zero feed intakes which can result in weight stasis or even loss. In the current study, there were no significant dietary effects on FCR. This is in contrast to findings of Yokoyama *et al.* (1982) who reported enhanced growth performance in post-weaned pigs fed antibiotic supplemented diets. The authors postulated that intestinal *p*-cresol production may be responsible for a growth depressing effect in the weaner pig, and that dietary antibiotic supplementation could be promoting growth by inhibiting the bacterial production of this metabolite. A reduction in *p*-cresol production would conserve glucose and ATP that is utilised in the detoxification process prior to its excretion from the body (Yokoyama *et al.*, 1982). Dilute *p*-cresol solutions have been shown to inhibit the growth and metabolism of *Lactobacillus acidophilus* (Dreizen and Spies, 1948 as cited by Yokoyama *et al.*, 1982). This is supported by work conducted by Bernhart and Zilliken (1958) as cited by Yokoyama *et al.*, (1982), who illustrated an inverse relationship between the urinary excretion of volatile phenols (i.e.

phenol and *p*-cresol) and the weight gain of rats fed 10% tyrosine in a sucrose diet. Supplementing the diet with chlortetracycline or substituting lactose in place of sucrose decreased the urinary excretion of the volatile phenols and alleviated the growth depression. However, Wisman *et al.* (1957) and Schmidt *et al.* (1949) as cited by Yokoyama *et al.* (1982), found no relationship between the production of volatile phenolic and aromatic metabolites and the growth promotion induced by dietary antibiotic supplementation. A further proposed effect of dietary antibiotic supplementation is an improvement in the efficiency of energy utilisation (Francois, 1962). For example, Vervaeke *et al.* (1979) suggested that dietary antibiotics conserve glucose through reduced organic acid production by intestinal bacteria, enabling more energy to be partitioned towards growth. It has also been hypothesised that dietary antibiotics suppress the intestinal bacteria population, resulting in a reduced mass and turnover rate of the intestinal mucosal cells, thus conserving energy necessary to maintain the tissue Visek (1978). Alternatively dietary antibiotics may change the metabolism of intestinal microbes rather than the relative numbers of each species, although the exact mode of action will be specific to each class of antibiotic.

O'Quinn *et al.* (1997) proposed that ZnO possessed no additive or synergistic actions with that of antimicrobial agents. This supports the findings of the present study. However, in the study of O'Quinn *et al.* (1997), the pigs were between 12 and 15 d of age, and reared under high health conditions, and evidence suggests that the greatest responses are observed with animals produced in low health (or disease challenged) and low management environments. Furthermore, Mahan *et al.* (2000) reported synergistic growth responses to a combination of 1,500 mg Zn/kg from ZnO and the antimicrobial carbadox.

Liver Zn concentrations reflected both dietary Zn supplementation and the duration that the Zn was fed. Tissue Zn concentrations increased over time with the highest concentrations being reached on day 14 for all dietary treatments. As expected the mean Zn concentration was highest for animals fed the ZnO (diet 2) and ZnO + avilamycin diets (diet 4). The mean liver Zn concentration for animals fed diet 2 was over 5 times greater than that for control animals. In contrast, mean kidney Zn concentration for animals fed diet 2 was 1.5 times greater than the control animals. This higher concentration of Zn in the liver is related to the fact that the liver acts as a storage organ for Zn (Carlson *et al.*, 1999). Furthermore, Poulsen and Larsen (1995) reported that the kidneys and (or) gastrointestinal tract are involved in

homeostatic regulation that is supported by findings of Carlson *et al.* (1999). For example, Carlson *et al.* (1999) reported that in pigs fed 3000 ppm Zn for 28 days post-weaning, the kidney Zn concentration plateaued on approximately day 14 of the study representing a potential renal homeostatic role. The tissue Zn concentrations determined in this study were slightly higher than those obtained by Carlson *et al.* (1999). These authors reported an approximate 4-fold increase in liver Zn concentration in comparison to baseline animals and only a 1.4 fold increase for kidney concentrations. These differences could be due to a number of factors, for example, the study of Carlson *et al.* (1999) was conducted in the States, employing a different genotype of pig. Furthermore, the diets contained spray-dried animal plasma, fishmeal and dried skim milk all of which may contain significant amounts of Zn. For example, Hahn and Baker (1993) reported plasma zinc levels of between 0.85 and 1.29 mg / L for pigs fed the basal diet (containing 125 ppm Zn). In order to quantify these values somewhat, 3,000 ppm supplemental Zn achieved plasma Zn concentrations of between 1.30 and 2.78 from ZnO and $ZnSO_4$ sources respectively. Additionally, dietary spray-dried animal plasma has been reported to enhance daily gains and efficiency of feed utilisation (Stahly, 1996). The piglets were also group housed.

The kidney Zn concentrations determined for animals fed diets 1 and 3 (*i.e.* diets containing no supplemental Zn) are less than those obtained from animals fed the Zn-supplemented diets (diets 2 and 4). However, it is noteworthy, that they are of a relatively large magnitude in comparison to those from diets 2 and 4. Additionally, liver Zn concentrations obtained from animals fed diets 1 and 3 were almost identical and of a much lower order than those obtained from animals fed diets 2 and 4. These values are however, within the range of those reported by Carlson *et al.* (1999); between 28.4 and 46.6 µg/g for traditionally- (24.5 d of age) and early-weaned (11.5 d of age) pigs respectively. Furthermore, in the study of Carlson *et al.* (1999), mean kidney Zn concentrations were only modified to 17.4 and 25.6 µg/g for traditionally- and early-weaned pigs respectively after being fed 3,000 ppm supplemental Zn for a period of 2-weeks. Such modest changes in kidney Zn concentration are also supported by data of Pejsak *et al.* (1998). In the current study, all samples were analysed in triplicate and in a randomised manner in order to minimise any effects due to experimental technique or contamination. That is, a single run contained samples from both liver and kidney tissues, from animals of different ages and diets. As expected, kidney Zn concentration increased throughout the experiment. This could be related to the increasing feed intake levels, which

would also result in a greater intake of Zn. Since it is known that the kidney is involved in Zn homeostasis, it follows that any excess Zn that is to be eliminated from the body (via the urine and faeces) would have to pass through the kidneys that may account for the enhanced Zn concentrations reported.

Blood urea nitrogen (BUN) levels were not affected by dietary treatment nor did they change over time. Urea is the principal end product of nitrogen metabolism in mammals, and constitutes the largest fraction of the non-protein nitrogen component of the blood, and is produced in the liver and excreted through the kidneys in the urine. Consequently, the circulating levels of urea depend upon protein (and hence amino acid) intake, protein metabolism and kidney function. No dietary effects on BUN levels indicates that all 4 diets were providing adequate amino acids and hence protein to meet the piglets' requirements. This further suggests that all animals were achieving adequate total feed intake levels.

In the present study, no incidence of scouring was observed in any treatment group. As such the effects of ZnO and/or antibiotic treatment on the incidence of scours could not be investigated. However, in a study conducted by Schell and Kornegay (1996), scouring was only observed in control pigs, and the authors suggested that the feeding of high concentrations of Zn (3,000 ppm ZnO) might reduce scouring in weanling pigs.

In conclusion, very limited effects of ZnO and avilamycin supplementation were observed, with no significant modifications of intestinal morphology or the microflora species monitored post-weaning. It should however be noted that the gut ecosystem is extremely complex, containing at least 700 species, and effects on, or interactions with, other microflora species are possible. Although supplemental ZnO may stimulate feed intake, any enhanced growth is not solely a function of increased voluntary feed intake (Carlson *et al.* 1999). Further research is required to elucidate the mechanism for any feed intake response; certainly it is difficult to identify cause and effect, and the growth-promoting efficacy of ZnO. Moreover, environmental aspects of spreading faeces with a high content of ZnO must also be considered. Proposed legislation is also suggesting a ban on the inclusion of zinc oxide at pharmacological levels (3.1 kg/tonne) in the diets of post-weaned piglets by 2006.

4.9 FUTURE WORK / IMPROVEMENTS

Although the study reported in this chapter was conducted at the time using the most appropriate techniques and methods, the results from this study have highlighted a number of areas where further work or modifications to the protocol could be employed.

An inverse relationship between absorption and dietary Zn intake has been reported in animal models (Jackson *et al.,* 1981; Coppen and Davies, 1987). However, at extremely high intakes, absorption efficiency was found to be no longer affected and Zn homeostasis was regulated principally by excretion. Therefore, it would be interesting to measure the Zn content of faeces and/or urine. However, it must also be borne in mind that faeces and urine would also contain endogenously secreted Zn that has been excreted from the body. However, such as procedure would involve a balance study and the collection of faeces / urine may require a catheter which would be very difficult for weaner pigs due to their smaller size and the associated ethical implications. Although this would provide an indication as to the amount of dietary Zn being absorbed/retained, the endogenous Zn would still need to be quantified.

Since considerable Zn can be stored in the bones, and it has been suggested that this pool can be mobilised (Hahn and Baker, 1993; Smith *et al.,* 1995b; Carlson *et al.,* 1999), it would be interesting to determine the Zn content of bones. Furthermore, the retention of mineral elements (*i.e.* Zn) in proportion to N retention may be an important indicator of physiological requirement, storage or deposition (Larsen and Poulsen, 1994).

Alkaline phosphatase activity also provides an indication of the physiological Zn status and can be easily determined.

CHAPTER 5A:
THE EFFECTS OF DIETARY YEAST-BASED NUCLEOTIDE SUPPLEMENTATION ON POST-WEANING PIGLETS

5A.1 INTRODUCTION

The post-weaning growth check still causes considerable economic losses in pig production. Some of the problems in susceptibility to disease have been associated with problems concerning immunoregulation. For example, immaturity of the neonatal immune system, stress-associated immunosuppression and pathogen-induced immunosuppression have all been linked to increased disease susceptibility throughout the post-weaning period. Thus there exists an inherent need to provide complementary or alternative therapeutic strategies to alleviate these problems. The removal of the 4 remaining in-feed antibiotics in 2006 within the European Union will further exacerbate the necessity for alternative therapies

Modern pig production systems are such that the spread of infectious agents is both widespread and relatively straightforward. Consequently a high proportion of animals encounter both clinical and subclinical infections. Infections have a strong negative impact on growth performance even when they are sub-clinical (Beisel, 1988), and reduction of infections, for example by specific pathogen free (SPF) conditions, have been reported to increase weight gains by 20 - 40% (Young *et al.*, 1959; Caldwell *et al.*, 1961). Since the deleterious effects of microorganisms are regulated by the immune system, it follows that the host's immune capacity should form an important consideration when improving health and performance of pigs reared under conventional systems.

Such modern systems generally result in the exposure of the animals to a number of infectious agents concurrently with other 'stressors'. Such managerial 'stressors' may include for example, weaning (and the associated mixing and moving etc.), and transportation (particularly in multi-site production systems), which can affect the host's immune defence system. Weaning pigs at a young age can result in a number of problems, for example passive antibody-mediated immunity, derived from colostral immunoglobulins, reaches a maximum in the pig at 24 to 36 h of age and then decreases logarithmically to very low levels when approximately 3 weeks-old (Speer *et al.* (1959)

Miller *et al.* (1962), Porter (1976)). This emphasises the immunological vulnerability of the young pig at a time when it is exposed to a variety of different stressors associated with weaning. Additionally, evidence suggests that a pig's decreased physiological maturity from the stress of weaning lowers antibody synthesis. A number of studies, such as Gwazdauskas *et al.* (1978), Haye and Et (1979), Blecha and Kelley (1981) have provided evidence that weaning impairs antibody-mediated immunity and have suggested that weaning may compromise cellular immune function in young pigs. More specifically, Blecha *et al.* (1983) reported that, immediately post-weaning, piglets showed reduced ability to react to the lymphocyte mitogen PHA administered either intradermally or when lymphocytes were cultured *in vitro*. Furthermore, in a study conducted by Blecha and Kelley (1981), the production of specific antibody was also reduced when sheep red blood cells were administered to piglets 1 day post-weaning compared to 2 weeks post-weaning. This apparent systemic immunosuppression has been suggested to be involved in the aetiology of post-weaning diarrhoea, since disease cannot be induced reliably in unweaned animals following a challenge by pathogenic organisms (Kenworthy and Allen, 1966).

At birth, the piglet has high cortisol levels due to the hormonal trigger for the induction of parturition, but these levels decline rapidly to normal adult levels within 5 days post-partum (McCauley and Hartmann, 1984). However, Dvorak (1972) reported high cortisol levels persisting for up to 60 days postpartum and suggested that the neonate is therefore immuno-compromised. High post-partum cortisol levels have been reported to cause reduced chemotactic and phagocytic responses in the neonatal calf (Pierce and Koster, 1980), but this has not been demonstrated in piglets. McCauley and Hartmann (1984) argued that leukocyte dynamics reflect a minimal suppression of the immune function of the newborn piglet as the neonate has a neutrophilia that persists longer than 48 hours. Furthermore, neutrophilia of newborns is also observed in other species (Archer and Jeffcott, 1977). A colostral factor (La Motte and Eberhardt, 1976) has been identified which promotes neutrophilia in suckling calves, but this has not yet been demonstrated in sows' milk. Neutrophils are powerful effector cells in a cytotoxic response dependent on specific antibodies (antibody-dependent cell-mediated cytotoxicity). The neutrophilia observed shortly after birth, together with the specific antibody present in colostrums, may be important defence mechanisms (Zarkower *et al.*, 1982) for the unprimed neonate.

Ascogen (Chemoforma Ltd, Augst, Switzerland), a yeast-based nucleotide source, consists of a biologically active complex of metabolites of nucleotide metabolism with pyrimidines being the most important item among them. Ascogen is completed by some additional products such as organic acids of the citric and aspartic acid cycle, vitamins and trace elements. Thermolysed dried yeasts serve as a carrier of this complex (Adamek, 1994), which explains why this product is frequently referred to as a 'yeast-based nucleotide source'.

Studies in both infants and animal models suggest that dietary nucleotides have significant effects on the immune and gastrointestinal systems (see Carver, 1999). It has been suggested that under conditions of limited nucleotide intake, rapid growth or certain disease challenges, dietary (or pre-formed) nucleotide may spare the cost of *de novo* nucleotide synthesis and optimise the metabolic function of rapidly dividing tissues such as those of the gastrointestinal and immune systems. Furthermore, in addition to serving as nucleic acid precursors, nucleotides play roles as intercellular and intracellular biological mediators (Carver, 1999). More specifically, in laboratory animal studies, supplemental dietary nucleosides and nucleotides have been shown to result in optimal growth and maturation of the gut (Nunez *et al.*, 1990; Uauy *et al.*, 1990), enhanced lymphocyte proliferation in response to mitogens (Rudolph *et al.*, 1990) and improved host resistance to bacterial (Kulkarni *et al.*, 1986; Adjei *et al.*, 1992) and fungal (Fanslow *et al.*, 1988) infections.

Many studies have investigated the role of dietary nucleosides and nucleotides in the maintenance of the cellular immune system. For example, the addition of dietary nucleosides and nucleotides has been shown to increase DTH (delayed-type hypersensitivity) responses (Kulkarni *et al.*, 1987) enhance host resistance to infection (Kulkarni *et al.*, 1986; Fanslow *et al.*, 1988; Adjei *et al.*, 1993), affected the number and function of T helper cells (Van Buren *et al.*, 1985; Kulkarni *et al.*, 1989; Rudolph *et al.*, 1990; Jyonouchi *et al.*, 1994) and enhanced the cytotoxic function of natural killer cells in BALB/c mice (Carver *et al.*, 1990; Kulkarni *et al.*, 1994a).

The role of dietary nucleotides in the functioning of the immune system has not yet been fully elucidated. However, immature lymphocytes are not capable of synthesising purine and pyrimidine bases required to produce nucleotides and must therefore rely on the *de novo* synthesis of nucleotides by other organs such as the liver (Rudolph *et al.*, 1990) or

an exogenous source such as the diet. Furthermore, evidence presented in the literature evaluating the effects of yeast-based nucleotide sources, or other immuno-potentiators in piglets, is extremely limited. Most research has concentrated on human, aquaculture and laboratory animal studies with some limited work performed with ducks. Accordingly, a study was designed to examine the effects of one such immuno-potentiator, Ascogen, on a number of parameters as outlined below.

5A.2 AIMS

To determine the effects of feeding a conventional starter diet supplemented with a yeast-based nucleotide source (Ascogen; Chemoforma Ltd, Augst, Switzerland) to piglets on gut physiology (in terms of villus height, width and crypt depth) microflora characteristics (numbers and ratios of several coliforms, lactobacilli, and bifidobacteria), immune status (lymphocyte proliferation responses to mitogens), and performance characteristics between 8 and 20 kg live-weight. It is postulated that dietary nucleotide-supplementation will improve small intestinal structure and enhance the ratio of beneficial microflora (lactobacilli and bifidobacteria) in the small intestine, and also enhance the host immune status post-weaning.

5A.3 METHODS

5A.3.1 Animals and housing

Thirty-six entire male piglets (Section 2.2) were used to evaluate the experimental diets. Animals were individually housed as described previously (Section 2.2.1), and as in previous studies 4 piglets where slaughtered on day 0 (weaning day) to provide baseline data.

5A3.2 Diets

A single-stage trial diet programme was used based on Provimi Ltd 'Multiwean' diet. Diets are based upon identical formulations but diet 2 was supplemented with 4 g Ascogen / kg (according to the manufacturers recommended inclusion rate).

Diet 1 No supplement; Conventional Starter Diet (Control)

Diet 2 Conventional Starter Diet + 4 g Ascogen /kg (Treatment)

Each of the two diets was fed to 16 piglets over an experimental period of 14 days. Both diets comprised the same raw ingredients as shown in Table 5A.1. The chemical composition of the diets used has been presented previously in Table 2.1 (section 2.3).

Table 5A.1: Diet specification

Ingredient	Inclusion (g/kg) Diet 1 Control	Diet 2 Treatment
Cooked cereals	473.8	469.8
Oilseeds	175.0	175.0
Fish Products	92.0	92.0
Whey Powders	212.0	212.0
Oils & Fats	25.0	25.0
Vitamins & Minerals	8.1	8.1
Amino Acids	4.1	4.1
Skim Milk	10.0	10.0
Ascogen	0.0	4.0

5A.3.3 Post-mortem procedure

The experimental design has been described previously (section 2.4, and 2.5), and any modifications to this procedure are described below.

On the day of weaning (day 0; $n = 4$), and on days 2, 4, 6 and 14 ($n = 8$; 4 piglets fed each of the experimental diets), piglets were slaughtered, and samples of small intestine taken for subsequent histological examination as described in Section 2.6.

Digesta samples were also removed for microbiological enumeration for lactobacilli, coliforms and bifidobacteria species (Section 2.8). Furthermore, a peripheral blood sample was removed for subsequent lymphocyte isolation (Section 2.9). In addition, the digesta samples obtained from the jejunum, duodenum, ileum, colon and rectum (S1-S5) for microbiological analyses also underwent pH analysis.

In addition to the faecal swabbing procedure that has been described previously (Section 2.8.2), all slaughter animals underwent faecal swabbing immediately prior to slaughter. This was to allow a comparison between intestinal and faecal microflora to be made.

5A.4 Statistical analyses

Statistical analyses were undertaken as described previously in Section 2.12.

5A.5 RESULTS

5A.5.1 Gut morphology

Tables 5A.2 and 5A.3 represent villus height data. It can be clearly seen that a degree of villus atrophy occurs immediately post-weaning between days 0 and 2. Villus lengthening then occurs up to day 14 (P=<0.001; <0.001(L)), and there was also a significant correlation between BVR and day (P=<0.001). There was a significant region effect with the distal regions (0.75 proportion) of the small intestine being the shortest (P=<0.001; overall dietary mean). Significant interactions between dietary treatments over time or BVR and diet were not evident. No significant differences were determined between dietary treatment or baseline animals compared to the remainder. Determination of the percentage difference in villus height between villi isolated from baseline and the remainder of the experimental animals demonstrates that a greater degree of villus atrophy (*i.e.* severity and duration) occurred in animals offered the control compared with the treatment diet (Figure 5A.1). The longer villi present in the proximal region of the small intestine decreased in height proportionately less than villi in the medial or distal domains of the gut (a decrease in height of the order 26-28% was apparent over the initial 4 days post-weaning).

Villus width followed a similar pattern; there was a strong trend towards a reduction in VW between those animals slaughtered on day 0 (baseline) and the remainder of the animals (P=0.066; Tables 5A.4 and 5A.5). In fact, the baseline VW was only achieved by day 14 for those animals fed diet1 (control) and it was never achieved by those fed diet 2 (treatment). Generally, VW increased throughout the experimental period (P=0.006; <0.001(L)), and there were significant relationships between diet and day (P=0.027; 0.074(L)), and BVR and day (P=0.018). Villus width measurements were not significantly different with respect to diet, region of intestine or BVR diet interactions.

Small intestine crypt depth increased throughout the experimental period (P=<0.001; 0.137(L); <0.001(Q)) for those animals fed diet 2 (treatment), with those fed diet 1 (control) following a similar pattern, but crypt depth declined between days 6 and 14 (Tables 5A.6 and 5A.7). As such there was a highly significant dietary effect (P=<0.001), and diet day (P=<0.001; <0.001(L); 0.003(Q)) interaction. Although not significant a trend was evident towards a region influence (P=0.093) with the medial (0.50 along the length of the small intestine) CD being deepest (overall mean) across all 3 groups (baseline, control and treatment groups). Measurements of crypt depth also determined a significant difference with respect to BVR (P=<0.001), BVR day (P=<0.001) and BVR diet (P=<0.001) interactions.

Baseline animals displayed the greatest villus height to crypt depth ratio (P=<0.001; VH:CD; tables 5A.8 and 5A.9) Furthermore, VH:CD was greatest on day 14 for both dietary groups (P=<0.001; <0.001(L); <0.001(Q)) and those fed diet 1 (control) had a greater overall VH:CD than those fed diet 2 (treatment; P=<0.001). A significant relationship between diet and day was also evident (P=<0.001; <0.001(L); 0.001(Q)).

Table 5A.2: Effect of dietary treatment on villus height (μm; $n = 16$ per diet; baseline $n = 4$)

Day	Baseline Proportion of Intestine 0.25	0.50	0.75	mean	Diet 1 Proportion of Intestine 0.25	0.50	0.75	mean	Diet 2 Proportion of Intestine 0.25	0.50	0.75	mean
0	446	446	436	443								
2					387	356	327	**357**	373	324	325	**341**
4					439	399	317	**385**	452	460	416	**443**
6					408	458	382	**416**	390	417	418	**408**
14					532	493	459	**495**	501	485	524	**503**
mean	**446**	**446**	**436**	***443***	**441**	**427**	**371**	***413***	**429**	**522**	**420**	***424***

Baseline = animals slaughtered on day 0 (weaning); diet 1 = control; diet 2 = treatment

Table 5A.3: A summary of the statistical analysis for villus height

Diet		Day		Diet*Day		Region		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
12.8	0.411	18.0	<0.001 <0.001 (L) 0.190 (Q)	25.5	0.199 0.988 (L) 0.613 (Q)	4.1	<0.001	21.0	0.261	24.3	<0.001	22.1	0.453

BVR = Baseline versus the rest; day refers to slaughter day

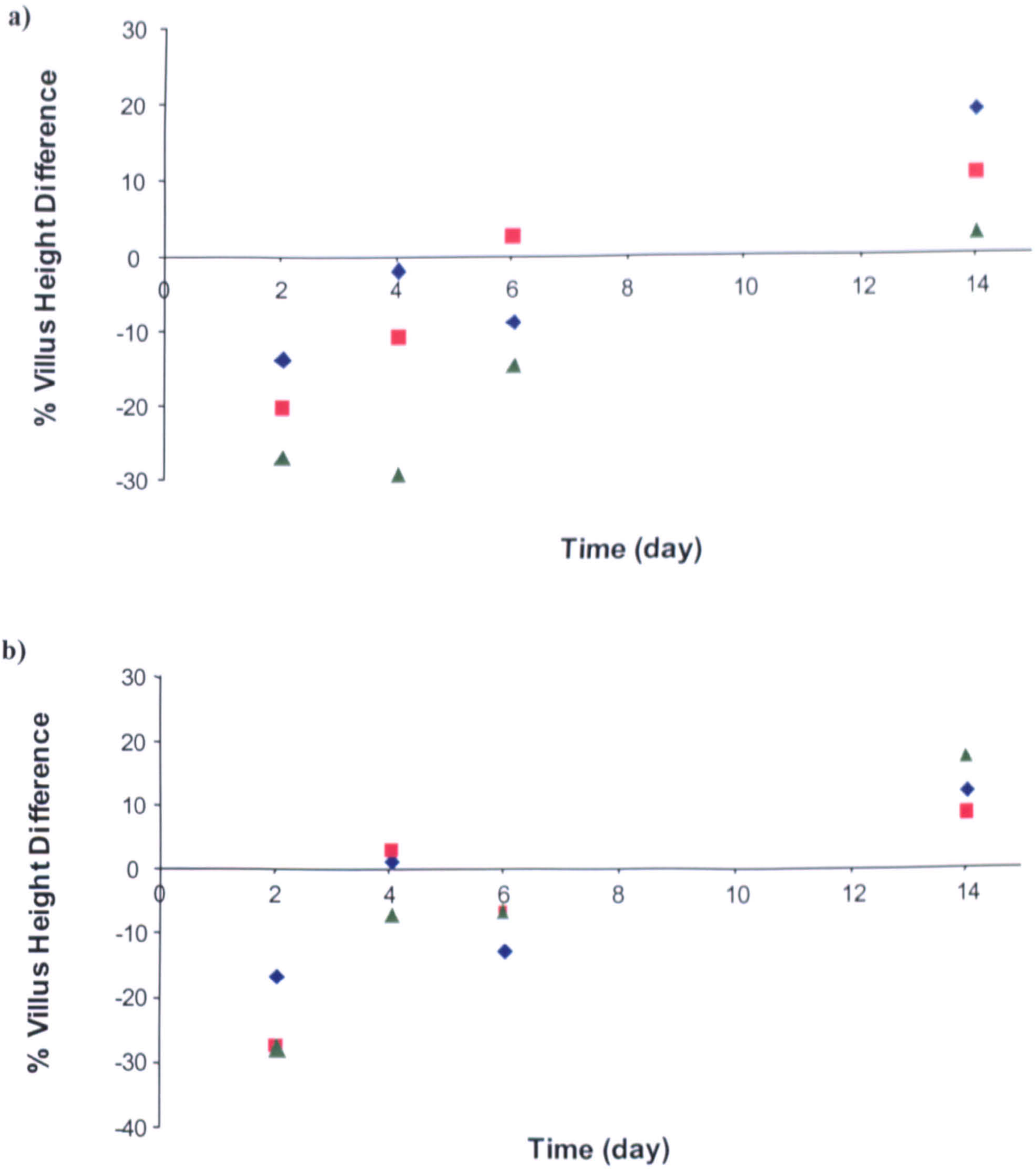


Figure 5A.1: Percentage difference in villus height between baseline values and those obtained from animals slaughtered on days 2, 4, 6 or 14 fed a) diet 1 (control) or b) diet 2 (treatment) at regions proportionately 0.25 (◆), 0.50 (■) and 0.75 (▲) along the length of the intestine from the gastric pylorus to the ileo-caecal valve.

Table 5A.4: Effect of dietary treatment on villus width (μm; $n = 16$ per diet; baseline $n = 4$)

	Baseline Proportion of Intestine				Diet 1 Proportion of Intestine				Diet 2 Proportion of Intestine			
Day	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean
0	88	84	80	84								
2					69	73	74	72	83	75	74	77
4					77	86	84	82	75	76	74	75
6					83	80	73	79	75	80	82	79
14					91	85	84	97	79	80	82	80
mean	88	84	80	*84*	80	81	79	*80*	78	78	78	*78*

Baseline = animals slaughtered on day 0 (weaning); diet 1 = control; diet 2 = treatment

Table 5A.5: A summary of the statistical analysis for villus width

Diet		Day		Diet*Day		Region		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
1.6	0.194	2.2	0.006 <0.001 (L) 0.441 (Q)	3.1	0.027 0.074 (L) 0.418 (Q)	1.1	0.765	2.7	0.066	3.1	0.018	2.8	0.256

BVR = Baseline versus the rest; day refers to slaughter day

Table 5A.6: Effect of dietary treatment on crypt depth (μm; $n = 16$ per diet; baseline $n = 4$)

Day	Baseline Proportion of Intestine 0.25	0.50	0.75	mean	Diet 1 Proportion of Intestine 0.25	0.50	0.75	mean	Diet 2 Proportion of Intestine 0.25	0.50	0.75	mean
0	136	161	130	143								
2					125	134	122	127	192	169	168	176
4					207	223	203	211	221	239	216	225
6					232	232	232	232	239	251	248	246
14					133	151	143	142	240	240	247	242
mean	136	161	130	*143*	174	185	175	*178*	223	225	220	*223*

Baseline = animals slaughtered on day 0 (weaning); diet 1 = control; diet 2 = treatment

Table 5A.7: A summary of the statistical analysis for crypt depth

Diet s.e.d	Diet P	Day s.e.d	Day P	Diet*Day s.e.d	Diet*Day P	Region s.e.d	Region P	BVR s.e.d	BVR P	BVR*Day s.e.d	BVR*Day P	BVR*Diet s.e.d	BVR*Diet P
6.8	<0.001	9.6	<0.001 0.137 (L) <0.001 (Q)	13.6	<0.001 <0.001 (L) 0.003 (Q)	3.7	0.093	9.6	<0.001	11.1	<0.001	10.1	<0.001

BVR = Baseline versus the rest; day refers to slaughter day

Table 5A.8: Effect of dietary treatment on villus height to crypt depth ratio (n = 16 per diet; baseline n = 4)

	Baseline				Diet 1				Diet 2			
	Proportion of Intestine				Proportion of Intestine				Proportion of Intestine			
Day	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean
0	3.7	3.0	3.7	3.5								
2					3.3	2.9	2.8	3.0	2.1	2.2	2.3	2.2
4					2.2	1.9	1.6	1.9	2.2	2.0	2.0	2.1
6					2.0	2.1	1.7	2.0	1.7	1.8	1.8	1.8
14					4.5	3.6	3.4	3.8	2.3	2.2	2.2	2.3
mean	3.7	3.0	3.7	3.5	3.0	2.6	2.4	2.7	2.1	2.1	2.1	2.1

Baseline = animals slaughtered on day 0 (weaning); diet 1 = control; diet 2 = treatment

Table 5A.9: A summary of the statistical analysis for villus height to crypt depth ratio

Diet		Day		Diet*Day		Region		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.11	<0.001	0.16	<0.001 <0.001 (L) <0.001 (Q)	0.23	<0.001 <0.001 (L) 0.001 (Q)	0.06	<0.001	0.18	<0.001	0.20	<0.001	0.16	<0.001

BVR = Baseline versus the rest; day refers to slaughter day

5A.5.2 Microbiological analyses

5A.5.2.1 Digesta samples

Intestinal coliform counts increased with advancing distance along the small intestine (S1-S5; P=<0.001; Tables 5A.10 and 5A.11). Animals offered diet 2 displayed significantly lower coliform counts (treatment; P=0.033), and total numbers decreased throughout the experimental period for both dietary groups (P=0.009; 0.002(L)). Evidence of a significant relationship between diet and day (P=0.067 (L)), BVR and day (P=0.008) and BVR and diet (P=0.032) was also apparent. No significant differences between the baseline (n = 4) and remainder of the animals (n = 32) were identified.

Intestinal lactobacilli counts were found to increase between days 2 and 14 (P=0.007; 0.395 (L); 0.008 (Q); Tables 5A.12 and 5A.13). No significant differences with respect to baseline animals, dietary treatment or relationship between diet and day were found. Results also demonstrated enhanced lactobacilli counts along the length of the intestine (S1-S5; P=<0.001). Significant relationships between BVR and day (P=0.008) and BVR and diet (P=0.032) were also apparent.

Bifdobacteria counts were similar for both dietary treatment groups and attained their numerical peak on slaughter day 4 (P=0.006; Tables 5A.14 and 5A.15). A significant difference between the baseline and remainder of the animals (P=<0.001) was also detected, in addition to a significant correlation between BVR and day (P=0.024). No significant difference with respect to sample site or interactions between diet and day, and BVR and diet were found.

The lactobacilli to coliform ratio increased significantly between days 2 to 14 (P=0.005; <0.001 (L)) for those animals fed both diets 1 (control) and 2 (treatment; Tables 5A.16 and 5A.17). Conversely, this ratio declined with increasing distance along the intestine (S1-S5; P=<0.001). No significant influence of diet or BVR or relationships between diet and day, or BVR and diet were found. There was however, a significant correlation between BVR and day (P=0.004).

Table 5A.10: Mean log coliform counts (cfu / g) obtained from digesta samples (S1-S5*) along the intestine (n = 16 per diet; baseline n = 4)

	Baseline						Diet 1						Diet 2					
	Sample site						Sample site						Sample site					
Day	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean
0	5.0	5.0	7.6	9.7	8.7	7.2												
2							6.8	7.6	9.3	9.0	9.4	8.4	4.9	5.2	8.1	8.4	8.6	7.0
4							8.0	7.8	10.7	9.2	9.1	9.0	5.1	6.0	8.6	9.5	8.6	7.6
6							6.8	5.8	10.1	9.6	8.9	8.2	4.5	4.9	7.9	7.4	7.3	6.4
14							5.1	3.1	5.4	6.3	8.5	5.7	6.1	5.5	5.9	7.2	6.8	6.3
mean	5.0	5.0	7.6	9.7	8.7	7.2	6.7	6.1	8.9	8.5	9.0	7.8	5.2	5.4	7.6	8.1	7.8	6.8

*S1 – S5 = duodenum, jejunum, ileum, colon and rectum respectively; baseline = animals slaughtered on day 0 (weaning); diet 1 = control; diet 2 = treatment

Table 5A.11: A summary of the statistical analysis for intestinal coliforms

Diet		Day		Diet*Day		Sample Site		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.44	0.033	0.63	0.009 0.002 (L) 0.549 (Q)	0.87	0.221 0.067 (L) 0.340 (Q)	0.36	<0.001	0.67	0.852	0.77	0.008	0.70	0.032

BVR = Baseline versus the rest; day refers to slaughter day; sample site refers to sites S1 – S5 (see above)

Table 5A.12: Mean log lactobacilli counts (cfu / g) obtained from digesta samples (S1-S5*) along the intestine (n = 16 per diet; baseline n = 4)

	Baseline						Diet 1						Diet 2					
	Sample site						Sample site						Sample site					
Day	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean
0	8.4	9.4	9.8	10.0	9.1	9.3												
2							7.1	8.2	7.7	8.2	9.1	8.1	6.8	7.4	7.4	8.1	7.9	7.5
4							8.0	8.9	9.4	9.4	8.7	8.9	8.9	8.4	9.7	9.8	9.7	9.3
6							8.4	8.3	9.3	9.6	9.3	9.0	8.0	7.7	8.5	9.3	8.9	8.5
14							5.8	7.6	9.7	9.3	9.4	8.4	7.2	7.5	9.7	9.8	9.5	8.7
mean	8.4	9.4	9.8	10.0	9.1	9.3	7.3	8.2	9.0	9.1	9.1	8.6	7.7	7.7	8.8	9.3	9.0	8.5

*S1 – S5 = duodenum, jejunum, ileum, colon and rectum respectively; baseline = animals slaughtered on day 0 (weaning); diet 1 = control; diet 2 = treatment

Table 5A.13: A summary of the statistical analysis for intestinal lactobacilli

Diet		Day		Diet*Day		Sample Site		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.24	0.785	0.34	0.007 0.395 (L) 0.008 (Q)	0.48	0.321 0.336 (L) 0.943 (Q)	0.18	<0.001	0.67	0.852	0.77	0.008	0.70	0.032

BVR = Baseline versus the rest; day refers to slaughter day; sample site refers to sites S1 – S5 (see above)

Table 5A.14: Mean log bifidobacteria counts (cfu / g) obtained from digesta samples (S1-S5*) along the intestine (n = 16 per diet; baseline n = 4)

	Baseline						Diet 1						Diet 2					
	Sample site						Sample site						Sample site					
Day	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean
0	7.9	8.2	7.6	9.4	8.7	8.4												
2							7.8	9.6	9.2	9.4	10.3	9.3	9.4	9.5	9.5	8.7	9.5	9.3
4							9.9	9.9	10.1	10.4	10.0	10.1	9.9	9.8	10.0	9.9	9.9	9.9
6							9.5	9.7	9.5	9.7	9.8	9.6	9.3	9.2	9.5	9.4	9.7	9.4
14							10.1	10.1	10.0	10.3	9.6	10.0	8.9	9.4	9.6	9.9	9.7	9.5
mean	7.9	8.2	7.6	9.4	8.7	*8.4*	9.3	9.8	9.7	9.9	9.9	*9.7*	9.4	9.5	9.6	9.5	9.7	*9.5*

*S1 – S5 = duodenum, jejunum, ileum, colon and rectum respectively; baseline = animals slaughtered on day 0 (weaning); diet 1 = control; diet 2 = treatment

Table 5A.15: A summary of the statistical analysis for intestinal bifidobacteria

Diet		Day		Diet*Day		Sample Site		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.13	0.111	0.18	0.006 0.172 (L) 0.195 (Q)	0.26	0.458 0.121 (L) 0.881 (Q)	0.17	0.108	0.23	<0.001	0.27	0.024	0.25	0.181

BVR = Baseline versus the rest; day refers to slaughter day; sample site refers to sites S1 – S5 (see above)

Table 5A.16: Mean log lactobacilli to coliform ratio for digesta samples (S1-S5*) along the intestine (n = 16 per diet; baseline n = 4)

	Baseline						Diet 1						Diet 2					
	Sample site						Sample site						Sample site					
Day	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean
0	1.9	2.0	1.4	1.0	1.0	1.5												
2							1.1	1.2	0.8	0.9	1.0	1.0	1.4	1.4	0.9	1.0	0.9	1.1
4							1.0	1.2	0.9	1.0	1.0	1.0	1.8	1.6	1.3	1.1	1.1	1.4
6							1.2	1.5	0.9	1.0	1.0	1.1	1.9	1.7	1.2	1.4	1.4	1.5
14							1.5	2.5	2.0	1.7	1.1	1.7	1.3	1.5	1.9	1.5	1.5	1.6
mean	1.9	2.0	1.4	1.0	1.0	*1.5*	1.2	1.6	1.2	1.2	1.0	*1.2*	1.6	1.6	1.3	1.2	1.2	*1.4*

*S1 – S5 = duodenum, jejunum, ileum, colon and rectum respectively; baseline = animals slaughtered on day 0 (weaning); diet 1 = control; diet 2 = treatment

Table 5A.17: A summary of the statistical analysis for intestinal lactobacilli to coliform ratios

Diet		Day		Diet*Day		Sample Site		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.11	0.134	0.15	0.005 <0.001 (L) 0.574 (Q)	0.22	0.242 0.130 (L) 0.173 (Q)	0.08	<0.001	0.16	0.269	0.18	0.004	0.17	0.123

BVR = Baseline versus the rest; day refers to slaughter day; sample site refers to sites S1 – S5 (see above)

5A.5.2.2 Faecal samples

Faecal coliform counts were significantly lower in the faeces of baseline compared to the remainder of the animals (P=<0.001; Tables 5A.18 and 5A.19). Faecal coliform numbers tended (P=0.079) to be greater in control than treatment animals. A strong trend towards enhanced faecal coliforms on day 14 was also evident (P=0.061; 0.055(L)). Significant relationships between diet and day (P=<0.001), BVR and day (P=<0.001) and finally BVR and diet (P=<0.001) were also demonstrated.

A similar pattern was determined for faecal lactobacilli counts which increased significantly throughout the experimental period (P=<0.001; <0.001(L); <0.001(Q); Tables 5A.20 and 5A.21). Furthermore, treatment animals displayed higher lactobacilli counts in comparison to those fed diet 1 (control; P=<0.001). Significant differences between BVR (P=<0.001), and significant relationships between diet and day (P<0.001), BVR and day (P=<0.001), and BVR and diet (P=<0.001) were evident.

Faecal bifidobacteria counts were not significantly different with respect to diet or BVR diet interactions (Tables 5A.22 and 5A.23). They did however increase significantly throughout the experimental period (P<0.001; <0.001(L); <0.001(Q)). Analysis also revealed a significant difference between BVR (P=<0.001), and relationships between diet and day (P=0.002; 0.001(L)), and BVR and day (P=<0.001).

Faecal lactobacilli to coliform ratio increased significantly throughout the experimental period (P=<0.001; <0.001(L); Tables 5A.24 and 5A.25). Significant correlations between diet and day (P=<0.001; 0.021(Q)), and BVR and day (P<0.001) were also determined. No significant differences between diet, BVR or a BVR and diet interaction was found.

A comparison between rectal and faecal samples illustrated highly significant differences in all 3 species of bacteria enumerated (P = 0.021; 0.009; <0.001 for bifidobacteria, coliforms and lactobacilli respectively). In the case of coliforms and lactobacilli, rectal samples displayed greater counts. Conversely, in the case of bifidobacteria, faecal swabs displayed higher bacterial numbers.

No significant differences between any of the parameters recorded (diet, day, BVR, diet and day, BVR and day or BVR and diet) on digesta pH values were evident (Tables 5A.26 and 5A.27). The average dietary pH was 6.02 ± 0.134.

Table 5A.18: Mean log coliform counts (cfu / swab) obtained from faecal samples (n = 16 per diet; n = 4 baseline)

Day	Baseline	Diet 1	Diet 2	mean
0	7.1			7.1
1		8.0	7.7	7.8
2		7.3	7.7	7.5
3		7.9	7.5	7.7
4		8.1	7.5	7.8
5		7.2	7.6	7.4
6		7.7	7.6	7.6
8		7.4	7.1	7.3
10		8.3	7.2	7.8
12		6.9	8.7	7.8
14		8.0	5.7	6.8
Mean	7.1	7.7	7.4	7.6

Baseline = animals slaughtered on day 0 (weaning); diet 1 = control; diet 2 = treatment

Table 5A.19: A summary of the statistical analysis for faecal coliforms

Diet		Day		Diet*Day		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.14	0.079	0.32	0.061 0.055 (L) 0.375 (Q)	0.46	<0.001 0.104 (L) 0.082 (Q)	0.19	<0.001	0.20	<0.001	0.25	<0.001

BVR = Baseline versus the rest; day refers to slaughter day

Table 5A.20: Mean log lactobacilli counts (cfu / swab) obtained from faecal samples (n = 16 per diet; n = 4 baseline)

Day	Baseline	Diet 1	Diet 2	mean
0	6.7			6.7
1		7.1	6.6	6.9
2		5.9	6.2	6.1
3		6.8	7.4	7.1
4		6.6	8.7	7.7
5		8.0	6.9	7.4
6		7.4	7.7	7.6
8		8.5	8.6	8.6
10		8.1	8.9	8.5
12		8.0	8.7	8.3
14		7.4	7.8	7.6
Mean	6.7	7.4	7.8	7.6

Baseline = animals slaughtered on day 0 (weaning); diet 1 = control; diet 2 = treatment

Table 5A.21: A summary of the statistical analysis for faecal lactobacilli

Diet		Day		Diet*Day		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.11	<0.001	0.25	<0.001 <0.001 (L) <0.001 (Q)	0.35	<0.001 0.210 (L) 0.526 (Q)	0.19	<0.001	0.20	<0.001	0.25	<0.001

BVR = Baseline versus the rest; day refers to slaughter day

Table 5A.22: Mean log bifidobacteria counts (cfu / swab) obtained from faecal samples (n = 16 per diet; baseline n = 4)

Day	Baseline	Diet 1	Diet 2	mean
0	9.0			9.0
1		9.1	9.2	9.1
2		9.5	9.9	9.7
3		10.2	11.1	10.7
4		9.9	10.3	10.1
5		10.1	10.5	10.3
6		10.8	9.8	10.3
8		12.1	12.2	12.2
10		10.4	9.7	10.0
12		11.8	11.0	11.4
14		11.3	11.0	11.1
Mean	9.0	10.5	10.5	*10.5*

Baseline = animals slaughtered on day 0 (weaning); diet 1 = control; diet 2 = treatment

Table 5A.23: A summary of the statistical analysis for faecal bifidobacteria

Diet		Day		Diet*Day		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.11	0.539	0.25	<0.001 <0.001 (L) <0.001 (Q)	0.36	0.002 0.001 (L) 0.575 (Q)	0.18	<0.001	0.19	<0.001	0.24	0.520

BVR = Baseline versus the rest; day refers to slaughter day

Table 5A.24: Mean log lactobacilli to coliform ratios obtained from faecal samples (n = 16 per diet; baseline n = 4)

Day	Baseline	Diet 1	Diet 2	mean
0	1.0			
1		0.8	1.0	0.9
2		0.9	0.7	0.8
3		0.8	1.1	1.0
4		0.9	1.3	1.1
5		0.9	0.9	0.9
6		1.0	1.0	1.0
8		1.3	1.2	1.2
10		1.1	1.2	1.1
12		1.3	0.9	1.1
14		1.0	1.5	1.3
mean	1.0	1.0	1.1	*1.0*

Baseline = animals slaughtered on day 0 (weaning); diet 1 = control; diet 2 = treatment

Table 5A.25: A summary of the statistical analysis for faecal lactobacilli to coliform ratio

Diet		Day		Diet*Day		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.06	0.248	0.06	<0.001 <0.001 (L) 0.443 (Q)	0.11	<0.001 0.862 (L) 0.021 (Q)	0.08	0.425	0.09	<0.001	0.08	0.131

BVR = Baseline versus the rest; day refers to slaughter day

Table 5A.26: Mean digesta pH obtained from digesta samples (S1-S5*) along the intestine ($n = 8$ per diet; baseline $n = 4$)

	Baseline						Diet 1						Diet 2					
	Sample site						Sample site						Sample site					
Day	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean
0	5.8	6.6	8.0	6.9	7.3	6.9												
2							6.5	6.5	7.8	6.9	7.0	6.9	6.3	6.7	7.9	7.0	7.0	7.0
4							6.3	6.8	8.2	6.9	7.4	7.1	6.3	6.3	7.9	6.6	7.1	6.8
6							6.2	6.6	8.0	6.8	6.6	6.8	5.9	6.8	8.1	6.8	6.9	6.9
14							6.6	6.6	8.0	6.8	6.8	6.9	6.2	7.2	8.0	6.6	6.7	6.9
mean	5.8	6.6	8.0	6.9	7.3	6.9	6.4	6.6	8.0	6.8	6.8	6.9	6.2	6.7	7.9	6.7	6.9	6.9

*S1 – S5 = duodenum, jejunum, ileum, colon and rectum respectively; baseline = animals slaughtered on day 0 (weaning); diet 1 = control; diet 2 = treatment

Table 5A.27: A summary of the statistical analysis for digesta pH

Diet		Day		Diet*Day		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.06	0.702	0.084	0.493 0.346 (L) 0.521 (Q)	0.119	0.099 0.274 (L) 0.537 (Q)	0.087	0.941	0.1	0.463	0.091	0.692

BVR = Baseline versus the rest; day refers to slaughter day

5A.5.3 Performance parameters

Average daily live-weight gain (DLWG) for individual animals are illustrated in figure 5A.2, and the statistical analysis included all 32 experimental animals. A trend (P=0.073) was evident towards increased DLWG for animals offered diet 1 (control; average = 257g / d) in comparison to treatment animals (average = 147 g / d). No significant difference between diets on feed intake (table 5A.28) levels was determined. As expected, feed intake increased over time (P=<0.001; <0.001(L)), and there was a significant relationship between day and diet (P=0.008; 0.013(L); 0.039(Q)). The dataset for feed conversion ratio (FCR) clearly illustrates the poor FCR that is observed over the immediate post-weaning period (up to day 2) (Figure 5A.3 and Table 5A.29). This is linked to the low levels of feed intake combined with poor growth at this time. As expected, FCR improved over the duration of the experiment (P=<0.001; <0.001(L); <0.001(Q)). A significant relationship between day and diet (P=<0.001) was determined but there was no significant difference between dietary treatments.

The time taken for each animal to take first feed and water were also recorded. The earliest time to first feed was 1h 40 min with 8 piglets taking over 6 hours. Conversely, one piglet drank water immediately upon being placed in the pen, and 5 piglets took greater than 6 hours to drink water, with the remaining animals being in between these 2 time periods.

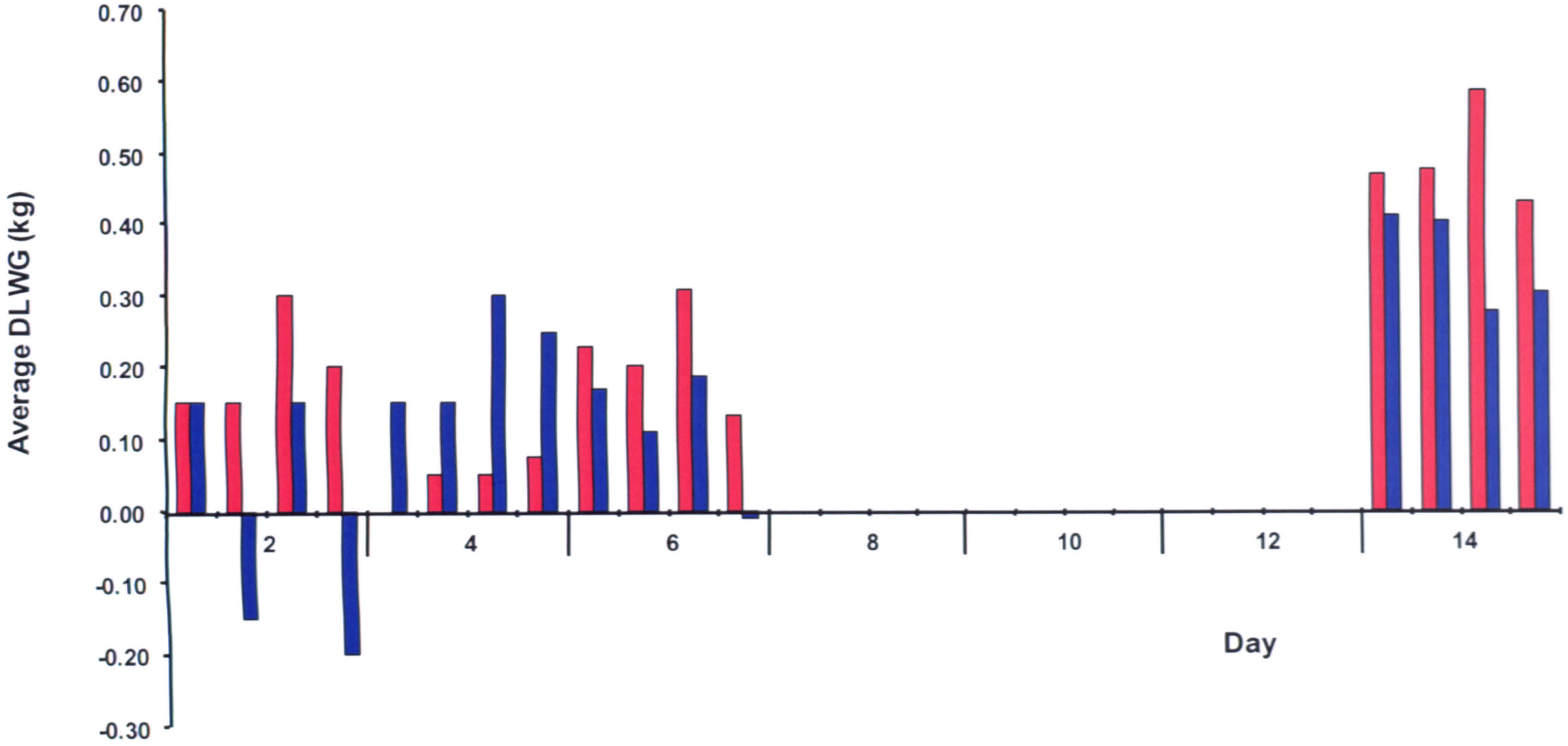


Figure 5A.2: Mean DLWG for individual animals fed diets 1(control) and 2 (treatment) grouped according to slaughter day (P=0.073; s.e.d = 0.06; n = 32)

Table 5A.28: Effect of dietary treatment on feed intake (g; n = 16 per diet) and associated statistical analysis

	Diet			Diet		Day		Diet*Day	
Day	1	2	Mean	s.e.d	P	s.e.d	P	s.e.d	P
2	187	150	169	22.2	0.638	31.5	<0.001	44.5	0.008
4	173	273	223				<0.001 (L)		0.013 (L)
6	312	339	326				0.266 (Q)		0.039 (Q)
14	594	461	527						
Mean	**316**	**306**	***311***						

diet 1 = control; diet 2 = treatment; day refers to slaughter day

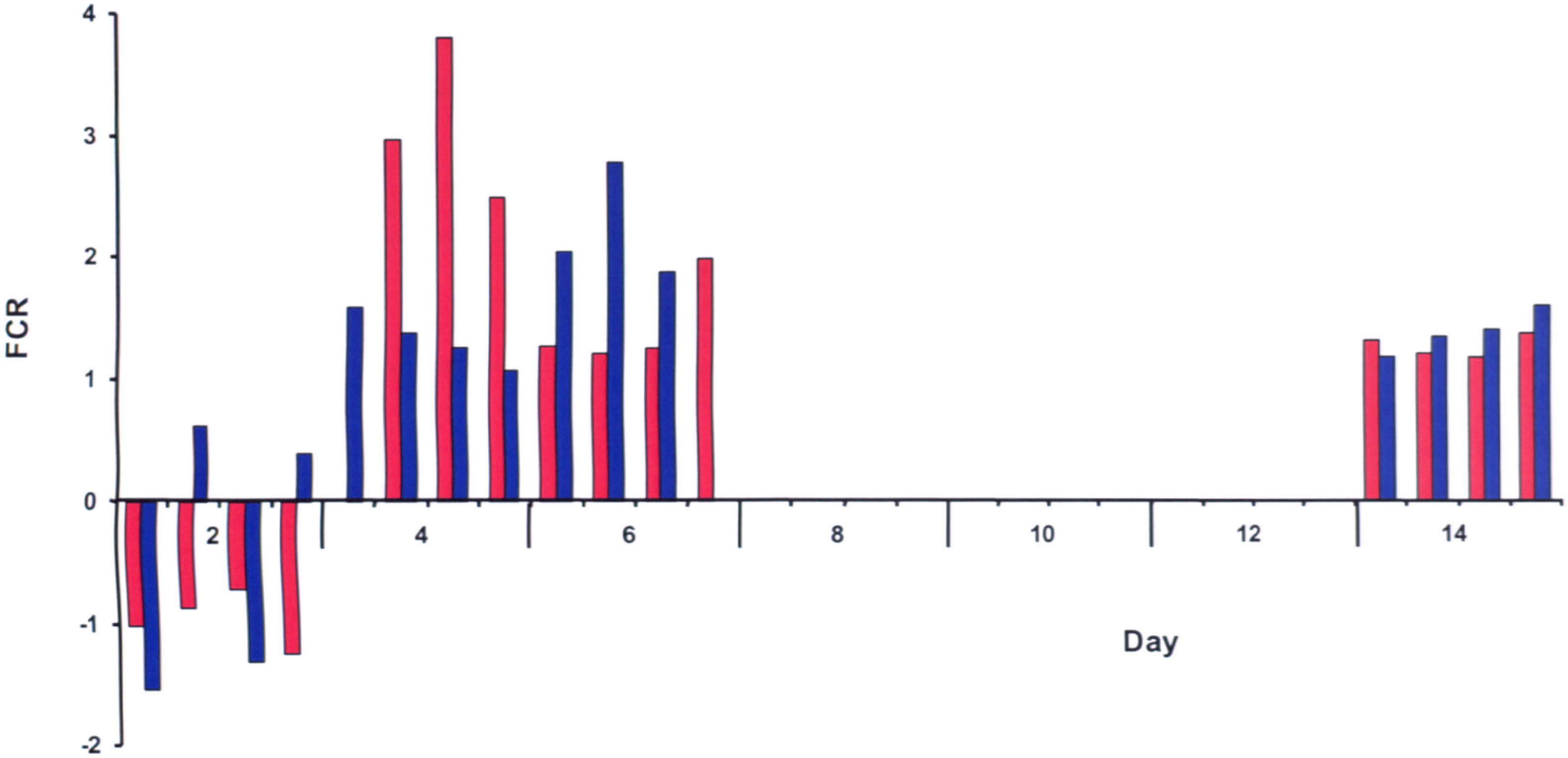


Figure 5A.3: Mean FCR for individual animals fed diets 1 (■ control) and 2 (■ treatment) grouped according to slaughter day (see table 5A.29 for statistical analysis)

Table 5A.29: A summary of the statistical analysis for FCR (n = 16 per diet)

Day	Diet 1	Diet 2	Mean	Diet s.e.d	Diet P	Day s.e.d	Day P	Diet*Day s.e.d	Diet*Day P
2	-1.0	-0.5	-0.7	0.18	0.649	0.26	<0.001	0.37	<0.001
4	3.1	1.3	2.2				<0.001 (L)		0.449 (L)
6	1.4	2.2	1.8				<0.001 (Q)		0.766 (Q)
14	1.3	1.4	1.3						
Mean	1.2	1.1	*1.2*						

5A.5.4 Lymphocyte proliferation

Figures 5A.4 – 5A.6 and Table 5A.30 represent peripheral blood lymphocyte (PBL) proliferation in response to 2 mitogens (Con A and PHA) at 3 different concentrations (10.0, 3.3 and 1.0 μg/ml) for piglets slaughtered on days 2, 4, 6 and 14. There are no data presented for baseline animals (slaughtered on day 0) due to insufficient lymphocyte isolation at slaughter, all subsequent individual PBL samples were pooled for treatment and slaughter day. A similar response was observed for both mitogens at all 3 concentrations. The maximum uptake of methyl-[^{3}H]-thymidine was achieved on day 4 for both mitogens, both dietary treatments, and for all 3 mitogen concentrations. This enhanced uptake was relatively short-lived and declined by day 6. There was a highly significant temporal effect (P=0.001; 0.079(L); 0.091(Q)), and a significant relationship between diet and day (P=0.012; 0.007(L); 0.020(Q)). Analysis determined no significant difference between dietary treatments on lymphocyte proliferation responses. There was a highly significant difference between mitogen sources (P<0.001) with mitogen 2 (PHA) achieving greater levels of uptake (3.134 and 3.476 (log cpm) for mitogen 1 and 2 respectively). As expected, a significant difference between mitogen concentration (P=<0.001) was observed, with concentration 1 (10 μg/ml) achieving the highest level of uptake, and concentration 3 the lowest (1.1 μg/ml).

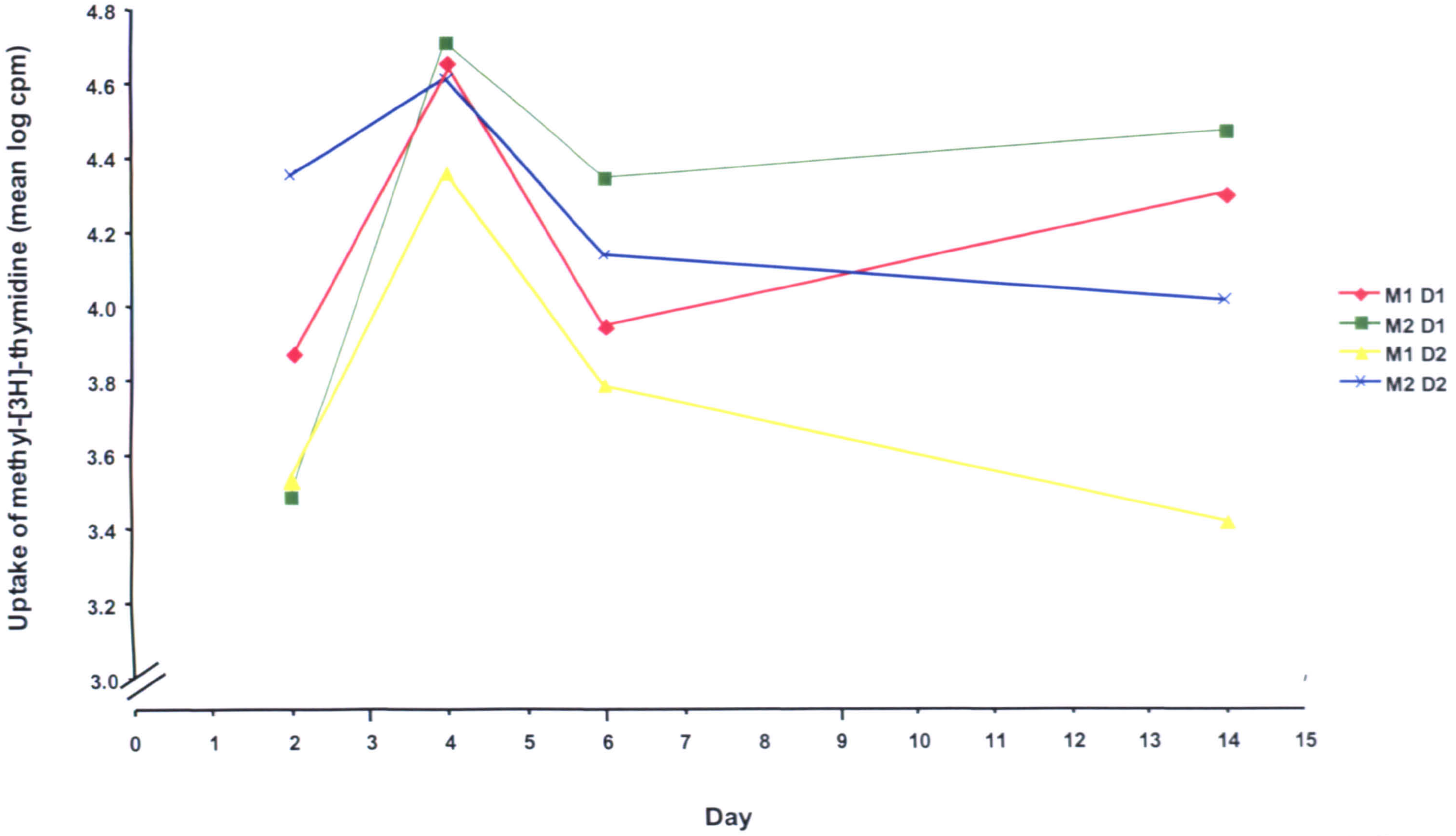


Figure 5A.4: The proliferation of PBLs from piglets fed diet 1 (D1; control) or 2 (D2; treatment) stimulated with Con A (M1) or PHA (M2) at a concentration of 10 μg/ml

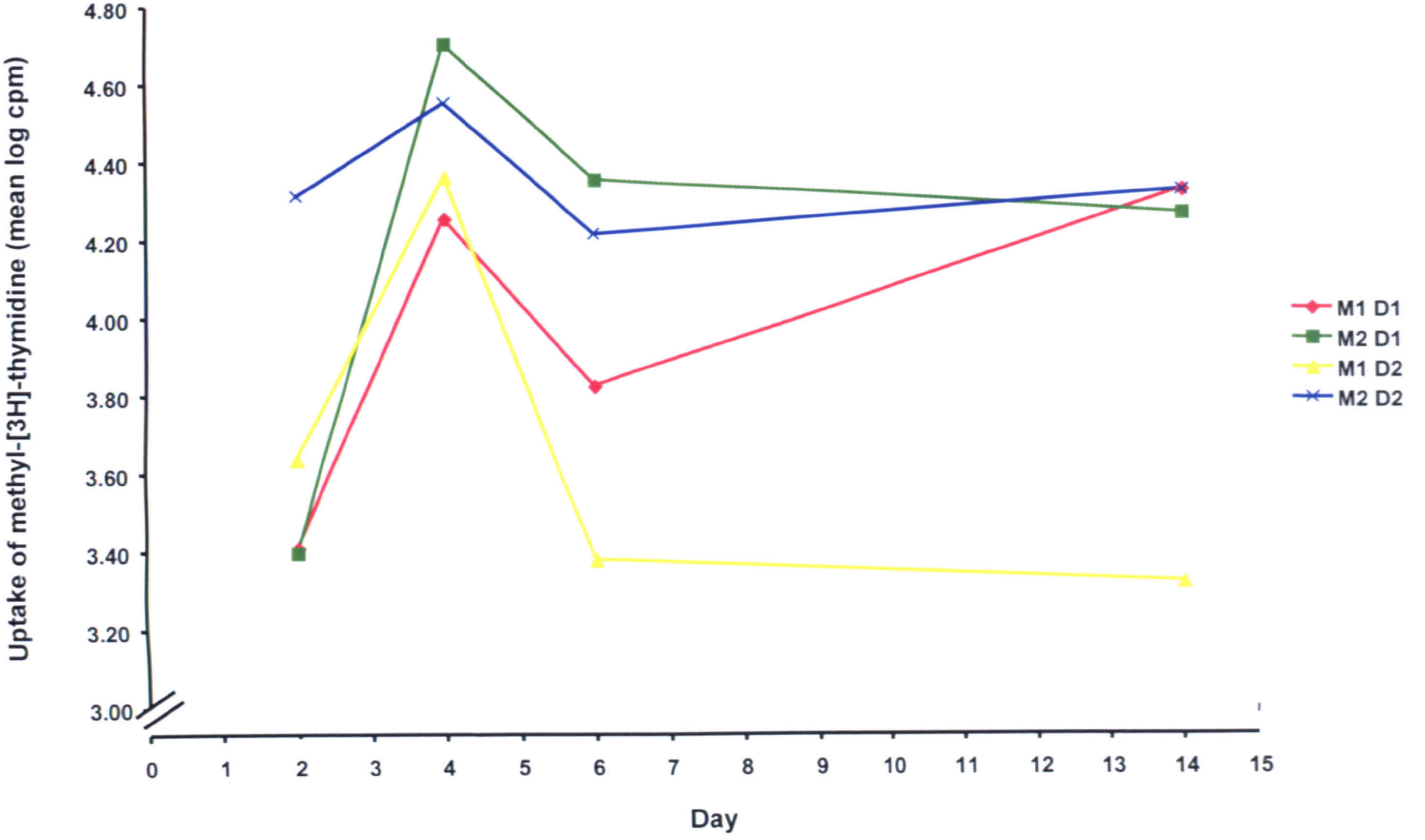


Figure 5A.5: The proliferation of PBLs from piglets fed diet 1 (D1; control) or 2 (D2; treatment) stimulated with Con A (M1) or PHA (M2) at a concentration of 3.3 µg/ml

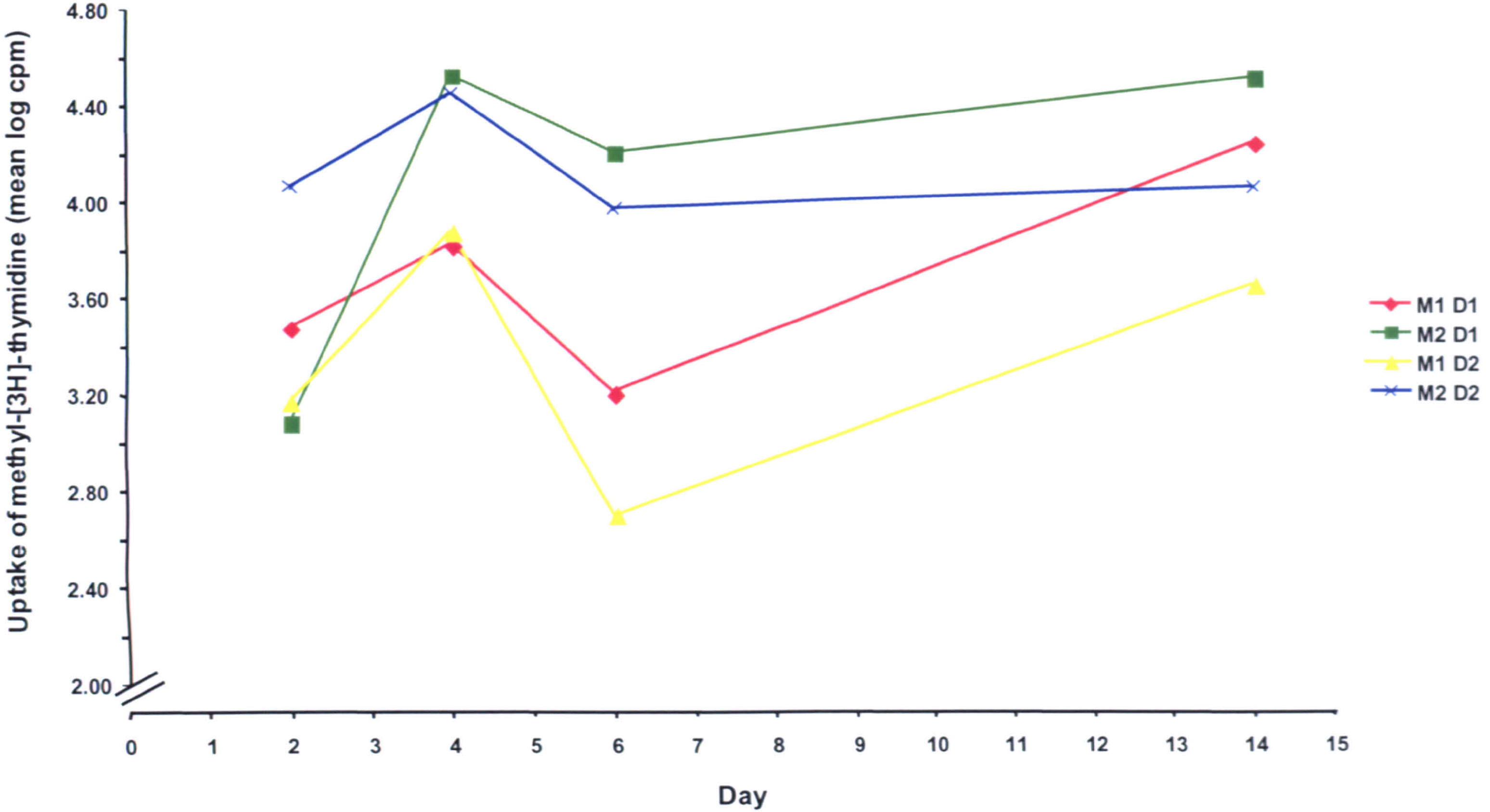


Fig 5A.6: The proliferation of PBLs from piglets fed diet 1 (D1; control) or 2 (D2; treatment) stimulated with Con A (M1) or PHA (M2) at a concentration of 1.1 μg/ml

Table 5A.30: A summary of the statistical analysis for the proliferation of PBLs

Diet		Day		Diet*Day		Mitogen		Concentration	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.06	0.097	0.08	0.001 0.079 (L) 0.091 (Q)	0.12	0.012 0.007 (L) 0.020 (Q)	0.06	<0.001	0.08	<0.001

Day refers to slaughter day; mitogen = either PHA or Con A; concentrations were 10.0, 3.3 and 1. μg / ml

5A.6 EXECUTIVE SUMMARY OF RESULTS

The current study found that villus atrophy, villus widening and crypt hyperplasia occurred in the immediate post-weaning period and responses by control and treatment animals were similar. Intestinal coliform counts were significantly lower in treatment animals and intestinal bifidobacteria and lactobacilli counts were similar for both dietary treatment groups. This resulted in the intestinal lactobacilli to coliform ratio increasing significantly throughout the experimental period. Conversely, faecal coliform counts were greatest in treatment animals (due to faecal shedding, which demonstrates elimination from the host GIT) and again, no significant differences in either faecal bifidobacteria or lactobacilli counts were evident with respect to dietary treatment. There was no significant difference between dietary treatments on DLWG or FCR. Lymphocyte proliferation responses were similar for both treatment groups, although enhanced responses to the mitogen PHA were observed in comparison to Con A.

The findings of the current study suggest that supplementing piglet diets post-weaning with a yeast-based nucleotide source may exert some effects on the host. Although villus atrophy did occur post-weaning, this was somewhat limited, with recovery of villli height commencing after 2 days post-weaning. A significant reduction in intestinal coliform numbers may have important implications for the post-weaned piglet, since these bacteria are thought to be non-beneficial and can be pathogenic. Possibly, the most important finding is the apparent stimulation of the immune system at a time when most piglets undergo a period of severe immuno-suppression (refer to chapter 6). Immunohistology determinations incorporating mucosal architecture (*i.e.* crypt cell production rate and mucosal mast cell enumeration) and intraepithelial lymphocyte enumeration can be used to quantify mucosal cell-mediated immune reactions (Mowat and Ferguson, 1982). Such reactions could possess important implications for the health and subsequent performance of the host animal.

CHAPTER 5B:

THE EFFECTS OF DIETARY YEAST-BASED NUCLEOTIDE SUPPLEMENT ON POST-WEANING PIGLETS PART 2: INFLUENCE OF CREEP FEEDING

5B.1 INTRODUCTION

It is well documented that *Escherichia coli* is among the earliest bacteria to colonise the gut in piglets (Drasar and Barrow, 1985) and thus frequently occur as commensals. However, these colonising *E. coli* strains must be able to compete for limiting nutrients and tolerate the prevailing conditions in order to grow and establish (Freter *et al.*, 1983). Pigs possess an extremely complex range of intestinal *E. coli* floras, and it has been suggested that some strains may be site specific (Thomlinson (1969), Hinton *et al.* (1985)). It is also well recognised that virtually all pigs carry pathogenic *E. coli* in their intestinal tracts and that, depending upon certain predisposing factors, these proliferate at weaning (Palmer and Hulland (1965), Svendsen *et al.* (1977) as cited by Miller *et al.* (1984)).

Frequently, *E. coli* present in the environment can be the principal cause of diarrhoeal infections in piglets (Katouli *et al.* (1995), Sainsbury (1995)). *E. coli* diarrhoea occurs when these pathogenic serotypes significantly increase in number thus altering the ratio between pathogenic and non-pathogenic mircoorganisms. The major problems arise from *E. coli* that promotes enteric colibacillois where the microorganisms are responsible for the release of toxins. Such toxins can target intestinal cells or the cells of other organs and subsequent digestive disorders are often observed. A common strain of this group is the enterotoxigenic *E. coli* (ETEC) generally detected in faecal samples of piglets with and perhaps more importantly without diarrhoea (Nabuurs, 1998). It is thought that the small intestine becomes more susceptible to enterotoxins immediately post-weaning which is related to the immature digestive physiology of the piglet combined with the removal of protective factors present in sow milk. The diarrhoea that develops during the first week post-weaning is more commonly known as porcine post-weaning diarrhoea (PWD) and has been described by Hampson (1994). Such post-weaning colibacillosis is the most common cause of post-weaning mortality, killing between 1.5% - 2.0% of piglets weaned (Hampson, 1994), and this

combined with reduced growth rates in less severe cases is a major cause of economic loss to pig producers, and represents a health and welfare issue.

The aetiology (Miniats and Roe (1968), McAllister *et al.* (1979), Mainil *et al.* (1989)), epidemiology (Kuhn *et al.* (1985), Nagy *et al.* (1990), Osek and Trusszczynski (1992)), and pathogenesis (Wilson and Francis (1986), Rose *et al.* (1987), Soderlind *et al.* (1988), Fairbrother *et al.* (1989)) of *E. coli* causing pre- and post-weaning diarrhoea have been the subject of many studies. For example, a study conducted by Katouli *et al.* (1995) utilised biochemical fingerprinting to monitor the persistence and colonisation of intestinal *E. coli* isolated from the faeces of sows and their litters. The authors isolated several biochemical phenotypes (BPTs) of *E. coli* and illustrated that the intestinal colonisation consisted of successive waves of different *E. coli* BPTs, the tenure of which varied from a few days to 2 weeks. Moreover, the majority of these BPTs disappeared in the succeeding samples and were not recovered again from the same piglets (*i.e.* they were transient strains). This is in agreement with data presented by Craven and Barnum (1971) who also reported a similar pattern of successive colonisation of *E. coli* serogroups in healthy piglets throughout the suckling and early post-weaning periods. However, in the study of Katouli *et al.* (1995), some *E. coli* strains, which colonised the piglets early in the suckling period, persisted for a long period and were referred to as resident BPTs. Each piglet carried more than one resident BPT (mean 2.4 BPTs per pig), some of which were also found in other piglets, indicating that both strain and host specificity are important for colonisation and persistence of *E. coli* in pigs (Katouli *et al.*, 1995). Furthermore, a huge similarity in *E. coli* floras between littermates throughout the suckling period was recorded, although each piglet developed a unique flora during post-weaning and fattening periods. Abrupt weaning in piglets has also been associated with a 100-fold drop in the numbers of lactobacilli in the intestine, and a 50-fold increase in the numbers of *E. coli* (Huis in't Veld and Havenaar, 1993).

At weaning, irrespective of age or incidence of PWD, a sudden increase in numbers of haemolytic *E. coli* throughout the small and large intestines is observed (Kenworthy and Crabb (1963), Hinton *et al.* (1985)). Furthermore, it is uncommon to detect these bacteria in the gastrointestinal tract of suckling pigs, or in mature animals (Hampson, 1994). However, Hampson *et al.* (1987) illustrated that such bacteria must be present since they can be carried into a new unused weaner house by healthy suckling pigs. Furthermore, Hampson *et al.*

(1987) also indicated that colonisation of weaner pigs can also occur following their exposure to the contaminated environment of weaner houses that have previously contained pigs.

It has been suggested that the physiological changes that occur post-weaning predispose piglets to develop diarrhoea (Hampson, 1994). For example, Schulman (1973) suggested that the gastric pH increases to non-bactericidal levels post-weaning, allowing ingested enterotoxigenic *E. coli* (ETEC) to survive and colonise the small intestine. Conversely, however, Hampson *et al.* (1985) reported that weaned pigs tended to have both more acidic gastric contents and fewer viable coliform bacteria in the stomach than did unweaned animals. Moreover, Hampson (1994) stated that the initiation of disease is most likely to result from an increase in numbers of bacteria that have already colonised the intestines of piglets before weaning. This is most likely to be a consequence of the introduction of solid post-weaning diets, and the stress associated with weaning which commonly results in perturbation of the gut ecosystem.

Changes in intestinal morphology such as villus atrophy, crypt hyperplasia, reduced absorptive and digestive capacities combined with decreased brush-border enzyme activity in the small intestine (McCracken *et al.*, 1999) occur post-weaning. The precise aetiology of these changes remains to be fully elucidated, although several proposals have been made. For example, they have been suggested to be the result of hypersensitivity to dietary antigens (Miller *et al.*, 1984c), to be the action of viruses (Leece *et al.*, 1983), to a lack of dietary intake ('luminal nutrition' concept) (Kelly *et al.*, 1984) or to be normal adaptive changes that are exaggerated by early weaning (Hampson (1983) as cited by Hampson (1994), Kelly *et al.* (1991a)). Whatever the exact causes these changes could predispose the piglets to post-weaning diarrhoea. A reduction in digestive and absorptive function of the small intestine would encourage the development of an osmotic diarrhoea, whilst unabsorbed dietary material might act as a substrate for ETEC in the gastrointestinal tract (Hampson, 1994).

Enterotoxigenic *E. coli* infections are common contributing factors to post-weaning scours in pigs. Predisposing factors in such infections include the removal of protective levels of IgA and other beneficial factors present in sow's milk, inadequate feed and water intake, inadequate gastric acid secretion, unstable microbiota and expression of membrane and mucin glyconjugates that serve as binding sites for enteropathogens (Kelly, *et al.* (1994),

Thacker (1999), Kelly and Coutts (2000b), Kelly and King (2001a), Kelly and King (2001c)). Post-weaning malnutrition predisposes to infection by compromising the barrier and immune functions of the gut. Concurrently, the infections adversely influence dietary intake, absorption and cause loss of endogenous nutrients (Calder and Jackson, 2000). Niewold *et al.* (2000) proposed that a fundamental predisposing factor to post-weaning diarrhoea is intestinal ischaemia. Intestinal blood supply may be unable to meet the metabolic demands of the rapidly growing intestine undergoing hyper-regenerative villus repair. It is suggested that the resulting ischaemia leads to intestinal acidosis and increased permeability for enterotoxigenic *E. coli* toxins (Niewold *et al.*, 2000).

Pre-weaned pigs have small, underdeveloped large intestines, which rapidly increase in size and content following weaning at 3 weeks of age (Hampson, 1987). The large intestine is a principal site of water and electrolyte absorption in the weaned pig (Hamilton and Roe, 1977), and this activity is facilitated by the absorption of volatile fatty acids (VFAs) (Argenzio, 1982). Newly weaned pigs are immature in terms of their digestive physiology and their intestinal microflora is incompletely developed. Fermentation in the large intestine is therefore limited, so that water and electrolyte movement associated with absorption of VFAs may also be diminished (Hampson, 1987). Conversely, Etheridge *et al.* (1984) proposed that the consumption of poorly digestible diets by weaner pigs results in increased bacterial fermentation, with an increased lactic acid and VFA production, (the products of bacterial fermentation, along with undigested and unabsorbed food residues as well as minerals, increase the osmolarity of the intestinal contents, resulting in a lack of water reabsorption and a continued influx of water into the intestinal lumen) which in turn causes an osmotic diarrhoea.

Evidence concerning the interaction between dietary intake, both pre- and post-weaning, and the occurrence of diarrhoea is equivocal. For example, Miller *et al.* (1984a) demonstrated that the consumption of small amounts of creep food before weaning may 'prime' intestinal hypersensitivity reactions to dietary antigens post-weaning, and exacerbate PWD. Miller *et al.* (1984a) demonstrated that piglets given a small amount of creep food as a 'priming' dose before weaning went on to show a significantly greater depression in their ability to absorb xylose 8 days post-weaning and suffered more diarrhoea than did other weaned pigs.

Conversely, most authors have not been able to substantiate this. For example, Hampson and Smith (1986), illustrated that all weaned pigs displayed a reduction in their ability to absorb xylose 1 week post-weaning, but that this was not influenced by the pattern of creep feeding before weaning. This protective effect of withholding creep food was associated with a low dietary intake after weaning with this regimen. Pigs which developed diarrhoea tended to be those that consumed more feed after weaning than their contemporaries. Haemolytic enterotoxigenic *E. coli* and rotaviruses were present in the faeces of most pigs post-weaning, but in those animals that ate too much (hyper-alimentation) and developed diarrhoea, excretion of the *E. coli* continued for approximately twice as long as in animals that remained healthy. This is also supported by investigations of Hampson *et al.* (1988), Kelly *et al.* (1990), Kelly (1990) and Sarmiento *et al.* (1990).

Kelly (1990) regulated the intake of creep feed through gastric intubation throughout the suckling period and piglets were orally infected with 10^6 enteropathogenic *E. coli*. Although piglet response varied considerably, consumption of creep feed did not significantly affect the prevalence, duration or severity of experimentally-induced diarrhoea. Intake of creep feed is probably the most important factor in maximising weaning live-weight that subsequently reduces the susceptibility of developing PWD. Restriction of dietary intake after weaning alone, or in combination with an increased dietary fibre content, reduced both the proliferation of haemolytic *E. coli* and the occurrence of PWD (Smith and Halls, 1968). There is evidence to suggest that feeding liquid weaner diets can reduce the number of coliform bacteria within the intestinal tract and prevent both post-weaning growth check (Leece *et al.*, 1979) and PWD. Kelley (1982) reported that when weaned piglets are exposed to cold or fluctuating temperatures, their susceptibility to enterotoxigenic *E. coli* (ETEC) infections is increased. This reduction in resistance is probably due to changes in immune function caused by stress. Rotavirus has also been associated with PWD often in conjunction with haemolytic *E. coli* (Leece and King (1978), Leece *et al.* (1983), Nabuurs *et al.* (1993)).

A marked increase in the number of haemolytic *E. coli* has been reported to occur from 2 days post-weaning (Kenworthy and Crabb, 1963). Furthermore, Kenworthy and Crabb (1963) also reported that haemolytic strains proliferate in the intestinal tract of healthy pigs post-weaning, although this population was smaller than in littermates that developed diarrhoea (Kenworthy and Crabb, 1963). Thomlinson (1969) stated that abrupt dietary

change, often at weaning, movement of animals such as mixing litters, unrest and irregular feeding, inadequate drinking water supply and other so-called stress factors such as damp and cold housing, are just some of the factors responsible for the multiplication and dominance of *E. coli* serotypes in pigs. Miller *et al.* (1984b) and Kelly (1990) reported an increased susceptibility of pigs to bacterial and toxin-mediated infections at weaning. The removal of immunologic factors present in sows milk may contribute to this susceptibility. However, the developmental process in the gut is accompanied by subtle changes in the microvillar membranes which may influence disease susceptibility. The maturity of the enterocyte influences its binding characteristics for lectins and this glycosylation shift might increase binding of bacteria to the mucosal surface (Etzler and Branstrator, 1979). King and Kelly (1990) in addition to Pang *et al.* (1987), have provided conclusive evidence indicating that modification of carbohydrate moieties of the microvillar membrane occurs during development in the rat and pig. This may be reflected in the heightened susceptibility to infection (precocious shedding of *E. coli* in the faeces) observed with some of the animals weaned at 3 weeks old in the study conducted by Kelly (1990), perhaps involving changes in the expression and maturation of bacterial receptor sites on the mucosal surfaces. The susceptibility of rabbit intestine to colonisation by *E. coli* is temporally related to the appearance of receptors (Boedeker and Cheney, 1984). It is, therefore, suggested that the process of weaning encourages the expression of receptors including those that are recognised by enteric pathogens. This possibility, considered in conjunction with physiological immaturity of the immune system of the young pig at 2 to 3 weeks old, may explain the high incidence of enteric infection associated with the practice of early weaning (Kelly, 1990).

A series of changes in small intestinal structure and enterocyte brush border enzyme activities after weaning has, as a net effect, a temporary reduction in intestinal digestive and absorptive function. This would facilitate the development of an osmotic diarrhoea, and unabsorbed dietary material could act as a potential substrate for enterotoxigenic *E. coli* (ETEC) within the gastrointestinal tract which could result in PWD. It has been proposed that newly weaned pigs are susceptible to develop diarrhoea because they are immature in terms of their digestive physiology (Corring *et al.*, 1978), and their intestinal microlfora is incompletely developed. As such they are ill equipped to cope with (digest) solid diets.

Fermentation is therefore limited, so that water and electrolyte movement associated with absorption of VFAs may also be diminished (Hampson, 1987).

In a study conducted by Kelly (1990), the experimental strain of *E. coli* was consistently isolated from the faeces during bouts of diarrhoea, the total counts being in the range of 10^8 to 10^{10} organisms g^{-1} of fresh faeces. Smith and Linggood (1971) have reported similar numbers. Shedding did not correlate with the severity of diarrhoea. This finding supports the observations of Sarmiento *et al.* (1988) who concluded that faecal shedding was not a reliable indicator of colonisation of the small intestine because of the uncertainty regarding the proliferative or pathogenic activity of these bacteria during transit in the small intestine and in the hind-gut.

In the present study, an *E. coli* infection occurred, the source of which was later identified as the drinking water supply within the experimental unit. Once the infection became apparent, the header tank was immediately sanitised with Virkon™ (non-toxic disinfectant) that was drawn through the system via the nipple drinkers. Although this infection was not expected, the experimental protocol was immediately modified to incorporate *E. coli* identification and quantification. Furthermore, veterinary advice was sought, the animals examined instantly and antibiotic intervention was not deemed necessary by the veterinary surgeon. It should also be emphasised that no animal suffered any pathological effects in any way as a result of the infection.

5B. 2 AIMS

To determine the effects of creep feeding and dietary immuno-potentiator supplementation on gut physiology, microflora characteristics and immunological parameters in post-weaned piglets. It is postulated that dietary nucleotide supplementation pre- and post-weaning will reduce the extent and duration of small intestinal damage associated with the weaning process. Such effects may also enhance growth performance post-weaning.

5B.3 METHODS

5B.3.1 Animals and housing

Thirty-six entire male piglets (Section 4.3.1) were used to evaluate the experimental diets. Animals were offered the diets from 14 days pre-weaning whilst still suckling the sow (*i.e.*

on a litter or group basis) in farrowing accommodation (up to day 0 (weaning)). At weaning, the animals were transferred to the experimental unit, individually housed as described previously (Section 4.3.1), and 4 piglets were slaughtered on day 0 (weaning day) to provide baseline data. The animals received the same diets pre- and post-weaning.

5B3.2 Diets

The same 2 diets were formulated as described for the previous study (section 5A3.2), and Ascogen was included at the same inclusion rate of 4g/kg. Both experimental diets were fed from 14 days pre-weaning (day –14; on the site of birth) through to 14 days post-weaning (day 14). For complete diet specification refer to Table 5A.1.

5B3.3 Post-mortem procedure

The post-mortem procedure has been described previously in sections 2.4, and 4.3.3. The only modification to this procedure was the investigation for signs of gastric ulceration. At slaughter, the stomach was removed, cut open and any gastric contents washed off with cold water. The stomach wall was then inspected for any signs of gastric ulceration and photographs taken for subsequent investigation.

5B3.4 Microbiological analyses

The microbiological analysis has been described previously in sections 2.8 and 5A3.3. Due to the *E. coli* outbreak during this study, faecal swabs were undertaken for *E. coli* determination from day 7 to 14 and from days 9 to 14 for lactobacilli and bifidobacteria species. In addition, digesta samples also underwent *E. coli* enumeration and identification throughout the experimental period (day 0 to 14).

5B3.4.1 *E. coli* serotype isolation

E. coli isolated from intestinal samples underwent serotype isolation and determination. Serial dilutions were prepared in maximum recovery diluent (MRD, Oxoid). *E. coli* and coliforms were enumerated using *E. coli* Petri film (3M, MA, USA) that was incubated at 37°C for 24 h.

5B3.4.1.1 Maintenance of organisms

E. coli, isolates were sub-cultured onto Luria Bertani agar (Difco, USA) plates and incubated at 37°C for 24 h. Colonies (2-3) were then transferred to micro-bank bead tubes (Pro-Lab, Surrey, UK) according to the manufacture's instructions. The tubes were stored at –20°C until required for DNA fingerprint studies.

Due to limited resources, faecal swabs were only performed in the study to monitor the *E. coli* load of the piglets once an *E. coli* infection had been established. As such, not all piglets employed in this study underwent faecal swabbing procedures.

5B3.4.1.2 ERIC-DNA fingerprinting

E. coli colonies from each sample were grown overnight on Luria Bertani agar (Difco) at 37°C. Genomic DNA was recovered from the cells by using a modified method to that described by Polyzou *et al.* (2000). A colony was removed from the agar plate and was suspended in 0.2 ml TE buffer and placed in a boiling water bath for 10 min. Cell debris was removed by centrifugation (13, 000 x *g* for 10 min) and the supernatant containing the DNA decanted into a sterile eppendorf tube.

The various DNA preparations were typed using ERIC-PCR (Versalovic *et al.* 1991). The primers used were;

ERIC1 (forward) 5'-ATGTAAGCTCCTGGGGATTCAC-3'
ERIC2 (reverse) 5'-AAGTAAGTGACTGGGGTGAGCG-3'

The PCR reactions were carried out in a total reaction volume of 25µl. A 1 µl DNA sample was added to 24 µl master mix containing: 100 pM of each primer, 1 U of *Taq* DNA polymerase (Advanced Biotechnologies, Surrey, UK), 0.2 mM each of the deoxribonucleotide triphosphates; dATP, dCTP, dGTP and dTTP (Promega), 4 mM $MgCl_2$ (Advanced Biotechnologies), and Buffer II (X10) (Bochringer Mannheim). The reactions were carried out in a Techne thermocycler (Progene), at the following temperatures: one cycle for 3 min at 94°C, then 35 cycles comprising: 30 sec at 94°C, 1 min at 52°C, 4 min at 65°C. The final cycle was for 8 min at 65°C.

The PCR product was mixed with 5µl loading buffer and electrophoresed in a 2% agarose gel containing ethidium bromide (0.5 µg/ml) in Tris-acetate EDTA running buffer at 70 V for 2h.

5B3.4.1.3 DNA fingerprint analysis

Comparison of the DNA fingerprints was undertaken using Amersham Pharmacia, ImageMaster® 1D-Elite gel analysis and database software (Amersham Pharmacia Biotech, Buckinghamshire, UK). Dendrograms were constructed using the Dice similarity coefficient (S_D) and the UPGMA (unweighted pair group method using arithmetic averages) clustering algorithm (Sokal and Sneath, 1963).

5B3.5 Lymphocyte proliferation

The immunological analyses have been described previously in section 2.9. In the current study all proliferation experiments were performed with fresh lymphocytes.

5B3.6 Statistical analyses

Statistical analyses were undertaken as described previously in section 2.12. The between-treatment variation was partitioned into Baseline versus the Rest (BVR) as described for previous studies; no modifications were made. Due to the nature of the study, *i.e.* the experimental dietary regimens were introduced as creep feeds from 14 days pre-weaning, the dietary effects were analysed by *including* the baseline values and comparing treatments of all 36 animals. In this instance the baseline animals were classified as receiving either diet 1 (control) or diet 2 (treatment; nucleotide supplementation) in accordance with the remainder of the experimental animals. Animals were blocked according to litter in all analyses.

5B.4 RESULTS

No evidence of gastric ulceration was observed in any of the animals at slaughter.

5B.4.1 Gut morphology

Generally, villus height (Table 5B.2), villus width (Table 5B.4) and crypt depth (Table 5B.6) increased with increasing distance along the small intestine from proportionately 0.25 to 0.50 from the gastric pylorus to the ileo-caecal valve. Measurements taken at the region of 0.75

were generally less than those at 0.25 along the small intestine. This trend was apparent across all 5-time points (d 0, 2, 4, 6 & 14) for both dietary treatments.

Analysis of variance established that intestinal villi changed height significantly throughout the experimental period for both dietary treatments (P=0.032; P=0.004 (L); Table 5B.3). No significant difference with respect to diet and BVR, or relationships between diet and day, or BVR and diet were apparent. There was however, a significant difference in relation to intestinal region (P=<0.001), with villi at proportionately 0.50 along the intestine being tallest. There was also a significant correlation between BVR and day, with average VH being shorter on day 2, than day 0 and then increasing up to the tallest heights recorded on day 14.

The percentage difference in villus height between the baseline and the remainder of the experimental animals demonstrated that, although villus atrophy occurred to a greater extent in control animals, it occurred for a greater period of time in treatment animals (Table 5B.1). The animals (both control and treatment), which were slaughtered on the final day of the study (day 14), displayed the tallest villi in the distal region of the intestine.

VW remained within relatively narrow limits throughout the experimental period (Table 5B.5). There was however, a significant difference between regions of the intestine (P=0.039), with VW generally being greatest at proportionately 0.50 (medially) along the length of the small intestine. No significant differences were determined between diet, day, or BVR. Significant interactions between diet and day, BVR and day, and BVR and diet were not evident.

Analysis of CD revealed a significant increase in depth throughout the experimental period (P=<0.001; <0.001(L); 0.054(Q); Table 5B.7). A significant effect of region was demonstrated (P=0.017), with 0.75 being the most shallow when averaged over all 4-time points (days 2, 4, 6 and 14). No significant difference with respect to diet, BVR or BVR and diet interaction was apparent. Significant relationships between diet and day (P=0.033(Q)) and BVR and day (P=<0.001) were also demonstrated.

VH:CD ratio differed significantly across region (P=0.007), and there was also a significant relationship between diet and day (P=0.025 (Q); Table 5B.9). No significant differences were determined in relation to diet, day or BVR. Additionally, no significant relationships between diet and day, BVR and day or BVR and diet were observed.

Table 5B.2: Effect of dietary treatment on villus height (μm; $n = 16$ per diet; baseline (animals slaughtered on day 0); $n = 4$)

	Diet 1				Diet 2			
	Proportion of Intestine				Proportion of Intestine			
Day	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean
0	500	511	459	480	505	506	454	474
2	440	455	465	453	471	475	420	455
4	450	479	393	441	546	498	417	487
6	550	544	493	529	481	492	425	466
14	533	542	561	545	543	562	584	563
mean	493	505	478	492	510	507	461	493

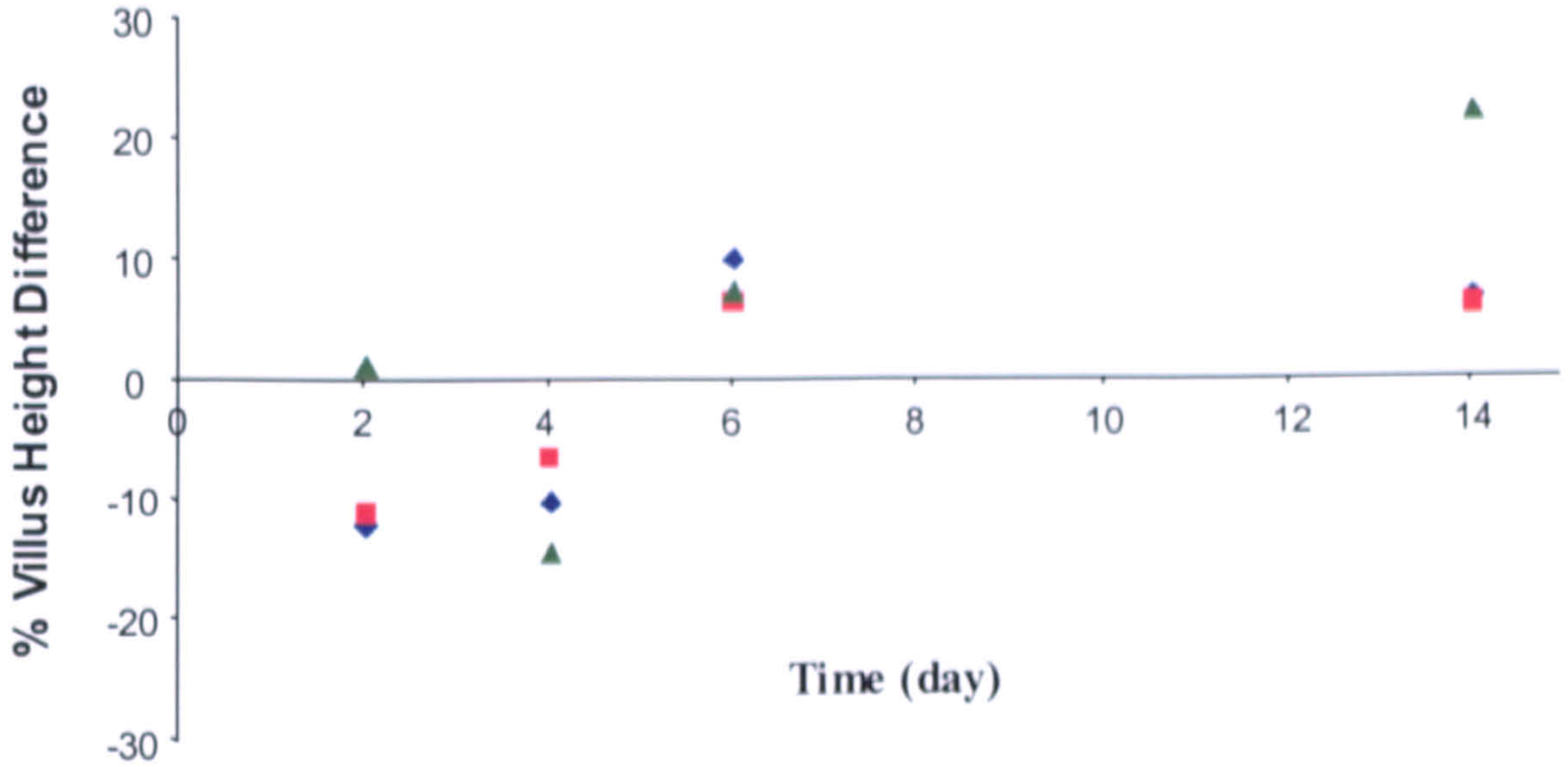
Diet 1 = control; diet 2 = treatment

Table 5B.3: A summary of the statistical analysis for villus height

Diet		Day		Diet*Day		Region		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
26.5	0.993	37.5	0.032 0.004 (L) 0.823 (Q)	53.1	0.506 0.874 (L) 0.386 (Q)	4.7	<0.001	37.0	0.661	42.7	0.033	39.0	0.973

BVR = Baseline versus the rest

a)



b)

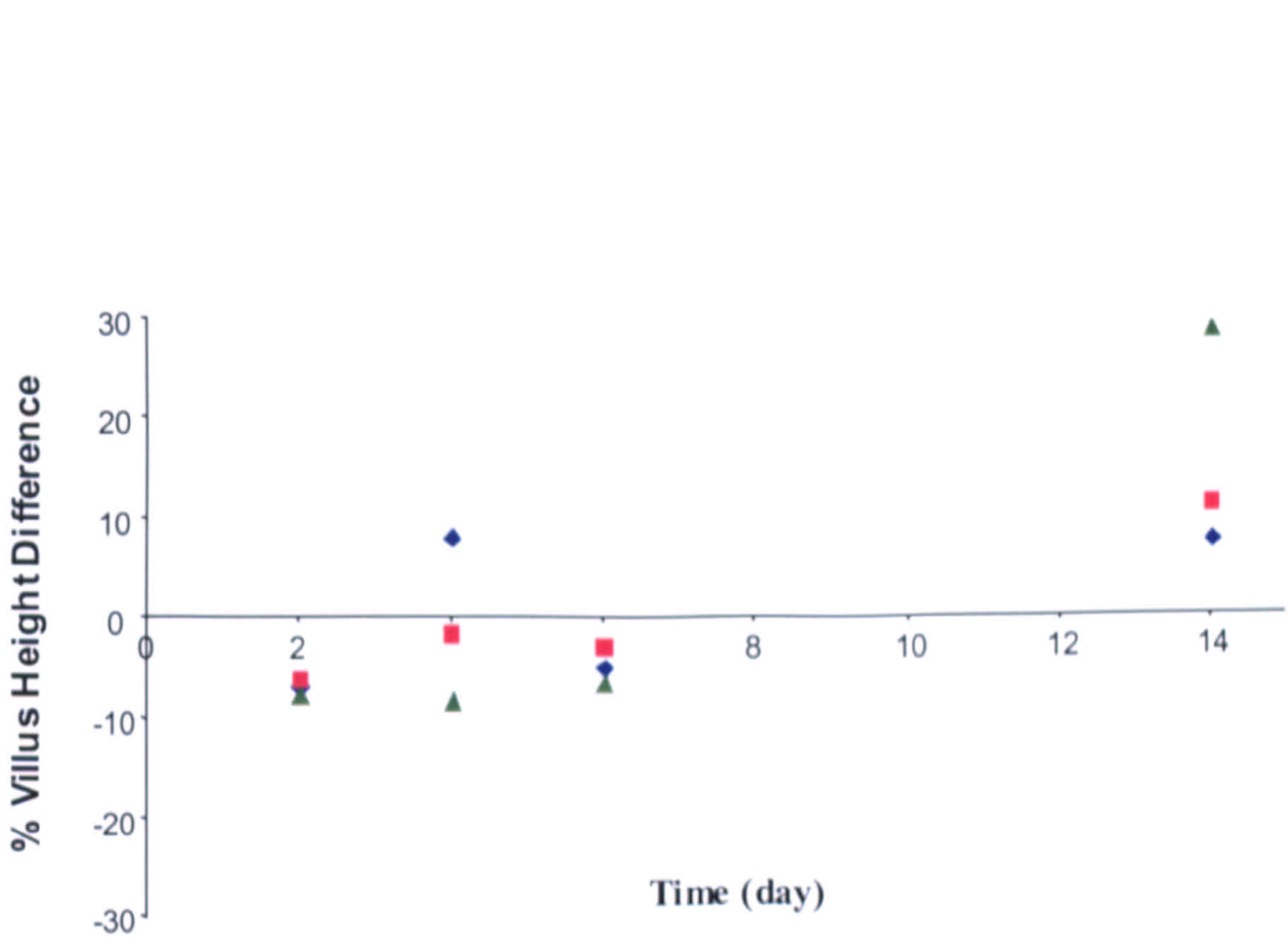


Figure 5B.1: Percentage difference in villus height between baseline values and those obtained from animals slaughtered on days 2, 4, 6 or 14 fed a) diet 1 (control) or b) diet 2 (treatment) at regions proportionately 0.25 (◆), 0.50 (■) and 0.75 (▲) along the length of the intestine from the gastric pylorus to the ileo-caecal valve.

Table 5B.4: Effect of dietary treatment on villus width (μm; $n = 16$ per diet; baseline (animals slaughtered on day 0); $n = 4$)

	Diet 1 Proportion of Intestine				Diet 2 Proportion of Intestine			
Day	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean
0	95	98	91	89	95	95	89	90
2	87	113	92	97	89	94	86	90
4	91	91	89	91	89	91	92	91
6	96	100	91	96	97	91	91	93
14	100	95	107	101	99	108	101	102
mean	93	100	95	*96*	93	96	93	*94*

Diet 1 = control; diet 2 = treatment

Table 5B.5: A summary of the statistical analysis for villus width

Diet		Day		Diet*Day		Region		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
3.9	0.578	5.5	0.226 0.060 (L) 0.627 (Q)	0.8	0.786 0.480 (L) 0.651 (Q)	2.4	0.039	5.5	0.359	6.3	0.202	5.8	0.560

BVR = Baseline versus the rest

Table 5B.6: Effect of dietary treatment on crypt depth (μm; n =16 per diet; baseline (animals slaughtered on day 0); n = 4)

	Diet 1 Proportion of Intestine				Diet 2 Proportion of Intestine			
Day	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean
0	217	207	203	187	218	221	211	219
2	199	203	206	203	184	195	177	185
4	198	190	199	196	218	210	219	215
6	223	230	206	219	222	235	225	227
14	271	238	226	245	240	241	229	236
mean	223	215	209	*216*	216	220	212	*216*

Diet 1 = control; diet 2 = treatment

Table 5B.7: A summary of the statistical analysis for crypt depth

Diet		Day		Diet*Day		Region		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
5.3	0.828	7.5	<0.001 <0.001 (L) 0.054 (Q)	10.6	0.062 0.622 (L) 0.033 (Q)	3.7	0.017	8.1	0.165	9.4	<0.001	8.6	0.947

BVR = Baseline versus the rest

Table 5B.8: Effect of dietary treatment on villus height to crypt depth ratio ($n = 16$ per diet; baseline (animals slaughtered on day 0); $n = 4$)

	Diet 1 Proportion of Intestine				Diet 2 Proportion of Intestine			
Day	0.25	0.50	0.75	mean	0.25	0.50	0.75	mean
0	2.5	2.6	2.4	2.7	2.5	2.4	2.3	2.3
2	2.3	2.2	2.3	2.3	2.6	2.5	2.4	2.5
4	2.3	2.5	2.0	2.3	2.5	2.3	1.9	2.3
6	2.5	2.4	2.5	2.5	2.2	2.1	1.9	2.1
14	2.0	2.3	2.5	2.3	2.3	2.3	2.6	2.4
mean	2.2	2.4	2.3	2.3	2.4	2.3	2.2	2.3

Diet 1 = control; diet 2 = treatment

Table 5B.9: A summary of the statistical analysis for villus height to crypt depth ratio

Diet		Day		Diet*Day		Region		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.13	0.919	0.18	0.686 0.940 (L) 0.421 (Q)	0.26	0.15 0.919 (L) 0.025 (Q)	0.06	0.007	0.17	0.793	0.2	0.877	0.18	0.973

BVR = Baseline versus the rest

5B.4.2 Microbiological analyses

Intestinal coliform counts (log cfu / g) declined significantly (P=0.001; P=<0.001(L)) throughout the experimental period (Tables 5B.10 and 5B.11). They also increased significantly (P=<0.001) along the length of the intestine (*i.e.* from the duodenum to the rectum). A significantly higher count was determined from the baseline (mean = 10.6 cfu / g), than the remainder of the animals (P=0.005). No significant differences between diet, or correlations between diet and day, or BVR and diet were evident. The relationship between BVR and day was however, significant (P=<0.001).

No significant temporal or dietary differences were determined with respect to intestinal lactobacilli counts (Tables 5B.12 and 5B.13). Similarly, analysis revealed no significant relationships between diet and day, BVR and day or BVR and diet. A significant difference between baseline and the remainder of the animals (P=0.031) was apparent, with baseline values being higher. Lactobacilli counts also increased significantly along the length of the small intestine (P=<0.001).

Bifidobacteria counts fluctuated over time (P=0.012), but were highest on days 2 and 4 for both treatment groups (Tables 5B.14 and 5B.15). A significant increase (P=0.020) in counts along the length of the intestine was also evident. Conversely, no significant differences in relation to dietary treatment, BVR, or correlations between diet and day, or BVR and day were demonstrated. There was however a significant relationship between BVR and day (P=0.012), with the mean baseline value being less than the overall means for both treatment groups over time.

Total mean *E. coli* counts (log cfu / g) declined significantly throughout the experimental period (P=0.001; <0.001(L)), and in comparison to the baseline animals (P=0.005; Tables 5B.16 and 5B.17). No significant difference between dietary treatments or relationship between diet and day, or BVR and diet was demonstrated. *E. coli* counts increased significantly along the length of the intestine (P=<0.001), and there was a significant correlation between BVR and day (P=<0.001). The *E. coli* isolated from the water system was determined at the concentration of 10^6 organisms / ml. The *E. coli* strain isolated from the water was also isolated from the intestines of the pigs. A total of 5 *E. coli* isolates were detected in the intestines of the experimental animals, with a similarity co-efficient of 0.95.

An example of a dendrogram illustrating some of the strains of *E. coli* isolated from the pigs is presented in Figure 5B.3. Watery contents of the intestine were also evident at slaughter.

Intestinal lactobacilli to coliform ratios increased significantly with respect to time (P=0.005; 0.001(L)) and following an inverse quadratic pattern/hyperbola across sample site (P=<0.001) *i.e.* decreased then increased (Tables 5B.18 and 5B.19). No significant differences between dietary treatment, BVR or relationships between diet and day, and BVR and diet were demonstrated.

Table 5B.10: Mean log coliform counts (cfu / g) obtained from digesta samples (S1-S5*) along the intestine (n = 16 per diet; baseline (animals slaughtered on day 0); n = 4)

	Diet 1						Diet 2					
	Sample site						Sample site					
Day	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean
0	8.7	9.2	12.2	11.3	11.9	10.7	7.9	8.0	13.4	11.6	12.1	10.6
2	5.6	6.8	11.0	11.1	11.3	9.1	4.1	7.5	11.1	11.5	11.1	9.1
4	5.9	6.9	8.5	10.7	10.5	8.5	6.8	7.7	11.9	11.1	11.3	9.7
6	4.8	3.9	9.7	8.8	7.4	6.9	5.9	6.1	3.6	6.7	4.4	5.3
14	4.6	4.2	6.6	5.0	3.1	4.7	5.7	4.3	11.1	6.4	3.3	6.2
mean	5.2	5.4	8.9	8.9	8.1	7.3	5.6	6.4	9.4	8.9	7.5	7.6

*S1 – S5 = duodenum, jejunum, ileum, colon and rectum respectively; diet 1 = control; diet 2 = treatment

Table 5B.11: A summary of the statistical analysis for intestinal coliforms

Diet		Day		Diet*Day		Sample Site		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.72	0.698	1.01	0.001 <0.001 (L) 0.127 (Q)	1.43	0.436 0.497 (L) 0.407 (Q)	0.76	<0.001	1.02	0.005	1.18	<0.001	1.08	0.683

BVR = Baseline versus the rest

Table 5B.12: Mean log lactobacilli counts (cfu / g) obtained from digesta samples (S1-S5*) along the intestine (n = 16 per diet; baseline (animals slaughtered on day 0); $n = 4$)

	Diet 1						Diet 2					
	Sample site						Sample site					
Day	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean
0	7.4	6.3	8.8	8.4	8.3	7.8	6.3	6.3	9.3	8.8	8.1	7.8
2	6.2	5.3	5.5	7.7	7.9	6.5	5.8	5.5	5.7	7.1	7.2	6.3
4	5.4	6.0	5.8	6.5	5.7	5.9	5.6	5.9	5.8	6.4	6.7	6.1
6	4.9	4.6	4.7	6.8	7.1	5.6	6.4	5.3	8.1	8.8	8.9	7.5
14	5.6	5.0	6.9	8.8	7.6	6.8	5.9	5.9	6.6	7.8	8.6	7.0
mean	5.5	5.2	5.7	7.5	7.1	6.2	5.9	5.7	6.5	7.5	7.8	6.7

*S1 – S5 = duodenum, jejunum, ileum, colon and rectum respectively; diet 1 = control; diet 2 = treatment

Table 5B.13: A summary of the statistical analysis for intestinal lactobacilli

Diet		Day		Diet*Day		Sample Site		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.41	0.238	0.58	0.477 0.219 (L) 0.828 (Q)	0.82	0.294 0.895 (L) 0.085 (Q)	0.24	<0.001	0.58	0.031	0.67	0.430	0.61	0.213

BVR = Baseline versus the rest

Table 5B.14: Mean log bifidobacteria counts (cfu / g) obtained from digesta samples (S1-S5*) along the intestine (n = 16 per diet; baseline (animals slaughtered on day 0); n = 4)

	Diet 1 Sample site						Diet 2 Sample site					
Day	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean
0	8.7	9.2	12.2	11.3	11.9	10.7	7.9	8.0	13.4	11.6	12.1	10.6
2	12.0	11.1	11.7	11.7	12.3	11.8	11.9	12.4	11.8	12.1	12.7	12.2
4	12.3	12.4	12.5	12.4	12.2	12.4	12.6	13.2	12.7	13.0	12.9	12.9
6	11.2	10.8	11.5	11.6	12.3	11.5	12.3	10.4	11.9	11.9	12.2	11.7
14	12.4	11.4	13.1	11.9	11.8	12.1	12.4	12.0	12.1	12.4	12.5	12.3
mean	12.0	11.4	12.2	11.9	12.1	*11.9*	12.3	12.0	12.1	12.4	12.6	*12.3*

*S1 – S5 = duodenum, jejunum, ileum, colon and rectum respectively; diet 1 = control; diet 2 = treatment

Table 5B.15: A summary of the statistical analysis for intestinal bifidobacteria

Diet		Day		Diet*Day		Sample Site		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.20	0.102	0.28	0.012 0.896 (L) 0.334 (Q)	0.39	0.920 0.561 (L) 0.930 (Q)	0.19	0.020	0.30	0.367	0.34	0.012	0.31	0.102

BVR = baseline versus the rest

Table 5B.16: Mean log *E. coli* counts (cfu / g) obtained from digesta samples (S1-S5*) along the intestine (n = 16 per diet; baseline (animals slaughtered on day 0); n = 4)

	Diet 1						Diet 2					
	Sample site						Sample site					
Day	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean
0	5.2	5.2	7.9	9.1	7.9	7.1	5.5	6.0	9.5	10.0	9.1	8.0
2	5.6	6.8	11.0	11.1	11.3	9.1	4.1	7.5	11.1	11.5	11.1	9.1
4	5.9	6.9	8.5	10.7	10.5	8.5	6.8	7.7	11.9	11.1	11.3	9.7
6	4.8	3.9	9.7	8.8	7.4	6.9	5.9	6.1	3.6	6.7	4.4	5.3
14	4.6	4.2	6.6	5.0	3.1	4.7	5.7	4.3	11.1	6.4	3.3	6.2
mean	5.2	5.4	8.9	8.9	8.1	7.3	5.6	6.4	9.4	8.9	7.5	7.6

*S1 – S5 = duodenum, jejunum, ileum, colon and rectum respectively; diet 1 = control; diet 2 = treatment

Table 5B.17: A summary of the statistical analysis for intestinal *E. coli*

Diet		Day		Diet*Day		Sample Site		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.72	0.698	1.01	0.001 <0.001 (L) 0.127 (Q)	1.43	0.436 0.497 (L) 0.407 (Q)	0.76	<0.001	1.02	0.005	1.18	<0.001	1.08	0.683

BVR = Baseline versus the rest

Representative *E. coli* isolates derived from the intestinal samples were subject to DNA fingerprinting and subsequently sub-dived into genotypes. The fingerprint patterns generated by ERIC-PCR typically resulted in 4 to 7 bands ranging between 150 to 1000bp (Figure 5B.2). The reproducibility of the DNA fingerprints was confirmed by inclusion of an *E. coli* type strain (JM 109) in every ERIC-PCR run. On this basis a similarity level of >80% was set to designate *E. coli* strains belonging to the same genotype. For example, Figure 5B.3 illustrates a representative dendrogram obtained from comparing the isolates recovered from intestinal samples. The genotype recovered from the water system, shared a high level of similarity to those present in piglet intestinal and faecal samples (Figure 5B.3).

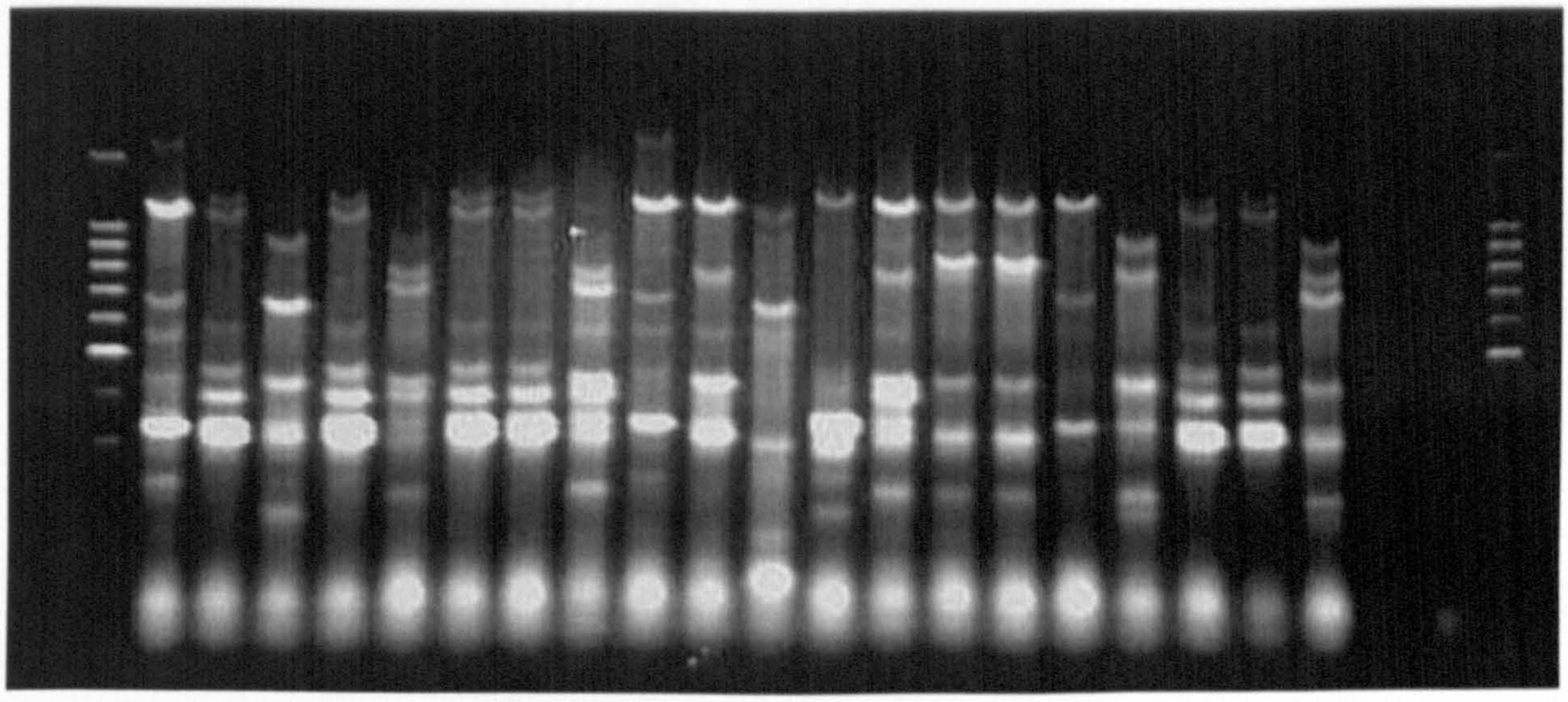


Figure 5B.2: Representative ERIC-PCR DNA fingerprints derived from *E. coli* isolates. Outer lanes are 100bp ladder. Lanes 2-8 and 19-20 *E. coli* isolates derived from duodenal and water samples. Lanes 9-10 derived from jejunal and lanes 11-18 from ileal samples. Lane 21 is an isolate recovered from the caecum of slaughter animals. The gels were 2% agarose – 1xTris-acetate-EDTA and contained 0.5μg of ethidium bromide per ml to stain the DNA.

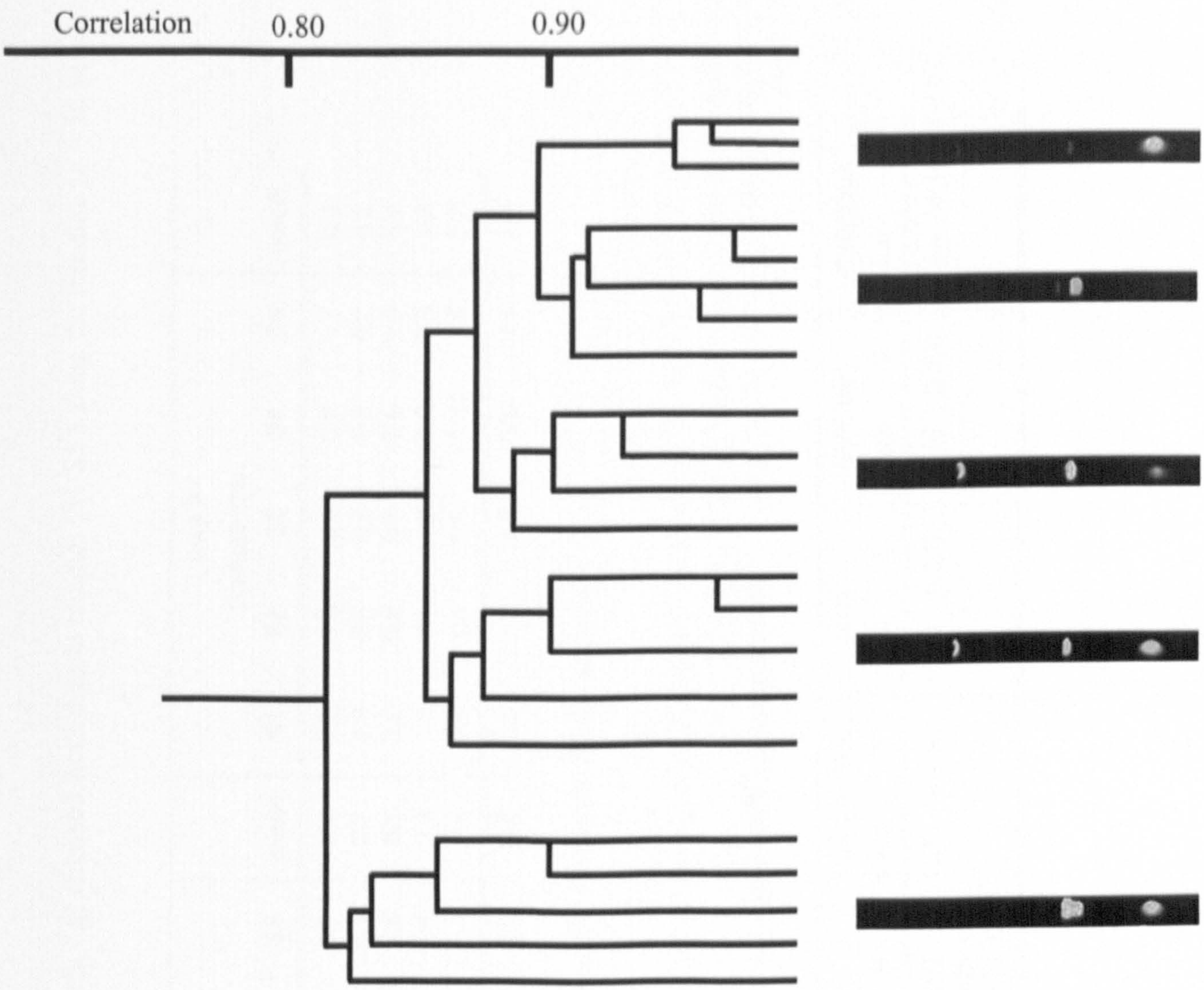


Figure 5B.3: Dendrogram representing the similarity co-efficient between different *E. coli* isolates.

Table 5B.18: Mean log lactobacilli to coliform counts (cfu / g) obtained from digesta samples (S1-S5*) along the intestine (n = 16 per diet; baseline (animals slaughtered on day 0); n = 4)

	Diet 1						Diet 2					
	Sample site						Sample site					
Day	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean
0	0.8	0.7	0.7	0.7	0.7	0.7	0.9	0.9	0.6	0.8	0.7	0.8
2	1.0	0.7	0.5	0.7	0.7	0.7	1.3	0.8	0.5	0.6	0.7	0.8
4	0.8	0.8	0.5	0.6	0.6	0.7	0.9	0.8	0.5	0.6	0.6	0.7
6	1.0	0.8	0.4	0.8	1.1	0.8	1.2	1.1	1.7	1.0	0.9	1.2
14	1.3	1.1	1.2	0.8	1.5	1.2	1.4	1.4	0.6	1.1	1.5	1.2
mean	1.0	0.9	0.7	0.7	1.0	*0.8*	1.2	1.0	0.8	0.8	0.9	*1.0*

*S1 – S5 = duodenum, jejunum, ileum, colon and rectum respectively; diet 1 = control; diet 2 = treatment

Table 5B.19: A summary of the statistical analysis for intestinal lactobacilli to coliform ratio

Diet		Day		Diet*Day		Sample Site		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.10	0.277	0.14	0.005 0.001 (L) 0.681 (Q)	0.20	0.569 0.965 (L) 0.249 (Q)	0.07	<0.001	0.06	0.323	0.10	0.003	0.14	0.193

BVR = Baseline versus the rest

A trend was observed towards a reduction in faecal coliform and counts over time (P=0.056; Table 5B.20). Faecal *E. coli* counts decreased significantly over time (P=0.013; 0.010(Q); Table 5B.23). No significant differences between dietary treatments was evident, however there was a significant linear correlation between diet and day (P=0.027(L)). No significant differences between dietary treatment or relationships between diet and day were found for faecal coliform, lactobacilli and bifidobacteria counts. Conversely, faecal lactobacilli and bifidobacteria counts increased significantly lactobacilli; P=<0.001; <0.001(L); <0.001(Q); bifidobacteria; P=<0.001; <0.001(L); <0.001(Q)) throughout the experimental period (Tables 5B.21 and 5B.22)).

Analysis of intestinal pH revealed no significant differences with respect to time or BVR (Tables 5B.24 and 5B.25). A significant difference (P=0.009) between dietary treatments was evident, with animals fed diet 1 (control) displaying lower digesta pH values. The pH also differed significantly across sample site (P=<0.001), for both dietary treatments with S1 (duodenum) being the most acidic and S3 (ileum) the least. A significant correlation between BVR and diet (P=0.008), but none with respect to diet and day, or BVR and day was determined. The mean dietary pH was 6.02 ± 0.13.

Table 5B.20: Mean log coliform counts (cfu / swab) obtained from faecal samples (n = 16 per diet) and associated statistical analysis

	Diet			Diet		Day		Diet*Day	
Day	1	2	mean	s.e.d	P	s.e.d	P	s.e.d	P
7	3.9	2.4	3.2	0.79	0.797	0.91	0.056	1.36	0.365
9	3.4	3.3	3.4				0.592 (L)		0.085 (L)
11	4.5	5.2	4.8				0.035 (Q)		0.913 (Q)
13	1.3	3.0	2.1						
mean	3.3	3.5	*3.4*						

Diet 1 = control; diet 2 = treatment

Table 5B.21: Mean log lactobacilli counts (cfu / swab) obtained from faecal samples (n = 16 per diet) and associated statistical analysis

	Diet			Diet		Day		Diet*Day	
Day	1	2	mean	s.e.d	P	s.e.d	P	s.e.d	P
9	5.6	5.5	5.6	0.26	0.606	0.24	<0.001	0.39	0.604
11	5.7	5.2	5.5				<0.001 (L)		0.966 (L)
13	5.8	5.9	5.9				<0.001 (Q)		0.277 (Q)
mean	4.3	4.2	*4.2*						

Diet 1 = control; diet 2 = treatment

Table 5B.22: Mean log bifidobacteria counts (cfu / swab) obtained from faecal samples (n = 16 per diet) and associated statistical analysis

	Diet			Diet		Day		Diet*Day	
Day	1	2	mean	s.e.d	P	s.e.d	P	s.e.d	P
9	5.6	6.1	5.9	0.15	0.609	0.22	<0.001	0.31	0.447
11	6.1	6.0	6.0				<0.001 (L)		0.526 (L)
13	6.5	6.4	6.5				<0.001 (Q)		0.427 (Q)
mean	4.5	4.6	*4.6*						

Diet 1 = control; diet 2 = treatment

Table 5B.23: Mean log *E. coli* counts (cfu / swab) obtained from faecal samples (n = 16 per diet) and associated statistical analysis

	Diet			Diet		Day		Diet*Day	
Day	1	2	mean	s.e.d	P	s.e.d	P	s.e.d	P
7	4.1	6.2	5.1	0.97	0.284	0.45	0.013	1.12	0.134
9	2.6	4.4	3.5				0.739 (L)		0.027 (L)
11	4.2	4.7	4.5				0.010 (Q)		0.879 (Q)
13	4.5	4.8	4.6						
mean	3.9	5.0	*4.4*						

Diet 1 = control; diet 2 = treatment

Table 5B.24: Mean digesta pH obtained from digesta samples (S1-S5*) along the intestine (n = 16 per diet; baseline (animals slaughtered on day 0); n = 4)

	Diet 1						Diet 2					
	Sample site						Sample site					
Day	S1	S2	S3	S4	S5	mean	S1	S2	S3	S4	S5	mean
0	6.5	7.0	7.5	7.0	7.0	7.0	5.3	7.3	7.5	6.6	6.5	6.5
2	5.3	7.3	7.5	6.6	6.5	6.6	6.8	8.0	7.9	6.8	7.1	7.3
4	5.3	7.3	8.5	6.0	7.0	6.8	5.8	7.3	8.3	6.5	7.0	7.0
6	5.9	7.5	7.8	6.3	7.0	6.9	6.5	8.2	7.8	6.5	6.8	7.2
14	5.3	6.0	8.1	6.3	6.5	6.4	5.7	6.9	8.1	6.5	6.7	6.8
mean	5.4	7.0	8.0	6.3	6.7	6.7	6.2	7.6	8.0	6.6	6.9	7.0

*S1 – S5 = duodenum, jejunum, ileum, colon and rectum respectively; diet 1 = control; diet 2 = treatment

Table 5B.25: A summary of the statistical analysis for digesta pH

Diet		Day		Diet*Day		Sample Site		BVR		BVR*Day		BVR*Diet	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.13	0.009	0.18	0.133 0.042 (L) 0.379 (Q)	0.26	0.514 0.697 (L) 0.247 (Q)	0.18	<0.001	0.19	0.648	0.22	0.123	0.20	0.008

BVR = Baseline versus the rest

5B.4.3 Performance parameters

No significant effect of dietary treatment was evident on daily live-weight gain (DLWG). Figure 5B.4 illustrates the variability among animals, and the weight loss that commonly occurs in the immediate post-weaning period.

No significant diet or diet and day interaction was found with respect to FCR (Figure 5B.5 and Table 5B.26). Figure 5B.5 illustrates the poor FCR displayed at day 2, with the values increasing significantly over time (P=0.049; 0.012 (Q)) and becoming more consistent. The poor and often negative FCR observed for animals slaughtered on day 2 is due to the low and sometimes negligible feed intakes observed over the initial days post-weaning (Table 5B.27). As expected, feed intake increased significantly over time (P=<0.001; <0.001(L); <0.001(Q)), and was highly variable over the early stages of the experimental period (refer to Appendix 2 for individual feed intake data). No significant differences between dietary treatment or relationships between diet and day were evident.

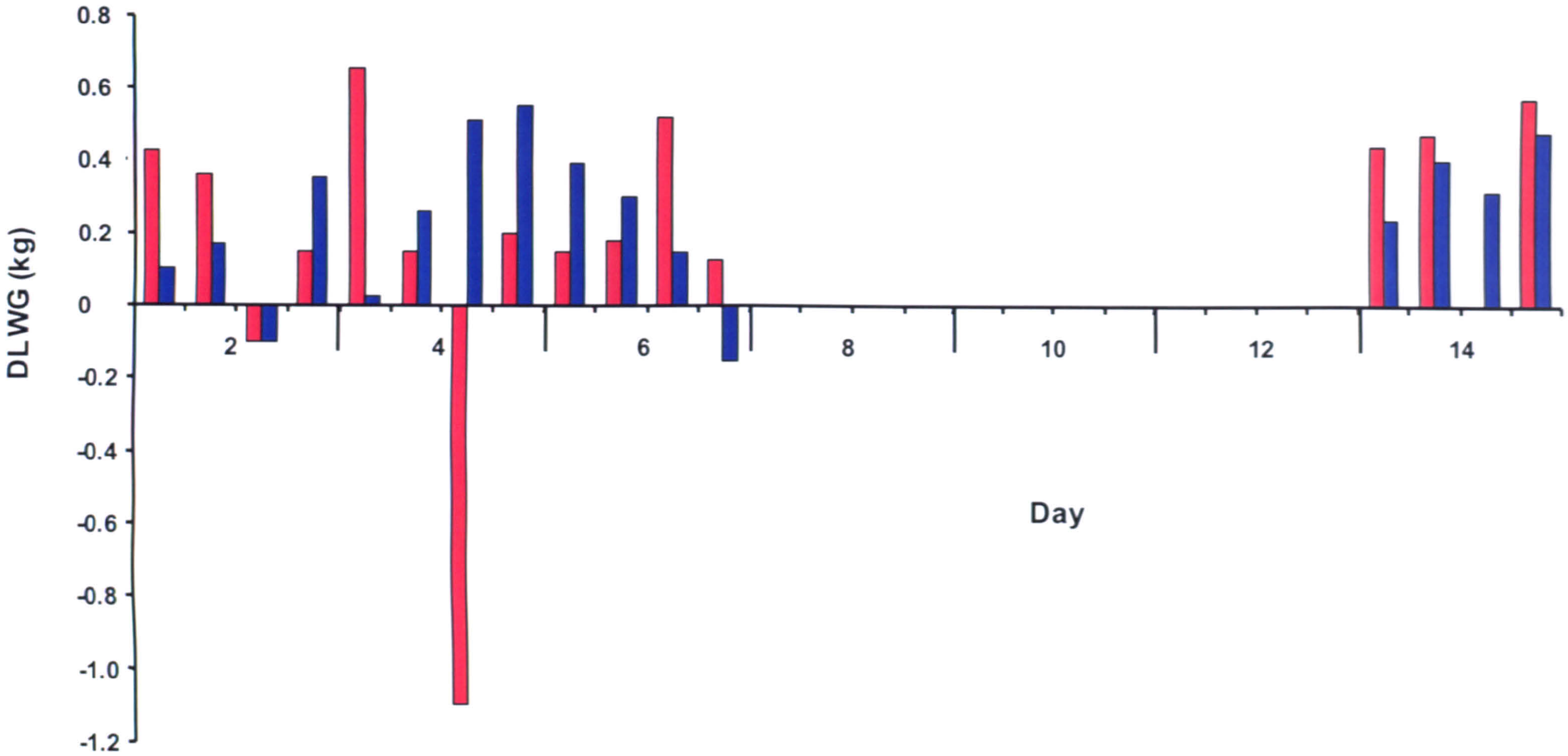


Figure 5B.4: Mean DLWG for individual animals fed diets 1 (■ control) and 2 (■ treatment; P=0.278; s.e.d = 0.11), grouped according to slaughter day

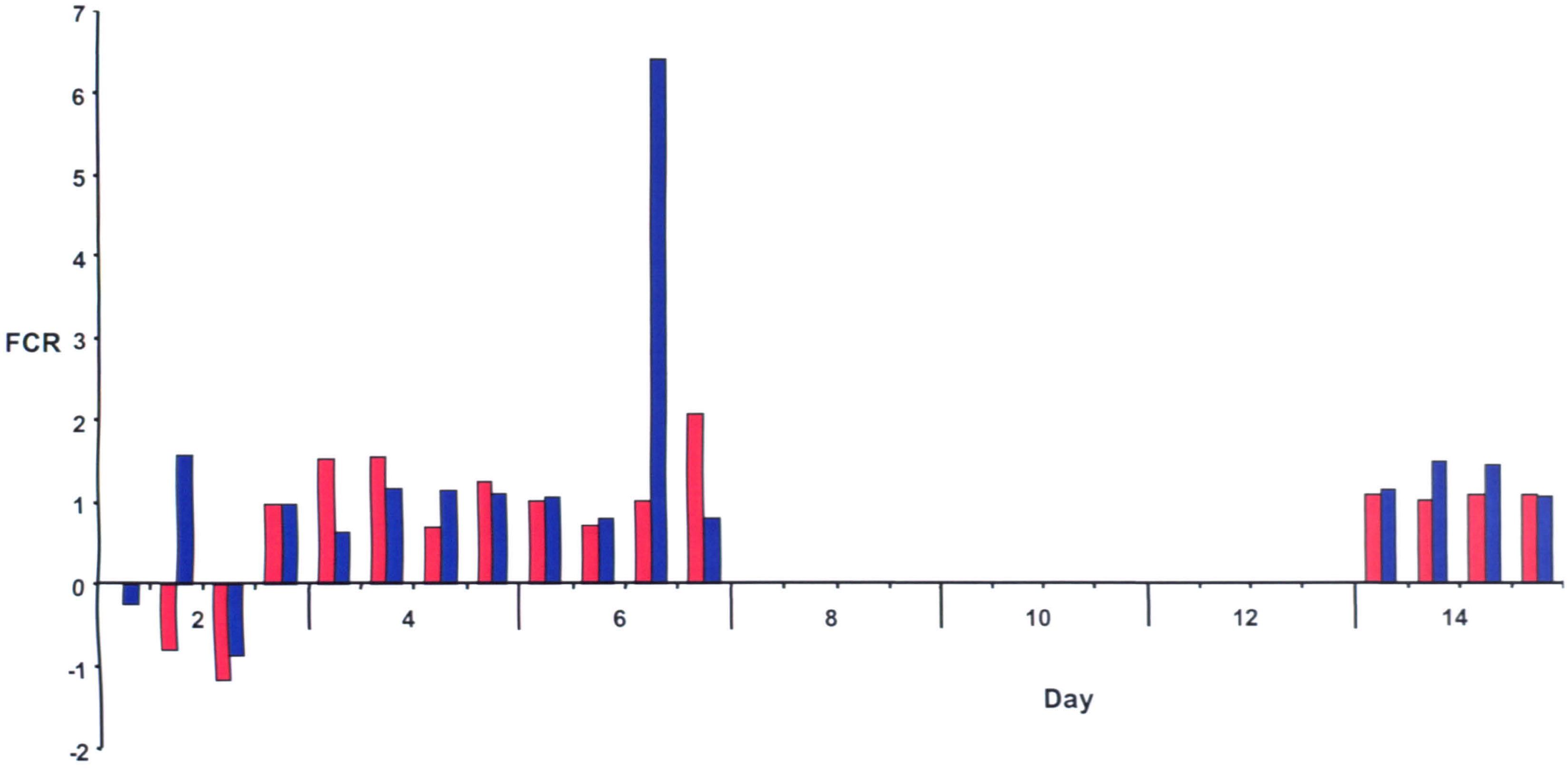


Figure 5B.5: Mean FCR for individual animals 1 (■ control) and 2 (■ treatment; see Table 6.26 for statistical analysis) grouped according to slaughter day

Table 5B.26: A summary of the statistical analysis for mean FCR (n = 16 per diet)

Day	Diet 1	Diet 2	Mean	Diet s.e.d	Diet P	Day s.e.d	Day P	Diet*Day s.e.d	Diet*Day P
2	-0.3	0.4	**0.0**	0.41	0.305	0.59	0.049	0.83	0.697
4	1.3	1.0	**1.1**				0.195 (L)		0.862 (L)
6	1.2	2.3	**1.7**				0.012 (Q)		0.806 (Q)
14	1.1	1.3	**1.2**						
Mean	**0.8**	**1.2**	***1.0***						

Diet 1 = control; diet 2 = treatment

Table 5B.27: Effect of dietary treatment on mean feed intake (g; n = 16 per diet) and associated statistical analysis

Day	Diet 1	Diet 2	Mean	Diet s.e.d	Diet P	Day s.e.d	Day P	Diet*Day s.e.d	Diet*Day P
2	165	212	**189**	39.9	0.597	56.4	<0.001	79.8	0.539
4	264	208	**236**				<0.001 (L)		0.344 (L)
6	332	324	**328**				<0.001 (Q)		0.367 (Q)
14	470	572	**521**						
Mean	**308**	**329**	***318***						

Refer to Appendix 1 for actual feed intake data (individual animals); diet 1 = control; diet 2 = treatment

5B.4.4 Lymphocyte proliferation

Figures 5B.6 to 5B.8, and Table 5B.28 represent the proliferation responses of peripheral blood lymphocytes (PBL) to 2 mitogens (Con A and PHA) at 3 different concentrations (10.0, 3.3 and 1.0 μg/ml). All lymphocyte proliferation cultures were performed using lymphocytes isolated from individual animals *i.e.* they were not pooled as for those used in study 3 (Figures 5A.3-5A.5). A similar shaped response was observed for all 3 mitogen concentrations and with both mitogens. The figures illustrate an enhanced uptake of methyl-[^{3}H]-thymidine initially (between days 0 to 4) which then declines between days 4 and 14. As such there was a highly significant difference with respect to day (P=<0.001; <0.001(L); <0.001(Q)), and BVR (P=0.004). No significant relationships between BVR and diet, or dietary treatment alone were evident. Significant correlations between diet and day (P=0.021; 0.010(L)), BVR and day (P=<0.001), BVR and mitogen (P=<0.001), and BVR and mitogen concentration (P=<0.001) were determined. As expected, significant differences between both mitogen (P<0.001) and mitogen concentration (P=<0.001) were also found.

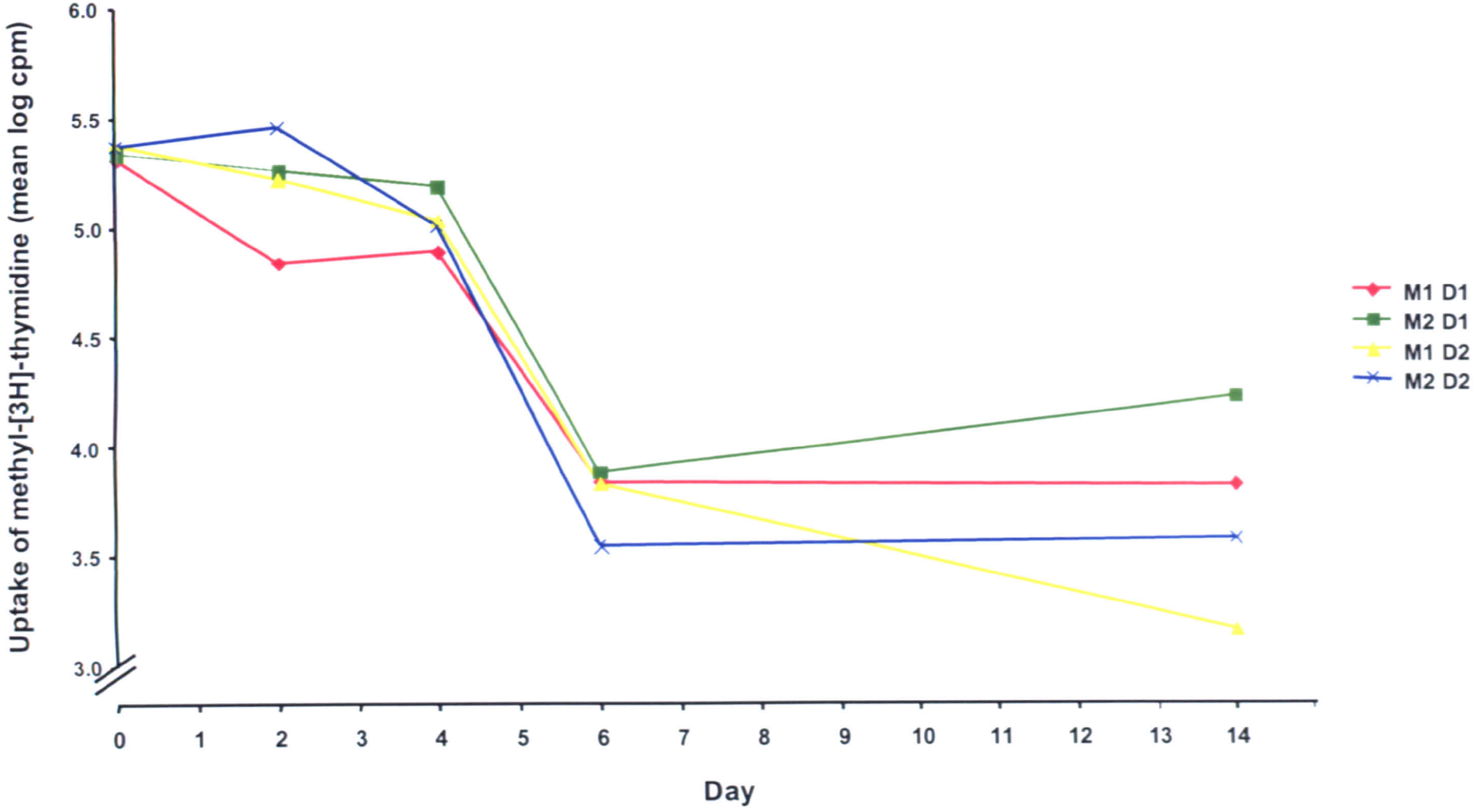


Figure 5B.6: The proliferation of PBLs from piglets fed diet 1 (D1; control) or 2 (D2; treatment) stimulated with Con A (M1) or PHA (M2) at a concentration of 10 μg/ml

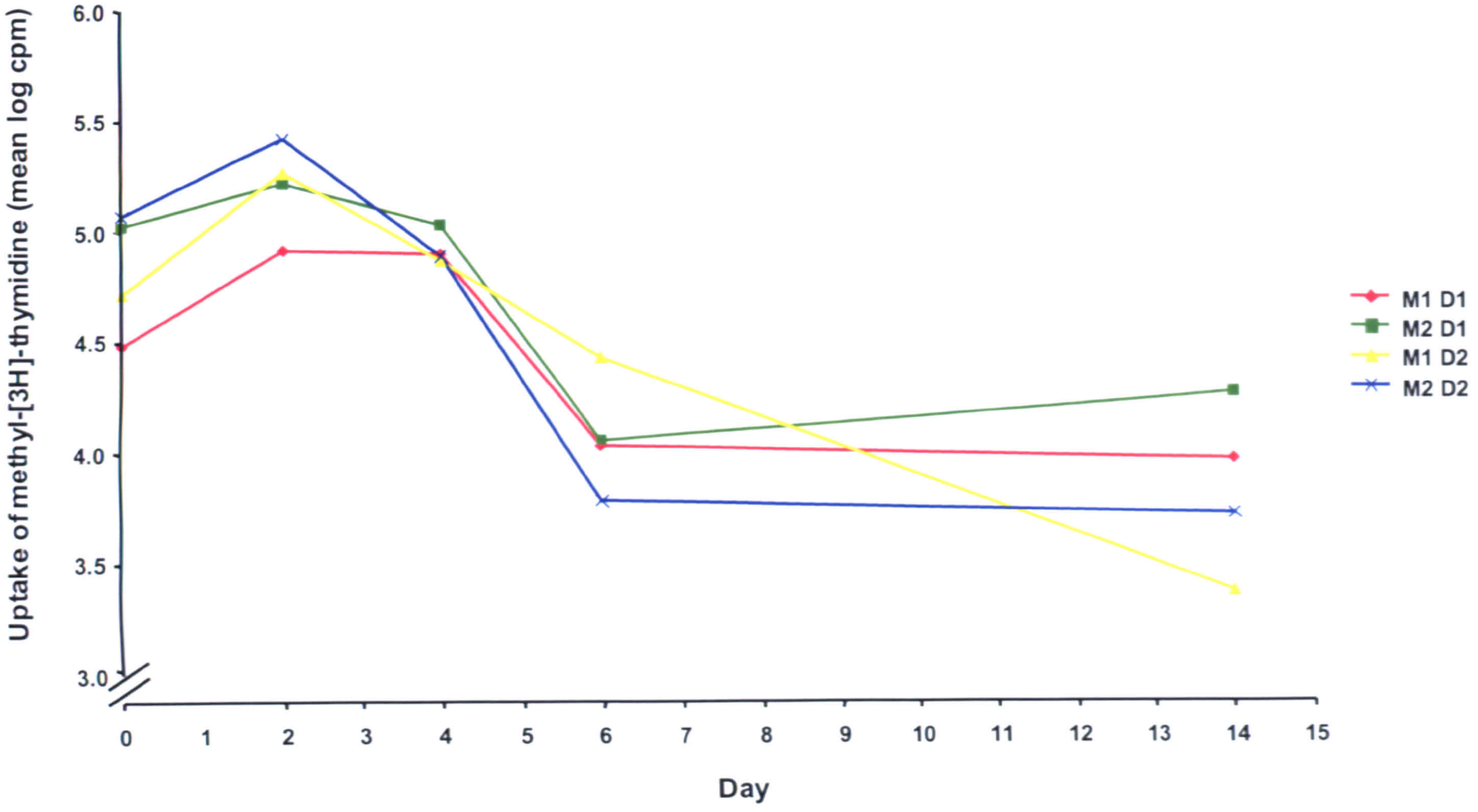


Figure 5B.7: The proliferation of PBLs from piglets fed diet 1 (D1; control) or 2 (D2; treatment) stimulated with Con A (M1) or PHA (M2) at a concentration of 3.3 μg/ml

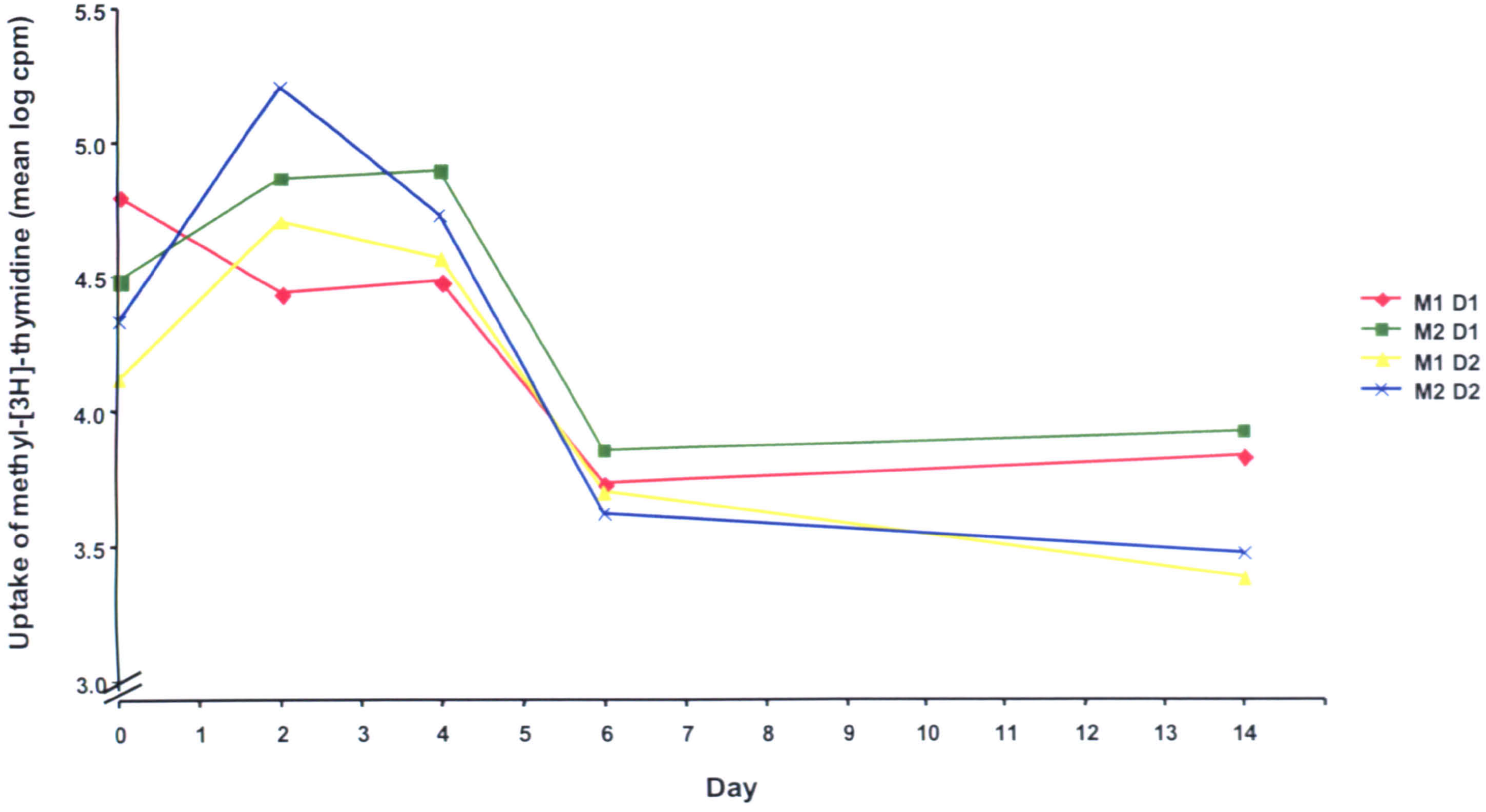


Figure 5B.8: The proliferation of PBLs from piglets fed diet 1 (D1; control) or 2 (D2; treatment) stimulated with Con A (M1) or PHA (M2) at a concentration of 1.1 μg/ml

Table 5B. 28: A summary of the statistical analysis for PBL proliferation responses

Diet		Day		Diet*Day		Mitogen		Mitogen Conc	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.08	0.384	0.12	<0.001 <0.001 (L) <0.001 (Q)	0.16	0.021 0.010 (L) 0.461 (Q)	0.04	<0.001	0.05	<0.001

BVR		BVR*Day		BVR*Diet		BVR*Mitogen		BVR*Mitogen Conc	
s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P	s.e.d	P
0.12	0.004	0.14	<0.001	0.17	0.657	0.13	<0.001	0.15	<0.001

BVR = Baseline versus the rest; conc = concentration

5B. 5 EXECUTIVE SUMMARY OF RESULTS

In the current study a degree of villus atrophy was apparent in the immediate post-weaning period in both dietary groups, although recovery occurred earlier (after 2 days post-weaning) in the treatment than the control-animals. Concurrent with the observations of study 3, villus widening and crypt hyperplasia also occurred throughout the post-weaning period. However, in contrast to the results obtained in study 3, there were no significant differences between dietary treatments on any of the microbiological species (both intestinal and faecal samples) investigated (*i.e.* coliforms, *E. coli*, lactobacilli and bifidobacteria), or DLWG, feed intake levels or FCR. Treatment animals displayed more alkaline intestinal pH than control animals. Proliferation responses of peripheral blood lymphocytes to mitogens exhibited no significant differences due to diet, although a significant relationship between day and diet was evident.

These results suggest that the introduction of creep feed from 14 days pre-weaning may exert a beneficial influence on the gut ecosystem (in terms of stabilisation of the microflora). Creep feeding pre-weaning allows a period of adaptation to occur to the diets before the stressful event of weaning occurs. Although those animals offered the treatment diets displayed more alkaline digesta pH values, any effects of this were not manifest on the gut microbial populations (the specific species investigated in the present study). The lymphocyte proliferative responses may indicate that the animals were experiencing some degree of hypersensitivity reaction in response to the dietary antigens, although there is also some suggestion that the observed response is age-dependent.

It should also be borne in mind that the *E. coli* infection encountered in the current study, would have affected the gut ecosystem and host immune status in some manner. Kelly (1990) stated that host predisposition to *E. coli* infection is considered a principal factor in the initiation of a sudden proliferation of enteropathogenic strains of *E. coli* and in the subsequent onset of diarrhoea. The underlying factors which have been implicated include inflammatory metabolites of bacterial origin (Kenworthy, 1976), immune-mediated damage to the small intestine (Miller *et al.*, 1983), damage mediated by rotavirus (Leece *et al.*, 1983) and genetic factors. Additionally, pigs are often immuno-suppressed at the time of weaning, which will further enhance their susceptibility to infection. Although the context of the current *E. coli* infection is somewhat different to that considered by Kelly (1990), the adverse changes in small intestinal structure observed in the current study, may nonetheless have facilitated the

colonisation of these animals by the pathogen. A further important consideration highlighted in the study of Kelly (1990) was the individual variation observed in the response of pigs to the *E. coli*.

In the study conducted by Kelly (1990), piglets were infected with pathogenic strains of *E. coli*, the onset and severity of the diarrhoea was not alleviated by prior exposure to a high level of creep feed pre-weaning. This contrasts with the findings of Miller *et al.* (1985), that pigs encouraged to consume a tolerising dose of creep feed did not succumb to PWD (Miller *et al.*, 1984a;b). The failure to demonstrate prophylaxis against *E. coli* infection by high pre-weaning intakes at either 2 or 3 weeks of age (Kelly, 1985 as cited by Kelly, 1990 and Kelly *et al.*, 1986) and the absence of a pre-weaning treatment effect on the morphology and function of the gut (Kelly *et al.*, 1986; Kelly *et al.*, 1990) are inconsistent with the hypothesis of delayed hypersensitivity. The present results of Kelly *et al.* (1986) support those of Hampson *et al.* (1988), who compared abruptly weaned pigs with animals with those fed low levels of creep feed (primed) and observed no differences in the susceptibility or severity of diarrhoea between the two groups.

The *E. coli* counts obtained in the current study were slightly higher than those reported by Sasaki *et al.* (1987) for control animals, but were generally within the same range (Table 5B.28). The current study also revealed that the ileum contained the highest *E. coli* load in comparison to the dudodenum and jejunum, which is also supported by findings of Sasaki *et al.* (1987). Furthermore, Sasaki *et al.* (1987) reported no significant differences between the intestinal *E. coli* counts observed from piglets 5 and 6 weeks of age.

Table 5B.29: *E. coli* counts obtained from intestinal samples for control animals in the current study at 6 and 14 days post-weaning

Region of Intestine	mean *E. coli* count (log cfu g^{-1})			
	Age 31 d (d 6 p-w)		Age 39 d (d 14 p-w)	
	Diet 1	Diet 2	Diet 1	Diet 2
Duodenum	4.8	5.9	4.6	5.7
Jejunum	3.9	6.1	4.2	4.3
Ileum	9.7	3.6	6.6	11.1

Table 5B.30: *E. coli* counts obtained from intestinal samples for control animals in the study of Sasaki *et al.* (1987)

Region of Intestine	*E. coli* count (log cfu g-1)			
	Five weeks		Six weeks	
	Mean	Range	Mean	Range
Duodenum	1.2	(0.0 - 4.6)	1.1	(0.0 - 4.3)
Jejunum	2.0	(0.0 - 4.0)	1.4	(0.0 - 5.6)
Ileum	7.0	(3.6 - 10.0)	6.9	(5.3 - 9.3)

From Sasaki *et al.* (1987), 0 indicates $<10^{3.3}$ organisms, data shown for control animals only.

Sasaki *et al.* (1987) also reported occasional diarrhoea, a reduction in live-weight gain and watery contents of the colon at slaughter.

Faecal shedding and intestinal *E. coli* counts correlated reasonably well ($r^2 = 0.759$) in the current study. Generally the mean intestinal count was slightly higher that the faecal count. This could be related to the fact that faecal *E. coli* load was enumerated via the technique of faecal swabs. As such, the faecal count was performed on a swab and not weight basis, thus rendering comparison between faecal and intestinal samples somewhat limited. Faecal grab samples could have been taken to provide enumeration on a fresh weight basis, although aerobic conditions and interaction with the environment can affect the results obtained. In the

study of Kelly (1990), shedding did not correlate with severity of diarrhoea, which supports the observations of Sarmiento *et al.* (1988) who concluded that faecal shedding was not a reliable indicator of *E. coli* colonisation of the small intestine because of the uncertainty regarding the proliferative or pathogenic activity of these bacteria during transit in the small intestine and in the hind gut.

CHAPTER 5C:

THE EFFECT OF FEEDING A NUCLEOTIDE-SUPPLEMENTED DIET ON PIG POST-WEANING PERFORMANCE

5C.1 INTRODUCTION

Previous work has demonstrated that the nucleotide source Ascogen can have beneficial effects on the intestinal wall and can be associated with improved daily live-weight gain (Uauy *et al.*, 1990). This study involved a total of 96 animals and was intended to investigate the effects, if any, of feeding Ascogen pre- / post-weaning on the growing / finishing performance of pigs. As such it was intended to monitor the performance of the pigs through to slaughter at 90 kg. Unfortunately, all animals were removed from the trial on day 26 post-weaning due to the incidence of severe enteric disease and the requirement for antibiotic therapy.

Evidence suggests that growth rates achieved during the post-weaning period are reflected in later life. For example, Tokach *et al.* (1992) and Azain (1993) reported that piglets achieving good growth rates (225-340 g / d) during the 1st week post-weaning reached much greater weights 10 to 28 days later when compared to piglets achieving poor growth (0-110 g / d). Regardless of the age at weaning, piglets lose some 100 to 250g on the first day after weaning, but the loss of body weight (Bwt) is recovered 2 to 4 d post-weaning (Le Dividich and Seve, 2000). However, although reduced overall growth rates post-weaning are observed this is not true for some individual body parts. Le Dividich *et al.* (1998) reported a 25% increased relative weight of the small intestine at 3-7 d post-weaning and a 52% increase at 10-14 days. Corresponding increases in the relative weight of the pancreas were 17 and 30% respectively. The increased weight of the GIT relative to Bwt occurs regardless of age at weaning and is caused by the change in the type of diet. This is demonstrated by the knowledge that gastrointestinal growth is, to some extent, dependent on the amount of feed intake during the immediate post-weaning period (Kelly *et al.* (1991b), Pluske *et al.* (1997)). Additionally, small intestinal growth is greater in piglets fed solid diet than those fed liquid milk substitutes (McCracken *et al.*, 1995).

5C.2 AIMS

The purpose of the present study was to evaluate the performance of post-weaned pigs fed diets based on a standard Provimi Ltd feed programme between weaning and 25 days post-weaning when given Ascogen pre- and post-weaning under commercial conditions. It was hypothesised that animals fed ascogen both pre- and post-weaning would exhibit enhanced performance post-weaning.

5C.3 METHODS

5C.3.1 Animals and housing

Ninety-six pigs were housed in groups of 6 piglets per pen (total = 16 groups) immediately after weaning (prior to weaning, all animals were housed on a litter basis with the sow in farrowing accommodation). All pigs were weighed pre-weaning and allocated to pens in such a way as to ensure that each pen contained either large (8-9 kg), medium (6-7 kg), or small (5 kg) piglets. The average weight at weaning was approximately 6.5 kg, and all animals were kept in the same groups from weaning through to slaughter.

5C3.2 Diets

A single-stage trial diet programme was used based on Provimi Ltd Multiwean (containing no zinc oxide or any other digestive enhancer). Animals fed pre-weaning were offered the diets on a group (litter) basis whilst housed in farrowing accommodation (*i.e.* suckling the sow). The dietary treatments were as follows;

Diet 1	Negative control; conventional starter diet (fed from weaning (d 0) to 25 days (d 25) post-weaning)
Diet 2	+ Ascogen; fed from 7 days pre-weaning (d –7) to weaning
Diet 3	+ Ascogen; fed from weaning to 25 days post-weaning
Diet 4	+ Ascogen; fed from 7 days pre-weaning to 25 days post- weaning

Pigs were fed the starter diet for the duration of the trial up to 25 days post-weaning (day 25). Ascogen was included at a rate of 4 kg/t where it was applied (diets 2, 3 and 4). One of the

four diets was fed to 4 groups of 6 piglets (*i.e.* 24 piglets in total). All 4 diets comprised of the same raw ingredients as shown below in Table 5C3.1 (refer to Table 2.1 for dietary chemical composition).

Table 5C3.1: Diet specification (refer to table 2.1 for chemical composition)

Ingredient	Inclusion (g/kg)
Cooked cereals	497.6
Oilseeds	173
Fish Products	87.0
Whey Powders	215.5
Oils & Fats	12.0
Vitamins & Minerals	15.0
Amino Acids	2.9

5C3.3 Growth performance parameters

Pigs were weighed individually on the day of weaning (day 0), 8 days post-weaning (d 8), and at 25 days post-weaning (d 25). Feed intakes were also recorded throughout the trial on a pen (group) basis and food conversion data calculated. The trial was expected to conclude at slaughter at approximately 90 kg. Unfortunately, all animals were removed from the trial on day 26 post-weaning due to the incidence of severe enteric disease and the requirement for antibiotic therapy.

5C3.4 STATISTICAL ANALYSES

Statistical analyses were undertaken as described previously in section 2.12. In this study conducted on a commercial unit, each group or pen of animals was one replicate (*i.e.* 4 pens per diet). A total of 96 animals were involved in the study, with 4 groups of animals (6 pigs per group) for each of the 4 dietary treatment (*i.e.* a total of 24 animals per dietary treatment).

5C.4 RESULTS

Analysis revealed no significant differences with respect to dietary treatment on both daily feed intake and feed conversion ratio (Tables 5C.2, 5C.3 and 5C.4). As expected, daily feed intake increased significantly throughout the experimental period ($P<0.001$; $P<0.001$ (L)) and FCR also improved significantly over time ($P<0.001$; $P<0.001$ (L)). There was no significant relationship between dietary treatment and day on daily feed intake, although a trend was observed for FCR ($P=0.083$).

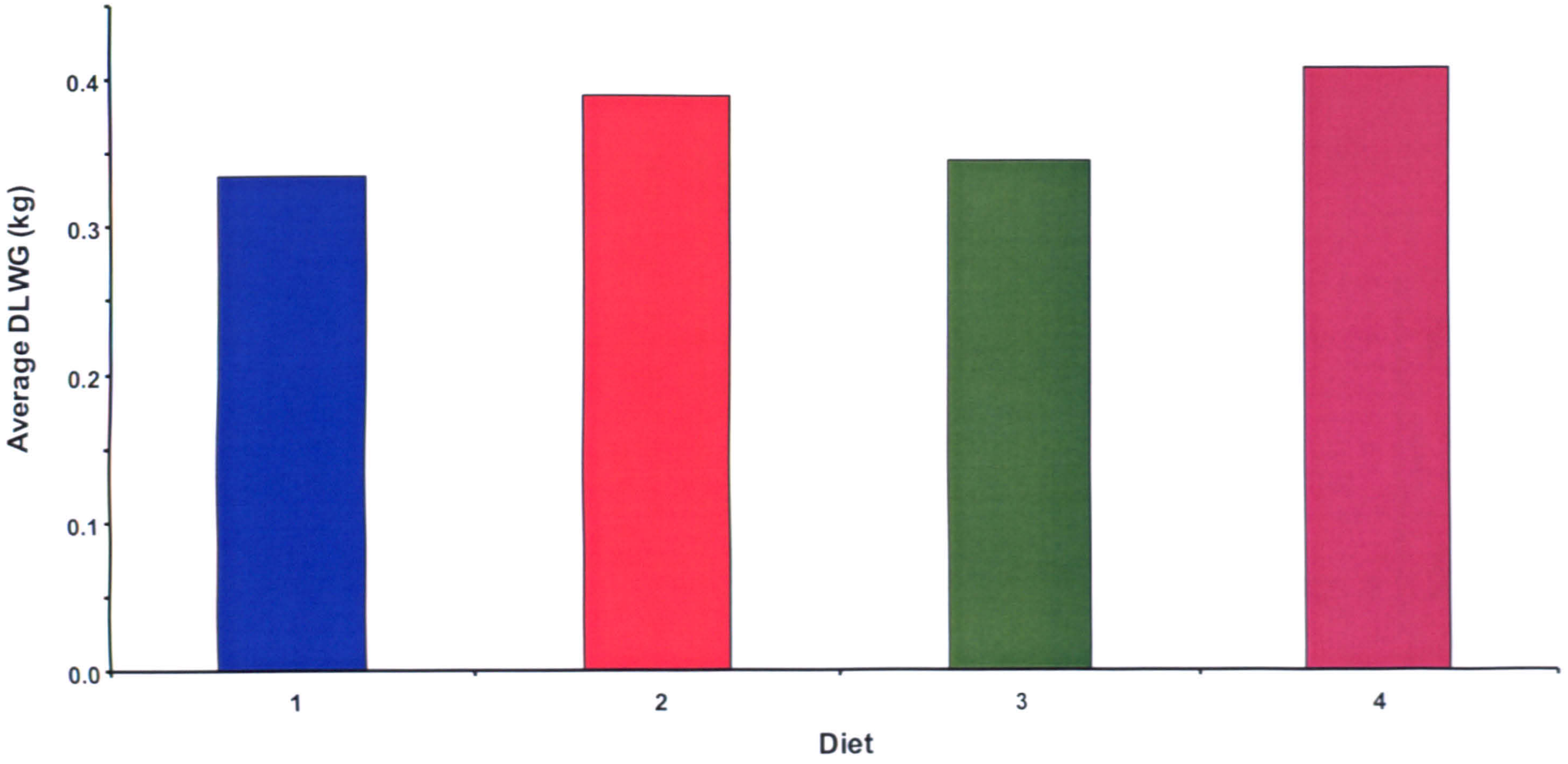


Figure 5C.3.1: Average DLWG (from weaning to 25 days post-weaning) for group-housed animals fed one of four diets ($P<0.001$; s.e.d = 0.185; $n = 4$ pens per diet (with 6 animals per pen))

Table 5C4.2: Mean feed intake (kg) and FCR (per group; n = 4 groups per diet; n = 6 animals per group)

Diet	Feed Intake	FCR
1	8.8	1.42
2	9.75	1.12
3	9.63	1.36
4	10.0	0.90

Diet 1 = control diet fed from d 0 to +25; diet 2 = treatment diet fed from d –7 to 0; diet 3 = treatment diet fed from d 0; diet 4 = treatment diet fed from d –7 to +25.

Table 5C4.3: A summary of the statistical analysis for mean feed intake (n = 4 groups (24 animals) per diet)

Diet		Day		Diet*Day	
s.e.d	P	s.e.d	P	s.e.d	P
0.20	0.119	0.12	<0.001 <0.001 (L)	0.26	0.201 0.201 (L)

Diet 1 = control diet fed from d 0 to +25; diet 2 = treatment diet fed from d –7 to 0; diet 3 = treatment diet fed from d 0; diet 4 = treatment diet fed from d –7 to +25.

Table 5C4.4: A summary of the statistical analysis for mean FCR (n = 4 groups (24 animals) per diet)

Diet		Day		Diet*Day	
s.e.d	P	s.e.d	P	s.e.d	P
0.23	0.446	0.13	<0.001 <0.001 (L)	0.29	0.083 0.083 (L)

Diet 1 = control diet fed from d 0 to +25; diet 2 = treatment diet fed from d –7 to 0; diet 3 = treatment diet fed from d 0; diet 4 = treatment diet fed from d –7 to +25.

5C. 5 EXECUTIVE SUMMARY OF RESULTS

Animals fed diet 4 (dietary regimen; Ascogen supplementation from –7d pre-weaning through to 25 days post-weaning), displayed the greatest rates of DLWG, followed by those fed dietary regimen 3 (Ascogen fed from 7 days pre-weaning). This suggests that the use of creep-feed from 7 days pre-weaning could be an important parameter in determining subsequent animal performance. This is most likely due to the fact that the introduction of creep-feed pre-weaning should result in a greater weight at weaning (although this is dependent on pre-weaning feed intake levels). Weaning weight is well established to correlate with performance later in life (Wolter and Ellis, 2001; Varley, personal communication). The dietary regimens that involved the introduction of creep feed pre-weaning (diets 2 and 4) also possess the additional benefit of allowing the piglets an adaptation period prior to weaning. As such weaning may not be such a stressful event (in comparison to those offered no creep feed pre-weaning), and the characteristic post-weaning anorexia (or low and variable levels of feed intake) may not have occurred. Although not significant (P=0.119), it is noteworthy that those animals on dietary regimen 2 and 4 (creep-feed), exhibited higher levels of feed intake post-weaning than those not offered creep feed (dietary regimen 1 and 3). Since feed intake or more specifically luminal nutrition is known to be an important factor influencing digestive physiology post-weaning, any enhanced (or even modest) levels of feed intake over the initial 14 days post-weaning are likely to exert some impact on the gut ecosystem of the host animal. Due to the disease outbreak and subsequent premature termination of the study, the effects of creep feeding on growing / finishing performance was unable to be investigated.

The introduction of creep-feed pre-weaning may also exert beneficial effects post-weaning through the reduction of stress associated with weaning that may ultimately result in a decreased duration and severity in immuno-suppression, which commonly accompanies weaning. This supported by data generated in study 4 (Chapter 5B).

CHAPTER 5:
DISCUSSION

Although a direct comparison between studies 5A and 5B cannot be made, there are nevertheless a number of common factors between the two. However, such comparisons should be interpretated with caution as the studies were performed in isolation from one another, at different periods of time (involving distinct animal batches), and encountered differing disease environments / pathogenic challenge. It should also be noted however that the same source of animals of the same genotype were used in both studies.

More specifically, seasonal changes in gut microflora could influence individual studies. For example, Hillman (2001) demonstrated indications of seasonal changes in the populations of *Lactobacillus* spp, *Bifidobacterium* spp, coliform bacteria and *Clostridium* spp, though not in total anaerobic bacteria or *Bacteroides* spp, in the colonic digesta of pigs. Furthermore, Hillman (2001) suggested that the effects of any attempt at gut manipulation may depend on the time of year at which it is applied. Hillman (2001) also suggested that this would be particularly true in the case of feed additives which include, or which are intended to affect, coliform or lactic acid bacteria.

These variations in the intestinal microflora, together with other known sources of variation such as animal age, herd and individual differences and dietary effects, would probably be entirely predictable if their causes were better understood. In particular, any immunoregulatory or general immune system responses will depend upon a number of factors including the function and physiological status of the animals themselves. Currently, it is not possible to take account of these variabilities within animal studies (more importantly, in comparing data from different studies) because the effect of many of these sources of variation has yet to be fully investigated. It is, however, important to appreciate that replication of experimental studies will be influenced by all of these factors, and possibly by many other factors not yet identified. It is thus possible that two discrete studies investigating the effect of a feed additive or supplement on the growth of an animal species may yield different results, although both datasets would be valid within the current state of knowledge of all these variables (Hillman, 2001).

Villus atrophy and crypt hyperplasia are phenomena that are known to occur at the time of weaning (Pluske *et al.* (1997), van Beers-Schreurs *et al.* (1998), McCracken *et al.* (1999), Tang *et al.* (1999)). Although in the present studies villus atrophy only occurred immediately post-weaning, this is probably what would occur under normal production conditions since the responses of both control and treatment animals were similar. In a study conducted by Hampson (1986), it was determined that villus height decreased to 75% pre-weaning values (940 to 694 µm) within 24 hours of weaning at 21 days. Subsequent reductions in villus height were smaller but continued to decline until the fifth day post-weaning, at which point villus height at most sites along the gut was 50% of the initial weaning value. However in the current studies 5A and 5B, villi were much shorter at the start of the experiment, and underwent less severe atrophy post-weaning, with the maximum villus atrophy of 29% occurring in control animals in study 5A. A comparison between the percentage differences in villus height obtained in studies 5A and 5B, clearly illustrates that in study 5B, the severity of villus atrophy was less in comparison to that observed in study 5A. Since the same dietary specifications were utilised in both studies, this apparent alleviation of villus atrophy could be attributed to the creep feeding regimen (diets fed from 14 days pre-weaning in study 5B). However, although the same dietary specifications were used, the actual batches of raw materials were different, and as such the dietary compositions may have differed slightly.

In study 5A, the longer villi present in the proximal part of the small intestine decreased in height proportionately less than villi in the medial or distal part of the gut (a decrease in height of the order 26-28% was apparent). This is in contrast to results reported by Cera *et al.* (1988) where the longer villi present in the proximal part of the small intestine decreased in height proportionately more than the villi towards the distal part of the gut. Conversely, the findings of study 5B are not so clear since the largest extent of villus atrophy was recorded in the proximal and medial regions for those fed diet 1 and the proximal and distal regions of the intestine for those offered diet 2.

There is a considerable amount of evidence indicating that nucleosides / nucleotides are essential for the growth and maturation of gut epithelial cells. For example, nucleoside supplementation has been shown to increase the rate of gastrointestinal maturation and growth in young rats as determined by mass, RNA, DNA, and protein concentrations and activity of brush border enzymes (Uauy *et al.*, 1990). Further, evidence also indicates that nucleotide supplementation has the capacity to restore the biochemical atrophy of the

small intestine at proximal and distal sites, and improve intestinal development after chronic diarrhoea (Nunez *et al.* (1990), Bueno *et al.* (1994)). Additionally, Adjei *et al.* (1994) observed that intraperitoneal and oral (Adjei and Yamamoto, 1995) administration of nucleosides and nucleotides inhibited the incidence of endotoxin-induced bacterial translocation, enhanced survival, decreased intestinal injury (*i.e.* villus height, crypt depth and total wall thickness were more developed), and reduced the recovery of colony forming units of both Gram-negative and Gram-positive enteric and facultative microorganisms in protein-deficient mice. Iijima *et al.* (1993) also illustrated that supplementation of total parenteral nutrition solutions with a nucleotide-nucleoside mixture prevented intestinal mucosal atrophic changes observed in non-supplemented rats, and that the effect was most evident in the ileum. A preliminary study conducted by Espinoza *et al.* (1992) indicated that a nucleotide-supplemented diet reduced the incidence of diarrhoea in young infants. Furthermore, Nunez *et al.* (1990) demonstrated that dietary nucleotides modulate the intestinal development after lactose-induced chronic diarrhoea in malnourished rats, and Uauy *et al.* (1990) reported that diets free of nucleosides and nitrogenous bases may have adverse effects on the gut, further suggesting that dietary nucleotides and nucleosides may have an important implication in the growth and maturation of the small intestine.

However, despite this body of evidence, the current studies 5A and 5B produced no significant effects of dietary treatment on gut morphology (in terms of VH and VW), although a significant CD (which was also manifested in the VH:CD ratio) effect was observed in study 5A. This lack of dietary effect could be associated with both the level and duration that the nucleotide-supplemented diets were fed. However, it should also be noted that there could be species differences since most studies investigating nucleotide supplementation focus on the laboratory rodent. The fact that the degree of villus atrophy was over a relatively short period, and that a similar response was observed for animals in both dietary groups (control and treatment) and in both studies (5A and 5B), suggests that this may be related to the intake of feed. This is a well-established phenomenon; indeed Diamond and Karasov, (1983) stated that that the presence of food in the lumen or, in particular, nutrient flows along the gut is one of the most potent stimuli for proliferation of the gastrointestinal tract. Furthermore, in a review by Kelly *et al.* (1992), it was stated that the structural and functional maintenance of the intestinal mucosa requires the intake of food through the oral cavity *and* the actual physical presence of the food within the gut. The luminal nutrition theory relates specifically to villus atrophy post-weaning. This is

supported by data presented by other researchers, such as Kelly *et al.* (1984), McCracken and Kelly (1984), and van Beers-Schreurs *et al.* (1998) who suggested that mucosal atrophy post-weaning might be related more to the lack of a continuous supply of substrate than to any antigenicity in the diet or to inherently low levels of digestive enzyme activity. More specifically, McCracken *et al.* (1995), and Pluske (1996a;b) illustrated the interdependence between voluntary food intake and mucosal architecture.

Pluske (1993) as cited by Pluske (2000) reasoned that if the nutritional stress of interrupted intake at weaning could be overcome, then the transition from sow's milk to solid food would be less traumatic and piglet growth would increase. Since an increase in food intake in the immediate post-weaning period is likely to exert potent stimulatory effects on mucosal growth and function, this may preserve the integrity of the small intestine and promote growth through an enhancement and (or) preservation of digestive and absorptive capacity (Pluske, 2000). Pluske *et al.* (1996a; c) demonstrated that piglets given cow milk *ad libitum* (2.5 times maintenance) possessed similar villus heights pre- and post-weaning. Furthermore, the provision of milk at the same level of energy intake as from *ad libitum* provision of a dry diet resulted in similar gut damage (in terms of villus atrophy and crypt hyperplasia). This suggests that when food/energy intake is maintained after weaning, the typical villus atrophy at 4-5 days post-weaning can be avoided (Bruininx *et al.*, 2001). Bruininx *et al.* (2001) also stated that the combination of damaged gut architecture and decline in digestive enzyme activity, which often occurs at weaning, is probably due to a decreased supply of nutrients and energy. Hence it follows that the prevention of the reduction of nutrient intake is crucial for maintaining both gut integrity and digestive capacity. This is important for realising the optimum performance and health status of the weaned pig (Bruininx *et al.*, 2001).

Melin *et al.* (2001) stated that the gut is a dynamic ecosystem of major complexity, and gastrointestinal health can be defined as the ability to maintain a balance within this system. Furthermore, there is believed to be a positive correlation between environmental stability, high diversity and high community stability. Therefore, a flora with a high diversity is considered to reflect a stable microbial community with a higher colonisation resistance. Weaning introduces a number of factors that could be regarded as environmental instability, resulting in a decreased diversity of the enteric microflora. The bacterial composition of the intestine of mammalian species has been reviewed by a number of authors (e.g. Moughan *et al.*, 1992; Maxwell and Stewart, 1994).

A high diversity of the normal flora is believed to stabilise the intestinal microecosystem (Hentges, 1983). If this stability is disturbed, e.g., stress induced by the process of weaning or by administration of antibiotics, the endogenous as well as exogenous bacteria may colonise the intestine and overgrow, resulting in a low diversity. Hence Katouli *et al.* (1994) stated that the evaluation of the diversity of the normal flora is useful for understanding the functional status of the intestine under conditions inducing bacterial translocation. However, studying the diversity of a bacterial population by conventional methods requires cultural analysis of a large number of bacteria from each sample which is laborious and time consuming.

Attempts were made to characterise, identify and enumerate certain microbial species throughout the intestine. It is however important to note that the intestinal microflora comprise an estimated 400 different bacterial species. Furthermore, several microhabitats exist within the intestines that exert a selective influence on the local composition and metabolic activity of the microflora. These microniches are found in the proximal and distal intestine associated with the villus surface, crypts, epithelial associated mucins and luminal mucus (Kelly and King, 2001c). The existence of these microhabitats further complicates the ability to isolate a representative population of microbial species.

In both studies 5A and 5B, the total mean number of intestinal coliforms declined significantly throughout the experimental period, whilst also increasing along the length of the intestine. This decline is most likely an age-dependent effect, and is supported by data of Katouli *et al.* (1999). Furthermore, Howe *et al.* (1976) reported that, during the suckling period, the population of coliform bacteria within the gut is rarely static as several bacteria of different serotypes (Howe *et al.*, 1976), biotypes (Hinton *et al.*, (1982), Hinton *et al.*, (1985)) and genotypes (Nagy *et al.*, 1990) are present at any one time. Katouli *et al.* (1999) reported that the diversity of coliforms increased after birth, until at weaning when one dominant but unique coliform phenotype appeared within each pig. In the current studies, general coliform counts were determined but classification into individual serotypes was not possible.

The current study 5A found that intestinal coliform load was responsive to dietary nucleotide-supplementation, causing a decline in numbers. This was not, however, observed in the animals from study 5B. This could be explained by the dietary treatment regimen; from day 0 in study 5A, and day –14 in study 5B. An acclimatisation period for

both the animal and its microflora to solid diets is considered necessary, and it appears plausible that the animals in study 5B had adapted to the diets and as such possessed a more stable microflora. Additionally, the animals in study 5B underwent a period of *E. coli* infection that could also affect the dynamics of the gut ecosystem and consequently the resident microflora. An increase in the intestinal *E. coli* load would result in enhanced competition for energy and nutrients at the gut level, possibly to the detriment of other microbial species. *In vitro* studies suggest that dietary nucleotides are capable of modifying the type and growth of the intestinal microflora *in vivo*. For example Tanaka and Mutai (1980), demonstrated enhanced growth of *Bifidobacterium* when a basal medium was supplemented with nucleic acids (in addition Gil *et al.*, 1986a).

Dietary nucleotide supplementation exerted a significant impact on faecal coliform numbers; in study 5A, treatment animals possessed higher faecal counts. This is in direct contrast to the intestinal coliform counts that were significantly lower in treatment animals and conflicts with the results documented by Gil *et al.* (1986a). However, the current results illustrate that treatment animals also exhibited greater numbers of faecal lactobacilli, which is in agreement with Gil *et al.* (1986a). Conversely the bifidobacteria counts were similar for both control and treatment groups which again conflict with findings of Gil *et al.* (1986a). Possible explanations for the differences reported between the 2 current studies and between those of Gil *et al.* (1986a) could be due to the initial microbial and pathogenic load (both within the intestinal tract and surrounding environment) and consequently the immunological status of the experimental animals. Furthermore, since individual serotypes were not identified within the current studies, there may have been differences at the serotype level, which were not investigated. Although equivocal, results from the current studies highlight that dietary nucleotide addition may have several benefits for the development, maturation, and repair of the GIT and may also influence the gut microflora. However it must also be borne in mind that, within animal production, each herd (and indeed individual animals within a herd) will likely possess its own distinct microflora and pathogenic load, which will in turn hamper the establishment of a common dietary supplement which is universally beneficial to all pig production units.

In direct contrast to intestinal coliforms, intestinal lactobacilli counts increased significantly throughout the experimental period and also along the length of the intestine in study 5A. This is consistent with findings of Pollmann *et al.* (1980). This latter pattern

was also observed in study 5B, although the lactobacilli numbers remained relatively constant throughout the experimental period. These results are in direct contrast to what one would expect, particularly in those animals employed in study 5A which were weaned abruptly from a liquid milk diet to a solid post-weaning one. At weaning a large population of lactic-acid producing bacteria would be expected (to break down the lactose present in maternal milk), with this population declining throughout the post-weaning period due to the removal / reduction of the principal substrate lactose (present in large quantities in milk). Again, since the animals in study 5B had been exposed to solid diets for a period of time prior to weaning, both the animals (particularly the digestive system and the relevant enzymes) and their microflora would be expected to have adapted (to some extent) to solid diets. This may explain the relatively constant temporal lactobacilli numbers. Alternatively, the increase in intestinal lactobacilli counts may represent an inter-relationship between intestinal coliform and lactobacilli populations possibly through competition between the 2 species. Furthermore, colon lactobacilli counts were considerably lower in studies 5A and 5B than those presented by Khaddour *et al.* (1998).

The results presented for study 5B (control and treatment diets fed pre-weaning) indicate that both the animals and their microflora had acclimatised to the diets. The temporal changes that were observed in intestinal coliform, and *E. coli* are most likely to be age-dependent since it is well documented that coliforms and specifically commensal *E. coli* species are initial colonisers immediately after birth and numbers decline over time. Specifically, Katouli *et al.* (1995) reported a greater number of biochemical phenotypes (BPTs) of *E. coli* isolated from the faeces of sows than their offspring. This suggests that, during the suckling period, only a few phenotypes of *E.coli* colonise the gut, but the number of phenotypes increase as the piglet ages (Katouli *et al.*, 1995). Moog (1979) and Okerman (1987) postulated that this could be the result of redifferentiation of the mucosal epithelium in piglets during the suckling period. Furthermore, the dietary changes associated with weaning significantly alter the intestinal environment, a consequence of which is modification of the composition of the indigenous flora.

In a study conducted by Melin *et al.* (1997), pigs at weaning (32 days of age) were exposed to a pathogenic strain of *E. coli*. No clinical signs of diarrhoea were reported, although the majority of the infected pigs shed the challenge strain for 6 days post-weaning (63% of animals excreted the challenge strain between days 3 and 5 post-weaning). Furthermore, a continuous decline in the number of excreted microorganisms

per gram faeces (in terms of *E. coli*, enterococci and *C. perfringens*) was also observed, which is consistent with observations in healthy piglets (Smith and Crabb, 1961; Kenworthy and Crabb, 1963; Melin *et al.*, 1997). This is also consistent with the findings of the current study 5B. Moreover, in the same study Melin *et al.* (1997), observed a higher proliferative response during the 1st week post-weaning by PBLs isolated from *E. coli* infected pigs. Furthermore, it was suggested that although the *E. coli* infection was subclinical, it did affect the immune reactivity of the pigs. Indeed, endotoxin is one of the most potent initiators of acute-phase responses inducing increased cytokine production, which has been demonstrated in various porcine models (Kramer *et al.*, 1993; Nakajima *et al.*, 1995).

It has been suggested that piglets housed together develop similar coliform floras (Melin *et al.*, 1997). Although in the current studies the animals were housed individually, there was olfactory and limited tactile contact between animals which may have permitted some cross contamination to occur.

An additional factor affecting the indigenous flora is the incidence of diarrhoea. Both diarrhoea and weaning are critical phases in terms of bacterial colonisation because high-level peristaltic movements accompanying diarrhoea and changes in nutritional status of the animal after weaning may greatly affect the physiological status of the gut and hence bacterial colonisation (Katouli *et al.*, 1995). This is demonstrated in the study of Katouli *et al.* (1995) where most resident strains were not found in the samples taken during the diarrhoea outbreak and 1 week after weaning. It was suggested therefore that, during these periods, the resident phenotypes were present in small numbers in faecal samples and escaped detection. In the current study 5B, the incidence of diarrhoea was slight, although the majority of animals displayed watery intestinal contents which may have exerted some influence on the gut ecosystem and more specifically bacterial populations and dynamics.

Bifidobacteria (member of Actinomycetaceae family) are numerically abundant in the normal intestinal flora in comparison to lactic acid bacteria (Moore and Holdeman, 1974). This abundance is primarily due the metabolic production of acids (primarily acetate and lactate) and the subsequent reduction of gut pH to levels at which other bacteria are unable to effectively compete (Tamura, 1983). However, in the current studies, no significant differences with respect to intestinal bifidobacteria counts were evident. The

levels of bifidobacteria reported in the colon of the animals in the current studies are consistent with those reported by Khaddour *et al.* (1998).

In study 5B, there was a significant dietary effect on intestinal pH with control animals displaying more acidic pH values. The bifidobacteria population cannot explain this reduction in pH since no significant difference was reported between the 2 dietary groups. Intestinal bifidobacteria counts tended to fluctuate throughout the experimental period for both control and treatment groups in both studies. As such, it is extremely difficult to draw any firm conclusions from these data. Studies conducted by Gibson and Wang (1994) illustrated that various bifidobacteria species possess the capacity to secrete an anti-microbial substance with a broad spectrum of activity affecting species belonging to the genera *Salmonella, Listeria, Campylobacter, Shigella* and *Vibrio cholerae.*

Specifically, Wang and Gibson (1993) reported that bifidobacteria grew at the expense of bacteriodes, clostridia and coliforms that were maintained at low levels or reduced. Although this could explain the reduction in coliform numbers throughout the experimental period which was observed in both studies 5A and 5B this is unlikely due to the lack of a dietary response. It is however possible that the dietary treatments did exert some (although non-significant) effect on the intestinal microflora load, albeit somewhat transient and short-lived. This would explain the significant dietary response observed in study 5A (treatment animals displayed significantly lower coliform numbers), and no dietary effect in study 5B. However, this temporal reduction in coliform population could also be an age-dependent effect (Katouli *et al.*, 1999) rather than one related to the dietary treatments.

Gil *et al.* (1986b) reported significantly higher faecal bifidobacteria counts in newborn infants (24 - 48 h after birth), suckled on maternal colostrum in comparison to milk formula. It is well documented that maternal colostrum and milk contain higher concentrations of nucleotides than formula milk; no difference was reported in terms of lactobacilli counts. Evidence suggests that nucleotides possess the ability to modify the type and growth of the intestinal microflora. For example, Gil *et al.* (1986a) observed that young infants fed nucleotide-supplemented formula had higher percentages of faecal bifidobacteria and lactobacilli, and lower percentage of Gram-negative enterics (such as enterobacteria) compared to formula-fed infants (in addition Tanaka and Mutai (1980)). However, these studies/data do not determine whether the decreased percentage of

enterobacteria in the stools is due to a direct effect of nucleotides or is a result of growth competition by the bifidobacteria.

Lymphocytes are the key cells controlling the immune response and can be characterised either by their surface proteins or the stimuli that trigger their division. The most important of these proteins are lectins that bind to cell-surface glycoproteins inducing subsequent division. These lectins are commonly isolated from plants, with examples including phytohemagglutinin (PHA) obtained from the red kidney bean (*Phaseolus vulgaris*), concanavalin A (Con A) obtained from the jack bean (*Canavalis ensiformis*), and pokeweed mitogen (PWM) obtained from the pokeweed plant (*Phytolacca americana).* In studies 5A and 5B, mitogen-induced lymphocyte proliferation was used as an *in vitro* index of cellular immune function, as lymphocyte stimulation in the presence of mitogens is recommended for testing the capacity of cell mediated immunity (Hoskinson *et al.,* 1990; Becker and Misfeldt, 1993). It is assumed that greater proliferation *in vitro* indicates a more effective cell function (Cohen and Herbert, 1996).

Lectins specifically bind sugar residues on glycoprotein side chains. For example, Con A binds α-mannose and α-glucose, and PHA binds *N*-acetyl-galactosamine. However, not all lymphocytes respond equally well to all lectins. Consequently, PHA primarily stimulates T-cell division, although it has a slight effect on B-cells, Con A stimulates T-cells alone, whereas PWM acts on both T- and B-cells. Although the mechanisms of mitogenicity are unclear, the plant lectins are known to open Ca^{2+} channels in the cell membrane with subsequent calcium influx into the cell (Tizard, 2000).

Van Buren *et al.* (1983; 1985) provided evidence that any effects of nucleotide supplemention would be exerted on the T- and not the B-lymphocytes. Hence, the lymphocyte proliferative studies would be expected to detect any effects of dietary nucleotide-supplement. Furthermore, Kulkarni *et al.* (1987) provided evidence of a down-regulation of the T-cell mediated response by a nucleotide-free diet. This has also been confirmed by *in vivo* functional activity studies (Kulkarni *et al.,* 1994a).

In both lymphocyte proliferation experiments (studies 5A and 5B), the same general response was observed. In study 5A (diets fed from weaning (d 0) to d 14), the uptake of thymidine peaked at day 4 post-weaning and declined thereafter. In study 5B (diets fed from d −14 pre-weaning through to d 14 post-weaning), the uptake of thymidine was

elevated from day 0 (weaning) until day 4. The pattern of thymidine uptake observed on day 14 in both studies was; (greatest first) PHA diet 1, Con A diet 1, PHA diet 2 and Con A diet 2. The differential uptake of thymidine, which was observed between the mitogens PHA and Con A, is explained by the response of different lymphocytes to these lectins. It appears logical therefore that the uptake of thymidine by PHA would be greater than that of Con A, since this mitogen targets a broader range of lymphocytes. Kanitz *et al.* (2002) reported that T-lymphocytes appear to be more affected by post-weaning stress than B-lymphocytes (Wallgren *et al.* (1994), Dominguez-Gerpe and Lefkovits (1996), Tuchscherer *et al.* (1998)), and as a result the activity of T-lymphocytes is more suppressed. The results from studies 5A and 5B support this concept since the uptake of thymidine was significantly less for the lymphocytes stimulated by Con A than PHA ($P=<0.001$). Con A stimulates T-cells alone.

Blecha and Kelley (1981), suggested that environmental stressors or adverse environments (such as cold), alter the capability of young pigs to mount an effective immune response (through the alteration of physiological control systems that regulate immunological events). Evidence also exists that stress and social events affect specific lymphocyte proliferation responses, (for example, Raab *et al.* (1986), Hardy *et al.* (1990), Laudenslager *et al.* (1990), Boccia *et al.* (1992), McGlone *et al.* (1993), Hessing *et al.* (1994), Barnard *et al.* (1996), Clarke *et al.* (1996)). This indicates that any immunosuppression could be related more to the stress associated with weaning rather than to any direct effect, such as abrupt dietary change. This is also supported by the fact that cortisol has been shown to affect lymphocyte function adversely, although results are equivocal (Roth and Kaeberle (1982), Westly and Kelley (1984)).

The initial low uptake of thymidine observed in study 5A at day 2 post-weaning, could be related to the period of introduction and the duration that the diets were fed. Additionally, the palatability of the diets may also exert some effect since this would influence feed intake levels. A period of adaptation to the solid diets is required (in terms of digestive system, enzymes and gut microflora) for feed intake levels to reach threshold and for the pool of dietary nucleotides to build up (in the case of animals offered diet 2). Although dietary treatment did not exert a significant effect in study 5A, there was a trend towards enhanced thymidine uptake by the lymphocytes isolated from animals fed diet 1. This could be related to the degree of functional maturity, development and activity of the lymphocytes. For example, it is well documented that immunosuppression is associated

with weaning (e.g. Blecha and Kelley (1981), Hammerberg *et al.* (1989), Puppe *et al.* (1997), Wattrang *et al.* (1998)) and it has been suggested that this could be overcome in the presence of nucleotide supplementation. Klasing (1988) reported that changes in the immune response are often a consequence of a change in the availability of that nutrient for its structure or metabolic function in cells of the immune system. This suggests that immunosupression associated with weaning could be induced via low feed intakes or anorexia in the immediate post-weaning period. Although it is also possible that anorexia or feed deprivation could induce stress which could subsequently result in immunosupression. Blecha and Kelley (1981) and Blecha *et al.* (1983; 1985) reported impaired antibody-mediated immunity and subsequent physiological modifications detrimental to cellular immune reactivity as a consequence of weaning. Such immunosuppression, could explain the low uptake of methyl-thymidine immediately post-weaning which was observed in the current study.

More specifically, Blecha *et al.* (1983) conducted a study in which pigs were weaned at 2, 3, 4 and 5 weeks of age; PWM and PHA stimulated lymphocyte blastogenesis was performed in comparison to unweaned controls at all 4 ages. The authors reported a suppressed *in vivo* and *in vitro* cellular immune response (manifested as lower lymphocyte blastogenic and delayed-type hypersensitivity responses compared to non-weaned littermates) when pigs were weaned at less than 5 weeks of age. In particular, lymphocyte blastogenic responses to PHA were decreased ($P<0.01$) in pigs weaned at 2 and 3 weeks of age. This is further supported by data of Kanitz *et al.* (2002). In the current studies (5A and 5B), pigs were weaned at 25 days of age which is again consistent with published data reporting reduced immunological responses at this age. However Blecha *et al.* (1983) reported no significant effect of weaning on PHA-induced blastogenesis in 5-week-old pigs. This indicates that the post-weaning immunosuppression is age-dependent, and is perhaps evidence to suggest that piglets would benefit from a later weaning age. Indeed, this is soon to be the case, since legislation has recently been proposed for a minmium weaning age of 4 weeks (28 days).

An earlier study conducted by Blecha and Kelley (1981) suggested that the onset of this post-weaning immunosuppression is rapid, occurring by 2 days post-weaning and that the duration of this immunosuppression is relatively short-lived (no immunosuppression reported in pigs weaned at 5 weeks of age). This is also supported by later data of Blecha *et al.* (1985). Furthermore, it has also been suggested that the capability of young pigs to

synthesise antigen-specific antibody increases with age (Brown (1961), Miller *et al.* (1962)). Since a non-significant response was observed for both control and treatment animals in study 5A (P = 0.097), the enhanced lymphocyte blastogenic response observed at day 4 post-weaning, could be an age-dependent effect. However, if this were true, one would expect this elevated response to be maintained over a greater period of time (the response declined between days 4 and 6).

Furthermore, indirect evidence suggests that weaning may compromise cellular immunity. For example, Osaba and Miller (1964) reported that mice lacking a functional cellular immune system could not synthesise antibodies to sheep erythrocytes. Additionally, Haye and Et (1979) demonstrated that pigs immunised at weaning (20 d old) displayed decreased antibody titers to sheep erythrocytes compared with pigs immunised 5d before weaning. This is consistent with findings of Blecha and Kelley (1981).

The physiological and immunological mechanism(s) responsible for weaning-impaired cellular immunity are unknown. Data generated from murine models indicate that glucocorticoids are involved in stress-induced suppression of cellular immune response to sheep erythrocytes (Blecha *et al.*, 1982). Recent *in vitro* data have shown that physiological concentrations of cortisol added to porcine lymphocyte cultures caused suppression of mitogen-stimulated lymphocyte blastogenesis (Kelley *et al.*, 1982). Worsaae and Schmidt (1980) reported that plasma cortisol concentrations were higher in early-weaned that in non-weaned controls. Moreover, Blecha *et al.* (1983) deduced that elevated plasma corticosteroid concentrations were responsible for the changes observed in cellular immune responses. However other factors, such as prostaglandins (Goodwin *et al.* 1981) serotonin (Bliznakov, 1980) and sympathoadrenal neurochemical changes (Stanton and Mueller, 1976), might also have been involved in this weaning-induced immunosuppression.

Hammerberg *et al.* (1989) demonstrated that conventionally raised piglets (weaned between 4 and 5 weeks of age) exhibited a deficiency in their humoral response that extended from birth up to the fourth (generally the time of weaning in the EU) and sometimes sixth postnatal week. This was based on results from an *in vivo* inoculation with a low dose of lysozyme, a T-cell-dependent antigen, and *in vitro* PWM induction of immunoglobulin production in BMC (blood mononuclear cells). Moreover, at the same time (as this humoral deficiency) deficiency in the primary *in vitro* cellular proliferative

response to lysozyme was observed with BMC. The authors suggested that this represented a T-cell deficient response in pigs up to 4 weeks of age, as Schwartz *et al.* (1975) stated that the *in vitro* proliferative response to a T cell-dependent antigen is attributed to the induction of T-cell proliferation.

Furthermore, Hammerberg *et al.* (1989) also illustrated that within the initial 4 weeks after birth, the porcine T- and B-cell components exhibited reduced responses to specific antigenic challenge exposure. The authors reasoned that this observation, particularly the decreased *in vitro* response of the T-cells to the antigen, combined with work conducted by Symons and Clarkson (1979; 1983) that B-cells from neonatal pigs are fully competent, indicates a deficiency in the T-cell component attributable to either a lack of helper T-cells or to the presence of suppressor T-cells. Furthermore the ability of T-cells obtained from pigs during this age period, to respond to PHA, suggests that these T-cells were fully competent, given the appropriate stimulant. The lack of fully functional T-cells in response to a low antigen dose could also be attributed to a low production of the lymphokine, IL-2. However, further analyses led Hammerberg *et al.* (1989) to conclude that the putative antigen-specific T-cell deficiency was probably due more to a subtle difference rather than a general lack of mature functional T-cells and probably was determined by the amount of antigen present to stimulate the T-cells from neonatal pigs.

In both studies 5A and 5B, the uptake of thymidine declined with age, which is supported by data from other researchers. For example, Hoskinson *et al.* (1990) reported a high proliferation response of PBLs to mitogen stimulation in the newborn piglet, which declined rapidly with increasing age (proliferation response was 10-fold greater at approx. 3 days than at 6 wks of age). These differences could not be assay-related since each included piglets of different age groups. Similar age-related differences have been reported previously, such as Nielsen (1987) where PBL proliferation responses increased with increasing gestation in fetal piglets. The exact mechanism is unclear, although one suggestion is that PBLs from newborn piglets are in an inactivated state. Alternatively, PBLs from newborn pigs could be in an immature state and may therefore possess an inherently greater capacity for proliferation. Becker and Misfeldt (1993) also demonstrated that numbers of leukocytes ($P<0.08$) and lymphocytes ($P<0.006$) increased with age (between d 1 and 30), whereas numbers of neutrophils did not change. This is supported by work conducted by Blecha *et al.* (1983), where *in vitro* lymphocyte blastogenesis showed a linear age effect ($P<0.01$) with younger pigs exhibiting greater

cellular immune responses, but those effects probably were not physiological and might have reflected the critical periods of incubation of lymphocyte cultures. Blecha *et al.* (1983) also reported that it is likely that the optimal incubation period for lymphocyte cultures varies with animal age. Jensen and Christensen (1981) demonstrated that lymphocyte cultures from older pigs peaked later than did cultures from young pigs; it was concluded that it is, however, unlikely that the higher [^{3}H]-thymidine incorporation in lymphocyte cultures from older pigs would alter the interpretation of data. Such an effect is not thought to be apparent in the current studies due to the relatively short-time scale involved (14 days). The studies of Jensen and Christensen (1981) and Blecha *et al.* (1983) investigated the effects on a weekly basis over a greater age range (from birth up to 5 weeks post-weaning) than the current studies.

The *E. coli* infection encountered in study 5B would further alter the dynamics of the immune system. For example, such an infection could induce proliferation of the lymphocytes *in vivo*, although it is not clear whether such influences would be manifested over the relatively short experimental period (14 d). Although bacterial enumeration results demonstrate that *E. coli* colonisation occurred at weaning, clinical signs of disease were not observed until 6 days post-weaning. It is possible therefore that the low proliferation responses of lymphocytes isolated from day 6 and 14 could be due to the fact that these lymphocytes had already been stimulated *in vivo* by the *E. coli* pathogen. It should also be borne in mind that this is occurring concomitantly with the immunosuppression of the pigs associated with the process of weaning. Other factors influencing the immune system include the level of exposure of the animals to the *E. coli* infection. The exposure level of the *E. coli* in the water was 10^6 cfu / ml. This would appear to be similar to levels used to experimentally induce *E. coli* infection (Kelly (1990), Melin *et al.* (2000)). Once the *E. coli* was detected in the drinking water system (day 6), it was immediately sanitised thus removing the infectious agent.

In the animals that were offered the nucleotide-supplemented diet, it is possible that the T-cells had already achieved optimum development and activation (due to the supply of preformed pyrimidines which are required for the proper development and activation of T-cells (Kulkarni *et al.*, 1994b). It seems plausible therefore that lymphocytes isolated from treatment animals could be at a more advanced stage of development and activation than those isolated from control animals. Consequently, such lymphocytes (from the treatment group) would possess a reduced capacity to develop and proliferate when

compared to lymphocytes isolated from animals fed non-nucleotide supplemented diets. This is further supported by data of Kulkarni *et al.* (1984), who demonstrated that feeding a nucleotide-free diet caused functional impairment of T-lymphocytes in mice. This could be due to the defect in differentiation and maturation of T-lymphocytes associated with immunodeficiency. However, this is unlikely to have occurred in the current studies since the diets may not have been fed for sufficient duration for this to be effective. This is further supported by findings of Becker and Misfeldt (1993) who suggested that low PBL proliferation responses could be affected by stress such as that of weaning and associated management practices. One would expect such responses to be relatively short-term and transient in nature. Furthermore, it is also noteworthy that the control diet was not specifically nucleotide-free merely not nucleotide-supplemented, and as such was formulated to meet the nutritional requirements of the animals and was not deficient in any manner. This may explain the non-significant dietary responses observed in the current studies, since all the animal's nutritional requirements were being met.

Dritz *et al.* (1995) indicated that immune activation could limit growth performance. If this phenomenon is true, the data from study 5A (but not study 5B where no significant dietary effects were reported) support this, since a trend (P=0.073) was observed towards an enhanced growth response for animals fed the control diet. This is further supported by the elevated growth performance commonly associated with segregated early-weaning production systems (as a consequence of reduced pathogen load). This enhanced performance (see Harris (1988) and Dritz *et al.* (1995)) has been attributed to suppression or elimination of inflammatory cytokine production. The infectious load present in segregated production systems is less, hence it is postulated that the enhanced growth is a consequence of reduced pathogenic organism load and a subsequent reduction in immune system stimulation. This combination of reduced pathogenic load and immune stimulation result in lower inflammatory cytokine production. Furthermore, studies conducted by Klasing *et al.* (1987) and Blecha *et al.* (1994) have demonstrated that increased levels of the inflammatory cytokine interleukin-1 have resulted in decreased growth performance. Furthermore, Klasing (2001) suggested that 6% of the net energy ingested is directed towards mounting an immune response. It is logical to assume that, immediately post-weaning, piglets possess enhanced requirements for energy and nucleotides (due to their involvement in protein synthesis and cell division) since the dietary supply has declined, because weaning is generally associated with a reduced feed intake. Antigenic challenge, which is common under regular weaning conditions, may further increase the demand for

nucleotides (Jyonouchi, 1994). However, this was not substantiated by the results of Sijben *et al.* (1998) who reported that the provision of dietary nucleotides did not reduce the piglets' energy demand. Furthermore, the same study demonstrated enhanced animal performance in terms of growth and energy retention in control compared to those fed nucleotide-supplemented (4 g/kg) diets. An explanation for these results could be that the availability of nucleotides in the control group in both the present study and that of Sijben *et al.* (1998) was adequate, so depletion did not occur. This appears possible since Jyonouchi (1994) demonstrated that a healthy human only utilises less than 5% of total dietary nucleotides. A second possible explanation for the enhanced performance of the control group could be that the *de novo* synthesis of nucleotides in this group increased compared to the treatment group. However, this explanation was discredited in the study of Sijben *et al.* (1998) since, for this to occur, ME_m (ME for maintenance) would be expected to be larger in the control animals because *de novo* synthesis is considered energetically expensive (Jyonouchi, 1994). Sijben *et al.* (1998), indicated that ME_m was in fact larger in treatment than control animals. Finally, a third possible explanation could be a negative effect of supplemental dietary nucleotides as a result of an overdose, which is known to be toxic. However, the concentration of supplemental nucleotides (4 g/kg) utilised in both the current study and that of Sijben *et al.* (1998), was according to the manufacturers recommendations and also below the maximum safety level, when this is converted from man to piglet expressed in metabolic weight, as recommended by the Protein-caloric advisory group (1975).

Carver (1999) reported that dietary nucleotides play a role in the maintenance of the immune response. While the mechanism is unknown, data suggest that exogenous nucleotides supplied by the diet may contribute to the nucleotide pool available to stimulated leukocytes, which rapidly turn over and thus have increased nucleotide requirements. Furthermore, Perignon *et al.* (1987) found a limited capacity of lymphocytes to salvage pyrimidines, while Marijnen *et al.* (1989) suggested that the nucleotide salvage pathway may not be capable of providing sufficient purine nucleotides for proliferating lymphocytes. Activation of lymphocytes causes a rapid increase in the synthesis of nucleotides, which are required first for the increase in energy metabolism and later as precursors for nucleic acid synthesis. These studies suggest that proliferating lymphocytes require an exogenous supply of nucleotides for optimum function. Induction of lymphocyte proliferation is accompanied by a dramatic increase in intracellular

nucleotide pools and the expression of large numbers of transmembrane nucleotide / nucleoside transporters (Carver and Walker, 1995).

Wattrang *et al.* (1998) reported a significant ($P<0.05$) decrease in Con A-induced proliferation after weaning and again after transfer to the finishing unit. This supports the view that the proliferation response is reduced during periods of 'stress' such as weaning, and consequently that the piglets are immunocompromised at such times. These data support the response observed in the present study (5A) where Con A-induced proliferation was reduced/compromised at weaning. Additionally, in the study of Wattrang *et al.* (1998), the Con A-induced proliferation response increased between days 2 and 7 post-weaning and decreased slightly between days 17 and 21 post-weaning. In the present study (5A), the enhanced proliferation was only short-term since the response declined by day 6 post-weaning. Differences observed between the two studies may be due to the different temporal sampling pattern (present study; 0, 2, 4, 6 and 14 days post-weaning; Wattrang *et al.* (1998); –6, 0, 3, 7 and 21 days post-weaning). Other factors include the disease status of the animals, effect of transport (albeit a very short distance); in the study of Wattrang *et al.* (1998) the animals were group housed after weaning and retained on the same unit, compared to individually-housed and transported to the experimental unit in the present study.

Suganuma *et al.* (1986) presented evidence that T-lymphocytes might suppress the differentiation of B lymphocytes into plasma cells *in vivo*; T-lymphocytes from suckling pigs were added to cultures of PBL from adult pigs and underwent mitogen stimulation. Suppressor activity of T-lymphocytes was consistently demonstrated throughout the neonatal and suckling period, although this declined with age and disappeared at 5 weeks of age. Furthermore, the addition of adult T-lymphocytes enhanced the differentiation of adult PBL more strongly than that of T-lymphocytes isolated from suckling pigs at 6 weeks of age. Moreover, this period of suppressor T-lymphocyte (up to 5 weeks of age) activity closely correlated to the period of poor antibody production. If suppression of B-lymphocyte differentiation were to occur, this could further enhance post-weaning immunosuppression with subsequent enhanced susceptibility to local infections.

In study 5B, thymidine uptake was elevated at the time of weaning (day 0), and remained so until day 4. Conversely, in study 3 the response was compromised at weaning but increased after 2 days post-weaning. Although no firm conclusions can be drawn from a

comparison of studies 5A and 5B, the response observed in study 5B could be a direct effect of the dietary regimen as both diets were fed from day 14 pre-weaning. No significant differences between dietary treatments were evident. This suggests that the response of PBL to mitogen stimulation could be a function of feed intake *per se* and not related to the actual dietary components (*i.e.* nucleotide addition). Additionally, it should be noted that in the study of Wattrang *et al.* (1998) pigs were creep fed from 7 days pre-weaning and weaned at 28 days of age and a reduced response to Con A post-weaning was observed. The mitogen induced responses may be related to the duration that creep feed was offered and the subsequent levels of feed intake. Unfortunately, creep feed intake levels are exceptionally difficult to record (piglets are not housed individually pre-weaning, and feed is inevitably spilled and wasted) and were not recorded in either the current study or that of Wattrang *et al.* (1998). The average weaning weight was 6.50 and 8.89 kg for animals in studies 5A and 5B respectively. Since the animals in both studies were the same genotype and reared under the same management regimen, the enhanced weaning weight achieved in study 5B is most likley attributable to the intake of creep feed pre-weaning. This suggests that in study 5B, the piglets were in fact consuming significant quantities of creep feed. This appears to be in contrast to findings of Pluske (1995) who stated that, generally, dry creep feeds have little or no effect on weaning weight due to low intake levels. It should also be noted that in study 5B, the piglets were undergoing a disease challenge with *E. coli* which would stimulate the immune system resulting in more nutrients and energy being partitioned towards establishing the immune system throughout the immediate post-weaning period (thus exhibiting lower rates of growth for both control and treatment groups). It is however not possible to ascertain whether any enhanced feed intake in study 5B was attributable to the disease challenge or related to familarity of the piglets to the diet. Any enhanced feed intake is however likely to be related in part to the larger body size of the animals utilised in study 5B due to the intake of creep feed pre-weaning; it follows that a larger animal requires more nutrients for maintenance. Poor FCRs were recorded in both studies 5A and 5B throughout the initial post-weaning period. However, in study 5B only 4 animals displayed negative FCR (due to weight loss), compared to 6 in study 5A, which again is most likely attributed to the creep feed intake in study 5B. It should also be noted however that both fasting and excess feed intake have been demonstrated to contribute to immunosupression (Klasing, 1988).

It may also be postulated in study 5B that, since the animals had undergone a 14 d period of adaptation to the diets (*i.e.* creep feed), the process of weaning may not have been as abrupt or as stressful an event compared to animals not offered creep feed (*i.e.* study 3). Thus, the severity of immunosuppression experienced by the piglets as a consequence of weaning may have been less. This is demonstrated by the elevated uptake of thymidine that was observed at day 0 (weaning) and is further supported by data presented from study 3, which has already been discussed. If this were a dietary effect *per se*, one would expect this to be a relatively transient response since the animals would adapt to the solid post-weaning diets, which indeed appeared to be the case in both studies. The PBL proliferation responses to mitogen stimulation could be further explained by the 'antigenicity' theory associated with post-weaning diets. Evidence concerning this issue is somewhat equivocal. For example, Miller *et al.* (1984a;c) stated that transient hypersensitivity to the antigenic components of the weanling diets may induce post-weaning diarrhoea (PWD) and effect modifications to both gut morphology and the immunological system. The authors reasoned that while the consumption of small amounts of creep feed (less than 100g) 'primes' hypersensitivity and exacerbate PWD, adequate consumption of creep feed before weaning may protect pigs from reactions to dietary antigens post-weaning and eliminate PWD.

It is possible therefore that the animals involved in study 5B could have been experiencing or combating some degree of transient hypersensitivity due to low levels of creep feed intakes (although evidence suggests that the duration and severity of transient hypersensitivity would depend on the level of feed intake pre-weaning) at the time of and immediately post-weaning. Miller *et al.* (1984a) reported that a pre-weaning intake of at least 400g of feed was necessary to prevent post-weaning diarrhoea. In study 5A, the diets were not introduced until day 0, hence such a hypersensitivity response could be somewhat delayed due to the incidence of anorexia immediately post-weaning. This is supported by findings of McCracken *et al.* (1999) (discussed above). Furthermore, malnutrition or nutritional deficiency (post-weaning anorexia) is reported to exert important and significant influences on cell-mediated immunity. Such effects of anorexia are more likely to occur in study 5A than 5B since mean feed intake was greater in study 4 throughout the initial 6 days post-weaning. In a study conducted by Pizzini *et al.* (1990), the effects of protein malnutrition and starvation were tested in animals fed nucleotide-free or yeast RNA-supplemented diets. In the protein malnutrition model the animals were fed the protein-free diet for 7 to 10 d and then offered protein-free, nucleotide-free,

commercial diet or yeast-RNA diets. Popliteal lymph node assays were performed and, although the nucleotide-free animals regained as much weight as the other groups (except protein-free), the nucleotide-free animals did not regain significant popliteal lymph node reactivity. In the starvation study, PHA- and Con A-stimulated blastogenesis assays demonstrated that animals previously maintained on the yeast-RNA diet displayed a significantly higher response. These studies clearly demonstrate that in periods of nutritional stress dietary nucleotides, in addition to energy or protein alone, are required to restore and maintain the cellular immune system. Moreover, Hampson and Kidder (1986) observed that the intake of creep feed pre-weaning did not encourage greater intake in the initial 5 days post-weaning (however feed intake levels pre-weaning fell well short of the 600g recommended by English (1980)).

Stokes *et al.* (2001) postulated that a transient aberrant immune response to antigens in the post-weaning diet may predispose to bacterial infection and disease. Numerous researchers have suggested that immune responses to dietary antigen, especially those derived from soya protein, are an important cause of local inflammation and subsequent villus atrophy (Dunsford *et al.* (1989), Li *et al.* (1990), Li *et al.* (1991), Bailey *et al.* (1993), Miller *et al.* (1994)). McCracken *et al.* (1999) concluded that soya-induced inflammation, if present, is likely to compromise intestinal morphology due to local inflammation caused by anorexia in the immediate post-weaning period. Extensive research is required into the possible cellular mechanisms linking hypersensitivity and the shaping of the villus epithelium at weaning. Although hypersensitivity is a contentious issue, there are sound nutritional reasons why soya protein should not be used at too a high concentration in weaner diets; 2- to 3-week old pigs cannot utilise bean meal effectively because they lack adequate levels of the digestive enzyme systems needed to break down complex proteins and carbohydrates (although this is influenced by the degree of heat treatment to which the soya-beans are exposed) (Kelly and King, 2001b). In the current studies soya was included at a rate of less than 200 g/kg which is generally considered acceptable for piglets of this age to degrade. However, most workers have not been able to substantiate this suggestion of hypersensitivity (Hampson and Smith (1986), Hampson *et al.* (1988), Kelly *et al.* (1990)).

No PWD was observed in study 5A, and that encountered in study 5B was attributed to *E. coli* infection. Stokes *et al.* (1987) reported increases in enterocyte turnover and villus atrophy in reponse to transient hypersensitivity reactions, with subsequent proliferation of

E. coli and the induction of post-weaning diarrhoea. In study 5B, the water system was identified as the source of *E. coli* infection. The conclusions of Stokes *et al.* (1987) do not however appear to be consistent with the findings of study 5B, where the severity of villus atrophy (in comparison to study 5A) appeared to be less. It is however also possible that any beneficial effects in terms of gut morphology due to dietary treatment and regimen, could have been counterbalanced by consequences of hypersensitivity reactions.

Miller *et al.* (1984b) suggested that *E. coli* is an opportunist rather than a primary pathogen. It has also been suggested that the negative influence of weaning itself on immune functions might contribute to the development of PWD (Bailey *et al.* (1992), Hessing *et al.* (1995), Wattrang *et al.* (1998)). This suggests that in study 5B, the *E. coli* infection may have been exacerbated by the process of weaning and the occurrence of any hypersensitivity reactions. Furthermore, if soybeans have not been appropriately heat treated, the young piglet may be unable to digest fully the soya-bean protein resulting in undigested protein reaching the duodenum supporting more bacterial growth with subsequent gastrointestinal damage (Miller *et al.*, 1984b). However in studies reported, transient hypersensitivity reactions were also accompanied by adverse effects on host performance. This cannot be ascertained from the current study since there was no negative control which was not offered creep feed pre-weaning. However in comparison to the animals in study 5A, the growth rates were slightly higher in study 5B, although this is likely to be an effect of body size. The results of Li *et al.* (1990) support the hypothesis that orally administered antigens evoke a local immune response with subsequent inferior performance of pigs at 28 days of age. This is also supported by data of Barratt *et al.* (1978) and Klasing (1988). Furthermore, in the study of Klasing (1988) no proliferative responses of blood or intestinal lymphocytes to purified soya protein were reported indicating that antigenic proteins of soya proteins do not evoke cell-mediated immune responses.

A number of mechanisms could explain the positive responses obtained from feeding supplementary dietary nucleotides reported in the literature; first, eliciting specific immune reactions could evoke the response. Second, a nucleotide-induced enhancement in growth could be the consequence of enhanced non-specific immunity. Third, because tolerance to oral antigens can be increased (Mowat (1987), Stokes *et al.* (1997) see also Miller *et al.* (1984a;b;c)), including a dietary immuno-potentiator such as a nucleotide source post-weaning might alleviate soybean-induced delayed-type hypersensitivity by

enhancing tolerance to soya-bean proteins. Finally, supplementary nucleotides could reduce inflammatory cytokine responses (Poutsiaka *et al.*, 1993), thereby allowing energy and nutrients to be partitioned towards growth (Klasing *et al.*, 1987).

Jyonouchi *et al.* (1994) suggested that exogenous nucleotide and nucleosides can be rapidly incorporated into the tissue nucleotide pool and rapidly restore impaired T-helper cell functions (*i.e.* accomplished in a relatively short period of time (8 days)). However, it should also be noted that in the studies of Jyonouchi *et al.* (1994), the dietary treatments were administered intraperitoneally ensuring more rapid absorption into the nucleotide pool than if administered in the feed. In the current studies, the nucleotide was incorporated into the diet and this combined with the low level of feed intake immediately post-weaning, indicates that nucleotide absorption would be somewhat slower than that in the study of Jyonouchi *et al.* (1994). As such, it follows that it may be more beneficial to feed the nucleotide-supplemented diets for a longer period of time than that employed in study 5A. It is further suggested that feeding nucleotide sources as a creep feed would yield optimum results, which would have the benefit of enhancing DLWG throughout the initial post-weaning period (due to increased feed intake) and reducing the impact of any potential transient hypersensitivity responses to the dietary components (if feed intakes of >100g can be achieved pre-weaning).

Brown-Borg *et al.* (1993) reported that pigs with high baseline and stimulated cortisol concentrations expressed reduced lymphocyte proliferative responses to mitogens *in vitro*. Furthermore, weaning has been shown to increase serum cortisol concentration so it follows that such animals immediately post-weaning may have reduced proliferation responses. Brown-Borg *et al.* (1993) went on to suggest that neonatal pigs with low cortisol response to stress may have enhanced ability to respond immunologically and, therefore, may have a definite advantage over stress-prone pigs. As such it may be possible that, in the present studies, cortisol levels exerted some influence on the mitogen-induced proliferation reponses.

In conclusion, the studies reported in this chapter have contributed to the limited knowledge of dietary nucleotide-supplementation in the post-weaned piglet. Furthermore, the studies were the first to investigate the effects of nucleotide-supplementation on aspects of gut health and function. The exact mechanism of dietary nucleotide effects on gut morphology and immunity are unknown. Additional studies are thus required to

elucidate the complicated interactions between gut microflora, physiology, hormones, and immunity, and their contribution to post-weaning growth check in the newly weaned piglet.

CHAPTER 6

GENERAL DISCUSSION

The studies detailed within this thesis investigated the contribution and significance of nutritional influences, on the complex and multi-factorial problem of post-weaning growth check in the piglet. Multi-disciplinary investigations focussed upon the relationship between post-weaning nutrition and the gut ecosystem with specific emphasis on gut physiology, immunity and microflora. This chapter encompasses all the main findings in this thesis, adopting a holistic perspective of the research and suggests possible studies for the future.

Whilst the specific limitations and difficulties encountered during the current study are detailed in individual chapters, common concerns include the availability and uniformity of the experimental animals used, *i.e.* physiological and immunological status and gut microflora population. Increases in animal numbers and treatment replicates, in addition to sampling frequency and microflora characterisation are recommended. Further improvements involve the sampling of animals throughout the pre-weaning period, immunohistology and the refinement of key techniques, such as intestinal microflora recovery and enumeration. These recommendations should be incorporated into future studies.

The pigs used in this study were raised under conventional conditions that exposed them to varying environmental factors and possibly to other unknown variables. Because of this, the subsequent conclusions only suggested a particular immunologic or physiologic tendency to such pigs. An attempt to limit genetic variability was made by using the same pigs which were from a multiplier breeding herd where genetic variability would be less than conventional commercial units, at the various times selected for testing. However, the relatively small number of pigs and litters might not have been sufficient to control all variables and thus placed limitations on the interpretation of the data.

It is well established that, in post-weaned piglets, growth check is irrevocably linked to post-weaning feed intake and luminal nutrition, which consequently has a hypotrophic effect on epithelial growth, development and differentiation (Pluske, 2000). The actual ingestion of food and its physical presence in the gut *per se* are essential for the structural

and functional maintenance of the intestinal mucosa (see Kelly *et al.*, 1992). As such, the weaning transition is generally accompanied by adverse changes in intestinal morphology and reduced absorptive capacity (see Pluske *et al.* (1997), van Beers-Schreurs *et al.* (1998) McCracken *et al.* (1999), Tang *et al.* (1999)). Furthermore, perturbation of the gut ecosystem commonly occurs at the time of weaning, and is known to exert a major impact on subsequent growth and performance of the post-weaned piglet (see Kelly and King, 2001a). Despite this, a direct link between solid feed intake and gut physiology has not been established, in that an optimum or threshold level has not been determined below which adverse changes in intestinal morphology occur. However, Pluske *et al.* (1996a; b) was able to preserve the structure and function of the small intestine post-weaning through the provision of a liquid milk diet throughout this period. Conversely, in the same study, piglets offered a dry starter diet displayed villus atrophy and crypt hyperplasia. Indeed generally the current studies appear to limit the duration of villus atrophy post-weaning to the initial 6 days post-weaning. Hampson (1986) demonstrated that villus atrophy occurred until the fifth day post-weaning which is further supported by findings of Cera *et al.* (1988) who reported a reduction in the microvilli length 3 to 7 days post-weaning. Generally, villus height begins to increase from 5 to 8 days post-weaning compared to unweaned pigs who experience only a slight reduction in villus height.

Evidence suggests that such adverse changes only occur after weaning if there is continuous absence from the sow. Hampson (1983) as cited by Pluske (2001), conducted a study with pigs that were weaned for 2 days and then returned to the dam for 3 days. These pigs displayed crypt elongation, which was only equivalent to that of pigs weaned for 2 days. Furthermore, Hampson (1983) as cited by Pluske (2001) also suggested that the consumption of food after weaning is necessary for crypt hyperplasia to occur, but a lack of intake may not be necessary for villus atrophy to occur.

The preliminary study investigating the acid binding-capacity (ABC) of post-weaning solid diets (Chapter 3), demonstrated that it is possible to improve intestinal morphology characteristics. The ABC of the diet is thought to influence the stomach pH with subsequent beneficial effects on digestion and creating an adverse environment for potentially pathogenic microorganisms. Despite the improvements in intestinal structure post-weaning, these effects were not manifested in increased performance, *i.e.* DLWG. The improvements in intestinal structure may not have been of significant magnitude to influence performance parameters.

The major finding of the ZnO study was the distinct positive relationship between dietary ZnO supplementation and post-weaning growth performance (Chapter 4). This is consistent with findings of Mavromichalis *et al.* (2000), however the mechanisms by which dietary ZnO exerts its effects are poorly understood. Nevertheless, the current study did indicate a positive influence of dietary ZnO supplementation on feed intake levels that may account, in part, for the enhanced growth performance. This finding was not however manifested through modifications of intestinal morphology or the lactobacilli and coliform populations studied. It appears possible, however, that dietary ZnO exerts an effect either luminally or systemically (Carlson *et al.*, 1999). Further research is required to determine the mechanism responsible for the enhanced feed intake response. The current study highlights the complex nature of the growth promotant properties of dietary ZnO supplementation, and the requirement for further research to investigate the multi-disciplinary and complex interactions responsible.

The dietary nucleotide-supplemented studies (Chapter 5) demonstrated that modifications to the gut ecosystem in terms of intestinal morphology and microflora are probably due to the dietary feed intake *per se*, and not the actual dietary composition. This theory has been demonstrated by a number of researchers, *i.e.* Kelly *et al.* (1984), McCracken and Kelly (1984), Kelly *et al.* (1991b), Pluske *et al.* (1996c). Quantifying the possible contribution of food intake *per se* on intestinal morphological and biochemical modifications is onerous due to the necessity to measure feed intake. Kelly *et al.* (1984) and McCracken and Kelly (1984) overcame this through the adoption of gastric intubation in order to regulate feed consumption and thus minimise individual variation in feed intake post-weaning. Voluntary feed intake throughout the initial post-weaning period has been shown to be both low and highly variable. Indeed, many piglets often fail to consume adequate food to meet their maintenance energy requirement (Bark *et al.* (1986), Le Dividich and Seve (2000)). However, a number of researchers have provided evidence that nucleotide supplementation has a direct effect on intestinal structure and function. Specifically, Uauy *et al.* (1990) reported enhanced mucosal protein, DNA, villus height and disaccharidase activities in the intestine of weanling rats fed a nucleotide-supplemented diet (8 g/kg). Additionally, other investigators report an accelerated intestinal recovery following food deprivation in rats fed nucleotide-supplemented diets (Ortega *et al.*, 1995). Pickering *et al.* (1998) reported that infants fed nucleotide-supplemented formula had a significantly lower incidence of diarrhoea compared with infants fed unsupplemented formula.

A focal point for future research is both to identify and achieve the critical feed intake levels at which villus atrophy, crypt hyperplasia and associated reductions in digestive enzyme levels and activities do not occur. In essence, this is probably only achievable through the introduction of creep feeds pre-weaning in order to attain sufficient levels of feed intake post-weaning without inducing hypersensitivity reactions. The introduction of creep feeds across the UK pig industry is most likely to occur in the near future due to EU legislation increasing the minimum weaning age to 4 weeks.

The first current nucleotide study (5A) demonstrated a reduction in intestinal coliform count post-weaning in piglets offered the nucleotide-supplemented diet. This is considered beneficial to the host since these species are often considered detrimental to the health and well being of the host and are frequently pathogenic in nature. Such an effect was not demonstrated in the second nucleotide study (5B), which may be attributable to the fact that the diets were offered from 14 days pre-weaning allowing the gut microflora to adapt and stabilise in response to the dietary change. Additionally, these animals were exposed to an *E. coli* challenge which may have exerted some influence on the gut microflora populations and may have resulted in the maintenance of the *E. coli* population which would otherwise have declined in response to both the dietary supplements and with advancing age of the host. Both factors are likely to have exerted an influence.

The findings of study 5A, also suggest that the piglets were immunosupressed at the time of weaning. A number of authors (Blecha and Kelley, (1981), Hammerberg *et al.* (1989), Puppe *et al.* (1997)) have demonstrated this phenomenon. Conversely, in study 5B, no evidence of immunosuppression was indicated. This could be a direct effect of the creep feed both in terms of allowing the piglets a 14 day period to adapt to the diets prior to weaning, or it may represent a transient hypersensitivity reaction to either the dietary antigens as a consequence of low levels of feed intake pre-weaning or to the colonising *E. coli* bacteria. Makinde *et al.* (1997) suggested that dietary antigens might be responsible for inducing villus atrophy. Hence, any diminished growth may therefore be due to reduced digestive and absorptive capacities as a result of intestinal mucosal damage. This is unlikely to be a major influence in the current nucleotide studies since villus atrophy was only apparent in the initial few days post-weaning. Such concepts have been discussed previously in chapter 5. Study 5B suggests that creep feeding may be beneficial in terms of reducing the stress associated with abrupt weaning and its consequences, in

addition to enhancing feed intake levels and DLWG throughout the post-weaning period. This latter benefit is supported by the results of study 5C where enhanced DLWG was observed in animals offered a nucleotide-supplemented diet from 7 days pre-weaning through to 25 days post-weaning. It has also been suggested that adverse changes in intestinal morphology is a consequence of local immune responses (Mowat and Ferguson, 1982). Furthermore, these results reported by Mowat and Ferguson (1982) are comparable with those which occurred within the intestine of weaned piglets (Kenworthy and Allen, 1966; Kenworthy *et al.*, 1967; Kenworthy, 1976), an observation which has lead to the suggestion that the changes may have an immunological basis (Miller *et al.*, 1984c).

It has also been proposed by several authors e.g. Hampson and Smith (1986), Kelly (1990), Kelly *et al.* (1990) that villus atrophy and crypt hyperplasia may be related to the withdrawal of 'intrinsic factors', such as IgA, in sows' milk which may also contain an array of biologically active peptides (e.g. epidermal growth factor) and compounds (e.g. polyamines) that may stimulate protein and DNA synthesis and have functional roles in intestinal differentiation. The causes of morphology modifications are mostly likely to be a combination of the factors discussed above.

However, despite the vast research attention directed towards low feed intakes and anorexia post-weaning, over-consumption or hyper-alimentation also presents problems. For example, Leece *et al.* (1983) demonstrated that newly weaned pigs challenged with enteropathogenic *E. coli* and rotavirus were more likely to develop prolonged diarrhoea if they were subjected to a dietary regimen involving a high nutrient intake fed 3 times a day. Conversely, a less severe response occurred if this daily total intake was divided into 24 portions and offered hourly, and the least severe response was observed in piglets fed one third of this total nutrient intake. The rationale, which explains this, is the knowledge that newly weaned pigs suffer alterations in digestive and absorptive capacity, thus a large nutrient intake may be insufficiently digested and absorbed and hence contribute an osmotic component to any pathogen-induced diarrhoea after weaning. Indeed, this phenomenon has long been recognised by pig producers who often temporarily restrict dietary intakes in the period immediately after weaning. This is also supported by findings of Hampson and Smith (1986). Furthermore, Klasing (1988) also reported that over consumption of feed could result in impaired cellular immunity.

Hillman (2001) stated that reports concerning effective growth-promoting substances, which have included microbiological investigation of the gut microflora, show that improved gut health is linked to an increase in the numbers of lactic acid bacteria and/or a decrease in the numbers of coliform bacteria. As the coliform bacteria typically comprise about 1% of the total microbial population of the intestine, while *Lactobacillus* species account for more than 90% of the population (Khaddour *et al.*, 1998), it is not immediately apparent why a reduction in number of coliforms, or an increase in lactobacilli, should make much difference to the absorption of digested material by the gut.

Hillman (2001) reasoned that the explanation for this might lie in both the formation of lactic acid by lactobacilli and the sugar-utilising qualities of coliforms. The principal product of metabolism of sugars by *Lactobacillus* spp. is lactic acid, which may be absorbed and used by the animal. The degradation of feed in the gut by lactobacilli, therefore, need not result in a great deal of energy loss to the animal. Alternatively, the coliform bacteria use oxygen, which is present at appreciable quantities in the piglet intestine (Hillman *et al.*, 1993). The coliform bacteria have a complete set of tricarboxylic acid cycle enzymes, so that the products of aerobic sugar breakdown by coliforms will be carbon dioxide and water, resulting in complete loss of the feed energy.

In this scenario, any reduction in the numbers and/or activity of coliform bacteria would be beneficial to the animal. Additionally, any increase in lactic acid bacteria in relation to the total bacterial load could also prove beneficial, since these bacteria are unable to extract more than a small fraction of the available energy from the feed. Acidification as a result of the activity of *Lactobacillus* spp. may also assist digestion by chemical hydrolysis of certain feed components, and would inhibit the activity of coliform pathogens.

Hillman *et al.* (1994) developed an *in vitro* fermentation system capable of maintaining at least part of the microbial population of the contents of the porcine ileum. The counts obtained from the fermenter at 50µmol/l dissolved oxygen over the pH range 6.0 – 8.0 approximated to the relative viable counts of the lactobacilli and coliforms reported *in vivo* in the posterior regions of the ileum (Smith and Jones, 1963). The numbers of coliforms are generally around $10^3 - 10^5$ in the anterior ileum of healthy pigs, and in this region of the gut the predominance of lactobacilli over coliforms in healthy animals may

be 1 or 2 orders of magnitude (Pollmann *et al.*, 1980; Pollmann *et al.*, 1984). The coliform levels recorded in the ileum in the current studies (Chapters 4 & 5) were slightly higher than those reported by (Pollmann *et al.*, 1980; Pollmann *et al.*, 1984), although this could be an age-related effect since coliform numbers decline with advancing age. The current results are however, consistent with those determined by Khaddour *et al.* (1998). Further, this concept is supported by the findings of the current studies and also Katouli *et al.* (1999). Lactobacilli counts followed a similar but opposite pattern, with numbers increasing both throughout the experimental period and along the length of the intestine. Again this is consistent with the investigations of (Pollmann *et al.*, 1980; Pollmann *et al.*, 1984).

The pH of the small intestine and stomach is important as it affects the activity of enzymes (which function within an optimum range) and the ability of potentially pathogenic microorganisms to colonise these structures. However, in a study conducted by Hillman *et al.* (1994), it was observed that changes in pH over the range 6.0 – 8.5 had relatively little effect on the total numbers of aerobic and anaerobic bacteria in the porcine ileum. Most studies have demonstrated a rise in pH in the small intestine, and in the caecum and colon, with pH values typically between 6.0 and 7.7 (Stewart *et al.*, 1993). However, Vervaeke *et al.* (1973) recorded higher pH values in animals between 3 and 7 weeks of age of 5.3 and 8.4 in the stomach and posterior small intestine respectively. Hillman (2001) reported alkaline pH values (typically pH 8.5 to 9.2) *in situ* in the caecum and colon. Both the pH values presented by Vervaeke *et al.* (1973) and Hillman (2001) are slightly more alkaline that those observed in studies 5A and 5B. Hillman (2001) also observed that the pH of the gut contents fell rapidly on withdrawal from the gut, which could account in part for the lower pH values reported by other investigators. Vervaeke *et al.* (1973) also documented between 10^7 and 10^9 anaerobes in the small intestine of piglets, which is further supported by findings of Hillman *et al.* (1994).

The current studies suggest that dietary modification can influence significantly host intestinal health and performance. However, this method of enhancing immune status and gut health is somewhat limited due to the impact of environmental factors such as pathogen load and physiological and subsequent physiological immune status of the animals at the time of weaning.

The current work has focussed upon nutritional influences on the gut ecosystem, and has highlighted the complex nature of this. Furthermore, factors contributing to changes in intestinal structure and microflora load and their interactions have also been highlighted. In particular, the extensive influence of luminal nutrition on intestinal structure has been emphasised and certainly warrants further research. Due to the nature of the weaning process in commercial pig production, Pluske *et al.* (1997) quite rightly suggested that the interaction between dietary growth, 'protective' factors and pathogenic microorganisms with the cell epithelium are likely to be important determinants of the way in which the weaned pig digests and absorbs consumed feed. A detailed examination within the immunobiology discipline, together with the interactions between cytokines and the mucosal immune system, will provide invaluable knowledge into the mechanisms controlling these factors. Other fundamental aspects of research should include the use of exogenous amines such as glutamine (Pierzynowski *et al.*, 2000) and polyamines (Kelly *et al.* (1991c), Bardocz *et al.* (2000)). Current molecular methods demonstrate that approximately 60% of the microflora associated with the porcine gut wall remains uncharacterised (Kelly and King, 2001b). Evidence is now emerging which emphasises the impact of the indigenous non-pathogenic flora on both innate and adaptive immunity. Within this gut microflora, diverse activities have been identified with substantial ramifications in relation to gut health and performance of pigs. The mechanisms of interaction between the gut mucosa and bacteria are emerging as research areas of paramount importance in relation to both human and animal health. Unravelling the cellular and molecular basis of bacterial colonisation, host recognition and the modulatory effects of bacteria on intestinal cell signalling and gene expression will provide the platform for the development of safer, targeted therapeutics to prevent disease and promote intestinal health (Kelly and King, 2001b). Unfortunately, the development of therapeutic agents, which target specific pathogens, is somewhat limited as a consequence of the rapid reproduction cycle and evolutionary mechanisms of such microbial pathogens. In light of this, enhancing the immune status and defence mechanisms would afford a greater degree of protection and allow the acquisition of 'natural' immunity.

APPENDIX 1:

DAILY LIVE WEIGHT GAIN CALCULATION

Genstat program used to calculate daily live weight gain

The following is an example of the program used to calculate daily live weight gain (DLWG) and to determine differences between treatments. The data consisting of a column for PIG, WEIGHT and DAY for all animals are read in from an Excel spreadsheet first (Diet and Sex columns are ignored). The diet and sex factor is declared later for the *36* calculated weight gain values.

```
scalar dlwtg[1...36],intercept
read[setn=y] missing
*
:
for dum1=1...36;dum2=dlwtg[1...36]
restrict Weight;Pig .EQ. dum1
if (nobs(Weight) .gt. 1)
model Weight
fit Day
rkeep residuals=r;estimates=c
equate old=c;new=!p(intercept,dum2)
else
equate new=intercept;old=missing
equate new=dum2;old=missing
endif
endfor
variate [nvalues=36] alldlwtg
equate new =alldlwtg;old=!p(dlwtg[1...36])

Factor [levels=2;values=4(0),16(1),16(2)]Diet
treat Diet

anova[fprob=y]alldlwtg
```

Note that figures in *italics* are altered depending upon the number of pigs and treatment under analysis, e.g. the *diet* term could be changed to *sex*

APPENDIX 2:

FEED INTAKE LEVELS

Table A2.1: Feed scale appropriate for *ad libitum* feeding regimen for piglets

Live-weight (kg)	Each meal allowance (kg)	Meal allowance/day (kg)
9 – 12	0.248	0.495
12 – 15	0.440	0.880
15 - 18	0.468	0.935

Piglets at this live-weight and age have extremely variable voluntary feed intakes. These figures are only a guide (Zarkadas, 1999).

Table A2.2: Daily feed intakes levels for individual animals in study 2 (Chapter 4)

Pig	Slaughter Day	Diet	Day 1	2	3	4	5	6	7	8	9	10	11	12	13	14	Total FI	FCR
9	14	1	49	265	445	448	468	635	462	557	640	1339	857	736	861	820	8582	1.38
10		2	124	483	641	480	500	395	793	595	699	1309	946	772	755	891	9383	1.14
11		3	36	96	392	470	448	500	557	440	542	1208	836	887	844	680	7936	1.50
12		4	42	295	582	500	500	400	605	559	511	1113	650	778	577	628	7740	1.36
17		1	33	561	417	500	670	700	481	666	656	1175	680	830	940	949	9258	1.32
18		2	61	345	566	470	662	569	626	615	699	1231	832	774	852	847	9149	1.37
19		3	155	242	373	373	587	317	442	440	496	1038	747	829	740	764	7543	1.64
20		4	63	372	544	500	700	700	618	759	687	1295	801	869	612	906	9426	1.33
1	6	1	68	479	431	281	380	420									2059	1.72
2		2	102	296	591	406	397	390									2182	1.45
3		3	155	173	475	262	253	322									1640	1.17
4		4	128	202	484	386	423	394									2017	1.12
21		1	52	178	209	142	474	242									1297	-6.48
22		2	163	301	478	450	666	434									2492	1.31
23		3	156	123	305	447	675	381									2087	1.39
24		4	77	135	324	337	652	428									1953	1.78
5	4	1	39	44	44	242											369	-0.46
6		2	116	74	23	23											236	•
7		3	39	273	548	303											1163	2.91
8		4	42	112	510	338											1002	3.34
13		1	29	76	473	352											930	2.33
14		2	28	87	310	346											771	-3.86
15		3	53	368	538	448											1407	2.01
16		4	37	26	350	320											733	2.62
29	2	1	14	301													315	-3.15
30		2	30	276													306	1.53
31		3	23	110													133	-0.44
32		4	16	54													70	-0.35
33		1	51	151													202	-1.01
34		2	26	21													47	-0.06
35		3	22	181													203	2.03
36		4	12	29													41	-0.21

Table A2.3: Feed intake levels for individual animals in study 3 (Chapter 5A)

Pig	Slaughter Day	Diet	Day 1	2	3	4	5	6	7	8	9	10	11	12	13	14	Total FI	FCR
1	14	1	201	284	438	478	500	600	750	790	780	569	715	912	611	773	8401	1.31
2		1	38	25	264	418	490	612	657	599	687	602	660	883	593	807	7335	1.20
3		1	415	341	500	500	485	750	702	907	427	676	913	1000	1000	991	9607	1.19
4		1	224	430	318	457	500	634	582	692	631	502	688	930	639	677	7904	1.39
13		2	204	144	295	327	464	465	454	523	509	510	620	874	531	703	6623	1.18
14		2	109	235	313	389	500	580	602	508	586	549	663	913	537	740	7224	1.34
15		2	98	233	184	226	309	475	365	522	559	450	520	536	269	455	5201	1.41
16		2	26	193	241	351	500	520	553	557	561	455	761	700	691	666	6775	1.61
17	6	1	23	329	350	333	405	337									1777	1.27
18		1	19	225	365	458	196	179									1442	1.20
19		1	70	299	500	500	525	377									2271	1.26
20		1	215	352	246	490	360	327									1990	1.99
29		2	28	286	409	580	750	403									2456	2.05
30		2	133	224	230	455	679	226									1947	2.78
31		2	136	226	259	505	750	378									2254	1.88
32		2	22	78	116	368	619	287									1490	0.00
21	4	1	77	8	322	265											672	0.00
22		1	28	88	210	266											592	2.96
23		1	29	28	297	407											761	3.81
24		1	30	13	338	365											746	2.49
25		2	25	33	424	474											956	1.59
26		2	29	54	361	385											829	1.38
27		2	20	488	500	500											1508	1.26
28		2	21	131	480	443											1075	1.08
5	2	1	102	203													305	-1.02
6		1	111	149													260	-0.87
7		1	238	196													434	-0.72
8		1	260	239													499	-1.25
9		2	193	271													464	-1.55
10		2	159	25													184	0.61
11		2	216	178													394	-1.31
12		2	89	68													157	0.39

Table 2A.4: Individual feed intake levels for animals in study 4 (Chapter 5B)

Pig	Slaughter Day	Diet	Day 1	2	3	4	5	6	7	8	9	10	11	12	13	14	Total FI	FCR
1	14	2	60.5	60.5	104	124	167	177	290	318	329	416	395	308	372	370	3491	1.13
3		2	1572.5	1572.5	306	364	419	459	499	493	507	491	556	603	704	761	9307	1.50
4		1	194.5	194.5	297	256	365	374	297	370	317	359	375	414	507	606	4926	1.07
5		2	1465	1465	314	493	520	556	543	513	509	453	589	821	551	826	9618	1.44
8		1	185	185	309	350	408	520	645	587	675	647	719	889	862	1000	7981	1.01
10		1	18.5	18.5	299	326	405	460	500	422	490	513	558	595	589	683	5877	1.07
19		2	323.5	323.5	757	561	599	665	647	668	677	799	789	898	941	978	9626	1.06
20		1	226	226	334	354	442	609	601	639	651	563	633	792	737	701	7508	1.07
2	6	1	159.5	159.5	435	531	551	673									2509	1.00
11		2	226	226	243	325	473	409									1902	1.06
15		2	253	253	391	431	632	549									2509	0.78
17		2	198	198	246	272	219	152									1285	6.43
18		1	76	76	181	216	293	310									1152	0.72
25		2	230	230	380	385	465	379									2069	0.80
26		1	301	301	323	397	426	480									2228	1.01
28		1	271	271	417	632	44	436									2071	2.07
7	4	2	156	156	405	466											1183	0.62
9		2	239.5	239.5	121	102											702	1.17
12		1	21.5	21.5	469	406											918	1.53
13		2	98	98	277	321											794	1.13
16		1	98.5	98.5	271	314											782	1.56
21		2	65	65	210	315											655	1.09
22		1	214	214	581	520											1529	0.70
24		1	190	190	290	332											1002	1.25
6	2	1	277	277													554	•
14		1	120	120													240	-0.80
23		2	262.5	262.5													525	-0.24
27		2	158	158													316	1.58
33		2	88	88													176	-0.88
34		1	118.5	118.5													237	-1.19
35		2	341	341													682	0.97
36		1	145.5	145.5													291	0.97

APPENDIX 3:

REAGENTS AND RECIPES

CHAPTER FOUR

4.5 Analysis of blood urea nitrogen

At slaughter, blood was collected from the cranial vena cava region and stored overnight at 4^0C. The blood was then centrifuged at 2,000 x *g* for 12 minutes and the serum removed. The serum fraction was then centrifuged for a further 12 minutes at 3,000 x *g* and the serum removed once again and frozen for subsequent analysis. Serum urea nitrogen was determined using a Sigma Diagnostics INFINITY™ BUN Reagent kit. The diagnostic kit protocol was utilised where 1 ml aliquot of BUN reagent was added to a cuvette plus 10 microlitres of sample achieving a 1:100 sample: reagent ratio. Once prepared, the sample was left to stand for 30 sec and the absorbance read (A1). The sample was then left for a further 60 sec and the second absorbance (A2) read and recorded.

Calculations

Results were calculated by the spectrophotometer as follows;

$$\text{Urea} = [(\text{A/min of Unknown}) / (\text{A/min of Calibrator})] \times \text{Calibrator Value}$$

$$\text{A/min} = (A2 - A1)$$

Where: A1 = Absorbance at first read time

A2 = Absorbance at last read time

Example:

	A1	A2	A/min
Unknown	1.7	1.6	0.1
Calibrator	1.8	1.66	0.14

Value of Calibrator = 40 mg/dL Urea Nitrogen

Urea Nitrogen, mg/dL = [0.10 / 0.14] x 40 = 29 mg/dL

CHAPTERS FOUR AND FIVE

2.8.2 Maximum recovery diluent

Maximum recovery diluent (MRD) (4.75g, Oxoid) was was made up to a final volume of 500 ml with reverse osmosis water and autoclaved (121°C, 15 min).

2.8.5.1 Columbia agar base

This Columbia agar base was modified according to the method of Beerens (1990); 39g of the agar base, 5g glucose and 5g cysteine hydrochloride was suspended in 1 litre of distilled water, and dissolved completely by boiling on hotplates with agitation. The medium was then allowed to cool to 70 ^{0}C and 2.5 ml propionic acid added followed by 18.5ml molar sodium hydroxide under aseptic conditions to adjust the pH to 5.0. The medium was the transferred to a water bath at 50 ^{0}C before the plates were poured into petri-dishes.

2.8.5.2 Rogosa agar

The ingredients (82g) were dissolved in 1 litre of distilled water and brought to the boil to dissolve completely. 1.32 ml glacial acetic acid (Fisher Scientific) was added and mixed thoroughly. The agar was the heated to 90-100 ^{0}C for 2-3 minutes with frequent agitation and transferred to a 50 ^{0}C water bath for cooling before the plates were poured.

2.8.5.2 Petrifilm

The 3M Petrifilm Coliform Count (CC) plate is a ready-made culture medium system which contains modified Violet Red Bile (VRB) nutrients, a cold-water-soluble gelling agent, and a tetrazolium indicator that facilitates colony enumeration.

5B3.4.1.2 Luria Bertani agar

Luria Bertani agar (15.5g, Difco) was made up to a final volume of 1000 ml with reverse osmosis water and autoclaved (121°C, 15 min).

BUFFER COMPOSITIONS; CHAPTER 5B (5B3.4.1.2)

Buffer I: 5X stock: maleic acid (500 mM, Sigma) and NaCl (150 mM, BDH) dissolved in reverse osmosis water and adjusted to pH 7.5 with sodium hydroxide pellets (BDH).

Buffer II: 10X stock: Blocking reagent (Bochringer Manheim) dissolved in 1X buffer (10 % w/v) and stored at 20^0C. On use the stock solution was diluted with 1X buffer 1 (1% w/v) and stored at 4^0C.

Ethidium bromide solution (0.5 mg ml^{-1}): ethidium bromide (50 mg, Sigma) was dissolved in reverse osmosis water (100 ml).

Loading buffer: EDTA (0.2) and glycerol (50% v/v) adjusted to pH 8.5 to which bromophenol blue (0.05% w/v) was added

TE buffer: Tris-HCl (10 mM, pH 9.5), EDTA (1mM, pH 8.0) was adjusted to pH 8.0 and autoclaved (121°C, 15 min).

Agarose gel (1.5%); 3 g agarose was added into 160 ml TE buffer and brought to the boil to dissolve. Ethidium bromide was added

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