# EFFECT OF CEREAL TYPE AND COMMENSAL BACTERIA ON AVAILABILITY OF METHIONINE SOURCES AND INTESTINAL PHYSIOLOGY IN PIGS

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**ABSTRACT**

An investigation was conducted to determine the contribution of the gastrointestinal microbiota to variation in bioefficacy of methionine sources and the interrelationship between intestinal microbiota and cereal grain type with respect to gastrointestinal physiology. Apparent gastrointestinal absorption of DL-methionine (MET) and 2-hydroxy-4-methylthiobutanoic acid (MHA-FA), post-weaning intestinal morphology, digestive physiology, mucin dynamics and digesta flow were studied in a series of experiments using conventional and gnotobiotic pigs. At 14 d of age, sow - reared conventional (CON) pigs and isolator - reared monoassociated gnotobiotic pigs (EF) were weaned to corn or wheat/barley based diets supplemented with MET or MHA-FA. At 24 d of age, after an overnight fast, pigs were fed experimental diet supplemented with $10^7$ Bq of either $^3$H-L-MET or $^3$H-L-MHA-FA per kg of feed and chromic oxide (0.5% wt/wt). Pigs were killed 3 h after consuming the meal to collect digesta and tissue samples from the stomach and along the small intestinal (SI) length. Conventional pigs fed a wheat/barley-based diet had increased ($P < 0.05$) total aerobes, whereas supplementation with MHA-FA increased ($P < 0.05$) total aerobes and lactobacilli populations in proximal SI. Among the gnotobiotic pigs, 8 pigs (2 isolators) were monoassociated with a bacteria closely related to *Providencia* spp. and 16 pigs (4 isolators) were monoassociated with *Enterococcus faecium (EF).* Species of bacterial contaminant and diet composition did not affect residual MET or MHA-FA in digesta. Decreased ($P < 0.05$) apparent residual MET in digesta compared with MHA-FA in CON but not monoasscoiated pigs, along with significantly higher ($P<0.05$) MET associated radioactivity at 5% SI tissue suggested that microbial metabolism of MHA-FA increases

its retention in small intestinal digesta and contributes in part to the lower bioefficacy of MHA-FA compared to MET. A comparison of CON and EF pigs showed that wheat/barley diets increased digesta viscosity ($P < 0.01$) and proliferating cell nuclear antigen (PCNA) expression ($P < 0.001$) and tended to decrease ($P < 0.07$) aminopeptidase N (APN) activity. Monoassociation decreased ($P < 0.01$) body weight, relative spleen weight, crypt depth, PCNA expression, caspase-3 activity, sucrase expression, total goblet cells in crypts and mucin gene expression and increased ($P < 0.01$) relative SI length, digesta viscosity, villus height, APN and sucrase activity. Interactive effects between cereal grain type and microbial status were observed only as trends ($P < 0.1$) for PCNA, Muc2, APN and sucrase suggesting these effects were mediated indirectly through microbial changes. Decreased % retained chromic oxide in digesta at all SI locations and no chromic oxide at 95% SI length in monoassociated pigs indicated slower small intestinal transit of digesta in monoassociated pigs. We successfully developed the chromic oxide microassay for estimating chromic oxide in $1/20^{th}$ of original sample size (2.0 g). Results of this study indicate that microbial metabolism of MHA-FA contributes in part to the lower bioefficacy of MHA-FA compared to MET. Monoassociation had major effects on intestinal physiology whereas limited indirectly mediated effects of cereal type were observed suggesting no major influences of cereal grain type during the short early post-weaning phase.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| A: G | activity: gene expression |
| ADF | acid detergent fibre |
| ANOVA | analysis of variance |
| APN | aminopeptidase N |
| ATP | adenine triphosphate |
| BB | brush border |
| BSA | bovine serum albumin |
| cAMP | cyclic adenosine monophosphate |
| casp3 | caspase 3 |
| cDNA | complementary deoxyribonucleic acid |
| CFU | colony forming units |
| CMC | carboxymethylcellulose |
| CON | conventional |
| Cpm | counts per minute |
| cpn60 | chaperonin 60 |
| cpnDB | chaperonin 60 database |
| DF | dietary fibre |
| DM | dry matter |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleotide triphosphate |
| EC | *Escherichia coli* |
| EDTA | ethylene diamine tetra acetate |
| EML | epithelial mucosal layer |
| GAPDH | glyceraldehyde-phosphate dehydrogenase |
| GF | germfree |
| GIT | gastrointestinal tract |
| GLM | general linear models |
| GLP-2 | glucagon like peptide-2 |
| GLUT2 | glucose tranporter-2 |
| HPLC | high pressure liquid chromatography |
| IgG | immunoglobulin G |
| IL-1β and IL-6 | interleukin 1β and interleukin-6 |
| LF | *Lactobacillus fermentum* |
| LI | large intestine |
| LPH | lactase phlorizin hydrolase |
| LPS | lipopolysaccharide |
| MA | monoassociated |
| MET | methionine |
| MHA-FA | 2-hydroxy-4-methyl thiobutanoic acid |
| MHC | major histocompatability complex |
| mRNA | messenger ribonucleic acid |
| NDC | nondigestible carbohydrates |
| NDF | neutral detergent fibre |
| NF-κB | nuclear factor kappa B |

| | |
|---|---|
| NSC | non-starch carbohydrates |
| NSP | non-starch polysaccharides |
| ODC | ornithine decarboxylase |
| PBS | phosphate buffered saline |
| PBS-T | phosphate buffered saline + Tween |
| PCNA | proliferating cell nuclear antigen |
| PCR | polymerase chain reaction |
| PDV | portal-drained viscera |
| PMSF | phenylmethylsulfonyl fluoride |
| PSA | polysaccharide A |
| qPCR | quantitative PCR |
| RNA | ribonucleic acid |
| RT-PCR | reverse transcriptase polymerase chain reaction |
| SCFA | short chain fatty acid |
| SE | standard error |
| SGLT-1 | sodium glucose cotransporter |
| SI | small intestine |
| TNF-α | tumour necrosis factor- α |
| UT | universal target |

## 1.0 INTRODUCTION

Dietary carbohydrates constitute a major part of swine diets but with diverse composition originating from various cereal grain sources (Bach Knudsen, 1997). Considering the purported health benefits of dietary fibre and whole cereal grains (Slavin, 2003), dietary strategies promoting gastrointestinal health are becoming more popular. Feeding diets based on corn, wheat or barley as carbohydrate source alters gut microbial population composition (Hill *et al.*, 2005). These enteric bacteria play an important role in modifying the structure, biochemistry and physiology of the gastrointestinal tract of the host. Gastrointestinal tract morphology and physiology are altered by enteric bacteria which in turn could affect the digestion and absorption of nutrients as well as host defenses against pathogens.

Another factor generating increased interest in the role of the commensal gastrointestinal microbiota in livestock health and performance is consumer concern regarding antibiotic resistance in zoonotic pathogens arising from sub-therapeutic use of antibiotics (Ratcliff, 2000). Since the growth promotion associated with sub-therapeutic antibiotic use has at least in part been attributed to modification of the gastrointestinal microbial composition, efforts in search of alternatives to antibiotics have considered alternative means of modifying intestinal microbial composition. While these efforts have been limited by a lack (but improving) of understanding of the desirable characteristics of a health/growth-promoting intestinal microbiota, approaches have included management practices, feed ingredient selection, prebiotics and probiotics.

Methionine is supplemented as DL-methionine (DL-MET) or methionine hydroxy-analogue - free acid (MHA-FA), chemically identified as DL-2-hydroxy-4-

methylthiobutanoic acid in pig diets. A significant amount of literature has demonstrated that DL-MHA-FA has lower bioefficacy as a methionine source than DL-MET (Jansman *et al.,* 2003). Various mechanisms might be responsible for this reduced bioefficacy including variation in efficiency of intestinal transport, conversion of MHA-FA to methionine and metabolism by intestinal microbiota. In chickens, there is evidence that intestinal bacteria metabolize MHA-FA making it less available to the bird (Drew *et al*., 2003). However, there is no direct evidence to support this in the pig where gastrointestinal physiology, digesta passage and microbial populations are considerably different than in chicken.

Availability of a gnotobiotic pig model in our laboratory provides us a unique opportunity to study the interactions of dietary components, intestinal microbial composition and host physiology. Earlier work has reported that early colonizing commensal bacteria differentially affect digestive function of the host and the proliferative and apoptotic activity which was associated with the induced expression of antinflammatory cytokine and death ligands (Shirkey et al., 2006; Willing and Van Kessel, 2007). Inclusion of fibre in diet produces changes in intestinal physiology and digestion of nutrients (Wenk, 2001). Furthermore, studies in other laboratories have suggested that physiological responses to dietary fibre are mediated indirectly by gut microbiota (Komai *et al.,* 1982; Sakata, 1986). With this background, this thesis was developed based on the exploration of two hypotheses. Firstly, we hypothesized that intestinal microbial metabolism contributes to variation in bioavailability of methionine sources in the pig. Secondly, we hypothesized that cereal grain type differentially affects

gastrointestinal physiology and that these effects are mediated by changes in gut microbiota.

To test these hypotheses we provided gnotobiotic and conventional pigs diets based on corn or wheat and barley. Furthermore diets were supplemented with one of two methionine sources, DL-methionine and DL-MHA-FA. Research objectives included the determination of the effects of cereal type and microbial status on apparent retention of the two methionine sources in intestinal contents and determination of the effect cereal type and microbial status on digesta flow, intestinal physiology and mucin dynamics.

## 2.0. LITERATURE REVIEW

### 2.1. Composition of feed ingredients

Cereal grains and their by-products are the major feed ingredients in pig diets worldwide (FAO, 2001). However, the cereal sources used vary from region to region which influences the carbohydrate fraction of feed (Bach Knudsen, 1997). Carbohydrates are the main constituents of plant ingredients with starch as the major carbohydrate. The non-starch carbohydrates (NSC)/nondigestible carbohydrates (NDC) include non-starch polysaccharides (NSP) and other carbohydrates such as pectins and glycoproteins that make up plant cell walls. These polysaccharides are indigestible by vertebrate enzymes and are the major constituents of dietary fibre. Waxes, cutin, lignin and even resistant starch are also considered to be a component of dietary fibre (Theander and Aman, 1979) while others also include phenolic esters, proteins and gums from feed additives (Selvendran, 1984).

Despite extensive research on the significance of dietary fibre (DF) in monogastric nutrition over the last quarter of the 20$^{th}$ century, no universal agreement over the definition of DF has been reached (De Vries *et al.*, 1999). In terms of practical application of dietary fibre concept in animal nutrition, the physiological definition of dietary fibre by Theander and Aman (1979) seems to be the most appropriate. Theander and Aman (1979) define DF as ‘a group of polysaccharides and other polymers in plant material in the diet which are neither digested by normal secretions nor absorbed in the upper gastrointestinal tract’. So accordingly, the carbohydrate constituents of DF are the cell wall non digestible carbohydrates which include cellulose, hemicelllulose, pectic substances and also other non-structural plant substances (Baker *et al.,* 1979). The term

non-starch polysaccharide (NSP) covers a large variety of polysaccharide molecules excluding starch. The NSPs fall into three main groups including cellulose, non-cellulosic polymers and pectic polysaccharides (Bailey, 1973). Of all the dietary plant polysaccharides ingested by vertebrate animals only starch can be digested by host enzymes. A substantial portion of this escapes small intestinal digestion and enters the large intestine where it undergoes bacterial fermentation.

### 2.1.1. Chemical structure of dietary fibre components in cereal grains

Polysaccharides are polymers of 11 or more monosaccharides joined through glycosidic linkages (Theander *et al.,* 1989). The monosaccharides commonly present in cereal cell walls are five carbon sugars (pentoses) such as L-arabinose and D-xylose and six carbon sugars (hexoses) as D-glucose, D-galactose and D-mannose. The acidic sugars are D-galacturonic acid, D-glucuronic acid and its 4-O-methyl ether. These have a carboxyl group on C-5 of the ring structure. The deoxyhexoses are rhamnose and fucose with a methyl group on C-5.

The main polysaccharides of plant cell walls are cellulose, arabinoxylans, mixed linked β-(1-3) (1-4)-D-glucans, xyloglucans and rhamnogalacturonans (Selvendran, 1984). The NSP in cereal grains are composed predominantly of arabinoxylans (pentosans) and beta-glucans which form an amorphous matrix around cellulose microfibrils closely associated with glucomannans (Selvendran, 1984). Pectic polysaccharides represent a complex group of polysaccharides in which D-galacturonic acid is a major component with small amounts of sugars along with some uronic acids as methyl esters. Only small amounts of pectic polysaccharides are found in the stem and

leaves of cereal grains. Lignin is a polymer of phenyl propane and is not digested in the gastrointestinal tract. The relative proportion of NSP content varies in different cereal ingredients (Table 2.1). Corn and sorghum contain very low levels of NSP, whereas wheat, rye and triticale contain substantial amounts of both soluble and insoluble NSP. The main soluble NSP in these grains are arabinoxylans, whereas barley and oats have mostly β-glucans. In barley, β-(1-3), (1-4) glucans make up 30-60g/kg DM (Fincher and Stone, 1986). In rye, arabinoxylans are present at around 100g/kg (Chesson, 1995). In wheat arabinoxylans are largely located in the cell walls of aleurone layer and are present around 50-80 g/kg (Posner, 2000). Arabinoxylans in corn vary from 43-66g/kg, mostly present in bran and are largely insoluble (Choct and Annison, 1990).

**Table 2.1. The types and concentration (g/kg dry matter) of polysaccharides, fibrous components and lignin in some cereal grains[1]**

| | Wheat | Barley | Corn | Soybean meal | Oats | Triticale | Rye |
|---|---|---|---|---|---|---|---|
| Starch | 651 | 587 | 690 | 27 | 468 | na | 613 |
| Cellulose | 20 | 43 | 22 | 62 | 82 | 25 | 16 |
| NCP[2] | | | | | | na[4] | |
| Soluble | 25 | 56 | 9 | 63 | 40 | | 42 |
| Insoluble | 74 | 88 | 66 | 92 | 110 | | 94 |
| Arabinoxylans | 81 | 79 | 52 | na | na | 108 | 89 |
| b-glucans | 8 | 43 | na | na | na | 17 | 20 |
| T-NSP[3] | 119 | 187 | 97 | 217 | 232 | na | 152 |
| Lignin | 19 | 35 | 11 | 16 | 66 | na | 21 |
| Dietary fibre | 138 | 222 | na | 233 | 298 | na | 174 |

[1]Adapted from Choct (1997) and Chesson (1995)

[2]Non cellulose polysaccharides

[3]Total Non-starch polysaccharides

[4]Not available

## 2.2. Physico-chemical properties of cereal fibre components and implications

The physical and chemical associations of fibre components with non-carbohydrate fractions in plant cell wall have a large influence on the use of plant sources as an animal feed ingredient. These associations can influence the physico-chemical properties of dietary fibre components and consequently their action in the gastrointestinal tract of pigs affecting availability of nutrients to the animal.zzz

### 2.2.1. Physical properties

Different dietary fibre compounds have different chemical composition and thereby have special physical properties that affect the efficiency of digestive functions in different ways (Bach Knudsen, 2001). The major physico-chemical properties of dietary fibre are the cation exchange capacity, hydration properties, viscosity and organic compound absorptive properties (Bach Knudsen, 2001).

Water holding/binding capacity (WHC/WBC) reflects the ability of a fibre source to incorporate water in its matrix. It is determined by the structure of the molecules, pH and electrolyte concentration of the surrounding fluid. The majority of polysaccharides form viscous solutions when dissolved in water (Morris, 1992). The viscosity is dependent on the molecular weight and concentration of the polymer. Branched structure of arabinoxylans allows them to absorb water and form viscous solutions in digesta of poultry (Chesson, 1995) and pigs (Johansen, 1996). The solubility of NSP also affects the physical properties and thereby has physiological significance. With high soluble fibre in diet, the viscosity of digesta is increased and pH is stabilized at low levels (Wenk C, 2001).

### 2.2.2. Digesta transit

High fibre content of digesta is thought to increase peristaltic action and therefore, reduces the passage time in the small and large intestine giving lesser time for absorption of nutrients. However different studies reported variable effects of fibre on digesta passage rate. High soluble fibre content in the diet will cause more water binding thus increasing volume of digesta thereby reducing transit time in stomach (Wenk, 2001). Jorgenson *et al.,* (1996) reported that pigs fed high dietary fibre had a five to six fold increase in the flow rate of digesta through the terminal ileum. In another study, both guar gum and cellulose were found to reduce digesta passage rate in growing pigs (Owusu-Asiedu *et al.,* 2006). Wilfart *et al.,* (2007) reported no influence of wheat bran content of diet on gastric emptying, however, decreased mean retention time of solid phase in the small intestine was observed.

Transit in the hindgut is generally reported to be increased by dietary fibre content (Wenk, 2001). Another study in pigs found that dietary NSP stimulate digesta passage through the GIT especially the large intestine (Van Leeuwen and Jansman, 2007). Le Goff *et al.,* (2002) suggested that fibre supplementation exerts a direct physical action in the hind gut of pigs stimulating propulsive colonic motility due to greater bulk of digesta. Fukomoto *et al.,* (2003) reported that SCFAs stimulate colonic transit in rats.

### 2.2.3. Nutritional influences

Increased viscosity of digesta associated with soluble NSP in the diet has been shown to decrease digestibility of starch, fat and proteins in broiler chickens (Smits and Annison, 1996) and to affect energy value of cereal grains in pigs (Bach Knudsen and Hansen, 1991). Also s reduction of protein, amino acids and mineral digestion occurs when fibre is added to diet of pigs (Eggum, 1995). High gut viscosity decreases rate of diffusion of substrates and digestive enzymes hindering their effective interaction at the mucosal surface in rats (Ikegami *et al.,* 1990). Furthermore, soluble NSPs may interact with glycocalyx of the intestinal brush border and thicken the rate-limiting unstirred water layer of the mucosa (Johnson and Gee, 1981). Soluble NSPs also change gut functions by modifying endogenous secretions of water, proteins, electrolytes and lipids (Angkanaporn *et al.,*1994) and increasing digestive secretions in pigs (Dierick *et al.,* 1989).

NSP can also bind nutrients and digestive enzymes and some regulatory proteins in the gut. In addition, certain NSP can bind bile salts, lipids and cholesterol (Vahouny *et al.,* 1981) causing their continuous drainage from the intestine. These effects could lead to major changes in the digestive and absorptive dynamics of the gut leading to poor nutrient assimilation by the animal.

An increased intake of dietary fibre could reduce total tract digestibility and leads to increased proportion of energy being digested in the large intestine (Jorgenson *et al.,* 1996; Just *et al.,* 1983). This leads to less energy being absorbed as monosaccharides from the small intestine and more energy being provided as SCFA and lactic acids.

### 2.2.4. Physiological implications

Major effects of NSP are linked with the viscous nature of polysaccharides. These effects are mediated by altered ingesta passage rate, interaction with gut microbiota, modification of intestinal mucosa and changes in hormonal regulation due to altered rate of nutrient absorption (Vahouny, 1982).

Feeding diets high in fibre have been shown to alter intestinal morphology in chickens (Langhout, 1998; Iji *et al.,* 2001), rats (Southon *et al.,* 1985) and humans (Malkki and Virtanen, 2001). High fibre diets have also been shown to cause hypertrophy of gastrointestinal tract in pigs (Anugwa *et al.,* 1989 and McDonald *et al.,* 2001). Jin *et al.,* (1994) reported that feeding diets containing high levels of insoluble fibre such as wheat straw increased cell proliferation in intestinal crypts of the jejunum and colon and increased epithelial cell death rate in jejunum and ileum of growing pigs. McCullough *et al.,* (1998) reported that feeding a diet containing a mixture of fermentable dietary fibre sources to rats enhanced crypt fusion in the proximal colon. Any change in quantity and quality of mucus secretion may have important physiological implications (Rhodes, 1989). It was shown that neutral, acidic and sulphated mucins vary widely in various diseases in man (Smith and Podolsky, 1986). Cassidy et al., (1990) reported an increase in sulphomucins and total mucins in small intestine and colon of conventional rats fed with many soluble or insoluble dietary fibres postulating that the changes in mucin composition contributed to protective effects of dietary fibre in the incidence of colon cancer. Dietary supplementation with fibre is reported to increase the secretory activity of goblet cells in rats (Vahouny *et al.,* 1985; Satchithanandam *et al.,* 1990 and Satchithanandam *et al.,* 1996). Increased turnover of jejunal mucins was observed in pigs

fed diets based on fibre (More *et al.*, 1987). Satchithanandam *et al.,* (1996) observed that addition of 5% citrus fibre in diet of rats caused increased concentration of luminal mucin in the stomach (3.5 fold) and small intestine (2-fold). Increased number of mucous cells was reported in small and large intestine of rats fed fermentable fibre (McCullough *et al.*, 1998). Christelle Piel *et al.*, (2005) reported 30% increase in the total number of goblet cells/villus and 56% higher crude mucin output in piglets fed carboxymethyl cellulose (CMC) at 40 g/kg compared to cellulose based control diet.

Apart from age and diet, microbial flora is known to affect the chemical composition of intestinal mucins. Sharma and Schumacher (1995) observed differences in the relative proportion of acidic and neutral mucins in the goblet cells of rats fed different diets. Germ-free rats fed diets based on cereal fibre showed neutral mucins predominantly. Surface mucus staining was more intense in conventional than germ-free rats. Fontaine *et al.,* (1996) reported that germ-free rats fed a diet supplemented with inulin at 100 g/kg had increased amount of neutral mucins in caecal content and higher sulfated mucins in the colonic contents. It also increased neutral mucins and sulphomucins in caecal mucosa and decreased the amount of sialomucins. In heteroxenic rats having human flora, inulin decreased acidic mucins and increased sulphated mucins in caecal contents and mucosa. Kleesen *et al.,* (2003) demonstrated that both intestinal bacteria and diet have an influence on mucin composition of epithelial mucous layer. Bacterial association of germ-free rats elevated acidic mucins in their colon. Among the microbiota associated groups, the group fed fructan-inulin based diet had sulfomucins predominantly compared to sialomucins in commercial diet fed group. Bacterial

colonization of germ-free rats also caused an increase in neutral mucin in distal jejunum and colon.

### 2.3. Fermentative properties of dietary fibre

Diet is one of the most influential environmental factors affecting the GIT microbiota. The chemical composition of digesta as determined by the chemical composition of the diet is one of the major determinants of the makeup of GIT microbiota (Apajalahti and Bedford, 2000). Microbial density increases to $10^8$-$10^9$/gm of digesta in distal small intestine presumably due to slow passage rate and large amount of digesta (Jensen and Jorgensen, 1994). This microbiota is responsible for digestion of a substantial portion of NSP (Fadel *et al.,* 1989). The slower passage rate of digesta in large intestine creates an ideal physico-chemical environment with beneficial pH, temperature and humidity for bacterial proliferation and fermentation. Several studies report that nondigestible nutrients such as soluble dietary fibres, resistant carbohydrates (starch) and resistant protein modify the gastrointestinal physiology, mainly through fermentation in the large bowel (Campbell *et al.,*, 1997 and Roberfroid and Delzenne, 1998). Short chain fatty acids have been proposed to be the mediators of the systemic effects of such nutrients. There is not much information available on the involvement of other

fermentation/metabolic products, namely polyamines and bioactive peptides in the physiological effects of nondigestible carbohydrates.

### 2.3.1 Digestion of dietary fibre

Cereal dietary fibrous components can not be digested by host digestive enzymes. The enzyme activities in the small intestine are specific for α-linked units and are inactive against β-linked glucose polymers in dietary fibre (Chesson, 1987). However, proximal small intestine has some fibre degrading bacteria which can disrupt cell walls (Graham *et al.,* 1986) potentially affecting digestibility of nutrients. Apparent digestibility of fibrous components in swine diets is quite variable and may range from 0 to 97% (Rerat, 1978). Digestion of DF is commonly believed to occur primarily in the large intestine yet 10-62% of NSP disappear in upper intestine (Fadel *et al.*, 1989) suggesting considerable small intestinal bacteria-mediated digestion. In the large intestine, digestion is accomplished either by enzymes from small intestine or by microbial fermentation. The main sites for DF degradation are the caecum and proximal colon (Gdala *et al.,* 1997 and Glitso *et al.*, 1998). Depending on the origin, dietary fibre is digested to various degrees. The amount of cereal NSP reported to be digested in the large intestine varies from 48-95% (Bach Knudsen *et al.,* 1993; Gdala *et al.,* 1997, Jorgensen *et al.,* 1996). Total tract cereal cellulose digestibility varies from 2-84% depending on its source (Bach Knudsen, 2001). Pectic substances and hemicellulose are digested to a greater extent than cellulose. β-glucans appear to be completely digested in the gastrointestinal tract (Bach Knudsen *et al.,* 1993b). This is because of partial depolymerisation of β-glucans making those readily available sources for microbiota in the large intestine (Johansen *et al.,* 1997). For soluble

NSP, swelling and high WBC cause increased surface area for microbial action encouraging degradation. Cereal diets containing linear and relatively soluble β-glucans show higher digestibility in the range of 17-73 % in oats (Bach Knudsen *et al.*, 1993a, b) and 70%-97% in barley (Graham *et al.*, 1986, 1989). Branched chain arabinoxylans from wheat, rye and oats have digestibility values ranging from 8% to 19% in wheat, oat or rye products (Bach Knudsen *et al.*, 1991 and Glitso *et al.*, 1998).

### 2.3.2. Bacterial fermentation products

The main end products of microbial fermentation are lactic acid, short chain fatty acids and various gases (hydrogen, carbon dioxide and methane) (Bach Knudsen *et al.*, 1991). Organic acids are well known examples of luminal factors that affect gut functions. The SCFAs are absorbed in the large intestine and contribute to the energy supply of the pig (Fonty and Gouet, 1989). The three major SCFA produced in the colon are acetate, propionate and butyrate. The SCFAs are absorbed rapidly by passive diffusion in the caecum and colon as suggested by decreasing SCFA concentration from the caecum and proximal colon to the distal colon in pigs (Bach Knudsen *et al.*, 1991; Ruppin *et al.*, 1980). Individual SCFAs are ultimately taken up by different organs and have different metabolic fates. Acetate is carried to the liver and then taken up by peripheral tissues such as skeletal and cardiac muscles (Cummings and Farlane, 1997) and can also be used by adipocytes for lipogenesis (Bergman, 1990). Butyrate is used primarily by colonic epithelium which derives 60-70% of its energy from butyrate (Roediger, 1980; 1982; Rerat *et al.*, 1987). Propionate is transported to liver, converted to glucose for gluconeogenesis in ruminants (Bergman, 1966) but not much is known about

its role in other species. The mean supply of net energy from SCFA to net energy for maintenance is about 15-24% for growing and finishing pigs (Dierick *et al.,* 1989 and McBurney and Sauer, 1993) and 5-10% for man (Nordgaard *et al.,* 1995).

Apart from nutritional value, SCFAs have important effects on other aspects of gut physiology. SCFA, particularly butyrate, have been implicated to have a role in human and animal health (Skata and Inagaki, 2001). SCFAs also stimulate epithelial cell proliferation and differentiation (Skata, 1987 and Kripke *et al.,* 1989). Epithelial cell proliferation was found to be less active in small and large intestine of germ-free rats than in conventional animals (Komai *et al.,* 1982 and Sakata, 1987). Absence of intestinal proliferation in germ-free rats fed diets supplemented with fibre has shown the importance of intestinal microbiota in intestinal proliferation (Komai *et al.,* 1982; Goodlad *et al.,* 1989). Use of non-fermentable dietary bulk (Kaolin) neither stimulates epithelium nor modifies the effect of SCFA (Sakata, 1986) suggesting that it is not likely that physical abrasion stimulates epithelial proliferation. However, in one study, addition of high viscosity non fermentable carboxymethyl cellulose (CMC) at 40 g/kg air dried diet; 400-800mPa/s for a solution of 20g/l) to cooked rice diet for weanling piglets led to decreased villus length and increased crypt depth (McDonald *et al.,* 2001). SCFAs are the predominant anions in the colon and stimulate the resorption of water and sodium (Hume, 1997). In an acidic environment these are capable of inhibiting the growth of some intestinal bacterial pathogens such as *Escherichia coli, Clostridium difficile* in pigs (Prohaska, 1986; May *et al.,* 1994). Systemic SCFAs have also been reported to increase plasma GLP-2, mRNA abundance of ileal proglucagon, GLUT2, SGLT-1 and increased expression of early response genes involved in the control of the cell cycle and

proliferation (Tappenden *et al.,* 1997 and Tappenden and McBurney, 1998). Short chain fatty acids have also been looked into for their role in influencing gene expression in human colonocytes (Basson *et al.,* 2000).

### 2.3.3 Possible influences on microbiota

One of the most important factors influencing microbial population and activity in the gastrointestinal tract is the type diet including its structural composition, solubility (Hogberg and Lindberg, 2004) and amount of substrate available (McFarlane and Cummings, 1991). Source of dietary fibre influences the digestion site, gut environment and thereby conditions for the proliferation of microbes in the gastro-intestinal tract (Hogberg and Lindberg, 2004). Cereal grain fibre components are important energy substrates for microbes. Inclusion of these in the diet shifts the enzymatic digestion of α-glycosidic linkages in the small intestine to β-glycosidic linkages fermentation by microbiota in the large intestine (Chesson, 1987; Bach Knudsen and Jensen, 1991). Feeding a high fibre diet containing wheat bran (102g NSP/kg feed) or oat bran (93gNSP/kg feed) to pigs increased the microbial activity in GIT by 5.5 times as measured by ATP concentration (Jorgenson and Just, 1988). The maximum microbial activity was observed at the end of the caecum or in the proximal part of the small intestine compared to the end of the small intestine in low fibre diet fed pigs. Increased (5-9 times) carbon dioxide and methane production in GIT of pigs fed high fibre diet also reflected increased microbial activity. Jensen and Jorgenson (1994) observed greater microbial activity in the stomach, last third of small intestine, caecum and proximal colon based on higher bacterial counts, ATP concentration, adenylate energy charge and low

pH in pigs fed pea fibre and pectin. Increased numbers of cellulolytic bacteria were found in the colon of growing pigs and adult animals by prolonged feeding of high fibre diets (Varel, 1987).

Higher (2-3 log) anaerobic microbial counts were reported in the ileum of birds fed rye or pectin enriched diets compared to corn-soy diets (Wagner and Thomas, 1978). Varel *et al.,* (1982) observed an initial suppression of gastrointestinal tract microbiota in pigs when exposed to high-fibre diet (50% alfalfa meal). However, after prolonged feeding (17 weeks) total bacterial counts as well as the number of cellulolytic bacteria increased significantly in lean genotype pigs. Pigs fed diets based on cooked white rice and different carbohydrate sources showed different ATP concentration in the digesta (Pluske *et al.*, 1998). The pigs fed diets containing sources of resistant starch and guar gum+ starch had higher conc. of ATP in the caeca than pigs fed all other diets.

There are few studies studying the effects of various dietary cereals on the gastrointestinal microbiota composition in pigs. Drew *et al.,* (2002) studied the influence of feeding diets based on corn, wheat or barley as main carbohydrate source to weaned pigs and reported that the bacterial populations were significantly related with ADF and NDF contents of the diets. In ileum, the barley based diet lowered the number of Enterobacteria and increased Lactobacilli compared to the corn diet. The wheat diet also increased the number of *Lactobacillus spp*. In the caecum barley based diet increased number of total anaerobes, *Lactobacillus spp*. and *Bifidobacterium spp*. compared to the corn diet. The wheat diet increased the number of *Bifidobacterium spp*. and decreased total aerobes and Clostridium spp. compared to barley diets. This variation in bacterial composition with different cereal grains in diet was further confirmed using a

chaperonin-60 based phylotype profiling of intestinal contents and quantitative PCR (Hill *et al.,* 2005).

Increased bacterial counts were observed except for *Bifidobacteria spp*. in grower pigs fed diets containing high NSP (Owusu-Asiedu *et al.,* 2004). Lactobacilli, Bifidobacteria, Clostridia, Streptococcus and Enterobacteria counts were highest for pigs fed 14% NSP (7% guar-gum+ 7% cellulose).

### 2.4. Microbiota and its significance to host physiology

The gastrointestinal tract is inhabited by the largest, most complex and dynamic collection of microorganisms in nature. In fact, the prokaryotic cells in the body outnumber human cells by a factor of 10 (Savage, 1977). Microbial densities in the proximal and middle small intestine are relatively low but increase drastically in the distal small intestine ($10^8$ bacteria/ml of luminal content) and colon ($10^{11}$-$^{12}$/g) (Savage, 1977; Swords *et al.,* 1993) and comprise an estimated > 500 different bacterial species (Moore *et al.,* 1987; Hill *et al.,* 2002 and Leser *et al.,* 2002). The intestinal ecosystem is shaped by the interactions between its microbes, intestinal epithelium, mucosal immune system, microvasculature and enteric nervous system (Gordon *et al.,* 1997). Host-bacterial interactions play an important role in modifying the structure, chemistry and physiology of the GIT (Hooper and Gordon, 2001). The gut microbiota provides the host with nutritional, proliferative and protective benefits. But all these benefits have a cost involved with them in form of competing for nutrients, generating toxic compounds, increasing host maintenance requirements due to altered morphology and stimulating intestinal inflammatory responses.

### 2.4.1 Microbial contributions to the host

Commensal/symbiotic relationships of host and bacteria are based on metabolic capabilities of one or both partners to exploit an otherwise unavailable nutrient. Two complimentary mechanisms seem to work for benefiting the host. Commensal microbiota is responsible for metabolizing dietary substances to nutrients that can be absorbed and utilized by the host. Also the presence of microbes can alter the intrinsic metabolic activities of host cells resulting in more efficient nutrient absorption and assimilation (Hooper *et al.*, 2002).

Mammals are well equipped to absorb monosaccharides and hydrolyze certain disaccharides and starch, but are limited in their ability to hydrolyze and utilize other polysaccharides. Carbohydrates represent the biggest fraction accounting typically for 300-500g/kg of dry solid passing from the small to the large intestine. The most active sites for dietary carbohydrate digestion are the caecum and proximal colon (Canibe and Bach Knudsen, 1997; Glitso *et al.,* 1998). Because of the anaerobic environment, carbon and monosaccharides released from carbohydrate polymers are converted to pyruvate via glycolysis resulting in net production of ATP. The prominent end products of bacterial fermentation in the gut are short chain fatty acids.

Gut commensals protect the host from pathogens by suppressing colonization by newly entering bacteria and pathogenic microbiota (Van der Waaij *et al*., 1971). The phenomenon of competitive exclusion works as commensals compete for epithelial adhesion sites, nutrients, stimulate immune system and intestinal motility, secrete anti-microbial compounds such as organic acids (Ewing and Cole, 1994; Kelly and King,

2001). Commensal bacteria can also inhibit inflammation (Kelly *et al*., 2004), which may be protective to the host as excessive inflammation can lead to disruption of the epithelial barrier.

Gastrointestinal microbiota also contribute to amino acid homeostasis particularly in ruminants (Virtanen, 1966). In humans 1-20% of circulating plasma lysine and threonine may be derived from intestinal microbiota (Metges *et al.,* 2000). Pigs have also been shown to absorb some of the amino acids synthesized by their microbiota (Torrallardona *et al.,* 2003). High concentrations of urea are found in the colon of germ-free rats indicating microbial role in nitrogen recycling in the gut (Moreau *et al.,* 1976) as urea is hydrolysed to ammonia by the intestinal microbiota. Yamanaka *et al.,* (1974) examined effects of mono-association of mice on protein digestion and retention. Colonization with a *Staphylococcus sp*. caused the host to increase nitrogen retention. Colonization with other species like *Bacteroides sp, E coli, Lactobacillus sp, Staphylocccus epidimis* or *St. faecalis* had no effect on nitrogen retention.

Synthesis of vitamins by gut microbes has been known for many years. Germ-free rodents need vitamin K in their diets as compared to conventionally raised (Wostmann, 1981). Also some germ-free animals require higher amounts of vitamin B (example B12, biotin, folic acid and pantothenic acid) as compared to conventional animals (Sumi *et al.,* 1977).

Microbial induction of intestinal genes that facilitate the recovery of nutrients has been shown by *Bacteroides thetaiotaomicron* colonization of germ-free mice. This monoassociation produced changes in expression of a number of host genes involved in the processing and absorption of carbohydrates e.g. increased ileal expression of

Na+/glucose co-transporter (Hedeger *et al.,* 1987). There was concerted increase in different proteins/factors involved in host's lipid absorption (Lowe *et al.,* 1998). This also changed the expression of genes involved in regulated absorption of dietary metal ions (Hooper *et al.*, 2001).

### 2.4.2 Nutrient digestion, absorption and bioavailability

Absorption of nutrients from the small intestine in essence depends on the functional capabilities of intestinal mucosa as a whole and of individual enterocytes and the conditions in the lumen. Gut commensals have direct as well as indirect effects on the activity of digestive enzymes (Corring *et al.,* 1981). The direct effects include microbial synthesis of enzymes that are comparable to enzymes of host resulting in increased total enzyme activity. Microbial synthesis of enzymes not produced by host, such as cellulase, improves the nutrient utilization by the host (Cranwell, 1968). The microbiota may indirectly influence host enzymatic activity through changes in luminal $_{P}$H, alteration of secretory and absorptive functions and changes in intestinal epithelial cell renewal rates (Bruckner and Szabo, 1984). Recently, in the gnotobiotic pig, bacteria have been shown to affect expression of digestive enzymes in a manner anti-parallel to changes in specific activity (Willing and Van Kessel, 2008).

Total enzyme activity could also decrease due to splitting or inhibition of host enzymes by microbiota. The microbiota apparently does not affect the concentration and secretion of pancreatic enzymes including trypsinogen, chymotrypsinogen, amylase, lipase, elastase and carboxypeptidase A and B (Bruckner and Szabo, 1984). But these enzymes undergo a progressive microbial inactivation. As the bacterial density increases

from small intestine to large intestine in the conventional animal, host intestinal enzymatic activity decreases accordingly (Reddy *et al.,* 1969). Luminal peptidase and disaccharidase (except lactase) activity has been found to decrease progressively from small intestine to caecum and colon in conventional pigs, rats and chickens. Whereas, in germ-free animals, activities of these enzymes in lumen of large intestine were similar to those found in small intestine (Corring *et al.,* 1981). In contrast pancreatic lipase activity appears to be identical in germ-free and conventional animals (Reddy *et al.,* 1969).

There are also inconclusive reports of increased peptidases and disaccharidases activity in small intestinal epithelial cells of germ-free rats and piglets (Bruckner and Sazbo, 1984). In germ-free animals slower epithelial renewal results in more mature epithelial cells with better developed brush borders which may result in increased concentration and activity of brush border enzymes including lactase, maltase, sucrase and alkaline phosphatase (Wostmann, 1981).

Many *in vitro* absorption studies indicated that absorption of some nutrients was impaired by the presence of microorganisms (Heneghan, 1963). The passive or carrier-facilitated transport appeared to be greater in a germ-free gut (Heneghan, 1984). Presence of longer villi and slower intestinal propulsion in germ-free animals may enhance absorption. Generally, ATP-driven active absorption seem to be unaffected by microbiota. Yokota and Coates (1982) investigated the effects of intestinal bacteria on nutrients uptake in *in vivo* chicken jejunal loop model. Germ-free, conventional and monoassociated chicks (*Streptococcus faecium*) were compared for their ability to absorb L-[$^{3}$H]-methionine and D-[$^{14}$C]-glucose. Uptake of both nutrients/g of intestinal tissue

was less in conventional and mono-associated groups compared to germ-free chicks but no differences were detected when uptake was expressed per unit length of intestine.

Microbes can hydrolyse conjugated bile salts in the intestine and hydroxylate primary bile acids (Andrieaux *et al.,* 1989). This microbial metabolism of bile acids was reported to impair the host's ability to absorb lipids and could modulate the absorption rate of fat soluble compounds. The microbiota not only affects the absorption of fatty acids but also the type of dietary and endogenous fatty acids excreted in faeces (Bruckner and Szabo, 1984).

Several observations have suggested that gut microbiota could be affecting nutrient bioavailability by affecting enzymatic digestion and absorption by intestinal mucosa. Intestinal bacteria are capable of hydrogenating unsaturated fatty acids thereby, may decrease intestinal fat absorption. Cobb *et al.*, (1991) examined the role of gut microbiota in ascorbic acid catabolism using conventional and germ-free guinea pigs and reported that hepatic decarboxylation and gut microbiota, in tandem contributed to ascorbic acid decarboxylation. Grolier *et al.,* (1998) observed that the bioavailability of both α and β-carotene was improved when the intestinal microbiota was absent or partially destroyed in rats. The bioavailability of chlorogenic acid, a polyphenol in human diets with antioxidant and anticarcinogenic properties, was shown to depend largely on its metabolism by the gut microbiota (Gonthier *et al.,* 2003).

Several studies have tried to determine the effect of microbiota on host's protein status. The results have consistently indicated that the digestion of protein in the small intestine of germ-free and conventional animals is very similar but there are significant differences in the lower bowel (Bruckner and Szabo, 1984). Increased concentration of

urea, amino acids, mucoproteins and peptides were observed in caecal contents of germ-free rats and chickens compared to conventional counterparts (Bruckner and Szabo, 1984). This was suggested to be due to production of microbial proteolytic enzymes resulting in an increase in the host's ability to utilize undigested feed residues and endogenous nitrogenous compounds. Microbiota could utilize nitrogen available in form of dietary amino acids, endogenous amino acids, and urea and also from non-protein-nitrogenous compounds like urea, purines, pyrimidines, polyamines and amino sugars etc. The contribution of each of these sources to microbial protein is not clear. Depending on the maximum contribution from dietary amino acids or urea and other non-protein nitrogen compounds, microbial *de novo* amino acid synthesis could be deleterious or beneficial to the host (Fuller, 1998).

Also the GIT microbiota competes with host enzymes for substrates. Non protein nitrogen compounds are degraded and incorporated into bacterial protein (Braun and Campbell, 1989; Mead, 1989). Earlier studies compared absorption of MET and MHA-FA (Figure 2.1) in conventional chicken by determining the amount of each compound remaining in digesta following intestinal passage.


Methionine Methionine hydroxy-analogue (MHA-FA)

**Figure 2.1. Structure of methionine and methionine hydroxy-analogue**

It was reported that about 2.8% of MET and 9.2% of MHA-FA remained in the digesta after intestinal passage (Rostagno and Barbosa, 1995). This approach, however, did not account for possible conversion of MET and MHA-FA to other compounds by bacterial metabolism resulting in an overestimate of absorption. Other studies in chickens (Esteve Garcia and Austic 1993; Lingens and Molnar, 1996; Maenz and Engele Schaan, 1996a) used radiolabelled MET and MHA-FA to overcome this problem and reported that 10-20% of the original radiolabelled MHA-FA activity in the feed was present in the distal sections of the small intestine compared to 3.5-5% for MET. Drew *et al.*, (2003) reported that the competition for nutrients between the host and gut bacteria can have significant effect on nutrients availability to the host. It was found that the residual $^{3}H$ activity in ileum of conventional chickens was higher (10-15%) compared to the germ-free group (4-8%) demonstrating that intestinal bacteria significantly reduce the apparent absorption of MHA-FA from the intestinal tract of broiler chickens.

Liu *et al.*, (2003) reported significant inter- and intra-species differences in the metabolism of amino acids by lactic acid bacteria. Some amino acids were utilized, whereas others were produced. Some species like *Lactobacillus brevis* and *L. fermentum* were the most metabolically active whereas Leuconostocs were the least active. *In vitro* bacterial metabolism of methionine and MHA-FA was studied by Hegedus *et al.,* (1993). They reported that none of the three lactic acid bacteria they studied *(Lactobacillus plantarum, L. casei* and *Leuconostoc mesenteroides)* could utilize MHA-FA though all could use methionine.

### 2.4.3 Mucus secretion and composition

Apart from age and diet, presence of gut microbiota also alters the mucin secretion and composition. Germ-free rats and new born children were shown to excrete large amounts of mucin with faeces as compared to conventional rats and healthy adult humans (Carlstedt-Duke *et al.,* 1986). Wostmann (1996) indicated 35-50% more goblet cells in germ-free rats, dogs and piglets than their conventional counterparts. Kleesen *et al.,* (2003) reported that bacterial association of germ-free rats with *Bacteroides vulgatus* and *Bifidobacterium longum* or with a human faecal flora and diet based on fructans and inulin increased the thickness of epithelial mucous layer (EML) and number of goblet cells per crypt in the intestine.

Gastrointestinal microbiota also alters mucin composition. It is suggested that acidic mucins protect against bacterial translocation as are less degraded by bacterial glycosidases and host proteases. This idea is supported by findings where intestinal regions with high population of microbes express acidic mucins predominantly (Roberton, 1997; Amerogenon *et al.,* 1998). In germ-free rodents, increased neutral to acidic mucin ratio was observed in the colon (Sharma and Schumacher, 1995; Meslin *et al.,* 1999). Also sulfomucins were increased while sialomucins showed a decrease. The small intestine of germ-free rats showed fewer sialylated mucins than conventionally raised rats on a commercial diet (Sharma and Schumacher, 1995). Heteroxenic rats (germ-free rats conventionalized with human microbiota) showed larger number of more sulfomucin-containing cells in small intestine and more sialomucin containing cells in the large intestine than in germ-free rats. The ability of bacteria to bind mucin carbohydrates could avoid their expulsion and these mucus resident microbes prevent pathogenic

microbes from colonizing the mucus layer (Deplancke and Gaskins, 2001). Thus it would be advantageous for the commensal and pathogenic bacteria to regulate mucus synthesis, mucin secretion and composition.

Mucin molecules consist of a protein core to which various carbohydrate chains are attached by glycosidic linkages. In humans, eighteen mucin genes encoding human mucin glycoproteins have been assigned to Muc gene family (Moniaux *et al.,* 2001). The regulation of goblet cell responses by intestinal signals is not very well understood. Most of the information for microbial modulation of mucin secretion comes from *in vitro* studies with pathogens. Cholera-toxin of *Vibrio cholerae* has been shown to trigger heavy mucin release either directly (Epple *et al.,* 1998) or via a cAMP dependent mechanism (Lencer *et al.,* 1990). Similar findings were reported with *Entamoeba histolytica* (Chadee and Meerovitch, 1985) and *Listeria monocytogenes* (Coconnier *et al.,* 1998). Expression of non-epithelial mucin genes Muc 2 and Muc 5AC was upregulated in cultured airway epithelial cells by exposure to Gram positive (*Staphylococcus aureus, St. epidermis* and *Streptococcus pyogenes*) and Gram negative (*Pseudomonas aeruginosa* and *E coli* (Dohrman *et al.,* 1998). Exposure of HT-29 intestinal epithelial cells to an enteropathogenic *E coli* did not alter Muc 2 or 3 gene expression (Mack *et al.,* 1999). However, the probiotic strains *Lactobacillus plantarum* 299v and *L. rhamnosus* GG increased expression of both Muc2 and 3 in HT-29 colon cell culture. Bacteria may also inhibit mucus production in the gut. Exposure of human gastric cell line KA-70 III to *Helicobacter pylori* inhibited mucin synthesis and also suppressed expression of Muc1 and 5AC genes (Byrd *et al.,* 2000).

Administration of lipopolysaccharides of indigenous E coli strain to germ-free rats caused an increase of colonic neutral mucins (Enss *et al.,* 1996). This concept was consistently proved by Bry *et al.,* (1996). They showed that monoassociation of germ-free mice with common intestinal bacteria *Bacteroides thetaitaomicron* signalled the host epithelial cell to produce fucosylated glycol conjugates on small intestinal epithelial cells. Host derived cytokines have also been shown to affect mucin synthesis and secretion. IL-1 induced rapid mucin release from LS 180 goblet cell lines (Enss *et al.,* 2000). TNF-α and IL-6 also increased expression of Muc 2 and Muc 5B and Muc 2, Muc 5B and Muc 6, respectively. These pro-inflammatory cytokines stimulated the release of less glycosylated mucins which might be due to reduced glycosylation in Golgi apparatus because of accelerated passage.

### 2.4.4 Nutrient requirements

#### 2.4.4.1 Intestinal histology and morphology

Gastrointestinal tissues represent only approximately 5% of body weight, but consume a very high proportion of (~15–35%) of whole-body oxygen consumption and protein turnover (Ebner *et al.,* 1994). In conventional animals the normal cell turnover rate has been calculated to be about $10^8$ cells/d which varies with patho-physiological conditions (Norin and Midtvedt, 2000). The higher rates of cellular metabolism, proliferation and renewal of gut tissues explain their large proportional impact on whole body metabolism despite being only 3-6% of the body weight. In absence of bacteria, the transit time of an epithelial cell from crypt to tip of villus may be increased by a factor of two (Abrams *et al.,* 1963). Reduced weight of small intestine and cecum was found in

germ-free animals (Gordon *et al.,* 1966), chickens (Reyniers *et al.,* 1960) and pigs (Miniats and Valli, 1973), respectively. Furuse and Yokota (1984) reported shorter absolute and relative length (length/body weight) of the small intestine of germ-free chicks. The difference in weight was largely attributed to thinner intestinal wall and reduced lymphoid tissue in lamina propria and reduced submucosal depth (Shurson *et al.,* 1990).

Apart from weight, intestinal morphology differs substantially in germ-free animals. Bacterial load and the species colonizing the gut affect epithelial cell morphology and the rate of turnover in the small intestine in mouse (Khoury *et al.,*1969) and pigs (Kenworthy, 1970), respectively. Because of reduced crypt depth in GF animals, the villus height to crypt depth ratio is higher than conventional animals (Wostmann, 1996). Shirkey *et al.,* (2006) reported conventional pigs to have shorter villi and significantly deeper crypts thereby, a significantly lower villus height/ crypt depth compared to germ-free pigs. Monoassociated group with *Lactobacillus fermentum* had a villus height/crypt depth closer to GF group and EC group had even less villus height.

Intestinal mucosa utilizes substantial amounts (40–60%) of dietary amino acids (Stoll *et al.,* 1998). This higher rate of amino acid utilization by portal drained viscera (PDV) can have a significant impact on systemic availability of amino acids. Recent studies have shown that the portal-drained viscera (PDV) largely comprised of gastrointestinal tissue, accounts for 25-50% of whole body protein and amino acid metabolism (Stoll *et al.,* 1998; Yu *et al.,* 2000). In young pigs, 30-60% of total dietary intake of some limiting amino acids such as lysine, threonine and methionine could be used by gastrointestinal tissue (Stoll *et al.,* 1998).

Commensal microbiota has long been known for its nutritional contributions to the host, however, the area studying nutrients utilization by microbes is still in its infancy. Based on studies using radiolabelled $^{14}C$, Fuller (2003) suggested that less than 4% of amino acids in ileal digesta are derived from *de novo* synthesis, the rest coming from pre-formed amino acids from diet or endogenous secretions. Due to bacterial utilization of dietary amino acids, nitrogen utilization by the host is reduced (March *et al.,* 1978 and Furuse and Yokota, 1985). It is becoming increasingly apparent that nutritional and gut environment conditions that stimulate bacterial growth or metabolism may lead to limited dietary essential amino acid availability for growth. Yu *et al.,* (2000) showed that parasitic infection in sheep increased leucine oxidation and utilization by PDV tissues, reducing systemic availability of amino acids by 20-30%. Thus, it is highly likely that the presence and load of commensals directly affects the intestinal nutrient requirements, which in turn limits the availability of dietary nutrients.

As we know the type of diet fed to the animals influences the population and activity of intestinal microbiota (Drew *et al.,* 2002; Hill *et al.,* 2005), this in turn could affect the protein digestibility and amino acids availability to the host. Therefore, the diet and thereby quantitative and qualitative changes in the gastrointestinal tract are likely to play an integral role in overall growth and maintenance of health in pigs.

#### 2.4.4.2 Intestinal motility

Digestibility is influenced by interactions between various processes including digesta transit, digestion, absorption and the physico-chemical conditions of gut and feed (Wilfart *et al.,* 2007). Many studies have reported slower digesta passage rate through the small intestine in germ-free animals. Sacquet *et al.,* (1971) observed a slower small intestinal digesta passage rate in germ-free rats but the differences disappeared after caecectomy. Abrams and Bishop (1967) established by using a radiolabelled marker that the caecum of germ-free mice retained greater % of the marker and the passage of marker into faeces was also slower. However, Ford (1971) found no significant differences in the transit time of food through gastrointestinal tract in germ-free chicks compared to conventional chicks. Germ-free rats were reported to have a slower and restricted spatial and temporal transport of migrating motor complexes in small intestine than in conventional animals (Falk *et al.,* 1998). Because of slower passage of food in the gut in germ-free animals, intestinal digestion has been suggested to be more efficient as the enzymes are in contact with food for longer times (Corring *et al.,* 1981).

## 2.5. Summary

The relative bioefficacy value of MHA-FA compared to methionine is subject of considerable controversy however there is general agreement that DL-MHA-FA has a lower bioefficacy compared to DL-methionine (Jansman *et al*., 2003). Still not much is known about the mechanisms responsible for this variation especially in pigs. Also in our quest to develop ‘optimal microbiota’ to achieve maximum animal health and production potential in this new century without the use of antibiotics , many new

strategies have to be explored. Use of variation in fibre content and composition of commonly used dietary cereal ingredients in this effort might be one of the most useful ideas. Based on these observations, the overall objectives of this study were to elucidate whether metabolism of MHA-FA by gut microbiota could be responsible for its reduced bioefficacy compared to DL-methionine and whether use of different cereal grains in diet influence GI physiology and if so, whether these effects were carried out by altered microbiota.

# 3.0 APPARENT ABSORPTION OF METHIONINE AND 2-HYDROXY-4-METHYLTHIOBUTANOIC ACID FROM GASTROINTESTINAL TRACT OF CONVENTIONAL AND GNOTOBIOTIC PIGS

## 3.1. Abstract

The effect of commensal microbiota and feeding corn or wheat/barley-based diets on the apparent gastrointestinal absorption of DL-methionine (MET) and 2-hydroxy-4-methylthiobutanoic acid (MHA-FA) was studied in conventional (n = 32) and gnotobiotic pigs (n = 24). Conventional pigs were vaginally delivered and sow-reared until weaning at 14 d of age. Gnotobiotic pigs were derived by caesarian section and reared in HEPA ( high efficiency particulate air) -filtered isolator units with *ad libitum* access to a milk-based formula. Corn or wheat/barley-based diets were fed to all pigs from 14 to 24 d of age. At 24 d of age, after an overnight fast, pigs were fed 20 g/kg BW of experimental diet supplemented with $10^7$ Bq of either $^3$H-L-MET or $^3$H-L-MHA-FA per kg of feed and chromic oxide (0.5% wt/wt). Pigs were killed for sample collection 3 hours after consuming the meal. Residual $^3$H-MET and $^3$H-MHA-FA were estimated in gastrointestinal contents as the ratio of $^3$H:chromic oxide in digesta samples to the ratio of $^3$H:chromic oxide in feed. In conventional (CON) pigs, feeding a wheat/barley-based diet increased ($P < 0.05$) total aerobes, whereas supplementation with MHA-FA increased ($P < 0.05$) total aerobes and lactobacilli populations in proximal small intestine (SI). Among the gnotobiotic pigs, bacterial contamination occurred such that 8 pigs (2 isolators) were monoassociated with a Gram negative bacteria closely related to *Providencia* spp. and 16 pigs (4 isolators) were monoassociated with Gram positive *Enterococcus faecium.*

Species of monoassociated bacterial contaminant and diet composition did not affect residual MET or MHA-FA in digesta. In both CON and monoassociated (MA) pigs, MET and MHA-FA were retained in stomach (92%) but disappeared rapidly from proximal SI. Residual MET and MHA-FA in digesta was not different in MA pigs, however, in CON pigs less ($P < 0.01$) apparent residual MET was found in digesta recovered at 25% (from cranial to caudal) and 75% of SI length compared with MHA-FA. Apparent residual MET was 16 and 8 % compared with 34 and 15% for MHA-FA, at the 25 and 75% locations, respectively. In proximal SI tissue significantly ($P<0.05$) higher radioactivity (cpm/mg wet issue) was associated with MET pigs (8.56±0.47) as compared to MHA-FA (5.45±0.50). This study suggests that microbial metabolism of MHA-FA increases retention in small intestinal digesta relative to MET and contributes in part to the lower bioefficacy of MHA-FA compared to MET.

### 3.2. Implications

This work has shown that the intestinal microbiota in pigs can metabolize even readily available nutrients such as supplemented amino acids, hence affecting their availability to the host. Significant amount of literature has demonstrated that DL-2-hydroxy-4-methylthiobutanoic acid. (MHA-FA) has lower bioefficacy as a methionine source than DL-methionine (MET). We provide evidence that in swine diets the lower ioefficacy of MHA-FA is, in part, due to its high microbial metabolism relative to MET. However, we did not find any effect of diet composition on the degree of microbial metabolism of MET or MHA-FA, indicating that changes in microbial composition do not necessarily reflect a significant change in the level of metabolism of supplemented amino acid source.

### 3.3. Introduction

There is increasing realization that along with a nutritional contribution mainly by the hindgut communities, the upper gut microbiota competes with animal cells for a wide variety of nutrients including the amino acids (Savage, 1986). Conventional microbiota were reported to catabolise ascorbic acid and chlorogenic acid (Cobb *et al.,* 1991; Gonthier *et al.,* 2003) and can also take up and incorporate amino acids into microbial protein (Salter *et al.,* 1974) or utilize the carbon skeleton as an energy source. Liu *et al.,* (2003) concluded that the various *Lactobacillus* species, which predominate in the pig small intestine (Hill *et al.,* 2005) show a wide diversity in their abilities to metabolise amino acids in *in vitro* experiments.

Crystalline amino acids are commonly supplemented in livestock diets to provide optimum essential amino acid balance with minimum nitrogen excretion. DL-methionine (DL-MET) and hydroxy-analogue of methionine, DL-2-hydroxy-4-methylthiobutanoic acid (MHA-FA) are alternate sources for supplementation of methionine. The Dutch Central Bureau for Livestock Feeding (Jansman *et al.,* 2003) summarized the available literature and reported bio-efficacy values of DL-MHA-FA as 77% in broiler chickens, 83% in layers and 82% in piglets on equimolar basis compared to DL-MET.

The mechanisms responsible for the lower bio-efficacy of MHA-FA could involve efficiency of intestinal transport, efficiency conversion of MHA-FA to L-methionine and microbial metabolism. Studies in poultry have reported lower apparent absorption of MHA-FA associated with low affinity, low velocity brush border transport mechanisms compared to MET (Maenz and Engele Schaan, 1996b). Earlier results from our laboratory using the germ-free chicken suggested that the more slowly absorbed

MHA-FA may be metabolised by the intestinal microbiota reducing host availability (Drew *et al.,* 2003). Therefore, the present study was designed to examine whether gastrointestinal microbiota similarly affect the apparent absorption of MET and MHA-FA in conventional and gnotobiotic pigs. Since diet composition, and specifically cereal grain variety, affects microbial composition in the pig small intestine, (Hill *et al.*, 2005; Pieper *et al.,* 2008), we also investigated whether diets formulated on corn or barley and wheat affected apparent absorption of methionine sources.

## 3.4. Materials and methods

### 3.4.1. Source and synthesis of radiotracers

L-[methyl-$^{3}$H] methionine (93% pure) was purchased from Amersham Biosciences (Oakville, ON, Canada). L-[methyl-$^{3}$H] MHA-FA was synthesized by nitrous deamination of L-[methyl-$^{3}$H] methionine according to the procedure of Winitz *et al.,* (1956) except that sodium nitrite was used at 5.68 mol/L to improve yield. The method for the purification and separation of L-[methyl-$^{3}$H] methionine and MHA-FA was first developed and validated with non radioactive L-methionine (Sigma chemicals) and MHA-FA (Novus International Inc.) on HPLC using an ultraviolet post column detection system at 210 nm. For HPLC analysis, a C-18 column (Phenomenex Luna 3µ; 150x4.6mm) with a mobile phase of 17.5% methanol (pH 3.8) at a flow rate of 0.8 mL/min was used. L-[methyl-$^{3}$H] methionine was isolated from contaminant radioactivity by single peak elution on HPLC. Elution profiles of L-[methyl-$^{3}$H] methionine (Amersham Biosciences) and synthesized L-[methyl-$^{3}$H] MHA-FA were determined by scintillation counting (Beckman LS6000 TA liquid scintillation counter, Beckman

Instruments, Fullerton, CA). Aliquots of purified L-[methyl-$^{3}$H] MET (99%) and L-[methyl-$^{3}$H] MHA-FA] (99%) were stored in liquid nitrogen and used within 3 weeks.

### 3.4.2. Experimental diets

Dietary treatments included diets based on corn or wheat and barley and supplemented with DL-methionine or its hydroxy-analogue (MHA-FA) as the methionine source. Diet formulations, calculated and analysed nutrient composition are presented in Table 3.1. Diets were formulated to meet or exceed nutrient requirements (NRC, 1998). Methionine was added as either DL-methionine (0.26% of 99% pure product) or MHA-FA (0.295% of 88% pure product)) on an equimolar basis generating 4 experimental diets. For gnotobiotic experiments diets were sterilized by gamma-irradiation at 5 Mrads (MDS Nordion, Canadian Irradiation Centre, Laval, Quebec). All protocols were approved by the University of Saskatchewan Animal Care and Use Committee and Radiation Safety Committee.

**Table 3.1. Diet formulations used in the experiment**

| Ingredients (%) | Corn-soy-MET/MHA-FA | Wheat-barley-soy MET/MHA-FA |
|---|---|---|
| Corn | 41.53 | 0.00 |
| Soybean meal (48%) | 35.00 | 29.70 |
| Wheat | 0.00 | 28.50 |
| Barley | 0.00 | 18.00 |
| Whey powder | 12.00 | 12.00 |
| Canola oil | 4.00 | 4.16 |
| Blood cells | 2.40 | 2.47 |
| Celite[1] | 1.90 | 1.90 |
| Dicalcium phosphate | 0.73 | 0.65 |
| Calcium carbonate | 0.67 | 0.73 |
| Vitamin-mineral premix[2] | 1.00 | 1.00 |
| DL-methionine, 99% | 0.26 or | 0.26 or |
| DL-MHA-FA, 88% | 0.295 | 0.295 |
| L-Lysine HCl | 0.24 | 0.33 |
| L-Threonine | 0.13 | 0.17 |
| L-Tryptophan | 0.04 | 0.03 |
| Pro-bond | 0.10 | 0.10 |
| ***Nutrients (calculated)*** | | |
| ME (Mcal/Kg) | 3.35 | 3.35 |
| Digestible Lysine | 1.45 | 1.45 |
| Digestible Methionine | 0.56 | 0.55 |
| ***Nutrients (analysed)*** | | |
| CP | 26.83 | 28.05 |
| ADF | 4.23 | 5.26 |
| NDF | 7.45 | 10.43 |

[1]Celite Corporation, Lompoc, California.

[2]Provided (per kg of diet): Zn, 100 mg as zinc sulphate; Fe, 80 mg as ferrous sulphate; Cu, 50 mg as copper sulphate; Mn, 25 mg as manganous sulphate; I, 0.50 mg as calcium iodate; Se, 0.10 mg as sodium selenite.

Vitamin A, 8250 IU; Vitamin D, 825 IU; Vitamin E, 40 IU; niacin, 35 mg; D-pantothenic acid, 15 mg; menadione, 4 mg; folacin, 2 mg; thiamine, 1 mg; D-biotin, 0.2 mg; Vitamin $B_{12}$, 25 µg.

### 3.4.3. Conventional pig experimental design

All experiments were conducted using Large White x White Duroc pigs sourced from from Prairie Swine Centre, Inc. (Saskatoon SK). In two replicate experiments a total of 32 vaginally delivered, sow-reared pigs were weaned at 14 d of age and randomly assigned to one of four experimental diets (4 pigs per diet in each experiment) balanced for litter of origin, sex and body weight. Ten *d* after weaning, and following a 10 hour overnight fast, pigs were offered a test meal (20 g/kg BW) of the corresponding experimental diet with added chromic oxide (0.5%) and $10^7$ Bq of HPLC purified L-[methyl-$^3$H] MET or L-[methyl-$^3$H] MHA-FA per kilogram feed. At 3 hours following the meal, pigs were killed by asphyxiation with carbon dioxide and exsanguinated.

### 3.4.4. Preparation of gnotobiotic isolators

Gnotobiotic isolators (Class Biologically Clean Ltd., Madison, WI) were maintained under positive pressure, and ventilation occurred through High Efficiency Particulate Air (HEPA) filters to maintain sterility. All components of the isolators and experimental materials placed inside were sterilized by autoclaving at 121˚C for 30 minutes or by spraying with 1:10:1 (base:water:activator) clidox (Pharmacal Research laboratories, Naugatuck, CT). The isolators were further sterilized with a 5% solution of peracetic acid (35%; FMC Corp., Philadelphia, PA), sealed for 24 hours, and vented for a minimum of at least 36 hours prior to pig placement.

### 3.4.5. Gnotobiotic pig derivation and maintenance

Pigs were delivered by caesarian section and aseptically passed through a betadine filled (10% povidone-iodine; Purdue Pharma, Stanford, CT) dip tank into a sterile HEPA-filtered transfer unit. Pigs from 2 sows were revived in the transfer unit, dried and then transferred aseptically to gnotobiotic isolators (4 pigs/isolator), balancing for litter of origin, sex and body weight. Each of the piglets were bottle fed 100 ml of γ-irradiated colostrum within the first 24 hours and thereafter fed infant formula (Similac, Abbott Laboratories, Abbott Park, IL) as similac:water as 2:1 (vol/vol) containing 3.13g/100 mL protein, 8.13g/100 mL lipid, 16.2g/100 mL carbohydrate as fed. Pigs were fed *ad libitum* in 3 equal feedings at 8 hours intervals until weaning at 14 d of age. Room temperature was maintained at 34˚C on day 1 and reduced by 1˚C every 2 d until 30˚C.

### 3.4.6. Gnotobiotic pig experimental design

In each of 2 gnotobiotic trials, 16 pigs were assigned to one of four gnotobiotic isolators each with a capacity of rearing 4 pigs. At 14 d of age, one pig in each isolator received one of the 4 sterilized experimental diets such that there were 4 pigs per treatment in each experiment. Sterile water was available *ad libitum*. At 10 d post weaning pigs, were fasted overnight and fed the corresponding experimental diets containing a $^{3}$H-labelled methionine source as a meal using an identical protocol as described for conventional pigs.

### 3.4.7. Sample collection

Immediately following exsanguination, an incision was made along ventral midline of abdomen, and after clamping at the pylorus and ileo-cecal junction, the small intestine was dissected from mesentery. The stomach and caecum were removed separately. The length of the small intestine was measured and regions corresponding to 5, 25, 50, 75 and 95% of length beginning at the pyloric sphincter were identified. Digesta was collected from stomach and a 30 cm segment at each small intestinal location, snap-frozen in liquid nitrogen and frozen at –80˚C for subsequent analysis. Approximately 10 cm tissue segment was also collected from 5% of SI length, snap-frozen in liquid nitrogen and frozen at -80˚C for analysis of $^{3}H$ activity.

### 3.4.8. Microbial identification and enumeration

Digesta was sampled (100 mg) aseptically from 25 and 75% of SI location, placed in 15 mL sterile plastic tubes containing 1 ml of 0.1% sterile peptone buffer with 5 g/L of cysteine hydrochloride (Sigma Chemical Co., St. Louis, MO) and kept on ice until diluted for culture within 3 hours of collection. For conventional pigs, total aerobes and anaerobes were enumerated on BBL blood agar base (VWR Int., Mississauga, ON, Canada) containing 5% sheep blood using an automated spiral plater (Autoplate, Spiral Biotech inc., Bethesda, MD) and incubated aerobically and anaerobically (10% $CO_2$, 10% $H_2$, and 80% $N_2$) respectively, for 24 hours at 37 ˚C. Lactobacilli were enumerated by culture on de Man, Rogosa and Sharpe agar (Becton, Dickinson and Co., Sparks, MD, USA) anaerobically for 48 hours at 37 ˚C. Digesta samples were also cultured aerobically on MacConkey's agar (Becton, Dickinson and Co., Sparks, MD, USA) and Bile esculin

agar (Becton, Dickinson and Co., Sparks, MD, USA) for the enumeration of enterobacteria and enterococci, respectively. For gnotobiotic pigs, only total aerobes and anaerobes were enumerated. All dilutions were plated using an automated spiral plater (Autoplate, Spiral Biotech inc., Bethesda, MD). Results were expressed as $\log_{10}$ colony forming units per gram of wet intestinal contents.

For gnotobiotic experiments, colonies cultured from digesta samples were isolated and further cultured in trypticase soy (TS) broth (Difco, Becton Dickinson and Co. Sparks, MD, USA) followed by freezing in 15% glycerol at -80 ˚C. Colony taxonomic identification was by *cpn*60 universal target (UT) sequencing (Hill *et al.,* 2004). Briefly, after overnight culture from frozen stocks in TS broth, bacterial cells were harvested by centrifugation and genomic DNA was extracted with phenol-chloroform-isoamyl alcohol according to previously described methods (Dumonceaux *et al.*, 2006). The *cpn*60 UT was amplified by PCR using 1µl of extracted DNA, 0.5 U of Taq polymerase, 50mM MgCl2, 10mM of dNTPs and 0.375 µM for each of the degenerate primers H729 and H730 (Hill *et al.,* 2002). Reactions were subject to 95˚C for 3 minutes, followed by 40 cycles of 1minute at 95˚C, 1 minute at 46˚C, 1 minute at 72˚C and a terminal 5 minutes extension at 72˚C. PCR products were purified using QIAquick Gel extraction Kit (Qiagen) and sequenced directly (National Research Council, Plant Biotechnology Institute, Saskatoon, SK). Nucleotide sequences were trimmed of plasmid and primer sequence and identified by comparison to typed strain sequences in cpnDB (http://cpndb.cbr.nrc.ca) using BlastN (Hill *et al.*, 2004).

### 3.4.9. Measurement of apparent retention of L-[methyl-$^3$H] MET and L-[methyl-$^3$H] MHA-FA in digesta

Chromic oxide ($Cr_2O_3$) was estimated in diets and freeze-dried digesta according to the procedure of Fenton and Fenton (1979) adapted for small sample sizes. Approximately, 100 mg of freeze-dried digesta or 400 mg of feed was digested with the digestion mixture (Fenton and Fenton, 1979) following by overnight ashing in muffle furnace at 450˚C, further dilution to 10ml with distilled water, centrifugation ( 3000g, 10 minutes). Absorbance was measured at 440 nm in spectrophotometer (Spectramax, Molecular Devices Corp., Sunnyvale, CA). Chromic oxide concentration was calculated from the regression equation developed from a standard curve ranging from 250 µg to 3 mg chromic oxide (Anachemia Canada Inc. Lachine, Quebec). $^3$H activity in freeze dried digesta and feed samples was determined according to methods described previously (Drew *et al.*, 2003). Briefly, 20 mg of freeze-dried digesta or approximately 50 mg of diet was mixed with 1 mL of BTS-450 tissue solubilizer (Beckman Coulter Inc., Mississauga Ontario) in 18 mL scintillation vials and the mixture was incubated for 2 hours at 40 $^0$C followed by addition of 0.5 mL isopropanol and 0.2 mL hydrogen peroxide. After 10 min. at room temperature and a further 2 h at 40 $^0$C, the mixture was diluted with 5 mL deionized water, combined with 10 mL scintillation cocktail (Beckman Coulter Inc., Mississauga Ontario) and counts per minute (cpm) recorded using a Beckman LS600 TA liquid scintillation counter. Apparent retention of methionine and MHA-FA in digesta was determined using chromic oxide as indigestible marker according to following equation:

Apparent retention = ($^3$H activity$_{digesta}$**:** $Cr_2O_{3digesta}$) / ($^3$H activity$_{feed}$**:** $Cr_2O_{3feed}$) X 100%

### 3.4.10. Analysis of $^{3}H$ activity in intestinal tissue

To measure $^{3}H$ activity in intestinal tissue, frozen samples were coarsely ground under liquid nitrogen with mortar and pestle. An 80-100 mg homogenous sub-sample was weighed and mixed with 1ml of BTS-450 tissue solubilizer (Beckman Coulter Inc.). The tissue was incubated at 40˚C for 3-4 hours with frequent vortexing. Thereafter 10 mL of scintillation cocktail and 70 µL of glacial acetic acid was added. Radioactivity was determined using a Beckman LS600 TA liquid scintillation counter (Beckman Coulter Inc.) and reported as cpm per mg wet tissue.

### 3.4.11. Statistical analyses

Monoassociation of isolator-reared pigs with either of two bacterial species required a reconsideration of data analysis. Analysis of data from monoassociated pigs by one-way ANOVA using a GLM (SPSS Inc, Chicago IL, USA) indicated no effect of monoassociated species on any parameter. Similarly a separate one-way ANOVA indicated no replication effect in the conventional pig experiments. Consequently data was analyzed as 2 x 2 x 2 factorial using a GLM procedure with fixed main effects of cereal grain (corn vs. wheat and barley), methionine source (DL-methionine vs. DL-MHA-FA) and microbial status (conventional vs. monoassociated), plus interactions as sources of variation. Because of significant ($P<0.07$) methionine source by microbial status interaction at 75% location and absence of data at 95% SI length, data were analyzed separately for conventional and monoassociated pig experiments as 2 X 2 factorial with cereal grain and methionine source as main effects.

## 3.5. Results

### 3.5.1. Health and microbial status

In the conventional pig experiment one pig was euthanized at 21 d of age due to anorexia without other clinical signs. Three isolator reared pigs were emaciated after failing to consume the post-weaning diet and were euthanized at 24 days of age. All other CON and MA pigs appeared healthy based on visual appearance and appetite.

### 3.5.2. Microbial enumeration - conventional pigs

Standard plate counts on selective agars revealed significant differences only for digesta collected at the 25% SI location. Total aerobic counts (log cfu/g digesta) in digesta collected at 25% of SI length for pigs fed wheat-barley diets (5.48±0.14 ) was higher ($P<0.05$) compared to corn- based diets (4.81±0.23). Total aerobe count was also significantly ($P<0.05$) higher with MHA-FA supplementation (5.42±0.19) compared to with MET supplementation (4.90±0.20). Similarly, lactobacilli counts increased significantly in digesta from the 25% SI location for the MHA-FA group (6.22±0.15) compared to the MET group (5.63±0.19). Cereal grain type or source of methionine did not affect total anaerobes, enterobacteria or enterococci at 25% or 75% SI location.

### 3.5.3. Microbial enumeration and identification - monoassociated pigs

Culture of small intestinal contents at 25 and 75% locations showed bacterial contamination of isolator-reared pigs. Morphologic examination of the colonies on aerobic and anaerobic blood agar suggested the presence of a single bacterial species.

Identification by *cpn*60 UT sequence analysis of 2-3 colonies from each pig confirmed that pigs in two isolators were monoassociated with a Gram negative *Providencia like spp.* (98 % identity to *Providencia rettgeri*). Pigs in the remaining isolators were monoassociated with Gram positive *Enterococcus faecium* (100% identity).

Among Gram-negative monoassociated pigs, total aerobic counts were 7.24±0.21 and 8.31±0.17 log cfu/g at 25 and 75% of SI length, respectively. Anaerobic plate counts were 7.10±0.21 at 25% and 8.26±0.20 log cfu/g at 25 and 75% SI locations, respectively. For the Gram-positive monoassociated pigs, total aerobe counts (log cfu/g) were 7.66±0.24 and 8.76±0.09, whereas total anaerobic counts were 7.98±0.31 and 8.88±0.07 at 25 and 75% of SI length, respectively.

### 3.5.4. Apparent retention of L-[methyl-$^{3}$H] MET and L-[methyl-$^{3}$H] MHA-FA in digesta

Residual L-[methyl-$^{3}$H] MET and L-[methyl-$^{3}$H] MHA-FA activity in contents of conventionally raised and monoassociated pigs is shown in Table 3.2. In stomach of CON and MA pigs both methionine sources were present at similar high levels (92 to 99%) which were not affected by cereal source or microbial status. Both Methionine sources disappeared rapidly from proximal small intestine such the less than 50% residual activity was observed at the 5% location. Approximately 12-14% residual activity was observed in the distal small intestine. Cereal grain did not affect the residual amounts of either methionine source. At 75% of SI length an interaction (P=0.06) between methionine source and microbial status was observed. Furthermore, no data could be determined for the 95% of SI length in MA pigs because chromic oxide concentration

was below detection at this location at 3 hours after feeding. Separate analysis of residual activity for CON pigs revealed that residual L- MHA-FA was significantly ($P<0.01$) higher (34% , 15% and 13%) compared to L-MET (16% , 8% and 7.8%) at 25%, 75% and 95% of SI length, respectively (Figure 3.1A). In MA pigs, the apparent retention of MET and MHA-FA was similar at all small intestinal locations excepting at the 75% SI location where a trend ($P< 0.07$) to increase residual MHA-FA was observed (Figure 3.1B).

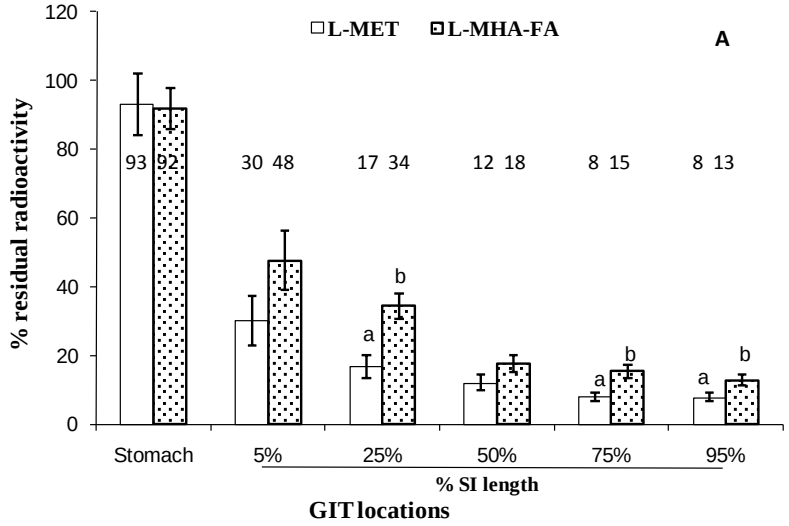
### 3.5.5. Apparent tissue associated L-[methyl-$^3$H] MET and L-[methyl-$^3$H] MHA-FA activity

Radioactivity in small intestinal tissue associated with feeding L-[methyl-$^3$H] MET and L-[methyl-$^3$H] MHA-FA was detectable only at the 5% SI location (Figure 3.2 A and B). A significant ($P< 0.01$) methionine source by microbial status interaction was observed for tissue radioactivity (Bq/ mg wet weight) such that at the 5% SI location activity associated with L-[methyl-$^3$H] MET feeding (8.56±0.47) was significantly ($P<0.05$) higher as compared to L-[methyl-$^3$H] MHA-FA feeding (5.45±0.50) in CON pigs only (Figure 3.2A). Cereal grain type did not affect tissue radioactivity levels.

**Table 3.2. Percent retained radioactivity in gastrointestinal tracts of conventional and monoassociated pigs fed corn and wheat-barley based diets supplemented with DL-methionine or DL-MHA-FA on equimolar basis**

| | Stomach | 5% | 25% | 50% | 75% |
|---|---|---|---|---|---|
| **Methionine source** | | | | | |
| DL-MET | 91.79 | 41.01 | 20.75 | 12.03 | 8.99 |
| DL-MHA-FA | 91.52 | 44.87 | 30.19 | 16.70 | 14.60 |
| Pooled SEM | 2.04 | 3.18 | 1.58 | 0.99 | 0.65 |
| **Cereal type** | | | | | |
| Corn | 95.05 | 45.96 | 26.29 | 12.86 | 11.95 |
| Wheat-barley | 88.26 | 39.93 | 24.65 | 15.87 | 11.63 |
| Pooled SEM | 1.98 | 3.03 | 1.77 | 1.03 | 0.79 |
| **Microbial status** | | | | | |
| Conventional[1] | 92.23 | 37.53 | 23.85 | 15.07 | 11.54 |
| Monoassociated[2] | 91.07 | 48.36 | 27.09 | 13.66 | 12.04 |
| *P. rettgeri*[3] | 92.38 | 55.77 | 26.71 | 10.81 | 8.60 |
| *E. faecium*[3] | 90.75 | 42.69 | 28.05 | 14.61 | 13.31 |
| Pooled SEM | 2.04 | 3.17 | 1.75 | 1.05 | 0.79 |
| **P-value** | | | | | |
| Cereal type | 0.121 | 0.402 | 0.599 | 0.146 | 0.810 |
| Methionine source | 0.950 | 0.591 | 0.004 | 0.027 | 0.000 |
| Microbial status | 0.787 | 0.142 | 0.30 | 0.491 | 0.707 |
| Methionine source*microbial status | 0.878 | 0.323 | 0.224 | 0.882 | 0.062 |

[1] n= 8 except wheat-barley MHA-FA treatment where n= 7; [2] n=6 except wheat-barley MET and Corn-MET treatments where n=5; [3]Retained radioactivity was not different between pigs monoassociated with different bacterial species such that observations were combined.



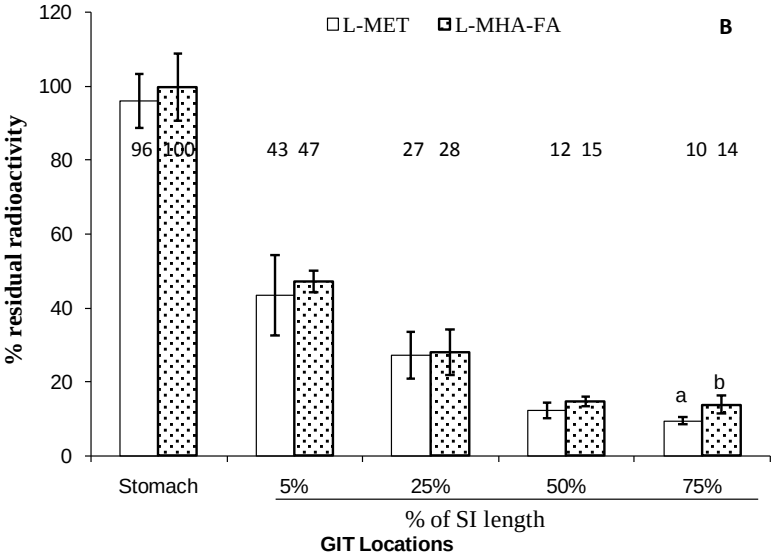
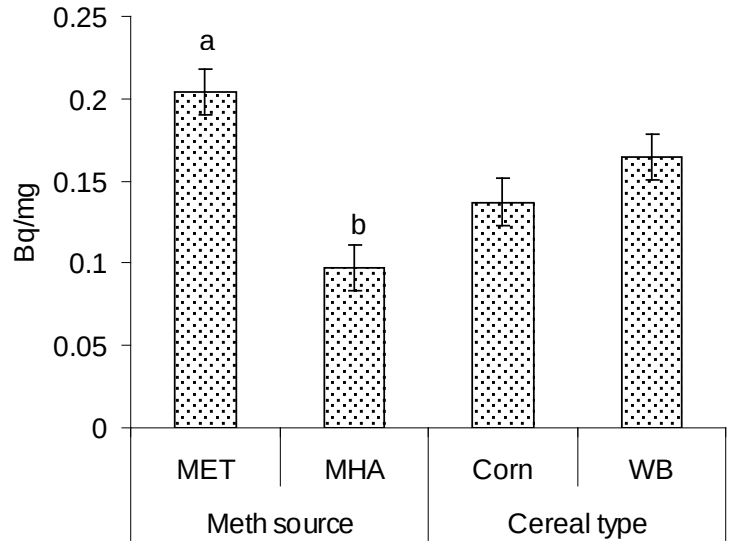
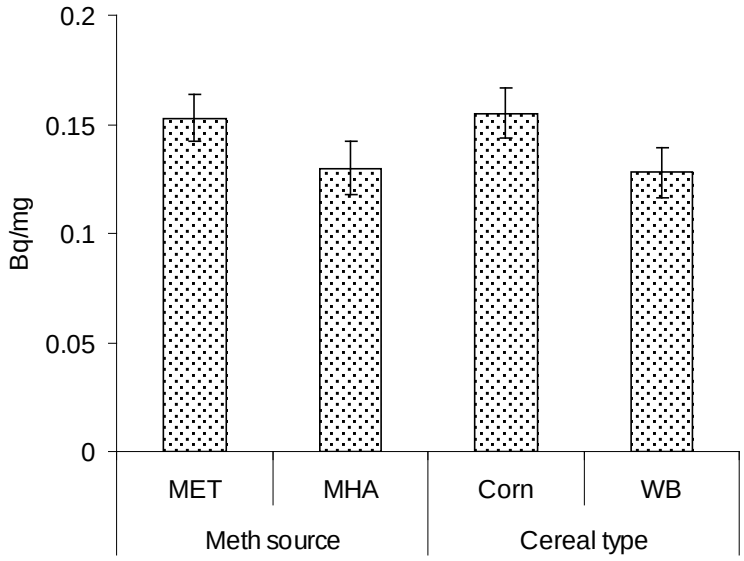




**Figure 3.1. Apparent retention of L-[methyl-$^3$H] MET and L-[methyl-$^3$H] MHA-FA in digesta in conventional (A) and monoassociated pigs (B) fed corn or wheat-barley based post-weaning diets. Values over each bar are mean percent retained radioactivity. The vertical bars depict SE. Bars with a different letters are significantly different at P<0.05 (panel A) or P<0.07 (panel B).**




**A**




**B**

**Figure 3.2. Apparent tissue associated L-[methyl-3H] MET and L-[methyl-3H] MHA-FA activity in conventional (A) and monoassociated pigs (B) fed corn or wheat-barley (WB) based post-weaning diets. The vertical bars depict SE. Bars with different letters are significantly different (P<0.05)**

### 3.6. Discussion

The highly complex enteric microbiota play an important role in modifying the structure, biochemistry and physiology of the gastrointestinal tract impacting nutrition and health of the host (Falk *et al.,* 1998). Gnotobiotic animals, where gastrointestinal bacterial populations are clearly defined, offer an indispensable tool to study a broad range of specific host-bacterial and host-nutrient-bacterial interaction. Here we utilized the gnotobiotic model to establish in the pig the role of the intestinal microbiota in determining the availability for absorption of supplemented amino acids, particularly supplemented methionine and MHA-FA. Since different cereal grains are known to affect the composition of intestinal microbiota in conventionally reared animals (Hill *et al.,* 2005), the study compared corn and wheat/barley based diets.

In the present study, residual $^{3}$H-MHA-FA activity observed in intestinal contents was significantly higher than $^{3}$H-MET activity along the entire SI in the conventionally reared pigs. In contrast, in monoassociated pigs, residual $^{3}$H-MHA-FA and $^{3}$H-MET activity in small intestinal contents was similar. We hypothesize that the increase in residual MHA-FA activity in digesta of conventional pigs, but not monoassociated pigs, reflects a preferential metabolism of MHA-FA, relative to methionine, by the complex consortia of bacteria present in the intestine. This increase in microbial metabolism of MHA-FA could reduce the amount of MHA-FA available for absorption and contribute to the lower bio-efficacy of MHA-FA (Jansman *et al.,* 2003). Our results in pigs are in agreement with an earlier report by our laboratory in the chicken where conventional chickens had increased residual $^{3}$H-MHA-FA in intestinal contents compared with the germ-free chicken (Drew *et al.,* 2003).

MHA-FA is transported by non-stereospecific intermediate affinity $H^+$-dependent system whereas methionine is transported by $Na^+$ dependent system B transporter with higher substrate affinity and maximal velocity compared to $H^+$-dependent system (Maenz and Engele Schaan, 1996b). We have previously suggested that the slower rate of transport of MHA-FA may prolong the opportunity for microbial metabolism relative to methionine (Drew *et al.,* 2003).

Many studies in chicken (Esteve Garcia and Austic 1993; Lingens and Molnar, 1996; Maenz and Engele Schaan, 1996a) used radiolabelled MET and MHA-FA and estimated intestinal absorption based on residual radioactivity in intestinal contents. This method is not affected by bacterial metabolism of MET and MHA-FA since the radiotracer would remain within the intestinal contents even following metabolism. All three studies reported that 10-20% of the original radiolabelled MHA-FA activity in the feed was present in the distal sections of the small intestine compared to 3.5-5% for MET, thus in agreement with our results. Moreover, HPLC analysis of gut contents from the distal ileum showed that only 10% of the residual radioactivity was associated with MHA-FA suggesting that the compound had been metabolized during intestinal transit (Maenz and Engele Schaan, 1996a) and further supporting metabolism by gut microbiota.

We found that MHA-FA supplementation increased the number of total aerobes and lactobacilli in the upper small intestine, a response which may have contributed to the increased residual MHA-FA activity in upper small intestinal contents. Among lactic acid bacteria, which are dominant in the upper small intestine, there is significant inter- and intra-species differences in their utilization of various amino acids (Liu *et al.,* 2003). Hegedus *et al.,* (1993) reported that of the three lactobacilli species *(Lactobacillus*

*plantarum, L. casei* and *Leuconostoc mesenteroides)* studied none could utilize MHA-FA though all could use methionine. In this regard, further characterization of the proximal gut microbiota and species variation in metabolizing various amino acids is required.

Although the methionine sources disappeared rapidly from the upper small intestine in the pig, interestingly, the residual activity in intestinal contents of both methionine sources was much lower (9-15%) in the upper small intestine of chicken (Drew *et al.,* 2003) as compared to the pig (35-45%). The rate of absorption of methionine sources may be slower in the upper SI of pig compared to chicken. Also both MET and MHA-FA were essentially completely retained in the pig stomach in contrast to a report by Richards *et al.,* (2005) in the chicken in which only 15% of supplemented MHA-FA could be detected by HPLC in digesta recovered just prior to the duodenum. The authors suggested 85% of supplemented MHA-FA was passively absorbed from the upper tract of the chicken (crop, proventriculus), a phenomenon that does not appear to occur appreciably in pig stomach. Alternatively, it is possible that the low recovery of MHA-FA by HPLC in chicken proventriculus could be partly explained by microbial metabolism to alternate products not detected by HPLC.

Wester *et al.,* (2006) reported that in lambs, small intestinal tissues are capable of converting MHA-FA to methionine and further that MHA-FA-derived methionine is preferentially retained in the intestine to support anabolism, sparing absorbed methionine from dietary protein for transport to the liver. This raised the possibility that in the current study increased radioactivity observed in digesta in the MHA-FA supplemented pigs may be due to preferential in situ metabolism which could contribute to higher radioactivity associated with endogenous losses. In the current study, however, increased

upper small intestinal tissue radioactivity was associated with the MET supplemented diet compared to MHA-FA. This is consistent with greater apparent absorption of MET from the upper small intestine (lower residual radioactivity) and suggests, although not conclusively, that the increased residual radioactivity in the digesta observed for MHA-FA supplementation could not be accounted for by increased radioactivity in endogenous losses.

Chromic oxide content in digesta from 95% of SI length was below the assay detection limit for monoassociated pigs preventing determination of residual radioactivity in digesta. In contrast, chromic oxide content in conventional pigs was always highest at 95% length of small intestine. This could be due to a slower transit rate from stomach to distal small intestine in MA pigs resulting in negligible chromic oxide content. Previous reports indicate that the commensal microbiota decrease the intestinal transit time in rats (Riottot *et al.,* 1980).

Gut microbiota is influenced by many factors including age, weaning and diet. Nutrients in the gut affect the microbiota by providing preferential substrates for bacterial growth (Jensen and Jorgensen, 1994 and Hill *et al.,* 2005). Although we did not conduct an extensive molecular examination of the composition of the intestinal microbiota in conventional animals in the current study, limited culture-based analysis detected an effect of cereal grain type. Interestingly, wheat-barley based diets increased total aerobes in upper GI, a location for greatest opportunity for microbial metabolism of supplemented methionine source, given the rapid absorption. Nevertheless no effect of cereal grain source on residual activity of MHA-FA or MET was observed.

Our results support the hypothesis the intestinal microbiota compete with the host for metabolism of readily available nutrients such as supplemented amino acids. Our previous results of increased residual $^{3}$H-MHA-FA in intestinal contents of conventional but not germ-free chicken and current similar results in conventional and monoassociated pigs suggest that microbial metabolism of MHA-FA reduces the availability of MHA-FA for absorption relative to MET. Whether MHA-FA is a preferred substrate for intestinal bacteria or the slower absorption kinetics relative to MET provide greater opportunity for microbial metabolism is not clear. Microbial metabolism of MHA-FA is, however, a likely contributor to reduced bio-efficacy of MHA-FA relative to methionine.

# 4.0. POST WEANING INTESTINAL PHYSIOLOGY AND MUCIN DYNAMICS IS INFLUENCED BY CEREAL GRAIN TYPE AND COMMENSAL MICROBIOTA

## 4.1. Abstract

Mechanisms by which diet composition and commensal microbiota influence post-weaning intestinal physiology were studied using conventional and gnotobiotic pigs in a 2x2 factorial design. Caesarean-section derived germ-free pigs (n=16) were reared in HEPA-filtered isolator units (4 pigs/unit) and fed sterilized sow colostrum (120 mL/pig) followed by infant formula (2:1; forumula: water) *ad libitum*. Conventional (CON) pigs (n=32) were vaginally delivered and sow-reared. At 14 d of age all pigs were weaned to diets formulated to meet nutrient requirements using corn or wheat/barley. At 24 d of age, pigs were killed and digesta and tissue collected at 75% (cranial to caudal) of small intestinal (SI) length. Contamination of germ-free pigs resulted in monoassociation with *Enterococcus faecium.* Villus height, crypt depth and number of goblet cells (acidic, neutral and total) were determined using stained formalin-fixed tissue cross-sections taken at 75% of SI length. Proliferative and apoptotic activity were assessed by analysis of proliferating cell nuclear antigen (PCNA) expression and activated caspase-3 activity, respectively. Aminopeptidase and sucrase activities and expression were determined as indicators of digestive function. Mucin cell counts and expression of membrane associated mucin genes Muc 1, Muc 13 and secreted type Muc 2 was also determined. Wheat/barley diets increased digesta viscosity ($P < 0.01$) and PCNA expression ($P < 0.001$) and tended to decrease ($P < 0.07$) APN activity. Monoassociation reduced ($P <$

0.01) body weight, relative spleen weight, crypt depth, PCNA expression, active caspase 3 abundance, sucrase expression, neutral, acidic and total goblet cells in crypts and neutral goblet cell in villi and mucin gene expression. Monoassociation increased ($P <$ 0.01) relative SI length, digesta viscosity, villus height, APN and sucrase activity and tended to increase acidic mucin cells in the villi. Interactive effects were observed only as trends ($P < 0.1$) such that increased PCNA expression was evident only in conventional wheat-barley-fed pigs, Muc 2 expression was lower only in monoassociated pigs fed wheat-barley and APN activity was highest in monoassociated pigs fed corn. In conclusion, as expected, monoassociation markedly influenced intestinal physiology. Limited effects of cereal type were observed. Only the increase in expression of PCNA in wheat-barley diets appeared microbially mediated.

### 4.2. Introduction

The mammalian gut harbors a vast and complex community of microbiota in a continuous and dynamic relationship with the host. There is increasing evidence that commensal bacteria, through intimate contact with the mucosa, play a crucial role in gene expression and functional development of several components of the immune and gastrointestinal systems (Hooper, 2004). Gnotobiotic animals represent a valuable model for evaluating the effects of single species or defined populations of microorganisms on host response. Earlier studies in gnotobiotic pigs in our laboratory have reported significant differences in intestinal morphology and gene expression in pigs monoassociated with different bacterial species (Shirkey *et al.,* 2006; Willing and Van Kessel, 2007; Siggers *et al.,* 2008; Danielsen *et al.,* 2007).

Diet composition and specifically cereal grain type can affect microbial composition in the pig small intestine (Drew *et al.,* 2004; Hill *et al.,* 2005; Pieper *et al.,* 2008). Furthermore, various studies have fed different fibre sources in an effort to optimize gut environment and health at weaning in pigs (Pluske *et al.,* 1998; Montagne *et al.,* 2004 and Hedemann *et al.,* 2006). Dietary fibre has been reported to alter gastrointestinal physicochemical environment, morphology (Jin *et al.,* 1994) and digestive enzyme activity in pigs (Hedemann *et al.,* 2006). Increasing evidence in monogastric animals suggest that nature and origin of fibre also influences mucin synthesis, secretion and composition (Sharma and Schumacher, 1995 and Christelle Piel *et al.,* 2005). The physiological responses associated with changes in diet composition and fibre content have largely been assumed as indirect, associated with changes in microbial composition (Flint *et al.,* 2007). We therefore investigated whether the whole cereal grains influenced gastrointestinal physiology and if so, whether these effects were direct or associated with altered microbial status.

## 4.3. Materials and methods

### 4.3.1 Experimental Design and Diets

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

Experimental diets (Table 3.1) were formulated using corn or wheat and barley using supplemented DL-methionine or its hydroxyl-analog (MHA-FA) to meet or exceed nutrient requirements (NRC, 1998). For the gnotobiotic experiments, diets were vacuum

sealed and sterilized by gamma-irradiation at 5 Mrads (MDS Nordion, Canadian Irradiation centre, Laval, Quebec). Pigs in all treatments groups had *ad libitum* access to feed and water. All the parameters were examined at 75% SI location consistent with the site of maximum influence of microbiota on intestinal morphology as previously observed (Shirkey *et al*., 2006).

### 4.3.2. Conventional pig experimental design

In two replicate conventional pig (Large White x White Duroc; Prairie Swine Centre, Inc., Saskatoon SK) experiments (16 pigs per replicate) were vaginally delivered and sow reared until 14 *d* of age. Pigs were then weaned and randomly assigned to one of four experimental diets (4 pigs per diet in each experiment) balanced for litter of origin, sex and body weight. Ten days after weaning, pigs were killed by asphyxiation with carbon dioxide and exsanguinated. This is the same group of 32 conventional piglets as reported in chapter 3.3.3

### 4.3.3 Gnotobiotic pig experiments

Enterococus faecium (EF)-monoassociated piglets described in Chapter 3, sections 3.3.4., 3.3.5. and 3.3.6 were utilized in this experiment.

### 4.3.4. Monitoring of Microbial Status

Peri-anal swabs were taken from gnotobiotic pigs during the course of the experiment to monitor microbial status. Swabs and the intestinal contents collected aseptically at 75% of SI length of SI following euthanasia were cultured aerobically and anaerobically on tryticase soy (TS) agar (Difco, Becton Dickinson and Co. Sparks, MD, USA), for enumeration and identification of colonizing bacteria. Bacterial populations were expressed as $\log_{10}$ colony forming units per gram of wet intestinal contents.

In the case of gnotobiotic pigs, isolated bacterial colonies were identified by *cpn*60 universal target (UT) sequencing (Hill *et al.,* 2004). Briefly, bacterial DNA was extracted from overnight culture in TS broth with phenol-chloroform-isoamyl alcohol according to previously described methods (Dumonceaux *et al.,* 2006). PCR reactions were set up using 1µl of extracted DNA, 0.5 U of Taq polymerase, 50mM MgCl2, 10mM of dNTPs and 0.375 µM for each of the degenerate primers H729 and H730 (Hill *et al.,* 2002). The protocol included denaturation at 95˚C for 3 min, followed by 40 cycles of 1min at 95˚C, 1 min at 46˚C, 1 min at 72˚C and finally a 5 min extension at 72˚C. PCR products were purified using QIAquick Gel extraction Kit (Qiagen) and sequenced directly by cycle sequencing at the NRC Plant Biotechnology Institute core facility by using M13 forward and reverse sequencing primers. PreGap4 (version 1.1) and Gap4 (version 4.6) in the Staden software package (release 2000: J. Bonfield, K. Beal, M. Betts, M. Jordan, and R. Staden, 2000) were used to assemble raw data. Contig sequences were compared to cpnDB (http://cpndb.cbr.nrc.ca) using FASTA (Hill *et al.,* 2004).

### 4.3.5. Sample collection

At 24 d of age piglets were removed from isolators, weighed and killed by submersion in $CO_2$ and exsanguination. An incision was made along ventral midline of abdomen, and after clamping at the pylorus and ileo-cecal junction, the small intestine was dissected from mesentery and length recorded. A 2 cm segment obtained at 75% of the small intestinal length was placed in 10% buffered formalin for 24 hours and subsequently transferred to 70% ethanol before embedding in paraffin and staining with hematoxylin and eosin for histological analysis. Two 10 cm segments for mRNA and protein analysis were obtained at 75% of the small intestinal length, snap frozen and stored at -80°C. Digesta was also collected from 75% small intestinal location for estimation of pH and viscosity. Liver, spleen and heart were dissected from the peritoneum, blotted and weighed.

### 4.3.6. Digesta physicochemical properties

The pH and viscosity of digesta samples at 75% of small intestinal location were measured immediately after collection. For measuring viscosity, 1-2 g of contents was centrifuged at 14000 rpm for 3 minutes and viscosity (centipoise) was determined using a digital viscometer (Brookfield digital viscometer, Model LVTDVCP-
II, Brookfield Engineering Laboratories, Stoughton, MA., USA). For estimation of pH, the ileal digesta (1-2 g) was vortexed well to make slurry and pH was determined using digital pH/mV/°C meter (Cole Palmer Instrument Company, Chicago, IL, USA). The probe of pH meter was washed well with deionized water between samples.

### 4.3.7. Intestinal morphology

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

### 4.3.8. Intestinal goblet cell histochemistry

All tissue sections were stained in 1 batch to minimize differences in technical manipulations. Tissue sections were stained with alcian blue for acidic mucin, periodic acid-Schiff reaction for neutral mucin and alcian blue plus periodic acid-Schiff reaction for total mucin by Prairie Diagnostic Services (University of Saskatchewan). All slides were coded and examined by a blinded observer. Mucin containing cells were counted in 10 full sized villi and crypts, using an Axiostar plus light microscope (Carl Zeiss Canada Ltd., Toronto, ON) and analyzed using AxioVision 3.1 measurement software (Carl Zeiss Canada Ltd.).

### 4.3.9. Gene expression analysis

Whole intestinal segments were ground using a mortar and pestle and total RNA was extracted from 20-30 mg of tissue using an RNeasy Mini Kit (Qiagen, Mississauga, ON). Genomic DNA was removed from RNA using RNase-free DNase Set (Qiagen). RNA was quantified by optical density at 260/280 nm using a spectrophotometer (Ultrospec 2000, Pharmacia Biotech, Baie d'Urfe, PQ) and 1 μg of RNA was used to generate first strand cDNA using SuperScript$^{TM}$ III First-Strand Synthesis System

(Invitrogen, Carlsbad, CA). Porcine PCNA sequence was not available thus a segment of the gene was sequenced by amplification with primers designed based on human sequence (GenBank accession BC062439). The resulting sequence (GenBank accession DQ473295) showed 93% identity to human PCNA transcript. Primers for APN, LPH, sucrase, PCNA, and housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) were designed (Table 4.1) using Oligo 6 (Molecular Biology Insights, Inc., Cascade, CO) and Beacon Designer (PREMIER Biosoft International, Palo Alto, CA) software. Primers for mucin genes Muc 1 (accession number AY24350801; product size 194bp), Muc 2 (accession number AK231524.1; product size 168bp) and Muc 13 (accession number AK231169.1; product size 189bp) were designed using software Primer 3 (Table 4.1). Target specificity of the PCR primers was confirmed by comparison against the Genbank database using BLAST (National Centre for Biotechnology Information, Bethesda, MD). Transcript abundance was measured by qPCR using SYBR Green detection (iCycler iQ Real-Time PCR detection system, Bio-Rad). Respective RT-PCR products were cloned using the pGEM-T® Easy Vector System II (Promega Co., Madison, WI) followed by purification (Qiagen Plasmid Mini Kit, Qiagen) and quantification using PicoGreen DNA Quantitation Kit (Molecular Probes, Eugene, OR) to generate standard curves (range $10^1$-$10^7$ copies target gene). The PCR products for all the genes were confirmed by sequencing. Transcript numbers are reported per 100 copies of GAPDH except for mucin genes where transcript numbers are reported per 10,000 copies of GAPDH.

**Table 4.1. Quantitative real - time PCR primers for all genes**

| Gene | Forward (5'-3') | Reverse (5'-3') |
|---|---|---|
| APN | CAATATGCCGCCCAAAGGTTC | CCGGATCAGGACGCCATTT |
| LPH | CCAAGTTCTACGCCTCCATAGTC | TCCAAGAAGCAGAAGAGCAAAGA |
| PCNA | TACGCTAAGGGCAGAAGATAATG | CTGAGATCTCGGCATATACGTG |
| GAPDH | GTTTGTGATGGGCGTGAAC | ATGGACCGTGGTCATGAGT |
| Sucrase | TGGCATCCAGATTCGAAGAA | GATCTCGCTTAAATGCCGTGT |
| Muc 1 | CGGAAGCAGGCACCTATAAC | TCACGGCTGCTTTCTTGACA |
| Muc 2 | CGGCTCTCCAGTCTACTCGT | CTCACAACGTTCTTCACGGT |
| Muc13 | GGTGATTGCATTCGTCCTCT | CCAGTCGGTGTCTTAGGGAT |

### 4.3.10. Protein and enzyme activity assays

Intestinal tissue at 75% of SI length was ground over liquid nitrogen using a mortar and pestle. The pulverized tissue (200 mg) was homogenized in 10 mL of 1% Triton X-100 with a Brinkman Homogenizer and centrifuged at 1500g for 5 min. The supernatant was used for estimating protein content, lactase phlorizin hydrolase, aminopeptidase N and sucrase activity. Enzyme activities are reported per minute per gram of protein determined using the Bio-Rad protein microassay procedure (Bio-Rad Laboratories) with a bovine serum albumin standard (Bradford, 1976).

APN activity was determined by modification of a previously reported method (Maroux *et al.,* 1973). Substrate for the APN assay was 10 mM L-alanine-4-nitroanilide with 50 mM TRIS HCl at pH 7.3. The standards included a range from 6.25 to 200 μM of 4-nitroaniline with 10 μl boiled protein homogenate, with the blank containing 200 μl substrate and 10 μl boiled homogenate. Substrate (200 μl) was added to 10 μl of homogenate and the color development measured at 405 nm (Spectramax, Molecular Devices Corp., Sunnyvale, CA) after 5 minutes at 37°C.

Lactase and sucrase activity was measured by determining the amount of glucose liberated from lactose or sucrose, respectively, as modified from Dahlqvist (1964). β-lactose or sucrose (20 μL of 0.1 M solution) was added to 20 μL of homogenate and incubated in a 96 well plate for 30 minutes at room temperature. Glucose liberated was then measured using the Wako Glucose Assay Kit (Wako Bioproducts, Richmond, VA) according to manufacturer's instructions.

### 4.3.11. Caspase-3 activity

Caspase-3 like enzyme activity was measured in homogenized tissue based on fluorescence emission following Asp-Glu-Val-Asp (DEVD) specific cleavage using the EnzChek® Caspase 3 Assay Kit (Molecular Probes, Inc., Eugene, OR). Briefly, 5 mg pulverized intestinal tissue at 75% SI length was lysed in 200 μL of lysis buffer (10 mM TRIS, pH 7.5, 0.1 M NaCl, 1 mM EDTA, 0.01% TRITON™ X-100) using a freeze thaw cycle in liquid nitrogen. Then the lysed cells were centrifuged at 5000 rpm for 5 minutes and 50 μL of the resulting lysate was placed in duplicate in a 96 well plate. To each sample well, 50 μL of 2X reaction buffer [20 mM PIPES, pH 7.4, 2 mM EDTA, 0.2% CHAPS, 0.02 mM Z (benzyloxycarbonyl group)-DEVD-AMC (7-Amino-4-methylcoumarin)] was added and incubated for 10 min at room temperature. Fluorescence emission (excitation/emission wavelength - 342/441 nm) was determined using a Fluoroskan Ascent fluorometer (Thermo Labsystems, Helsinki, Finland). Assay linearity was confirmed by analysis of fluorescence when serial 2-fold dilutions of lysed tissue homogenate were used. Specificity of fluorescence emission to DEVD cleavage was confirmed by addition of a non-fluorescent reversible aldehyde substrate inhibitor (Ac-DEVD-CHO). A standard curve was prepared using AMC ranging from 6.25-100 μM.

### 4.3.12. Statistical analysis

A separate one-way ANOVA using general linear models (GLM, SPSS software v. 12.0, SPSS Inc, Chicago IL, USA) showed no replication effect for conventional pigs. Data were subsequently analyzed as a 2x2x2 ANOVA using GLM with main effects of

cereal grain (corn vs. wheat/barley), microbial status (conventional vs. monoassociated) and methionine source (MET versus MHA-FA) plus interactions as sources of variation. Source of methionine did not affect any parameter studied and was removed from the model. Therefore, the final model was a 2x2 factorial with main effects of cereal grain and microbial status plus interactions as sources of variation. Where significant interactions were observed between cereal grain type and microbial status, data was aggregated as a single main effect and means were separated using REGWF with significance of $P < 0.05$.

## 4.4. Results

### 4.4.1. Health, body weight, organ weights and SI length

Generally all pigs were in good health except one conventional pig fed corn (replicate 1) and one gnotobiotic pig fed wheat/barley were euthanized due to postweaning anorexia. All other pigs appeared healthy based on visual appearance, appetite, fecal consistency and final body weight (Table 4.2). Initial body weights of gnotobiotic pigs were not recorded to avoid an increased possibility of introducing contamination. Final body weight (kg) at 24 d of age was markedly greater ($P< 0.0001$) in conventional pigs compared with monoassociated pigs. Also, pigs fed wheat barley were heavier ($P< 0.05$) than corn-fed pigs. Mean relative length of SI and relative heart and liver weight was greater ($P< 0.05$) in monoassociated pigs as compared to CON pigs. Relative spleen weight was greatest in CON pigs. Diet composition did not affect relative organ length or weight.

**Table 4.2. Body weights and average relative lengths of small intestine, weights of liver, spleen and heart in conventional and monoassociated pigs fed corn or wheat-barley diets**

| | Body wt. (kg) | SI length (m/kg) | Liver (g/kg) | Spleen (g/kg) | Heart (g/kg) |
|---|---|---|---|---|---|
| **Cereal type** | | | | | |
| Corn | 4.23 | 1.85 | 23.03 | 1.72 | 6.33 |
| Wheat-barley | 4.70 | 1.71 | 23.75 | 1.89 | 6.18 |
| Pooled SEM | 1.02 | 0.42 | 1.26 | 0.27 | 0.66 |
| **Microbial status** | | | | | |
| Conventional | 5.62 | 1.25 | 22.54 | 2.03 | 5.76 |
| EF | 3.32 | 2.32 | 24.25 | 1.59 | 6.74 |
| Pooled SEM | 0.64 | 0.16 | 1.73 | 0.40 | 0.57 |
| **P value** | | | | | |
| Cereal type | 0.042 | 0.057 | 0.254 | 0.225 | 0.587 |
| Microbial status | 0.001 | 0.001 | 0.008 | 0.002 | 0.004 |
| Cereal*microbial status | 0.140 | 0.196 | 0.903 | 0.837 | 0.619 |

### 4.4.2. Microbial Status

Culture of peri-anal swabs indicated contamination of gnotobiotic pigs at 1 d of age. Morphologic examination of colonies observed on aerobic and anaerobic blood agar from cultured swabs or digesta collected at 25% and 75% of SI length suggested the presence of a single bacterial species. Furthermore, the nucleotide sequence for the *cpn*60 UT amplified from all selected colonies showed 100% identity to the Gram positive bacterium, *Enterococcus faecium* (EF) confirming monoassociation. For monoassociated pigs, total aerobic counts at 25% and 75% of SI location (log cfu/g) were not significantly different for corn (7.73±0.27, 8.82±0.26) and wheat-barley (7.61±0.25, 8.71±0.25) diets, respectively. Total anaerobic counts at 25% and 75% of SI length also did not differ between corn (8.17±0.34, 8.99± 0.23) and wheat-barley (7.76±0.34, 8.78 ±0.22) fed pigs, respectively.

For conventional pigs, total aerobe counts (log cfu/g) at 25% location were significantly ($P<0.05$) higher in wheat-barley fed pigs (5.47±0.19) compared to corn (4.81±0.19). Total aerobes at 75% location (6.35±0.19, 6.26±0.20) and total anaerobes at 25% (5.70± 0.14, 5.92± 0.15) and 75% location (6.48± 0.21, 6.42± 0.23) for corn and wheat/barley, respectively, were not significantly different for corn or wheat/barley.

### 4.4.3. Intestinal environment

Ileal pH at 75% of small intestinal length was not significantly different for cereal grain type or the type of microbiota (Table 4.3). However, both the type of the cereal and microbial status influenced the ileal digesta viscosity measured at 75% of small intestinal

length (Table 4.3). Higher viscosity was found in monoassociated pigs ($P<0.001$) and wheat-barley fed pigs ($P<0.01$) compared to CON and corn fed pigs, respectively.

### 4.4.4. Enterocyte replacement

Microscopic examination of the small intestinal cross-sections at 75% length revealed that conventional pigs had shorter ($P<0.001$) villi and longer ($P<0.001$) crypts than monoassociated pigs (Table 4.3). Diet composition did not affect villus height or crypt depth. PCNA transcript abundance (copies per 100 copies GAPDH) was higher ($P=0.001$) in conventionally raised pigs (Table 4.3) consistent with deeper crypts. Interestingly, PCNA transcript abundance was higher ($P<0.001$) in pigs fed wheat/barley compared to corn. The effect was associated with a trend ($P<0.1$) towards a cereal type by microbial status interaction (Figure 4.1, panel A) such that PCNA expression was highest in conventional pigs fed wheat barley. Caspase-3 activity in SI tissue, as an indicator of apoptotic activity, was increased ($P<0.001$) in conventional pigs, compared to monoassociation with EF (Table 4.3). Dietary composition did not affect caspase-3 activity.

### 4.4.5. Digestive enzyme activity and expression

Aminopeptidase expression was relatively consistent among treatment groups except that monoassociated pigs tended to have higher ($P=0.07$) expression (Table 4.4). There was a trend ($P=0.1$) towards a significant interaction between microbial status and APN activity. (Table 4.4, Figure 4.1, Panel B). Monoassociated pigs had increased APN activity relative to conventional pigs however this effect was most pronounced in corn

than wheat/barley-fed pigs. Sucrase expression and activity were markedly affected by microbial status such that in monoassociated pigs expression was decreased ($P<0.001$) and activity was increased ($P<0.001$) altering ($P<0.001$) the ratio. Examination of the trend ($P<0.1$) towards a significant interaction for sucrase expression did not reveal a marked shift from this pattern (Figure 4.1, Panel C). Cereal type did not affect sucrase parameters. No significant differences were observed for LPH gene expression, activity or the ratio of activity to expression (data not shown).

**Table 4.3. Effect of cereal type and microbial status on pH, viscosity and intestinal morphology in conventional and monoassociated pigs fed corn or wheat-barley diets**

| | Ileal pH | Viscosity (centipoise) | Villous height (μM) | Crypt depth (μM) | PCNA abundance (no. of copies/100 copies GAPDH) | Caspase - 3 (nmoles/ g tissue/min) |
|---|---|---|---|---|---|---|
| **Cereal type** | | | | | | |
| Corn | 6.94 | 1.85 | 385 | 172 | 34.6 | 20.8 |
| Wheat-barley | 7.02 | 2.21 | 359 | 174 | 70.23 | 23.9 |
| Pooled SEM | 0.23 | 0.42 | 45.6 | 30.2 | 15.1 | 9.30 |
| **Microbial status** | | | | | | |
| Conventional | 7.05 | 1.48 | 333 | 202.3 | 63.9 | 31.6 |
| EF | 6.90 | 2.58 | 411 | 144.9 | 41.1 | 13.1 |
| Pooled SEM | 0.23 | 0.26 | 53.6 | 24.6 | 27.1 | 9.60 |
| **P-value** | | | | | | |
| Cereal type | 0.42 | 0.01 | 0.22 | 0.86 | 0.01 | 0.34 |
| Microbial status | 0.15 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| Cereal*microbial status | 0.57 | 0.94 | 0.45 | 0.60 | 0.09 | 0.26 |

**Table 4.4. Effect of cereal type and microbial status on digestive enzymes activity and expression in conventional and monoassociated pigs fed corn or wheat-barley diets**

| | APN expression (no. of copies/100 copies of GAPDH) | APN activity (µmoles p-nitroaniline liberated/g/min) | APN activity: expression | Sucrase expression(log no. of copies /100 copies of GAPDH) | Sucrase activity (Log µmoles glucose liberated /g/min) | Sucrase activity: expression |
|---|---|---|---|---|---|---|
| **Cereal type** | | | | | | |
| Corn | 7894 | 267 | 3.11 | 3.88 | 0.92 | 0.35 |
| Wheat-barley | 8271 | 215 | 2.84 | 3.64 | 0.96 | 0.35 |
| Pooled SEM | 1212 | 81 | 0.80 | 1.24 | 0.22 | 0.16 |
| **Microbial status** | | | | | | |
| Conventional | 7474 | 208 | 3.07 | 5.53 | 0.74 | 0.13 |
| EF | 8691 | 275 | 2.89 | 1.99 | 1.15 | 0.57 |
| Pooled SEM | 1486 | 54.7 | 1.29 | 0.54 | 0.19 | 0.04 |
| **P value** | | | | | | |
| Cereal type | 0.576 | 0.07 | 0.60 | 0.25 | 0.64 | 0.99 |
| Microbial status | 0.07 | 0.02 | 0.73 | 0.01 | 0.01 | 0.01 |
| Cereal*microbial status | 0.15 | 0.09 | 0.22 | 0.09 | 0.92 | 0.50 |

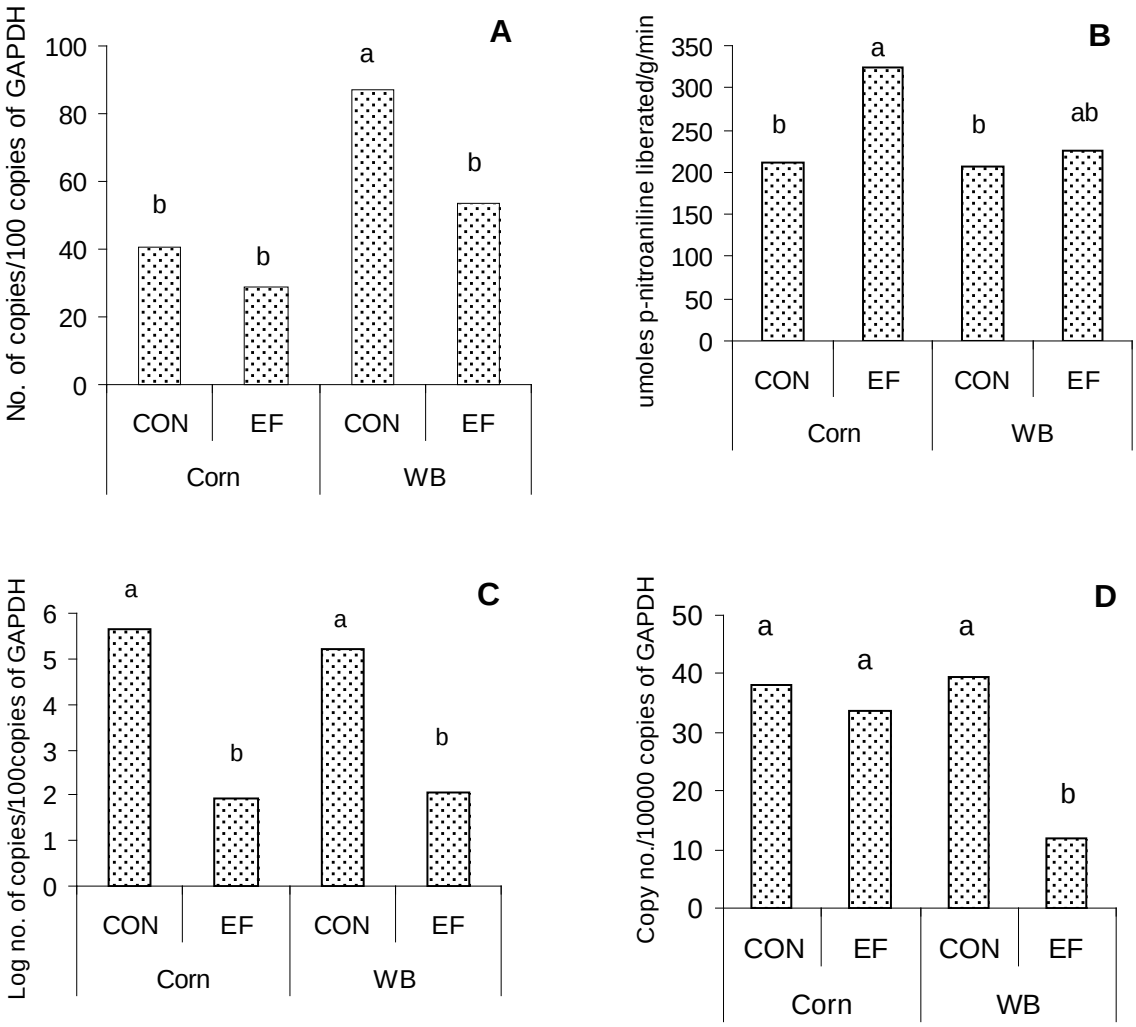



**Figure 4.1. PCNA expression (A), APN activity (B), sucrase expression (C) and Muc 2 expression (D) in conventional (CON) and monoassociated (EF) pigs fed corn or wheat-barley based post-weaning diets. Bars with different letters are significantly different (P<0.05)**

### 4.4.6. Goblet cell histochemistry and mucin expression

The total number of goblet cells per villus was not significantly affected by microbial status, however, monoassociation decreased ($P<0.001$) the number of neutral mucin cells per villi and tended ($P<0.1$) to increase the number of acidic mucin cells compared with conventional pigs. In crypts, monoassociation decreased ($P<0.001$) the number of neutral and acidic goblet cells as well as the total goblet cell number. Type of cereal grain did not affect the number or type of goblet cells in villi or crypts.

Relative transcript abundance for three mucin genes in whole intestinal tissue at 75% SI length is presented in Table 4.5. Based on the number of copies/10,000 copies of GAPDH, Muc 13 was the most highly expressed followed by Muc 2 and Muc 1. The expression of membrane associated mucin genes (Muc 1 and Muc 13) was significantly reduced ($P<0.001$) in monoassociated compared to conventional pigs. Relative expression of the secreted mucin (Muc 2) was also significantly ($P<0.05$) lower in monoassociated pigs, however, analysis of the interaction trend ( $P<0.1$) for this gene revealed that the reduction in Muc 2 expression was more marked in monoassociated pigs fed wheat/barley compared to monoassociated pigs fed corn ( Figure 4.1, Panel D).

**Table 4.5. Effect of cereal type and microbial status on goblet cells staining pattern and mucin gene expression in conventional and monoassociated pigs fed corn or wheat-barley diets**

| | Number/10 villi | | | Number/10 crypts | | | Mucin genes (copy no./10000 copies of GAPDH) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Neutral | Acidic | Total | Neutral | Acidic | Total | Muc 1 | Muc 2 | Muc 13 |
| **Cereal type** | | | | | | | | | |
| Corn | 16.1 | 42.0 | 120.1 | 19.74 | 12.23 | 88.38 | 0.06 | 35.90 | 118.9 |
| Wheat-barley | 15.4 | 39.9 | 122.5 | 19.35 | 10.14 | 85.71 | 0.05 | 25.66 | 103.4 |
| Pooled SEM | 5.33 | 8.81 | 8.19 | 6.28 | 4.69 | 10.24 | 0.001 | 0.47 | 2.63 |
| **Microbial status** | | | | | | | | | |
| Conventional | 22.3 | 37.0 | 121.7 | 25.85 | 14.83 | 99.18 | 0.08 | 38.73 | 157.8 |
| EF | 9.21 | 44.9 | 120.9 | 13.24 | 7.54 | 74.91 | 0.03 | 22.83 | 64.5 |
| Pooled SEM | 5.11 | 8.36 | 9.74 | 6.49 | 5.38 | 7.55 | 0.001 | 0.47 | 2.39 |
| **P value** | | | | | | | | | |
| Cereal type | 0.72 | 0.63 | 0.57 | 0.87 | 0.25 | 0.32 | 0.85 | 0.11 | 0.64 |
| Microbial status | 0.01 | 0.07 | 0.85 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| Cereal*microbial status | 0.41 | 0.17 | 0.73 | 0.73 | 0.34 | 0.68 | 0.35 | 0.07 | 0.59 |

### 4.5. Discussion

These experiments were initially planned to examine the effect of cereal grain type on post weaning digestive physiology in conventional and germ-free pigs. This approach enabled the separation of responses mediated directly by the cereal grain constituents (including but not limited to the difference in ADF and especially NDF content of different cereal grains) versus those mediated indirectly through microbial fermentation. We hypothesized that cereal grain type affects digestive physiology and these would be mediated indirectly by changes in the composition of intestinal microbial community (Hill *et al.*, 2005) or fermentation products. Unfortunately, contamination of the germ-free pigs with *E. faecium* occurred. Fortunately, however, based on viable plate counts, the number of *E. faecium* in intestinal contents was not affected by cereal-type. Thus, cereal type did not influence bacterial colonization in monoassociated pigs in contrast to conventional pigs in which the mixed microbial population was altered based on viable plate counts. Testing of the original hypothesis therefore remains essentially valid.

Our finding of heavier conventional pigs at the end of the experiment is in agreement with results reported by Landy and Ledbetter (1966) and Waxler and Drees (1972) where conventional pigs were found to be heavier than germ-free and isolator reared contaminated pigs at three weeks of age. Raising of conventional pigs with sows until weaning compared to gnotobiotic pigs which were kept in isolators from day 0 probably added to the difference in their growth rates. Although gamma irradiation is the preferred method of diet sterilization resulting in less severe nutrient loss versus other methods (Sickel *et al.*, 1969) some nutrient loss is still accepted. Thus, some caution should be used in assessing the effects of monoassociation versus a conventional microbiota. While

the pigs were of the same chronological age, the observed differences in growth rate could have affected developmental age. Results of relative comparison of organs are also in agreement with Waxler and Drees, (1972) with a heavier heart in germ-free pigs with no difference in liver weight. Reduced weight of spleen in gnotobiotic pigs is consistent with limited immune system development and less antigenic stimulation (Rothkotter, 1991). Relative SI length was greater in monoassociated pigs in contrast to the hypothesis that an increase in SI length could be a host response to improve competition with bacteria for nutrients. Mochizuki and Makita (1997) found that small intestine was significantly shorter only in female SPF swine compared to conventional swine. Length of germfree rat small intestine was found to be comparable to its conventional equivalents (Wostmann, 1996). Thus there is little support to suggest increased SI length imparts an advantage in nutrient competition with the microbiota. Numerous studies examining dietary fibre effects on physicochemical environment and gastrointestinal morphology of the gut indicate variation in results due to different fibre sources, levels and duration of fibre feeding. For example McDonald *et al.,* (2001) found decreased villus height and crypt depth with addition of CMC whereas others found no or only small visible effects on morphology by feeding high levels of non-fermentable dietary fibre (Anugwa et al, 1989; Vahouny, 1987). Nevertheless, it is generally accepted that soluble fibre increases digesta viscosity and reduces pH in the SI associated with increased microbial enzymatic degradation of NSP and fermentation to lactic and volatile fatty acids (Drochner and Coenen, 1986; Van der Meulen and Bakker, 1991; Jensen and Jorgensen, 1994 and Hogberg and Lindberg, 2004). We did not find significant cereal grain type or microbial status effects on ileal pH. This was somewhat surprising in terms

of microbial status in that we expected a higher pH in monoassociated pigs associated with reduced fermentative capability. However, *Enterococci* spp. are among the lactic acid bacteria which produce lactic acid as a major fermentation product. Although we did not measure digesta lactic acid or VFA content it is possible that lactic acid levels were comparable in monoassociated and conventional pigs. Digesta viscosity was increased in wheat/barley fed pigs consistent with the high soluble fibre content. Pigs associated with EF also demonstrated increased viscosity independent of diet consistent with a limited capacity of EF in fibre degradation. Although fibre degradation is often discussed as primarily a function of the hindgut microbiota, our results do confirm a microbial contribution to small intestinal fibre degradation and digesta viscosity.

In the present study, there was good agreement among changes in villus height, crypt depth, expression of PCNA and caspase-3 activity as indicators of intestinal morphology. Conventional pigs had short villi and longer crypts associated with higher PCNA expression and caspase-3 activity in agreement with earlier studies in pigs (Willing and Van Kessel, 2007). Wheat-barley fed conventional pigs were found to have higher PCNA expression primarily associated with a conventional microbiota. Previous studies have reported that high dietary fibre alters the rate of intestinal cell turnover as well as intestinal morphology in growing pigs (Jin *et al.,* 1994). Furthermore, McCullough *et al.,* (1998) concluded that dietary fibre has direct and indirect effects on the gut including higher crypt cell production and decreased enteroendocrine cells in small intestine of conventional rats. We observed that wheat-barley diets increased total aerobes significantly ($P<0.05$) (chapter 3.4.2.) based on conventional culture analysis. Our finding of higher PCNA transcript abundance in conventional and wheat-barley diet fed

pigs along with a trend of significant interaction suggests that at the intestinal cellular level dietary fibre effects were mediated by gut microbiota and also supporting the hypothesis that high dietary fibre increases intestinal proliferation. Surprisingly, however, cereal grain fibre had no effect on crypt depth pointing to the fact that cell proliferation might not be limited to the crypts in pigs as has already been reported in chicken (Uni *et al.,* 1998).

The trophic effects of dietary fibre may be caused directly by physicochemical changes in the gut environment or indirectly mediated by bacterial fermentation products and/or the stimulation of trophic hormones. Use of non-fermentable dietary bulk (Kaolin) neither stimulates epithelium nor modifies the effect of SCFA (Sakata, 1986) suggesting that it is not likely that physical abrasion stimulates epithelial proliferation. Other studies, however, have reported decreased villus length and increased crypt depth with addition of high viscosity non fermentable carboxymethyl cellulose (CMC) to the diet of weanling piglets (McDonald *et al.,* 2001) and increased villus height in pigs fed high insoluble dietary fibre (Hedemann *et al.,* 2006). Absence of increased intestinal proliferation in EF pigs in this study is in accordance with earlier studies in germ-free rats suggesting the importance of intestinal microbiota in intestinal proliferation (Komai *et al.,* 1982; Goodlad *et al.,* 1989 and Fuller *et al.,* 1993). Short chain fatty acids are the main end products of bacterial fermentation but the mechanisms of their trophic action on intestinal tissue have not been clarified. The findings here indicate that at molecular level effects of cereal grain type on gut morphology were indirectly mediated by conventional microbiota. SCFAs were reported to increase functional capacity of bowel by increasing the mRNA abundance of nutrient transporters including facilitative glucose transporter

(GLUT2), brush-border sodium/glucose cotransporter (SGLT-1) (Tappenden, 1997) and by increasing epithelial surface area (Bartholome *et al.,* 2003). Other studies revealed that SCFA induced intestinal adaptation was associated with upregulation of ileal proglucagon mRNA abundance as well as plasma GLP-2 concentrations (Tappenden and McBurney, 1998 and Tappenden *et al.,* 1998). Intestinal microbiota has also been shown to differentially affect the proglucagon gene expression (Siggers *et al.,* 2008). However, the specific attributes of dietary fibre that affect these processes are unclear.

In the present study, aminopeptidase and disaccharidases activity and gene expression was estimated in whole intestinal tissue at 75% length of small intestine. Intestinal microbiota can affect digestive physiology by altering digestive enzymes directly by microbial synthesis or inhibition of enzymes (Borgstrom *et al.,* 1959) or indirectly through alterations in luminal pH, secretory and absorptive functions and epithelial turnover (Lesher *et al.,* 1964 and Khoury *et al.,* 1969). We found reduced activity of brush border enzyme aminopeptidase N in conventional pigs in agreement with previous studies (Borgstrom *et al.,* 1959 and Willing and Van Kessel, 2009). However, in contrast to the previous study (Willing and Van Kessel, 2009) where in conventional pigs decreased APN activity was found concomitantly with increased expression, we observed that APN expression tended to be higher in monoassociated pigs. These studies were carried out in 24 d old post weaned pigs on a solid corn-soy or wheat-barley soy diets and therefore, different experimental design including age of pigs, weaning status and dietary factors might be responsible for the change in expression. In this study, presence of higher number of mature enterocytes as indicated by finding of longer villi in monoassociated pigs could be responsible for higher APN enzyme activity as postulated

in germ-free rats (Reddy and Wostmann, 1966). Alternatively, in conventional pigs microbial products could inactivate digestive enzymes leading to reduced activity as reported earlier (Corring *et al.,* 1981). It is not clear why the increase in APN activity in EF pigs tended to be less in wheat-barley compared to corn-fed pigs. It is possible however, that the physico-chemical properties of wheat-barley relative to corn impacted APN activity directly.

We found reduced sucrase activity along with higher expression in conventional pigs suggesting increased turnover of this enzyme. These results agree with earlier results of increased expression and decreased activity of APN in milk fed conventionalized pigs (Willing and Van Kessel, 2007). In this study, reduced villus height, increased PCNA expression and higher caspase-3 activity in conventional pigs indicate reduced mature cell number and hence might be responsible for reduced activity. For higher expression of sucrase some other mechanism might play a role. It is known that postnatal maturation of intestinal digestive enzymes can be regulated at the levels of transcription (Krasinski *et al.,* 1980), synthesis (Seetharam *et al.,* 1980), post-translational glycosylation (Beaulieu *et al.,* 1989) and turnover (Seetharam *et al.,* 1980). Ontogenic expression of sucrase isomaltase is primarily regulated at transcriptional level in rats (Leeper and Henning, 1990) and humans (Traber *et al.,* 1992). Various exogenous and endogenous factors including nutrients, growth factors and glucocorticoids can affect brush border enzyme activities by influencing these processes (Kodolvsky, 1981). This increased expression could be as a feedback response to reduced activity or as a direct response to microbial colonization in conventional pigs as suggested for APN (Willing and Van Kessel, 2007).

Cereal grain type was not found to affect sucrase activity to expression ratio despite higher PCNA expression in wheat-barley fed pigs.

Our results of no significant effects of cereal grain type or the microbial status on the activity or expression of LPH in post-weaned pigs can be explained by reports that LPH activity diminishes around weaning because of reduced gene expression and protein translation and increase enzyme turnover in rats (Tsuboi *et al.,* 1992) and pigs (Torp *et al.,* 1993).

In chapter 3.0 we reported that MHA-FA supplemented pigs had higher number of total aerobes and lactobacilli in the upper small intestine. Absence of any significant effects of methionine source in spite of changes in microbial composition suggest that these changes were not sufficient for influencing GI physiology as studied by the parameters here.

We estimated the number of different types of mucin cells in the villi as well as crypts. Conventional pigs had higher neutral mucin goblet cells and a trend of significantly higher acidic cells in their villi in monoassociated pigs. Though not analyzed statistically, we also observed more acidic than neutral goblet cells in villi in conventional as well as monoassociated pigs. This is in agreement with a report by Deplancke and Gaskins (2001) which indicated that the ratio of neutral to acidic mucins increases between birth and weaning and decreases after weaning. Also the higher number of acidic than neutral mucins in villi agree with the studies indicating that the intestinal regions densely populated by microbes predominantly express acidic mucins as these appear less degradable by bacterial glycosidases and host proteases (Roberton and Wright, 1997 and Deplancke *et al.,* 2000). In crypts, we found a higher number of acidic,

neutral as well as total mucin cells in conventional compared to monoassociated pigs in agreement with previous studies reporting that germfree rodents had few and smaller sized goblet cells (Enss *et al.,* 1992; Kandori *et al.,* 1996 and Meslin *et al.,* 1999). In the crypts we also observed increased number of neutral compared to acidic mucin cells in all pigs. Increased number of goblet cells in conventional pigs can be explained as mucus offers a number of ecological advantages to the host such that goblet cell number and presumably mucus secretion is increased in response to intestinal microbes. It was also reported that both commensals and pathogenic bacteria regulate mucus synthesis, secretion and composition from host goblet cells (Deplancke and Gaskins, 2001). Cereal grain type was not found to affect mucin cell number or mucin composition in villi or crypts suggesting the microbial composition changes associated with cereal type did not markedly change mucin composition or goblet cell number.

The chemical composition of mucin within cells undergoes changes as the goblet cells mature in mid-crypt region and migrates both upwards toward the villi and downward into deeper regions of crypts (Cheng and Bjerknes, 1980). The production of different types of mucins is dependent not only on number of goblet cells and their mucin content but also on the extent of secretion of preformed mucins and onset and rate of biosynthesis of different mucin types (Brown *et al.,* 1988). In addition to changes in goblet cell counts and types, expression of mucin genes was also influenced by gut microbial composition. Significantly higher expression of membrane bound Muc 1 and Muc 13 in conventional pigs suggested increased capacity for mucin synthesis at the molecular level. As membrane bound mucins, this expression activity is likely associated with enterocytes rather than goblet cells and suggests an important role for these mucins

in the host response to microbial colonization. Interestingly, expression of the secreted mucin (Dharmani et al, 2009) Muc 2, tended to be lower in monoassociated pigs fed wheat-barley but not corn. This contrasts the finding for goblet cell number and indicates that goblet cell number and mucin synthesis may not always be associated.

The regulatory mechanisms that mediate microbiota induced changes in goblet cell number, composition and mucin genes expression are poorly understood. Two hypotheses have been put forward including direct microbial effects through the release of bioactive factors or indirectly by activating the host cells (Deplancke and Gaskins, 2001). Bacterial products like lipopolysaccharides and flagellin A from Gram negative and lipoteichoic acid from Gram positive bacteria were reported as modulators of Muc 2 and Muc 5AC (Dharmani *et al.*, 2009). Others have reported a direct cross-talk between microbes and host epithelium suggesting that the host capacity for synthesizing diverse carbohydrates may have evolved in part from their need both to evade pathogenic relationships and to coevolve in symbiotic relationships with nonpathogenic microbes resident in gut (Hooper and Gordon, 2001). To our knowledge, there are no reports studying expression of mucin genes in pigs. Detailed investigation of mucin dynamics in monoassociated gnotobiotic animals could shed more light on these regulatory mechanisms.

In summary, the intestinal microbiota has major influences on intestinal morphology and digestive physiology including increased viscosity, villus height, APN and sucrase activity along with a decrease in body weight, spleen weight, crypt depth, PCNA expression, caspase-3 activity, sucrase expression, goblet cell counts and mucin genes expression. This study also demonstrates that the post-weaning decline in expression and

activity of LPH is not influenced by the intestinal microbiota. Cereal grain type was found to influence gastrointestinal physiology although differences were relatively minor. Interactions between cereal grain type and microbial status were observed for APN activity and PCNA, sucrase and Muc 2 expression. The nature of those interactions suggested that the microbiota were required for the increase in PCNA expression associated with wheat-barley diets and is consistent with microbial compositional changes associated with fibre content relative to corn. For the other interactions observed it is unclear whether differences were affected directly by components of the cereal or indirectly through microbial fermentation. This study confirms previous studies demonstrating a marked microbial influence on intestinal development. Limited physiological differences were associated with cereal grain type in the diet suggesting that dietary cereal ingredient selection, other than for supply of essential nutrients may not be important for the early (10 days) post weaning phase.

# 5.0. DIGESTA FLOW IN UPPER GASTROINTESTINAL TRACT IN CONVENTIONAL AND GNOTOBIOTIC PIGS

## 5.1. Abstract

Two studies were conducted using sixteen, 14 d old weanling conventional (CON) and sixteen gnotobiotic pigs. For both studies, the experimental diets were based on corn-soybean meal or wheat-barley-soybean meal and supplemented with DL-methionine or its hydroxy-analogue (MHA-FA) as the methionine source to yield four experimental treatments. At 24 d of age after an overnight fast, piglets were offered predetermined (20g/kg BW) meal of their respective experimental diets containing 0.5% chromic oxide. CON pigs were killed at 3 (3 pigs per treatment), 4 (3 pigs per treatment) and 5 (2 pigs per treatment) hours and gnotobiotic pigs were killed at 3 hours after the start of the meal. Intestinal digesta samples were collected at 5, 25, 50, 75 and 95% of small intestinal length. The pH and viscosity were measured in digesta samples at 75% of small intestinal location immediately after collection. An assay was modified and validated to be suitable for detection of chromic oxide in small digesta samples. Percent recovered chromic oxide in digesta samples was estimated at all locations in CON and MA pigs. In CON pigs, corn based diets produced higher pH ($P<0.05$) and lower viscosity ($P<0.05$) compared with wheat-barley based diets. Cereal grain type did not affect the pH or viscosity in MA pigs. Chromic oxide concentration increased distally in the small intestine in both conventional and MA pigs. In conventional pigs for both diets the chromic oxide concentration in small intestine was not significantly different at different time points. Compared with CON, MA pigs showed decreased percent chromic

oxide in digesta at all locations suggesting smaller boluses of feed exited the stomach of MA pigs. Chromic oxide at 95% SI length in MA pigs was not detectable suggesting a slower small intestinal transit of digesta in these pigs. The type of cereal grain in the diet did not affect the percent chromic oxide recovered in CON or MA pigs. In conclusion, a novel chromic oxide microassay was developed and demonstrated an microbial impact on gastric chyme emptying and small intestinal transit time

## 5.2. Introduction

Nutrient digestibility is a function of passage rate of digesta especially through the stomach and upper small intestine. Digestion in turn is affected by animal factors (Noblet and Shi, 1994) and physico-chemical characteristics of the feed (Le Goff and Noblet, 2001).

Various indigestible markers are used for digestibility studies including titanium oxide ($TiO_2$), ytterbium oxide ($Yb_2O_3$), chromium-EDTA (Cr-EDTA), acid insoluble ash (AIA) and chromic oxide ($Cr_2O_3$). Use of chromic oxide as an indigestible marker in this study required large sample sizes for its estimation posing a constraint for its use, especially in individual small pigs, when sampling proximal intestinal segments and when multiple analyses are proposed. Therefore, a microassay for estimation of chromic oxide in small sample size needed to be developed.

Many studies have evaluated effects of dietary fibre on digestive processes but there is limited literature available describing fibre effects on digesta transit through the gastrointestinal tract. For example, Owusu-Asiedu *et al.,* (2006) reported that increasing non starch polysaccharides in the diet in form of guar gum (soluble) and cellulose

(insoluble) reduced the digesta passage rate in the small intestine. Increasing the insoluble dietary fibre in diet decreased the mean retention time in small intestine (Wilfart *et al.,* 2007). Furthermore, indigenous microbiota have been shown to influence intestinal motility in rats (Tennant *et al.,* 1969) suggesting a possible relationship between microbiota and digesta rate of passage. Rate of passage of digesta through the gastrointestinal tract has important implications affecting opportunity for the host for nutrient digestion and absorption and particularly at locations where digesta is retained for longer periods of time, affect opportunity for microbial fermentation as well. Therefore, the current study was designed to determine the effects of corn vs. wheat/barley and commensal microbiota on digesta flow in the pig.

## 5.3. Materials and Methods

### 5.3.1. Chromic oxide microassay

The chromic oxide microassay was based on the procedure of Fenton and Fenton (1979) with modifications to accommodate small sample size. Briefly, 100 mg of freeze-dried digesta or 600 mg of freeze-dried diet were placed at 450$^{o}$C overnight to eliminate all organic matter. These samples were then digested with 5 mL of digestion mixture (2 % sodium molybdate, 40% of 70% perchloric acid and 30% of sulfuric acid in distilled water) on a pre-heated hotplate at 300ºC until the color became yellow or red and then further heated for an additional 15 min. After cooling, the digested samples were adjusted to 10 mL using distilled water and centrifuged at 3000 rpm for 10 min. The absorbency of the supernatant was measured using a spectrophotometer (Spectramax, Molecular Devices Corp., Sunnyvale, CA) at 440 nm after transferring 250 µL in triplicates into 96

well plates ( Nalge Nunc International, USA). A blank and series of chromic oxide standards (Anachemia Canada Inc. Lachine, Quebec) ranging from 250-3000 µg were prepared according to the same assay procedure. Chromic oxide was weighed using a microbalance (Mettler Instrumente AG, Switzerland). Chromic oxide concentration (µg/mg or mg/g of digesta) was calculated using a linear regression equation describing the relationship between the mass of chromic oxide and optical density at 440 nm. Chromic oxide concentration (µg chromic oxide/mg digesta dry matter) in digesta was normalized to (divided by) concentration of chromic oxide in feed (µg chromic oxide/mg digesta dry matter) to correct for any variation in endogenous secretions.

#### 5.3.1.1. Validation of chromic oxide microassay

##### 5.3.1.1.1. To confirm linearity and repeatability

Chromic oxide standards were prepared by weighing aliquots of chromic oxide as described above. Aliquots were prepared in triplicates within 10% of 250, 500, 1000, 1500, 2000 and 3000 µg. Actual mass was recorded and used in the calculations. Calibration standards were prepared on 3 separate days and assays used to determine chromic oxide content in a representative high and low content digesta sample (determine by visual inspection). For each calculation series a linear equation was fitted to the plot of chromic oxide mass versus optical density (440nm). The slope, intercept and correlation coefficient ($R^2$) for each line were recorded. To compare the modified procedure with the standard assay described by Fenton and Fenton (1979), four experimental diets were assayed in triplicates using both procedures.

### 5.3.2. Animal experiments

Two studies involving conventional and germ-free pigs were conducted. The experimental protocol was approved by the University of Saskatchewan Animal Care and Use Committee (IACUC) and was performed in accordance with recommendations of the Canadian Council on Animal Care (1993) as specified in the Guide to the Care and Use of Experimental Animals. For both studies, the experimental diets were based on corn-soybean meal and wheat-barley-soybean meal and supplemented with either DL-methionine or its hydroxy-analogue (MHA-FA) as the methionine source to yield four experimental treatments. Diets were formulated to meet or exceed nutrient requirements of pigs (NRC, 1998) and pelleted. Table 3.1 depicts the diet formulations and calculated as well as analyzed nutrients composition.

#### 5.3.2.1. Conventional pig study

The experimental design for conventional pigs was as described in Chapter 3 section 3.3.3 (Conventional pig experimental design).

Pigs were weighed and humanely killed at 3 (3 pigs per treatment), 4 (3 pigs per treatment) and 5 (2 pigs per treatment) hours after the start of the meal. Intestinal digesta samples were collected at 5, 25, 50, 75 and 95% of small intestinal length, kept on ice and then stored at -20˚C until further analysis. Separate digesta samples were also collected aseptically to confirm bacterial status as described in Chapter 3 section 3.3.7 (Sample collection).

#### 5.3.2.2. Gnotobiotic pig study

Gnotobiotic pigs were the same pigs as described in Chapter 3 section 3.3.4 (Preparation of gnotobiotic isolators), 3.3.5 (Gnotobiotic pig derivation and maintenance) and 3.3.6 (Gnotobiotic pig experimental design). At 24 d of age piglets were offered meal corresponding to 20g/kg BW of their respective experimental diets containing 0.5% chromic oxide. At 3 hours after the start of the meal pigs were weighed and killed by submersion in $CO_2$ and exsanguination. The small intestine was dissected from the mesentery and digesta was collected from 5, 25, 50, 75 and 95% of small intestinal length, kept on ice and then stored at -20˚C until further analysis. Digesta samples were also collected aseptically to confirm bacterial status (see Chapter 3, section 3.3.8. Microbial identification and enumeration).

### 5.3.3. Laboratory Analysis

#### 5.3.3.1. pH and viscosity measurement

The pH and viscosity of digesta samples at 75% of small intestinal location were measured immediately after collection. For measuring viscosity, 1-2 g of content was centrifuged at 14000 rpm for 3 minutes and viscosity (centipoise) was determined using a digital viscometer (Brookfield digital viscometer, Model LVTDVCP-II, Brookfield Engineering Laboratories, Stoughton, MA., USA). For estimation of pH, the ileal digesta (1-2 g) was vortexed well to make slurry and pH was determined using digital pH/mV/°C meter (Cole Palmer Instrument Company, Chicago, IL, USA). The probe of pH meter was washed well with deionised water between samples.

### 5.3.4 Statistical analysis

In a separate one-way ANOVA using the general linear model, source of methionine did not affect any of the parameters studied for conventional or gnotobiotic pigs and therefore was excluded from both final models. Conventional pigs data was analyzed as 2X3X5 factorial using the general linear models procedure of SPSS software (v. 12.0, SPSS Inc, Chicago IL, USA) with main effects of cereal grain type (corn vs. wheat and barley) and time after the meal (3 vs. 4 vs. 5 hrs.) and SI location (5, 25, 50, 75 and 95% of SI length) and interactions as source of variation. For gnotobiotic pigs, data was analyzed as 2X5 factorial with main effects of cereal grain type (corn vs. wheat-barley) and SI location and interactions as sources of variation. Treatment means were

separated using the Least Square Difference and differences were considered significant where $P<0.05$.

## 5.4. Results

### 5.4.1. Validation of chromic oxide microassay

The $R^2$ for the linear regression of each series of chromic oxide standards against absorbance ranged from 0.9917 to 0.9988 as indicated (Figure 5.1). The slope of the calibration curve ranged from 5600 to 5900 µg/unit O.D. (450 nm) and the y-intercept from 11 to 65 µg chromic oxide. Triplicate determination of chromic oxide mass for the same relatively high content digesta sample on each calibration curve produced a mean (±SEM) chromic oxide mass of 18.23±0.12, 17.36±0.11 and 18.01±0.11 µg providing a CV of 2.53 %. For a representative low concentration digesta sample determinations on each curve were 2.89±0.02, 2.83±0.02 and 3.30±0.03 µg with a CV of 8.44%. Table 5.1 shows the CV for chromic oxide determinations in digesta samples analyzed using the original procedure of Fenton and Fenton, (1979) and the modified microassay. The CV varied between 0.86 to 4.26 %.

### 5.4.2. Microbial status of gnotobiotic pigs

Conventional cultural and further molecular sequence analysis (Hill *et al.*, 2004) confirmed that pigs in the four isolators were monoassociated with Gram positive *Enterococcus faecium* (100% identity).

### 5.4.3. Digesta flow

Figure 5.2 (panels A and B) present chromic oxide recovered in digesta as a percent of chromic oxide in diet for conventional and monoassociated pigs, respectively. Percent recovered chromic oxide increased distally in the small intestine in both conventional and monoassociated pigs. Interestingly, in conventional pigs percent chromic oxide recovered declined at the 95% location. In conventional pigs for both diets the chromic oxide concentration in the small intestine was not significantly different at different time points following the meal, nor did time following the meal show any significant interaction with other main effects. In monoassociated pigs percent chromic oxide recovered in intestinal contents was much lower than for conventional pigs. Also we failed to detect any chromic oxide at 95% length of small intestine at 3 hours following the meal. The type of cereal grain in the diet did not affect the percent chromic oxide recovered in conventional or monoassociated pigs.

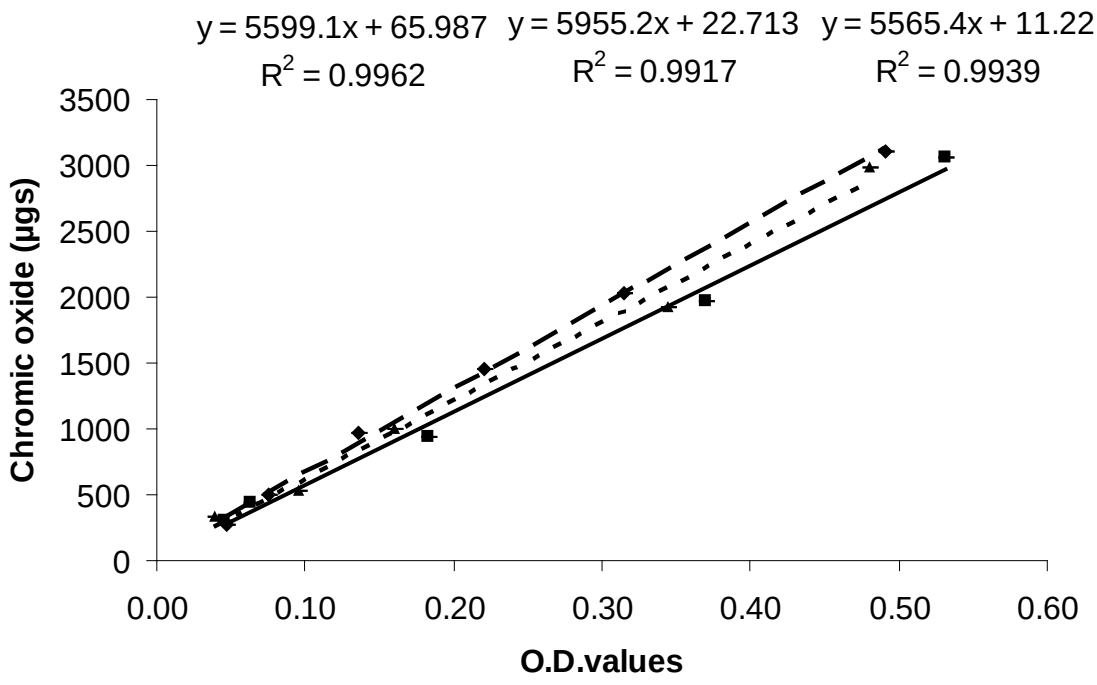



**Figure 5.1. Calibration curves for three experiments showing relation between absorbance (440 nm) and chromic oxide mass using the modified microassay procedure. The linear equation and $R^2$ for each series is given at the top of the figure.**

**Table 5.1. Comparison of chromic oxide content (μg/mg) in the four experimental diets using the original Fenton and Fenton (1979) procedure or the modified procedure**.

| Type of diet | Original procedure | Modified procedure | CV[a] |
|---|---|---|---|
| Corn-Soy-MET | 5.6 | 5.74 | 1.75 |
| Wheat-Barley-Soy-MET | 5.8 | 6.16 | 4.26 |
| Corn-Soy-MHA-FA | 5.7 | 5.77 | 0.86 |
| Wheat-Barley-Soy-MHA-FA | 5.8 | 5.92 | 1.45 |

[a] Coefficient of variance

### 5.4.4. Intestinal environment

The corn and wheat-barley diets were found to significantly alter digesta pH and viscosity. In conventional pigs, corn diets had higher pH ($P<0.05$) and lower viscosity ($P<0.05$). In monoassociated pigs cereal grain type did not affect the pH or viscosity. Though a statistical comparison could not be made, monoassociated pigs had higher viscosity and lower pH for both corn and wheat-barley diets than conventional pigs.

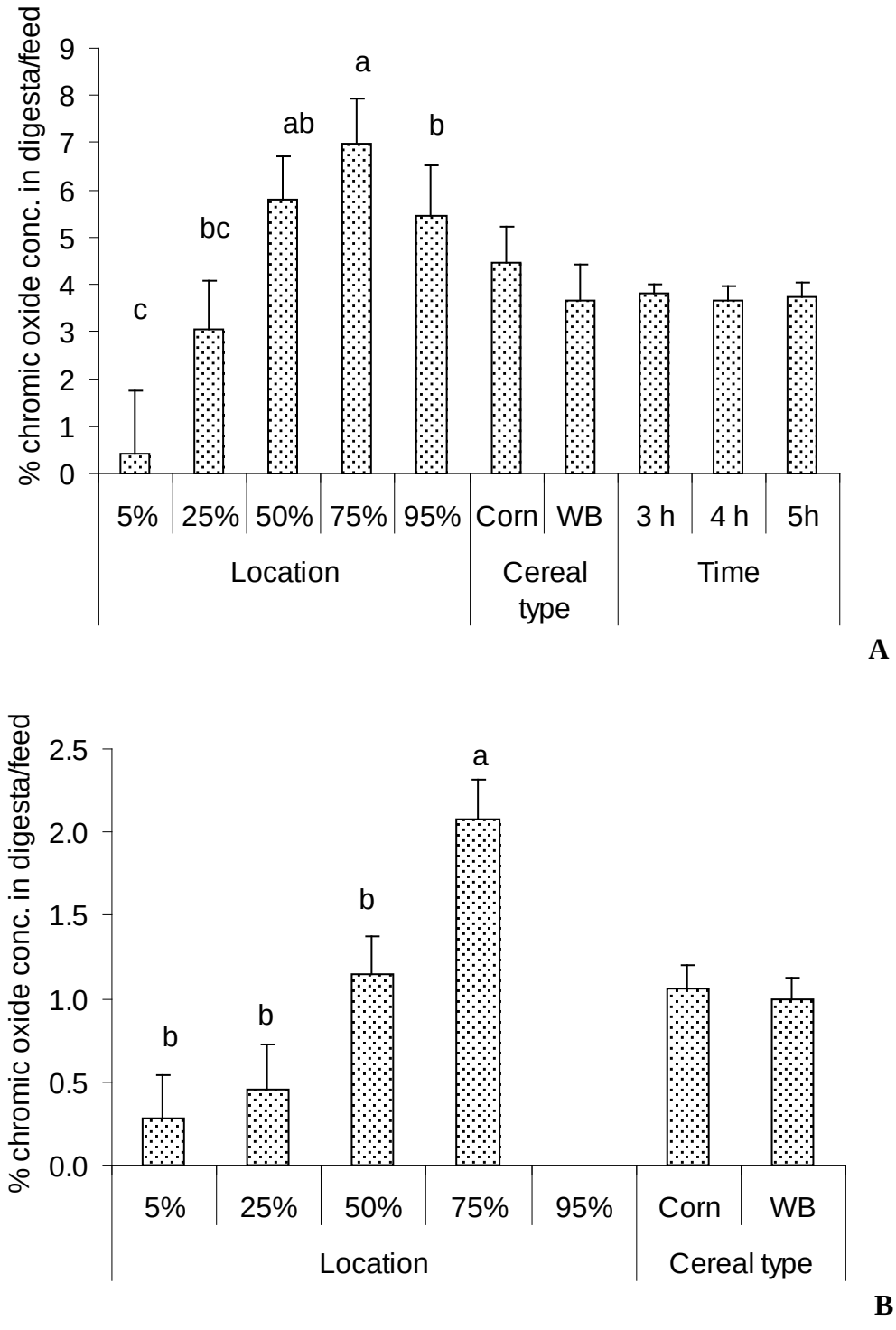



**Figure 5.2. Percent chromic oxide recovered in digesta at 5, 25, 50, 75 and 95% length of SI at 3, 4 and 5 hrs. in CON (A) and 5, 25, 50, 75% SI length in MA pigs (B) at 3 hrs. after feeding a corn or wheat-barley based meal. The vertical bars depict SE. [a, b] Means with a different letter are significantly different (P<0.05)**

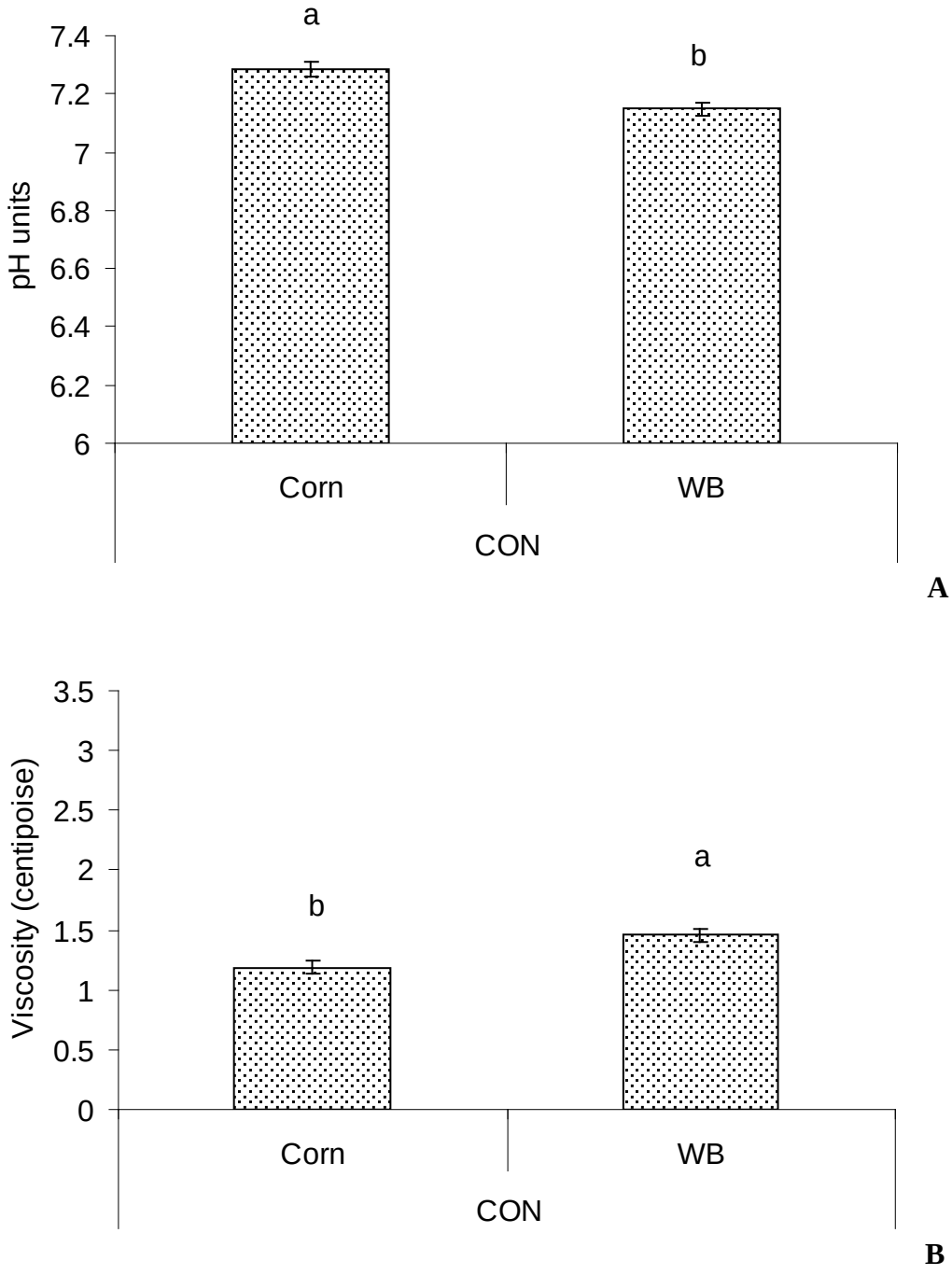



**Figure 5.3. pH (A) and viscosity (B) in digesta at 75% of small intestinal length in conventional pigs fed corn or wheat-barley based diets. The vertical bars depict SE. [a, b] Different lowercase letters on each bar indicate significant difference (P<0.05)**

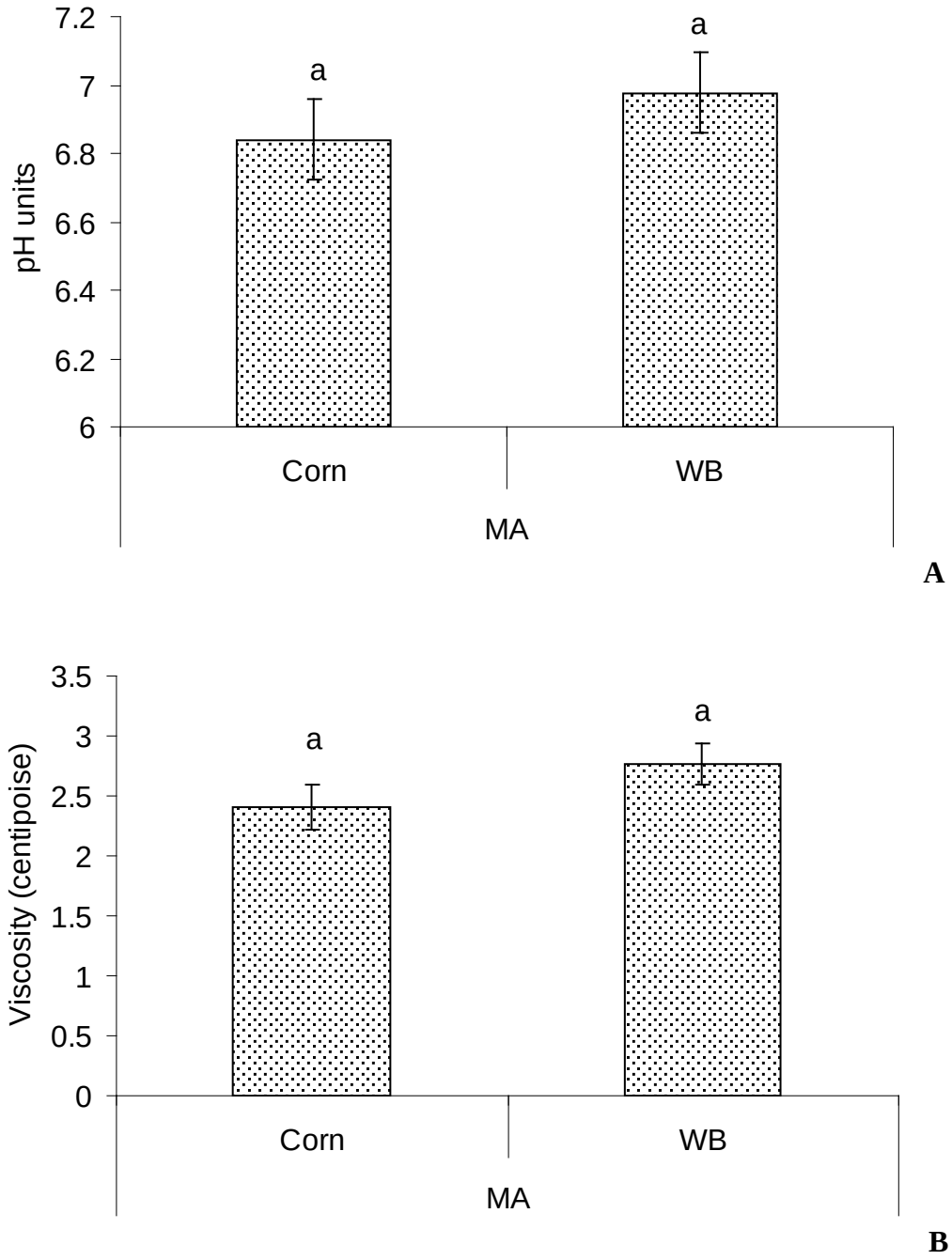



**Figure 5.4. pH (A) and viscosity (B) in digesta at 75% of small intestinal length in monoassociated pigs fed corn or wheat-barley based diets. The vertical bars depict SE. [a, b] Different lowercase letters on each bar indicate significant difference ($P<0.05$).**

### 5.5. Discussion

The flow of dry matter through the gastrointestinal tract has been estimated by measurement of an indicator in intestinal contents or faeces at regular intervals. Chromium oxide does not fullfill all of the ideal characteristics of an indicator (Faichney, 1975; Beever *et al.*, 1978) nevertheless it is one of the most commonly used markers of dry matter flow in swine digestibility studies. The limitations of chromic oxide as an indigestible marker include interference of phosphorus with $Cr_2O_3$ estimation, possibility of environmental pollution due to heavy metal chromium (Cr) and hazardous nature of perchloric acid used in this procedure. We were successfully able to scale down the assay to estimate chromic oxide in $1/20^{th}$ of original assay sample size. The correlation coefficient of the plots between chromic oxide values and optical density was above 0.99 indicating linearity. Although the CV between assays increased at lower chromic oxide concentrations interassay variation was in an acceptable range. The coefficient of variance between the original assay of Fenton and Fenton (1979) and the microassay ranged from 0.86 to 4.26% showing good agreement with the original assay.

We used chromic oxide concentration in digesta as a percent of chromic oxide in feed along the small intestine as a measure of flow of digesta through the upper gut so as to correct for endogenous secretions and any changes of biomass. Because we did not collect total mass of digesta and therefore, we could not calculate absolute % of chromic oxide at each location.

In conventional pigs, the amount of marker recovered in digesta was not different for different cereal grains or at different time points indicating steady emptying of stomach over that period. Gastric emptying is represented as a mass-action law driven by

total dry matter mass (Usry *et al.,* 1991) or protein mass (Bastianelli *et al.,* 1996). Gastric emptying is likely to be faster for about 30 minutes after ingestion because of higher intragastric pressure (Laplace and Tomassone, 1970). However, after this initial phase of rapid gastric emptying, the flow rate would be slower because of the feedback from the small intestine. Ultimately gastric emptying will be dependent on the rate of passage of digesta along the small intestine and rate of digestion of food (Potkins *et al.,* 1991). Many studies investigating effects of dietary fibre on the passage rate of digesta (Le Goff *et al.,* 2002, Leeuwen and Jansman, 2007; Wilfart *et al.,* 2007) report decreased mean retention time with increasing dietary fibre. However, a lot of variability has been reported in the results associated with animal factors, the nature and origin of dietary fibre, use of different markers and calculation differences. Use of guar gum and pectin (soluble polysaccharides) accelerated gastric emptying and digesta passage rate along small intestine but did not significantly alter overall transit time whereas bran and oatmeal by-product increased rate of passage of digesta through the large intestine resulting in faster passage rate through gastrointestinal tract (Potkins *et al.,* 1991). Our results of no effects of type of cereal grains (corn versus wheat-barley) on digesta flow through small intestine are in accordance with previous studies by Latymer *et al.,* (1985) and Potkins *et al.,* (1991) which reported that wheat bran and oatmeal by-product had no significant effects on digesta passage rate in the small intestine. Use of whole cereal grains instead of soluble or insoluble fibre sources in this study, might have been responsible for absence of any significant effects on small intestinal digesta passage rate. In particular, fibrous components in the cereal matrix would be expected to take longer to solubilize reducing opportunity for impact in the upper gastrointestinal tract relative to purified fibres.

Our results of higher recovery of chromic oxide in all locations of small intestine of conventional pigs suggest that the bolus size leaving the stomach was larger. In this study, percent chromic oxide recovered across different locations of small intestine increased distally in both conventional and monoassociated pigs. This response probably reflects the addition of dry matter to digesta in proximal intestine from bile and pancreatic secretions, possibly mucus secretion, plus the removal of dry matter as nutrient absorption moving distally. Interestingly, chromic oxide recovery declined at the 95% SI location which we speculate could reflect the contribution of microbial biomass to digesta dry matter.

Percent chromic oxide recovered at any time point in conventional pigs was higher than percent chromic oxide recovered in monoassociated pigs at 3 hours following test meal. We did not detect any chromic oxide in the distal small intestine in MA pigs at 3 hours post meal suggesting slower stomach emptying or slower small intestine transit in these pigs. Earlier studies have found that germfree rats had significantly reduced gastric emptying of oleic acid and triolein and subsequently reported reduced gastrointestinal motility (Tennant, *et al.*, 1969). In this study based on % chromic oxide values in digesta to feed we concluded that the flow rate of digesta was slower in monoassociated pigs. Availability of total mass of digesta in stomach and at each small intestinal location and hence calculation of absolute mass of chromic oxide would have added further valuable information.

Increased viscosity in monoassociated pigs could also contribute to slower digesta passage caused by higher water holding capacity resulting in less bulkiness (Le Goff *et al.*, 2002). Another study reported that the spatial and temporal spread of migrating motor

complexes (MMC) is more restricted in the small intestines of germfree rats and slower than in conventional animals (Falk *et al.,* 1998). Husebye *et al.,* (1994) concluded from studies with conventionalization of germfree rats that the intestinal microbiota exerts a marked stimulatory influence on the spatial and temporal spread MMC in the small intestine. Also in another study (Caenepeel *et al.,* 1989) the myoelectric complex was found to migrate slowly in germ-free rats which became faster when the germ-free rats were associated with a limited microbiota. While the mechanisms by which bacteria influence small bowel motor function are not understood, our results suggest that the influence of the microbiota on peristalsis and rate of passage of digesta can also be extended to the pig.

Bacterial colonization might influence the types and amounts of neurotransmitters and hormones or the enteric neuronal plexus. The role of other mediators like short chain fatty acids can not be ruled out as these have been shown to stimulate contractions in the terminal ileum in dogs (Kamath *et al.,* 1987) and humans (Kamath *et al.,* 1988). It seems plausible that complex interactions exist between bacteria, with their metabolic pathways, toxins (Mathias and Clench, 1985) and antigenic properties, and the host's responses. Lower pH observed in germ-free pigs could also be a mediator for the slower ileal motility in accordance with a previous in vitro study using rat tissues showing that the contractile effects of short chain fatty acids were brought out by pH changes (Cherbut *et al.,* 1996).

Our finding of higher viscosity and low pH in wheat-barley fed conventional pigs are in accordance with previous studies by Jensen and Jorgensen (1994) and Wenk (2001). It was reported that a diet with high soluble fibre content will cause more water

binding leading to increased viscosity and lower pH (Wenk, 2001) because of higher production of SCFAs by microbial fermentation. No cereal grain effects were observed in MA pigs because of absence of bacterial digestion and fermentation of cereal grain fibre. However, as noted in chapter 4, the pH of ileal digesta from MA pigs was lower than in conventional pigs and may have increased rate of passage compared to the germ-free state. The lower pH in MA pigs could reflect lactic acid production by *E. faecium*.

In conclusion, we were able to successfully modify and develop the microassay for estimation of chromic oxide in $1/20^{th}$ of original sample size. We have also provided data to suggest that the commensal microbiota influence passage rate of digesta in the pig small intestine, extending previous findings primarily from rodents.

## 6.0. GENERAL DISCUSSION AND CONCLUSIONS

There is growing amount of interest and need for researching gastrointestinal microbiota to study three way interaction including microbe-microbe interactions, microbe-host interactions and microbe –nutrient interactions. These interactions could potentially affect host nutrient availability, immune status and gut health and may be expressed differentially based on the host's age, genetics and environment. Therefore, an understanding of interactions of various cereal grains in diet with the intestinal microbiota and their effect on host nutrient supply and physiology will help in developing alternative management and dietary measures to maintain animal health and performance.

Availability of a gnotobiotic pig model in our laboratory provides us a unique opportunity to study the interactions of dietary components with the desired microbial populations and the mechanisms involved. Our hypotheses for this body of work explored the specific role of the intestinal microbiota in the host:microbiota:nutrient interaction relationship as it applies to outcomes related to animal nutrition and health. To investigate the role of the microbiota we utilized an *in vivo* gnotobiotic model. The approach allows the study of experimental treatments on the host in an environment where microbiota do and do not contribute to the host response. A comparison of responses with and without a microbial influence permits a clear delineation of the microbial contribution to host response. Clearly, this model is disadvantaged by the significant technical requirements to maintain a gnotobiotic environment and the significant constraints this places on experimental design. Constraints include availability of isolator space, raising enough experimental units and the inability to study pathways involved. For this reason microbial influences on host response are often

studied indirectly or using *in vitro* models. The gnotobiotic model does, however, deliver a number of advantages compared with *in vitro* approaches; these include the ability to study specific host responses to colonization with a single or a group of bacteria. Therefore, gnotobiotic animals provide an indispensable tool to study importance of gastrointestinal microbiota and host response to microbial colonization.

In previous work we reported the effect of an intestinal microbiota in 14 day old pigs fed infant formula (Shirkey *et al.*, 2006; Willing and Van Kessel., 2007). In the present work we extended our gnotobiotic pig model from pre-weaning to post weaning by introducing γ - irradiated cereal based diets from 14 to 24 days of age. This was obviously an important extension of the model necessary to examine the effect of cereal type and allow further investigation of effects of feed ingredients on host physiology beyond the preweaning phase. Furthermore, the model was improved by using γ-irradiated sow colostrum, a source of passive antibody protection versus porcine serum. While pigs in previous studies were in good health, analysis of serum antibodies levels indicated marginal passive protection and a possible area to improve the model.

In chapter 3, we hypothesized that microbial metabolism contributes to variation in bioavailability of methionine sources in pigs. Objectives of this research were to determine the small intestinal retention of methionine and its hydroxy- analogue in conventional and germ-free pigs and whether it was affected by cereal grain composition of the diet. Here we found increased retained MHA-FA associated radioactivity in conventional but not in monoassociated pigs along with decreased MHA-FA tissue radioactivity suggesting that microbial metabolism of MHA-FA could be responsible for its reduced relative availability for absorption. We extended previous findings of

microbial metabolism of MHA-FA in chickens to pigs (Drew *et al*., 2003). However, it was not clear whether MHA-FA is preferentially metabolized or its comparatively slower transport because of low affinity and low velocity $H^+$ nonstereospecific transport system compared to a system B transporter for D- and L-methionine (Maenz and Engele Schaan, 1996b), leads to increased metabolism by gut microbiota.

We found higher counts of total aerobes and lactobacilli in MHA-FA supplemented pigs but whether these bacteria could be involved in its metabolism, is not clear. We did *in vitro* experiments to investigate which gut bacteria could have metabolised $^3$H- MHA-FA or $^3$H-MET starting with *E. coli* and *L. fermentum* but unfortunately the methodology didn't work. Our results also indicate that even for readily available nutrients where absorption occurs rapidly in upper small intestine, there is potential for microbial competition and catabolism. This may have implications for amino acid supplementation. We we did not see any effect of cereal type on apparent absorption of MET or MHA-FA. Our cereals differed primarily in fibre content more likely to impact distal rather than proximal intestinal microbial community structure. One wonders whether differences in abundance of readily available carbohydrates may have had a greater impact on microbial catabolism of rapidly absorbed dietary supplements.

In chapter 4, we hypothesized that the cereal grain type alters gastrointestinal physiology and mucin dynamics and that these effects are mediated indirectly by altered microbiota. Gnotobiotic pigs were monoassociated in the current study, however, the viable counts of contaminating bacteria were not influenced by cereal grain type and therefore, we could still test whether effects of cereal grain type were indirectly mediated by changes in gut microbiota.

In this chapter, we learned that type of cereal grain used influenced gastrointestinal morphology and physiology and these limited effects were indirectly brought about by changes in gut microbial composition as indicated by interactive effects for PCNA, APN activity, sucrase and Muc 2 expression. In this study we elected to compare cereal grain type as an approach to changing dietary fibre composition. Alternatively we could have used a number of available synthetic or purified fibre sources such as carboxymethyl cellulose, pectin, β-glucan. These various purified sources would have permitted an examination of soluble versus insoluble fibre effects and/or allowed a more precise examination of effects of fibre without confounding impact of differences in other whole cereal components. We chose to utilize cereal grain for two reasons. Firstly because fibre in livestock diets is normally fed in the context of the whole grain matrix which is likely to impact its physicochemical properties along the length of the intestine. Secondly, and related to that, use of whole grain diets is more relevant to the commercial industry. Inclusion of fibre in diet causes changes in intestinal secretions and altered morphology (Wenk, 2001) and digestive physiology (Hedemann *et al.*, 2006). The increased intestinal epithelial turnover, as observed here with the higher fibre wheat/barley diet, can lead to a reduction of digestion of various complex nutrients such as proteins and starch as well as in absorption of their digestion products, and other dietary components (minerals, vitamins). This suggests that the relationship between digestibility of a feed ingredient and its chemical composition may not solely reflect the relative ease of releasing absorbable monomers (e.g. amino acids, glucose) but also differences in digestive and/or absorptive capacity mediated indirectly by host responses (e.g. epithelial cell turnover rate) to changes in microbial composition.

Intestinal cell proliferation will have implications for digestive physiology as well as host intestinal health. Our findings of reduced villus height, longer crypts along with higher PCNA expression and caspase-3 activity in post-weaned conventional pigs confirm similar earlier findings of increased intestinal cell turnover in unweaned pigs (Willing and Van Kessel, 2007), however the differences in this study were less dramatic which could be because of solid diet in postweaned conventional and monoassociated pigs.

The mucus gel layer present on intestinal epithelial surface acts as a protective barrier and any change in quality and quantity of mucus secretion may have important physiological implications. In view of the ever changing animal husbandry, appropriate dietary modifications could correlate with the protection of intestinal mucosa from various infections. Dietary factors are known to affect the number of goblet cells and composition of intestinal mucins (Sharma and Schumaccher., 1995). One objective of this study was also to compare conventional and germ free animals to gain some insight into the levels to which dietary effects on mucin dynamics are mediated by indigenous microbiota. It is well known that effects of dietary fibre on mucin parameters vary depending on the nature, level and duration of fibre feeding. In this study, cereal grain type was not found to affect goblet cell numbers in intestinal crypts or villi, mucin composition or mucin gene expression and therefore, we were limited to a discussion of effects of microbiota on mucin dynamics. Based on the results of increased total and different types of mucin cells in conventional animals we were able to confirm that mucin secretion is enhanced with microbial colonization. Our novel work of investigation of mucin genes expression also indicated that conventional pigs had higher capacity of

mucin secretion at molecular level again consolidating the fact that mucin secretion is enhanced with microbial colonization to provide the host with various protective advantages. Our results of more acidic mucin cells in crypts of conventional pigs and higher acidic than neutral mucin in villi of all animals are in agreement with studies reporting that the intestinal regions densely populated by microbes predominantly express acidic mucins as these appear less degradable by bacterial glycosidases and host proteases (Roberton and Wright, 1997 and Deplancke *et al.,* 2000).

In chapter 5, we set out to examine the effects of dietary cereal grains and commensal microbiota on digesta flow in newly-weaned pigs. We successfully developed a microassay for estimation of chromic oxide in small sample sizes which was essential for our study because of low sample recovery from the proximal locations of small intestine. However, we understand that use of chromic oxide as indigestible marker is diminishing because of hazardous nature of perchloric acid used in the assay. Still, the microassay may be of value where small sample size is an issue.

We found evidence of a slower rate of passage of digesta along small intestine in monoassociated pigs extending these finding from rodents (Riottot *et al.,* 1980). Although the exact mechanisms of bacterial interactions with intestinal motility are not known, it seems plausible that complex interactions exist between bacteria, with their metabolic pathways, toxins (Mathias and Clench, 1985) and antigenic properties, and the host's responses and these interactions have implications for nutrient digestibility. Nutrient digestion and absorption including that of amino acids is typically enhanced with slower passage rate of digesta because of ample time for enzymatic hydrolysis and absorption (Murray *et al*., 1977; Corring *et al*., 1991). In the case of comparable residual

radioactivity for MET and MHA-FA in gnotbiotic pigs, perhaps the slower rate of passage offered more opportunity for MHA-FA absorption via the slower, low affinity lactate transport system as compared to the system B transporter for methionine. Whether rate of passage is a major contributor here is subject to debate. Few interactions were observed between intestinal morphology and cereal type such that speculation on the role of rate of passage of digersta is probably not warranted.

In the present studies we accepted our hypotheses by concluding that intestinal microbial metabolism could be responsible for reduced availability of MHA-FA for absorption and that the cereal grain type had limited effects on intestinal morphology and physiology which were indirectly mediated via gut microbial changes. Also microbiota was found to have major influences on intestinal physiology and mucin dynamics and digesta flow rate.

### 6.1. Limitations of present studies

In the present studies, gnotobiotic pigs grew poorly compared to conventional pigs which were raised with sows until weaning at 14 d of age. Use of infant formula compared to sow's milk during pre-weaning period may have accounted for much of the performance differences because lactose is the main energy source in infant formula compared to fat in sow's milk (Veum and Odle, 2001). Also the absence of maternal transfer of bioactive factors, leucocytes and cytokines in isolator reared monoassociated pigs might have contributed to reduced immune response and hence reduced growth (Bocci *et al*., 1993; Ellis *et al*., 1997).

We also experienced greater difficulty with adjusting isolator reared pigs to solid feed intake compared with conventional pigs. The difference in final weight of conventional and monoassociated pigs was significant and raised the question whether comparison of these groups examined a microbial influence or an impact of physiological age (chronological age was controlled). Another option to avoid this outcome could have been to compare gnotobiotic pigs with isolator reared conventionalized pigs as reported previously (Shirkey *et al*., 2006). But in this case, availability of isolator space, raising enough experimental units in each trial favored the selection of earlier mentioned experimental design. Furthermore, the sow-reared conventional pigs used in the present study reflected an animal model more consistent with a commercial pig and the application of our finding to the commercial swine industry.

As we know, the gnotobiotic animal research has difficulties including caesarian–section delivery, sterile derivation of pigs, resuscitation, maintaining gnotobiotic conditions in isolators and providing appropriately sterilized feed and water. In the current study, pigs were contaminated with one or more bacterial species during the course of trial 1 and with a single bacterial species in trial 2. Presence of a pin point hole was thought to be responsible for contamination in the first trial while we couldn't pinpoint the reason for contamination in second trial. Another possible source of contamination is unobserved respiration in caesarian section derived pigs prior to being passed through the iodine bath into the sterile transfer unit for resuscitation.

Another factor to be kept in mind while interpreting results of gnotobiotic research is that contaminating bacteria might colonize at much higher levels due to absence of microbial competition as found in this study where MA bacterium *E. faecium*

counts varied from 7.61±0.25 to 8.82±0.26 log cfu/g of digesta compared to lower levels of conventional microbiota ranging from 4.81±0.19 to 6.48± 0.21 log cfu/g. In other cases, contaminants might colonize at lower levels than in a conventional environment because of their dependency on co-habitants. Also a single contaminating bacterium may not be generating same fermentation products as in a conventional setting due to absence of cross-feeding. There is also a possibility that the contaminating bacterium might have a different gene expression profile in absence of conventional microbiota and hence the host response to a single bacterium might be different than in the presence of conventional microbiota.

### 6.2. Future research

In the current study, we only investigated microbial influences on the host availability of methionine from different sources. However, complex interactions in the gut warrant further studies to understand microbial effects on availability of nutrients to the host and its implications on animal performance. As mentioned earlier, it was not clear whether MHA-FA is preferentially metabolized or its comparatively slower transport leads to increased metabolism by gut microbiota. Therefore, the mechanisms involved in microbial metabolism of MHA-FA need to be elucidated. Also the bacterial species involved in metabolism of MHA-FA need to be identified probably by first investigating the responsible bacteria in *in vitro* studies and then by verifying the results *in vivo*.

The implications of changes in gastrointestinal physiology and mucin type need to be elucidated in terms of animal health and performance. Along with GI physiology and

mucin composition, studying commensal and pathogen colonization patterns with varied dietary composition will contribute to the development of an ‘optimal microbiota’ correlated to animal health. Effects and relationship of various dietary components with flow rate of digesta need to be investigated. This interrelationshiop will influence digestion and absorption of nutrients and hence their availability to the host which will have implications for animal performance.

This is a new era in nutrition where consumers are shaping the science of animal nutrition leading to increasing restrictions on subtherapeutic use of antibiotics as growth promoters. Thus new methods are needed to allow for the development of ‘optimal microbiota’. Our findings contribute to a better understanding of interactions between dietary constituents, gut microbiota and host physiology and will help us in formulating diets so as to improve animal health and performance.

## 7.0. LIST OF REFERENCES


Abrams, G. D. and Bishop, J. E. 1967. Effect of the normal microbial flora on gastrointestinal motiltity. Proc. Soc. Exp. Biol. Med. 126: 301.

Abrams, G. D., Bauer, H., and Sprinz, H. 1963. Influence of the normal flora on mucosal morphology and cellular renewal in the ileum. A comparison of germfree and conventional mice. Lab. Invest. 12: 355-364.

Andrieux, C., Gadelle, D., Leprince, C. & Sacquet, E. 1989. Effects of some poorly digestible carbohydrates on bile acid bacteria transformations in the rat. Br. J. Nutr. 62**:** 103-119.

Angkanaporn, K., Choct, M., Bryden, W. L., Annison, E. F. and Annison, G. 1994. Effects of wheat pentosans on endogenous amino acid losses in chickens. J. Sci. Food Agric. 66: 399-404.

Anugwa, F. O. I., V. H. Varel, J. S. Dickson, W. G. Pond, and L. P. Krook. 1989. Effects of dietary fiber and protein concentration on growth, feed efficiency, visceral organ weights and large intestine microbial populations of swine. J. Nutr. 119:879-886.

Apajalahti, J., and M. Bedford. 2000. Impact of dietary and environmental factors on microbial communities of the avian GI tract. Online 4/8/2002. http://www.poultry-health.com/fora/inthelth/apabed01.htm.

Bach Knudsen, K E.2001. The nutritional significance of ‘dietary fibre’ analysis. Anim. Feed Sci. Tech. 90:3-20.

Bach Knudsen, K. E. and Hansen, I. 1991. Gastrointestinal implications in pigs of wheat oat fractions. 2. Microbial activity in the gastrointestinal tract. Br. J. Nutr. 65: 233–248.

Bach Knudsen, K. E., Jensen, B. B. and Hansen, I. 1993a. Oat bran but not a beta-glucan-enriched oat fraction enhances butyrate productionin the large intestine of pigs. J. Nutr.123: 1235-1247.

Bach Knudsen, K. E., Jensen, B. B., Andersen, J. O. and Hansen, I. 1991. Gastrointestinal implications in pigs of wheat and oat fractions. 2. Microbial activity in the gastrointestinal tract. Br. J. Nutr. 65: 233–248.

Bach Knudsen, K.E. and Hansen, I. 1991. Gastrointestinal implications in pigs of wheat and oat fractions 1. Digestibility and bulking properties of polysaccharides and other major constituents. Br. J. Nutr. 65: 217–232.

Bach Knudsen, K.E. and Jensen, B.B. 1991. Effect of source and level of dietary fibre on microbial fermentation in the large intestine of pigs. In: Proceedings of the Fifth International Symposium on Digestive Physiology in Pigs. Verstegen, M.W.A., Huisman, J., den Hartog, L.A. (eds.), , 24–26 April 1991, Wageningen, The Netherlands, pp. 389–393.

Bach Knudsen, K.E., 1997. Carbohydrate and lignin contents of plant materials used in animal feeding. Anim. Feed Sci. Technol. 67: 319–338.

Bach Knudsen, K.E., Jensen, B.B. and Hansen, I. 1993b. Digestion of polysaccharides and other major components in the small and large intestine of pigs fed diets consisting of oat fractions rich in β-D-glucan. Br. J. Nutr. 70: 537-556.

Bailey, R. W. 1973. Chemistry and Biochemistry of Herbage. 1:157-211

Baker, D., Norris, K. H. and Li, B. W. 1979. Food fibre analysis: Advances in methodology. Pages 67-68 in: Dietary fibres: Chemistry and nutrition. G. E. Inglett and I. Falkehag, ed. Academic Press, London.

Bartholome, A. L., Albin, D. M. ad Tappenden, K. A. 2003.Intestinal epithelial proliferation and villus height increased by short-chain fatty acid supplemented total parenteral nutrition following 80% jejunoileal resection in piglets. FASEB J. 77: 1-2.

Bassoon, M. D., Liu, Y. W., Hanly, A. M., Emenaker, N. J., Shenoy, S. G. and Rothberg, B. E. G. 2000. Identification and comparative analysis of human colonocytes short-chain fatty acid response genes. J. Gastrointestin. Surgery. 4: 501-512.

Bastianelli, D., Sauvant, D. and Rerat, A. 1996. Mathetical modeling of digestion and nutrient absorption in pigs. J. Anim. Sci. 74: 1873-1888.

Beaulieu, J. F., Nichols, B. and Quaroni, A. 1989. Posttranslational regulation of sucrase-isomaltase expression in intestinal crypt and villus cells. J. Biol. Chem. 264: 20000–20011.

Beckmann, L., Vahjen, W. and Simon, O. 2000. Isolation of an 1,3-1,4-β-glucan degrading Enterococcus faecium from the intestinal tract of chicken and partial characterization of its novel 1,3-1,4- β-glucanase. J. Basic. Microbiol. 40: 303-310.

Beever, D. E., Kellaway, R. C., Thomson, D. J. MacRae , J. C., Evans, C. C. and Wallace, A. S.1978. A comparison of two non-radioactive digesta marker systems for the measurement of nutrient flow at the proximal duodenum of calves. J. Agric. Sci. 90: 157-163.

Bergman, E. N. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol. Rev. 70**:** 567–590.

Bergman, E. N., Roe, W. E. and Kon, K. 1966. Quantitative aspects of propionate metabolism and gluconeogenesis in sheep. Am. J. Physiol. 211**:** 793-799.

Bocci, V., von Bremen, K., Corradeschi, F., Franchi, F., Luzzi, E. and Paulesu, L., 1993. Presence of interferon-gamma and interleukin-6 in colostrum of normal women. Lymphokine Cytokine Res. 12: 21–24.

Borgstrom, B., Dahlquist, A., Gustafsson, E., Lundh, G. and Malmquist, J. 1959. Trypsin, invertase and amylase content of feces of germfree rats. Proc. Soc. Exp. Bio. Med. 102: 154.

Bradford, M. 1976. Rapid and sensitive method for quantification of microgram quantities of protein utilizing principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Braun, E. J. and Campbell, C. E. 1989. Uric acid decomposition in the lower gastrointestinal tract. J. Exp. Zool. Suppl. 3: 70-74.

Brown, P. J., Miller, B. G., Stokes, C. R., Blazquez, N. B. and Bourne, F. J. 1988. Histochemistry of mucins of pig intestinal secretory epithelial cells before and after weaning. J. Comp. Path. 98:313-323.

Bruckner, G. and Szabo, J. 1984. Nutrient absorption in gnotobiotic animals. In: Adv. Nutr. Res. 6: 271-332.

Bry, L., Falk, P. G., Midvedt, T., and Gordon, J. I. 1996. A model of host-microbial interactions in an open mammalian ecosystem. Science. 273: 1380-1383.

Byrd, J. C., Yunker, C. K., Xu, Q. S., Sternberg, L. R. and Bresalier, R. S. 2000. Inhibition of gastric mucin synthesis by *Helicobacter pylori*. Gastroenterology. 118: 1072–1079.

Caenepeel, P. H., Janssens, J., Vantrappen, G., Eyssen, H. and Coremans, G. 1989. Interdigestive myoelectric complex in germ-free rats. Dig. Dis. Sci. 34: 1180-1184.

Campbell, J. M., Fahey, Jr. G. C. and Wolf, B. W. 1997. Selected indigestible oligosaccharides affect large bowel mass, cecal and fecal short-chain fatty acids, pH and microflora in rats. J. Nutr. 127:130–136.

Canibe, N. and Bach Knudsen, K. E. 1997. Apparent digestibility of non-starch polysaccharides and short-chain fatty acids production in the large intestine of pigs fed dried or toasted peas. Acta. Agric. Scand. 47: 106-116.

Carlstedt-Duke, B., Høverstad, T., Lingaas, E., Norin, K, E., Saxerholt, H., Steinbakk, M. and Midtvedt, T. 1986. Influence of antibiotics on intestinal mucin in healthy subjects. Eur. J. Clin. Microbiol. 5: 634–638.

Chadee, K. and Meerovitch, F. 1985.The pethology of experimentally induced cecal amebiasis in gerbils (Meriones unguiculatus). Liver changes and amebic liver abscess formation. Am. J. Pathol. 119: 485-494.

Cheng, H. and Bjerknes, M. 1980. The stem-cell zone of the mouse small intestine epithelium. *In*: Cell Proliferation in the Gastrointestinal Tract.Appleton, D. R., Sunter, J. P. and Watson, A. J. Eds. Pitman Medical Ltd., Tunbridge Wells.

Cherbut, C., Aube, A. C., Blottiere, H. M., Pacaud, P., Scarpignato, C. and Galmiche, J. P. 1996. *In vitro* contractile effects of short chain fatty acids in the rat terminal ileum. Gut. 38: 53-58.

Chesson, A. 1995. Dietary fibre. Pages 547-576 in: Food polysaccharides and their applications. A. M. Stephen, ed. Marcel Dekker, Inc., New york.

Chesson, A., 1987. Supplementary enzymes to improve the utilization of pig and poultry diets. In: Haresign, W., Cole, D.J.A. (Eds.), Recent Advances in Animal Nutrition. Butterworts, London, pp. 71–89.

Choct, M. 1997. Feed non-starch polysaccharides: Chemical structures and nutritional significance. Feed Milling International. June. 13-26.

Choct, M. and Annison, G. 1990. Antinutritive activity of wheat pentosans in broiler diets. Br. Poult. Sci. 31: 811-821.

Christelle, P., Montage, L., Bernard, S. and Jean-Paul, L. 2005. Increasing digesta viscosity using carboxymethylcellulose in weaned piglets stimulates ileal goblet cell numbers and maturation. J. Nutr. 135: 86-91.

Coates, M. E. 1975. Gnotobiotic animals in research : Their uses and limitations. Lab. Animals. 9: 275-282.

Cobb, M. M., Holloway, B.A. and Rivers, J. M. 1991. Ascorbic acid catabolism by gut microflora: studies in germ-free and conventional guinea pigs. J. Nutr. Biochem. 2: 635-643.

Coconnier, M. H., Dlissi, E., Robard, M., Laboisse, C. L., Gaillard, J. L. and Servin, A. L. 1998. *Listeria monocytogenes* stimulates mucus exocytosis in cultured human

polarized mucosecreting intestinal cells through action of listeriolysin O. Infect Immun. 66: 3673–81.

Corring, T., Juste, C. and Simoes-Nunes, C. 1981. Digestive enzymes in the germ-free animal Reprod. Nutr. Dev. 21: 355-70.

Cranwell, P.D. 1968. Microbial fermentation in the alimentary tract of the pig. Nutrition Abstracts and Reviews. 38: 721-730.

Cummings J. H. and Macfarlane, G. T. 1997. Colonic microflora: nutrition and health. Nutrition. 13: 3476–3478.

Dahiya, J. P., Hoehler, D., Van Kessel, A. G. and Drew, M. D. 2007. Effect of different dietary methionine sources on intestinal bacterial populations in broiler chickens. Poult. Sci. 86: 2358-2366.

Danielsen, E. M., Cowell, G. M., Sjostrom, H. and Noren, O. 1986. Translation control of an intestinal microvillar enzyme. Biochem. J. 235: 447-451.

Danielsen, M., Hornshoj, M., Siggers, R. H., Borg, Jensen, B., Van Kessel, A. G. and Bendixen, E. 2007. Effects of bacterial colonization on the porcine intestinal proteome. J. Proteome Res. 6: 2596-2604.

Deplancke, B. and Gaskins, H. R. 2001. Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. Am. J. Clin. Nutr. 73: 1131S-41S.

Deplancke, B., Hristova, K. R., Oakley, H. A., McCracken, R., Aminov, R., Mackie, I. and Gaskins, H.R. 2000. Molecular ecological analysis of the succession and diversity of sulfate-reducing bacteria in the mouse gastrointestinal tract. Appl. Environ. Microbiol. 66: 2166-2174.

DeVries, J.W., Prosky, L., Li, B. and Cho, S., 1999. A historical perspective on defining dietary fiber. Cereal Foods World**.** 44: 367–369.

Dharmani, P., Srivastava, V., Kissoon-Singh, V. and Chadee, K. 2009. Role of intestinal mucins in innate host defense mechanisms against pathogens. J. Innate Immun. 1: 123-135.

Dierick, N. A., Vervaeke, I. J., Demeyer, D. I. and Decuypere, J. A. 1989. Approach to the energetic importance of fibre digestion in pigs. 1. Importance of fermentation in the overall energy supply. Anim. Feed Sci. Tech. 23: 141-

Dohrman, A., Miyata, S. and Gallup, M. 1998. Mucin gene (*MUC 2* and *MUC 5AC*) upregulation by Gram-positive and Gram-negative bacteria. Biochim. Biophys. Acta. 1406: 251–9.

Drew, M.D., A. G. Van Kessel, and D. D. Maenz. 2003. Absorption of methionine and 2-hydroxy-4-methylthiobutoanic acid in conventional and germ-free chickens. Poult. Sci. 82:1149-1153.

Drew, M.D., A. G. Van Kessel., A. E. Estrada, E. D. Ekpe and R. T. Ziljstra. 2002. Effect of dietary cereal on intestinal bacterial populations in weaned pigs. Can. J. Anim. Sci. 82: 607-609.

Drochner, W. and Coenen, M. 1986. Pflanzliche Strukturstoffe in der Schweineernährung, Übers. Tierernährg. 14: 1–50.

Dumonceaux, T.J., Hill, J.E., Briggs, S.A., Amoako, K.K., Hemmingsen, S.M. and Van Kessel, A.G. 2006. Enumeration of specific bacterial populations in complex intestinal communities using quantitative PCR based on the chaperonin-60 target. J. Microbiol. Methods. 64: 46-62.

Ebner, S., Schoknecht, P. A., Reeds, P. J. and Burrin, D. G. 1994. Growth and metabolism of gastrointestinal and skeletal muscle tissues in protein-malnourished neonatal pigs. Am. J. Physiol. 267: R221–7.

Eggum, B. O. 1995. Dietary fiber and protein digestion and utilization. *In* :Dietary Fiber in Health and Disease. D. Kritchevsky and C. Bonfield (eds.). Eagen Press, St. Paul, MN. pp. 146–157.

Ellis, L. A., Mastro, A. M. and Picciano, M. F. 1997. Do milk-borne cytokines and hormones influence neonatal immune cell function. J. Nutr. 127:985S-988S.

Enss, M. L., Cornberg, M., Wagner, S. 2000. Proinflammatory cytokines trigger *MUC* gene expression and mucin release in the intestinal cancer cell line LS180. Inflamm.Res. 49:162–9.

Enss, M. L., Gross-Siestrup, S., Schmidt-Wittig, U. and Gartner, K. 1992. Changes in colonic mucins of germfree rats in response to the introduction of a “normal” rat microbial flora.Rat colonic mucin. J. Exp. Anim. Sci. 35: 110-119.

Enss, M. L., Muller, H., Schmidt-Wittig, U., Kownatzki, R., Coenen, M. and Hedrich, H. J.1996. Effects of perorally applied endotoxin on colonic mucins of germfree rats. Scand. J. Gastroenterol. 31:868–74.

Enss, M. L., Schmidtwittig, U. and Muller, H. 1996. Orally applied endotoxin stimulates colonic mucin releasing cells in germfree rats. J. Anim. Exp. Anim. Sci. 37: 138-148.

Epple, H. J., Kreusel, K. M., Hanski, C., Schulzke, J. D., Riecken, E. O. and Fromm, M. Differential stimulation of intestinal mucin secretion by cholera toxin and carbachol. Pflugers Arch. 433: 638–47.

Esteve Garcia, E. and R.E. Austic. 1993. Intestinal absorption and renal excretion of dietary methionine sources by the growing chicken. J. Nutr. Biochem. 4: 576-587.

Ewing, W. N. and Cole, D. J. A. 1994. The Living Gut. An Introduction to microorganisms in Nutrition. Context, Ireland, UK.

Fadel, J. G., Newman, R. K., Newman, C. W**.** and Graham, H. 1989. Effects of baking hulless barley on the digestibility of dietary components as measured at the ileum and in the feces of pigs. J. Nutr.119: 722-726.

Faichney, G. J. 1975. The use of markers to partition digestion within the gastrointestinal tract of ruminants. In: digestion and metabolism in the ruminant. I. W. McDonald and A. C. I. Warner (eds.). University of New England, Armidale, Australia, pp 277-291.

Falk, P. G., Hooper, L. V., Midvedt, T. and Gordon, J. I. 1998. Creating and maintaining the gastrointestinal ecosystem: what we know and need to know from gnotobiolgy. Micrbiol. and Mol. Biol. Rev. 62: 1157-1170.

FAO. Food and Agriculture Organization of the United Nations. 2001. FAOSTAT

Fenton, T. W. and Fenton, M. 1979. An improved procedure for the determination of chromic oxide in feed and faeces. Can. J. Anim. Sci. 59: 631-634.

Flint, H. J., Duncan, S. H., Scott, K. P. and Louis, P. 2007. Interactions and competition within the microbial community of the human colon : links between diet and health. Environ. Microbiol. 9: 1101-1111.

Fontaine, B. N., Meslin, J. C., Lory, S. and Andrieux, C. 1996. Intestinal mucin distribution in the germ-free rat and in the heteroxenic rat harbouring a human bacterial flora: effect of inulin in the diet. Br. J. Nutr. 75: 881-892.

Fonty, G. and Gouet, Ph. 1989. Fibre degrading microorganisms in the monogastric digestive tract. Anim. Feed Sci. Tech. 23: 91-107.

Ford, D. J. 1971. Some aspects of digestive physiology of germ-free and conventional chicks. PhD Thesis. University of Reading. Berkshire, UK.

Fukomoto, S., Tatewaki, M., Yamada, T., Fujimiya, M., Mantyh, C., Voss, M., Eubanks, S., Harris, M., Pappas, T. N. and Takahashi, T. 2003. Short-chain fatty acids stimulate colonic transit via intraluminal 5-HT release in rats. Am. J. Physiol. 284: 1269-1276.

Fuller, M. F. 1998. Nitrogen cycling in the gut. Ann. Rev. Nutr. 18: 385-411.

Furuse, M. and Yokota, H. 1984. Effect of the gut microflora on the size and weight of organs of chicks fed diets of different protein content. British Poult. Sci. 25: 429–439.

Gaskins, H. R. 1997. Immunological aspects of host/microbiota interactions at the intestinal epithelium. *In*: Gastrointestinal microbiology. Mackie RI, White BA, Isaacson RE, eds. New York: Chapman & Hall, Vol 2: 537–87.

Gdala, J., Jansman, A. J. M., Buraczewska, L., Huisman, J. and van Leeuwen, P. 1997. The influence of alpha-galactosidase supplementation on the ileal digestibility of lupin seed carbohydrates and dietary protein in young pigs. Anim. Feed Sci. Tech. 67: 115–125.

Glitso, L. V., Brunsgaard, G., Hojsgaard, S., Sandstrom, B. and Bach Knudsen, K. E. 1998. Intestinal degradation in pigs of rye dietary fibre with different structural characteristics. Br. J. Nutr. 80: 457-468.

Gonthier, M. P., Verny, M. A., Besson, C., Remesy, C. and Scalbert, A. 2003. Chlorogenic acid bioavailability largely depends on its metabolism by the gut microflora in rats. J. Nutr. 133:1853-1859.

Goodlad, R. A., Ratcliffe, B., Fordham, J. P. and Wright, N. A. 1989. Does dietary fibre stimulate intestinal epithelial cell proliferation in germ free rats? Gut. 30:820-825.

Gordon, H. A.1955. Germfree research: a basic study in host-contaminant relationship. III. Morphologic characterization of germfree life. N. Y. Acad. Med. 31: 239–242.

Gordon, H., Bruckner.E, Staley, T., Wagner, M. and Wostmann, B. 1966. Characteristics of germfree rat. Acta. Anat. 64: 367-372.

Gordon, J. I., Hooper, L. V., McNevin, M. S., Wong, M. and Bry, L. 1997. Epithelial cell growth and differentiation. III. Promoting diversity in the intestine: conversations between the microflora, epithelium and diffuse GALT. Am. J. Physiol. Gastrointest. Liver physiol. 273: G565-G570.

Graham, H., Fadel, J. G., Newman, C. W. and Newman, P. K. 1989. Effect of pelleting and beta-glucanase supplementation on the ileal and fecal digestibility of a barley-based diet in the pig. J. Anim. Sci. 67: 1293-1298.

Graham, H., Hesselman, K., Johnsson, E. and Aman, P. 1986. Influence of beta-glucanase supplementation on digestion of a barley based diet in the pig gastrointestinal tract. Nutr. Rep. Int. 34: 1089-1096.

Grolier, P., P. Borel, C. Duszka, S. Lory, M. C. Alexandre-Gouabau, V. Azais-Braesco and L. Nugon-Bauson.1998. The bioavailability of α- and β- carotene is affected by gut microflora in the rat. Br. J. Nutr. 80: 199-204.

Hedemann, M. S., Eskildsen, M., Laerke, H. N., Pedersen, C., Lindberg, J. E., Laurinen, P. and Bach Knudsen, K. E. 2006. Intestinal morphology and enzymatic activit innewly weaned pigs fed contrasting fibre concentrations and fibre properties. J. Anim. Sci. 84: 1375-1386.

Hegedus, M., E. Andrasofszky., E. Brydll., T. Veresegyhazy., and J. Tamas. 1993. Biological activity of methionine derivatives: I. Microbiological activity of methionine derivatives. Mag. Alla. Lapja. 48: 527-531.

Henegan, J. B. 1963. Influence of microbial flora on xylose absorption in rats and mice. Am. J. Physiol. 205: 417-420.

Heneghan, J. B. 1984. Physiology of the alimentary tract. *In*: The germ-free animal in biomedical research**.** Coates, M. E**.** & Gustafsson, B. E. (eds.) London. Laboratory Animals Ltd. pp. 169-191.

Hill, J. E., Seipp, R. P., Betts, M., Hawkins, L., Van Kessel, A. G., Crosby, W. L. and Hemmingsen, S. M. 2002. Extensive profiling of a complex microbial community using high throughput sequencing. Appl. Environ. Microbiol. 68: 3055-3066.

Hill, J.E., S. L. Penny, K.G. Crowell, S.H. Goh, and S.M. Hemmingsen. 2004. cpnDB: A chaperonin sequence database. Genome Research 14: 1669-1675.

Hill, J.E., S. M. Hemmingsen., G. G. Blair., T. J. Dumonceaux.., J. Klassen., R. T. Zijlstra., S. H. Goh, and A. G. Van Kessel. 2005. Comparison of ileum microflora of pigs fed corn, wheat or barley based diets by chaperonin-60 sequencing and quantitative PCR. Appl. Envir. Microbiol. 71: 867-875.

Hogber, A., and Lindberg, J. E. 2004. Influence of cereal non-starch polysaccharides on digestion site and gut environment in growing pigs. Livestock Prod. Sci. 87: 121-130.

Högberg, A. and Lindberg, J. E. 2004. Influence of cereal non-starch polysaccharides and enzyme supplementation on digestion site and gut environment in weaned piglets. Anim. Feed Sci. Tech. 116: 113-128.

Hooper, L. V. 2004.Bacterial contribution to mammalian gut. Trends Microbial. 12: 129-134.

Hooper, L. V. and Gordon, J. I. 2001. Glycans as legislators of host-microbial interactions: spanning the spectrum from symbiosis to pathogenecity. Glycobiology. 11: 1R- 10R.

Hooper, L. V., Midtvedt, T. and Gordon, J. I. 2002. How host-microbial interactions shape the nutrient environment of the mammalian intestine. Ann. Rev. Nutr. 22: 283-307.

Hooper, L. V**.,** Wong, M. H., Thelin, A., Hansson, L., Falk, P. G. and Gordon J. I. 2001. Molecular Analysis of Commensal Host-Microbial Relationships in the Intestine. Science**.** 291: 881 – 884.

Hume, I.D. 1997. Fermentation in the hindgut of mammals. *In* :Gastrointestinal Microbiology. Vol. 1. Gastrointestinal Ecosystems and Fermentations. R. I. Mackie and B.A. White. (eds.) Chapman & Hall, New York. pp. 84–115.

Husebye, E., Hellstrom, P. M. and Midtvedt, T. 1994. Intestinal microflora stimulates myoelectric activity of rat small intestine by promoting cyclic initiation and aboral propagation of migrating myoelectric complex. Dig. Dis. Sci. 39: 946-956.

Ihara, T., Tsujikawa, T., Fujiyama, Y. and Bamba, T. 2003. Alterations in the DNA binding activity of transcriptional factors activator protein-1, Sp1, and hepatocyte nuclear factor-1 in rat jejunum during starvation and refeeding. J. Gastroenterol. Hepatol. 18: 705-711.

Iji, P. A., Saki, A. and Tivey, D. R. 2001. Body and intestinal growth of broiler chicks on a commercial starter diet. 1. Intestinal weight and mucosal development. Brit. Poult. Sci. 42: 505-513.

Ikegami, S., Tsuchihashi, F., Harada, H., Tsuchihashi, N., Nishide, E. and Innami, S., 1990. Effect of viscous indigestible polysaccharides on pancreatic-biliary secretion and digestive organs in rats. J. Nutr. 120: 353–360.

Jansman, A. J. M., C. A. Kan, and J. Wiebenga. 2003. Comparison of the biological efficacy of DL-methionine and Hydroxy-4-Methylbutanoic acid (HMB) in pigs and poultry. CVB documentation Report No. 29, Centraal Veevoederbureau, Lelystad, NL.

Jensen, B. B. and Jorgensen, H. 1994. Effect of dietary fibre on microbial activity and microbial gas production in various regions of the gastrointestinal tract of pigs. Appl. Environ. Microbiol. 60: 1897-1904.

Jin, L., Reynolds, L. P., Redmer, D. A., Caton, J. S. and Crenshaw, J. D. 1994. Effects of dietary fiber on intestinal growth, cell proliferation, and morphology in growing pigs. J. Anim. Sci. 72: 2270-2278.

Johansen, H. N., Bach Knudsen, K. E., Sandstrom, B. and Skjoth, F. 1996. Effects of varying content of soluble dietary fibre from wheat flour and oat milling fractions on gastric emptying in pigs. Brit. J. Nutr. 75: 339-351.

Johansen, H. N., Bach Knudsen, K. E., Wood, P. J. and Fulcher, R. G. 1997. Physicochemical properties and the degradation of oat bran polysaccharides in the gut of pigs, J. Sci. Food Agric. 73: 81–92.

Johnson**,** I. T. and Gee, J. M. 1981. Effect of gel-forming gums on the intestinal unstirred layer and sugar transport *in vitro*. Gut. 22: 398-403**.**

Jørgensen, H. and Just, A. 1988. Effect of different dietary components on site of absorption/site of disappearance of nutrients. *In*: Proceedings of the Fourth International Seminar at the Institute of Animal Physiology and Nutrition. Buraczewska, L., Buraczewski, S., Pastuszewska, B., Zebrowska, T. (eds.). Jablonna, Poland. pp. 230–239 .

Jørgensen, H., Zhao, X.-Q. and Eggum, B.O. 1996. The influence of dietary fibre and environmental temperature on the development of the gastrointestinal tract, digestibility, degree of fermentation in the hind-gut and energy metabolism in pigs. Br. J. Nutr. 75: 365–378.

Just, A., Fernández, J.A. and Jørgensen, H. 1983. The net energy value of diets for growing pigs in relation to the fermentative processes in the digestive tract and the site of absorption of nutrients. Livestock Prod. Sci. 10: 171–186.

Kamath, P. S., Hoepfner, M. T. and Phillips, S. F. 1987. Short-chain fatty acids stimulate motility of the canine ileum. Am. J. Physiol. 253: G 427-G 433.

Kamath, P. S., Phillips, S. F., O'Connor, M. K. and Brown, M. L. and Zinsmeister, A. R. 1988. Short-chain fatty acids stimulate ileal motility in humans. Gastroenterol. 95: 1496-1502.

Kandori, H., Hirayama, K., Takeda, M. and Doi, K.1996. Histochemical, lectin-histochemical and morphological characteristics of intestinal goblet cells of germfree and conventional mice. Exp. Anim. 45: 155-60.

Kelly, D. and King, T.P. 2001. Luminal bacteria: regulation of gut function and immunity. *In*: Manipulation of the Gut Environment in Pigs. Piva, A., Bach Kudsen, K.E and Lindberg, J.E. (eds). Nottingham University Press, Nottingham, UK, pp. 113–131.

Kelly, D., Campbell, J. I., King, T. P., Grant, G., Jansson, E. A., Coutts, A. G. P., Pettersson, S., and Conway, S. 2004. Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-gamma and RelA. Nat. Immunol. 5: 104-112.

Kenworthy, R. 1970. Effect of Escherichia coli on germ-free and gnotobiotic pigs. I. Light and electron microscopy of the small intestine. J. Comp. Pathol. 80: 53–63.

Khoury, K. A., Floch, M. H. and Hersh, T. 1969. Small intestinal mucosal cell proliferation and bacterial flora in the conventionalization of the germfree mouse. J. Exp. Med. 130: 659-670.

Kirwan, W. O., Smith, A. N., McConnell, A. A., Mitchell, W. D. and Eastwood, M. A. 1974. Action of different bran preparations on colonic function. Brit. Med. J. 4: 187-189.

Kleesen, B., Hartmann, l. and Blaut, M. 2003. Fructans in the diet cause alterations of intestinal mucosal architecture released mucins and mucosa-associated bifidobacteria in gnotobiotic rats. Brit. J. Nutr. 89: 597-606.

Kodolvsky, O. 1981 Developmental, dietary and hormonal control of intestinal disaccharidases in mammals (including man). *In*: Carbohydrate metabolism and its disorders. Randle PJ, Steiner DF, Whelan WJ (eds). Academic Press, London, pp 481–522.

Komai, M., Takehisa, F., and Kimura, S. 1982. Effect of dietary fiber on intestinal epithelial cell kinetics of germ-free and conventional mice. Nutr. Rep. Int. 26: 255-261.

Kripke S. A., Fox A. D., Berman J. M., Settle R. G. and Rombeau J. L. 1989. Stimulation of intestinal mucosal growth with intracolonic infusion of short-chain fatty acids. J. Parent. Enteral Nutr.13: 109-116

Landy, J. J., and Ledbetter, R. K.1966. Delivery and maintenance of the germ-free pig. In Swine in biomedical research. Richland,Washington : Pacific Northwest Laboratory. pp. 619-632.

Langhout, D. J. 1998. The role of the intestinal flora as affected by non-starch polysaccharides in broiler chicks. Ph.D. thesis. Wageningen Agricultural University, TNO Nutrition and Food Research Institute, Department of Aninaml Nutrition and Physiology, Wageningen, The Netherlands.

Laplace, J. P. and Tomassone, R. 1970. Gastro-duodenal emptying in the pig. Chronic fistulation through extra-pleural thoracic pathway: study of a technique for mathematical analysis of emptying. Annals de zootechnie. 19: 303-332.

Latymer, E. A., Low, A. G. and Woodley, S. C. 1985. The effect of dietary fibre on the rate of passage through different sections of the gut in pigs. *In*: Proceedings of the 3rd International Seminar on Digestive Physiology in the Pig, pp 215-218 (A.

Just, H. Jorgensen and J. A. Fernandez, editors). Copenhegen: National Institute of Animal science.

Le Goff, G. and Noblet, J. 2001. Comparative digestibility of dietary energy and nutrients in growing pigs and adult sows. J. Anim. Sci. 79: 2418-2427.

Le Goff, G., Van Milgen, J. and Noblet, J. 2002. Influence of dietary fibre on digestive utilization and and rate of passage in growing pigs, finishing pigs and adult sows. J. Anim. Sci. 74: 503-515.

Leeper, L. L. and Henning, S. J. 1990. Development and tissue distribution of sucrase-isomaltase mRNA in the rat. Am. J. Physiology. 21: G52-G58.

Lencer, W. I., Reinhart, F. D. and Neutra, M. R.1990. Interaction of cholera toxin with cloned human goblet cells in monolayer culture. Am. J. Physiol. 258: G96–102.

Leser, T. D., Amenuvor, J. Z., Jensen, T. K., Lindecrona, R. H., Boye, M. and Moller, K. 2002. Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Appl. Environ. Microbiol. 68: 673-690.

Lesher, S., Walburg, H. E. Jr., and Sacher, G.A. Jr. 1964. Generation cycle in the duodenal crypt cells of germfree and conventional mice. Nature (London). 202: 884-886.

Lingens, G. and S. Molnar. 1996. Studies on metabolism of broilers by using $^{14}$C-labelled DL-methionine and DL-methionine hydroxy analogue Ca-salt. Arch. Anim. Nutr. 49:113-124.

Liu, S.-Q., R. Holland, and V.L. Crow. 2003. The potential of dairy lactic acid bacteria to metabolise amino acids via non-transaminating reactions and endogenous transamination. International J. Food Microbiol. 86: 257-269.

Lowe, M. E., Kaplan, M. H., Jackson-Grusby, L., D'Agostino, D. and Grusby, M. J. 1998. Decreased Neonatal Dietary Fat Absorption and T Cell Cytotoxicity in Pancreatic Lipase-related Protein 2-Deficient Mice. J. Biol. Chem. 273: 31215-31221.

Mack, D. R., Michail, S., Wei, S., McDougall, L. and Hollingsworth, M. A.1999. Probiotics inhibit enteropathogenic *E. coli* adherence in vitro by inducing intestinal mucin gene expression. Am. J. Physiol. 276: G941–50.

Maenz, D.D. and C.M. Engele Schaan. 1996a. Methionine and 2-hydroxy-4-methylthiobutanoic acid are partially converted to nonabsorbed compounds during passage through the small intestine and heat exposure does not affect small intestinal absorption of methionine sources in broiler chickens. J. Nutr. 126: 438-1444.

Maenz, D.D. and C.M. Engele Schaan. 1996b. Methionine and 2-hydroxy-4-methylthiobutanoic acid are transported by distinct Na+-dependent and H+-dependent systems in the brush border membrane of the chick intestinal epithelium. J. Nutr. 126: 529-536.

Malkki, Y. and Virtanen, E. 2001. Gastrointestinal effects of oat bran and oat gum - a review. Lebensm Wiss. Technol. 34: 337–347.

Marion, J., Peterson, Y. M., Rome, V., Thomas, F., Sangild P. T., Dividich, J. L. and huerou-Luron, I. L. 2005. Early weaning stimulates intestinal brush border enzyme activites in piglets, mainly at the posttranscriotional level. J. Pediatric Gastroenterol. Nutr. 41: 401-410.

Maroux, S., Louvard, D., and Baratti, J. 1973. Aminopeptidase from hog intestinal brush border. Biochem. Biophys. Acta. 321: 282-295.

Mathias, J. R. and Clench, M. H. 1985. Pathophysiology of diarrhoea caused by bacterial overgrowth of the small intestine. Am. J. Med. Sci. 289: 243-248.

May, T., Mackie, R. I., Fahey, G. C. Jr., Cremin J. D. and Garleb, K. A. 1994. Effect of fiber source on short-chain fatty acid production and on the growth and toxin production by *Clostridium difficile*. Scand. J. Gastroenterol. 19: 916-922.

McBurney, M. I. and Sauer, W. C. 1993. Fiber and large bowel energy absorption: validation of the integrated ileostomy - fermentation model using pigs. J. Nutr. 123: 721-727.

McCullough, J. S., Ratcliffe, B., Mandir, N., Carr, K. E. and Goodlad, R. A. 1998. Dietary fibre and intestinal microflora: effects on intestinal morphometry and crypt branching. Gut. 42: 799-806.

McDonald, D.E., Pethick, D.W., Mullan, B.P. and Hampson, D.J. 2001. Increasing viscosity of the intestinal contents alters small intestinal structure and intestinal growth, and stimulate proliferation of enterotoxigenic *Escherichia coli* in newly-weaned pigs. Br. J. Nutr. 86: 487–498.

Mcfarlane, G. T. and Cummings, J. H. 1991. Control and consequences of bacterial fermentation in the human colon. J. Appl. Bacteriol. 70: 443-459.

Mead, G.C. 1989. Microbes of the avian caecum: types present and substrates utilized. J. Exp. Zool. Suppl. 3: 48-54.

Meslin, J. C., Fontaine, N. and Andrieux, C. 1999.Variation of mucin distribution in the rat intestine, caecum and colon: effect of the bacterial flora. Comp. Biochem. Physiol. 123: 235-239.

Metges, C. C. 2000. Contribution of microbial amino acid homeostasis of the host. J. Nutr. 130, Suppl 1: 1857S-1864S.

Miniats, O. P. and Valli, E.P. 1973.The gastrointestinal tract of gnotobiotic pigs, In : Germ-free research (J.B. Heneghan, ed.), p. 575, Academic Press, New York.

Moniaux, N., Escande, F., Porchet, N., Aubert, J. P. and Batra, S. K. 2001. Structural organization and classification of human mucin genes. Front. Biosci. 6: D1192-1206

Montagne, L., Cavaney, F. S., Hampson, D. J., Lallies, J. P. and Pluske, J. R. 2004. Effect of diet composition on postweaning colibacillosis in piglets. J. Anim. Sci. 82: 2364-2374.

Moore, W. E. C., Moore, L. V. H., Cato, E. P., Wilkins, T. D. and Kornegay, E. T. 1987. Effect of high-fibre and high-oil diets on the fecal flora of swine. Appl. and Environ. Microbiol. 53: 1638-1644.

More, J., Fioramonti, J., Benazet, F. and Bueno, L. 1987. Histochemical characterization of glycoproteins present in jejunal and colonic goblet cells of pigs on different diets. Histochem. 87: 189-94.

Moreau, M.-C., Ducluzeau, R. and Raibaud, P. 1976. Hydrolysis of urea in the gastrointestinal tract of 'monoxenic' rats: effect of immunization with strains of ureolytic bacteria. Infect. Immun. 13: 9-15.

Morris, E. R. 1992. Physico-chemical properties of food polysaccharides. In: Dietray fibre: a component of food: nutritional function in health and disease. Schweizer, T. F., Edwards, C. A. (eds.), Springer, London, pp. 41-45.

Murray, A. G., Fuller, M. F. and Pirie, A. R. 1977. The effect of fibre in the form of various polysaccharides on the apparent digestibility of protein in the pig. Anim. Product. 24: 139.

Noblet, J. and Shi, X. S. 1994. Effect of body weight on digestive utilization of energy and nutrients of ingredients and diets in pigs. Livestock Prod. Sci. 37: 323-338.

Nordgaard, I., Mortensen, P. B. and Langkilde, A. M. 1995. Small intestinal malabsorption and colonic fermentation of resistant starch and resistant peptides to short-chain fatty acids. Nutrition. 11: 129-137.

Norin, E. and Midtvedt, T. 2000. Interactions of bacteria with the host. Alteration of microflora-associated characteristics of the host; non-immune function. Microbial Ecology in Hlth. Dis. Suppl. 2: 186-193.

NRC, Nutrient Requirements of Swine. 1998, 10$^{th}$ edition. Washington, DC: National Academic Press.

Owusu-Asiedu, A., Patience, J. F., Laarveld, B., Van Kessel, A. G., Simmins, P. H. and Zijlstra, R.T. 2006. Effects of guar gum and cellulose on digesta passage rate, ileal microbial populations, energy and protein digestibility, and performance of grower pgs. J. Anim. Sci. 84: 843-852.

Pieper, R., Jha, R., Rossnagel, B., Van Kessel, A. G., Souffrant, W. and Leterme, P. 2008. Effect of barley and oat cultivars with different carbohydrate composition

on the intestinal bacterial community composition in weaned piglets. FEMS Microbial Ecology. 66: 556-566.

Pluske, J. R., Durmic, Z., Pethick, D. W., Mullan, B. P. and Hampson, D. J. 1998. Confirmation of the Role of Rapidly Fermentable Carbohydrates in the Expression of Swine Dysentery in Pigs after Experimental Infection. J. Nutr. 128: 1737-1744.

Posner, E.S. 2000. Wheat. *In*: Handbook of Cereal science and technology, second edition, Revised and expanded. K. Kulp and J. G. Ponte, Jr., ed. Marcel Dekker, Inc. New York, NY. pp: 1-29

Potkins, Z. V., Lawerence, T. L. J. and Thomlinson, J. R. 1991. Effects of structural and non-structural polysaccharides in the diet of growing pig on gastric emptying rate and rate of passage of digetsa to the terminal ileum and through the total gastrointestinal tract. Br. J. Nutr. 65: 391-413.

Prohaska, L. 1986. Antibacterial mechanism of volatile fatty acids in the intestinal tract of pigs against *Escherichia coli*. Zentbl. Vetmed. Reihe B. 33:166–173.

Ratcliff, J. 2000. Antibiotic bans - European perspective. In: 47th Maryland Nutrition Conference for Feed Manufacturers. pp. 135-152.

Reddy, B. S., Pleasants, J. R. and Wostmann, B. S. 1969. Pancreatic enzymes in germfree and conventional rats fed chemically defined water-soluble diet free from natural substrates. J. Nutr. 97: 327-334.

Reddy, B., and Wostmann, B. 1966. Intestinal disaccharidase activities in growing germfree and conventional rats. Arch. Biochem. Biophys. 113: 609-616.

Rerat, A. 1978. Digestion and absorption of carbohydrates and nitrogenous matters in the hind gut of the omnivorous, non-ruminant animal. J.Anim. Sci. 46:1808-1837.

Rerat, A., Fiszlewicz, M., Giusi, A. and Vaugelade, P. 1987. Influence of meal frequency on postprandial variations in the production and absorption of volatile fatty acids in the digestive tract of conscious pigs. J. Anim. Sci. 64: 448–456.

Reyniers, J. A., Wagner, M., Luckey, T. D. and Gordon, H. A. 1960. Survey of germfree animals: the White Wyandotte Bantam and White Leghorn chicken. In: Lobound Reports No. 3 (J. A. Reyniers, ed.). University of Notre Dame press, Notre Dame, Ind. p. 1

Rhodes, J. M. 1989.Colonic mucus and mucosal glycoproteins: the key to colitis and cancer ? Gut. 30: 1660-1666.

Richards, J. D., Atwell, C. A., Vazquez-Anon, M. and Dibner, J. J. 2005. Comparative *in vitro* and *in vivo* absorption of 2-hydroxy-4(methylthio) butanoic acid and methionine in the broiler chicken. Poult. Sci. 84: 1397-1405.

Riottot, M., Sacquet, E., Vila, J. P. and Leprince, C. 1980. Relationship between small intestine transit and bile acid metabolism in axenic and holoxenic rats fed different diets. Reprod. Nutr. Develop. 20: 163-171.

Roberfroid M. B. and Delzenne N. M. 1998. Dietary fructans. Ann. Rev. Nutr. 8: 117-143.

Roberton, A. M. and Wright, D. P.1997. Bacterial glycosulphatases and sulphomucin degradation. Can. J. Gastroenterol. 11: 361-366.

Roediger, W. E. W. 1980. Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man. Gut. 21: 793-798.

Roediger, W. E. W. 1982. Utilization of nutrients by isolated epithelial cells of the rat colon. Gastroenterol. 83: 424–429.

Rostagno, H. S. and W.A. Barbosa. 1995. Biological efficacy and absorption of DL-methionine hydroxy analogue free acid compared to DL-methionine in chickens as affected by heat stress. Brit. Poult. Sci. 36: 303-312.

Rothkotter, H.J., Ulbrich, H. and Pabst, R. 1991. The postnatal development of gut lamina propria lymphocytes: Number, proliferation, and T and B cell subsets in conventional and germ-free pigs. Ped. Res. 29: 237-242.

Ruppin, H., Bar-Meir, S., Soergel, K. H., Wood, C. M. and Schmitt, M. G. 1980. Absorption of short-chain fatty acids by the colon. Gastroenterol. 78: 1500-07.

Sacquet, E., Raibaud, P. and Garnier, J. 1971. Comparative study of the microbial flora of the stomach, small intestine and cecum in "holoxenic" (conventional) rats and its changes after various surgical interventions: blind jejunal loops or bile duct fistulae. Ann. Inst. Pasteur (Paris). 120: 501-24.

Sakata, T. 1986. Effects of indigestible dietary bulk and short chain fatty acids on the tissue weight and epithelial cell proliferation rate of the digestive tract in rats. J. Nutr. Sci. Vitaminol. 32: 355–362.

Sakata, T. 1987. Stimulatory effect of short-chain fatty acids on epithelial cell proliferation in the rat intestine: a possible explanation for trophic effects of fermentable fibre, gut microbes and luminal trophic factors. Br. J. Nutr. 58: 95-103.

Sakata, T. and Inagaki, A. 2001. Organic acid production in the large intestine: implication for epithelial cell proliferation and cell death. *In* :The Gut environment of Pigs. Piva, A., Bach Knudsen, K.E., Lindberg, J.E. (eds.). Nottingham University Press, Nottingham, pp. 85–94.

Salter, D. N., M. E. Coates, and D. Hewitt. 1974. The utilization of protein and excretion of uric acid in germ-free and conventional chicks. Br. J. Nutr. 31: 307-318.

Sanderson, I. R. 1999.The physicochemical environment of the neonatal intestine. J. Clin. Nutr. 69: 1028S-34S.

Satchithanandam, S., Klurfeld, D. M., Calvert, R. J. and Cassidy, M. M. 1996. Effects of dietary fibres on gastrointestinal mucins in rats. Nutr. Res. 16: 1163-1177.

Satchithanandam, S., Vargofcak-Apker, M., Calvert, R. J., Leeds, A. R. and Cassidy, M. M. 1990. Alteration of gastrointestinal mucin by fibre feeding in rats. J. Nutr.120: 1179-84.

Savage, D. 1977. Microbial ecology of gastrointestinal tract. Ann. Rev. Microbiol. 31: 107-133.

Savage, D. C. 1986. Gastrointestinal microflora in mammalian nutrition. Ann. Rev. Nutr. 6: 155-178.

Seetharam, B., Yeh, Y. Y. and Alpers, D. H. 1980. Turnover of intestinal brush-border proteins during postnatal development in rat. Am. J. Physiol. 239: G524–G531.

Selvendran, R. R., 1984. The plant cell wall as a source of dietary fibre: chemistry and structure. Am. J. Clin. Nutr. 39: 320–337.

Sharma, R. and Schumacher, U. 1995. Morphometric analysis of intestinal mucins inder different dietary conditions and gut flora in rats. Dig. Dis. Sci. 40: 2532-2539.

Shirkey, T. W., Siggers, R. H., Goldade, B. G., Marshall, J. K., Drew, M. D., Laarveld, B., and A. G. Van Kessel. 2006. Effects of commensal bacteria on intestinal morphology and expression of proinflammatory cytokines in the gnotobiotic pig. Exp. Biol. Med. 231: 1333-1345.

Shurson, G.C., Ku, P.K., Waxler, G.L., Yokoyama, M.T. and Miller ER. 1990. Physiogical relationships between microbiological status and dietary copper levels in the pig. J. Anim. Sci. 68: 1061–1071.

Siggers, R. H., Shirkey, T. W., Drew, M. D., Laarveld, B. and Van Kessel, A. G. 2008. Cloning of porcine proglucagon and effect of commensal bacteria on relative gene expression in the intestine of gnotobiotic pigs. Can. J. Anim. Sci. 88: 429-438.

Slavin, J. 2003. Why whole grains are protective: biologic mechanisms. Proc. Nutr. Soc. 62: 129-134.

Smith, A. C. and Podolsky, D. K. 1986. Colonic mucin glycoproteins in health and disease. Clin. Gastroenterol. 15: 815-837.

Smits, C. H. M. and Annison, G. 1996. Non-starch plant polysaccharides in broiler nutrition - towards a physiologically valid approach to their determination. World's Poult. Sci. J. 52: 203-221.

Southon, S**.,** Livesey, G., Gee, J. M. and Johnson, I. T. 1985**.** Intestinal cellular proliferation and protein synthesis in zinc-deficient rats. Brit. J. Nutr. 53: 595-603.

Stoll, B., Henry, J., Reeds, P.J., Yu, H., Jahoor, F. and Burrin, D. G. 1998. Catabolism dominates the first-pass intestinal metabolism of dietary essential amino acids in milk protein-fed piglets. J. Nutr. 128: 606–14.

Sumi, Y., Miyakawa, M., Kanzaki, M. and Kotake, Y. 1977. Vitamin $B_6$ deficiency in germfree rats. J. Nutr. 107: 1707-1714.

Swords, W. E., Wu, C. C., Champlin, F. R. and Buddington, R. K. 1993. Postnatal changes in selected bacterial groups of the pig colonic microflora. Biol. Neonate. 63: 191-200.

Tappenden, K. A. and McBurney, M. I. 1998. Systemic short-chain fatty acids rapidly alter gastrointestinal structure, function, and expression of early response genes. Dig. Dis. Sci. 43: 1526-1536.

Tappenden, K. A., Drozdowski, L. A., Thomson, A. B. and McBurney, M. I. 1998. Short-chain fatty acid-supplemented total parenteral nutrition alters intestinal structure, glucose transporter 2 (GLUT2) mRNA and protein, and proglucagon mRNA abundance in normal rats. Am. J. Clin. Nutr. 68: 118-125.

Tappenden, K. A., Thomson, A. B., Wild, G. E. and McBurney, M. I. 1997. Short-chain fatty acid-supplemented total parenteral nutrition enhances functional adaptation to intestinal resection in rats. Gastroenterol. 112: 792-802.

Tennant, B., Reina-Guerra, M., Harrold, D. and Goldman, M. 1969. Influence of microorganisma on intestinal absorption: Oleic acid $^{131}$I and triolein $^{131}$I absorption by germfree and conventional rats. J. Nutr. 97: 65-69.

Theander, O. and Aman, P. 1979. The chemistry, morphology and analysis of dietary-fibre components. In: dietary fibres: chemistry and nutrition. G. E. Inglett and I. Falkehag, ed. Academic Press, London,pp 215-244.

Theander, O. E., Westerlund, E., Aman, P. and Graham, H. 1989. Plant cell walls and monogastric diets. Anim. Feed Sci. Technol. 23: 205-225.

Thorbecke, G. J., Gordon, H. A., Wostman, B., Wagner, M. and Reyniers, J. A. 1957. Lymphoid tissue and serum gamma globulin in young germfree chickens. J. Infect. Dis. 101(3):237–251.

Torp, N., Rossi, M., Troelsen, J.T., Olsen, J. and Danielsen, E.M. 1993. Lactase-phlorizin hydrolase and aminopeptidase N are differentially regulated in the small intestine of the pig. Biochem. J. 295: 177-182.

Torrallardona, D., Harris, C. I. and M.F. Fuller. 2003. Pigs' gastrointestinal microflora provide them with essential amino acids. J. Nutr. 133: 1127-1131.

Traber, P. G., Yu, L., Wu, G. D. and Judge, T. A. 1992. Sucrase-isomaltase gene expression along crypt-villus axis of human small intestine is regulated at level of mRNA abundance. Am. J. Physiol. 262: G123–G130.

Tsuboi, K. K., Kwong, L. K., Sunshine, P. and Castillo, R. O. 1992. Mechanism of maturational decline of rat intestinal lactase-phlorizin hydrolase. Biochem. J. 282: 107–113.

Uni, Z., Platin, R. and Sklan, D. 1998. Cell proliferation in chicken intestinal epithelium occurs both in the crypt and along the villus. J. Comp. Physiol. 168: 241-247.

Usry, J. L., Turner, L. W., Stahly, T. S., Bridges, T. C. and Gates, R. S. 1991. GI-tract stimulation model of the growing pig. Trans. Am. Soc. Agric. Eng. 34: 1879-1880.

Vahouny, G. V. 1982. Dietary fiber, lipid metabolism, and atherosclerosis. Fed. Proc. 41: 2801-6.

Vahouny, G. V., Connor, W. E., Roy, T., Lin, D. S. and Gallo, L. L.1981. Lymphatic absorption of shellfish sterols and their effects on cholesterol absorption Am. J. Clin. Nutr. 34: 507-513.

Vahouny, G. V., Le, T., Ifrim, L., Satchithanandam, S. and Cassidy, M. M. 1985. Stimulatiom of intestinal cytokinetics and mucin turnover in rats fed wheat bran or cellulose. Am. J. Clin. Nutr. 41: 895-900.

Van der Meulen, J. and Bakker, J. G. M. 1991. Effect of various sources of dietary fibre on chemico-physical characteristics of digesta in the stomach and the small intestine of the pig. In: Proceedings of the Fifth International Symposium on Digestive Physiology in Pigs. Verstegen, M.W.A., Huisman, J., den Hartog, L.A. (eds.), 24–26 April 1991, Wageningen, The Netherlands, pp. 440–445.

Van Leeuwen, P. and Jansman, A. J. M. 2007. Effect of dietary water holding capacity and level of fermentable organic matter on digesta passage in various parts of the digestive tract in growing pigs. Livestock Sci. 109: 77-80.

Van Leeuwen, P. and Jansman, A. J. M. 2007. Effects of dietary water holding capacity and level of fermentable organic matter on digesta passage in various parts of the digestive tract in growing pigs. Livestock Sci. 109: 77-80.

Vander Waaij, D., J. M. berghius-de Vries. and J. E.Lekkerkerk-Van der Wees. 1971. Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. J. Hygiene. 69: 405-411.

Varel, V. H. 1987. Activity of fiber-degrading microorganisms in the pig large intestine. J. Anim. Sci. 65: 488-496.

Varel, V. H., Pond, W. G., Pekas, J. C. and Yen, J. T. 1982. Influence of high-fiber diet on bacterial populations in gastrointestinal tracts of obese- and lean-genotype pigs. Appl. Environ. Microbiol. 44:107-12.

Virtanen, A. I. 1966. Milk production of cows on protein-free feed. Science. 153: 1603-1614.

Wanger, D.D and Thomas, O. P. 1978. Influence of diets containing rye or pectin on the intestinal flora of chicks. Poult. Sci. 57: 971-975.

Waxler, G. L. and Drees, D. T. 1972. Comparison of body weights, organ weights and histological features of selected organs of gnotobiotic, conventional and isolator-reared contaminated pigs. Can. J. Comp. Med. 36: 265-274.

Wenk, C. 2001.The role of dietary fibre in the digestive physiology of the pig. Anim. Feed Sci. Tech. 90: 21-33.

Wester, T. J., Vazquez-Anon, M., Dibner, J., Parker, D. S., Calder, A. G. and Lobley, G. E. 2006. Hepatic metabolism of 2-hydroxy-4-methylthiobutyrae in growing lambs. J. Dairy Sci. 89: 1062-1071.

Wilfart A., Montagne l., Simmins H., Noblet J and J. van Milgen. 2007. Effect of fibre content in the diet on the mean retention time in different segments of the digestive tract in growing pigs. Livestock Sci. 109: 27-29.

Wilfart, A., Montagne, L., Simmins, H., Noblet, J. and van Milgen, J. 2007. Digesta transit in different segments of the gastrointestinal tract of pigs as affected by insoluble fibre supplied by wheat bran. Brit. J. Nutr. 98: 54-62.

Willing, B. P. and Van Kessel, A. G. 2007. Enterocyte proliferation and apoptosis in the caudal small intestine is influenced by the composition of colonizing commensal bacteria in the neonatal gnotobiotic pig. J. Anim. Sci. 85: 3256-3266.

Willing, B. P. and Van Kessel, A. G. 2009. Intestinal microbiota differentially affect brush border enzyme activity and expression in the neonatal gnotobiotic pig. J Anim. Physiol. Nutr. (in press: DOI: 10.1111/j.1439-0396.2008.00841.x).

Winitz, M., L. Bloch-Frankenthal., N. Izumiya., S. N. Birnabaum., C. G. Barker., and J. P. Greenstein. 1956. Studies on diastereoiomeric a-amino acids and correseponding a-hydroxy acids. VII. Influence of a-configuration on enzymatic susceptibility. J. Am. Chem. Soc. 78**:** 2423-2430.

Wostmann, B. S. 1981. The germ-free animal in nutritional studies. Ann. Rev. Nutr. 1: 257-279.

Wostmann, B. S. 1996. Germfree and Gnotobiotic Animal Models: Background and Applications. CRC Press, Inc.: Boca Raton, FL, USA.

Yamanaka, M., Nomura, T., Kametaka, M. 1974. Role of intestinal microbes on body nitrogen accumulation in germfree, gnotobiotic and conventional mice. Nutr. Sci. Vitaminol. 20:389-400.

Yokota, H. and Coates, M. E. 1982. The uptake of nutrients from the small intestine of gnotobiotic and conventional chicks. Brit. J. Nutr. 47: 349-356.

Yu, F., Bruce, L. A., Calder, A. G., Milne, E., Coop, R. L. Jackson, F., Horgan, G. W. and MacRae, J. C. 2000. Subclinical infection with the nematode Trichostrongylus colubriformis increases gastrointestinal tract leucine metabolism and reduces availability of leucine for other tissues. J. Anim. Sci. 78: 380-390.

# 8.0. APPENDICES

## 8.1. Appendix A

Chapter 3.0 is reproduced with permission of the Animal Consortium (www.animal-journal.eu) as journal article: Malik, G., Hoehler, D., Rademacher, M., Drew, M.D. and Van Kessel, A.G. 2009. Apparent absorption of methionine and 2-hydroxy-4-methylthiobutanoic acid from gastrointestinal tract of conventional and gnotobiotic pigs. Animal. 3: 1378-1386.