IMPACT OF TYLOSIN PHOSPHATE, FLAXSEED, AND FLAXSEED FRACTIONS ON SMALL INTESTINAL MICROBIAL PROFILES IN PIGS

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ABSTRACT

Understanding how antimicrobial growth promoters (AGPs) affect small intestinal microbiota may help to discover effective alternatives. The impact of dietary supplementation with tylosin phosphate on small intestinal microbial profiles was investigated in growing pigs, and compared with the microbial profile of pigs fed flaxseed or its fractions. Eighteen ileal-cannulated barrows (33.1 ± 2.4 kg) received either the control diet (C, wheat, peas and soybean meal), or C plus 22 mg/kg tylosin phosphate (T), 20% whole flaxseed (WF), 18% hot-water extracted flaxseed (HWE), 4% flaxseed hulls (H), or 8% flaxseed oil (O) during three 21-d periods in a change over design. Ileal digesta (100 mL) was collected on d 16 and 17 of each period. Two chaperonin-60 universal target (*cpn*60 UT) libraries were constructed from pooled ileal digesta DNA extracted from the C and T diets. A total of 1634 nucleotide sequences were determined, and 117 different *cpn*60 UT sequences identified. Microbial diversity was greatest in the C library compared to T. Taxonomic composition between libraries differed, and included Lactobacillales (94% of C and 86% of T sequences), Enterobacteriaceae (3% of C and 13% of T), Clostridiales, Bacillales and Bifidobacterium taxa. T had a reduced ratio of Lactobacillales: Enterobacteriaceae sequences (6:1) compared to C (35:1). Lactobacilli: enterobacteria plate count ratios were highest in WF compared to C or T diets. *Lactobacillus johnsonii* genomes detected by qPCR were increased by 17.2 and 12%, in T and WF diets, respectively, compared to C. Numbers of *L. amylovorus* genomes were 25% lower in the H diet compared to C. Numbers of *Escherichia coli* and *Streptococcus alactolyticus* genomes were unaffected by dietary treatment, despite differences in library clone frequency for these species. Increased *L. johnsonii* colonization with tylosin suggests possible probiotic properties of this bacterium. Only inclusion of whole flaxseed resulted in a similar increase in *L. johnsonii*. Overall, ileal microbial profiles of

growing pigs were similar and remained mostly unaffected by dietary tylosin or flaxseed inclusion.

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LIST OF ABBREVIATIONS

AGP	antimicrobial growth promoter
ATCC	American type culture collection
bp	base pair
cfu	colony forming units
*cpn*60	*chaperonin*-60
°C	degrees Celsius
CO_2	carbon dioxide
d	day
DNA	deoxyribonucleic acid
g	grams
h	hour
HWE	hot water extracted
H_2	hydrogen gas
µg	microgram
µL	microlitre
min	minutes
mL	millilitre
mM	millimolar
mol	mole
N_2	nitrogen
nM	nanomolar
nt	nucleotide
PCR	polymerase chain reaction
RNA	ribonucleic acid
s	seconds
sp.	species
UT	universal target
vol	volume
wk	week

1. INTRODUCTION

Antibiotics are commonly fed at sub-therapeutic levels in swine diets to improve growth rate, feed efficiency and reproductive performance, and to reduce mortality and morbidity (Cromwell, 2002). However, there is significant concern from the public and scientific communities that imprudent antibiotic use in animal feed may contribute to the development and spread of antimicrobial resistant bacterial pathogens posing a threat to human health (McDermott, 2002b). With the assumption that all antimicrobial growth promoter (AGP) use in livestock feeding is imprudent, and that the human health hazard is real, the EU has adopted the 'precautionary principal', and a widespread ban of AGPs in livestock feeds took full effect in January 2006. A comprehensive report released by the World Health Organization (2003) has concluded (using Denmark as an example) that there have been no serious negative effects of the ban on food animal production, animal health, food safety or consumer prices.

There is currently very little regulatory activity regarding AGP use in the US and Canada, although commercial and consumer pressure in these countries may force producers to voluntarily remove AGPs from animal feeds (Dibner, 2005). Despite heated debate among scientists, and increasing public scrutiny of the issue, there is still no consensus on the level of risk posed to human health by AGP use animals and its contribution to the development and dissemination of antibiotic resistance (McDermott, 2002b). In light of the ongoing controversy, most animal scientists in North America have renewed interest in understanding exactly how AGPs function to improve growth with the goal of discovering effective alternatives.

Several explanations have been suggested for the growth promoting-effects of antimicrobials. The effects may be the result of some type of mediation of the intestinal microbiota and subsequent improvements in nutrient digestion and absorption, since antimicrobials have no growth promoting effects in germ-free animals (Coates, 1963). Other

explanations stem from the ability of AGPs to suppress the growth of pathogens and prevent sub-clinical disease. Some alternatives for AGPs that have been suggested or are already in use include probiotics, prebiotics, organic acids, fermented liquid feeds, zinc, and dietary ingredient manipulation (Gaskins, 2002). However, no single replacement or feed additive has been able to completely and successfully mimic the performance benefits achieved with AGPs thus far. Therefore, an increased understanding of exactly how AGPs impact intestinal microbial populations is necessary prior to the identification of a suitable replacement.

Animal nutritionists are interested in exploring the impact of novel feed ingredients on intestinal microbial populations as a 'natural' approach to replacing AGPs. The Western Canadian provinces make up the largest flaxseed-producing region in the world (Bhatty, 1995). Flaxseed is unique among oilseeds for several reasons. It is the richest terrestrial source of alpha-linolenic acid, and has a high content of mucilaginous water-soluble polysaccharides and lignans. It has been successfully incorporated into swine and poultry diets to alter the omega-3 fatty acid content of meat and eggs (Lewis, 2000; Romans, 1995). Incorporating flaxseed in human diets has been receiving more interest in Europe and North America based on evidence that it may have anticancer and cardiovascular benefits (Kolodziejczyk, 1995). There is also evidence to indicate that the fatty acid and water-soluble polysaccharide fractions of flaxseed could exhibit antibacterial effects on intestinal microbial populations, and/or promote a healthy gut environment by acting as substrates for certain commensal bacteria species.

2. LITERATURE REVIEW

2.1 Subtherapeutic antibiotic use in swine nutrition

Antibiotics have been used extensively in swine nutrition to assist in maintaining the equilibrium between intestinal health and growth efficiency. Since their discovery over 50 years ago, the use of antibiotics as feed additives has steadily increased. In 2004, 21.7 million pounds of antibiotics for use in farm and companion animals were sold in the USA alone (Phillips, 2005). Of the total, only 5% of antibiotics were reportedly used for health promotion, rather than therapeutic treatment of disease (Phillips, 2005). It has been estimated that anywhere from 20-90% of swine rations are medicated in Canada, depending on the age of pig (Dunlop, 1998). There are ten antimicrobial drugs registered for use in swine to improve growth promotion, weight gain and/or feed efficiency (Health Canada, 2002). Of these, five are also registered for use both in therapy and disease prevention, prophylaxis and/or control. There is very little publicly available data on the quantities of antimicrobial drugs used in food animals in Canada, and there is currently no mechanism by which this data could be collected (Health Canada, 2002). One of the recommendations of the Advisory Committee on Animal Uses of Antimicrobials and Impact on Resistance and Human Health (Advisory Committee) was to design and implement a national monitoring program of antimicrobial use in food animals, and to make the data publicly available (Health Canada, 2002).

2.1.1 Benefits to pig performance

The performance benefits of feeding sub-therapeutic levels of antimicrobial drugs are well documented (Cromwell, 2001). Improvement in average daily gain is 16.4, 10.6, and 4.2%, and feed efficiency is improved by 6.9, 4.5 and 2.2% for starter, grower, and finisher pigs respectively (Cromwell, 2002). Young, growing pigs show the greatest improvement in growth rate and feed efficiency when fed AGPs compared to finishing pigs, since they are more

susceptible to disease and are under more stress following weaning. AGPs are likely more effective in the farm setting than in research centres were facilities may be cleaner, the environment less stressful and the disease load is diminished (Cromwell, 2002). The economic benefits producers receive feeding AGPs stem from the improvements in daily gain, feed efficiency and reduced post-weaning mortality and has been estimated in the United States to be USD $2.99 net return per pig (Cromwell, 2002).

2.1.2 Growth promoting modes of action

Although the positive impact of AGPs on growth performance is well known, the mechanisms by which antibiotics improve growth are not completely understood. However, there are several proposed explanations: 1) control of sub-clinical infections, 2) reduction of microbial metabolites that depress growth, 3) reduction of microbial nutrient use, 4) enhanced nutrient uptake via thinner gut wall (Anderson, 2000; Gaskins, 2002; Visek, 1978). Each of these explanations assumes that the presence of bacteria in the gut depresses growth of the host, which is supported by the observation that antibiotics have no growth-promoting effects in germfree animals (Coates, 1963; Gaskins, 2002). It has also been suggested that the overall effect of AGPs is achieved through a reduction in the total number or the number of species of bacteria present in the gut (Collier et al., 2003b; Gaskins, 2002).

Many AGPs are indicated for use to prevent certain diseases in addition to improving animal performance. For instance, tylosin phosphate is approved as an aid in the prevention of swine dysentery and ileitis, and for improving feed efficiency and growth performance in swine. However, the recommended dosages required for disease prophylaxis are higher than for growth promotion only. There is a tendency to assume that feeding AGPs at the level required for growth promotion will also control any sub-clinical infections that may be impairing performance. However, because sub-clinical disease is difficult to detect and monitor, there are

few studies that have proven the above is true. The sudden removal of AGPs in Europe and the coinciding deterioration in animal welfare due to increased mortality, morbidity and enteric infections indicates that AGPs also play an important role in food animal disease prophylaxis (Casewell et al., 2003).

AGPs may improve pig performance by reducing the number of (specific) bacteria and subsequently reducing the quantity of microbial metabolites that may increase gut mucosa turnover and depress growth (Anderson, 2000). For example, lactobacilli are largely responsible for bile-salt hydrolase activity in the intestine. Bile-salt hydrolase-producing bacteria are able to deconjugate bile acids resulting in free bile acids that are toxic to enterocytes (Tannock, 1989). Free bile acids also impair protein, carbohydrate, and especially lipid absorption, since the reduced conjugated bile acid molecule concentrations could limit micelle formation. Feighner and Dashkevicz (1987) discovered that birds fed antibiotics had lower bacterial cholytaurine hydrolase activity and improved performance compared to non-medicated birds. The authors suggest that antibiotic feed additives elicit their effects by reducing the numbers of bile-salt hydrolyse-producing bacteria. Similar results were found in pigs, where antibiotic treatment reduced lithocholic acid concentrations and improved growth and feed efficiency (Tracy, 1987).

Intestinal bacteria utilize and degrade amino acids to obtain carbon, nitrogen and energy, thus reducing their availability to the host, and producing a variety of potentially toxic catabolites (Gaskins, 2001). Amines are produced by decarboxylation of amino acids and include histamine, cadaverine, tyramine and putrescine, which are active in the gut (Gaskins, 2001). Elevated levels of amines, particularly putrescine and cadaverine, in the gut have been linked to diarrhoea in young pigs (Porter, 1969). A reduction in the number of bacteria responsible for amino acid decarboxylation (*Bacteroides, Clostridium, Enterobacterium, Lactobacillus*, and *Streptococcus*) would reduce the amount of amines produced, and may lower the incidence of diarrhoea.

Ammonia is also a product of bacterial catabolism of amino acids, and has been shown to depress growth, likely by increasing gut epithelial cell turnover (Visek, 1978). The reduction of bacterial ammonia production has been proposed as a possible mechanism for the growth promoting effect of antimicrobials (Visek, 1978). Phenols and indoles are produced by the bacterial catabolism of aromatic amino acids. Yokoyama et al. (1982) found that feeding antibiotics reduced the excretion of phenols in urine and increased weight gain in weaning pigs and suggested that bacterial production of phenols and indoles depresses growth in young pigs. The benefits to pig performance that are achieved may be due in part to the improvements seen in protein metabolism (Anderson, 2000), or the increase in serum insulin-like growth factor I that has been observed when AGPs are fed (Hathaway, 1996).

Although the commensal intestinal microbiota make a significant positive contribution to extracting the maximum amount of nutrients from the host's diet, some competition for nutrients does occur, especially in the small intestine (Hooper et al., 2002). The word commensal comes from the Latin *commensalis* literally means "at table together". The nutrients required to maintain both the host and the microbiota originate from the diet of the host, so while each feed from the same 'dish' some competition for nutrients occurs. For instance, lactobacilli compete directly with the host for glucose to produce lactic acid, which increases intestinal motility and thus reduces nutrient utilization via an increase in digesta passage rate (Gaskins, 2001; Saunders, 1982).

A stable microbial population develops and is maintained with a significant energetic cost to the host. The number, type and distribution of bacteria influence innate host defence mechanisms such as increased epithelial cell turnover rate and mucus production in the gut; functions which are provided at the expense of animal growth efficiency (Gaskins, 2001). Gastrointestinal tissues represent only 5% of total body weight, but consume 15-35% of body

oxygen and protein turnover due to high epithelial cell sloughing and mucus secretion (Ebner, 1994; Reeds, 1993). Although colonic fermentation by bacteria does contribute energy to the pig, there is an energetic cost to maintaining a voluminous, densely populated colon (Gaskins, 2001). The resulting dilemma is in discovering a balance between the optimal microbial population for intestinal health and maintaining body and intestinal growth efficiency. AGP play a vital role in maintaining this balance.

Tylosin has been reported to improve apparent nitrogen digestibility by 3%, nitrogen retention by 6% and nitrogen utilization efficiency by 5%, while decreasing nitrogen excretion by 10% (Weldon, 1997). These improvements can be partly explained by considering that a lower bacterial load in the intestine would result in less endogenous protein loss. For instance, bacterial degradation of the intestinal mucus layer represents a considerable energy and protein loss for the host (Gaskins, 2001), since mucus must be continually secreted by the host and goblet cells and intestinal epithelium constantly turned over. In fact, the amount of energy and amino acids required for cell turnover is lower in germ-free mice than in those with commensal microflora (Abrams, 1963). Tylosin treatment in poultry decreased the proportion of mucolytic bacteria (Collier et al., 2003a), which would spare the host the additional nutrient requirement of continually regenerating the protective mucus layer.

Indigenous microflora are necessary for the normal development of the intestinal mucosal immune system. Derivation of germfree animals has revealed that without normal microflora, the Peyer's patches, mesenteric lymph nodes, and lamina propria are underdeveloped, and numbers of lymphocytes and goblet cells are decreased (Gaskins, 2001; McCracken, 1999; Rothkotter, 1991; Woolverton, 1992). Indeed, germfree animals are highly sensitive to enteric infections (Bealmear, 1980), and germfree pigs require colonization with bacteria to stimulate the secretion of natural antibodies (Butler et al., 2002), further indicating the importance of normal intestinal

microorganisms in the development of host defence mechanisms. AGP may function to improve growth by decreasing the number of immunogenic bacteria residing in the small intestine saving the host the energetic 'cost' associated with continually maintaining a low level of gut inflammation (Anderson, 2000). Pigs raised to slaughter weight under specific pathogen free conditions had lower maintenance energy requirements than their conventionally raised littermates (Anderson, 2000).

2.1.3 Impact of AGPs on intestinal microbial populations

A more comprehensive understanding of the mechanisms of action of AGPs would be gained by an elucidation of their effects on intestinal microbial composition, however, the microbial populations that are influenced by AGPs have never been completely identified (Tannock, 1997), and are only recently being investigated. Tylosin phosphate is indicated for use in swine and poultry diets for increased rate of gain and feed efficiency, and the prevention and/or control of ileitis and dysentery in swine (CFIA, 2002). Tylosin administration reduced the concentration of *Clostridium. perfringens* and associated lesions, and improved intestinal barrier function in a chick model of necrotic enteritis (Collier et al., 2003a). In a mixed anaerobic continuous fermentation culture of chicken gastrointestinal microorganisms, tylosin phosphate treatment reduced total volatile fatty acid (VFA) production and facilitated the persistence of *E. coli* 0157:H7, while untreated cultures had higher VFA concentrations and were able to clear the pathogen (Poole et al., 2003).

In the small intestine of growing pigs, continuous tylosin phosphate treatment (40 g/ton, manufacturer's recommended dose for growth promotion) reduced the number of bacterial species and increased the homogeneity of microbial populations for the initial 14 d of treatment (Collier et al., 2003b). Interestingly, the percentage of lactobacilli, specifically *Lactobacillus. gasseri,* was selectively increased after 14 d of continuous tylosin treatment (Collier et al.,

2003b). In the same study, a continuous rotation treatment of antibiotics (wk 1, chlorotetracycline sulfathiazole penicillin; wk 2, bacitracin and roxarsone; wk 3, lincomycin; wk 4, carbadox; wk 5, virginiamycin) decreased *L. johnsonii* and *Streptococcus infantarius*, and *L. gasseri* was not detected. The continuous rotation treatment increased microbial homogeneity throughout the experiment, but did not selectively increase the percentage of lactobacilli. The authors concluded that homogenization of the pig ileal microbiota and selection for lactobacilli may be the mechanism for growth promotion by AGPs, despite the potentially deleterious effects of bile-salt hydrolase production by lactobacilli. From these examples, it is clear that antibiotic inclusion could have either potentially beneficial or detrimental effects on intestinal health and microbial populations.

2.2 Declining subtherapeutic antibiotic use

2.2.1 Antibiotic resistance

Antimicrobial use at a sub-therapeutic level over long periods is favourable to the selection and preservation of resistant bacteria. Microorganisms that are able to survive in the presence of the selection pressure applied by antimicrobial drugs will thrive and have potential to distribute resistance to others (McEwen, 2002). It has been shown repeatedly that continuous feeding of subtherapeutic antimicrobials to food animals increases resistance levels and multiple resistance (Tannock, 1997). Equally, if not more concerning, is evidence that food animals could act as a pool for resistant bacteria or genes that may be passed on either directly (via resistant zoonotic organisms) or indirectly (via transmission of resistance genes in animals to human pathogens) to the human population, resulting in an increase in antimicrobial resistant infections. Antimicrobial resistant infections may be more difficult or expensive to treat, more virulent, numerous, and severe than sensitive infections (Health Canada, 2002).

An example of direct transmission of antimicrobial drug resistance occurred in the US following the approval of fluoroquinolones for use in poultry in the US in 1995 (Smith, 1999). Domestically acquired resistance to ciprofloxacin in *Campylobacter* species first emerged in human campylobacteriosis patients in the first two years following the initiation of fluoroquinolone use in poultry. Contaminated poultry meat was identified as an important reservoir of the resistant *Campylobacter jejuni* (Smith, 1999). When broilers were fed US Food and Drug Administration approved doses of fluoroquinolones, *C. jejuni* resistance developed rapidly and persisted; the minimum inhibitory concentration of ciprofloxacin increased from 0.25 μg/ml to 32 μg/ml during 5 d of treatment (McDermott, 2002a). Lagoons holding waste from swine facilities have also been blamed for facilitating the contamination of local environments with resistant bacteria (Chee-Sanford et al., 2001). Antibiotic resistant strains of *Enterococcus* have been isolated from pork (Hayes et al., 2003), although some researchers have found no direct connection between the antibiotic resistance patterns in vancomycin-resistant enterococci (VRE) isolates found on pork and VRE in humans with nosocomial infections (Klein et al., 1998). Multi drug-resistant *Salmonella* have been isolated from swine faeces (Gebreyes and Altier, 2002) and commercial pork samples (Chen et al., 2004). These examples indicate that antimicrobial resistant human pathogens could be transferred to vehicles of infection (meat, soil) and then to humans.

Evidence of indirect transmission of resistance genes from animals to human pathogens has been demonstrated. Researchers were able to show that resistance genes from porcine *Enterococcus faecium* could be transferred at a high frequency to human commensal bacteria of the same species in the digestive tract of gnotobiotic mice (Moubareck et al., 2003). Genetic analysis of human and animal methicillin-resistant *Staphylococcus aureus* (MRSA) isolates

revealed that some of the genomes were very closely related, suggesting that animal food products may be a potential source of human MRSA infection (Lee, 2003). Researchers in the Netherlands examined the genotypes of vancomycin resistant enterococci (VRE) in poultry and poultry farmers. The study found indications that poultry farmers' commensal enterococci populations had acquired resistance genes from the farmers' poultry flocks by horizontal gene transfer (van den Bogaard et al., 2002). A study examining *qacA,* an antibiotic resistance gene coding for a multi-drug efflux pump (Mitchell et al., 1999), and β-lactamase, an enzyme produced by bacteria that breaks down penicillin (Lyon, 1987), found that the genes were >99.9% identical in *S. aureas* and *Staphylococcus haemolyticus* from human and animal isolates (Anthonisen et al., 2002). This suggests that staphylococcal species that colonize humans and animals are able to exchange genes that cause resistance to antibiotics and disinfectants. Onan and Lapara (2003) examined the numbers and types of cultivable tylosin-resistant bacteria from agriculture soils and found that the percentage of bacteria resistant to tylosin was higher at sites where antibiotics were used sub-therapeutically. The authors hypothesized that the presence of antimicrobial-resistant bacteria in the soil was either due to indigenous soil bacteria acquiring resistance from non-indigenous faecal bacteria carrying resistance genes or developing resistance in the presence of antimicrobial residues in the soil.

There is evidence to suggest that feeding antimicrobial growth promoters to animals may pose a threat to human health. However, some scientists maintain that the risk to human health from feeding AGP to livestock is still theoretical, and that the clinical consequences of resistant strains actually reaching humans are small (Phillips et al., 2004). Indeed, if antimicrobial resistant human pathogens contaminate meat or other food, proper handling and cooking procedures would kill them. Also, the contribution of imprudent antimicrobial use by humans often tends to be downplayed in favour of 'pointing the finger' at the livestock industry.

2.2.2 Outcome of banning sub-therapeutic antimicrobials

Sweden was the first country to ban the use of AGPs in 1986. Since then, Denmark has also legislated bans of AGPs, with remaining EU member nations to follow suit by 2006. Although there are no legislated bans on AGP in North America, major fast-food companies McDonalds Corporation and KFC have declared that they will not purchase the meat of animals that have been fed AGPs that fall in the same class of compounds used in human medicine (McDonald's Corporation, 2003; KFC, 2005). When faced with this consumer pressure, producers are forced to discontinue feeding AGPs if they wish to market to major companies or export to countries where a legislated ban is in place. The World Health Organization (WHO) released a report evaluating the impact of terminating antimicrobial growth promoters in Denmark (WHO, 2003). The report outlined the economic impact of the ban, how it affected the usage of antimicrobials, antimicrobial resistance, animal health, welfare, and production. The net cost associated with discontinuing the feeding of AGPs was estimated at $1.53 CAD per pig produced, or a 1% increase in cost of production (WHO, 2003). It was suggested that the costs may be outweighed by increased consumer confidence and improvements to public health. In Denmark, the ban on AGPs reduced the total amount of antimicrobials fed to poultry and pigs from 204,686 kg in 1994 to 94,200 kg in 2001. The amount of time that pigs are exposed to AGPs throughout their lifespan also decreased from nearly continuous exposure to an average of 7.9 d. Based on the overall amount and potency of antimicrobials in relation to the pig population, researchers have found that the ban has not increased their therapeutic use (Arnold, 2004) especially for those antimicrobials that are particularly important in human medicine (cephalosporins, fluoroquinolones) (WHO, 2003).

The main goal of banning AGPs was to reduce the reservoir of resistance genes in food animals. Aarestrup et al. (2001) determined that it was possible to reduce the overall level of resistance in pigs and poultry by removing the source of selective pressure. They found that the incidences of avilamycin, erythromycin, vancomycin and virginiamycin resistance in *E. faecium* and *Enterococcus faecalis* isolates from pigs and broilers were reduced following the banning of these substances. In addition, when the use of these antimicrobials increased, the incidence of resistance increased as well. A study conducted in the US found that the incidence of erythromycin-resistant enterococci was higher on farms where tylosin phosphate was used as a growth promoter (59%) compared to farms where it was used therapeutically (28%) (Jackson et al., 2004), however, resistance also existed on farms where no antimicrobials were used (2%). There is extensive data available from Denmark indicating that the AGP ban there reduced the food animal reservoir of resistant enterococci, and therefore reduced the pool of resistance genes (WHO, 2003). It is unclear if the ban on AGPs and the subsequent decrease in resistance genes in food animals has resulted in a parallel decrease in the carriage of resistance in the intestinal tract of humans.

The ban of AGPs in Denmark resulted in a 0.7% increase in pig mortality during the nursery period (7-30 kg), while no increases in mortality were recorded for the finishing period (Callesen, 2002). Sweden also reported a 1.5% increase in post-weaning mortality during the first year following the ban (Wierup, 2001). Following the ban, Denmark reported a marked increase in the incidence of post-weaning diarrhoea, mirrored by an increase in usage of antimicrobials indicated for diarrhoea treatment, particularly for *Lawsonia intracellularis* (Larsen, 2002). A similar increase in diarrhoea treatments occurred in finisher pigs, however treatment levels had returned to normal levels seven months following the ban.

Discontinuing the use of AGPs in Denmark had no negative effects on the average daily gain or mortality in finisher pigs (Callesen, 2002), and the total volume of pork production continued to increase during the period following the ban (WHO, 2003). Weaner pigs experienced a decrease in average daily gain and took longer to reach 30 kg (Callesen, 2002) in Denmark, while Sweden experienced a 5-6 d increase in age at 25 kg (Wierup, 2001). The impact of the banning AGPs was inconsistent in Sweden, where more progressive pig producers reported improved production following the ban, and others did not recover production levels achieved prior to the ban (Wierup, 2001).

2.2.3 Overview of antibiotic alternatives

Animal nutritionists have become highly motivated to discover effective alternatives to feeding antimicrobial growth promoters since their general ban, which took full effect in the European Union in 2005. The potential alternatives often target the gastrointestinal microbiota, as its modification is the most widely accepted theory of how AGPs function to improve growth. For example, probiotics have received significant attention as potential replacements for AGP. By Fuller's definition, probiotics are "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 1989). Although it is unclear exactly what the ideal microbial balance would be for pigs, probiotic strains capable of competitively excluding pathogens and thus preventing disease and maintaining a "steady state" within the gastrointestinal tract would be considered desirable. Shu et al. (2001) found that treating weaned pigs with the probiotic *Bifidobacterium lactis* HN019 reduced the severity of diarrhoea by reducing the concentrations of *E. coli* and rotavirus, and enhancing the piglets' immune-mediated responses to gastrointestinal tract infection. Pollmann et al. (2005) showed that administering a probiotic strain of *E. faecium* to chlamydia-positive sows during pregnancy reduced the rate of infection of the intracellular pathogen in their offspring. In a field study of a

low-health status farm, treatment of weaning pigs with 10^7 viable spores of a probiotic strain of *Bacillus licheniformis* resulted in reduced incidence and severity of post-weaning diarrhoea syndrome, lower mortality, reduced detection of enterotoxigenic *E. coli*, and improved piglet performance (Kyriakis et al., 1999). The positive effects of feeding *B. licheniformis* in a 1:1 ratio with *Bacillus subtilis* was found to extend into the growing and finishing stages as well, where pigs treated throughout the production cycle with at least 1.28 X 10^6 viable spores had lower overall mortality and morbidity, and improved weight gain, feed efficiency, and carcass quality (Alexopoulos et al., 2004).

Widespread probiotic use is limited in part by the problems associated with maintaining viable bacteria through high temperature feed processing, storage, ingestion and digestion by the pig, and in establishing colonization beyond the termination of feeding. Although lactobacilli have been successfully used as probiotics in humans, their use in animal feeds is limited since they are unstable during feed processing and storage (Simon, 2003). Typically, once the probiotic is no longer being fed, the number of probiotic bacteria falls below the limit of detection or returns to pre-test levels. For example, in a study where humans consumed milk products containing a probiotic strain of *Lactobacillus rhamnosus* DR20 for six months, the probiotic strain was not detected at levels above 10^2 cells/g faeces in the three months following the test in all but one of the subjects (Tannock et al., 2000). While piglets were fed a probiotic strain of *Lactobacillus reuteri*, faecal lactobacilli counts were higher than controls, although the numbers declined slowly following the end of probiotic feeding (Chang, 2001). Since there is significant variation in effectiveness among strains and species (Reid, 1999) (their ability to adhere to cells, exclude pathogens, persist and multiply, produce products antagonistic to pathogen growth and, cohabitate with autochthonous flora) it is challenging to maintain consistent products and to monitor their quality.

Some nutrients are capable of promoting growth of 'beneficial' bacteria, without simultaneously increasing pathogen populations as well. These substances have been termed prebiotics, and are often used in combination with the probiotic strains of bacteria with the intention of providing a preferred fermentation substrate to promote colonization. Gibson and Roberfroid (1995) define prebiotics as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon". This definition should perhaps be elaborated to include effects on species resident in the small intestine, since this region is typically targeted for modification in livestock. Non-digestible oligosaccharides can be utilized preferentially by lactobacilli and bifidobacterial species (Kaplan and Hutkins, 2000), resulting in an increase in short-chain fatty acid and lactic acid production, which lowers the pH in the large intestine and may prevent colonization by *Salmonella* (Naughton et al., 2001). Fructooligosaccharides (FOS) included at 0.1% in the diet of chicks reduced the birds' susceptibility to *Salmonella* colonization (Fukata, 1999). Similar findings were reported in pigs, where supplementation of drinking water with FOS reduced faecal shedding of *Salmonella typhimurium* (Letellier, 2000). In early weaned pigs, the inclusion of 0.5% FOS was detrimental to growth performance and lowered serum IGF-I concentrations compared to controls (Estrada, 2001). These results suggest that the utility of FOS as growth-promoting feed additives for weaned pigs is limited.

Organic short chain fatty acids have been called the "most promising" compounds to replace AGPs thus far. Their antimicrobial activity arises from their supposed ability to lower dietary pH, and shift between undissociated and dissociated forms with changes in environmental pH. In the undissociated form, organic acids are able to enter the microorganism's cytoplasm where the higher pH will cause them to dissociate and disrupt decarboxylases, catalases, and nutrient transport systems (Partanen, 1999). When considering data from several experiments,

formic, fumaric and citric acids appeared to improve growth performance and feed efficiency in weaned pigs, although the effectiveness did not equal that of AGPs and the growth response varied from –58 g/d to +106 g/d (Partanen, 1999). In finishing pigs, formic and fumaric acids were effective in improving growth performance and feed efficiency, however the growth response varied from –50 g/d to +58 g/d (Partanen, 1999). High levels of propionic acid tended to depress feed intake and was not as effective in promoting growth (Partanen, 1999). Franco et al. (2005) found that intestinal coliform counts were lower in piglets fed a mixture of formic and lactic acids (1:1 or 1:2 ratio) compared to controls and those fed formic acid alone, while Risley et al. (1993) reported that feeding fumaric or citric acid did not improve piglets' response to an *E. coli* challenge.

Medium chain fatty acid-containing triacylglycerols (MCTAG) used in combination with supplemental lipase are degraded *in situ* to form medium chain fatty acids (C6-C12) that have antibacterial activities (Decuypere, 2003), but are not used alone because of their poor odour and taste. Preliminary research with feeding *Cuphea* seeds (naturally high in MCTAG) and lipase to piglets showed improvements in feed intake and efficiency, growth rate, and higher villus: crypt ratios (Dierick, 2003). Unfortunately, the widespread application of this approach to replacing AGPs may be hampered by the limited availability of *Cuphea* seeds, and the high price of commercially produced MCTAG oils.

Fermented liquid feed has been investigated as a possible solution to digestive disorders and poor growth experienced by some swine producers following the ban on AGPs (Pedersen et al., 2004). Feeding fermented liquid diets to grower and finisher pigs increased the number of lactic acid bacteria and lactic acid production, and lowered gastric pH and the number of enterobacteria compared to pigs fed liquid diets (Canibe and Jensen, 2003). However, there was no benefit to pig performance by feeding a fermented liquid diet, since feed intake and daily gain

was lower in those pigs. The authors suggested that synthetic lysine added to fermented diets is decomposed during the fermentation resulting in poor production (Pedersen et al., 2004). Subsequently, fermenting only the grain in liquid diets improved production values in finishing pigs by 11%; a result of improved growth rate and feed efficiency (Pedersen et al., 2004). Weaner pigs fed diets with fermented wheat had higher villus heights and villus:crypt ratios (Scholten, 2002).

Including pharmacological levels of zinc oxide (2000-3000 ppm) in the diet of both conventionally and early-weaned pigs enhances their growth performance (Carlson et al., 1999; Hill et al., 2001; Smith et al., 1997), may enhance epithelial morphology in the small intestine (Li, 2001), and reduce the severity of post-weaning diarrhoea by up to 50% (Poulsen, 1995). Carlson and Poulsen (2003) found that piglets fed high levels of zinc after weaning had enhanced mucosal function, and did not conjure the same secretory response to toxin producing intestinal bacteria, which may explain their resistance to diarrhoea. Denmark has adopted legislation preventing producers from including more than 250-ppm zinc in pig diets to prevent soil ecosystem and crop yield disruptions that may arise from the application of manure containing high levels of heavy metals.

Researchers faced with the removal of antibiotics from animal feeds have also turned to 'nutrition' in order to control enteric disease (Pluske, 2002). Several studies have shown that certain dietary constituents can pre-dispose animals to the development of enteric infections. For example, feed ingredients that greatly increase the viscosity of intestinal contents are associated with increased faecal shedding of enterotoxigenic haemolytic *E. coli* in weaned piglets (McDonald, 2001). Additional factors that may contribute to *E. coli* infection in piglets include gut transit time, feed form, presence of non-starch polysaccharides, and the ratio of lactobacilli:coliforms (Pluske, 2002). A diet low in soluble non-starch polysaccharides (NSP)

and resistant starch (RS) may protect pigs against infection with *Brachyspira hyodysenteriae,* the bacterium associated with swine dysentery (Pluske et al., 1998). More recently, however, it was reported that a diet low in NSP and RS did not prevent the development of swine dysentery in challenged pigs (Lindecrona, 2003). The mechanisms by which dietary components may impact clinical enteric diseases are poorly understood, therefore there is a need for increased understanding of how 'nutrition' influences the gut environment and interacts with commensal and pathogenic gut microorganisms (Pluske, 2002).

In order to further understand how diet composition impacts intestinal microbial profiles, the *cpn*60 bacterial identification method has been used in combination with qPCR to profile and quantify the small intestinal microflora of pigs fed diets based on corn, wheat, and barley (Hill et al., 2005). A total of 372 unique nucleotide sequences were identified, and the most abundantly recovered sequences were 90-100% identical to *L. amylovorus.* The corn library contained more Clostridiales-like sequences than the wheat or barley libraries, and *Streptococcus*-like sequences were found in greater relative abundance in the barley library. In addition to differences in relative sequence abundance between diets, there was significant sequence diversity within the taxonomic groups, which would not have been elucidated by culture-based methods. The results of the qPCR revealed the same relative abundances of taxa as determined by the library sequencing approach. The study also revealed that the frequency of recovery of clones within a taxon correlated well with the relative abundance of the taxon in the original sample. Thus, the library approach provides both a qualitative and semi-quantitative profile of the members of the community under study. Potential uses for *cpn*60 sequence libraries include their use as a tool for development of methods to quantify members of complex communities, for detecting pathogens or specific organisms, for monitoring treatment differences, or population changes over time (Hill et al., 2002). The ability to accurately profile microbial populations will improve

our understanding of the gut microflora, potentially allowing us to promote intestinal environments that support beneficial bacteria, and protect the host from intestinal disease.

2.3 Flaxseed

2.3.1 Chemical composition including anti-nutritional factors

In 2005, Western Canadian flaxseed (No.1 C.W.) contained 44% oil and 22.6% protein (DeClercq, 2005). Alpha linolenic acid (ALA 18:3n-3) is the most abundant fatty acid in flaxseed representing 60.5% of total fatty acids in this year's crop to date. Flaxseed also contains 4.9% palmitic acid (16:0), 3.1% stearic acid (18:0), 15.3% oleic acid (18:1), and 15.6% linoleic acid (18:2) (DeClercq, 2005). Flaxseed has a high content of mucilaginous water-soluble polysaccharides, which are contained mostly in the outermost cells of the hull. The hull is difficult to separate from the endosperm and cotyledons because of the seed's small size and shape, but makes up from 38-40% of the total seed (Bhatty, 1995).

Flaxseed contains a quantitatively minor but physiologically important level of cyanogenic glycosides (Mazza, 1995). These secondary plant metabolites are derived from valine and isoleucine and are able to liberate hydrogen cyanide upon hydrolysis (Mazza, 1995). Linustatin is the most abundant cyanogen found in flax, and the total content varies significantly between cultivars. Fortunately, the hydrogen cyanide content of flaxseed can be significantly reduced by autoclaving, microwave roasting, and pelleting (Feng, 2003). Another antinutritional factor that has limited the use of raw flaxseed in poultry diets is linatine, a vitamin B_6 antagonist that is present in the cotyledons and can be removed by demucilating the flaxseed (Bhatty, 1995). Flaxseed meal also has some trypsin inhibitor activity, although they are significantly lower than levels found in raw soybean and canola (Bhatty, 1995).

2.3.2 Human health benefits

Flaxseed has been consumed as a food and for its medicinal properties since it was first cultivated around 5000 BC (Judd, 1995; Oomah, 2001). The unique chemical composition of flaxseed has made it the most widely studied oilseed as a functional food. It is rich in α-linolenic acid (18: 3n-3), mucilaginous water-soluble polysaccharides and lignans, all of which have been found to confer numerous health benefits to humans. Flaxseed and its components may protect against cardiovascular disease, inhibit pro-inflammatory mediators, lower cholesterol, benefit renal function and bone health, and reduce the risk of hormone-related cancers in women (Oomah, 2001).

Flaxseed oil contains 59.3 % α-linolenic acid (ALA) on average (DeClercq, 2005), making it the richest terrestrial plant source of this essential fatty acid. ALA is a precursor to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and ALA supplementation via flaxseed or flaxseed oil increases tissue EPA, and decreases arachidonic acid (ARA) (Calder, 2001; Cunnane, 1995; Mantzioris et al., 1995). An increase in tissue EPA suppresses the production of ARA-derived eicosanoids and increases the production of EPA-derived eicosanoids, which are considered less inflammatory (Calder, 2001). Both epidemiological and clinical studies have linked consumption of diets rich in ALA to a reduction in cardiovascular risk (Ascherio et al., 1996; Hu et al., 1999). In addition to lowering total serum cholesterol, LDL cholesterol and triglycerides, researchers have suggested that a diet rich in ALA reduces cardiovascular disease risk by also inhibiting vascular inflammation and endothelial activation (Zhao et al., 2004).

Consumption of whole flaxseed has also been shown to confer health benefits to humans. Females who consumed 50 g of ground raw flaxseed perdfor 4 wkss experience lowered serum total cholesterol and low-density lipoprotein cholesterol (Cunnane et al., 1993). In the same

group of studies, females who consumed flaxseed flour or flaxseed mucilage had an improved post-prandial glucose response compared to those who consumed white bread. The inclusion of flaxseed oil in the diet has been shown to inhibit the production of pro-inflammatory cytokines interleukin-1β and tumour necrosis factor-α in humans (Caughey et al., 1996; James et al., 2000).

Flaxseed is a rich source of the phytoestrogen and plant lignan secoisolariciresinol diglycoside (SDG), which is converted to the precursors for mammalian lignans enterolactone and enterodiol by colonic bacteria (Chen, 2002). Breast cancer patients, and those at high risk for breast and colon cancer excrete lower levels of these mammalian lignans in their urine (Thompson, 1995). Flaxseed supplementation has been shown to inhibit the initiation, promotion, progression, and metastasis of mammary carcinogenesis in rats (Chen, 2002)

2.3.3 Flaxseed in swine nutrition

The utilization of flaxseed in animal feeds has been limited thus far to production of specialty food products such as omega-3 fatty acid-rich eggs (Lewis, 2000) and meat (Kouba et al., 2003). Finisher pigs fed diets with up to 15% ground flaxseed for the last 25 d prior to slaughter did not experience any reductions in growth performance or changes in carcass traits (Romans, 1995). Feeding flaxseed increased the amounts of alpha-linolenic acid and eicosapentaenoic acid in backfat, raw belly, and cooked bacon. A taste panel was unable to distinguish between meat from pigs fed flaxseed and those on control diets.

2.3.4 Potential for antimicrobial activity

In anticipation of future restrictions on feed antibiotics, our research has focused on understanding the intestinal microbial ecosystem, with particular interest in the impact of nutrition and novel feed ingredients on small intestinal microbial populations in pigs. The unique chemical composition of flaxseed and its specific fractions suggests that it may have desirable

nutritional and antimicrobial properties. Flaxseed is a very rich in α-linolenic acid, and contains a high content of mucilaginous water-soluble polysaccharides (Bhatty, 1995). Unsaturated fatty acids such as α-linolenic acid have been shown to exhibit antibacterial properties (Kodicek, 1945). Indeed, high concentrations of free α-linolenic acid inhibited the growth of bacteria in vitro, and altered bacterial adhesion sites on Caco-2 cells (Kankaanpaa et al., 2001). In arctic charr, feeding linolenic acid promoted colonization by lactic acid bacteria, which are considered beneficial residents of the fish intestinal ecosystem (Ringø, 1998). High concentrations of α-linolenic acid may alter the attachment sites for intestinal bacteria by altering the fatty acid composition of the intestinal wall (Kankaanpaa et al., 2001; Ringø, 1998).

The mucilaginous water-soluble polysaccharides in whole flaxseed render it unsuitable for poultry feed as its high water-holding capacity and viscosity may reduce digestion and absorption of nutrients (Alzueta, 2003), however pigs are less susceptible to alterations in intestinal viscosity. The mucilage fraction is contained mostly within the hull, and can be removed from the flaxseed by hot water extraction (Mazza, 1989). Flaxseed mucilage consists of acidic and neutral fractions (Cui, 1994; Fedeniuk, 1994). The acidic fraction consists of mainly pectic-like polysaccharides, containing L-rhamnose, D-galactose, and D-galacturonic acid, while the neutral fraction is mostly a β-D-(1,4)-xylan backbone of arabinoxylan component (Cui, 1994). Researchers have found higher *Lactobacillus* plate counts in poultry fed whole flaxseed (Alzueta, 2003) compared to demucilaged flaxseed, suggesting that the water-soluble polysaccharide fraction impacts microbial growth. *Bifidobacterium* spp. and *Bacteroides* spp. are able to effectively utilize oligosaccharides such as arabinoxylans (van Laere, 2000). Arabinoxylan breakdown has been shown to increase propionate production and *Bacteriodes fragilis* plate counts during *in vitro* studies with human intestinal microbiota (Hopkins et al., 2003). Low molecular weight galactooligosaccharides have been shown to stimulate the growth

of *Bifidobacteria* and *Lactobacilli* in the intestine of infants (Moro, 2002). Swine faecal microflora are able to effectively ferment a wide array of galactooligosaccharides including stachyose and raffinose (Smiricky-Tjardes et al., 2003). Oligogalacturonic acids, or dietary fibre pectins present in flax, are effectively degraded by pure cultures of *Bacteroides thetaiotaomicron* (Dongowski et al., 2000). This is of particular interest because *B. thetaiotaomicron* has recently been found to selectively induce the expression of *ang4*, a protein that is secreted by paneth cells into the gut lumen where it exhibits bactericidal activity against intestinal microbes in mice (Hooper, 2003). *B. thetaiotaomicron* may also activate an anti-inflammatory mechanism in the gut (Kelly et al., 2004). Volatile fatty acids, which are produced during the fermentation of carbohydrates in the intestine, may inhibit the growth of pathogens such as *Salmonella* (Williams, 2001). There is sufficient evidence to indicate that the fatty acid and water-soluble polysaccharide fractions of flaxseed could exhibit antibacterial effects on intestinal microbial populations, and/or promote a healthy gut environment by acting as substrates for certain commensal bacteria species.

2.4 Summary

Despite heated debate among scientists, and increasing public scrutiny of the issue, there remains no consensus on the level of risk posed to human health by AGP use in animals and its contribution to the development and dissemination of antibiotic resistance. Considering that AGP use in livestock feeds in North America will likely decline as it has in the European Union, whether through legislated or voluntary means, the task remains to determine how producers will maintain the current level of production in the future. Countless alternatives to AGPs have been investigated, including probiotics, prebiotics, organic acids, fermented liquid feeds, zinc, and dietary ingredient manipulation, although no single method seems capable of completely matching the impact achieved with AGPs. Thus, a more detailed understanding of how AGPs

impact intestinal microbial populations is necessary prior to the identification of a single, suitable replacement, since the small intestinal microbiota seems the most likely target for modification by AGPs. Armed with this information, researchers can continue to investigate and discover potential AGP alternatives, such as flaxseed, by comparing microbial profiles, intestinal health parameters, and animal performance data to that achieved with AGP treatment.

3. INVESTIGATION OF EFFECT OF TYLOSIN PHOSPHATE, FLAXSEED, AND FLAXSEED FRACTIONS ON SMALL INTESTINAL MICROBIAL PROFILES IN PIGS.

3.1 Introduction

In anticipation of future restrictions on feed antibiotics, researchers are focusing on understanding the intestinal microbial ecosystem, with particular interest in the impact of AGPs and novel feed ingredients on small intestinal microbial populations. APGs such as tylosin phosphate, a macrolide antibiotic that inhibits protein synthesis in Gram-positive bacteria, likely elicit their growth promoting effects by altering the microbial ecosystem, rather than directly affecting host metabolism. Therefore an improved understanding of the effect of AGPs on intestinal microbial ecology, aided by molecular enumeration methods, could help identify microbial colonization profiles associated with improved health and productivity. Previous studies examining the impact of tylosin on microbial populations in the pig ileum showed increased homogeneity of microbial populations, and an increase in the percentage of lactobacilli (Collier et al., 2003b). The authors suggested that homogenization of the pig ileal microbiota and selection for lactobacilli may be the mechanism for growth promotion by AGPs. However, a more precise and specific elucidation of the impact of tylosin on ileal microbial populations in pigs is necessary to aid in the selection of alternatives to AGPs.

The unique chemical composition of flaxseed and its specific fractions suggests that it may have desirable nutrient and antimicrobial characteristics, however its impact on intestinal microbial populations in pigs in unknown. Flaxseed is a rich source of α-linolenic acid, and contains a high content of mucilaginous water-soluble polysaccharides (Bhatty, 1995). Unsaturated fatty acids such as α-linolenic acid havebeen shown to exhibit antibacterial properties (Kodicek, 1945), and researchers have found higher *Lactobacillus* plate counts in

poultry fed whole flaxseed (Alzueta, 2003) compared to demucilaged flaxseed, suggesting that the water-soluble polysaccharide fraction impacts microbial growth.

To overcome the limitations of culture-based methods for profiling microbial populations, a combination of high throughput sequencing and quantitative real-time PCR (qPCR) based on *chaperonin*-60 (*cpn*60) has been used successfully to describe differences in the small intestinal microbial communities in pigs fed corn, wheat, or barley-based diets (Hill et al., 2005). It was hypothesised that dietary inclusion of flaxseed or its fractions will beneficially alter small intestinal microbial populations in pigs and thus serve as an effective replacement for sub-therapeutic antibiotics in swine diets. The impact of sub-therapeutic antibiotic inclusion was compared to the impact of flaxseed or flaxseed fraction inclusion on small intestinal microbial profiles in pigs using the *cpn*60 bacterial identification method. The first objective was to determine the effect of dietary tylosin phosphate inclusion on small intestinal microbial profiles in pigs. Culture-based analysis was performed and two *cpn*60 clone libraries were constructed to compare and contrast the small intestinal microbial profiles of pigs fed diets with and without tylosin phosphate, a commonly used growth-promoting antibiotic in swine diets. The second objective was to compare the effect of tylosin phosphate inclusion with the inclusion of flaxseed or its fractions on colonization by certain commensal bacteria and zoonotic pathogens. Quantitative PCR was used to enumerate organisms that were affected by tylosin phosphate inclusion as identified by the *cpn*60 library comparison.

3.2 Materials and Methods

3.2.1 Animals and sample collection

The animal protocol was approved by the University of Saskatchewan Committee on Animal Care and Supply (protocol # 19980106), and followed principles established by the Canadian Council on Animal Care (1993). The study utilized 18 ileal-cannulated barrows (33.1 ± 2.4 kg), fed one of six diets (Table 3.1, Control diet: basal diet based on wheat, peas and soybean meal (C), Whole flax diet: basal diet plus 20% ground whole flax seed (WF), Hull diet: basal diet plus 4% flax hulls (H), Hot water extracted flax diet: basal diet plus 18% hot water extracted flax seed (HWE), Oil diet 5: basal diet plus 8% flaxseed oil (O), Tylosin phosphate diet (Elanco Animal Health, Guelph, ON): basal diet with 22 mg/kg tylosin phosphate (T) during three consecutive 21-d periods in a change over design. Two weeks of each period were designated for acclimatization to the experimental diets. Pigs were housed in 1.5 x 1.5 m metabolism pens and had free access to water. Diets were provided twice daily at 0800 and 1600h in a 1:1 ratio with water and fed at 2.5 x maintenance requirements. On days 16 and 17 of each period, 100 ml of digesta was collected from the open cannula in bags containing N_2 immediately following the morning feeding, resulting in 9 observations per treatment for each collection day. Figure 3.1 outlines the handling of ileal digesta samples.

3.2.2 Flaxseed fractions

All flaxseed and fractions thereof (except oil) used in experimental diets were obtained from the same supply of seed. The flaxseed was first dry roasted in a rotating drum dryer to 130ºC, and then held in an insulated storage chamber for 10-15 min. The flaxseed was then autoclaved at 121 ºC and dried for 10 min before undergoing further processing. The hot-water extracted flax was prepared following the procedure of Cui et al. (1994). Briefly, whole autoclaved flaxseed and water (13:1 ratio of water to flaxseed) were heated in a steam jacketed

kettle with continuous stirring at 85ºC for 3 h. Following this, the seeds and mucilage were separated using a 40 mesh screen and the seeds were washed with 85ºC water to remove any adhering mucilage. The seeds were then dried at 55ºC for 48 h.

Flaxseed de-hulling took place at the Prairie Agricultural Machinery Institute's Crops Processing Development Centre (Humbolt, SK). The procedure employed is based on the Tangential Abrasive Dehulling Device (TADD) described by Rieckher et al. (1986). Whole flax, HWE flax and flax hulls were ground (Pizzey's Milling Angusville, MB) using a 2.8 mm round screen size. Organic cold-pressed flaxseed oil was purchased at a local supermarket (Herbal Select, Canada).

3.2.3 Culture based enumeration

Culture-based microbial enumeration was conducted using samples of freshly collected ileal contents diluted to 10^{-3} and 10^{-5} (w/v) in an anaerobic chamber using sterile peptone (0.1% peptone, 0.5% NaCl, 0.05% cysteine HCl). Samples were plated on selective agars in an aerobic environment using a spiral plater (AP 4000, Spiral Biotech Inc., Bethesda, MD). Selective agars employed were total anaerobes, blood agar; total aerobes, blood agar; enterobacteria, MacConkey; lactobacilli, MRS; *C. perfringens*, blood agar and neomycin; Streptococcus, bile esculin; bifidobacteria, Bereen's agar. Aerobic organisms (enterobacteria, total aerobes, and streptococci) were incubated for 30 h at 37ºC. Plates enumerating total anaerobes (bifidobacteria, *C. perfringens*, total anaerobes, and lactobacilli) were incubated in an anaerobic chamber (10% CO_2, 10% H_2, and 80% N_2) at 37ºC for 30 h.

Table 3.1 Ingredient and nutrient composition of basal diet

Ingredient	%
Wheat	65.13
Peas	20.00
SBM	10.40
Canola oil	1.00
Limestone	1.00
Di-calcium phosphate	0.80
Salt	0.40
Vitamin mix	0.40
Mineral mix	0.40
Lysine HCl	0.30
L-threonine	0.11
DL-methionine	0.06
Calculated nutrient composition	
Crude protein (analysed, DM basis)	22.80
DE (Mcal/kg)	3.40
D Lysine (g/Mcal DE)	2.65
Ca	0.68
P	0.55

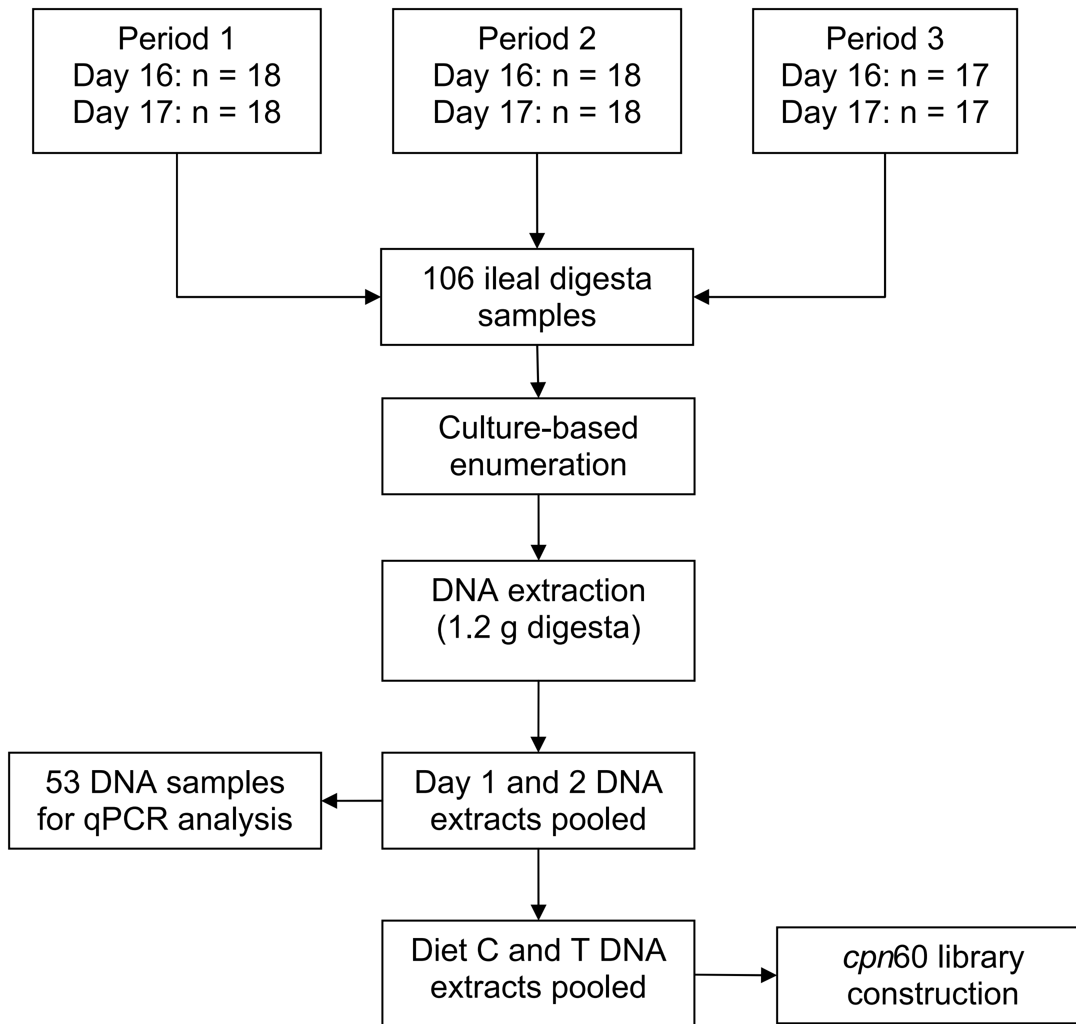


Figure 3.1 Outline of sample collection and handling. Ileal digesta was collected from 3 pigs × 6 diets on both days of each period, except for period 3, where one pig from Diet O was omitted from the experiment due to illness; therefore 17 ileal digesta samples were collected.

3.2.4 Genomic DNA extraction

DNA was extracted from 1.2 g of digesta using a modified phenol-chloroform-isoamyl alcohol bead-beating method designed to maximise cell lysis (Apajalahti et al., 1998; Hill et al., 2005). Faecal samples (0.2 g per tube) were placed in a bead-beating tube (Mo-Bio Laboratories, Solano Beach, CA) and incubated at 37°C for 30-60 min with RNase A (73 µg), lysozyme (750 µg), and proteinase K (400 µg) in 0.365 ml of buffer containing 50 mM Tris-Cl, 50 mM EDTA, 0.5% Tween and 0.5% Triton X-100. Following this, 0.135 ml of a buffer containing 3 mM guanidine-HCl and 20% Tween 20 was added and the samples were incubated at 50°C for 30 min. Following a freeze-thaw cycle (-70°C/25°C), samples were placed in a bead beater (Bio101 ThermoSavant FP120) and shaken three times for 20 s (setting 5) with 700 µl of 25: 24:1 phenol/chloroform/isoamyl alcohol. The samples were centrifuged at 13,000 x g for 15 min, and the top aqueous phase was transferred to a new tube. Nucleic acids were precipitated with 1 vol isopropanol and 0.1 vol 3 M Na acetate, pelleted by centrifugation for 15 min, washed in 1 ml of 70% ethanol, air dried, and resuspended in 100 µl of TE buffer (10 mM Tris-Cl and 1 mM EDTA). Samples collected on consecutive days within each period were pooled and purified using an anion exchange resin (Qiagen Tip/20G) according to the manufacturer's protocol and dissolved in 0.1 ml TE buffer.

3.2.5 Library construction

Two *cpn*60 clone libraries were constructed from ileal DNA samples collected from pigs fed the basal diet with (T) and without (C) tylosin phosphate. Genomic DNA was pooled across diets and subjected to PCR amplification of the *cpn*60 universal target (*cpn*60 UT). The PCR reactions (50 µL total reaction volume) contained 20 mM Tris-HCl pH 8.4, 50 mM KCl, 2.5 mM magnesium chloride, 0.2 mM dNTPs, 2.0 units of *taq* polymerase (Invitrogen), 100 nM primers H279 (5'-GAI III GCI GGI GAY GGI ACI ACI AC-3') and H280 (5'-YKI YKI TCI CCR AAI

CCI GGI GCY TT-3'), and 300 nM primers H1612 (5'-GAI III GCI GGY GAC GGY ACS ACS AC-3') and H1613 (5'-CGR CGR TCR CCG AAG CCS GGI GCC TT-3'). The 1:3 mixture of degenerate, universal *cpn*60 primers (H279 and H280) and primers that efficiently amplify high G+C templates (H1612 and H1613) have been shown to improve template representation in *cpn*60 PCR product libraries generated from complex templates (Hill et al., 2006). The PCR protocol consisted of 2 min at 95ºC, followed by 40 cycles of denaturing at 95ºC for 30 sec, annealing at either 42, 46.5, 50.4, or 56ºC for 30 sec, and a final extension for two minutes at 72ºC. Four annealing temperatures were utilized to limit any PCR amplification bias that could occur at any one temperature (Hill et al., 2006; Hill et al., 2002; Hill et al., 2005). All reactions were done in parallel on an iCycler (Bio-Rad, Hercules, CA).

The *cpn*60 UT products amplified at the four annealing temperatures were pooled and 10 µl was subjected to agarose gel electrophoresis. Excised bands were purified using the QiaQuick Gel extraction kit according to the manufacturer's instructions (Qiagen, Mississauga, ON). Purified PCR products were A-tailed in a 10-µL reaction containing 0.5 mM dNTPs, 2.5 units of *Taq* polymerase (Invitrogen), 2.5 mM magnesium chloride, and 100 ng of template DNA incubated at 72°C for 15 min and ligated into plasmid cloning vector pGEM®-T Easy (Promega Corporation, Madison, WI). The ligation reactions (10 µL total volume) contained 5 µL 2X Rapid Ligation Buffer, 50 ng pGEM®-T Easy vector (Promega), 30 ng of PCR product, and 3 Weiss units of T4 DNA ligase, and were incubated overnight at 4°C. The entire ligation reaction was used to transform 250 µL of *E. coli* JM 109 competent cells (Promega). Transformed *E. coli* were cultured on LB agar containing 100 µg of ampicillin and spread with IPTG and X-Gal (100 µl of 100 mM IPTG and 20 µl 50 mg/ml X-Gal) at 37°C overnight. A total of 1152 colonies were picked with sterile wooden toothpicks and transferred into 96-well plates containing 100 µL of LB broth with 50 µg/ml ampicillin and incubated overnight at 37°C. Subsequently, 100 µL of

LB broth with 15 % (v/v) dimethyl sulfoxide (Sigma Chemicals, St. Louis, MO) was added to all wells and plates were stored at -80°C.

The DNA template for sequencing was prepared using TempliPhi™ DNA Amplifiction (Amersham Biosciences) according to manufacturer's instructions. The sequencing reactions were executed as described previously by Hill et al. (2002). Sequence data analysis was performed using software available at the Canadian Bioinformatics Resource (http://cbr.nrc.ca). First, raw sequence data were assembled into contigs using PreGap4 and Gap4 and vector and primer sequences were removed. The sequence data was compared with the *cpn*60 database (Hill et al., 2004), cpnDB (http://cpndb.cbr.nrc.ca) using FASTA and BLASTP, and were assigned a nearest neighbour based on sequence similarity. Phylogenetic analysis was executed with software in the PHYLIP package (Felsenstein, 2004. PHYLIP [Phylogeny Inference Package] version 3.6, distributed by the author, Department of Genetics, University of Washington, Seattle, WA). Sequence alignments were done with Clustalw, distance matrices were computed with Dnadist, and phylogenetic trees were constructed using Neighbor and viewed with TreeView (Hill et al., 2004; Page, 1996). The number of *cpn*60 UT clones within a taxonomic group were calculated and expressed as a percent of total clone numbers within diet.

3.2.6 Selection of bacterial targets, design and validation of qPCR assays

Real time qPCR assays were developed to enumerate *L. amylovorus*, *S. alactolyticus*, and clusters of closely related bacteria identified by phylogenetic analysis and designated group L1 (90% similar to *Lactobacillus delbrueckii*), group L2 (88% similar to *Lactobacillus vaginalis*), and group B1 (74% similar to *Bacillus thuringiensis*). Signature Oligo software (Life Intel Inc., Port Moody, Canada) was employed to identify *cpn*60 nucleotide signature sequences that were unique to the target organism or cluster when compared to closely related taxa. Diagnostic PCR primers (Table 3.2) were designed to anneal at these sequence locations using Oligo 6 Primer

Table 3.2 Sequences of the PCR primers and PCR cycling conditions used to detect *cpn*60 UT sequences of selected bacterial species and target groups

Target group	**Primer 1 (5’ -3’)**	**Primer 2 (5’- 3’)**	**PCR cycling conditions**[5]
B1	CGGGAGCTAATCCAATAACGA	CGCAGCTACGTTAGCAATTGA	40 × (95 °C, 30 s; 58 °C, 30 s, 72 °C, 30 s)
L1	CTATTTCAAGTGCTTCAAGCG	GGAGTCTTCGATGGTGATGAC	40 × (95 °C, 30 s; 58 °C, 30 s, 72 °C, 30 s)
L2	TGGGATCGACAAGGCTACGC	CGTCGGCAATCAGCTTACCAA	40 × (95 °C, 30 s; 58 °C, 30 s, 72 °C, 30 s)
S. alactolyticus	CGAATCTGCGGTTTCAGTAGC	GATGATACGGCAGCAACTTGG	40 × (95 °C, 30 s; 55 °C, 30 s, 72 °C, 30 s)
L. amylovorus	CATCTAAAGAAGTTGGTGACT	CAATGATTAACAAAGCCTTAC	40 × (95 °C, 30 s; 55 °C, 30 s, 72 °C, 30 s)
E. coli[1]	GGCTATCATCACTGAAGGTCTG	TTCTTCAACTGCAGCGGTAAC	40 × (94 °C, 30 s; 61 °C, 30 s, 72 °C, 30 s)
L10[2]	AAGCTGCCGTTGATGAATTAC	AGCGTCAGCGATTAAGTCACC	40 × (94 °C, 30 s; 61 °C, 30 s, 72 °C, 30 s)
C. perfringens[3]	AAATGTAACAGCAGGGGCA	TGAAATTGCAGCAACTCTAGC	40 × (94 °C, 30 s; 59 °C, 30 s, 72 °C, 30 s)
L. johnsonii[4]	TACTATTGAAGAATCAAAGGG	TCAGTAATCAAAATGTAAGGG	40 × (95 °C, 30 s; 55 °C, 30 s, 72 °C, 30 s)

[1] TaqMan probe sequence (5’6-carboxyfluorescein; 3’ Black Hole Quencher® 1) TGTTGCTGCGGGCATGAACC
[2] TaqMan probe sequence TTTAGATGCTGATGAAACAGCAGCTACGTT
[3] TaqMan probe sequence ATGTCTTCTTTTCCATTTACAGGCTTAGAA
[4] TaqMan probe sequence TAAGTCTGCTTCCATCTTGTCGTT
[5] All PCR assays began with one cycle at 95°C for 1.5 min, except for *C. perfringens* and *L. johnsonii,* which began with 1 × 94 °C for 2 min, and 1 × 95 °C for 3 min, respectively

Analysis software (version 6.0, Molecular Biology Insights, 2003). Primers were checked for specificity by comparison to C and T library sequence data and typed strain reference data in cpnDB using BLAST. Following this, primers were tested for specificity by PCR amplification of plasmid DNA containing the appropriate target *cpn*60 UT and a mixed panel of *cpn*60 UT DNA templates (plasmid DNA containing *cpn*60 UT or genomic DNA) representing 23 bacterial species commonly isolated from the intestine (Table B1, Appendix B). qPCR assays for *L. johnsonii*, *E. coli*, *C. perfringens* and the L10 group were designed and validated as described in previous studies (Dumonceaux et al., 2006; Hill et al., 2005). The *E. coli* PCR primers were notable to differentiate between *E. coli* and *Shigella flexneri* since the *cpn*60 universal target sequences for these two species are identical (Dumonceaux et al., 2006). The L10 primers were designed to amplify an abundant group of bacteria identified in the ileal contents of pigs fed corn, wheat or barley diets that are 95-100% similar to *L. amylovorus* ATCC 33198 (Dumonceaux et al., 2006; Hill et al., 2005). The specificity of each primer pair against the mixed panel is shown in Figure 3.2.

3.2.7 Quantitative PCR

All qPCR assays were performed with the iCycler iQ Real-Time PCR detection system (Bio-Rad Laboritories, Hercules, CA). For B1, L1, L2, *L. amylovorus,* and *S. alactolyticus* the final reaction mix contained Platinum® SYBR® Green qPCR Supermix UDG (Invitrogen), 500 nM of each primer, plasmid calibration standards of genomic DNA from ileal contents and ddH_2O adjusted to a final volume of 25 μl. For *E. coli,* L10*, C. perfringens*, and *L. johnsonii* reaction mixes contained Platinum® qPCR Supermix UDG (Invitrogen), 500 nM of each primer, 200 nM of probe, plasmid calibration standards or genomic DNA from ileal contents and ddH_2O

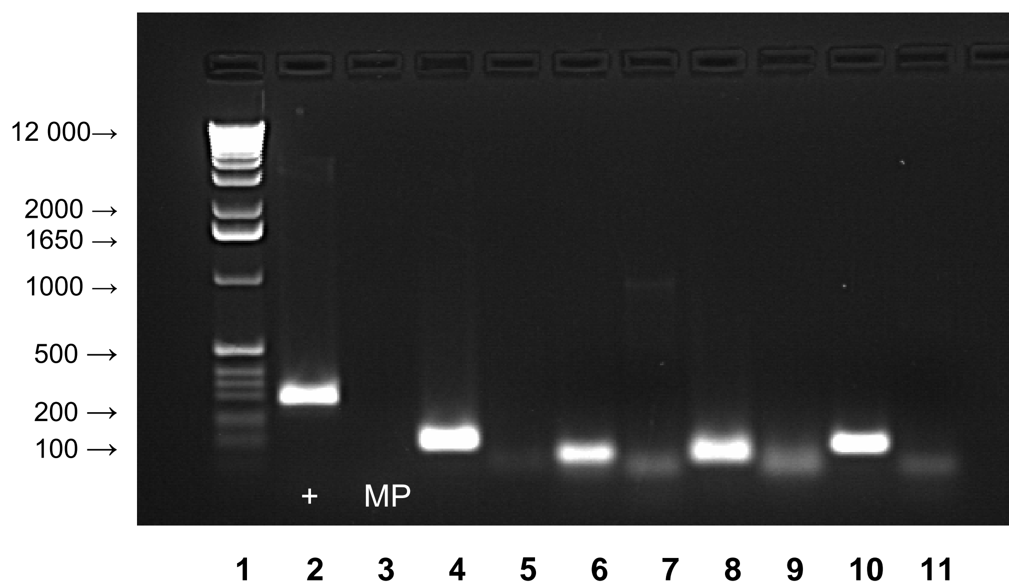


Figure 3.2 Agarose gel showing specificity of qPCR primers for target bacteria. PCR amplification of plasmid encoded *cpn*60 UT for the target bacteria and a mixed panel of *cpn*60 UT (MP, Appendix 2) without target *cpn*60 UT representation is shown. Target bacteria amplified include *L. amylovorus* (lanes 2 and 3), L2 (lanes 4 and 5), L1 (lanes 6 and 7), *Streptococcus alactolyticus* (lanes 8 and 9), or B1 (lanes 10 and 11). Molecular weights are indicated to the left of the 1 kB DNA ladder (lane 1).

adjusted to a final volume of 25 µl. The PCR cycling conditions are shown in Table 3.2. Data collection was set at the annealing/extension step. All samples were assayed in duplicate.

Standard curves for all qPCR assays were prepared using a 10-fold dilution series of plasmid DNA containing *cpn*60 UT sequences for the bacterial species in question. Plasmid DNA was purified from 5 mL overnight cultures of *E. coli* in LB broth using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. Plasmid DNA concentrations were determined using PicoGreen (Molecular Probes) with a Fluoroskan Ascent FL fluorometer (Thermo Labsystems). Plasmid copy number was calculated as the weight of plasmid DNA in each standard divided by plasmid molecular weight (2.31 X 10^6 g/mol with 3550-bp plasmid size) (Dumonceaux et al., 2006). Threshold cycle values were plotted against the calibration standard copy number to generate a standard curve. Threshold values for unknowns were interpolated against the standard curve using software (iCycler iQ optical system software, version 3.0) to provide copy number and were reported as target bacteria genomes per gram of ileal contents.

3.2.8 Statistical analysis

All data were analysed as a univariate ANOVA using the general linear models (GLM) procedure in SPSS (SPSS Inc., Chicago, IL, USA, version 13.0). Sources of variation included were dietary treatment and period. Where period was not significant, it was removed from the statistical model, and data was analysed with dietary treatment as the source of variation. For plate count data collected over two consecutive days, the mean was calculated and used for statistical analysis. Means were separated by Ryan-Einot-Gabriel-Welsch multiple F test. Significance was considered when $P < 0.05$, and trends were reported when $P < 0.10$.

3.3 Results

3.3.1 Culture-based enumeration

The inclusion of flaxseed hulls in the diet of growing pigs increased lactobacilli plate counts (Table 3.3) by 6.4% ($P < 0.05$) compared to the control diet. A similar, but non-significant response was observed for diets containing flaxseed oil and tylosin which each resulted in a 5.1% increase. The experimental diets had no impact on the number of total aerobes, total anaerobes, streptococci, bifidobacteria, enterobacteria, lactobacilli, or *C. perfringens* ($P > 0.05$). The ratio between lactobacillus and enterobacteria plate counts was greatest for diets containing flaxseed fractions (ranging between 1.57:1 and 2.04:1) compared to control or tylosin diets (1.43:1). This ratio was significantly different ($P > 0.05$) for whole flax compared to control and tylosin diets only.

3.3.2 Comparison of library profiles in control and tylosin treatment groups

From the 2304 randomly selected PCR product clones submitted for sequencing, 1634 high quality, full-length *cpn*60 gene segments were obtained (Table 3.4). Within this collection were 117 different nucleotide sequences. The diversity for both libraries, calculated as the number of different nucleotide sequences as a percentage of the total number of sequences examined, was 7.2 overall (a higher number indicates greater diversity). The control library (C) contained 709 sequences, 59 of which were different. The diversity for C was 8.3. The tylosin library (T) contained a greater number of total sequences (925), however, a lower proportion of these (49 or diversity index 5.3) were different compared to the C library. Less than 7% of the different sequences were shared between the C and T libraries (9 sequences in total), however these sequences were recovered frequently and accounted for 80% the C library sequences and 78% of the T library. Table A1 in Appendix A lists the names, nearest neighbour within cpnDB, and frequency of recovery for all clones from the C and T libraries.

Table 3.3 Mean bacterial number (log cfu) per gram of ileal contents from pigs fed diets with and without tylosin phosphate, flaxseed or flaxseed fractions as determined by viable count on selective media

Target[1]	**Control**	**Whole Flax**	**Hulls**	**HWE**	**Oil**	**Tylosin**	**Pooled SEM**	***P*-value**
Lactobacilli[2]	7.97[a]	8.13[ab]	8.52[b]	7.95[a]	8.41[ab]	8.40[ab]	0.14	0.019
C. perfringens	1.59	2.57	1.86	2.36	2.27	2.36	0.28	0.410
Enterobacteria[3]	5.58	4.61	4.99	5.13	5.40	5.92	0.49	0.218
Non-lactose fermenters[4]	3.67	3.80	4.68	4.17	4.25	4.25	0.45	0.915
Streptococci	6.10	6.43	6.50	6.32	5.92	6.73	0.31	0.181
Bifidobacteria	7.33	7.19	7.29	7.46	7.44	7.65	0.15	0.363
Total aerobes	6.12	6.19	6.31	6.31	6.38	6.62	0.35	0.859
Total anaerobes	7.95	7.88	8.09	7.92	8.05	8.22	0.16	0.475
L:E ratio[2, 5]	1.43[a]	2.04[b]	1.91[ab]	1.63[ab]	1.57[ab]	1.43[a]	0.14	0.016

[1] Listed targets are those presumed to grow on the selective media utilized; Lactobacilli, MRS; *C. perfringens*, blood agar with neomycin; coliforms and non-lactose fermenters, MacConkey agar; Streptococci, bile esculin; Bifidobacteria, Bereen's agar, total aerobes and anaerobes, blood agar

[2] Means within a row with different letter superscripts are different ($P < 0.05$)

[3] Lactose fermenting colonies on MacConkey agar

[4] White colonies on MacConkey agar

[5] Ratio between lactobacilli and enterobacteria plate counts

Table 3.4 Number of sequences generated for each library

Library	No. of sequences examined	No. of different nucleotide sequences	Diversity[1]
Control	709	59	8.3
Tylosin	925	49	5.3
C + T	1634	117 (9)[2]	7.2

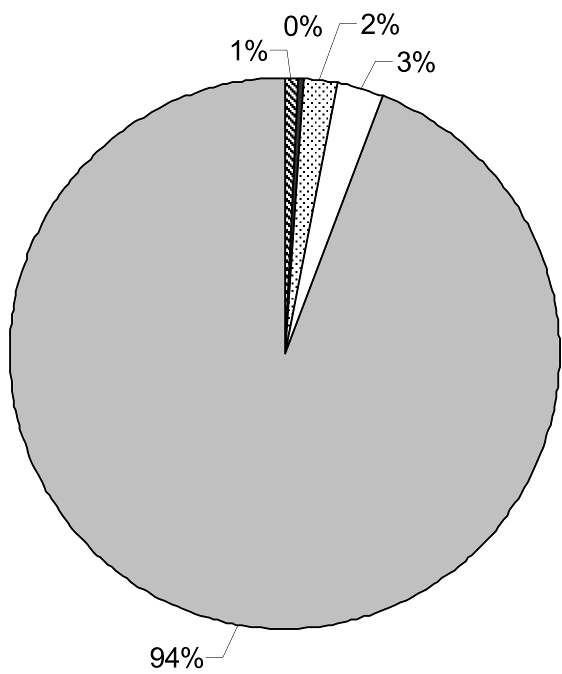
[1] Calculated as the number of different sequences as a percentage of examined sequences
[2] 117 is the total number of different sequences (59 + 49 +9 = 117), 9 is the number of different sequences that were found in both the C and T libraries

Table 3.5 lists nearest neighbour assignments and clone names for *cpn*60 UT sequences that were recovered at least 10 times from either library. The three most frequently recovered sequences were 99.6-100% similar to *L. amylovorus* ATCC 33198, and had at most two nucleotide differences between any pair of these three sequences. Eight of the 14 cloned sequences listed in Table 3.5 were recovered independently from both libraries. All eight independently recovered sequences were 99-100% similar to *L. amylovorus* ATCC 33198.Figure 3.3 shows the taxonomic composition of the C and T libraries generated from pooled pig ileal contents fed control and tylosin diets. Lactobacillales-like sequences dominated both libraries. Nearest neighbour analysis of the *cpn*60 UT revealed that 89% of clones had sequences that were between 85 and 100% similar to *L. amylovorus*. More clones belonging to the Lactobacillales taxon were recovered from the C library (94% of clones) than the T library (86% of clones). Enterobacteriaceae-like sequences were more abundant in the T library (13.4% of clones) compared to C (2.7% of clones). The ratio between Lactobacillales- and Enterobacteriaceae-like clones was 35:1 for the C library and 6:1 for the T library. Five clones belonging to the Bacillales taxon were found in the C library, while none appeared in the T library. The C library also had more clones from the Clostridiales and bifidobacteria taxa (2 and 0.3% compared to 0.1 and 0.75% for T, respectively).

Table 3.5 Names, nearest reference sequences, and frequency of recovery for clones recovered from C and T libraries at least 10 times

			Frequency (no. of clones)				
Clone name	**Nearest cpn60 neighbour**	**% DNA identity**	**C**	**T**	**Total**	**% C[1]**	**% T[2]**
cl0_cn29	*Lactobacillus amylovorus* ATCC33198	100	198	490	688	27.93	52.97
cl0_cn26	*Lactobacillus amylovorus* ATCC33198	99.819	223	55	278	31.45	5.95
cl0_cn22	*Lactobacillus amylovorus* ATCC33198	99.638	53	42	95	7.48	4.54
cl7_cn15	*Lactobacillus delbrueckii* subsp. *lactis* ATCC12315	89.674	31	57	88	4.37	6.16
cl0_cn17	*Escherichia coli* C175-94	100	0	81	81	0.00	8.76
cl0_cn35	*Lactobacillus amylovorus* ATCC33198	99.819	26	44	70	3.67	4.76
cl0_cn34	*Lactobacillus amylovorus* ATCC33198	99.457	15	14	29	2.12	1.51
cl0_cn32	*Lactobacillus amylovorus* ATCC33198	99.638	12	11	23	1.69	1.19
cl0_cn27	*Lactobacillus amylovorus* ATCC33198	99.819	8	10	18	1.13	1.08
cl7_cn14	*Lactobacillus delbrueckii* subsp. *lactis* ATCC12315	89.674	16	0	16	2.26	0.00
cl26_cn1	*Streptococcus alactolyticus* ATCC43077	99.819	0	13	13	0.00	1.41
cl354_cn3	*Escherichia coli* O157:H7 RIMD 509952	99.459	12	0	12	1.69	0.00
cl3_cn21	*Lactobacillus amylovorus* ATCC33198	99.819	0	12	12	0.00	1.30
cl0_cn16	*Escherichia coli* C175-94	99.820	0	10	10	0.00	1.08

[1]Clone frequency as a percentage of total C library clones
[2]Clone frequency as a percentage of total T library clones



Bacillales

Bifidobacterium

Clostridiales

Enterobacteriaceae

Lactobacillales

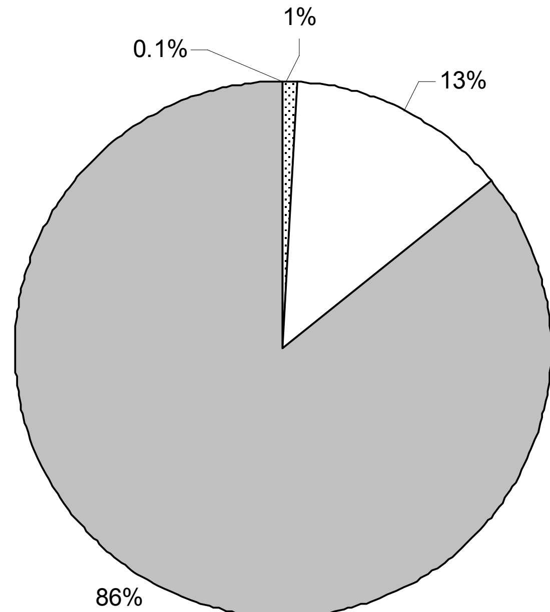
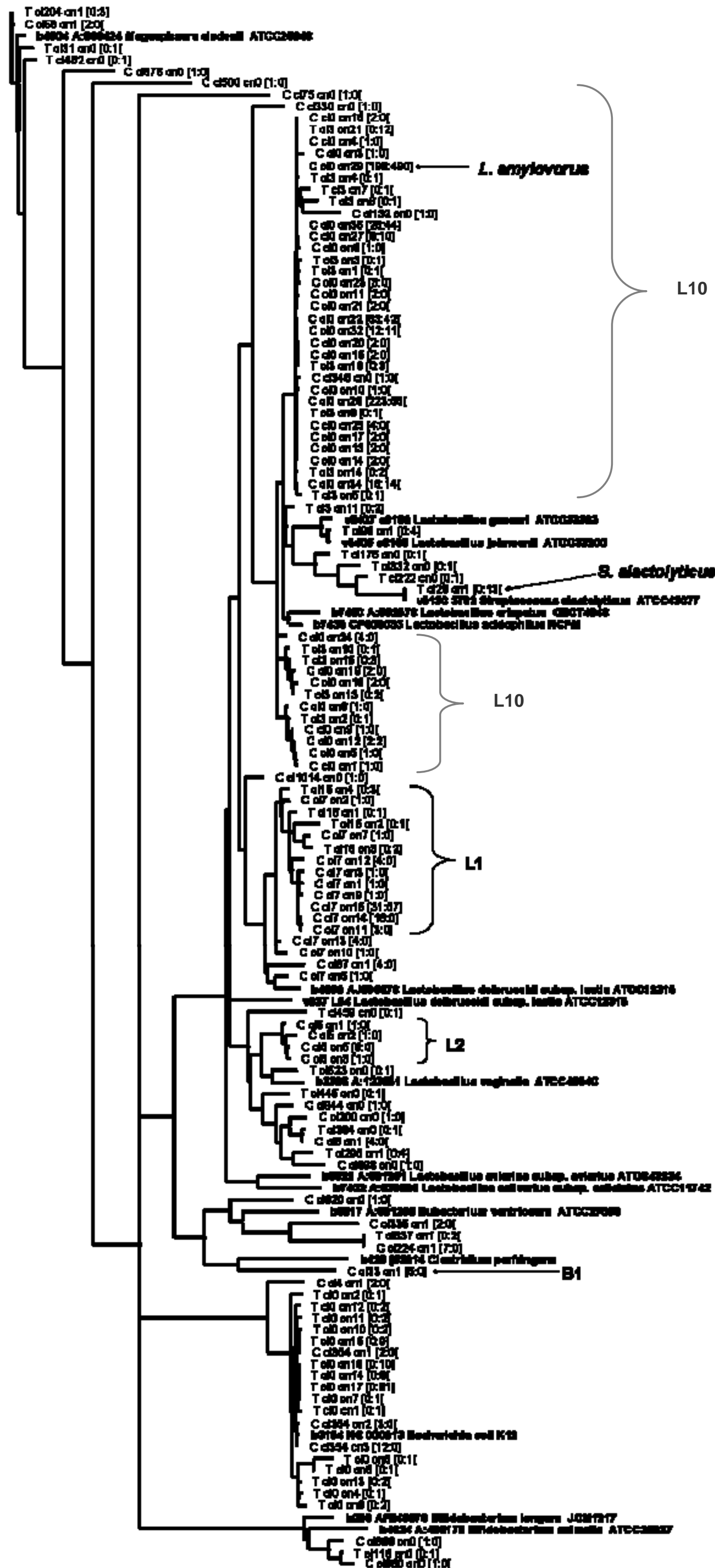


Figure 3.3 Taxonomic composition of C and T clone libraries

A phylogenetic tree constructed from a pool of all 117 different *cpn*60 UT sequences is shown in Figure 3.4. Selected ATCC type strains were included in the tree construction for reference. The frequency that each sequence occurred in the libraries is indicated by the C:T ratio in parentheses following the clone name. Some clusters of closely related sequences (determined by distance) are defined based on their nearest neighbour. For example, the L1 group is loosely related to *L. delbrueckii* (89.7%), the L2 group is loosely related to *L. vaginalis* (88.9%), and B1 is 74% similar to *B. thuringiensis*.

The overall average percent DNA identity for all sequences in both libraries was 93%, indicating that the majority of sequences recovered were dissimilar to those previously catalogued in cpnDB. This is especially the case for sequences where the nearest neighbour belonged to the Clostridiales or Bacillales taxa, where the average percent DNA identities were 83 and 74%, respectively. The average percent DNA identities for sequences in the bifidobacteria, Lactobacillale*s*, and Enterobacteriaceae groups were 97, 93, and 98%, respectively.

Differences in library composition were identified by comparison of C:T clone frequency ratios (adjusted for total sequence number in each library) for clusters of closely related organisms. The combined frequency ratio for sequences assigned to the cluster designated L1 was 59:64 (C:T) representing 8.3 and 6.9% of sequences recovered in the C and T libraries, respectively. The L2 group was recovered mostly in the C library (12:1 or 1.7 and 0.1% of sequences), and the B1 group had 5 sequences recovered from the T library only. *S. alactolyticus* and *L. johnsonii cpn*60 UT sequences were recovered only in the T library (0:13 and 0:4 clone frequency ratios, respectively).



L. amylovorus
L10
S. alactolyticus
L10
L1
L2
B1
0.1

Figure 3.4 Phylogenetic analysis of 117 unique nucleotide sequences recovered from the control and tylosin libraries. Groups or individual bacterial species targeted for primer design and qPCR analysis are indicated: *L. amylovorus*, *S. alactolyticus*, L10, L1, L2, and B1. Reference sequences (in bold text) were included for information purposes. Numbers following the clone names indicate the number of clones recovered from the C:T libraries.

3.3.3 Quantitative PCR

The examination and comparison of clone frequency values for single sequences and clusters of closely related sequences was the basis for qPCR target selection. *L. johnsonii* and *S. alactolyticus* (represented by cl26_cn1 and cl98_cn1 respectively) were selected as qPCR targets since they were only recovered from the T library. *E. coli* was selected as a target since 124 Enterobacteriaceae-like sequences were recovered from T, while only 19 were recovered from C. *L. amylovorus* and the L10 group were selected because a higher proportion of Lactobacillales-like sequences were recovered from C than T. The L1 and L2 groups were both recovered at a higher frequency in the C library and were therefore also selected as qPCR targets.

L10 genomes were abundant in ileal contents of pigs irrespective of diet composition. Mean L10 genomes detected for all diets was 10.6 $\log_{10}$ per gram of intestinal contents as illustrated in Figure 3.5. Detection of L10 group bacteria was highest in intestinal contents from pigs fed flaxseed oil diets, and lowest in pigs fed whole flax, flaxseed hulls, and control diets ($P < 0.05$).

The mean number of *L. amylovorus* genomes recovered across all diets was 9.00 $\log_{10}$ genomes per g of intestinal contents (Figure 3.6). Pigs fed diets containing flaxseed hulls had the lowest number of *L. amylovorus* genomes. Feeding flaxseed hull diets lowered the number of *L. amylovorus* genomes by 25% compared to the control diet ($P < 0.05$), 23.6% compared to the whole flaxseed diet ($P < 0.05$), 24% compared to the flaxseed oil diet ($P < 0.05$), and 25% compared to the tylosin diet ($P < 0.05$).

The average number of *L. johnsonii* genomes recovered across all diets was 5.94 $\log_{10}$ per g of intestinal contents (Figure 3.7). The highest level of *L. johnsonii* detection was in pigs fed tylosin and whole flaxseed diets. Tylosin treatment resulted in a 17.2% increase in *L. johnsonii* detection compared to the control ($P < 0.05$). Whole flaxseed inclusion resulted in a 12%

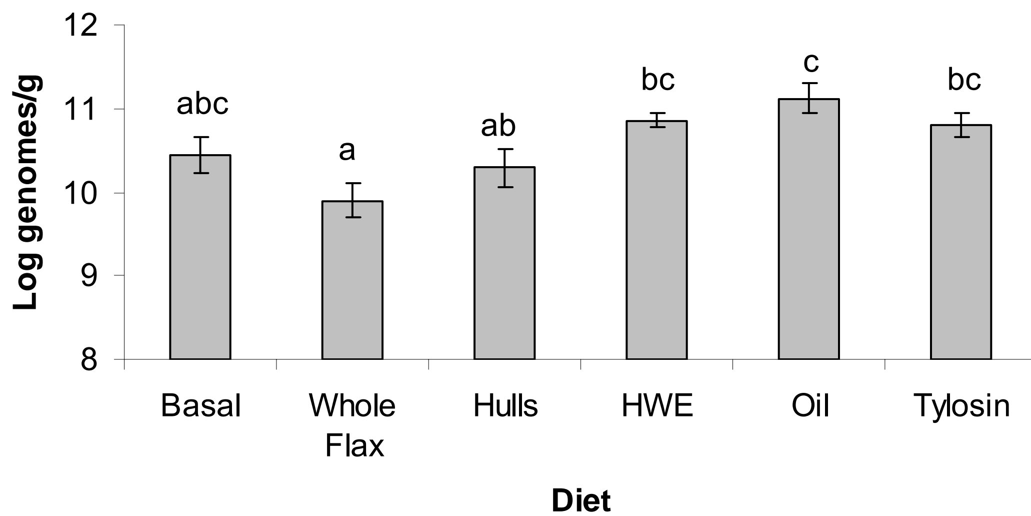


Figure 3.5 Mean (±SE) number of L10 group genomes detected in ileal contents of pigs fed diets with and without tylosin phosphate, flaxseed or flaxseed fractions. Columns with different letters are significantly different (*P* <0.05). Pooled SEM is 0.17.

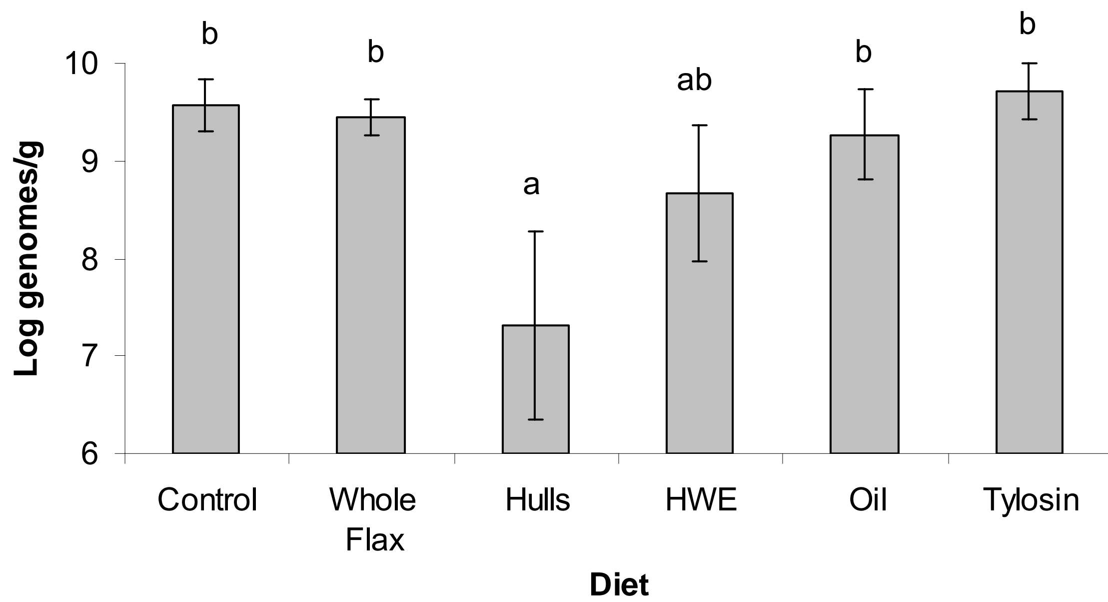


Figure 3.6 Mean (±SE) number of *L. amylovorus* genomes detected in ileal contents of pigs fed diets with and without tylosin phosphate, flaxseed or flaxseed fractions. Columns with different letters are significantly different ($P < 0.05$). Pooled SEM is 0.53.

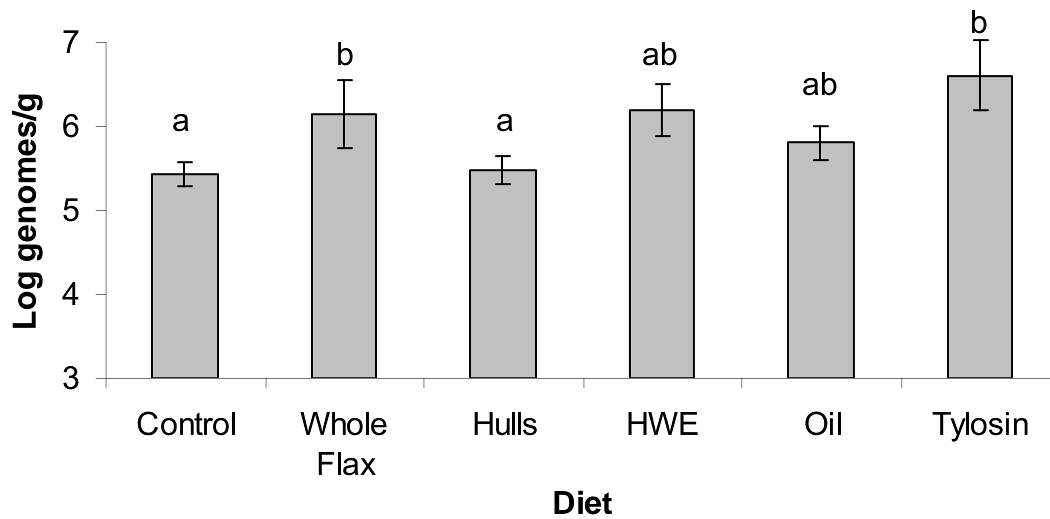


Figure 3.7 Mean (±SE) number of *L. johnsonii* genomes detected in ileal contents of pigs fed diets with and without tylosin phosphate, flaxseed or flaxseed fractions. Columns with different letters are significantly different ($P < 0.05$). Pooled SEM is 0.27.

increase ($P < 0.05$). There was a significant diet × period interaction, where the greatest increase in *L. johnsonii* detection in whole ground flaxseed and tylosin diets occurred in periods one and two (Figure 3.8).

The average number of L1, L2 and B1 group genomes recovered across all diets was 7.87, 7.45, and 7.39 $\log_{10}$ per g of intestinal contents, respectively (Table 3.6). *S. alactolyticus, E. coli,* and *C. perfringens* genomes were detected at 7.12, 7.32, and 4.55 $\log_{10}$ per g of intestinal contents, respectively. The inclusion of flaxseed, its fractions or tylosin had no impact on the level of detection of these target groups or species ($P > 0.05$).

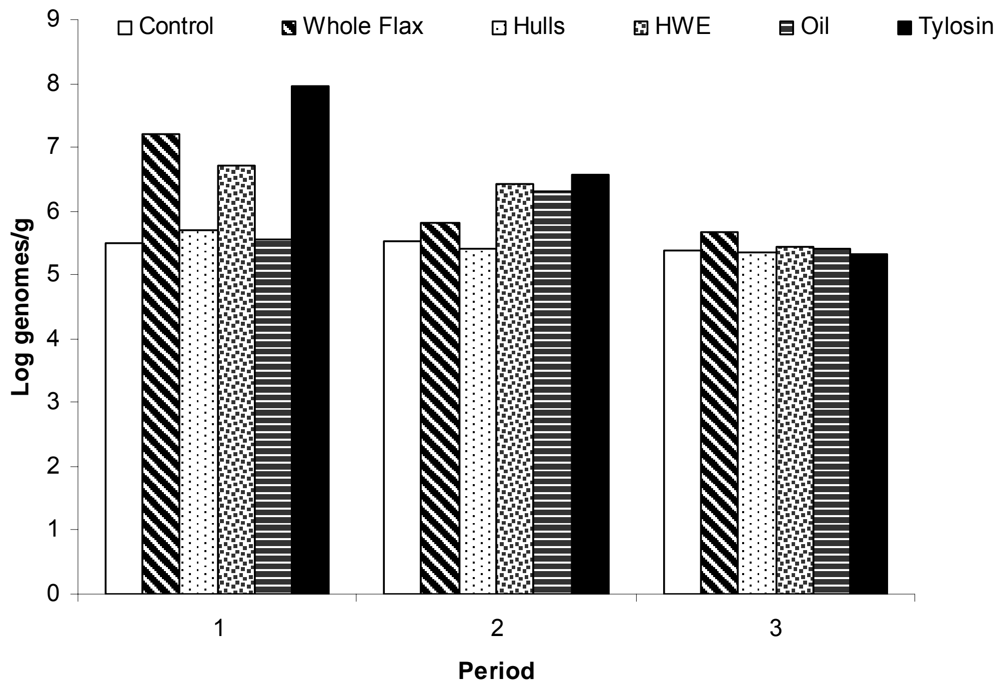


Figure 3.8 Mean number of *L. johnsonii* genomes detected in each period in ileal contents of pigs fed diets with and without tylosin phosphate, flaxseed or flaxseed fractions. Diet × period interaction, $P < 0.05$.

Table 3.6 Copy number (log no. of genomes) of target bacteria in one gram of ileal contents from pigs fed diets with and without tylosin phosphate, flaxseed or flaxseed fractions as determined by qPCR

Target	**Control**	**Whole Flax**	**Hulls**	**HWE**	**Oil**	**Tylosin**	**Pooled SEM**	***P*-value**
B1[1]	7.85	7.62	7.52	7.68	6.98	6.68	0.30	0.054
L1[2]	7.83	7.84	7.94	7.76	7.93	7.92	0.13	0.646
L2[3]	7.48	7.29	7.57	7.79	7.55	7.04	0.20	0.180
S. alactolyticus	6.93	7.21	7.43	7.33	6.64	7.19	0.37	0.728
E. coli	7.29	7.44	7.42	7.14	7.09	7.53	0.21	0.684
C. perfringens	4.52	4.26	4.96	4.48	4.27	4.82	0.21	0.100

[1] Represents C_cl33_cn1, 74% similarity to *Bacillus thuringiensis* var. *morrisoni* HD518 in Appendix 1
[2] Represents C_cl7_cn14, 89.7% similarity to *Lactobacillus delbrueckii* subsp. *lactis* ATCC12315/NCC946 in Appendix 1
[3] Represents C_cl5_cn5, 88.9% similarity to *Lactobacillus vaginalis* ATCC49540 in Appendix 1

3.4 Discussion

Small intestinal microbial communities have not been well characterised in animals despite their potential to compete with the host animal for nutrients and affect critical host responses such as enterocyte turnover, and mucus production (Gaskins, 2001). The goal of the current study was to determine how the microbial communities present in the pig ileum are affected by the AGP, tylosin phosphate, and to compare this response with dietary inclusion of flaxseed or flaxseed fractions as a method of identifying flaxseed components with potentially beneficial microbial attributes. Until recently, identifying microorganisms required their isolation in pure culture, followed by testing for physiological and biochemical traits (Amann, 1995). These traditional cultivation methods are limited and biased because only bacteria with known nutritional and growth requirements are culturable. Microbiologists have frequently observed that most microscopically visible bacterial cells are viable, but do not form colonies on selective media; subsequently, it has been estimated that only 0.001 to 15% of the microorganisms present in a population can be cultured in the laboratory (Amann, 1995). Cultivation techniques are also tedious, labour intensive, and lack precision.

To overcome limitations associated with culture-based methods, molecular-based approaches to profiling microbial communities are increasingly being used to improve the understanding of intestinal host-microbial interactions (Vaughan, 2000). Here, we used a combination of traditional microbial culture, high-throughput sequencing of *cpn*60 PCR product libraries, and qPCR to profile and quantify the ileal microbial community of pigs fed diets with and without AGP, flaxseed and its fractions. We utilized the *cpn*60 UT as a molecular target for this purpose, since it can be amplified with universal PCR primers, the UT region (549-567 bp) is easily sequenced, and generally contains sufficient phylogenetic information to differentiate closely related species, subspecies and serotypes more effectively than 16S rRNA sequences

(Brousseau et al., 2001; Goh et al., 1996; 1997; 1998; 2000; Hill et al., 2005). Tylosin phosphate was selected for study since it is commonly used in swine diets for growth promotion. The ileal contents were studied because microbial populations resident in the small intestine have the most potential to compete with the host for nutrients, and limit digestive and absorptive processes by affecting enterocyte physiology (Gaskins, 2001).

In any survey of a microbial community, including a molecular-based survey, significant effort is required to ensure adequate representation of all members of the community. For *cpn*60 clone sequence library construction, we used an established combination of physical, chemical and enzymatic steps (Dumonceaux et al., 2006) to ensure broad species representation in genomic DNA preparations from ileal contents. We also employed a mixture of universal PCR primers that have been shown to improve template representation in *cpn*60 PCR product libraries generated from complex templates such as those generated on DNA extraction from environmental samples (Hill et al., 2006). Under-representation of high G + C organisms such as *Bifidobacterium* spp., which are known to be present in porcine faeces in significant numbers (Estrada, 2001), has been criticism of both 16S rDNA and *cpn*60 based libraries (Hill et al., 2006; Hill et al., 2002; Leser et al., 2002). The clone libraries reported here contain sequences representative of the *Bifidobacterium* genus suggesting a more comprehensive library representation than our previous *cpn*60-based library studies. However, the overall taxonomic composition of these libraries is consistent with previous surveys of ileal microflora of pigs fed corn, wheat and barley diets (Hill et al., 2005).

Tylosin: impact on microbial composition and homogeneity

Examination of C and T *cpn*60 sequence library composition revealed that dietary inclusion of tylosin for 16-17 d altered the overall taxonomic composition of ileal microbial flora and increased its homogeneity. Studies that have examined the impact of tylosin or other AGP

on homogeneity of intestinal microbial populations in pigs are relatively few in number. Collier et al (2003b) reported that pigs fed tylosin had fewer PCR-DGGE bands than control pigs after d 14 of treatment, indicating that the antibiotic treatment increased the homogeneity of ileal microbial populations. However, after d 21 tylosin-fed pigs were not different from controls in terms of homogeneity (Collier et al., 2003b), suggesting that the impact of tylosin treatment on microbial diversity is not continued over long periods and thus may not contribute to the long term growth promoting response. Using PCR-DGGE to examine shifts in mouse intestinal microbiota caused by 14 d of cefoxitin treatment (a broad-spectrum cephalosporin antimicrobial), McCracken et al. (2001) found that overall microbial diversity was unaffected by the treatment, but that different bands were present in samples of cefoxitin-treated mice compared to controls. Although we found an increase in microbial homogeneity with tylosin treatment, which is contrary to the mouse study, we also observed low levels of similarity between C and T libraries where only 8 of the different cloned sequences were shared between these treatments. Interestingly, all 8 of the different, shared sequences were 99-100% similar to *L. amylovorus* ATCC33198, the dominant species identified in pig ileum here and previously (Hill et al. 2006). A similar relatively low level of identity was noted between *cpn*60 UT sequences recovered in libraries prepared from ileal contents of pigs fed corn, wheat or barley (Hill et al. 2005). This occurs despite high similarity in the taxonomic groups represented in each library at the genus and species level and indicates considerable genetic variation in the intestinal microbiota at the species and subspecies levels.

Tylosin: impact on Enterobacteriaceae

Tylosin phosphate is a macrolide antibiotic that inhibits protein synthesis in Gram-positive bacteria (Collier et al., 2003b) and as such the greater abundance of Gram-negative Enterobacteriaceae-like clones discovered in the T library was not surprising. Interestingly,

qPCR enumeration of *E. coli* genomes, and coliform plate counts did not reveal the same increase in this species with tylosin inclusion. Due to a lack of *cpn*60 UT sequence diversity in the Enterobacteriaceae taxon, we were unable to design specific PCR primers to enumerate the specific sub-group of *E. coli*-related organisms that were increased by tylosin inclusion. The *E. coli* primers utilized here are 100% complementary to annealing sites of five reference strains of *E. coli* and 3 reference strains of *Shigella flexneri*. In addition, the primers show between 95-100% nt similarity at the annealing sites to nearly all Enterobacteriaceae organisms recovered from both the C and T libraries, and thus likely amplify most members of this group. The *cpn*60 UT sequences for *Escherichia* and *Shigella* are between 98-100% identical to each other, limiting the opportunity to differentiate even between these genera let alone a specific sub-group of *E. coli*. Therefore, the primers used here were not able to specifically detect the sequences responsible for the observed difference in library clone frequency. It could be speculated that the high frequency of *E. coli* sub-group sequences recovered in the T library represents selection for a phenotype most capable of occupying a new environmental niche created by dietary tylosin phosphate inclusion.

Our clone libraries were constructed from a pool of DNA extracted from ileal contents of all representatives of the C and T diets, while the qPCR analyses were performed on DNA samples from individual pigs fed each of the experimental diets during all three periods. Thus, some dissimilarity in the DNA template represented did occur. For example, qPCR analysis of *E. coli* genomes in the same pool used for library construction detected 7.94 $\log_{10}$ *cpn*60 UT copies in the diet C pool, and 8.83 $\log_{10}$ copies in T, which correlates well to the clone frequency data. However, qPCR analysis of individual pig samples (not pooled by diet) did not reveal similar treatment differences, demonstrating how errors associated with sub-sampling and pooling of DNA extracts remains a limitation of molecular techniques, since particular experimental groups

or individual animals can easily be misrepresented. By pooling DNA from digesta samples collected over two days, thorough mixing of both digesta and DNA extracts, and extracting DNA from 1.2 g of ileal contents for subsequent analyses, we were able to ensure that individual samples were as representative as possible without becoming cost and time prohibitive.

Tylosin: impact on Lactobacillales

Lactobacilli counts on MRS agar were higher for tylosin-treated pigs compared to the control, while library sequence analysis showed that tylosin-treated pigs had a lower percentage of clones from the Lactobacillales taxon. This difference although initially appearing contradictory likely reflects one of the limitations of culture-based enumeration, since the specificity of MRS media is likely not limited to lactobacilli nor does it allow for growth of all members of the Lactobacillales taxon. Hartemink and Rombouts (1999) concluded that 95% of colonies on MRS that were examined microscopically did not have conformations typical of lactobacilli (straight, non-spore-forming rods), and instead resembled bifid shaped bacteria, streptococci, yeasts, spore-forming anaerobes and *Megasphera* species. Compared to Rogosa and LAMVAB media, MRS consistently supported the highest number of cfu per g, demonstrating its reduced selectivity (Hartemink and Rombouts, 1999). The higher plate counts observed in the present study reflect an increase in the lactobacilli that grow on MRS, while the clone frequency data suggested that tylosin treatment reduced the number of Lactobacillales-like clones as a percentage of the represented *cpn*60 gene segments. Thus, it is inappropriate to compare absolute viable counts to clone frequency data since the approaches enumerate widely different parameters. The phenotypic criteria used for microbial characterisation, such as growth on selective media, do not always enable phylogenetic identification, which is a disadvantage of culture-based enumeration methods. Interestingly, the ATCC lactobacilli reference strains contained in cpnDB are mostly strains that grow on MRS, while 13% of the Lactobacillales-like

clones recovered here are only 78-96% similar to a reference lactobacillus, and may not thrive on MRS, possibly accounting for the discrepancy.

Collier et al. (2003a) reported an increase in the percentage of members of the *Lactobacillus* genus, as measured by qPCR, after d 14 and continuing until d 35 in tylosin-treated pigs. This is contrary to our library data, where a lower percentage of Lactobacillales-like clones were recovered from the T library than the C library, but not surprising since the parameters being measured are not equivalent (genus vs. entire taxon). We also reported no differences in *L. amylovorus* or L10 group genome detection in tylosin treated pigs compared to the control. Ileal samples for the current study were collected after 16 and 17 d of tylosin treatment, so a similar increase in percent of lactobacilli could be expected. The discrepancy may be due to differences in DNA template utilised for analysis (pools vs. individual DNA samples), as discussed previously. Knarreborg et al. (2002) used PCR-DGGE and viable counts to elucidate bacterial community changes in the ileum of broiler chickens fed a combination of avilamycin (everninomicin class antimicrobial that targets Gram-positive bacteria) and salinomycin (coccidiostat) or different fat sources. They found that lactobacilli were decreased by antimicrobial treatments on d 14 and 21, but not on d 35, and *C. perfringens* counts and PCR-DGGE bands were additively reduced by soy oil and avilamycin treatment (Knarreborg et al., 2002). Tylosin administration reduced the concentration of *C. perfringens* and improved intestinal barrier function in a chick model of necrotic enteritis (Collier et al., 2003a). Our clone frequency data agree with Knarreborg study, since we also showed a decrease in the proportion of Lactobacillales-like sequences after 16 d of tylosin treatment. However, our qPCR analysis of *C. perfringens* did not demonstrate its decrease with tylosin treatment, likely due to the increased level of sensitivity gained through qPCR compared to DGGE, which has a threshold detection limit of only 10^7 to 10^8 cfu per g (Simpson et al., 1999).

Hill et al. (2005) reported that qPCR analysis confirmed the abundances of taxa that were targeted based on the library sequencing approach, although for some targets 5.4 $\log_{10}$ genomes were detected by qPCR when the frequency of corresponding clones was zero. We had similar findings, where *L. johnsonii* was detected at 5.43 $\log_{10}$ genomes per g in the C diet and the clone frequency was zero, confirming that clone frequency data can provide a useful indication of relative taxa abundances for abundant bacteria, but are less useful for bacteria present in smaller numbers. Our data also support our previous findings indicating that the limit of detection for a 1000 clone *cpn*60-based library is near 10^5 genomes per g (Hill et al., 2005).

The current study revealed an increase in *L. johnsonii* with tylosin treatment compared to the control diet, although there was a diet × period interaction for *L. johnsonii* detection. In periods 1 and 2, *L. johnsonii* detection was highest in tylosin-treated pigs, but in period 3, tylosin-treated pigs were not different from the control, and pigs fed whole flaxseed had the highest levels of *L. johnsonii*. This discrepancy is likely a product of substantial individual variation amongst pigs, and is also reflective of natural changes occurring in the intestinal microflora as the pig ages and the adult microflora is established. Antibiotics may affect the sequence of bacterial succession by selecting for specific bacterial populations (Swords, 1993), so theoretically, the effects of tylosin administration may continue beyond the termination of treatment. However, although the effects of tylosin on *L. johnsonii* decreased with time, the trend remained and the limited number of observations in each period prevents any definitive conclusions to be made regarding possible carry-over effect. The change-over design employed in this experiment assumes that no effects from one dietary treatment are 'carried over' into the following period. Acclimatization periods less than 15 days in duration have been used successfully in previous digestibility experiments (Diebold et al., 2005). In an experiment where five-wk old barrows were continuously fed tylosin for 42 d, Collier et al (2003b) found that for the first 14 d of

treatment the number of bacterial species detected as indicated by PCR-DGGE bands was decreased. However, by 21 d of tylosin treatment, the number of bands was not different from control pigs. This indicates that the bacterial population recovered from tylosin administration between 14 and 21 d following the start of treatment, and contributed to our rationale for employing the change-over design in the current study.

Tylosin: mode of action

The inclusion of tylosin in the diet may have resulted in the exclusion of sensitive species, which could allow for secondary species to gain a competitive advantage. Of the species targeted in this study, *L. johnsonii* was the only organism to be increased in the presence of tylosin as determined by qPCR and clone frequency data. *L. johnsonii*, a close relative of *L. gasseri* (96.6% similarity between *cpn*60 UT regions), has been studied as a potential probiotic because of its immune system modulation (Haller et al., 2000; Ibnou-Zekri et al., 2003), pathogen inhibition (Bernet et al., 1994), and epithelial cell attachment abilities (Granato et al., 1999). *L. johnsonii* has been used successfully as a competitive exclusion agent to control *C. perfringens* in 20-d old specific pathogen free chicks (La Ragione et al., 2004). *L. johnsonii* lacks genes for the biosynthesis of amino acids, purine nucleotides and cofactors (Pridmore, 2004). Instead, it has many transporters, peptidases and proteases, making it well suited to the small intestine where the environment is rich in free amino acids and peptides, and also suggesting a strong dependency on the host or other intestinal bacteria for nutrition. Since it lacks enzymes for carbohydrate breakdown, *L. johnsonii* would likely not survive competition with other bacteria in the colon that possess these enzymes (Pridmore, 2004). *L. johnsonii* also has genes encoding surface molecules like mucus-binding proteins, IgA protease, and glycosylated fimbriae that may assist its binding to mucosal surfaces and modulation of the intestinal immune system (Pridmore, 2004). Its survival in the gut is further enhanced by three bile salt hydrolase genes, a unique bile

transporter, and genes that encode bacteriocins (Pridmore, 2004). Considering these physiological details, it is apparent that *L. johnsonii* is highly suited to a very specific niche in the small intestine, and relies heavily on other bacteria and the host's diet for survival. The gut environment conditions created with tylosin treatment seem favourable to 'opening up' this niche, perhaps by eliminating species that would normally out-compete *L. johnsonii.* Collier et al. (2003b) found that *L. gasseri* was increased in the presence of tylosin. Although a similar increase was not observed in this study (*L. gasseri* was below the limit of detection), it is very closely related to *L. johnsonii*, and could occupy a very similar niche in the small intestine.

Patterson et al. (2005) found that tylosin treated mice had higher average daily gain, higher haemolytic bacteria populations in the caecum and colon, and lower numbers of lumen and adherent lactic acid bacteria in the ileum, caecum, colon and faeces compared to control mice. The authors suggested that while the population of beneficial lactobacilli was decreased, there was likely sufficient "critical mass" remaining to provide benefit to the host. Based on the above study, and other research (Feighner, 1987; Tannock, 1989) that has implicated lactobacilli as the primary contributors to bile salt hydrolase activity and have shown that AGPs can reduce this activity, it is possible that the growth promoting effects of tylosin may be achieved by reducing the lactobacilli populations sufficiently to reduce bile salt hydrolase activity, but maintaining this dominant group to competitively exclude opportunistic pathogens. Bile-salt hydrolase-producing bacteria are able to deconjugate bile acids resulting in free bile acids that are toxic to enterocytes (Tannock, 1989). Free bile acids also impair protein, carbohydrate, and especially lipid absorption, since the reduced conjugated bile acid molecule concentrations could limit micelle formation. Since *L. johnsonii* is highly dependent on the host's diet for nutrition, high colonization levels may lead to competition for amino acids resulting in potentially negative effects on the host. Lactobacilli also compete directly with the host for glucose to produce lactic

acid, which increases intestinal motility and thus reduces nutrient utilization via an increase in digesta passage rate (Gaskins, 2001; Saunders, 1982).

Generally, a more diverse (or less homogeneous) ecosystem is considered more stable and therefore more desirable (Backhed et al., 2005). This can be explained by the "insurance hypothesis", which assumes that a biodiverse ecosystem is more resilient to stress because it has many species filling similar ecological roles and therefore a broader selection of stress responses (Yachi and Loreau, 1999). This hypothesis is supported by studies of humans suffering from active inflammatory bowel disease, where microbial diversity (measured by number of DGGE bands) was reduced by 50% in Crohn's patients and by 30% in those with ulcerative colitis compared to non-inflammatory controls (Ott, 2004). In piglets fed parenterally, microbial diversity was reduced and susceptibility to *C. perfringens* colonization was increased (Deplancke et al., 2002). During weaning, the piglet's microbial ecosystem changes from a being a simple, unstable community into a complex and stable one. It is during this stressful transition period that piglets are most susceptible to intestinal disease. These examples illustrate how less diverse intestinal ecosystems are more susceptible to internal and external stressors.

If a more diverse, less homogeneous intestinal ecosystem is considered more stable and better able to respond to stress, it seems counterintuitive that AGP treatment, which is designed to improve intestinal health and growth performance, actually reduces microbial diversity. It is possible that the presence of tylosin promotes a gut environment where stability is allowed despite the reduction in diversity, thus the combination of stability and low diversity is beneficial to the host. Alternatively, subtherapeutic tylosin treatment may promote a less diverse intestinal ecosystem composed of highly adapted species that are capable of performing a wide variety of tasks and stress responses. Thus, fewer species exist, but they effectively occupy the majority of available niches and therefore prevent colonization by opportunistic pathogens under the

conditions supported by AGP treatment. *L. johnsonii* is a good example of a highly adapted species promoted by tylosin, since it is able to competitively exclude and inhibit pathogens and attach to intestinal epithelial cells, as previously discussed. The *cpn*60 clone library approach used here may not provide a true representation of microbial diversity, since only species present at approximately 10^5 genomes per g are detected in a library of this size. However, our libraries do illustrate the microbial diversity present among the most predominant and functionally relevant species in the ileum.

It has been suggested that piglets with a higher lactobacillus: enterobacteria or *E. coli* ratio may experience improved growth and performance, and are more resistant to intestinal disorders (Demeckova et al., 2002; Demeckova, 2003; Muralidhara et al., 1977). This idea stems from research indicating that the growth of enterotoxigenic *E. coli* species is inhibited by lactic acid bacteria (Jin, 2000). The Lactobacillales: Enterobacteriaceae ratio for the T library was much lower than the C library (6:1 compared to 35:1 respectively), indicating that, in this case, tylosin does not seem to promote an appropriate "healthy" balance between lactic acid bacteria and coliforms, or alternatively, it does not function to promote growth by specifically altering this ratio.

Flaxseed and fractions

The second objective of the current study was to determine the effect of flaxseed and its fractions on small intestinal microbial population in pigs. By comparison with the effect of tylosin, a beneficial microbial response may be identified. The inclusion rates for each of the flaxseed fractions were selected based on the proportion they contribute to the whole seed, with 20% whole flaxseed as the maximum inclusion rate to avoid negatively impacting feed intake. Flaxseed fractions were studied in addition to whole flax to more accurately elucidate which chemical component of the seed impacted microbial growth. For *cpn*60 UT libraries with 1000

clones, we have found that the frequency of clone recovery in each library correlates well with organism abundance as determined by qPCR (Hill et al., 2005). Therefore, we used clone recovery frequency data from the C and T libraries as a selection tool to identify specific organisms for qPCR enumeration in individual pigs fed diets containing flaxseed or flaxseed fractions. This approach enabled comparison of colonization profiles for pigs fed flaxseed and tylosin-treated pigs.

The inclusion of whole flaxseed, flaxseed hulls, and oil resulted in lactobacilli counts similar to those for tylosin. Interestingly, of all the experimental subjects, pigs fed the hull diet had the lowest level of *L. amylovorus* genome detection by qPCR, but the highest viable counts for lactobacillus on MRS. Clones with *L. amylovorus* as their nearest neighbour were the most abundantly recovered sequences from both the C and T libraries, indicating its importance as the most predominant lactobacillus in the porcine gut, similar to findings by Hill et al (2005). Alzueta et al. (2003) found the broilers fed whole flaxseed had higher viable counts for lactobacilli compared to those fed demucilaged flaxseed, suggesting that the mucilage (which is contained mostly in the hull) caused the increase. Our viable plate counts agree with these findings, since the hulls alone caused a greater increase in lactobacilli than whole flax compared to the control or HWE flaxseed.

The apparent discrepancy between viable plate count and qPCR data for lactobacilli numbers in pigs fed flaxseed hulls may be explained by a closer examination of which bacterial species thrive on MRS media. As discussed previously, a study that compared media used to detect lactobacilli in pig ileum contents found that the vast majority of the colonies from MRS media that were examined microscopically were not typical of lactobacilli conformation (Hartemink and Rombouts, 1999). Therefore, it is likely that the colonies counted on MRS agar in this study were not all lactobacilli. This is particularly true for the hull diet, which appears to

support a population of bacteria that thrive on MRS, but are likely not all closely related to *L. amylovorus*.

The inclusion of whole flaxseed, HWE flax and or flaxseed oil resulted in an increase in *L. johnsonii* similar to the observed increase in tylosin-treated pigs. Whole flax is 21.8% protein, 44.2% oil (DeClercq, 2005), a very rich source of α-linolenic acid, and contains a high content of mucilaginous water-soluble polysaccharides (Bhatty, 1995). Pigs fed diets containing added whole ground flaxseed, HWE flaxseed, or flaxseed oil would have a greater concentration of α-linolenic acid and soluble fibre entering the small intestine than pigs fed the control diet. *L. johnsonii* relies heavily on the host's diet and other bacteria for amino acids and carbohydrates, which would be readily available in the small intestine following the catabolism of the WF, HWE or O diets, and also present in higher concentrations than in the control diet. It is likely that the protein and oil components benefited *L. johnsonii* colonization most significantly, since the hull fraction does not appear to promote *L. johnsonii* growth. The HWE flax fraction had very similar colonization levels to whole flax, and the oil was intermediate.

There is sufficient evidence to indicate that the fatty acid and water-soluble polysaccharide fractions of flaxseed could exhibit antibacterial effects on intestinal microbial populations, or promote a healthy gut environment by acting as substrates for certain commensal bacterial species, like *L. johnsonii* or *L. amylovorus*. Unsaturated fatty acids such as α-linolenic acid have been shown to exhibit antibacterial properties (Kodicek, 1945). Indeed, high concentrations of free α-linolenic acid inhibited the growth of bacteria *in vitro*, and altered bacterial adhesion sites on Caco-2 cells (Kankaanpaa et al., 2001). In arctic charr, feeding linolenic acid promoted colonization by lactic acid bacteria, which are considered beneficial residents of the fish intestinal ecosystem (Ringø, 1998). High concentrations of α-linolenic acid may alter the attachment sites

for intestinal bacteria by altering the fatty acid composition of the intestinal wall (Kankaanpaa et al., 2001; Ringø, 1998).

The water soluble fibre present in flaxseed consists of acidic and neutral fractions (Cui, 1994; Fedeniuk, 1994). The acidic fraction consists of mainly pectic-like polysaccharides, containing L-rhamnose, D-galactose, and D-galacturonic acid, while the neutral fraction is mostly a β-D-(1,4)-xylan backbone of arabinoxylan component (Cui, 1994). *Bifidobacterium* spp. and *Bacteroides* spp. are able to utilize oligosaccharides such as arabinoxylans effectively (van Laere, 2000). Swine faecal microflora are able to ferment a wide array of galactooligosaccharides including stachyose and raffinose effectively (Smiricky-Tjardes et al., 2003). Oligogalacturonic acids, or dietary fibre pectins present in flax, are effectively degraded by pure cultures of *B. thetaiotaomicron* (Dongowski et al., 2000). This is of particular interest because *B. thetaiotaomicron* has recently been found to selectively induce the expression of *ang4*, a protein that is secreted by paneth cells into the gut lumen where it exhibits bactericidal activity against intestinal microbes in mice (Hooper, 2003). *B. thetaiotaomicron* may also activate an anti-inflammatory mechanism in the gut (Kelly et al., 2004). It would be interesting to determine in the future if flaxseed or its fractions indeed promote *B. thetaiotaomicron* colonization in faecal microflora.

In this study, the ratio between lactobacillus and coliform viable counts was highest in pigs fed diets containing whole ground flaxseed compared to the control or tylosin diets. Pigs fed HWE, hulls and oil had intermediate lactobacillus: coliform ratios. This may suggest that whole flaxseed, and possibly its fractions, promote a healthier balance in the microbial population than pigs fed tylosin.

3.5 Conclusion

Inclusion of tylosin phosphate in the diet of growing pigs at the level intended for growth promotion resulted in a reduction in microbial diversity, an increase in *L. johnsonii* genomes detected and colonies on MRS agar, and reduced the Lactobacillales:Enterobacteriaceae ratio compared to the control diet. No single flaxseed fraction promoted a microbial response that was identical to tylosin. However, whole flaxseed, HWE flaxseed and the oil fraction were each responsible for significantly altering at least one of the parameters measured. Whole ground flaxseed most closely mirrored the effects on intestinal microbial colonization achieved with tylosin inclusion, especially increasing *L. johnsonii* genome detection, although a more favourable lactobacilli: enterobacteria ratio was observed for pigs fed whole flaxseed compared to the tylosin diet. Response to the HWE flaxseed diet was similar to whole flaxseed, although lactobacilli:enterobacteria ratios and *L. johnsonii* genome detection were lower than whole flax. Pigs fed diets containing flaxseed oil had high colonization by *L. amylovorus* and L10 group bacteria, but *L. johnsonii* was not selectively increased. The flaxseed hull diet imparted the least response among the measured parameters, although it supported a unique population of lactobacilli that thrived on MRS media, but were not detectable by qPCR enumeration of *L. amylovorus*.

A more detailed investigation of whole or HWE flaxseed's impact on microbial homogeneity in the pig ileum may provide further support for their use in swine diets to reduce AGP usage, since data presented here suggest that tylosin's mode of action relates to its ability to homogenize the microbial population and select for highly adapted microbial species that outcompete pathogens and have good IEC attachment, such as *L. johnsonii*. Limitations in the cross-over study design, the relatively short sampling period and lack of pig performance data prevent definitive conclusions from being made regarding the ideal flaxseed fraction to serve as a

replacement for AGP in swine diets. It would be interesting to investigate the longer-term effects of dietary flaxseed compared to AGP on growth performance and incidence of intestinal disease in grower and finisher pigs in a large-scale commercial study. Overall, ileal microbial profiles of growing pigs were similar and remained mostly unaffected by dietary tylosin or flaxseed inclusion, indicating the importance of host factors in establishing microbial colonization patterns. However, considering the numerous potential human health benefits realized with the consumption of pork enriched with omega-3 fatty acids, and the positive impact of whole and HWE extracted flaxseed shown here on intestinal populations in pigs, further investigation of flaxseed usage in swine diets is certainly warranted.

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5. APPENDIX A

Table A1. Names, nearest reference sequences and frequency of recovery for all sequences

			Frequency (number of clones)		
Clone Name	**Nearest cpn60 Neighbour**	**% DNA Identity**	**C**	**T**	**Total clones**
C_cl0_cn1	*Lactobacillus amylovorus* ATCC33198	92.210	1	0	1
C_cl0_cn10	*Lactobacillus amylovorus* ATCC33198	99.096	1	0	1
C_cl0_cn11	*Lactobacillus amylovorus* ATCC33198	99.457	2	0	2
C_cl0_cn12	*Lactobacillus amylovorus* ATCC33198	92.935	2	2	4
C_cl0_cn13	*Lactobacillus amylovorus* ATCC33198	99.457	2	0	2
C_cl0_cn14	*Lactobacillus amylovorus* ATCC33198	99.457	2	0	2
C_cl0_cn15	*Lactobacillus amylovorus* ATCC33198	99.275	2	0	2
C_cl0_cn16	*Lactobacillus amylovorus* ATCC33198	99.819	2	0	2
C_cl0_cn17	*Lactobacillus amylovorus* ATCC33198	99.457	2	0	2
C_cl0_cn18	*Lactobacillus amylovorus* ATCC33198	91.848	2	0	2
C_cl0_cn19	*Lactobacillus amylovorus* ATCC33198	93.116	2	0	2
C_cl0_cn20	*Lactobacillus curvatus* Ingeldew	99.638	2	0	2
C_cl0_cn21	*Lactobacillus amylovorus* ATCC33198	99.457	2	0	2
C_cl0_cn22	*Lactobacillus amylovorus* ATCC33198	99.638	53	42	95
C_cl0_cn23	*Lactobacillus amylovorus* ATCC33198	99.457	3	0	3
C_cl0_cn24	*Lactobacillus amylovorus* ATCC33198	94.928	4	0	4
C_cl0_cn25	*Lactobacillus amylovorus* ATCC33198	99.457	4	0	4
C_cl0_cn26	*Lactobacillus amylovorus* ATCC33198	99.819	223	55	278
C_cl0_cn27	*Lactobacillus amylovorus* ATCC33198	99.819	8	10	18
C_cl0_cn29	*Lactobacillus amylovorus* ATCC33198	100.00	198	490	688
C_cl0_cn3	*Lactobacillus amylovorus* ATCC33198	98.551	1	0	1
C_cl0_cn32	*Lactobacillus amylovorus* ATCC33198	99.638	12	11	23
C_cl0_cn34	*Lactobacillus amylovorus* ATCC33198	99.457	15	14	29
C_cl0_cn35	*Lactobacillus amylovorus* ATCC33198	99.819	26	44	70
C_cl0_cn4	*Lactobacillus amylovorus* ATCC33198	99.819	1	0	1
C_cl0_cn5	*Lactobacillus amylovorus* ATCC33198	92.391	1	0	1
C_cl0_cn6	*Lactobacillus amylovorus* ATCC33198	94.746	1	0	1
C_cl0_cn8	*Lactobacillus amylovorus* ATCC33198	99.275	1	0	1
C_cl0_cn9	*Lactobacillus amylovorus* ATCC33198	93.297	1	0	1
C_cl1014_cn0	*Lactobacillus amylovorus* ATCC33198	84.964	1	0	1

C_cl132_cn0	*Lactobacillus amylovorus* ATCC33198	91.304	1	0	1
C_cl200_cn0	*Lactobacillus ingluviei* LMG 20380	80.399	1	0	1
C_cl224_cn1	*Eubacterium ventriosum* ATCC27560	72.694	7	0	7
C_cl33_cn1	*Bacillus thuringiensis* var. morrisoni HD518	73.913	5	0	5
C_cl330_cn0	*Lactobacillus amylovorus* ATCC33198	86.980	1	0	1
C_cl335_cn1	*Eubacterium ventriosum* ATCC27560	73.418	2	0	2
C_cl346_cn0	*Lactobacillus amylovorus* ATCC33198	99.094	1	0	1
C_cl354_cn1	*Escherichia coli* ATCC35401 H10407	99.640	2	0	2
C_cl354_cn2	*Escherichia coli* O157:H7 RIMD 509952	99.099	3	0	3
C_cl354_cn3	*Escherichia coli* O157:H7 RIMD 509952	99.459	12	0	12
C_cl4_cn1	*Shigella flexneri* 2a strain 2457T	88.468	2	0	2
C_cl5_cn1	*Lactobacillus vaginalis* ATCC49540	88.043	1	0	1
C_cl5_cn2	*Lactobacillus vaginalis* ATCC49540	88.043	1	0	1
C_cl5_cn3	*Lactobacillus vaginalis* ATCC49540	88.587	1	0	1
C_cl5_cn5	*Lactobacillus vaginalis* ATCC49540	88.949	9	0	9
C_cl500_cn0	*Selenomonas ruminantium* subsp. ruminantium ATCC12561	81.193	1	0	1
C_cl58_cn1	*Megasphaera elsdenii* ATCC25940	96.558	2	0	2
C_cl6_cn1	*Lactobacillus vaginalis* ATCC49540	81.522	4	0	4
C_cl67_cn1	*Lactobacillus delbrueckii* subsp. indicus NCC665	89.312	4	0	4
C_cl698_cn0	*Lactobacillus vaginalis* ATCC49540	78.947	1	0	1
C_cl7_cn1	*Lactobacillus delbrueckii* subsp. lactis ATCC12315/NCC946	88.949	1	0	1
C_cl7_cn10	*Lactobacillus delbrueckii* subsp. lactis ATCC12315/NCC946	86.413	1	0	1
C_cl7_cn11	*Lactobacillus delbrueckii* subsp. lactis ATCC12315/NCC946	89.674	3	0	3
C_cl7_cn12	*Lactobacillus delbrueckii* subsp. bulgaricus ATCC11842/NCC641	93.297	4	0	4
C_cl7_cn13	*Lactobacillus delbrueckii* subsp. lactis ATCC12315/NCC946	87.500	4	0	4
C_cl7_cn14	*Lactobacillus delbrueckii* subsp. lactis ATCC12315/NCC946	89.674	16	0	16
C_cl7_cn15	*Lactobacillus delbrueckii* subsp. lactis ATCC12315/NCC946	89.674	31	57	88
C_cl7_cn2	*Lactobacillus delbrueckii* subsp. lactis ATCC12315/NCC946	88.949	1	0	1
C_cl7_cn3	*Lactobacillus delbrueckii* subsp. lactis ATCC12315/NCC946	88.587	1	0	1
C_cl7_cn5	*Lactobacillus delbrueckii* subsp. lactis ATCC12315/NCC946	92.935	1	0	1
C_cl7_cn7	*Lactobacillus delbrueckii* subsp. delbrueckii ATCC9649/NCC621	88.768	1	0	1
C_cl7_cn9	*Lactobacillus delbrueckii* subsp. lactis ATCC12315/NCC946	89.493	1	0	1
C_cl75_cn0	*Clostridium thermocellum* NCIMB 10682	70.430	1	0	1
C_cl820_cn0	*Clostridium scindens* ATCC35704	80.944	1	0	1
C_cl860_cn0	*Bifidobacterium thermacidophilum* subsp. suis P3-11	96.007	1	0	1
C_cl876_cn0	*Megasphaera elsdenii* ATCC25940	83.063	1	0	1

C_cl899_cn0	*Bifidobacterium boum* JCM1211	98.732	1	0	1
C_cl944_cn0	*Lactobacillus vaginalis* ATCC49540	82.790	1	0	1
T_cl0_cn1	*Escherichia coli* C175-94	99.459	0	1	1
T_cl0_cn10	*Escherichia coli* ATCC35401 H10407	99.457	0	2	2
T_cl0_cn11	*Escherichia coli* ATCC35401 H10407	98.919	0	2	2
T_cl0_cn12	*Shigella flexneri* 2a strain 2457T	99.279	0	2	2
T_cl0_cn13	*Escherichia coli* O157:H7 RIMD 509952	97.477	0	2	2
T_cl0_cn14	*Escherichia coli* C175-94	99.640	0	8	8
T_cl0_cn15	*Escherichia coli* ATCC35401 H10407	100.00	0	9	9
T_cl0_cn16	*Escherichia coli* C175-94	99.820	0	10	10
T_cl0_cn17	*Escherichia coli* C175-94	100.00	0	81	81
T_cl0_cn2	*Citrobacter freundii* ATCC8090	99.640	0	1	1
T_cl0_cn4	*Shigella flexneri* 2a strain 2457T	97.658	0	1	1
T_cl0_cn5	*Klebsiella pneumoniae* JCM1662	99.820	0	1	1
T_cl0_cn6	*Klebsiella pneumoniae* JCM1662	96.036	0	1	1
T_cl0_cn7	*Escherichia coli* C175-94	99.640	0	1	1
T_cl0_cn9	*Escherichia coli* ATCC35401 H10407	96.216	0	2	2
T_cl116_cn0	*Bifidobacterium thermacidophilum* suis P3-14	98.004	0	1	1
T_cl15_cn1	*Lactobacillus delbrueckii* subsp. lactis ATCC12315/NCC946	89.493	0	1	1
T_cl15_cn2	*Lactobacillus delbrueckii* subsp. lactis ATCC12315/NCC946	86.775	0	1	1
T_cl15_cn3	*Lactobacillus delbrueckii* subsp. delbrueckii ATCC9649/NCC621	87.138	0	2	2
T_cl15_cn4	*Lactobacillus delbrueckii* subsp. lactis ATCC12315/NCC946	88.406	0	3	3
T_cl175_cn0	*Lactobacillus amylovorus* ATCC33198	90.090	0	1	1
T_cl204_cn1	*Megasphaera elsdenii* ATCC25940	96.745	0	3	3
T_cl222_cn0	*Streptococcus alactolyticus* ATCC43077	90.450	0	1	1
T_cl26_cn1	*Streptococcus alactolyticus* ATCC43077	99.819	0	13	13
T_cl295_cn1	*Lactobacillus vaginalis* ATCC49540	80.797	0	4	4
T_cl3_cn1	*Lactobacillus amylovorus* ATCC33198	99.457	0	1	1
T_cl3_cn10	*Lactobacillus amylovorus* ATCC33198	94.022	0	1	1
T_cl3_cn11	*Lactobacillus amylovorus* ATCC33198	95.652	0	2	2
T_cl3_cn13	*Lactobacillus amylovorus* ATCC33198	92.935	0	2	2
T_cl3_cn14	*Lactobacillus amylovorus* ATCC33198	99.275	0	2	2
T_cl3_cn15	*Lactobacillus amylovorus* ATCC33198	94.022	0	3	3
T_cl3_cn16	*Lactobacillus amylovorus* ATCC33198	99.275	0	3	3
T_cl3_cn2	*Lactobacillus amylovorus* ATCC33198	94.384	0	1	1
T_cl3_cn21	*Lactobacillus amylovorus* ATCC33198	99.819	0	12	12

T_cl3_cn3	*Lactobacillus amylovorus* ATCC33198	98.913	0	1	1
T_cl3_cn4	*Lactobacillus amylovorus* ATCC33198	99.638	0	1	1
T_cl3_cn5	*Lactobacillus amylovorus* ATCC33198	98.732	0	1	1
T_cl3_cn6	*Lactobacillus amylovorus* ATCC33198	96.014	0	1	1
T_cl3_cn7	*Lactobacillus amylovorus* ATCC33198	98.175	0	1	1
T_cl3_cn8	*Lactobacillus amylovorus* ATCC33198	99.457	0	1	1
T_cl31_cn0	*Megasphaera elsdenii* ATCC25940	94.394	0	1	1
T_cl332_cn0	*Lactobacillus johnsonii* NCC 533	88.406	0	1	1
T_cl384_cn0	*Lactobacillus vaginalis* ATCC49540	80.874	0	1	1
T_cl445_cn0	*Lactobacillus vaginalis* ATCC49540	85.326	0	1	1
T_cl459_cn0	*Lactobacillus vaginalis* ATCC49540	83.906	0	1	1
T_cl462_cn0	*Megasphaera elsdenii* ATCC25940	94.746	0	1	1
T_cl523_cn0	*Lactobacillus vaginalis* ATCC49540	89.493	0	1	1
T_cl537_cn1	*Eubacterium ventriosum* ATCC27560	72.545	0	2	2
T_cl98_cn1	*Lactobacillus johnsonii* NCC 533	99.275	0	4	4

6. APPENDIX B

Table B1. Mixed panel of intestinal tract organisms for PCR primer validation

Organism	Source	Growth Medium
Genomic DNA prepared from cultured cells		
Salmonella enterica	Isolate[1]	Nutrient broth 37°C
Clostridium perfringens	chicken isolate[1]	Brain heart infusion 37°C anaerobic
Campylobacter jejuni	ATCC 33291	Preston agar
Enterobacter aerogenes	ATCC 13048	Nutrient broth 30°C
Enterobacter cloacae	ATCC 13047	Nutrient broth 30°C
Citrobacter freundii	ATCC 43864	Nutrient broth 37°C
Bifidobacterium longum	ATCC 15707	TPY broth 37°C anaerobic
Bifidobacterium animalis	ATCC 27536	TPY broth 37°C anaerobic
Enterococcus faecalis	ATCC 49332	Brain heart infusion 37°C
Enterococcus avium	ATCC 14025	Brain heart infusion 37°C
Enterococcus gallinarum	ATCC 49608	Brain heart infusion 37°C
Klebsiella pneumoniae	ATCC 13883	Nutrient broth 37°C
Lactobacillus amylovorus	ATCC 33198	Lactobacilli MRS broth 37°C
Streptococcus hyointestinalis	ATCC 49169	Brain heart infusion 37°C
Bacteroides eggerthii	ATCC 27754	Cooked meat medium 37°C anaerobic
Prevotella corporis	ATCC 33574	Cooked meat medium 37°C anaerobic
Peptostreptococcus asaccharolyticus	ATCC 14963	Cooked meat medium 37°C anaerobic
Fusobacterium perfoetens	ATCC 25556	Cooked meat medium 37°C anaerobic
Eubacterium limnosum	ATCC 8486	Cooked meat medium 37°C anaerobic
Plasmid consisting of cloned, sequenced *cpn*60 UT[2]		
Escherichia coli ETEC H10409	ATCC 35401	
Enterococcus faecium	ATCC 19434	
Streptococcus agalactiae	ATCC 2386	
Staphylococcus aureus	ATCC 25923	

[1] Identity verified by biochemical typing and/or by sequencing of *cpn*60 UT

[2] Plasmid sources included genomic DNA at concentration equal to 1/1000 of total DNA