EFFECT OF A FERMENTED YEAST PRODUCT ON THE GASTROINTESTINAL TRACT MICROBIAL DIVERSITY OF WEANED PIGS CHALLENGED WITH *SALMONELLA ENTERICA* TYPHIMURIUM DT104

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ABSTRACT

Gastrointestinal tract (GIT) microorganisms play important roles in animal health, including providing energy and vitamins, improving the host immune response and preventing pathogenic microorganisms from colonizing. Prebiotic feed supplementation offers an alternative to antimicrobial growth promoters by stimulating key populations of the GIT bacteria that can ferment these non-digestible compounds, producing various short chain fatty acids used by the animal. The objective of this study was to quantify the effects of a proprietary *Saccharomyces cerevisiae* fermentation product (XPC, Diamond V Mills, Inc., Cedar Rapids, IA) inclusion in nursery diets on the microbial diversity and growth performance of pigs before, during and after an oral challenge with *Salmonella*. Pigs (n= 40) were weaned at 21 d of age, blocked by body weight (BW) and assigned in a 2×2 factorial arrangement consisting of diet (control or 0.2% XPC) and inoculation (broth or *Salmonella*). Diet had no effect on pig growth performance prior to inoculation; however, consumption of XPC altered the composition of the gastrointestinal microbial community resulting in increased growth performance prior to inoculation. After *Salmonella* infection, XPC altered the composition of the gastrointestinal microbial community resulting in increased ($P < 0.05$) populations of *Bacteroidetes* and *Lactobacillus*. Infection with *Salmonella* and treatment of the piglets with ceftiofur-HCl resulted in alterations to the species richness and abundance of key members of the GIT community. The addition of XPC to the diets of weaning pigs results in greater compensatory gains after infection with *Salmonella* and an increase in beneficial bacteria within the GIT.

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Dedication

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Attribution

Dr. Monica A. Ponder: (Food Science and Technology) major advisor, assisted with proposal and thesis development as well as sample collection

Dr. Jeffery Escobar: (Animal and Poultry Science) committee member, designed animal experiment layout, assisted in animal trail sample collections as well as statistical data analysis.

Dr. Robert C. Williams: (Food Science and Technology) committee member, provided valuable feedback and support throughout M.S. project

Gabriela Lopez-Velasco: (Food Science and Technology graduate student) assisted in fecal DNA extraction and provided technical instruction on denaturant gradient gel electrophoresis (DGGE) and real-time PCR

Kathryn L. Price: (Animal and Poultry Science graduate student) assisted in animal trail with animal care, sample collection, screening feed for *Salmonella*, media preparation, growth performance data analysis, and intestinal morphology.

Matt Utt: (Animal and Poultry Science) assisted in animal trial with animal care, sample collection, and intestinal morphology.

Hanbae Lee: (Animal and Poultry Science graduate student) assisted in animal trial with animal care, sample collection, and media preparation

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Chapter 1: Introduction and Justification

Piglets at the weaning stage often are under the stress of changing diets and moving to new grower herd facilities. This stress makes them more susceptible to infections such as salmonellosis. Piglets infected with *Salmonella* often exhibit lethargy, diarrhea, fever, and weight loss (Funk, 2004). Small piglets may succumb to infection resulting in profit loss along with medicine and veterinary expenses (Jansen et al., 2007). Pigs that fail to put on efficient weight also cost the grower extra feed and time to move the product. The general hygiene protocol of herd movement in large swine facilities is known as all-in-all-out. Once a previous herd moves out of a grower facility, pens undergo a thorough disinfection and handling precautions enacted to prevent contaminating the incoming herd (Funk, 2004). A sick pig can corrupt the entire herd in an all-in-all-out facility. Producers typically provide antibiotics as a prophylactic, preventing infectious disease and therefore increasing the animal's growth performance. However, increasing prevalence of antibiotic resistant pathogens and the presence of antibiotic residues in food animals has led the European Union to ban the use of antibiotics for growth promotion in animal feeds (Williams et al., 2005). Therefore, alternatives to antibiotic supplemented feed that would allow for good animal growth performance while minimizing disease are actively sought.

Prebiotic compounds offer an attractive alternative due to the stimulation of beneficial bacteria that can provide the host additional carbon sources and vitamins while out-competing other bacteria, including pathogens, for colonization. Prebiotic supplements are compounds that are not digestible by the host animal but are capable of being broken down by gut bacteria. Elements of yeast, mannanoligosaccharides, and β glucans all have prebiotic qualities that benefit host immunity (Jensen et al., 2008; Leterme, 2000; Pieper et al., 2008). Bacteria

belonging to the genera *Bacteroides* and *Lactobacillus* are beneficial members that aid in producing lean, healthy pigs and can be stimulated by such compounds (De Angelis et al., 2006; Guo, 2008).

The purpose of this project was to determine the benefits of a prebiotic compound (XPC) on weanling pigs challenged with *Salmonella enterica* Typhimurium DT104. Pigs were accessed for dietary benefits during *Salmonella* infection by body weight, rectal temperatures and changes in abundance of the bacteria within the gastrointestinal tract.

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Chapter 2: Literature Review

Weaning Practices in U.S. Swine Production

During weaning young pigs are segregated from their mothers and begin the transition from a liquid to a solid diet. In the U.S. commercial swine operations, pigs undergo weaning between 14-21 days of age (National Pork Board, 2002). This is earlier than the European weaning practice of 21-28 days. This is to keep the sow in good weight condition because nursing is very taxing on the animal, as well as reduces the risk of transmitting diseases to its offspring. In high production animal husbandry, sows can yield up to 10 litters of piglets in a lifetime averaging over two litters per year (Katouli and Wallgren 2005). Weaned pigs are sorted by either litter, sex or body weight and placed in nursery pens with 10-20 other piglets until they are 8-12 weeks old (National Pork Board, 2002). Pigs move into fattening facilities at 10-14 weeks (Katouli and Wallgren, 2005). This group will move together until finishing using all in: all out barn hygiene practices to decrease the risk of contaminating the herd. This is a stressful period for the animal due to separation from the mother, change in diet, introduction to new pigs and new surroundings. Many weanlings will lose weight or not gain weight during this period causing producers to lose profits getting the pigs up to market weight. These factors are compounded by the fact that the piglet's immune system is poorly formed increasing the chances of illness and death.

It is important to provide recently weaned pigs with clean water to prevent dehydration while transitioning to dry feed because water absorption is poor during the first 2 weeks of weaning (National Pork Board, 2002; Katouli and Wallgren, 2005). The carbohydrate and protein components of feed are a dramatic change from maternal milk. Most commercial swine diets have a composition of 63.5% corn, 28% soybean meal, 5.2% fat, 1.9% deflourinated

phosphate, 0.9% vitamins, 0.35% NaCl, 0.25% CaCO3 and 0.05% $CuSO_4$ (National Pork Board, 2002) whereas milk has a composition of 5-6.5% protein, 5.5-6.5% lactose and 5.5-6.5% fat along with IgA (Katouli and Wallgren, 2005). Producers frequently use pelleted feed because of it consists of small particle size, decreases dust, decreases segregation of ingredients, increases bulk density and most importantly, improves feed: gain efficiency compared to its meal form (Den Angelis et. al., 2006). However, the sudden loss of protective IgA during feed consumption increases the risk of disease and illness (Katouli and Wallgren, 2005) resulting in a need for antimicrobial feed additives.

The Porcine Intestinal Tract

The intestinal tract environment is one of the primary active organs responsible for controlling livestock health and well-being (Dowd et al., 2008). In a newborn pig, the sole diet is milk and requires the high lactase activity of the small intestine (Katouli and Wallgren, 2005). During the weaning process, the consumption of cereals decreases the lactase ability and increasing α-amylase in the saliva as well as the pancreatic enzymes as milk is replaced (Katouli and Wallgren, 2005). The stomach and large intestine increase in size to utilize fermentation of feed and absorb electrolytes (Katouli and Wallgren, 2005). The porcine stomach is comprised of 10^7 to 10^9 CFU/gram of bacteria and the population increases as one moves further down to the colon with 10^{10} to 10^{11} CFU/gram (De Angelis et al., 2006). Each organism plays an important role either in digestion/fermentation of feed, synthesis of nutrients or vitamins, or assisting the animals resistance to enteric pathogens (Vanbelle et al., 1990). Defense against intestinal invaders depend on the composition and metabolic activity of the bacteria along with the barrier function of the mucosa (Bovee-Oudenhoven et al., 2003).

When the pig is born, its intestine is essentially sterile until the piglet suckles the mother receiving bacteria from the milk, skin, and possibly fecal matter (Katouli and Wallgren, 2005). Comparisons of fecal samples of piglets to their dams show little variation in bacterial members suggesting that sows are the initial source of microbiota for piglets (Katouli and Wallgren, 2005). In just 5 to 6 days after birth the porcine digestive tract is colonized with up to 500 different bacterial strains for a total population of 10^{14}; many of its residents are non-culturable (Vanbelle et al., 1990). However, the intestinal tract does not develop fully until the pig has reach 5 to 6 weeks of age (De Angelis, Siragusa et al. 2006). During weaning the stress and abrupt change in diet causes the diversity of the intestinal members to decrease sharply, especially during the first 3 days of weaning lowering their protection (Katouli and Wallgren, 2005). During this decrease of diversity, other members within the gut utilize the vacancy to proliferate resulting in no change to bacterial population size (Melin et al., 1997). Interaction with other pen mates causes the bacterial community to mirror that of the pen mates until the pig matures (Katouli et al., 1995).

Bacterial members of the gut microbiota

The predominant microorganism within the gut are anaerobic and outnumber aerobes by at least 3 to 5 logs per gram of fresh weight intestinal tissue content (Katouli and Wallgren, 2005). The five most prevalent genera resident within the porcine intestinal tract include *Clostridium, Lactobacillus, Streptococcus, Sarcina,* and *Helicobacter* (De Angelis et al., 2006; Thanantong et al., 2006; Dowd et al., 2008). Other organisms include *Peptococcus, Eubacterium, Bacteroides* and *Bifidobacteria* (De Angelis et al., 2006). Included in the *Clostridium spp.* isolates were *C. glycolicum, C. disporicum, C. septicum, C. kuyveri, C.*

perfringens and *C. difficile* (Dowd et al., 2008). However; not all of these organisms are beneficial to the host, *Clostridium perfringens* and *C. difficile* are known pathogens that inhabit the intestinal tract. *Bifidobacteria boum* and *B. suis* are known to ferment lactose from maternal milk (Mikkelsen et al., 2003). In adults and young pigs *Bifidobacteria* numbers range from 10^7 to 10^8 bacteria per gram of feces (Mikkelsen et al., 2003; Dowd et al., 2008). Surprisingly, the population of *Bifidobacteria* determined by PCR using genus specific primers, makes up less than 1% of the porcine intestinal flora (Mikkelsen et al., 2003). Lactobacilli establish early in the piglet intestine and remain one of the predominant residents in the gut flora community as the pig matures (De Angelis et al., 2006). Lactic acid bacteria produce short chain fatty acids or volatile fatty acids that can inhibit pathogen growth as well as additional energy for the host. Ruminococci are another important organisms that make up the swine intestinal tract in developing pigs and are responsible for breaking down cellulose during fiber fermentation in the hindgut (Varel, 1984). Recent data suggests that ruminococci make up 15.2% of the ileum microbiota (Dowd et al., 2008). Research examining obese and lean pigs found that obese pigs had significantly lower numbers of *Bacteroides* and *Bacteroidetes* present within the gut bacteria and was possibly attributed to fat storage (Guo, 2008). Factors affecting the gut bacteria are dietary, microbial interaction and host interaction origins (Dowd et al., 2008).

Microbial Ecology of the Swine Gut

Within the gastrointestinal tract, a diverse community of bacteria is constantly interacting with their host and each other. The establishment of the gut flora depends on the composition of native residents, the degree of bacteria present on ingested feed, the fermentation of digesta, the sow's skin and pen environment (Katouli and Wallgren, 2005). Some organisms are transient

and only pass through the intestinal tract, whereas other organisms become established and colonize (Katouli and Wallgren, 2005). This ebb and flow of members continues as the pig ages. During diet changes from nursing to solid feed and then to a high energy fattening diet, the microbiota becomes more diverse. This may be due to environmental changes, interactions with other pigs or the fermentation of the feed (Katouli and Wallgren, 2005). However, in the early stages of life the organisms dominating the pig intestine are capable of utilizing various carbon sources compare to the organisms found in intestine during the post-weaning and fattening period (Katouli and Wallgren, 2005).

In the first 3 days after weaning, the microbial diversity decreases dramatically, this can cause problems since high diversity correlates to protection from foreign microorganisms (Kuhn et al., 1993). If barn hygiene and animal management is implemented the microbiota will be restored after 2-3 weeks (Katouli et al., 1999). Pen mates will exhibit similar intestinal populations during this phase suggesting exposure through animal contact as well as environmental settings (Katouli et al., 1999). At the fattening stage in healthy pigs, the fermentative capacity of the microbiota is lower than pigs at the nursing stage; however, there is a higher diversity of enteric microbes during fattening (Kuhn et al., 1993). When pigs reach the fattening stage they are both physically and immunlogically mature resulting in little disruption due to enteric pathogens (Kuhn et al., 1993).

Stress can cause changes to the intestinal bacteria resulting in a decreased diversity that makes the animal more vulnerable to disease (Katouli and Wallgren, 2005). Stress is more dramatic in younger pigs such as during weaning, grouping, changes in diet, and transportation resulting in diarrheic events and a decrease in microbial diversity (National Pork Board, 2002; Katouli and Wallgren, 2005). However not all diarrheic events are due to decreases in microbial

diversity, enteric pathogens such as *E. coli* (serotypes O138, O139, and O141), *Salmonella, Yersinia enterocolitica,* and *Clostridium perfringens* are known to cause diarrhea in newborn and weanling pigs (Berschinger, 1999; Katouli and Wallgren, 2005).

The native bacteria provide protection against foreign bacteria that attempt to colonize the gut, especially pathogenic bacteria (Katouli and Wallgren, 2005). Native bacteria have several strategies for preventing pathogens from colonizing such as production of bacteriocins, defensins and short chain fatty acids and competition for attachment sites and energy sources (Bovee-Oudenhoven et al., 2003; Katouli and Wallgren, 2005; De Angelis et al., 2006; Niba et al., 2009). In some instances, the pathogen still may be able to colonize the gut by expressing virulence factors (Katouli and Wallgren, 2005). The pathogen may also take advantage of a weakened host immune system or a suppressed microbiota due to antibiotic treatment (Katouli and Wallgren, 2005). When the normal microbiota is suppressed by antibiotic treatment the competition for attachment and energy sources are no longer present allowing pathogenic bacteria to colonize (Tannock, 1995). Strategies to stabilize the microbiota examine feed composition, antibiotic growth performers, zinc oxide supplementation, probiotics, and prebiotics (Katouli and Wallgren, 2005).

Microbial Community Analysis Techniques

Analysis of a microbial community involves identifying the members present as well as the quantity in which they are present. One of the major components of studying microbial communities involves examining microbial diversity within a specific environment such as food, soils or the intestine (Juste` et al., 2008). Microbial diversity refers to the number of different genera within a sample. Several molecular techniques exist for examining microbial diversity

such as denaturant gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single-strand conformation polymorphism (SSCP), terminal restriction fragment length polymorphism (T-RFLP), amplified ribosomal DNA restriction analysis (ARDRA) also know as restriction fragment length polymorphism (RFLP), and (automated) ribosomal intergenic spacer analysis ((A)RISA) (Juste` et al., 2008). Both DGGE and TGGE implement electrophoresis of similar length (700 bp) PCR products of conserved sequences, such as 16S, on acrylamide gels. The difference between these two techniques is DGGE using a gradient of denaturing chemicals while TGGE implements a temperature gradient to denature the DNA both properties rely on the guanidine: cytosine (GC) content of the DNA to separate the fragments, since high concentrations of GC resist denaturation and have a high melting temperature (Muyzer et al., 1993). Single-strand conformation polymorphism also uses electrophoresis to separate PCR products based on conformational differences of folded single strand products, however, during electrophoresis it is possible for the DNA to re-anneal (Juste` et al., 2008). Terminal restriction fragment length polymorphism is frequently used for comparative community analysis in which marker genes are fluorescently labeled then undergo restriction digestion before being separated and detected (Juste` et al., 2008). In this process only terminal restriction fragments are detected and used to compare the community; a standard labeled with a different fluorescent allows the comparison of fragment lengths, differences in the fragment lengths indicate a more complex community (Juste` et al., 2008). Restriction fragments are also the basis for ARDRA or RFLP but unlike T-RFLP, all the fragments are detected increasing the revolution of the analysis, however, multiple restriction digestions must be used to obtain an accurate representation of the community and interpreting the patterns can be a complex task (Tiedje et al., 1999). The staining sensitivity of RFLP is narrow resulting in the loss of bands, resulting in lower community

abundance (Tiedje et al., 1999). Lastly, (A) RISA consists of PCR amplification of DNA between the 16S and 23S ribosomal genes known as an intergenic spacer region (Juste` et al., 2008). These 400 to 1200 bp spacers undergo separation by electrophoresis with each band corresponding to at least one organism; however, some members may have more than one copy of the targeted genome resulting in multiple bands (Fisher and Triplett, 1999).

Denaturant gradient gel electrophoresis

Denaturant gradient gel electrophoresis (DGGE) is a community analysis tool that allows researchers to compare the similarity between different communities. Universal primers to amplify broad phylogenetic groups, such as all Bacteria (16s rDNA, *rpo*B), all Fungi (18s rDNA) or specific members of the community also contain a GC-rich clamp at the 5' end of the forward primer (Juste` et al., 2008; Muyzer et al., 1993). This GC-rich clamp is essential to retarding the band migration allowing discrimination between community members based on the GC content of the organism's sequence. (Muyzer et al., 1993). Touchdown PCR increases specificity by using an annealing temperature above the standard in the first few cycles to limit primer annealing to sequences with exact matches (Muyzer et al., 1993). Unlike standard horizontal agarose gel electrophoresis, DGGE separates the fragments (200-700 bp) through a gradient (20-80%) consisting of formamide and urea, which will denature the bonds between the double stranded fragments (Muyzer et al., 1993). Fragments that contain larger numbers of G and C nucleotides will require more energy, thus more denaturant; to break the three bonds between guanine and cytosine nucleotides. Therefore, the further a fragment travels through a gradient, the larger the GC content of the fragment. After electrophoresis is complete, gels undergo staining with either SYBR Green, SYBR Gold, silver staining or ethidium bromide then

visualized under ultra violet light (Muyzer et al., 1993). DGGE does not provide the identities of individual members of the community, unless individual bands are excised and sequenced.

An individual band rarely belongs to one organism due to the high conservation of the genes used to amplify the members of the community. For this reason, a band is typically believed to represent a group of organisms with similar GC content, resulting in reduced richness estimates (Yang and Crowley, 2000). Microheterogenity, the presence of multiple, slightly different genes within one organism, frequently results in more than one band within a gel representing an individual organism, resulting in increased richness estimates (Nakatsu et al., 2000). Despite these limitations, DGGE can still be used to determine the richness of a community (number of bands) and compare the diversity between samples (location and number of bands) (Muyzer et al., 1993). Sequencing excised bands provides a more accurate description of the community richness (Nakatsu et al., 2000). The sensitivity of DGGE is further reduced by the ability to visualize the fragments within a polyacrylamide gel. Low copy number fragments will appear faint or not at all when stained and therefore not be represented within the profile (Juste` et al., 2008). The extraction method used to isolate DNA can also influence the community profile presented by DGGE (Carrigg et al., 2007). Discrepancies in community analysis can be reduced by using a second technique such as sequencing, hybridization with probes or real-time PCR (Carrigg et al., 2007; Stephen et al., 1998). Molecular finger printing methods such as DGGE and T-RFLP are only semi quantitative and require another method if the quantification of the microbial community is to be addressed (Juste` et al., 2008).

Quantitative real time polymerase chain reaction

The use of polymerase chain reaction (PCR) is a faster and cheaper way to detect pathogens in food, fecal or water samples (Bohaychuk et al., 2007). Conventional methods can take up to five or more days for results while real-time PCR only requires 52-54 hours from pre-enrichment to PCR reaction (Bohaychuk et al., 2007). While culture based methods do not effectively reflect true microbial counts, PCR is able to identify members that are termed "viable but non culturable". Low bacterial numbers in a fecal sample may require the use of an enrichment step to dilute inhibitors and aid in recovering injured cells (Feder et al., 2001). Inhibitors are capable of creating false negatives while DNA from dead cells can create false positives, both of these issues are resolved when a 2 step enrichment or the enriched sample is diluted to reduce the concentration of problematic items (Krascsenicsova et al., 2008).

Quantitative real-time PCR (qrt-PCR) allows the starting amount of template DNA to be determined whereas qualitative rt-PCR only detects presence/absence of the targeted DNA sequence (Bio-Rad Laboratories). Polymerase chain reaction amplifies multiple copies of template DNA to using specific primers designed for either a certain gene or sequence that are able to target specific taxonomic designations from domain to species (Smith and Osborn, 2009). Primers bind to specific portions of the DNA that will undergo amplification using temperature changes by first denaturing the double stranded DNA, then annealing or adding new nucleotides to the single strand, and finally extension of two new double stranded DNA molecules. This procedure repeats many times generating 30 to 40 cycles ensuring the product has enough copies to be detectable for downstream applications. During the qrt-PCR reaction, fluorescent dyes, such as SYBR Green, are added to intercalate with the newly formed DNA strand, the fluorescence increases as the target DNA amplifies (Hein et al., 2006; Smith and Osborn, 2009).

Once the amplified product has reached a detectable limit over the background, or threshold cycle (Ct value), the measurement is recorded (Bio-Rad Laboratories; Smith and Osborn, 2009). Therefore, samples that have higher initial concentrations of target DNA will only have to undergo a few amplification cycles to achieve a detectable fluorescence, resulting in a low Ct value. By including known concentrations of target DNA or standards at 10 fold dilutions, the number of copies is extrapolated from the Ct values. This is possible by graphing the Ct values of the standard dilutions and, using the slope, r^2, and y-intercept of the plotted line, the log copy number of the specific gene or bacteria within the sample can be calculated. The benefit of qrt-PCR is that results are available after amplification therefore reducing carry over contamination that may occur while loading agarose gels and as well as saving time (Hein et al., 2006; Schmittgen, 2001). The results are easily reproducible and highly sensitive, unlike DGGE (Smith and Osborn, 2009; Nakatsu et al., 2000).

The ability to quantify specific phyla or genera makes qrt-PCR an important tool in microbial community analysis. Yet the reaction is only as specific as the primers selected. Since SYBR Green binds to any double stranded DNA present including non-specific amplicons and primer dimers, primer sensitivity and concentration must be optimized to produce accurate results (Smith and Osborn, 2009). Melting curves performed after the amplification run to confirm that only the desired target is generating fluorescence (Smith and Osborn, 2009). This analysis works on the basis that as the double stranded DNA denatures the fluorescent agent is lost resulting in a decrease in measured fluorescence, multiple peaks within a melt curve analysis indicate various melting points, therefore different amplicons were formed (Bio-Rad Laboratories).

Fluorescent probes such as *Taq*Man are an alternative to SYBR Green. The probe is fluorescently labeled at the 5' end and contains a quencher molecule at the 3' end. While the two parts are close together, no fluorescence occurs (Smith and Osborn, 2009). During the annealing step of each cycle of the PCR, primers and the probe bind to their target sequences. At the extension phase of the reaction, the 5' exonuclease activity of the *Taq* polymerase enzyme cleaves the fluorophore from the *Taq*Man probe and fluorescence can be measured (Smith and Osborn, 2009). Unlike SYBR Green, the specificity of the probe ensures the specificity of the amplified DNA (Smith and Osborn, 2009).

Salmonella **Overview**

Salmonella is typically a routine gastrointestinal pathogen. Current knowledge on the genetics and pathogenesis of *Salmonella* is limited to just a few specific serotypes. However, the genus *Salmonella* consists of 2 species, 7 subspecies, and more than 2500 different serotypes, many of these strains have been implicated in food-borne disease. Only one species of *Salmonella*, *enterica*, is associated with clinical disease. The majority of *Salmonella* isolates from humans belong to subspecies I, although subspecies IIIa and IIIb infections do occur in humans and result in not only gastrointestinal distress but also extra-intestinal infections such as septicemia and chronic urinary tract infections (Feder et al., 2001; Mahajan et al., 2003; Schroter et al., 2004). *Salmonella enterica* is ingested in contaminated food and water, enters the stomach and invade the mucosal cells to adhere to the intestinal villi causing inflammation of the intestinal mucosa (Salyers, 2002). The inflammation results in abdominal pain due to the release in prostaglandins from the tissues responding to the lipopolysacchrides surrounding the pathogen (Salyers, 2002). To adhere to the intestinal wall *Salmonella* uses structures called fimbria.

Salmonella invades the cells by causing "membrane ruffling" which forces the host cells to engulf the bacteria changing the actin filaments on the host surface (Salyers, 2002). The pathogen clears the intestinal mucosa lining and injects virulence proteins into the endothethial cell using a type III secretion system encoded by genes within the *inv, spa, prg,* and *org* cassettes *(*Salyers, 2002*)*. Salmonellosis can occur in all age groups; however, those who are very young, elderly or immuno-compromised frequently experience more severe illness, leading to hospitalization and even death. Symptoms of the infection include acute stomach pain, diarrhea and fever with an onset of 1 to 3 days after consumption of a contaminated food product. If *Salmonella* migrates across the intestinal epithelium into the bloodstream, septicemia can occur, causing serious complications. In 2005 serotypes of *Salmonella enterica* accounted for 45,322 culture confirmed illnesses within the U. S. (CDC, 2007). Approximately 6-9% of these human cases can be attributed to the consumption of fresh or processed pork products (Frenzen, 1999). Other studies estimate that *Salmonella* is present on 10 -16% of raw pork products due to fecal contamination of carcasses (Wonderling et al., 2003). The zoonotic aspect of salmonellosis makes it a serious issue of public health for the pork industry, the handlers in the swine barns and the consumer.

Salmonella infections in swine

Infections by serovars of *Salmonella* in pigs significantly affect the swine industry due to poor weight gain in pigs, profit loss due to extra veterinary treatment and extra feed costs for farmers and potentially contaminated meat for consumers (Jansen et al., 2007). Salmonellosis has a high mortality and low morbidity in weaned pigs less than 5 months old. It has been estimated that between 25 and 48% of the U.S. swine herds may be colonized with various

serotypes of *Salmonella enterica* (Wonderling et al., 2003; Callaway et al., 2008). The two most often isolated serotypes isolated from pigs in the U.S. are *Salmonella enterica* sv. Typhimurium and *Salmonella enterica* sv. Worthington (**Table 2.1**). Septicemia in pigs is often caused by *S.* Choleraesuis, particularly biotype kunzendorf, (Feder et al., 2001) while infection by the majority of other *Salmonella* serotypes results in diarrhea (Fedorka-Cray et al., 1994; Callaway et al., 2008). In the United Kingdom and Ireland, *S.* Typhimurium and *S.* Derby are the most common serotypes (Mannion et al., 2007). In a 17 year study conducted in the Netherlands, serotype *S.* Typhimurium strain pt 150 was the most prevalent serotype phage type in humans and pigs (van Duijkeren et al., 2002*)*. The multi-drug resistant *S.* Typhimurium DT104 serotype is found in humans, pigs and cattle (van Duijkeren et al., 2002*)*. While *S.* Typhimurium isolates have declined in humans, poultry and cattle, it remains stable in the porcine population (van Duijkeren et al., 2002*)*. According to the CDC, the most common serotypes in humans are *S.* Enteriditis, *S.* Typhimurium and *S.* Heidelberg. It is interesting that *S.* Typhimurium is also the highest isolated in swine (**Table 2.1**). The serotype most frequently isolated from pigs in the Netherlands (1996-2001) provides supporting evidence of the ubiquitous nature of the *S.* Typhimurium (van Duijkeren et al., 2002*)*. The prevalence of serotypes in swine depends on the region's climate, *S.* Derby is more predominate in Europe and while *S.* Typhimurium is more predominate in North America (Wonderling et al., 2003). Other emerging serotypes may be the result of environmental contamination from feed or neighboring domestic animals or livestock (Fedorka-Cray et al., 1994; Callaway et al., 2005; Amaechi, 2006).

Salmonella was isolated from 38% of the swine farms surveyed by the National Animal Health Monitoring System (Wonderling et al., 2003). Each isolate underwent subtyping by pulsed field gel electrophoresis (PFGE). Isolates from this survey belonged to 32 different PFGE

fingerprint (pulsetypes). Epidemiological comparisons by PFGE of porcine isolates of *S.* Typhimurium, *S.* Choleraesuis, *S.* Heidelberg, *S.* Derby, and *S.* Agona revealed that 19/24 of these isolates matched the pulsetype of a strain associated with human disease. However, other PFGE patterns, not associated with clinical illness were observes for serotypes *S.* Derby and *S.* Typhimurium (Wonderling et al., 2003).

Risk factors associated with *Salmonella* infections include improper farm hygiene, not following all-in all-out practices when moving herds, contaminated feed, shipping stress and domestic or wildlife animals near the farm. Farm hygiene involves disinfecting barn floors and walls between installments of pigs. This is crucial since *Salmonella* is capable of surviving for years in wet environments and several months in dry environments if it is not exposed to high temperatures or direct sunlight (Gay, 1999). *Salmonella* Typhimurium (10^5 CFU/mL), inoculated into pig manure can survive 34 days in the summer and 58 days in the winter, indicating that manure is a potential environmental reservoir (Amaechi, 2006; Mannion et al., 2007). Other studies have shown that *Salmonella* can remain viable for 7 weeks in the soil at organic pig farms or more than a year in soil under natural simulated conditions present in the swine environment (Feder et al., 2001; Jensen et al., 2006) .

Swine are capable of shedding asymptomatically less than 10 CFU/ gram of feces (Feder et al., 2001). Sow-to-pig transmission of *Salmonella* serotypes may occur, resulting in infection or carriage in pigs moved to new facilities for finishing (Funk 2004). Stress related to weaning or shipping increases fecal shedding of *Salmonella* serotypes (Davies et al., 2004; Gebreyes et al., 2004; Scherer et al., 2008). To reduce contamination by these events most production barns utilize the all in- all out movement of pigs where pigs from a single shipment remain together and move as a unit to prevent infection. A Danish study showed that farms that used all in- all

out production and had areas for handlers to change boots and clothing prior to entering had herds that were three times less likely to be seropositive for *Salmonella* (Funk, 2004).

Environmental factors such as temperature and presence of neighboring animals increase the risk of Salmonellosis in swine. Increased variation in daily high temperatures have been shown to increase the incidence of *Salmonella* in North Carolina finisher pigs possibly due to heat stress (Funk, 2004). High temperatures alone, are not responsible for increased shedding on farm as, the highest fecal shedding rates for swine and cattle are reported from October to December (Callaway et al., 2008). Birds that roost near barns, and feed from the animal troughs or livestock droppings can potentially become contaminated or transfer a pathogen to the troughs. This continuing cycle can potentially infect the whole herd. When fecal or cloacal samples of wildlife and domestic animals surrounding the swine barns were evaluated, starlings and rodents were shown to carry the same strains of verocytotoxin producing *E. coli* (Nielsen et al., 2004) or the same *Salmonella* serotypes that were present in the infected cattle or pigs (Funk, 2004; Nielsen et al., 2004). Once infection has occurred, the pathogen will be present in feces for several days but will survive in the lymph nodes for many weeks (Amaechi, 2006). Prevalence and carrier rate of pigs in different age groups from 5 different farms indicated that fattening pigs had the highest carriage rate of *Salmonella spp. (*25%) while weaning pigs had a rate of 20%, piglets showed a carriage rate of 13.3% (Amaechi, 2006). This same study also revealed that 18.4% of healthy pigs are carriers of *Salmonella* spp. especially, *S.* Typhimurium and *S.* Enteriditis.

Use of Prebiotics and Probiotics

An animal's diet can greatly affect the microbial community of its gastrointestinal tract. Pathogenicity of *Salmonella* spp. can be specific between animal species *(*Rodenburg et al., 2007*)*. Diet affects *Salmonella* colonization, diets low in calcium and high in fat result in low resistance to food-borne bacterial infections *(*Rodenburg et al., 2007*)*. Fat provides a protective effect for the pathogen as it passes through the stomach and intensify diarrhea. Calcium phosphate promotes lactobacilli providing resistance to *Salmonella* infections *(*Bovee-Oudenhoven et al., 2003*)*. Normal host defenses are capable of eliminating > 80% of organisms from the gut within hours (Deng et al., 2008*)*. Prebiotic feeds have become a major research interest for stimulating the bifidobacteria or lactobacilli of in the intestinal tract. Prebiotics are compounds, generally non digestible dietary supplements, that stimulate the growth of beneficial intestinal bacteria (Mikkelsen et al., 2003). Because the compounds do not undergo digestion in the host small intestine, they are available as energy sources for bacteria in the large intestine. Many of these substrates are believed to be fermented in the cecum (Santos et al., 2006). The metabolic activity necessary to generate the organic acids that lower the pH in the intestine are important in creating the anti-infectious property (Silva et al., 2004). This feed focus is appealing because of its ability to naturally promote beneficial bacteria without the health issues that confront the use of antimicrobial residues in food animals (Ojha et al., 2007). Currently, food components such as inulin-type fructans seem to produce the best prebiotic effect (Santos et al., 2006). The nature of the prebiotic determines the nature of the fermented substrates. Fructans are resistant to digestion by *Clostridium* species. Along with fructans, xylo-oligosacchrides (XOS) and lactulose are fermented by *Bifidobacterium, Lactobacillus* and *Bacteroides*.

Probiotics are 'live microorganisms' that benefit the host when dispensed in sufficient amounts (De Angelis et al., 2006). In general, probiotics stimulate the immune system, out-compete pathogens for resources or inhibit pathogens from colonizing. *Lactobacillus* populations have the most promise for fulfilling this description. *Lactobacillus, Lactococcus, Streptococcus* and *Bifidobacterium* are acidogenic bacteria and have shown inhibitory activity towards pathogen growth, as well as the ability to adhere to intestinal wall cells while promoting host health (Silva et al., 2004; Santos et al., 2006; Dowd et al., 2008). In humans bifidobacteria are considered better probiotic organisms than lactobacilli for prevention or treatment of intestinal disorders (Silva et al., 2004). The peptidoglycan structure of bifidobacteria stimulates the innate immune response enhancing phagocytosis, lymphocyte and cytokine production along with the humoral immune response (Silva et al., 2004).

Determining which microorganisms have the potential to be used as probiotics for pigs consists of isolating suitable habitants, the safety of the strain, in vitro characterization, technological adaptation, obtaining high levels of a viable organism as a mixture, the strain's ability to survive and persist in the host and the strain's capability to produce compounds that inhibit pathogen growth (De Angelis et al., 2006). Once a strain is determined to be a potential probiotic, the issue becomes how to incorporate the strain into a feed source. *Bifidobacterium longum* has an oxygen tolerance that is advantageous to production (Silva et al., 2004). Lactobacilli orally administered separately from feed in freeze-dried and liquid forms and indicate a high persistence and colonization in the intestinal level as well as decreased infection by *Salmonella* Typhimurium and *Enterobacteriaceae* in pigs (De Angelis et al., 2006). Pelleted feed involves a heating step that could potentially damage most probiotic strains. However; recently in an experiment performed by De Angelis et *al.* in 2006 involving probiotics in pelleted

feed, after just 24 hours of incubation in the ileum, *E. coli* strains decreased from 5.5 to 3.0-4.1 log CFU/g (De Angelis et al., 2006). Methods for controlling *E. coli* shedding in ruminants reported a reduction of *E. coli* O157 by using *Streptococcus faecium* with lactic acid bacteria (Sargeant et al., 2007). However, pigs are monogastric animals and these results may not mirror a monogastric model. This previous study indicated that average daily gain and gain- to-feed ratio were higher for the probiotic feed groups compared to control trial. The difficulty with probiotics is that they are not a long term fix, as the gut flora reverts back to a set point once disruptions are resolved or probiotic diets are no longer followed (Silva et al., 2004; Barman et al., 2008). Probiotics do not colonize the digestive tract of animals with complex microbial communities, therefore, daily intake is needed to keep them at a high enough level to be functional(Silva et al., 2004).

Prebiotic effects on the intestinal tract flora and shedding

In a study using 8 different oligosaccharides to examine prebiotic significance, the total anaerobe counts increased in all the mice on experimental diets (Santos et al., 2006). Prebiotic diets also decreased clostridial bacteria in the large bowel; this was especially evident in the diet containing xylo-oligosacchrides (XOS). Unfortunately, failure to continue the diet results in loss of all the prebiotic effects. After 6 months, the microbial counts return to the state exhibited on a basal diet. The total number of anaerobes, lactobacilli, and bifidobacteria decrease while the total number or aerobes, enterobacteria, and clostridia exhibit an increase in abundance.

Fructo-oligosacchrides (FOS) and lactulose do not break down by host digestion in the small intestine. Fructo-oligosacchrides are rapidly fermented by microbes in the cecum, a pouch like structure that connects the ileum to the ascending colon, making the contents acidic (via

short chain fatty acids and lactic acid production) and inhibit colonization of pathogens. Even though these compounds inhibit colonization by *Salmonella*, FOS and lactulose enhance its translocation across the gut mucosa that occurs via ileal M cells located within Peyer's patches (Bovee-Oudenhoven et al., 2003). This indicates that the mechanisms of colonization resistance and translocation are different along the intestinal tract. *Salmonella* infected mice fed the FOS or lactulose supplements showed an increase inflammatory response in the cecum and colon mucosa. Since the mice did not shed the pathogen it is likely that the inflammation of mucosa responded by increasing mucus excretion but as the oligosaccharides impaired the gut barrier with its short chain fatty acids the organism was able to move across the barrier in the cecum and colon to evade the immune response (Bovee-Oudenhoven et al., 2003). FOS compounds decreased the daily amount of fecal matter and the pH of the fecal water in comparison to other non-digestible carbohydrates (Bovee-Oudenhoven et al., 2003). Lactulose and FOS stimulate the excretion of fecal mucin. Lactulose and FOS also increased the amount of fecal lactobacilli, bifidobacteria and the number of enterobacteria in feces. Mice fed the FOS diet also had reduced fecal shedding of *Salmonella* showing that: "the colonization resistance is inversely related to fecal pathogen excretion in time" (Bovee-Oudenhoven et al., 2003). Since FOS stimulated enterobacteria, a non-beneficial microbe group that can cause septicemia, it does not follow the criteria of a probiotic (Bovee-Oudenhoven e et al., 2003; De Angelis et al., 2006). β-glucans found in cereals are also known to promote lactobacilli in weaning pigs (Jonsson 1991). Soluble non-starch polysaccharides like β-glucans can increase digesta retention time in the gastrointestinal tract and endogenous nitrogen excretion affecting the digestibility of other nutrients including starches and proteins so that the flow is increased to the large intestine (Leterme, 2000; Pieper 2008).

Conclusions

Weaning is a stressful time in young animals. Their under-developed immune system is not prepared for the surrounding environment resulting in morbidity and mortality that result in profit losses for the producer. The addition of prebiotic compounds to animal feed stimulates existing beneficial gut microbes may strengthen the intestinal community against enteric pathogens such as *Salmonella.* Examining the fluctuations of specific community members during the consumption of prebiotics as well as a *Salmonella* infection will provide insight on the porcine intestinal tract.

Table 2.1. Top 10 most frequently isolated *Salmonella* serotypes from swine as reported to the USDA (2001-2005) and from humans as reported to the CDC (2003-2005) (This table was adapted from USDA and CDC data) (USDA:APHIS 1997; CDC 2004; CDC 2005; CDC 2006). Serotypes in bold are present in both categories.

Rank	USDA Surveillance Data *Salmonella* isolates from swine (2001-2005)	CDC Human Isolates (2003-2005) n=406443
1	**Typhimurium**	**Typhimurium**
2	Worthington	**Enteritidis**
3	Cubana	Newport
4	**Infantis**	Heidelberg
5	**Enteriditis**	Javiana
6	Mbandaka	Montevideo
7	Newport	Muenchen
8	Senftenberg	Oranienburg
9	Choleraesuis var. kunzendorf	Agona
10	Kentucky	**Infantis**

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Chapter 3 Use of *Saccharomyces cerevisiae* fermentation product on growth performance and microbiota of weaned pigs during *Salmonella* infection

ABSTRACT

Fermented yeast products are rich in mannanoligosaccharides, β-glucans, and other nutritional metabolites that may optimize gut health and immunity, which can translate into better growth performance and a lower risk of food borne pathogens. The objective of this study was to quantify the effects of *Saccharomyces cerevisiae* fermentation product (XPC, Diamond V Mills, Inc., Cedar Rapids, IA) inclusion in nursery diets on pig performance before, during, and after an oral challenge with *Salmonella*. Pigs (n=20) were weaned at 21 d of age, blocked by BW and assigned in a 2×2 factorial arrangement consisting of diet (control or 0.2% XPC) and inoculation (broth or *Salmonella*). Pigs were fed a 3-phase nursery diet (0-7 d, 7-21 d, and 21-35 d) with *ad libitum* access to water and feed. Growth performance and alterations in the gastrointestinal microbial ecology were measured during pre-inoculation (PRE 0-14 d),sick (SICK 14-21 d), and post-inoculation (POST 21-35 d). Rectal temperature (RT), BW, and ADG were measured weekly and daily during SICK. On d 14, pigs were orally inoculated with 8.8 $X10^8$ CFU of *Salmonella enterica* serovar Typhimurium or sterile broth. From d 17-20, all pigs received 10 mg/kg BW i.m. ceftiofur-HCl. Diet had no effect on BW, ADG or RT during any period (P = 0.12 to 0.95). Inclusion of XPC tended ($P < 0.10$) to increase *Salmonella* shedding in feces during SICK. Consumption of XPC altered the composition of the gastrointestinal microbial community resulting in increased ($P < 0.05$) populations of *Bacteroidetes* and *Lactobacillus* after *Salmonella* infection. Pigs inoculated with *Salmonella* had decreased ADG and BW, and elevated RT during SICK ($P < 0.001$). Furthermore, fecal *Salmonella* CFU (log_{10}) was modestly

correlated ($P < 0.002$) with BW (r = -0.22), ADFI (r = -0.27), ADG (r = -0.36), Gain:Feed ratio (r = -0.18), and RT (r = 0.52), during SICK. Following antibiotic administration, all *Salmonella*-infected pigs stopped shedding. During POST, an interaction between diet and inoculation ($P = 0.009$) on ADG indicated that pigs infected with *Salmonella* grew better eating XPC than control diet. The addition of XPC to the diets of weaning pigs results in greater compensatory gains after infection with *Salmonella* than pigs fed conventional nursery diets due to an increase in beneficial bacteria within the gastrointestinal tract.

Key words: pig growth performance, *Salmonella*, yeast culture, pre-biotic feed supplement, gastrointestinal microbial ecology

INTRODUCTION

Fermented yeast products may offer an alternative to antibiotic growth promoters (**AGP**) in food animal production. Diamond V XPC Yeast Culture is a rich source of mannanoligosaccharides, β-glucans, and other yeast fermentation metabolites. These prebiotic compounds provide additional nutrients that can be fermented by gastrointestinal bacteria providing energy, vitamins and antimicrobial agents (Casey et al., 2004; Niba et al., 2009). Prebiotic compounds may prevent the interaction between pathogenic bacteria and intestinal cells, as well as strengthening the immune system (Burkey et al., 2004). A current working hypothesis states that a healthier gut in conjunction with a robust immune system should translate into better growth performance of pigs (Branner and Roth-Maier, 2006). Currently, this is a key component for the swine industry because the use of AGP is not permissible in the

European Union. Additionally, several international and domestic markets are starting to demand animal-derived products from antibiotic-free animals.

With the increasing demand for "safer" products of animal origin, consumers are not only stipulating AGP-free eggs, dairy, and meat products but are also demanding a lower prevalence of food-borne pathogens. The improved growth performance in response to AGP was linked to the presence of environmental pathogens more than 40 years ago (Coates et al., 1963). Thus, with the anticipated ban of AGP, many are concerned that the pathogenic load of farm animals can drastically increase. This scenario can lead to consumer apprehension towards animal products. Therefore, the quest for alternatives to the use of AGP in the animal industry is not only to prevent a potential reduction in growth performance but also to avert a possible increase in food borne pathogens in eggs, dairy, and meat products. The objective of this study was to quantify the effects of XPC (Diamond V Mills, Inc., Cedar Rapids, IA) inclusion in nursery diets on pig performance and gastrointestinal microbial ecology associated with an oral challenge with *Salmonella*. We hypothesize that *Salmonella* shedding will be increased as well as species abundance will be in pigs consuming the 0.2% prebiotic diet XPC.

MATERIALS AND METHODS

All procedures received approval from both Virginia Tech Institutional Animal Care and Use Committee and Biosafety Committee and took place in a Biosafety Level (**BSL**)-2 facility. In addition, all analytical and bacterial analyses took place in BSL-2 laboratories.

Bacterial strains and culture

Salmonella enterica subspecies *enterica* serovar Typhimurium DT104 was obtained from the American Type Culture Collection (**ATCC**, BAA-185, Manassas, VA). This strain was resuscitated in 10 mL of TSB at 37°C for 24 h and plated onto tryptic soy agar (**TSA**). A single colony was picked and then was subjected to procedures that rendered its progeny resistant to the antibiotics Nalidixic acid (Acros Organics, Morris Plains, NJ) and Novobiocin (BD Bioscience) through sequential transfer onto TSA plates of increasing concentrations until achieving a final resistance to 20 μg/mL and 25 μg/mL, respectively (*S.* Typhimurium $Nal^{R}Nov^{R}$). The final strain was tested for susceptibility to 10 mg/kg ceftiofur-HCl (Pfizer Animal Health, New York, NY) to insure antibiotic efficacy of pig treatment. *Salmonella* Typhimurium $Nal^{R}Nov^{R}$ was cultured overnight at 37°C in TSB medium on an orbital shaker at 150 rpm and bacterial populations were estimated by spectrophotometry at 600 nm. For inocula preparation, *S.* Typhimurium $Nal^{R}Nov^{R}$ were harvested at 7,500×g for 10 min at 4°C, and re-suspended in sterile TSB.

The following cultures served as positive controls to generate standard curves to quantify select phylum and genera within the pig feces using real time PCR: *Lactobacillus acidophilus* NCFM™ strain (ATCC 700396) grown anaerobically in tryptic soy broth (TSB) at 37°C, *Bacteroides thetaiotaomicron* (ATCC 29741) grown anaerobically in pre-reduced anaerobically sterilized (**PRAS**) cooked meat broth (BD Biosciences) at 37°C, and *Flavobacterium sp.* grown aerobically using R2A agar (BD Biosciences) at 25°C.

Animals, housing, diets, and experimental protocol

Crossbred pigs were obtained from a commercial swine farm (Waverly, VA) and used to

assess the effect of a proprietary *Saccharomyces cerevisiae* fermentation product (**XPC**, Diamond V Mills, Inc., Cedar Rapids, IA) inclusion in nursery diets on pig growth performance before, during, and after an oral challenge with *S.* Typhimurium $Nal^{R}Nov^{R}$. Twenty pigs per trial were used in 2 replicates, for a total of 40 pigs (10 pigs per treatment). Pigs were weaned at 21 d of age (7.02 ± 0.27 kg), transported to Virginia Tech, blocked by BW, and randomly assigned to treatments. Individual rectal swabs were collected at arrival to the BSL-2 facility to screen for the presence of *Salmonella*. All samples were incubated at 37°C for 24 h in Gram-negative Hajna broth for enrichment, followed by plating onto BGA to screen for *Salmonella* indicative colonies.

Pigs were housed in individual pens and segregated in 2 identical rooms according to their assigned inocula (*Salmonella* or sterile broth) to minimize the potential for cross-contamination. Inocula conditions were tested in both rooms during the 2 trials of the study to ensure no room effect. Rooms were discretely ventilated with 100% clean air (i.e., no recirculation), were under negative pressure at all times, and automated systems controlled the temperature and lighting (18 h light:6 h dark with lights on at 0600) of each individual room. Each pen contained plastic coated expanded metal floor, a nipple waterer, and a self-feeder. Feed ingredients, mixed diets, and initial fecal samples underwent enrichment in Gram-negative Hanja broth at 37°C for 24 h (BD Bioscience, Franklin Lake, NJ) before plating onto Brilliant Green Agar (**BGA**, BD Bioscience) plates for qualitative determination of *Salmonella spp*. Dietary treatments commenced immediately upon arrival and pigs had *ad libitum* access to water and feed unless otherwise indicated. Pigs were fed a 3-phase nursery diet (**Table 3.1**; phase 1, 0-7 d; phase 2, 7-21 d; phase 3, 21-35 d) after weaning. Pigs were fed either control or XPC diets for 2

wk before oral inoculation with *S.* Typhimurium $Nal^{R}Nov^{R}$ and continued on their respective diets after inoculation.

A corn-soybean meal basal diet was made that exceeded recommendations for nutrients and contained no antibiotics (National Research Council, 1998). From the basal diet, the experimental diet consisted of displacing 0.2% of the corn with XPC. Prior to feeding, all feed ingredients and mixed diets were screened for the presence of *Salmonella* and were negative.

The experimental protocol was designed to simulate an enteric disease outbreak and treatment in a nursery facility after weaning. Thus, pigs were weaned, inoculated, allowed to develop clinical signs of disease, treated with antibiotics, and allowed to recover. The experiment consisted of 3 periods: pre-inoculation (**PRE**; 0-14 d), sick (**SICK**; 14-21 d), and post-inoculation (**POST**; 21-35 d). Pigs and feeders were weighed every 7 d to determine average daily gain (ADG), average daily feed intake (ADFI), and gain: feed ratio. Rectal temperatures (**RT**) were measured weekly during PRE and POST, and every 12 h during SICK. On d 14, conscious pigs were given orally 5 mL of tryptic soy broth (**TSB**) containing 10^{9} colony forming units (**CFU**) of *S.* Typhimurium $Nal^{R}Nov^{R}$ or 5 mL of sterile TSB.

Daily fecal grab samples were collected after inoculation (d 14-21) to determine fecal shedding of *S.* Typhimurium $Nal^{R}Nov^{R}$. On d 17, all pigs were treated daily with 5 mg/kg BW i.m. ceftiofur-HCl for 4 d. On d 35, pigs were euthanized with a lethal dose of 120 mg/kg BW of sodium pentobarbital i.v. (Beuthanasia-D, Schering-Plough, Union, NJ). Carcasses were disposed as regulated medical waste in accordance to university, local, state, and federal regulations.

Enumeration *of S. Typhimurium* Nal^{R}Nov^{R}

During each morning chore, ~ 10 g of feces were collected from each pig daily during d 14-21 using a sterile fecal loop, contents were placed in a sterile filter bag, and immediately processed with 90 ml of buffered peptone water (**BPW**, BD Biosciences) in a stomacher for 2 min to create a fecal slurry. The fecal slurry was then serially diluted and plated, in duplicate, onto BGA plates containing 20 μg/mL nalidixic acid and 25 μg/mL novobiocin. The plates were allowed to air dry and then incubated at 37°C for 24 h. Plates were then inspected for white colonies with red-pink halos, indicative of *Salmonella*. Initial presumptive positive plates were plated onto a second selective media, xylose lysine tergitol 4 (**XLT-4**) agar, the presence of black round colonies was indicative of *S.* Typhimurium Nal^{R}Nov^{R}. The total number of *Salmonella* colonies on each plate was quantified to determine daily shedding rates for each pig. Fecal slurries were then stored at -20°C until fecal DNA could be extracted.

Fecal scores

During the SICK period, fecal scores were determined. Pigs having a watery stool were given a score of 3, semi-solid stool 2, and those with solid stool were scored as 1. All fecal scores were determined by the same person and recorded daily during the morning (0800 to 1000).

Intestinal morphology

Upon euthanasia, intestinal samples were collected for morphology. A tissue sample from the duodenum, jejunum, and ileum (2-3 cm in length) were placed in 15-ml plastic conical tubes containing 10 ml of phosphate-buffered formalin (Fisher Scientific, Fairlawn, NJ). Tissue

sections were sent to a commercial histology laboratory (Histo-Scientfic Reserach Laboratories, Mt. Jackson, VA) for microscope slide preparation and staining. Three random cuts from each tissue section were mounted on microscope slides and stained with Alcian blue and Periodic acid-Schiff. One evaluator per intestinal section was used to obtain morphological data. Evaluators randomly reviewed slides without knowledge of treatments. For each segment sample, four (4) different readings per cut in each of three (3) cuts per microscope slice (i.e., 12 readings per tissue section) were collected. The following endpoints were measured: villus height (μm), villus width (μm), crypt depth (μm), number of goblet cells in the villus perimeter, and number of goblet cells in each crypt. Villus perimeter (**VP**) was calculated as follows: $VP = h \times 2 + w$, where h is villus height and w is villus width. A modified cylinder area equation was used to calculate villus area (**VA**) as follows: $VA = [\pi \times (w \div 2)^2] + (\pi \times w) \times h$; where, w is width of villus and h is height of villus. Data from the three tissue cuts per tissue section were averaged to create a single value for each of the described endpoints.

Community profiling

Product amplification: Fecal DNA was extracted using the UltraClean Fecal DNA kit (Mo Bio Laboratories, Carlsbad, CA) per manufacturer's instructions. Bacterial members of the microbial community were targeted by amplifying the 16S rRNA gene from the total fecal DNA (50 ng/μL) to generate a 566 bp fragment using the primers 341-F (5'-CCT ACG GGA GGC AGC AG-3') and 907-R (5'-CCG TCA ATT CMT TTG AGT TT-3'). The forward primer was modified to add a 40 nucleotide GC clamp at the 5' end (5'-CGC CCG CCG CGC GCG GGC GGG GCG GGG GCA CGG GGG G-3'). Each 25 μL reaction contained 1.5 mM of $MgCl_2$, 50 mM of KCl, 0.2 mM of each dinucleotide, 1% of dimethylsulfoxide (**DMSO**), 25 mM

of Tris-HCl (pH 8), 1 U/μL of HotStart-IT FideliTaq DNA polymerase (USB, Cleveland, OH), 0.5 μM of each primer, and 50 ng of DNA. The PCR protocol consisted of 94°C for 5 min, followed by 19 cycles of: 94°C for 1 min, amplification at 64°C for 1 min (decreasing 1°C every second cycle, touchdown) and elongation at 72°C for 3 min; followed by 9 additional cycles of denaturation at 94°C for 1 min, amplification at 55°C for 1 min, and elongation at 72°C for 3 min; finally 1 cycle of 94°C for 1 min, amplification at 55°C for 1 min, and a final elongation step at 72°C for 10 min. The size and intensity of PCR products were electrophoretically confirmed using 0.9% agarose gels (Fisher-Scientific, Atlanta, GA).

Denaturing Gradient Gel Electrophoresis (**DGGE**) Conditions: The PCR products were run on a 8% polyacrylamide gel in a 30-60% denaturant gradient of urea and formamide [100% denaturant corresponds to 7 M urea plus 40% (vol/vol) of deionized formamide] using the Bio-Rad DCode™ Universal Detection System (Bio-Rad, Hercules, CA). Twenty-two microliters of PCR products were separated at constant voltage of 85 V and temperature of 60°C for 17 hours. The DNA bands were visualized by staining with ethidium bromide (5 μg/mL) and photographed using the Molecular Imager GelDoc XR (Bio-Rad). Two different gels were analyzed for each sample and the number of bands present within each sample determined species richness.

Quantification of Select Gastrointestinal Bacteria: Real-time PCR was performed to quantify the abundance of total bacteria, and bacteria belonging to specific phyla (*Bacteroidetes* and *Firmicutes*), and genera (*Bacteroides* and *Lactobacillus*) in fecal samples obtained from pigs in both inoculums and diets on d 14, 15, 17, 18, (SICK) and 35 (POST).

Standard curves were produced from DNA isolated using the Puregene DNA purification kit (GENTRA Systems, Minneapolis, MN) per manufacturer's instructions. DNA was serially diluted 10-fold from 100-ng/μL to 0.1-ng/μL. Standard curves for real time PCR amplification were prepared using primers (**Table 3.2**) consisting of: 100 ng, 10 ng, 1 ng, and 0.1 ng. Each 25 μL reaction contained a respective amount of DNA template, 12.5 μL of HotStart-IT SYBR Green qPCR Master Mix 2× which contains 5 mM $MgCl_2$ and 0.4 mM of nucleotides (USB, Cleveland, OH), 10 nM of fluorescein as passive reference dye (USB) and 0.5 μM of forward and reverse primers (**Table 3.2**). The PCR conditions were denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing for 30 s at appropriate temperature (**Table 3.2**), and elongation at 72°C for 1 min. Each concentration in the standard curve was done in triplicate using separated plates. Melting curve analysis of the PCR products was conducted following each assay to confirm that the fluorescence signal was originated from specific PCR product. Amplification was carried out with an iQ5 Optical system Real Time PCR detection system (Bio-Rad).

Statistical analysis

Growth performance and RT data were analyzed with the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) as a complete randomized block design in a 2×2 using replicate as a random effect and pig as repeated measures (Kaps and Lamberson, 2004). The model included ADG, ADFI, BW, and RT across the 3 periods (PRE, SICK, and POST). Fecal analysis of *Salmonella* shedding, number of copies of bacterial species (both $\log_{10}$) per g of feces, and fecal score were also determined with PROC MIXED with replicate as a random effect. Least squares means were obtained using Tukey adjustment. The DGGE bands were visualized using

Quantity One-1D analysis software (Bio-Rad) and the DGGE profiles were clustered based on similarity using the un-weighted pair group method with mathematical averages (UPGMA; Dice coefficient of similarity) using Gelcompare II (Applied Maths, TX) and reported as dendrograms.

RESULTS

Rectal temperature, fecal score, and Salmonella shedding

All pigs were negative for *Salmonella* presence in feces before inoculation. Furthermore, pigs that received sterile broth never shed *Salmonella* at a detectable limit or developed a febrile response. These results clearly indicate that non-infected pigs remained free of detectable *Salmonella* or any other pathogen that would have triggered a systemic inflammatory response. Experimental infection with *Salmonella* resulted in a marked increase ($P < 0.001$) in RT during SICK (**Figure 3.1**). This febrile response is a clear indication that pigs were clinically sick and had developed a systemic immune response. A linear reduction in RT of *Salmonella*-infected pigs was observed from d 17 (start of i.m. ceftiofur) to the end of antibiotic treatment on d 21. Inclusion of XPC in the diet tended ($P < 0.10$) to increase *Salmonella* shedding in feces (**Figure 3.2**). Inoculation with *Salmonella* increased ($P < 0.001$) fecal scores (i.e., less firm stool), however, diarrheic episodes were not observed. Inclusion of XPC had no effect on the average fecal score ($P = 0.21$, **Figure 3.3**). .

Growth performance

Inclusion of XPC had no effect on ADG, Gain:Feed ratio, BW, or RT during the PRE period (**Table 3.3**). During this period, however, pigs consuming XPC tended ($P = 0.06$) to

reduce ADFI that resulted in numerical improvement (5%) in Gain:Feed ratio. Inoculation with *Salmonella* drastically reduced ($P < 0.001$) ADG, ADFI, and BW of pigs compared to non-inoculated pigs. Gain:Feed ratio tended ($P = 0.07$) to be lower in *Salmonella*-infected pigs mainly due to a numeric reduction in pigs consuming control diet. During the recovery (i.e., POST) period, there was a diet×inoculation interaction ($P < 0.01$) indicating that infected pigs consuming XPC gained more weight than infected pigs consuming the control diet. Overall tendency interactions ($P < 0.10$) for Gain: Feed ratio and RT, indicate an advantage of consuming XPC. Growth performance was modestly correlated ($P < 0.002$) with fecal shedding of *Salmonella* during SICK: BW (r = -0.22), ADFI (r = -0.27), ADG (r = -0.36), and Gain: Feed ratio (r = -0.18). These negative correlations suggest that increased fecal shedding of *Salmonella* is partially associated with poorer growth performance.

Intestinal morphology

Intestinal measurements were taken 2 wk after pigs stopped shedding *Salmonella* in feces and had clinically recovered from infection. The numbers of goblet cells present in the duodenal vili crypt were reduced ($P < 0.04$) in pigs previously experiencing a clinical response to *Salmonella* despite a 2 wk period of recovery. Furthermore, the jejunum villus height of pigs that recovered from *Salmonella* infection were reduced in pigs fed XPC, however the villi height of non-infected pigs fed XPC increased. Diet×inoculation interactions ($P < 0.10$) were observed for duodenum villus height ($P <0.003$) and for doudenum villus/crypt ratio (**VCR**) ($P < 0.10$) pointing to markedly different effect of XPC inclusion in healthy pigs compared to recovered from a previous infection with *Salmonella*. In the jejunum, however, a diet×inoculation interaction ($P < 0.06$) shows a beneficial effect of XPC consumption on villus area in pigs with

resolved *Salmonella* infections. In the ileum, the main site of *Salmonella* infection, XPC tended to reduced crypt depth ($P < 0.07$), number of goblet cells per villus ($P < 0.11$), and villus area ($P < 0.11$) in pigs recovered from a previous infection with *Salmonella*. Overall, it appears that inclusion of XPC has diverse effects on the intestinal morphology of the small intestinal tract.

Denaturing gradient gel electrophoresis

The overall total species richness, determined by the number of DGGE bands, was not different between the pigs fed a control diet and those fed XPC. However, noticeable shifts in community composition and apparent abundance were apparent by band position and intensity. Several members, indicated by band pattern, are present within both diets representing stable community members. The number of bands in the DGGE profiles varied from 16-20 for fecal samples (results not shown). The similarity indices between individual animals consuming the same diet ranged from 65-70%. Infection of the pigs with *Salmonella* resulted in a shift in composition of the fecal community in samples 1 d post-infection. The DGGE profiles of feces from infected vs. non-infected pigs clustered individually and were only 30-40% similar to each other (**Figure 3.4**). In pigs challenged with *Salmonella* the groupings of the individual pigs were more similar with d 14 and 15 clustered together, yet distinctly different from d 18, with the exception of Pig 21 (**Figure 3.4**). Marked shifts in the species richness of all pigs occur at d 18, which corresponds to 1 d after administration of the antibiotic, ceftiofur-HCl.

Bacterial populations

Over the entire trial, inclusion of XPC in the diet increased ($P < 0.001$, log transformed data) the number of copies in feces of *Bacteroides* by 2.6 fold (**Figure 3.5**, 3.3×10^5 for control vs. 8.5×10^5 for XPC) and *Lactobacillus spp.* by 3.5 fold (**Figure 3.8**, 3.3×10^7 for control vs. 1.2×10^8 for XPC), while reducing *Firmicutes* by 50% (**Figure 3.6**, 1.2×10^{10} for control vs. 5.7×10^9 for XPC). In addition, a diet×inoculation ($P < 0.001$) interaction resulted in a 2.8-fold increase ($P < 0.01$) in *Bacteroidetes* copies in *Salmonella*-infected pigs consuming XPC (**Figure 3.7**, 2.2×10^6 for control vs. 6.2×10^6 for XPC). Prior to inoculation, there was no difference in total bacteria abundance (**Figure 3.9**). During SICK, consumption of XPC increased ($P < 0.01$) the populations of *Bacteroides*, *Bacteroidetes*, and *Lactobacillus* (**Table 3.5**). A tendency ($P < 0.07$) for diet×inoculation interaction during POST suggest a greater increase in total bacterial copies in the feces of *Salmonella*-infected pigs consuming XPC compared to infected animals eating the control diet (**Table 3.5**).

DISCUSSION

Prebiotic compounds, offer an attractive alternative to the use of antibiotic growth promoters. Growth promotion associated with prebiotics is believed to result from enhanced energy gained by the fermentation of these compounds within the lower GIT allowing the host animal to generate muscle mass, effectively producing a desirable market weight (Branner and Roth-Maier, 2006). Other health benefits such as stimulation of intestinal mobility and mineral absorption, elimination of ammonium, direct stimulation of the immune system, and the inhibition of toxin binding are associated with host/prebiotic synergy (Macfarlane et al., 2008). However, the greatest protection against pathogenic bacterial infections are achieved by

stimulating GIT bacteria to produce short chain fatty acids that are inhibitory to some pathogens and increase in numbers, therefore reducing attachment sites for pathogens on the intestinal mucosa (Niba et al., 2009).

Prebiotics act by stimulating diverse communities of microorganisms to colonize the gastrointestinal tract. Culture independent analyses reveal that inclusion of prebiotic compounds significantly alters the abundance of key members of the fecal microbial flora but not the overall species richness. Prebiotic compounds have been previously shown to increase the composition of beneficial bacteria, such as *Bifidobacterium* and *Lactobacillus,* in the colon of humans in mice (Tzortzis et al., 2005). Few studies, have characterized changes to microbial communities of swine fed prebiotic diets. In this study, we show that the inclusion of a commercial prebiotic dietary supplement, XPC, containing mannanoligosaccharides and β-glucans results in an increase in the number of copies for *Bacteroides* and *Lactobacillus* present in the feces of pigs compared to controls ($P < 0.005$). Increased amounts of β-glucans contained within XPC have been shown to increase the digesta retention time in the small intestine, affecting the digestibility of other nutrients, particularly protein and starch, and increasing the flow into the large intestine (Leterme et al., 2000). Inclusion of mixed-linked β-glucans in the diet of rats (Snart et al., 2006) and pigs (Pieper et al., 2008) corresponded with increased population of *Lactobacillus* (Jonsson and Hemmingsson, 1991), which conforms with the findings of this study.

The majority of the swine fecal microbiota (70%) is dominated by members of the *Firmicutes* (including *Clostridium* spp., *Lactobacillus* spp., and *Streptococcus* spp), while *Bacteroidetes* averaged about 9% of the total microbiota (Dowd et al., 2008; Guo et al., 2008b). The ability to stimulate certain members of these populations is associated with increased weight gain. In mice, increased numbers of *Firmicutes* have been associated with increased obesity

(Ley et al., 2005). In contrast, increased fecal populations of *Bacteroides* and *Bacteroidetes* have been negatively correlated with obesity in humans, mice (Ley et al., 2005; Turnbaugh et al., 2009), and increased backfat thickness in pigs (Guo et al., 2008a). By stimulating select members of this microbiota, it may be possible to improve the recovery of sick animals, while maintain the optimum ratio of muscle to fat. In the present study, *Salmonella* infected pigs were associated with lower populations of *Firmicutes* and elevated populations of *Bacteroidetes,* which was associated with decreased ADG. An increase in *Lactobacillus sp,* but not all members of the *Firmicutes* as well as increases in beneficial *Bacteroidetes may* be responsible for th*e* improved the growth performance of pigs consuming XPC after a challenge with *Salmonella* compared to those consuming the control diet.

Increased abundance of efficient fermenters such as *Lactobacillus*, likely resulted in increased growth performance in the post illness period. However, increased numbers of *Firmicutes* and *Bacteroides* was also associated with pigs inoculated pigs fed the XPC diet during the sick period. This may be due to the ability of these bacteria to produce sort chain fatty acids and bind to mucosal surfaces. Isolates of *Lactobacillus* have been identified that are able to inhibit the invasion of tissue culture cells by enteric pathogens such as *Salmonella enterica* and *E. coli* O157:H7 *in vitro* (Casey et al., 2004; Silva et al., 2004). *Bifidobacter lactis* and *Lactobacillus rhamnosous* cultures, resulted in decreased adherence of *Salmonella, E. coli* and *Clostridium* to intestinal mucosa (Collado et al., 2007). Weanling pigs supplemented with a mixture of probiotic bacteria (3 species of *Lactobacillus* and *Pediococcus* sp), subsequently challenged with *Salmonella* Typhimurium showed reduced incidence, shedding and duration of diarrhea (Casey et al., 2007). It is possible that the prebiotic compound itself possesses antimicrobial activity. XPC consists of fermented yeast metabolites of *Saccharomyces*

cervesiae. Extracts from *Saccharomyces cerevisiae* were associated with growth inhibition of *Candida tropicalis* and *Escherichia coli* in vitro, likely due to competition for nutrients (Jensen et al., 2008). Soluble compounds may also be increasing the composition of other members of the microbial community not considered in this study. Microbial communities of fecal samples appear significantly affected by the presence of *Salmonella*, as indicated by DGGE analysis. Similarity indexes of the DGGE profiles consider both alterations to abundance, as indicated by band intensity, and species richness, as indicated by band location. These differences may be due to alterations in the members of other phylogenetic groups that were not considered in this study. Enteric salmonellosis has been reported to alter the microbial ecology, specifically increasing the numbers of *Clostridia*, of the murine gastrointestinal tract preceding the onset of diarrhea, suggesting the involvement of pathogen-commensal interactions and/or host responses unrelated to diarrhea (Barman et al., 2008).

Changes in bacterial populations and intestinal morphology could be acting concomitantly to enhance the recovery of pigs experimentally infected with *Salmonella*. The tendency to increase *Salmonella* shedding when consuming XPC can be interpreted to suggest a rapid elimination of the pathogen from the gastrointestinal systems, which may result in lower infection rates and enhanced clearance of the pathogen (Bovee-Oudenhoven et al., 2003). The choice of a Nalidixic acid and Novobiocin resistant strain was to greatly reduce the possibility of detecting *Salmonella* that were not introduced in this experiment. Pigs infected with *Salmonella* in the present study were febrile for 5.5 d after inoculation (i.e., 2.5 d after i.m. treatment ceftiofur-HCl) compared to 1-3 d in a similar study (Burkey et al., 2004). This comparison suggests that the stain of *Salmonella* Typhimurium used in the present study appears to be more pathogenic to pigs. This observation is important to consider because pigs did not naturally

recover from the disease and instead received treatment with an antibiotic *Salmonella* was specifically tested to be sensitive. Yet, it took 2.5 d of i.m. antibiotic treatment to return RT to PRE levels and 3 d to eliminate shedding in feces. Taking into account the beneficial changes in bacterial populations induced by XPC intake during SICK, we hypothesized that inclusion of XPC may have a stronger effect on growth performance in animals suffering from longer infections than the one used in this study. In other words, inclusion of *Saccharomyces cerevisiae* fermentation products in commercial diets for pigs may enhance the growth performance of pigs since they are more likely to naturally clear infections or have chronic exposure to pathogens.

IMPLICATIONS

Inclusion of *Saccharomyces cerevisiae* fermentation product to the diets of weaning pigs can result in greater compensatory gains after infection with *Salmonella* than pigs fed conventional nursery diets. Prebiotics such as XPC may enhance the recovery of animals after an infection by altering gastrointestinal morphology and by maintaining or enhancing the populations of beneficial bacteria within the gastrointestinal tract, which may in turn contribute to improve the lean growth of pigs.

Table 3.1. Composition of basal diet, as fed basis

Item	Phase 1	Phase 2	Phase 3
Ingredient, %			
Ground corn[1]	39.73	58.00	64.41
Soybean meal, 47.5% CP	20.00	28.25	30.40
Soy protein concentrate[2]	4.00	- - -	- - -
Fish meal	8.00	4.60	- - -
Dried whey	25.00	5.00	- - -
Soy oil	2.00	1.80	2.00
Limestone, ground	0.28	0.60	0.80
Dicalcium phosphate	- - -	0.70	1.13
Salt	0.30	0.30	0.30
Vitamin premix[3]	0.25	0.25	0.25
Trace mineral premix[4]	0.15	0.15	0.15
L-Lys-HCl %	0.20	0.25	0.29
L-Threonine	0.07	0.10	0.20
DL-Methionine	0.02	- - -	0.07
Calculated composition			
ME, kcal/kg	3,420	3,419	3,422
CP, %	23.7	22.0	20.2
Lys, %	1.64	1.47	1.31
Met, %	0.44	0.38	0.38
Thr, %	1.05	0.95	0.95
Trp, %	0.28	0.26	0.24
Arg, %	1.40	1.38	1.30
Ca, %	0.82	0.78	0.68
Available P, %	0.47	0.37	0.28

[1]XPC added at the expense of corn.
[2]Soycomil-P, ADM, Decatur, IL.
[3]Provided the following per kilogram of diet: vitamin A, 7,335 IU; vitamin D_3, 1,010 IU; vitamin E, 42 IU; menadione (as menadione sodium bisulfite complex), 3.3 mg; riboflavin, 6.4 mg; D-pantothenic acid, 21 mg; niacin, 31 mg; vitamin B_{12}, 27.6 μg; D-biotin, 0.3 mg; choline, 550 mg; folic acid, 1.4 mg.
[4]Provided the following per kilogram of diet: Zn, 180 mg; Fe, 180 mg; Mn, 55 mg; Cu, 11 mg; I, 0.5 mg; and Se, 0.3 mg.

Table 3.2. Primer sets used to quantify abundance of key members of swine microbial community using real-time PCR.

Genera of Interest	Primer sequence	Species Amplified	Annealing Temperature, °C	Reference
Bacteroides	Forward: AllBac296F, 5'-GAGAGGAAGGTCCCCCAC-3' Reverse: AllBac412R, 5'-CGCTACTTGGCTGGTTCAG-3'	*Bacteroides thetaiotaomicron*, *Eubacterium rectale*	60.0	Layton et al., 2006
Bacteroidetes	Forward: Bact934F, 5'-GGARCATGTGGTTTAATTCGATGAT-3' Reverse: Bact1060R, 5'-AGCTGACGACAACCATGCAG-3'	*Flavobacteria*	60.0	Guo et al., 2008
Firmicutes	Forward: 5'- GCA GTA GGG AAT CTT CCG-3' Reverse: 5'- ATTACCGCGGCTGCTGG-3'	*Lactobacillus acidophilus*	60.0	Fierer et al., 2005
Lactobacillus	Forward:5'-AGAGGTAGTAACTGGCCTTTA-3' Reverse:5'-GCGGAAACCTCCCAACA-3'	*Lactobacillus acidophilus*	58.5	Malinen et al., 2003
Total Bacteria	Forward:5'-ACTCCTACGGGAGGCAGCAG-3' Reverse:5'-ATTACCGCGGCTGCTGG-3'		60.0	Lane, 1991; Muyzer et al., 1993

Table 3.3. Growth performance and rectal temperature of pigs consuming post-weaning diets without (control) or with (XPC) *Saccharomyces cerevisiae* fermentation product and orally gavaged with *Salmonella* Typhimurium $Nal^{R}Nov^{R}$ or sterile broth.

	Broth		*Salmonella*			P-value		
	Control	0.2% XPC	Control	0.2% XPC	SEM	Inoc.	Diet	Inoc.×Diet
Pre-inoculation[1]								
ADG, kg	0.087	0.104	- - -	- - -	0.015	- - -	0.4166	- - -
ADFI, kg	0.394	0.295	- - -	- - -	0.037	- - -	0.0610	- - -
Gain: Feed ratio	0.236	0.248	- - -	- - -	0.242	- - -	0.5685	- - -
BW, kg	7.48	7.60	- - -	- - -	0.129	- - -	0.4947	- - -
Rectal temperature, °C	39.34	39.45	- - -	- - -	0.05	- - -	0.1499	- - -
Sick[2]								
ADG, kg	0.478	0.485	0.285	0.272	0.042	0.0001	0.9499	0.8167
ADFI, kg	0.939	0.826	0.668	0.641	0.045	0.0001	0.1229	0.3395
Gain: Feed ratio	0.554	0.590	0.233	0.529	0.106	0.0728	0.1190	0.2227
BW, kg	9.79	10.04	8.66	8.94	0.187	0.0001	0.1555	0.9236
Rectal temperature, °C	39.49	39.60	40.15	40.15	0.04	0.0001	0.1899	0.2043
Post-sick[3]								
ADG, kg	0.603	0.476	0.559	0.592	0.030	0.2292	0.1225	0.0094
ADFI, kg	1.08	1.11	0.879	0.938	0.047	0.0001	0.3405	0.7638
Gain: Feed ratio	0.599	0.466	0.715	0.734	0.058	0.0013	0.3299	0.1911
BW, kg	15.88	15.78	13.97	14.26	0.383	0.0001	0.8025	0.6184
Rectal temperature, °C	39.73	39.85	39.92	39.73	0.07	0.6028	0.5488	0.0198
Overall								
ADG, kg	0.441	0.422	0.316	0.323	0.028	0.0001	0.8184	0.6329
ADFI, kg	0.898	0.816	0.657	0.651	0.031	0.0001	0.1604	0.2251
Gain: Feed ratio	0.577	0.545	0.344	0.614	0.091	0.3723	0.1936	0.0992
BW, kg	10.77	10.86	9.65	9.94	0.19	0.0001	0.3106	0.6072
Rectal temperature, °C	39.49	39.60	40.01	39.98	0.03	0.0001	0.2290	0.0672

[1]From weaning to before pigs were orally gavage with sterile broth or 10^{9} CFU *S.* Typhimurium $Nal^{R}Nov^{R}$ on d 14 post-weaning.
[2]Day 14 to 21 post-weaning. All pigs were treated with 5 mg/kg BW i.m. ceftiofur-HCl on d 17-20.
[3]Day 21 to 35 post-weaning.

Table 3.4. Small intestinal morphology of pigs consuming post-weaning diets without (control) or with (XPC) *Saccharomyces cerevisiae* fermentation product and orally gavaged with *Salmonella* Typhimurium $Nal^{R}Nov^{R}$.

	Broth		*Salmonella*			P-value		
	Control	0.2% XPC	Control	0.2% XPC	SEM	Inoc.	Diet	Inoc.×Diet
Duodedum								
Villus height, μm	539.0	616.7	609.6	539.1	23.3	0.8819	0.8762	0.0031
Crypt depth, μm	374.6	355.9	369.5	356.7	15.1	0.8855	0.3047	0.8491
Villus/Crypt ratio	1.47	1.76	1.68	1.63	0.10	0.7065	0.2595	0.0982
Villus width, μm	148.0	140.6	134.1	139.7	4.5	0.1066	0.8375	0.1589
No. goblet cell/villus	24.9	29.5	24.3	22.9	2.4	0.1431	0.5213	0.2314
Villus goblet, No./μm	0.021	0.020	0.017	0.018	0.001	0.0959	0.9457	0.6344
No. goblet cell/crypt	20.3	20.6	18.7	17.3	1.2	0.0435	0.6412	0.4627
Crypt goblet, No./μm	0.055	0.058	0.051	0.049	0.003	0.0645	0.9545	0.4409
Villus area, mm^2	0.267	0.288	0.286	0.251	0.015	0.5738	0.6323	0.0621
Jejunum								
Villus height, μm	478.9	456.7	471.6	499.0	16.5	0.2962	0.8762	0.1438
Crypt depth, μm	304.8	300.8	294.1	321.9	10.3	0.6157	0.2575	0.1322
Villus/Crypt ratio	1.59	1.58	1.61	1.57	0.08	0.9829	0.7992	0.8805
Villus width, μm	136.4	130.5	125.8	132.0	2.8	0.1155	0.9584	0.0400
No. goblet cell/villus	20.3	21.6	21.6	19.0	2.2	0.7708	0.7799	0.3865
Villus goblet, No./μm	0.019	0.020	0.018	0.018	0.001	0.2649	0.5315	0.6143
No. goblet cell/crypt	21.6	23.3	22.9	24.6	1.4	0.3451	0.2091	0.9959
Crypt goblet, No./μm	0.075	0.078	0.072	0.072	0.004	0.3210	0.6790	0.7997
Villus area, mm^2	0.219	0.200	0.203	0.221	0.009	0.7910	0.9577	0.0564
Ileum								
Villus height, μm	421.6	421.2	466.4	426.1	17.5	0.1559	0.2447	0.2527
Crypt depth, μm	305.0	277.8	295.3	286.0	9.4	0.9392	0.0619	0.3498
Villus/Crypt ratio	1.43	1.55	1.58	1.47	0.07	0.6606	0.9991	0.1163
Villus width, μm	140.9	136.3	132.7	128.9	2.9	0.0120	0.1565	0.8862

No. goblet cell/villus	24.1	22.7	26.6	21.5	2.0	0.7492	0.1075	0.3506
Villus goblet, No./μm	0.025	0.023	0.024	0.022	0.002	0.7610	0.2256	0.8111
No. goblet cell/crypt	23.1	19.9	21.8	22.0	1.4	0.7865	0.2777	0.2261
Crypt goblet, No./μm	0.076	0.071	0.077	0.075	0.004	0.5283	0.3629	0.8129
Villus area, mm^2	0.198	0.196	0.208	0.185	0.007	0.9822	0.1020	0.1649

Table 3.5. Overall LS mean comparisons of Diet * Inoculum interactions on fecal microbiota abundance.

Inoculum	Diet	Inoculum	Diet	P-value				
				Bacteroides	***Firmicutes***	***Bacteroidetes***	***Lactobacillus***	**Total Bacteria**
Broth		*Salmonella*		0.2679	0.0002	0.5195	0.0068	0.0099
	Control		XPC	0.0011	0.0077	0.5752	0.0077	0.4246
Broth	Control	Broth	XPC	0.1722	<0.0001	0.0011	0.0402	0.8510
Broth	Control	*Salmonella*	Control	0.8083	<0.0001	0.0187	0.0275	0.0102
Broth	Control	*Salmonella*	XPC	0.0018	<0.0001	0.9527	0.0002	0.1977
Broth	XPC	*Salmonella*	Control	0.1166	0.4289	0.3949	0.9764	0.0173
Broth	XPC	*Salmonella*	XPC	0.0750	0.4710	0.0005	0.0959	0.2724
Salmonella	Control	*Salmonella*	XPC	0.0010	0.9316	0.0118	0.0812	0.1904

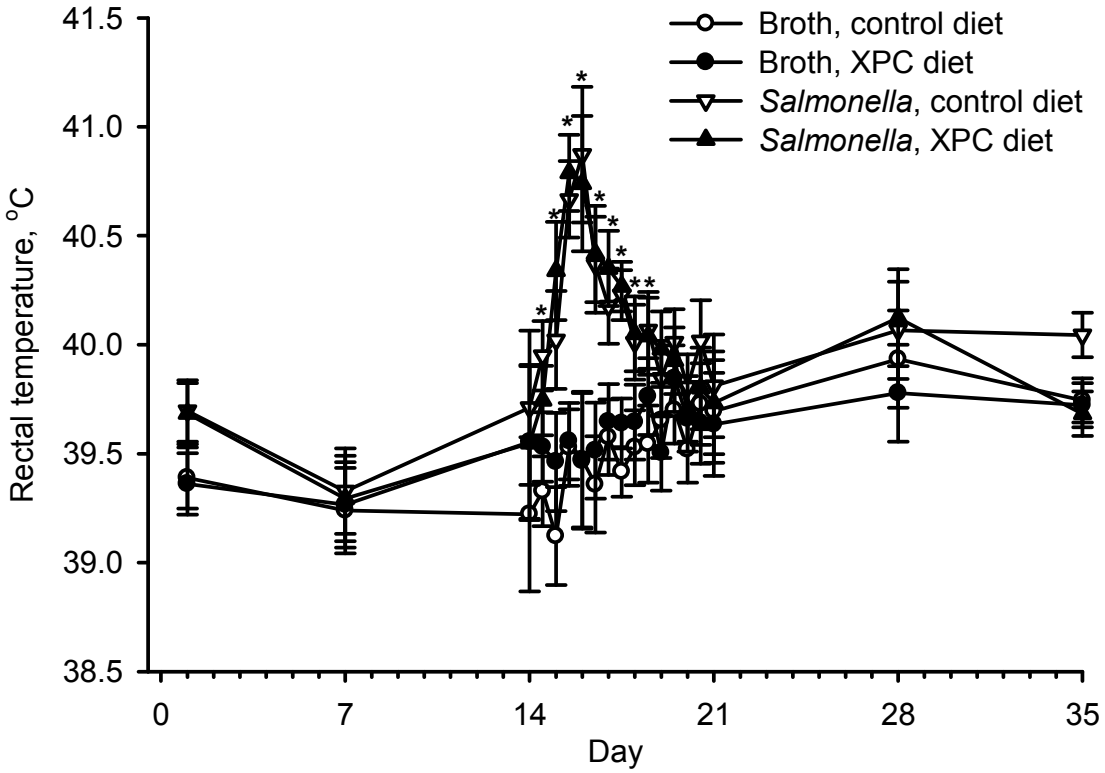

Figure 3.1. Effect of infection with *Salmonella* and consumption of XPC on rectal temperature. From weaning (d = 1) pigs had ad libitum access to a nursery diet with (**XPC**) or without (**Control**) 0.2% XPC inclusion. Pigs were inoculated with 5 mL containing 10^9 CFU *S.* Typhimurium $Nal^R Nov^R$ (***Salmonella***) on d 14 after weaning or received 5 mL of sterile broth (**Broth**). Pigs were treated daily with 10 mg/kg BW i.m. ceftiofur-HCl on d 17 through 20. Inoculation with *Salmonella* increased rectal temperature (* $P < 0.01$) on d 14.5 through 19 compared to non-infected controls.

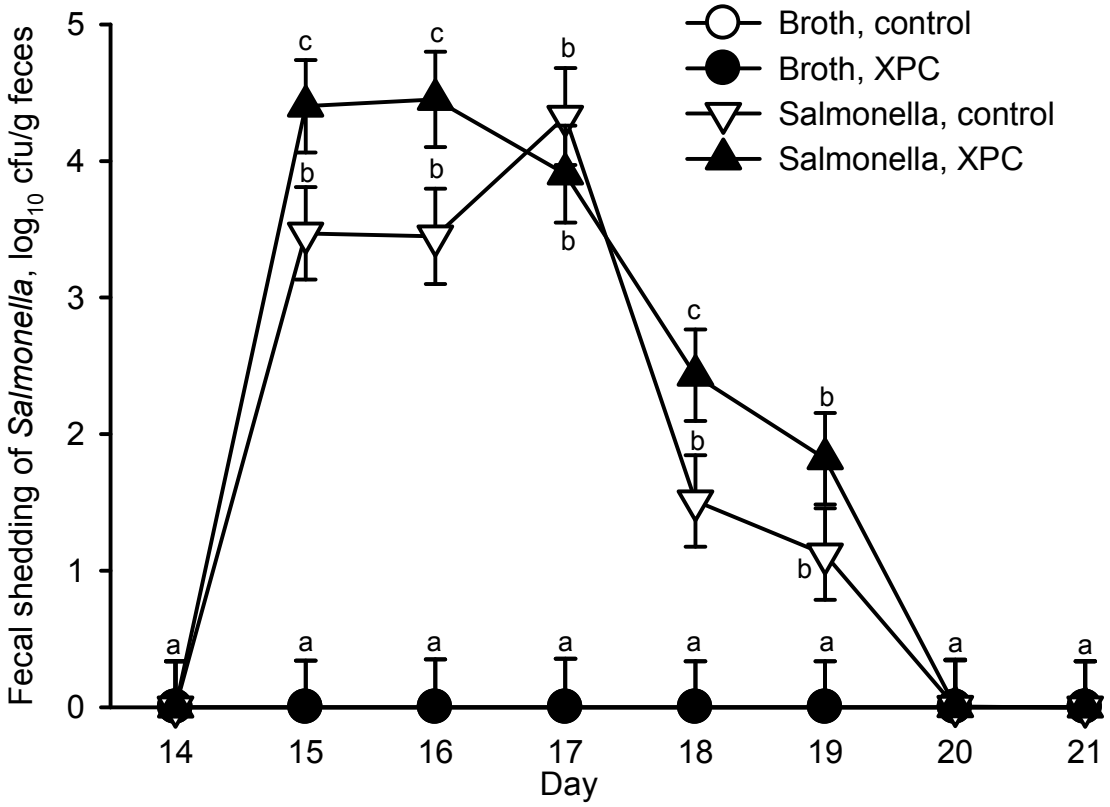

Figure 3.2. Effect of infection with *Salmonella* and consumption of XPC on fecal shedding of *Salmonella*. From weaning (d = 1) pigs had ad libitum access to a nursery diet with (**XPC**) or without (**Control**) 0.2% XPC inclusion. Pigs were inoculated with 5 mL containing 10^9 CFU *S.* Typhimurium $Nal^R Nov^R$ (***Salmonella***) on d 14 after weaning or received 5 mL of sterile broth (**Broth**). Pigs were treated daily with 10 mg/kg BW i.m. ceftiofur-HCl on d 17 through 20. Different letters corresponds to significance ($P <$ 0.5) among treatments within d.

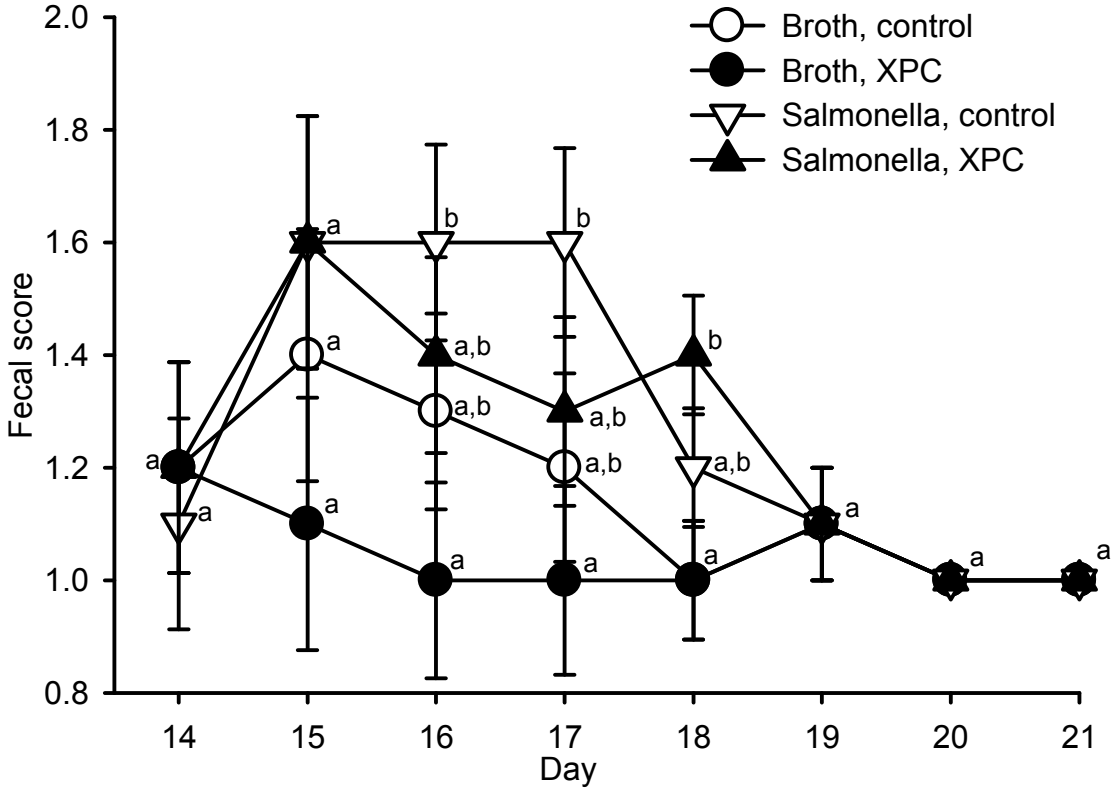

Figure 3.3. Effect of infection with *Salmonella* and consumption of XPC on fecal score. From weaning (d = 1) pigs had ad libitum access to a nursery diet with (**XPC**) or without (**Control**) 0.2% XPC inclusion. Pigs were inoculated with 5 mL containing 10^9 CFU *S.* Typhimurium $Nal^R Nov^R$ (***Salmonella***) on d 14 after weaning or received 5 mL of sterile broth (**Broth**). Pigs were treated daily with 10 mg/kg BW i.m. ceftiofur-HCl on d 17 through 20. Pigs having a watery stool were given a score of 3, semi-solid stool a 2, and those with solid stool were scored as a 1. Values not sharing common letters indicate significance ($P < 0.05$) within day.

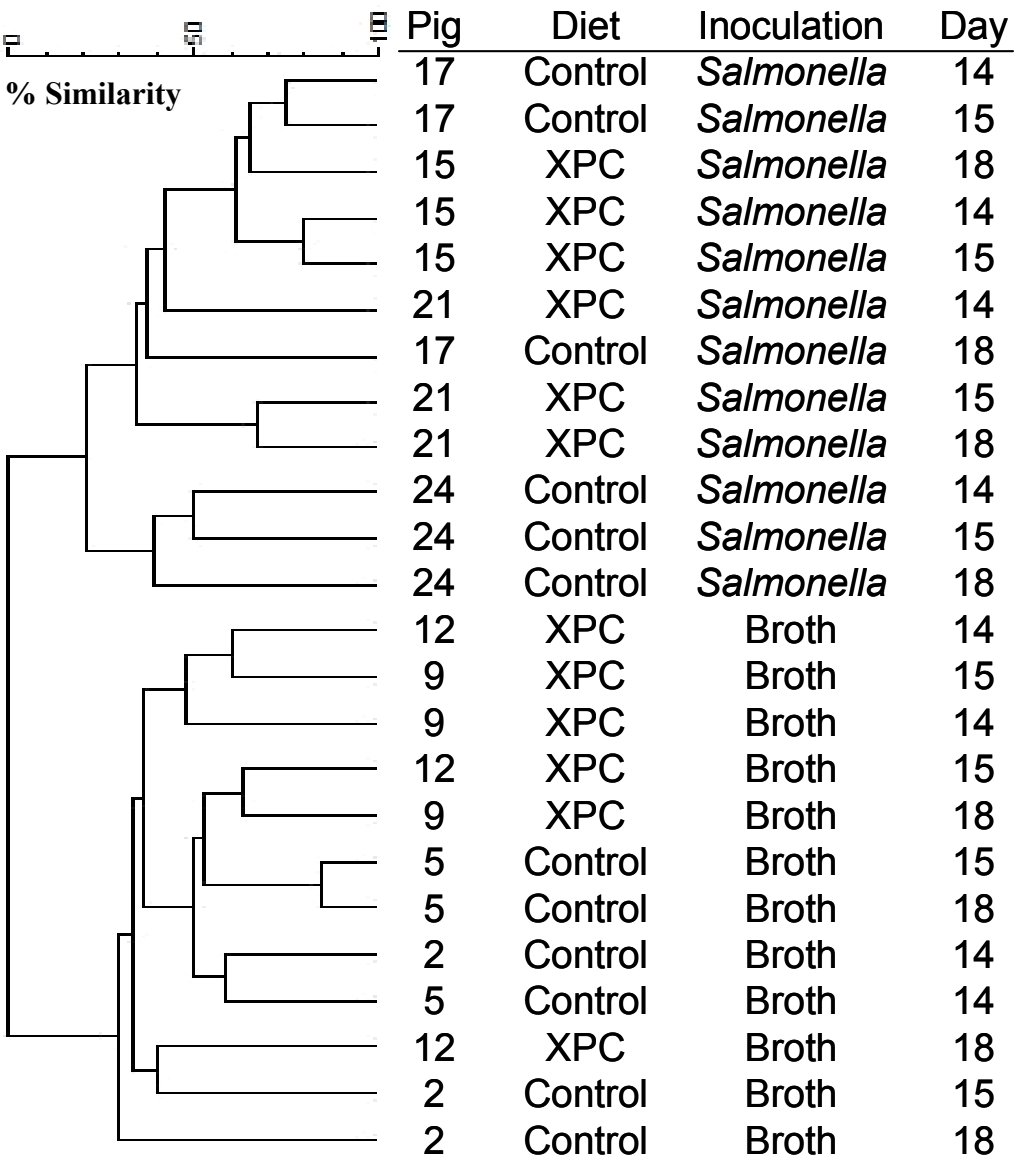

Figure 3.4. Effect of infection with *Salmonella* and consumption of XPC on fecal species richness determined by denaturing gradient gel electrophoresis. From weaning (d = 1) pigs had ad libitum access to a nursery diet with (**XPC**) or without (**Control**) 0.2% XPC inclusion. Pigs were inoculated with 5 mL containing 10^9 CFU *S.* Typhimurium $Nal^R Nov^R$ (***Salmonella***) on d 14 after weaning or received 5 mL of sterile broth (**Broth**).

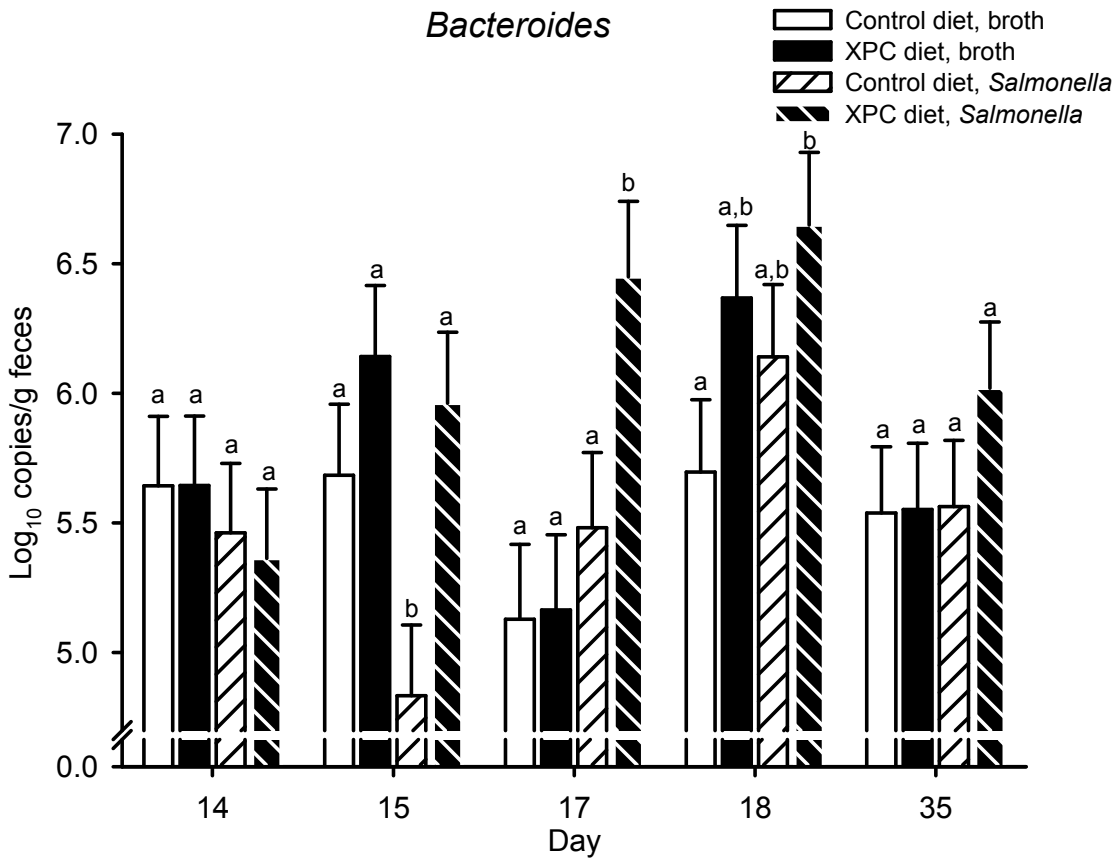

Figure 3.5. Effect of infection with *Salmonella* and consumption of XPC on the number of *Bacteroides* copies (log_{10}) determined by QRT-PCR from fecal samples. From weaning (d = 1) pigs had ad libitum access to a nursery diet with (**XPC**) or without (**Control**) 0.2% XPC inclusion. Pigs were inoculated with 5 mL containing 10^9 CFU *S.* Typhimurium $Nal^R Nov^R$ (***Salmonella***) on d 14 after weaning or received 5 mL of sterile broth (**Broth**). Values not sharing common letters indicate significance (P <0.05) within day.

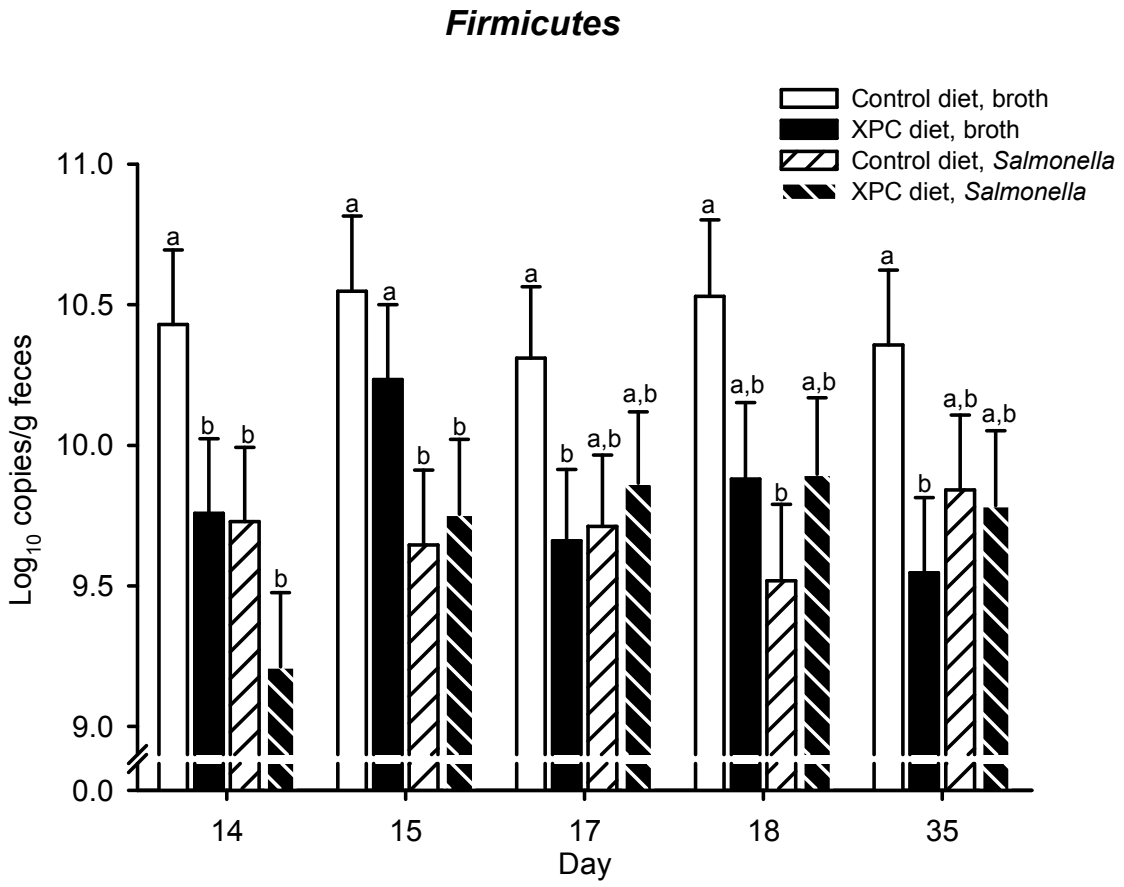

Figure 3.6. Effect of infection with *Salmonella* and consumption of XPC on the number of *Firmicutes* copies (log_{10}) determined by QRT-PCR from fecal samples. From weaning (d = 1) pigs had ad libitum access to a nursery diet with (**XPC**) or without (**Control**) 0.2% XPC inclusion. Pigs were inoculated with 5 mL containing 10^9 CFU *S.* Typhimurium $Nal^R Nov^R$ (***Salmonella***) on d 14 after weaning or received 5 mL of sterile broth (**Broth**). Values not sharing common letters indicate significance (P <0.05) within day.

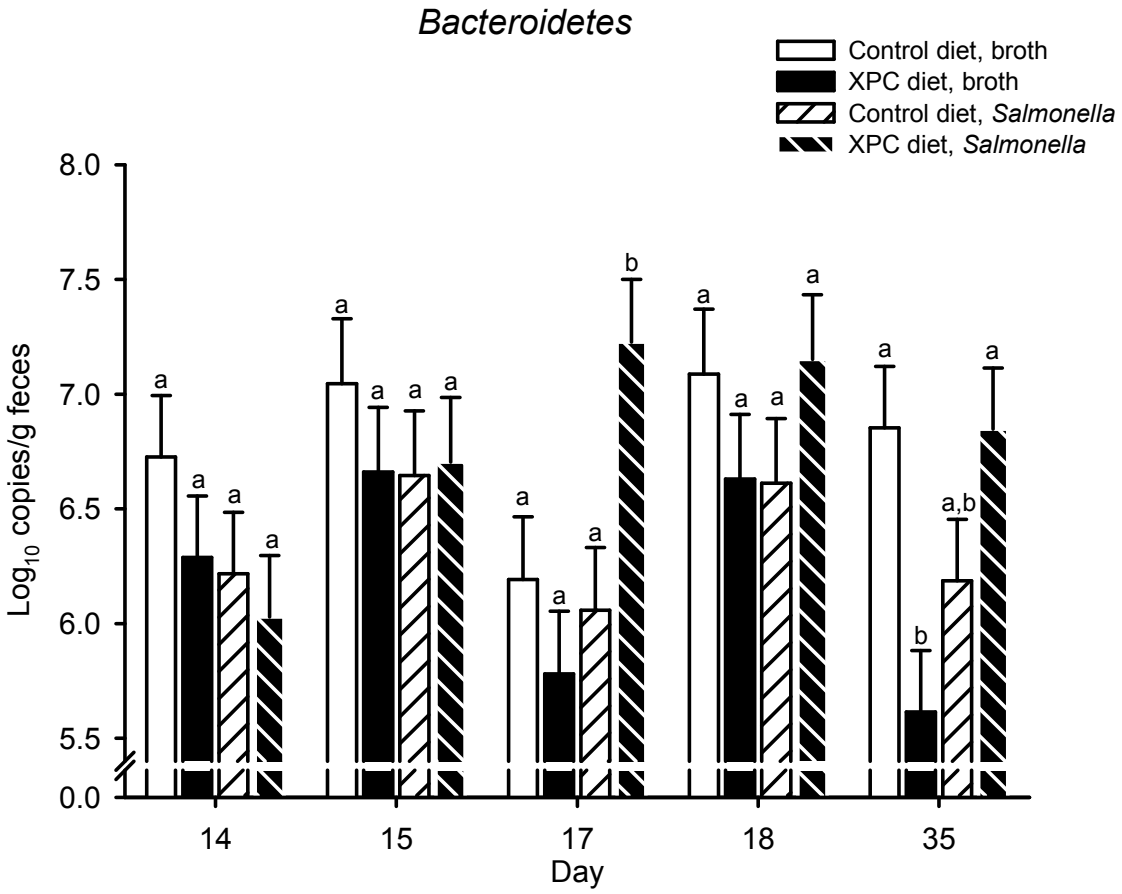

Figure 3.7. Effect of infection with *Salmonella* and consumption of XPC on the number of *Bacteroidetes* copies (log_{10}) determined by QRT-PCR from fecal samples. From weaning (d = 1) pigs had ad libitum access to a nursery diet with (**XPC**) or without (**Control**) 0.2% XPC inclusion. Pigs were inoculated with 5 mL containing 10^9 CFU *S.* Typhimurium $Nal^R Nov^R$ (***Salmonella***) on d 14 after weaning or received 5 mL of sterile broth (**Broth**). Values not sharing common letters indicate significance (P <0.05) within day.

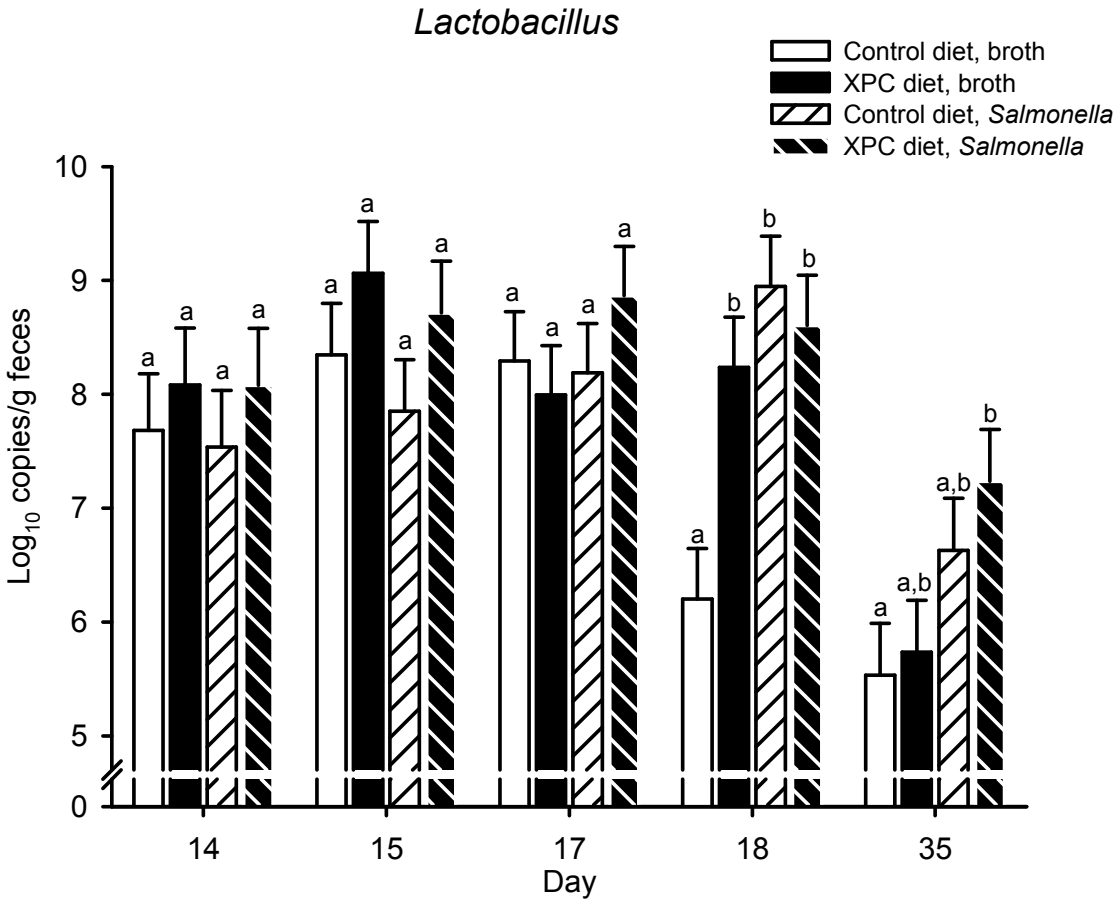

Figure 3.8. Effect of infection with *Salmonella* and consumption of XPC on the number of *Lactobacillus* copies (log_{10}) determined by QRT-PCR from fecal samples. From weaning (d = 1) pigs had ad libitum access to a nursery diet with (**XPC**) or without (**Control**) 0.2% XPC inclusion. Pigs were inoculated with 5 mL containing 10^9 CFU *S.* Typhimurium $Nal^R Nov^R$ (***Salmonella***) on d 14 after weaning or received 5 mL of sterile broth (**Broth**). Values not sharing common letters indicate significance (P <0.05) within day.

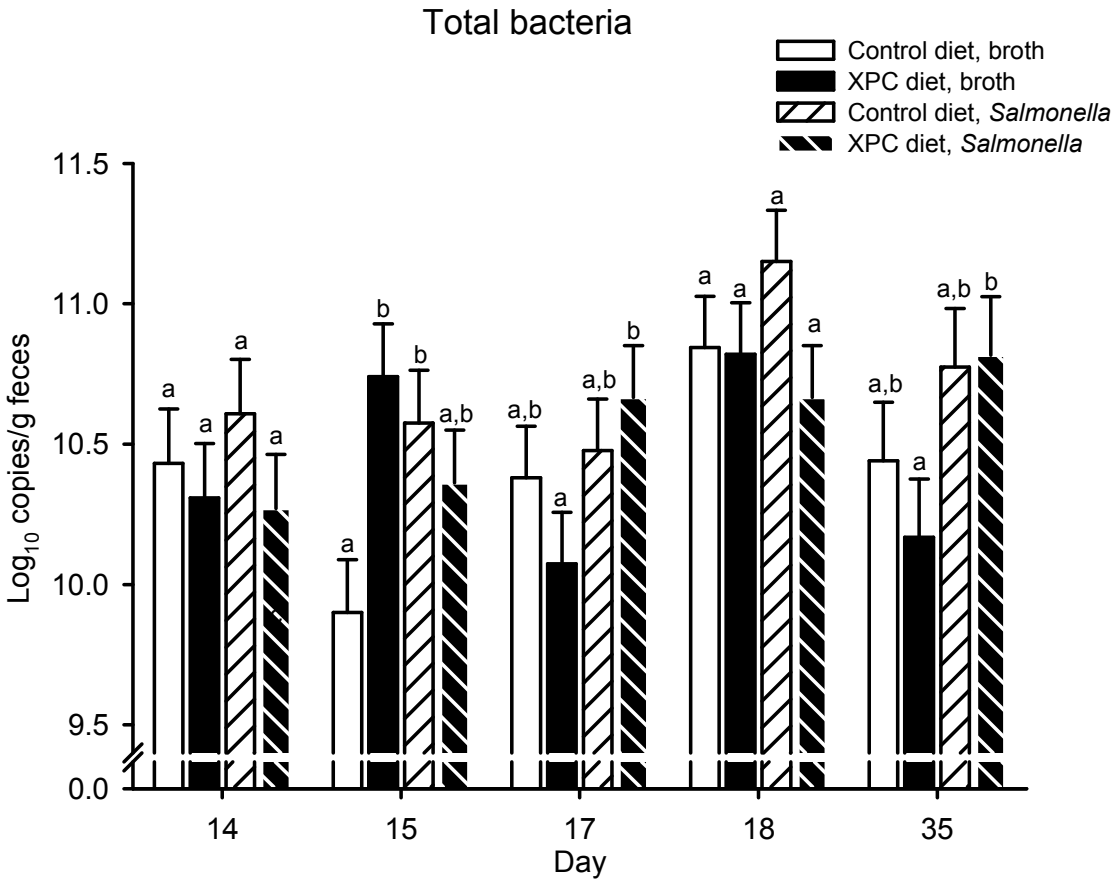

Figure 3.9. Effect of infection with *Salmonella* and consumption of XPC on the number of total bacterial copies (log_{10}) determined by QRT-PCR from fecal samples. From weaning (d = 1) pigs had ad libitum access to a nursery diet with (**XPC**) or without (**Control**) 0.2% XPC inclusion. Pigs were inoculated with 5 mL containing 10^9 CFU *S.* Typhimurium $Nal^R Nov^R$ (***Salmonella***) on d 14 after weaning or received 5 mL of sterile broth (**Broth**). Values not sharing common letters indicate significance (P <0.05) within day.

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Chapter 4: Conclusions and Future Directions

The purpose of this research project was to determine the effects of a prebiotic compound (XPC) on weanling pigs challenged with *Salmonella enterica* Typhimurium DT104 in relation to the diversity of the gastrointestinal tract and animal growth performance. Infection with *Salmonella* decreased body weight, average daily gain, and average daily feed intake supporting previous findings. Species richness of pigs 1 d post inoculation was affected by the presence of *Salmonella*. All profiles show a decrease in richness on d 18 due to antibiotic treatment. *Salmonella* inoculated pigs consuming XPC have increased shedding rates of *Salmonella* in feces, compared to a control diet. This may suggest that fewer *Salmonella* were able to colonize the intestinal mucosa, possibly due to increased competition for mucosal lining (Bovee-Oudenhoven et al., 2003). However, diet had no effect on the degree of febrile response to the infection.

During infection, a diet×inoculation interaction yielded a significant increase in *Bacteroidetes, Bacteroides*, and *Lactobacillus* abundance ($P<0.01$) suggesting the ability of these members to utilize the yeast product was associated with increased fecal shedding of *Salmonella.* To determine if these bacterial groups were associated with decreased colonization it would be necessary to directly observe *Salmonella* in association with the intestinal tissue. This would necessitate that the animals are euthanized, intestinal samples obtained and microscopy is used to visualize *Salmonella* that are differentiated by fluorescent *in situ* hybridization techniques. As the intention of this project was to determine the effect of prebiotic supplementation on growth performance post infection only fecal shedding was determined.

Results of this study indicated that supplementation of the XPC compound resulted in alterations to the gastrointestinal microbial community though overall species richness and diversity of the pigs was not different between the diets. However, increases in animal growth performance were significant only for XPC fed pigs that were previously infected with *Salmonella* infection than the control ($P < 0.01$). The increase in weight correlates to the 2.8 fold increase in *Bacteroidetes,* a phylum that has been positively associated with lean healthy animals (Guo et al., 2008b). Once pigs cleared the *Salmonella* infection, those on XPC demonstrated an increase in total bacteria abundance compared to the control diet, suggesting that these increased populations could generate more energy. To confirm if additional energy is available for the pig it would be necessary to obtain digesta samples and to compare levels of fermentation products (volatile fatty acids) and rates of absorption.

Although this experiment mirrored typical nursery care protocols in regards to antibiotic treatment, future assessments on the impact of *Salmonella* infection on the intestinal microbiota without antibiotic intervention could more thoroughly examine the changes in community members without the disruption caused by the antibiotic. Another possible research direction could investigate the effect of a longer supplementation phase prior to the oral challenge with *Salmonella* in weanling pigs or investigating the benefits of the XPC supplement consumed by more mature pigs in which the microbial community is more established. Lastly, to determine whether fecal samples yield accurate representation of the true intestinal community, the correlation between microbial community members present in fecal DNA and intestinal tissue DNA samples is another area of research in need of examination.

References

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Appendix A: DGGE gels used to create cluster analysis

Preparation: Fecal DNA was extracted using the UltraClean Fecal DNA kit (Mo Bio Laboratories, Carlsbad, CA) per manufacturer's instructions. Bacterial members of the microbial community were targeted by amplifying the 16S rRNA gene from the total fecal DNA (50 ng/μL) to generate a 566 bp fragment using the primers 341-f (5'-CCT ACG GGA GGC AGC AG-3') and 907r (5'-CCG TCA ATT CMT TTG AGT TT-3'). The forward primer was modified to add a 40 nucleotide GC clamp at the 5' end (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3'). Each 25 μL reaction contained 1.5 mM of $MgCl_2$, 50 mM of KCl, 0.2 mM of each dinucleotide, 1% of dimethylsulfoxide (**DMSO**), 25 mM of Tris-HCl (pH 8), 1 U/μL of HotStart-IT FideliTaq DNA polymerase (USB, Cleveland, OH), 0.5 μM of each primer, and 50 ng of DNA. The PCR protocol consisted of 94°C for 5 min, followed by 19 cycles of: 94°C for 1 min, amplification at 64°C for 1 min (decreasing 1°C every second cycle, touchdown) and elongation at 72°C for 3 min; followed by 9 additional cycles of denaturation at 94°C for 1 min, amplification at 55°C for 1 min, and elongation at 72°C for 3 min; finally 1 cycle of 94°C for 1 min, amplification at 55°C for 1 min, and a final elongation step at 72°C for 10 min. The size and intensity of PCR products were electrophoretically confirmed using 0.9% agarose gels (Fisher-Scientific, Atlanta, GA).

Method: The PCR products were run on a 8% polyacrylamide gel in a 30-60% denaturant gradient of urea and formamide [100% denaturant corresponds to 7 M urea plus 40% (vol/vol) of deionized formamide] using the Bio-Rad DCode^{TM} Universal Detection System (Bio-Rad, Hercules, CA). Twenty-two microliters of PCR products

were separated at constant voltage of 85 V and temperature of 60°C for 17 hours. The DNA bands were visualized by staining with ethidium bromide (5 μg/mL) and photographed using the Molecular Imager GelDoc XR (Bio-Rad). The DGGE bands were visualized using Quantity One-1D analysis software (Bio-Rad) and the DGGE profiles were clustered based on similarity using the un-weighted pair group method with mathematical averages (UPGMA; Dice coefficient of similarity) using Gelcompare II (Applied Maths, TX) and reported as dendrograms. Two different gels were analyzed for each sample and the number of bands present within each sample determined species richness (**A.1** and **A.2)**.

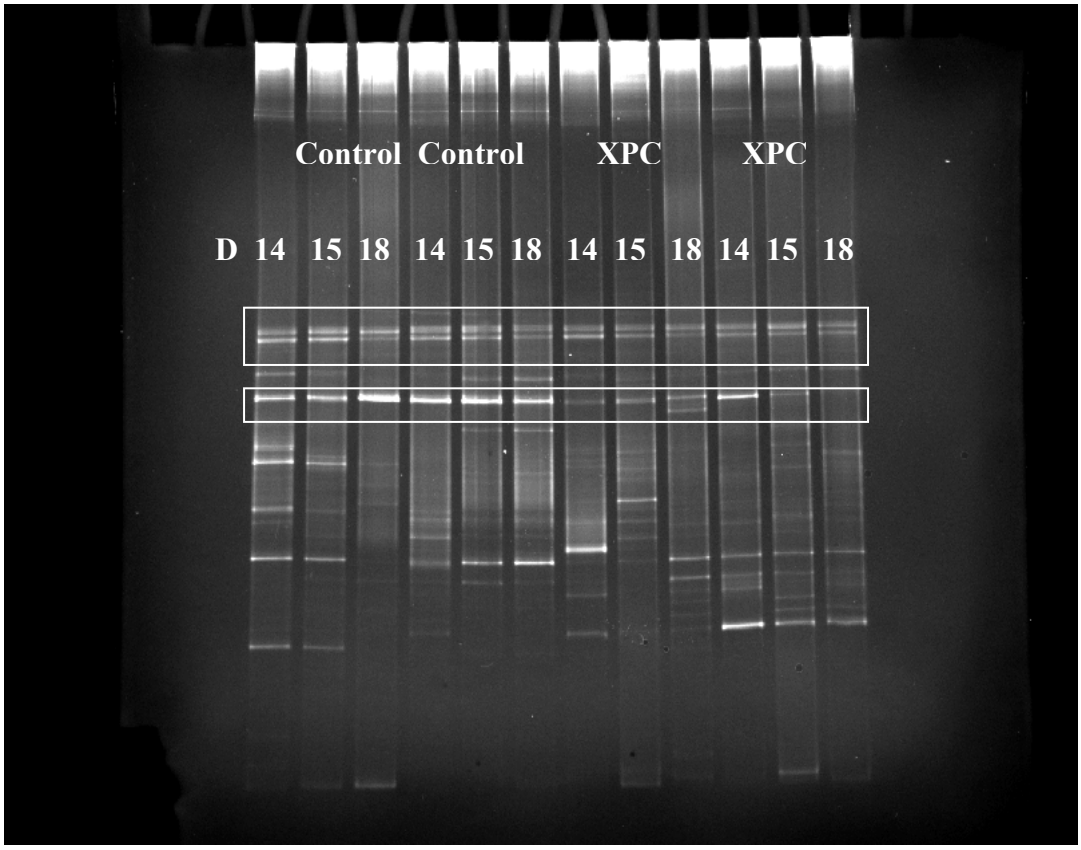

Figure A.1. Denaturant gradient gel electrophoresis (DGGE) of bacterial members of non-infected swine days 14, 15, and 18 from control and XPC diet regimens. Similar band patterns are outlined in boxes.

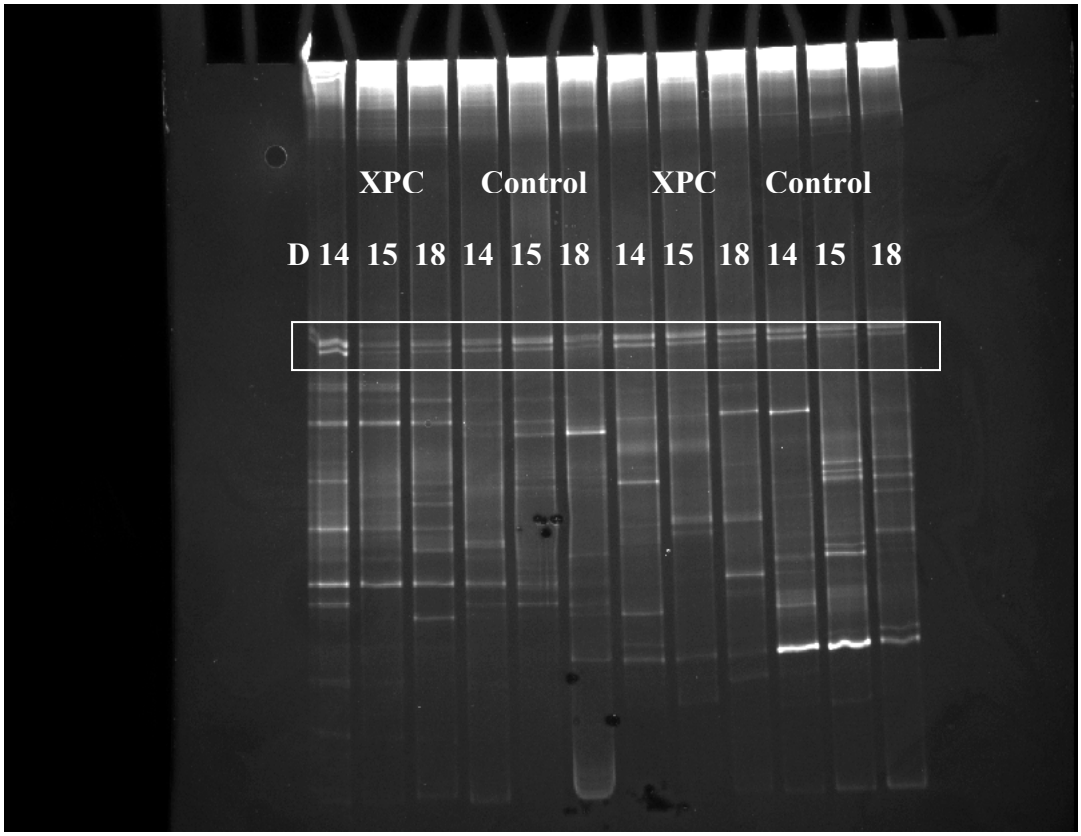

Figure A.2. Denaturant gradient gel electrophoresis (DGGE) of bacterial members of *Salmonella enterica* sv. Typhimurium $Nal^{R}Nov^{R}$ infected swine days 14, 15, and 18 from control and XPC diet regimens. Similar band patterns are outlined in boxes.

Appendix B: Quantitative real-time data of *Bifidobacteria* and *Ruminococcus* abundance

The quantification of *Bifidobacteria* and *Ruminococcus* were to be included within the manuscript. However, after several attempts, gross differences in abundance between *Salmonella* inoculated pigs and non-inoculated pigs on d 14 (prior to inoculation) could not be resolved. While animal variation often common in biological research, total bacterial data suggests this was not the case. Although storage at -20°C should not affect fecal DNA, the non-inoculated samples did not undergo extraction until several months after the inoculated samples. These samples also received a different lot of HotStart-IT SYBR Green qPCR Master Mix 2× for real-time analysis however; the equipment calibration factors remained the same throughout the real-time assay. The abundance per gram of feces for both species is included (**Table B.2** and **Table B.3)**.

Method: Real-time PCR was performed to quantify the abundance of *Bifidobacteria* and *Ruminococcus* in fecal samples obtained from pigs in both inoculums and diets on d 14, 15, 17, 18, (SICK) and 35 (POST)..

Standard curves were produced from DNA isolated using the Puregene DNA purification kit (GENTRA Systems, Minneapolis, MN) per manufacturer's instructions. DNA was serially diluted 10-fold from 100-ng/μL to 0.1-ng/μL. Standard curves for real time PCR amplification were prepared using primers (**Table B.1**) consisting of: 100 ng, 10 ng, 1 ng, and 0.1 ng. Each 25 μL reaction contained a respective amount of DNA template, 12.5 μL of HotStart-IT SYBR Green qPCR Master Mix 2× which contains 5 mM $MgCl_2$ and 0.4 mM of nucleotides (USB, Cleveland, OH), 10 nM of fluorescein as

passive reference dye (USB) and 0.5 μM of forward and reverse primers (**Table B.1**). The PCR conditions were denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing for 30 s at appropriate temperature (**Table B.1**), and elongation at 72°C for 1 min. Each concentration in the standard curve was done in triplicate using separated plates. Melting curve analysis of the PCR products was conducted following each assay to confirm that the fluorescence signal was originated from specific PCR product. Amplification was carried out with an iQ5 Optical system Real Time PCR detection system (Bio-Rad).

Table B.1 Primer sets used to quantify abundance of *Bifidobacteria* and *Ruminococcus* within the swine microbial community using real-time PCR.

Genera of Interest	Primer sequence	Species Amplified	Annealing Temperature, °C	Reference
Bifidobacterium	Forward:5'-CTCCTGGAAACGGGTGG-3' Reverse:5'-GTGTTCTTCCCGATATCTACA-3'	*B. adolescentis* *B. angulatum* *B. bifidum* *B. breve* *B. catenulatum* *B. infantis* *B. longum*	62.5	Matsuki et al., 2002
Ruminococcus	Forward:5'-GGTGGCAAAGCCATTCGGT-3' Reverse:5'-GTTACGGGACGGTCAGAG-3'	*R. productus*	64.0	Malinen et al., 2003

Table B.2. Least square means of abundance of *Bifidobacteria* copies per gram of feces (log_{10}) according to real-time PCR

Inoculum	Diet	Day	LS mean Estimate	Standard Deviation
Broth	**Control**	**14**	5.9483	0.9088
Broth	**Control**	**15**	7.7700	0.9955
Broth	**Control**	**17**	8.8980	0.9955
Broth	**Control**	**18**	6.5267	0.9088
Broth	**Control**	**35**	6.4917	0.9088
Broth	**XPC**	**14**	6.3633	0.9088
Broth	**XPC**	**15**	6.7433	0.9088
Broth	**XPC**	**17**	6.5000	0.9088
Broth	**XPC**	**18**	6.7233	0.9088
Broth	**XPC**	**35**	6.4417	0.9088
Salmonella	**Control**	**14**	5.9000	0.9088
Salmonella	**Control**	**15**	5.9433	0.9088
Salmonella	**Control**	**17**	5.7667	0.9088
Salmonella	**Control**	**18**	8.3140	0.9955
Salmonella	**Control**	**35**	5.9533	0.9088
Salmonella	**XPC**	**14**	5.4280	0.9955
Salmonella	**XPC**	**15**	3.4700	0.9088
Salmonella	**XPC**	**17**	4.0483	0.9088
Salmonella	**XPC**	**18**	4.1933	0.9088
Salmonella	**XPC**	**35**	3.5250	0.9088

Table B.3. Least square means of abundance of *Ruminococcus* copies per gram of feces (log_{10}) according to real-time PCR

Inoculum	Diet	Day	LS mean Estimate	Standard Deviation
Broth	**Control**	**14**	5.6900	1.2029
Broth	**Control**	**15**	4.2683	1.0981
Broth	**Control**	**17**	4.3330	1.0981
Broth	**Control**	**18**	4.0367	1.0981
Broth	**Control**	**35**	3.2117	1.0981
Broth	**XPC**	**14**	4.0133	1.0981
Broth	**XPC**	**15**	3.9250	1.0981
Broth	**XPC**	**17**	3.6600	1.0981
Broth	**XPC**	**18**	3.4933	1.0981
Broth	**XPC**	**35**	2.9367	1.0981
Salmonella	**Control**	**14**	3.0800	1.0981
Salmonella	**Control**	**15**	3.0450	1.0981
Salmonella	**Control**	**17**	3.1100	1.0981
Salmonella	**Control**	**18**	7.0840	1.2029
Salmonella	**Control**	**35**	2.2217	1.0981
Salmonella	**XPC**	**14**	1.7267	1.0981
Salmonella	**XPC**	**15**	2.4450	1.0981
Salmonella	**XPC**	**17**	2.0350	1.0981
Salmonella	**XPC**	**18**	5.5920	1.2029
Salmonella	**XPC**	**35**	1.8050	1.0981

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