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Investigation of aerobic and anaerobic microflora and volatile fatty acids in the jejunum, ileum, and cecum of the weanling pig

Mark Ashburn Franklin

To the Graduate Council:

I am submitting herewith a dissertation written by Mark Ashburn Franklin entitled "Investigation of aerobic and anaerobic microflora and volatile fatty acids in the jejunum, ileum, and cecum of the weanling pig." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

Alan Mathew, Major Professor

We have read this dissertation and recommend its acceptance:

Neal Shrick, Dick Heitmann, David Golden, Craig Reinemeyer

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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INVESTIGATION OF AEROBIC AND ANAEROBIC MICROFLORA AND VOLATILE FATTY ACIDS IN THE JEJUNUM, ILEUM, AND CECUM OF THE WEANLING PIG

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Mark Ashburn Franklin
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Abstract

Experiment 1. The purpose of this experiment was to characterize aerobic and anaerobic microflora, volatile fatty acids (VFA) , pH, dry matter, and lactate (L+ and D-) concentrations in the jejunum, ileum, and cecum of pigs weaned at 17 or 24 d of age. Pigs were surgically fitted with T-cannulas in either the jejunum, ileum, or cecum at approximately 14 d of age. After a 3 d recovery period, pigs were randomly assigned a weaning age and intestinal and fecal contents were sampled at 17, 20, 24, 27, 31, and 34 d of age. Noncannulated littermates were also sacrificed at weaning with intestinal, fecal, and blood samples taken to determine effects of cannulation. No differences ($P > .05$) were noted in serum cortisol or microbial populations between cannulated and noncannulated pigs. Dry matter percentage and pH levels were higher ($P < .05$) in the cecum than in the ileum or jejunum. Unweaned pigs maintained higher ($P < .05$) lactobacilli populations than weaned pigs; however, populations declined ($P < .05$) in both groups postweaning. Fecal populations of *E. coli* and lactobacilli declined ($P < .05$) postweaning; whereas, fecal bifidobacteria populations increased ($P < .05$) regardless of weaning age. VFA levels were higher ($P < .05$) in the cecum versus the jejunum or ileum and lactate (L+) tended ($P < .07$) to be higher in the jejunum and ileum versus the cecum. Weaning and weaning age were determined to have significant effects on microflora and VFA concentrations.

Experiment 2. The purpose of this experiment was to investigate the effect of dietary galactose on aerobic and anaerobic microflora, volatile fatty acids (VFA), pH, dry matter, and lactate (L+ and D-) concentrations in the ileum and cecum of weanling pigs. Pigs were surgically fitted with T-cannulas in either the ileum or cecum at approximately 14 d of age. After a 4 d recovery period, pigs were weaned at 18 d of age and randomly assigned either a control or treatment diet.

The control diet was a phase starter diet based on corn starch and the treatment diet was similar except for galactose replacing a portion (13%) of the corn starch. The diets were mixed with water and offered twice daily. Intestinal and fecal samples were obtained at 18, 21, 25, 28, 32, and 35 d of age. Digesta pH increased ($P < .05$) following weaning regardless of collection site. Dry matter percentage was higher ($P < .01$) in cecal contents compared to ileal contents. Galactose did not affect populations of microflora or VFA concentrations, except for a tendency ($P < .08$) to prolong *E. coli* populations postweaning and to decrease acetate production. Lactate (L+) concentrations were greater ($P < .05$) in ileal versus cecal contents. Results indicate that galactose fed at this level incurs only minimal changes in microflora and VFA concentrations in the ileum and cecum of weanling pigs.

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PART 1

LITERATURE REVIEW

Economic Impact of Weaning Disorders

Postweaning diarrhea is a major source of economic loss for swine producers with scours accounting for 15% of total nursery phase pig deaths (1). Based on a national annual production of 98 million pigs, nursery losses are estimated to account for over 2.5 million pig deaths per year (2). If one conservatively assumes a $20 production cost per weaned pig and a potential net profit of $10 per finished pig (3), the monetary loss to swine producers exceeds $80 million annually. In addition to lost income due directly to death, poor feed efficiency, stunted growth, and chronic diarrhea, postweaning diarrhea results in higher feed, fuel, and medication costs, increased days to market, and an interruption in the flow of production.

Microbial Environment in the Gastrointestinal Tract

The intestinal tract of humans and animals is normally sterile at birth, but it soon becomes colonized by microorganisms from the surrounding environment (4, 5). The majority of these bacteria probably come from the dam (6). The intestinal microflora of humans and animals consists of two types: resident and transient (7). Transient species are defined as microflora which are only occasionally found in the intestine, possibly introduced by food, but not permanent members of the intestinal flora. Resident species are defined as those microflora that become established in the host for an extended period of time. In order for a species to be classified as a resident, it must colonize the intestine. Several mechanisms are employed by the animal to resist this colonization including mucus production and peristaltic action. Mechanical removal by peristaltic action, aided by the secretion of mucus, is probably the main fate of viable organisms that enter the

small intestine (8). Colonization does occur and can be detrimental or beneficial to the host animal depending on the colonizing microorganism. Colonization by commensal species is beneficial to the animal because these organisms may suppress proliferation of pathogenic species (9, 10). In other circumstances, pathogenic species are able to establish and produce some type of disease state (11, 12). Vital to this initial colonization is an initially high stomach pH at birth which permits rapid multiplication of bacteria (11). These bacteria then pass into the small intestine where proliferation can continue. The pH of the stomach subsequently decreases and acts as a barrier to colonization by most microorganisms. However, lactobacilli and streptococci have been found in the stomach. These organisms have been shown to be intimately associated with the non-secretory region (*pars esophagea*) of the pig (13). It has been suggested that this flora is responsible for the decline in pH after the first day of life by producing lactic acid before the onset of HCl production by the host, and this decreased pH holds in check the growth of *Escherichia coli* (*E. coli*) (14). Even after the onset of HCl production, pH microenvironments have been suggested to occur (14) which may allow local proliferation of coliforms in the stomach at a pH of 4.5 and below. However, the stomach generally acts as a barrier to most microorganisms once the pH is established. Several researchers have shown a difference in the diversity of microfloral populations between the anterior versus posterior gastrointestinal tract. McAllister et al. (15) found that all bacterial groups were present in lowest numbers in the stomach or anterior small intestine and all tended to increase from anterior small intestine to spiral colon. It has been suggested that the only major groups of microorganisms present in the anterior small

intestine of the pig are lactobacilli, streptococci, and coliforms (11, 14). The posterior gastrointestinal tract contains a more complex microbial population (14), with an adult human postulated to have more than 400 different bacterial species present in the intestinal flora (16). This diversity is thought to be a stabilizing force on the intestinal ecosystem, with a decrease in diversity possibly leading to intestinal disturbances and increased susceptibility to various diseases (5). However, the continual presence of microflora in the gastrointestinal tract does seem to be species specific. In the pig, unlike the human, the small intestine is constantly occupied by a microbial flora which varies between 10^6 and 10^9 microorganisms per gram wet content (12). The presence of microflora in the gastrointestinal tract in conventionally raised versus germfree (gnotobiotic) pigs has also been shown to result in a greatly increased surface area (17). Anaerobic microorganisms have been shown to greatly outnumber aerobic microorganisms in the mammalian gastrointestinal tract (15). In addition, anaerobic bacteria as well as resident strains of facultative anaerobes have been shown to control populations of other bacteria (18, 19). In humans the majority of adult viable stool organisms are obligate anaerobes (20).

Commensal Microflora

Two genre of bacteria considered to be commensal are lactobacilli and bifidobacteria. Lactobacilli are present in the intestines of pigs as one of the most predominant microorganisms (6, 21). The presence of lactobacilli as a constituent of the normal microflora of the gastrointestinal tract is generally considered to be beneficial to the porcine host (22, 23, 24). One benefit of lactobacilli may come from the production

of lactic acid which can play a role in maintaining gastrointestinal tract pH. By establishing a lower pH, lactobacilli may exclude potential pathogenic bacteria from colonizing. Additionally, lactobacilli have been shown to block possible intestinal receptors of *E. coli* and discourage bacterial proliferation by secreting toxic metabolites (25). As reviewed by Bezkorovainy (26), bifidobacteria were first isolated from the feces of breast fed infants by Tissier in 1899. Bifidobacteria were classified as lactobacilli well in the 1960's and even the 1970's (26). Indeed, bifidobacteria have been shown to have similar protective roles as that of lactobacilli (27). In addition, lactobacilli and bifidobacteria have been shown to have anti-carcinogenic properties in the gastrointestinal tract (28).

Pathogenic Microflora

Several microorganisms are capable of producing a disease state in the pig, but none have been as well studied as *E. coli*. First described by Theodor Escherich in the late 1800's (29), these gram negative bacteria are known to be responsible for diarrhea in swine. Not all *E. coli* are responsible for or capable of producing a disease state, rather most are considered to be members of the normal flora in pigs (30). However, bacterial counts have shown that pathogenic strains of *E. coli* greatly outnumber non-pathogenic strains in diseased versus healthy pigs (11). The pathogenicity of *E. coli* depends on their production of colonization factors, enterotoxins, and endotoxins (30). In swine, diarrhea primarily affects young animals, having its greatest effect on newborn and weanling pigs (31). Diarrhea caused by enterotoxigenic *E. coli* (ETEC) is associated with several pilus antigens which include K88(F4), K99(F5), 987P(F6), and F41 in neonatal swine (32).

Certain strains are known to produce these proteinaceous filaments, or pili, which allow their attachment to the small intestinal mucosa (33, 34). Interaction with mucosal surfaces is a vital step for infection (35). These pili are thought to be vital for colonization of the small intestine because they promote adherence that resists clearing actions of the intestinal tract (36, 37). These clearing actions are the result of peristaltic movements of the small intestine and have their greatest force in the anterior portion of the intestinal tract (38).

Volatile Fatty Acid Production

Volatile fatty acids (VFA) are produced by the fermentation of digesta by bacteria (39). These organic acids are thought to play several important roles in the overall health of the gastrointestinal tract as well as the whole animal. The major substrates for this fermentation, as reviewed by Bergman (39), include cellulose, hemicellulose, pectin, starches, dextran, and soluble carbohydrates such as mono- and disaccharides. The major end products are acetate, propionate, butyrate, carbon dioxide, and methane. Volatile fatty acids have been found in the gastrointestinal tracts of all herbivores, most omnivores, and some carnivores dependent upon diet. The major sites of VFA production in the pig include the cecum and colon (40, 41); however, significant amounts of VFA were found in the stomach and small intestine. It is logical that the greatest VFA production would follow bacterial concentrations and was first demonstrated by Elsden et al. (41). The major site of VFA production in the pig is the large intestine with acetate, propionate, and butyrate making up most of the VFA (42). The concentrations of these acids in the cecum, colon, rectum, and feces of all animals have been measured in the

range of 30-240 mM but average 70-120 mM (39). Total VFA concentrations have been shown to be greater in the pig in the lower gastrointestinal tract than in the upper gastrointestinal tract (43).

Volatile Fatty Acid Absorption

Volatile fatty acids are readily absorbed from all segments of the lower digestive tract, where absorption seems to be passive and is dependent upon pH and VFA concentration (44). In the large intestine of the pig, acetate is produced and absorbed in greater quantities than propionate or butyrate and accounts for at least one half of the caloric value of the three (42). Bergman (39) stated that 90-99% of the VFA are present in the anionic form because the pH of the fermentation chambers is near neutral and the pKa of the acids is around 4.8. The absorption of the acid form (nondissociated) occurs with luminal accumulation of bicarbonate and increase in pH, while appearance of bicarbonate is not seen when the dissociated form is absorbed, with the aid of sodium (45).

Volatile Fatty Acid Metabolism

Much more is known about VFA metabolism in ruminant species than in nonruminant species. The metabolism first starts at the site of production and VFA may be transported to peripheral tissues. Stevens (46) showed that metabolism by the rumen epithelium accounted for 45% of the acetate, 65% of the propionate, and 85% of the butyrate absorbed from the lumen bath. Most of the butyrate is used by rumen tissue to form carbon dioxide or ketone bodies (47). This has also been shown in the colonic mucosa of pigs (42). The remaining VFA not used during absorption are then transported

to the liver where the majority of the propionate (95%) is taken up. Propionate is the only VFA that can be used as a major source of glucose production (48). Most of the acetate (90-98%) is utilized in the peripheral tissues (39). In the pig, portal blood has a greater concentration of VFA than does hepatic or arterial blood because of direct VFA absorption through the gut epithelium (42). Pethick et al. (49) also stated that there is substantial gut utilization of acetate from the blood with the majority being used by smooth muscle in the gut wall.

Effects of Volatile Fatty Acids

Volatile fatty acids have been shown to have effects that play a role in the overall health of animals. These effects include the exclusion of pathogenic bacteria, energy production, and changes in gastric motility. Several researchers have shown that VFA can promote the proliferation of commensal organisms (50, 51, 52). Wolin (50) found that VFA do not inhibit the growth of *E. coli* at a pH of 7.0 but a marked reduction in growth was seen when the growth medium was adjusted to pH 6.0. Bergheim (53) also found that above pH 6.5 these acids had relatively little effect on inhibition and below pH 4.0, the pH in itself becomes important in bacterial inhibition. He found that in the pH range of 5.0-6.0, where the pH in itself is not inhibitory, the fatty acids even in low concentrations have a marked effect upon growth of *E. coli*. Using a mouse model, Bohnoff and Miller (54) demonstrated that antibiotic fed mice became highly susceptible to oral *Salmonella* infection and they believed antibiotics eliminated members of the normal flora which were capable of forming VFA in vivo. However, not all fatty acids are equal in their ability to deter pathogenic growth. Sheu and Freese (55) found that the

lipopolysaccharide layer of gram-negative bacteria protected them against inhibition by intermediate and long-chain fatty acids. In addition, they found that these gram-negative bacteria were not resistant to short-chain fatty acids up to hexanoate and only slightly resistant against octanoate. In a later study, Fay and Farias (52) found the toxicity of fatty acids to *E. coli* to be dependent on the given concentration of fatty acids, the chain length of the fatty acids, and culture medium in which the bacteria were grown.

Volatile fatty acids have been suggested as energy sources for the intestinal mass as well as for the whole animal itself. A rabbit study by Henning and Hird (56) showed that VFA serve as the primary energy source to the intestinal mass, contributing up to 70% of the required maintenance needs. Large intestinal VFA production in the pig has been estimated to contribute between 5 and 28% of the total maintenance energy requirement (42, 57). The ruminant animal has been shown to rely even more on VFA for energy, with an estimate of 70-80% of the animal's energy requirement supplied by VFA (58). In addition to energy production, VFA have been shown to be involved in cholesterol metabolism, potent stimulators of insulin secretion, gastrointestinal blood flow, and epithelial cell proliferation as reviewed by Bergman (39). Malbert et al. (59) reported that ileal VFA play a role in gastric emptying and serve to decrease intestinal motility. Mathew et al. (60) postulated this may play a role in satiety and affect feed intake patterns of pigs immediately postweaning.

Effects of Weaning on Microflora

The process of weaning presents a challenge to the young pig. The diet is changed from easily digested milk to a dry feed of different nutrient content (61). In

addition, when weaned at 3 to 4 weeks of age, pigs are removed from their source of immunity (milk) before producing sufficient antibodies themselves for protection from enteric diseases (62). This sometimes results in a postweaning lag and may lead to diarrhea. Generally postweaning lag has been characterized as a period of poor feed intake, unthriftiness, and failure to thrive for 7-10 days following weaning. Smith (6) reported that the fecal bacterial flora of calves, lambs, piglets, human babies, and rabbits were remarkably similar in early life, but as the animals aged, considerable dissimilarities became progressively more apparent. As an example, he states that the feces of calves 6 months of age frequently contained 10,000 times fewer viable bacteria than they did when the calves were only a few weeks old. By contrast, no decrease was noted with aging in the human baby. He further proposes that the similarities may be due to the presence of a milk diet, but upon weaning, diets change dramatically as does gastrointestinal function among ruminants, nonruminants, and hindgut fermenters. In pigs, several changes in microfloral populations have been noticed. Research has shown *E. coli* populations to increase after weaning (4, 61, 63). Other researchers have shown no weaning effect on coliform populations (64). This difference seems to be related to weaning environment. Lactobacilli have been generally reported to decline after weaning (15, 63). McAllister et al. (15) also noted that several populations of bacteria generally declined upon weaning. The above studies looked at pigs weaned over a variety of ages, from 2 days to 8 weeks; so it can be concluded that weaning is a major factor in influencing the microbial makeup of the gastrointestinal tract.

Effects of Weaning on Volatile Fatty Acids

The effect of weaning on VFA production in the pig has been investigated by only a few researchers in limited areas of the gastrointestinal tract. Most of the VFA research has been conducted with weaned pigs fed a variety of diets to determine effects on VFA concentrations. Volatile fatty acids have been generally noticed to decline following weaning (63, 65). These decreases have been observed mainly for acetate, propionate, and butyrate. These three acids make up the majority of the VFA found in the gastrointestinal tract of pigs and acetate makes up the vast majority of all VFA found (63, 65). This loss may be significant to the young pig because of the possible energy loss associated with a decline in VFA concentrations (42, 57). In addition, the protective role of VFA against pathogenic microflora may also be compromised (52, 53).

Gastric Function

The development of digestive capacity in the pig has been well studied. Digestive capacity in the pig has been observed to increase with age with the exception of lactase (66). The production of HCl necessary for the initiation of digestive processes has been shown to be variable among pigs. Cranwell et al. (67) found that acid secretion in the stomach occurred in some pigs from 1 d of age, but in others significant quantities of HCl were not produced before 24 d of age. They suggested that this may be due to the presence of lactic acid from bacterial fermentation having a negative response on HCl production. As a possible mechanism, he postulated this could be due to a suppression of gastrin by bacterial fermentation products. As reviewed by Manners (66), increasing amounts of pepsin are produced as pigs get older and the full activation of pepsin by HCl

has to await the production of sufficient acid to overcome the buffering power of the stomach contents. Small intestinal sucrase and trehalase are not found in the newborn pig; however, the levels of both enzymes in the mucosa increase throughout much of the life of the pig. Maltase levels were found to be low at birth and increased to 56 d of age with a subsequent decline. Bile volume has been observed to increase slowly until 6 or 7 kg with more rapid increases above this body weight. Likewise, pancreatic secretions were 500 mL/d at 5-6 weeks of age and increased to over 8 L/d at 7-8 months of age. Over the first two months of life, the levels in pancreatic tissue of lipase, alpha-amylase, chymotrypsin, and trypsin have been shown to increase considerably. Research has shown that weaning causes an increase in gastrointestinal tract pH of pigs weaned at 3 weeks of age (68). This group did not notice a decrease in ileal pH in pigs on the same experiment weaned at 28 d of age. In another study by Mathew et al. (63), pigs weaned at 31 d of age showed no increase in pH after weaning. These results are consistent with the above studies showing significant HCl production later in the pig's life. This postweaning pH increase in early weaned pigs mimics the neonatal pig. The high pH after birth allows for proliferation of microflora, as does the increase in pH postweaning. This may allow for the establishment of a disease state in postweaned pigs and/or explain part of the postweaning lag so often observed in young weaned pigs.

The small intestinal structure has also been observed to change over the course of weaning. It was found that the process of weaning, even in the absence of a disease state, caused a temporary decrease in the absorption of fluid and electrolytes (69) and a reduction in villus height and an increase in crypt depth (70). Measurement of crypt

depth gives a general indication of the likely maturity and functional capacity of enterocytes on the villi (70). Crypt elongation was seen predominantly in the distal small intestine while the loss of villus height was greatest in the proximal small intestine (70). This suggests an overall reduction in enterocyte maturity; and a lower net absorption was observed in pigs in which the villi had recently regenerated (69), suggesting that absorption is a function of enterocyte maturity rather than overall length. In addition, it has been shown that the binding capacity of the enterocytes of three week old weaned pigs for *E. coli* heat stable toxin a (STa) was nearly three times greater than that of unweaned pigs (71). Taken together, these results suggest that the increased pH may allow for a proliferation of pathogenic bacteria which can produce toxins that bind more easily to immature enterocytes expressed after weaning, thus potentially leading to a disease state in the small intestine of early weaned pigs.

Antibodies for protective immunity are vital to neonatal pigs. The young pig receives its first antibodies from colostrum and a study by Miller et al. (62) showed that the baby pig is capable of oral absorption of antibodies from serum through 36 hours of age and that these absorbed antibodies are retained in measurable concentrations for as long as seven days. Speer et al. (72) have shown colostral antibody absorption to be insignificant after 24 h of age. Miller et al. (62) have also shown that there was little or no active production of antibodies until pigs reached 3 weeks of age. Brown et al. (73) emphasized the critical period of low immunity in the pig at 3 weeks of age. They also showed the capability of the baby pig to actively produce antibodies increased thereafter and by 6 weeks of age the mean antibody response was 20 times greater than at 3 weeks

of age. These observations become interesting viewed in light of current industry trends to wean pigs at 3 weeks of age or younger.

Oxygen Sensitivity of Microorganisms

Microflora have been shown to have varying degrees of oxygen sensitivity (74, 75). A classification of bacteria on their ability to use oxygen has been proposed as follows: (i) obligate aerobes, (ii) facultative anaerobes, (iii) aerotolerant anaerobes, (iv) obligate anaerobes, and (v) microaerophilic organisms (76). Damage to the microorganism arises from the production of the superoxide anion, hydrogen peroxide, singlet oxygen, and hydroxyl radicals. Superoxide dismutase, catalase, and peroxidase are the major enzymes responsible for the protection of the cell against these toxic oxygen reduction products. The protection of microorganisms from these toxic products has been proposed to follow several mechanisms. In 1971, McCord et al. (77) determined the distribution of superoxide dismutase (SOD) and catalase in aerobes, strict anaerobes, and aerotolerant anaerobes. They found that strict anaerobes contained SOD and catalase activity, while aerotolerant organisms contained some SOD but not catalase, and they concluded that the main function of SOD was protection of oxygen-metabolizing organisms against the potentially detrimental effects of the superoxide anion. There are two known isoforms of SOD, conferring protection from both endogenously and exogenously produced oxygen radicals. An iron-containing SOD protects organisms from exogenously produced superoxide anions while a manganese-containing form protects the organism from endogenously produced superoxide anions. Rolfe et al. (74) stated that the differences in oxygen tolerance among anaerobes cannot be accounted for

solely on the basis of the presence of these enzyme levels. They proposed the rate at which bacteria reduce oxygen to form toxic products is probably an important factor related to their survival in air. In addition, work by Gregory and Fridovich (78) showed that certain strains of lactobacilli, which lacked catalase and SOD, were very resistant to oxygen simply because they utilized no oxygen and therefore produced no toxic oxygen intermediates. However, protection against exogenously produced superoxide anions could be particularly important for anaerobic bacteria that establish in well oxygenated tissues (74).

Anaerobic Growth Conditions

Loesche (75) described strict anaerobes as those species incapable of agar surface growth at pO_2 levels greater than 0.5% with moderate anaerobes including those species capable of growth in the presence of oxygen levels as high as 2 to 8% and tolerating exposure to room atmosphere for 60 to 90 minutes without appreciable loss of viability. Patel et al. (79) stated that many anaerobes have substantial tolerance to O_2 and that even strict anaerobes may be handled aerobically for short periods without jeopardizing their growth potential. Several techniques are used to obtain anaerobic media including: pouring in an anaerobic chamber immediately after autoclaving or boiling and pouring aerobically with the presence of reducing agents in the media with a prereduction period in an anaerobic chamber before plating. Patel et al. (79) also stated that aerobically poured medium which has been prereduced for 24 hours can even be inoculated aerobically and incubated in an anaerobic environment without much loss in cell viability. It has also been reported that auto-oxidation of cysteine, a reducing agent, results in the

production of hydrogen peroxide which is toxic to some anaerobes (80, 81); however, Rolfe et al. (74) found no evidence that oxidized products of cysteine affected the survival of the anaerobes used in their studies.

Oxygen Content in the Gastrointestinal Tract

Vervaeke et al. (82) measured the oxidation-reduction potential (Eh) in the gastrointestinal tract of young pigs and found that the values ranged from 265 mv in the stomach to -214 mv in the cecum with an ever decreasing Eh from the stomach to the cecum. However, a negative Eh value was not obtained until the ileum was reached (-43 mv). These Eh values also increased from the cecum to the rectum (-35 mv). Therefore, they concluded that the total counts of anaerobes are higher in the cecum than in other segments of the digestive tract and confirmed this with actual bacterial counts. In comparison, the Eh of the rumen is reported to be about -350 mv (83). The pig intestinal environment is therefore more oxidative than that of the rumen. In a direct measurement of dissolved oxygen in the gastrointestinal tract of pigs, Hillman et al. (84) found that dissolved oxygen was observed to increase from 108 μmol/L in the stomach to 188 μmol/L in the jejunum followed by a gradual decline to 85 μmol/L at the midpoint of the ascending colon. This group also found that diet had little effect on the dissolved oxygen concentrations in the gut and it has been postulated that these levels of dissolved oxygen are maintained physiologically as a result of diffusion from the bloodstream (85). These levels suggest that the growth of anaerobic bacteria would be nearly impossible, yet anaerobic bacteria do persist in the gastrointestinal tract of pigs. Hillman et al. (84) proposed that the predominance of facultative and aerotolerant bacteria in the small

intestine of the pig gut (11) is probably related to the higher concentrations of dissolved oxygen present and to the rapid flow of digesta in this part of the gut (86). This flow rate would discourage the development of microniches where anaerobic organisms might be able to persist; however, in the cecum and colon, the flow rate is reduced (86) and the survival of anaerobes would probably depend on the existence of anaerobic microniches (87).

Effects of Acid Supplementation on Microbes

Organic acids have been used in attempts to acidify diets to prevent microbial proliferation of pathogens. The first attempts to use diet acidification was directed at the alleviation of postweaning scours (88). This study showed that lactic and propionic acids reduced *E. coli* populations in the duodenum and jejunum of weanling pigs. Additionally, Mathew et al. (89) showed a decrease in ileal *E. coli* populations when supplementing with propionic acid. The addition of fumaric or citric acid was also observed to decrease *E. coli* and anaerobic microbes in the intestinal tract of starter pigs (90). However, no response was observed on ETEC in challenged pigs (91) or on lactobacilli or *E. coli* populations (43). Inorganic acids such as hydrochloric, sulfuric, and phosphoric have also been used, but results showed no clear benefits for the reduction of pathogens (92).

Effects of Probiotic Supplementation on Microbes

As reviewed by Fuller (93), the term probiotic was originally used to describe substances produced by one protozoan which stimulated another, but was later used to describe animal feed supplements which had a beneficial effect on the host animal by

affecting its gut flora. Fuller rewrote this definition to read, " A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". He stressed the term live, as this has been the greatest problem in delivering probiotics to pigs, because viability of the preparation and gastric passage are both essential to the efficacy of probiotics (93). Several different organisms have been given to pigs as probiotics, with lactobacilli and bifidobacteria being the most common. The justification for the use of lactobacilli stems from studies which show that as developing gut flora increase in lactobacilli, other organisms decrease (6). The use of a bifidobacterial preparation has also been shown to protect piglets against diarrhea (94).

Effect of Carbohydrate Supplementation on Microbes

Several carbohydrate sources have been used in pig diets in an attempt to manipulate enteric microflora. Mathew et al. (65) found that pigs fed galactan had lower ileal *E. coli* populations and pigs fed galactose had higher ileal lactobacilli populations than did pigs on a control diet. Brown et al. (95) found fecal bifidobacteria in greater concentrations in pigs fed a high amylose corn starch versus a low amylose cornstarch. He stated that high amylose starch acts as a prebiotic in promoting the fecal excretion of probiotic organisms. The term prebiotic can be described as nondigestible food ingredients that affect the host beneficially by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health (96). Another group of compounds that are considered prebiotics are the fructooligosaccharides (FOS). As reviewed by Fishbein et al. (97), FOS are naturally occurring compounds that have been reported in a variety of plants. They are basically

sucrose molecules to which 1, 2, or 3 additional fructose units have been added by β (2, 1) glycosidic linkage. These FOS have been used in an attempt to improve gastrointestinal health because bifidobacteria selectively utilize FOS (98).

Methods of Microbial Study

Several techniques have been employed to study microfloral populations in the gastrointestinal tract. The most noninvasive of these being the analysis of fecal samples. However, there is some question as to whether the fecal microflora are indicative of large intestinal activity, much less small intestinal activity. Early researchers in this area employed the sacrifice of littermate pigs to obtain digesta samples. This technique is improved over fecal collection, especially for small intestinal analyses; however, pig to pig variation may mask treatment effects (4, 5). Cannulation techniques have also been used to study microfloral changes in the intestine (63, 68). Several points need to be addressed when using this technique. The cannula should not interfere with the normal microfloral populations. If interference does occur, it should be reversible in a short period of time before collection starts. Additionally, there has been some question as to the collection of the important microbes when taking only a luminal sample of digesta (15, 99, 100), as adherent bacteria are left behind. Scraping the intestinal mucosa through the cannula would not allow an unbiased sample to be obtained at the next collection. Clearly, all of these methods have advantages and disadvantages, and a study utilizing a combination of methods could give greater results over that of any single method.

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PART 2

CHARACTERIZATION OF MICROFLORA AND VOLATILE FATTY ACID CONCENTRATIONS IN THE JEJUNUM, ILEUM, AND CECUM OF PIGS WEANED AT 17 VERSUS 24 DAYS OF AGE

Abstract

In a series of five 17 d replicate trials, a total of 54 cannulated and 12 noncannulated pigs were used to determine the effects of weaning age on pH, dry matter percentage, aerobic and anaerobic microflora, lactate, and volatile fatty acid (VFA) concentrations in the jejunum, ileum, and cecum of weanling pigs. At approximately 14 d of age, cannulated pigs were surgically fitted with T-cannulas in the jejunum (n = 20), ileum (n = 18), or cecum (n = 16). All pigs were randomly assigned to either 17 or 24 d weaning. Upon weaning, cannulated pigs were individually caged in an environmentally controlled room with ad libitum access to a phase starter diet and water. Noncannulated pigs were sacrificed at weaning and samples were collected from the jejunum, ileum, and cecum. Digesta and fecal swabs from cannulated pigs were collected twice weekly. The 12 noncannulated pigs served as controls to determine effects of cannulation. Serum cortisol levels were determined for cannulated and noncannulated groups and no differences ($P > .05$) were observed. Populations of microbes did not differ ($P > .05$) between cannulated and noncannulated pigs. The pH of cecal contents differed ($P < .05$) from that of the jejunal or ileal contents and dry matter percentage was higher ($P < .05$) for cecal contents than that observed in the jejunal or ileal contents. Pigs weaned at 24 d of age had increased ($P < .05$) *E. coli* populations 3 d postweaning compared to preweaning populations, regardless of site of collection; whereas this increase was not observed in pigs weaned at 17 d of age. Unweaned pigs maintained higher ($P < .05$) lactobacilli populations than weaned pigs; however, populations declined ($P < .05$) in both groups by 3 d postweaning, with pigs weaned at 24 d of age having lactobacilli

populations greater than pigs weaned at 17 d of age. Fecal populations of *E. coli* and lactobacilli declined ($P < .05$) postweaning regardless of weaning age; whereas, fecal bifidobacteria populations increased ($P < .05$) postweaning regardless of weaning age. Populations of total fecal anaerobes declined ($P < .05$) in pigs weaned at 17 d of age but were maintained in pigs weaned at 24 d of age. Concentrations of VFA were greater ($P < .05$) in the cecum than in the jejunum or ileum, and acetic acid concentrations decreased ($P < .05$) postweaning regardless of weaning age. A tendency for L+ lactate concentrations to be greater ($P < .07$) in the ileum and jejunum versus the cecum was observed. Results indicate that weaning and weaning age have significant effects on microflora and VFA concentrations.

Key Words: Pigs, Intestinal Microflora, Weaning, Jejunum, Ileum, Cecum

Introduction

The process of weaning can be a challenge for the young pig. With decreased weaning age, the young pig may be less adaptable to these changes in diet, environment, and social structure. Mixing litters at weaning can also expose pigs to more disease as well as social stresses. Enzyme function seems to be associated with the age of the pig and has been shown to be less than optimal in pigs less than 30 d of age (1). In addition, HCl production needed for proper gastric function, has been shown to be insignificant in pigs before 24 d of age (2). In a weaning age study, Mathew et al. (3) showed an increase in ileal pH in pigs following weaning at 21 d of age, but not in pigs following weaning at 28 d of age. Loss of maternal mediated protection conferred from passive immunity may

also affect pigs weaned at early ages, as the active immune function offers little protection until about 28 d of age (4).

Intestinal microfloral populations of young pigs have been studied primarily through the sacrifice of littermates (5, 6) and the use of ileal cannulas (3, 7). The sacrifice of littermates does not account for individual variation among pigs. Variation in microfloral populations among pigs in the same litter has been observed (5, 6). Ileal cannulation techniques allow for repeated samplings over the course of the weaning transition; however, observations are only possible in one area of the gastrointestinal tract. Therefore, the objective of this study was to characterize microflora and VFA concentrations in the jejunum, ileum, and cecum of pigs during the weaning transition.

Materials and Methods

Animals:

A total of 54 weanling pigs from 9 litters were used in five replicate trials in this study. At approximately 14 d of age, pigs were cannulated in the jejunum (n = 20), ileum (n = 18), or cecum (n = 16), in accordance with the Institutional Animal Care and Use Committee of The University of Tennessee, Knoxville. Following surgery, pigs were returned to their respective sows and allowed a minimum 3 d recovery period. Pigs were then randomly assigned to a weaning age with half of the pigs being weaned at approximately 17 d of age while half remained on the sow until 24 d of age. At weaning, pigs were housed individually in stainless steel cages in an environmentally controlled room and allowed ad libitum access to a phase starter diet and water. Orts and pig weights were taken for the last two replications to determine feed intake and growth rates.

Surgical Procedure:

After pigs were fasted for 24 h and moved to the surgical suite, they were pre-anesthetized by intramuscular injection of 1 mL (100 mg) of ketamine hydrochloride (Ketaset, Fort Dodge Laboratories, Fort Dodge, IA) plus .1 mL (1 mg) acepromazine maleate and .3 mL (3 mg) of atropine sulfate (Butler Company, Columbus, OH). Halothane (Fluothane, Fort Dodge Laboratories) anesthetic gas was delivered by a non-rebreathing delivery system, initially at 2% level and a flow rate of 1.5 L/min of O_2. Initial delivery of halothane was made using a cone mask until the proper plane of anesthesia was reached, after which pigs were intubated with a 3.0 mm I.D., cuffed, Murphy Eye tracheal tube (Mallinkrodt Critical Care, Glens Falls, NY). The left flank or lateral midline of the pig was prepared for surgery by shaving, followed by 3 betadine and alcohol scrubs. A 3 cm dorsoventral incision, using a number 10 scalpel blade attached to a number 3 handle, was made approximately 2 cm posterior to the last rib for jejunal, ileal, and cecal cannulations. For duodenal cannulations, a 5 cm lateral midline incision from the tip of the sternum toward the navel was made. The intestine was exteriorized and placed on a sterile 4x4 gauze pad soaked with sterile saline to keep the intestine moist during the surgical procedure. A purse string suture was placed in the muscularis layer of the intestine using 3-0 PDS I I monofilament suture with a Taper RB-1 needle (Ethicon, Somerville, NJ). A 1 cm incision was made into the lumen between the stitches of the purse string suture, the flange of the cannula was inserted into the incision, and the purse string suture was drawn up to secure the intestinal wall to the cannula. The purse string was tied using a surgeon's knot followed by 3 square knots.

Using a brass cork borer, a hole, 1 cm in diameter, was cut into the abdominal wall approximately 1.5 cm dorsal to the incision for the jejunal, ileal, and cecal cannulations. This hole was cut on the right flank immediately posterior to the last rib for the duodenal cannulations. The stem of the cannula was brought out through this opening by securing the stem of the cannula with Allis forceps and pulling it through the hole. A washer threaded onto the exposed cannula was turned down in order to draw the intestine, muscle layers, and abdominal wall securely together without twisting the intestine or applying too much pressure to the skin or muscle.

The incision was closed using 1-0 PDS I I monofilament suture with a Taper TP-1 needle (Ethicon) in a simple continuous pattern for the peritoneum and muscle layers. A separate skin closure was performed using 3-0 PDS I I monofilament with a Taper RB-1 needle (Ethicon) in a continuous subcuticular pattern. All sutures were secured with a surgeon's knot and 3 square knots.

The surgical procedure for ileal cannulations has been previously described in the literature (8), and cannula placement was determined by locating the mesenteric attachment bridging the distal end of the cecum to the final ileal loop of the small intestine. This loop is approximately 10 cm proximal to the ileal-cecal junction in pigs of this age; and ileal cannulas were inserted at this location to ensure consistent placement along the small intestine. Duodenal, jejunal, and cecal cannulations were a modification of the ileal cannulation technique described by Walker et al. (9). Cecal cannula placement was determined by locating the blind end of the cecum and cannulas were placed at the midpoint of the blind end of the cecum to ensure consistent placement.

Jejunal cannula placement was determined by locating the ileum as described above and moving in a cranial direction until the terminal portion of the mesenteric lymph nodes was located. Cannulas were placed in the jejunum on the cranial side of the terminal portion of the mesenteric lymph nodes. This corresponded to cannulas being approximately 30 cm from the ileo-cecal junction in pigs of this age. Duodenal cannulas were placed between the stomach and the ligature of Treitz; however, this proved to be problematic in pigs this age because of the distance required to exteriorize behind the last rib. Therefore, cannulations in the duodenum were discontinued in this experiment. Cannulas were specially constructed (Mechanical Engineering Department, Purdue University, West Lafayette, IN) from Delrin 600 plastic, and were similar in design to those described by Walker et al. (9).

At the end of surgery, the use of halothane was discontinued and the endotracheal tube was removed following evidence of a swallowing reflex. Antibiotic ointment was applied prior to placing a 4x4 gauze pad over the incision. Pigs were wrapped with 2 inch cling gauze and 3 inch Elasticon tape (Johnson and Johnson, New Brunswick, NJ) in order to protect the incision and cannula. The pigs were observed until fully recovered from the effects of the anesthesia, at which time they were returned to the Blount Swine Farm and placed on their respective sow for a recovery period. Pigs were observed daily and bandages were changed as necessary until weaning.

Anaerobic Chamber Establishment:

In order to investigate anaerobic microflora, an anaerobic chamber was constructed from one-half inch plexiglas and assembled by the Biological Services

Facility at The University of Tennessee, Knoxville. This chamber was equipped with a palladium catalyst system (Coy Laboratory Products, Detroit, MI) to remove trace oxygen from the chamber environment. Oxygen levels were monitored using an oxygen analyzer (Coy Laboratory Products) capable of detecting from 0 to 2000 ppm oxygen. Gas used for the chamber environment consisted of 90% nitrogen, 5% carbon dioxide, and 5% hydrogen and was purchased pre-mixed (National Welders Supply, Knoxville, TN) in order to avoid explosive hydrogen levels in the chamber. Seals on the port used to introduce and remove items from the chamber were not capable of withstanding the normal negative pressures associated with evacuating oxygen from the port. Therefore, a modified procedure was used incorporating routine flushing with the mixed gas to remove oxygen from the port before entering the chamber and this method proved to be satisfactory. The catalyst was changed twice weekly for adequate oxygen removal. Initially an incubator was installed in the chamber; however, this proved to be problematic because the humidity level was excessive in the presence of the incubator. Thus, the incubator was removed and plates were placed in glass jars with sealable lids and removed from the chamber for incubation.

Sampling:

Intestinal contents were collected at 17, 20, 24, 27, 31, and 34 d of age in both weaning groups, and collection was initiated at approximately 0700 of each sampling day. At that time a sterile rubber balloon with no head space was placed on the opened cannula and intestinal contents were allowed to collect until approximately 10 g of digesta had accumulated. Because digesta flow was intermittent, balloons were replaced

approximately every 30 min until digesta began to flow consistently. Typically, once the digesta started flowing, approximately 15 min were needed for sufficient collection of material. Samples were immediately placed on ice until further analysis. This procedure was followed to minimize the possibility of bacterial proliferation and/or fermentation in the balloons. Fecal swabs were taken when the intestinal sampling was complete.

Sample Preparation:

In preparation for analysis, one gram of digesta was weighed into a test tube and fecal swabs were weighed to determine the amount of sample collected. Fecal swabs were then placed in a test tube and 9 mL of phosphate buffered saline (PBS) were added to the fecal and digesta test tubes. These initial mixtures were immediately placed in an anaerobic chamber. Fecal tubes were agitated for 1 min and ten-fold dilutions were then made from both sets of tubes using prereduced PBS as the diluent. Collection and preparation were typically completed within 2 h of the initiation of collection. Sample pH was determined from the remaining digesta using a Corning #345 pH meter (Corning, New York, NY) with a high performance glass electrode (cat. # 476390). Digesta were then centrifuged in a Beckman model J2-HS centrifuge (Palo Alto, CA) with a JA20 rotor at 27,000 x g at 4°C for 15 min. The resulting supernatant was collected and stored at -80°C until VFA and lactate analyses could be performed.

Microbial Analysis:

For anaerobic culture, 50 μL of each serial dilution were plated in duplicate onto prereduced media inside the anaerobic chamber. Inoculated Petri dishes were then placed in canning jars, the lids partially tightened, and jars subjected to a vacuum prior to

sealing. Jars were then placed in convection incubators for appropriate culture conditions. For culture of aerobes, serial dilutions were removed from the anaerobic chamber and Petri dishes were inoculated and placed directly into incubators.

Four media were utilized in this study and included: 1) Lactose MacConkey agar (Difco, Detroit, MI) for enumeration of *E. coli*, 2) Rogosa media (Difco) for enumeration of lactobacilli, 3) Modified Columbia media (10) for enumeration of bifidobacteria and 4) Media 10 (11) for enumeration of total anaerobes. Initially, another medium was investigated for the enumeration of bifidobacteria. Lithium chloride-sodium propionate agar (12) was used to isolate bifidobacteria; however, microscopic evaluation of all colonies was required to identify isolates and this proved to be limiting in our laboratory. Therefore, the Modified Columbia medium was used with a modified enzyme test (Patterson et al., 1997 unpublished results) to detect the fructose-6-phosphate phosphoketolase enzyme unique to bifidobacteria (see Appendix 3). *E. coli* were incubated aerobically at 37°C for 24 h. Anaerobic culture included bifidobacteria, which were incubated at 40°C for 48 h, and lactobacilli and total anaerobes, which were incubated at 37°C for 48 h. Bacteria were enumerated by visual count, disregarding atypical colonies. Previous work in our laboratory has confirmed typical *E. coli* colony growth on MacConkey media (3), and in that study, selected colonies were subjected to biochemical analysis (API 20, Vitek BioMerieux, Syosset, NY) to identify isolates to the species level. In all cases, colonies suspected of being *E. coli* were determined to be *E. coli* by the biochemical analysis. The majority (96%) of isolates not phenotypical of *E. coli* were determined to be other species. All large white colonies were counted as

lactobacilli on Rogosa media as described by Krause et al. (13). Large white colonies were counted as bifidobacteria on Modified Columbia media (10) and all colonies were counted as total anaerobes on Media 10 (11).

VFA Analysis:

Volatile fatty acid concentrations were determined using a gas chromatographic method modified from Playne (14). Briefly, one and one-half milliliters of supernatant were mixed with 300 μL of 25% metaphosphoric acid (5:1 ratio) and incubated at room temperature for 30 min. Following centrifugation to remove the precipitate, 1μL of sample was injected into a Hewlett Packard model 5890 gas chromatograph (Hewlett Packard, Avondale, PA) with an HP-FFAP 10-m x .53 mm x 1-μm capillary column packed with cross-linked polyethylene glycol-TPA. A flame ionization detector was used with an oven temperature of 200°C, and a detector temperature of 250°C, for determination of acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate concentrations. The lower detectable limit for all VFA was .1 mmol/L.

Lactate Analysis:

Lactate concentrations were determined using a lactate analysis kit (#826-B; Sigma Chemical, St. Louis, MO). In this assay, 200 μL of the intestinal content supernatant was mixed with 400 μL of ice-cold 8% perchloric acid. Samples were incubated on ice for 30 min and centrifuged to remove precipitate. The resulting supernatant was assayed according to Sigma, using the appropriate lactate dehydrogenase enzymes in separate assays for determination of D(-) and L(+) lactate. A modification of volumes was implemented to allow the use of microtiter plates and associated equipment.

In the modified analysis, 200 μL of NAD-glycine solution was pipetted into a flat bottom Serocluster 96-well microtiter plate (Costar Corp., Cambridge, MA). To that solution, 20 μL of each sample supernatant was added to triplicate wells. Absorbance was read at 340 nm on a model EL 340 Bio Kinetics microtiter plate reader (Bio-Tek Instruments, Wiooski, VT). Following the initial reading, 10 μL of the appropriate lactate dehydrogenase (L+ or D-) enzyme was added to each well. Plates were incubated for 15 min at 37°C followed by incubation for 15 min at room temperature. Following the final incubation, absorbance was read a second time. Regression analysis using a standard curve was used to convert absorbance readings to lactate concentrations. The lower detectable limit for the lactate analysis was .05 mmol/L.

Cortisol Analysis:

Blood samples were obtained by vena cava puncture from both cannulated and noncannulated pigs, upon weaning and again at one week postweaning. Samples were allowed to clot at 4°C for one hour prior to centrifugation at 800 x g for 15 min. Serum was removed and stored at -20°C until cortisol analyses could be performed. Serum concentrations of cortisol were determined by solid phase radioimmunoassay (15) using a standardized kit (Diagnostic Products Corporation, Los Angeles, CA). Twenty-five microliters of sample were used and sensitivity of the assay was 2 ng/mL. Coefficients of variation were less than 8% for all samples tested.

Statistical Analysis:

The statistical model consisted of a randomized complete block design using repeated measures analysis with the individual pig serving as the experimental unit. Data

were analyzed using the Mixed Model Procedure of SAS (16). Differences between least squares means were separated using pairwise t-tests. Differences between days were determined using Pdmix procedures. Microbial and lactate concentrations were transformed (log_{10}) prior to statistical analysis.

Results

Pigs consumed 279 g/d of the phase starter diet and gained 144 g/d during this experiment. No differences ($P > .05$) were observed between weaning ages for intake or gain data. Cortisol concentrations did not differ ($P > .05$) between cannulated and noncannulated pigs, nor did cortisol differ ($P > .05$) in pigs weaned at 17 d of age (40 ng/mL) compared to pigs weaned at 24 d of age (56 ng/mL). There were no differences ($P > .05$) in tested microbial populations between cannulated and noncannulated animals. The pH of the cecal contents differed ($P < .05$) from that of the ileal and jejunal contents; however, pH did not differ ($P > .05$) between ileal or jejunal contents (Table 1; all Tables located in Appendix 1). A similar pattern for dry matter percentage was also observed, with the cecal contents containing more (11.1%) ($P < .05$) dry matter than that of the ileum (5.5%) or jejunum (5.3%); with no differences observed ($P > .05$) between the ileum or jejunum. A day effect ($P < .002$) was observed in dry matter percentage regardless of site, as dry matter increased with increasing age of pig (Figure 1; all Figures located in Appendix 2).

In pigs weaned at 17 d of age, *E. coli* populations did not change ($P > .05$) postweaning; whereas, populations increased ($P < .05$) 3 d postweaning in pigs weaned at 24 d of age regardless of site (Table 2). There was a tendency ($P < .08$) for greater

lactobacilli populations in the cecum (9.75 $\log_{10}$ cfu/g) versus the ileum (9.08 $\log_{10}$ cfu/g), with populations in the jejunum being intermediate. Unweaned pigs maintained greater ($P < .05$) lactobacilli populations than weaned littermates, but once weaned, populations declined (Table 2). Pigs weaned at 24 d had greater ($P < .05$) lactobacilli populations 3 d postweaning than 17 d old pigs (Table 2). Bifidobacteria populations were greater ($P < .05$) in the cecum (6.59 $\log_{10}$ cfu/g) compared to those observed in the ileum (5.71 $\log_{10}$ cfu/g) or jejunum (5.60 $\log_{10}$ cfu/g).

Populations of *E. coli* and lactobacilli in fecal material declined ($P < .05$) after weaning regardless of weaning age; however, fecal bifidobacteria populations increased ($P < .05$) after weaning regardless of weaning age (Table 3). Populations of total anaerobes found in feces declined ($P < .05$) in pigs weaned at 17 d of age, but were maintained in pigs weaned at 24 d of age (Table 3).

Concentrations of VFA were observed to be greater ($P < .05$) in the cecum than the ileum or jejunum for all analyzed fatty acids (Table 4), with no differences noted ($P > .05$) between ileal and jejunal levels. Total VFA concentrations observed in the jejunum, ileum, and cecum were 36.15, 41.75, and 116.55 mmol/L respectively. Acetic acid constituted 80, 80, and 61% of the total acid respectively in the jejunum, ileum, and cecum. Concentrations of acetic acid decreased ($P < .05$) after weaning regardless of weaning age (Table 5); whereas, no postweaning declines were observed for other VFA. L+ lactate concentrations tended to be higher ($P < .07$) in the ileum (11.04 mmol/L) and jejunum (10.56 mmol/L) versus the cecum (2.68 mmol/L) (Table 6). Additionally, there was an increase ($P < .05$) in L+ lactate over the course of the study for pigs weaned at 17

d but not for pigs weaned at 24 d of age (Table 7). No differences ($P > .05$) in D- lactate were observed (Tables 6 and 7).

Discussion

The effects of weaning age on microflora and VFA concentrations were investigated in this study. Additionally, we were interested in ascertaining if differences occurred between cannulated and noncannulated pigs; therefore, we investigated microfloral and cortisol levels for both groups. No differences were observed. This is in agreement with an earlier study in our laboratory, where we determined no differences existed between ileal cannulated and noncannulated pigs (17). Cortisol was used as a general indicator of a stress response by animals in both studies (18). We conclude that cannulation had little effect on microfloral populations in the jejunum, ileum, or cecum in this study. The pH values observed in this study compare well with those found by other investigators (3, 19). The distance between the ileal and cecal cannulas was approximately 15 cm in pigs in this study and the difference in pH between these sites is substantial. Dry matter percentage also varies considerably over this distance, as the cecum contained more than twice the dry matter of the ileum or jejunum.

Initially, we also wanted to investigate microflora and VFA concentrations in the duodenum in order to characterize levels found in this area of the gastrointestinal tract. Some success was obtained with duodenal cannulas; however, maintaining these pigs for repeated sampling proved to be difficult. In retrospect, we should have exteriorized through the rib cage at a point closer to the cannulation site because the duodenum is anchored at both the stomach and the ligature of Treitz. This would have placed less

stress on the intestine than exteriorizing behind the last rib and could have allowed longer maintenance of duodenally cannulated pigs.

No postweaning increases in *E. coli* were observed in pigs weaned at 17 d of age, in agreement with previous studies (20, 21), but in contrast with others (3, 8, 22) where *E. coli* populations increased after weaning. Mathew et al. (21) postulated the absence of an *E. coli* increase may be due to weaning pigs into a highly sanitized, environmentally controlled room with limited contact among pigs. We also observed *E. coli* populations to be lower in pigs remaining on the sow, as have other investigators (3, 20). Lactobacilli populations declined in both groups of pigs, but populations fell to lower levels in pigs weaned at the earlier age compared to pigs weaned at the later age. This may indicate that pigs weaned at older ages are more adept at dealing with changes over the course of weaning. Cranwell et al. (2) found HCl production to be an age related factor, with HCl production to be more stable in pigs at least 24 d of age. This may have direct implications to lactobacilli which prefer lower pH (23). In another study, similar results were found with regard to pH and lactobacilli populations in pigs weaned at 21 d versus 28 d of age (3). Fecal populations of lactobacilli and *E. coli* followed patterns typical of those observed in the more anterior portions of the gastrointestinal tract in this study. However, fecal bifidobacteria experienced increases postweaning, possibly due to the decrease in lactobacilli and *E. coli* in the posterior gastrointestinal tract. The loss of direct competition may benefit other bacterial populations, including bifidobacteria. An increased fermentable substrate concentration may also have resulted from the decline in certain bacterial populations or from more substrate reaching the large intestine in pigs

consuming weaning diets. Because anaerobes far outnumber aerobes in the gastrointestinal tract (22), we looked at total anaerobic concentrations as an overall indicator of stability in the gastrointestinal tract. Total fecal anaerobe populations returned to preweaning levels by the end of the study in pigs weaned at 24 d of age, whereas populations in pigs weaned at 17 d decreased through the end of the study. No differences were observed in total anaerobes in cannulated sites of the gastrointestinal tract. Evidently, these changes are restricted to regions posterior to our cannula placement in this study. These data also seem to indicate total anaerobes recover more quickly from weaning stresses in pigs weaned at later ages than pigs weaned at younger ages.

Cecal concentrations of VFA indicate more fermentation occurs in this region than in the ileum or jejunum. The ileal, jejunal, and cecal concentrations of VFA found in this study agree with those from another study in our laboratory (21); however, cecal and jejunal VFA levels were only obtained by sacrificing animals at the end of that experiment. Thus, direct comparisons of these studies over the course of the weaning are not possible.

The increased VFA levels coincide with increased microbial populations found in the cecum, thus indicating greater microbial fermentation. Elsden et at. (24) demonstrated that VFA production follows bacterial populations, and Imoto and Namioka (25) showed the major site of VFA production in the pig to be the large intestine. Changes in fermentation patterns postweaning are also indicated by shifts in lactate production (21), as was observed in this study for the L+ isomer which increased after

weaning. Pigs in this study consumed typical amounts of feed daily and appeared to grow at a rate typical of pigs of this age (26). This study indicates VFA decline postweaning, which may have significant effects on the energy available to the intestinal mass. Large intestinal VFA production in the pig has been estimated to contribute between 5 and 28% of the total maintenance energy requirement (25, 27). In other species, VFA have been shown to be the primary energy source to the intestinal mass, contributing up to 70% of the required maintenance needs (28). Because of the importance of the microflora, their activities, and byproducts, further research is warranted to determine optimal conditions to maintain the gastrointestinal health of the weaned pig.

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APPENDIX 1
DATA TABLES EXPERIMENT 1

TABLE 1
pH of Jejunal, Ileal, and Cecal Contents of Weanling Pigs on Various Days*

Age, d	Jejunum	Ileum	Cecum
17	7.21[a#]	7.23[a]	6.61[bcd]
20	7.03[ab]	7.39[a]	6.37[d]
24	7.44[a]	7.13[ab]	6.44[cd]
27	7.28[a]	7.06[ab]	6.15[d]
31	7.24[a]	7.33[a]	6.60[bcd]
34	7.05[abc]	7.24[ab]	6.66[bcd]
SEM		.27	

* Data represent least squares means from five replicates.
# a, b, c, d Least squares means with different superscripts differ ($P < .05$).
SEM = maximum standard error of the mean observed among all test periods and sites.

TABLE 2
Intestinal Microbial Populations on Various Days in Pigs Weaned at 17 Versus 24 Days of Age*

	E. coli		Lactobacilli		Bifidobacteria		Total Anaerobes	
Age, d	W17#	W24	W17	W24	W17	W24	W17	W24
17	8.41^{ab} **	8.53^{ab}	9.75^{bc}	10.23^{ab}	6.00^{ab}	5.08^{b}	9.81^{ab}	9.82^{ab}
20	8.95^{a}	7.64^{cd}	7.47^{f}	10.93^{a}	6.07^{ab}	7.18^{a}	9.87^{ab}	10.36^{a}
24	8.16^{abc}	7.92^{bc}	8.31^{ef}	10.58^{a}	5.79^{ab}	6.33^{ab}	9.25^{b}	10.05^{a}
27	7.46^{cd}	8.77^{a}	9.44^{bcd}	9.14^{cde}	6.12^{ab}	5.48^{b}	9.90^{ab}	10.28^{a}
31	7.48^{cd}	6.85^{d}	8.87^{de}	9.52^{bcd}	6.37^{ab}	6.18^{ab}	9.68^{ab}	9.74^{ab}
34	7.20^{cd}	6.70^{d}	8.80^{cde}	9.35^{bcde}	5.78^{ab}	5.23^{b}	9.94^{ab}	9.63^{ab}
SEM	.46		.57		1.04		.47	

* Log_{10} colony forming units per gram of intestinal contents, wet basis. Data represent least squares means from five replicates.
# W17 = pigs weaned at 17 d of age. W24 = pigs weaned at 24 d of age.
SEM = maximum standard error of the mean observed among all days and weaning groups.
** a, b, c, d, e Least squares means within bacterial groups with different superscripts differ ($P < .05$).

TABLE 3
Fecal Microbial Populations on Various Days in Pigs Weaned at 17 Versus 24 Days of Age*

	E. coli		Lactobacilli		Bifidobacteria		Total Anaerobes	
Age, d	W17 #	W24	W17	W24	W17	W24	W17	W24
17	8.84^{ab} **	8.57^{ab}	8.67^{b}	8.36^{bcd}	5.32^{cde}	4.49^{e}	9.86^{bc}	9.78^{abcd}
20	9.25^{a}	8.68^{ab}	8.15^{bcd}	9.60^{a}	5.67^{bcde}	5.65^{bcde}	10.21^{ab}	9.55^{bcd}
24	8.77^{ab}	8.80^{ab}	7.85^{cd}	8.76^{b}	5.88^{abcd}	5.25^{de}	9.68^{bcd}	10.35^{a}
27	7.30^{c}	8.50^{ab}	8.68^{bc}	8.34^{bcd}	6.51^{ab}	5.92^{abcd}	9.21^{cde}	10.02^{abc}
31	7.57^{c}	7.73^{c}	8.37^{bcd}	7.68^{d}	6.54^{abc}	7.13^{a}	9.05^{de}	9.12^{de}
34	7.23^{c}	8.02^{bc}	8.13^{bcd}	8.20^{bcd}	6.83^{abcd}	6.03^{abcde}	8.60^{e}	9.30^{cde}
SEM	.51		.43		1.23		.37	

* Log_{10} colony forming units per gram of intestinal contents, wet basis. Data represent least squares means from five replicates.
# W17 = pigs weaned at 17 d of age. W24 = pigs weaned at 24 d of age.
SEM = maximum standard error of the mean observed among all days and weaning groups.
** a, b, c, d, e Least squares means within bacterial groups with different superscripts differ ($P < .05$).

TABLE 4

Volatile Fatty Acid Concentrations at Various Sites Along the Gastrointestinal Tract*

Site	Acetate	Propionate	Butyrate	Valerate	Isobutyrate	Isovalerate
Cecum	71.55[a #]	27.71[a]	10.41[a]	2.84[a]	1.64[a]	2.40[a]
Ileum	33.36[b]	3.66[b]	2.78[b]	.47[b]	.87[b]	.61[b]
Jejunum	28.78[b]	3.55[b]	2.19[b]	.45[b]	.65[b]	.53[b]
SEM	7.15	2.47	1.25	.75	.31	.51

* Data are mmoles/L and represent least squares means from three replicates.

a, b Least squares means with different superscripts within VFA differ ($P < .05$).

SEM = maximum standard error of the mean observed among all sites.

TABLE 5

Acetate Concentrations on Various Days in Pigs Weaned at 17 Versus 24 Days of Age*

Age, d	W17#	W24
17	63.24[b**]	59.59[bc]
20	37.87[cd]	74.41[ab]
24	35.89[d]	93.59[a]
27	31.00[d]	23.41[d]
31	35.55[d]	24.08[d]
34	26.26[d]	29.84[d]
SEM	12.66	

* Data are mmoles/L and represent least squares means from three replicates.

W17 = pigs weaned at 17 d of age. W24 = pigs weaned at 24 d of age.

** a, b, c, d Least squares means with different superscripts differ ($P < .05$).

SEM = maximum standard error of the mean observed among all periods and weaning groups.

TABLE 6

Lactate Concentrations at Various Sites Along the Gastrointestinal Tract in Weanling Pigs*

	Lactate (L+)	Lactate (D-)
Jejunum	$10.56^{b\#}$	6.00^{a}
Ileum	11.04^{b}	5.87^{a}
Cecum	2.68^{a}	4.09^{a}
SEM	3.57	1.41

* Data are mmoles/L and represent least squares means from three replicates.

#a, b Least squares means with different superscripts within lactate isomer differ ($P < .07$).

SEM = maximum standard error of the mean observed among all sites.

TABLE 7

Lactate Concentrations on Various Days in Pigs Weaned at 17 Versus 24 Days of Age*

	L+ isomer		D- isomer	
Age, d	W17#	W24	W17	W24
17	2.98[b]**	3.54[b]	4.11[ab]	4.41[ab]
20	16.24[ab]	3.28[b]	5.45[ab]	1.78[b]
24	10.42[ab]	4.29[b]	6.36[ab]	4.46[ab]
27	18.74[a]	14.17[ab]	9.27[a]	7.51[ab]
31	8.36[ab]	4.07[b]	6.38[ab]	4.15[ab]
34	.87[ab]	10.13[ab]	4.98[ab]	4.95[ab]
SEM	5.74		2.53	

* Data are mmoles/L and represent least squares means from three replications.

W17 = pigs weaned at 17 d of age. W24 = pigs weaned at 24 d of age.

** [a, b] Least squares means with different superscripts within lactate isomer differ ($P < .05$).

SEM = average standard error of the mean observed among all periods and weaning groups.0

Appendix 2
Figures for Experiment 1

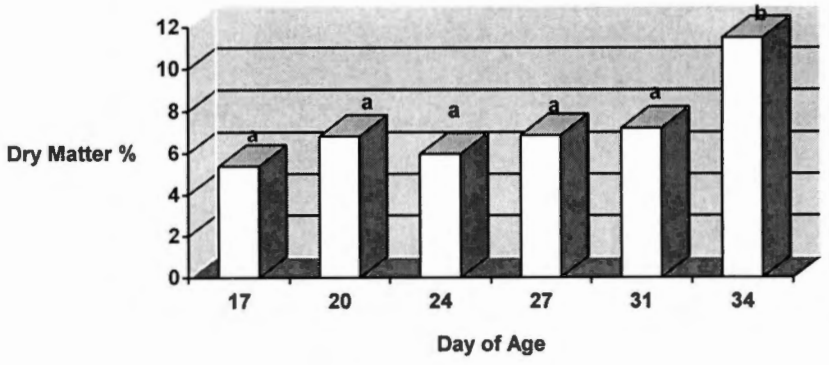

Figure 1. Dry Matter Percentage on Various Days in Weanling Pigs

APPENDIX 3
MODIFIED ENZYME TEST

Reagents and Procedure:

Reagents:

1. Cetab (hexadecyltrimethylammonium bromide), 0.45 mg/mL in water.
2. Phosphate buffer (KH2PO4), 0.05M, and cysteine, 500 mg/L (H2O). Mix the phosphate and cysteine 1:1 (Vol/Vol) and adjust pH to 6.5 with fresh NaOH.
3. Sodium fluoride (NaF), 6 mg/mL, and potassium or sodium iodoacetate, 10 mg/mL. Mix at 1:1 (Vol/Vol).
4. Hydroxylamine-HCl, 13 g/100 mL. Neutralize with fresh NaOH to a pH of 6.5.
5. Trichloroacetic acid (TCA), 15% (Wt/Vol) in water.
6. Hydrochloric acid (HCl), 4 N.
7. Ferric chloride*6H2O, 5% (Wt/Vol) in 0.1 N HCl.
8. Fructose-6-phosphate (Na salt, 70% purity), 80 mg/mL in water.

Procedure:

1. Grow cells in liquid medium (Reinforced Clostridial medium, 10 mL).
2. Wash cells twice with Reagent 2 by centrifugation at 10,000 rpm for 15 min.
3. Add 0.4 mL Reagent 1.
4. Add 0.25 mL Reagent 3.
5. Add 0.25 mL Reagent 8 and shake to mix.
6. Incubate at 37°C for 30 min.
7. Add 1.5 mL Reagent 4.
8. Allow to stand for 10 min at room temperature.
9. Add 1 mL Reagent 5.
10. Add 1 mL Reagent 6.
11. Add 1 mL Reagent 7 and shake to mix.

Remarks:

A reddish-violet color will develop immediately with the addition of Reagent 7. This is taken as a positive result. If the color remains yellow, the test is negative. The intensity of the color formed may vary depending on the strain of bifidobacteria. For strains that produce light color, the color will be more evident visually after incubation at room temperature to allow debris and protein to settle. A blank tube containing bacteria but without Reagent 8 or Reagent 1 can be used for comparison. In some cases, a slight color may develop without addition of Reagent 1. This will occur if significant cell disruption occurs during the cell-washing process.

PART 3

EFFECT OF GALACTOSE SUPPLEMENTATION ON ILEAL AND CECAL MICROFLORAL POPULATIONS IN WEANLING PIGS

Abstract

In a 17 d trial, pigs weaned at approximately 18 d of age were used to investigate the effects of galactose inclusion in the diet on pH, dry matter percentage, aerobic and anaerobic microflora, lactate, and volatile fatty acid (VFA) concentrations in the ileum and cecum. Pigs were cannulated at approximately 14 d of age in either the ileum or cecum. The treatment diets consisted of: 1) a corn starch based control diet and 2) a similar diet with galactose added at 13%, replacing corn starch. Digesta were collected twice weekly and fecal swabs were obtained at the time of digesta collection. Fecal material and digesta were analyzed for populations of *E. coli*, lactobacilli, bifidobacteria, and total anaerobes. Digesta were also analyzed for pH. Digesta pH increased ($P < .05$) following weaning regardless of collection site. Dry matter percentage was nearly two times greater ($P < .01$) in the cecal contents compared to the ileal contents. Intestinal *E. coli* populations decreased ($P < .05$) following weaning and remained at these lower populations through the end of the study. Intestinal lactobacilli, bifidobacteria, and total anaerobe populations initially decreased ($P < .05$) following weaning, with lactobacilli and bifidobacteria returning to preweaning levels by the end of the study. Fecal *E. coli* and fecal total anaerobes decreased ($P < .05$) over the course of the study; however, fecal lactobacilli and bifidobacteria increased ($P < .05$) over the same interval. Galactose did not affect ($P > .05$) populations of microflora tested in this study at either sampling site, except for a tendency ($P < .08$) for galactose to prolong high *E. coli* populations postweaning. Acetate, propionate, butyrate, and isovalerate concentrations differed ($P < .001$) in the ileum versus the cecum. These same acids along with isobutyrate also decreased ($P < .05$) after weaning regardless of site or treatment. There was a tendency ($P < .08$) for galactose to decrease acetate production;

otherwise, no treatment effects were observed. Lactate (L+) concentrations were greater (P = .05) in ileal contents than concentrations observed from cecal contents. These data indicate that galactose fed at this level incurs only minimal changes in microflora or VFA concentrations in the ileum and cecum of pigs.

Key Words: Pigs, Intestinal Microflora, Volatile Fatty Acids, Ileum, Cecum, Weaning

Introduction

Weaning is a time of difficult transition for the young pig. The shift from a milk-based diet to a plant-based diet has been shown to induce many problems, including unthriftiness, scours, and mortality (1, 2). Intestinal microfloral populations have been observed to fluctuate during this transition period (3, 4). This fluctuation may be due to the change in substrate available to the microbes, including a decrease in galactose in the postweaning diet, which may cause a shift in microbial populations. Changes in microbial populations may affect both commensal and pathogenic bacteria. It has been reported that certain species of lactobacilli and bifidobacteria, considered to be commensal microflora (5, 6), preferentially select certain carbohydrates for their growth (7, 8).

Fermentation of carbohydrates by bacteria provides the intestine with VFA, which can be used directly as an energy source for the intestinal mass (9, 10). This energy source could be significant to the pig, thus a shift in fermentation patterns resulting from changes in microbial populations may account for some of the postweaning problems mentioned earlier. Previous work has shown VFA to affect gastric motility patterns (11), and Mathew et al. (4) suggested this may give the newly weaned pig a sensation of satiety

which would reinforce the typical decreased intake seen in the early postweaning period. Decreased intakes may also cause a shift in microbial populations, thus affecting VFA concentrations.

Earlier studies investigated microflora (12, 13) and VFA production (14, 15) at specific sites along the gastrointestinal tract. However, the majority of these studies have been limited to the sacrifice and comparison of littermates. One concern of these studies is that pigs, even of the same age, may be at different stages of intestinal development. Cranwell et al. (16) demonstrated HCl production occurred in some pigs at 1 d of age but not in others until 24 d of age. Thus, repeated measures from a single animal may be advantageous. Previous studies in our laboratory have investigated aerobic microfloral and VFA concentrations in the same animal over time, but have concentrated primarily on the ileum (3, 4). A more descriptive approach may be necessary to determine effects of microbial substrates in swine diets at various sites of the GI tract. Therefore, the objective of this study was to characterize anaerobic and aerobic microfloral populations and VFA concentrations in the ileum and cecum of weanling pigs fed diets with differing amounts of galactose.

Materials and Methods

Animals:

A total of 24 weanling pigs from 4 litters were used in two replicate trials in this study. At approximately 14 d of age, 12 pigs were cannulated in the ileum and 12 were cannulated in the cecum, in accordance with the Institutional Animal Care and Use Committee of The University of Tennessee, Knoxville. The surgical procedure for ileal

cannulations has been previously described in the literature (17). Ileal cannula placement was determined by locating the mesenteric attachment bridging the distal end of the cecum to the final ileal loop of the small intestine. This loop is approximately 10 cm proximal to the ileal-cecal junction in pigs of this age; and cannulas were inserted at this location to ensure consistent placement along the small intestine. Cecal cannulations were a modification of the ileal cannulation technique described by Walker et al. (18). Cecal cannula placement was determined by locating the blind end of the cecum and cannulas were placed at the midpoint of the blind end of the cecum to ensure consistent placement. Cannulas were specially constructed (Mechanical Engineering Department, Purdue University, West Lafayette, IN) from Delrin 600 plastic, and were similar in design to those described by Walker et al. (18). Following surgery, pigs were returned to their respective sows and allowed a 4 d recovery period prior to weaning at approximately 18 d of age. At weaning pigs were housed individually in stainless steel cages in an environmentally controlled room and randomly assigned to one of two test diets.

Diets:

The diets consisted of a corn starch base with or without supplemental galactose (Table 1; all Tables located in Appendix 1). Starch (Roquette, Des Moines, IA) was ground in a Wiley mill with a 6 mm screen before mixing with other ingredients. At feeding, treatment diets were mixed with water to form a gruel in order to control dust and promote intake. Feed was provided twice daily with the amount fed based on consumption from the previous feeding. Water was available ad libitum between the two

feedings. Orts were collected and dried, then weighed to determine total intake for all pigs.

Sampling:

Ileal and cecal contents were collected on the day of weaning and at 3, 7, 10, 14, and 17 d postweaning, corresponding to 18, 21, 25, 28, 32 and 35 d of age. Collection was initiated at approximately 0700 of each sampling day. At that time, a sterile rubber balloon with no head space was placed on the opened cannula and intestinal contents were allowed to collect until approximately 10 g of digesta had accumulated. Because digesta flow was intermittent, balloons were replaced approximately every 30 min until digesta began to flow consistently. Typically, once the digesta started flowing, approximately 15 min were needed for sufficient collection of material. Samples were immediately placed on ice until further analysis. This procedure was followed to minimize the possibility of bacterial proliferation and/or fermentation in the balloons. Fecal swabs were taken when the intestinal sampling was complete.

Sample Preparation:

In preparation for analysis, one gram of digesta was weighed into a test tube and fecal swabs were weighed to determine the amount of sample collected. Fecal swabs were then placed in a test tube and 9 mL of phosphate buffered saline (PBS) were added to the fecal and digesta test tubes. These initial mixtures were immediately placed in an anaerobic chamber. Tubes containing fecal samples were agitated for 1 min and ten-fold dilutions were then made from both sets of tubes using prereduced PBS as the diluent. Collection and preparation were typically completed within 2 h of the initiation of

collection. Sample pH was determined from the remaining digesta using a Corning #345 pH meter (Corning, New York, NY) with a high performance glass electrode (cat. # 476390). Digesta were then centrifuged in a Beckman model J2-HS centrifuge (Palo Alto, CA) with a JA20 rotor at 27,000 x g at 4°C for 15 min. The resulting supernatant was collected and stored at -80°C until VFA and lactate analysis could be performed.

Microbial Analysis:

For anaerobic culture, 50 µL of each serial dilution were plated in duplicate onto prereduced media inside the anaerobic chamber. Inoculated Petri dishes were then placed in canning jars, the lids partially tightened, and jars subjected to a vacuum prior to sealing. Jars were then placed in convection incubators for appropriate culture conditions. For culture of aerobes, serial dilutions were removed from the anaerobic chamber and Petri dishes were inoculated and placed directly into incubators.

Four media were utilized in this study and included: 1) Lactose MacConkey agar (Difco, Detroit, MI) for enumeration of *E. coli*, 2) Rogosa media (Difco) for enumeration of lactobacilli, 3) Modified Columbia media (19) for enumeration of bifidobacteria and 4) Media 10 (20) for enumeration of total anaerobes. *E. coli* were incubated aerobically at 37°C for 24 h. Anaerobic culture included bifidobacteria, which were incubated at 40°C for 48 h, and lactobacilli and total anaerobes, which were incubated at 37°C for 48 h. Bacteria were enumerated by visual count, disregarding atypical colonies. Previous work in our laboratory has confirmed typical *E. coli* colony growth on MacConkey media (4), and all typical colonies were counted as *E. coli*. In that study (4), selected colonies were subjected to biochemical analysis (API 20, Vitek BioMerieux, Syosset, NY) to identify

isolates to the species level. In all cases, colonies suspected of being *E. coli* were determined to be *E. coli* by the biochemical analysis. The majority (96%) of isolates not phenotypical of *E. coli* were determined to be other species. All large white colonies were counted as lactobacilli on Rogosa media (21). Large white colonies were counted as bifidobacteria on Modified Columbia media (19) and all colonies were counted as total anaerobes on Media 10 (20).

VFA Analysis:

Volatile fatty acid concentrations were determined using a gas chromatographic method modified from Playne (22). Briefly, one and one-half milliliters of supernatant were mixed with 300 μL of 25% metaphosphoric acid (5:1 ratio) and incubated at room temperature for 30 min. Following centrifugation to remove the precipitate, 1μL of sample was injected into a Hewlett Packard model 5890 gas chromatograph (Hewlett Packard, Avondale, PA) with an HP-FFAP 10-m x .53mm x 1-μm capillary column packed with cross-linked polyethylene glycol-TPA. A flame ionization detector was used with an oven temperature of 200°C, and a detector temperature of 250°C, for determination of acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate concentrations. The lower detectable limit for all VFA was .1 mmol/L.

Lactate Analysis:

Lactate concentrations were determined using a lactate analysis kit (#826-B; Sigma Chemical, St. Louis, MO). In this assay, 200 μL of the intestinal content supernatant was mixed with 400 μL of ice-cold 8% perchloric acid. Samples were incubated on ice for 30 min and centrifuged to remove precipitate. The resulting

supernatant was assayed according to Sigma, using the appropriate lactate dehydrogenase enzymes in separate assays for determination of D(-) and L(+) lactate. A modification of volumes was implemented to allow the use of microtiter plates and associated equipment. In the modified analysis, 200 μL of NAD-glycine solution was pipetted into a flat bottom Serocluster 96-well microtiter plate (Costar Corp., Cambridge, MA). To that solution, 20 μL of each sample supernatant was added to triplicate wells. Absorbance was read at 340 nm on a model EL 340 Bio Kinetics microtiter plate reader (Bio-Tek Instruments, Wiooski, VT). Following the initial reading, 10 μL of the appropriate lactate dehydrogenase (L+ or D-) enzyme was added to each well. Plates were incubated for 15 min at 37°C followed by incubation for 15 min at room temperature. Following the final incubation, absorbance was read a second time. Regression analysis using a standard curve was used to convert absorbance readings to lactate concentrations. The lower detectable limit for the lactate analysis was .05 mmol/L.

Statistical Analysis:

The statistical model consisted of a randomized complete block design using repeated measures analysis with the individual pig serving as the experimental unit. Data were analyzed using the Mixed Model Procedure of SAS (23). Differences between least squares means were separated using pairwise t-tests. Differences between days were determined using Pdmix procedures. Microbial and lactate concentrations were transformed (log_{10}) prior to statistical analysis.

Results

There were no differences ($P > .05$) in the amount of feed consumed on either the galactose diet (388 g/day) or control diet (369 g/day), nor were differences ($P > .05$) in gain noted between pigs on the galactose diet (220 g/day) and those consuming the control diet (219 g/day). The pH differed between the ileal (7.11) and cecal (6.10) contents ($P < .01$); however, no differences ($P > .05$) were observed between treatments at either site. Cecal content pH increased by 21 d of age but returned to preweaning levels by 25 d of age; whereas, the pH of ileal contents remained elevated after weaning (Table 2). Dry matter percentage of cecal contents (6.93%) was nearly twice that ($P < .01$) of ileal contents (3.84%). A day effect ($P < .04$) was observed for dry matter percentage, with dry matter decreasing on the first sampling postweaning for both sites and treatments, but returning to preweaning levels by the end of the study (Figure 1; all Figures located in Appendix 2). No main effect differences ($P > .05$) in dry matter percentage were observed between treatments. Pigs consuming the galactose diet tended ($P < .08$) to maintain *E. coli* populations longer than those consuming the control diet (Table 3). A day effect was observed for some microflora populations with *E. coli*, bifidobacteria, and total anaerobes decreasing by 7 d postweaning regardless of treatment or site (Table 4). Total anaerobes and *E. coli* remained at these lower levels through the end of the experiment; whereas, bifidobacteria returned to preweaning levels by the end of the study. A day effect was also observed for fecal *E. coli* ($P < .004$) , total fecal anaerobes ($P < .004$), and lactobacilli ($P < .001$) with populations of these organisms

decreasing by 3 d postweaning. In contrast, fecal bifidobacteria ($P < .004$) and fecal lactobacilli ($P < .001$) were observed to increase during this time (Table 4).

The concentrations of acetic acid differed ($P < .001$) between ileal and cecal contents, with the cecum (77.51 mmol/L) containing more than twice that of the ileum (35.89 mmol/L). A similar pattern was observed ($P < .001$) for propionic (27.6 mmol/L versus 4.33 mmol/L), butyric (12.28 mmol/L versus 1.79 mmol/L) and isovaleric (1.26 mmol/L versus 0.36 mmol/L) acid concentrations. A day effect was also observed postweaning in intestinal contents, with acetate ($P < .001$), isobutyrate ($P < .03$), butyrate ($P < .001$), and isovalerate ($P < .001$) concentrations decreasing postweaning regardless of site or treatment (Tables 5 and 6). There was a tendency ($P < .08$) for the galactose diet to decrease acetate production (Table 7), otherwise no treatment effects were observed. Comparisons of valeric acid in cecal and ileal contents was not possible due to undetectable levels in ileal contents. Total concentrations of VFA in the cecum were 170.9 mmol/L and 82.8 mmol/L in the ileum at 18 d of age, and were 100.2 mmol/L in the cecum and 31.4 mmol/L in the ileum at 21 d of age. Acetate made up the largest proportion of these concentrations in both the cecum (60%) and ileum (80%).

Lactate (L+) concentrations were greater ($P = .05$) in ileal contents (9.37 mmol/L) than concentrations observed from cecal contents (3.40 mmol/L). At 21 d of age, concentrations of L+ and D- lactate were greater in the ileum versus the cecum (Table 8).

Discussion

Dietary galactose inclusion was investigated in this study to determine possible effects the decrease of this sugar at weaning may have on enteric microflora. Commensal

bacterial groups have been shown to prefer certain carbohydrates, with bifidobacteria selectively utilizing kestoses, fructooligosaccharides, and galactose (7, 8, 24); however, galactose had no effect on bifidobacteria populations in this study. Microfloral populations, pH, dry matter, lactate, and VFA concentrations were observed at two different sites in the intestinal tract over the course of weaning in the same animal. Bifidobacteria and total anaerobe populations followed a postweaning pattern typically observed for lactobacilli in our previous studies (3, 4), with populations decreasing markedly following weaning. Populations of *E. coli* were not observed to increase after weaning, as has been observed in previous studies, and this may be due to the housing conditions of pigs in this study, which consisted of a highly sanitized, environmentally controlled room (25).

The tendency for galactose to maintain *E. coli* populations in this study after weaning may be due to that species' ability to utilize galactose more efficiently. The increase in fecal bifidobacteria immediately following weaning could be due to an overall increase in the amount of carbohydrate reaching the large intestine with pigs consuming a weaning diet versus a milk diet. The maintenance of *E. coli* populations and the increase in fecal bifidobacteria may also be due to a decrease in competition for substrate with the decline observed in other bacterial populations including lactobacilli and total anaerobes. Other work in our laboratory has suggested that galactose may decrease degradation of intestinal mucins, which typically contain a high proportion of galactosyl carbohydrate moieties (unpublished results). Populations of bacteria may be altering degradation of intestinal mucins, thus shifting fermentation patterns in the presence of dietary galactose.

The cecal pH values we observed were similar to those of Smith and Jones (26), who found the pH in the cecum to be 6.1. However, the pH they reported in the ileum (8.0) was somewhat higher than we observed in this and other studies (3, 4). Mathew et al. (3) reported increased ileal pH levels in pigs weaned at 3 weeks of age compared to pigs weaned at 4 weeks of age. Increased ileal and cecal pH was also observed in this study with pigs weaned at 18 d of age, which agrees with the data of Cranwell et al. (16) who suggested that significant quantities of HCl were not produced before 24 d of age.

Ileal VFA concentrations observed in this study were similar to other work in this laboratory (4) and cecal VFA levels were similar to those reported by Sutton et al. (27). The decrease in some VFA immediately postweaning could be due to the pigs decreased feed intake during the first few days after weaning. A concomitant rise in lactate was also observed and may indicate a shift in the fermentation patterns of the microbes (25). These observations may indicate that intestinal microbes need an adjustment period similar to that observed in ruminants, when dramatic diet changes are induced.

We realize that the anaerobes isolated in this study were exposed to some oxygen during collection, which may have killed the more fastidious organisms; however, other workers have shown that a short term exposure to oxygen had no detrimental effect on bacteria that were otherwise considered strict anaerobes (28, 29). Patel et al. (29) also demonstrated that agar medium containing reducing agents and previously reduced for 24 h can be inoculated aerobically and incubated in an anaerobic environment without significant loss in cell viability. For anaerobic culture in the present study, organisms were inoculated anaerobically onto media that had been previously reduced for 24 h and

incubated anaerobically. Methods to obtain a totally anaerobic sample and introduce it into an anaerobic environment would be beneficial in characterizing microfloral populations in young pigs. Additionally, this was not intended to be an exhaustive characterization of all anaerobes in the intestinal tract of swine, but rather a comparison of dietary components.

We also do not feel that cannulation had an adverse effect on microbes and VFA sampled in this study. Pigs performed well on both diets and intakes were typical of pigs this age (30). In a similar study, we found no differences in microbial populations or cortisol concentrations between pigs cannulated in the jejunum, ileum or cecum versus noncannulated littermates (unpublished results). Concentrations of VFA were also similar to those found in noncannulated pigs in earlier studies (25). Thus, we feel our data are representative of pigs weaned at this age. The use of intestinal cannulations appears to have greater benefits than sacrificing littermates because sampling the same animal over the course of weaning is possible, thus reducing pig to pig variation previously observed among littermates (31, 32).

We were unable to detect differences in selected microfloral populations with addition of galactose in the diet. However, other species not tested may have been affected. Additionally, galactose concentrations may not have been sufficient to alter populations. It is likely that galactose concentrations in the GI tract of nursing pigs are greater than that of pigs fed the galactose diet in this study. We chose this level to replace a reasonable proportion of the galactose lost when pigs are weaned, and our inclusion level replaced 25% of the total starch in the control diet.

Future work needs to address the declines in commensal microflora and VFA levels observed during the postweaning period. Some problems associated with postweaning lag may be alleviated by promoting commensal microflora and VFA levels immediately postweaning. Eliminating postweaning lag, decreasing disease incidence, and increasing efficiency of production would have a significant and positive impact on the industry.

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APPENDIX 1
DATA TABLES EXPERIMENT 2

TABLE 1
Composition of Diets (as-fed basis)*

Ingredient	Control kg	Treatment kg
Corn Starch	51.80	38.80
Galactose	0.00	13.00
Soybean meal, 48% CP	30.00	30.00
Dextrose	7.50	7.50
Bloodmeal, spray dried 88.8% CP	4.00	4.00
Fishmeal, Menhaden 62.9% CP	4.00	4.00
Limestone	.65	.65
Dicalcium phosphate	1.20	1.20
Salt	.35	.35
Vitamin/Mineral Premix#	.50	.50
Total	100	100

*Diets were formulated to meet or exceed NRC recommendations for 5 to 10 kg pig.
#Contained (per kilogram of diet): 849 mg Ca; 150 mg Zn; 132 mg Fe; 20 mg Mn; 12 mg Cu; .31 mg Se; 1,298 IU Vit. A; 3,260 IU Vit. D; 2.4 IU Vit. E; 143 µg Menadione (sodium bisulphite form); 3.3 µg Vit. B12; 880 µg Riboflavin; 2.6 mg d-Pantothenic acid; 4.4 mg Niacin.

TABLE 2
pH of Ileal and Cecal Contents of Weanling Pigs on Various Days*

Age, d	Ileum	Cecum
18	$6.53^{c\#}$	6.02^{e}
21	6.90^{b}	6.41^{cd}
25	7.22^{ab}	5.98^{e}
28	7.30^{a}	6.13^{de}
32	7.44^{a}	6.03^{de}
35	7.29^{ab}	6.03^{de}
SEM		.20

* Data represent least squares means from two replicate trials (n = 6/treatment). Pigs were weaned at 18 d of age.

a,b,c,d,e Least squares means with different superscripts differ at $P < .05$.

SEM = maximum standard error of the mean observed among all test periods and sites.

TABLE 3

Populations of Intestinal *E. coli* in Weanling Pigs on Various Days*

Age, d	Control	Treatment
18	$9.73^{a\#}$	9.81^{a}
21	8.58^{b}	10.0^{a}
25	7.54^{c}	7.67^{bc}
28	7.57^{bc}	7.27^{c}
32	7.52^{c}	7.26^{c}
35	7.34^{bc}	6.19^{c}
SEM		.75

* Data represent least squares means from two replicate trials (n = 6/treatment) and are expressed as $\log_{10}$ colony forming units (CFU)/g of intestinal contents, wet basis. Pigs were weaned at 18 d of age.

a,b,c Least squares means with different superscripts differ at $P < .05$.

SEM = maximum standard error of the mean observed among all test periods and diets.

TABLE 4

Fecal and Intestinal Microbial Populations on Various Days in Weanling Pigs*

Age, d	Total lactobacilli		Total bifidobacteria		Total *E. coli*		Total Anaerobes	
	$F^{a\#}$	I^{a}	F^{b}	I^{c}	F^{b}	I^{b}	F^{b}	I^{a}
18	8.68^{e**}	10.96^{c}	6.09^{e}	8.18^{c}	9.67^{c}	9.77^{c}	10.24^{c}	10.86^{c}
21	8.82^{de}	10.11^{d}	7.66^{c}	7.80^{cde}	9.01^{d}	9.29^{c}	9.68^{de}	10.39^{cd}
25	8.95^{cde}	10.59^{cd}	6.90^{d}	7.22^{ef}	7.68^{f}	7.60^{d}	8.84^{f}	9.96^{d}
28	9.25^{cd}	9.96^{d}	7.57^{c}	7.30^{def}	8.61^{de}	7.42^{d}	9.80^{cd}	9.69^{d}
32	9.41^{c}	10.39^{cd}	6.91^{d}	7.04^{f}	8.42^{de}	7.39^{d}	9.64^{de}	9.99^{d}
35	9.06^{cde}	10.50^{cd}	7.83^{c}	8.17^{cd}	7.82^{ef}	6.77^{d}	8.96^{ef}	9.34^{d}
SEM	.32	.42	.46	.48	.41	.49	.53	.64

TABLE 5
Ileal and Cecal Acetate, Propionate, and Butyrate Concentrations on Various Days in Weanling Pigs*

	acetate		propionate		butyrate	
Age, d	I**	C	I	C	I	C
18	67.8$^{bc\#}$	107.9^{a}	6.8^{d}	30.2^{ab}	6.3^{cd}	21.1^{a}
21	23.3^{d}	60.9^{c}	3.6^{d}	25.3^{bc}	1.1^{e}	8.5^{c}
25	25.5^{d}	77.1^{b}	2.6^{d}	33.3^{ab}	.7^{e}	14.6^{b}
28	32.0^{d}	68.5^{bc}	6.0^{d}	21.9^{c}	.6^{e}	9.4^{c}
32	34.4^{d}	74.6^{bc}	4.4^{d}	18.6^{c}	.8^{e}	9.5^{bc}
35	32.4^{d}	76.1^{b}	2.6^{d}	36.2^{a}	1.2^{de}	10.7^{bc}
SEM	6.6		4.0		2.5	

* Data are mmoles/L and represent least squares means from two replicate trials (n = 6/treatment). Pigs were weaned at 18 d of age.
** I = ileum, C = cecum.
# $^{a, b, c, d, e}$ Least squares means with different superscripts within VFA differ ($P < .05$).
SEM = maximum standard error of the mean observed among all periods and sites.

TABLE 6
Ileal and Cecal Valerate, Isobutyrate, and Isovalerate Concentrations on Various Days in Weanling Pigs *

	valerate		isobutyrate		isovalerate	
Age, d	I**	C	I	C	I	C
18	.5$^{d\#}$	5.6^{a}	.7^{c}	2.9^{a}	.7^{bc}	3.2^{a}
21	1.2^{cd}	3.5^{b}	1.9^{ab}	.8^{bc}	.3^{c}	1.2^{b}
25	.2^{cd}	4.2^{ab}	.6^{c}	.6^{c}	.3^{c}	.5^{bc}
28	ND##	3.0^{bc}	1.1^{bc}	.9^{bc}	.2^{bc}	1.0^{bc}
32	ND	2.0^{bcd}	.7^{c}	.7^{bc}	.4^{bc}	.8^{bc}
35	.3^{cd}	2.3^{bcd}	1.6^{bc}	.5^{c}	.4^{bc}	.8^{bc}
SEM	1.3		.5		.6	

* Data are mmoles/L and represent least squares means from two replicate trials (n=6/treatment). Pigs were weaned at 18 d of age.

** I=ileum, C=cecum.

a, b, c, d Least squares means with different superscripts within VFA differ ($P < .05$).

SEM= maximum standard error of the mean observed among all periods and sites.

ND = not detected.

TABLE 7
Volatile Fatty Acid Concentrations at Various Sites Along the Gastrointestinal Tract*

Diets	Acetate	Propionate	Butyrate
Control	60.84[a #]	16.97[a]	7.56[a]
Treatment	52.56[b]	14.96[a]	6.51[a]
SEM	2.23	1.46	.83

* Data are mmoles/L and represent least squares means from two replicates.
[# a, b] Least squares means with different superscripts within VFA differ ($P < .08$).
SEM = maximum standard error of the mean observed among both diets.

TABLE 8

Ileal and Cecal Lactate Concentrations on Various Days in Weanling Pigs. *

	L+ Lactate		D- Lactate	
Age, d	Ileum	Cecum	Ileum	Cecum
18	$5.73^{bc\,\#}$	5.18^{bc}	4.47^{abc}	5.36^{abc}
21	18.29^{a}	3.74^{bc}	8.26^{a}	3.54^{bc}
25	12.45^{ab}	5.35^{bc}	7.30^{ab}	6.20^{abc}
28	8.08^{bc}	1.61^{c}	5.28^{abc}	1.66^{c}
32	8.03^{bc}	2.04^{bc}	4.89^{abc}	2.45^{abc}
35	3.65^{bc}	2.47^{bc}	3.27^{abc}	1.94^{c}
SEM	4.62		2.67	

* Data are mmoles/L and represent least squares means from two replicate trials (n = 6/treatment). Pigs were weaned at 18 d of age.

a, b, c Means within lactate isomer with different superscripts differ, $P = .05$.

SEM= maximum standard error of the mean observed among all test periods and sites.

Appendix 2
Figures for Experiment 2

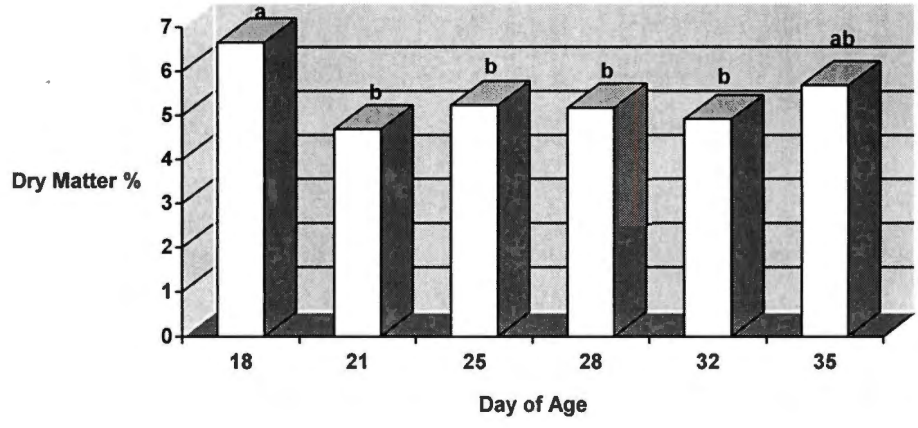

Figure 1. Dry Matter Percentage on Various Days in Weanling Pigs

VITA

Mark Ashburn Franklin was born July 29, 1971, and is the son of James Dale and Evetta Franklin. He received his elementary and secondary education in Dandridge, Tennessee, graduating from Jefferson County High School in May of 1989. He then entered the University of Tennessee, Knoxville majoring in Animal Science. Mark graduated from the University of Tennessee with a Bachelor of Science in Animal Science in May, 1994. He returned in June of 1994 to the University of Tennessee to begin his graduate work. Mark received his Master of Science with a major in Animal Science in May, 1996. He continued his graduate education at the University of Tennessee and received the Doctor of Philosophy with a major in Animal Science in December of 1998.