A MICROBIOME AND METABOLOME ANALYSIS OF ANTIBIOTIC GROWTH PROMOTORS AND ANTIBIOTIC ALTERNATIVES FED TO SWINE

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Abstract

The mechanisms of action for antibiotic growth promotion are poorly understood, making it difficult to select effective alternatives capable of providing similar responses. The objective of this thesis was to identify mechanism of action when feeding growth promoting levels of antibiotics and antibiotic alternatives. The first trial identified tylosin-responsive metabolites in growing pigs. This experiment identified that feeding tylosin increased the concentration of the secondary bile acids hyodeoxycholic acid and lithocholic acid and multiple bacteria in the *Clostridia* family. These findings suggest the mechanism of growth promotion involves an alteration in bile acid metabolism. The second experiment evaluated the growth performance, metabolic responses, and changes in intestinal microbiome composition of nursery pigs fed a variety of feed additives and antibiotic controls. Only the antibiotic group had improved growth performance over the negative control. Although none of the feed additives tested had an effect on alpha or beta microbiome diversity, supplementing multiple herbal blends increased the relative abundance of cecal bacteria in the genus *Lactobacillus*. In addition, the effect of experiment location had a greater influence on the microbiome than the effect of dietary treatment. Overall, these experiments identified that antibiotic growth promotion could use a mechanism involving bile acid metabolism, but this was not consistent when a different antibiotic was used. In addition, the proposed antibiotic alternatives had a minimal impact on animal growth, microbiome, and metabolism, but experiment location significantly impacted these parameters and this effect should be further evaluated.

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Chapter 1: Literature Review

Introduction

Pork production has become more efficient in recent years compared with previous years as a result of genetic improvements and precision nutrition technologies which include the use of growth promoting feed additives (1,2). Historically, improvements in nutritional efficiency in pork production systems was achieved by using subtherapeutic doses of antibiotics to improve growth performance in food producing animals, but the use of antibiotic growth promoters (AGPs) in animal agriculture has contributed to the development of antibiotic-resistant bacteria which poses a human and animal health risk (3). This public health concern led to regulatory action to develop and implement the Veterinary Feed Directive (VFD) in the United States (4). The VFD restricts the use of AGPs in food animal production, which has led to an urgent need to identify and evaluate the effectiveness of alternatives to maintain optimal animal growth and nutritional efficiency. Unfortunately, the biological mechanisms by which AGPs enhance growth and health of pigs are not completely understood, which creates challenges to identify alternative feed additives that provide comparable responses.

The proposed mechanisms of action from adding AGPs to swine diets include modifying the intestinal microbiome and altering metabolism to improve feed efficiency (1,5). Ideally, alternatives to AGPs should mimic these growth performance and biological responses. Promising AGP alternatives that have been demonstrated to provide beneficial effects by altering the microbiome and animal metabolism include botanical extracts and yeast products (6). Unfortunately, the biological and growth responses from

feeding either botanicals or yeast products to swine have been inconsistent (6–9). For this reason, the objective of this review is to evaluate the history and use of antibiotic growth promotors, their impact on the intestinal microbiome and animal metabolism, and describe our current knowledge of the use of selected alternative growth promoting feed additives.

Role of the microbiome in swine health and productivity

The gastrointestinal microbiome in pigs

Millions of microbes reside in the gastrointestinal tract (GIT) of swine and they collectively play a role in nutrient digestion, pathogen protection, immune system modulation, and intestinal epithelium function (10,11). Although bacteria are often the focus of microbiome research in pigs, other microbes including viruses, protozoa, and fungi are also present in the GIT, but their interaction with the bacterial microbiome is still not well characterized (12). When focusing specifically on the bacteria in the microbiome, a meta-analysis of 20 different studies showed that 99% of pig fecal samples included the bacterial genera *Prevotella, Clostridium, Alloprevotella*, and *Ruminococcus*, indicating that pigs have common constituents or a "core microbiome" that is relatively consistent among commercial pigs around the world (13).

When evaluating the function of these core bacteria, the bacterial genera *Prevotella* has been correlated with diets abundant in carbohydrates and fiber and, in swine specifically, their increased relative abundance in the intestine has been correlated with improved growth performance (14,15). Taking this into consideration, increased relative abundance of bacteria in the genera *Prevotella* may be beneficial for nursery pigs as they transition from milk-based to plant-based diets at weaning. When evaluating the

relationship between *Prevotella* and other bacteria genera, a network analysis of the microbiome of 18 pigs that were monitored from birth to 174 days of age showed that *Prevotella* are connected to other bacteria in the GIT microbiome (16). This indicates *Prevotella* is involved in many complex cooperative and competitive relationships with other bacteria in the gastrointestinal tract, which may potentially be difficult to manipulate if only relative abundance of *Prevotella* affect growth performance.

The bacterial genera *Clostridium* is also a core bacterium in the pig's GIT microbiome (13). Despite its classification as a significant bacterial genera in the swine GIT microbiome, its function is not well understood (13,17). Much of the research focusing on *Clostridium* focuses on potentially pathogenic strains in this genus. Bacterial species including *Clostridium perfringens* and *Clostridium difficile* are both intestinal pathogens in swine associated with diarrhea, enteritis, and reduced growth performance (18). However, to better understand the role of *Clostridium* spp. in the core microbiome, more research is needed in understanding the functionality of other species in this genus that are not pathogenic.

The bacterial genera *Alloprevotella* is another significant bacterium in the pig's core microbiome, and has been associated with short chain fatty acid (SCFA) production, specifically acetic acid and succinic acid (19). When *Alloprevotella* are relatively abundant in the swine intestinal microbiome, it has been correlated with improved BW and ADG (20). Although a clear mechanism of the role that *Alloprevotella* may play in improving pig growth has not been established, it is possible that increased SCFA production provides additional energy that can be utilized by the animal. Previous research has shown that supplementing SCFA in diets of germ free pigs that are lacking a

gut microbiome alters lipid and glucose metabolism primarily through GLUT-2 and GYS2 expression (21). When taking into consideration the SCFA production of *Alloprevotella*, this bacterial genera may play a key role in glucose and lipid metabolism in pigs.

The bacterial genera *Ruminococcus* is another key bacterium in the core swine microbiome that has multiple species of cellulolytic and hemicellulolytic bacteria capable of fermenting dietary fiber to provide energy to the pig (22). Similar to *Alloprevotella, Ruminococcus* in the intestine of pigs also has a positive correlation with ADG and BW (20). The association with *Ruminococcus* and *Alloprevotella* with a growth improvement and their ability to ferment fiber to produce SCFA for energy, suggests that these core components of the intestinal microbiome may be important biomarkers for assessing improvements in fermentation and subsequent energy production and metabolism for pigs.

Variation in bacterial composition in the swine GIT microbiome

Apart from the core microbiome, bacteria from other genera have been identified in swine fecal samples that vary dramatically between experiments, farm location, diet, age, and genetic line of the pigs (13). When evaluating the variation between different pig breeds, groups of Durocs, Large White, and Landrace pigs that were kept on the same farm and fed the same diet had significantly different relative abundance of *Catenibacterium*, *Clostridium*, and *Bacteroides* (23). Given that these breeds also demonstrated different ADG and ADFI, these researchers suggested that the microbiome not only varies by breed, but also plays a key role in shaping the production parameters

(23). When this effect was evaluated within a Jinhua pig population, a PCoA plot representing the beta diversity in the fecal microbiome showed that genetic strain and weaning age had a higher impact on the microbiome composition compared with farm location (24). These findings suggest genetic predisposition plays an important role in shaping the pig's microbiome, and as we move toward a definition of a "good" or "bad" microbiome for describing growth performance responses to nutritional interventions, considerations should be based on microbiome variation among genetic lines of pigs.

Farm has also been shown to affect the alpha diversity of the colon microbiome in growing pigs (25). A meta-analysis of 20 studies showed that the impact of study was greater than the impact of age of pigs or sampling location (13). These findings suggest that apart from the core microbiome, there is a variable population of different bacterial genera and species in the pig microbiome that is heavily influenced by the environment. Therefore, microbes may be influenced by exposure to various environmental conditions such as barn temperature or bacterial populations within a barn. However, more research is needed to validate this effect and determine the factors and management practices within a barn that have the greatest likelihood of affecting the pig's gut microbiome.

Microbiome development in pigs

The colonization of the intestinal microbiome begins immediately after an animal is born (26). Genetically encoded chemical epitopes, or attachment sites, expressed in the intestinal mucous play a role in determining which bacterial species colonize each section of the gastrointestinal tract (1). This initial colonization appears to be heavily influenced by the environment of the animal early in life (26). In humans, the similarity in bacterial composition of infant fecal samples and the breast milk/vaginal swab from the mother

indicates that the bacterial population is influenced by the specific bacteria that the baby is exposed to early in life (26). These findings indicate that the maternal environment was a major contributor to the initial microbial colonization of the intestinal tract, which is a pattern that may be similar in swine.

After the initial colonization, age is the most significant modifier in GIT microbiome composition (27). It is possible that the changes observed in pigs and other mammals relative to the developing microbiome are driven by the changes in diet as an animal matures. For example, in the growing pig, *Bacteroides*, *Butyricimonas, Clostridiales* and *Escherichia/Shigella* all decline as the pig ages (28). A similar pattern of decline, specifically in the genera *Bacteroides*, has also been shown to occur in human infants (26). Previous research results have suggested that bacterial species from these genera decline as the animal transitions from a milk-based diet to grain-based diets because these bacterial species are better adapted to use the high concentrations of milk oligosaccharides present in the infant diet as a unique carbon source (28). Therefore, initial environmental exposure and diet play important roles in influencing the microbiome in animal gastrointestinal tracts early in life.

Although it is important to understand how the GIT microbiome develops over time, it is also necessary to understand specific changes in the microbiome composition that may be beneficial or harmful to the animal. An experiment conducted by Mach et al. (2015) evaluated the fecal microbiome of piglets from birth through weaning and correlated the fecal microbiome data with growth performance measures. In this experiment, they found that the bacterial communities in piglets co-evolve with their hosts towards two different groups, primarily distinguished by *Ruminococcaceae* or

Prevotella genera abundance in the microbiome (28). Before weaning, all piglets had high concentrations of *Ruminococcaceae* but after weaning, there was a shift in microbiota composition that moved half of the pigs to the *Prevotella* dominated group (28). When the microbiome composition was correlated with growth performance measures, pigs in the *Ruminococcaceae* group had greater average daily gain (ADG) before weaning than pigs in the *Prevotella* group (28). However, after weaning, pigs in the *Prevotella* group had increased body weights and ADG compared with the pigs in the *Ruminococcaceae* group. These results indicate that pigs with a distinct change in the microbiome at weaning grew faster after weaning compared with pigs that did not experience this transition to the *Prevotella* group. This microbiome shift supports the concept of bacterial adaptation to diets, previously described for *Prevotella* and *Ruminococcaceae* species, suggesting that if the pig's microbiome can adapt during weaning to better utilize nutrients, growth performance may be improved (29).

Another study evaluated fecal samples from 518 healthy pigs and found that pigs with an increased concentration of bacteria in the genera *Prevotella* and *Mitsuokella* in their intestinal microbiome, had a positive correlation with improved body weight and ADG (30). Results from a separate experiment showed that *Treponema* abundance in the microbiome was also associated with improved feed efficiency compared to other defining microbiome characteristics (31). Similarly, others have observed that *Christensenellaceae*, *Oscillibacter*, and *Cellulosilyticum* were positively correlated with improved feed efficiency in pigs (32). Authors of these studies suggested that these bacteria, in addition to *Prevotella* and *Mitsuokella*, improve the pig's ability to utilize nutrients in the diet and ultimately increase body weight gain (28,30). Although it is still

unclear how the development of the intestinal microbiome impacts the pig's ability to utilize dietary nutrients, further research that integrates microbiome, metabolome, and animal performance data may increase our capabilities to identify patterns that will elucidate mechanisms of action of AGPs and other growth promoting feed additives.

Microbiome dysbiosis

Certain bacteria are more prone to causing an infection, or disrupt the GIT microbiome than others, and common swine pathogenic bacteria include *Salmonella* and *Escherichia coli* strains (33). The pathogenicity of bacteria depends on species, strain, genes, and relative abundance in the intestines (34). Upon infection of the GIT, these pathogenic bacteria produce toxins that cause diarrhea, dehydration, destruction of the intestinal epithelium, and reduced growth performance (35). A healthy microbiome can provide resistance against the colonization of a pathogenic bacterium (36). This protective effect of the microbiome is mediated through direct killing, reducing competition for limited nutrients, and enhancement of immune responses (37). However, a disruption or reduction in bacterial diversity, such as treatment with antibiotics, can make the host vulnerable to infection by pathogenic bacteria (36). For this reason, it is important to understand the bacterial species that create a resilient microbiome so that feed additives that are effective in protecting animals from pathogenic bacterial infections can be developed and used in swine diets.

Antimicrobials

The history of antibiotics as growth promoters

A timeline summary of the history of antibiotic use in livestock production is shown in Figure 1. Penicillin was the first antibiotic to be discovered and mass produced to treat war time casualties during World War II (38). As the benefits of penicillin became clear across multiple scientific disciplines and applications, more antibiotics were discovered and produced, including chlortetracycline, streptomycin, and virginiamycin (39–41). As antibiotics became more available for use in animal agriculture, research was conducted to determine if dietary antibiotic inclusion interacted with vitamin synthesis by intestinal bacteria in chicks (40). This experiment produced unexpected results where the chicks grew faster when fed antibiotics compared with those fed without antibiotics (40). Further investigations showed that adding chlortetracycline to the diet promoted growth in growing pigs (42). Since the discovery of antibiotic growth promotion in 1946, many more antibiotics were evaluated for their ability to improve growth during various pork production phases.

The first antibiotics evaluated for their growth promotion effects included penicillin, streptomycin, aureomycin, virginiamycin, chlortetracycline, and neomycin (Table 1; 39–41). Antibiotics including penicillin, virginiamycin, neomycin, and chlortetracycline continued to be heavily utilized in both United States and Canadian swine production systems for many years (43,44). When including antibiotics in swine diets from 1945 to 1955, ADG was increased up to 30% and feed:gain improved by as much as 26% (Table 1). It also became apparent that such improvements in growth were present even when different antibiotics were used (45). As research on the use of

antibiotics as growth promoters continued and improved growth performance responses were re-evaluated, the magnitude of growth performance improvements from feeding antibiotics began to diminish. For example, although the positive impact of antibiotics was maintained in nursery pigs, by 1979 the growth promoting effects AGP on ADG decreased in effectiveness by over 10% from 1956 to 1977 (46). This decline may be attributed to advancements in biosecurity, health, nutrition, and genetics during this time period.

Despite the decreasing return on investment after their discovery and initial use, antibiotics continued to be included in swine diets after 1980 (43). In 1999, 51% of swine farms surveyed in the United States added antibiotics to diets on a continuous basis for disease prevention and growth promotion (43). In a survey conducted in Ontario in 1993, 37% of pork producers reported that they included antibiotics in diets for growth promotion purposes (47). However, by 2006, only 18% of antibiotics were used in animal production for growth promotion purposes, while 82% was for disease treatment and prevention in the United States (2). Currently, antibiotics are not used for growth promotion or disease prevention purposes, and the United States Food and Drug Administration reported that from 2016 to 2018, antibiotic use in swine decreased by 24% and totaled 2,374,348 kg of antibiotics used per year (48). This decrease in antibiotics use in swine has contributed to the increased demand for antibiotic alternatives that can product a similar growth promoting response.

Mechanism of Action for Antibiotic Growth Promotion

Despite many years of adding antibiotics to swine diets to improve growth performance, the mechanisms of action are still not well understood. Various researchers

have proposed that antibiotics improve growth by preventing pathogen infection, decreasing immune system activity, altering intestinal morphology, improving epithelium function, and improving feed efficiency (1,5,49). It is likely that each of these biological changes are interrelated and contributed to the overall improvement in the health and growth of pigs fed AGPs.

Effects of antibiotics on the gut microbiome

Reduction of subclinical disease and growth promotion

It is well established that a healthy, disease-free herd has an increased capacity for growth, and providing a clean and disinfected environment will improve growth performance (50,51). Growth performance of germ-free pigs has been shown to be greater than for conventionally reared pigs because of the absence of microflora (52). On commercial farms, the concept of AGP's suppressing pathogens was demonstrated by the increase in disease pressure when subtherapeutic doses of antibiotics were banned in Europe (51). When Sweden banned the use of AGPs, there was an overall increase in demand for feed with therapeutic levels of antibiotics because of increased health and disease challenges (51). Similar findings have been observed in controlled experiments, where feeding antibiotics at growth promotion levels decreased mortality in nursery pigs from 4.3% to 2% (2). An even more dramatic decrease in mortality was observed across five different trials where a decrease from 15.6% to 3.1% in nursery pigs was observed under high disease conditions pigs were fed diets containing AGPs (2). These collective findings suggest that AGPs improve growth performance through enhanced immune protection.

When pathogens are present, even at a subclinical level, growth depression can be associated with an immune response (53). For example, a systemic innate immune response increases the synthesis of acute phase proteins and initiates muscle catabolism which requires more energy than a transient humoral response (53). An immune challenge is associated with decreased feed intake, changes in hormones, alteration of trace mineral metabolism and requirements, and causes an overall reduction in feed efficiency (53). Feeding antibiotics limits the presence of bacterial pathogens that have the capacity to induce pro-inflammatory cytokine release, signaling an immune response (51). These pro-inflammatory cytokines have a known catabolic effect and are associated with reduced appetite and increase fatigue (54–57). Therefore, by simply reducing the number of bacteria that could cause a release pro-inflammatory cytokines, dietary antibiotic supplementation can improve lean growth in pigs.

Improved energy and nutrient digestibility with dietary AGPs

Energy digestibility

Dietary AGPs have been reported to improve the metabolizable energy (ME) in swine diets (58). For example, including antibiotics in a piglet nursery diet was as effective in increasing dry matter and energy digestibility as pelleting the diets (58). Lin (2011) theorized that feeding AGP's changes the diversity of the microbiome in the animal's intestine to create a favorable microbiome composition for utilizing energy in the diet and improve growth performance. This concept is based on the idea that some bacteria are more equipped to provide short chain fatty acids to the animal than others, and antibiotics could reduce competitive bacteria which allows energy producing bacteria

to proliferate. However, Lin (2011) indicated that it is unclear if the main effects of the microbiome on improving ME content is through the production of short chain fatty acids, or through less glucose utilization from bacteria. An *in vitro* experiment was used to calculate the amount of glucose spared from bacteria in the intestine that could instead be used for growth, and results suggested that adding subtherapeutic levels of virginiamycin provided 2.68% additional energy available for growth, while adding subtherapeutic levels of spiramycin provided only 1.56% additional energy that could be utilized for growth purposes in pigs (59). The combined results suggest that if the number of intestinal bacteria that utilize glucose are reduced, more energy may be available for use by pigs and diet energy digestibility may be improved. However, *in vivo* studies are needed to confirm that supplementing antibiotics at subtherapeutic levels in swine diets reduces the total number of bacteria in the microbiome.

Lipid digestibility

Much of the early research on the effect of feeding antimicrobials on lipid digestion was conducted in broilers. Supplementation of broiler diets with the ionophores, which are classified as antibiotics but not used as therapeutic antibiotics, improved lipid digestibility in the ileum (60). Lipid digestibility has been shown to be correlated with bile acid conjugation and the change in the GIT microbiome in broilers, where the concentration of conjugated bile acids (taurocholic and taurochenodeoxycholic acids) were greater in birds fed diets containing the antimicrobial bacitracin compared with those fed non-antibiotic diets (60). These changes in bile acid concentrations were correlated with a decrease in *Lactobacillus salivarius,* a bacterial isolate capable of

deconjugating bile acids in culture (60). These researchers hypothesized that although *L. Salivarius* can inhabit the gut of young broilers, AGP administration suppresses these bacteria and their associated bile acid hydrolase activity, which increases the concentration of conjugated bile acids and improves lipid digestion and absorption (60). In addition, other results from this study showed that birds raised on the floor had lower concentrations of taurocholic acid in the ileal contents and slower growth rates than birds raised in cages (60).

Similar observations of improved lipid digestibility from feeding AGPs have also been made in swine. When feeding the antibiotic tylosin to growing pigs, there was an increase in secondary bile acids lithocholic acid and hyodeoxycholic acid in fecal contents (61). This change was correlated with changes in a specific genus of microbes in the *Clostridia* family (61). These findings support the idea that antibiotics reduce microbial competition for resources in the intestine and increase the concentration of bacteria that conjugate bile acids, which also increases secondary bile acid production and potentially improves lipid digestibility of the diet. Further research results showed that feeding a combination of the antibiotics chlortetracycline and tiamulin encouraged microbial production of secondary bile acids in the large intestine of nursery pigs (62). Results from this experiment also showed that the changes in secondary bile acid production altered bile acid signaling in the intestine, liver, and adipose tissue; and had the overall effect of improved metabolic efficiency in the pig (62). These findings suggest antibiotics play a role in changing the intestinal microbiome and increasing the proportion of bacteria that deconjugate bile acids which contribute to the mechanism of growth promotion for at least some antibiotics.

Protein digestibility

Interactions between the microbiome and proteins in the diet are not always beneficial to the host. Bacteria in the microbiome typically compete with their host for many nutrients, including amino acids (63,64). Some bacteria can incorporate amino acids from the diet into bacterial proteins, while other bacteria utilize amino acids for energy through fermentation (63,64). However, there is still limited information on how the composition of the intestinal microbiome may be altered by antibiotics to change dietary protein digestibility. Some empirical evidence has suggested that feeding tylosin and avilamycin increased nitrogen retention in growing-finishing pigs by improving nitrogen digestibility and decreasing urinary nitrogen, which suggests that some AGP's may improve protein digestibility (65). However, results from another study showed that feeding avoparcin did not improve apparent ileal or fecal digestibility of either crude protein or amino acids (66). This discrepancy is possibly due to differences in the antibiotic being added to the diet because different antibiotics target different bacterial species which likely influences their mechanism of action.

Mineral digestibility

Adding AGP's to swine diets may also affect the digestibility of phosphorus, but the results are inconsistent. For example, although feeding a diet containing virginiamycin improved phosphorus digestibility in growing pigs fed a phosphorous deficient diet, this response was difficult to repeat (67). However, when these findings were correlated to the microbiome, virginiamycin decreased *Lactobacilli* in diets containing adequate phosphorus content, but did not affect the concentration of

Lactobacilli in a phosphorus depleted diet, suggesting that *Lactobacilli* are involved in improved phosphorus utilization (67). In another study, although virginiamycin improved calcium, phosphorus, magnesium, iron, and zinc digestibility in high fiber diets fed to growing pigs, it had minimal effect on the same mineral digestibility in corn-soybean diets (68). In contrast, feeding different antibiotics, such as tylosin and bacitracin, did not improve dry matter, energy, phosphorus, nitrogen, or calcium digestibility in growing pigs (69). This variation in response suggests that the effect of AGP's on mineral digestibility seems to be dependent on both diet composition and nutrient levels and type of antibiotic being fed.

The development of antibiotic resistance

Not long after the discovery of antibiotics, antibiotic resistant bacteria began to be identified in hospitals (39,70). Bacteria can develop resistance to antibiotics through two main mechanisms: in response to environmental pressure, and mutation (71). When resistance develops though environmental pressure, a gene in a specific bacterium will mutate, replicate, and be vertically transmitted to the next generation resulting in an increased number of bacteria with that gene in the environment (71). Antibiotic resistant genes can also be transferred horizontally through conjugation, transformation, and transduction (71). Conjugation is the most common mechanism of transmission for antibiotic resistance in bacteria, and is mediated by bacterial plasmids and cell to cell junctions that allow DNA transfer between cells (71,72). Transformation is the uptake, integration, and expression of free DNA that may contain antibiotic resistant genes (72). Finally, transduction is the transfer of DNA through a specific vector, such as

bacteriophages (72). The biological mechanism for antibiotic resistance is commonly through antibiotic destruction, antibiotic efflux, or antibiotic receptor modification (71). The current understanding of why some bacteria develop resistance to specific antibiotics and other bacteria remain susceptible to antibiotics is limited.

Once bacteria develop resistance to an antibiotic there are limited reversion opportunities to make that bacteria population susceptible to the antibiotics again. A literature review evaluating 36 studies that removed antibiotics from poultry diets found no effect on reversion to the antibiotic sensitive phenotype (73). These results validate the importance of novel interventions rather than simply developing more antibiotics, because resistance to antibiotics is impossible to reverse. Additionally, current research suggests that antibiotic resistance genes can be associated with multiple antibiotics, indicating that a gene with a resistant phenotype to a specific antibiotic may also be resistant to a different antibiotic without previous exposure to that bacteria colony (74).

Antibiotic use in livestock production systems has been heavily criticized for contributing to the development of antibiotic resistant bacteria (75). This criticism has been strengthened by the identification of antibiotic resistant strains of bacteria in animals that were fed diets containing antibiotics (76). The main concern with antibiotic resistant bacteria in livestock is their ability to be transmitted to human-adapted pathogens via people, contaminated food, and the environment (75). Fortunately, results from one study showed no differences in antibiotic resistant *Campylobacter* bacteria contamination on carcasses between antibiotic-free vs. conventionally reared swine or poultry, suggesting that more research is needed to directly prove that antibiotic use in livestock leads to increased antibiotic resistant bacteria contamination on food products (73). In contrast,

the same study identified differences in antibiotic resistant *Campylobacter* concentrations present on the farm of conventionally raised pigs compared with antibiotic-free pigs, suggesting farm workers could be at risk for infections with antibiotic resistant bacteria (73).

Ultimately, concerns over antibiotic resistance must be solved using a One Health approach, which is a collaborative effort of multiple disciplines including animal science, veterinary medicine, public health, human medicine, crop science, and environmental science as well as incorporating people in all geographic areas (75). In the World Health Organization's global action plan, people from all sectors and disciplines are encouraged to address antimicrobial resistance in their line of work (77). The first step in reducing antimicrobial resistance is prevention, because every prevented infection will eliminate the need for using antimicrobials (77). The second step is access, where veterinarians need to have the appropriate tools, training, and knowledge to make appropriate diagnosis that will allow for responsible use of antimicrobials (77). Third is sustainability, which requires that each country develop a long-term antimicrobial use plan rather than focusing only on a short-term solution (77). Finally, the last recommendation is to establish incremental targets for implementation, where countries at different stages of developing a plan for resistance can make create attainable targets to guide continued improvement (77). In the area of animal science, understanding how to incorporate these recommendations into management of pork production systems will be critical for reducing the threat of antimicrobial resistant bacteria.

Overview of antibiotic regulations in various countries

Concerns over antibiotic resistance in human medicine and the increasing pressure to use less antibiotics in livestock production has sparked global legislative action. Sweden was the first country to take steps in limiting antibiotic use in 1986 (78). During this time, a national ban was implemented on the use of antibiotic growth promoters and antimicrobials in food animal production without a prescription (78). Immediately following this ban, no decline in finishing pig growth performance was observed, but nursery pig growth performance rapidly declined (78). In addition, an increase in clinical health problems in nursery pigs created a demand for medicated piglet feed containing therapeutic doses of antibiotics (78).

The second country to take a regulatory action against antibiotic use was the Netherlands, which banned the antimicrobials olaquindox and carbadox in 1997, and required veterinary prescriptions for all other antibiotics (79). About the same time as the new regulations were implemented in the Netherlands, Denmark passed similar laws eliminating the use of antibiotic growth promoters, banning the use of avoparcin, and limiting veterinarians profit on antibiotic sales (80). After the antibiotic ban in Denmark, there was a limited effect on growth performance of growing pigs, but nursery pig growth performance declined and the production costs resulting from excluding antibiotics in swine diets increased by $1.18 USD per pig in Denmark (80). The European Union eventually implemented a complete ban on the use of AGP's in animal feed in 2006, and following the E.U.'s AGP ban, Taiwan also required a prescription for antibiotic use and eliminated the use of antibiotics for growth promotion (79). In general, countries that applied a complete ban on AGPs reported similar findings on swine growth performance,

which were minimal impacts on finishing pigs and increased mortality and morbidity of pigs during the nursery stage leading to increased use of therapeutic levels of antibiotics (79). In China, restrictions on AGP use were not discussed until 2017, where a plan was implemented to end AGP addition to swine diets by the year 2020 (81).

Antibiotic legislation in the United States

As the threat of antibiotic resistant bacteria increased, so did the demand for stricter regulations on antibiotic use in livestock production in the United States. This led to the final rule of the Veterinary Feed Directive (VFD) in October of 2015 (4), which went into effect on January 1, 2017, and required all antibiotics deemed medically important in human medicine to no longer be used in livestock feeds at subtherapeutic levels. In addition, if antibiotics are to be used for treatment of disease (therapeutic levels), they must be administered by a veterinarian and through a valid veterinary-client-patient relationship (4). A survey was conducted in 2016 to collect information on the cost and challenges of implementing the VFD and its potential impacts on the swine industry (82). Results from this survey showed that the largest change producers would make was through modification of vaccination and biosecurity protocols to keep their herd protected from disease (82). In addition, 12.4% of respondents indicated that they would be utilizing more non-antibiotic alternatives in diets on their farms (82). In the same survey, most respondents believed that the VFD would decrease antibiotic use, with the greatest proportion (34.7%) estimating a 21 to 30% reduction in antibiotic use (82). It has yet to be determined if this decrease in antibiotic use is accurate, and if this decline is effective in reducing the future development of antibiotic resistance.

Antibiotic alternatives

With the elimination of antibiotics in swine diets, there was a rise in health challenges on E.U. pig farms (78). With the increased disease pressure came an increased interest in natural feed additives that could help improve animal health, avoid the regulation of antibiotics, and appeal to consumers. Promising feed additives that can accomplish each of these tasks include botanical products and yeast products. Such products are readily accepted by consumers because of they are familiar components of food (e.g., yeast, oregano, thyme), are easily approved for organic production, and have several research studies supporting their potential animal health benefits when utilized in the correct situations (7,83–85). However, the main limitation with these products is their inability to produce a consistent growth or health response across studies and various production conditions. To better understand how to strategically use these feed additives to produce a reliable response, more research is needed to understand their modes of action in pigs, which should include focusing on the gut microbiome and metabolism when they are included in the diet.

Botanicals

Botanical products are defined by the United States Food and Drug Administration as products that include plant materials, algae, macroscopic fungi, and combinations thereof (86). Further classification of botanical products includes herbs and spices or essential oils. Herbs and spices are botanical products that are whole or dried products added to diets, while essential oils are created from further processing such as extraction through

cold expression or distillation (9,86). Botanical products can be added to swine diets in a variety of forms. The term “botanical” or “phytogenic” are often used interchangeably when describing feed additives, and simply refer to a product of plant origin (9). Further classification of phytogenic or botanical products can be done either by how the product was processed or by taxonomical classification of the plant that was used to derive the feed additive (9). The most common forms of botanical products are essential oils, herbs, and spices.

Essential oils consist of various secondary plant metabolites which are typically delivered using an oil-based carrier (87). These oils are obtained from plants through a variety of processes which usually involve either steam or dry distillation (87). Essential oils can be extracted from multiple parts of the plant, including flowers, leaves, rhizomes, roots, seeds, bark, and fruit (87). The types and concentrations of chemical components in essential oils vary based on a variety of factors including species, location of collection, climate conditions, harvest time, and method of isolation (88). Essential oils are the most commonly used form of botanical products in swine nutrition because of their high concentration of plant metabolites compared with simply grinding and using the entire plant (89).

Apart from essential oils, herbs and spices are botanical products that are added to diets in whole or dried form (86). Herbs can generally be defined as plants that are used for their leaves, stems, flowers, and roots, while spices include aromatic seeds (90). Common herb and spice products that have been previously evaluated for use in swine nutrition include oregano, thyme, sage, garlic, coriander, pepper, and blends of multiple products (9). To achieve a health or growth improvement in animals, these products

generally must be added at higher concentrations in the diet (1.0 to 40.0 g/kg) compared with essential oils (0.02 to 0.1 g/kg) (9). Regardless of whether these products are herbs and spices or essential oils, many botanicals have similar active compounds that contribute to antioxidative and antimicrobial effects, including phenolic compounds, terrapins, and aldehydes (9,84,88,91). However, the concentration of these active compounds will vary based on the herb or spice being used (84,92).

Active components of botanical products

There are hundreds of chemical components in botanical products that vary based on plant origin. One class of biologically active chemical compounds in botanical products are terpenes, which are volatile, unsaturated hydrocarbons (87). Terpenes have desirable properties including antiseptic, anti-inflammatory, bactericidal, and antiviral effects (87). For example, piperine is a terpene which is present in some botanical products that have been added to swine diets to improve growth (93). Additional important components of botanical products are phenolic compounds, including thymol and carvacrol, which are the active components in thyme- and oregano-based products (9,94). Botanical products also typically include alcohols, which have antibacterial and antiviral properties (87). Aldehydes and acids have anti-inflammatory properties, and they are present in botanical products like citral from citrus extracts and cinnamic acids. Ketones have a capacity to improve cell proliferation specifically in wound healing, but they are not always a desirable component in botanical products for animal nutrition because many of them are toxic in animals (87). Lastly, esters are common components of botanical products and provide beneficial effects including antimicrobial and sedative effects (87).

Antimicrobial activity of essential oils

The chemical components in botanical products that are the most effective antimicrobials were identified in a review of 6 studies evaluating *in vitro* antimicrobial activity against either *E. coli, S. enteritidis, S. typhimurium, S. aureus, B. cereus,* and coliform bacteria (85). Carvacrol was the most effective antimicrobial in 3 studies, while cinnamaldehyde and citronellal were the most effective each of two other studies (85). Therefore, these specific compounds appear to be desirable components in botanical products added to swine diets if the goal is to use a feed additive with antimicrobial activity. Research has also suggested that some botanical products have antimicrobial activity against pathogens of high economic importance such as *Streptococcus suis* (95). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined for 4 different essential oils against 30 different strains of *Streptococcus suis*, and red thyme, common thyme, oregano, and cinnamon all had antimicrobial activity against *Streptococcus suis* (95). However, although these responses need to be validated in an *in vivo* experiment, it appears that some botanical products have antimicrobial properties that may be effective in controlling selected bacterial species.

The antimicrobial properties of botanical products are highly dependent on the plant of origin, but there is also considerable variation in composition and concentrations between samples of the same plant or between different parts of the plant such as flower vs. leaf (91). For example, when evaluating the antimicrobial activity (based on the size of the zone of inhibition) of sage oil extracted from 21 different sage plants, there was a

26% variation in their antimicrobial capacity depending on which specific plant was used (91). These differences were attributed to variation in the chemical composition of each individual plant (91). When comparing the antimicrobial activity of sage oil from the flowers or the leaves of the same plant, there was up to 30% variation in the zone of bacterial inhibition (91). If the driving mechanism of action when supplementing botanical products in swine diets is antimicrobial activity, these results are concerning. This variation in antimicrobial activity emphasizes the need for increased quality control in the production of essential oils to produce a consistent product. Without these measures, variation in product composition will likely not produce consistent growth responses in pigs.

Antioxidant activity of botanical products

Oxidative stress occurs in animals when pro-oxidants exceed antioxidant protection within the body. In this situation, excess free radicals will react with lipids and proteins in the cell membrane to create tissue damage (96). Common dietary antioxidant supplements, including vitamin E and blends of ethoxyquin and propyl gallate, have been shown to reduce oxidative stress and improve growth and health of pigs (97).

The *Labiatae* family of plants, including sage, lavender, mint, rosemary, thyme, and oregano are especially known for their antioxidant properties (90). This activity is attributed to the high concentration of monoterpenes in this family of plants, and more specifically, the presence of the hydroxyl group on the aromatic ring of monoterpenes (90). Other botanicals, such as oregano, thyme, and clove based products, have antioxidant capacity attributed to other phenolic compounds such as flavonoids and

terpenes present in the products (98). Although phenolic compounds have been extensively studied for their antioxidant effects, botanical products have an abundance of other metabolites that also act as antioxidants. For example, rosemary, thyme, oregano and marjoram have 26 different compounds with antioxidant activity, ginger has more than 40 antioxidant compounds, and allspice has over 25 different antioxidant compounds (99). Given the high concentration of antioxidants in many botanical products, these results suggest that the primary mechanism of action may likely be associated with their antioxidant effects.

In addition to evaluating the presence of molecules that can act as antioxidants in botanical products, it is also necessary to confirm that these products have antioxidant effects when fed to animals. When thyme oil was added to the diet of aging rats, there was increased brain superoxide dismutase and glutathione peroxidase activities compared with rats fed the negative control diet (94). Superoxide dismutase and glutathione peroxidase are enzymes that serve as antioxidants through dismutation of superoxide and lipid peroxides, and measuring their activity in tissue is a useful evaluation of the oxidative status of animals (100–102). Using the same measurements to evaluate the effect of oregano oil in swine diets, there was also an increase in the activity of superoxide dismutase and glutathione peroxidase in the serum and liver of pigs fed the essential oil compared with feeding a not supplemented control diet, indicating that oregano oil had a beneficial effect on reducing oxidative stress (103). These results indicate that certain botanical products can improve the oxidative status of pigs, but this response depends on the type of botanical product used and the concentrations of its specific antioxidant components. For example, a direct relationship between the

concentration of phenolic compounds (e.g., thymol and borneol) in the product and the antioxidant index suggests that a significant component for antioxidant capacity is specifically related to the phenolic compounds in the oil (91). By identifying the components in botanical products that produce an antimicrobial and antioxidant effect, it may be possible for suppliers of these products to develop processes to ensure consistent concentrations of such compounds in commercial products.

Growth performance effects from feeding botanicals to weaned pigs

The growth response when adding botanicals, including essential oils, herbs, and plant extracts to nursery pig diet is inconsistent. A summary of 365 experiments found that while feeding botanicals improved ADG in 32% of the experiments, there was no difference in 67% of experiments, and 1% of the experiments reported a negative response (104). When comparing ADFI, 84% of the experiments showed no difference when feeding a botanical product, and 77% of experiments showed no effect on G:F when feeding a botanical (Figure 2). Another review of 15 different studies evaluating essential oils, herbs, and spices found that feeding botanical products changed ADG in weaned pigs between -5 to 33% (89). The variation in performance responses can likely be explained by the different types of oils, purity, composition, and dietary doses that were tested across experiments. In the case of one specific essential oil blend, three experiments tested blends of 5% carvacrol, 3% cinnamaldehyde, and 2% capsicum added at 300 mg/kg to nursery pig diets. While results from two of these experiments showed an improvement in ADG and ADFI, the third experiment resulted in a 5 to 6% decrease in ADG and ADFI (89). This suggests that even though the same three essential oils

(carvacrol, cinnamaldehyde, and capsicum) were included at the same concentrations in each of the three experiments, growth responses varied based on other factors. Furthermore, because many of these botanical products have antimicrobial and antioxidant properties it is likely that these products will produce a growth performance improvement only when in a disease challenge and/or oxidative stress situation. If the pigs are healthy, products that reduce oxidative stress or pathogen pressure, such as essential oils, will likely have less of an impact on improving growth performance.

Yeast products

Commercially available yeast products and derivatives can be classified into categories including viable yeast products, yeast cultures, nutritional yeast products, specialty yeast products, and fractioned yeast products (7). Live yeast products have a known probiotic effect that can prevent pathogen growth and improve fiber digestibility, but the components of yeast cells alone may also be beneficial to the animal (105). Yeast cell walls contain mannan oligosaccharides and β-glucans, and when supplemented in diets for nursery pigs, these compounds improve ADG, ADFI, and F:G in addition to their prebiotic effects, and also have an ability to enhance the immune response (106–108). Yeast cells also can provide a concentrated supply of nucleotides to the diet, which contain significantly higher concentrations of nucleotides than found in meat by-products and other plant-based ingredients (109). Adding nucleotides alone to swine diets has been shown to improve growth, intestinal function, and immune responses, but unfortunately such positive responses have been difficult to repeat because of the specific product used, product concentration, and experimental conditions (110). This lack of repeatability has

made it difficult to determine the roles that nucleotides, β-glucans, or mannan oligosaccharide provide toward the functionality of yeast-based feed additives.

Nucleotides

Nucleotides are a class of molecules that make up nucleic acids and play a critical role in the transfer and utilization of ATP in energy metabolism (110,111). Nucleotides are obtained through synthesis by the body or absorbed from the diet (111). Dietary nucleotides cannot be stored in the body and supplementation in the diet ensures nucleotides are available to intestinal cells at all times (112). Research results suggest that supplementing nucleotides in diets and maintaining an available nucleotide pool for DNA and RNA synthesis, animals will have improved intestinal morphology, immune responses, and overall growth (110,113).

In swine nutrition, AGP's are most frequently utilized in weaned pig diets because of the high amount of stress that occurs during weaning and makes nursery pigs more susceptible to disease. During weaning, the shift from liquid milk-based diets to solid diets containing plant-based feed ingredients is an insult to the immature intestine, but by 4 to 5 days after weaning, the intestine starts a recovery period (114). During this injury and recovery period, intestinal cells utilize the salvage pathway to supply nucleotides to intestinal cells and reduce the energy demand (109). Researchers have suggested that supplementing nucleotides during this stress period and may accelerate the recovery after an insult, such as weaning (115,116). For example, after 5 days of fasting, rats fed a nucleotide supplemented diet had sucrase and maltase activity return to baseline values after 3 days, while animals fed nucleotide-free diets required 6 days to

return to baseline (113). In the same experiment, ATP concentrations in both jejunum and ileum returned to baseline after 3 days in rats fed nucleotide supplemented diets compared with 6 days in rats fed nucleotide-free diets (113). Similar results were observed in cell culture experiments, where cell death was prevented and cell growth was promoted in caco-2 cells grown in nutritionally stressed conditions with nucleotide supplements provided to the media (117). Despite research in rodents and in cell culture, the impact of nucleotide supplementation alone on improving growth performance and health status of nursery pigs is still largely unknown, and results from limited research studies have provided mixed results (110). Future studies should focus on evaluating 1) the effect of supplementing purified nucleotides without the confounding effects of other compounds in yeast products, and 2) the appropriate dose and type of nucleotides (i.e., adenine, guanine, cytosine). By better understanding the potential role that nucleotides may play in overcoming the stresses associated with the weaning transition in nursery pigs, it may be possible to develop yeast-based feed additives that effectively exploit the potential health benefits of nucleotides.

Mannan oligosaccharides

Yeast cell walls are known to have a high concentration of mannan oligosaccharides (MOS), which are complex carbohydrates. These MOS have been supplemented in pig feeds for many years and have well-established beneficial effects on growth performance and health (7,118). Bio-Mos is one of the most popular MOS products that is commercially available, and has been demonstrated through multiple experiments to increase feed intake in swine (119). Results from another meta-analysis

confirmed these findings, but also showed that while the addition of MOS to swine diets increased growth performance for slow growing pigs during the first 2 weeks post-weaning, it had no effect on improving growth performance of pigs with normal growth rate (108). These findings suggest that MOS is more effective during disease challenge periods or for pigs experiencing additional stress or difficulty recovering after weaning.

Mannan oligosaccharides have been suggested to have prebiotic effects and a positive impact on the microbiome (120). Unfortunately, the current scientific literature does not strongly support this hypothesis and results are highly variable (118). Although the results suggesting feeding diets containing MOS can dramatically impact the microbiome are inconsistent, there is consistent evidence that MOS additions to nursery pig diets are beneficial during and after a pathogen challenge (118). Therefore, based on our current knowledge of adding MOS to nursery diets, the greatest effect appears to involve reduced pathogen colonization and improved gastrointestinal health recovery after a challenge.

β-glucans

The β-glucans are a class of glucose polymers that are found in the cell wall of yeast, other microbes, and cereal grains (121). There are limited experiments where β-glucans alone were supplemented in swine diets, because the health and growth performance effects of β-glucans are typically evaluated as a component of yeast and cereal grains including oats, barely, and rye (122). However, when β-glucans were supplemented to growing pig diets, improvements in growth performance, nutrient digestibility, carcass length, and pork quality have been observed (123). In contrast, a

separate publication outlined two experiments where β-glucans were supplemented to nursery pig diets with or without antibiotics (124), and results showed that the adding β-glucans to diets did not improve growth performance compared to pigs fed the negative control diets (124). Like many of these non-antibiotic growth promoting compounds, more research is needed to gain a better understanding of the conditions when β-glucans may provide an improvement in growth performance.

Growth performance effects from feeding yeast products

Extensive research has been conducted to evaluate feeding a variety of yeast products and the impact on swine growth performance (Figure 3). A review of 98 studies evaluating the growth response when weaned pigs were fed yeast products showed that in 24% of the experiments, an increase in ADG was observed when pigs were fed diets containing a yeast product, and 16% of experiment reported an increase in ADFI and G:F (104). In contrast, results from 75% of experiments showed no effect on ADG, 83% showed no effect on ADFI, and 84% showed no effect on G:F when feeding yeast products (104). With most studies reporting no effect on pig growth performance, more research is necessary to understand the growth promotion mechanism of action to better understand how yeast products can be incorporated into swine diets to produce a repeatable growth improvement.

Novel ways to evaluate feed additives

With the limitations around feeding antibiotics for growth promotion purposes, there is a need to better understand how to strategically and effectively utilize dietary

antibiotic alternatives in swine production. Growth promotion responses from the addition of feed additives to pig diets are complex and involve multiple dynamic biological systems. For this reason, it is challenging to select feed additives that provide consistent improvements in growth responses to economically justify their use in swine diets. The reasons for inconsistent growth responses among experiments evaluating various feed additives are numerous. In order to understand these inconsistencies in animal growth, it is essential to understand what biological systems are driving growth performance, which requires analysis of multiple animal systems. Microbiome and metabolome analyses offer an opportunity to study these complex interactions of biological systems in the pig.

The cost of sample analysis using microbiome and metabolome platforms has decreased, making these powerful analytical approaches accessible for use in animal nutrition and production research. The use of these powerful platforms results in very large data sets that can be evaluated using multiple data mining approaches. By incorporating these in-depth approaches into traditional on-farm growth performance experiments, greater insights into the mechanisms of action of feed additives may be achieved, and the strategic use of those additives may be facilitated.

By conducting metabolomics and microbiome analysis, more information is obtained to deduce mechanisms of action compared with using traditional growth performance experiments which are only intended to evaluate production parameter responses. Metabolomics and microbiome data can provide a better indication of when a feed additive will likely result in a growth or health response and how it may interact with other dietary and environmental factors. However, the dynamic and complex nature

of pig biology can make it difficult to interpret data and apply findings from these types of analytical platforms. For this reason, it is important to understand the benefits and limitations of microbiome and metabolome technologies available for swine nutrition research.

Microbiome evaluation

Within the last 10 years, the ability to performe16s rRNA sequencing to identify bacterial composition from intestinal contents or fecal samples has greatly improved as a result of large amounts of microbiome data and microbiome-based experiments in multiple fields (125). However, there are still knowledge gaps in the microbiome literature and limitations of current methods for evaluating the microbiome. To better understand how to utilize these new technologies, there needs to be a clear understanding of how these methods and analysis may be used in animal experiments.

16s rRNA sequencing

The most utilized technologies when analyzing the microbiome of a pig is16s rRNA sequencing and shotgun sequencing. The foundation of 16s rRNA gene sequencing is based on the 16s rRNA gene that is present in all bacteria (126). The 16S rRNA gene is approximately 1,500 bp, and thus large enough to be used for informatics (information science) purposes (126). When sequencing this gene, a polymerase chain reaction (PCR) is completed on a specific region of bacterial DNA that is highly variable between different bacterial species (127). There are different regions of the bacterial gene that are either conserved or variable between different species. By sequencing a variable region

of the 16S rRNA gene, this information can be used to identify the bacteria that are present in a sample. After sequencing a variable region of the 16S rRNA gene, the sequencing reads are cleaned and aligned to microbial genomic databases using bioinformatic software to identify the bacterial species present in the sample (127). The sequencing of the 16s rRNA gene is ultimately a sufficient proxy for the full-length sequence previously needed for multiple community analyses, such as those based on a phylogenetic tree (128). When analyzing data from 16s rRNA sequencing, multiple bioinformatic tools are available for the analysis and there are many variations in the sequencing platform, method of taxonomic classification, and further analysis of data. During this analysis, it is advised to utilize either university core facilities or collaborate with a professional with extensive expertise and experience in bioinformatics or microbiome research.

Although recent availability and reduced cost of 16s rRNA sequencing has made it an ideal option for analysis of samples in a variety of experiments, it also has its limitations. This method has low power for making conclusions of the phylogenic history at the species level (126). This is especially problematic when investigating two species that recently diverged, which is especially apparent in the genera *Bacillus, Bordetella, Campylobacter, Neisseria, Pseudomonas*, and *Streptococcus* (126). The second main limitation to 16s rRNA sequencing is that it requires databases to classify bacteria. When using these databases, only bacteria that have previously been classified and are publicly available can be identified in the analysis.

Shotgun metagenomic sequencing

When compared to 16s rRNA sequencing, shotgun metagenomic sequencing uses a different approach to categorize bacteria. In this method, DNA from the entire sample is broken into random fragments, sequenced, and then pieced back together using bioinformatics tools (129). One of the advantages of using this method is that it also provides information on microorganisms outside of bacteria (129). In addition, provides the capability of sequencing pieces of the genome that might be indicative of microbial function in addition to classifying them (129). One of the main benefits of shotgun sequencing is its ability to resolve species-level and strain-level classification, which is sometimes limited with 16s rRNA sequencing (127). These sequencing methods provide information on genome content, functional potential, and information on organisms present at a very low abundance (127).

One of the limitations of shotgun sequencing, especially in animal studies, is its sensitivity to contamination (127). In swine experiments, sample collection is often done on farms without highly sanitary conditions, which can be problematic given the high sensitivity of shotgun sequencing. Shotgun sequencing is also limited by its relatively high cost of analysis, making it difficult to use in experiments with large numbers of animals or samples. In addition to its high laboratory cost compared to 16s rRNA sequencing, shotgun sequencing requires substantially more complex and computationally expensive analytic approaches (127). Overall, shotgun sequencing may be ideal for studies focusing on the functionality of the microbiome, but its high cost and complexity is a limitation for many swine trials that are simply investigating the composition of the microbiome.

Additional microbiome analysis

After sequences have been identified and classified, either through 16s rRNA sequencing or shotgun sequencing, a table can be created outlining the bacterial species present in a sample, and the relative abundance of those bacterial species in the sample. This information is provided in the amplicon sequence variant table, which can then be further analyzed to identify trends and patterns in the data set. A summary of different approaches to analyze the microbiome is outlined in Table 2.

Limitations in microbiome experiments

Current literature evaluating the microbiome is lacking one significant conclusion, the definition of a "good" microbiome. This is a complicated and dynamic question that does not have a straightforward answer. For this reason, research questions that compare the microbiome of different treatment groups should be avoided unless other data are being collected to quantify positive or negative responses. Previous research in poultry has identified specific intestinal bacteria that have a positive and negative association with growth performance. In swine, research studies have provided somewhat mixed results on which bacteria are consistently associated with increased growth.

Analyzing a pig's intestinal microbiome is complicated because of the many variables that interact and impact it including early environmental exposure, variation by individual animal, and diet (13,24,31). Because of the number of interactions, it is difficult to isolate the impact of each individual variable. This makes it incredibly challenging to make singular microbiome recommendations that will consistently and robustly improve growth performance.

Metabolome analysis

A metabolite can be defined as any small molecule that is an intermediate or end-product of metabolism. A common platform for analyzing metabolites is liquid chromatography–mass spectrometry (LC-MS) and there are many opportunities to utilize either LC-MS analysis or another method of mass spectrometry to evaluate biological responses from feed additives in swine nutrition experiments.

Methods for metabolite analysis

Liquid chromatography–mass spectrometry measures the concentration or relative abundance of small molecules in tissue or intestinal contents with both high sensitivity and specificity. The liquid chromatography (LC) portion of the analysis converts the targeted molecules to a charged state (130). In addition to LC, gas chromatography or nuclear magnetic resonance can also be used to physically separate metabolites before analyzing their mass (130). This analysis is then followed by an immediate analysis of the ions and fragmented ions based on their mass to charge ratio, which is the mass spectrometry (MS) part of the analysis (130). This mass to charge ratio can be used to identify a specific metabolite. There are different methods used for both ionization of molecules and ion analysis including an electrospray ionization source, atmospheric pressure chemical ionization source, and atmospheric pressure photo ionization (130). The method of ionization may change based on the type of sample or target metabolite of interest. Electrospray ionization source tends to be the most common method and is ideal for biological molecules, which are typical metabolites in samples collected from swine nutrition experiments (130).

Following the detection of mass to charge ratio, an accurate mass is also determined. This is done using metal rods called quadrupoles. In the quadrupole analyzers, changing the voltage in the metal rods allows for metabolites with a specific mass to charge ratio to move through the quadrupoles at a specific time. By changing these voltages and letting specific metabolites through the quadrupole at a time, the system creates a mass gradient that can be used to identify metabolites (130). Depending on the targeted metabolite, collision cells can also be implemented between quadrupoles to improve disassociation and transmission of specific ions (130). Another method of mass determination is a time of flight (TOF) analyzer. This analyzer accelerates the target ions through a high voltage chamber. The mass to charge ratio of the metabolite will ultimately affect the amount of time it takes the ion to reach the other end (130). The time of flight analyzer has a high sensitivity and high mass accuracy, making it ideal for identifying the mass of small molecules (130). These methods can also be combined in a hybrid analyzer. Using this approach, one of the quadrupole chambers can be replaced with a time of fight chamber, producing a quadrupole time of flight analyzer (QTOF) (130). This hybrid method is especially useful for proteomics measurements. The equipment and methods used in metabolomics are complex and specific to each experiment, and additional expertise in the area will help ensure proper data collection. A summary of metabolomics analysis is outlined in Table 3.

After an analysis is complete in the LC-MS, metabolites are identified and quantified. During an untargeted analysis, every measurable metabolite is used in the analysis to identify if there are distinguishable trends in the data (131). This can be done through a multivariate analysis and data mining techniques that are appropriate for very

large datasets. Untargeted analysis is ideal for discovery of patterns and novel metabolites, and is only limited by detection sensitivities in the methods or machine used (131). On the other hand, a targeted analysis involves measurement of metabolites in a defined group of metabolites. When conducting a targeted analysis, the methods can be adjusted and optimized for the specific metabolites of interest (131), and standards can be included for quantification of metabolites. For example, if an experiment is only interested in evaluation serum amino acids, a targeted analysis can be used to only evaluate serum amino acids. Unfortunately, a targeted analysis will only provide information on a subset of metabolites and not the full spectra of metabolites that comprise an untargeted analysis. For example, in the previously mentioned amino acid scenario, the analysis will not provide any information on serum triglycerides or other metabolites.

Limitations of metabolome experiments

Despite the many advantages of metabolomics analysis of tissues or intestinal contents, there are a few limitations to using this approach. The most limiting factor is the biochemistry, physiology, and organic chemistry expertise required to successfully interpret data from the analysis and derive meaningful conclusions related to regulation of metabolism. If an expert with this type of knowledge or experience is not already involved in a collaboration utilizing metabolomics-based methods, it is advised to seek collaboration or consultation with professionals with expertise in these essential scientific disciplines.

When conducting an untargeted analysis, there is also the potential limitation for a metabolite to not be identified using retention time and adjusted mass. Further analysis can be used to fragment and identify the structure of the unknown metabolite, but this requires additional resources to disassociate the unstable ions in the molecule and repeat the mass spectrometry analysis. In addition to unidentified metabolites, metabolomics analyses do not provide information on the source of metabolites. For example, if there is an increased concentration of methionine in the intestinal contents of a pig, it is impossible to determine if that methionine was from the diet and unabsorbed, produced by bacteria in the intestine, or derived from endogenous losses from the animal. This makes it difficult to make conclusions about the mechanism of action when the source of the metabolite cannot be identified without the use of other methods (e.g, such as stable isotopes).

Applications of metabolome research in swine nutrition

At the University of Minnesota, multiple experiments have been conducted to integrate microbiome, metabolome, and animal health data. For example, an experiment was conducted to identify the mode of action for the antibiotic tylosin in diets for growing-finishing pigs. During this analysis, the antibiotic was fed to growing pigs for 12 weeks and fecal samples were collected from pigs fed diets with and without the antibiotic (61). A principle component analysis of the metabolites identified in these fecal samples showed the main difference between treatments was attributed to the bile acids hyodeoxycholic acid and lithocholic acid (61). The trend in fecal metabolites, identified in this experiment, led to more specific follow up studies focused on bile acids. These

follow up studies ultimately identified a proposed mode of action for growth promotion that involved changes in bile acid metabolism when feeding antibiotics (62). A similar approach can be utilized to evaluate the mechanism of action for other feed additives as growth promoters.

Using metabolomics analysis platforms has also been useful in evaluating alternative feed ingredients and providing information that will be useful when using feed additives in diets containing such ingredients. For example, when feeding rapeseed meal to Norwegian landrace nursery pigs, an untargeted LC-MS analysis of intestinal contents identified a significant increase in sinapine and choline (132). Using this information to guide further analysis into potential metabolic pathways, subsequent evaluation indicated that this choline was released through hydrolysis of the sinapine in rapeseed meal and converted to the microbial metabolite trimethylamine (TMA) followed by conversion to trimethylamine N-oxide (TMAO) (132). Previous research has also demonstrated that TMAO supplemented in swine diets can improve the growth performance(133). Taking these findings into account, such information can be used to further investigate novel feed supplements including TMAO. In addition, this study also led to the discovery of increased levels of multiple oxidized metabolites and aldehydes and decreased levels of ascorbic acid and docosahexaenoic acid–containing lipids in the liver and serum (134). These collective findings indicate that feeding rapeseed meal could disrupt the redox balance in nursery pigs, and feed additives that have antioxidant capacity may be useful when feeding rapeseed-based diets (134). Metabolomic data can be extremely valuable in identifying mechanisms of action in swine nutrition trials, and when used in conjunction

with intestinal microbiome analysis, a more complete understanding of biological mechanisms of how specific feed additives may improve growth in pigs.

Summary and future research

Antibiotics have been included in swine diets for decades to improve growth performance of pigs. Unfortunately, supplementing low doses of antibiotics over extended periods of time has created an ideal environment for the development of antibiotic resistant bacteria, which is a serious risk to public health. Pork producers are committed to continuing to raise pigs safely, efficiently, and humanely, which has created a demand for new feed additives that can be used to potentially achieve the same benefits AGP's previously provided. The mechanisms of action from feeding AGPs have not been clearly defined, but the improvement in growth performance appears to involve 1) alterations of the microbiome, 2) improvements in nutrient digestibility, 3) reductions of subclinical disease, and 4) improvements in intestinal health. However, there are still many questions that need to be answered in future research to confirm these mechanisms. A review of previous literature has suggested that the microbiome, metabolism, and overall health of pigs plays a critical role in a growth promotion response, and for this reason it is essential to use a systems approach to characterize changes in these components when attempting to identify the mechanisms of these growth promotion responses.

Various feed additives have been proposed to provide similar growth promotion effects to antibiotics. Botanical products have been reported to alter the microbiome, reduce oxidative stress, and improve intestinal health. Yeast-based products also show

potential as antibiotic alternatives by providing prebiotic effects, improving intestinal health, and aiding in recovery after an intestinal challenge. However, a general lack of correlated microbiome, metabolomic, animal growth, and animal health-based data make it difficult to determine associated causes of highly variable growth responses when feeding these products to weaned pigs. To determine if dietary yeast and botanical products have a significant effect on the microbiome of weaned pigs, microbiome research needs to include an evaluation of both alpha and beta diversity and identify microbial biomarkers that are associated with growth improvements. Metabolomics data can then be used to identify metabolic pathways significantly affected by including these feed additives in weaned pig diets, which may include bile acid metabolism and measures of oxidative status. Finally, correlated responses between the microbiome and metabolome with animal health and growth responses may provide a better understanding of the conditions necessary for effectively utilizing yeast and botanical products to improve growth performance and health of weaned pigs.

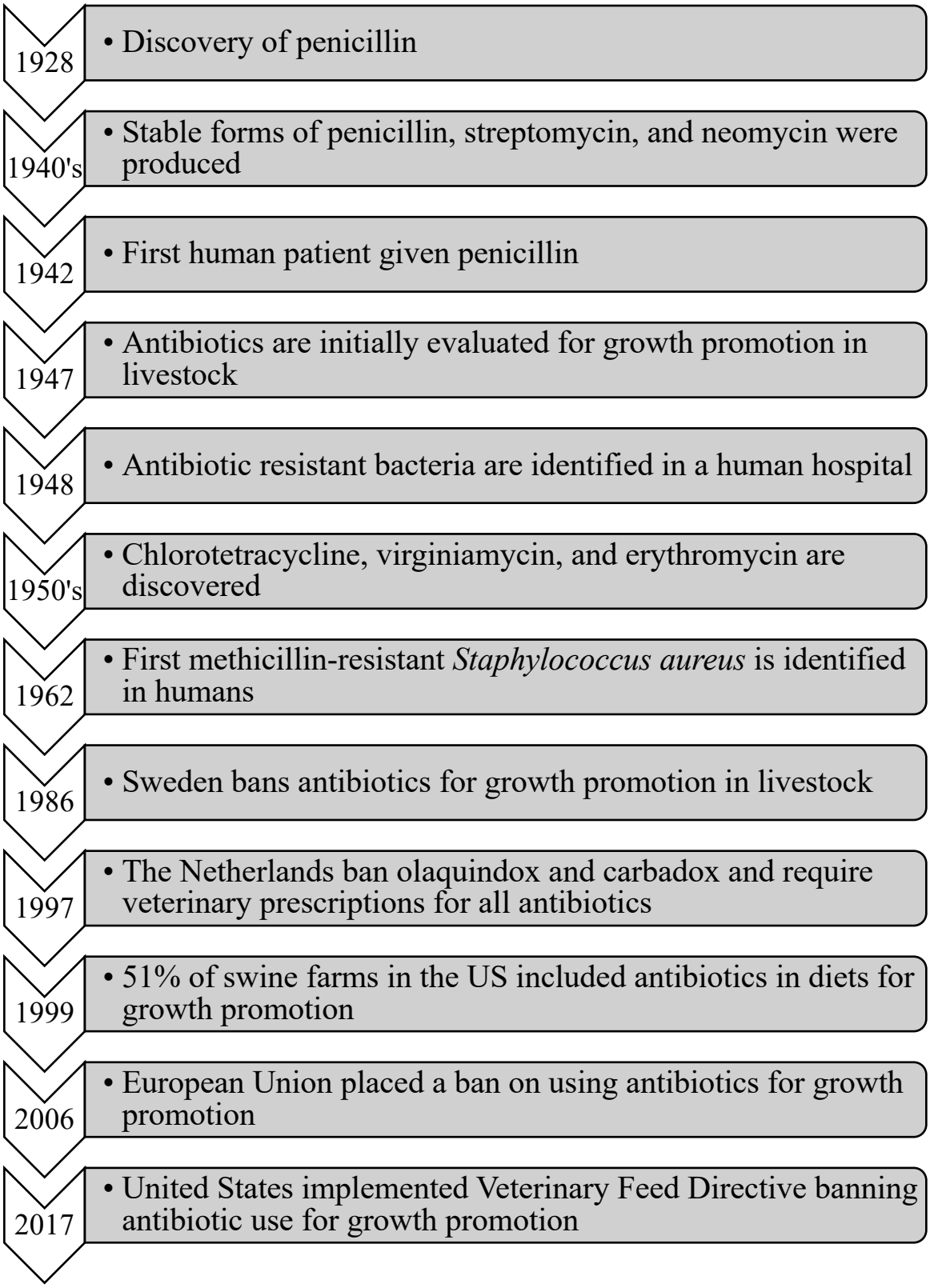
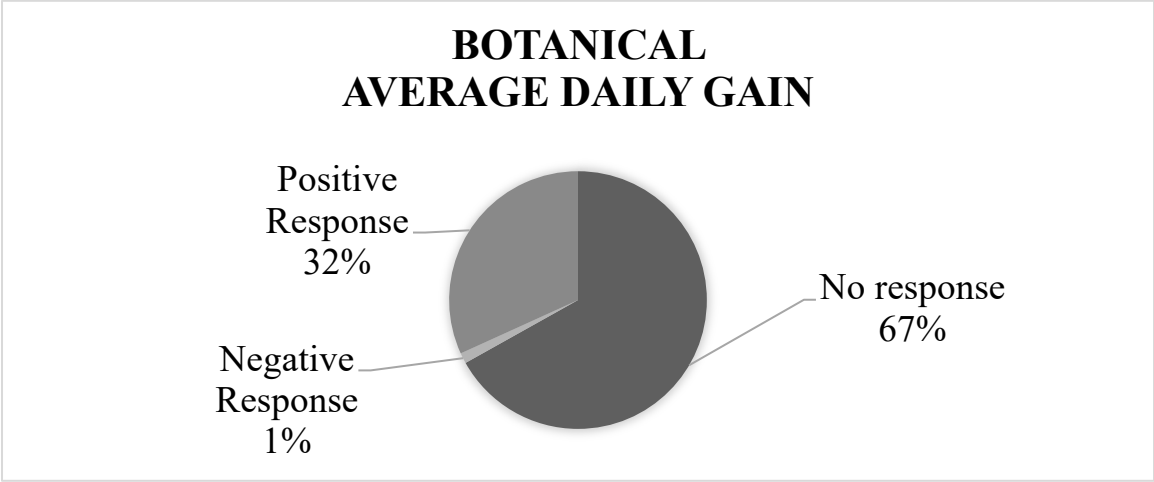


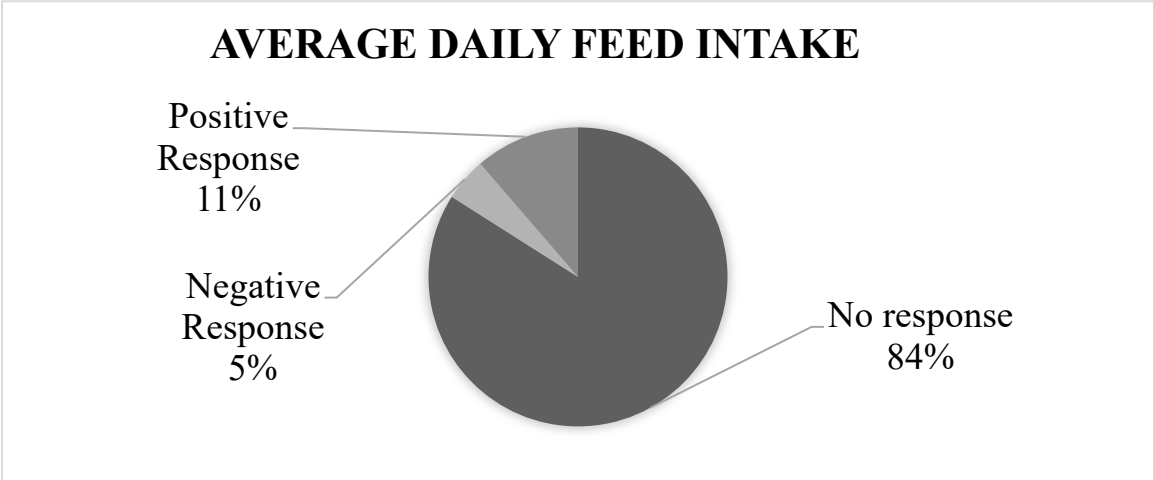
Figure 1 Timeline of Antibiotic discovery, antibiotic resistance, and international legislative bans restricting use for growth promotion.

Table 1 Summary of early research from 1945-1955 on the growth response when supplementing antibiotics.

Reference	Antibiotic		Growth Performance[1]		Significance[2]
	Type	Amount (mg/kg)	ADG (g)	F/G	
(135)	Penicillin	2.2	C: 562 T: 671	C: 3.14 T: 3.03	ADG: $P < 0.05$ G/F: NS
(136)	Penicillin	5	C: 358 T: 476	C: 4.15 T: 3.51	ADG: NS G/F: NS
(137)	Penicillin	13	C: 485 T: 490	C: 3.96 T: 4.00	ADG: NS G/F: NS
(136)	Streptomycin	5.5	C: 358 T: 521	C: 4.15 T: 3.29	ADG: $P < 0.05$ G/F: NS
(135)	Streptomycin	22	C: 562 T: 626	C: 3.14 T: 3.09	ADG: NS G/F: NS
(138)	Streptomycin	33	C: 735 T: 816	C: 3.92 T: 3.34	ADG: $P < 0.05$ G/F: $P < 0.05$
(139)	Aureomycin	16.5	C: 594 T: 725	C: 3.44 T: 3.84	ADG: $P < 0.05$ G/F: NS
(135)	Aureomycin	22	C: 562 T: 680	C: 3.14 T: 3.05	ADG: $P < 0.05$ G/F: NS
(140)	Aureomycin	22, 110, and 220	22: 707 110: 776 220: 726	22: 3.42 110: 3.56 220: 3.17	ADG: $P < 0.05$ G/F: NS
(138)	Aureomycin HCL	22	C: 735 T: 839	C: 3.92 T: 3.22	ADG: $P < 0.05$ G/F: $P < 0.05$
(41)	Virginiamycin	22 and 44	C: 522 10: 585 20: 576	0: 3.36 10: 3.17 20: 3.12	ADG: NS G/F: NS
(141)	Chlortetracycline	110	C: 698 T: 843	C: 3.25 T: 3.19	ADG: $P < 0.05$ G/F: NS
(135)	Neomycin	22	C: 562 T: 381	C: 3.14 T: 3.11	ADG: NS G/F: NS

[1] C: negative control, T: antibiotic treatment
[2] NS: Not significant





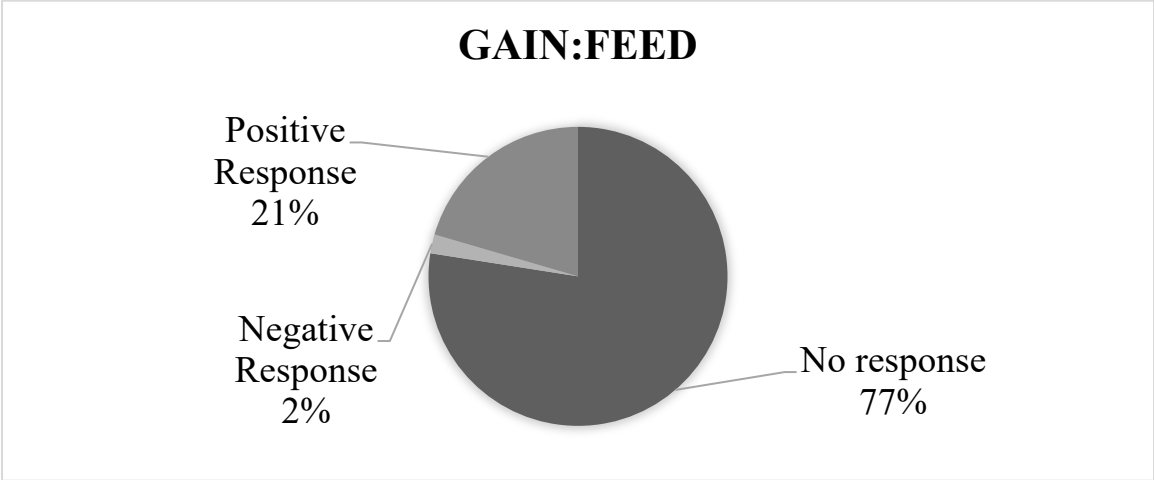
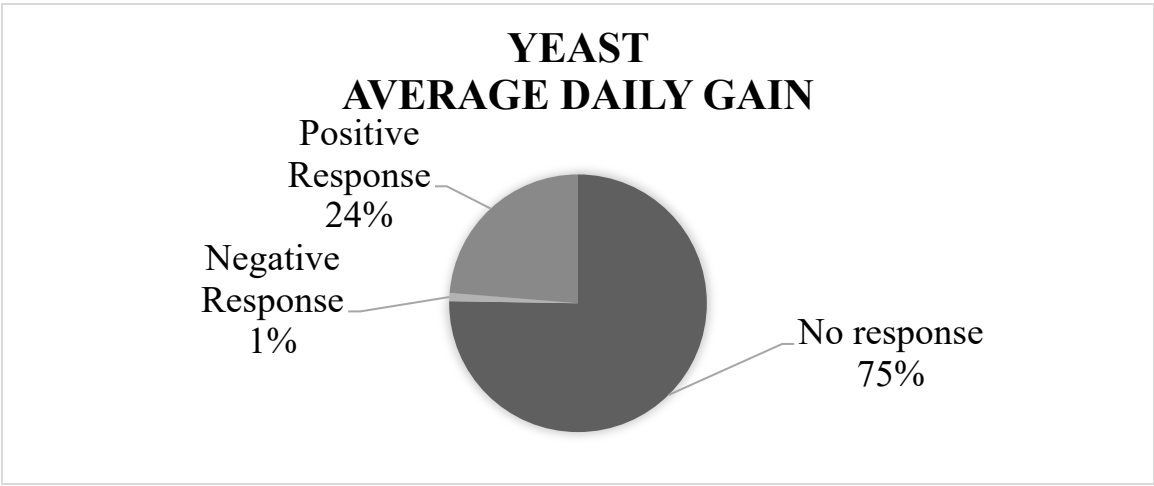
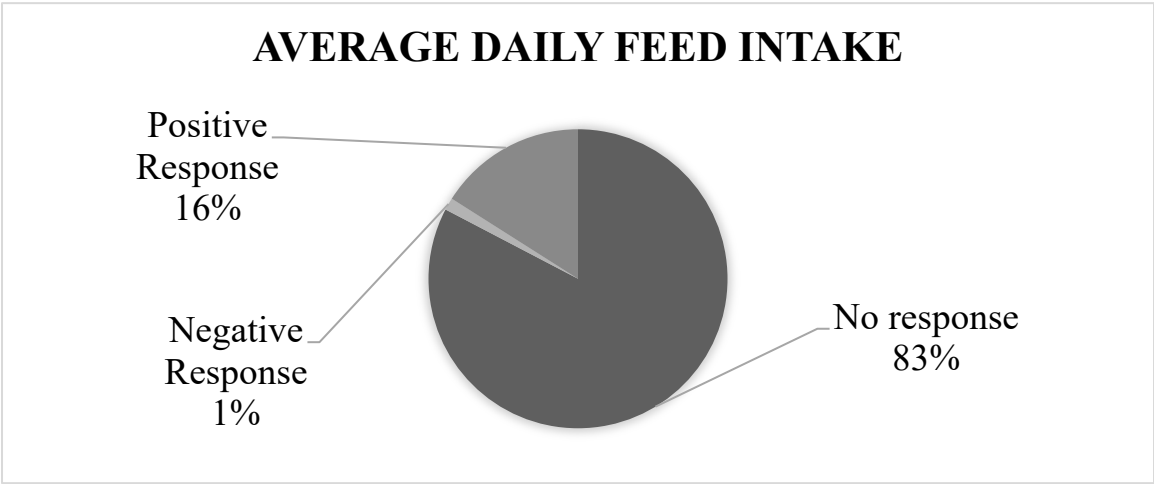


Figure 2 Summary of growth performance responses when feeding essential oil and botanical based products to pigs. Positive response indicates a significant increase ($P < 0.05$), negative response indicates significant decrease ($P < 0.05$), and no response indicates no significant difference from the negative control. Adapted from (104).





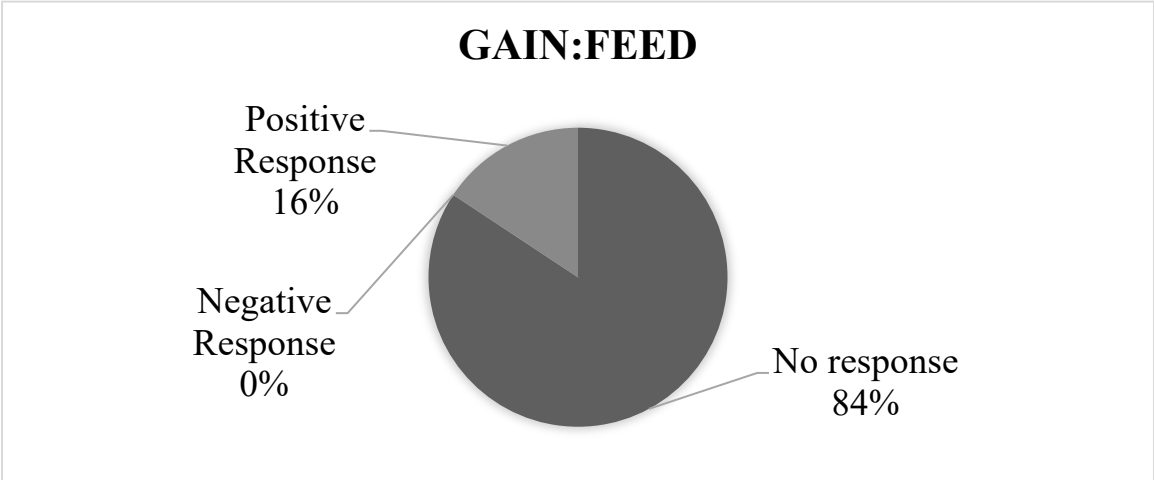


Figure 3 Summary of growth performance responses when feeding yeast products to pigs. Positive response indicates a significant increase ($P < 0.05$), negative response indicates significant decrease ($P < 0.05$), and no response indicates no significant difference from the negative control. Adapted from (104).

Table 2 Common data analysis methods for microbiome and metabolome data.

Method	Output	Ref.
Chao 1 index	Abundance-based estimator of species richness or the total number of species present in a sample.	(142)
Shannon index (H)	Alpha diversity, or the diversity within a single sample. Estimator of species richness and species evenness with more weight on species richness.	(143)
Simpson index (D)	Alpha diversity, or the diversity within a single sample. Estimator of species richness and species evenness with more weight on species evenness.	(144)
Bray-Curtis	Beta diversity, or the diversity among different samples. Accounts for species presence/absence and species abundance.	(145)
Jaccard index	Beta diversity, or the diversity among different samples. Uses species presence/absence data.	(146)
Indicator values	Identifies biomarkers with a high specificity and fidelity to a given group.	(147)
Random forest analysis	A classification algorithm that uses decision trees to identify similarities. Can also be used to rank variables of importance.	(148)
Sourcetracker	Predicts the source of a microbial community. Uses qiime2 software.	(149)
FEAST	Predicts the source of a microbial community. Uses R software.	(150)
Principle Component Analysis (PCA)	Pattern recognition. Used to visualize relatedness between populations. The model does not know what sample is assigned to each treatment (unsupervised).	(151)
Partial Least-Squares Discriminant Analysis (PLS-DA)	Pattern recognition. Used to visualize relatedness between populations. The model does know what sample is assigned to each treatment (supervised).	(151)
Loading plot	Used in combination with PCA or PLS-DA. Shows how strongly a characteristic influences a PCA plot.	(151)
Heat maps	Graphical representation of relative abundance using color intensity.	(152)
Correlation matrix	Correlates any two data sets with each other or within itself. Can be used to corelate metabolites and microbiome, or metabolites/microbiome with growth performance.	(153)
Network analysis	Uses correlations to breakdown the relationships of specific metabolites or microbes. Builds a network of these relationships.	(154)
Pathway analysis	Determines which specific pathways, including cell processes, genes, or metabolites, are enriched.	(155)

Table 3 Summary of metabolomics methods.

Method	Method of separation	Benefits	Limitations
Liquid chromatography-mass spectrometry (LC-MS)	physical separation of liquid chromatography incorporates liquids in the mobile phase	high sensitivity high separation efficiency high reproducibility	difficult to identify metabolites time consuming analysis
Gas chromatograph-mass spectrometry (GC-MS)	physical separation of gas chromatography incorporates inert gasses in the mobile phase	high sensitivity high separation efficiency high reproducibility ideal for low molecular weight metabolites easy identification of metabolites	time consuming analysis
Direct-infusion mass spectrometry (DIMS)	sample extracts are directly infused into mass spectrometer	high sensitivity high reproducibility reduced analysis time compared to LC-MS	cannot distinguish isomers difficult to identify metabolites
Capillary electrophoresis mass spectrometry (CE-MS)	physical separation of capillary electrophoresis uses electrokinetic separation methods	high sensitivity high separation efficiency ideal for polar and ionic metabolites	low reproducibility time consuming analysis difficult to identify metabolites
Nuclear Magnetic Resonance (NMR)	intermolecular magnetic field is used to identify structure and functional group	very high reproducibility easy identification of metabolites	low sensitivity limited to about 100 metabolites

Table adapted from (156)

Chapter 2: Fecal Hyodeoxycholic Acid is Correlated with Tylosin-induced Microbiome Changes in Growing Pigs

Summary

The changes in the gut microbiome play an important role in the promoting effects of antibiotics, such as tylosin, to the health and productivity of farm animals. Microbial metabolites are expected to be key mediators between antibiotics-induced microbiome changes and growth-promoting effects. The objective of this study was to extend the identification of tylosin-responsive microbes to the identification of tylosin-responsive metabolites in growing pigs. The feeding trial was conducted on a commercial farm using two pens of pigs fed diets with and without tylosin (40 mg/kg of diet). Fecal samples were collected from 10 pigs per pen at weeks 10, 13, 16, 19, and 22 of age, and subsequently analyzed using liquid chromatography-mass spectrometry (LC-MS) analysis. The multivariate model of LC-MS data showed that time-dependent changes occurred in the fecal metabolome of both control and tylosin-treated pigs. More importantly, the metabolomic profiles were similar between the tylosin treatment and control groups in weeks 10 and 22, but diverged during weeks 13-19. Subsequent analyses of the fecal metabolites contributing to the separation of two groups of pigs showed that hyodeoxycholic acid (HDCA), together with tylosin and its metabolites in feces, was greatly increased during weeks 13-19 ($P < 0.05$) in the group of pigs fed tylosin. The integration of current metabolomics data and the microbiome data from a previous study revealed the consistency between HDCA and a specific genus of microbes in the *Clostridia* family. Further studies are required to determine the causative relations between tylosin-elicited changes in HDCA and the microbiome as well as the role of HDCA in the growth promoting effects of tylosin.

Introduction

Current food animal production systems have been able to supply animal products (e.g., milk, eggs, meat) at lower cost than ever before. Likewise, efficiency of food animal production is greater in modern production systems than in the past, while also decreasing environmental impact (157). Modern farms attained such efficiencies in productivity in part because of the implementation of technologies such as utilization of antibiotics as growth promoters. Sub-therapeutic levels of antibiotics in feeds have been used in swine and poultry diets since the 1940s to improve growth performance of animals while also reducing sub-clinical disease (1,158). However, the use of antibiotics also increases selective pressures responsible for the evolution of antibiotic resistant bacteria (159,160). The One Health framework suggests that animal health is closely linked to human health and consequently, the use of antibiotic growth promoters increases the risk of antibiotic resistant bacteria in humans (161). Therefore, it is necessary to develop strategies that maintain and improve animal productivity while reducing the usage of antibiotics in the production of livestock.

The mechanism(s) whereby antibiotics improve growth and efficiency of pigs is still not completely understood, making it difficult for nutritionists, veterinarians, and food animal producers to identify antibiotic alternatives that can produce similar improvements in growth performance without using sub-therapeutic levels of antibiotics. Early experiments in poultry showed that germ-free chicks fed sub-therapeutic levels of antibiotics did not have improved growth compared to the ones fed control diets, indicating that the microbiome plays a significant role in the growth promotion process (162). The microbiome affects numerous physiological processes of animals including

protection against some pathogens, development of the immune system and stimulation of immune responses, development of the epithelium, nutrient digestion, and nutrient metabolism (163). Because of the multiple roles ascribed to the microbiome in animals and the complexity of the composition of the microbiome, it has been difficult to define specific mechanisms of antibiotic growth promotion. To fully understand the impact of sub-therapeutic levels of antibiotics on animal growth, research is needed that integrate growth with the metabolome.

Previous studies identified that pigs fed the antibiotic tylosin, had prominent shifts in their fecal microbiome in both abundant and less abundant species compared with the pigs fed an antibiotic-free control diet (164). These results also showed that the composition of the microbiome converged over time, and tylosin appeared to increase the rate at which the microbiome matured. We hypothesized that this shift in microbiome maturation and ultimate convergence would also be represented in the functionality of the microbiome, especially the production of specific bacterial metabolites (165). The objective of this study was to determine tylosin-induced changes in the fecal metabolome of growing pigs and also to correlate these metabolic changes with tylosin-induced changes in the microbiome for a better understanding of the mechanisms mediating antibiotic growth promotion.

Materials and Methods

Animal experiment

The animals were housed in conventional confinement facilities on a commercial farm located in southwestern Minnesota for the duration of the experiment (farm 2) (164).

Only samples collected from farm two of the experiment previously reported by Kim et al were used for further analysis (164). Two pens containing 50 pigs each were used in the experiment. Ten pigs in each pen were randomly chosen, ear tagged for identification and were sampled throughout the sampling period. Pigs in one pen received tylosin in their feed at a concentration of 40 mg/kg beginning at 10 weeks of age and continuing for 12 weeks. Tylosin was chosen as the antibiotic because of its frequent use for growth promotion in the swine industry. The second pen of pigs served as a control and pigs were fed the same feed except that tylosin was not included in it. None of the pigs were given any additional antimicrobials through the duration of the experiment, and all pigs were fed the same standard commercial corn-soybean meal diet. Fresh feces were collected directly from the rectum of the 20 pigs at 10, 13, 16, 19, and 22 weeks of age. Samples were stored at -80 °C until analysis. The stability of bile acids in fecal samples after long-term storage has been demonstrated in a previous study (166). This study was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Minnesota (Protocol 0705A09361).

Metabolomics analysis

Chemicals and Reagents

LC−MS-grade water and acetonitrile (ACN) were purchased from Fisher Scientific (Houston, TX); triphenylphosphine (TPP) and 2- hydrazinoquinoline (HQ) from Alfa Aesar (Ward Hill, MA); 2,2′-dipyridyl disulfide (DPDS) from MP Biomedicals (Santa Ana, CA); tylosin tartrate from Ark Pharm (Arlington Heights, IL); acetic acid-d_4 from Sigma-Aldrich (St. Louis, MO); glycocholic acid-$^{13}C_1$ from C/D/N Isotopes

(Quebec, Canada). The metabolite standards used for structural confirmation were from Sigma-Aldrich, Fisher Scientific, AlfaAesar, Ark Pharm (Libertyville, IL), respectively.

Fecal Sample Preparation

Fifty mg of pig fecal samples were mixed with 50% aqueous ACN containing 5 μM glycocholic acid-$^{13}C_1$ in 1:10 (w/v) ratio and sonicated for 10 min. The samples were then subjected to further mixing using a vortex mixer and then were centrifuged at 18,000 ×g at 4°C for 10 min to obtain fecal sample extracts. The extracts were stored at −80 °C prior to further analysis.

Derivatization of Short-Chain Fatty Acids (SCFAs) in Fecal Samples

Short-chain fatty acids (SCFAs) in the pig fecal samples were derivatized with HQ prior to LC-MS analysis using a modification of Lu, Yao, & Chen (167). Two microliters of the extract supernatant were mixed with 70 μL of acetonitrile containing 7.5 μM acetic acid-d_4, 10 μL DPDS, 10 μL TPP, and 10 μL HQ. The mixture was incubated at 60 °C for 30 min, chilled on ice, and mixed with 100 μL H_2O. The mixture was then centrifuged at 18,000 ×*g* for 10 min. Five microliters of the supernatant were injected into the UPLC system.

LC−MS Analysis

Fecal extracts were analyzed in both non-derivatized form and derivatized form. Non-derivitized fecal extracted were separated a BEH C18 column (Waters, Milford, MA) using a mobile phase gradient containing 0.1% formic acid (A) and ACN containing

0.1% formic acid (B). For SCFAs analysis, HQ-derivitized fecal samples were separated a BEH C18 column (Waters, Milford, MA) using a mobile phase gradient containing 2 mM ammonium acetate and 0.05% acetic acid, v/v (A), and H_2O/ ACN = 5:95, v/v, containing 2 mM ammonium acetate and 0.05% acetic acid, v/v (B). The LC eluant was introduced into a Xevo-G2-S quadrupole time-of-flight mass spectrometer (Waters) for accurate mass measurement and ion counting. Capillary voltage and cone voltage for electrospray ionization was maintained at 0.1 kV and 5 V for negative-mode detection, and at 3 kV and 30 V for positive-mode detection. Source temperature and desolvation temperature were set at 120 and 350 °C, respectively. Nitrogen was used as both cone gas (50 L/h) and desolvation gas (800 L/h), and argon was used as collision gas. For accurate mass measurement, the mass spectrometer was calibrated with sodium formate solution with a mass-to-charge ratio (m/z) of 50-1000 and monitored by the intermittent injection of the lock mass leucine enkephalin ($[M + H]^+$ = 556.2771 *m/z* and $[M - H]^-$ = 554.2615 *m/z*) in real time. Mass chromatograms and mass spectral data were acquired and processed by MassLynx software (Waters) in centroided format. The concentration of individual compounds was determined by calculating the ratio between the peak area of compound and the peak area of internal standard and fitting with a standard curve using QuanLynx software (Waters).

Chemometric Analysis and Biomarker Identification

The chromatographic and spectral data of fecal extracts were deconvoluted by MarkerLynx software (Waters). A multivariate data matrix containing information on sample identity, ion identity (retention time and *m/z*), and ion abundance was generated

through centroiding, deisotoping, filtering, peak recognition, and integration. The intensity of each ion was calculated by normalizing the single-ion counts (SIC) versus the total-ion counts (TIC) in the whole chromatogram. The data matrix was further exported into SIMCA-P+ software (Umetrics, Kinnelon, NJ) and transformed by *Pareto* scaling, and then analyzed by unsupervised principal component analysis (PCA), supervised partial least squares-discriminant analysis (PLS-DA), and supervised orthogonal partial least squares-discriminant analysis (OPLS-DA). Major latent variables in the data matrix were described in a scores scatter plot of the established multivariate model. Metabolites affected by tylosin were identified by analysis of ions contributing to the separation of tylosin and control samples in the loadings plot the models. The chemical identities of compounds of interest were determined by accurate mass measurement, elemental composition analysis, MSMS fragmentation, and comparisons with authentic standards if available.

Microbiome correlation analysis

Isolation of DNA**,** PCR amplicon production, sequencing, and analysis were all completed and analyzed previously (164). Since the microbiome data analysis only used pooled data, the average relative abundance of metabolites for each time point and treatment were used in this calculation. This allowed the data sets to be equally compared for the correlations analysis. The average values for the metabolomics samples were only used for the correlation analysis. Weighted Bray-Curtis beta diversity metrics were calculated using the vegan package in the statistical software R (168). The dissimilarity distance matrix for both the microbiome and metabolome data was calculated after relative abundance transformations to account for non-normal distributions. Correlations

between microbiome and metabolome data were calculated using the mantel test and procrustes analyses, also within the vegan R package (168). A multiple correlation analysis approach, based on Spearman correlation coefficients and adjusted using false discovery rate (fdr) methods for multiple testing using the microbiome R package (169), was also conducted to assess how the abundance of identified bacterial taxonomic units covaried with the abundance of identified bile acid metabolites.

Results

Metabolomic comparisons

The distribution of fecal samples in the score plot of a PLS-DA model showed that time-dependent changes in the fecal metabolome occurred in both control and tylosin-treated pigs (Figure 4). Between the two treatment groups, the metabolome profiles were comparable at 10 and 22 weeks of age, but different during weeks 13-19 (Figure 4). The metabolites contributing to the separation between control and tylosin groups in 13, 16, and 19 weeks of pigs were defined in the S-plot of a OPLS-DA model (Figure 4). As expected, tylosin and its metabolites contributed to the separation of two groups of pigs in the models (Figure 4). More importantly, HDCA, a bile acid, was identified as another prominent marker associated with tylosin feeding (Figure 4).

Following the observation of HDCA as a tylosin-responsive metabolite, the levels of bile acids in feces were quantified. Based on their concentrations, HDCA and lithocholic acid (LCA) are major bile acids while deoxycholic acid (DCA) and cholic acid (CA) are minor ones in pig feces (Figure 5). More importantly, the results showed that the concentrations of fecal bile acids were relatively stable in the control group

between week 10 and 22, but significantly and differently affected by tylosin in the treatment group (Figure 5). HDCA and CA shared a comparable time-course profile, since the concentrations of both bile acids were elevated by tylosin during weeks 13 and 19, but became comparable to the controls on week 22 (Figure 5). In contrast, DCA and LCA were only increased by tylosin treatment during weeks 19 and 22 (Figure 5). Besides bile acids, short chain fatty acids (SCFA) in these fecal samples, including acetic acid, propionic acid, butyric acid, and valeric acid, were also quantified. The acetic acid concentrations were different ($P < 0.05$) between the control and treatment group at the 10 weeks of age (Figure 6). This pre-existing difference between groups cannot be simply explained by tylosin treatment because the antibiotic was only added a few hours before the fecal samples were collected. Aside from this difference at a single time point, there were no differences in the concentration of any SCFA between the treatment and control group (Figure 6).

Comparison of metabolome and microbiome

There was a positive correlation ($r = 0.78$, $P = 0.001$) between the microbiome composition and metabolomic patterns at all time points for both treatments, revealed by a Mantel test. Procrustes analysis based on the Spearman method further confirmed this correlation (correlation in a symmetric Procrustes rotation = 0.88, $P = 0.001$, m12 squared= 0.22). Multiple correlation analysis was also used to detect associations between identified metabolites and OTUs in the microbiome. Significant correlations ($Q < 0.05$) were observed between bile acid metabolites and limited OTUs. HDCA is the most common bile acid in pigs and was associated with the abundance of the family

Lachnospiraceae, which belong to order of Clostridiales. LCA was associated with *Coprococcus* and *Ruminococcus* in both treatment and control groups (Table 4). To further evaluate these relationships over time, the concentrations of bile acid metabolites were plotted against the abundances of their correlated bacterial species (Figure 7). For LCA, even though metabolite concentration between treatment and control deviated in later weeks, the abundance of *Coprococcus* remained similar between groups but increased over time. The association between LCA and *Ruminococcus* appeared to be more direct, meaning the concentration of LCA increased over time in the tylosin treatment group, as did the abundance of *Ruminococcus.* There was an initial increase in the levels of *Lachnospiraceae* and HDCA in pigs fed tylosin, but at subsequent time points, the levels of both decreased to match the control group at 22 weeks.

Discussion

Because metabolites can function as energy carriers and signaling initiators of the growth and wellbeing of host and gut microbes, metabolomic analysis could provide useful insights on the connections between growth promoting effects and microbiome modulating effects of antibiotics. In this study, the composition of the fecal metabolome was similar between tylosin-treated pigs and control pigs at weeks 10 and 22, but different at weeks of 13, 16, and 19 of age (Figure 4). This observation resembles our previous observation on the fecal microbiome of these pigs, because the differences in the distribution and quantity of microbes between the control and tylosin treated group were also observed at weeks 13, 16, and 19, but not weeks 10 and 22 (164). This phenomenon suggested that tylosin might cause the microbiome to mature at a faster rate and then

stabilize by week 22 (164). Interestingly, this "maturation" of the microbiome was observed in a different set of animals and samples (farm 1) compared to the samples used for the metabolomics analysis presented in this paper (farm 2) (164). The authors explained this variation in microbiome between farms as a technical issue from more in-depth sequencing on farm two, variation in the microbiome between farms that could respond differently to antibiotics, or inaccuracy of the maturation hypothesis. Though this pattern was not as clear in the microbiome on farm two, our results from metabolomic analysis still support this hypothesis, showing similar metabolite compositional patterns between groups at 10 weeks and 22 weeks, with convergence of the metabolome profiles at 22 weeks. When evaluating this phenomenon from an ecological perspective, it has been proposed that the microbiome is always driven to return to a stable state, even after the impact of a stressor, such as antibiotic exposure (170,171). Our results suggest that the functionality of the microbiome may also follow this pattern, as reflected by convergence of metabolome profiles between the tylosin treated pigs and control pigs.

Because of the role that the microbiome plays in converting primary bile acids to secondary bile acids, we hypothesized that the concentration of secondary bile acids would be altered after exposure to tylosin (172). Previous studies have shown that antibiotics can impact secondary bile acid secretion in humans and rats (173,174). Furthermore, previous research has also identified that variation in the gut microbiome between germ-free and conventional mice impacts primary bile acid synthesis in the liver through interactions between gut microbes and the nuclear receptor Farnesoid X receptor (175). For this reason, it was also hypothesized that we would observe variations in primary bile acid secretion between the tylosin treatment and the control group. Although

the concentration of CA (primary bile acid) was different between treatment and control group, this difference was only present for weeks 16 and 19. It is still unknown which species of bacteria are most involved in the regulation of the Farnesoid X receptor pathway, and we were unable to confirm if antibiotic induced changes in the microbiome impacted this pathway.

Our data also showed differences in HDCA concentration between the tylosin treatment and control group. Because HDCA is produced in germ-free pigs, it has been considered to also be a primary bile acid (176). However, previous research has also demonstrated that a healthy microbiome is capable of producing significant amounts of HDCA, indicating it could also be considered to be a secondary bile acid (177). Based on these conflicting results, it is unclear if HDCA should be considered a primary or secondary bile acid. For this reason, it is difficult to determine which mechanism may be impacting the increased concentration of HDCA in our experiment (i.e. action from the microbiome or interaction with the liver and primary bile acid production). We also found differences in the concentration of LCAs between treatments, which is another secondary bile acids in pigs. The abundance of bacterial class *Clostridia* have been shown to be correlated with intestinal secondary bile acids (178). It has also been reported that bacteria from the family *Clostridia* plays a critical role in bile acid deconjugation (179). In our experiment, we identified a significant, positive correlation with the secondary bile acids LCA and HDCA that were associated with three bacterial species in the class *Clostridia* (Class *Coprococcus, Ruminococcus,* and *Lachnospiraceae*). Thus, we suggest that feeding sub-therapeutic levels of Tylosin may lead to increases in the abundance of *Clostridia*, and ultimately increasing the production

of secondary bile acids. It is also worth noting that the majority of previous studies that have reported changes in bile acid metabolism, fed greater antibiotic doses compared to sub-therapeutic levels of tylosin fed in the current study (173,178,180). Our experiment found similar alterations in the microbiome, leading to consequential changes in the animals' metabolome, even with a relatively low dose of tylosin.

SCFA are a major group of microbial metabolites in the large intestine (181). Influences of antibiotic exposure on SCFA production have been observed in both human and animal studies. For example, the concentrations of SCFA in feces were reduced by the 6-day treatment of a variety of antibiotics in healthy human subjects (182), while feeding sub-therapeutic levels of antibiotics increased concentrations of SCFA in cecum of treated mice (183). In contrast to these observations, no difference in fecal SCFA concentrations was observed between control and tylosin-treated pigs in this study. It is possible that bacteria responsible for SCFA production might not be sensitive to the dose of tylosin in this study. Tylosin is macrolide-class broad spectrum antibiotic commonly used for its activity against gram-negative bacteria, but is also effective against a select number of gram-positive bacteria (184). Specific gram-positive bacteria from the families *Propionibacteriaceae, Bifidobacteriaceae* and *Veillonellaceae* have been shown to play a role in SCFA production (185). It is possible that tylosin did not have a bacteriostatic effect on some of these bacteria, allowing them to continue SCFA production without major changes.

One of the main limitations of our experiment was our inability to correlate the change in bile acid synthesis to a change in growth performance or health of pigs, because body weights, mortality, and morbidity data were not collected during the

experiment. There is currently limited research available that has reported a direct change in growth performance of swine as a result of increased bile acid synthesis, but some previous research suggests that the mechanism for growth promotion when feeding antibiotics are due to changes in bile biotransformation (186,187). However, this proposed mechanism suggests that increased bile acid secretion decreases average daily gain in the animal, which has been demonstrated in swine with LCA(180). Our results showed that the concentrations of LCA and other bile acids increased in pigs fed tylosin, which suggests that these differences may be specific to tylosin. Various antibiotics target different types of bacteria, which suggests that the mechanisms of growth promotion through modulation of the gut microbiome will vary between antibiotics used (1,175,178). Without growth performance data being available from this experiment, we are unable to determine the impact of the altered bile acid concentrations on growth of these pigs.

Conclusion

In conclusion, inclusion of sub-therapeutic levels of tylosin in the diet of growing pigs impacted bile acid concentration in the feces, but this change tended to diminish in subsequent time periods. These observations warrant further investigation to better understand the role of bile acids in growth and development of pigs, and whether these observations may be correlated with the mechanisms of growth promotion when supplementing diets with sub-therapeutic levels of antibiotics for pigs.

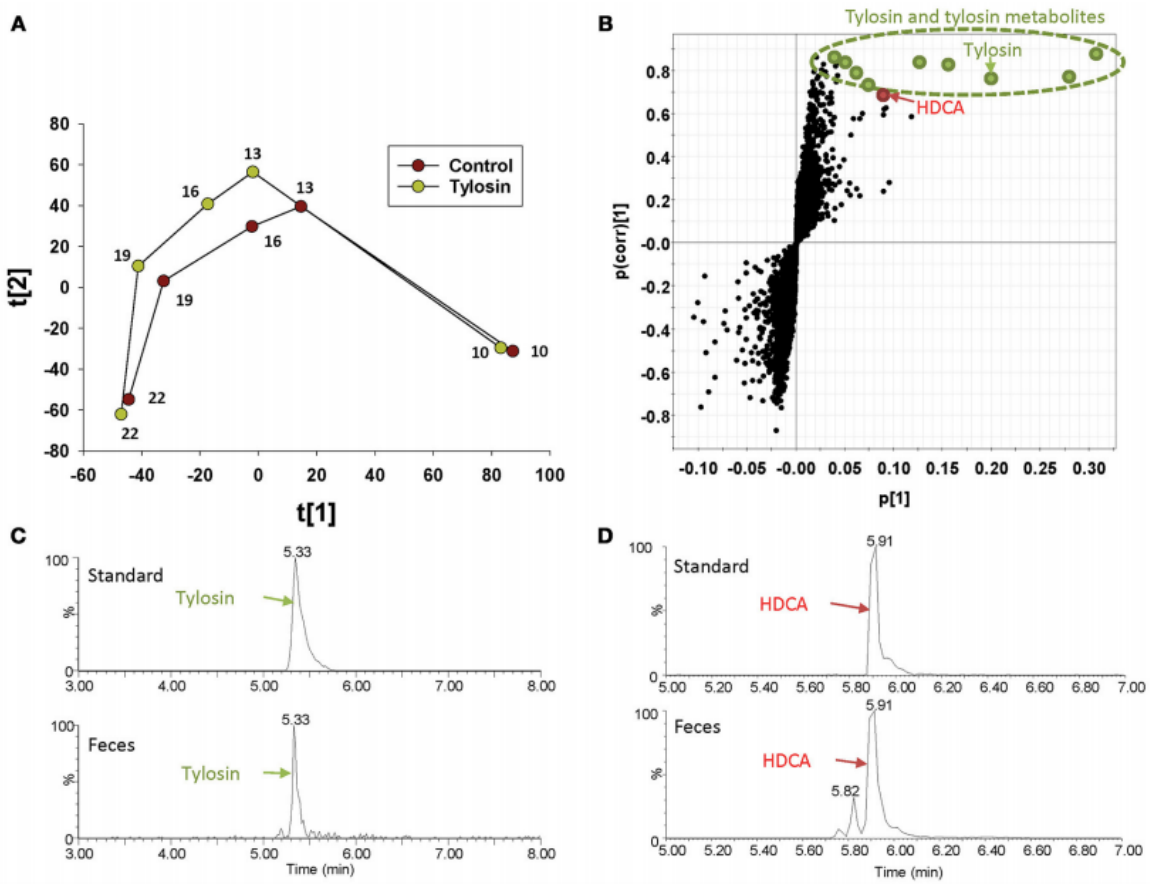


Figure 4 Identification of fecal metabolites induced by tylosin treatment through LC-MS-based metabolomics. (A) Scores plot of a PLS-DA model on fecal samples from the tylosin-treated and control pigs. The t[1] and t[2] values represent the scores of each data point in the principal component 1 and 2 of the model, respectively. These values are the averages of 10 pigs under the same treatment at weeks 10, 13, 16, 19 and 22. (B) S-plot of an OPLS model on week 13-19 control and tylosine treatment samples. The fecal metabolites contributing to the separation of two groups of pigs are labeled. The p[1] axis represents the magnitude of the fecal ions. The p(corr)[1] axis represents the correlation of the ions towards the predictive variation induced by tylosin treatment. (C) Extracted chromatograms of tylosin standard and a fecal sample. (D) Extracted chromatograms of HDCA standard and a fecal sample.

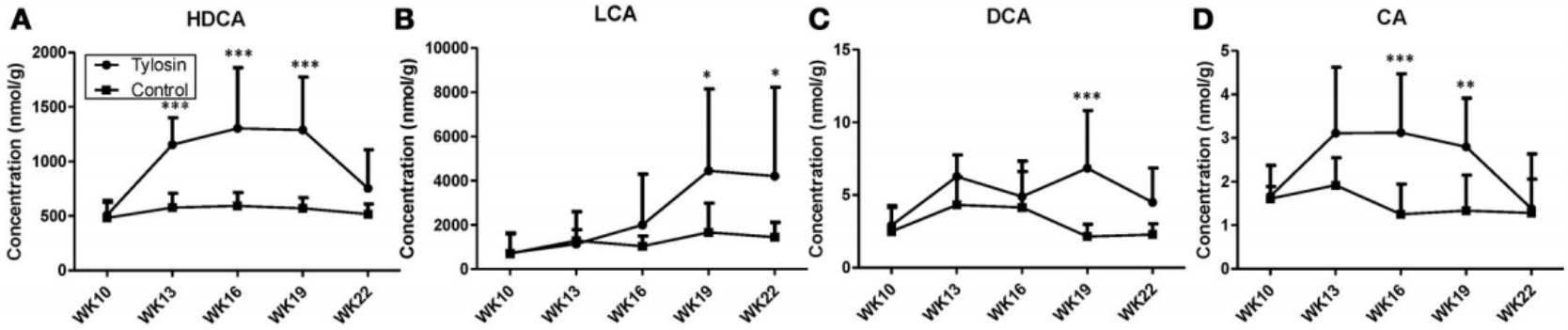


Figure 5 Concentrations of bile acids in fecal samples from control and tylosin-treated pigs from week 10 to week 22. (A) HDCA. (B) LCA. (C) deoxycholic acid (DCA). (D) CA. Values are mean ± S.D. (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

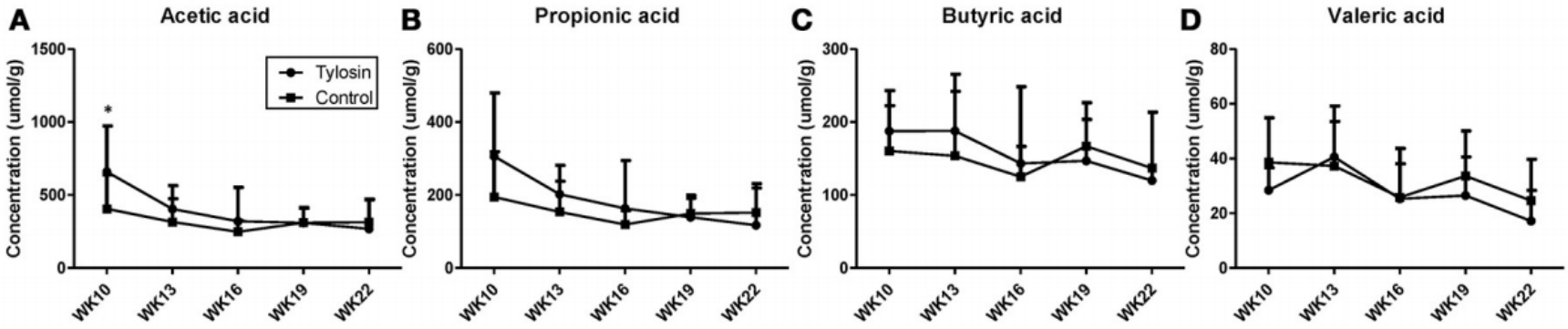


Figure 6 Concentrations of SCFAs in fecal samples from control and tylosin-treated pigs from week 10 to week 22. (A) acetic acid. (B) propionic acid. (C) butyric acid. (D) valeric acid. Values are mean ± S.D. (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

Table 4 Significant correlations (Q < 0.05) between tylosin-responsive bile acids and tylosin-responsive families in swine feces.

Taxa	Metabolite	r-value	Q-value[1]
k__Bacteria..p__Firmicutes..c__Clostridia..o__Clostridiales..f__Lachnospiraceae..g__*Coprococcus*..s__.60	LCA	0.939394	< 0.0001
k__Bacteria..p__Firmicutes..c__Clostridia..o__Clostridiales..f__Ruminococcaceae..g__*Ruminococcus*..s__.102	LCA	0.951515	< 0.0001
k__Bacteria..p__Firmicutes..c__Clostridia..o__Clostridiales..f__Lachnospiraceae.79	HDCA	0.963263	0.0313

[1]Adjusted for multiple comparisons in the model through false discovery rate.

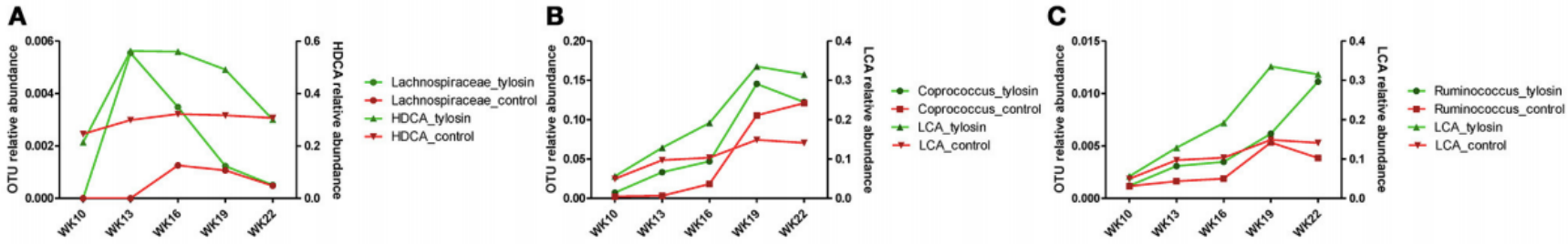


Figure 7 Comparison of relative abundance for significantly correlated bacterial species over time for HDCA (A) and LCA (B) (C).

Chapter 3: Using metabolomics and microbiome analyses to understand variation in growth promotion responses in pigs from feeding subtherapeutic levels of antibiotics

Summary

The mechanisms of growth promoting responses from feeding antibiotics are poorly understood. As a result, identifying feed additives that provide similar responses is challenging. The objective of this study was to evaluate the growth performance, metabolic profile, and changes in intestinal microbiome composition of healthy nursery pigs fed diets containing subtherapeutic concentrations of antibiotics. Diets formulated to contain 0.01% chlortetracycline and 0.01% sulfamethazine (AB) or no antibiotics (NC) were fed to pigs weaned at 20-d of age. Three experiments were conducted using at least 8 replicates per dietary treatment in a completely randomized block design with the main effects of time, diet, and experiment. Experiment 1 and 2 were completed in the same nursery facility and experiment 3 was conducted in a wean-finish facility. Both facilities were located on the same research farm. Weaned pigs of a common genotype from the same source herd were weighed and feed disappearance was determined on d 10, d 21, and d 42 post-weaning to calculate average daily gain (ADG), average daily feed intake (ADFI), and gain:feed (G:F). On d 42, one pig/pen was selected for blood, cecal and ileum content collection. Targeted and untargeted metabolomic profiles were assessed via liquid chromatography-mass spectrometry (LC-MS) in serum and cecal contents. The composition of bacterial communities in intestinal content samples was determined by sequencing the V4 region of the 16s rRNA bacterial gene. Metabolomics and microbiome data were analyzed using principal component analysis, indicator values, and random forest analysis. Feeding AB increased ($P < 0.05$) overall ADG and ADFI compared with

NC. Body weight, ADG, and G:F differed ($P < 0.05$) by experiment. The serum metabolome was different between experiments ($P < 0.05$), but not between dietary treatments. In the cecal and ileal microbiome, there were no differences in alpha diversity, but beta diversity was different ($P < 0.05$) between experiments. Percentage error from the random forest analysis indicated that most of the variation (8% error) in the microbiome was explained by the facility where the experiments were conducted. These findings suggest that facility had a greater effect on growth performance, serum and cecal metabolites, and microbiome than feeding diets containing subtherapeutic levels of antibiotics. In conclusion, antibiotics had a minimal impact on the metabolome and microbiome of nursery pigs compared with the facility where the experiments were conducted.

Introduction

Pork production continues to become more efficient as a result of genetic improvements and the use of nutritional interventions such as feed additives (1,2,188–191). Historically, the use of subtherapeutic levels of antibiotics (antibiotics as growth promoters, AGPs) in swine diets was effective in improving health and growth performance, especially under poor sanitary housing conditions (1,135,192). However, the use of AGPs in animal agriculture has been shown to contribute toward the development of antibiotic-resistant bacteria, which is a significant human health risk (3,79,80,193,194). These concerns have triggered global action to reduce antibiotic use in both humans and livestock (79). In the United States, legislative action was taken to restrict AGPs use in animal diets through the implementation of the Veterinary Feed Directive (VFD)(4). As a result, there is tremendous interest in identifying, developing,

and using feed additives that are as effective as subtherapeutic dietary levels of antibiotics for improving animal growth and health.

By better understanding how AGPs improve growth, it may be possible to identify feed additives that provide similar responses when added to animal diets. Unfortunately, the growth improvement when adding antibiotics to swine diets is not consistent (45,158,192). Results from a meta-analysis of 495 peer reviewed studies evaluating pigs in all growth stages showed that 71% of studies reported no differences in ADG when AGPs were fed (104). Results from previous research have also suggested there is a variation in an animal's intestinal microbiome between different experiments (13). When considering the interaction between AGPs and the intestinal microbiome, some of the variation in growth promotion may be explained by different composition of the intestinal microbiome at the beginning of the experiments (13). In addition, experiments are often conducted on one farm or barn, which limits our ability to understand the role of the research site on observed effects.

We hypothesized that feeding diets containing common AGPs would improve growth performance through a modification of microbiome diversity and bacterial metabolite concentrations in the gastrointestinal tract. Therefore, the objective of this study was to evaluate the growth response, metabolic changes, and gastrointestinal tract microbial changes in nursery pigs fed diets with or without AGPs across three separate experiments, conducted in two different facilities.

Materials and Methods

The feeding experiments and sample collection were conducted at the Purina Animal Nutrition Research Farm (Gray Summit, MO, USA) using approved animal care

and use protocols and supervised by Purina Animal Nutrition research personnel. All metabolome and microbiome laboratory analyses, as well as associated data and statistical analyses were conducted at the University of Minnesota (St. Paul, MN, USA).

Animals, housing, and experimental design

Experiment (exp) 1 occurred from June 11th to July 26th, 2018; exp 2 was conducted from August 20th to October 4th, 2018; and exp 3 was performed from July 16th to August 30th, 2018. Experiments 1 and 2 were completed in the same environmentally controlled nursery facility, while exp 3 was conducted in an environmentally controlled wean-to-finish facility located on the same research farm. The nursery facility included plastic flooring, five-hole plastic feeders, and nipple waterers. The wean-to-finish facility used in experiment 3 included concrete slatted flooring, metal feeders, and cup waterers. In both barns, room temperature was maintained at approximately 30°C during the first week after weaning and decreased 1.5°C per week for each 6 wk experiment. Before each experiment, the barns were all washed and sanitized using the same chemicals and standard operating procedures established by Purina Animal Nutrition.

The minimum number of replicates was determined by calculating the necessary sample size (n) to achieve statistical significance at $P \leq 0.05$ and power of 0.80. Data (mean and standard deviation) from previous studies of microbiota and metabolomic analyses were used as inputs to G*Power 3.1 (Kiel University, Germany), which indicated that 8 replicates were required to detect significant differences. Experiment 1 and 3 utilized eight replicates per treatment, while exp 2 included nine replicates per treatment. Each exp used a completely randomized block design and pigs were blocked by initial BW and sex.

All pigs were from the same genetic line (PIC Camborough × PIC 337, Hendersonville, TN, USA), and were obtained from the same farm and sow unit (Purina Animal Nutrition). Average initial body weight (BW) of weaned pigs in each exp was 6.5 kg at a weaning age of 20-d. Pigs were vaccinated for *Streptococcus suis* and *Mycoplasma hyorhinis* (Autogenous Bacterin, Philbro Animal Health, Teaneck, NJ) at 5 to 7-d of age and received a booster inoculation at weaning. Pigs were also vaccinated for *Haemophilus parasuis* (ParaSail, Newport Laboratories, Worthington, MN), and *Salmonella typhimurium* (Enterisol-Salmonella T/C; BI, St. Joseph, MO) 7-d prior to weaning; and Circovirus Type 2 (Fostera PCV Chimera, Zoetis, Charles City, IA) at weaning. Throughout the study, pigs were monitored for health status and any medication treatments used were recorded. In exp 1, two pigs were removed from the AB group because of *Streptococcus suis* infection and one pig was removed from the NC group because of lameness. In exp 2, one pig in AB group and one pig in the NC treatment were removed because of lameness. In exp 3, one pig was removed from the AB group and one pig from the NC group for assumed *Streptococcus suis* infection.

Dietary treatments

Each exp used a 3-phase feeding program consisting of feeding phase 1 diet from weaning (d 0) to d 10 post-weaning; phase 2 diet from d 10 to d 21; and phase 3 diet from d 21 to d 42 post-weaning. All diets were formulated to exceed the National Research Council (2012) energy and nutrient requirements for nursery pigs and were manufactured in pelleted form at the Purina Research Manufacturing Unit (Gray Summit, MO, USA). Two dietary treatments were fed, where the antibiotic diets (AB) contained 0.5% Aureo

Mix 10-10S (Zoetis; Charles City, IA) providing 0.01% chlortetracycline and 0.01% sulfamethazine throughout each 42-d exp, and negative control diets (NC) contained no antibiotics. All pigs had *ad libitum* access to feed and water throughout each exp.

Statistical analysis of growth performance data

Growth performance data (BW, ADG, ADFI, and G:F) were analyzed for absence of outliers and normal distribution using the UNIVARIATE procedure of SAS (SAS Institute, Cary, NC). Pen was considered the experimental unit and experimental data were analyzed as a randomized complete block design using the GLIMMIX procedure of SAS with time as repeated measure with autoregressive 1 variance structure. Degrees of freedom were calculated using the Kenward-Roger method. Replicate was considered as random effect, while fixed effects included dietary treatments and experiment. The effect of facility was not included as a main effect because barn was not an independent variable from the experiment. The main effects included treatment, experiment, and time, along with the interactions of treatment × time, experiment × time, experiment × treatment, and experiment × treatment × time. Significant differences were declared at $P \leq 0.05$.

Sample collection

For metabolomic and microbiome analyses, one pig with BW closest to the median BW of the pen was selected for blood and intestinal content collection. Blood samples were collected via venipuncture of the jugular vein in Vacutainer® blood collection tubes (BD, Franklin Lakes, NJ, USA) and then centrifuged at 2,000 × *g* for 15 min at 4ºC. Serum was then aliquoted and stored at -80ºC. To collect digesta samples, pigs were

euthanized using CO_2 gas and exsanguination. The entire intestinal tract was removed, placed on a sterile surface, and sterilized utensils were used to collect intestinal content. Approximately 1.5 mL of cecal contents were collected from the lateral side of the cecum and 1.5 mL of ileum contents were collected 30 cm proximal to the ileocecal junction. Each sample was snap frozen in liquid nitrogen and stored at -80ºC.

Metabolomics

Liquid chromatography–mass spectrometry (LC-MS) based metabolomic analysis involved several steps including sample preparation, chemical derivatization, LC-MS analysis, data deconvolution and processing; multivariate data analysis, and marker characterization and quantification (195). Deproteinization of serum was conducted by mixing one volume of serum with 19 volumes of 66% aqueous acetonitrile (ACN) followed by centrifugation at 18,000 × *g* for 10 min at room temperature. Cecal content samples were mixed with 50% aqueous ACN containing 5 μM glycocholic acid-$^{13}C_1$ in 1:10 (w/v) ratio. Samples were sonicated for 10 min followed by mixing using a vortex mixer. After mixing, they were centrifuged at 18,000 × *g* for 10 min and the supernatant was collected to obtain cecal sample extracts.

For detection of metabolites containing amino groups in their structure, samples were derivatized with dansyl chloride (DC) prior to the LC-MS analysis. Briefly, 5 μL of samples or standards were mixed with 5 μL of 100 μM *p*-chlorophenylalanine (internal standard), 50 μL of 10 mM sodium carbonate, and 100 μL of DC (3 mg/mL in acetone). The mixtures were incubated at 25°C for 15 min, centrifuged (18,000 × *g*) for 10 min, and the supernatant was transferred into high-performance liquid chromatography vials for LC-MS analysis.

Samples were derivatized with 2-hydrazinoquinoline (HQ) prior to the LC-MS analysis for identification of carboxylic acids, aldehydes, and ketones species. Briefly, 2 μL of sample were added into a 100 μL of freshly prepared acetonitrile solution containing 1 mM 2,2′-dipyridyl disulfide (DPDS), 1 mM triphenylphosphine (TPP), and 1 mM HQ. The reaction mixture was incubated at 60°C for 30 min, chilled on ice and then mixed with 100 μL of ice-cold H_2O. After centrifugation at 21,000 × *g* for 10 min, the supernatant was transferred into a HPLC vial for LC-MS analysis.

A 5 μL aliquot of sample prepared from serum or cecal fluid was injected into an Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA) and separated by a gradient of mobile phase ranging from water to 95% aqueous acetonitrile containing 0.1% formic acid over a 10 min run. The LC eluant was introduced into a Xevo-G2-S quadrupole time of flight mass spectrometry (QTOFMS, Waters) for mass measurement and ion counting. Capillary voltage and cone voltage for electrospray ionization was maintained at 3 kV and 30 V for positive-mode detection, or at -3 kV and -35 V for negative-mode detection, respectively. Source temperature and desolvation temperature were set at 120°C and 350°C, respectively. Nitrogen was used as both cone gas (50 L/h) and desolvation gas (600 L/h), and argon was used as collision gas. For accurate mass measurement, the mass spectrometer was calibrated with sodium formate solution (range *m/z* 50–1,000) and monitored by the intermittent injection of the lock mass ($[M+H]^+$ = *m/z* 556.2771 and ($[M+H]^-$ = *m/z* 554.2615) in real time. Mass chromatograms and mass spectral data were acquired and processed using MassLynx™ software (Waters, Milford, MA, USA) in centroided format.

After data acquisition in the UPLC-QTOFMS system, chromatographic and spectral data of samples were deconvoluted by MarkerLynx™ software (Waters, Milford, MA, USA). A multivariate data matrix containing information on sample identity, ion identity (RT and *m/z*) and ion abundance was generated through centroiding, deisotoping, filtering, peak recognition, and integration. The intensity of each ion was calculated by normalizing the single ion counts (SIC) *versus* the total ion counts (TIC) in the whole chromatogram.

In the untargeted metabolite analysis, the processed data matrix was exported into Rstudio software (Boston, MA, USA) and then analyzed by unsupervised principal components analysis (PCA). The vegan package was used to determine the weighted Bray-Curtis matrix (196). Major latent variables in the data matrix were determined as the principal components of PCA model.

For the targeted analysis, individual metabolite concentrations were calculated using the ratio between the peak area of metabolite and the peak area of internal standard and fitting with a standard curve using QuanLynx software (Waters, Milford, MA). The concentration of targeted analysis was evaluated for normality using the Shapiro test in Rstudio. An analysis of variance between treatments was completed using the Kruskal-Wallis rank sum test using the stats package in Rstudio. If significant, multiple comparisons were performed using the pgirmess package in Rstudio (197). Differences of group means were considered significant if $P < 0.05$ and a trend was based on $0.05 \geq P \leq 0.10$.

Microbiome

All cecal (n = 52) and ileum (n = 52) content samples were submitted to the University of Minnesota Genomics Center for DNA extraction and sequencing of the 16S rRNA gene V4 region. The extractions were performed using a DNeasy PowerSoil DNA extraction kit (Qiagen, Hilden, Germany) following manufacturers' instructions. The DNA samples were then submitted to the University of Minnesota Genomics Center where they were processed using their standardized methods. Library prep was completed using a previously described dual-indexing method (198). Marker gene sequencing was then completed using the Illumina MiSeq Next Generation platform (Illumina, San Diego, CA, USA) with a targeted average sequencing depth of 100,000 reads per sample.

Amplicon Sequence Variants (ASV) were identified from raw Illumina sequence reads using cutadapt, fastx, and qiime2 bioinformatics tools (199–201). The adapter sequences, barcodes and primers were removed from the pair-end reads and low-quality reads were discarded. Primers were removed using the default parameters of the cutadapt program. Empty lines were then removed from the output and then the sequences were filtered using a quality score of 50 and all other parameters set to default settings. The paired ends were then merged using bbmap merger and singletons were discarded (202). Data were then imported into Qiime 2, where sequences were demultiplexed and processed through the dada2 pipeline to identify ASVs. Taxonomic analysis of the ASVs obtained was then completed using a pre-trained classifier. After data processing, 72% of the reads were retained from the raw reads.

After obtaining the ASV table, data were transferred into Rstudio where the rarefied richness and Simpson alpha diversity were determined using the vegan package.

In addition, the vegan package was used to determine the weighted Bray-Curtis matrix. This matrix was used to calculate beta diversity between treatments. Finally, species indicator values were calculated using the labdsv package. The indicator value is a product of frequency and abundance of a given taxon across all samples belonging to a specific treatment. When an indicator value is close to 1, the taxon is present in many samples of that treatment and in high abundance compared with other treatment groups. A good indicator value is generally greater than 0.8, and only indicator values greater than this threshold are reported in this manuscript. Indicator values were reported to identify representative species for AB and NC, and for facility. A random forest analysis was also completed using the randomForest package in Rstudio (203). Confusion matrices and percentage error were reported for comparisons between treatments, between exp, and between the facilities where the exp were conducted.

Results

Growth performance

There was no dietary treatment effect on BW or G:F (Table 5). There was, however, an increase ($P < 0.05$) in ADG and ADFI in pigs fed diets with AB. As expected, BW, ADG, and ADFI increased with time. In addition, there was a time × treatment interaction ($P < 0.05$) for ADG which indicates that AB was more effective at improving ADG during the 21-d to 42-d period compared with the other growth periods (Table 5). However, this interaction was not observed for BW, ADFI, or G:F.

There were no differences in initial BW across the three experiments. Overall, pigs in exp 3 had less ($P < 0.05$) final BW and G:F compared with pigs in exp 1 and 2,

indicating that those housed in the wean-finish facility had reduced growth performance compared with pigs housed in the nursery facility (Table 6). There were no interactions of exp × time or exp × treatment, indicating that the effect of exp on BW, ADG, ADFI, or G:F was independent of treatment and time. The interaction between treatment × exp × time was only significant for G:F, indicating that the relationship between gain efficiency in the NC and AB group varied based on the experiment and time point ($P = 0.047$; Table 7).

Metabolome

Pigs were fed AB had greater ($P < 0.05$) serum concentrations of arginine, histidine, lysine, phenylalanine, and valine compared with those fed NC (Table 8). However, in the untargeted analysis comparing all identified metabolites, there was no clear separation between the metabolome of pigs fed AB compared with those fed NC (Figure 8). In contrast, there was separation ($P < 0.05$) between the metabolic profile of pigs in different exp, indicating that the exp itself had a stronger effect on the serum metabolome than dietary treatment (Figure 8).

A similar pattern to the metabolome serum profile was observed in the metabolomic profile of cecal contents. When comparing targeted metabolites, there were no differences in short chain fatty acids and bile acid concentrations between pigs fed AB and NC treatments (Table 9). Although there was separation of untargeted metabolites between the AB and NC (Figure 9), this separation was largely due to the antibiotic metabolites present in the cecal samples. When evaluating the untargeted metabolites,

greater differences were evident when comparing the three exp than differences between AB and NC.

Microbiome

Feeding AB had no effect on the alpha diversity, which was assessed using the Simpson index, or the rarefied richness of the cecal or ileal contents (Figure 10). In addition, there was no clear separation of bacterial species between pigs fed AB compared with those fed NC (Figure 11). Although a treatment effect was not detected, there was a difference ($P < 0.05$) in beta diversity between experiments for both cecal and ileal samples (Figure 11).

Despite a minimal impact on the alpha and beta diversity of the microbiome when antibiotics were fed, indicator values were used to identify potential microbial indicators of treatments. Microbial indicators may be useful for detecting bacterial species that were highly affected by feeding antibiotics. Indicators were only reported if they had values greater than 0.8 and a different relative abundance between the dietary treatment groups. In the cecal samples, two bacteria from the family *Clostridiaceae* were identified (Table 10). These bacterial variants were both increased by feeding AB compared with NC. There were no bacterial variants identified that had high indicator values for pigs fed NC. In the ileal samples, three bacteria were identified to be increased in pigs fed AB: *Streptococcaceae* (family) *Streptococcus* (genus), *Clostridiales* (order) *Peptostreptococcaceae* (family), and *Leuconostocaceae* (family) *Weissella* (genus).

A random forest analysis was used to compare the ability of the cecal microbiome dataset to predict a pigs' dietary treatment, experiment, or facility. When using the data to

predict the dietary treatment fed, there was a 40% error rate. This indicates that there were not enough differences in the microbiome between dietary treatments to make accurate predictions (Table 11). When this analysis was repeated to make predictions by exp, the error rate was reduced to 28%. Finally, when a random forest analysis was conducted to compare the facility in which the exp was conducted, the error rate was only 8%.

Multiple microbial biomarkers were identified for each facility (Table 12). In the cecal samples, these included bacterial variants from the family *Veillonellaceae* and *Lachnospiraceae* in the nursery barn, and bacterial variants from the family *Clostridiaceae* in the wean-finish barn. For ileal content samples, bacterial variants from the family *Lactobacillaceae* were more abundant in the nursery facility while bacterial variants from the family *Clostridiacea* were more abundant in the wean-finish facility. These differences show that the location of the exp can have a dramatic impact on the microbiome, and differences are caused by only a few variants of bacteria.

Discussion

One of the many challenges of determining the relative effectiveness of feeding AGPs or other feed additives in swine diets is the lack of reproducibility of responses among experiments (45). To gain insight on how AGPs improve growth, we fed nursery diets without antibiotics (NC) and with antibiotics (AB) in three separate exp to determine and compare growth, metabolic, and microbial responses in weaned pigs.

Overall, there was an increase in ADG and ADFI when pigs were fed diets containing AB compared with those fed NC, where the increased ADG was a result of enhanced feed intake but there was not a major improvement in gain efficiency. These

findings were somewhat surprising because feeding AGPs have been shown to improve both ADG and G:F in many experiments (1,158,192). The interaction between diet and time for ADG also indicated that pigs responded differently to AB as they increased in age. These findings contradict previous research which has shown that the growth promotion responses from feeding antibiotics are greater immediately after weaning compared with subsequent time points during pig growth (1,158). The observed lack of growth improvement during the first phase post-weaning in the present study may indicate the animals in our experiments did not experience significant weaning stress or immune challenge, because these factors have been shown to result in significant growth responses to dietary antibiotics (204).

When feeding antibiotics, there was a significant increase in the amino acids lysine, histidine, and phenylalanine concentration in the serum. In this study, feeding AB increased ADFI by 6.7% which is equivalent to 0.45 g more lysine consumed per day based on the average dietary lysine content in the overall nursery phase. When relating this to serum metabolite values, serum lysine concentration increased by 25%. Results from a previous study showed that a change in serum lysine concentration can be directly related to dietary supply of lysine (205). However, serum lysine concentration is not a measure of absorption, and it must also be considered that lysine catabolism is slower than that of other essential amino acids (206). This slowed catabolism may explain the greater magnitude of increase in serum lysine concentration than the increase in feed intake. The increased feed intake in pigs fed AB could also explain increased serum levels of histidine, as the concentration of histidine has also been previously reported to have a direct correlation to feed intake (207). In the case of phenylalanine, the increased

concentration in the serum could be reflective of increased health status when pigs are fed an antibiotic. Because of its concentration in acute phase proteins, phenylalanine is considered the first limiting amino in health challenged pigs (208). Taking this into account, increased plasma concentrations of phenylalanine could indicate the pigs fed AB were not experiencing the negative inflammatory effects of a health challenge, but further research would be needed to confirm this hypothesis.

Despite the addition of AGPs to the diet, there were no effects on the cecal metabolome in the targeted or untargeted analyses. We hypothesized that pigs fed AB would have differences in bacterial metabolites including short chain fatty acid (SCFA) and bile acid (BA) concentrations. Surprisingly, there were no differences in SCFA and BA concentrations in the cecal contents of pigs fed AB or NC. Results from previous studies have shown that adding antibiotics to pig diets increases secondary BA concentrations in the intestine, and this response has been linked to the mechanism of growth promotion for antimicrobials (62). However, this effect was not observed when supplementing AGPs in the present experiments. This lack of response may be due to different antibiotics fed (e.g., tylosin and chlortetracycline) in previous studies compared with feeding a combination of chlortetracycline and sulfamethazine in the present experiments (61,62). Because tylosin and sulfamethazine utilize a different mechanism of action to kill bacteria, it is likely that they may have a different mechanism than increased BA concentrations.

Similar to the metabolomic analysis results, there were no differences in the microbiome rarefied richness, and alpha or beta diversity between treatments in either cecal or ileal samples. The lack of differences in the microbiome may be explained by

animal contact between pens of different treatments because the barriers between pens in the experimental facilities consisted of bars with open spaces that allowed partial contact. In poultry, alpha diversity of the cecal microbiome was not different between the control treatment and birds fed antibiotics when the experiment was conducted in facilities that separated pens with mesh screens (209). However, when the same experiment was conducted in facilities with solid barriers between pens or in isolation facilities, the alpha diversity of the cecal microbiome was significantly altered by the dietary treatment (209). These findings suggest the type of pen partitions may have limited our ability to detect differences in this study, because pigs had the opportunity for contact with other pigs in adjacent pens and across treatments.

Despite a lack of dietary treatment differences in alpha and beta diversity, supplementing AGPs changed a small proportion of specific bacteria. These results are not unusual, where results from other studies have shown that feeding AGPs to pigs caused changes in only *Proteobacteria* and *Escherichia coli* in the entire fecal microbiome (210). Results from a study that evaluated the effects of feeding antibiotics to pigs on the microbiome in multiple sites of the intestinal tract, showed that in the ileum, *Lachnobacterium* was the only genus that contributed to separation between positive and negative control animals (211). Similarly, the abundance of only a few bacteria variants changed in the present study, with some bacteria identified as good indicators for the AB treatment including *Clostridiaceae* (exp 2), *Streptomycetaceae* (exp 3), *Streptococcaceae* (exp 1), *Peptostreptococcacea* (exp 2), and *Leuconostocaceae* (exp 2).

One of the significant findings from this study was the dramatic effect of experiment on growth performance, serum and cecal metabolome, and cecal and ileum

microbiome. Experiment significantly affected BW, ADG, and G:F responses, suggesting that differences in growth performance were be due to environmental differences between the wean-finish facility compared to the nursery barn where these experiments were conducted, although previous research results have shown that no differences in ADG, ADFI, or feed efficiency were observed for pigs raised in either wean-finish facilities or nursery facilities (212). The impact of exp could also be influenced by season, maternal factors, or batch of pigs because each experiment was conducted at a different time between July-October and piglets were obtained from different groups of sows. However, if this were the case, the random forest analysis would have resulted in the lowest error when comparing each experiment separately. Instead, the results from the random forest analysis suggest that the greatest variation in the microbiome is explained by the facility where the exp was conducted, because pigs used in the two experiments conducted in the nursery facility had a similar microbiome. Other studies have reported a similar effect to those observed in the present study. A meta-analysis of 20 studies that evaluated 16s rRNA sequencing data found that the most significant factor that affected composition and structure of the swine gut microbiota was the study itself (13). Using this information, interpreting results from future experiments that involve both growth performance and changes in gastrointestinal microbiome when feeding various feed additives to pigs should involve considerations for potential differences in location and experiment. In addition, studies are needed to investigate the environmental factors within a swine research facility that significantly influence the microbiome, which may include temperature, humidity, environmental microbes, facility materials (e.g., steel, aluminum, plastic), age of barn, barn size, or ventilation system. A better understanding

of how these variables impact the animal's microbiome may facilitate better control of these variables in future experiments.

Conclusions

Overall, supplementing diets with subtherapeutic levels chlortetracycline and sulfamethazine had a minimal effect on the growth performance, metabolism, and gastrointestinal microbiome of weaned pigs. When comparing the effect of the antibiotic treatment across the different exp, exp had an effect on animal metabolism, and the facility where the exp was performed had a greater impact on the microbiome than the antibiotic treatment itself. These findings indicate that environmental characteristics of swine research facilities can have a significant effect on growth performance and microbiome of animals that must be accounted for in future evaluations of feed additives.

Table 5 Effects of dietary antibiotic supplementation on growth performance parameters at various time points.

	Antibiotic[1,2]		SEM	*P*-value		
	No	Yes		Treatment	Time	Treatment × Time
Body weight (kg)						
Day 0	6.49	6.48	0.416	0.20	$P < 0.05$	0.27
Day 10	8.02	7.95				
Day 21	12.92	13.10				
Day 42	25.77	26.97				
Mean	15.57	16.01				
Average daily gain (kg)						
Day 10	0.15	0.15	0.01	$P < 0.05$	$P < 0.05$	$P < 0.05$
Day 21	0.44	0.47				
Day 42	0.61[a]	0.66[b]				
Mean	0.40[a]	0.42[b]				
Average daily feed intake (kg)						
Day 10	0.17	0.17	0.01	$P < 0.05$	$P < 0.05$	0.07
Day 21	0.51	0.53				
Day 42	0.89	0.95				
Mean	0.52[a]	0.55[b]				
Gain:Feed						
Day 10	0.87	0.85	0.01	0.98	$P < 0.05$	0.19
Day 21	0.87	0.89				
Day 42	0.69	0.70				
Mean	0.81	0.81				

[1] Data are presented as least squared means. Experimental data were analyzed as a randomized complete block design using the GLIMMIX procedure of SAS with time as repeated measurements with autoregressive 1 variance structure. Degrees of freedom were calculated using Kenward-Roger method. Random effects included replicate, while fixed effects included dietary treatments and experiment.

[2]Different letters within the same row differ at $P < 0.05$.

Table 6 Effect of experiment on growth performance parameters at various time points.

	Experiment[1,2]			SEM	*P*-value			
	1	2	3		Experiment (Exp)	Time	Exp × Time	Exp × Treatment
Body weight (kg)								
Day 0	6.68	6.58	6.20					
Day 10	8.37	8.04	7.54	0.52	$P < 0.05$	$P < 0.05$	0.84	0.84
Day 21	13.75	12.92	12.35					
Day 42	27.07	26.74	25.32					
Overall	16.40[a]	15.90[a]	15.07[b]					
Average daily gain (kg)								
Day 10	0.17	0.14	0.13					
Day 21	0.49	0.44	0.44	0.13	$P < 0.05$	$P < 0.05$	0.06	0.20
Day 42	0.63	0.67	0.62					
Overall	0.43[a]	0.41[ab]	0.40[b]					
Average daily feed intake (kg)								
Day 10	0.18	0.17	0.16					
Day 21	0.55	0.50	0.51	0.02	0.67	$P < 0.05$	0.17	0.08
Day 42	0.90	0.92	0.93					
Overall	0.54	0.53	0.54					
Gain:Feed								
Day 10	0.91	0.85	0.82					
Day 21	0.89	0.89	0.86	0.01	$P < 0.05$	$P < 0.05$	0.10	0.10
Day 42	0.71	0.72	0.66					
Overall	0.84[a]	0.82[a]	0.78[b]					

[1] Data are presented as least squared means. Experimental data were analyzed as a randomized complete block design using the GLIMMIX procedure of SAS with time as repeated measurements with autoregressive 1 variance structure. Degrees of freedom were calculated using Kenward-Roger method. Random effects included replicate, while fixed effects included dietary treatments and experiment

[2]Different letters within the same row differ at $P < 0.05$.

Table 7 Interactive effects of dietary antibiotic supplementation, experiment, and time point on growth performance measures.

Experiment x Time x Treatment interaction[1,2]											
Experiment	1		2		3		SEM	*P*- value			
Antibiotic	No	Yes	No	Yes	No	Yes		Treatment (Trt)	Time	Experiment (Exp)	Trt x Time x Exp
Body weight (kg)											
Day 0	6.69	6.68	6.58	6.58	6.19	6.20					
Day 10	8.48	8.27	8.10	7.99	7.48	7.60	0.73	0.20	$P < 0.05$	$P < 0.05$	0.97
Day 21	13.89	13.61	12.78	13.07	12.08	12.61					
Day 42	26.86	27.26	26.22	27.26	24.24	26.4					
Average daily gain (kg)											
Day 10	0.18	0.16	0.15	0.14	0.13	0.14					
Day 21	0.49	0.49	0.42	0.46	0.42	0.46	0.02	$P < 0.05$	$P < 0.05$	$P < 0.05$	0.93
Day 42	0.62	0.65	0.64	0.68	0.58	0.65					
Average daily feed intake (kg)											
Day 10	0.19	0.18	0.17	0.17	0.16	0.17					
Day 21	0.56	0.54	0.49	0.51	0.49	0.53	0.03	$P < 0.05$	$P < 0.05$	0.67	0.71
Day 42	0.89	0.90	0.90	0.95	0.87	1.00					
Gain:Feed											
Day 10	0.94[a]	0.88[b]	0.87	0.83	0.80	0.84					
Day 21	0.88	0.91	0.87	0.90	0.86	0.85	0.02	0.98	$P < 0.05$	$P < 0.05$	$P < 0.05$
Day 42	0.69	0.72	0.72	0.72	0.67	0.66					

[1] Data are presented as least squared means. Experimental data were analyzed as a randomized complete block design using the GLIMMIX procedure of SAS with time as repeated measures with autoregressive 1 variance structure. Degrees of freedom were calculated using Kenward-Roger method. Random effects included replicate, while fixed effects included dietary treatments and experiments

Table 8 Effect of dietary antibiotic supplementation on serum amino acid concentrations.

Experiment	1		2		3		Combined		*P*-value[1]
Free amino acids (mg/g)	Antibiotic	Negative control	Antibiotic	Negative control	Antibiotic	Negative control	Antibiotic	Negative control	
Alanine	0.32	0.31	0.47	0.45	0.35	0.38	0.39	0.38	0.99
Arginine	0.53	0.46	0.60	0.51	0.52	0.46	0.55	0.48*	0.01
Asparagine	0.17	0.15	0.20	0.16	0.18	0.19	0.18	0.17	0.47
Aspartic acid	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.87
Citrulline	0.06	0.04	0.06	0.06	0.07	0.08	0.06	0.06	0.45
Glutamic acid	0.28	0.25	0.27	0.36	0.46	0.43	0.33	0.35	0.73
Glutamine	1.17	1.06	1.30	1.09	1.12	1.09	1.20	1.08	0.15
Glycine	1.14	1.06	1.31	1.20	1.21	1.13	1.22	1.13	0.15
Histidine	0.09	0.07	0.12	0.09	0.10	0.08	0.10	0.08*	0.01
Leucine/Isoleucine	0.36	0.31	0.43	0.39	0.37	0.35	0.39	0.35	0.17
Lysine	0.49	0.42	0.65	0.57	0.62	0.42	0.59	0.47*	0.02
Methionine	0.03	0.03	0.02	0.02	0.02	0.03	0.03	0.03	0.66
Ornithine	0.12	0.10	0.17	0.16	0.17	0.16	0.15	0.14	0.30
Phenylalanine	0.24	0.21	0.30	0.24	0.26	0.21	0.27	0.22*	0.01
Proline	0.58	0.56	0.73	0.69	0.59	0.58	0.64	0.61	0.45
Serine	0.19	0.18	0.33	0.27	0.22	0.20	0.25	0.22	0.23
Taurine	0.06	0.03	0.28	0.16	0.04	0.03	0.13	0.08	0.11
Threonine	0.42	0.51	0.54	0.46	0.34	0.29	0.44	0.42	0.74
Tryptophan	0.13	0.11	0.18	0.16	0.15	0.12	0.15	0.13	0.14
Tyrosine	0.17	0.15	0.17	0.15	0.11	0.11	0.15	0.14	0.46
Valine	0.38	0.33	0.49	0.43	0.38	0.32	0.42	0.36	0.05

[1] *P*-value from Kruskal Wallis one-way analysis of variance comparing all antibiotic treated pigs to all negative control pigs.* Values are significantly different ($P < 0.05$) than the negative control within the same experiment.

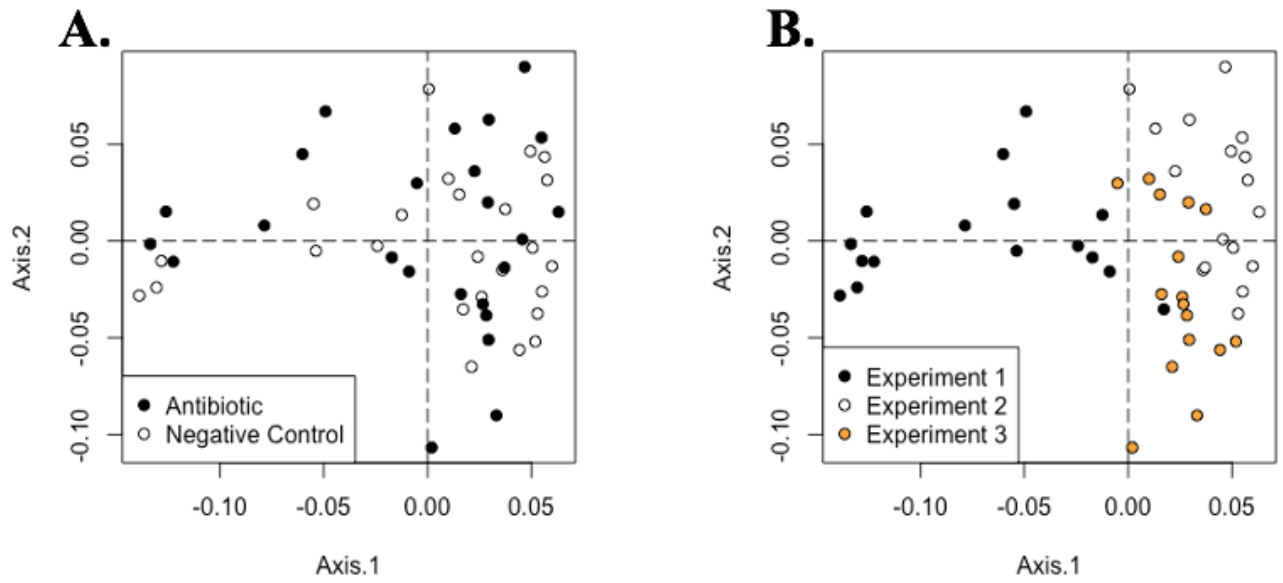


Figure 8 Metabolite analysis of serum samples through weighted Bray-Curtis distance ordination of metabolite beta diversity in serum samples colored by A) treatment and B) experiment. Lack of separation indicates no major difference in the metabolome among dietary treatments (PERMANOVA, $R^2 = 0.03$, $P = 0.19$), but clear separation by experiment (PERMANOVA, $R^2 = 0.26$ $P = 0.01$).

Table 9 Effects of dietary antibiotic supplementation on amino acid, short-chain fatty acid, and bile acid profiles in cecal contents.

Experiment	1		2		3		Combined		
Treatment	Antibiotic	Control	Antibiotic	Control	Antibiotic	Control	Antibiotic	Control	P-value
Amino acids, µg/g									
Alanine	0.64	0.60	0.28	0.30	0.63	0.49	0.51	0.46	0.55
Arginine	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.97
Asparagine	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.29
Aspartic acid	0.32	0.28	0.17	0.23	0.27	0.20	0.25	0.23	0.75
Citrulline	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.32
Glutamic acid	1.18	0.96	0.56	0.68	0.98	0.64	0.89	0.75	0.50
Glutamine	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.83
Glycine	0.12	0.09	0.03	0.04	0.07	0.04	0.07	0.06	0.52
Histidine	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.80
Leucine	0.03	0.04	0.01	0.01	0.03	0.02	0.02	0.03	0.65
Lysine	0.15	0.17	0.06	0.08	0.13	0.09	0.11	0.11	0.87
Methionine	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.31
Ornithine	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.70
Phenylalanine	0.05	0.07	0.02	0.03	0.05	0.04	0.04	0.04	0.78
Proline	0.05	0.06	0.03	0.03	0.04	0.04	0.04	0.04	0.66
Serine	0.02	0.03	0.01	0.01	0.02	0.01	0.01	0.02	0.53
Taurine	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.22
Threonine	0.04	0.04	0.02	0.03	0.03	0.02	0.03	0.03	0.79
Tryptophan	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.90
Tyrosine	0.06	0.10	0.03	0.05	0.03	0.02	0.04	0.06	0.36
Valine	0.04	0.05	0.02	0.02	0.05	0.03	0.03	0.03	0.99
Short chain fatty acids, mg/g									
Acetic acid	32.44	29.60	33.38	34.22	41.22	36.13	35.59	33.35	0.48
Propionic acid	20.97	20.78	19.99	21.88	26.91	23.72	22.52	22.12	0.87

Butyric acid	22.15	17.41	22.23	25.04	28.48	27.28	24.20	23.32	0.72
Valeric acid	6.05	3.67	5.33	7.54	7.69	7.42	6.31	6.26	0.96
Isovaleric acid	57.49	124.64	22.48	66.65	32.77	19.21	36.98	70.02	0.42
Bile acids, µg/g									
Cholic acid	0.10	0.05	0.00	0.05	0.36	0.00	0.15	0.03	0.11
Chenodeoxy cholic acid	19.48	11.88	17.84	7.98	30.03	15.46	22.27	11.62	0.29
Lithocholic acid	111.33	101.16	59.08	138.33	151.86	151.29	105.49	130.58	0.23
Taurocheno deoxycholic acid	0.13	0.19	0.39	0.89	0.00	0.00	0.18	0.38	0.42
Hyodeoxycholic acid	495.28	413.82	393.44	472.09	764.63	697.79	544.81	525.67	0.78

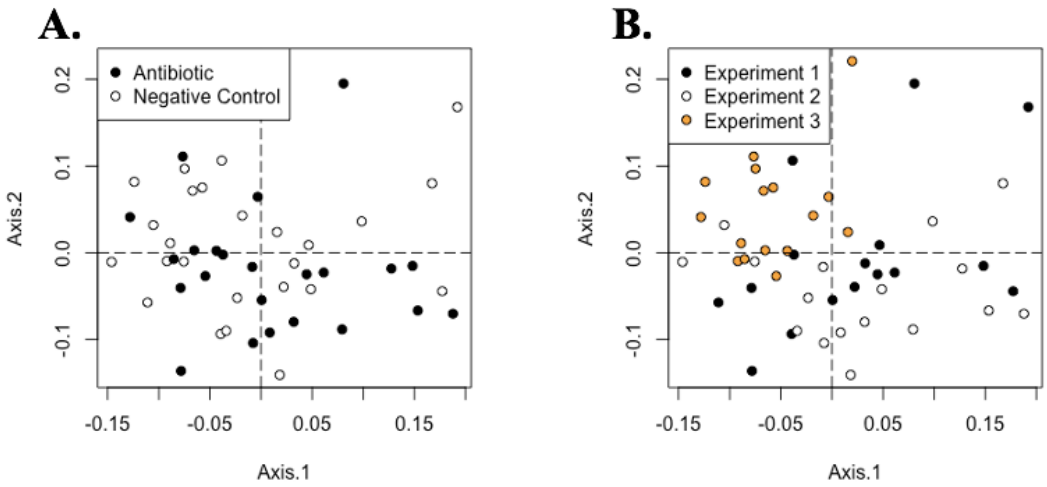


Figure 9 Metabolite analysis of cecal samples through weighted Bray-Curtis distance ordination of metabolite beta diversity in cecal samples colored by A) treatment and B) experiment. Clear separation indicates differences in the cecal metabolome by treatment (PERMANOVA, $R^2 = 0.04$, $P = 0.04$) and by experiment (PERMANOVA, $R^2 = 0.16$, $P = 0.01$).

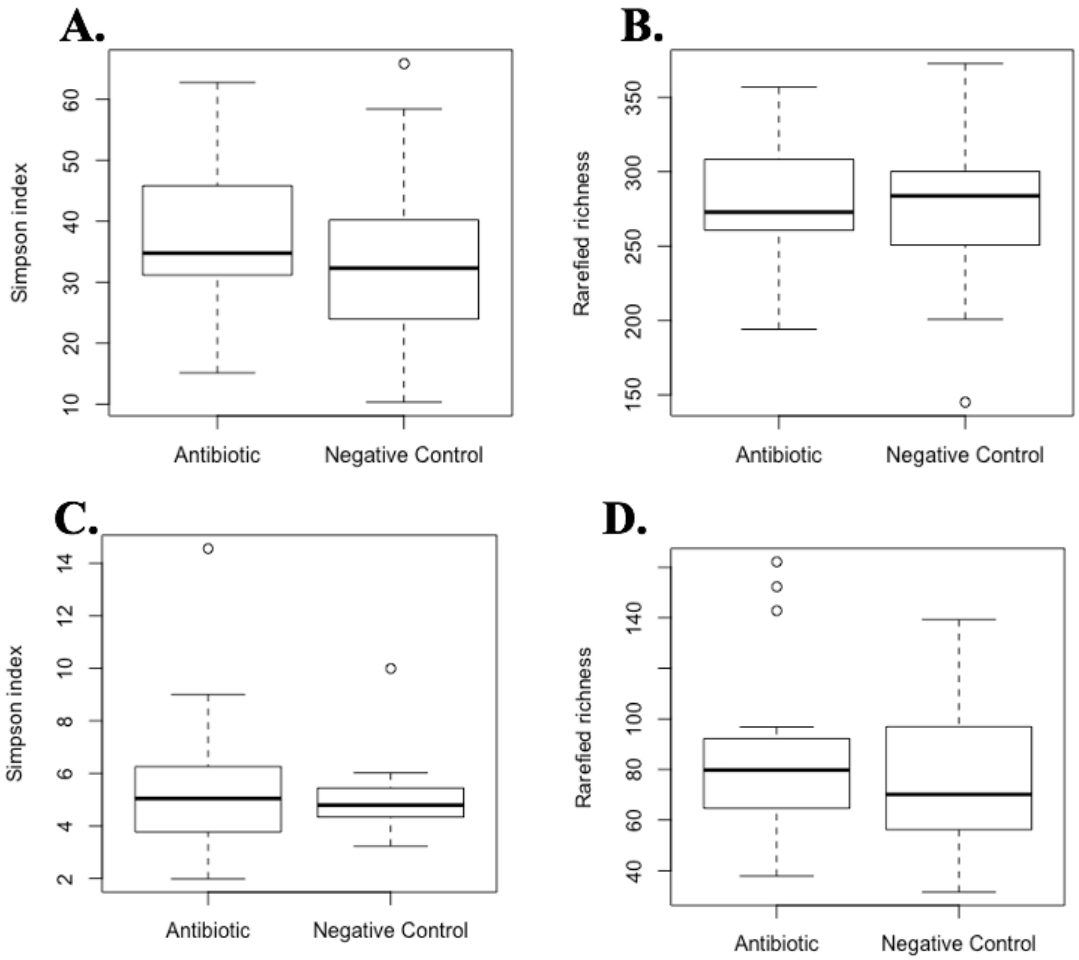


Figure 10 Microbiome analysis of bacterial composition of cecal and ileal samples. Treatments include antibiotic treatment and negative control A) The Simpson alpha diversity index for each treatment in cecal content. There were no significant differences in the Simpson diversity index between treatments (P = 0.16). B**)** The rarefied richness for each treatment in the cecal content. There were no significant differences in the rarefied richness between treatments (P = 0.60). C) The Simpson alpha diversity index for each treatment in the ileal content. There were no significant differences in the rarefied richness between treatments (P = 0.48). D) The rarefied richness for each treatment in the ileal content. There were no significant differences in the rarefied richness between treatments (P = 0.53).

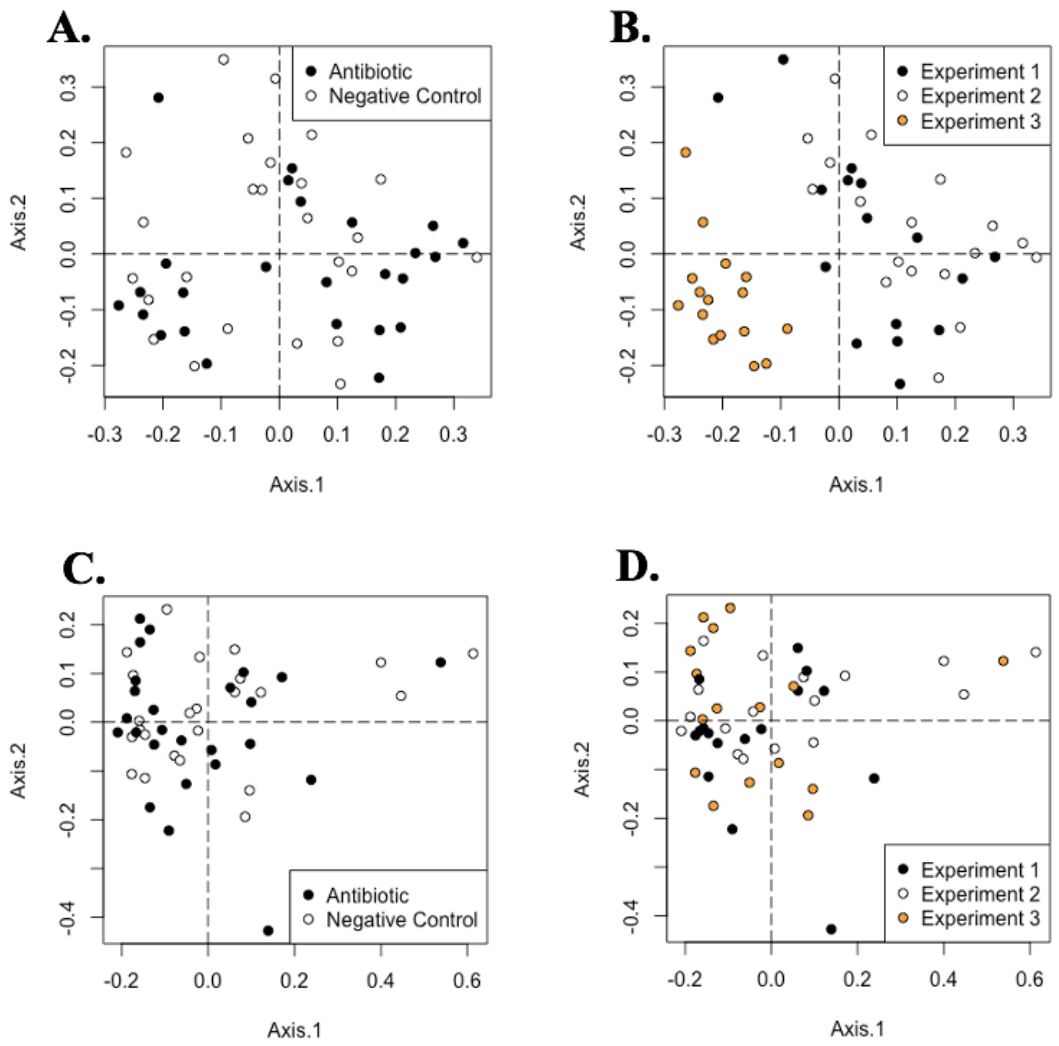


Figure 11 Microbiome analysis of cecal and ileal samples through weighted Bray-Curtis distance ordination of microbiome beta diversity in cecal samples colored by A) treatment and B) experiment. Clear separation indicates differences in the cecal microbiome by experiment (PERMANOVA, $R^2 = 0.18$, $P = 0.01$) but not by treatment (PERMANOVA, $R^2 = 0.02$, $P = 0.19$). The weighted Bray-Curtis distance ordination of microbiome beta diversity in ileal samples colored by C) treatment and D) experiment. Clear separation indicates differences in the ileal microbiome by experiment (PERMANOVA, $R^2 = 0.11$, $P = 0.0$) but not by treatment (PERMANOVA, $R^2 = 0.0$, $P = 0.48$).

Table 10 Cecal and ileal microbial biomarkers for dietary antibiotic treatment and mean relative abundance comparison for each treatment.

Cecal content microbial biomarkers					
Amplicon Sequence Variant[1]	Experiment	Indicator Value	Negative control (mean[2] ± standard deviation)	Antibiotic (mean[2] ± standard deviation)	*P*-value [3]
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Clostridiaceae g__SMB53	2	0.82	0.50 ± 0.47	2.26 ± 1.61	0.01
k__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Streptomycetaceae	3	1.00	0 ± 0	0.03 ± 0.02	0.01
Ileal content microbial biomarkers					
Amplicon Sequence Variant	Experiment	Indicator Value	Negative control (mean[1] ± standard deviation)	Antibiotic (mean[1] ± standard deviation)	*P*-value
k__Bacteria p__Firmicutes c__Bacilli o__Lactobacillales f__Streptococcaceae g__Streptococcus	1	0.85	0.17 ± 0.34	0.98 ± 0.99	0.05
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Peptostreptococcace	2	0.83	0.87 ± 1.06	4.36 ± 4.46	0.05
k__Bacteria p__Firmicutes c__Bacilli o__Lactobacillales f__Leuconostocaceae g__Weissella	2	0.83	0.01 ± 0.04	0.06 ± 0.05	0.01

[1]k represents kingdom, p represents phylum, c represents class, o represents order, f represents family, g represents genus, and s represents species. Bacteria are classified to be as specific as possible with the sequences available.
[2]Means expressed as percentage relative abundance.
[3]*P*-value from Kruskal Wallis one-way analysis of variance.

Table 11 Percentage error and confusion matrix from random forest analysis in cecal microbiome samples.

Confusion matrix by treatment (error: 40%)

		Predicted outcome		
		Negative control	Antibiotic treatment	Total
True outcome	Negative control	9	4	13
	Antibiotic treatment	6	6	12
	Total	15	10	25

Confusion matrix by experiment (error: 28%)

		Predicted outcome			
		Experiment 1	Experiment 2	Experiment 3	Total
True outcome	Experiment 1	8	1	1	10
	Experiment 2	4	4	0	8
	Experiment 3	1	0	6	7
	Total	13	5	7	25

Confusion matrix by barn (error: 8%)

		Predicted outcome		
		Nursery	Wean-Finish	Total
True outcome	Nursery	18	0	18
	Wean-Finish	2	5	7
	Total	20	5	25

Table 12 Cecal and ileal microbial biomarkers barn and mean relative abundance comparison for each location.

Cecal samples[1]					
Amplicon Sequence Variant[2]	Facility	Indicator value	Nursery (mean[3] ± standard deviation)	Wean-Finish (mean[3] ± standard deviation)	*P*-value[4]
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Veillonellaceae g__Acidaminococcus	Nursery	0.93	0.40 ± 0.40	0.01 ± 0.01	0.03
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Veillonellaceae g__Megasphaera	Nursery	0.92	2.72 ± 1.75	0.21 ± 0.34	0.01
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Lachnospiraceae g__Roseburia s__faecis	Nursery	0.91	2.65 ± 2.61	0.23 ± 0.23	0.02
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Peptostreptococcaceae	Nursery	0.86	0.29 ± 0.23	0.03 ± 0.05	0.01
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Clostridiaceae g__Clostridium s__celatum	Wean-finish	0.99	0.01 ± 0.01	0.38 ± 0.36	0.01

k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Clostridiaceae g__Clostridium s__butyricum	Wean-finish	0.96	0.01 ± 0.012	0.15 ± 0.09	0.01
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Lachnospiraceae g__Lachnospira	Wean-finish	0.88	0 ± 0	0.68 ± 0.68	0.01
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Veillonellaceae	Wean-finish	0.88	0 ± 0	0.21 ± 0.19	0.01
k__Bacteria p__Bacteroidetes c__Bacteroidia o__Bacteroidales f__S24-7	Wean-finish	0.88	0 ± 0	0.02 ± 0.02	0.01
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales	Wean-finish	0.88	0 ± 0	0.21 ± 0.26	0.01
k__Bacteria p__Bacteroidetes c__Bacteroidia o__Bacteroidales f__S24-7	Wean-finish	0.86	0.02 ± 0.05	0.88 ± 1.25	0.01
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Peptostreptococcaceae	Wean-finish	0.85	0.02 ± 0.04	0.09 ± 0.06	0.00
k__Bacteria p__Firmicutes	Wean-finish	0.84	0.06 ± 0.05	0.26 ± 0.32	0.01

c__Clostridia o__Clostridiales f__Lachnospiraceae g__Blautia					
k__Bacteria p__Bacteroidetes c__Bacteroidia o__Bacteroidales f__Prevotellaceae g__Prevotella s__stercorea	Wean-finish	0.82	0.05 ± 0.08	0.19 ± 0.19	0.01

Ileal samples[1]

Amplicon Sequence Variant[2]	Facility	Indicator value	Nursery (mean[3] ± standard deviation)	Wean-Finish (mean[3] ± standard deviation)	*P*-value[4]
k__Bacteria p__Firmicutes c__Bacilli o__Lactobacillales f__Lactobacillaceae g__Lactobacillus	Nursery	0.81	11.43 ± 9.49	2.60 ± 3.25	0.02
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Clostridiaceae	Nursery	0.81	0.90 ± 0.73	0.21 ± 0.14	0.02
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Clostridiaceae	Wean-finish	1.00	0 ± 0	0.37 ± 0.08	0.00
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Clostridiaceae g__Clostridium s__celatum	Wean finish	0.99	0.02 ± 0.05	2.08 ± 1.08	0.00

k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Clostridiaceae	Wean-finish	0.97	0.01 ± 0.06	0.42 ± 0.11	0.00
k__Archaea p__Euryarchaeota c__Methanobacteria o__Methanobacteriales f__Methanobacteriaceae g__Methanosphaera	Wean-finish	0.96	0.02 ± 0.04	0.42 ± 0.22	0.00
k__Bacteria p__Actinobacteria c__Coriobacteriia o__Coriobacteriales f__Coriobacteriaceae g__Collinsella s__aerofaciens	Wean-finish	0.90	0.01 ± 0.03	0.12 ± 0.07	0.00
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Ruminococcaceae	Wean-finish	0.89	0.02 ± 0.01	0.05 ± 0.05	0.01
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Clostridiaceae g__Clostridium s__celatum	Wean-finish	0.88	0 ± 0	0.24 ± 0.20	0.00
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Lachnospiraceae g__Coprococcus	Wean-finish	0.82	0.01 ± 0.01	0.04 ± 0.03	0.00
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales	Wean-finish	0.81	0.02 ± 0.02	0.09 ± 0.05	0.00

f__Lachnospiraceae g__Blautia					
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Clostridiaceae	Wean-finish	0.80	0.11 ± 0.30	0.46 ± 0.13	0.01

[1]Facility comparison done only with negative control treatment group.
[2]k represents kingdom, p represents phylum, c represents class, o represents order, f represents family, g represents genus, and s represents species. Bacteria are classified to be as specific as possible with the sequences available.
[3]Means expressed as percent relative abundance.
[4]*P*-value from Kruskal Wallis one-way analysis of variance.

Chapter 4: Use of metabolomics and microbiome approaches to determine and compare biological responses of antibiotics, phytogenic extracts, volatile & semivolatile milk substances, and yeast-based feed additives in diets fed to weaned pigs

Summary

Determining the mode of action of improved growth and health when feeding various feed additives is essential for their strategic and optimal use. The objective of this study was to evaluate the growth performance, metabolic profiles, and intestinal microbiome composition of nursery pigs fed a variety of feed additives. The feed additives evaluated included: chlortetracycline and sulfamethazine antibiotics (PC), herbal blends (HB1, HB2, HB3), turmeric (TUM), garlic (GAR), bitter orange extract (BOE), sweet orange extract (SOE), volatile and semivolatile milk substances (MS1, MS2), yeast nucleotide products (YN1, YN2) and a yeast cell wall (YCW) product compared with a negative control (NC). Growth performance responses from feeding diets containing these additives were evaluated using three separate experiments involving at least 8 replicates for each treatment. Pigs were individually weighed and feed disappearance was determined on d-10, d-21, and d-42 post-weaning of each experiment, and average daily gain (ADG), average daily feed intake (ADFI), and gain:feed (G:F) conversion were calculated. On d-42, one pig per pen was selected for blood, cecal and ileum content sample collection to assess changes in the metabolome via liquid chromatography-mass spectrometry (LC-MS). The microbial composition of bacterial communities in ileal and cecal contents was determined by extracting DNA and sequencing the V4 region of the 16s rRNA bacterial gene using the MiSeq platform.

Metabolomics and microbiome data were analyzed using multivariate and data mining approaches, and growth performance data were analyzed using a generalized mixed model. The PC was the only treatment group that had improved ($P < 0.05$) ADG and ADFI compared with NC across all experiments. In addition, principal component analysis for both the microbiome and metabolome showed no clear differences among non-antibiotic treatments. Although none of the feed additives affected alpha or beta microbiome diversity, the abundance of specific bacterial taxa increased significantly in the cecal content when compared to the negative control. Supplementing HB1, HB2, HB3, and GAR increased the relative abundance of bacteria in the genus *Lactobacillus* in the cecum. These findings indicate that feeding nursery diets containing these non-antibiotic feed additives had no effect on growth performance and metabolome but increased the relative abundance of a protective bacteria genus in the ileal and cecal microbiome of pigs in a non-disease challenge environment.

Introduction

Many commercial feed additives are marketed with the goal of improving health and growth performance of nursery pigs. Poor growth performance after weaning is often associated with low feed intake and subsequent chronic intestinal disorders, which many of these products are intended to ameliorate (213). Despite the health and growth performance benefits provided by many feed additives, there is limited information on the mechanisms in which they affect biological systems in pigs – including metabolism and the microbiome. This mechanistic information is essential for ensuring that feed

additives are strategically used in situations where they will have a greater chance of consistently providing growth performance benefits.

Although some studies have shown growth performance improvements from feeding herbal blends, phytogenic extracts, yeast-based products, and milk substances; growth responses have been variable (6,7,9,84,91,93,214,215). Herbal blends are botanical products that consist of multiple types of herbal plants, while phytogenic extracts are products from single plants, spices, and fruits (9). Both herbal blends and phytogenic extracts have antimicrobial, anti-inflammatory, and antioxidant capabilities that are mostly attributed to phenolic compounds, flavonoids, and carotenoids (9,189,215–217).

Variable growth responses have also been observed when feeding yeast products. Yeast products have been shown to improve average daily gain (ADG) and gain:feed (G:F) in nursery pigs, but the magnitude of response is variable (8,110,114). Differences in relative growth responses of pigs fed these yeast products may be due to a variety of yeast characteristics including prebiotic and probiotic effects (105,218), mannan oligosaccharide concentration, (108,118,119) or nucleotide concentrations (109,219,220). Unfortunately, little is known about how types of diets, animal health, and production conditions may impact the efficacy of these products.

Milk substances are produced during milk fermentation and commonly include butyric and acetic acid (221). Although it is unclear if these milk substances improve the palatability of diets to improve feed intake in nursery pigs, previous research has shown that adding organic acids alone, including butyric and acetic acid, to diets improves growth performance, nutrient digestibility, and reduces intestinal pathogen infections

(98,222,223). Butyric acid plays a key role in providing energy to the intestinal epithelium, which is important during recovery from intestinal damage during weaning (191,224). Despite these potential advantages, the mechanisms of action when feeding milk substances or their impact on growth performance have not been elucidated, making it difficult to justify their inclusion in commercial swine diets.

The variation in growth responses when supplementing herbal blends, phytogenic extracts, milk substances, and yeast products in weaned pig diets is potentially due to differences in active compounds and their concentrations in these products. However, our limited knowledge of the mechanisms of responses makes it difficult to determine which of these active compounds are the most beneficial and how they can be used to reliably improve growth. By evaluating the microbiome and metabolism of pigs, patterns can be potentially identified that may help explain the animal's response when these additives are included in diets. For this reason, the objective of this experiment was to evaluate the overall impact of supplementing herbal blends, phytogenic extracts, milk substances, or yeast products on the growth performance, microbiome, and metabolome of nursery pigs. Our hypothesis was that the feed additives will improve growth performance of nursery pigs through modulation of the intestinal microbiome and metabolism of the pigs.

Materials and Methods

The feeding experiments and sample collection were conducted at the Purina Animal Nutrition Research Farm (Gray Summit, MO, USA) and animal care protocols were approved and supervised by Purina Animal Nutrition. All other metabolomics and

microbiome analysis and procedures were performed at the University of Minnesota (St. Paul, MN, USA).

Animals, housing, and experimental design

Three separate animal feeding experiments (exp) were conducted, where exp 1 and 2 were performed in the same nursery facility, and exp 3 was conducted in a wean-to-finish facility. The nursery facility was environmentally controlled and individual pens included plastic flooring, five-hole plastic feeders, and nipple waterers. Similarly, individual pens in the wean-to-finish facility contained slatted (concrete) flooring, metal feeders, and cup waterers. Room temperature in both facilities was maintained at about 30°C on the first week and decreased 1.8°C per week during the 6-wk nursery feeding period.

The minimum number of replicates was determined by calculating the necessary sample size (n) to achieve statistical significance at $P \leq 0.05$. Data (mean and standard deviation) from previous studies of microbiota and metabolomic analysis were used as input to G*Power 3.1 (Kiel University, Germany) to determine that 8 replicates per dietary treatment were required to detect differences with a power of 0.8.

Experiment 1, 2, and 3 utilized 504, 516, and 520 newly weaned pigs, respectively. All nursery pigs were from the same genetic line (PIC Camborough × PIC 337; Hendersonville, TN, USA), and pigs in all 3 exp were weaned at 20-d of age and had an average initial body weight (BW) of 6.5 kg. Pigs were blocked by initial BW and gender and blocks were randomly allotted to treatments to provide at least 8 pens/treatment. In exp 1 and 2, pigs were placed in pens with a stocking density of 8 to 9 pigs/pen (0.36 to 0.41 m^2/pig), while 12 to 14 pigs per pen (0.58 to 0.63 m^2/pig) were used in exp 3.

All pigs were vaccinated for *Streptococcus suis* and *Mycoplasma hyorhinis* (Autogenous Bacterin, Philbro Animal Health, Teaneck, NJ) at 5 to 7-d of age (booster at weaning), for *Haemophilus parasuis* (ParaSail, Newport Laboratories, Worthington, MN) and *Salmonella typhimurium* (Enterisol-Salmonella T/C; BI, St. Joseph, MO) 7-d prior to weaning, and Circovirus Type 2 (Fostera PCV Chimera, Zoetis, Charles City, IA) at weaning. Throughout all three exps, pigs were monitored daily for health status, but no health concerns were observed that required medication administration.

Dietary treatments

A 3-phase feeding program was used that consisted of feeding phase 1 diets from weaning (d 0) to d 10 post-weaning; phase 2 diets from d 10 to d 21 post-weaning; and phase 3 diets from d 21 to d 42 post-weaning. All diets were formulated to meet or exceed the NRC (2012) recommendations for energy and nutrient requirements of nursery pigs and were manufactured in pelleted form at the Purina Research Manufacturing Unit. Pens were assigned randomly to dietary treatments. The positive control (PC) was medicated with Aureomix 10-10S (Zoetis, Charles City, IA) at 0.5% of the diet to provide 0.01% chlortetracycline and 0.01% sulfamethazine. Herbal blend products HB1, HB2, and HB3, were different proprietary herbal blends that were recommended to be added to diets at a rate of 0.03%, 0.1%, and 0.02%, respectively, by the manufacturers. Phytogenic extract dietary treatments included 0.01% turmeric (TUM), 0.015% garlic (GAR), 0.03% bitter orange extract (BOE), and 0.037% to 0.018% sweet orange extract (SOE) depending on dietary phase. Proprietary volatile and semi-volatile milk substances (0.05% MS1 and 0.03% MS2) were added to diets based on

manufacturers recommendations. Yeast-derived additives including yeast nucleotide products (YN1 and YN2) and a yeast cell wall product (YCW) were added to diets at inclusion rates of 0.1% to 0.05%, depending on the dietary phase. All pigs were provided *ad libitum* access to feed and water throughout each exp.

Chemometric analysis of additives

The feed additives were analyzed for chemical composition in original and microwave digested forms. Chemometric analysis of the digested forms involved hydrolyzing each additive using HCl followed by microwave digestion. The hydrolysates were dried with N_2 and reconstituted using 50% acetonitrile. Chemometric analysis of the original forms of these additives involved extraction using 50% acetonitrile followed by analyzing these extracts using three different LC-MS methods: positive mode, negative mode, and dansylation. Data were then subjected to a multivariate analysis, and the major chemical components found in each additive were identified using the Metlin (metlin.scripps.edu) database search.

Growth performance data analysis

Growth performance data were analyzed for overall structure, absence of outliers, and normal distribution using the UNIVARIATE procedure of SAS (SAS Institute, Cary, NC). Pen was considered the experimental unit for body weight (BW), ADG, average daily feed intake (ADFI), and G:F. Experimental data were analyzed as a randomized complete block design using the GLIMMIX procedure of SAS with time as a repeated measures and Autoregressive 1 variance structure. Degrees of freedom were calculated

using Kenward-Roger method. Random effects included replicate, while fixed effects included all dietary treatments. Significant differences were declared at $P \leq 0.05$ and trends at $P \leq 0.10$.

Sample collection

For metabolomic and microbiome analysis, one pig with BW closest to the median BW in the pen was selected for blood and ileum and cecal content collection. Blood samples were collected via venipuncture of the jugular vein using Vacutainer® tubes (BD, Franklin Lakes, NJ, USA) and then centrifuged at 2,000 × *g* for 15 min at 4ºC. Serum was then aliquoted and stored at -80ºC. To collect digesta samples, pigs were euthanized using CO_2 gas and exsanguination. The entire intestinal tract was removed and placed on a sterile surface and sterilized utensils were used to collect intestinal content. Approximately 1.5 mL of cecal contents were collected from the lateral side of the cecum, and 1.5 mL of ileum contents were collected 30 cm proximal to the ileocecal junction. Each sample was then snap frozen in liquid nitrogen and stored at -80ºC.

Metabolomics

Liquid chromatography–mass spectrometry (LC-MS)-based metabolomic analysis involved several steps including sample preparation, chemical derivatization, LC-MS analysis, data deconvolution and processing; multivariate data analysis, and marker characterization and quantification (195). Deproteinization of serum was conducted by mixing one volume of serum with 19 volumes of 66% aqueous acetonitrile (ACN) followed by centrifugation at 18,000 × *g* for 10 min at room temperature. Cecal content

samples were mixed with 50% aqueous ACN containing 5 μM glycocholic acid-$^{13}C_1$ in 1:10 (w/v) ratio. Samples were sonicated for 10 min followed by mixing using a vortex mixer. After mixing, they were centrifuged at 18,000 × *g* for 10 min to obtain cecal sample extracts.

For detection of metabolites containing amino groups in their structure, samples were derivatized with dansyl chloride (DC) prior to the LC-MS analysis. Briefly, 5 μL of samples or standards were mixed with 5 μL of 100 μM *p*-chlorophenylalanine (internal standard), 50 μL of 10 mM sodium carbonate, and 100 μL of DC (3 mg/mL in acetone). The mixture was incubated at 25°C for 15 min and centrifuged (18,000 × *g*) for 10 min, the supernatant was transferred into a high-performance liquid chromatography vial for LC-MS analysis.

Samples were derivatized with 2-hydrazinoquinoline (HQ) prior to the LC-MS analysis for identification of carboxylic acids, aldehydes, and ketones species. Briefly, 2 μL of sample were added into 100 μL of freshly prepared acetonitrile solution containing 1 mM 2,2′-dipyridyl disulfide (DPDS), 1 mM triphenylphosphine (TPP) and 1 mM HQ. The reaction mixture was incubated at 60°C for 30 min, chilled on ice and then mixed with 100 μL of ice-cold H_2O. After centrifugation at 21,000 × *g* for 10 min, the supernatant was transferred into a HPLC vial for LC-MS analysis.

A 5 μl aliquot of sample prepared from serum or cecal fluid was injected into an Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA) and metabolites were separated by a gradient of mobile phase ranging from water to 95% aqueous acetonitrile containing 0.1% formic acid over a 10 min run. The LC eluant was introduced into a Xevo-G2-S quadrupole time of flight mass spectrometry

(QTOFMS, Waters) for mass measurement and ion counting. Capillary voltage and cone voltage for electrospray ionization was maintained at 3 kV and 30 V for positive-mode detection, or at -3 kV and -35 V for negative-mode detection, respectively. Source temperature and desolvation temperature were set at 120°C and 350°C, respectively. Nitrogen was used as a cone gas (50 L/h) and desolvation gas (600 L/h), and argon was used as a collision gas. For accurate mass measurement, the mass spectrometer was calibrated with sodium formate solution (range *m/z* 50–1,000) and monitored by the intermittent injection of the lock mass ($[M+H]^+$ = *m/z* 556.2771 and ($[M+H]^-$ = *m/z* 554.2615) in real time. Mass chromatograms and mass spectral data were acquired and processed using MassLynx software (Waters, Milford, MA, USA) in centroided format.

After data acquisition in the UPLC-QTOFMS system, chromatographic and spectral data of samples were deconvoluted by MarkerLynx™ software (Waters, Milford, MA, USA). A multivariate data matrix containing information on sample identity, ion identity (RT and *m/z*) and ion abundance was generated through centroiding, deisotoping, filtering, peak recognition, and integration. The intensity of each ion was calculated by normalizing the single ion counts (SIC) *versus* the total ion counts (TIC) in the whole chromatogram.

Individual metabolite concentrations were calculated using the ratio between the peak area of metabolite and the peak area of internal standard and fitting with a standard curve using QuanLynx software (Waters Corp, Milford, MA, USA). These concentrations were then compared across treatments using the aov function in the statistical analysis package Rstudio (225). Pairwise comparisons with the negative control were completed using a Wilcoxon test. For analysis of the untargeted metabolites, the processed data matrix was

exported into Rstudio software (Boston, MA, USA) and then analyzed by unsupervised principal components analysis (PCA). The vegan package was used to determine the weighted Bray-Curtis matrix (196). The separation between treatment groups was quantified using a PERMANOVA value calculated using the adonis function from the vegan package in Rstudio (168). Finally, metabolite indicator values were calculated using the labdsv package in Rstudio (226). The indicator value is the product of frequency and abundance of a given metabolite across all samples belonging to a specific treatment. The closer to 1 the indicator value is, means that the metabolite is present in multiple samples of that treatment and in high abundance compared to other treatment groups. A high indicator value is generally greater than 0.8. Only high indicator values were included in the results. The chemical identities of interested compounds were determined by accurate mass measurement, elemental composition analysis, and then a database search using Metlin search engine (metlin.scripps.edu).

Microbiome

All cecal (n = 150) and ileum (n = 150) samples were submitted to the University of Minnesota Genomics Center for DNA extraction and sequencing of the 16s V4 region. The extractions were preformed using a DNeasy PowerSoil DNA extraction kit (Qiagen, Hilden, Germany) using manufacturers procedures. The DNA samples were then submitted to the University of Minnesota Genomics Center where they were analyzed using their standardized methods. Library prep was completed using a previously described dual-indexing method (198). Marker gene sequencing was then completed

using the Illumina MiSeq Next Generation platform with a targeted average sequencing depth of 100,000 reads per sample.

Amplicon Sequence Variants (ASV) were identified from raw Illumina sequence reads using cutadapt, fastx, and qiime2 bioinformatics tools (199–201). The adapter sequences, barcodes and primers were removed from the pair-end reads and low-quality reads were discarded. Primers were removed using the default parameters of the cutadapt program. Empty lines were then removed from the output, sequences were filtered using a quality score of 50, and all other parameters were set to default settings. The paired ends were then merged using bbmap merger and singletons were discarded (202). Data were then imported into Qiime 2, where sequences were demultiplexed and processed through the dada2 pipeline to identify ASVs. Taxonomic analysis of the ASVs obtained was then completed using a pre-trained classifier. After data processing, 72% of the reads were retained from the raw reads.

After obtaining the ASV table, data were transferred into R studio where the rarefied richness and Simpson alpha diversity were determined using the vegan package. In addition, the vegan package was also used to determine the weighted Bray-Curtis matrix. This matrix was used to calculate beta diversity between treatments. Finally, species indicator values were calculated using the labdsv package and only indicator values greater than 0.8 are reported in this manuscript.

Results

Chemical composition of feed additives

Based on chemometrics analysis of the products evaluated in exp 1, all the herbal blends HB1, HB2, and HB3 had relatively high concentrations of choline, glycine and pipecolic acid. The herbal blend HB1 had a relatively high amount of piperine, whereas HB2 contained curcumin. Curcumin was also identified as a unique component in the phytogenic extract TUM, while GAR contained ibervirin. The phytogenic extracts BOE and SOE were distinguished from other products due to greater relative abundance of flavonoids compared with the other products evaluated. Quinic acid was a notable compound in the MS2 product, while the yeast products were characterized by greater relative abundance of free amino acids and dipeptides compared with the other feed additives evaluated (Table 14).

Growth performance responses

Exp. 1

There was an interaction ($P < 0.05$) between time and treatment for G:F, indicating the products (HB1, HB2, HB3, TUM, GAR) had a variable effect on gain efficiency depending on the age of the pigs (Table 15). Gain:feed was lower ($P < 0.05$) for pigs in the PC group during the first 10-d postweaning period compared with the those fed NC, HB1, TUM, and GAR treatments. In phase 2 (d 10 to 21), pigs fed PC had greater ($P < 0.05$) G:F than pigs fed HB1.

Exp. 2

There were no differences between the products (BOE, SOE, MS1, MS2) in BW, ADG, ADFI, or G:F during the first 10-d feeding period (Table 16) . However, during

phase 2 (d 10 to 21), pigs fed PC had greater ($P < 0.05$) BW than those fed SOE, and also had greater ($P < 0.05$) ADG than pigs fed NC, BOE, SOE, and MS2 (Table 16). Pigs fed PC diets also had a greater ($P < 0.05$) ADFI than pigs fed SOE and MS2 from d 10 – d 22. This pattern continued through the final period (d 22 – d 42), where the PC group had a greater ($P < 0.05$) BW than pigs fed NC, BOE, SOE, and MS2.

Exp. 3

Pigs fed PC were heavier ($P < 0.05$) at 42-d compared with those fed NC, YN1, YN2, and YCW (Table 17). However, there were no differences in ADG or ADFI among dietary treatments during the entire 42-d feeding period. Pigs fed PC had greater ($P < 0.05$) G:F compared with pigs fed YN2 only during the d 0 to d 10 feeding period. None of the yeast products evaluated affected growth performance of weaned pigs compared with feeding the NC diets.

Targeted metabolomics

Serum

In the analysis of the concentration of targeted metabolites in the serum metabolome, there were no differences in circulating amino acids for any of the dietary treatments in exp 1 (Table 18) and 2 (Table 19). In exp 3, pigs in the PC group had greater ($P < 0.05$) serum lysine concentration compared with pigs fed the NC and yeast products (Table 20). The concentration of serine was greater ($P < 0.05$) in pigs fed YCW compared with those fed NC (Table 20). Serum metabolite biomarkers were identified for

each treatment, and the only biomarker with high indicator value identified was sulfadimidine, an antibiotic metabolite, in pigs fed PC diets (Table 21).

Cecal contents

Metabolite biomarkers in the cecal contents were identified for each treatment. Similar to the findings for the serum metabolites, antibiotic metabolites including sulfadimidine and chlortetracycline were identified in the PC samples of cecal contents (Table 22). In addition, metabutamine was identified as a biomarker for HB2, and butyramide was detected as a biomarker for HB3. Ibervirin was identified as biomarker when feeding GAR (Table 22), which was also identified as a component of the GAR product from the chemometrics results. The YN2 was the only yeast product treatment that was associated with a biomarker with high indicator value (phloionolic acid).

Microbiome

Despite the addition of antibiotics and various feed additives to nursery pig diets, there were no differences among treatments in either the Simpson alpha diversity index, rarefied richness, or beta diversity in the cecal content samples or ileal content samples in any of the experiments (Figure 12, Figure 13, Figure 14, Figure 15). However, some specific microbial biomarkers were identified in the cecal contents for bacterial strains that were affected by the dietary treatments. Feeding HB1, HB2, HB3, and GAR increased the relative abundance of *Lactobacillaceae Lactobacillus* compared with pigs fed NC (Table 23). Feeding diets containing YN1 increased the relative abundance of *Veillonellaceae Megasphaera* and *Prevotella Stercorea* compared with pigs fed NC

(Table 23). In the same exp (exp 3), *Actinomycetales Streptomycetaceae* was identified as a good biomarker for PC because it was only present in the intestinal tract when the antibiotics were fed to pigs (Table 23).

Discussion

Active compounds in feed additives

Based on the chemometric analysis, HB1 was unique from the other herbal blends because of its high concentrations of the black pepper alkaloid, piperine. Although research is limited, feed additives containing piperine have been shown to improve ADG and ADFI in nursery pigs (93). The herbal blend HB2 and phytogenic extract TUM contained relatively high concentrations of curcumin. Curcumin is a polyphenolic compound typically found in the spice turmeric (227). Turmeric has been reported to provide anti-oxidant, anti-inflammatory, and anti-microbial effects in rodent models (227–229). In addition, previous feeding trials have demonstrated improved growth of nursery pigs when fed turmeric powder, but it is unclear of this effect was due to curcumin alone or the other plant substances in turmeric including tumerones, sesquiterpenes, stigmasterole, β-sitosterole, and cholesteroland (230,231). All three herbal blends (HB1, HB2, and HB3) had relatively high concentrations of quinic acid, which has been shown to increase tryptophan and nicotinamide production in the gastrointestinal tract and enhance DNA repair and immune function via NF-kB inhibition (232). However, we did not observe any differences in tryptophan concentrations in the cecal contents in our study, even when herbal blends were included in the diet. The herbal blend HB3 was unique compared with other herbal blends because of its relatively high

concentrations of betaine, which is a derivative of glycine and functions as a methyl group donor as well as having osmotic properties that may be beneficial to the intestinal epithelium of pigs (233). When betaine was added at a rate of 0.15% in the diet, ADG has been shown to increase up to 15% in pigs (233). Despite these potential advantages, the limited amount of betaine in HB3 and the relatively low (0.02%) diet inclusion rate, there was not enough betaine in the diet to elicit an improvement in growth performance. It is also worth noting that betaine is an oxidized form of choline, which was present in herbal blends but not phytogenic extracts, milk substances, or yeast products. Previous research has shown that unabsorbed choline can be metabolized by intestinal microbes to form trimethylamine (TMA), which will eventually be converted to trimethylamine N-oxide (TMAO) in the liver (132). One study has shown that TMAO improves ADG and G:F when supplemented to diets of finishing pigs (133). However, it is unknown if the amount of choline present in these products is enough to produce the same concentrations of TMAO that improved growth performance in this previous study.

Bitter orange extract and sweet orange extract were similar in chemical composition and contained relatively high concentrations of flavonoids including equol, nobiletin, and tangeritin. Flavonoids may be beneficial in the swine diets due to their anti-oxidant, anti-inflammatory, anti-microbial, and anti-carcinogenic properties that may improve growth performance (234–236). In a previous study, feeding diets containing 0.1% to 0.2% citrus plant extracts to nursery pigs resulted in greater ADG compared with pigs fed the negative control diets (214). In a separate study, the addition of Chinese herbal medicine blends, that contained high concentrations of flavonoids and polyphenols, were fed to nursery pigs at an inclusion rate 0.3% of the diet and had no

effect on ADG or F:G but increased feed intake compared with pigs fed the negative control diets (237). The variation in growth performance results reported in the literature may be explained by differences in the concentration or chemical form of other bioactive molecules included in the feed additives. In our study, both SOE and BOE were included in the diet at a concentration below 0.04%, which is less than doses of 0.1% to 0.3% that resulted in growth improvements in other experiments (214,237).

In exp 2, we evaluated milk substances fed to nursery pigs. Unfortunately, the methods used for the chemometrics analysis did not result in identification of any unique compounds in these products, making it difficult to determine if they contained any potentially beneficial compounds that may promote growth in pigs.

The yeast-based products evaluated using chemometrics analysis contained significant concentrations of free amino acids and dipeptides but no other unique chemical compounds. However, the methods used for characterizing the compounds present in yeast were not appropriate for measuring nucleotides, mannan oligosaccharides, and β-glucans which are known to be present in yeast cell walls (7).

Growth performance responses

There were no differences in growth performance between non-antibiotic dietary treatments and the negative control for each experiment. The lack of growth performance improvements from feeding these additives may have been due to the high health of pigs and hygiene conditions of facilities used during these experiments. Several studies have shown that growth improvements from feeding diets containing antibiotic growth promoters (AGP) are greater on commercial farms than in university or research settings

(2). These differences in growth performance responses have been attributed to cleaner facilities, less stress, and less disease pressure on research farms compared to commercial farms (2). When taking into consideration there was no mortality observed in any of the three exp in this study, it is possible that the additives simply provided minimal benefits under near optimal health, environment, and nutrition provided.

Serum metabolite responses

Higher concentrations of lysine were observed in serum of pigs fed PC compared with those fed other dietary treatments (YN1, YN2, and YCW) in exp 3 only. Previously studies have suggested that a change in serum lysine concentration can be directly related to dietary supply of lysine, and it is likely that the increased lysine concentration may be a result of increased feed intake observed in exp 3 (205). In this study, feeding AB increased ADFI by 6.7%, while serum lysine concentration increased by 25%. However, serum lysine concentration is not a measure of absorption, and it must also be considered that lysine catabolism is slower than that of other essential amino acids (206). A slower rate of catabolism may explain the magnitude of change in serum lysine being greater than the increase resulting from increased feed intake.

Other than serum lysine concentrations, antibiotic metabolites were the only other metabolites identified as biomarkers from feeding the PC diets. Despite the potential for feeding diets containing HB1, HB2, HB3, TUM, GAR, SOE, and BOE to reduce oxidative stress, there were no serum metabolites such as protein carbonyls, malondialdehyde, superoxide dismutase, or glutathione peroxidase that indicated pigs were experiencing oxidative stress (102,238). However, because there was also not a

marked increase in these oxidative stress biomarkers in pigs fed NC diets, it appears that none of the pigs were experiencing oxidative stress throughout the experiments.

Cecal metabolite and microbiome response

When feeding GAR, ibervirin was the only metabolite identified in both the chemometric analysis and as a biomarker for the GAR treatment in the cecal contents, suggesting that ibervirin was not absorbed in the small intestine. Ibervirin is a thiocynate that is used as a biomarker for root vegetable consumption in humans, but there is no information available on its impact on animal health and nutrition (239). However, the minimal effect that GAR had on the microbiome of pigs suggests that ibervirin does not have substantial anti-microbial effects at the dietary concentration that was used in this experiment. None of the other metabolites identified in the chemometrics analysis had high indicator values in any of the other treatments. For example, curcumin was identified as being abundant in TUM, but it did not have a significant indicator value in the cecal contents of the pigs fed this product. Curcumin is poorly digested and if included in the diet, it should be present in the intestinal contents to influence the microbiome (240). It is possible that if TUM was included at a higher dietary inclusion rate, the curcumin may have influenced the cecal microbiome. A previous experiment used a dietary dose of 0.03% curcumin compared with 0.01% turmeric used in our study (241). In the experiment using 0.03% curcumin, the curcumin treatment decreased the concentration of *Escherichia coli* in the ileum and reduced inflammation (241). Because our experiment used both a lower dose and turmeric instead of pure curcumin, it is possible that 0.01% turmeric does not provide enough curcumin to produce a health

response. Differences in diet inclusion rates of active compounds is also a potential explanation for why flavonoids in BOE and SOE, or the quinic acid in PHY06 and MS2, were also not identified in the cecal contents of pigs fed additives containing these compounds and why there was minimal impact on the cecal microbiome.

There were no differences in the rarefied richness or alpha diversity between treatments in either cecal or ileal content samples for any of the three experiments (Figure 4.2, Figure 4.4). Furthermore, there were no differences in the weighted bray-curtis beta-diversity measurements among feed additives for both cecal and ileal contents in all three experiments (Figure 4.1, Figure 4.3). This lack of microbiome response may be explained by the exposure of pigs fed one dietary treatment to pigs fed a different dietary treatment in adjacent pens because barriers between pens consisting of bars with open spaces instead of solid barriers. In poultry, alpha diversity of the cecal microbiome was not different between birds fed negative control diets compared with those fed positive control diets containing medium chain fatty acids when the experiment was conducted in facilities that separated pens with mesh screens (209). However, when the same experiment was conducted in facilities with solid barriers between pens or in isolation facilities, the alpha diversity of the cecal microbiome was significantly decreased by feeding the diet containing medium chain fatty acids (209). These findings suggest the type of pen divisions used in the facilities where these experiments were conducted may have limited our ability to detect differences in microbiome composition due to dietary treatment because pigs had the opportunity to be in contact with pigs in adjacent pens fed other dietary treatments.

Despite a lack of difference in alpha and beta diversity, feed additives did affect specific taxa of bacteria, which has been previously reported where feeding AGPs to pigs caused a change in the microbiome that was driven by *Proteobacteria* and *Escherichia coli* (210). Results from a similar research trial showed that feeding a combination of the antibiotics chlortetracycline, sulfamethazine, and penicillin to pigs resulted in differences in *Lachnobacterium* in the ileum compared with pigs fed positive and negative control diets (211). Overall, these results suggest that including feed additives in nursery pig diets appear to only affect a few select species of microbes compared with a dramatic change on the entire microbial composition.

To help determine the bacterial species that were increased by feed additive supplementation, indicator values were calculated. For the cecal content samples in exp 3, bacteria from the family *Streptomycetaceae* had a high indicator value in pigs fed the positive control diets compared with all other dietary treatments. This family of bacteria is commonly found in soil, but it is also commonly used for the production of commercial antibiotics(242). In addition to changes in bacteria from the family *Streptomycetaceae*, there were also interesting patterns observed with the bacteria from genus *Lactobacillus* when feeding the additives (HB1, HB2, HB3, TUM, GAR) evaluated in exp 1. *Lactobacillus* spp. have been extensively studied and evaluated as probiotics or direct fed microbials in swine diets. Multiple studies have reported that when supplementing various strains of *Lactobacillus spp.* in diets for nursery pigs, growth rate and feed efficiency were improved, along with reduced diarrhea and increased resistance to gastrointestinal pathogens (243). In our study, there was one strain of *Lactobacillus* that had increased concentrations when the phytogenic products were supplemented in the

feed. There was no difference in the concentration of this bacterial strain when the antibiotics were fed, but when HB1, HB3, or GAR were added to the diet, there was an increase in the relative abundance of this *Lactobacillus* bacteria. Although this increase was not associated improved growth performance in this study, it is possible that in a health challenge situation, the increase in *Lactobacillus* may have a protective effect. Adding various *Lactobacillus* strains to swine and poultry diets has been shown to have an antagonistic effect on pathogenic bacteria in the intestine including *Salmonella* (105,244,245). This means that weaned pigs may benefit from the addition of HB1, HB3, or GAR to diets if they encounter a pathogen challenge.

Conclusion

Of the 13 feed additives that were evaluated, none of them were effective for improving growth performance of nursery pigs under the high health and environmental hygiene conditions used in this study. In addition, these feed additive products had a minimal impact on the serum and cecal metabolite profile, and on ileal and cecal microbiome. Despite a minimal overall impact on the microbiome, supplementing the herbal blends HB1, HB3, and the phytogenic extract GAR increased the relative abundance of bacteria in the genus *Lactobacillus*. This increase in *Lactobacillus* could provide a protective effect, indicating these feed additives may be useful antibiotic alternatives in health challenged pigs, but more research is necessary to confirm this hypothesis.

Table 13 Description of dietary treatments.

Additive category	Treatment name	Experiment	Treatment description	Inclusion	Pens/ treatment
Controls	Positive control (PC)	All	Antibiotic (AB; Aureomix S10/10)	0.5%	25
	Negative control (NC)	All	No AB or test products	NA	25
Herbal blends	HB1	Exp 1	NC + Essential oil and herb mixture 1	0.03%	8
	HB2	Exp 1	NC + Essential oil and herb mixture 2	0.1%	8
	HB3	Exp 1	NC + Essential oil and herb mixture 3	0.02%	8
Phytogenic extract	TUM	Exp 1	NC + Turmeric	0.01%	8
	GAR	Exp 1	NC + Garlic	0.015%	8
	BOE	Exp 2	NC + bitter orange extract	0.03%	9
	SOE	Exp 2	NC + sweet orange extract	0.037-0.018%	9
Milk substances	MS1	Exp 2	NC + Volatile Milk Substances	0.05-0.03%	9
	MS2	Exp 2	NC + Volatile & Semi-volatile Milk Substances	0.05-0.03%	9
Yeast products	YN1	Exp 3	NC + Yeast nucleotide 1	0.1-0.05%	8
	YN2	Exp 3	NC + Yeast nucleotide 2	0.1-0.05%	8
	YCW	Exp 3	NC + Yeast cell wall	0.1-0.05%	8

Table 14 Identified chemical composition of antibiotic alternatives.

Product[1]	Herbal blends			Phytogenic extracts				Milk substances		Yeast products		
	HB1	**HB2**	**HB3**	**TUM**	**GAR**	**BOE**	**SOE**	**MS1**	**MS2**	**YN1**	**YN2**	**YCW**
Free amino acids										+	+	+
Choline	+	+	+									
Glycine	+	+	+									
Pipecolic acid	+	+	+									
Dipeptides										+	+	+
Betaine	+	+	+			+						
Flavonoids (Nobiletin, tangeritin, quercetin)						+	+					
Piperine	+											
Quinic acid									+			
Curcumin		+		+								
Ibervirin					+							

[1]Compounds were proprietary botanical herb extracts 1, 2, and 3, phytogenic and herb extracts based on turmeric (TUM), garlic (GAR), bitter orange (BOE), and sweet orange (SOE), volatile milk substances (MS1), and volatile and semi-volatile milk substances (MS2). yeast nucleotide mixtures 1 (YN1), 2 (YN2), and yeast cell wall mixture (YCW). All products curtesy of Purina Animal Nutrition (Gray Summit, MO, USA)

Table 15 Body weight, average daily gain, average daily feed intake, and gain:feed of nursery pigs fed diets with essential oils and phytogenic extracts additives.

Measure	Experiment 1 Dietary Treatments							SEM	*P*-value		
	Positive control	Negative control	HB1	HB2	HB3	TUM	GAR		Trt	Time	Trt × Time
BW, kg											
Day 0	6.69	6.69	6.69	6.69	6.69	6.68	6.70				
Day 10	8.26	8.48	8.58	8.40	8.36	8.50	8.53	1.33	0.641	0.001	0.565
Day 21	13.61	13.89	13.97	13.63	13.66	14.06	14.1				
Day 42	27.29	26.86	26.8	26.56	26.75	27.29	27.66				
ADG, kg											
Days 0-10	0.16	0.18	0.19	0.17	0.17	0.18	0.18	0.02	0.489	0.001	0.617
Days 10-21	0.49	0.49	0.49	0.47	0.48	0.50	0.50				
Days 21-42	0.65	0.62	0.61	0.62	0.62	0.63	0.65				
ADFI, kg											
Days 0-10	0.18	0.19	0.20	0.19	0.18	0.19	0.2	0.04	0.316	0.001	0.712
Days 10-21	0.54	0.56	0.58	0.55	0.55	0.57	0.58				
Days 21-42	0.90	0.89	0.89	0.90	0.91	0.92	0.93				
G:F											
Days 0-10	0.88^{a}	0.94^{b}	0.93^{b}	0.91^{ab}	0.91^{ab}	0.93^{b}	0.95^{b}	0.01	0.828	0.001	0.049
Days 10-21	0.91^{a}	0.88^{ab}	0.84^{b}	0.88^{ab}	0.89^{ab}	0.89^{ab}	0.87^{ab}				
Days 21-42	0.72	0.69	0.69	0.69	0.69	0.69	0.69				

[1]Treatments (Trt) include antibiotic (PC), no antibiotic (NC), essential oil and herb mixture 1 (HB1), essential oil and herb mixture 2 (HB2), essential oil and herb mixture 3 (HB3), turmeric (TUM), and garlic (GAR) All products curtesy of Purina Animal Nutrition (Gray Summit, MO, USA)

[ab]Means within a row with different superscripts differ ($P < 0.05$)

Table 16 Body weight, average daily gain, average daily feed intake, and gain:feed of nursery pigs fed diets supplemented with phytogenic extracts and milk substances.

Measure	Experiment 2 Dietary Treatments						SEM	*P*-value		
	Positive control	Negative control	BOE	SOE	MS1	MS2		Trt	Time	Trt × Time
BW, kg										
Day 0	6.60	6.60	6.61	6.61	6.60	6.61				
Day 10	7.98	8.10	7.96	7.82	7.97	7.92				
Day 21	13.06[a]	12.78[ab]	12.54[ab]	12.15[b]	12.66[ab]	13.06[ab]	1.63	0.038	0.001	0.161
Day 42	27.26[a]	26.22[b]	26.11[b]	24.97[c]	26.54[ab]	25.83[b]				
ADG, kg										
Days 0-10	0.14	0.15	0.14	0.12	0.14	0.13	0.04	0.001	0.001	0.625
Days 10-21	0.46[a]	0.42[b]	0.42[b]	0.40[b]	0.43[ab]	0.42[b]				
Days 21-42	0.68[a]	0.64[bc]	0.65[ab]	0.61[c]	0.66[ab]	0.63[bc]				
ADFI, kg										
Days 0-10	0.17	0.17	0.17	0.16	0.16	0.17	0.05	0.035	0.001	0.456
Days 10-21	0.51[a]	0.49[ab]	0.47[ab]	0.45[b]	0.49[ab]	0.46[b]				
Days 21-42	0.95[a]	0.90[bc]	0.90[ab]	0.85[c]	0.92[abc]	0.90[bc]				
G:F										
Days 0-10	0.83	0.87	0.81	0.77	0.83	0.78	0.02	0.085	0.001	0.318
Days 10-21	0.90	0.87	0.89	0.86	0.87	0.90				
Days 21-42	0.72	0.72	0.72	0.72	0.72	0.71				

[1]Treatments include antibiotic (PC), no antibiotic (NC), bitter orange extract (BOE), sweet orange extract (SOE), volatile milk substances (MS1), and volatile and semi-volatile milk substances (MS2). All products curtesy of Purina Animal Nutrition (Gray Summit, MO, USA)

[abc]Means within a row with different superscripts differ ($P < 0.05$)

Table 17 Body weight, average daily gain, average daily feed intake, and gain:feed of nursery pigs fed diets supplemented with yeast products.

Measure	Experiment 3 Dietary Treatments					SEM	*P*-value		
	Positive Control	Negative Control	YN1	YN2	YCW		Trt	Time	Trt × Time
BW, kg									
Day 0	6.21	6.19	6.20	6.20	6.20				
Day 10	7.60	7.48	7.53	7.41	7.50				
Day 21	12.61	12.08	12.08	12.18	12.00	1.79	0.029	0.001	0.041
Day 42	26.40[a]	24.24[b]	24.56[b]	24.15[b]	24.20[b]				
ADG, kg									
Days 0-10	0.14	0.13	0.13	0.12	0.13	0.05	0.001	0.001	0.460
Days 10-21	0.45[a]	0.42[ab]	0.41[ab]	0.43[ab]	0.41[b]				
Days 21-42	0.65[a]	0.58[b]	0.60[b]	0.57[b]	0.58[b]				
ADFI, kg									
Days 0-10	0.17	0.16	0.16	0.15	0.16	0.06	0.017	0.001	0.219
Days 10-21	0.54	0.48	0.5	0.5	0.49				
Days 21-42	1.00[a]	0.87[b]	0.88[b]	0.90[b]	0.89[b]				
G:F									
Days 0-10	0.84[a]	0.80[ab]	0.82[ab]	0.77[b]	0.79[ab]	0.02	0.054	0.001	0.640
Days 10-21	0.85	0.86	0.83	0.86	0.83				
Days 21-42	0.66	0.67	0.68	0.63	0.65				

[1]Treatments include antibiotic (PC), no antibiotic (NC), yeast nucleotide product 1 (YN1), yeast nucleotide 2 (YN2), and yeast cell wall (YCW) products. All products curtesy of Purina Animal Nutrition (Gray Summit, MO, USA)
[ab]Means within a row with different superscripts differ ($P < 0.05$)

Table 18 Amino acids concentration in the serum of pigs in experiment 1.

Amino acid, mg/g	Experiment 1 Dietary Treatments[1]							*P*-value[2]
	NC	PC	HB1	HB2	HB3	TUM	GAR	
Alanine	0.313	0.324	0.368	0.302	0.283	0.285	0.348	0.466
Arginine	0.458	0.531	0.533	0.413	0.422	0.424	0.456	0.734
Asparagine	0.153	0.170	0.216	0.165	0.141	0.125	0.196	0.610
Aspartic acid	0.005	0.005	0.006	0.006	0.006	0.008	0.007	0.707
Citrulline	0.035	0.062	0.064	0.056	0.044	0.048	0.051	0.504
Glutamic acid	0.253	0.278	0.284	0.272	0.273	0.309	0.351	0.845
Glutamine	1.063	1.166	1.204	1.064	1.012	0.970	1.197	0.909
Glycine	1.059	1.137	1.120	0.958	0.991	0.940	1.062	0.956
Histidine	0.067	0.089	0.085	0.065	0.067	0.061	0.070	0.677
Leucine-Isoleucine	0.313	0.357	0.364	0.290	0.278	0.280	0.340	0.506
Lysine	0.417	0.490	0.524	0.358	0.398	0.412	0.451	0.883
Ornithine	0.098	0.121	0.120	0.102	0.098	0.093	0.114	0.835
Phenylalanine	0.207	0.242	0.238	0.212	0.182	0.177	0.202	0.533
Proline	0.557	0.584	0.621	0.524	0.523	0.489	0.602	0.778
Serine	0.184	0.194	0.238	0.214	0.167	0.161	0.231	0.646
Taurine	0.027	0.063	0.040	0.028	0.045	0.042	0.030	0.267
Threonine	0.511	0.418	0.418	0.349	0.273	0.298	0.403	0.132
Tryptophan	0.108	0.132	0.130	0.094	0.093	0.093	0.099	0.539
Tyrosine	0.149	0.173	0.170	0.136	0.118	0.109	0.149	0.365
Valine	0.328	0.376	0.370	0.288	0.281	0.281	0.331	0.409

[1]Treatments include no antibiotic (NC), antibiotic (PC), essential oil and herb mixture 1 (HB1), essential oil and herb mixture 2 (HB2), essential oil and herb mixture 3 (HB3), turmeric (TUM), and garlic (GAR). All products curtesy of Purina Animal Nutrition (Gray Summit, MO, USA)

[2]*P*-value from Kruskal Wallis one-way analysis of variance

Table 19 Amino acid concentration in the serum of pigs in experiment 2.

Amino acid, mg/g	Experiment 2 Dietary Treatments[1]						*P*-value[2]
	PC	NC	BOE	SOE	MS1	MS2	
Alanine	0.471	0.449	0.429	0.483	0.39	0.417	0.3846
Arginine	0.601	0.509	0.518	0.586	0.493	0.497	0.3404
Asparagine	0.196	0.163	0.184	0.186	0.142	0.154	0.8184
Aspartic acid	0.007	0.007	0.007	0.009	0.006	0.009	0.6069
Citrulline	0.057	0.055	0.057	0.067	0.067	0.061	0.8842
Glutamate	0.267	0.363	0.345	0.362	0.26	0.345	0.4063
Glutamine	1.303	1.094	1.187	1.421*	1.122	1.138	0.0784
Glycine	1.31	1.196	1.318	1.217	1.097	1.263	0.2582
Histidine	0.119	0.087	0.084	0.101	0.089	0.086	0.3695
Leucine-Isoleucine	0.426	0.393	0.37	0.411	0.357	0.385	0.7709
Lysine	0.649	0.566	0.489	0.591	0.426	0.537	0.3009
Methionine	0.022	0.023	0.02	0.032	0.014	0.017	0.3376
Ornithine	0.169	0.158	0.153	0.169	0.135	0.167	0.7637
Phenylalanine	0.294	0.235	0.236	0.281	0.257	0.228	0.5562
Proline	0.731	0.688	0.651	0.72	0.603	0.63	0.2027
Serine	0.333	0.267	0.253	0.336	0.222	0.272	0.3624
Taurine	0.279	0.156	0.147	0.185	0.154	0.202	0.1269
Threonine	0.537	0.458	0.479	0.576	0.416	0.396	0.2239
Tryptophan	0.178	0.159	0.137	0.161	0.14	0.122	0.5144
Tyrosine	0.165	0.146	0.130	0.170	0.138	0.129	0.5619
Valine	0.493	0.426	0.400	0.428	0.369	0.417	0.4316

[1]Treatments include no antibiotic (NC), antibiotic (PC), bitter orange extract (BOE), sweet orange extract (SOE), Volatile Milk Substances (MS1), and Volatile and Semi-volatile Milk Substances (MS2). All products curtesy of Purina Animal Nutrition (Gray Summit, MO, USA)

[2]*P*-value from Kruskal Wallis one-way analysis of variance

* Indicates value different from negative control at $P < 0.05$

Table 20 Amino acid concentration in the serum of pigs in experiment 3.

Amino acid, mg/g	Experiment 3 Dietary Treatments[1]					*P*-value
	NC	PC	YCW	YN1	YN2	
Alanine	0.383	0.349	0.369	0.356	0.395	0.444
Arginine	0.463	0.518	0.438	0.460	0.444	0.144
Asparagine	0.185	0.180	0.177	0.166	0.188	0.993
Aspartic acid	0.009	0.008	0.008	0.007	0.007	0.427
Citrulline	0.083	0.073	0.055*	0.100	0.081	0.118
Glutamic acid	0.428	0.464	0.361	0.348	0.381	0.649
Glutamine	1.094	1.116	0.998	1.006	1.042	0.517
Glycine	1.132	1.205	1.128	1.112	1.233	0.899
Histidine	0.076	0.103	0.084	0.085	0.085	0.580
Leucine-Isoleucine	0.345	0.374	0.342	0.351	0.375	0.735
Lysine	0.417[a]	0.620[b]	0.439[a]	0.482[a]	0.482[a]	0.037
Ornithine	0.158	0.171	0.152	0.163	0.162	0.849
Phenylalanine	0.208	0.255	0.218	0.198	0.198	0.207
Proline	0.581	0.587	0.563	0.548	0.588	0.868
Serine	0.200	0.217	0.258*	0.211	0.251	0.120
Taurine	0.034	0.043	0.024	0.063	0.043	0.210
Threonine	0.287	0.338	0.296	0.333	0.307	0.924
Tryptophan	0.119	0.147	0.119	0.124	0.119	0.548
Tyrosine	0.115	0.105	0.117	0.115	0.117	0.972
Valine	0.319	0.382	0.332	0.368	0.343	0.351

[1]Treatments include no antibiotic (NC), antibiotic (PC), yeast nucleotide product 1 (YN1), yeast nucleotide 2 (YN2), and yeast cell wall (YCW) products. All products curtesy of Purina Animal Nutrition (Gray Summit, MO, USA)

[a,b] Means within a row with different superscripts differ ($P < 0.05$)

* Indicates value different from negative control at $P < 0.05$

Table 21 Serum metabolites that have high abundance and specificity to pigs fed antibiotics, botanical extracts, phytogenic and herb extracts, milk substances, and yeast products compared to the negative control treatment.

Metabolite[1]	Metabolite Identification[2]	Experiment	Treatment[3]	Indicator value[4]	Negative control mean[5] ± standard deviation	Treatment mean[5] ± standard deviation	*P* value[6]
3.31_321.1015 DC		1	PC	0.997	0 ± 0	0.007 ± 0.002	0.001
3.04_279.0921 HQ	Sulfadimidine	1	PC	0.996	0 ± 0.001	0.055 ± 0.021	0.001
3.04_279.0921 HQ	Sulfadimidine	2	PC	0.985	0.001 ± 0.003	0.066 ± 0.017	0.000
7.90_221.0204 HQ		2	BOE	0.875	0 ± 0.001	0.003 ± 0.002	0.002
3.04_279.0921 HQ	Sulfadimidine	3	PC	0.980	0.001 ± 0.003	0.062 ± 0.014	0.001
4.45_264.0803 DC		3	PC	0.875	0.003 ± 0	0.079 ± 0.059	0.003

[1]Metabolite characteristics listed as retention time_mass method of derivatization used [either negative mode (ne), positive mode (po), dansyl chloride (DC), or 2-hydrazinoquinoline (HQ)]
[2]Metabolite identification based on database search of the adjusted mass. Identification was left blank if no metabolite was identified with this mass.
[3]Treatments include antibiotic (PC) and bitter orange extract (BOE) products. All products curtesy of Purina Animal Nutrition (Gray Summit, MO, USA)
[4]The indicator value is a product of frequency and abundance. A good indicator value is generally greater than 0.8.
[5]Means expressed as relative abundance with respect to all metabolites (%)
[6]*P*-value from Kruskal Wallis one-way analysis of variance

Table 22 Cecal metabolites that high abundance and specificity to pigs fed antibiotics, botanical extracts, phytogenic and herb extracts, milk substances, and yeast products compared to the negative control treatment.

Metabolite[1]	Metabolite Identification[2]	Experiment	Treatment[3]	Indicator value[4]	Negative control mean[5] ± standard deviation	Treatment mean[5] ± standard deviation	*P* – value[6]
2.95_279.0918_po	Sulfadimidine	1	PC	1.000	0 ± 0	0.764 ± 0.192	0.001
3.29_321.1024_po	N4-Acetylsulfadimidine	1	PC	1.000	0 ± 0	0.082 ± 0.051	0.001
3.74_479.1220_po	Chlortetracycline	1	PC	1.000	0 ± 0	0.275 ± 0.063	0.001
3.31_479.1221_po	Chlortetracycline	1	PC	1.000	0 ± 0	0.214 ± 0.076	0.001
4.28_479.1227_po	Chlortetracycline	1	PC	1.000	0 ± 0	0.356 ± 0.078	0.001
2.06_237.1603_po	Metabutethamine	1	HB2	0.940	0.004 ± 0.003	0.064 ± 0.071	0.041
5.43_321.1267_dc	Butyramide	1	HB3	0.834	0.033 ± 0.038	0.168 ± 0.071	0.001
6.31_300.1031_dc		1	HB3	0.815	0.034 ± 0.041	0.149 ± 0.067	0.002
1.76_181.9900_ne	Ibervirin	1	GAR	1.000	0 ± 0	0.051 ± 0.017	0.001
2.95_279.0918_po	Sulfadimidine	2	PC	1.000	0 ± 0	0.855 ± 0.169	0.001
3.29_321.1024_po	Acetylsulfadimidine	2	PC	1.000	0 ± 0	0.094 ± 0.04	0.001
3.29_321.1024_po	Acetylsulfadimidine	3	PC	1.000	0 ± 0	0.059 ± 0.026	0.001
3.74_479.1220_po	Chlortetracycline	3	PC	1.000	0 ± 0	0.197 ± 0.066	0.001
3.31_479.1221_po	Chlortetracycline	3	PC	1.000	0 ± 0	0.103 ± 0.029	0.001
4.28_479.1227_po	Chlortetracycline	3	PC	1.000	0.003 ± 0	0.286 ± 0.107	0.001
2.95_279.0918_po	Sulfadimidine	3	PC	0.993	0.003 ± 0.014	0.716 ± 0.17	0.001
4.09_331.2475_ne	Phloionolic acid	3	YN2	0.875	0.011 ± 0	0.048 ± 0.026	0.001

[1]Metabolite characteristics listed as retention time_mass method of derivatization used [either negative mode (ne), positive mode (po), dansyl chloride (DC), or 2-hydrazinoquinoline (HQ)]
[2]Metabolite identification based on database search of the adjusted mass. Identification was left blank if no metabolite was identified with this mass.
[3]Treatments include antibiotic (PC), essential oil and herb mixture 2 (HB2), essential oil and herb mixture 3 (HB3), garlic (GAR), and yeast nucleotide 2 (YN2). All products curtesy of Purina Animal Nutrition (Gray Summit, MO, USA)
[4]The indicator value is a product of frequency and abundance. A good indicator value is greater than 0.8
[5]Means expressed as relative abundance (%)
[6]P-value from Kruskal Wallis one-way analysis of variance

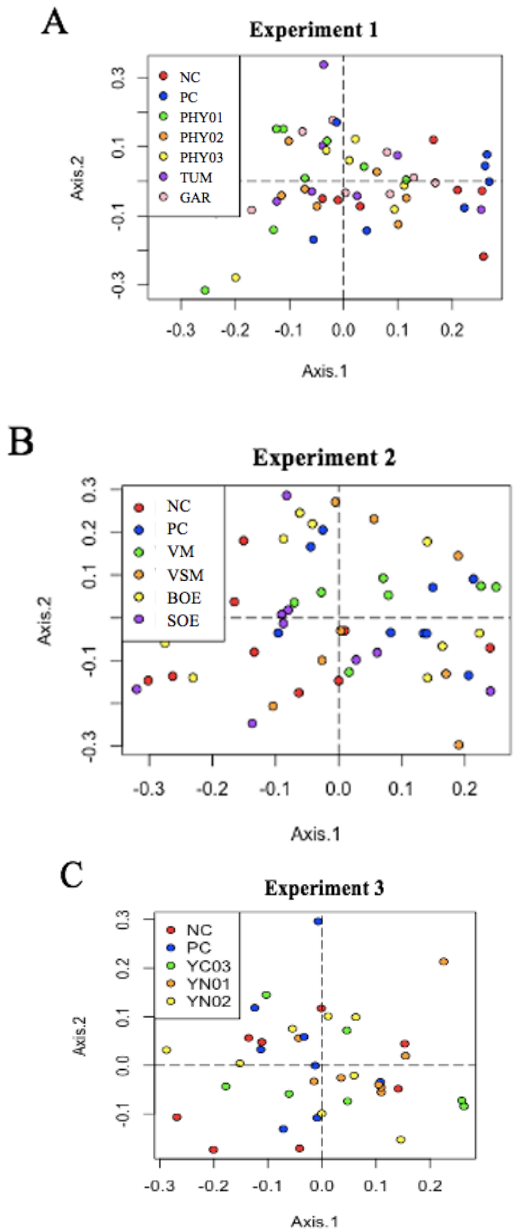


Figure 12 Microbiome beta diversity analysis of cecal samples. A) antibiotic (PC), no antibiotic (NC), essential oil and herb mixture 1 (PHY01), essential oil and herb mixture 2 (PHY02), essential oil and herb mixture 3 (PHY03), turmeric (TUM), and garlic (GAR). All products courtesy of Purina Animal Nutrition (Gray Summit, MO, USA). Weighted bray-curtis distance ordination of bacterial beta diversity in cecal samples. Lack of separation indicates no major difference in the microbiome among treatments (PERMANOVA, $R^2 = 0.12707$, $p = 0.05$). B) antibiotic (PC), no antibiotic (NC), bitter orange extract (BOE), sweet orange extract (SOE), volatile milk substances (VM01), and volatile & semi-volatile milk substances (VSM02). Lack of separation indicates no major difference in the microbiome among treatments (PERMANOVA, $R^2 = 0.0982$, $p = 0.383$). C) antibiotic (PC), no antibiotic (NC), yeast nucleotide product 1 (YN01), yeast nucleotide 2 (YN02), and yeast cell wall (YC03) products. Weighted bray-curtis distance ordination of bacterial beta diversity in cecal samples (PERMANOVA, $R^2 = 0.11835$, $p = 0.147$)

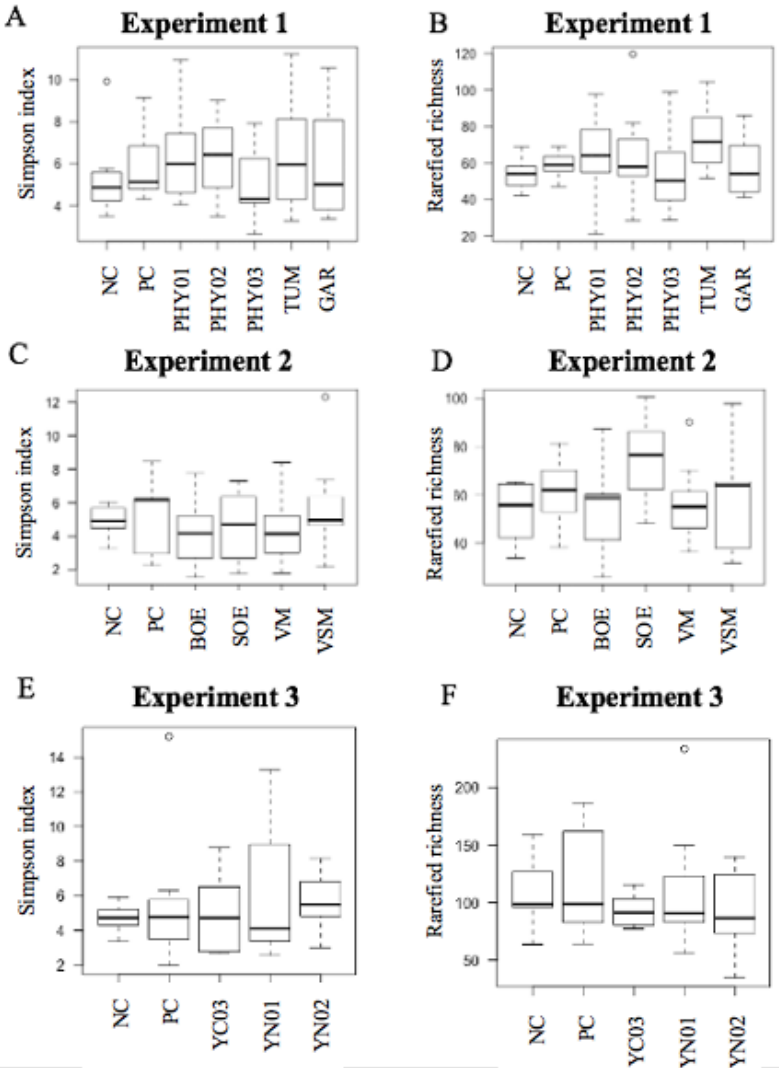


Figure 13Microbiome alpha diversity analysis of cecal samples. Treatments include antibiotic (PC), no antibiotic (NC), essential oil and herb mixture 1 (PHY01), essential oil and herb mixture 2 (PHY02), essential oil and herb mixture 3 (PHY03), turmeric (TUM), garlic (GAR), bitter orange extract (BOE), sweet orange extract (SOE), volatile milk substances (VM01), and volatile & semi-volatile milk substances (VSM02), yeast nucleotide product 1 (YN01), yeast nucleotide 2 (YN02), and yeast cell wall (YC03) products. A) The Simpson alpha diversity index for each treatment in experiment 1. There were no significant differences in the Simpson diversity index between treatments. B) The rarefied richness for each treatment in experiment 1. There were no significant differences in the rarefied richness between treatments. C) The Simpson alpha diversity index for each treatment in experiment 2. There were no significant differences in the Simpson diversity index between treatments. D) The rarefied richness for each treatment in experiment 2. There were no significant differences in the rarefied richness between treatments. E) The Simpson alpha diversity index for each treatment in experiment 3. There were no significant differences in the Simpson diversity index between treatments. F) The rarefied richness for each treatment in experiment 3. There were no significant differences in the rarefied richness between treatments. All products courtesy of Purina Animal Nutrition (Gray Summit, MO, USA)

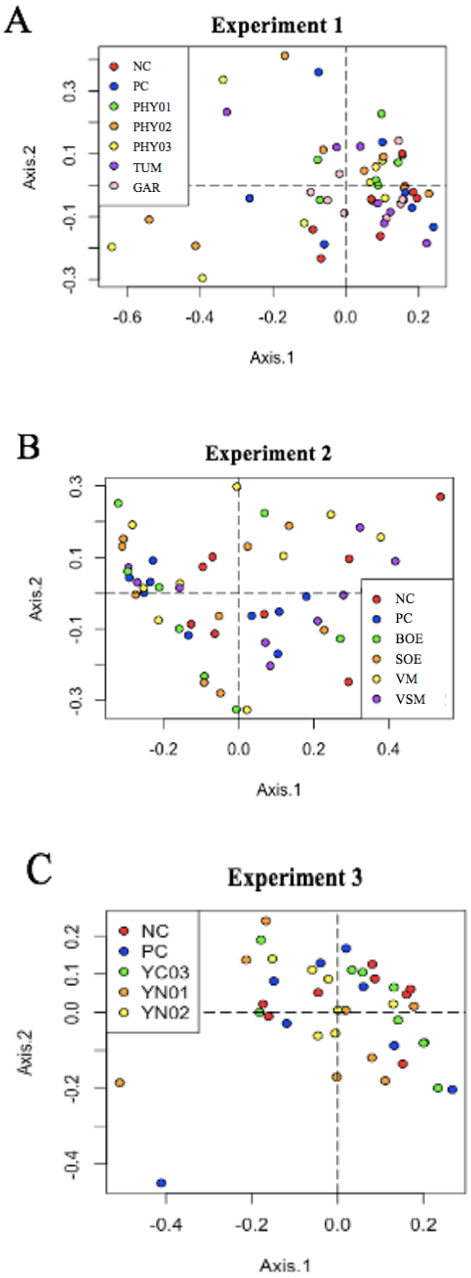


Figure 14 Microbiome beta diversity analysis of Ileal samples. A) Weighted bray-curtis distance ordination of bacterial beta diversity in ileal samples. Treatments include antibiotic (PC), no antibiotic (NC), essential oil and herb mixture 1 (PHY01), essential oil and herb mixture 2 (PHY02), essential oil and herb mixture 3 (PHY03), turmeric (TUM), and garlic (GAR). Lack of separation indicates no major difference in the microbiome among treatments (PERMANOVA, $R^2 = 0.11233$, $p = 0.417$). B) Weighted bray-curtis distance ordination of bacterial beta diversity in ileal samples. Treatments include antibiotic (PC), no antibiotic (NC), bitter orange extract (BOE), sweet orange extract (SOE), Volatile Milk Substances (VM01), and volatile & semi-volatile milk substances (VSM02). Lack of separation indicates no major difference in the microbiome among treatments (PERMANOVA, $R^2 = 0.10134$, $p = 0.334$). C) Weighted bray-curtis distance ordination of bacterial beta diversity in ileal samples (PERMANOVA, $R^2 = 0.07836$, $p = 0.855$). Treatments include antibiotic (PC), no antibiotic (NC), yeast nucleotide product 1 (YN01), yeast nucleotide 2 (YN02), and yeast cell wall (YC03) products. All products courtesy of Purina Animal Nutrition (Gray Summit, MO, USA)

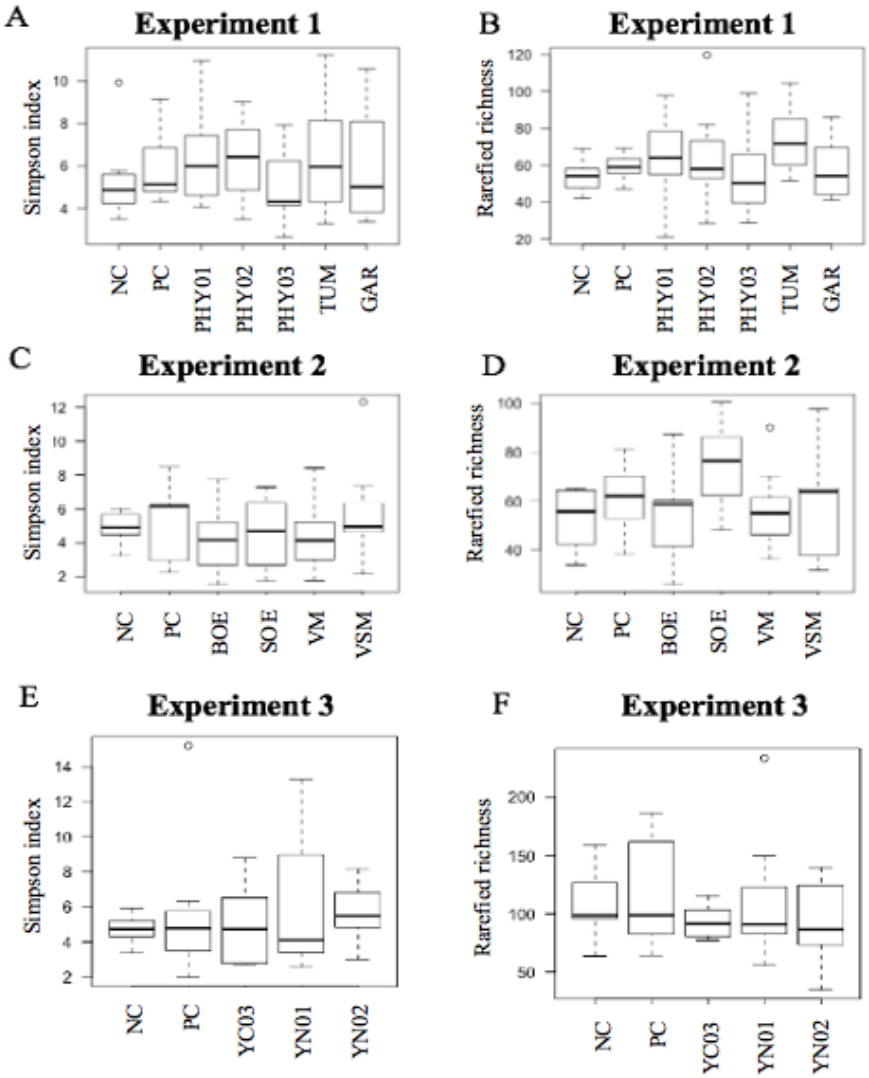


Figure 15 Microbiome alpha diversity analysis of Ileal samples. Treatments include antibiotic (PC), no antibiotic (NC), essential oil and herb mixture 1 (PHY01), essential oil and herb mixture 2 (PHY02), essential oil and herb mixture 3 (PHY03), turmeric (TUM), garlic (GAR), bitter orange extract (BOE), sweet orange extract (SOE), volatile milk substances (VM01), and volatile & semi-volatile milk substances (VSM02), yeast nucleotide product 1 (YN01), yeast nucleotide 2 (YN02), and yeast cell wall (YC03) products. A) The Simpson alpha diversity index for each treatment in experiment 1. There were no significant differences in the Simpson diversity index between treatments. B) The rarefied richness for each treatment in experiment 1. There were no significant differences in the rarefied richness between treatments. C) The Simpson alpha diversity index for each treatment in experiment 2. There were no significant differences in the Simpson diversity index between treatments. D) The rarefied richness for each treatment in experiment 2. There were no significant differences in the rarefied richness between treatments. E) The Simpson alpha diversity index for each treatment in experiment 3. There were no significant differences in the Simpson diversity index between treatments. F) The rarefied richness for each treatment in experiment 3. There were no significant differences in the rarefied richness between treatments. All products courtesy of Purina Animal Nutrition (Gray Summit, MO, USA)

Table 23 Cecal bacterial strains that have high abundance and specificity to pigs fed antibiotics, botanical extracts, phytogenic and herb extracts, milk substances, and yeast products compared to the negative control treatment.

Bacterial strain	Indicator value[1]	Exp	Treatment[2]	Negative control mean[3] ± standard deviation	Treatment mean[3] ± standard deviation	*P*-value[4]
k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus	0.878	1	HB1	0.755 ± 0.614	5.449 ± 4.621	0.019
k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus	0.940	1	HB3	0.755 ± 0.614	11.925 ± 10.770	0.010
k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus	0.835	1	GAR	0.755 ± 0.614	3.835 ± 3.681	0.046
k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Ruminococcus	0.821	1	GAR	0.094 ± 0.16	0.434 ± 0.342	0.032
k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Clostridiaceae; g__SMB53	0.8203	2	PC	0.495 ± 0.474	2.26 ± 1.606	0.009
k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Streptomycetaceae	1.000	3	PC	0 ± 0	0.033 ± 0.019	0.001

k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Megasphaera	0.915	3	YN1	0.228 ± 0.321	2.438 ± 2.175	0.019
k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__stercorea.	0.817	3	YN1	0.035 ± 0.045	0.157 ± 0.12	0.024

[1]The indicator value is a product of frequency and abundance. A good indicator value is greater than 0.8

[2]Treatments include antibiotic (PC), no antibiotic (NC), essential oil and herb mixture 1 (HB1), garlic (GAR), and yeast nucleotide product 1 (YN1). All products curtesy of Purina Animal Nutrition (Gray Summit, MO, USA)

[3]Means expressed as relative abundance (%)

[4]P-value from Kruskal Wallis one-way analysis of variance

Table 24 Effects of dietary feed additive supplementation on amino acid, short chain fatty acid, and bile acid concentrations in cecal samples from experiment 1.

Metabolite[1]	Experiment 1 treatments							
	NC	PC	PHY01	PHY02	PHY03	TUM	GAR	*P*-value[3]
Amino Acids, mg/g								
Alanine	0.597	0.640	0.491	0.609	0.740	0.726	0.497	0.551
Arginine	0.017	0.015	0.008	0.015	0.007	0.123	0.008	0.675
Asparagine	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.806
Aspartic acid	0.275	0.317	0.212	0.286	0.277	0.188	0.198	0.580
Citrulline	0.006	0.024	0.011	0.020	0.016	0.007	0.003*	0.181
Glutamic acid	0.966	1.182	0.971	0.782	1.104	0.886	1.410	0.941
Glutamine	0.003	0.004	0.002	0.003	0.002	0.087	0.001	0.921
Glycine	0.090	0.122	0.074	0.083	0.110	0.131	0.094	0.916
Histidine	0.006	0.008	0.003	0.004	0.005	0.030	0.004	0.830
Leucine- Isoleucine	0.044	0.028	0.022	0.043	0.038	0.074	0.017	0.312
Lysine	0.171	0.147	0.112	0.164	0.177	0.146	0.103	0.491
Methionine	0.003	0.002	0.001	0.002	0.002	0.003	0.001	0.081
Ornithine	0.002	0.002	0.005	0.005	0.014*	0.003	0.008	0.079
Phenylalanine	0.069	0.053	0.044	0.064	0.065	0.107	0.037	0.403
Proline	0.060	0.047	0.039	0.060	0.074	0.082	0.034*	0.229
r-amino-n-butyric	0.003	0.003	0.004	0.002	0.003	0.002	0.002	0.664
Serine	0.029	0.023	0.014	0.029	0.020	0.079	0.009*	0.275
Taurine	0.000	0.001	0.000	0.000	0.001	0.003	0.000	0.240
Threonine	0.040	0.040	0.027	0.040	0.034	0.068	0.019	0.641
Tryptophan	0.002	0.001	0.000	0.001	0.001	0.016	0.001	0.735
Tyrosine	0.099	0.057	0.039	0.082	0.075	0.150	0.036	0.328
Short chain fatty acids, mg/g								
Acetic acid	29.59	32.43	30.13	23.69	24.14	24.36	23.34	0.562
Propionic acid	20.77	20.97	17.38	14.32	16.30	15.80	14.43	0.624
Butyric acid	17.40	22.15	18.13	14.84	15.49	15.52	15.04	0.603
Valeric acid	3.669	6.048	4.002	3.504	4.258	3.564	3.360	0.573

Isovaleric acid	124.63	57.48	45.70	49.04	130.33	58.09	23.88	0.692
Bile acids[2], µg/g								
Cholic acid	0.051	0.102	0.153	0.000	0.051	0.000	0.000	0.515
Chenodeoxycholic acid	11.87	19.48	10.01	26.25	36.60	72.33	21.49	0.846
Deoxycholic acid	ND	ND	ND	ND	ND	ND	ND	N/A
Lithocholic acid	101.15	111.32	95.13	119.23	99.13	130.10	55.07	0.379
Glycocholic acid								
Glycochenodeo-xycholic acid	ND	ND	ND	ND	ND	ND	ND	N/A
Glycodeoxycho-lic acid	ND	ND	ND	ND	ND	ND	ND	N/A
Taurocholic acid	ND	ND	ND	ND	ND	ND	ND	N/A
Taurochenodeo-xycholic acid	0.187	0.125	0.125	0.000*	1.811	0.625	0.062	0.091
Taurodeoxycho-lic acid	ND	ND	ND	ND	ND	ND	ND	N/A
Hyodeoxycholic acid	413.81	495.27	485.90	576.44	454.25	563.97	405.72	0.801

[1] Treatments include antibiotic (PC), no antibiotic (NC), essential oil and herb mixture 1 (PHY01), essential oil and herb mixture 2 (PHY02), essential oil and herb mixture 3 (PHY03), turmeric (TUM), and garlic (GAR). All products curtesy of Purina Animal Nutrition (Gray Summit, MO, USA)

[2]ND: not detected

[3]P-value from Kruskal Wallis one-way analysis of variance

*Significantly different from negative control at $P < 0.1$

Table 25 Effects of dietary feed additive supplementation on amino acid, short chain fatty acid, and bile acid concentrations in cecal samples in experiment 2.

Metabolite[1]	Experiment 2 treatments						
	PC	NC	BOE	SOE	VM01	VSM02	*P*-value[3]
Amino Acids, mg/g							
Alanine	0.028	0.030	0.026	0.036	0.041	0.028	0.7340
Arginine	0.032	0.035	0.085	0.049	0.034	0.049	0.7024
Asparagine	0.018	0.021	0.042	0.022	0.024	0.019	0.7502
Aspartic acid	0.023	0.025	0.053	0.024	0.026	0.026	0.7142
Citrulline	0.033	0.048	0.102	0.036	0.053	0.042	0.7511
Glutamic acid	0.002	0.001	0.007	0.001	0.001	0.001	0.5504
Glutamine	0.007	0.006	0.004	0.003	0.005	0.003	0.4900
Glycine	0.002	0.002	0.019	0.005	0.003	0.002	0.5830
Histidine	0.064	0.081	0.145	0.065	0.059	0.087	0.3862
Leucine- Isoleucine	0.173	0.226	0.271	0.208	0.175	0.220	0.6622
Lysine	0.000	0.000	0.044	0.001	0.000	0.000	0.4335
Methionine	0.570	0.679	0.712	0.716	0.572	0.805	0.9478
Ornithine	0.002	0.001	0.048	0.004	0.001	0.001	0.4415
Phenylalanine	0.001	0.001	0.005	0.001	0.001	0.001	0.4782
Proline	0.005	0.010	0.044	0.007	0.006	0.008	0.4333
r-amino-n-butyric	0.018	0.027	0.039	0.022	0.018	0.019	0.5928
Serine	0.004	0.001	0.003	0.001	0.006	0.002	0.5979
Taurine	0.026	0.026	0.053	0.032	0.027	0.026	0.5561
Threonine	0.002	0.006	0.003	0.004	0.002	0.007	0.3583
Tryptophan	0.001	0.007	0.033	0.003	0.005	0.009	0.5119
Tyrosine	0.009	0.013	0.038	0.011	0.011	0.012	0.5973
Short chain fatty acids, mg/g							
Acetic acid	33.4	34.2	29.9	28.6	26.4*	32.7	0.5480
Propionic acid	20.0	21.9	18.7	16.8	16.2*	22.3	0.3721
Butyric acid	22.2	25.0	19.2	17.8*	17.2*	20.2	0.1309
Valeric acid	5.3[ab]	7.5[a]	4.5[ab]	4.4[ab]	4.2[b]	4.5[ab]	0.0391
Isovaleric acid	22.5	66.7	48.2	129.9	60.1	157.9	0.1641

Bile acids[2], µg/g							
Cholic acid	0.0	0.0	0.2	0.0	0.0	0.0	0.3935
Chenodeoxycholic acid	17.8	8.0	42.6	21.3	17.1	65.9	0.5377
Deoxycholic acid	ND	ND	ND	ND	ND	ND	N/A
Lithocholic acid	59.0	138.3*	76.1	96.6	71.0	110.6	0.2791
Glycocholic acid	0.0	0.0	0.0	0.0	0.0	0.0	N/A
Taurocholic acid	ND	ND	ND	ND	ND	ND	N/A
Taurochenodeox-ycholic acid	0.4	0.9	0.1	0.1	0.5	7.3	0.3849
Hyodeoxycholic acid	393.4	472.1	455.8	500.1	366.6	457.2	0.8975

[1]Treatments include antibiotic (PC), no antibiotic (NC), bitter orange extract (BOE), sweet orange extract (SOE), volatile milk substances (VM01), and volatile & semi-volatile milk substances (VSM02). All products curtesy of Purina Animal Nutrition (Gray Summit, MO, USA)

[2] ND: not detected

[3]P-value from Kruskal Wallis one-way analysis of variance

[a,b,c] Means within a row with different superscripts differ ($P < 0.05$)

* Indicates value different from negative control at $P < 0.05$

Table 26 Effects of dietary feed additive supplementation on amino acid, short chain fatty acid, and bile acid profile in cecal samples in experiment 3.

Metabolite[1]	Experiment 3 treatments					
	NC	PC	YN01	YN02	YC03	*P*-value[3]
Amino Acids, mg/g						
Alanine	0.493	0.634	0.502	0.382	0.405	0.060
Arginine	0.002	0.002	0.005	0.001	0.001	0.095
Asparagine	0.000	0.000	0.000	0.000	0.000	0.694
AsparticAcid	0.203	0.266	0.204	0.129	0.205	0.196
Citrulline	0.006	0.013	0.001*	0.002	0.004	0.051
GlutamicAcid	0.642[b]	0.982[a]	0.586[b]	0.360[c]	0.582[b]	0.046
Glutamine	0.000	0.000	0.001	0.000	0.000	0.179
Glycine	0.045	0.068	0.050	0.027	0.033	0.175
Histidine	0.001[a]	0.002[b]	0.001[a]	0.001[a]	0.001[a]	0.039
Leucine- Isoleucine	0.020[a]	0.030[b]	0.022[a]	0.013[a]	0.018[a]	0.032
Lysine	0.095[a]	0.128[b]	0.087[a]	0.047[a]	0.083[a]	0.013
Methionine	0.001	0.002	0.002	0.001	0.001	0.076
Ornithine	0.002	0.006	0.003	0.001	0.001	0.396
Phenylalanine	0.036[a]	0.046[b]	0.039[a]	0.023[a]	0.030[a]	0.012
Proline	0.039	0.042	0.038	0.029	0.037	0.351
r-amino-n-butyric	0.014[a]	0.015[a]	0.015[a]	0.005[b]	0.012[a]	0.028
Serine	0.000	0.001	0.000	0.001	0.000	0.407
Taurine	0.019	0.025	0.022	0.010	0.021	0.103
Threonine	0.000	0.001	0.000	0.000	0.000	0.059
Tryptophan	0.023	0.034	0.025	0.012	0.017	0.126
Tyrosine	0.027[a]	0.045[b]	0.029[s]	0.015[a]	0.023[a]	0.004
Short chain fatty acids, mg/g						
Acetic acid	36.126	41.217	36.983	36.794	41.955	0.480
Propionic acid	23.718	26.910	23.988	23.787	25.336	0.574
Butyric acid	27.283	28.476	26.138	24.987	30.918	0.340
Valeric acid	7.420	7.686	7.130	6.419	8.942	0.181
Isovaleric acid	19.213	32.771	9.294	6.064	8.987	0.979

Bile Acids[2], µg/g						
Cholic acid	0.000[a]	0.357[b]	0.000[a]	0.000[a]	0.000[a]	0.002
Chenodeoxycholic acid	15.457	30.032	15.163	8.735	7.606	0.775
Deoxycholic acid	ND	ND	ND	ND	ND	N/A
Lithocholic acid	151.291	151.856	126.531	115.704	117.022	0.535
Glycocholic acid	ND	ND	ND	ND	ND	N/A
Glycochenodeoxycho-lic acid	ND	ND	ND	ND	ND	N/A
Glycodeoxycholic acid	ND	ND	ND	ND	ND	N/A
Taurocholic acid	ND	ND	ND	ND	ND	N/A
Taurochenodeoxycho-lic acid	0.000	0.000	0.062	0.000	0.000	0.406
Taurodeoxycholic acid	ND	ND	ND	ND	ND	N/A
Hyodeoxycholic acid	697.793	764.628	614.274	542.483	589.787	0.981

[1]Treatments include antibiotic (PC), no antibiotic (NC), yeast nucleotide product 1 (YN01), yeast nucleotide 2 (YN02), and yeast cell wall (YC03) products. All products curtesy of Purina Animal Nutrition (Gray Summit, MO, USA)

[2]ND: not detected

[3]P-value from Kruskal Wallis one-way analysis

[a,b,c] Means within a row with different superscripts differ ($P < 0.05$)

* Indicates value different from negative control at $P < 0.05$

Overall summary

The overall goal of this research was to determine a mechanism of action for antibiotic growth promotors and antibiotic alternatives. By evaluating multiple parameters and animal systems, information can be integrated to develop a mechanism of action. Previous research suggests that the mechanism of action for antibiotic growth promotors is based on an alteration of the microbiome which provides more resources for animal growth. For this reason, an evaluation of the microbiome is a key aspect in understanding the mechanism of action. Additionally, a metabolomics analysis of serum and intestinal content will provide information on how other animal systems respond to the microbiome alteration, or how the animal responds to the additive itself. This makes metabolomics data an important part, in conjunction with the microbiome data, to drawing conclusions about a mechanism of action. Therefore, this research consisted of feeding the antibiotics tylosin and a mixture of chlortetracycline and sulfamethazine to pigs, and evaluating the growth, microbiome, and metabolic response. After identifying a mechanism of action, recommendations can be made to pork producers on how to effectively use the feed additives.

Results in chapter 2 suggest the antibiotic tylosin alters the microbiome and improves growth by changing the concentration of bile acids. More specifically, the concentration of the secondary bile acid hyodeoxycholic acid was higher in the fecal metabolome of pigs fed tylosin than the negative control pigs. The concentration of Hyodeoxycholic acid was also positively correlated with the relative abundance of bacteria in the genus of *Clostridia*. Hyodeoxycholic acid is a secondary bile acid that is

produced when the intestinal bacteria deconjugate primary bile acids. The increased concentration of hyodeoxycholic acid is hypothesized to increase bile acid signaling, and activate pathways associated with protein and lipid metabolism and protection from pathogens. However, after 9 weeks of supplementing antibiotics, this effect was no longer present, suggesting the microbiome will eventually adapt to the tylosin in the diet. This is an important finding for evaluating feed additives, because a feed additive with a diminishing growth response should not be kept in the diet longer than it is effective.

The results from chapter 3 suggest that different antibiotics have a different mechanism of growth promotion, because the combination of chlortetracycline and sulfamethazine fed to nursery pigs had no impact on bile acid concentrations in the intestinal content. When evaluating the cecal and ileal microbiome, there were no differences in the alpha or beta diversity between the pigs fed an antibiotic and the negative control. In addition, there were no differences in the serum or cecal metabolome between the antibiotic and negative control group. In this experiment, pigs were healthy with no mortality, suggesting the antibiotics could not provide an additional benefit to the animal that ideal nutrition and housing was not already providing. These findings suggest that the mechanism of growth promotion when feeding chlortetracycline and sulfamethazine is dependent on the health status of the farm. Despite the minimal impact the antibiotic had on the animal's microbiome and metabolome, location had a significant impact on both of these parameters. These findings suggest that even pigs managed with the same practices will have different baseline microbiomes and metabolic profiles if they are housed in different barns. This variability has not been well described in previous research and the high impact location has on these parameters calls for more research

identifying which barn characteristics shape the animals microbiome. For example, the impact on the microbiome or metabolome caused by the number of animals per pen, materials used to build pens, ventilation, temperature, humidity, or pen size. By understanding how these factors shape the microbiome and metabolome, we can better understand how to influence these systems to improve animal growth.

Because of the significant threat of antibiotic resistant bacteria, antibiotics can no longer be utilized in swine for growth promotion purposes. For this reason, multiple categories of feed additives are being evaluated for their ability to improve health and growth in the absence of antibiotics, including yeast products, essential oils, and herbs and spices. When evaluating 13 of these products, none of them were able to improve growth performance when fed to nursery pigs at the tested usage level. In addition, none of these products impacted alpha or beta diversity of the cecal or ileal microbiome or the beta diversity of the cecal or serum metabolome. However, four of the products increased the relative abundance of bacteria in the genus *Lactobacillus,* a bacteria family well known for its protective effect against pathogens. These findings suggest that these products could not provide an additional benefit that was not already being provided by a complete diet and good management practices, but it is hypothesized they could produce a protective effect during a gastrointestinal pathogen challenge or under stressful conditions.

As demonstrated in chapters 2, 3, and 4, microbiome and metabolomics analyses are useful tools for understanding how a feed additive is impacting an animal. However, experiments utilizing these tools need to be carefully designed to provide useful data. This includes thoroughly reading through the literature, selecting appropriate

experimental designs, choosing a relevant dose and experimental setting, and collecting samples that will answer the research question. When an experiment uses an appropriate approach, research in animal science can move beyond just evaluating a growth response and instead evaluate the response of multiple animal systems. This approach will continue to provide information that can be used to develop a mechanism of action for current and future feed additives which will help ensure their optimal use in swine diets.

References

1. Gaskins HR, Collier CT, Anderson DB. Antibiotics as growth promotants: mode of action. Anim Biotechnol. 2006;13(1):29–42.
2. Cromwell GL. Why and how antibiotics are used in swine production. Anim Biotechnol. 2006;13(1):1532–2378.
3. van Den Bogaard AEA, Stobberingh EE. Epidemiology of resistance to antibiotics. Link between animals and humans. Int J Antimicrob Agents. 2000;14(4):327–35.
4. Veterinary Feed Directive [Internet]. Food and Drug Administration; 2015. Available from: https://www.fda.gov/animal-veterinary/development-approval-process/veterinary-feed-directive-vfd
5. Dibner JJ, Richards JD. Antibiotic Growth Promoters in Agriculture : History and Mode of Action. 2005;634–43.
6. Thacker PA. Alternatives to antibiotics as growth promoters for use in swine production: a review. J Anim Sci Biotechnol [Internet]. 2013 Dec 14;4(1):35. Available from: https://jasbsci.biomedcentral.com/articles/10.1186/2049-1891-4-35
7. Shurson GC. Yeast and yeast derivatives in feed additives and ingredients: Sources, characteristics, animal responses, and quantification methods [Internet]. Vol. 235, Animal Feed Science and Technology. Elsevier; 2018. p. 60–76. Available from: https://doi.org/10.1016/j.anifeedsci.2017.11.010
8. van der Peet-Schwering CMC, Jansman AJM, Smidt H, Yoon I. Effects of yeast culture on performance, gut integrity, and blood cell composition of weanling pigs. J Anim Sci [Internet]. 2007 Nov 1;85(11):3099–109. Available from: https://academic.oup.com/jas/article/85/11/3099/4779206
9. Windisch W, Schedle K, Plitzner C, Kroismayr A. Use of phytogenic products as feed additives for swine and poultry. J Anim Sci. 2008;86.
10. Aluthge ND, Van Sambeek DM, Carney-Hinkle EE, Li YS, Fernando SC, Burkey TE. BOARD INVITED REVIEW: The pig microbiota and the potential for harnessing the power of the microbiome to improve growth and health1. J Anim Sci [Internet]. 2019 Sep 3;97(9):3741–57. Available from: https://academic.oup.com/jas/article/97/9/3741/5524612
11. Mohajeri MH, Brummer RJM, Rastall RA, Weersma RK, Harmsen HJM, Faas M, et al. The role of the microbiome for human health: from basic science to clinical applications. Eur J Nutr [Internet]. 2018 May 10;57(S1):1–14. Available from: http://link.springer.com/10.1007/s00394-018-1703-4
12. Cresci G, Izzo K. Gut Microbiome. In: Adult short bowel syndrome. 2019. p. 45–54.
13. Holman DB, Brunelle BW, Trachsel J, Allen HK. Meta-analysis To Define a Core Microbiota in the Swine Gut. Bik H, editor. mSystems [Internet]. 2017 Jun 27;2(3):1–14. Available from: https://msystems.asm.org/content/2/3/e00004-17
14. Amat S, Lantz H, Munyaka PM, Willing BP. Prevotella in pigs: The positive and negative associations with production and health. Microorganisms. 2020;8(10):1–27.
15. Precup G, Vodnar DC. Gut Prevotella as a possible biomarker of diet and its

eubiotic versus dysbiotic roles: A comprehensive literature review. Br J Nutr. 2019;122(2):131–40.
16. Wang X, Tsai T, Deng F, Wei X, Chai J, Knapp J, et al. Longitudinal investigation of the swine gut microbiome from birth to market reveals stage and growth performance associated bacteria. Microbiome. 2019;7(1):1–18.
17. Lamendella R, Santo Domingo JW, Ghosh S, Martinson J, Oerther DB. Comparative fecal metagenomics unveils unique functional capacity of the swine gut. Vol. 11, BMC Microbiology. 2011. p. 1–39.
18. Baker AA, Davis E, Rehberger T, Rosener D, Icrobiol APPLENM. Prevalence and Diversity of Toxigenic Clostridium perfringens and Clostridium difficile among Swine Herds in the Midwest . Appl Environ Microbiol. 2010;76(9):2961–7.
19. Downes J, Dewhirst FE, Tanner ACR, Wade WG. Description of Alloprevotella rava gen. nov., sp. nov., isolated from the human oral cavity, and reclassification of Prevotella tannerae Moore et al. 1994 as Alloprevotella tannerae gen. nov., comb. nov. Int J Syst Evol Microbiol. 2013;63(PART4):1214–8.
20. Tang S, Xin Y, Ma Y, Xu X, Zhao S, Cao J. Screening of Microbes Associated With Swine Growth and Fat Deposition Traits Across the Intestinal Tract. Front Microbiol. 2020;11(October):1–11.
21. Zhou H, Yu B, Sun J, Liu Z, Chen H, Ge L, et al. Short-chain fatty acids can improve lipid and glucose metabolism independently of the pig gut microbiota. J Anim Sci Biotechnol. 2021;12(1):1–14.
22. Varel VH, Yen JT. Microbial Perspective on Fiber Utilization by Swine. J Anim Sci. 1997;75(10):2715–22.
23. Bergamaschi M, Tiezzi F, Howard J, Huang YJ, Gray KA, Schillebeeckx C, et al. Gut microbiome composition differences among breeds impact feed efficiency in swine. Microbiome. 2020;8(1):1–15.
24. Yang H, Xiao Y, Wang J, Xiang Y, Gong Y, Wen X, et al. Core gut microbiota in Jinhua pigs and its correlation with strain, farm and weaning age. J Microbiol. 2018;56(5):346–55.
25. Vigors S, O' Doherty J V., Sweeney T. Colonic microbiome profiles for improved feed efficiency can be identified despite major effects of farm of origin and contemporary group in pigs. Animal. 2020;14(12):2472–80.
26. Palmer C, Bik EM, DiGiulio DB, Relman DA, Brown PO. Development of the Human Infant Intestinal Microbiota. Ruan Y, editor. PLoS Biol [Internet]. 2007 Jun 26;5(7):e177. Available from: https://dx.plos.org/10.1371/journal.pbio.0050177
27. Soler C, Goossens T, Bermejo A, Migura-García L, Cusco A, Francino O, et al. Digestive microbiota is different in pigs receiving antimicrobials or a feed additive during the nursery period. Zoetendal EG, editor. PLoS One [Internet]. 2018 May 25;13(5):e0197353. Available from: https://dx.plos.org/10.1371/journal.pone.0197353
28. Mach N, Berri M, Estellé J, Levenez F, Lemonnier G, Denis C, et al. Early-life establishment of the swine gut microbiome and impact on host phenotypes. Environ Microbiol Rep. 2015;7(3):554–69.
29. Flint HJ, Scott KP, Duncan SH, Louis P, Forano E. Microbial degradation of complex carbohydrates in the gut. Gut Microbes. 2012;3(4):289–306.

30. Ramayo-Caldas Y, Mach N, Lepage P, Levenez F, Denis C, Lemonnier G, et al. Phylogenetic network analysis applied to pig gut microbiota identifies an ecosystem structure linked with growth traits. ISME J. 2016;10(12):2973–7.
31. Yang H, Huang X, Fang S, He M, Zhao Y, Wu Z, et al. Unraveling the Fecal Microbiota and Metagenomic Functional Capacity Associated with Feed Efficiency in Pigs. Front Microbiol [Internet]. 2017 Aug 15;8(AUG):1–11. Available from: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01555/full
32. McCormack UM, Curião T, Buzoianu SG, Prieto ML, Ryan T, Varley P, et al. Exploring a Possible Link between the Intestinal Microbiota and Feed Efficiency in Pigs. Dudley EG, editor. Appl Environ Microbiol [Internet]. 2017 Aug 1;83(15):1–16. Available from: https://aem.asm.org/lookup/doi/10.1128/AEM.00380-17
33. VanderWaal K, Deen J. Global trends in infectious diseases of swine. Proc Natl Acad Sci [Internet]. 2018 Nov 6;115(45):11495–500. Available from: http://www.pnas.org/lookup/doi/10.1073/pnas.1806068115
34. Ehrlich GD, Hiller NL, Hu FZ. What makes pathogens pathogenic. Genome Biol. 2008;9(6).
35. Helke KL, Ezell PC, Duran-Struuck R, Swindle MM. Biology and Diseases of Swine. In: Laboratory Animal Medicine [Internet]. Third Edit. Elsevier; 2015. p. 695–769. Available from: http://dx.doi.org/10.1016/B978-0-12-409527-4.00016-X
36. Baümler AJ, Sperandio V. Interactions between the microbiota and pathogenic bacteria in the gut. Nature. 2016;535(7610):85–93.
37. Pickard JM, Zeng MY, Caruso R, Nunez G. Gut Microbiota: Role in Pathogen Colonization, Immune Responses and Inflammatory Disease. immunol Rev. 2017;176(5):139–48.
38. Gustafson RH, Bowen RE. Antibiotic use in animal agriculture. Vol. 83, Journal of Applied Microbiology. 1997. p. 531–41.
39. Barber M, Rozwadowska-Dowzenko M. Infection by penicillin resistant staphlycocci lancet. Lancet. 1948;2:641–4.
40. Moore P, Evenson A, Luckey T, McCoy E, Elvehjem C, Hart E. Use of sulfasuxidine, streptothricin, and streptomycin in nutritional studies with the chick. J Biol Chem [Internet]. 1946 Oct;165(2):437–41. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20276107
41. Jones JR, Pond WG. Effect of the Addition of Lysine and Virginiamycin to Corn-Soybean Meal Rations on Performance of Weanling Pigs. J Anim Sci [Internet]. 1963 Nov 1;22(4):1033–7. Available from: https://academic.oup.com/jas/article/22/4/1033-1037/4701286
42. Lepley KC, Catron D V., Culbertson CC. Dried Whole Aureomycin Mash and Meat and Bone Scraps for Growing-Fattening Swine. J Anim Sci [Internet]. 1950 Nov 1;9(4):608–14. Available from: https://academic.oup.com/jas/article/9/4/608/4728632
43. Dewey CE, Cox BD, Straw BE, Bush EJ, Hurd S. Use of antimicrobials in swine feeds in the United States. J Swine Heal Prod. 1999;7(1):19–25.
44. Rajić A, Reid-Smith R, Deckert AE, Dewey CE, McEwen SA. Reported antibiotic use in 90 swine farms in Alberta. Can Vet J = La Rev Vet Can [Internet]. 2006

May;47(5):446–52. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16734370
45. Hays VW. Effectiveness of Feed Additive Usage of Antibacterial Agents in Swine and Poultry Production. [Internet]. The Hays Report. Long Beach, CA: Rachelle Laboratories; 1979. Available from: https://archive.org/details/effectivenessoff00hays/page/n7/mode/2up
46. United States. Congress. Office of Technology Assessment. Drugs in livestock feed [Internet]. 1979. Available from: http://www.princeton.edu/~ota/disk3/1979/7905/7905.PDF
47. Dunlop RH, McEwan SA, Meek AH, Friendship RM. Extent of feed additive use in the Ontario swine industry. In: 13th Annual Swine Research Update. 1994. p. 58–60.
48. US Food and Drug Administration. Summary report on antimicrobials sold or distributed for use in food-producing animals. J Linguist [Internet]. 2018;3(2):139–57. Available from: https://www.euskalit.net/archivos/201803/modelogestionavanzada_2018.pdf?1%0Ahttps://dialnet.unirioja.es/servlet/articulo?codigo=4786739%0Ahttps://www2.deloitte.com/content/dam/Deloitte/mx/Documents/human-capital/HCT-2018.pdf%0Ahttp://pepsic.bvsalud.org/pd
49. Brüssow H. Growth promotion and gut microbiota: Insights from antibiotic use. Vol. 17, Environmental Microbiology. 2015. p. 2216–27.
50. Young G, Underdahl N, Sumpton L, Peo E, Olsen L, Kelley G, et al. Swine repopulation. I. Performance within a disease-free experiment station herd. J Am Vet Med Assoc [Internet]. 1959 Jun 1;134(11):491–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/13654135
51. Comission on Antimicrobial Feed Additives. Antimicrobial Feed Additives [Internet]. Stolkholm; 1997. Available from: https://www.government.se/contentassets/f09ed76c354441b6b5e4d51f1f637101/chapter-1-4-antimicrobial-feed-additives
52. Shurson GC, Ku PK, Waxler GL, Yokoyama MT, Miller ER. Physiological relationships between microbiological status and dietary copper levels in the pig. J Anim Sci [Internet]. 1990 Apr 1;68(4):1061–71. Available from: https://academic.oup.com/jas/article/68/4/1061/4704718
53. Klasing KC, Laãœrin DE, Peng RK, Fry DM. Nutrition and Immunology Immunologically Mediated Growth Depression in Chicks: Influence of Feed Intake , Corticosterone and Interleukin-1. J Nutr. 1987;(85):1629–37.
54. Souza E. Corticotropin-Releasing Factor and Interleukin-1 Receptors in the Brain-Endocrine-Immune Axis Role in Stress Response and Infection. Ann N Y Acad Sci [Internet]. 1993 Oct;697:9–27. Available from: http://doi.wiley.com/10.1111/j.1749-6632.1993.tb49919.x
55. Angioni S, Petraglia F, Gallinelli A, Cossarizza A, Franceschi C, Muscettola M, et al. Corticotropin-releasing hormone modulates cytokines release in cultured human peripheral blood mononuclear cells. Life Sci [Internet]. 1993 Jan;53(23):1735–42. Available from: https://linkinghub.elsevier.com/retrieve/pii/0024320593901605
56. Niewold TA. The Nonantibiotic Anti-Inflammatory Effect of Antimicrobial

Growth Promoters, the Real Mode of Action? A Hypothesis. Poult Sci [Internet]. 2007 Apr;86(4):605–9. Available from: http://dx.doi.org/10.1093/ps/86.4.605
57. Paulsen Ø, Laird B, Aass N, Lea T, Fayers P, Kaasa S, et al. The relationship between pro-inflammatory cytokines and pain, appetite and fatigue in patients with advanced cancer. Scheede-Bergdahl C, editor. PLoS One [Internet]. 2017 May 25;12(5):e0177620. Available from: https://dx.plos.org/10.1371/journal.pone.0177620
58. Gorrill ADL, Bell JM, Williams CM. Ingredient and processing interrelationships in swine feeds. Can J Anim Sci [Internet]. 1960 Dec 1;40(2):83–92. Available from: http://www.nrcresearchpress.com/doi/10.4141/cjas60-014
59. Vervaeke IJ, Decuypere JA, Dierick NA, Henderickx HK. Quantitative in Vitro Evaluation of the Energy Metabolism Influenced by Virginiamycin and Spiramycin used as Growth Promoters in Pig Nutrition. J Anim Sci [Internet]. 1979 Sep 1;49(3):846–56. Available from: https://academic.oup.com/jas/article/49/3/846-856/4699304
60. Guban J, Korver DR, Allison GE, Tannock GW. Relationship of dietary antimicrobial drug administration with broiler performance, decreased population levels of Lactobacillus salivarius, and reduced bile salt deconjugation in the ileum of broiler chickens. Poult Sci. 2006;85(12):2186–94.
61. Trudeau MP, Zhou Y, Leite FL, Gomez A, Urriola PE, Shurson GC, et al. Fecal Hyodeoxycholic Acid Is Correlated With Tylosin-Induced Microbiome Changes in Growing Pigs. Front Vet Sci. 2018;5(August):1–7.
62. Ipharraguerre IR, Pastor JJ, Gavaldà-Navarro A, Villarroya F, Mereu A. Antimicrobial promotion of pig growth is associated with tissue-specific remodeling of bile acid signature and signaling. Sci Rep [Internet]. 2018 Dec 12;8(1):13671. Available from: http://www.nature.com/articles/s41598-018-32107-9
63. Diether N, Willing B. Microbial Fermentation of Dietary Protein: An Important Factor in Diet–Microbe–Host Interaction. Microorganisms [Internet]. 2019 Jan 13;7(1):19. Available from: http://www.mdpi.com/2076-2607/7/1/19
64. Salter DN, Coates ME, Hewitt D. The utilization of protein and excretion of uric acid in germ-free and conventional chicks. Br J Nutr [Internet]. 1974 May 9;31(3):307–18. Available from: https://www.cambridge.org/core/product/identifier/S0007114574000407/type/journal_article
65. Roth FX, Kirchgessner M. Influence of Avilamycin and Tylosin on Retention and Excretion of Nitrogen in Growing Pigs. J Anim Physiol Anim Nutr (Berl) [Internet]. 1993 Jan 8;69(1–5):175–85. Available from: http://doi.wiley.com/10.1111/j.1439-0396.1993.tb00803.x
66. Yin Y-L, McEvoy JD, Schulze H, McCracken KJ. Effects of xylanase and antibiotic addition on ileal and faecal apparent digestibilities of dietary nutrients and evaluating HCl-insoluble ash as a dietary marker in growing pigs. Anim Sci [Internet]. 2001 Feb 18;72(1):95–103. Available from: https://www.cambridge.org/core/product/identifier/S1357729800055594/type/journal_article
67. Agudelo JH, Lindemann MD, Cromwell GL, Newman MC, Nimmo RD.

Virginiamycin improves phosphorus digestibility and utilization by growing-finishing pigs fed a phosphorus-deficient, corn-soybean meal diet. J Anim Sci [Internet]. 2007 Sep 1;85(9):2173–82. Available from: https://academic.oup.com/jas/article/85/9/2173/4778378
68. Ravindran V, Kornegay ET, Webb KE. Effects of Fiber and Virginiamycin on Nutrient Absorption, Nutrient Retention and Rate of Passage in Growing Swine. J Anim Sci [Internet]. 1984 Aug 1;59(2):400–8. Available from: https://academic.oup.com/jas/article/59/2/400-408/4745966
69. Lindemann MD, Quant AD, Monegue JS, Wang M, Cromwell GL, Newman MC. Evaluation of antibiotic effects on phosphorus digestibility and utilization by growing-finishing pigs fed a phosphorus-deficient, corn-soybean meal diet1,2. J Anim Sci [Internet]. 2010 May 1;88(5):1752–8. Available from: https://academic.oup.com/jas/article/88/5/1752-1758/4745607
70. Crofton J, Mitchison DA. Streptomycin Resistance in Pulmonary Tuberculosis. BMJ [Internet]. 1948 Dec 11;2(4588):1009–15. Available from: https://www.bmj.com/lookup/doi/10.1136/bmj.2.4588.1009
71. Alanis AJ. Resistance to antibiotics: Are we in the post-antibiotic era? Vol. 36, Archives of Medical Research. 2005. p. 697–705.
72. Thomas CM, Nielsen KM. Mechanisms of, and Barriers to, Horizontal Gene Transfer between Bacteria. Nat Rev Microbiol [Internet]. 2005 Sep;3(9):711–21. Available from: http://www.nature.com/articles/nrmicro1234
73. McCrackin MA, Helke KL, Galloway AM, Poole AZ, Salgado CD, Marriott B p. Effect of Antimicrobial Use in Agricultural Animals on Drug-resistant Foodborne Campylobacteriosis in Humans: A Systematic Literature Review. Crit Rev Food Sci Nutr [Internet]. 2016 Oct 2;56(13):2115–32. Available from: http://www.tandfonline.com/doi/full/10.1080/10408398.2015.1119798
74. Johnson TA, Stedtfeld RD, Wang Q, Cole JR, Hashsham SA, Looft T, et al. Clusters of Antibiotic Resistance Genes Enriched Together Stay Together in Swine Agriculture. MBio [Internet]. 2016 May 4;7(2):1–11. Available from: https://mbio.asm.org/content/7/2/e02214-15
75. Robinson TP, Bu DP, Carrique-Mas J, Fèvre EM, Gilbert M, Grace D, et al. Antibiotic resistance is the quintessential One Health issue. Trans R Soc Trop Med Hyg [Internet]. 2016 Jul;110(7):377–80. Available from: https://academic.oup.com/trstmh/article-lookup/doi/10.1093/trstmh/trw048
76. Starr MP, Reynolds DM. Streptomycin Resistance of Coliform Bacteria from Turkeys Fed Streptomycin. Am J Public Heal Nations Heal [Internet]. 1951 Nov;41:1375–80. Available from: http://ajph.aphapublications.org/doi/10.2105/AJPH.41.11_Pt_1.1375
77. World Health Organization. Global action plan on antimicrobial resistance [Internet]. 2015. Available from: https://www.who.int/antimicrobial-resistance/publications/global-action-plan/en/
78. Wierup M. The Swedish experience of the 1986 year ban of antimicrobial growth promoters, with special reference to animal health, disease prevention, productivity, and usage of antimicrobials. Microb Drug Resist. 2001;7(2):183–90.
79. Maron DF, Smith TJS, Nachman KE. Restrictions on antimicrobial use in food animal production: An international regulatory and economic survey. Global

Health. 2013;9(1).
80. Levy S. Reduced Antibiotic Use in Livestock: How Denmark Tackled Resistance. Environ Health Perspect [Internet]. 2014 Jun;122(6):160–5. Available from: https://ehp.niehs.nih.gov/doi/10.1289/ehp.122-A160
81. Hu YJ, Cowling BJ. Reducing antibiotic use in Livestock, China. Bull World Health Organ. 2020;98(5):360–1.
82. Schulz LL, Rademacher CJ. Food and Drug Administration Guidance 209 and 213 and Veterinary Feed Directive regulations regarding antibiotic use in livestock: A survey of preparation and anticipated impacts in the swine industry. J Swine Heal Prod. 2017;25(5):247–55.
83. Chambers V E, Chambers IV E, Castro M. What Is "Natural"? Consumer Responses to Selected Ingredients. foods. 2018;7(65).
84. Craig WJ. Health-promoting properties of common herbs. Am J Clin Nutr [Internet]. 1999 Sep 1;70(3):491s-499s. Available from: https://academic.oup.com/ajcn/article/70/3/491s/4714940
85. Zhai H, Liu H, Wang S, Wu J, Kluenter A-M. Potential of essential oils for poultry and pigs. Anim Nutr [Internet]. 2018 Jun;4(2):179–86. Available from: https://doi.org/10.1016/j.aninu.2018.01.005
86. Food and Drug Administration. Botanical Drug Development Guidance for Industry Botanical. FDA Guid. 2016;1.
87. Hanif MA, Nisar S, Khan GS, Mushtaq Z, Zubair M. Essential Oils. In: Essential Oil Research. 2019. p. 3–17.
88. Máthé A. Essential oils–biochemistry, production and utilisation. In: Phytgenics in animal nutrition: natural concepts to optimize gut health and preformance. 2009. p. 1–18.
89. Zeng Z, Zhang S, Wang H, Piao X. Essential oil and aromatic plants as feed additives in non-ruminant nutrition: a review. J Anim Sci Biotechnol [Internet]. 2015 Dec 24;6(1):7. Available from: https://jasbsci.biomedcentral.com/articles/10.1186/s40104-015-0004-5
90. Cuppett S, Hall C. Antioxidant activity of Labiatae. In: Taylor S, editor. Advances in Food and Nutrition Research. Academic Press; 1998. p. 245–71.
91. Franz C, Baser K, Windisch W. Essential oils and aromatic plants in animal feeding - a European perspective. A review. Flavour Fragr J [Internet]. 2010 Sep;25(5):327–40. Available from: http://doi.wiley.com/10.1002/ffj.1967
92. Gong J, Yin F, Hou Y, Yin Y. Review: Chinese herbs as alternatives to antibiotics in feed for swine and poultry production: Potential and challenges in application. Can J Anim Sci. 2014;94:223–41.
93. Silva Júnior CD, Martins CCS, Dias FTF, Sitanaka NY, Ferracioli LB, Moraes JE, et al. The use of an alternative feed additive, containing benzoic acid, thymol, eugenol, and piperine, improved growth performance, nutrient and energy digestibility, and gut health in weaned piglets. J Anim Sci [Internet]. 2020 May 1;98(5). Available from: https://academic.oup.com/jas/article/doi/10.1093/jas/skaa119/5819383
94. Youdim KA, Deans SG. Effect of thyme oil and thymol dietary supplementation on the antioxidant status and fatty acid composition of the ageing rat brain. Br J Nutr [Internet]. 2000 Jan;83(1):87–93. Available from:

http://www.ncbi.nlm.nih.gov/pubmed/10703468
95. Lucía A, Carmen S, Inmaculada T, Maldonado A, Belén ÁG. Antimicrobial activity of selected essential oils against Streptococcus suis isolated from pigs. 2018;(February):1–6.
96. Machlin LJ, Gordon RS, Meisky KH. The Effect of Antioxidants on Vitamin E Deficiency Symptoms and Production of Liver "Peroxide" in the Chicken. J Nutr [Internet]. 1959 Feb 1;67(2):333–43. Available from: https://academic.oup.com/jn/article/67/2/333-343/4777628
97. Lu T, Harper AF, Zhao J, Estienne MJ, Dalloul RA. Supplementing antioxidants to pigs fed diets high in oxidants: I. Effects on growth performance, liver function, and oxidative status. J Anim Sci [Internet]. 2014 Dec 1;92(12):5455–63. Available from: https://academic.oup.com/jas/article/92/12/5455/4703275
98. Costa L, Luciano F, Miyada V, Gois F. Review article: Herbal extracts and organic acids as natural feed additives in pig diets. S Afr J Anim Sci [Internet]. 2013 Jun 7;43(2):181–93. Available from: http://www.ajol.info/index.php/sajas/article/view/91221
99. Nakatani N. Phenolic antioxidants from herbs and spices. BioFactors. 2000;13(1–4):141–6.
100. Sies H. Oxidative stress: oxidants and antioxidants. Exp Physiol [Internet]. 1997 Mar 1;82(2):291–5. Available from: http://doi.wiley.com/10.1113/expphysiol.1997.sp004024
101. Bayne A-C V., Sohal RS. Effects of superoxide dismutase/catalase mimetics on life span and oxidative stress resistance in the housefly, Musca domestica. Free Radic Biol Med [Internet]. 2002 Jun;32(11):1229–34. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0891584902008493
102. Padurariu M, Ciobica A, Hritcu L, Stoica B, Bild W, Stefanescu C. Changes of some oxidative stress markers in the serum of patients with mild cognitive impairment and Alzheimer's disease. Neurosci Lett [Internet]. 2010 Jan;469(1):6–10. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0304394009014992
103. Cheng C, Liu Z, Zhou Y, Wei H, Zhang X, Xia M, et al. Effect of oregano essential oil supplementation to a reduced-protein, amino acid-supplemented diet on meat quality, fatty acid composition, and oxidative stability of Longissimus thoracis muscle in growing-finishing pigs. Meat Sci [Internet]. 2017 Nov;133:103–9. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0309174017300761
104. Schweer W, Gabler N. Alternatives to Growth-promoting Antibiotics. Natl Pork Board Rep. 2017;
105. Liao SF, Nyachoti M. Using probiotics to improve swine gut health and nutrient utilization [Internet]. Vol. 3, Animal Nutrition. Elsevier Taiwan LLC; 2017. p. 331–43. Available from: https://doi.org/10.1016/j.aninu.2017.06.007
106. Ryan MT, Collins CB, O'Doherty J V., Sweeney T. Effects of dietary β-glucans supplementation on cytokine expression in porcine liver. J Anim Sci [Internet]. 2012 Dec 1;90:40–2. Available from: https://academic.oup.com/jas/article/90/suppl_4/40/4704230
107. Russo P, López P, Capozzi V, de Palencia PF, Dueñas MT, Spano G, et al. Beta-

Glucans Improve Growth, Viability and Colonization of Probiotic Microorganisms. Int J Mol Sci [Internet]. 2012 May 18;13(5):6026–39. Available from: http://www.mdpi.com/1422-0067/13/5/6026
108. Miguel JC, Rodriguez-Zas SL, Pettigrew JE. Efficacy of a mannan oligosaccharide (Bio-Mos®) for improving nursery pig performance. Vol. 12, Journal of Swine Health and Production. 2004. p. 296–307.
109. Che L, Hu L, Liu Y, Yan C, Peng X, Xu Q, et al. Dietary Nucleotides Supplementation Improves the Intestinal Development and Immune Function of Neonates with Intra-Uterine Growth Restriction in a Pig Model. Simeoni U, editor. PLoS One [Internet]. 2016 Jun 15;11(6):e0157314. Available from: https://dx.plos.org/10.1371/journal.pone.0157314
110. Sauer N, Mosenthin R, Bauer E. The role of dietary nucleotides in single-stomached animals. Nutr Res Rev. 2011;24(1):46–59.
111. Koolman J, Roehm K-H. Color Atlas of Biochemistry. Stuttgart, Germany: Thieme Medical Publishers; 2013. 178–185 p.
112. Sánchez-Pozo A, Gil A. Nucleotides as semiessential nutritional components. Br J Nutr [Internet]. 2002;87 Suppl 1(May):S135-7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11895150
113. Ortega MAA, Nunez MAC, Gil A, Nchez-pozo ASÃ. Biochemical and Molecular Roles of Nutrients Dietary Nucleotides Accelerate Intestinal after Food Deprivation in Old Rats. Biochem Mol Roles Nutr. 1995;(July 1994):1413–8.
114. Carlson M, Veum T, Turk J. Effects of yeast extract versus animal plasma in weanling pig diets on growth performance and intestinal morphology. J Swine Heal Prod [Internet]. 2005;13(August):204–9. Available from: http://www.aasv.org/shap.html.
115. Uauy R, Quan R, Gil A. Role of Nucleotides in Intestinal Development and Repair: Implications for Infant Nutrition. J Nutr [Internet]. 1994 Aug 1;124(suppl 8):1436S-1441S. Available from: https://academic.oup.com/jn/article/124/suppl_8/1436S/4730470
116. Bueno J, Torres M, Almendros A, Carmona R, Nufiez MC, Rios A, et al. Effect of dietary nucleotides on small intestinal repair after diarrhoea. Histological and ultrastructural changes. Gut. 1994;35(7):926–33.
117. He Y, Chu SH, Walker W a. Nucleotide supplements alter proliferation and differentiation of cultured human (Caco-2) and rat (IEC-6) intestinal epithelial cells. J Nutr [Internet]. 1993 Jun;123(6):1017–27. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8505661
118. Halas V, Nochta I. Mannan Oligosaccharides in Nursery Pig Nutrition and Their Potential Mode of Action. Animals. 2012;2:261–74.
119. Spring P, Wenk C, Connolly A, Kiers A. Review Article A review of 733 published trials on Bio-Mos®, a mannan oligosaccharide, and Actigen®, a second generation mannose rich fraction, on farm and companion animals. J Appl Anim Nutr. 2017;3(May):1–11.
120. Duan X, Tian G, Chen D, Huang L, Zhang D, Zheng P, et al. Mannan oligosaccharide supplementation in diets of sow and (or) their offspring improved immunity and regulated intestinal bacteria in piglet. J Anim Sci. 2019;97(11):4548–56.

121. Volman JJ, Ramakers JD, Plat J. Dietary modulation of immune function by β -glucans. Physiol Behav. 2008;94(2008):276–84.
122. Shurson GC, Hung YT, Jang JC, Urriola PE. Measures matter—determining the true nutri-physiological value of feed ingredients for swine. Animals. 2021;11(5):1–50.
123. Luo J, Zeng D, Cheng L, Mao X, Yu J, Yu B, et al. Dietary β-glucan supplementation improves growth performance, carcass traits and meat quality of finishing pigs. Anim Nutr [Internet]. 2019 Dec;5(4):380–5. Available from: https://linkinghub.elsevier.com/retrieve/pii/S2405654518303202
124. Hahn T, Lohakare JD, Lee SL, Moon WK, Chae BJ. Effects of supplementation of β -glucans on growth performance, nutrient digestibility, and immunity in weanling pigs Effects of supplementation of β -glucans on growth performance, nutrient digestibility, and immunity in weanling pigs. J Anim Sci. 2016;84(April):1422–8.
125. Ranjan R, Rani A, Metwally A, McGee HS, Perkins DL. Analysis of the microbiome: Advantages of whole genome shotgun versus 16S amplicon sequencing. Biochem Biophys Res Commun [Internet]. 2016 Jan;469(4):967–77. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0006291X15310883
126. Janda JM, Abbott SL. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. Vol. 45, Journal of Clinical Microbiology. 2007. p. 2761–4.
127. National Academies of Sciences, Engineering and M. Environmental Chemicals, the Human Microbiome, and Health Risk [Internet]. Washington, D.C.: National Academies Press; 2018. Available from: https://www.nap.edu/catalog/24960
128. Hamady M, Knight R. Microbial community profiling for human microbiome projects: Tools, techniques, and challenges. Genome Res [Internet]. 2009 Jul 1;19(7):1141–52. Available from: http://genome.cshlp.org/cgi/doi/10.1101/gr.085464.108
129. Mande SS, Mohammed MH, Ghosh TS. Classification of metagenomic sequences: methods and challenges. Brief Bioinform. 2012;13(6):669–81.
130. Pitt JJ. Principles and Applications of Liquid Chromatography- Mass Spectrometry in Clinical Biochemistry. Clin Biochem Rev. 2009;30(February):19–34.
131. Roberts LD, Souza AL, Gerszten RE, Clish CB. Current Protocols in Molecular Biology [Internet]. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, et al., editors. Vol. 30, Curr protoc mol biol. Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2001. 1–34 p. Available from: http://doi.wiley.com/10.1002/0471142727
132. Chen H, Peng L, Perez de Nanclares M, Trudeau MP, Yao D, Cheng Z, et al. Identification of Sinapine-Derived Choline from a Rapeseed Diet as a Source of Serum Trimethylamine N-Oxide in Pigś. J agriultural food Chem. 2019;67:7748–54.
133. Overland M, Rørvik KA, Skrede A. Effect of trimethylamine oxide and betaine in swine diets on growth performance, carcass characteristics, nutrient digestibility, and sensory quality of pork. J Anim Sci [Internet]. 1999;77(8):2143. Available from: https://academic.oup.com/jas/article/77/8/2143-2153/4653367
134. Chen C, Pérez de Nanclares M, Kurtz JF, Trudeau MP, Wang L, Yao D, et al.

Identification of redox imbalance as a prominent metabolic response elicited by rapeseed feeding in swine metabolome. J Anim Sci. 2018;96(5):1757–68.
135. Luecke RW, Thorp F, Newland HW, Mcmillen WN. The Growth Promoting Effects of Various Antibiotics on Pigs. J Anim Sci [Internet]. 1951 May 1;10(2):538–42. Available from: https://academic.oup.com/jas/article/10/2/538/4728600
136. Bridges JH, Dyer IA, Burkhart WC. Effects of Penicillin and Streptomycin on the Growth Rate and Bacterial Count in the Feces of Pigs. J Anim Sci [Internet]. 1952 Aug 1;11(3):474–9. Available from: https://academic.oup.com/jas/article/11/3/474/4761087
137. Lucas IAM, Calder AFC. The growth of pigs kept to one level of feeding, in two environments, and fed diets with and without an antibiotic. J Agric Sci [Internet]. 1955 Oct 27;46(3):307–19. Available from: https://www.cambridge.org/core/product/identifier/S0021859600040247/type/journal_article
138. Briggs JE, Beeson WM. Further Studies on the Supplementary Value of Aureomycin, Streptomycin, and Vitamin B12 in a Plant Protein Ration for Growing-Fattening Pigs. J Anim Sci [Internet]. 1951 Nov 1;10(4):820–7. Available from: https://academic.oup.com/jas/article/10/4/820/4760998
139. Perry TW, Beeson WM, Vosteen BW. The Effect of an Antibiotic or a Surfactant on the Growth and Carcass Composition of Swine. J Anim Sci [Internet]. 1953 May 1;12(2):310–5. Available from: https://academic.oup.com/jas/article/12/2/310/4761149
140. Berry ME, Schuck C. The Effect of Aureomycin on Growth and Protein Utilization in the Rat. J Nutr [Internet]. 1954 Oct 1;54(2):271–84. Available from: https://academic.oup.com/jn/article/54/2/271-284/4722679
141. Wahlstrom RC. The Effect of High Level Antibiotic Supplementation during Part or All of the Growing-Fattening Period of Swine. J Anim Sci [Internet]. 1956 Nov 1;15(4):1059–66. Available from: https://academic.oup.com/jas/article/15/4/1059/4761685
142. Chao A. Nonparametric Estimation of the Number of Classes in a Population. Scand J Stat. 1984;11(4):265–70.
143. Magurran A. Measuring Biological Diversity. Blackwell Science Ltd, Oxford, United Kingdom.; 2004.
144. Simpson EH. Measurement of Diversity. Nature. 1949;163(1943):688.
145. Bray JR, Curtis JT. An Ordination of the Upland Forest Communities of Southern Wisconsin. Ecol Monogr [Internet]. 1957 Feb;27(4):325–49. Available from: http://doi.wiley.com/10.2307/1942268
146. Jaccard P. Contribution au problème de l'immigration post-glaciaire de la flore alpine. Bull la Société Vaudoise des Sci Nat. 1900;(36):87–130.
147. Dufrene M, Legendre P. Species Assemblages and Indicator Species: The Need for a Flexible Asymmetrical Approach. Ecol Monogr [Internet]. 1997 Aug;67(3):345. Available from: http://www.jstor.org/stable/2963459?origin=crossref
148. Tin Kam Ho. The random subspace method for constructing decision forests. IEEE Trans Pattern Anal Mach Intell [Internet]. 1998;20(8):832–44. Available from: http://ieeexplore.ieee.org/document/709601/

149. Knights D, Kuczynski J, Charlson E, Zaneveld J, Mozer MC, Collman RG, et al. Bayesian community-wide culture-independent microbial source tracking. Nat Methods. 2013;8(9):761–3.
150. Shenhav L, Thompson M, Joseph TA, Briscoe L, Furman O, Bogumil D, et al. FEAST: fast expectation-maximization for microbial source tracking. Nat Methods [Internet]. 2019 Jul 10;16(7):627–32. Available from: http://www.nature.com/articles/s41592-019-0431-x
151. Worley B, Powers R. Multivariate Analysis in Metabolomics Bradley. Curr metabolomics. 2015;1(1):92–107.
152. Benton P, Ivaisevic J, Rinehart D, Epstein A, Kurczy M, Boska M, et al. An Interactive Cluster Heat Map to Visualize and Explore Multidimensional Metabolomic Data. Metabolomics. 2015;11(4):1029–34.
153. Schober P, Boer C, Schwarte LA. Correlation Coefficients. Anesth Analg [Internet]. 2018 May;126(5):1763–8. Available from: http://journals.lww.com/00000539-201805000-00050
154. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape : A Software Environment for Integrated Models of Biomolecular Interaction Networks. Genome Res. 2003;13:2498–504.
155. Jassal B, Matthews L, Viteri G, Gong C, Lorente P, Fabregat A, et al. The reactome pathway knowledgebase. Nucleic Acids Res. 2019;48:498–503.
156. Gonzalez-Dominguez A, Duran-Guerrero E, Fernandez-Recamales A, Lechuga-Sancho AM, Sayago A, Schwarz M, et al. An Overview on the Importance of Combining Complementary Analytical Platforms in Metabolomic Research. Curr Top Med Chem. 2017;17(30):3289–95.
157. Tubiello FN, Salvatore M, Rossi S, Ferrara A, Fitton N, Smith P. The FAOSTAT database of greenhouse gas emissions from agriculture. Environ Res Lett [Internet]. 2013 Mar 1;8(1):e015009. Available from: https://iopscience.iop.org/article/10.1088/1748-9326/8/1/015009
158. Dibner JJ, Richards JD. Antibiotic growth promoters in agriculture: history and mode of action. Poult Sci [Internet]. 2005 Apr;84(4):634–43. Available from: https://linkinghub.elsevier.com/retrieve/pii/S003257911944577X
159. Ghosh S, LaPara TM. The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. ISME J. 2007;1(3):191–203.
160. Martinez JL. Environmental pollution by antibiotics and by antibiotic resistance determinants [Internet]. Vol. 157, Environmental Pollution. Elsevier Ltd; 2009. p. 2893–902. Available from: http://dx.doi.org/10.1016/j.envpol.2009.05.051
161. Van Den Bogaard AE, Stobberingh EE. Epidemiology of resistance to antibiotics: Links between animals and humans. Int J Antimicrob Agents. 2000;14(4):327–35.
162. Coates ME, Fuller R, Harrison GF, Lev M, Suffolk SF. A comparision of the growth of chicks in the Gustafsson germ-free apparatus and in a conventional environment, with and without dietary supplements of penicillin. Br J Nutr [Internet]. 1963 Feb 9;17(1):141–50. Available from: https://www.cambridge.org/core/product/identifier/S0007114563000167/type/journal_article
163. Zoetendal EG, Cheng B, Koike S, Mackie RI. Molecular microbial ecology of the

gastrointestinal tract: from phylogeny to function. Curr Issues Intest Microbiol [Internet]. 2004 Sep;5(2):31–47. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15460065
164. Kim HB, Borewicz K, White BA, Singer RS, Sreevatsan S, Tu ZJ, et al. Microbial shifts in the swine distal gut in response to the treatment with antimicrobial growth promoter, tylosin. Proc Natl Acad Sci [Internet]. 2012;109(38):15485–90. Available from: http://www.pnas.org/cgi/doi/10.1073/pnas.1205147109
165. Donia MS, Fischbach MA. Small molecules from the human microbiota. Science (80-) [Internet]. 2015 Jul 24;349(6246):1254766–1254766. Available from: https://www.sciencemag.org/lookup/doi/10.1126/science.1254766
166. Obuseng VC, Moshoeshoe M, Nareetsile F. Bile acids as specific faecal pollution indicators in water and sediments. Eur Sci J. 2013;9(12):273–86.
167. Lu Y, Yao D, Chen C. 2-Hydrazinoquinoline as a Derivatization Agent for LC-MS-Based Metabolomic Investigation of Diabetic Ketoacidosis. Metabolites [Internet]. 2013 Oct 31;3(4):993–1010. Available from: http://www.mdpi.com/2218-1989/3/4/993/
168. Dixon P. VEGAN, a package of R functions for community ecology. J Veg Sci. 2003;14(6):927–30.
169. Lahti L, Shetty S. Tools for microbiome analysis in R. [Internet]. Microbiome package version 1.1.10012. 2017. Available from: http://bioconductor.org/packages/release/bioc/manuals/microbiome/man/microbiome.pdf
170. Beisner BE, Haydon DT, Cuddington K. Alternative Stable States in Ecology. Front Ecol Environ [Internet]. 2003 Sep;1(7):376. Available from: http://doi.wiley.com/10.1890/1540-9295(2003)001[0376:ASSIE]2.0.CO;2
171. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. Nature. 2012;489(7415):220–30.
172. Staels B, Fonseca VA. Bile acids and metabolic regulation: mechanisms and clinical responses to bile acid sequestration. Diabetes Care. 2009;32 Suppl 2:237–45.
173. Antunes LCM, Han J, Ferreira RBR, Lolić P, Borchers CH, Finlay BB. Effect of Antibiotic Treatment on the Intestinal Metabolome. Antimicrob Agents Chemother [Internet]. 2011 Apr;55(4):1494–503. Available from: https://aac.asm.org/content/55/4/1494
174. Swann JR, Want EJ, Geier FM, Spagou K, Wilson ID, Sidaway JE, et al. Systemic gut microbial modulation of bile acid metabolism in host tissue compartments. Proc Natl Acad Sci [Internet]. 2011 Mar 15;108(Supplement_1):4523–30. Available from: http://www.pnas.org/cgi/doi/10.1073/pnas.1006734107
175. Sayin SI, Wahlström A, Felin J, Jäntti S, Marschall H-U, Bamberg K, et al. Gut Microbiota Regulates Bile Acid Metabolism by Reducing the Levels of Tauro-beta-muricholic Acid, a Naturally Occurring FXR Antagonist. Cell Metab [Internet]. 2013 Feb;17(2):225–35. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1550413113000119
176. Haslewood G. Bile salts of germ-free domestic fowl and pigs. Biochem J. 1971;123:15–8.
177. Eyssen HJ, De Pauw G, Van Eldere J. Formation of Hyodeoxycholic Acid from

Muricholic Acid and Hyocholic Acid by an Unidentified Gram-Positive Rod Termed HDCA-1 Isolated from Rat Intestinal Microflora. Appl Environ Microbiol [Internet]. 1999 Jul 1;65(7):3158–63. Available from: https://aem.asm.org/content/65/7/3158
178. Zhang Y, Limaye PB, Renaud HJ, Klaassen CD. Effect of various antibiotics on modulation of intestinal microbiota and bile acid profile in mice. Toxicol Appl Pharmacol. 2014;277(2):138–45.
179. Ridlon JM, Hylemon PB. Identification and characterization of two bile acid coenzyme A transferases from Clostridium scindens, a bile acid 7α-dehydroxylating intestinal bacterium. J Lipid Res [Internet]. 2012 Jan;53(1):66–76. Available from: http://www.jlr.org/lookup/doi/10.1194/jlr.M020313
180. Tracy JD, Jensen AH. Effects of a Dietary Antimicrobial (Carbadox) on Liver Cholesterol 7α-Hydroxylase Activity and Bile Acid Patterns in the Young Pig. J Anim Sci [Internet]. 1987 Oct 1;65(4):1013–8. Available from: https://academic.oup.com/jas/article/65/4/1013-1018/4662590
181. Macfarlan G, Gibson G. Microbial aspects of the production of short-chain fatty acids in the large bowel. In: Cummings J, Rombeau J, Sakata T, editors. Physiological and Clinical Aspects of Short-Chain Fatty Acids. Cambridge University Press; 1995. p. 87–106.
182. Høverstad T, Carlstedt-Duke B, Lingaas E, Norin E, Saxerholt H, Steinbakk M, et al. Influence of Oral Intake of Seven Different Antibiotics on Faecal Short-Chain Fatty Acid Excretion in Healthy Subjects. Scand J Gastroenterol [Internet]. 1986 Jan 8;21(8):997–1003. Available from: http://www.tandfonline.com/doi/full/10.3109/00365528608996411
183. Cho I, Yamanishi S, Cox L, Methe BA, Zavadil J, Li K, et al. Antibiotics in early life alter the murine colonic microbiome and adiposity. Nature. 2012;488:621–9.
184. Giguere S, Prescott JF, Baggot JDJ, Walker RDR, Dowling PPM. Antimicrobial Therapy in Veterinary Medicine [Internet]. Giguère S, Prescott JF, Dowling PM, editors. Wiley; 2013. Available from: https://onlinelibrary.wiley.com/doi/book/10.1002/9781118675014
185. Ríos-Covián D, Ruas-Madiedo P, Margolles A, Gueimonde M, de los Reyes-Gavilán CG, Salazar N. Intestinal Short Chain Fatty Acids and their Link with Diet and Human Health. Front Microbiol [Internet]. 2016 Feb 17;7(FEB):1–9. Available from: http://journal.frontiersin.org/Article/10.3389/fmicb.2016.00185/abstract
186. Eyssen H. Role of the gut microflora in metabolism of lipids and sterols. Proc Nutr Soc [Internet]. 1973 Sep;32(2):59–63. Available from: http://www.ncbi.nlm.nih.gov/pubmed/4598411
187. Feighner SD, Dashkevicz MP. Effect of dietary carbohydrates on bacterial cholyltaurine hydrolase in poultry intestinal homogenates. Appl Environ Microbiol [Internet]. 1988 Feb;54(2):337–42. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3355130
188. Stanton TB. A call for antibiotic alternatives research. Trends Microbiol [Internet]. 2013;21(3):111–3. Available from: http://dx.doi.org/10.1016/j.tim.2012.11.002
189. DeLange CFM, Pluske J, Gong J, Nyachoti CM. Strategic use of feed ingredients and feed additives to stimulate gut health and development in young pigs. Livest

Sci. 2010;134:124–34.
190. Pluske JR. Feed- and feed additives-related aspects of gut health and development in weanling pigs. J Anim Sci Biotechnol [Internet]. 2013 Dec 7;4(1):1. Available from: https://jasbsci.biomedcentral.com/articles/10.1186/2049-1891-4-1
191. Liu Y, Espinosa CD, Abelilla JJ, Casas GA, Lagos LV, Lee SA, et al. Non-antibiotic feed additives in diets for pigs: A review. Anim Nutr [Internet]. 2018 Jun;4(2):113–25. Available from: https://doi.org/10.1016/j.aninu.2018.01.007
192. Thomke S, Elwinger K, Thomke S, Elwinger K, Growth I, Thomke S. Growth promotants in feeding pigs and poultry: Growth and feed efficiency responses to antibiotic growth promotants. Ann Zootech INRA/EDP Sci. 1998;47(2):85–97.
193. Zhu Y-G, Johnson TA, Su J-Q, Qiao M, Guo G-X, Stedtfeld RD, et al. Diverse and abundant antibiotic resistance genes in Chinese swine farms. Proc Natl Acad Sci [Internet]. 2013;110(9):3435–40. Available from: http://www.pnas.org/lookup/doi/10.1073/pnas.1222743110
194. Andersson DI, Hughes D. Antibiotic resistance and its cost: Is it possible to reverse resistance? Vol. 8, Nature Reviews Microbiology. Nature Publishing Group; 2010. p. 260–71.
195. Chen C, Gonzalez FJ. LC-MS-Based Metabolomics in Drug Metabolism. Drug metab rev. 2007;39(2–3):581–97.
196. Jari Oksanen F, Blanchet G, Friendly M, Kindt R, Legendre P, McGlinn D, et al. vegan: Community Ecology Package [Internet]. 2019. Available from: https://cran.r-project.org/web/packages/vegan/index.html
197. Giraudoux P. pgirmess: Spatial Analysis and Data Mining for Field Ecologists [Internet]. 2018. Available from: https://cran.r-project.org/web/packages/pgirmess/index.html
198. Gohl DM, Vangay P, Garbe J, Maclean A, Hauge A, Becker A, et al. Analysis Systematic improvement of amplicon marker gene methods for increased accuracy in microbiome studies. Nat Biotechnol. 2016;34(9):942–53.
199. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J [Internet]. 2011 May 2;17(1):10. Available from: http://journal.embnet.org/index.php/embnetjournal/article/view/200
200. Hannon GL. FASTX-Toolkit [Internet]. 2010. Available from: http://hannonlab.cshl.edu/fastx_toolkit/
201. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet C, Al-Ghalith GA, et al. QIIME 2: Reproducible, interactive, scalable, and extensible microbiome data science. PeerJ Prepr [Internet]. 2018 Oct;6:e27295v1. Available from: https://doi.org/10.7287/peerj.preprints.27295v1
202. Bushnell B. BBMap: A Fast, Accurate, Splice-Aware Aligner [Internet]. 2014. Available from: https://jgi.doe.gov/wp-content/uploads/2013/11/BB_User-Meeting-2014-poster-FINAL.pdf
203. Liaw A, Wiener M. Classification and Regression by randomForest. R News [Internet]. 2002;2(3):18–22. Available from: https://rdrr.io/cran/randomForest/man/randomForest.html
204. Verstegen MWA, Williams BA. alternatives to the use of antibiotics as growth promoters for monogastric animals. Anim Biotechnol [Internet]. 2002 Jul 11;13(1):113–27. Available from:

http://www.tandfonline.com/doi/abs/10.1081/ABIO-120005774
205. Regmi N, Wang T, Crenshaw MA, Rude BJ, Wu G, Liao SF. Effects of dietary lysine levels on plasma free amino acid profile in late-stage finishing pigs. Springerplus [Internet]. 2016 Dec 24;5(1):888. Available from: http://springerplus.springeropen.com/articles/10.1186/s40064-016-2463-3
206. Liao SF, Wang T, Regmi N. Lysine nutrition in swine and the related monogastric animals : muscle protein biosynthesis and beyond. Springerplus. 2015;4:147.
207. Li DF, Zhang JH, Gong LM. Optimum ratio of histidine in the piglet ideal protein model and its effects on the body metabolism: II. Optimum ratio of histidine in 10-20 kg piglet ideal protein and its effects on blood parameters. Arch Anim Nutr fur Tierernahrung. 2002;56(3):199–212.
208. Schweer W. Amino acid requirements of health challenged pigs. Iowa State Univ Digit Repos [Internet]. 2018;1–188. Available from: https://www.cambridge.org/core/product/identifier/CBO9781107415324A009/type/book_part
209. Kers JG, Velkers FC, Fischer EAJ, Hermes GDA, Lamot DM, Stegeman JA, et al. Take care of the environment: housing conditions affect the interplay of nutritional interventions and intestinal microbiota in broiler chickens. Anim Microbiome [Internet]. 2019 Dec 27;1(1):10. Available from: https://animalmicrobiome.biomedcentral.com/articles/10.1186/s42523-019-0009-z
210. Looft T, Johnson TA, Allen HK, Bayles DO, Alt DP, Stedtfeld RD, et al. In-feed antibiotic effects on the swine intestinal microbiome. Proc Natl Acad Sci U S A. 2012;109(5):1691–6.
211. Looft T, Allen HK, Cantarel BL, Levine UY, Bayles DO, Alt DP, et al. Bacteria, phages and pigs: The effects of in-feed antibiotics on the microbiome at different gut locations. ISME J. 2014;8(8):1566–76.
212. Fangman TJ, Abvp-shm D, Hardin LE, Grellner G, Carlson MS, Zulovich JM, et al. Case study Performance and disease status of pigs grown in a conventional nursery and grower- finisher facility. J swine Heal Prod. 2001;9(2):71–6.
213. Smith F, Clark JE, Overman BL, Tozel CC, Huang JH, Rivier JEF. Early weaning stress impairs development of mucosal barrier function in the porcine intestine. Am J Physiol. 2010;298(3).
214. Hong JW, Kim IH, Kwon OS, Min BJ, Lee WB, Shon KS. Influences of Plant Extract Supplementation on Performance and Blood Characteristics in Weaned Pigs. Asian-Australasian J Anim Sci [Internet]. 2004 Jan 1;17(3):374–8. Available from: http://ajas.info/journal/view.php?doi=10.5713/ajas.2004.374
215. Bravo D, Pirgozliev V, Rose SP. A mixture of carvacrol , cinnamaldehyde , and capsicum oleoresin improves energy utilization and growth performance of broiler chickens fed maize-based diet. 2014;1531–6.
216. Burt S. Essential oils : their antibacterial properties and potential applications in foods — a review. Int J Food Microbiol. 2004;94:223–53.
217. Liu Y, Song M, Che TM, Bravo D, Pettigrew JE. Anti-inflammatory effects of several plant extracts on porcine alveolar macrophages in vitro. J Anim Sci. 2012;90:2774–83.
218. Bajagai YS, Klieve AV, Dart PJ, Bryden WL. Probiotics in animal nutrition-production, impact, and regulation. Makkar H, editor. Rome: FAO Animal

Production and Health; 2016.
219. Jung B, Batal AB. Effect of dietary nucleotide supplementation on performance and development of the gastrointestinal tract of broilers. Br Poult Sci. 2012;53(1):98–105.
220. Domeneghini C, Di Giancamillo A, Savoini G, Paratte R, Bontempo V, Dell'Orto V. Structural patterns of swine ileal mucosa following L-glutamine and nucleotide administration during the weaning period. An histochemical and histometrical study. Histol Histopathol. 2004;19(1):49–58.
221. Pan DD, Wu Z, Peng T, Zeng XQ, Li H. Volatile organic compounds profile during milk fermentation by Lactobacillus pentosus and correlations between volatiles flavor and carbohydrate metabolism. J Dairy Sci. 2014;97:624–31.
222. Suiryanrayna M, Ramana J. A review of the effects of dietary organic acids fed to swine. J Anim Sci Biotechnol. 2015;6(45):1–11.
223. Papatsiros VG, Christodoulopoulos G, Filippopoulos LC. The use of organic acids in monogastric animals (swine and rabbits). J cell Anim Biol. 2012;6(10):154–9.
224. van der Aar PJ, Molist F, van der Klis JD. The central role of intestinal health on the effect of feed additives on feed intake in swine and poultry. Anim Feed Sci Technol [Internet]. 2017 Nov;233:64–75. Available from: https://doi.org/10.1016/j.anifeedsci.2016.07.019
225. R core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing [Internet]. Vienna, Austria; 2013. Available from: https://cran.r-project.org/doc/manuals/fullrefman.pdf
226. Roberts DW. Package 'labdsv' [Internet]. 2019. Available from: https://cran.r-project.org/web/packages/labdsv/labdsv.pdf
227. Suresh Babu P, Srinivasan K. Hypolipidemic action of curcumin, the active principle of turmeric (Curcuma longa) in streptozotocin induced diabetic rats. Mol Cell Biochem [Internet]. 1997 Jan;166(1–2):169–75. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9046034
228. Joe B, Lokesh BR. Prophylactic and therapeutic effects of n-3 polyunsaturated fatty acids, capsaicin, and curcumin on adjuvant induced arthritis in rats. J Nutr Biochem [Internet]. 1997 Jul;8(7):397–407. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0955286397000478
229. Dixit VP, Jain P, Joshi SC. Hypolipidaemic effects of Curcuma longa L and Nardostachys jatamansi, DC in triton-induced hyperlipidaemic rats. Indian J Physiol Pharmacol [Internet]. 1988;32(4):299–304. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3215683
230. Alagbe JO. Growth performance and blood parameters of weaner pigs fed diets supplemented with turmeric powder. J Agric Sci. 2017;7(2):57–61.
231. Benzie IFF, Wachtel-Galor S. Turmeric, the Golden Spice. In: Herbal Medicine: Biomolecular and Clinical Aspects [Internet]. 2nd editio. Boca Raton, FL: CRC Press/Taylor & Francis; 2011. Available from: https://www.ncbi.nlm.nih.gov/books/NBK92752
232. Pero RW, Lund H, Leanderson T. Antioxidant Metabolism Induced by Quinic Acid . Increased Urinary Excretion of Tryptophan and Nicotinamide. phytother res. 2009;346(May 2008):335–46.
233. Ratriyanto A, Mosenthin R, Bauer E, Eklund M. Metabolic, Osmoregulatory and

Nutritional Functions of Betaine in Monogastric Animals. Asian-Australasian J Anim Sci [Internet]. 2009 Aug 26;22(10):1461–76. Available from: http://ajas.info/journal/view.php?doi=10.5713/ajas.2009.80659
234. Barnard DL, Huffman JH, Meyerson LR, Sidwell RW. Mode of inhibition of respiratory syncytial virus by a plant flavonoid. Chemotherapy. 1993;39:212–7.
235. Kaul TN, Middleton E, Ogra PL. Antiviral effect of flavonoids on human viruses. J Med Virol [Internet]. 1985 Jan;15(1):71–9. Available from: http://doi.wiley.com/10.1002/jmv.1890150110
236. Silalahi J. Anticancer and health protective properties of citrus fruit components. Asia Pac J Clin Nutr [Internet]. 2002 Mar;11(1):79–84. Available from: http://doi.wiley.com/10.1046/j.1440-6047.2002.00271.x
237. Hui Shuang Y, Bor Chun W, Tu Fa L. Effects of Chinese traditional herbal medicine complex supplementation on the growth performance, immunity and serum traits of pigs. Anim Sci J [Internet]. 2011 Dec;82(6):747–52. Available from: http://doi.wiley.com/10.1111/j.1740-0929.2011.00897.x
238. Lindblom SC, Gabler NK, Dilger RN, Olson ZF, Loving CL, Kerr BJ. Influence of feeding thermally peroxidized soybean oil on oxidative status in growing pigs. J Anim Sci. 2018;96(2):545–57.
239. Yannai S. Dictionary of food compounds with CD-ROM: Additives, flavors, and ingredients. Boca Raton, FL: Chapman & Hall/CRC Press; 2004.
240. Shen L, Liu L, Ji H. Regulative effects of curcumin spice administration on gut microbiota and its pharmacological implications. food Nutr Res. 2017;61:1654–61.
241. Gan Z, Wei W, Li Y, Wu J, Zhao Y, Zhang L, et al. Curcumin and resveratrol regulate intestinal bacteria and alleviate intestinal inflammation in weaned piglets. Molecules. 2019;24(7).
242. Labeda DP, Goodfellow M, Brown R, Ward AC, Lanoot B, Vanncanneyt M, et al. Phylogenetic study of the species within the family Streptomycetaceae. Springer. 2012;101:73–104.
243. Valeriano VDV, Balolong MP, Kang D-K. Probiotic roles of Lactobacillus sp. in swine: insights from gut microbiota. J Appl Microbiol [Internet]. 2017 Mar;122(3):554–67. Available from: http://doi.wiley.com/10.1111/jam.13364
244. Tsai C, Hsih H, Chiu H, Lai Y. Antagonistic activity against Salmonella infection in vitro and in vivo for two Lactobacillus strains from swine and poultry. Int J Food Microbiol. 2005;102:185–94.
245. van Immerseel F, Cauwerts K, Devriese LA, Haesebrouck F, Ducatelle R. Feed additives to control Salmonella in poultry. Worlds Poult Sci J [Internet]. 2002;58(December):501–13. Available from: http://journals.cambridge.org/abstract_S0043933902000363