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PROBIOTIC EFFECTS ON BACTERIAL COMMUNITY STRUCTURE AND ANTIBIOTIC RESISTANCE OF GUT MICROBIOTA IN BROILER CHICKENS RAISED UNDER THREE DIFFERENT DIETARY CONDITIONS

A Thesis in

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by

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ABSTRACT

Antimicrobial resistance of bacterial pathogens has developed into a global issue and has become an imminent threat to both human and animal health. The sub-therapeutic levels of antimicrobial supplementation in food animal feed are consistently argued to contribute to antimicrobial resistance. Not only do poultry producers use antimicrobials as feed additives, but they also utilize probiotics as a live microbial feed supplement. Probiotic supplementation positively affects the chicken by improving its intestinal balance and can be used as an alternative to antimicrobial supplementation during poultry production.

The United States (U.S.) is the world's largest poultry producer and the second largest poultry exporter, with broiler chickens comprising over four-fifths of U.S. poultry production. Because broiler producers in the U.S. use antimicrobials for disease therapy, prophylaxis, and growth promotion, it is important to understand the contribution of antimicrobial use to the emergence of antimicrobial resistant bacteria and their associated genes as well as changes in resident microbiota. The effect of supplementing commercial broiler diet with probiotics, including conventionally-fed broiler chickens, is an important concept, as the resident microbiota may be significant for the prevention of such colonization.

Using a field-based trial approach, the gut microbiota and antibiotic resistance genes in broiler chickens fed with three commercial diet types: conventional (antibiotic-supplemented with and without probiotics), natural (antibiotic-free and probiotic-supplemented) and organic (antibiotic-free with organic standard probiotics), were

compared with real-time polymerase chain reaction assay using the Comparative Ct method. To determine phenotypic validation of antimicrobial resistance, microbial DNA recovered from broiler chicken feces was utilized for DNA library construction. Genotypic presence of resistance genes was compared among diet types using the Comparative Ct method.

It was hypothesized that the microbiota of conventionally-fed broiler chickens would be significantly different than both the microbiotas of chickens fed natural or organic diets. Furthermore, it was also hypothesized that there would be an increased amount of antimicrobial resistance genes found among broiler chickens that had been fed conventional diets when compared to broiler chickens fed a natural or organic diet.

At variable levels, antimicrobial resistance can be found among every microbiota from each diet type (conventional with and without probiotics, natural, and organic). The antimicrobial resistance found in both the antibiotic-free diets (organic and natural) was not a result of the supplementation of antimicrobials in feed. However, even with antimicrobial resistance found within each broiler chicken microbiota from each diet type, chickens fed with conventional diets contained more resistance genes in feces than chickens fed with natural and organic diets suggesting the addition of antimicrobials in diet may influence the microbial community structure and increase the antibiotic resistance gene repertoire in the commercial broiler gut.

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Chapter 1

Review of Literature

1.1 Antimicrobial Use in Poultry Production

1.1.1 Commercial broiler production in the U.S.

In 2013, broiler chicken production in the United States (U.S.) accounted for $30.7 billion, and the total commercial broiler live weight amounted to 50.6 billion pounds. Both values were up 24 and 2 percent, respectively, from 2012 (1). Below lists broiler chicken productivity by state:

Table 1: 2013 State Breakdown of Broiler Chicken Production and Value

Broiler Production and Value – States, United States, and 19 State Total: 2013

[Annual estimates cover the period December 1 previous year through November 30. Broiler production including other domestic meat-type strains. Excludes States producing less than 500,000 broilers]

State	Number produced	Pounds produced	Value of production
	(1,000 head)	(1,000 pounds)	(1,000 dollars)
Alabama	1,048,600	5,872,200	3,558,553
Arkansas	996,400	5,978,400	3,622,910
Delaware	215,600	1,530,800	927,665
Florida	64,400	392,800	238,037
Georgia	1,334,600	7,607,200	4,609,963
Kentucky	309,000	1,668,600	1,011,172
Maryland	305,200	1,617,600	980,266
Minnesota	48,100	283,800	171,983
Mississippi	734,000	4,477,400	2,713,304
Missouri	277,400	1,331,500	806,889
North Carolina	785,500	5,891,300	3,570,128
Ohio	70,100	406,600	246,400
Oklahoma	206,200	1,360,900	824,705
Pennsylvania	168,800	945,300	572,852
South Carolina	226,500	1,585,500	960,813
Tennessee	172,800	898,600	544,552
Texas	610,100	3,599,600	2,181,358
Virginia	249,600	1,347,800	816,767
West Virginia	96,800	387,200	234,643
Wisconsin	53,100	223,000	135,138
Other States [1]	552,000	3,220,600	1,951,683
United States	8,524,800	50,626,700	30,679,781
19 State Total [2]	8,222,700	49,008,600	29,699,212

The U.S. exported over 7.3 billion pounds of broiler chicken meat in 2013, with Mexico being their largest export market (1). The average broiler chicken consumption

was 81.9 pounds annually per capita (2). Chicken is considered a high quality protein because it contains the eight essential amino acids and a low amount of fat. The fat in chicken is primarily made up of unsaturated fat, which has the ability to provide a defense against heart disease. For these reasons, physicians and nutritionists both recommend chicken as an alternative to red meat (3).

1.1.2 Antimicrobial use in commercial broiler production

Between 1945 and 1999, the production of broiler chickens increased rapidly from 5 billion to 40 billion pounds per year (4). This dramatic rise in production led to fewer companies being responsible for larger amounts of poultry. This shift towards traditional poultry management caused standardization in practices, such as drug treatments to control and prevent infectious diseases. Antimicrobials were often utilized as a method to inhibit infectious disease (5). Ever since Fleming's discovery of penicillin in 1928, antimicrobials have been advertised for the management and treatment of microbial infections in both animals and humans.

Antimicrobials have greatly improved public health as well as animal health. Since their discovery, antimicrobials have significantly decreased mortality and morbidity due to infectious diseases in both humans and animals. In animal agriculture, antibiotics are used for disease treatment, prevention, control, and growth (6). However, use of antibiotics on food-producing animals, in particular for nontherapeutic purposes, is under heavy criticisms. For example, because tetracycline overuse has led to resistance, tetracyclines are considered ineffective treatments for *E. coli* infected- broiler chickens.

This acquired resistance has forced the field to develop new antimicrobials like the fluoroquinolones as treatment options for *E. coli*-infected poultry (7).

1.1.3 U.S. Food and Drug Administration and antimicrobial feed additives

Due to the benefits of promoted growth, improved feed efficiency, and control of endemic diseases in large groups of animals, the U.S. Food and Drug Administration (FDA) initially approved of antimicrobials as feed additives based upon the idea of improved animal safety (10). In 1977, the FDA determined that the abuse of antimicrobials in livestock and poultry threatened the effectiveness of many antimicrobial treatments when used in human medicine (11). The FDA issued an order that would have banned all non-medical usages of penicillin and tetracycline in livestock, unless drug companies could show the drugs were harmless. The proposal was criticized because, at that time, there was not adequate epidemiological evidence to show that antimicrobial resistant bacteria of animal origin were commonly transmitted to humans and caused serious illness. Congress directed FDA to conduct further studies related to the use of antimicrobials in animal feed and to delay on the implementation of the proposed antimicrobial withdrawal actions pending the outcome of these studies (12-14).

In 1998, The Animal Health Institute stated that 17.8 million pounds of antimicrobials were used in animal production; 83% of the antimicrobials were used for prevention and treatment, and 17% were used for growth promotion (1). The FDA now reports that there are more kilograms of antimicrobials sold in the U.S. for food-producing animals than for humans (15).

1.1.4 Early Concerns of Antimicrobial Use in Poultry

In 1951, a report emerged identifying antimicrobial resistant bacteria in turkeys after feeding streptomycin (16). Soon after, other studies began to show a link between bacterial resistance to tetracycline when chickens were fed growth promoting levels in their feed (17). In a report to the British government, there was a recommended ban on sub-therapeutic levels of antimicrobials in animal feed due to the early fear of developing antimicrobial resistance in human pathogens (18). Recommendations for the reduction or complete elimination of antimicrobials were presented to the U. S. in two reports by the Institute of Medicine (19-20). Further push for decreasing the amount and eventual eradication of antimicrobials came from both the Council for Agricultural Science and Technology (21) and the Committee on Drug Use in Food Animals (22). The World Health Organization commented that the use of antimicrobial growth promoters that are in antimicrobial classes also used in humans should be eliminated until the risks associated with their use were ruled out (23).

The antimicrobials used by animals and humans often belong to the same classes of antimicrobials, and therefore they use similar modes of action and bacterial cell targets. The similarities between antimicrobials used in both humans and animals have caused much scrutiny and worry. Bacteria can evolve to become resistant to antimicrobials that are used in animals and could possibly be transmitted to humans or spread their mechanisms of resistance. This transmission of resistance can cause the loss of therapeutic efficacy in both veterinary and human medicine (24).

Antimicrobial agents have the ability to change the bacterial environment by removing susceptible strains, and allowing antimicrobial resistant bacteria to live (25).

Evidence in the field shows positive associations between the occurrence of certain virulence genes and antimicrobial resistance determinants (26; 27). The effect of antimicrobial growth promoters on the increase of antimicrobial resistant bacteria has been the focus of several studies and has led to their ban in the European Union in 2006.

1.2 Antimicrobial Threats

The use of antimicrobials as growth promoters is negatively perceived because pathogenic bacteria of humans and animals have developed and shared a variety of antimicrobial resistance mechanisms that can be easily spread within microbial communities. A worldwide dissemination of antimicrobial resistance mechanisms resulting from antimicrobial selective pressures has unquestionably led to fewer therapeutic options and impaired treatment effectiveness in human medicine. For example, Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* has been transmitted from cows or pigs to humans and could cause diseases in humans (28-30). More recently, it was hypothesized that several cases of community-acquired urinary tract infections (UTI), caused by antimicrobial resistant bacteria, could be construed as outbreaks originating from food (31).

Studies in Canada suggest that poultry meats could play a role in human infections (31) and that chicken represented the most probable reservoir of extraintestinal pathogenic *E. coli*-causing UTI (33). Antimicrobial resistance can be spread in numerous ways, such as the exchange of genes or acquisition of resistant commensals or resistant pathogens from food and the environment. Because of these possibilities, global-coordinated actions are required (34; 35).

1.2.1 The Centers for Disease Control and Prevention's warning of antimicrobial abuse

One of the most serious health threats that exist today is antimicrobial resistance of pathogenic bacteria. Antimicrobial resistant bacterial infections have become common, some of which are now resistant to multiple types of antimicrobials. The Centers for Disease Control and Prevention (CDC) has reported that there have been more than two million people sickened each year by an antimicrobial resistant infection, of which at least 23,000 fatalities as a result. When both first-line and second-line antimicrobial options are exhausted due to resistance, potentially more toxic and expensive antimicrobials must be utilized, and this can be a dangerous last resort.

Antimicrobial resistance is a problem that spans the entire globe. The CDC's Antibiotic Resistance Threats in the United States report for 2013 warned that the abuse of antimicrobials as a feed additive has had a direct consequence on the emergence of antimicrobial resistant bacteria in food-producing animals (36). The resistant bacteria in food-producing animals are a cause of great concern not only from the threat of animals becoming potential carriers, but also due to the potential to contaminate the foods that come from those animals. A person who consumes these contaminated foods can develop antimicrobial resistant infections that can be life threatening (36).

Antimicrobial resistant bacteria are a major food safety concern. Antimicrobial resistant bacteria such as *Salmonella* or *E. coli* can infect humans through contact or consumption of contaminated food while non-pathogenic resistant isolates can transfer their resistant genes to human pathogenic bacteria. There are many factors influencing

the presence of antimicrobial resistant bacteria; however, practices contributing to the selection of antimicrobial resistant bacteria, including antimicrobial usage in livestock feed, and anxieties about food safety and reduced ability of antimicrobial treatment in human medicine, have moved many scientists to investigate further (35; 37).

Figure 1: Examples of How Antibiotic Resistance Spreads

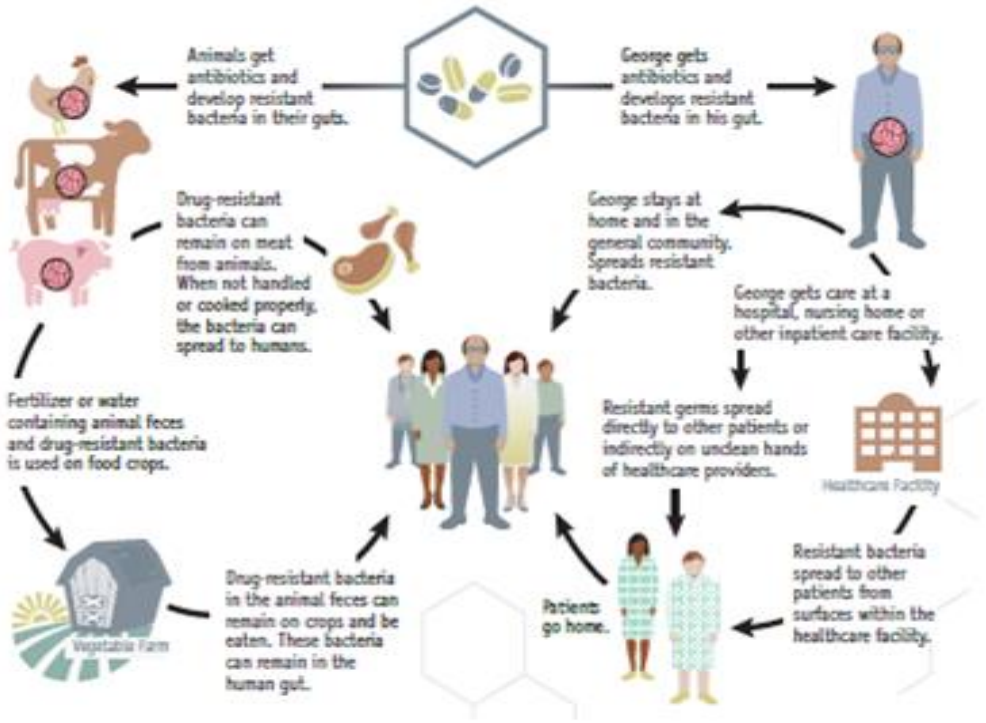

Figure 1: Demonstration of how the simple act of using antibiotics creates resistance. Figure from: CDC Antibiotic Resistance Threats in the United States, 2013.

Antimicrobial resistance has become a worldwide threat to public health. The USA National Antimicrobial Resistance Monitoring System (NARMS), assisted by the FDA and the United States Department of Agriculture (USDA), closely investigate antimicrobial susceptibility of enteric bacteria from humans, retail meats and food-producing animals in order to make judgments connected to the approval of safe and effective antimicrobial drugs for animals (38).

Fears for safe food and effective medical antimicrobials have pressured authorities for elimination of antimicrobials as growth promoters as well as those of medical importance in animal production (38). In spite of incomplete data and concrete facts, there were enough sincere and practical opinions for change and executing new regulations in the European Union. Comparable to the European Union, policies and recommendations in North America were founded on the precautionary principle. Due to the tie between antimicrobial use in food-producing animals and the emergence of antimicrobial-resistant infections in humans, the CDC strictly adheres to the belief that antimicrobials should only be used in food-producing animals under veterinary orders and to manage and treat infectious diseases, not to promote growth (39).

The CDC supports the FDA's strategy to promote the judicious use of antimicrobials that are invaluable in treating human bacterial infections. However, proficient control of foodborne pathogens remains a concern (40), and removal of non-therapeutic antimicrobials from animal production may possibly increase the prevalence of pathogens in the animal gut and the frequency of foodborne illnesses. Alternatives to antimicrobials are therefore required.

1.2.2. Modes of antimicrobial resistance spread

Many antimicrobial resistance genes in bacteria have been identified on mobile genetic elements such as plasmids, transposons and integrons. Transposons are also other mobile genetic elements that can contain antimicrobial resistance gene cassettes such as resistance integrons (44). Class 1 integrons, which can be spread through a wide variety bacterial species, are often found in bacteria associated with livestock and poultry (37).

Another vehicle for gene transfer across bacterial species of different taxa includes transduction or gene transfer mediated by bacteriophages (45).

These mobile genetic elements are readily disseminated among bacteria in the chicken gut or in extra-intestinal environments. Transformation and conjugation are mechanisms helping gene transfer among bacteria and are thought to play significant roles in the fast spread of antimicrobial resistance (41). In addition, the horizontal transfer of mobile genetic elements also contributes to the development of newly emerging bacterial pathogens through transfer of virulence genes. A variety of genetic materials, such as plasmids, can participate in this evolution (42). Furthermore, integrative and conjugative elements can be distributed through transferable elements like conjugative plasmids but can also integrate into the genome of new bacterial hosts (43).

Phenotypic recognition of inducible antimicrobial resistance may be problematic and can account for the silent dissemination of antimicrobial resistance genes in bacterial populations (46). In other words, bacterial isolates of animal origin might not be infectious to humans but they have the ability to carry and spread important antimicrobial resistance genes. As an example, the *vanA* gene cluster involved in vancomycin resistance could be detected in *Enterococci* of both human and animal origins, demonstrating horizontal transfer of gene clusters between *Enterococci* of different origins (47; 48). Similarly, multidrug resistant commensal *E. coli* colonizing animal intestinal tracts represent an important reservoir of antimicrobial resistance genes that can be transferred to other *E. coli* strains and other bacterial species through contact with other animals or humans and through contaminated food (49). Many food animals are

now broadly recognized as carriers of livestock-associated bacterial pathogens can cause diseases in the human host.

Not much is understood about the selection, distribution and dissemination of antimicrobial resistance genes in broiler chicken productions in relation to the use of specific therapeutic agents or antimicrobial growth promoters. In addition, the relative responsibility of selective pressures occasioned by human medicine, veterinary or agricultural practices is still unclear. Furthermore, metagenomics studies have established some links between resistance mechanisms found in clinical and environmental microorganisms (50), making even more difficult the identification of the primary cause of selective pressure and support arguments for multiple sources of antimicrobial resistance genes (51).

1.2.3 Antimicrobials and the gut microbiota

"The biological basis for antimicrobial effects on animal growth efficiency is most likely derived from effects on the intestinal microbiota, which in turn may reduce opportunistic subclinical infections, reduce the host response to the gut microflora, decrease competition for nutrients, and improve nutrient digestibility consequent to a reduction in some microbial fermentation by-products"(52). The lives of human beings, livestock and poultry are closely associated with microorganisms. The microbiota of the gut plays an important role in their overall health, productivity and well-being of humans and animals alike (53; 54). The growth of normal intestinal bacteria varies depending on the gut environment (55). Due to possible food safety and environmental health concerns,

the monitoring of microbiome changes as a function of chicken production practices is imperative.

The use of virginiamycin as a growth promoter was associated with increased bacterial abundance in the duodenal loop to proximal ileum and fewer bacteria affected in the ileocecal junction and cecum. These data indicates that virginiamycin modifies the composition of the chicken intestinal microbiota (56). Using the16S ribosomal RNA (rRNA) gene-based polymerase chain reaction followed by denaturing gradient gel electrophoresis profiling, dietary treatment with bacitracin can also alter the composition of the gut microbiota (57).

Lately, metagenomic sequencing techniques were able to show that salinomycin feeding had a profound influence on the dynamics of the chicken cecal microbiome (58). The salinomycin-fed group had an increased abundance of the *Elusimicrobia*, and a decreased amount of *Chloroflexi*, *Cyanobacteria,* and *Synergistetes*. The presence of *Bifidiobacterium spp.* and *Lactobacillus spp*. increased significantly in the salinomycin-fed birds compared to the untreated control group. Other researchers demonstrated the effect of antimicrobial growth promoters on the chicken gut microflora (59-61).

Pyrosequencing followed by phylogenetic analyses demonstrated that a diet supplemented with penicillin caused a rise in *Firmicutes* bacteria from 58.1 to 91.5% and a decreased amount of *Bacteroidetes* from 31.1 to 2.9% in the gut microflora of broilers compared to that observed in broilers fed with the control non-supplemented diet (60). Aside from the decrease of broiler ileal sucrose and maltase activities and the increase of ileal mucosal immunoglobulin A (IgA), the increase of *Lactobacillus* counts were

proposed to be one of the effects of bacitracin and oxytetracycline that could explain the improvement of feed efficiency seen in broiler chickens (62).

With such cascading effects, alternatives to antimicrobials administered for prevention or provided as growth promoters in feed are difficult to find. Several alternative strategies to antimicrobials in poultry and livestock production are under investigation (63-65). Individual strategies under examination include: direct-fed probiotics and live microbial feed supplements that improves the host's intestinal balance (66; 67); probiotics, indigestible feed ingredients that beneficially affect the host by selectively stimulating the activity of beneficial bacteria resident in the animal tract (68-70); vaccination (71) and immune-stimulation through cationic peptides and cytokines (72; 73); bacteriocins and antimicrobial peptides (74; 75); bacteriophages (76; 77); organic acids with antimicrobial activities; herbs, spices and other plant extracts (78); and controlled organic productions with emphasis on diet formulation and ingredient selection, cereal type and dietary protein source and level (79; 80). At this point, none of these approaches have been systematically implemented. Therefore, studies for new approaches to prevent poultry diseases and bacterial colonization of poultry by foodborne pathogens are ongoing globally.

1.3 Organic Poultry Production

1.3.1 Requirements for organic production in poultry industry

Public tension and concerns about food and environmental safety have driven researchers to seek out other approaches that could eliminate or decrease the use of antimicrobials while still maintaining production yields and low mortality in poultry

production. The drive for the expansion of organic meats is derived from the concerns about the use of antimicrobials and growth hormones in animal livestock, the environment, and the humane treatment of animals (81; 82).

There are basic requirements for the practice of organic poultry production in the U. S. The requirements are as follows: appropriate indoor and outdoor housing that allows for natural behavior to occur, certified organic feed and pasture is provided, no antimicrobials, no drugs or synthetic parasiticides, organic processing of meat and eggs, record-keeping system that enables poultry and products to be accounted for, organic system plan, a production that does not contribute to contamination of the soil or water, and no genetically modified organisms, ionizing radiation or sewage sludge (83; 84).

The Organic Foods Production Act of 1990 established national standards for organically produced commodities. The USDA began implementing these standards in October, 2002. All organic growers and handlers must be certified by state or private agencies under the USDA standards to be considered certified organic. The final retailers must also submit to all certified organic handler requirements for the organic product to keep the organic label. The USDA organic seal may only be used on agricultural products that are 100-percent organic or 95-percent organic. A fine of up to $10,000 per each infraction for those who falsify the USDA organic seal or do not maintain organic integrity is implemented by the U. S. government (85).

Organic poultry is much more expensive to grow than conventionally raised poultry due to feed costs, lower stocking amounts, the cost associated for providing outdoor access, and veterinary costs without the use of antimicrobials in intensively produced flocks of chickens. If there is a longer growing period for broilers, feed

efficiency can also deteriorate. Death is also a more common problem among organic poultry flocks. The cost of labor has the potential to increase, and recordkeeping may be an added expense, along with USDA certification fees. In order to offset production costs, organic poultry products do sell at a top-dollar price (86).

Organic meat continues to be one of the fastest growing parts of the organic food industry, with organic poultry meat making up two-thirds of the organic food industry. In the U. S., sales of organic poultry peaked at $161 million in 2005. Sales of organic poultry meat had quadrupled since 2003, and annual predicted growth rates range from 23 to 38 percent (87). Half of organic poultry sales were in natural food stores in 2003, 45 percent in mass market grocery stores (82). In 2005, approximately 12 percent of U.S. consumers reported purchasing organic poultry regularly (88).

1.4 Probiotic and Prebiotic Use in Poultry Production

1.4.1 Emergence of probiotic use in poultry production

Enteric diseases are an important concern to the poultry industry because of lost productivity, increased mortality, and the associated contamination of poultry products for human consumption. With increasing concerns about antimicrobial resistance, the ban on sub-therapeutic antimicrobial usage in Europe and the potential for a ban in the U. S., there has been an increasing interest to find an alternative to antimicrobial for poultry production.

Prebiotics and probiotics are two approaches that have potential to reduce enteric disease in poultry and subsequent contamination of poultry products. Probiotics are described as a “live microbial feed supplement which beneficially affects the host animal

by improving its intestinal balance" (89). Prebiotics are described as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (90). Combinations of both prebiotics and probiotics are known as symbiotics (91). Increased bacterial resistance to antimicrobials in humans has caused an increase in public and governmental interest in eliminating sub-therapeutic use of antimicrobials in livestock. An alternative to sub-therapeutic antimicrobials in livestock is the use of probiotic microorganisms, prebiotic substrates that enrich certain bacterial populations, or symbiotic combination of probiotics and prebiotics (92).

The concept of a balanced intestinal microbiota enhancing resistance to infection and reduction in resistance when the intestinal microbiota is disturbed is important in understanding the microbe-host relationship. Research over the last century has shown that lactic acid bacteria and certain other microorganisms can increase resistance to disease and that lactic acid bacteria can be enriched in the intestinal tract by feeding specific carbohydrates. More recently, there have been reports that probiotic organisms can be used as an alternative for growth stimulation and improved feed efficiency (93).

Chapter 2

Introduction

The total U.S. broiler chicken production in 2013 was valued at $30.7 billion, while exports of broiler chicken meat reached over 7.3 billion pounds (1). Since 1945, broiler chicken production has increased from 5 billion to 50 billion pounds (1). The increase in poultry production drove the industry to standardize practices and include regimes to ensure good animal health and welfare (4). The microbiota of the gut plays an important role in overall health, productivity and wellbeing of broiler chickens (56; 57). A balanced intestinal microbiota enhances resistance to infection and reduces resistance when the intestinal microbiota is disturbed (93). The broiler chicken microbiota is dependent upon many factors including: age, environment, and most importantly diet and feed additives (94; 95; 96; 97; 98; 99).

The lives of both human beings and poultry are closely associated with microorganisms (58). In commercial poultry production, broiler chickens are hatched in clean environments, resulting in a lack of natural microbiota. This lack of initial microbiota of hatchlings caused the industry to implement the common practice of using competitive exclusion products and probiotics for the early colonization of gut microbiota of broiler chickens with a healthy microbial community (100). At present, commercial broilers are raised under different management conditions; conventional, organic, and natural (107). A limited number of antibiotics are permitted in conventional feed whereas the use of probiotics has become a common practice in intensive commercial broiler

industry (108). Antibiotics and probiotics may influence the gut microbial community structures and therefore the disease resistance capabilities of broiler chickens. In order to determine how three commercial broiler chicken diets (conventional, natural and organic) and the presence of probiotics in each diet type influence the gut microbiota composition, we determined the relative quantification of bacterial genera by real-time PCR using the comparative Ct method.

The U.S. Food and Drug Administration (FDA) originally backed the use of antimicrobials as feed additives because of advantages such as: promoted growth, increased feed efficiency, and suppression of endemic diseases in large groups of animals (10). Growth promotion through the use of antimicrobial feed additives was eventually viewed negatively due to pathogenic bacteria of humans and animals developing and sharing antimicrobial resistance mechanisms that can be easily spread within microbial communities. The spread of antimicrobial resistance mechanisms caused from antimicrobial selective pressures has indisputably led to fewer therapeutic options and treatment effectiveness in human medicine (28-30). The addition and eventual abuse of antimicrobial feed additives partly led to The Centers for Disease Control and Prevention's (CDC) to release an Antibiotic Resistance Threats in the United States report for 2013, and it states that the overuse and misuse of antimicrobials as a feed additive has resulted in the rise of antimicrobial resistant bacteria in food-producing animals (36).

Antimicrobial resistance disseminates using genes that encode for resistance, resistant commensals, or resistant pathogens from food and the environment. Transformation and conjugation are the common mechanisms helping gene transfer

among bacteria and are thought to play significant roles in the fast spread of antimicrobial resistance (41). Also, the horizontal transfer of mobile genetic elements adds to the increase of emerging bacterial pathogens through the gain of virulence genes. Bacteria from animals may not be infectious to humans, but can still carry and spread important antimicrobial resistance genes (47; 48). The occurrence of resistance genes in commensals may perform as an indicator for the development of resistance in pathogens.

The resistant bacteria in food-producing animals are a cause for alarm not only because of the potential for the animals becoming resistant commensal carriers, but due to the danger of contamination in foods that come from those animals. Consuming these contaminated foods can cause antimicrobial resistant infections that could be both life threatening and difficult to treat due to antimicrobial resistance (36).

Fear over food and environmental safety has led to new feeding approaches that could lessen or eradicate the use of antimicrobials while sustaining production outputs and low mortality in poultry production. One approach to absolve public concerns over antimicrobial additives is organic meat production (81; 82). Another alternative to antibiotic-supplementation is probiotics. Probiotics may lower enteric disease in poultry and poultry products by changing the gut microbiota (89).

Chapter 3

Materials and Methods

3.1 Sample Collection

Three Pennsylvanian broiler houses were selected per diet type (antibiotic-supplemented, probiotic-supplemented and organic) for sampling sites. Each broiler house was unique based on its location in Pennsylvania, producer, and diet type. Chicken houses contained flock sizes varying from 8,000 to 25,000 broiler chickens. Broiler chickens from organic farms were fed diets containing: Actigen™ (Alltech, Lexington, KY), Primalac Poultry F/G (Star Labs, Inc, Clarksdale, MO), Enviva® Pro 01 GT (Danisco, Copenhagen, Dk), ZINPRO® E (Zinpro, Eden Prairie, MN), Allzyme® SSF (Alltech, Lexington, KY), GalliPro® Tect 1.0 (Chr. Hansen, Milwaukee, WI), XPC Green (Diamond V, Cedar Rapids, IA), Copper Sulfate (Old Bridge Chemicals, Old Bridge, NJ), Bio-D (Bio-D International, Eugene Oregon), Diatomaceeous Earth (DiaSource, Boise, ID), BIOSUPREME G^{MR} (Agroin, San Diego, CA), Mintrex® Zn:Mn (Novus, St. Charles, MO), and Enviro Supreme Green (Best Veterinary Solutions, Willmar, MN). Broiler chickens from probiotic-supplemented farms were fed diets containing: Opti-Bac® L (Huvepharma, St. Louis, MO), Gallipro® Tect 1.0 (Chr. Hansen, Milwaukee, WI), Primalac Poultry F/G (Star Labs, Clarksdale, MO), Calsporin® (QTI, Elgin, IL), Xtract® 6930 (Pancosma), Actigen (Alltech, Lexington,

KY), Biomin P.E.P. 125 (Biomin, Herzogenburg, AU), NutriFibe Complex (Ralco Nutrition, Marshall, MN), Gallinat +™ (JEFO, Saint-Hyacinthe, QC), Copper Sulfate (Old Bridge Chemicals, Old Bridge, NJ) , Intellibond C (Micronutrients, Indianapolis, IN), Micro-Aid (Distributor's Processing, Porterville, CA), and Bio Supreme PF (Agroin, San Diego, CA). Broiler chickens from convention farms who supplement antibiotics were fed diets containing: BMD (Zoetis, Florham Park, NJ), Maxiban® (Elanco, Indianapolis, IN), and Monteban® 45 (Elanco, Indianapolis, IN). Product descriptions can be found listed in Table 2 below.

Table 2: Diet Products and Descriptions

Product Name	Product Description
Monteban® 45 (Elanco, Indianapolis, IN).	Antimicrobial narasin
Maxiban® (Elanco, Indianapolis, IN)	Antimicrobials narasin and nicarbazin
BMD (Zoetis, Florham Park, NJ)	Antimicrobial bacitracin, mineral oil and calcium carbonate
Bio Supreme PF (Agroin, San Diego, CA)	*Yucca schidigera*
Micro-Aid (Distributor's Processing, Porterville, CA)	*Yucca*
Intellibond C (Micronutrients, Indianapolis, IN)	Basic Copper Chloride
Gallinat +™ (JEFO, Saint-Hyacinthe, QC)	Blend of essential oils and organic acids
NutriFibe Complex (Ralco Nutrition, Marshall, MN)	Prebiotic fibers, β glucans and *yucca*
Biomin P.E.P. 125 (Biomin, Herzogenburg, AU)	Oregano, anise and citrus essential oils
Actigen™ (Alltech, Lexington, KY)	Mannan oligosaccharide
Xtract® 6930 (Pancosma)	Capsicum oleoresin, cinnamaldehyde and carvacrol
Calsporin® (QTI, Elgin, IL)	Direct-fed microbial from Calpis utilizing viable spores of Bacillus subtilis C-3102
Primalac Poultry F/G (Star Labs, Clarksdale, MO)	*Lactobacillus acidophilus*, *Lactobacillus casei*, *Enterococcus faecium* and *Bifidobacterim bifidum*
Opti-Bac® L (Huvepharma, St. Louis, MO)	*Bacillus licheniformis* spores and calcium carbonate
Enviro Supreme Green (Best Veterinary Solutions, Willmar, MN)	*Bacillus subtilis*, *Bacillus licheniformis* and *Yucca schidigera*
Mintrex® Zn:Mn (Novus, St. Charles, MO)	Zinc and manganese
BIOSUPREME G^{MR} (Agroin, San Diego, CA)	*Yucca schidigera*
Diatomaceeous Earth (DiaSource, Boise, ID)	Amorphous non-crystalline silica
Bio-D (Bio-D International, Eugene Oregon)	Vitamin D
XPC Green (Diamond V, Cedar Rapids, IA)	*Saccharomyces cerevisiae*
GalliPro® Tect 1.0 (Chr. Hansen, Milwaukee, WI)	*Bacillus licheniformis*
Allzyme® SSF (Alltech, Lexington, KY)	*Aspergillus niger* produced phytase, xylanase,

	protease, cellulase, beta-glucanase and amylase
ZINPRO® E (Zinpro, Eden Prairie, MN)	Organic zinc
Enviva® Pro 01 GT (Danisco, Copenhagen, Dk)	*Bacillus subtilis* spores

Ten fecal samples were collected from each broiler house and separately bagged in Whirl-Paks® (Nasco, Fort Atkinson, WI). All samples were immediately transported back to the laboratory and stored at -80^{o}C.

3.2 Isolation of Bacterial DNA

Five fecal samples were combined to form one sample pool. To increase bacterial DNA yield, the pooled samples were lysed using the Mini-BeadBeater 16 (Biospec, Bartlesville, OK). Bacterial DNA was then extracted using QIAamp DNA Stool Kit (Qiagen, Valencia, CA). Concentration and purity of each pooled bacterial DNA sample was determined using Nanovue Plus nanodrop machine (GE Health Care, Fairfield, CT).

3.3 Microbiota Real-Time PCR

Amplification and detection of DNA by real-time PCR was performed with the 7500 Fast Real-Time PCR Systems (Life Technologies, Grand Island, NY). Duplicates of each pooled sample of bacterial DNA were run with each specific primer set at optimal annealing temperatures and conditions provided by the reference material with Power SYBR PCR Master Mix (Life Technologies). Table 3 gives the oligonucleotide sequence for each primer set of the specifically targeted groups.

Table 3: Targets and Sequences

Target Group	Oligonucleotide sequence (5'-3')	Reference	Amplicon size (bp)
Bacteriodes	GAGAGGAAGGTCCCCCAC CGCTACTTGGCTGGTTCAG	Layton *et al*. 2006	106
Clostridium difficile	TTGAGCGATTTACTTCGGTAAAGA CCATCCTGTACTGGCTCACCT	Rinttila *et al*. 2004	157
Clostridium perfringens	ATGCAAGTCGAGCGAGG	Rinttila *et al*. 2004	120

	TATGCGGTATTAATCTCCCTTT		
Enterobacteriaceae	CATGACGTTACCCGCAGAAGAAG CTCTACGAGACTCAAGCTTGC	Bartosch *et al.* 2004	195
Enterococcus spp.	CCCTTATTGTACTTCCCATTGT ACTCGTTGTACTTCCCATTGT	Rinttila *et al*. 2004	144
Genus *Prevotella*	CACGGTAAACGATGGATGCC GGTCGGGTTGCAGACC	Bekele *et al*. 2010	121
Lactobacillus spp.	GAGGCAGCAGTAGGGAATCTTC GGCCAGTTACTACCTCTATCC	Delroisse *et al*.2008	126
Eubac 16S rRNA	GTGSTGCAYGGYTGTCGTCAGTGSTGCA ACGTCRTCCMCACCTTCCTC	Maeda et al., 2003	150

PCR amplification protocol for *Bacteriodes* and *Enterobacteriaceae* consisted of 50°C for 2 min, followed by 95°C for 10 min and 50 cycles of 95°C for 30 s and 60°C for 45 s. PCR amplification for *Clostridium difficile* included an initial denaturation step at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 15 s, primer annealing at 58°C for 20 s and primer extension at 72°C for 45 s, with final extension step at 72°C for 5 min. PCR amplification for *Clostridium perfringens* included an initial denaturation step at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 15 s, primer annealing at 55°C for 20 s and primer extension at 72°C for 45 s, with final extension step at 72°C for 5 min. PCR amplification for *Enterococcus* spp. included an initial denaturation step at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 15 s, primer annealing at 61°C for 20 s and primer extension at 72°C for 45 s, with final extension step at 72°C for 5 min. Genus *Prevotella* had a thermal cycle consisting of denaturation at 95°C for 10 min , followed by 40 cycles of 95°C for 15 s, annealing at 55°C for 5 s and 72°C for 22 s. *Lactobacillus* spp. had a PCR amplification protocol consisting of initial denaturation step at 50°C for 2 min and 95°C for 10 min, followed by

45 cycles of 15 s at 95°C and 1 min at 60°C. Eubac 16S rRNA was used as an endogenous control and was run simultaneously with each primer pair for further comparative analysis by the comparative Ct method described below.

3.3.1 Comparative CT Analysis for Relative Quantification and Statistical Analysis

Relative quantification of each targeted bacterial species, group, genus and family were determined using the comparative C_T method (6). Fold change between each diet type was determined by the equation:

$$\text{Fold Change} = 2^{-\Delta\Delta CT} = [(C_T \text{ primer target} - C_T \text{ internal control}) \text{ sample A} - (C_T \text{ primer target} - C_T \text{ internal control}) \text{ sample B})]$$

Wilcoxon matched-pair tests and Mann-Whitney U tests were used for nonparametric analysis of non-Gaussian data with the GraphPad Prism Software 5.01 (GraphPad, La Jolla, CA). P values < 0.05 were considered statistically significant.

3.4 Three DNA Library Constructions with cloned broiler chicken fecal DNA

Construction of two DNA libraries was performed by cloning broiler chicken fecal DNA fragments into broad-host-range expression vectors pJN105 (with a gentamicin resistance marker) and pCF430 (with a tetracycline resistance marker). Strains were grown in Luria–Bertani (LB) medium with gentamicin (pJN105) or tetracycline (pCF430) to select for plasmid presence. Plasmid vectors were obtained from the National Institute of Genetics of Japan (Yata, Mishima, Shizuoka, Japan). Previously extracted broiler chicken fecal DNA was digested with the restriction enzymes EcoRI or

PstI, and fragments of 1.5 kb to 10 kb were gel purified and ligated into pJN105. The vector was prepared by digestion with the same enzymes and treated with shrimp alkaline phosphatase. Vector and DNA fragments digested with same restriction enzyme were ligated at 4°C overnight. Libraries were transformed into ElectroMAXTM DH10B cells (Gibco BRL) by electroporation. A third library was created as described above except that broiler chicken fecal DNA fragments were cloned into pCF430, and the resulting library was transformed into electrocompetent DH5α cells.

3.4.1 Isolation of transformants expressing antibiotic resistance

Pools of five transformants containing the appropriate plasmid from each of the three libraries were grow in LB medium with the selective antibiotic for two hours. 50 µl of pools from library one and two were plated on to LB agar containing inhibitory concentrations tetracycline (10 µg mL^{-1}), nalidixic acid (5 µg mL^{-1}), rifampicin (20 µg mL^{-1}), ampicillin (50 µg mL^{-1}), penicillin (10µg mL^{-1}), vancomycin (200 µg mL^{-1}), erythromycin (100 µg mL^{-1}), neomycin (10mg mL^{-1}), trimethoprim (2.5 mg mL^{-1}) and sulfisoxazole (9 mg mL^{-1}). Selections for aminoglycoside-resistant clones were conducted by plating 50 µl of pools from library three on to LB agar containing inhibitory concentrations of the aminoglycoside antibiotics amikacin (2.5 mg mL^{-1}), gentamicin (5 mg mL^{-1}), kanamycin (20 mg mL^{-1}), neomycin (10mg mL^{-1}), trimethoprim (2.5 mg mL^{-1}), streptomycin (20 mg mL^{-1}) and sulfisoxazole (9 mg mL^{-1}). Plates were incubated overnight at 37°C. Twelve antibiotic-resistant colonies were subcultured onto LB agar containing the plasmid-selective antibiotic (gentamicin or tetracycline) to confirm presence of correct plasmid with antibiotic-resistant insert and

grown overnight at 37°C. Colonies were subjected to plasmid rapid-screening and immediately run through a 1% agarose gel to visually determine the presence of an insert.

3.5 Real-Time PCR

Amplification and detection of DNA by real-time PCR was performed with the 7500 Fast Real-Time PCR Systems (Life Technologies, Grand Island, NY). Duplicates of each pooled sample of bacterial DNA were run with each specific primer set at optimal annealing temperatures and conditions provided by the reference material with Power SYBR PCR Master Mix (Life Technologies). Table Four gives the oligonucleotide sequence for each primer set of the specifically targeted genes.

Table 4: Gene Target and Primer Pair Sequences

Target Gene	Oligonucleotide sequence (5'-3')	Reference	Amplicon size (bp)
aacA-aphD	ATCCAAGAGCAATAAGGGCATAC GCCACACTATCATAACCACTACC	Khan *et al.* 2011	226
ermA	TCCTTACTTAATGACCGATGTACTCT TCTTCGCTTTCGCCACTTTGA	Sabet *et al.* 2007	146
mecA	AAGCGACTTCACATCTATTAGGTTAT TATATTCTTCGTTACTCATGCCATAC	Paule *et al.* 2005	402
msrC	AAGGAATCCTTCTCTCTCCG GTAAACAAAATCGTTCCCG	Reynolds *et al.* 2001	343

When targeting the *aacA-aphD* gene, a two-step amplification protocol was used involving an initial denaturation step of 98 °C for 2 min followed by 40 cycles of 98 °C for 1 s and 60 °C for 5 s. Amplification of the *ermA* gene was carried out by a 3 min at 95°C for initial denaturation, 30 cycles of 2 steps consisting of 30 s at 95°C for

denaturation, and 45 s at 55°C for annealing. The conditions for amplification of *mecA* gene consisted of an initial step at 95°C for 10 minutes followed by an amplification program for 40 cycles of 3 seconds at 95°C, 5 seconds at 61°C, and 20 seconds at 72°C. For detections and amplification of *msrC* gene was carried out with an initial denaturation step at 95°C for 10 min, followed by 40 cycles at 94°C denaturation for 45 s, 50°C annealing for 45 s, 72°C elongation for 60 s. Eubac 16S rRNA was used as an endogenous control and was run simultaneously with each primer pair for further comparative analysis by the comparative Ct method described below.

3.5.1 Comparative CT Analysis for Relative Quantification and Statistical Analysis

Relative quantification of each gene was determined using the comparative C_T method (6). Fold change between each diet type was determined by the equation:

$$\text{Fold Change} = 2^{-\Delta\Delta CT} = [(C_T \text{ primer target} - C_T \text{ internal control}) \text{ sample A} - (C_T \text{ primer target} - C_T \text{ internal control}) \text{ sample B})]$$

Wilcoxon matched-pair tests and Mann-Whitney U tests were used for nonparametric analysis of non-Gaussian data with the GraphPad Prism Software 5.01 (GraphPad, La Jolla, CA). P values < 0.05 were considered statistically significant**.**

Chapter 4

Results

4.1 Effect of Probiotic Addition to Antibiotic-Supplemented (Conventional) Diet when Compared to Organic and Natural Diets.

Figure 2: Fold Change in *Bacteriodes* Due to Conventional Diet with Probiotic Addition when Compared to Organic and Natural Diet.

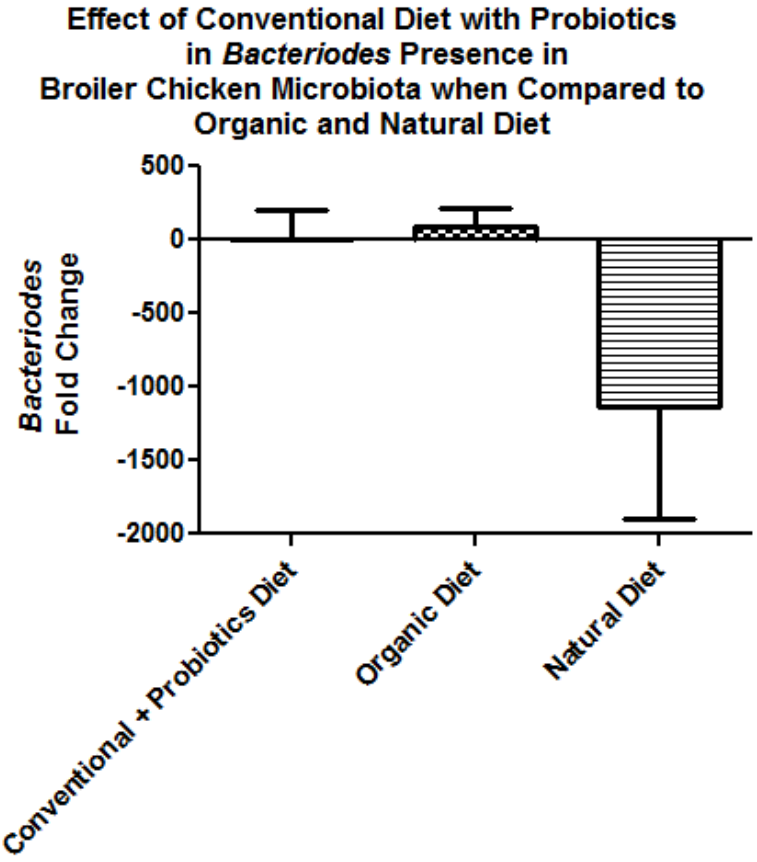

Figure 2 graphically illustrates the fold change observed in *Bacteriodes* when comparing three broiler chicken commercial diet types (conventional + probiotics, natural and organic) after utilizing the comparative Ct method. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *Bacteriodes* when comparing the conventional + probiotics broiler chicken to the broiler chicken organic diet

gave a p value of 0.8438. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *Bacteriodes* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken natural diet gave a p value of 0.5781. Using the Mann Whitney test, the fold change seen in *Bacteriodes* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken organic diet gave a p value of 0.5307. Using the Mann Whitney test, the fold change seen in *Bacteriodes* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken natural diet gave a p value of 0.9155.

An increased fold change of relative quantification of the *Bacteriodes* was observed from the comparison of the conventional plus probiotics broiler chicken diet to the organic broiler chicken diet, though this increase did not reach statistical significance. When the chickens were fed with the conventional plus probiotics broiler chicken diet compared to the natural broiler chicken diet, a severe decrease in relative quantification of *Bacteriodes* was observed. These stark decreases could have been due to the antimicrobial properties of the antibiotics in the conventional plus probiotic diet.

Figure 3: Fold Change in *Bacteriodes* Due to Conventional Diet without Probiotic Addition when Compared to Organic and Natural Diet.

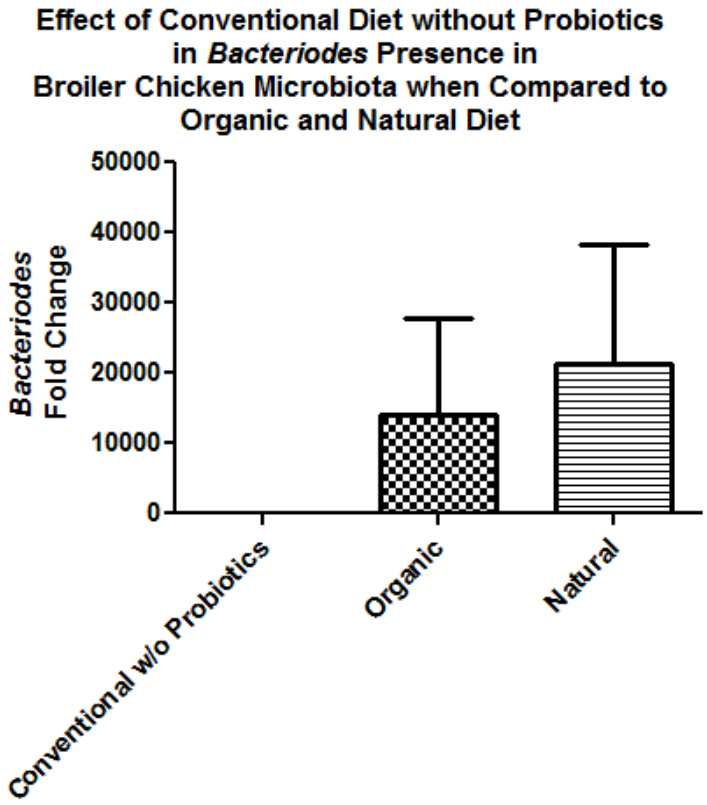

Figure 1 graphically depicts the fold change observed in *Bacteriodes* when comparing three broiler chicken commercial diet types (conventional without probiotics, natural and organic) after utilizing the comparative Ct method. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *Bacteriodes* when comparing the conventional without probiotics broiler chicken diet to the broiler chicken organic diet gave a p value of 0.2500. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *Bacteriodes* when comparing the conventional without probiotics broiler chicken diet to the broiler chicken natural diet gave a p value of 0.500.

An increased fold change in relative numbers of *Bacteriodes* was observed from the comparison of the conventional without probiotics broiler chicken diet to the organic broiler chicken diet, though these data were not significantly different. Furthermore, an increased fold change in relative quantification of *Bacteriodes* was also observed from

the comparison of conventional without probiotics broiler chicken diet to the natural broiler chicken diet. Antimicrobial supplements in the conventional diet may have created a favorable niche for antimicrobial resistant *Bacteriodes,* and could also account for the increased fold changes observed when compared to the antibiotic-free natural and organic diets. The presence of probiotics in the natural and organic diets could also have allowed for beneficial microorganisms other than *Bacteriodes* to compete for nutrients and not allow *Bacteriodes* to flourish.

Figure 4: Fold Change in *Enterobacteriaceae* Due to Conventional Diet with Probiotic Addition when Compared to Organic and Natural Diet

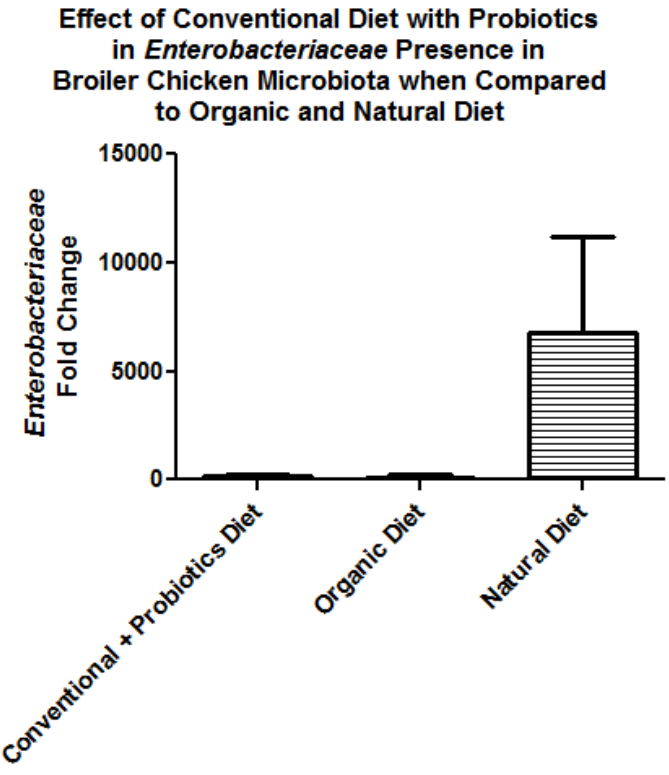

Figure 4 graphically illustrates the fold change observed in *Enterobacteriaceae* when comparing three broiler chicken commercial diet types (conventional + probiotics, natural and organic) after utilizing the comparative Ct method. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *Enterobacteriaceae* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken organic diet gave a P value of 0.9375. Using the Wilcoxon matched-pairs signed

rank test, the fold change seen in *Enterobacteriaceae* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken natural diet gave a P value of 0.3594. Using the Mann Whitney test, the fold change seen in *Enterobacteriaceae* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken organic diet gave a P value of 0.9155. Using the Mann Whitney test, the fold change seen in *Enterobacteriaceae* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken natural diet gave a P value of 0.1569.

The fold change in relative quantification of *Enterobacteriaceae* observed from the comparison of the conventional plus probiotics broiler chicken diet to the organic broiler chicken diet showed no significant fold change difference. The fold change in relative quantification of *Enterobacteriaceae* observed from the comparison of the conventional plus probiotics broiler chicken diet to the natural broiler chicken diet showed a severe increase. The antimicrobial properties in the conventional plus probiotic diet could account for the observed increase due to creating a favorable niche for antibiotic-resistant *Enterobacteriaceae*, that were otherwise inhibited by the specific probiotics found in the naturally-fed broiler chicken diet.

Figure 5: Fold Change in *Enterobacteriaceae* Due to Conventional Diet without Probiotic Addition when Compared to Organic and Natural Diet.

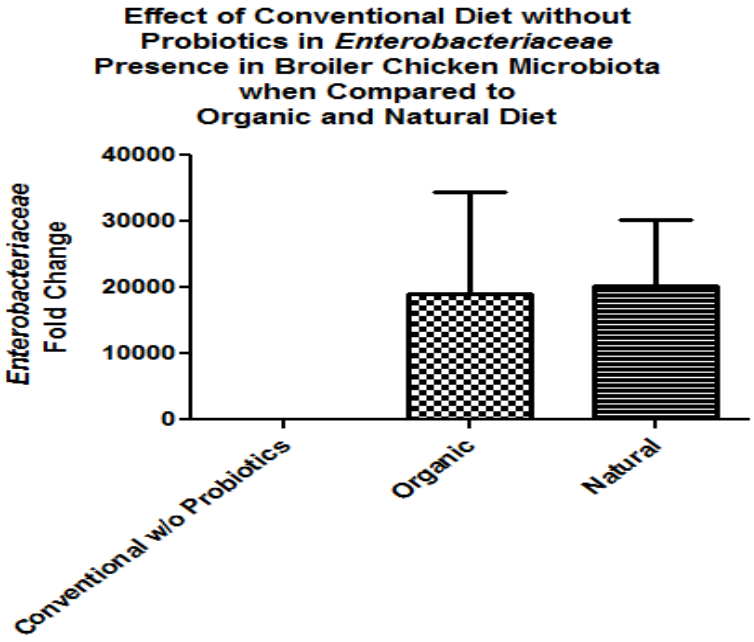

Figure 5 graphically depicts the fold change observed in *Enterobacteriaceae* when comparing three broiler chicken commercial diet types (conventional without probiotics, natural and organic) after utilizing the comparative Ct method. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *Enterobacteriaceae* when comparing the conventional without probiotics broiler chicken diet to the broiler chicken organic diet gave a P value of 0.500. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *Enterobacteriaceae* when comparing the conventional without probiotics broiler chicken diet to the broiler chicken natural diet gave a P value of 0.2500.

An increased fold change in relative quantification of *Enterobacteriaceae* was observed from the comparison of the conventional without probiotics broiler chicken diet to the organic broiler chicken diet, though these data are not statistically significant. Furthermore, an increased fold change in *Enterobacteriaceae* was also observed from the comparison of conventional without probiotics broiler chicken diet to the natural broiler chicken. Antimicrobial supplements in the conventional diet possibly created a favorable niche for antimicrobial resistant *Enterobacteriaceae*, and could also account for the increased fold changes observed when compared to the antibiotic-free natural and organic

diets. The presence of probiotics in the natural and organic diets could also have allowed for beneficial microorganisms to compete for nutrients and not allow *Enterobacteriaceae* to thrive.

Figure 6: Fold Change in Genus *Prevotella* Due to Conventional Diet with Probiotic Addition when Compared to Organic and Natural Diet.

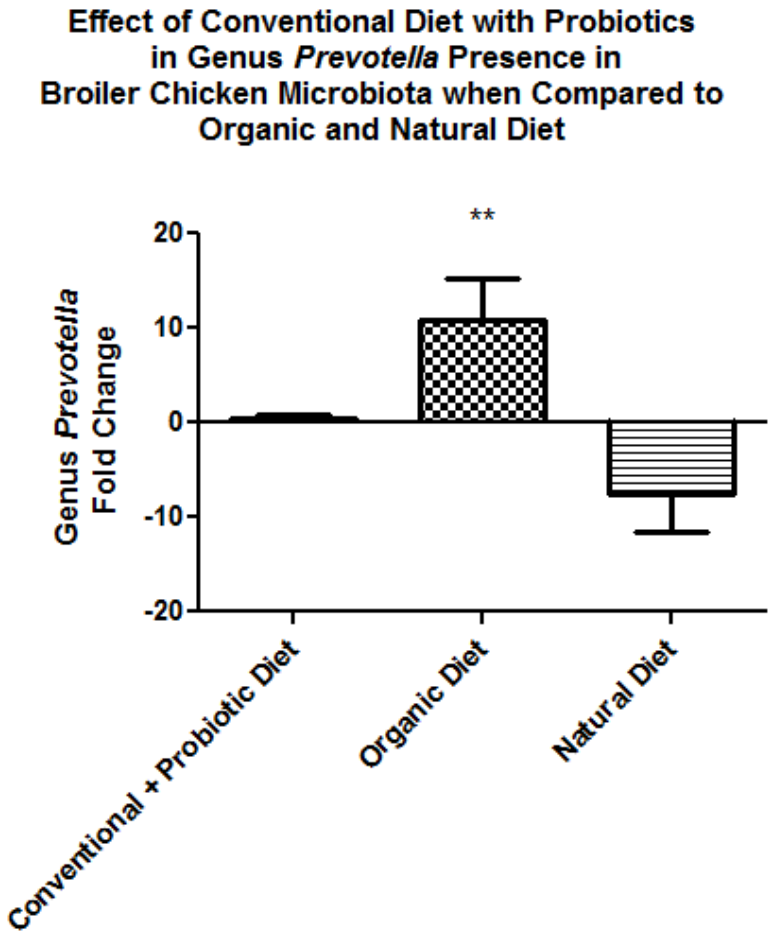

Figure 6 graphically illustrates the fold change observed in Genus *Prevotella* when comparing three broiler chicken commercial diet types (conventional + probiotics, natural and organic) after utilizing the comparative Ct method. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in Genus *Prevotella* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken organic diet gave a P value of 0.0117. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in Genus *Prevotella* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken

natural diet gave a P value of 0.0742. Using the Mann Whitney test, the fold change seen in Genus *Prevotella* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken organic diet gave a P value of 0.0011. Using the Mann Whitney test, the fold change seen in Genus *Prevotella* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken natural diet gave a P value of 0.2154.

The fold change in relative quantification of Genus *Prevotella* observed from the comparison of the conventional plus probiotics broiler chicken diet to the organic broiler chicken diet was significantly increased. This increased fold change could indicate that the antibiotics supplemented in the conventional diet created a niche for resistant *Prevotella*, even when probiotics were added. The fold change in relative quantification of Genus *Prevotella* observed from the comparison of the conventional plus probiotics broiler chicken diet to the natural broiler chicken diet showed a decrease, although it was not considered statistically significant. The antimicrobial properties in the conventional plus probiotic diet could account for the observed decrease due to creating an inhibition of *Prevotella*, plus the addition of probiotics to compete for nutrients within the gastrointestinal tract.

Figure 7: Fold Change in Genus *Prevotella* Due to Conventional Diet without Probiotic Addition when Compared to Organic and Natural Diet.

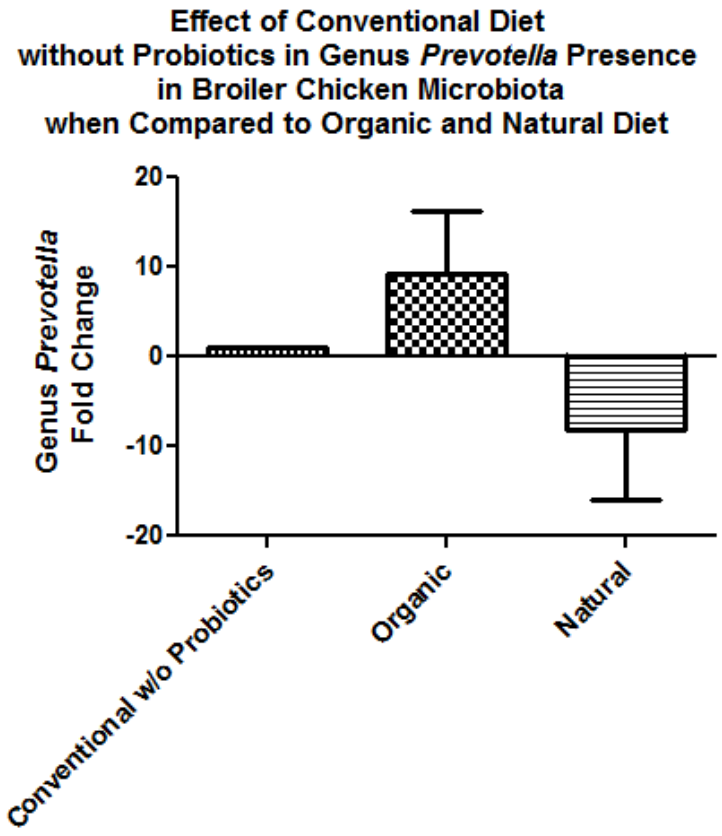

Figure 7 graphically depicts the fold change observed in Genus *Prevotella* when comparing three broiler chicken commercial diet types (conventional without probiotics, natural and organic) after utilizing the comparative Ct method. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in Genus *Prevotella* when comparing the conventional without probiotics broiler chicken diet to the broiler chicken organic diet gave a P value of 0.2500. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in Genus *Prevotella* when comparing the conventional without probiotics broiler chicken diet to the broiler chicken natural diet gave a P value of 0.500.

The increased fold change in relative quantification of the Genus *Prevotella* observed from the comparison of the conventional without probiotics broiler chicken diet to the organic broiler chicken diet was not significant. However, this increase could indicate that the antibiotics supplemented in the conventional diet created a niche for resistant *Prevotella*, even when probiotics were added. The fold change in Genus

Prevotella observed from the comparison of the conventional without probiotics broiler chicken diet to the natural broiler chicken diet showed a decrease, although it was not considered statistically significant. The antimicrobial properties in the conventional plus probiotic diet could account for the observed decrease due to possibly creating an inhibition of *Prevotella*, plus the addition of probiotics to compete for nutrients within the gastrointestinal tract.

Figure 8: Fold Change in *Clostridium perfringens* Due to Conventional Diet with Probiotic Addition when Compared to Organic and Natural Diet

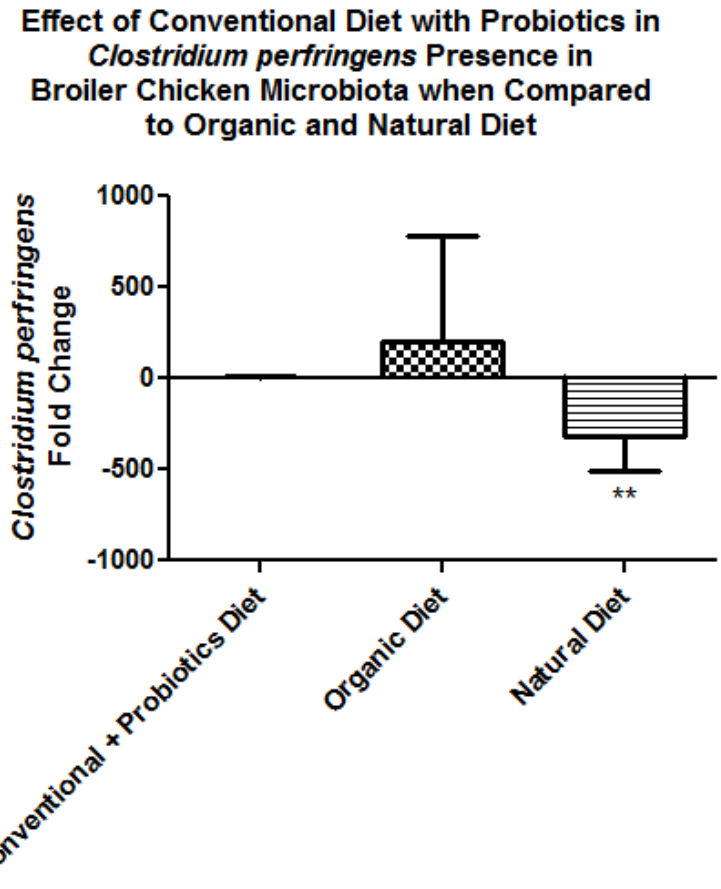

Figure 8 graphically illustrates the fold change observed in *Clostridium perfringens* when comparing three broiler chicken commercial diet types (conventional + probiotics, natural and organic) after utilizing the comparative Ct method. Using the Wilcoxon matched-pairs

signed rank test, the fold change seen in *Clostridium perfringens* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken organic diet gave a P value of 1.0000. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *Clostridium perfringens* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken natural diet gave a P value of 0.2500. Using the Mann Whitney test, the fold change seen in *Clostridium perfringens* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken organic diet gave a P value of 0.3762. Using the Mann Whitney test, the fold change seen in *Clostridium perfringens* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken natural diet gave a P value of 0.0381.

The decreased fold change in *Clostridium perfringens* observed from the comparison of the conventional with probiotics broiler chicken diet to the natural broiler chicken diet is statistically significant. This decrease may be because antibiotic supplementation in the conventional diet was able to inhibit *Clostridium perfringens* growth compared to the natural diet. The fold change in *Clostridium perfringens* observed from the comparison of the conventional with probiotics broiler chicken diet to the organic broiler chicken diet showed an increase, although it was not considered statistically significant. This could be due to antibiotic resistant *Clostridium perfringens* presence that was unable to be inhibited by antibiotics supplemented in the conventional diet and perhaps even thrived in a niche specifically induced by the addition of antibiotics to the broiler chicken microbiota.

Figure 9: Fold Change in *Clostridium perfringens* Due to Conventional Diet without Probiotic Addition when Compared to Organic and Natural Diet.

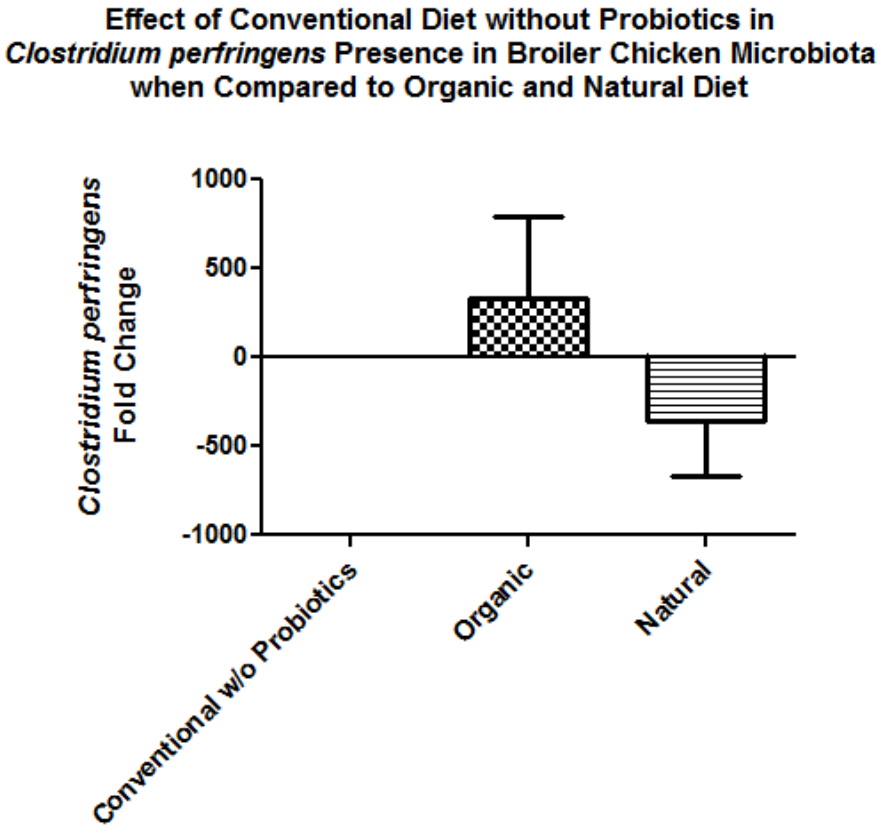

Figure 9 graphically depicts the fold change observed in *Clostridium perfringens* when comparing three broiler chicken commercial diet types (conventional without probiotics, natural and organic) after utilizing the comparative Ct method. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *Clostridium perfringens* when comparing the conventional without probiotics broiler chicken diet to the broiler chicken organic diet gave a P value of 1.0000. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *Clostridium perfringens* when comparing the conventional without probiotics broiler chicken diet to the broiler chicken natural diet gave a P value of 0.2500.

The decreased fold change in *Clostridium perfringens* observed from the comparison of the conventional without probiotics broiler chicken diet to the natural broiler chicken diet is not statistically significant. However, this decrease could be attributed to antibiotic supplementation in the conventional diet that was able to inhibit

Clostridium perfringens growth compared to the natural diet. The fold change in *Clostridium perfringens* observed from the comparison of the conventional with probiotics broiler chicken diet to the organic broiler chicken diet showed an increase, although it was not considered statistically significant. This could be due to antibiotic resistant *Clostridium perfringens* presence that was unable to be repressed by antibiotics supplemented in the conventional diet and perhaps even flourished in a niche specifically produced by the addition of antibiotics to the broiler chicken microbiota.

Figure 10: Fold Change in *Lactobacillus* spp. Due to Conventional Diet with Probiotic Addition when Compared to Organic and Natural Diet.

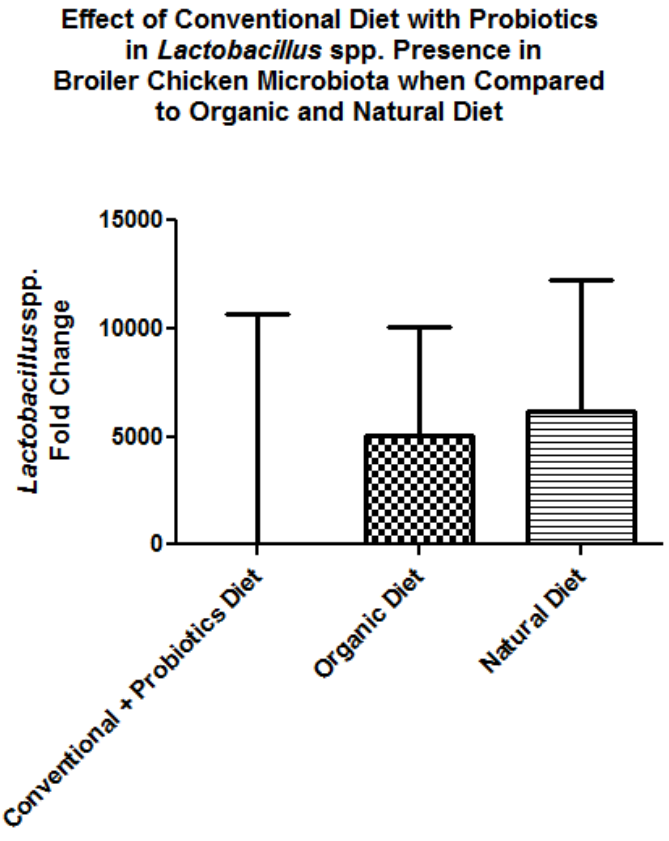

Figure 10 graphically illustrates the fold change observed in *Lactobacillus* spp. when comparing three broiler chicken commercial diet types (conventional + probiotics, natural

and organic) after utilizing the comparative Ct method. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *Lactobacillus* spp. when comparing the conventional + probiotics broiler chicken diet to the broiler chicken organic diet gave a P value of 0.8203. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *Lactobacillus* spp. when comparing the conventional + probiotics broiler chicken diet to the broiler chicken natural diet gave a P value of 0.7344. Using the Mann Whitney test, the fold change seen in *Lactobacillus* spp. when comparing the conventional + probiotics broiler chicken diet to the broiler chicken organic diet gave a P value of 0.5357. Using the Mann Whitney test, the fold change seen in *Lactobacillus* spp. when comparing the conventional + probiotics broiler chicken diet to the broiler chicken natural diet gave a P value of 0.5357.

The increased fold change in *Lactobacillus* spp. was observed from the comparison of the conventional with probiotics broiler chicken diet to the organic broiler chicken diet, however, it was not considered statistically significant. Furthermore, an increased fold change in *Lactobacillus* spp. was also observed from the comparison of conventional with probiotics broiler chicken diet to the natural broiler chicken. Probiotic supplementation, particularly of *Lactobacillus* species, in the conventional diet possibly surpassed the amount of probiotics supplemented in both natural and organic diets, and potentially explains the increased seen during the comparison.

Figure 11: Fold Change in *Lactobacillus* spp. Due to Conventional Diet with Probiotic Addition when Compared to Organic and Natural Diet.

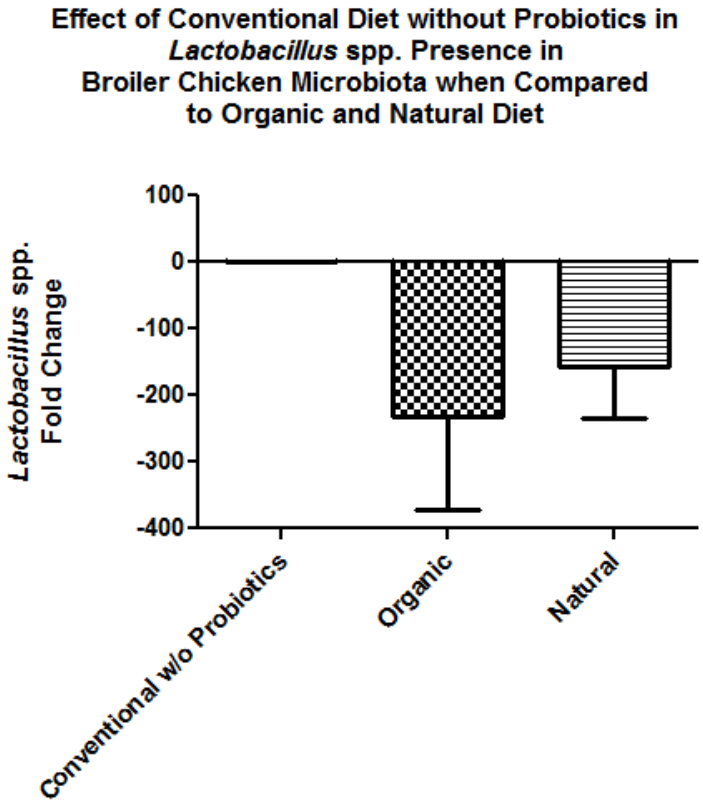

Figure 11 graphically depicts the fold change observed in *Lactobacillus* spp. when comparing three broiler chicken commercial diet types (conventional without probiotics, natural and organic) after utilizing the comparative Ct method. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *Lactobacillus* spp. when comparing the conventional without probiotics broiler chicken diet to the broiler chicken organic diet gave a P value of 0.2500. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *Lactobacillus* spp. when comparing the conventional without probiotics broiler chicken diet to the broiler chicken natural diet gave a P value of 0.2500.

The data was not considered statistically significant. However, a decreased fold change in *Lactobacillus* spp. was observed from the comparison of the conventional without probiotics broiler chicken diet to the organic broiler chicken diet. Furthermore, a decreased fold change in *Lactobacillus* spp. was also observed from the comparison of conventional without probiotics broiler chicken diet to the natural broiler chicken. The lack of probiotic supplementation in the conventional diet possibly accounts for the

decrease found when in comparing the amount of *Lactobacillus* in the conventionally-fed broiler chicken diet to both natural and organic diets.

4.2 Amplification of *Enterococcus* species in Four Commercial Diet Types (Conventional with Probiotics, Convention without Probiotics, Natural, and Organic)

Enterococcus spp. was detected in only one sample from a natural broiler chicken production, an organic broiler chicken production, and a conventional without probiotics broiler chicken production. There was no detection of *Enterococcus* spp. in any conventional with probiotic supplementation broiler chicken production. Due to lack of amplification in the majority of samples, comparative Ct method could not be utilized.

The lack of *Enterococcus* spp. amplification in the conventional with probiotic supplementation production could be attributed to both the antimicrobial properties from the antibiotics supplemented within the conventional diet inhibiting *Enterococcus* spp., and also the additional probiotics creating a more competitive microbiota in which *Enterococcus* spp. do not thrive as well.

4.3 Amplification of *Clostridium difficile* in Four Commercial Diet Types (Conventional with Probiotics, Convention without Probiotics, Natural, and Organic)

Clostridium difficile was not amplified in any samples tested. Due to lack of amplification in any samples, analysis by the comparative Ct method could not be carried out.

4.4.1 Phenotypic Expression of Resistance in Conventional with Probiotics Broiler Chicken Production

Transformants from conventional broiler chicken productions that utilized probiotics in their diets were found to be resistant to: kanamycin, amikacin, penicillin, neomycin, gentamicin, streptomycin, and sulfisoxazole. The conventional diet with probiotic supplementation yielded the only resistance to gentamicin found among samples.

4.4.2 Phenotypic Expression of Resistance in Conventional without Probiotics Broiler Chicken Production

Transformants from conventional broiler chicken productions that do not utilize probiotics in their diets were found to be resistant to: kanamycin, amikacin, penicillin, neomycin, streptomycin, and sulfisoxazole.

4.4.3 Phenotypic Expression of Resistance in Natural Broiler Chicken Production

Transformants from natural broiler chicken productions that use antibiotic-free and probiotic supplementation in their diet were found to be resistant to: kanamycin, amikacin, rifampicin, penicillin, neomycin, sulfisoxazole, erythromycin, and streptomycin. The natural diet yielded the only resistance to erythromycin found among samples.

4.4.4 Phenotypic Expression of Resistance in Organic Broiler Chicken Production

Transformants from organic broiler chicken productions that use antibiotic-free and organic standard probiotic supplementation were found to be resistant to: kanamycin, rifampicin, amikacin, penicillin, neomycin, sulfisoxazole, and streptomycin.

Table 5: Phenotypic Expression of Resistance

	Kanamycin	Tetracycline	Naladixic Acid	Gentamicin	Rifampicin	Amplicillin	Amikacin	Streptomycin	Penicillin	Vancomycin	Erythromycin	Neomycin	Trimethoprim	Sulfisoxazole
Library One														
Organic I	+	-	-	N/A	-	-	+	N/A	-	-	-	-	-	-
Organic II	-	-	-	N/A	+	-	-	N/A	-	-	-	+	-	+
Organic III	+	-	-	N/A	+	-	+	N/A	+	-	-	+	-	+
Probiotic I	-	-	-	N/A	-	-	-	N/A	-	-	-	-	-	+
Probiotic II	+	-	-	N/A	+	-	+	N/A	+	-	-	+	-	-
Probiotic III	-	-	-	N/A	-	-	+	N/A	-	-	-	-	-	-
Conventional + Probiotics I	-	-	-	N/A	-	-	+	N/A	+	-	-	+	-	-
Conventional + Probiotics II	+	-	-	N/A	-	-	+	N/A	-	-	-	+	-	-
Conventional w/o Probiotics	-	-	-	N/A	-	-	+	N/A	-	-	-	-	-	+
											-			
Library Two														
Organic I	+	-	-	N/A	-	-	+	N/A	+	-	-	-	-	-
Organic II	+	-	-	N/A	-	-	+	N/A	+	-	-	+	-	-
Organic III	-	-	-	N/A	-	-	+	N/A	-	-	-	-	-	+
Probiotic I	-	-	-	N/A	-	-	+	N/A	-	-	-	-	-	-
Probiotic II	-	-	-	N/A	-	-	+	N/A	-	-	+	-	-	+
Probiotic III	+	-	-	N/A	-	-	+	N/A	-	-	-	+	-	+
Conventional + Probiotics I	-	-	-	N/A	-	-	-	N/A	-	-	-	-	-	-
Conventional + Probiotics II	-	-	-	N/A	-	-	+	N/A	-	-	-	-	-	-
Conventional w/o Probiotics	+	-	-	N/A	-	-	+	N/A	+	-	-	+	-	+
Library Three														
Organic I	+	N/A	-	-	-	-	-	+	-	N/A	-	+	-	+
Organic II	+	N/A	-	-	-	-	+	+	+	N/A	-	+	-	+
Organic III	-	N/A	-	-	-	-	+	-	-	N/A	-	+	-	+
Probiotic I	+	N/A	-	-	+	-	+	+	+	N/A	-	+	-	+
Probiotic II	+	N/A	-	-	-	-	+	-	+	N/A	-	+	-	+
Probiotic III	+	N/A	-	-	-	-	+	-	+	N/A	-	+	-	+
Conventional + Probiotics I	+	N/A	-	+	-	-	+	+	+	N/A	-	+	-	+
Conventional + Probiotics II	-	N/A	-	-	-	-	+	+	+	N/A	-	+	-	+
Conventional w/o Probiotics	-	N/A	-	-	-	-	+	-	-	N/A	-	+	-	+

4.5. Fold Changes in Resistance Genes Comparing Conventional Broiler Chicken Diets with and without Probiotics to Organic and Natural Broiler Chicken Diets

Figure 12: Fold Change in *aacA-aphD* Due to Conventional Diet with Probiotic Addition when Compared to Organic and Natural Diet.

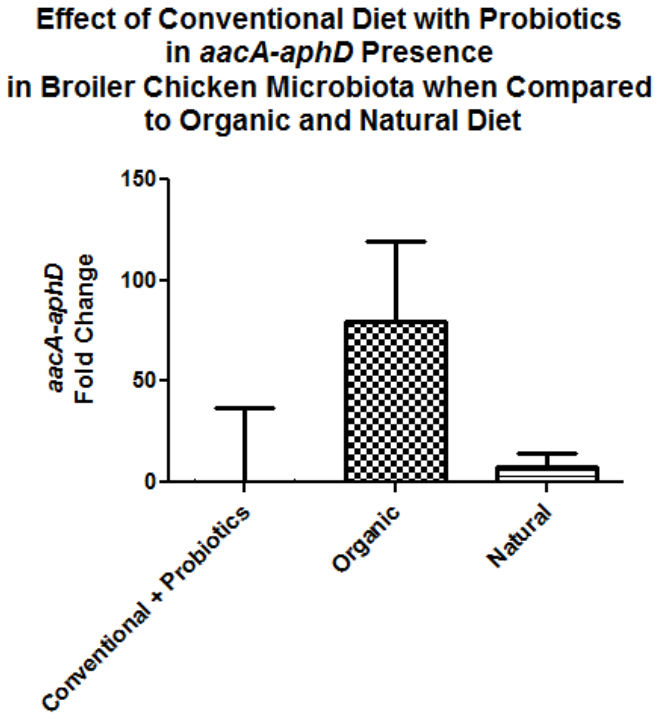

Figure 12 graphically illustrates the fold change observed in *aacA-aphD* when comparing three broiler chicken commercial diet types (conventional + probiotics, natural and organic) after utilizing the comparative Ct method. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *aacA-aphD*. when comparing the conventional + probiotics broiler chicken diet to the broiler chicken organic diet gave a P value of 0.1641. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *aacA-aphD* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken natural diet gave a P value of 1.0000. Using the Mann Whitney test, the fold change seen in *aacA-aphD* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken organic diet gave a P value of 0.0768. Using the Mann Whitney test, the fold change seen in *aacA-aphD* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken natural diet gave a P value of 1.0000.

The data was not considered statistically significant. However, an increased fold change in the presence of the *aacA-aphD* gene was observed from the comparison of the conventional with probiotics broiler chicken diet to the organic broiler chicken diet.

Furthermore, a slight increased fold change in the presence of the *aacA-aphD* gene was also observed from the comparison of conventional with probiotics broiler chicken diet to the natural broiler chicken diet. Antimicrobial supplements in the conventional diet possibly created a favorable niche for antimicrobial resistant microorganisms that carried and possibly horizontally transferred the *aacA-aphD* gene, and could also account for the increased fold changes observed when compared to the antibiotic-free natural and organic diets.

Figure 13: Fold Change in *aacA-aphD* Due to Conventional Diet without Probiotic Addition when Compared to Organic and Natural Diet

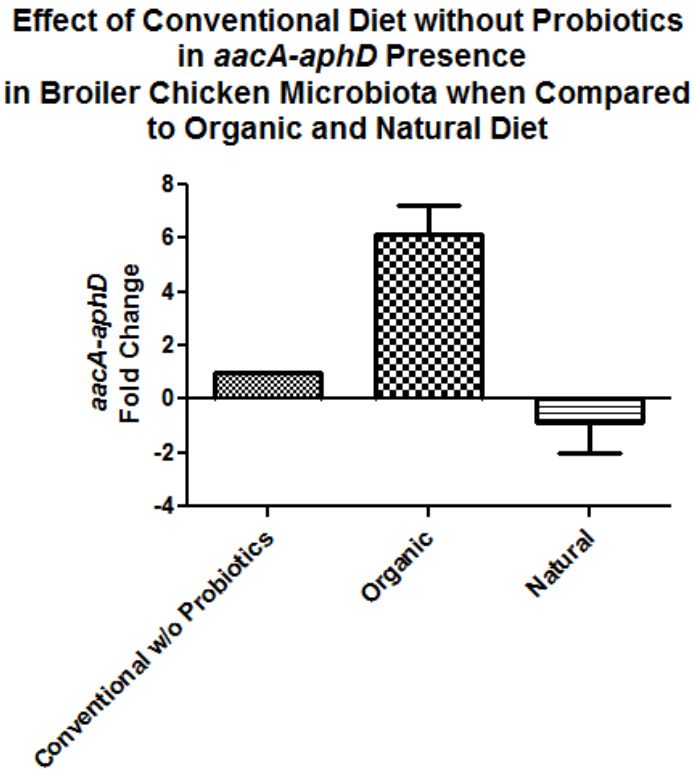

Figure 13 graphically depicts the fold change observed in *aacA-aphD* when comparing three broiler chicken commercial diet types (conventional without probiotics, natural and organic) after utilizing the comparative Ct method. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *aacA-aphD* when comparing the conventional without probiotics broiler chicken diet to the broiler chicken organic diet gave a P value of 0.2500. Using the Wilcoxon matched-pairs signed rank test, the

fold change seen in *aacA-aphD* when comparing the conventional without probiotics broiler chicken diet to the broiler chicken natural diet gave a P value of 0.5000.

Although the data was not considered statistically significant, an increased fold change in the presence of the *aacA-aphD* gene was observed from the comparison of the conventional without probiotics broiler chicken diet to the organic broiler chicken diet. However, a slight decreased fold change in the presence of the *aacA-aphD* gene was observed from the comparison of conventional without probiotics broiler chicken diet to the natural broiler chicken diet. Antimicrobial supplements in the conventional diet possibly created a favorable niche for antimicrobial resistant microorganisms and allowed for the persistence and horizontal transfer of the *aacA-aphD* gene. The spread of the resistance gene *aacA-aphD* to other microorganism could also account for the increased fold changes observed when compared to the antibiotic-free organic diets. The presence of probiotics in the natural and organic diets could also have allowed for beneficial microorganisms to compete for nutrients and not allow antimicrobial resistance microorganisms and their resistance genes to flourish.

Figure 14: Fold Change in *ermA* Due to Conventional Diet with Probiotic Addition when Compared to Organic and Natural Diet.

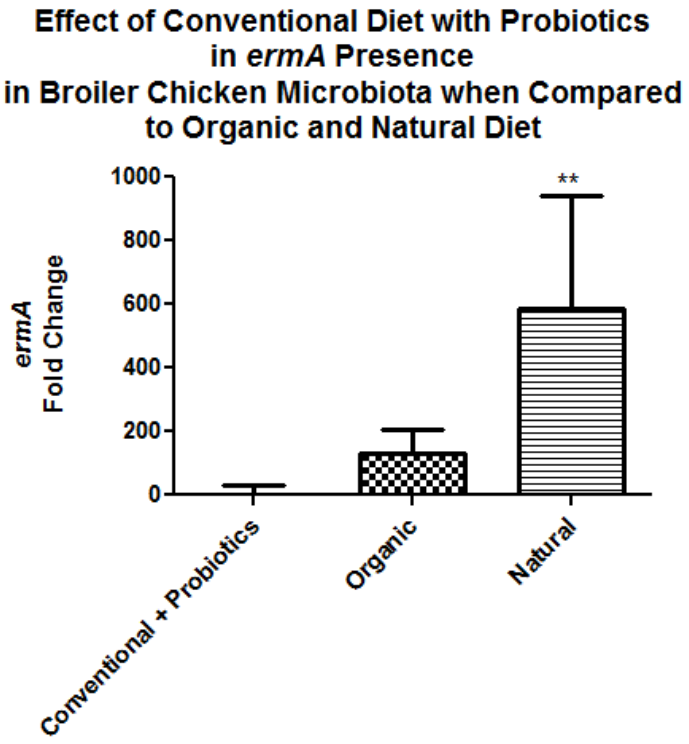

Figure 14 graphically illustrates the fold change observed in *ermA* when comparing three broiler chicken commercial diet types (conventional + probiotics, natural and organic) after utilizing the comparative Ct method. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *ermA* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken organic diet gave a P value of 0.2500. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *ermA* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken natural diet gave a P value of 0.0977. Using the Mann Whitney test, the fold change seen in *ermA* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken organic diet gave a P value of 0.1569. Using the Mann Whitney test, the fold change seen in *ermA* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken natural diet gave a P value of 0.0270.

A statistically significant increased fold change in the presence of the *ermA* gene was observed from the comparison of the conventional with probiotics broiler chicken diet to the natural broiler chicken diet. Furthermore, a non-statistically significant slight increased fold change in the presence of the *ermA* gene was also observed from the comparison of conventional with probiotics broiler chicken diet to the organic broiler chicken diet. Antimicrobial supplements in the conventional diet possibly created a

favorable niche for antimicrobial resistant microorganisms that carried and possibly horizontally transferred the *ermA* gene, and could also account for the increased fold changes observed when compared to the antibiotic-free natural and organic diets.

Figure 15: Fold Change in *ermA* Due to Conventional Diet without Probiotic Addition when Compared to Organic and Natural Diet.

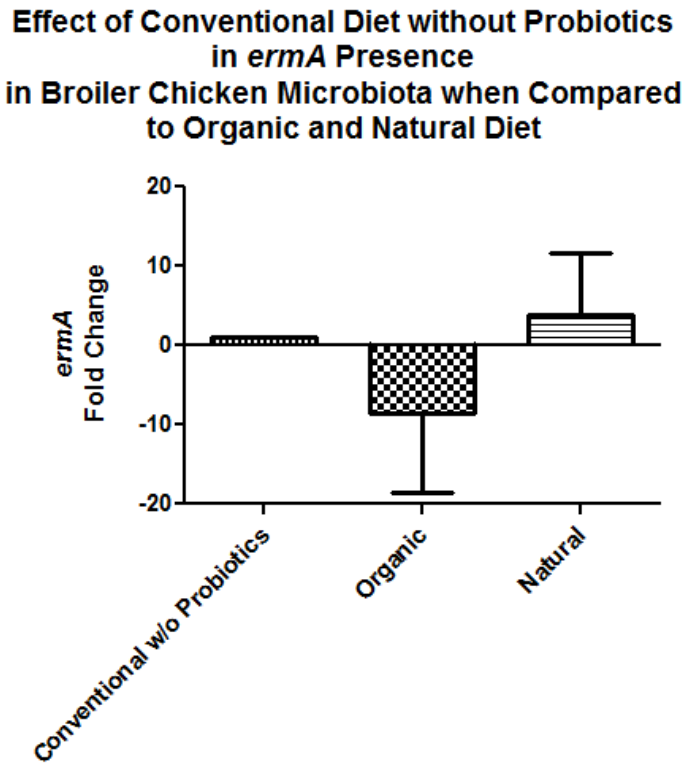

Figure 15 graphically depicts the fold change observed in *ermA* when comparing three broiler chicken commercial diet types (conventional without probiotics, natural and organic) after utilizing the comparative Ct method. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *ermA* when comparing the conventional without probiotics broiler chicken diet to the broiler chicken organic diet gave a P value of 0.7500. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *ermA* when comparing the conventional without probiotics broiler chicken diet to the broiler chicken natural diet gave a P value of 0.7500.

Although the data was not considered statistically significant, a decreased fold change in the presence of the *ermA* gene was observed from the comparison of the conventional without probiotics broiler chicken diet to the organic broiler chicken diet.

However, a slightly increased fold change in the presence of the *ermA* gene was observed from the comparison of conventional without probiotics broiler chicken diet to the natural broiler chicken diet. Antimicrobial supplements in the conventional diet possibly created a favorable niche for antimicrobial resistant microorganisms and allowed for the persistence and horizontal transfer of the *ermA* gene. The spread of the resistance gene *ermA* to other microorganism could also account for the increased fold changes observed when compared to the antibiotic-free natural diet. The presence of probiotics in the natural and organic diets could also have allowed for beneficial microorganisms to compete for nutrients and not allow antimicrobial resistance microorganisms and their resistance genes to flourish in the natural broiler chicken diet.

Figure 16: Fold Change in *msrC* Due to Conventional Diet with Probiotic Addition when Compared to Organic and Natural Diet.

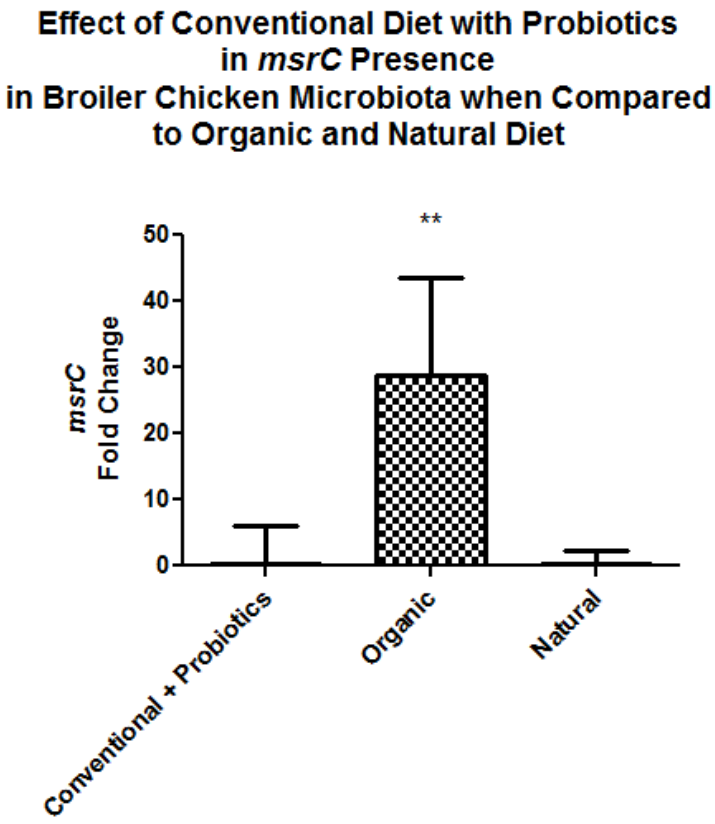

Figure 16 graphically illustrates the fold change observed in *msrC* when comparing three broiler chicken commercial diet types (conventional + probiotics, natural and organic) after utilizing the comparative Ct method. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *msrC* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken organic diet gave a P value of 0.0273. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *msrC* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken natural diet gave a P value of 0.6523. Using the Mann Whitney test, the fold change seen in *msrC* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken organic diet gave a P value of 0.1325. Using the Mann Whitney test, the fold change seen in *msrC* when comparing the conventional + probiotics broiler chicken diet to the broiler chicken natural diet gave a P value of 0.7234.

A statistically significant increased fold change in the presence of the *msrC* gene was observed from the comparison of the conventional with probiotics broiler chicken diet to the organic broiler chicken diet. Furthermore, a non-statistically significant fold change in the presence of the *ermA* gene was observed from the comparison of

conventional with probiotics broiler chicken diet to the organic broiler chicken diet. Antimicrobial supplements in the conventional diet possibly created a favorable niche for antimicrobial resistant microorganisms that carried and possibly horizontally transferred the *ermA* gene, and could also account for the statistically significant increased fold changes observed when compared to the antibiotic-free organic diet.

Figure 17: Fold Change in *msrC* Due to Conventional Diet without Probiotic Addition when Compared to Organic and Natural Diet.

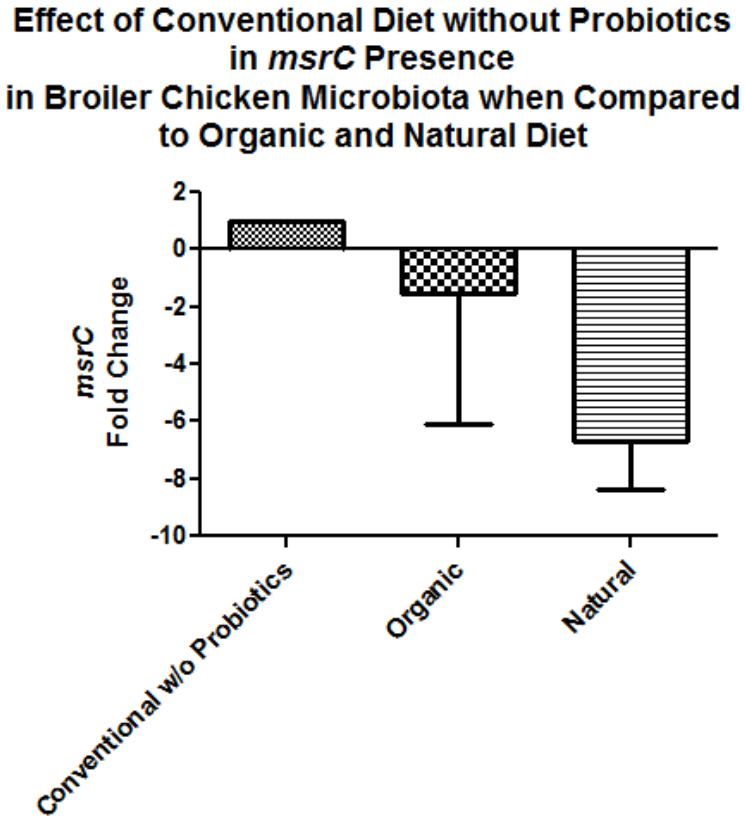

Figure 15 graphically depicts the fold change observed in *msrC* when comparing three broiler chicken commercial diet types (conventional without probiotics, natural and organic) after utilizing the comparative Ct method. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *msrC* when comparing the conventional without probiotics broiler chicken diet to the broiler chicken organic diet gave a P value of 1.0000. Using the Wilcoxon matched-pairs signed rank test, the fold change seen in *msrC* when comparing the conventional without probiotics broiler chicken diet to the broiler chicken natural diet gave a P value of 0.2500.

Although the data was not considered statistically significant, a decreased fold change in the presence of the *ermA* gene was observed from the comparison of the conventional without probiotics broiler chicken diet to the natural broiler chicken diet. Furthermore, a slightly decreased fold change in the presence of the *ermA* gene was observed from the comparison of conventional without probiotics broiler chicken diet to the organic broiler chicken diet.

4.5.1 Presence of *mecA* Resistance Gene

During the detection of the *mecA* through real-time PCR, only one sample amplified indicating the presence of *mecA* gene(s) are found within that sample. The sample that did amplify was from the conventionally-fed without probiotics broiler chicken production. Unfortunately the relative amount and fold change difference in comparison to the other samples was not possible due to no other samples amplifying.

Chapter 5

Discussion

When comparing the presence of Genus *Prevotella* in broiler chicken microbiota from a conventional production that uses antibiotics to an antibiotic-free organic production, there is a statistically significant increase in Genus *Prevotella* irrespective of probiotic supplementation in the conventional diet. These data suggest that resistance is being formed within the Genus *Prevotella* that allows propagation regardless of a more competitive environment caused by the probiotic addition.

The comparison of the presence of *Clostridium perfringens* in a conventionally-fed broiler chicken microbiota to an antibiotic-free naturally-fed broiler chicken microbiota yields a statistically significant decrease in *Clostridium perfringens* no matter if there was or was not probiotic supplementation in the conventional diet. This may suggest that antimicrobial properties in the antibiotics supplemented in both conventional types inhibited the colonization of *Clostridium perfringens*.

Large differences can be seen when comparing the presence of *Bacteriodes* in broiler chicken microbiota from a conventional production that uses probiotics to a conventional production that does not utilize probiotics. Probiotic addition to the conventional diet causes an alteration from a non-significant fold change, seen in comparison to organic-fed broiler chicken microbiota, to a severely decreased fold-change. Indeed, probiotic addition to the conventional diet also caused a shift from an increased fold change, seen in comparison to naturally-fed broiler chicken microbiota, to a severely decreased fold-change. These changes can be attributed to both the

antimicrobial properties from the antibiotics supplemented within the conventional diet inhibiting *Bacteriodes,* and also the additional probiotics creating a more competitive microbiota in which *Bacteriodes* do not thrive as well.

A notable difference can be seen when comparing the presence of *Enterobacteriaceae* in broiler chicken microbiota from a conventional production that uses probiotics to a conventional production that does not utilize probiotics. Probiotic addition to the conventional diet causes an alteration from an increased fold change, seen in comparison to organic-fed broiler chicken microbiota, to a minimal non-significant fold-change. Indeed, probiotic addition to the conventional diet also caused a shift from a severely increased fold change, seen in comparison to naturally-fed broiler chicken microbiota, to a lesser increased fold-change. These changes can be attributed to both the antimicrobial properties from the antibiotics supplemented within the conventional diet inhibiting *Enterobacteriaceae,* and also the additional probiotics creating a more competitive microbiota in which *Enterobacteriaceae* do not thrive as well.

When comparing *Lactobacillus* spp., great differences can be seen when in broiler chicken microbiota from a conventional production that uses probiotics to a conventional production that does not utilize probiotics. Probiotic addition to the conventional diet causes an alteration from a decreased fold change, seen in comparison to organic-fed broiler chicken microbiota, to a severely increased fold-change. Indeed, probiotic addition to the conventional diet also caused a shift from a decreased fold change, seen in comparison to naturally-fed broiler chicken microbiota, to a severely increased fold-change. These changes could be attributed to the additional probiotics used being of

Lactobacillus species and possibly a greater quantity is supplemented compared to organic and natural broiler chicken diets.

Phenotypic expression of antimicrobial resistance was presented by every sample in every diet type (organic, natural, conventional with probiotics, conventional without probiotics). Samples from broiler chickens fed organic diets showed resistance to the following antibiotic classes: aminoglycosides (kanamycin, amikacin, neomycin, streptomycin), rifamycins (rifampicin), β-lactams (penicillin), and sulfa drugs (sulfisoxazole). Samples from broiler chickens fed natural diets presented resistance to the following antibiotic classes: aminoglycosides (kanamycin, amikacin, neomycin, and streptomycin), rifamycins (rifampicin), β-lactams (penicillin), sulfa drugs (sulfisoxazole), and macrolides (erythromycin). Samples from broiler chickens fed conventional diets with probiotic supplementation showed resistance to the following antibiotic classes: aminoglycosides (gentamicin, kanamycin, amikacin, neomycin, and streptomycin), β-lactams (penicillin), and sulfa drugs (sulfisoxazole). Samples from broiler chickens fed conventional diets without probiotic supplementation presented resistance to the following antibiotic classes: aminoglycosides (kanamycin, amikacin, neomycin, and streptomycin), β-lactams (penicillin), and sulfa drugs (sulfisoxazole).

Regardless if a broiler chicken was fed a diet supplemented with antibiotics, resistance was still present among each sample. Resistance perhaps began elsewhere. A few possible reservoirs antimicrobial resistance could have come from the environment in which the broiler chickens were reared, transporter or slaughter. A broiler chicken can be exposed to fresh or new bedding depending on how many flocks have been previously grown on the same bedding. Water and feed, by cross-contamination, could provide the

ideal route for antimicrobial resistant bacteria to find their way into the chicken gut and microbiota. Antimicrobial resistant microorganisms might also find transportation through people if biohazard safety protocol is not followed every time. The ventilation systems in place to keep ammonia levels low and broiler chickens in a temperate environment provide an excellent way for airborne resistant pathogens to flow directly into the broiler chicken house.

When comparing *ermA* presence, great differences can be seen when in broiler chicken microbiota from a conventional production that uses probiotics to a conventional production that does not utilize probiotics. Probiotic addition to the conventional diet causes an alteration from a decreased fold change, seen in comparison to organic-fed broiler chicken microbiota, to a severely increased fold-change. Indeed, probiotic addition to the conventional diet also caused a shift from a decreased fold change, seen in comparison to naturally-fed broiler chicken microbiota, to a statistically increased fold-change. This may suggest that the bacteria that antimicrobials are targeting, are not the only bacteria present that can carry resistance genes and that these non-targeted bacterial carriers flourish with probiotic addition and promote the spread of macrolide drug class resistance.

When comparing *msrC* presence, notable shifts can be seen when in broiler chicken microbiota from a conventional production that uses probiotics to a conventional production that does not utilize probiotics. Probiotic addition to the conventional diet causes an alteration from a decreased fold change, seen in comparison to organic-fed broiler chicken microbiota, to a statistically significant increased fold-change. Indeed, probiotic addition to the conventional diet also caused a shift from a decreased fold

change, seen in comparison to naturally-fed broiler chicken microbiota, to no change detected. This may elude that the bacteria that antimicrobials are targeting are not the only bacteria present that can carry resistance genes and that these non-targeted bacterial carriers thrive with probiotic addition and encourage the dissemination of macrolide drug class resistance.

When comparing a*acA-aphD* presence, alterations can be seen when in broiler chicken microbiota from a conventional production that uses probiotics to a conventional production that does not utilize probiotics. Probiotic addition to the conventional diet causes an alteration from a notable increased fold change, seen in comparison to organic-fed broiler chicken microbiota, to a pointedly larger increased fold-change. Indeed, probiotic addition to the conventional diet also caused a shift from a slightly decreased fold change, seen in comparison to naturally-fed broiler chicken microbiota, to a slightly increased fold-change. This may elude that the bacteria that antimicrobials are targeting are not the only bacteria present that can carry resistance genes and that these non-targeted bacterial carriers thrive with probiotic addition.

Chapter 6

Conclusions and Future Directions

At varying levels, antimicrobial resistance can be found among every microbiota from each diet type (conventional with and without probiotics, natural, and organic). The antimicrobial resistance found in both the antibiotic-free diets (organic and natural) was not a product of the supplementation of antimicrobials within the feed. It could be hypothesized that antimicrobial resistance in broiler chicken production begins elsewhere. The commensal or pathogenic antimicrobial resistant bacteria is possibly transferred into the broiler chicken microbiota from cross-contamination occurring from exposure via hatcheries, houses, bedding, water, feed, improper biohazard precaution, ventilation, transport or slaughtering. However, even with antimicrobial resistance found within each broiler chicken microbiota from each diet, the addition of antimicrobials in conventional diets have had a direct side effect of changing the gut microbiota and increasing the amount of resistance genes present when compared to natural and organic diets.

This study led the way for many future studies, including sequencing of all antibiotic resistant transformants, which will allow us to search for existing and new antimicrobial resistance mechanisms and genes. A larger sample size will also allow for a greater statistical power. Real-time PCR for the amplification of rifampin and sulfa drug class resistance genes may result in more significant trends through the promising data from the phenotypic expression of resistance. Seeking out farms that use only vegetarian

diets with no probiotic or antibiotic supplementation in their broiler chickens could also serve as a new baseline for how every diet type changes this “wild type” chicken microbiota, also additional producers that use conventional diets without probiotic supplementation will be sought to increase sample size and statistical power. Additionally, the methods established in this study will allow for similar investigations into turkeys and laying hens in the future.

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