Selection of *Bacillus* strains for *Salmonella* and Ammonia Reduction within a Poultry House Environment

by

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Abstract

Salmonella species are a food safety concern associated with poultry products, causing thousands of gastrointestinal disorders every year. Currently, controlling *Salmonella* in poultry houses is not being performed adequately. It is assumed that chemical litter amendments used to control ammonia will also control *Salmonella*. It has been shown that these chemical amendments are transitively effective, while it is postulated that bacterial amendments could last indefinitely. A collection of *Bacillus* strains (n=244) was screened for inhibition of *Salmonella enterica* strains (n=10). The 30 *Bacillus* strains that had the highest degree of inhibition against the most *Salmonella* strains were selected for further evaluation. While no significant nitrification activity was observed, we did observe significant differences in the respective ability of different *Bacillus* strains to take up ammonia from a culture medium that contained ammonium chloride. Six strains were selected and applied in a litter trial at 10^7 CFU/g litter. No significant effects were observed with regards to *Salmonella* or ammonia levels for any treatment at any sampling time, but two *Bacillus* strains were re-isolated from the poultry litter demonstrating survival within the litter environment. Two of the best-performing *Bacillus* strains, AB01 and AP71, were selected for evaluation in feed. The genome for each of these strains was sequenced, *de novo* assembled and used to generate PCR primers unique to the strains for identification within poultry litter and intestinal samples. A poultry trial was conducted wherein the probiotic strains were fed to chickens at a dose of 10^7 CFU/g of feed, followed by inoculation by *Salmonella*. No *Salmonella* culturable counts were detected without

the use of enrichments in poultry samples, with or without probiotic feed, making it impossible to conclude whether the probiotic strains had the potential to reduce *Salmonella* levels. Future research will evaluate the potential of the probiotic strains to reduce ammonia levels in fresh poultry litter substrate, and to reduce *Salmonella* levels within the chicken intestinal microbiota using next-generation sequencing of 16S rRNA genes. These probiotic strains may be useful to the poultry industry in controlling ammonia and *Salmonella* levels within a poultry house environment.

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I. INTRODUCTION

The CDC estimates that 76 million illnesses are caused by foodborne pathogens annually in the United States, with poultry and its products being recognized risk factors for foodborne outbreaks of salmonellosis. The federal government has invested significant resources in diagnosis and prevention of foodborne disease and to a large degree much of the resources are dedicated to epidemiological investigations to identify the source of outbreaks. Significant resources are also invested in food inspection and compliance with slaughter and processing plant sanitation procedures intended to reduce pathogen levels in food. However, these surveillance networks do not address pre-harvest food safety, specifically the implementation of "on-farm" management practices for controlling *Salmonella*. The purpose of this research is to test the efficacy of *Bacillus* strains as a tool that can be applied for "on-farm" management of *Salmonella* in poultry and poultry litter. This work has great potential to transform the practice of poultry production in a way that is cost-effective, practical, environmentally sustainable, and beneficial to the economy of Alabama. The poultry industry is very significant in Alabama (~10% of the Alabama economy generating 75,000 jobs), any technology that promotes cost-effective and sustainable improvements to animal health and reduces the spread of foodborne pathogens could directly benefit this important industry and consumer health. The objectives of this research are to:

1. Identify strains of *Bacillus* that inhibit *Salmonella,*

2. Identify *Bacillus* strains that have nitrification activity in culture,

3. Identify *Bacillus* strains that reduce ammonia and/or *Salmonella* in poultry litter,

4. Evaluate efficacy of *Bacillus* strains in modifying chicken intestinal microbiota, reducing ammonia levels in litter, and promoting chicken health.

LITERATURE REVIEW

Probiotics

Probiotics have been produced and consumed by humans since the discovery of fermented foods. For thousands of years, fermentation has been a method of adding flavor and preserving food. However, during the turn of the twentieth century, biologist Ilya Metchnikoff theorized that aging was caused by harmful bacteria growing in the human intestine and that the introduction of lactic acid bacteria to the gut would inhibit the growth of these toxic inhabitants and thus slows the aging process (Metchnikoff 1907). Since then, the definition of probiotics has changed a number of times. The term was originally coined by Lilly and Stillwell in 1965, describing probiotics as "substances secreted by one microorganism which stimulates the growth of another" (Lilly and Stillwell 1965). In 1974, Parker used the term to describe a potential dietary supplement, defining it as "organisms and substances which contribute to intestinal microbial balance" (Parker 1974). Fuller believed this definition to be too vague and modified it, calling probiotics "a live microbial feed supplement which beneficially affects the host animal by improving its microbial balance" (Fuller 1989). The World Health Organization (WHO) produced a report in 2001, revising the definition to "live microorganisms, which when consumed in adequate amounts, confer a health effect on the host", from Schaafsma [1996]. This agrees with the definition posed by Havenaar et al. [1992] calling probiotics "A preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host." The definition will surely change as more is discovered about the gastrointestinal tract, its microbial flora, and the interactions between the diverse bacterial communities and their living habitat. However, a central question regarding probiotics

has changed little within the last century: Are probiotics a stable, safe, and reliable alternative to the conventional medicinal practices they have been theorized to replace (i.e. antibiotics)?

Probiotics in humans

In humans probiotics are primarily consumed either as an ordinary part of a food product, such as yogurt, or as a dietary supplement. However it is currently well documented that they do confer some health benefits, such as stimulation of the immune system (Isolauri et al. 2000), and a decrease in the duration and occurrence of diarrhea associated with *Clostridium difficile* (Segara-Newnham 2007) and traveler's diarrhea (Hilton et al. 1997). The most prominent probiotic candidates for human use are members of the genus *Bifidobacterium* and *Lactobacillus*, in particular *L. acidophilus, L. casei, L. rhamnosus*. It has also been reported that probiotics can improve lactose intolerance (Hove et al. 1999) and prevent cancer associated with the gastrointestinal tract (Geier et al. 2006).

The issue of public trust may be one of the greater obstacles for widespread probiotic use. With the revision of the food safety laws in the United States (DeWaal 2011) and a large focus on healthy eating habits, some consumers are suspicious of consuming active cultures. Compound this with the somewhat prototype nature of some probiotics, and some of their more ridiculous proposed therapeutic applications, such as treatment for autism and facilitation of nutrient utilization before a race for swimmers or marathon runners [Vanderhoof and Young, 2009]. It has even been found that some products do not contain the listed organisms, contain non-viable cultures, or contain unknown organisms. In order for the general public to begin to trust probiotics, potential products need to be tested, and their proposed effects verified, by established members of the scientific academic community. Even with these potential issues of

trust, there is a growing consumer interest in the benefits of microorganisms as an alternative to antibiotics and chemical pharmaceuticals.

Probiotics in Agriculture

The industrialization of agriculture has created a new wave of challenges due to bacterial pathogens. Large processing facilities packed full with livestock makes a breeding ground for veterinary disease and microbial contamination of what will eventually become food millions of people across the world will consume. In terms of federal regulation, very little is done to prevent contamination on the farm before reaching the slaughterhouses. Rapid transmission of pathogens in the tight livestock living quarters has lead to widespread use of veterinary therapeutic antimicrobials and sub-therapeutic doses of antibiotics are commonly used to improve the growth of the animals and the efficiency of their feed. These practices are causing concern over the natural evolution and spread of antibiotic resistant bacteria, pressing meat producing companies, government regulators, farmers and consumers to pursue alternative methods of pathogen control on the farm. Probiotics pose a possible solution to the problem. Currently, probiotics are not typically used during meat production, except in organic farms where an alternative to antibiotics is legally required for the food produced to be called organic (Griggs and Jacob 2005). As with genetically modified foods, consumers may be unwilling to eat meat that has been altered in a way that is not well understood and difficult to explain to the general public. It also may be challenging to convince farmers to switch from using antibiotics that keep their livestock healthy and improves the growth of the animal.

Probiotics in Poultry Production

As with all agriculture, the poultry production industry has grown dramatically within the last half century. An estimated 9 billion chickens are raised every year in the United States alone, and poultry represents the majority of meat consumed by Americans. The poultry industry struggles with many of the same challenges posed by all industrial livestock production. Very tight living spaces, the accumulation of feces, and the rapid spread of disease are serious issues for meat production as a whole, but the poultry industry carries a few unique challenges of its own. The majority of food-borne salmonellosis is caused by consumption of undercooked chicken and eggs (Crum-Cianflone 2008). Chickens can also be carriers of other food-borne pathogens, such as *Campylobacter*, *Clostridium*, and *Escherichia coli* (Kabir 2009). While some pathogens cause disease in chickens, and thus can be easily identified and removed, many, as in the case of *Salmonella,* are asymptomatic (Revolledo et al. 2006). The accumulation of chicken feces results in the release of massive amounts of ammonia, produced from the uric acid birds release as a nitrogenous waste. Volatilized ammonia poses a significant health hazard both for the chickens and the humans working around them (Ritz et al. 2004). While modern poultry management has a number of methods for overcoming these difficulties, the potential consequences of these conventional remedies often cause just as much concern as the problems they are meant to fix. Widespread use of antibiotics, whether therapeutic or not, results in the natural evolution of antibiotic-resistant bacteria, some of which can cause serious illness in humans (Apata 2009). Probiotic treatment may hold a more long-term solution for many of these problems.

According to Collado et al. [2010], in order for an organism to be called a probiotic it must meet the following five criteria:

1) The organism must have demonstrated a beneficial effect on the host.
2) The organism must be non-pathogenic, non-toxic, and free of adverse side effects. (In the case of an agricultural probiotic, this must be true for both the host and for humans consuming the product produced by the host.)
3) The organism must be able to survive the gastrointestinal tract. (It has also been suggested that the organism must be able to adhere to the gastrointestinal epithelium. [Chateau et. al. 1993])
4) The organism must be present in the product in an adequate number of viable cells to confer the health benefit.
5) The organism must be compatible with the product matrix, processing, and storage conditions to maintain the desired properties, and labeled correctly.

"A beneficial effect on the host" is very broad and can refer to a number of useful traits. One of the most sought after is the removal of pathogenic bacteria. The primary mechanism by which this takes place is competitive exclusion. Newly hatched chicks would be fed live cultures of bacteria native to the chicken microbiota, such as strains of *Lactobacillus*. The probiotic strain is given an opportunity to colonize a sterile gut. When *Salmonella* is introduced there is a community already in place, taking up all of the epithelial wall space, consuming available nutrients, and producing secondary metabolites that may be harmful to the pathogen. The first week after hatch is when a chicken is most susceptible to infection by *Salmonella,* when the gastrointestinal tract and its microbial population are not fully developed. As few as ten cells can initiate a *Salmonella* infection at this point in a chick's development. Blocking out infection at this point drastically reduces the chance of the development of salmonellosis, making the trait of effective competitive exclusion a top priority in the development of a poultry probiotic. However, the benefits of competitive exclusion are most effective when the probiotic is

colonizing an open, free gastrointestinal tract. Adding a few strains to an already established gut microbiota reduces the efficacy of the treatment, making this trait useless for rooting out an established pathogenic infection.

Another potential benefit of probiotic treatment is the improvement of the chicken's ability to utilize nutrients quickly and efficiently, allowing the bird to reach its maximal growth potential, with as little food as possible. A probiotic strain can achieve this by altering the intestinal microbiota, improving the growth of non-pathogenic resident communities important for digestion and production of essential nutrients, preventing gastrointestinal infections, and by directly improving the digestion and utilization of nutrients consumed by the host. Probiotics may also be able to stimulate the immune systems of the host they are used to treat and, once eliminated from the gastrointestinal tract, may be able to reduce the ammonia emissions that damage the health of the livestock and the people that work with them.

Selection of bacterial strains for development of a probiotic treatment involves a series of tests, *in vivo* and *in vitro*, that evaluate the ability of each individual strain to fulfill the essential criteria mentioned above and the traits and mechanisms with which they confer benefits to the health of the host (Figure 1). Netherwood et al. (1999) showed that a genetically modified strain of *Enterococcus faecium* reduced the populations of the pathogen *Enterococcus faecalis* in poultry. Higgins et al. (2007) demonstrated the use of lactic acid bacteria to reduce *Salmonella enterica* serovars Enteritidis and Typhimurium in newly hatched chicks. Yoruk et al. (2004) used a cocktail of *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Streptococcus* strains along with humate to improve feed efficiency and egg production and reduce mortality in egg laying hens. La Ragione et al. (2001) showed that oral inoculation of *Bacillus subtilis* spores could reduce intestinal colonization of *Escherichia coli* O78:K80 in chickens when challenged 24 hours after

treatment. It was found the efficacy of the *Bacillus* probiotic diminished over time, suggesting that the spores would need to be fed constantly to maintain protection. Tierney et al. (2004) demonstrated that *Lactobacillus* isolates could inhibit invasion of *Eimeria tenella*, the parasitic apicomplexan responsible for Coccidiosis. This protist is responsible for millions of dollars lost every year, and strains are growing more resistant to the drugs used to treat it.

Although a variety of applications for probiotics have been discovered, they are not widely used on the farm. A great deal of research has been conducted, but the molecular interactions that occur within the gastrointestinal tract between the host, beneficial microorganism, pathogen, and the resident intestinal microbiota is not well understood. This lack of understanding makes it difficult for companies and the farmers they employ to adopt a probiotic as an effective, safe, and cost-effective replacement for antibiotics. The rise of the organic food market in the United States and the European Union ban on the use of antimicrobial agents for anything other than treatment of a veterinary disease has done much to push for the development of better probiotics and better understanding of how they work.

Salmonella

Salmonella are a Gram negative, facultatively anaerobic genera of bacteria in the family Enterobacteriaceae, comprised of over 2500 different serotypes (Crum-Cianflone 2009). Most commonly associated with pathogenesis in humans is *S. enterica* subsp. Enterica. As a pathogen *Salmonella* generally causes a self-limiting case of gastroenteritis, however some strains, such as *S. typhi*, *S. typhimurium*, and *S. paratyphi*, are capable of systemic infections (Monack 2004).

Salmonellosis is the leading cause of food-borne illness in the United States. It is estimated to be responsible for over a million infections in the U.S., with over 93 million cases worldwide, leading to the deaths of over 150,000 deaths every year (Sanchez-Vargas et al. 2011). Non-Typhoidal salmonellosis, while uncomfortable, is usually only dangerous to the immunocompromised and the young and elderly. Infection is very common in developing countries in Asia, Africa, and South America, where it is an important cause of diarrhea in children.

In order to cause disease, *Salmonella* must survive the acidic gastric environment and the bile salts of the upper small intestine. It then invades the intestinal epithelial cells by inducing uptake in a process that resembles phagocytosis. *Salmonella* achieves this by producing a Type III secretion system, which it uses to attach to epithelial cells and inject effector molecules that manipulate the host cell's sensor pathways. The host cell begins the process of rearranging its cytoskeleton in order to engulf the pathogen. The bacterium, contained within a vacuole, then begins to replicate, using its effector molecules to prevent fusion of the *Salmonella*-containing vacuole with the host cell's lysosomes (Fierer and Swancut 2000). Some strains of *Salmonella*, such as *S. typhi*, are able to survive in this manner within the macrophages of the host, allowing them to spread to the spleen and lymph nodes (Monack et al. 2004).

In terms of disease, *Salmonella* generally splits into two groups: Typhoid and Non-Typhoid. Typhoid fever is caused by *S. typhi* and *S. paratyphi*, two pathogens highly adapted to the human host, which represents their only reservoir (Monack 2012). Typhoid infections are spread via the fecal-oral route, and outbreaks are usually associated with infected food handlers contaminating their wares (Sanchez-Vargas et al. 2011). *S. typhi* can infect and persist in individuals asymptomatically, causing the host to constantly shed the organism. This led to the

infamous “Typhoid Mary” outbreak after the turn of the twentieth century (Sanchez-Vargas et al. 2011). Typhoid fever, also known as enteric fever, is a systemic infection that can be life threatening if left untreated. Treatment typically involves an antibiotic regimen; however there is growing concern over antibiotic resistance. Strains of Typhoid *Salmonella* have been discovered in Asia that are resistant to a variety of beta-lactam antibiotics as well as trimethoprim-sulfamethoxazole, chloramphinacol, quinolone, and ciprofloxacin (Crum-Cianflone 2009).

Non-typhoid *Salmonella* cause a self-limiting gastroenteritis, causing nausea, non-bloody diarrhea, and vomiting. It is rarely life-threatening in healthy adults, but can cause serious illness in infants, the elderly, and the immunocompromised (Monack 2012). Poultry products represent the prime suspect in Non-Typhoidal Salmonellosis. In the 1970’s through the 1990’s, outbreaks were primarily associated with contaminated raw eggs. One outbreak in 1994 involved the contamination of ice cream premix transported in a tanker that had previously been improperly sanitized and that had been used to transport liquid egg (Crum-Cianflone 2008). The ice cream was shipped across the United States, resulting in over 200,000 cases of Salmonellosis. The sheer number of Salmonellosis cases associated with egg products resulted in an effort by the combined forces of the FDA and USDA to minimize the risk from eggs. It was discovered that *Salmonella* is inoculated transplacentally into an egg by a contaminated egg-laying hen. A new process of pasteurization was developed specifically for eggs, which involved heating at lower temperatures to kill the bacteria while maintaining the quality of the raw egg, and a variety of procedures were adopted to prevent inoculation of the hen flocks. These factors, combined with a nationwide campaign to educate consumers on the importance of proper storage and preparation, resulted in a significant drop in the occurrence of egg-associated *Salmonella* infections (Braden 2004). Despite these improvements, *Salmonella* still represents one of the most economically

important food-borne pathogens in the U.S. As with many other pathogens, the key to stopping infection in humans may lie in eliminating it from its reservoir, and with *Salmonella* this has proven extremely challenging.

***Bacillus* as probiotics**

Bacillus is a genus of Gram positive bacteria from the family Bacillaceae. Their most notable physiological feature is the ability to form endospores that are capable of withstanding extremes in temperature and pH, desiccation, starvation, toxins, and radiation (Cutting 2010). The genus *Bacillus* contains a number of pathogens, such as *B. anthracis*, the causative agent of Anthrax, and *B. cereus*, which causes a mild food-borne illness. Closely related to the latter is *B. thuringiensis*, a bacterium that produces a potent endotoxin inside its spores that punctures the gut of flying insects and has been extensively used as biological control for mosquitoes and crop pests (Vilas-Boas, Peruca, and Arantes, 2007). The genus *Bacillus* also contains a group of more innocuous organisms, so closely related that categorizing them into the classical taxon of species has proven difficult, causing them to be lumped together into the "*Bacillus subtilis* group" comprised of *B. amyloliquefaciens, Bacillus atrophaeus, Bacillus axarquiensis, Bacillus malacitensis, Bacillus mojavensis,* and *Bacillus vallismortis*, the *B. subtilis* group is primarily soil-borne. *Bacillus subtilis* has been shown to play a role in the production of coffee and cocoa during the processing that take place after harvest (Logan 2011). Members of the group have also been found associated with plant roots and even isolated from their tissues (Reva et al. 2002), and it is believed they play a role in nitrogen fixation, biological control of pathogens, and interactions with symbiotic bacteria (Logan 2011). Some members of this group have also been found within the gastrointestinal tract of animals, adapted to the host environment, rather than being transient residents of the intestine (Fakhry et al. 2008). A strain of *B. subtilis* isolated from

the gut of a catfish was show to be useful for biological control of the catfish pathogen *Edwardsiella ictaluri* (Ran et al. 2012).

One advantage many members of the *Bacillus* genus have over *Lactobacillus* is the ability to survive the acidic environment of the stomach via sporulation. For an orally administered probiotic, both in humans and livestock, this is a key trait. The heat tolerance of spores allows them to be stored at room temperature for long periods of time with no special equipment. The result is a variety of probiotic products already on the market. *B. clausii*, also known by the trade name Enterogermina, has been available for human consumption since 1958 and is recommended for use in preventing infantile diarrhea (Cutting 2011). *B. coagulans*, formerly classified as *Lactobacillus sporogenes*, is a probiotic for human consumption that has been shown to prevent antibiotic-associated diarrhea (Jurenka 2011) and may alleviate symptoms of rheumatoid arthritis (Mandel 2010). *Bacillus mojavensis* strain KJS-3 has shown potential as a biological control agent protecting plants from fungal pathogens and reducing accumulation of mycotoxins (Choi et al. 2010). While probiotics for humans have a large place in the market as a potential alternative to conventional medicine, *Bacillus* probiotics are not commonly used in industrial poultry production. However, interest is growing and a number of probiotics are currently in development. Supplementation of *B. subtilis* strain LS 1-2 improved the growth performance, nutrient retention, intestinal villus height, and reduction in *Clostridium* and coliform counts (Sen et al. 2011). Gil De Los Santos et al. [2005] demonstrated that *B. cereus* var. toyoii improved the feed efficiency in poultry when added to feed as a supplement.

Ammonia in the Poultry Industry

Nitrogen is a fundamental component of plants and animals. In poultry, nitrogen is consumed via feed, which is made up of a mixture of corn soy, wheat, and other grains. The

amino acids and proteins in feed are digested and metabolized by the chicken, but approximately one-third of the nitrogen consumed is actually incorporated into the chicken's physiology and the rest is excreted in its urine as uric acid (Ritz et al. 2004). The uric acid in the excreta is quickly converted into ammonia by uricolytic bacteria in the litter (Schefferle 1965). Industrial poultry production often involves very tightly packed cages in order to fit as many birds as possible. Broiler meat production has chickens raised communally on bedding that reduces farmer costs and is changed infrequently. These factors, combined with the often high temperature inside poultry houses, drastically increases the volume of ammonia produced, resulting in potentially dangerous conditions for poultry and the people who work around them daily. Current Occupational Safety and Health Administration standards limit exposure to 8 hours when ammonia concentrations are at 50ppm for poultry workers (Ritz et al. 2004). Detrimental effects on the growth performance and overall health of the birds have been observed at concentrations as low as 20ppm (Anderson et al. 1964). Prolonged exposure to ammonia concentrations exceeding this amount can lead to a decrease in feed efficiency, increased susceptibility to disease, loss of cilia in the lungs, and eye damage. These conditions have caused concern among animal welfare groups and poultry product retailers, calling for a lower industrial ammonia concentration standard. There is also growing concern over the effects volatilized ammonia may be having on the environment. Once released into the atmosphere, ammonia reacts with acidic molecules in the atmosphere, generating aerosolized ammonium that condenses and pollutes the soil and bodies of water. This easily available nitrogen source can cause algal blooms, acidify soils and water sources, and damage aquatic life (Galloway and Cowling 2002). This has resulted in an effort to discover litter treatments and adjustments that can be made to the conditions in order to reduce the amount of ammonia produced.

Ordinarily ammonia concentrations inside the poultry house are reduced by improving ventilation in the building. While this may improve conditions for the poultry and workers, it does not address the environmental issues. Also ventilation is reduced in the winter to preserve the temperature inside the house, so air quality will vary with the seasons. One strategy involves optimizing the poultry diet so as to reduce the amount of excess nitrogen ingested by the chickens, focusing on the amino acid requirements of the chicken (Keshavarz and Jackson 1992). The most commonly implemented strategy to reduce the amount of ammonia produced in the litter involves acidifying agents that lower the pH of the litter to inhibit the activity of uricolytic bacteria (Ritz et al. 2004). It has also been suggested that inhibitors of microbial and enzymatic activity be added to prevent the uricolytic reaction. It was shown that addition of zinc sulfate to poultry manure drastically decreased volatilization of ammonia from the litter (Kim and Patterson 2003). One potential strategy that is as yet untested is the use of a biological control agent in litter, either to prevent the outgrowth of ammonia-producing bacteria or to metabolize the ammonia being produced. The benefit of such a treatment lies in taking advantage of processes that take place naturally.

Conclusion

While much about probiotics and biological control agents is still poorly understood, these treatments have the potential to overcome many of the hurdles raised by the development of industrial agriculture and antibiotic resistance. The two issues of concern in the poultry industry are the presence of the human food-borne pathogens *Salmonella* and *Campylobacter*, and the high levels of ammonia within the poultry house environment. The key to making probiotics work is a better understanding of how bacterial communities interact with each other, with their host, and with newcomers, whether pathogenic or symbiotic. This research was

initiated in order to identify specific *Bacillus* strains that could serve as probiotics to reduce the levels of foodborne pathogens and ammonia within a poultry house environment.

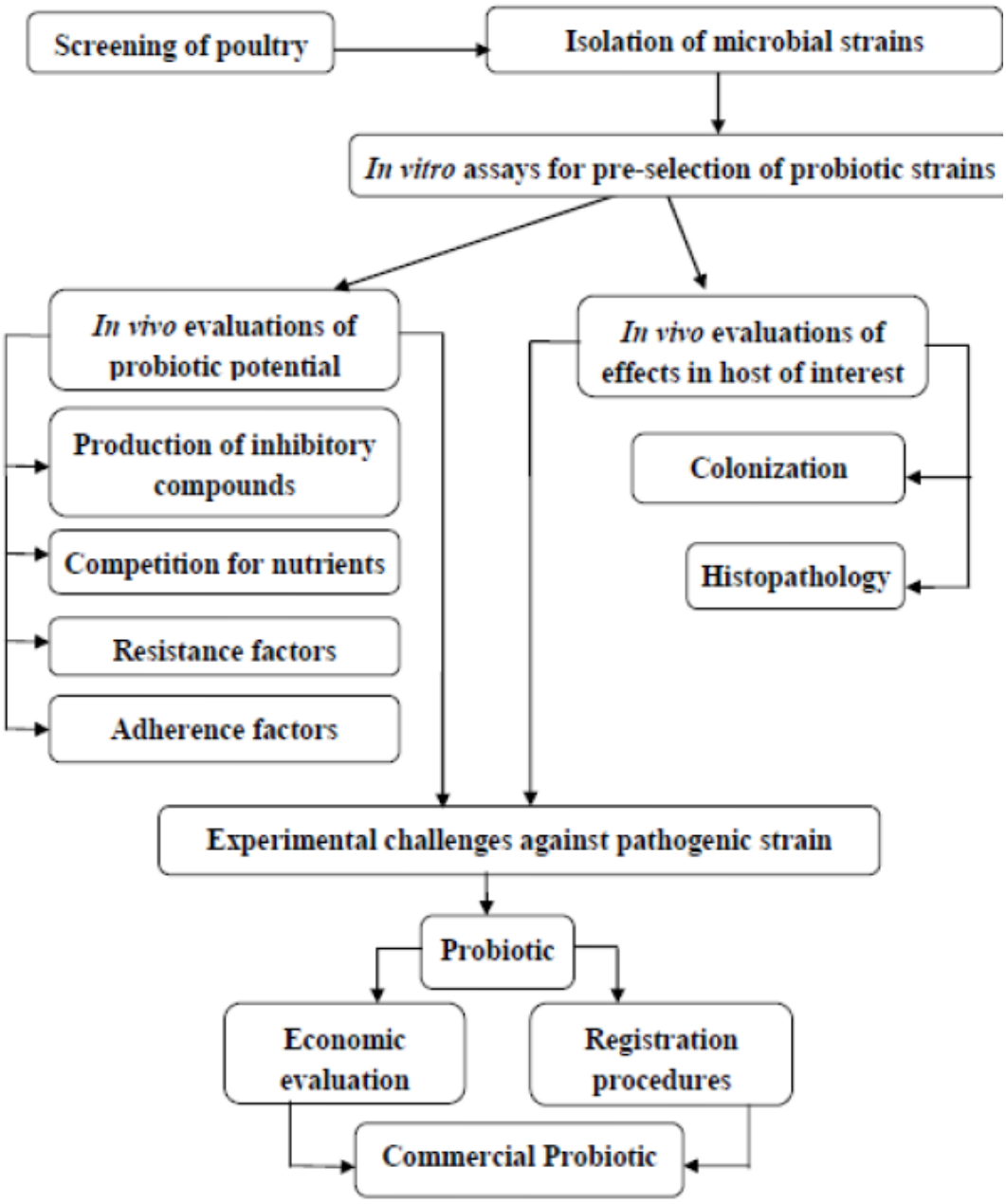

Figure 1: Diagram for the Selection of Probiotic Strains from Kabir 2009.

Selection of *Bacillus* strains for *Salmonella* reduction within a poultry house environment.

Abstract:

Salmonella species are a food safety concern associated with poultry products, causing thousands of gastrointestinal disorders every year. Currently, controlling *Salmonella* in poultry houses is not being performed adequetly. It is assumed that the chemical litter amendments used to control ammonia will also control *Salmonella*. It has been shown that these chemical amendments are transitively effective, while it is postulated that bacterial amendments could last indefinitely. A collection of *Bacillus* strains (n=244) was screened for inhibition of *Salmonella* species (n=10). The 30 *Bacillus* strains that had the highest degree of inhibition against the most *Salmonella* species were selected for further evaluation. While no significant nitrification activity was observed, we did observe significant differences in the respective ability of different *Bacillus* strains to take up ammonia from a culture medium that contained ammonium chloride. Six strains were selected and applied in a litter trial at 10^7 CFU/g litter. No significant effects were observed with regards to *Salmonella* or ammonia levels for any treatment at any sampling time, but two *Bacillus* strains were re-isolated from the poultry litter demonstrating survival within the complex litter environment. Two of the best-performing *Bacillus* strains, AB01 and AP71, had been selected for evaluation in feed. The genome for each of these strains had been sequenced, assembled and used to generate PCR primers unique to the strains for identification within poultry. Results from the poultry trial are as of yet inconclusive, due to an error in sequencing. Further work involving resequencing and the evaluation of the *Bacillus* spores efficacy in fresh litter will yield an answer as to whether these strains may be useful to the poultry industry.

Introduction:

Salmonella represents one of the most persistent food borne threats in the United States. The CDC estimates that 76 million illnesses are caused by food borne pathogens annually in the United States, with poultry and its products being recognized risk factors for food borne outbreaks of salmonellosis (Sanchez-Vargas et al. 2011). The federal government has invested significant resources in diagnosis and prevention of food borne disease and to a large degree much of the resources are dedicated to epidemiological investigations to identify the source of outbreaks. Significant resources are also invested in food inspection and compliance with slaughter and processing plant sanitation procedures intended to reduce pathogen levels in food. We initiated this study in order to identify probiotic bacteria within the genus *Bacillus* that have the capacity to 1.) prevent the colonization of the poultry gastrointestinal tract by *Salmonella*, 2.) reduce ammonia emissions from poultry litter, and 3.) perform these functions when applied as a probiotic feed addititive.

Materials and Methods:

Bacterial strains

A collection of 244 strains of *Bacillus subtilis* had been previously isolated from soil or the catfish gastrointestinal tract (Ran et al. 2012). Each bacterial culture was maintained as a cryopreserved pure culture within a -80^{o}C freezer. Pure cultures were grown on Tryptic Soy

Agar (TSA) at 30^{o}C for 24 hours to provide isolated colonies that could be used for further evaluation in bioassays. In some experiments a spore preparation of each respective strain was used as an inoculum.

In vitro screening for inhibition of *Salmonella enterica* growth

Each bacterial strain was screened for *in vitro* inhibitory activity against the top ten strains of *Salmonella enterica* responsible for salmonellosis in the United States as well as *Campylobacter jejuni*. Holes were bored in water agar plates and filled with TSA to limit the surface area upon which the *Bacillus* could grow. 100 µL of spore preparation from each *Bacillus* strain was spotted onto the TSA and incubated for 48 hours at 30˚C. Each strain of *S. enterica* was grown on TSA for 24 hours at 37˚C and then the log-phase culture was inoculated into a TSA soft agar (0.7% agar) that had been cooled to 42^{o}C. A 7 ml *S. enterica* soft agar suspension was then poured over the plates containing the *Bacillus* cultures. These plates were then incubated at 37˚C for 24 hours. Any clearing of *Salmonella* around the *Bacillus* was measured and recorded as a mm zone of inhibition.

Poultry litter trials

Poultry litter cleaned from chicken floor pens was placed in a walk-in incubator, water was added to 36 kg of litter to bring the litter moisture up to 30%, and then incubated for 5 days at 35˚C. 3 kg of litter was placed into 12 plastic tubs. Ammonia levels were determined directly before addition of the spore treatments. A mixture of all the tested strains at equal concentrations was added to the litter at a dose of 10^{7} CFU/ml. Ammonia measurements were taken at 0, 1, 2, 3, 4, and 7 days post-inoculation, and litter samples for bacterial heterotrophic counts were taken at 0, 1, 4, and 7 days post-inoculation. A portion of each litter sample was weighed and phosphate

buffer was added to make a tenfold dilution. The dilution was placed in a stomacher for 2 minutes, diluted further, and spread plated on plate count agar. Plates were incubated for 24 hours at 37˚C and counted. Suspect *Bacillus* colonies were identified by colony morphology, isolated, and cultured. The DNA of the suspect cultures was isolated and PCR performed to amplify the universal bacterial 16S rDNA gene. PCR products were Sanger sequenced and identified with BLAST.

The second poultry litter trial was performed the same as the first, with a few exceptions. The *Bacillus* strains used were adjusted according to the results of the uric acid gradient assay and 10mL of water was added to each tub every 24 hours to prevent the litter from drying out. Also the trial was conducted over a two week period, instead of one week. Ammonia measurements were taken at 0, 1, 2, 3, 4, 10, and 14 days post-inoculation, and litter samples for bacterial heterotrophic counts were taken at 0, 1, 2, 3, 4, 10, and 14 days post-inoculation.

Selection of strain-specific PCR primers

Genome sequences for strains AP71 and AB01 were determined by preparing bar-coded genomic libraries using the Nextera library prepapration kit (Illumina, San Diego). The bar-coded genomic libraries were sequenced at a final concentration of 12 pM on an Illumina MiSeq using a 2 x 150 paired-end sequencing kit. The sequence reads were exported into CLC Genomics Workbench and trimmed for quality at a setting of 0.01. A *de novo* assembly was conducted using both the CLC Genomics Workbench and the VelvetOptimiser software package (courtesy of Prof. Scott Santos, Auburn University Dept. of Biological Sciences). The contigs from each of these genomes were mapped against reference genomes from *B. subtilis* strains AP 193, AP 143, GB126, AB-01 for AP71 and vice versa, and the un-mapped reads that did not

match any of these bacterial genomes were used for a *de novo* assembly. The strain-specific contigs were used to generate strain-specific oligonucleotide primers using the PrimerBLAST algorithm available at the National Center for Biotechnology Information (NCBI) and primers were selected based on the criteria: 1.) Size: a primer set 18-20 bps long in order to have high specificity, 2.) lack of self-complementarity: a primer set that would not bind to themselves, each other, or the 16S primers, and 3.) Product size: a primer set that would generate product 1000 bps in length or less, so the product could easily be distinguished from the 16S product on a gel. Custom primers were synthesized by Operon (Huntsville, AL) and evaluated in PCR to determine if they produced a single, robust amplicon of the expected size. The thermalcylcing conditions used were 95ºC for the melting step, 70ºC dropping down to 55ºC at one degree per cycle for the annealing step, and 72ºC for the extension step. The custom primers that worked for strain AP71 were [CAATGCCAATGGGTCTGCTC] and [TGTGTAGCGATTGCGGGATT] and for strain AB01 were [CGGGAAGAGATGACCCGTTT] and [CGTCATACATTCCGCGACAC]. These primers were evaluated in a multiplex PCR reaction with 16S rRNA universal bacterial primers 27F and 1492R using template DNA isolated from AP71, AB-01, and DNA isolated from chicken feces. The primers were selected based on their ability to produce strong product only from the *Bacillus* strain for which they were designed.

Uric Acid Gradient Assay

A TSA gradient agar plate containing 10% uric acid (w/w) at the highest concentration was prepared. Each of the seven candidate *B. subtilis* strains was grown across the surface of the uric acid gradient and incubated for 24 hours at 30˚C. Growth was monitored and each plate was photographed to document the respective ability of each strain to grow in the presence of uric acid.

Spore Preparation

To produce spores, sporulation broth (peptone, beef extract, NaCl, K2HPO4, KCl, MgS04, MnSO4, Lactose) in screw top flasks was inoculated with a given *Bacillus* strain and incubated at 30˚C for 14 days. The culture was pipetted into a 50 ml centrifuge tube and subjected to centrifugation at 10,000 x *g* for 10 min. The bacterial supernatant was aspirated and the culture was washed with sterile milliQ water two times. The culture was re-suspended in 50 ml milliQ water and heated to 80˚C for 15 minutes to kill vegetative cells. The heated mixture was allowed to cool, then serially diluted and spread plated onto TSA for quantification. Spores were stored at 5˚C until use.

Poultry Trial

Spores were grown, quantified, and mixed into the feed at 10^7 CFU/g of feed. Seventy-five chickens were taken immediately upon hatching and placed in battery cages for the trial with five chickens per cage. The chickens were split into five treatment groups. One group received feed amended with AP71 spores and were challenged with *Salmonella* Enteritidis. The second group received feed amended with AB01 spores and was challenged with *Salmonella* 3. The third group received feed amended with a mixture of AP71 and AB01 together equaling a concentration of 10^7 CFU/g of feed and was challenged with *Salmonella*. The fourth group received feed containing no amendment and was challenged with *Salmonella* 3 weeks into the study. The last group received feed containing no amendment and was not challenged with *Salmonella*. After three weeks the three treatment groups and one of the controls were given a gavage containing *Salmonella enterica* serovar enteritidis. At four weeks the chickens were sacrificed and weighed. Their bursa was measured, and their spleen and ceca were removed for

Salmonella quantification. A portion of their small intestines was removed and split. Gut content and mucosa scraping samples were taken for DNA isolation, PCR, and sequencing. The spleens and ceca were initially stomached in phostphate buffer and the solution diluted for quantification of *Salmonella* on XLT4 media. The plates were incubated at 37˚C for 48 hours and the quantified. If there was inadequate growth, the spleens and ceca were then enriched in tetrathionate broth and the broth was streaked onto XLT4 media to verify the presence or absence of *Salmonella*.

DNA Isolation

DNA was extracted from the poultry guts samples using the E.Z.N.A. Stool DNA Kit from Omega Bio-Tek. A KLECO root homogenizer was used in lieu of a vortexer for the glass bead lysis step. Genomic DNA was stored in aliquots at -80^{o}C until use in PCR.

PCR/ Next Generation Sequencing

PCR to amplify the 16S rRNA genes from diverse bacteria was carried out using EconoTaq plus Green (Lucigen Corporation, Middleton, WI), with a unique oligonucleotide barcode and Illumina universal adapter sequence at the 5' end of the 806R primer and the Illumina adapter sequence added to the 515F primer (Klindworth et al. 2012). The PCR amplicons were analyzed via agarose gel electrophoresis (1% agarose gel in 1x TBE buffer resolved at 120V for 1 hour) and amplicons of the expected size were were pooled at approximate equimolar concentration, gel extracted using E.Z.N.A. Gel Extraction Kit from Omega Bio-Tek, and then quanitified using a Qubit fluorometer (Life Sciences). The quanitified pooled amplicons were sequenced on an Illumina MiSeq at a final concentration of 6 pM and using a 30% spike-in of the PhiX control genome.

Sequence Analysis

The 16S rRNA gene sequences were analyzed by QIIME (Quantitative Insights into Microbial Ecology). The sequences were fed into the Python script split_libraries_fastq.py, which separated sequences into their original sample groups by matching the barcode in the sequence with the barcode used for the sample during PCR, provided in a metadata mapping file. The demultiplexed sequences were then used to run the script pick_reference_otus_through_otu_table.py, which clusters the sequences into operational taxonomic units (OTUs) and classifies them by comparing representative sequences to QIIME's sequence database, in this case, Greengenes. The OTUs were picked at a 97% similarity with the Greengenes OTUs. The resulting OUT table was then used to generate relative abundance plots and statistical data.

Results:

In vitro screening for inhibition of *Salmonella* growth

The *Bacillus* strains (n=244) used in this study were isolated from soil and the catfish gastrointestinal tract (Ran et al. 2012). Each of the 244 *Bacillus* strains was tested against each of the ten *Salmonella enterica* strains responsible for the greatest incidence of Salmonellosis in the United States. There were a total of 56 *Bacillus* strains that significantly inhibited at least 1 of the *Salmonella* strains (Table 1).

The 30 *Bacillus* strains that had the highest degree of inhibition against the most *Salmonella* species were selected for further evaluation. All but three of these *Bacillus* strains

were capable of *Campylobacter jejuni* inhibition, with varying degrees of antagonistic activity (data not shown).

Ability to grow in the presence of uric acid and remove ammonia from cultures

The best-performing 30 strains were then evaluated to identify strains that had the potential to assimilate ammonia during growth. Each strain was grown in M9 minimal media with added 3% ammonium chloride for 3 days at 30^{o}C while shaking at 200 rpm. The cultures were then subjected to centrifugation and the supernatant was tested for the presence of nitrate and nitrate with the Greiss reagent (Oxford Biomedical Research, Oxford, MI). The Macklin laboratory also directly measured the ammonia present in the cell free supernatant (Table 1). While no significant nitrification activity was observed, we did observe significant differences in the respective ability of different *Bacillus* strains to assimilate ammonia (Table 2). Six strains (designated AP71, AP183, AP185, AP206, AP294, and AP302) were selected for further study based on the number of *Salmonella* strains inhibited and the magnitude of ammonia incorporation.

In order to determine the ability of these top *Bacillus* candidate strains to survive in a poultry litter environment, the six *Bacillus* strains were grown on a gradient agar containing uric acid with a concentration ranging from 0% to 10% (w/w). Another strain AB01 isolated from a study of fish probiotics (Ran et al. 2012) was also added to the study. Six of the seven strains tested showed positive growth across the entire rage of uric acid concentration. AP294 only showed growth at the 0% concentration and was eliminated from further study.

Poultry litter trials

In order to evaluate the efficacy of direct amendment of the *Bacillus* strains to poultry litter, a trial was conducted so as to test the effects amendment would have on the populations of *Salmonella* and the ammonia volatilized from the litter. One previous study showed significant reduction of *Salmonella* at 10^9 spores added (Figure 1). However in later trials at lower spore counts, no significant effects were observed with regards to *Salmonella* or ammonia levels for any treatment at any sampling time, but two *Bacillus* strains AB01 and AP71 were re-isolated from the poultry litter demonstrating survival within the complex litter environment. The colonies on the plate were evaluated using 16S rRNA sequences and compared with the known sequences from these strains to confirm their respective identity. Two of the best-performing *Bacillus* strains, AB01 and AP71, were then selected for evaluation in feed.

Development of *Bacillus* strain-specific primers

The genomes for strains AB01 and AP71 were sequenced and the contigs from these 2 strains were used for reference genome assemblies against related *Bacillus* strains in order to identify strain-specific genetic loci. These strain-specific contigs were then used to generate PCR primers unique to the strains for identification within poultry samples. The validation of these primers was conducted using genomic DNA isolated from pure bacterial strains (as positive controls) as well as related bacterial cultures (as negative controls) and poultry feces-extracted genomic DNA that had not had prior exposure to these probiotic strains (as negative controls), and the results indicated the ability of these primer sets to detect the respective strains (Figure 2).

Poultry Trial

The next step was to evaluate the efficacy of the remaining two strains as probiotics in an *in vivo* poultry trial. Neither of the two *Bacillus* strains, nor both combined, had any significant effect, either positive or negative, on the growth performance and immune function of the birds. There were no bird fatalities for the duration of the trial. Quantification of *Salmonella* by serial dilution and plating on XLT4 media was inconclusive due to there being too few to count. We resorted to tetrathionate broth enrichment followed by streaking on XLT4 media in order to detect whether or not *Salmonella* was present, regardless of numbers (Table 3). These data show no consistent trend in the effect of the two *Bacillus* strains on protection against *Salmonella* colonization. However, they also show no deleterious effects on the health, growth performance, immune function, or mortality in the birds.

Sequence Analysis

The 16S rRNA gene sequence analysis of the poultry gastrointestinal microbiota will permit the determination of whether the probiotic amendment had a significant effect on the gut microbial communities. However, the first attempt at generating 16S rRNA sequences from PCR amplicons yielded very little sequencing data (only 2,700 bacterial rRNA sequences), with the rest being the PhiX control. The rRNA sequences generated from the poultry gut were analyzed to determine the relative abundance of bacterial taxa in the different treatment groups (Figure 2). Even though the numbers of sequences are not sufficiently large enough to encompass the diversity of the bacterial taxa within the chicken intestine, there are some differences in the probiotic treatment groups relative to control samples. For strain AB01, the bacterial taxa that are increased more than 3% relative to the negative control are Pseudomonads, whereas the bacterial

taxa reduced more than 3% relative to the negative control are *Peptostreptococcus* and members of the family Clostridiaceae. For strain AP71, the bacterial taxa that are increased more than 3% relative to the negative control are *Turicibacter, Cetobacterium,* and *Acinetobacter*, whereas the bacterial taxa reduced more than 3% relative to the negative control are Pseudomonads and members of the family Clostridiaceae. Importantly, there was no *Salmonella* detected based on this analysis, but without reaching a greater degree of bacterial taxa diversity we cannot conclude that the chicken intestinal community lacked *Salmonella*. Further sequencing will be necessary to attain a greater degree of sensitivity and make stronger conclusions regarding changes in the chicken intestinal microbiota in response to the probiotic amendments.

Discussion

The purpose of this research was to identify specific *B. subtilis* strains with the potential to be applied as a poultry probiotic to prevent colonization of *Salmonella* in the gut, reduce ammonia emissions from the litter, improve the growth performance of the birds, and stimulate their immune system. Out of the collection of *Bacillus* strains, strains were found that showed inhibitory activity against *Salmonella* and that could assimilate nitrogenous wastes from their surrounding environment. While the *in vitro* studies to identify the probiotic strains have yielded some possible candidates, the *in vivo* studies to evaluate their use in either poultry litter or as a feed additive have not demonstrated beneficial effects. There are several reasons for why this may have happened.

In the case of poultry litter, the *Bacillus* strains may need to be applied to litter before it has been used in order for ammonia emissions to be effectively reduced. Our poultry litter trials involved the addition of *Bacillus* spores litter that had already been used by chickens and

removed for composting. The result is an extremely hostile environment, with a high pH, toxic excreta by-products, and an established microbial community thriving on the chicken's waste. It is our goal in the future to perform a new poultry litter trial, where *Bacillus* spores will be applied to fresh litter to be used by poultry and then tested for ammonia and the presence of *Salmonella*. Given the success of AB01 at higher concentrations, there is also a need to determine an appropriate dose for future experiments.

In the case of the chicken gastrointestinal tract, these *Bacillus* strains are not adapted to the gastrointestinal tract environment. Many of the probiotics that have been developed in the last few decades were isolated from the gastrointestinal tract of the host for which they were developed. The two strains used in the poultry trial, AP71 and AB01, were isolated from soil and the gastrointestinal tract of a catfish, respectively. This may suggest that they are unable to attach to the intestinal epithelial wall and grow. Association with the intestinal wall seems to be what causes the majority of the host-probiotic interactions that improve growth performance and immune stimulation. However, this does not mean that strain AP71 and AB01 cannot be useful in preventing *Salmonella* colonization and reduction of ammonia emissions from the litter. Regarding the determination of *Salmonella* during the probiotic feeding study, the tetrathionate enrichment may not provide a reliable representation of the effect of strains AB01 and AP71 on the levels of *Salmonella* during the challenge. Tetrathionate enrichment of the spleen and ceca can show a positive result for *Salmonella* with theoretically as little as one viable bacterial cell. While it tells us whether or not there was any *Salmonella* present, it does not tell us what affect the addition of *Bacillus* may have had on the population. With very low numbers of *Salmonella* cells within these samples there could be substantial variability and unreliability in the quantification of *Salmonella* from these samples using the enrichment method. The 16S rRNA

gene sequencing data is required to get a more sensitive determination of the bacterial communities within the chicken intestine and how these microbiota were changed as a result of treatment. In the future, we will repeat the next-generation sequencing of the 16S rRNA gene amplicons obtained from the chicken intestinal samples, using a higher concentration of amplicon DNA, and obtain a better representation of the chicken intestinal microbiota. While it is not apparent that the *Bacillus* treatments had any effect on the growth performance or immune function of the chickens, this study did indicate that these strains are not toxic and do not appear to disrupt the healthy microbiota present within the chicken intestine. Further *in vivo* studies will be needed in order to determine the potential of these probiotic strains in reducing foodborne pathogens of humans and ammonia within a poultry house environment.

Strain	*S. enteritidis*	*S. typhimurium*	*S. montevideo*	*S. kentucky*	*S. heidelberg*	*S. thompson*	*S. stanley*	*S. newport*	*S. newington*	*S. paratyphi*	*C. jejuni*	Ammonia
AB-01	-	-	3.0mm	2.0mm	-	3.0mm	-	-	-	2.0mm	8.5 mm	110
AP-3	-	-	-	-	-	-	-	-	-	2.0mm		
AP-7	-	-	-	-	-	-	-	-	-	4.0mm		
AP-18	-	-	2.0mm	-	-	1.0mm	-	-	-	4.0mm	8.9 mm	119
AP-67	-	-	-	3.0mm	-	2.0mm	-	-	-	5.0mm	10 mm	141
AP-71	2.0mm	3.0mm	3.0mm	-	-	-	-	-	-	5.0mm	8.5 mm	66
AP-75	-	5.0mm	-	4.0mm	-	-	-	-	-	5.0mm	8.9 mm	71
AP-76	4.0mm	-	3.0mm	4.0mm	-	-	-	-	-	4.0mm	9.1 mm	118
AP-77	-	-	3.0mm	4.0mm	3.0mm	2.0mm	-	-	2.0mm	3.0mm	11.5 mm	110
AP-78	3.0mm	-	-	4.0mm	-	3.0mm	3.0mm	-	-	6.0mm	14.5 mm	96
AP-79	-	-	2.0mm	-	-	-	-	-	-	5.0mm		
AP-80	-	-	-	4.0mm	-	-	-	-	-	3.0mm		
AP-81	-	-	-	-	3.0mm	-	3.0mm	-	3.0mm	7.0mm	9 mm	90
AP-86	4.0mm	-	4.0mm	3.0mm	-	-	-	-	-	6.0mm	11.75 mm	142
AP-87	2.0mm	-	-	-	3.0mm	-	-	-	-	1.0mm	8.6 mm	144
AP-92	-	-	-	-	-	-	-	-	-	2.0mm		
AP-99	1.0mm	7.0mm	3.0mm	5.0mm	-	-	-	-	-	8.0mm	7.5 mm	113
AP-100	-	-	-	-	-	-	-	-	-	5.0mm		
AP-102	-	-	2.00mm	-	-	2.0mm	-	-	-	4.0mm	7.75 mm	98
AP-111	-	-	-	-	4.0mm	-	-	2.0mm	-	5.0mm	9.6 mm	124
AP-112	-	-	-	4.0mm	-	2.0mm	2.0mm	-	2.0mm	4.0mm	10 mm	123
AP-113	-	-	-	-	-	-	-	-	-	3.0mm		
AP-115	-	-	2.0mm	-	-	-	-	-	3.0mm	6.0mm	9.9 mm	91
AP-149	-	2.0mm	-	-	-	-	-	-	-	3.0mm		
AP-150	-	-	-	-	2.0mm	-	-	-	-	-		
AP-151	-	-	-	-	1.0mm	-	-	-	-	-		
AP-152	-	-	-	-	-	-	2.0mm	-	-	-		
AP-163	-	-	-	-	2.0mm	-	-	-	-	-		
AP-183	-	-	-	3.0mm	5.0mm	3.0mm	-	-	-	9.0mm	8.5 mm	91
AP-184	-	-	-	3.0mm	-	-	-	-	-	-		
AP-185	3.5mm	-	-	4.0mm	3.0mm	-	3.0mm	-	-	-	7 mm	85
AP-188	-	-	2.0mm	-	-	-	-	-	-	-		
AP-190	2.0mm	-	3.0mm	2.0mm	3.0mm	-	-	-	1.0mm	-	No inhibition	150
AP-192	3.0mm	1.0mm	-	-	2.0mm	-	-	-	-	-	No inhibition	127
AP-193	3.0mm	-	-	-	-	-	-	-	-	-		
AP-204	-	8.0mm	3.0mm	-	-	-	2.0mm	-	3.0mm	4.0mm	No inhibition	107
AP-205	-	-	-	-	-	1.0mm	2.0mm	-	-	1.0mm	11.1 mm	102
AP-206	-	-	-	3.0mm	-	4.0mm	3.0mm	-	-	3.0mm	9 mm	38
AP-215	-	-	-	3.0mm	-	-	-	-	-	3.0mm		
AP-216	-	-	2.5mm	-	4.0mm	-	-	-	2.0mm	-	8.25 mm	80
AP-217	-	-	-	1.0mm	-	-	-	-	-	-		
AP-218	-	-	1.0mm	-	1.0mm	-	-	-	-	6.0mm	10.75 mm	116
AP-219	-	-	4.0mm	-	-	-	-	-	-	5.0mm		
AP-242	-	-	-	-	5.0mm	-	-	-	-	2.0mm		
AP-254	-	-	-	-	3.0mm	-	-	-	-	4.0mm		
AP-256	-	-	-	-	-	-	-	1.0mm	-	-		
AP-278	-	7.0mm	-	-	1.0mm	-	-	-	-	-		
AP-279	-	10.0mm	-	-	3.0mm	-	-	-	-	-		
AP-294	1.5mm	-	2.0mm	2.0mm	-	3.0mm	1.0mm	-	-	4.0mm	5.25 mm	89
AP-295	2.8mm	-	-	1.0mm	-	3.0mm	3.0mm	-	-	5.0mm	9.25 mm	112
AP-301	-	-	3.0mm	2.0mm	-	3.0mm	5.0mm	-	1.0mm	-	12.5 mm	95
AP-302	4.0mm	-	4.0mm	-	-	4.0mm	2.0mm	-	2.0mm	-	8.25 mm	78
AP-303	-	-	-	-	2.0mm	-	-	-	1.0mm	-		
AP-304	-	-	2.0mm	-	-	-	-	-	-	5.0mm		
AP-305	3.0mm	-	4.0mm	2.0mm	-	-	-	-	-	-	ND	121
H57B	-	-	-	4.0mm	-	-	-	-	-	-		

Table 1: 56 *Bacillus* strains with inhibitory activity against at least one strain of *Salmonella*. Yellow highlighted strains represent *Bacillus* that showed inhibitory activity to at least three *Salmonella* strains. Strain names in red were used in the poultry litter trial.

Strain	AB-01	AP71	AP183	AP185	AP206	AP294	AP302
% Ammonia Reduced	23.6	54.2	36.8	41.0	73.6	38.2	45.8

Table 2: Percent reduction in ammonia for the top 7 *Bacillus* strains (compared to control)

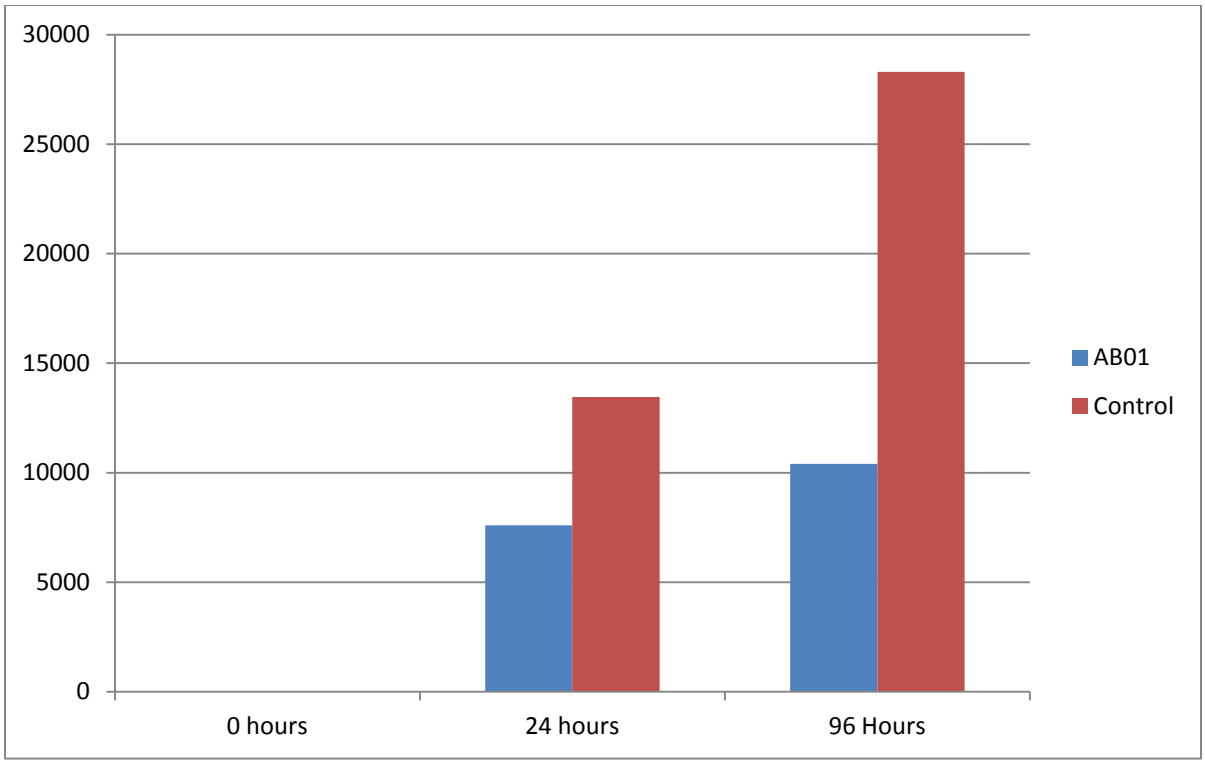

Figure 2: Effect of adding AB01 in high doses on *Salmonella* CFU counts

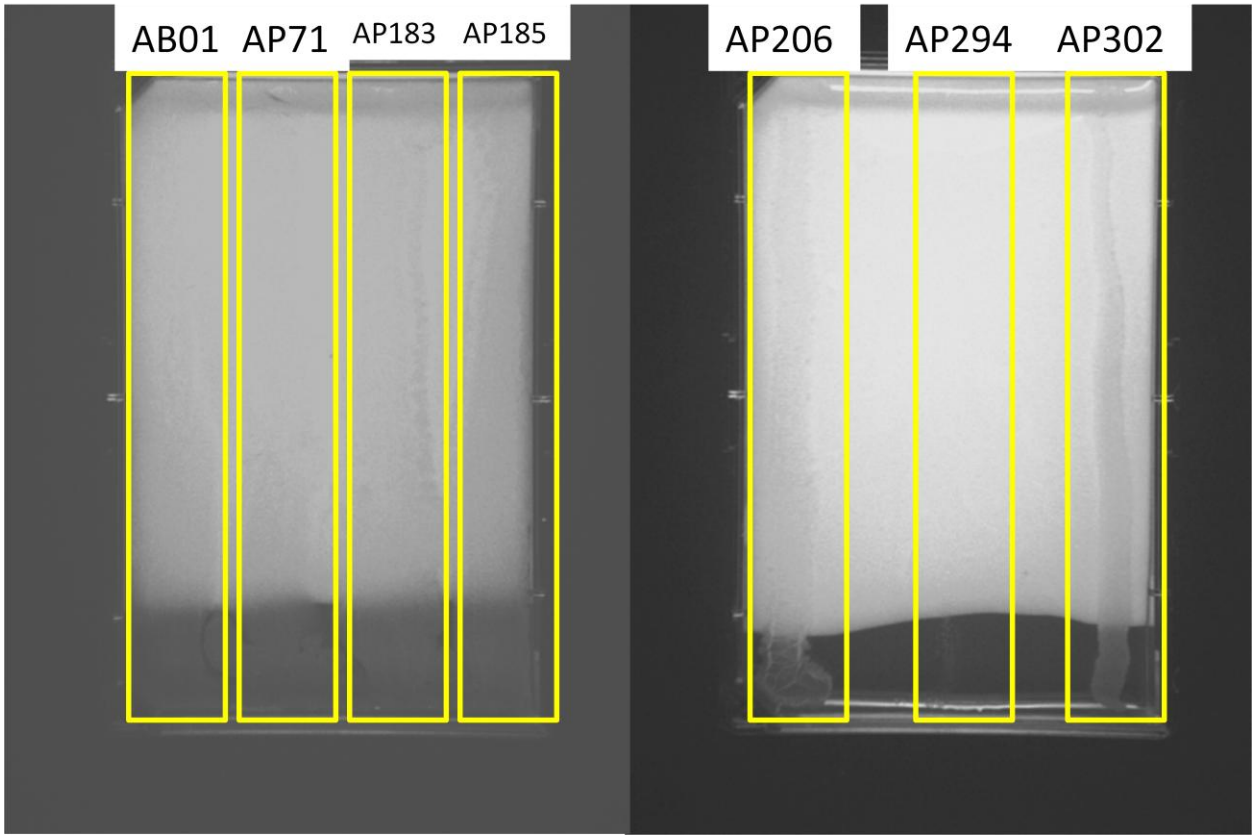

Figure 3: *Bacillus* strains growing on the surface of the uric acid gradient.

Treatment Group	# Birds w/ *Salmonella* from Cecum (out of 15)	# Birds w/ *Salmonella* from Spleen (out of 15)	Spleen/Body Weight Ratio
AB-01	9	8	0.0018
AP71	4	11	0.0015
AB-01 + AP71	6	11	0.0016
Positive Control	1	7	0.0017
Negative Control	0	0	0.0021

Table 3: Poultry trial enrichment and immune stimulation data

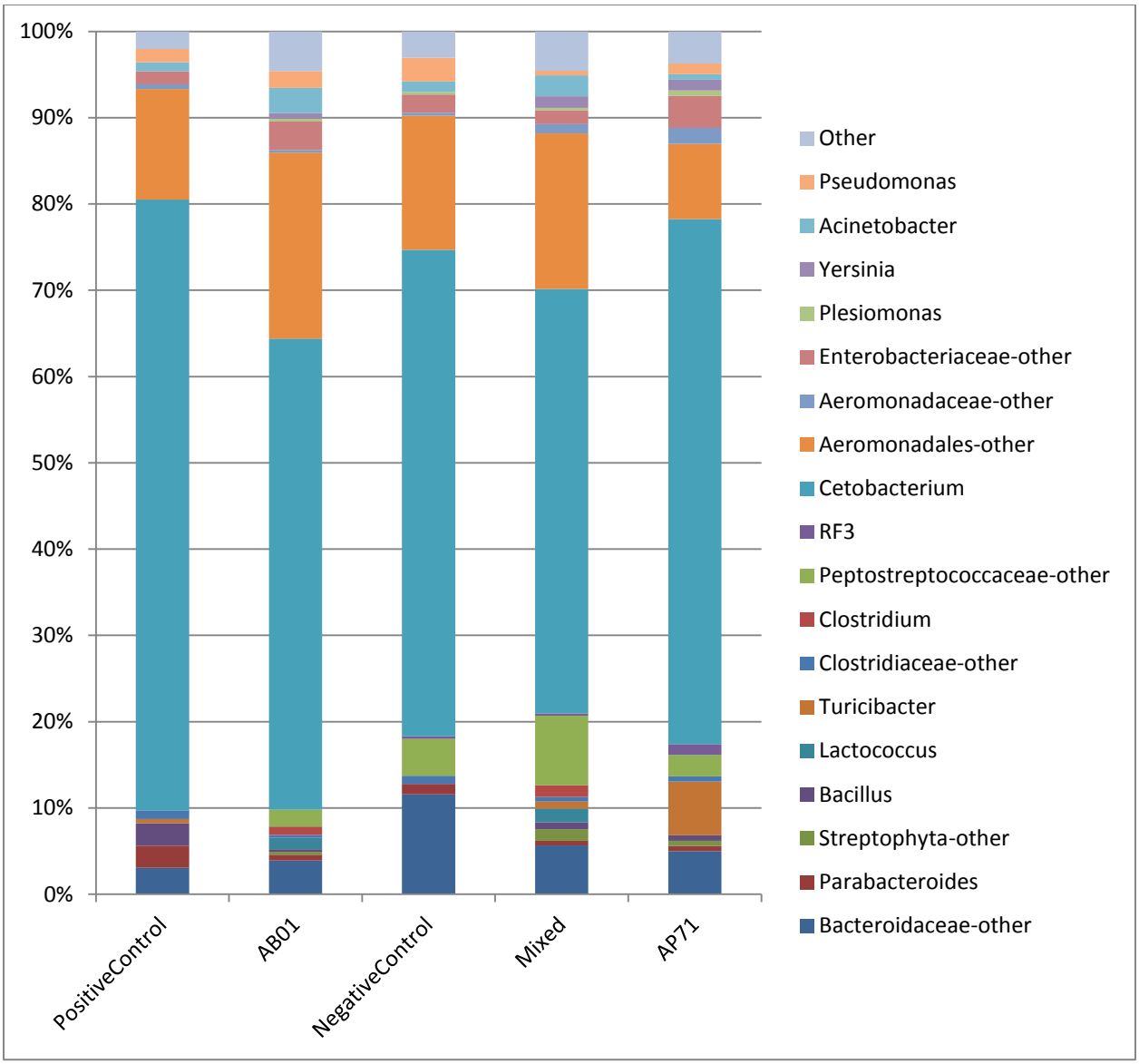

Figure 4: Relative abundance plot comparing bacterial communities between treatment groups

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