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# Poultry Performance and Food Safety Impacts of Selected Organic Acids and Probiotics

Anita Menconi

*University of Arkansas, Fayetteville*

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## Poultry Performance and Food Safety Impacts of Selected Organic Acids and Probiotics

Poultry Performance and Food Safety Impacts of Selected Organic Acids and Probiotics

A dissertation submitted in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy in Poultry Science

by

Anita Menconi  
UniPinhal  
Bachelor in Veterinary Medicine, 2005  
University of Arkansas  
Master of Science in Poultry Science, 2011

May 2014  
University of Arkansas

This dissertation is approved for recommendation to the Graduate Council.

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Dr. Billy M. Hargis  
Dissertation Director

---

Dr. Guillermo Tellez  
Committee Member

---

Dr. Lisa R. Bielke  
Committee Member

---

Dr. Luc R. Berghman  
Committee Member

---

Dr. Raphael L. Andreatti Filho  
Ex-Officio Member

## ABSTRACT

Improving production parameters and controlling foodborne pathogens have been challenges to the poultry industry. *Salmonella* has been the most common bacterial pathogen in laboratory confirmed foodborne illness cases, and contaminated poultry and poultry products have been identified as the most important source of transmission of *Salmonella* to humans. Therefore, research on effective interventions to reduce *Salmonella* transmission at the poultry production level has gained attention. Initially, a series of studies was conducted to evaluate the use of selected organic acids in controlling foodborne pathogens and improving poultry performance. Then, the characterization and application of lactic acid bacteria and *Bacillus* spp. based probiotics in poultry, and their combination, along with early nutrition, with glutamine supplementation were evaluated. In the first study, the use of organic acids *in vitro* and *in vivo* with broiler chicks (crop and cecal tonsil enumeration) reduced the incidence of *Salmonella* Typhimurium. In the second study, an organic acid product showed reductions in body weight loss during feed withdrawal and transportation, and meat quality improvement of broilers during commercial conditions. In the third study, organic acid mixtures were used in wash solutions for the reduction of spoilage and foodborne bacteria from chicken skin. The results demonstrated a reduction on pathogenic and spoilage bacteria from chicken skin, suggesting improvement of raw poultry safety properties. The fourth and fifth studies were conducted to identify and characterize probiotic strains of lactic acid bacteria and *Bacillus* spp., respectively. The evaluations included tolerance and resistance to acidic pH, high osmotic concentration of NaCl and bile salts, *in vitro* assessment of antimicrobial activity against enteropathogenic bacteria, and susceptibility to antibiotics. The last series of studies was carried out with the objective of evaluating the effects of glutamine supplementation in combination with a lactic acid based

probiotic, a *Bacillus subtilis* probiotic strain, and a commercial nutritional supplement for neonatal broilers and poults, on *Salmonella* Typhimurium colonization. The results showed increased body weight gain, villus height, villus width, and villus surface area index in chickens treated with neonatal nutrition and/or glutamine, and a reduction in *Salmonella* incidence and nitric oxide from ileal tissues of treated groups.

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- Menconi, A., Reginatto, A.R., Londero, A., Pumford, N.R., Morgan, M., Hargis, B. M., & Tellez, G. (2013). Effect of organic acids on *Salmonella* Typhimurium infection in broiler chickens. *International Journal of Poultry Science*, 12(2), 72-75.
- Menconi, A., Kuttappan, V. A., Hernandez-Velasco, X., Urbano, T., Matté, F., Layton, S., Kallapura, G., Latorre, J., Morales, B. E., Prado, O., Vicente, J. L., Barton, J., Andreatti Filho, R. L., Lovato, M., Hargis, B. M., & Tellez, G. (2014). Evaluation of a commercially available organic acid product on body weight loss, carcass yield, and meat quality during pre-slaughter feed withdrawal in broiler chickens: A poultry welfare and economic perspective. *Poultry Science*, 93, 448-455.
- Menconi, A., Shivaramaiah, S., Huff, G. R., Prado, O., Morales, J. E., Pumford, N. R., Morgan, M., Wolfenden, A., Bielke, L. R., Hargis, B. M., & Tellez, G. (2013). Effect of different concentrations of acetic, citric, and propionic acid dipping solutions on bacterial contamination of raw chicken skin. *Poultry Science*, 92, 2216-2220.
- Menconi, A., Kallapura, G., Latorre, J. D., Morgan, M. J., Pumford, N. R., Hargis, B. M., Tellez, G. (2014). Identification and characterization of lactic acid bacteria in a commercial probiotic culture. *Bioscience of Microbiota, Food and Health*, 33(1), 25-30.
- Menconi, A., Morgan, M. J., Pumford, N. R., Hargis, B. M., & Tellez, G. (2013). Physiological properties and *Salmonella* growth inhibition of probiotic *Bacillus* strains isolated from environmental and poultry sources. *International Journal of Bacteriology*, Article ID 958408, 8 pages.
- Menconi, A., Kallapura, G., Hernandez-Velasco, X., Latorre, J., Morgan, M., Pumford, N. R., Layton, S., Urbano, T., Caseres, M., Pixley, C., Barton, J., Hargis, B. M., & Tellez, G. (2013). Effect of Glutamine supplementation associated with probiotics on salmonella typhimurium and nitric oxide or glutamine with perinatal supplement on growth performance and intestinal morphology in broiler chickens. *Clinical Microbiology*, 2(5), 120.

## I. INTRODUCTION

Improving production parameters and controlling foodborne pathogens at all levels of production, in order to maintain the food safety of products, have been challenges to the poultry industry (Dickson et al., 1992; Berrang & Dickens, 2000; Harris et al., 2006; Lynch et al., 2006; Laury et al., 2009; Zhao et al., 2009). *Salmonella enterica* serotypes have been the most common bacterial pathogen in laboratory confirmed foodborne illness cases (Mani-López et al., 2012), accounting to approximately 1.0 million of the foodborne human diseases caused by bacteria per year in the United States (Voetsch et al., 2004a, 2004b; MMWR, 2011; Scallan et al., 2011).

*Salmonella* is a worldwide bacterium that is universally present in farm animals, and many of the more than 200 pathogenic serotypes are able to colonize the gastrointestinal tract of poultry (Mead et al., 2010). Moreover, contaminated poultry and poultry products have been identified by some researchers as the most important source of *Salmonella* transmission to humans (Lynch et al., 2006; Foley et al., 2011). Therefore, studies on effective interventions to minimize and possibly eradicate these bacteria at the poultry production have gained a tremendous focus by researchers and professionals of the poultry business.

Worldwide researchers have been working to develop alternatives for the ban of a wide range of drugs for animal production. Many preventive strategies have been developed to decrease the incidence of *Salmonella* colonization in broiler chickens, such as bacteriophage therapy, probiotics, prebiotics, synbiotics, water and feed acidifiers, and vaccines.

Many studies have focused on the use of organic acid in the feed or in the drinking water to improve chicken performance and/or to reduce foodborne pathogens at the poultry production level and its use as antimicrobial solution at the processing. Organic acid-based feed acidifiers have gained significant attention due to their high nutritional value and antimicrobial action

(Thompson & Hinton, 1997; Byrd et al., 2001; Kubena et al., 2001; Leeson et al., 2005; Tung & Petigrew, 2011; Companies and Markets, 2012; Islam, 2012). Moreover, the application of organic acids in the drinking water at critical periods of poultry growth has been reported to be helpful in maintaining a low pH in the crop, an intestinal development by the stability of the intestinal microflora, and eventually improving live production performance (Jarquin et al., 2007; Wolfenden et al., 2007a).

Probiotics have been used for many years to enhance intestinal health and treat intestinal diseases (Patterson and Burkholder, 2003; Nicholson, 2002; Laudanno et al., 2006; Aureli et al., 2010). Moreover, their use in the poultry industry has increased as potential alternatives to antibiotics used as growth promoters, and in select cases, for controlling specific enteric pathogens (Anadón et al., 2006; Cartman et al., 2008; Tellez et al., 2012; Ezema, 2013). The selection of a bacterial strain that possess probiotic effects includes the evaluation of characteristics such as survivability and persistence in the gastrointestinal tract, ability to attach to the intestinal mucosa, and competition with enteric pathogens (Bakari et al., 2011; Fontana et al., 2013). The most common probiotics that have been effective in poultry are lactic acid bacteria based (Tellez et al., 2012); however, some microorganisms such as *Saccharomyces boulardii* and *Bacillus* spp. have also been described as effective probiotics in poultry (Tellez et al., 2012). Furthermore, products containing *Bacillus* spores offer potential advantages over lactic acid bacteria products since they can be used as direct fed microbials (Anadón et al., 2006; Barbosa et al., 2005; Hong et al., 2005, 2008; Osipova et al., 2003; Williams, 2007; Wolken et al., 2003).

This dissertation and its literature review have the objective of studying and examining the use of selected organic acids in controlling foodborne pathogens and in improving poultry

performance, the characterization and application of lactic acid bacteria and *Bacillus* spp. based probiotics in the poultry industry, and their combination with glutamine supplementation as well as the combination of early nutrition and glutamine.

## **II. LITERATURE REVIEW**

### **A. POULTRY AND FOOD SAFETY**

Controlling foodborne pathogens such as *Salmonella enterica* serotypes, *Escherichia coli* O157:H7, and *Campylobacter* spp., at all levels of production, have been a challenge to the poultry industry (Dickson et al., 1992; Berrang & Dickens, 2000; Harris et al., 2006; Lynch et al., 2006; Laury et al., 2009; Zhao et al., 2009). Moreover, *Salmonella* has been the most common bacterial pathogen in laboratory confirmed foodborne illness cases reported by the Centers for Disease Control and Prevention, being considered a target pathogen for the food industry (Mani-López et al., 2012).

It is essential to maintain the food safety of poultry products in order to protect public health. Therefore, finding effective interventions to minimize and possibly eradicate these bacteria at the poultry production level has gained a tremendous focus by poultry researchers and professionals.

#### **1. Poultry products as a cause of human *Salmonella* infection**

*Salmonella*, a genus of the family Enterobacteriaceae, has been associated with infectious diseases (nontyphoid and typhoid salmonellosis) for many years, making it an important pathogen for both animals and humans (Su & Chiu, 2007; Lutful Kabir, 2010). It is estimated that 3.6 million foodborne human diseases per year, in the United States, are caused by bacteria,

and *Salmonella* spp. nontyphoidal accounts to about 1.0 million of these cases (Voetsch et al., 2004a, 2004b; MMWR, 2011; Scallan et al., 2011).

Approximately 80 *Salmonella* serotypes among all the 2,500 are often the most involved in *Salmonella* infections. *Salmonella enterica* subspecies *enterica* serotype Typhimurium and *Salmonella enterica* subspecies *enterica* serotype Enteritidis are the most frequent agents of salmonellosis; nevertheless, there has been increasing incidence and concern regarding the serotypes Heidelberg, Infantis, Agona, Hadar, Virchow (Chittick et al., 2006; Patchanee et al. 2008; Freitas et al., 2010), and Kentucky (Foley et al., 2011). Contaminated poultry and poultry products have been identified by some researchers as the most important source of transmission of *Salmonella* to humans (Lynch et al., 2006; Foley et al., 2011).

*Salmonella* is able to invade the gastrointestinal mucosa of poultry, as well as cecal tonsils and Peyer's patches, proliferate inside the macrophages and spread through blood or lymphatic circulation to other tissues such as liver and spleen, which are the primary site of invasion. Other tissues and organs susceptible to infection include ovary, oviduct, yolk sac, and lungs (Lutful Kabir, 2010; Foley et al., 2011). Once a chicken is infected, it can carry *Salmonella* normally in its gastrointestinal tract with no symptoms, and can cause cross-contamination to other birds and carcass contamination at the processing level (Mead et al., 2010). Contamination by *Salmonella* on live animals and carcasses can occur during transportation and processing (Bourassa et al., 2004; Parveen et al., 2007).

Moreover, increased pressure by consumers and regulatory agencies for reduced or even elimination of the use of antibiotics in food producing animals has created a challenge for the poultry industry to control *Salmonella* at the production level and within processing and

manufacturing plants (Hargis et al., 1995; Corrier et al., 1999a; Hinton et al., 2000; Mikolajczyk and Radkowski, 2002).

## **B. ORGANIC ACIDS IN POULTRY**

Worldwide potential for poultry acidifiers has been increasing due to higher demand for good quality poultry, which is also true for most of the other animal productions including swine and cattle (Berkhout, 2009). Increased utilization of feed or water acidifiers in emerging countries, coupled with escalating demand in the developing world, has expanded the market for these acidifiers (Companies and Markets, 2012). Organic acid based feed acidifiers have gained significant attention due to their high nutritional value and antimicrobial benefits (Companies and Markets, 2012).

Most research and subsequent applications have involved feed acidifiers as a preventive or treatment tool for disease management or to improve chicken performance by enhancing the nutrient digestibility and modulating the microbial populations in the digestive tract (Thompson & Hinton, 1997; Byrd et al., 2001; Kubena et al., 2001; Leeson et al., 2005; Tung & Petigrew, 2011; Islam, 2012).

However, the application of organic acids in the drinking water at critical periods of poultry growth such as during the first 7 days, feed changes, and feed withdrawal has been reported to be helpful to maintain a low pH in the crop, an intestinal development by the stability of the intestinal microflora, and eventually improving live production performance (Jarquin et al., 2007; Wolfenden et al., 2007a). Moreover, organic acids in the water have been frequently used as water sanitizers, reducing colonization of pathogenic bacteria in the gastrointestinal tract of chickens (Van Immerseel et al., 2006; Byrd et al., 2001).

## 1. The use of organic acids to control foodborne pathogens

Chickens contain large amounts of bacteria in their gastrointestinal tract, feathers, and feet; consequently, fecal bacteria could be present on chicken carcasses instantly after processing (Ramirez et al., 1997; Northcutt et al., 2003; Mani-López et al., 2012). Therefore, methods of intervention are needed to decrease populations of spoilage bacteria and foodborne enteropathogens in chicken meat.

Antimicrobial chemicals are commonly used during processing to reduce pathogen loads on carcasses. The most common antimicrobial treatment used for decontamination of poultry meat is sodium hypochlorite, commonly known as chlorine (Mountney & O'malley, 1965). Mountney & O'malley (1965) showed 1 to 2 log<sub>10</sub> reduction of *Salmonella* and *Campylobacter* on poultry carcasses treated with chlorine. While this may be sufficient to remove *Salmonella* from most poultry carcasses, chlorine may bind to organic matter, and be ineffective. In fact, the continued lack of decline in rates of foodborne illness (MMWR, 2011; Scallan et al., 2011) has suggested that chlorine treatment of carcasses in the processing facility is not effectively decreasing the incidence of *Salmonella* contamination. Moreover, difficulties in optimizing the disinfectant properties of chlorine (improper pH, concentration, or composition of incoming water) may reduce its efficacy. Chlorine treatment may also cause unpleasant and harmful odors due to the production of chlorine gas and trichloramines (Northcutt et al., 2005; Hinton et al., 2007; Northcutt et al., 2008).

For these reasons, the use of alternative methods, including organic acids, which are generally recognized as safe (GRAS) for meat products, to disinfect poultry carcass have been studied (Mani-López et al., 2012). Research based on the use of organic acids to spray or dip poultry carcasses has reported as much as 3 log<sub>10</sub> of *Salmonella* reduction (Vasseur et al., 1999;

Kubena et al., 2001; Hinton & Ingram, 2005; Lu et al., 2005; Harris et al., 2006; Van Immerseel et al., 2006).

Because most carcasses are considered to have about 100 *Salmonella* cells, carcass rinse applications that decrease *Salmonella* by 2 log<sub>10</sub> cfu/ml are considered effective (Jetton, et al., 1992). Yang et al. (1998) sprayed chicken carcasses with 2% lactic acid and recorded a 2 log<sub>10</sub> cfu reduction of *Salmonella* per carcass. Moreover, lactic acid and citric acid at concentrations of 1- 3% have been shown to reduce *Escherichia coli* O157:H7, *Salmonella enterica* serotypes, and *Listeria monocytogenes* when sprayed on beef and poultry carcasses (Vasseur et al., 1999).

Controlling foodborne pathogens, especially *Salmonella* at the poultry production level is also very important, and it directly reflects on the pathogenic bacteria levels found at the processing level. Berghaus et al. (2013) showed significant associations of *Salmonella* and *Campylobacter* prevalence between farms and processing plant in a study where they collected environmental samples from commercial broiler houses and evaluated the carcass rinse of chickens from the same flock at processing. Therefore, the use of organic acid in the feed or in the drinking water to reduce foodborne pathogens at the poultry production level has been a focus of many studies.

Organic acids can be used as feed preservatives to reduce the pH of the feed and consequently reduce bacterial contamination (Islam, 2012). Moreover, treatments with organic acids have shown a decrease in the number of *Salmonella* viable cells in animal feed and feed ingredients (Koyuncu et al., 2013). Organic acids, which are a readily available energy source for both chicken and bacteria, have been also applied in the feed or drinking water to reduce gastrointestinal bacterial pathogens. However, it is important that the administration of organic



acids occur in high enough concentrations to be bactericidal in the presence of organic matter, and low enough to be voluntarily consumed by the birds.

Organic acids in the poultry diet have shown modulation of the microbial populations in the digestive tract, especially in the crop, gizzard (Thompson & Hinton, 1997; Kubena et al., 2001) and small intestine (Cengiz et al., 2012). According to Alp et al. (1999), the dietary inclusion of an organic acid mix, composed by lactic, fumaric, propionic, citric, and formic acids, was able to decrease Enterobacteriaceae counts in the ileum of broiler chickens.

Application of organic acids in the drinking water during broiler's pre-slaughter feed withdrawal period has similarly demonstrated significantly reduction of *Salmonella* recovered from crops and cecal tonsils, and subsequently from the carcasses (Van Immerseel et al., 2006; Alali et al., 2010; Vandeplas et al., 2010). Byrd et al. (2001) used lactic acid in the drinking water during pre-slaughter feed withdrawal period, and reported a significant reduction of *Salmonella* and *Campylobacter* contamination of crops and carcasses at processing.

The antimicrobial efficacy and the effect on virulence of *Salmonella* differ with each organic acid treatment, and each organic acid has a unique effect on bacteria normally present in the crop and gastrointestinal tract (Furuse et al., 1991; Byrd et al., 2001; Castro Gonzalez et al., 2001; Kubena et al., 2001).

## **2. The use of organic acids during pre-slaughter feed withdrawal period**

In the poultry industry, different reasons can cause feed restriction; nevertheless, the most common feed restriction is the pre-slaughter feed withdrawal, which is a method employed to reduce fecal contamination of carcasses at processing (Corrier et al., 1999c; Byrd et al., 2001; Northcutt et al., 2003; Yi et al., 2005). Although this practice has showed efficacy in reducing

visible fecal/digesta contamination, it also leads to an imbalance in the natural population of lactic acid bacteria, causing an increase in pH, favoring pathogens as *Salmonella* to multiply in the crop. Moreover, carcass dehydration begins immediately after feed withdrawal (Benibo and Farr, 1985; Veerkamp, 1986), resulting in recommendations that slaughter take place within 4 to 8 h after feed withdrawal to minimize losses, which may be hard to achieve under commercial conditions. Consequently, scheduling managers need to consider feed withdrawal effects on both gut fullness and shrinkage. In addition to feed withdrawal, chickens must endure stress during catching, crating transport, and shackling (Gregory, 1994; Petracci et al., 2006). Moreover, feed restriction induces pecking of the contaminated litter, which may contaminate the crop (Corrier et al., 1999c), and it may contaminate raw poultry products if the crop is ruptured during processing (Hargis et al., 1995; Corrier et al., 1999b).

Drinking water acidification with organic acids has showed significant reduction of recoverable *Salmonella* in the crops and cecal tonsils, and consequently on the carcasses, when used during broiler's pre-slaughter feed withdrawal period (Byrd et al., 2001; Van Immerseel et al., 2006; Alali et al., 2010; Vandeplas et al., 2010). Wolfenden et al. (2007a) conducted a study in broiler chickens and showed that the drinking water administration of a commercially available organic acid product based on acetic, lactic, tannic, propionic, and caprylic acids, significantly reduced carcass condemnation at the processing plant and mortality during transportation, with consistent improvement of average body weight at the farm and at the processing plant in broiler chickens. In a similar study, treatment with the same organic acid product in the drinking water of commercial turkeys during feed withdrawal showed a significant reduction in the rate of weight loss during transportation and holding at the processing plant in

treated turkeys and improved average body weight in treated turkeys during 19 h with an average of 90 g difference (Pixley et al., 2010).

### **3. Antimicrobial mechanism of action of the organic acids**

The antimicrobial mechanism of action of organic acids depends on the type of acid, and each organic acid has a unique effect on bacteria (Kubena et al., 2001). It also depends on the target bacteria. For example, the minimal inhibitory concentration of acetic acid is 250 times lower for *Bacillus subtilis* than for *Lactobacillus* spp. (Hsiao and Siebert, 1999).

The antimicrobial effects of organic acids have been explained by the capability of these acids (in their undissociated form) to diffuse across the cell membrane, dissociating and acidifying the cell cytoplasm (Vasseur et al., 1999; Leeson et al., 2005; Van Immerseel et al., 2006). Additionally, the organic acid antimicrobial effect is increased when the pH is lower than the acid dissociation constant (pKa), which increases the concentration of H<sup>+</sup> ions, protonation, and diffusion of the acid across membranes (Mani-López et al., 2012).

The cytoplasm acidification will cause inhibition of bacterial growth by the presence of H<sup>+</sup> ions from the acids dissociation, membrane disruption, inhibition of metabolic reactions, accumulation of toxic anions, energy exhaustion to maintain homeostasis, and enzymes, proteins, and DNA alteration (Islam, 2012; Mani-López et al., 2012). Additionally, some organic acids (malic and citric acids) have shown the ability to chelate and disrupt the bacterial cell membrane (Mani-López et al., 2012).

Most of the antimicrobial properties of organic acids are related to changes in pH, but organic acid inhibitory effects may vary depending on their carbon chain, hydroxyl groups, and double bonds (Hsiao & Siebert, 1999). For instance, the variability of carbon chain of an organic

acid is proposed to be an important factor to differentiate a bacterial inhibition as bactericidal or bacteriostatic. According to Van Immerseel et al. (2006), the dose of 25 mM of a MCFA (medium chain fatty acid) was bacteriostatic to a *Salmonella* serotype Enteritidis strain; however, the same strain tolerated 100 mM of a SCFA (short chain fatty acid). These variabilities may explain the inconsistent results regarding the administration of organic acids and their antimicrobial effects in chickens (Cengiz et al., 2012).

### **C. POULTRY PERFORMANCE AND ANTIBIOTICS**

Antibiotic is considered as a growth promoter when administered at a non-therapeutic (low) concentration in the feed of food animals to stimulate growth and improve feed efficiency (Costa et al., 2011; Lin et al., 2013). Antibiotic as growth promoter (AGP) in the feed of different animal species has been used for more than 60 years in the United States as well as in other countries (Dibner & Richards, 2005; Costa et al., 2011), and its effect has been related to feed efficiency improvement and indirectly on growth improvement (Dibner & Richards, 2005).

The mechanisms on how antibiotics can promote growth are still unclear. The most common hypotheses are: (1) improvement of nutrients absorption by thinning the intestinal wall and villi and reducing intestinal size, which could be caused by a loss of mucosal cell proliferation due to the lack of short chain fatty acids in the lumen, which is provided through microbial fermentation; (2) protecting nutrients from being used by bacteria; (3) decreasing the number of bacteria and bacterial toxins; (4) reducing the incidence of subclinical infections; consequently, decreasing the metabolic cost of the immune system (Gaskins et al., 2002; Butaye et al., 2003; Dibner & Richards, 2005; Niewold, 2007; Costa et al., 2011).

Many studies regarding the mode of action of AGP have focused on interactions between the antibiotic and the intestinal microflora (Dibner & Richards, 2005). However, many scientists indicate that the main activity of several growth promoting antibiotics is due to an anti-inflammatory effect instead of an antimicrobial effect *per se*, and the microflora changes would be a consequence of the intestinal changes (Niewold, 2007; Buret, 2010; Costa et al., 2011).

Although the use of AGP has been a common practice of modern animal production (Butaye et al., 2003), its extensive use has contributed to the emergence of antimicrobial resistance in zoonotic pathogens (Costa et al., 2011; Lin et al., 2013). As a result, the European Union employed a ban on the administration of all AGP to livestock in January 1, 2006 (Anadon et al., 2006). Moreover, restrictions to the use of AGP in the United States has been anticipating a possible ban as well (Dibner & Richards, 2005; Costa et al., 2011). This ban has led to an increase in the cost of animal production, a decrease in livestock production (Costa et al., 2011), and an increase in the incidence of some animal diseases (Dibner & Richards, 2005), forcing animal husbandry to find alternatives such as enzymes, organic acids, probiotics, prebiotics, essential oils, and immunostimulants (Huyghebaert et al., 2011). The regulation of immune functions in the intestine is associated with the establishment of the microflora, which has led to the introduction of therapeutic interventions with the use of cultures of beneficial live microorganisms known as probiotics (Isolauri et al., 2001). Moreover, due to its characteristics and mode of action, probiotics have been extensively studied as an alternative for AGP in animal production (Chaucheyras-Durand & Durand, 2010).

## D. PROBIOTICS IN POULTRY

Probiotics, which are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO, 2002), have been used for many years to enhance intestinal health and treat intestinal disorders (Patterson & Burkholder, 2003; Nicholson, 2002; Laudanno et al., 2006; Aureli et al., 2010). The selection of a successful probiotic strain includes the evaluation of characteristics such as survivability and persistence in the gastrointestinal tract, ability to attach to the intestinal mucosa, and competition with enteric pathogens (Fontana et al., 2013). The probiotic microorganism, after being ingested, also needs to be resistant to low pH, gastric enzymes, bile salts, and other “insults” from the gastrointestinal tract (Bakari et al., 2011; Fontana et al., 2013). Moreover, features such as metabolic activity of a specific strain and concentration administered are important for a probiotic optimal efficacy (Chaucheyras-Durand & Durand, 2010; Huyghebaert et al., 2011).

The use of probiotics and direct fed microbials (DFM) in the poultry industry has increased as potential alternatives to antibiotics used as growth promoters and, in select cases, to control specific enteric pathogens (Anadón et al., 2006; Cartman et al., 2008; Tellez et al., 2012; Ezema, 2013). The most common probiotics that have been effective in poultry are lactic acid bacteria based, which are generally found in the gastrointestinal tract of vertebrates and invertebrates (Tellez et al., 2012). Lactic acid bacteria include, for example, *Lactobacillus* spp., *Bifidobacterium* spp., and *Enterococcus* spp. (Ljungh & Wadström, 2006). Some microorganisms such as *Saccharomyces boulardii* and *Bacillus* spp. that are not normally found in the gastrointestinal tract have also been described as effective probiotics in poultry (Tellez et al., 2012). Furthermore, both live and spore based probiotics from *Bacillus* spp. have earned

attention as a viable probiotic bacteria in the poultry industry (Cartman et al., 2008; Vila et al., 2009; Wolfenden et al., 2010; Shivaramaiah et al., 2011).

### **1. The use of lactic acid bacteria to control *Salmonella* spp. in poultry.**

Lactic acid bacteria (LAB) are Gram-positive bacteria, normally found in the gastrointestinal tract of humans and animals. The most common LAB used as a probiotic is the genus *Lactobacillus*, a nonspore-forming bacterium that has more than 125 species identified (Hori, 2010). *Lactobacillus* spp. has demonstrated, *in vitro* and *in vivo*, probiotic capability by innumerous studies in humans and animals.

For example, *Lactobacillus fermentum* and *Lactobacillus acidophilus* strains have shown, *in vitro*, the reduction on the attachment of *Salmonella* Typhimurium and *Salmonella* Pullorum to ileal epithelial cells (Jin et al., 1996). Also, in an *in vitro* study, Tsai et al. (2005) selected *Lactobacillus fermentum* from the cecum based on its ability to adhere intestinal epithelial cells and the ability to inhibit the growth of *Escherichia coli*, *Salmonella* Typhimurium, *Staphylococcus aureus*, and *Bacillus cereus*. A strain of *Lactobacillus crispatus* was also selected as a potential probiotic based on aggregation time and antibacterial activity against *Salmonella* Typhimurium, *Salmonella* Enteritidis, and *Escherichia coli* (Taheri et al., 2009).

In both *in vitro* and *in vivo* *Salmonella* enumeration from chicks' liver, spleen, and ceca studies, Van Coillie et al. (2007) demonstrated a reduction in *Salmonella* Enteritidis by *Lactobacillus* spp. strains previously isolated from the cloaca and vagina of laying hens. Moreover, a commercial probiotic consisting of *Lactobacillus reuteri*, *Enterococcus faecium*, *Bifidobacterium animalis*, *Pediococcus acidilactici*, and *Lactobacillus salivarius* showed 50% reduction of *Salmonella* Enteritidis enumerated from cecal ingesta (Mountzouris et al., 2009).

Laboratory and field research conducted with a commercial and defined lactic acid bacteria probiotic have demonstrated accelerated development of normal microflora and reduction in *Salmonella* colonization, in poultry (Tellez et al., 2006; Farnell et al., 2006; Higgins et al., 2007, 2008; Vicente et al., 2007a, 2007b; Wolfenden et al., 2007b, 2007c, 2008; Menconi et al., 2011). Rahimi et al. (2009) studied the intestinal morphology of turkey poultts treated, in the feed, with a commercial probiotic consisted of *Lactobacillus* spp and challenged with *Salmonella* serotypes Typhimurium, Kentucky, and Heidelberg. The authors observed changes in intestinal morphology regarding restoration of the villi loss or damage related to *Salmonella* infection (Rahimi et al., 2009).

## **2. The use of *Bacillus*-based probiotics in poultry**

The use of direct-fed microbials (DFM) has also earned attention as a viable alternative to traditional antibiotic therapies. Some commonly used DFM bacteria that have been successful in animals and humans include multiple strains of *Lactobacillus*, *Pediococcus*, *Bifidobacterium*, and especially *Bacillus* spp. (Zani et al., 1998; Ouwehand et al., 2002; O’Dea et al., 2006). *Bacillus* species are ubiquitous, Gram-positive bacteria (Nicholson, 2002) that have recently shown promise as DFM because of their capacity to form endospores, which can survive harsh environmental stress and transitions during storage and handling (Cartman et al., 2008). *Bacillus* species have been found in the normal intestinal flora of poultry, and some strains have shown to be capable of germinating and resporulating in chickens’ gastrointestinal tract (Hoa et al., 2000; Barbosa et al., 2005; Tam et al., 2006; Cartman et al., 2008). The administration of *Bacillus* spp. spores as feed additive offers many advantages, such as ease preparation, heat tolerance,



resistance to production processes, extended shelf-life, and low cost of production (Barbosa et al., 2005; Duc et al., 2004; Hong et al., 2005; Vila et al., 2010; Permponpattana et al., 2012).

Competitive exclusion of pathogens is a popular hypothesis to explain the action of probiotics (Patterson and Burkholder, 2003; Leser et al., 2008). Even though the process has been well demonstrated in *Lactobacillus* spp., some evidence exists that *Bacillus* spp. may have the same mode of action (Barbosa et al., 2005). Several studies have shown that either live vegetative cells or endospores of some isolates can prevent colon carcinogenesis (Malkov et al., 2006; Lee et al., 2007; Park et al., 2007) or produce antimicrobial substances against Gram-positive bacteria such as *Staphylococcus aureus*, *Enterococcus faecium*, and *Clostridium difficile* (O'Mahony et al., 2001; Urdaci et al., 2004).

The ability of a specific laboratory strain of *Bacillus subtilis* in inhibiting growth of different pathogens bacteria in chickens, such as *Escherichia coli*, *Clostridium perfringens*, and *Salmonella* Enteritidis has been shown (La Ragione, et al., 2001; La Ragione & Woodward, 2003). *In vitro* inhibition of *Clostridium perfringens* by a strain of *Bacillus subtilis* isolated from the gastrointestinal tract of chickens was also demonstrated by Teo & Tan (2005). *Salmonella* Enteritidis occurrence was reduced in broiler chickens in two experiments where a *Bacillus subtilis*-based probiotic was added to feed (Vila et al., 2009). Selected heat-resistant spore-forming *Bacillus* species, strain PHL-NP-122, showed markedly reduction of *Salmonella* and *Clostridium* in poultry (Wolfenden et al., 2010; Shivaramaiah et al., 2011; Wolfenden et al., 2011). Additionally, a *Bacillus subtilis* probiotic strain, DSM17299, reduced *Salmonella*-positive drag swabs and *Salmonella* Heidelberg incidence in the ceca of treated chickens (Knap et al., 2011). In addition to the efficacy of *Bacillus* as DFM in reducing *Salmonella* incidence in poultry, the ability of increasing feed consumption and body weight through the use of *Bacillus*-

based probiotics in poultry feed has also been demonstrated (Fritts et al., 2000; Vila et al., 2009; Wolfenden et al. 2010).

### **3. Mechanisms of action of probiotics**

Several mechanisms of action of probiotics have been proposed, including competition for receptor sites and nutrients, and production of antimicrobial substances such as bacteriocins, hydrogen peroxide, and volatile fatty acids (Patterson & Burkholder, 2003; Vandeplas et al., 2010). Also, probiotics have been described to cause a decrease in the intestinal pH by the production of organic acids, which in turn would create favorable conditions for the transient and resident microflora (Chaucheyras-Durand & Durand, 2010) and a production of nutrients and growth factors stimulating intestinal microflora (Delcenserie et al., 2008). Among all the mechanisms proposed for probiotic functions, the modulation of both innate and acquired immune systems has received a great attention (Jijon et al., 2004; Ng et al., 2009; Flore et al., 2010; Dicks & Botes, 2010; Socol et al., 2010). Moreover, it has been described that specific probiotic strains show anti-inflammatory properties, which has led to the research and discovery of new mechanisms of action of selected probiotic strains (Isolauri et al., 2002; Pagnini et al., 2010).

The bactericidal activity of LAB, for example, has been extensively studied, and the antimicrobial mechanism seems to be due to many factors. It is documented that lactic acid production and the resulting acidity is important (Fayol-Messaoudi et al., 2005; Makras et al., 2006); nevertheless, this characteristic is also complemented by other mechanisms such as synthesis of bacteriocins and other compounds (Ljungh & Wadström, 2006). Some *Lactobacillus* strains reduce nitrate ( $\text{NO}^{-3}$ ) to nitrite ( $\text{NO}^{-2}$ ) and nitric oxide under anaerobic conditions (Wolf

et al., 1990, 1991). Nitric oxide and  $\text{NO}^{-2}$  produced from  $\text{NO}^{-3}$  by *Lactobacillus* spp. may be a possible antimicrobial mechanism (Adawi et al., 1997, 1998). In *Lactobacillus* strains that produce hydrogen peroxide, the metabolites of lactic acid and hydrogen peroxide may act together to kill enteric pathogens (Cadieux et al., 2009; An et al., 2010; Atassi & Servin, 2010; Martin & Suarez, 2010). *Lactobacillus* are also known to produce antimicrobial biosurfactants (Reid et al., 1999; Reid, 2001; Portilla-Rivera et al., 2008; Walencka et al., 2008) and to increase the production of intestinal mucins that may function as a barrier and as a receptor for pathogens (Ljungh & Wadström, 2006).

Additionally, probiotic bacteria can exert immunomodulatory activities through their interactions with the host immune system. These interactions may cause enhancement of antigen-specific antibodies (Davies et al., 2009; Amit-Romach et al., 2010; Cai et al., 2010), activation or suppression of T-cells (Gorska et al., 2009; Sjogren et al., 2009; Starovoitova et al., 2009; Foligne et al., 2010), modulation of dendritic cell's phenotype and function (Drakes, et al., 2004; Hart et al., 2004; Thomas et al., 2009), and changes in cytokine expression profiles, which work in the induction and regulation of the immune response (Lutful Kabir, 2009; Shida et al., 2009; Nayak, 2010; Tsai et al., 2010). Moreover, research has shown that *Bacillus subtilis* spores, after oral ingestion, are immunogenic and are able to disseminate to the Peyer's patches and mesenteric lymph nodes (Duc et al., 2003a, 2003b; Permpoonpattana et al., 2012). According to Patterson and Burkholder (2003), the probiotic action mechanisms are not mutually exclusive, some bacteria may act using only one or several of the mechanisms.

Regarding the mode of action of probiotic products on intestinal inflammation, it has been proposed that the anti-inflammatory mechanisms of probiotic bacteria are induced by an improvement in barrier function, synthesis of antimicrobials, and a modulation of both

microflora and mucosal immune system, which, in general, occurs by decreasing the production of pro-inflammatory cytokines and increasing production of anti-inflammatory cytokines (Ewaschuk & Dieleman, 2006).

According to Isolauri et al. (2001, 2002), probiotic bacteria are able to equilibrate local pro-inflammatory and anti-inflammatory cytokine. These beneficial microorganisms have shown a reduction in lymphocyte proliferation and cytokine production by T cells, and also a reduction in intestinal inflammatory responses through the stimulation of secretory immunoglobulin A (IgA), which protects the mucosal surface by the non-activation of inflammatory responses (Isolauri et al., 2001). In a study on the influence of lactic acid bacteria on the intestinal mucosa of mice, Galdeano and Perdigon (2004) showed an increase in the number of interleukin - 10 (IL-10) cells and a stimulation of IgA production after the treatment with a viable *Lactobacillus casei* probiotic strain. There has been also *in vitro* description of the capability of probiotic bacteria in increasing the production of human anti-inflammatory cytokines such as IL-10 and transforming growth factor beta (TGF- $\beta$ ; Isolauri et al., 2002). Moreover, the use of probiotics has shown a decrease in the secretion of inflammatory cytokines by increasing the degradation of antigens in the intestine (Isolauri et al., 2002).

Okada et al. (2009) showed a downregulation of mRNA expression of IL-1  $\beta$  and tumor necrosis factor alpha (TNF- $\alpha$ ), which are released by macrophages during intestinal inflammation, by probiotic strains of *Bifidobacterium* species. Furthermore, the authors showed that the commensal *Enterococcus faecalis* (bacteria that have been described to play a role in inflammatory bowel disease (IBD) in both animals and humans) stimulated macrophages to produce IL-12 (cytokine that facilitates the differentiation of CD4<sup>+</sup> T helper cells through the activation of interferon gamma (IFN- $\gamma$ ) production) (Okada et al., 2009). Yan et al. (2011)

reported that a soluble protein derived from the probiotic strain of *Lactobacillus rhamnosus* was able to minimize the effects of dextran sodium sulfate (DSS) induced colitis (reducing intestinal epithelial apoptosis) in mice, through the activation of epidermal growth factor receptor.

Interestingly, genetically modified probiotic bacteria engineered to produce anti-inflammatory cytokines could also play a role in controlling intestinal inflammation. Steidler et al. (2000) showed a reduction in induced DSS colitis of mice treated with a strain of *Lactococcus lactis* genetically engineered to secrete IL-10. The stimulation of the release of IL-10 by a mixture of probiotic strains (*Bifidobacterium longum*, *Bifidobacterium infantis*, *Bifidobacterium breve*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus plantarum*, and *Streptococcus salivarius* subsp. *thermophilus*) known as VSL#3 was also described by Drakes et al. (2004) and Hart et al. (2004), in an *in vitro* model using dendritic cells culture. Moreover, the probiotic combination VSL#3 was tested in an *in vivo* experiment with mice and showed a stimulation of epithelial innate immunity (Pagnini et al., 2010). In addition, an increase in IL-10 has been described in mice fed *Lactobacillus delbrueckii* subspecies *bulgaricus* and *Lactobacillus casei* (Ghosh et al., 2004).

In a DSS or trinitrobenzenesulfonic acid induced colitis model in mice, *Bacillus polyfermenticus* demonstrated a reduction in the expression of inflammatory molecules such as chemokine (C-X-C motif) ligand 1, intercellular adhesion molecule, and TNF- $\alpha$ . The same strain also increased the expression of IL-10, decreasing colon inflammation (Im et al., 2009). In an *in vitro* experiment using human peripheral blood mononuclear cells, a probiotic combination of *Bacillus mesentericus*, *Clostridium butyricum*, and *Enterococcus faecalis* showed a decrease in TNF- $\alpha$  levels and an increase in IL-10 levels, increasing the number of T regulatory cells (Hua et al., 2010). Also, using human peripheral blood mononuclear cells, Imaoka et al. (2008) observed

an increase in the production of IL-10, and an inhibition of IL-8 (cytokine associated with inflammation in ulcerative colitis) secretion by a probiotic composed by *Bifidobacterium bifidum* and *Bifidobacterium breve*.

In a study conducted with IBD and healthy human patients, Shadnough et al. (2013) showed an increase in serum levels of IL-6 (pro-inflammatory cytokine) and IL-10 and a decrease in serum levels of IL-1 $\beta$  and TNF- $\alpha$  in IBD patients treated with a probiotic yogurt containing *Bifidobacterium* and *Lactobacillus*. Overall, probiotic bacteria could act in stabilizing intestinal inflammation by balancing the intestinal microflora, maintaining mucosal barrier, and modulating and improving the intestinal mucosal immune system, especially by keeping the balance of pro-inflammatory and anti-inflammatory cytokines and production of intestinal IgA (Isolauri et al., 2002; Hua et al., 2010; Ashraf & Shah, 2013; Zagato et al., 2014).

The following chapters represent research conducted towards finding sustainable antibiotic alternatives for improved welfare, production parameter, and control of *Salmonella* in poultry.

## **E. REFERENCES**

- Adawi, D., Kasravi, F. B., Molin, G., & Jeppsson, B. (1997). Effect of *Lactobacillus* supplementation with and without arginine on liver damage and bacterial translocation in an acute liver injury model in the rat. *Hepatology*, 25, 642-647.
- Adawi, D., Molin, G., & Jeppsson, B. (1998). Inhibition of nitric oxide production and the effects of arginine and *Lactobacillus* administration in an acute liver injury model. *Annals of Surgery*, 228, 748-755.
- Alali, W. Q., Thakur, S., Berghaus, R. D., Martin, M. P., & Gebreyes, W. A. (2010). Prevalence and distribution of *Salmonella* in organic and conventional broiler poultry farms. *Foodborne Pathogens and Disease*, 7(11), 1363-1371.
- Alp, M., Kocabagli, N., & Kahraman, R. (1999). Effects of dietary supplementation with organic acids and zinc bacitracin on ileal microflora, pH and performance in broilers. *Turkish Journal of Veterinary and Animal Sciences*, 23, 451-455.

- Amit-Romach, E., Uni, Z., & Reifen, R. (2010). Multistep mechanism of probiotic bacterium, the effect on innate immune system. *Molecular Nutrition & Food Research*, 54, 277-284.
- Anadón, A., Martínez-Larranaga, M. R., & Aranzazu Martínez, M. (2006). Probiotics for animal nutrition in the European Union. Regulation and safety assessment. *Regulatory Toxicology and Pharmacology*, 45(1), 91-95.
- An, H., Zhou, H., Huang, Y., Wang, G., Luan, C., Mou, J., Luo, Y., & Hao, Y. (2010). High-level expression of heme-dependent catalase gene *katA* from *Lactobacillus sakei* protects *Lactobacillus rhamnosus* from oxidative stress. *Molecular Biotechnology*, 45, 155-160.
- Ashraf, R., & Shah, N. P. (2013). Immune system stimulation by probiotic microorganisms. *Critical Reviews in Food Science and Nutrition*, 54(7), 938-356.
- Atassi, F., & Servin, A. L. (2010). Individual and co-operative roles of lactic acid and hydrogen peroxide in the killing activity of enteric strain *Lactobacillus johnsonii* NCC933 and vaginal strain *Lactobacillus gasseri* KS120.1 against enteric, uropathogenic and vaginosis-associated pathogens. *FEMS Microbiology Letters*, 304, 29-38.
- Aureli, P., Fiore, A., Scalfaro, C., Casale, M., & Franciosa, G. (2010). National survey outcomes on commercial probiotic food supplements in Italy. *International Journal of Food Microbiology*, 137, 265-273.
- Bakari, D., Tatsadjieu, N. L., Mbawala, A., & Mbofung, C. M. (2011). Assessment of physiological properties of some lactic acid bacteria isolated from the intestine of chickens use as probiotics and antimicrobial agents against enteropathogenic bacteria. *Innovative Romanian Food Biotechnology*, 8, 33-40.
- Barbosa T.M., Serra, C. R., La Ragione, R. M., Woodward, M. J., & Henriques, A. O. (2005). Screening for *Bacillus* isolates in the broiler gastrointestinal tract. *Applied and Environmental Microbiology*, 71(2), 968-978.
- Benibo, B., & Farr, A. (1985). The effects of feed and water withdrawal and holding shed treatments on broiler yield parameters. *Poultry Science*, 64, 920-924.
- Berghaus, R. D., Thayer, S. G., Law, B. F., Mild, R. M., Hofacre, C. L., & Singer, R. S. (2013). Enumeration of *Salmonella* and *Campylobacter* in environmental farm samples and processing plant carcass rinses from commercial broiler chicken flocks. *Applied and Environmental Microbiology*, 79(13), 4106-4114.
- Berkhout, N. (2009). Poultry feed acidifiers - global trends. *WorldPoultry*. Retrieved from <http://www.worldpoultry.net/Home/General/2009/12/Report-Poultry-feed-acidifiers---global-trends-WP004610W/>
- Berrang, M. E., & Dickens, J. A. (2000). Presence and level of *Campylobacter* spp. on broiler carcasses throughout the processing plant. *The Journal of Applied Poultry Research*, 9, 43-47.

- Bourassa, D. V., Fletcher, D. L., Buhr, R. J., Berrang, M. E., & Cason, J. A. (2004). Recovery of *Salmonellae* from trisodium phosphate-treated commercially processed broiler carcasses after chilling and after seven-day storage. *Poultry Science*, 83, 2079-2082.
- Buret, A. G. (2010). Immuno-modulation and anti-inflammatory benefits of antibiotics: the example of tilmicosin. *Canadian Journal of Veterinary Research*, 74(1), 1-10.
- Butaye, P., Devriese, L. A., & Haesebrouck, F. (2003). Antimicrobial growth promoters used in animal feed: effects of less well known antibiotics on gram-positive bacteria. *Clinical microbiology reviews*, 16(2), 175–188.
- Byrd, J. A., Hargis, B. M., Caldwell, D. J., Bailey, R. H., Herron, K. L., McReynolds, J. L., Brewer, R. L., Anderson, R. C., Bischoff, K. M., Callaway, T. R., & Kubena, L. F. (2001). Effect of lactic acid administration in the drinking water during preslaughter feed withdrawal on *Salmonella* and *Campylobacter* contamination of broilers. *Poultry Science*, 80, 278-283.
- Cadieux, P. A., Burton, J., Devillard, E., & Reid, G. (2009). *Lactobacillus* by-products inhibit the growth and virulence of uropathogenic *Escherichia coli*. *Journal of Physiology and Pharmacology*, 60(6), 13-18.
- Cai, S., Bay, B. H., Lee, Y. K., Lu, J., & Mahendran, R. (2010). Live and lyophilized *Lactobacillus* species elicit differential immunomodulatory effects on immune cells. *FEMS Microbiology Letters*, 302, 189-196.
- Cartman, S. T., La Ragione, R. M., & Woodward, M. J. (2008). *Bacillus subtilis* spores germinate in the chicken gastrointestinal tract. *Applied and Environmental Microbiology*, 74(16), 5254-5258.
- Castro Gonzalez, M. I., Montano Benavides, S., & Perez-Gil Romo, F. (2001). Fatty acids in sardine canned in tomato sauce from different fishing areas of the Mexican Pacific. *Archivos Latinoamericanos de Nutricion*, 51(4), 400-406.
- Cengiz, O., Koksall, B. H., Tatli, O., Sevim, O., Avci, H., Epikmen, T., Beyaz, D., Buyukyoruk, S., Boyacioglu, M., Uner, A., Onol, A. G. (2012). Influence of dietary organic acid blend supplementation and interaction with delayed feed access after hatch on broiler growth performance and intestinal health. *Veterinari Medicina*, 57(10), 515-528.
- Chaucheyras-Durand, F., & Durand, H. (2010). Probiotics in animal nutrition and health. *Beneficial Microbes*, 1(1), 3-9.
- Chittick, P., Sulka, A., Tauxe, R. V., & Fry, A. M. (2006). A summary of national reports of foodborne outbreaks of *Salmonella* Heidelberg infections in the United States: Clues for disease prevention. *Journal of Food Protection*, 69, 1150-1153.
- Companies and Markets. (2012). Feed acidifiers: A global strategic business report. Ban on animal feed antibiotics throws spotlight on feed acidifiers. Retrieved from



<http://www.companiesandmarkets.com/Market/Chemicals/Market-Research/Feed-Acidifiers-A-Global-Strategic-Business-Report/RPT933926>

- Corrier, D. E., Byrd, J. A., Hargis, B. M., Hume, M. E., Bailey, R. H., & Stanker, L. H. (1999a). Survival of *Salmonella* in the crop contents of market-age broilers during feed withdrawal. *Avian Diseases*, 43, 453-460.
- Corrier, D. E., Byrd, J. A., Hargis, B. M., Hume, M. E., Bailey, R. H., & Stanker, L. H. (1999b). Survival of *Salmonella* in the crop contents of market-age broilers during feed withdrawal. *Avian Diseases*, 43, 453-460.
- Corrier, D. E., Byrd, J. A., Hargis, B. M., Hume, M. E., Bailey, R. H., & Stanker, L. H. (1999c). Presence of *Salmonella* in the crop and ceca of broiler chickens before and after preslaughter feed withdrawal. *Poultry Science*, 78, 45-49.
- Costa, E., Uwiera, R., Kastelic, J. P., Selinger, L. B., & Inglis, G. D. (2011). Non-therapeutic administration of a model antimicrobial growth promoter modulates intestinal immune responses. *Gut pathogens*, 3(1), 1-15.
- Davies, J. M., Sheil, B., & Shanahan, F. (2009). Bacterial signalling overrides cytokine signalling and modifies dendritic cell differentiation. *Immunology*, 128, 805-815.
- Delcenserie, V., Martel, D., Lamoureux, M., Amiot, J., Boutin, Y., & Roy, D. (2008). Immunomodulatory effects of probiotics in the intestinal tract. *Current issues in Molecular Biology*, 10(1-2), 37-54.
- Dibner, J., & Richards, J. (2005). Antibiotic growth promoters in agriculture: history and mode of action. *Poultry Science*, 84(4), 634-643.
- Dicks, L. M. T., & Botes, M. (2011). Probiotic lactic acid bacteria in the gastro-intestinal tract: health benefits, safety and mode of action. *Beneficial Microbes*, 1(1), 11-29.
- Dickson, J. S., Siragusa, G. R., & Wray Jr, J. E. (1992). Predicting the growth of *Salmonella typhimurium* on beef by using the temperature function integration technique. *Applied and Environmental Microbiology*, 58, 3482-3487.
- Drakes, M., Blanchard, T., & Czinn, S. (2004). Bacterial probiotic modulation of dendritic cells. *Infection and Immunity*, 72, 3299-3309.
- Duc le, H., Hong, H. A., Barbosa, T. M., Henriques, A. O., & Cutting, S. M. (2004). Characterization of *Bacillus* probiotics available for human use. *Applied and Environmental Microbiology*, 70(4), 2161-2171.
- Duc, L. H., Hong, H. A., Fairweather, N., Ricca, E., & Cutting, S. M. (2003b). Bacterial spores as vaccine vehicles. *Infection and Immunity*, 71, 2810-2818.

- Duc, L. H., Hong, H. A., & Cutting, S. M. (2003a). Germination of the spore in the gastrointestinal tract provides a novel route for heterologous antigen presentation. *Vaccine*, 21, 4215-4224.
- Ezema, C. (2013). Probiotics in animal production: A review. *Journal of Veterinary Medicine and Animal Health*, 5(11), 308-316.
- Ewaschuk, J. B., & Dieleman, L. A. (2006). Probiotics and prebiotics in chronic inflammatory bowel diseases. *World Journal of Gastroenterology*, 12(37), 5941-5950.
- FAO (2002). Guidelines for the evaluation of probiotics in food. Food and Agriculture Organization of the United Nations. Retrieved from <ftp://ftp.fao.org/es/esn/food/wgreport2.pdf>
- Farnell, M., Donoghue, A., De Los Santos, F. S., Blore, P., Hargis, B. M., Tellez, G., & Donoghue, D. (2006). Upregulation of oxidative burst and degranulation in chicken heterophils stimulated with probiotic bacteria. *Poultry Science*, 85,1900-1906.
- Fayol-Messaoudi, D., Berger, C. N., Coconnier-Polter, M. H., Lievin-Le Moal, V., & Servin, A. L. (2005). pH-, Lactic acid-, and non-lactic acid-dependent activities of probiotic Lactobacilli against *Salmonella enterica* Serovar Typhimurium. *Applied Environmental Microbiology*, 71, 6008-6013.
- Flore, T. N., Francois, Z. N., & Felicite, T. M. (2010). Immune system stimulation in rats by *Lactobacillus* sp. isolates from Raffia wine (*Raphia vinifera*). *Cellular Immunology*, 260, 63-65.
- Foley, S. L., Nayak, R., Hanning, I. B., Johnson, T. J., Han, J., & Ricke, S. C. (2011). Population dynamics of *Salmonella enterica* serotypes in commercial egg and poultry production. *Applied and Environmental Microbiology*, 77(13), 4273-4279.
- Foligne, B., Dewulf, J., Breton, J., Claisse, O., Lonvaud-Funel, A., & Pot, B. (2010). Probiotic properties of non-conventional lactic acid bacteria: immunomodulation by *Oenococcus oeni*. *International Journal of Food Microbiology*, 140,136-145.
- Fontana, L., Brito, M. B., Diaz, J. P., Quezada, S. M., Gil, A. (2013). Sources, isolation, characterisation and evaluation of probiotics. *British Journal of Nutrition*, 109, S35-S50.
- Freitas Neto, O. C., Penha Filho, R. A. C., Barrow, P., & Berchieri Junior, A. (2010). Sources of human non-typhoid salmonellosis: a review. *Brazilian Journal of Poultry Science*, 12(1), 01-11.
- Fritts, C. A., Kersey, J. H., Motl, M. A., Kroger, E. C., Yan, F., Si, J., Jiang, Q., Campos, M. M., Waldroup, A. L., & Waldroup, P. W. (2000). *Bacillus subtilis* C-3102 (Calsporin) improves live performance and microbiological status of broiler chickens. *Journal of Applied Poultry Research*, 9, 149-155.

- Furuse, M., Yang, S. I., Niwa, N., & Okumura, J. (1991). Effect of short chain fatty acids on the performance and intestinal weight in germ-free and conventional chicks. *British Poultry Science*, 32, 159-165.
- Galdeano, C. M., & Perdigon, G. (2004). Role of viability of probiotic strains in their persistence in the gut and in mucosal immune stimulation. *Journal of Applied Microbiology*, 97(4), 673-681.
- Gaskins, H., Collier, C., & Anderson, D. (2002). Antibiotics as growth promotants: mode of action. *Animal Biotechnology*, 13(1), 29-42.
- Gorska, S., Jarzab, A., & Gamian, A. (2009). Probiotic bacteria in the human gastrointestinal tract as a factor stimulating the immune system. *Postępy Higieny i Medycyny Doświadczalnej*, 63, 653-667.
- Ghosh, S., Van Heel, D., & Playford, R. (2004). Probiotics in inflammatory bowel disease: is it all gut flora modulation? *Gut*, 53(5), 620-622.
- Gregory, N. (1994). Preslaughter handling, stunning and slaughter. *Meat Science*, 36, 45-56.
- Hargis, B. M., Caldwell, D. J., Brewer, R. L., Corrier, D. E., & DeLoach, J. R. (1995). Evaluation of the chicken crop as a source of *Salmonella* contamination for broiler carcasses. *Poultry Science*, 74, 1548-1552.
- Harris, K., Miller, M. F., Loneragan, G. H., & Brashears, M. M. (2006). Validation of the use of organic acids and acidified sodium chlorite to reduce *Escherichia coli* O157 and *Salmonella* typhimurium in beef trim and ground beef in a simulated processing environment. *Journal of Food Protection*, 69, 1802-1807.
- Hart, A. L., Lammers, K., Brigidi, P., Vitali, B., Rizzello, F., Gionchetti, P., Campieri, M., Kamm, M. A., Knight, S. C., & Stagg, A. J. (2004). Modulation of human dendritic cell phenotype and function by probiotic bacteria. *Gut*, 53, 1602-1609.
- Higgins, J., Higgins, S., Vicente, J., Wolfenden, A., Tellez, G., & Hargis, B. M. (2007). Temporal effects of lactic acid bacteria probiotic culture on *Salmonella* in neonatal broilers. *Poultry Science*, 86, 1662-1666.
- Higgins, S. E., Higgins, J. P., Wolfenden, A. D., Henderson, S. N., Torres-Rodriguez, A., Tellez, G., & Hargis, B. M. (2008). Evaluation of a lactobacillus-based probiotic culture for the reduction of *Salmonella* Enteritidis in neonatal broiler chicks. *Poultry Science*, 87, 27-31.
- Hinton Jr, A., & Ingram, K. D. (2005). Microbicidal activity of tripotassium phosphate and fatty acids toward spoilage and pathogenic bacteria associated with poultry. *Journal of Food Protection*, 68, 1462-1466.
- Hinton Jr, A., Northcutt, J. K., Smith, D. P., Musgrove, M. T., & Ingram, K. D. (2007). Spoilage microflora of broiler carcasses washed with electrolyzed oxidizing or chlorinated water using an inside-outside bird washer. *Poultry Science*, 86, 123-127.

- Hinton Jr, A., Buhr, R. J., & Ingram, K. D. (2000). Reduction of *Salmonella* in the crop of broiler chickens subjected to feed withdrawal. *Poultry Science*, 79, 1566-1570.
- Hoa, N. T., Baccigalupi, L., Huxham, A., Smertenko, A., Van, P. H., Ammendola, S., Ricca, E., & Cutting, S. M. (2000). Characterization of *Bacillus* species used for oral bacteriotherapy and bacterioprophyllaxis of gastrointestinal disorders. *Applied and Environmental Microbiology*, 66, 5241-5247.
- Hong, H. A., Huang, J. M., Khaneja, R., Hiep, L. V., Urdaci, M. C., & Cutting, S. M. (2008). The safety of *Bacillus subtilis* and *Bacillus indicus* as food probiotics. *Journal of Applied Microbiology*, 105(2), 510-520.
- Hong, H. A., Duc, L. H., & Cutting, S. M. (2005). The use of bacterial spores as probiotics. *FEMS Microbiology Reviews*, 29, 813-835.
- Hori, T. (2010). Probiotics recent human studies using *Lactobacillus casei* strain Shirota. In Cho, S.S., Finocchiaro. Handbook of prebiotics and probiotics ingredients: health benefits and food applications. CRC Press.
- Hsiao, C., & Siebert, K. J. (1999). Modeling the inhibitory effects of organic acids on bacteria. *International Journal of Food Microbiology*, 47, 189-201.
- Hua, M. C., Lin, T. Y., Lai, M. W., Kong, M. S., Chang, H. J., & Chen, C. C. (2010). Probiotic Bio-Three induces Th1 and anti-inflammatory effects in PBMC and dendritic cells. *World Journal of Gastroenterology*, 16(28), 3529-3540.
- Huyghebaert, G., Ducatelle, R., & Immerseel, F. V. (2011). An update on alternatives to antimicrobial growth promoters for broilers. *The Veterinary Journal*, 187(2), 182-188.
- Imaoka, A., Shima, T., Kato, K., Mizuno, S., Uehara, T., Matsumoto, S., Setoyama, H., Hara T., & Umesaki, Y. (2008). Anti-inflammatory activity of probiotic *Bifidobacterium*: enhancement of IL-10 production in peripheral blood mononuclear cells from ulcerative colitis patients and inhibition of IL-8 secretion in HT-29 cells. *World journal of gastroenterology*, 14(16), 2511-2516.
- Im, E., Choi, Y. J., Pothoulakis, C., & Rhee, S. H. (2009). *Bacillus polyfermenticus* ameliorates colonic inflammation by promoting cytoprotective effects in colitic mice. *The Journal of Nutrition*, 139(10), 1848-1854.
- Islam, K. M. S. (2012) Use of citric acid in broiler diets. *World's Poultry Science Journal*, 68, 104-118.
- Isolauri, E., Kirjavainen, P., & Salminen, S. (2002). Probiotics: a role in the treatment of intestinal infection and inflammation? *Gut*, 50(3), iii54–iii59.
- Isolauri, E., Sütas, Y., Kankaanpää, P., Arvilommi, H., & Salminen, S. (2001). Probiotics: effects on immunity. *The American Journal of Clinical Nutrition*, 73(2), 444s-450s.

- Jarquín, R. L., Nava, G. M., Wolfenden, A. D., Donoghue, A. M., Hanning, I., Higgins, S. E., & Hargis, B. M. (2007). The evaluation of organic acids and probiotic cultures to reduce *Salmonella* Enteritidis horizontal transmission and crop infection in broiler chickens. *International Journal of Poultry Science*, 6(3), 182-186.
- Jetton, J. P., Bilgili, S. F., Conner, D. E., Kotrola, J. S., & Reiber, M. A. (1992). Recovery of salmonellae from chilled broiler carcasses as affected by rinse media and enumeration method. *Journal of Food Protection*, 55, 329-332.
- Jijon, H., Backer, J., Diaz, H., Yeung, H., Thiel, D., McKaigney, C., De Simone, C., & Madsen, K. (2004). DNA from probiotic bacteria modulates murine and human epithelial and immune function. *Gastroenterology*, 126, 1358-1373.
- Jin, L. Z., Ho, Y. W., Ali, M. A., Abdullah, N., & Jalaludin, S. (1996). Effect of adherent *Lactobacillus* spp. on in vitro adherence of salmonellae to the intestinal epithelial cells of chicken. *Journal of Applied Bacteriology*, 81, 201-206.
- Knap, I., Kehlet, A. B., Bennedsen, M., Mathis, G. F., Hofacre, C. L., Lumpkins, B. S., Jensen, M. M., Raun, M., & Lay, A. (2011). *Bacillus subtilis* (DSM17299) significantly reduces *Salmonella* in broilers. *Poultry Science*, 90(8), 1690-1694.
- Koyuncu, S., Andersson, M. G., Löfström, C., Skandamis, P. N., Gounadaki, A., Zentek, J., & Häggblom, P. (2013). Organic acids for control of *Salmonella* in different feed materials. *BMC Veterinary Research*, 9(81), 1-9.
- Kubena, L. F., Byrd, J. A., Young, C. R., & Corrier, D. E. (2001). Effects of tannic acid on cecal volatile fatty acids and susceptibility to *Salmonella* Typhimurium colonization in broiler chicks. *Poultry Science*, 80, 1293-1298.
- La Ragione, R. M., Casula, G., Cutting, S. M., & Woodward, M. J. (2001). *Bacillus subtilis* spores competitively exclude *Escherichia coli* O78:K80 in poultry. *Veterinary Microbiology*, 79, 133-142.
- La Ragione, R. M., & Woodward, M. J. (2003). Competitive exclusion by *Bacillus subtilis* spores of *Salmonella enterica* serotype Enteritidis and *Clostridium perfringens* in young chickens. *Veterinary Microbiology*, 94, 245-256.
- Laudanno, O., Vasconcelos, L., Catalana, J., & Cesolari, J. (2006). Anti-inflammatory effect of bioflora probiotic administered orally or subcutaneously with live or dead bacteria. *Digestive Diseases and Science*, 51, 2180-2183.
- Laury, A. M., Alvarado, M. V., Nace, G., Alvarado, C. Z., Brooks, J. C., Echeverry, A., & Brashears, M. M. (2009). Validation of a lactic acid- and citric acid-based antimicrobial product for the reduction of *Escherichia coli* O157: H7 and *Salmonella* on beef tips and whole chicken carcasses. *Journal of Food Protection*, 72, 2208-2211.

- Lee, N. K., Park, J. S., Park, E., & Paik, H. D. (2007). Adherence and anticarcinogenic effects of *Bacillus polyfermenticus* SCD in the large intestine, *Letters in Applied Microbiology*, 44(3), 274-278.
- Leeson, S., Namkung, H., Antongiovanni, M., & Lee, E. H. (2005). Effect of butyric acid on the performance and carcass yield of broiler chickens. *Poultry Science*, 84, 1418-1422.
- Leser, T. D., Knarreborg, A., & Worm, V. (2008). Germination and outgrowth of *Bacillus subtilis* and *Bacillus licheniformis* spores in the gastrointestinal tract of pigs. *Journal of Applied Microbiology*, 104, 1025-1033.
- Lin, J., Hunkapiller, A. A., Layton, A. C., Chang, Y.-J., & Robbins, K. R. (2013). Response of intestinal microbiota to antibiotic growth promoters in chickens. *Foodborne Pathogens and Disease*, 10(4), 331-337.
- Ljungh, A., & Wadström, T. (2006). Lactic acid bacteria as probiotics. *Current Issues in Intestinal Microbiology*, 7(2), 73-89.
- Lutful Kabir, S. M. (2010). Avian colibacillosis and salmonellosis: a closer look at epidemiology, pathogenesis, diagnosis, control and public health concerns. *International Journal of Environmental Research and Public Health*, 7(1), 89-114.
- Lutful Kabir, S. M. (2009). The role of probiotics in the poultry industry. *International Journal of Molecular Sciences*, 10(8), 3531-3546.
- Lu, Z., Sebranek, J. G., Dickson, J. S., Mendonca, A. F., & Bailey, T. B. (2005). Application of predictive models to estimate *Listeria monocytogenes* growth on frankfurters treated with organic acid salts. *Journal of Food Protection*, 68, 2326-2332.
- Lynch, M., Painter, J., Woodruff, R., Braden, C., & Centers for Disease Control and Prevention. (2006). Surveillance for foodborne-disease outbreaks-United States, 1998-2002. *Morbidity and Mortality Weekly Report Surveillance Summaries*, 55, 1-42.
- Makras, L., Triantafyllou, V., Fayol-Messaoudi, D., Adriany, T., Zoumpopoulou, G., Tsakalidou, E., Servin, A., & De Vuyst, L. (2006). Kinetic analysis of the antibacterial activity of probiotic lactobacilli towards *Salmonella enterica* serovar Typhimurium reveals a role for lactic acid and other inhibitory compounds. *Research in Microbiology*, 157, 241-247.
- Malkov, S. V., Markelov, V. V., Polozov, G. Y., Barabanschikov, B. I., Kozhevnikov, A. Y., & Trushin M. V. (2006). Significant delay of lethal outcome in cancer patients due to peroral administration of *Bacillus oligonitrophilus* KU-1. *Scientific World Journal*, 8(6), 2177-2187.
- Mani-López, E., García, H. S., & López-Malo, A. (2012) Organic acids as antimicrobials to control *Salmonella* in meat and poultry products. *Food Research International*, 45, 713-721.

- Martin, R., & Suarez, J. E. (2010). Biosynthesis and degradation of H<sub>2</sub>O<sub>2</sub> by vaginal lactobacilli. *Applied and Environmental Microbiology*, 76, 400-405.
- Mead, G., Lammerding, A., Cox, N., Doyle, M. P., Humbert, F., Kulikovskiy, A., Panin, A., Pinheiro do Nascimento, V., Wierup, M., & the *Salmonella* on Raw Poultry Writing Committee. (2010). Scientific and technical factors affecting the setting of *Salmonella* criteria for raw poultry: a global perspective. *Journal of Food Protection*, 73(8), 1566-1590.
- Menconi A., Reginatto A. R., Londero A., Pumford N. R., Morgan M., Hargis B. M., & Tellez, G. (2013) Effect of organic acids on *Salmonella* Typhimurium infection in broiler chickens. *International Journal of Poultry Science*, 12, 72-75.
- Mikolajczyk, A., & Radkowski, M. (2002). *Salmonella* spp. on chicken carcasses in processing plants in Poland. *Journal of Food Protection*, 65, 1475-1479.
- MMWR (2011). Morbidity and Mortality Weekly Report, 60(22). Retrieved from <http://www.cdc.gov/mmwr>
- Mountney, G. J., & O'malley, J. (1965). Acids as poultry meat preservatives. *Poultry Science*, 44, 582-586.
- Mountzouris, K. C., Balaskas, C., Xanthakos, I., Tzivinikou, A., & Fegeros, K. (2009). Effects of a multi-species probiotic on biomarkers of competitive exclusion efficacy in broilers challenged with *Salmonella* Enteritidis. *British Poultry Science*, 50(4), 467-478.
- Nayak, S. K. (2010). Probiotics and immunity: a fish perspective. *Fish and Shellfish Immunology*, 29, 2-14.
- Ng, S. C., Hart, A. L., Kamm, M. A., Stagg, A. J., & Knight, S. C. (2009). Mechanisms of action of probiotics: recent advances. *Inflammatory Bowel Diseases*, 15, 300-310.
- Nicholson, W.L. (2002). Roles of *Bacillus* endospores in the environment. *Cellular and Molecular Life Sciences*, 59, 410-416.
- Niewold, T. (2007). The nonantibiotic anti-inflammatory effect of antimicrobial growth promoters, the real mode of action? A hypothesis. *Poultry Science*, 86(4), 605-609.
- Northcutt, J. K., Smith, D. P., Musgrove, M. T., Ingram, K. D., & Hinton Jr., A. (2005). Microbiological impact of spray washing broiler carcasses using different chlorine concentrations and water temperatures. *Poultry Science*, 84, 1648-1652.
- Northcutt, J. K., Smith, D., Huezo, R. I., & Ingram, K. D. (2008). Microbiology of broiler carcasses and chemistry of chiller water as affected by water reuse. *Poultry Science*, 87, 1458-1463.
- Northcutt, J., Berrang, M., Dickens, J., Fletcher, D., & Cox, N. (2003). Effect of broiler age, feed withdrawal, and transportation on levels of coliforms, *Campylobacter*, *Escherichia coli*

- and *Salmonella* on carcasses before and after immersion chilling. *Poultry Science*, 82, 169-173.
- O'Dea, E. E., Fasenko, G. M., Allison, G. E., Korver, D. R., Tannock, G. W., & Guan, L. L. (2006). Investigating the effects of commercial probiotics on broiler chick quality and production efficiency. *Poultry Science*, 85, 1855-1863.
- O'Mahony, L., Feeney, M., O'Halloran, S., Murphy, L., Kiely, B., Fitzgibbon, J., Lee, G., O'Sullivan, G., Shanahan, F., Collins, J. K. (2011). Probiotic impact on microbial flora, inflammation and tumour development in IL-10 knockout mice. *Alimentary Pharmacology & Therapeutics*, 15(8), 1219-1225.
- Okada, Y., Tsuzuki, Y., Hokari, R., Komoto, S., Kurihara, C., Kawaguchi, A., Nagao, S., & Miura, S. (2009). Anti-inflammatory effects of the genus *Bifidobacterium* on macrophages by modification of phospho-I kappaB and SOCS gene expression. *International Journal of Experimental Pathology*, 90(2), 131-140.
- Osipova, I. G., Makhailova, N. A., Sorokulova, I. B., Vasil'eva, E. A., & Gaiderov, A. A. (2003). Spore probiotics. [Sporovye probiotiki]. *Zhurnal Mikrobiologii, Epidemiologii, i Immunobiologii*, 3(3), 113-119.
- Ouweland, A. C., Salminen, S., & Isolauri, E. (2002). Probiotics: An overview of beneficial effects. *Antonie van Leeuwenhoek*, 82, 279-289.
- Pagnini, C., Saeed, R., Bamias, G., Arseneau, K. O., Pizarro, T. T., & Cominelli, F. (2010). Probiotics promote gut health through stimulation of epithelial innate immunity. *Proceedings of the National Academy of Sciences*, 107(1), 454-459.
- Park, E., Jeon, G. I., Park, J. S., & Paik, H. D. (2007). A probiotic strain of *Bacillus polyfermenticus* reduces DMH induced precancerous lesions in F344 male rat. *Biological & Pharmaceutical Bulletin*, 30(3), 569-574.
- Parveen, S., M. Taabodi, J. G. Schwarz, T. P. Oscar, J. Harter-Dennis, and D. G. White. (2007). Prevalence and antimicrobial resistance of *Salmonella* recovered from processed poultry. *Journal of Food Protection*, 70, 2466-2472.
- Patchanee, P., Zewde, B. M., Tadesse, D. A., Hoet, A., & Gebreyes, W. A. (2008). Characterization of multidrug-resistant *Salmonella enterica* serovar Heidelberg isolated from humans and animals. *Foodborne Pathogens and Diseases*, 5(6), 839-851.
- Patterson, J. A., & Burkholder, K. M. (2003). Application of prebiotics and probiotics in poultry production. *Poultry Science*, 82(4), 627-631.
- Permpoonpattana P., Hong, H. A., Khaneja, R., & Cutting, S. M. (2012). Evaluation of *Bacillus subtilis* strains as probiotics and their potential as a food ingredient. *Beneficial microbes*, 3(2), 127-135.



- Petracci, M., Bianchi, M., Cavani, C., Gaspari, P., & Lavazza, A. (2006). Preslaughter mortality in broiler chickens, turkeys, and spent hens under commercial slaughtering. *Poultry Science*, 85, 1660-1664.
- Pixley, C., Barton, J., Vicente, J., Wolfenden, A., Hargis, B., & Tellez, G. (2010). Evaluation of a commercially available organic acid product during feed withdrawal and its relation to carcass shrink in commercial turkeys. *International Journal of Poultry Science*, 9, 508-510.
- Portilla-Rivera, O., Torrado, A., Dominguez, J. M., & Moldes, A. B. (2008). Stability and emulsifying capacity of biosurfactants obtained from lignocellulosic sources using *Lactobacillus pentosus*. *Journal of Agriculture and Food Chemistry*, 5, 8074-8080.
- Rahimi, S., Grimes, J. L., Fletcher, O., Oviedo, E., & Sheldon, B. W. (2009). Effect of a direct-fed microbial (primalac) on structure and ultrastructure of small intestine in turkey poults. *Poultry Science*, 88(3), 491-503.
- Ramirez, G. A., Sarlin, L. L., Caldwell, D. J., Yezak Jr, C. R., Hume, M. E., Corrier, D. E., Deloach, J. R., & Hargis, B. M. (1997). Effect of feed withdrawal on the incidence of *Salmonella* in the crops and ceca of market age broiler chickens. *Poultry Science*, 76, 654-656.
- Reid, G., Heinemann, C., Velraeds, M., Van der Mei, H. C., & Busscher, H. J. (1999). Biosurfactants produced by *Lactobacillus*. *Methods in Enzymology*, 310, 426-433.
- Reid, G. (2001). Probiotic agents to protect the urogenital tract against infection. *American Journal of Clinical Nutrition*, 73, 437S-443S.
- Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., Jones, J. L., & Griffin, P. M. (2011). Foodborne illness acquired in the United States-major pathogens. *Emerging Infectious Disease*, 17, 7-15.
- Shadnoush, M., Shaker Hosseini, R., Mehrabi, Y., Delpisheh, A., Alipoor, E., Faghfoori, Z., Mohammadpour, N., et al. (2013). Probiotic yogurt affects pro-and anti-inflammatory factors in patients with inflammatory bowel disease. *Iranian Journal of Pharmaceutical Research*, 12(4), 929-936.
- Shida, K., Kiyoshima-Shibata, J., Kaji, R., Nagaoka, M., & Nanno, M. (2009). Peptidoglycan from lactobacilli inhibits interleukin-12 production by macrophages induced by *Lactobacillus casei* through Toll-like receptor 2-dependent and independent mechanisms. *Immunology*, 128, 858-869.
- Shivaramaiah, S., Pumford, N., Morgan, M., Wolfenden, R., Wolfenden, A., Torres-Rodriguez, A., Hargis, B., & Tellez, G. (2011). Evaluation of *Bacillus* species as potential candidates for direct-fed microbials in commercial poultry. *Poultry Science*, 90, 1574-1580.
- Sjogren, Y. M., Tomicic, S., Lundberg, A., Bottcher, M. F., Bjorksten, B., Sverremark-Ekstrom, E., & Jenmalm, M. C. (2009). Influence of early gut microbiota on the maturation of

- childhood mucosal and systemic immune responses. *Clinical & Experimental Allergy*, 39, 1842-1851.
- Socol, C. R., Vandenberghe, L. P. de S., Spier, M. R., Medeiros, A. B. P., Yamaguishi, C. T., Lindner, J. D. D., Pandey, A., et al. (2010). The potential of probiotics: a review. *Food Technology and Biotechnology*, 48(4), 413-434.
- Starovoitova, S. O., Tymoshok, N. O., Horchakov, V. I., & Spivak, M. I. (2009). Immunomodulation characteristics of *Lactobacillus* genus bacteria. *Mikrobiol. Z*, 71, 41-47.
- Steidler, L., Hans, W., Schotte, L., Neiryneck, S., Obermeier, F., Falk, W., Fiers, W., et al. (2000). Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. *Science*, 289(5483), 1352-1355.
- Su, L. H., & Chiu, C. H. (2007). *Salmonella*: clinical importance and evolution of nomenclature. *Chang Gung Medical Journal*, 30, 210-219.
- Taheri, H. R., Moravej, H., Tabandeh, F., Zaghari, M., & Shivazad, M. (2009). Screening of lactic acid bacteria toward their selection as a source of chicken probiotic. *Poultry Science*, 88(8), 1586-1593.
- Tam, N. K. M., Uyen, N. Q., Hong, H. A., Duc, L. H., Hoa, T. T., Serra, C. R., Henriques, A. O., & Cutting, S. M. (2006). The intestinal life cycle of *Bacillus subtilis* and close relatives. *Journal of Bacteriology*, 188, 2692-2700.
- Tellez, G., Pixley, C., Wolfenden, R. E., Layton, S. L., & Hargis, B. M. (2012). Probiotics/direct fed microbials for *Salmonella* control in poultry. *Food Research International*, 45, 628-633.
- Teo, A. Y. L., & Tan, H. M. (2005). Inhibition of *Clostridium perfringens* by a novel strain of *Bacillus subtilis* isolated from the gastrointestinal tracts of healthy chickens. *Applied and Environmental Microbiology*, 71, 4185-4190.
- Thomas, S., Przesdzing, I., Metzke, D., Schmitz, J., Radbruch, A., & Baumgart, D. C. (2009). *Saccharomyces boulardii* inhibits lipopolysaccharide-induced activation of human dendritic cells and T cell proliferation. *Clinical & Experimental Immunology*, 156, 78-87.
- Thompson, J. L., & Hinton, M. (1997) Antibacterial activity of formic and propionic acids in the diet of hens on salmonellas in the crop. *British Poultry Science*, 38, 59-65.
- Tung, C. M., & Pettigrew, J. E. (2011). Critical review of acidifiers. Practical ideas to address high feed and production costs. National Pork board web. Board, Des Moines, IA USA. Retrieved from <http://www.pork.org/ResearchDetail/541/CriticalReviewofAcid.aspx>
- Tsai, C. C., Hsih, H. Y., Chiu, H. H., Lai, Y. Y., Liu, J. H., Yu, B., & Tsen, H. Y. (2005). Antagonistic activity against *Salmonella* infection *in vitro* and *in vivo* for two

- Lactobacillus* strains from swine and poultry. *International Journal of Food Microbiology*, 102, 185-194.
- Tsai, Y. T., Cheng, P. C., Liao, J. W., & Pan, T. M. (2010). Effect of the administration of *Lactobacillus paracasei* subsp. *paracasei* NTU 101 on Peyer's patch-mediated mucosal immunity. *International Immunopharmacology*, 10(7), 791-798.
- Urdaci, M.C., Bressollier, P., & Pinchuk, I. (2004). *Bacillus clausii* probiotic strains: antimicrobial and immunomodulatory activities. *Journal of Clinical Gastroenterology*, 38(6) S86-90.
- Van Coillie, E., Goris, J., Cleenwerck, I., Grijspeerdt, K., Botteldoorn, N., Van Immerseel, F., De Buck, J., Vancanneyt, M., Swings, J., Herman, L., & Heyndrickx, M. (2007). Identification of lactobacilli isolated from the cloaca and vagina of laying hens and characterization for potential use as probiotics to control *Salmonella* Enteritidis. *Journal of Applied Microbiology*, 102(4), 1095-1106.
- Van Immerseel, F., Russell, J. B., Flythe, M. D., Gantois, I., Timbermont, L., Pasmans, F., Haesebrouck, F., & Ducatelle, R. (2006). The use of organic acids to combat *Salmonella* in poultry: a mechanistic explanation of the efficacy. *Avian Pathology*, 35, 182-188.
- Vandeplass, S., Dubois Dauphin, R., Beckers, Y., Thonart, P., & Thewis, A. (2010). *Salmonella* in chicken: current and developing strategies to reduce contamination at farm level. *Journal of Food Protection*, 73, 774-785.
- Vasseur, C., Baverel, L., Hebraud, M., & Labadie, J. (1999). Effect of osmotic, alkaline, acid or thermal stresses on the growth and inhibition of *Listeria monocytogenes*. *Journal of Applied Microbiology*, 86, 469-476.
- Veerkamp, C. (1986). Fasting and yield of broilers. *Poultry Science*, 65, 1299-1304.
- Vicente, J. L., Torres-Rodriguez, A., Higgins, S. E., Pixley, C., Tellez, G., Donoghue, A. M., & Hargis, B. M. (2008). Effect of a selected *Lactobacillus* spp.-based probiotic on *Salmonella enterica* serovar Enteritidis-infected broiler chicks. *Avian Diseases*, 52, 143-146.
- Vicente, J., Higgins, S., Bielke, L., Tellez, G., Donoghue, D., Donoghue, A., & Hargis, B. M. (2007a). Effect of probiotic culture candidates on *Salmonella* prevalence in commercial turkey houses. *Journal of Applied of Poultry Research*, 16, 471-476.
- Vicente, J. L., Aviña, L., Torres-Rodriguez, A., Hargis, B., & Tellez, G. (2007b). Effect of a *Lactobacillus* spp.-based probiotic culture product on broiler chicks performance under commercial conditions. *International Journal of Poultry Science*, 6 (3), 154-156.
- Vila, B., Fontgibell, A., Badiola, I., Esteve-Garcia, E., Jimenez, G., Castillo, M., & Brufau, J. (2009). Reduction of *Salmonella enterica* var. Enteritidis colonization and invasion by *Bacillus cereus* var. toyoi inclusion in poultry feeds. *Poultry Science*, 88(5), 975-979.

- Vila, B., Esteve-Garcia, E., & Brufau, J. (2010). Probiotic micro-organisms: 100 years of innovation and efficacy; modes of action. *World's Poultry Science Journal*, 65, 369-380.
- Voetsch, A. C., Angulo, F. J., Rabatsky-Her, T., Shallow, S., Cassidy, M., Thomas, S. M., Swanson, E., Zansky, S. M., Hawkins, M. A., Jones, T. F., Shillam, P. J., Van Gilder, T. J., Wells, J. G., Griffin, P. M., & Emerging Infections Program FoodNet Working Group. (2004a). Laboratory practices for stool-specimen culture for bacterial pathogens, including *Escherichia coli* O157:H7, in the FoodNet sites, 1995-2000. *Clinical Infectious Disease*, 38(3), S190-197.
- Voetsch, A. C., Van Gilder, T. J., Angulo, F. J., Farley, M. M., Shallow, S., Marcus, R., Cieslak, P. R., Deneen, V. C., Tauxe, R. V., & Emerging Infections Program FoodNet Working Group. (2004b). FoodNet estimate of the burden of illness caused by nontyphoidal *Salmonella* infections in the United States. *Clinical Infectious Disease*, 38(3), S127-34.
- Walencka, E., Rozalska, S., Sadowska, B., & Rozalska, B. (2008). The influence of *Lactobacillus acidophilus*-derived surfactants on staphylococcal adhesion and biofilm formation. *Folia Microbiologica*, 53, 61-66.
- Williams, P. (2007). *Bacillus subtilis*: A shocking message from a probiotic. *Cell Host & Microbe*, 1(4), 248-249.
- Wolf, G., Strahl, A., Meisel, J., & Hammes, W. P. (1991). Heme-dependent catalase activity of lactobacilli. *International Journal of Food Microbiology*, 12, 133-140.
- Wolf, G., Arendt, E. K., Pfahler, U., & Hammes, W. P. (1990). Heme-dependent and heme-independent nitrite reduction by lactic acid bacteria results in different N-containing products. *International Journal of Food Microbiology*, 10, 323-329.
- Wolfenden, A., Pixley, C., Higgins, J., Higgins, S., Vicente, J., Torres-Rodriguez, A., Hargis, B., & Tellez, G. (2007b). Evaluation of spray application of a *Lactobacillus*-based probiotic on *Salmonella* enteritidis colonization in broiler chickens. *International Journal of Poultry Science*, 6, 493-496.
- Wolfenden, A., Vicente, J., Higgins, J., Andreatti Filho, R., Higgins, S., Hargis, B. M., & Tellez, G. (2007c). Effect of organic acids and probiotics on *Salmonella* enteritidis infection in broiler chickens *International Journal of Poultry Science*, 6, 403-405.
- Wolfenden, A., Pixley, C., Vicente, J. L., Aviña, L., Hargis, B. M., & Tellez, G. (2007a) Effect of organic acids during feed withdrawal on mortality, shrinkage and carcass condemnation during transportation to the processing plant. *International Journal of Poultry Science*, 6, 497-500.
- Wolfenden, R., Pumford, N., Morgan, M., Shivaramaiah, S., Wolfenden, A., Pixley, C., Green, J., Tellez, G., & Hargis, B. (2011). Evaluation of selected direct-fed microbial candidates on live performance and *Salmonella* reduction in commercial turkey brooding houses. *Poultry Science*, 90, 2627-2631.

- Wolfenden, R., Pumford, N., Morgan, M., Shivaramaiah, S., Wolfenden, A., Tellez, G., & Hargis, B. (2010). Evaluation of a screening and selection method for *Bacillus* isolates for use as effective direct-fed microbials in commercial poultry. *International Journal of Poultry Science*, 9, 317-323.
- Wolken, W. A., Tramper, J., & van der Werf, M. J. (2003). What can spores do for us? *Trends in Biotechnology*, 21(8), 338-345.
- Yan, F., Cao, H., Cover, T. L., Washington, M. K., Shi, Y., Liu, L., Chaturvedi, R., Peek Jr R. M., Wilson K. T., & Polk, D. B. (2011). Colon-specific delivery of a probiotic-derived soluble protein ameliorates intestinal inflammation in mice through an EGFR-dependent mechanism. *The Journal of Clinical Investigation*, 121(6), 2242-2253.
- Yang, Z., Li, Y., & Slavik, M. (1998). Use of antimicrobial spray applied with an inside-outside birdwasher to reduce bacterial contamination on prechilled chicken carcasses. *Journal of Food Protection*, 61, 829-832.
- Yi, G., Allee, G., Knight, C., & Dibner, J. (2005). Impact of glutamine and oasis hatchling supplement on growth performance, small intestinal morphology, and immune response of broilers vaccinated and challenged with *Eimeria maxima*. *Poultry Science*, 84, 283-293.
- Zagato, E., Mileti, E., Massimiliano, L., Fasano, F., Budelli, A., Penna, G., & Rescigno, M. (2014). *Lactobacillus paracasei* CBA L74 metabolic products and fermented milk for infant formula have anti-inflammatory activity on dendritic cells *in vitro* and protective effects against colitis and an enteric pathogen *in vivo*. *Public Library of Science (PloS One)*, 9(2), e87615.
- Zani, J. L., da Cruz, F. W., dos Santos, A. F., & Gil-Turnes, C. (1998). Effect of probiotic CenBiot on the control of diarrhoea and feed efficiency in pigs. *Journal of Applied Microbiology*, 84, 68-71.
- Zhao, T., Zhao, P., & Doyle, M. P. (2009). Inactivation of *Salmonella* and *Escherichia coli* O157:H7 on lettuce and poultry skin by combinations of levulinic acid and sodium dodecyl sulfate. *Journal of Food Protection*, 72, 928-936.

### III. CHAPTER I

#### **Effect of Organic Acids on *Salmonella* Typhimurium Infection in Broiler Chickens.**

A. Menconi<sup>1</sup>, A. R. Reginatto<sup>2</sup>, A. Londero<sup>2</sup>, N. R. Pumford<sup>1</sup>, M. Morgan<sup>1</sup>, B.M. Hargis<sup>1</sup>, and G. Tellez<sup>1</sup>

<sup>1</sup>Department of Poultry Science, University of Arkansas, Fayetteville AR 72701

<sup>2</sup>Depto. de Medicina Veterinária – CCR Universidade Federal de Santa Maria, Brazil

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## **ABSTRACT**

An alternative to antibiotics is the use of certain organic acids for routinely encountered pathogens in the poultry industry. Direct acidification of drinking water with organic acids could significantly reduce the amount of recoverable *Salmonella* Typhimurium (ST) from the crop and cecal tonsils when used during the pre-slaughter feed withdrawal period. In the present study, *in vitro* and *in vivo* evaluations were conducted to compare a commercially available water acidifier (Optimizer<sup>®</sup>), *versus* two formulations of organic acid mix (OAM), made up of acetic, citric and propionic acids at a final concentration of either 0.031% or 0.062%, to reduce *Salmonella* Typhimurium in the crop and cecal tonsils of broiler chicks during a 24 h period. The two OAM showed better *in vitro* activity to reduce *Salmonella* when compared to control. *In vivo*, the OAM (0.062%) had a similar effect as Optimizer<sup>®</sup> showing a significant reduction in total number of ST positive cecal tonsils, and reducing the number of ST in the crop when compared with controls ( $P < 0.05$ ). All treatments reduced the number of ST recovered from crop contents at 24 h. This new formulation of OAM has great potential as a crop sanitizer and will be further evaluated under conditions similar to commercial chickens.

**Key words:** *Salmonella*, Organic Acid, Chickens

## INTRODUCTION

*Salmonella enterica* causes an estimated 1.4 million cases of foodborne illnesses annually in the United States, resulting in over 15,000 hospitalizations (Voetsch et al., 2004a, 2004b). Poultry and poultry products have been identified by some researchers as the most important source of transmission of *Salmonella* to the human population (Lynch et al., 2006). Increased pressure by consumers and regulatory agencies for reduced or even elimination of the use of antibiotics in food producing animals has created a need to find alternatives to maintain healthy and productive animals. These pressures are a challenge for the poultry industry for controlling *Salmonella* not only at the farm level, but also within processing and manufacturing plants (Hargis et al., 1995; Corrier et al., 1999a; Hinton et al., 2000; Mikolajczyk & Radkowski, 2002).

An alternative to antibiotics is the use of certain organic acids. Direct acidification of the water with organic acids could significantly reduce the amount of recoverable *Salmonella* on the carcasses or in the crops and cecal tonsils when used during the pre-slaughter feed withdrawal period (Van Immerseel et al., 2006; Alali et al., 2010; Vandeplas et al., 2010); however, previous research has suggested that administration of OA during the pre-slaughter feed withdrawal period could lead to carcass shrinkage (Byrd et al., 2001). While this evidence was shown when using lactic acid alone, Optimizer<sup>®</sup> was developed as a combination of organic acids used in combination at low individual concentrations so that water consumption was not discouraged (Jarquin et al., 2007; Wolfenden et al., 2007; Vicente et al., 2007a, 2007b, 2007c). Organic acids are a readily available energy source for both the chicken and the bacteria. Therefore, it is important that the organic acids be administered in high enough concentrations to be bactericidal in the presence of organic matter, and low enough to be voluntarily consumed by the birds.



In the present study, we compared a commercially available water acidifier (Optimizer<sup>®</sup>, Pacific Vet Group, Fayetteville, AR 72703), *versus* a new formulation of organic acid mix (OAM) to reduce *Salmonella* Typhimurium in the crop and cecal tonsils of broiler chicks.

## **MATERIALS AND METHODS**

### ***Salmonella Amplification***

A primary poultry isolate of *Salmonella* Typhimurium (ST) was used in these experiments. This isolate was selected for resistance to nalidixic acid (NA; Catalog No. N-4382, Sigma, St. Louis, MO 63178 USA). For these experiments, ST was grown in tryptic soy broth (TSB; Catalog No. N-4382, Sigma, St. Louis, MO 63178 USA) for approximately 8 h. The cells were washed three times with 0.9% sterile saline by centrifugation (3,000 x g), and the approximate concentration of the stock solution was determined spectrophotometrically at 625 nm. The stock solution was serially diluted and confirmed by colony counts of three replicate samples (0.1 mL/replicate) that were spread plated on brilliant green agar (BGA; Catalog No. N-4382, Sigma, St. Louis, MO 63178 USA) plates containing 25 µg/mL novobiocin (NO; Catalog No. N-1628, Sigma, St. Louis, MO 63178 USA) and 20 µg/mL nalidixic acid (NA). The colony-forming units of *Salmonella* determined by spread plating were reported as the concentration of *Salmonella* (in cfu/mL) for *in vitro* experiments and total colony-forming units for *in vivo* challenge experiments.

### ***Experimental Design - in vitro crop assay***

An assay previously described (Barnhart et al., 1999) was used with modifications. Briefly, 1.25 g of unmedicated chick starter feed was measured into 13×100 mm borosilicate

tubes and autoclaved. The feed was suspended in 4.5 mL sterile saline and inoculated with 0.5 mL of a *Salmonella* Typhimurium culture containing approximately  $10^4$  cfu/mL. The tubes were treated with either: 1) saline as a control; 2) OAM, having a final concentration of acetic, citric and propionic acids at 0.031% or; 3) OAM, having a final concentration of acetic, citric and propionic acids at 0.062%. Each sample was run as triplicate, each treatment had 5 replicates, and the entire assay was repeated in 2 additional trials. After administering the treatment, the tubes were vortexed and incubated at 37 °C for 30 minutes and an additional 6 h. The tubes were then agitated and 20 µL of the content was serially diluted and plated as triplicates on BGA containing novobiocin and nalidixic acid. Typical ST colonies were counted after 24 h of incubation.

### ***Experimental Design with chickens***

In experiment 1, 64 day-of-hatch broiler chicks were obtained from a local hatchery. Chicks were randomized and challenged with  $2 \times 10^5$  cfu/mL of ST. The chicks were then held in chick boxes for 1 h and then randomly assigned to 1) untreated control or continuous treatment in the drinking water with: 2) Optimizer<sup>®</sup> at commercial recommended doses; 3) OAM, having a final concentration of acetic, citric and propionic acids at 0.031% or; 4) OAM, having a final concentration of acetic, citric, and propionic acids at 0.062%. Chicks were housed in brooder batteries with food and water *ad libitum*. At 24 h post-challenge, chicks were humanely killed by CO<sub>2</sub> inhalation and crop, both ceca and cecal tonsils were aseptically harvested separately. *Salmonella* recovery procedures have been previously described by our laboratory and were followed with some modifications (Tellez et al., 1993). Briefly, crop and cecal tonsils were enriched in 10 mL of tetrathionate broth overnight at 37 °C. Following

enrichment, each sample was streaked for isolation on BGA plates containing 25 µg/mL NO and 20 µg/mL NA. The plates were incubated at 37 °C for 24 h and examined for the presence or absence of the antibiotic resistant ST. Ceca were weighed and then homogenized within sterile sample bags (Catalog No. N-1628, Sigma, St. Louis, MO 63178 USA) using a rubber mallet. Sterile saline (4X weight to volume) was added to each sample bag and hand stomached with the cecal contents. Dilutions were spread plated on BGA plates containing 25 µg/mL NO and 20 µg/mL NA. The plates were incubated at 37 °C for 24 h and cfu of ST per ceca were determined.

In experiment 2, 80 day-of-hatch broiler chicks were obtained from a local hatchery. Chicks were randomized and challenged with  $2 \times 10^5$  cfu/mL of ST. The chicks were then held in chick boxes for 1 h and then randomly assigned to 1) untreated control or continuous treatment in the drinking water with: 2) Optimizer<sup>®</sup> at commercial recommended doses; 3) OAM, having a final concentration of acetic, citric and propionic acids at 0.031% or; 4) OAM, having a final concentration of acetic, citric and propionic acids at 0.062%. Chicks were housed in brooder batteries with food and water *ad libitum*. At 24 h post-challenge, chicks were humanely killed by CO<sub>2</sub> inhalation and crops were aseptically harvested, weighed and were homogenized within sterile sample bags using a rubber mallet. Sterile saline (4X weight to volume) was added to each sample bag and hand stomached with the crop contents. Dilutions were spread plated on BGA plates containing 25 µg/mL NO and 20 µg/mL NA. The plates were incubated at 37 °C for 24 h and cfu of ST per crop were determined. Following this, crops were enriched with a 2X solution of tetrathionate broth overnight at 37 °C. Following enrichment, each sample was streaked for isolation on BGA plates containing 25 µg/mL NO and 20 µg/mL NA. The plates were incubated at 37 °C for 24 h and examined for the presence or absence of the antibiotic resistant ST.

### *Statistical Analysis*

The incidence of *Salmonella* recovery within experiments was compared using the chi-square test of independence (Zar, 1984) testing all possible combinations to determine significant ( $P < 0.05$ ) differences between control and treated groups. Cecal cfu data were converted to  $\log_{10}$  cfu numbers and then compared using the GLM procedure of SAS (SAS Institute, 2002) with significance reported at  $P < 0.05$ .

## **RESULTS AND DISCUSSION**

*Salmonella* colonization of poultry flocks can occur via horizontal transmission (Bailey et al., 2002; Kim et al., 2007; Alali et al., 2010; Vandeplass et al., 2010). Once cecal tonsil colonization is established, the bacterium is consistently shed in the feces (Bailey et al., 2002; Foley et al., 2008). Feed withdrawal induces pecking of the contaminated litter which may contaminate the crop (Corrier et al., 1999c) and if the crop is ruptured during processing, *Salmonella* may contaminate raw poultry products (Corrier et al., 1999b). Because the crop is more likely to rupture than the ceca, the crop represents an important source of *Salmonella* contamination to carcasses (Hargis et al., 1995; Corrier et al., 1999a). Table 1 summarizes the results of effect of OAM on ST in an *in vitro* crop assay. In 3 independent trials, the 0.031% OAM reduced ST by 6 h and the 0.062% OAM was also efficacious. However, when 0.062% OAM was tested in chickens, it had a similar effect as Optimizer<sup>®</sup> showing a significant reduction in total number of ST positive chickens in cecal tonsils (Table 2), and reducing the number of ST in the crop (Table 3) when compared with controls.

In the present study, Optimizer<sup>®</sup> reduced ST colonization in both crop and ceca (Tables 2 and 3) as has been previously reported (Jarquin et al., 2007; Wolfenden et al., 2007). In

experiment 1, treatment with OAM in the drinking water caused a significant reduction ( $P < 0.05$ ) in ST recovery from cecal tonsils when compared with the controls (OA treated=19% vs. controls=87%). Also, treatment with OAM reduced 2.21 logs of ST when compared with controls (Table 2). While any of the treatments reduced recovery of ST from the crop by enrichment, all treatments reduced the number of ST recovered from crop content at 24 h (Table 3). The organic acids used in this study (citric, acetic, and propionic) as well as others have been shown to be individually effective in reducing *Salmonella in vitro* (Van Immerseel et al., 2006).

The biocidal efficacy and the effect on virulence of *Salmonella* differ with each organic acid treatment and each organic acid has a unique effect on bacteria normally present in the crop and gastrointestinal tract (Furuse et al., 1991; Byrd et al., 2001; Castro Gonzalez et al., 2001; Kubena et al., 2001). Characteristics of organic acids such as chain length, side chain composition, pKa values, and hydrophobicity could be factors that effect biocidal activity (Van Immerseel et al., 2006). For these reasons, a mixture of organic acids was tested to reduce ST crop contamination. Further studies are being conducted to evaluate these new formulations of OAM during the pre-slaughter feed withdrawal period in commercial chickens to evaluate water consumption and bactericidal activity against *Salmonella* in the crop.

**TABLES**

**Table 1.** Effect of organic acid mix (OAM) on *Salmonella* Typhimurium (ST) in an *in vitro* crop assay.

	<b>Trial 1</b>		<b>Trial 2</b>		<b>Trial 3</b>	
	<b>30 minutes</b>	<b>6 hours</b>	<b>30 minutes</b>	<b>6 hours</b>	<b>30 minutes</b>	<b>6 hours</b>
<b>Control (ST)</b>	6.25 ± 0.13 <sup>a</sup>	7.09 ± 0.09 <sup>a</sup>	7.42 ± 0.03 <sup>a</sup>	7.07 ± 0.04 <sup>a</sup>	4.95 ± 0.13 <sup>a</sup>	5.99 ± 0.22 <sup>a</sup>
<b>0.031% OAM</b>	6.08 ± 0.8 <sup>a</sup>	5.98 ± 0.01 <sup>b</sup>	7.43 ± 0.03 <sup>a</sup>	5.86 ± 0.03 <sup>b</sup>	4.88 ± 0.24 <sup>a</sup>	4.56 ± 0.07 <sup>b</sup>
<b>0.062% OAM</b>	ND	ND	7.39 ± 0.04 <sup>a</sup>	6.24 ± 0.12 <sup>b</sup>	4.70 ± 0.22 <sup>b</sup>	4.56 ± 0.07 <sup>b</sup>

Organic acids mix = acetic, citric, and propionic acid

ND = Not determined

Data are expressed as log<sub>10</sub> mean ± standard error. Values within columns with different lowercase superscripts differ significantly (P < 0.05).

**Table 2.** Experiment 1, effect of Optimizer<sup>®</sup> or organic acids mix (OAM) on *Salmonella* Typhimurium (ST) infection in broiler chicks during 24 hours period.

<b>Treatment</b>	<b>Crop Enrichment culture</b>	<b>Cecal tonsils Enrichment culture</b>	<b>Log<sub>10</sub> ST / gram of ceca content</b>
<b>Control ST</b>	15/16 (94%)	14/16 (87%)	2.43 ± 0.35 <sup>a</sup>
<b>Optimizer<sup>®</sup></b>	13/16 (81%)	3/16 (19%) **	0.22 ± 0.22 <sup>b</sup>
<b>0.031% OAM</b>	16/16 (100%)	12/16 (75%)	2.02 ± 0.35 <sup>a</sup>
<b>0.062% OAM</b>	13/16 (81%)	8/16 (50%) *	1.34 ± 0.40 <sup>a</sup>

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Organic acids mix= acetic, citric, and propionic acid

Data of enrichment culture is expressed as positive/total chickens for each tissue sampled (%).

\* Indicates significant difference at P < 0.05. \*\* Indicates significant difference at P < 0.001.

Log<sub>10</sub> ST/gram of ceca content data is expressed as mean ± standard error. Values within columns with different lowercase superscripts differ significantly (P < 0.05).

**Table 3.** Experiment 2, effect of Optimizer<sup>®</sup> or organic acids mix (OAM) on *Salmonella* Typhimurium (ST) infection in broiler chicks during 24 hours period.

<b>Treatment</b>	<b>Crop Enrichment culture</b>	<b>Log<sub>10</sub> ST / gram of crop content</b>
<b>Control ST</b>	20/20 (100%)	5.21 ± 0.31 <sup>a</sup>
<b>Optimizer<sup>®</sup></b>	18/20 (90%)	3.73 ± 0.25 <sup>b</sup>
<b>0.031% OAM</b>	20/20 (100%)	3.96 ± 0.37 <sup>b</sup>
<b>0.062% OAM</b>	18/20 (90%)	3.89 ± 0.22 <sup>b</sup>

Organic acids mix= acetic, citric, and propionic acid

Data of enrichment culture is expressed as positive/total chickens for each tissue sampled (%).

Log<sub>10</sub> ST/gram of crop content is expressed as mean ± standard error. Values within columns with different lowercase superscripts differ significantly (P < 0.05).



## REFERENCES

- Alali, W. Q., Thakur, S., Berghaus, R. D., Martin, M. P., & Gebreyes, W. A. (2010). Prevalence and distribution of *Salmonella* in organic and conventional broiler poultry farms. *Foodborne Pathogens and Disease*, 7(11), 1363-1371.
- Bailey, J. S., Cox, N. A., Craven, S. E., & Cosby, D. E. (2002). Serotype tracking of *Salmonella* through integrated broiler chicken operations. *Journal of Food Protection*, 65, 742-745.
- Barnhart, E. T., Sarlin, L. L., Caldwell, D. J., Byrd, J. A., Corrier, D. E., & Hargis, B. M. (1999). Evaluation of potential disinfectants for preslaughter broiler crop decontamination. *Poultry Science*, 78, 32-37.
- Byrd, J. A., Hargis, B. M., Caldwell, D. J., Bailey, R. H., Herron, K. L., McReynolds, J. L., Brewer, R. L., Anderson, R. C., Bischoff, K. M., Callaway, T. R., & Kubena, L. F. (2001). Effect of lactic acid administration in the drinking water during preslaughter feed withdrawal on *Salmonella* and *Campylobacter* contamination of broilers. *Poultry Science*, 80, 278-283.
- Castro Gonzalez, M. I., Montano Benavides, S., & Perez-Gil Romo, F. (2001). Fatty acids in sardine canned in tomato sauce from different fishing areas of the Mexican Pacific. *Archivos Latinoamericanos de Nutricion*, 51(4), 400-406.
- Corrier, D. E., Byrd, J. A., Hargis, B. M., Hume, M. E., Bailey, R. H., & Stanker, L. H. (1999a). Survival of *Salmonella* in the crop contents of market-age broilers during feed withdrawal. *Avian Diseases*, 43, 453-460.
- Corrier, D. E., Byrd, J. A., Hargis, B. M., Hume, M. E., Bailey, R. H., & Stanker, L. H. (1999b). Survival of *Salmonella* in the crop contents of market-age broilers during feed withdrawal. *Avian Diseases*, 43, 453-460.
- Corrier, D. E., Byrd, J. A., Hargis, B. M., Hume, M. E., Bailey, R. H., & Stanker, L. H. (1999c). Presence of *Salmonella* in the crop and ceca of broiler chickens before and after preslaughter feed withdrawal. *Poultry Science*, 78, 45-49.
- Foley, S. L., Lynne, A. M., & Nayak, R. (2008). *Salmonella* challenges: prevalence in swine and poultry and potential pathogenicity of such isolates. *Journal of Animal Science*, 86, E149-162.
- Furuse, M., Yang, S. I., Niwa, N., & Okumura, J. (1991). Effect of short chain fatty acids on the performance and intestinal weight in germ-free and conventional chicks. *British Poultry Science*, 32, 159-165.
- Hargis, B. M., Caldwell, D. J., Brewer, R. L., Corrier, D. E., & Deloach, J. R. (1995). Evaluation of the chicken crop as a source of *Salmonella* contamination for broiler carcasses. *Poultry Science*, 74, 1548-1552.

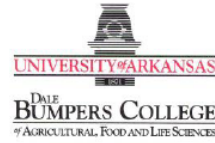
- Hinton Jr, A., Buhr, R. J., & Ingram, K. D. (2000). Reduction of *Salmonella* in the crop of broiler chickens subjected to feed withdrawal. *Poultry Science*, 79, 1566-1570.
- Jarquín, R. L., Nava, G. M., Wolfenden, A. D., Donoghue, A. M., Hanning, I., Higgins, S. E., & Hargis, B. M. (2007). The evaluation of organic acids and probiotic cultures to reduce *Salmonella* Enteritidis horizontal transmission and crop infection in broiler chickens. *International Journal of Poultry Science*, 6(3), 182-186.
- Kim, A., Lee, Y. J., Kang, M. S., Kwag, S. I., & Cho, J. K. (2007). Dissemination and tracking of *Salmonella* spp. in integrated broiler operation. *Journal of Veterinary Science*, 8, 155-161.
- Kubena, L. F., Byrd, J. A., Young, C. R., & Corrier, D. E. (2001). Effects of tannic acid on cecal volatile fatty acids and susceptibility to *Salmonella* Typhimurium colonization in broiler chicks. *Poultry Science*, 80, 1293-1298.
- Lynch, M., Painter, J., Woodruff, R., Braden, C., & Centers for Disease Control and Prevention. (2006). Surveillance for foodborne-disease outbreaks-United States, 1998-2002. *Morbidity and Mortality Weekly Report Surveillance Summaries*, 55, 1-42.
- Mikolajczyk, A., & Radkowski, M. (2002). *Salmonella* spp. on chicken carcasses in processing plants in Poland. *Journal of Food Protection*, 65, 1475-1479.
- SAS Institute Inc. (2002). SAS user's guide: statistics. SAS Institute Inc., Cary, N.C.
- Tellez, G., Dean, C. E., Corrier, D. E., Deloach, J. R., Jaeger, L., & Hargis, B. M. (1993). Effect of dietary lactose on cecal morphology, pH, organic acids, and *Salmonella* enteritidis organ invasion in Leghorn chicks. *Poultry Science*, 72, 636-642.
- Van Immerseel, F., Russell, J. B., Flythe, M. D., Gantois, I., Timbermont, L., Pasmans, F., Haesebrouck, F., & Ducatelle, R. (2006). The use of organic acids to combat *Salmonella* in poultry: a mechanistic explanation of the efficacy. *Avian Pathology*, 35, 182-188.
- Vandeplas, S., Dubois Dauphin, R., Beckers, Y., Thonart, P., & Thewis, A. (2010). *Salmonella* in chicken: current and developing strategies to reduce contamination at farm level. *Journal of Food Protection*, 73, 774-785.
- Vicente, J., Higgins, S., Bielke, L., Tellez, G., Donoghue, D., Donoghue, A., & Hargis, B. M. (2007a). Effect of probiotic culture candidates on *Salmonella* prevalence in commercial turkey houses. *Journal of Applied Poultry Research*, 16, 471-476.
- Vicente, J. L., Aviña, L., Torres-Rodriguez, A., Hargis, B., & Tellez, G. (2007b). Effect of a *Lactobacillus* spp.-based probiotic culture product on broiler chicks performance under commercial conditions. *International Journal of Poultry Science*, 6 (3), 154-156.
- Vicente, J., Wolfenden, A., Torres-Rodriguez, A., Higgins, S., Tellez, G., & Hargis, B. M. (2007c). Effect of a *Lactobacillus*-based probiotic and dietary lactose prebiotic on turkey poult

- performance with or without *Salmonella enteritidis* challenge. *Journal of Applied Poultry Research*, 16, 361-364.
- Voetsch, A. C., Angulo, F. J., Rabatsky-Her, T., Shallow, S., Cassidy, M., Thomas, S. M., Swanson, E., Zansky, S. M., Hawkins, M. A., Jones, T. F., Shillam, P. J., Van Gilder, T. J., Wells, J. G., Griffin, P. M., & Emerging Infections Program FoodNet Working Group. (2004a). Laboratory practices for stool-specimen culture for bacterial pathogens, including *Escherichia coli* O157:H7, in the FoodNet sites, 1995-2000. *Clinical Infectious Disease*, 38(3), S190-197.
- Voetsch, A. C., Van Gilder, T. J., Angulo, F. J., Farley, M. M., Shallow, S., Marcus, R., Cieslak, P. R., Deneen, V. C., Tauxe, R. V., & Emerging Infections Program FoodNet Working Group. (2004b). FoodNet estimate of the burden of illness caused by nontyphoidal *Salmonella* infections in the United States. *Clinical Infectious Disease*, 38(3), S127-34.
- Wolfenden, A., Vicente, J., Higgins, J., Andreatti Filho, R., Higgins, S., Hargis, B. M., & Tellez, G. (2007). Effect of organic acids and probiotics on *Salmonella enteritidis* infection in broiler chickens *International Journal of Poultry Science*, 6, 403-405.
- Zar, J. (1984). Pages 348-351 In: *Biostatistical Analysis*. 2nd ed. Prentice-Hall. Englewood Cliffs, NJ.

## APPENDIX



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To Whom It May Concern,

The first author of enclosed paper, “Effect of Organic Acids on *Salmonella* Typhimurium Infection in Broiler Chickens”, is Anita Menconi. Anita was primarily responsible for the work and research associated with this paper, and completed greater than 51% of the work.

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B.M. Hargis

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MEMORANDUM

TO: Lisa R. Bielke

FROM: Craig N. Coon, Chairman  
Institutional Animal Care  
And Use Committee

DATE: June 9, 2011

SUBJECT: IACUC PROTOCOL APPROVAL  
Expiration date : June 2, 2014

The Institutional Animal Care and Use Committee (IACUC) has APPROVED Protocol #11052-  
“USE OF GRAS APPROVED FEED INGREDIENTS FOR INCLUSION IN POULTRY  
DRINKING WATER TO SANITIZE CROP CONTENTS”. You may begin this study  
immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF  
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In granting its approval, the IACUC has approved only the protocol provided. Should there be  
any changes in the protocol during the research, please notify the IACUC in writing  
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## IV. CHAPTER II

### **Evaluation of a commercially available organic acid product on body weight loss, carcass yield, and meat quality during pre-slaughter feed withdrawal in broiler chickens: A poultry welfare and economic perspective**

A. Menconi,<sup>\*</sup> V. A. Kuttappan,<sup>\*</sup> X. Hernandez-Velasco,<sup>†</sup> T. Urbano,<sup>‡</sup> F. Matté,<sup>‡</sup> S. Layton,<sup>§</sup> G. Kallapura,<sup>\*</sup> J. Latorre,<sup>\*</sup> B. E. Morales,<sup>‡</sup> O. Prado,<sup>‡</sup> J. L. Vicente,<sup>¥</sup> J. Barton,<sup>¥</sup> R. L. Andreatti Filho,<sup>‡</sup> M. Lovato,<sup>‡</sup> B. M. Hargis,<sup>\*</sup> and G. Tellez<sup>\*</sup>

<sup>\*</sup>Department of Poultry Science, University of Arkansas, Fayetteville, AR 72701; <sup>†</sup>Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, México D.F. 04510.

<sup>‡</sup>Vetanco do Brasil, Rua Raimundo Zanella, 490D Distrito Industrial Flavio Baldissera, Brazil, CEP 89.801-973; <sup>§</sup>ARGENTINA VETANCO S.A. Chile 33 (B1603CMA) Vicente López. Buenos Aires, Argentina.

<sup>‡</sup>Departamento de Producción Agrícola y Animal, Universidad Autónoma Metropolitana, México D.F. 04960.

<sup>‡</sup>Facultad de Medicina Veterinaria y Zootecnia, Universidad de Colima, Colima, 28100, México.

<sup>¥</sup>Pacific Vet Group-USA, Inc. 2135 Creek View Drive Fayetteville, AR 72704.

<sup>‡</sup>Department of Veterinary Clinics, FMVZ UNESP Campus, Botucatu, Brazil.

<sup>‡</sup>Depto. de Medicina Veterinária – CCR Universidade Federal de Santa Maria, Brazil.

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## **ABSTRACT**

The effect of a commercial organic acid (OA) product on BW loss (BWL) during feed withdrawal (FW) and transportation, carcass yield, and meat quality was evaluated in broiler chickens. Two experiments were conducted in Brazil. Commercial houses were paired as control groups receiving regular water and treated groups receiving OA in the water. Treated birds had a reduction in BWL of 37 g in experiment 1 and 32.2 g in experiment 2. In experiment 2, no differences were observed in carcass yield between groups. Estimation of the cost benefit suggested a 1:16 ratio by using the OA. In experiment 3, conducted in Mexico, significant differences on water consumption, BWL, and meat quality characteristics were observed in chickens that were treated with the OA ( $P < 0.05$ ). These data suggests this OA product may improve animal welfare and economics concerns in the poultry industry by reducing BWL and improving meat quality attributes.

**Key words:** Organic acid, chicken, welfare, transportation, meat quality.



## INTRODUCTION

Global potential for poultry acidifiers, for both feed and water, are on the rise due to higher demand for top quality poultry, which is also true for most of the other animal production operations including swine and cattle (Berkhout, 2009). A global strategic business report on feed acidifiers have revealed that the growth potential in the feed acidifiers market is expected to remain robust and expected to rise, mostly attributed to the increasing demand for safe and high quality pork and poultry meat. Growing awareness and increasing adoption of the use of acidifiers in emerging countries, coupled with escalating demand in the developing world, has expanded the market for these acidifiers (Companies and Markets, 2012).

Europe continues to be the largest regional market with high demand for feed acidifiers in specific, primarily attributed to its large pig and poultry populations, supported largely by legislations that ban the use of antibiotics in feed. Organic acids (**OA**) based feed acidifiers have gained significance due to their high nutritional value and antimicrobial benefits. Major countries dominating the production scene for feed acidifiers include the US, China, Brazil, Mexico, and Japan, while demand is on the rise in developing regions like Latin America, Asia-Pacific, and Middle East (Companies and Markets, 2012).

Most of the research and subsequent applications has been involving feed acidifiers as a preventive or treatment tool for disease management or to improve bird performance. The inclusion of various OA or their salts to diets is shown to improve the growth performance by enhancing the nutrient digestibility and affecting the microbial populations in different parts of the digestive tract (Tung & Petigrew, 2011). The use of OA or other acidifiers in water management for poultry operations is a subject of much conversation between growers, veterinarians, and live production personnel. Further, research involving the establishment of

preferred pH for poultry, its effects on water consumption and eventually poultry welfare, has been limited. Using an OA based drinking water at critical periods of poultry growth is said to establish and maintain intestinal development by the stability of the intestinal microflora, eventually improving live production performance and cost.

Acidifying drinking water for poultry for the first seven days of life, when the birds are first placed into the house, is considered critical, since the crop and intestinal microbial morphology would still be under development. Maintenance of low crop pH by the lactic acid bacteria (**LAB**) in newly hatched poult and chicks is critical. The acidified drinking water provides a second layer of protection to the LAB and helps to establish them as a part of the crop's normal ecology. Once the crop's LAB population has been established, the bird will be able to maintain a low crop pH on its own as long as feed is available.

Feed withdrawal (**FW**) for various reasons or when chickens and turkeys are not eating for any reason, leads to an imbalance in the natural population of LAB, leading to an increase in pH, favoring pathogens like *Salmonella* to multiply in the crop. Pre-slaughter FW is a method commonly employed to reduce carcass contamination (Corrier et al., 1999; Byrd et al., 2001; Northcutt et al., 2003; Yi et al., 2005). However, carcass shrinkage (carcass dehydration) begins immediately after FW (Benibo & Farr, 1985; Veerkamp, 1986), resulting in recommendations that slaughter take place within 4 to 8 h after FW to minimize losses. Nevertheless, under commercial conditions, this time may be hard to achieve. Consequently, scheduling managers need to consider FW effects on both gut fullness and shrinkage.

In addition to FW, chickens must endure stress during catching, crating transport, and shackling (Gregory, 1994; Petracci et al., 2006). All these factors as well as the total time from FW to slaughter have important implications in welfare of the birds, economics for the poultry

industry and meat quality for the consumers (Gregory, 1996; Kannan et al., 1997; den Hertog-Meischke et al., 1997). In poultry and other species, transport-related economic losses are due to mortality, carcass shrinkage, and carcass condemnation (Veerkamp, 1986).

Previously, our laboratory conducted a study in broiler chickens showing that a commercially available water treatment product (Optimizer) significantly reduced carcass condemnation at the processing plant and mortality during transportation, with consistent improvement of average BW at the farm and at the processing plant in broiler chickens (Wolfenden et al., 2007a). In a similar study, the treatment with Optimizer in the drinking water of commercial turkeys during FW showed a significant reduction in the rate of weight loss during transportation and holding at the processing plant in the treated turkeys and improved average BW in treated turkeys during 19 h with an average of 90 g difference (Pixley et al., 2010). Both studies measured BW loss (**BWL**) during holding at the processing plant and the ability to mitigate that loss by treatment with OA prior to catching. It seems, likely, that dehydration progressively results in negative welfare for the animal, and the rate of BW change has the potential to be used as a metric in evaluating welfare status of commercial poultry (Warriss et al., 1993; Savenije et al., 2002; Rosenvold & Andersen, 2003; Pixley et al., 2010). Our research has shown the potential to reduce the rate of BWL by administering OA in the drinking water during FW and transportation to the processing plant. In the present study, Optimizer was used in different commercial broiler companies in Brazil and Mexico, to evaluate BWL during FW as well as during transportation to the processing plant. Carcass yield and meat quality during pre-slaughter feed withdrawal was also assessed, and the implications of poultry welfare and economic results are discussed.

## MATERIALS AND METHODS

### *Organic Acids*

An OA product (Optimizer™, Pacific Vet Group-USA, Inc., Fayetteville, Arkansas) was used in the drinking water during FW according to manufacturer's directions (4 L Optimizer/1,000 L of water). This commercial OA product is a combination of five different OA (lactic, acetic, tannic, propionic, and caprylic acids) that contains proprietary flavoring agents. This OA product has been shown to reduce *Salmonella* colonization in crop and cecal tonsils without affecting water consumption in chickens (Vicente et al., 2007; Wolfenden et al., 2007b; Jarquin et al., 2007).

### *Experimental Design*

*Experiment 1. Effect of the organic acid product on body weight loss during pre-slaughter feed withdrawal and transportation under commercial conditions in different states of Brazil.*

In experiment 1, five trials were conducted in five different commercial poultry farms located in 3 different states of Brazil (Table 1). Furthermore, the individual trials were done during the year 2012, with birds having different age and subjected to various feed withdrawal, transportation and total fasting periods. In all these trials, houses were designated as control groups receiving regular water and as treated groups receiving OA in the water at a concentration of 4 L/1,000 L of water (vol/vol) according to the manufacturer's directions. In trial 3, eight commercial chicken houses of market age broiler chickens were paired. In all other trials, two commercial chicken houses were paired. A total of 35 birds per house treatment were neck tagged and individually weighed before the feed withdrawal period and at the time of arrival to the

processing facility (after the transportation). The difference between the above two body weights was taken to determine the BWL under pre-slaughter commercial conditions. Later, a cost benefit analysis was performed based on the BWL to estimate the economic benefit in administering OA in broiler chickens.

***Experiment 2. Effect of the organic acid product on carcass yield in broiler chickens in Brazil.***

This experiment was performed in November 2012 in a poultry farm located in the state of Paraná. Sixteen commercial chicken houses of market age broiler chickens (47 days of age) were paired. In this experiment, eight houses were designated as control groups receiving regular water and 8 houses as treated groups receiving OA in the water at a concentration of 4 L/1,000 L of water (vol/vol), according to manufacturer's directions. At each farm, 40 tagged market age broilers per house treatment were individually weighed after FW period and at the time of arrival to the processing facility. Feed withdraw time was 8 h and transportation was of 2 h, being a total fasting time of 10 h. Carcass yield was also calculated for the same tagged 40 birds.

***Experiment 3. Effect of organic acid on water consumption, body weight loss, and meat quality measurements during 8 hours pre-slaughter feed withdrawal in broiler chickens from Mexico.***

A total of 240 forty-day-old female Cobb 500 broilers were obtained from a commercial farm (Colima, Mexico) and moved to isolation facilities at CVM, University of Colima, Mexico. Broilers were neck tagged and randomly assigned to 8 pens, 4 controls and 4 treated, each pen measuring 3 m<sup>2</sup> with 30 birds per pen and provided finisher feeder and water *ad libitum*. Broilers were kept in a temperature controlled room at 30 °C. At 42 d of age, all chickens were weighed and treatment was initiated to 4 pens by adding the OA in the drinking water. Control groups

receiving regular water and treated groups receiving OA in the water adjusted to a concentration of 4 L/1,000 L of water (vol/vol) according to manufacturer's directions. When treatment was initiated, feed was removed from the control and treatment pens and water consumption was monitored in all pens. After 8 h of treatment, all broilers were weighed and final water consumption recorded. Three birds from each pen were humanely killed by cervical dislocation. Breast muscles (pectoralis major) were removed immediately and stored individually in plastic bags at 4 °C for 24 h for further analysis of meat quality measurements.

### ***Meat Quality Measurements***

At 24 h post mortem, the breast meat pH was determined on individual fillets according to the method as described by Qiao et al. (2002). The pH was determined using a Model pH/ISE meter, calibrated at pH 4.0 and 7.0, and was conducted on the medial bone side as follows: a cut approximately 0.5 cm in length and depth was made in the meat, and a drop of deionized water was placed in the cut to improve contact with the pH probe. The probe was rinsed with deionized water and was dried with a filter between samples, and was cleaned with alcohol after every lot of 3 fillets.

The complete International Commission on Illumination (**CIE**) system color profile of lightness ( $L^*$ ), redness ( $a^*$ ), and yellowness ( $b^*$ ) was measured on the cranial and medial surface (bone side) using a reflectance colorimeter (Minolta Chroma Meter CR-10, Minolta, Osaka, Japan), in an area free of obvious color defects (bruises, blood spots, or surface discolorations) at room temperature ( $25 \pm 2$  °C), immediately after samples were tagged. Measurements were made on the medial surface to avoid breast fillet surface discolorations due to possible over scalding in the plant. Color values were calibrated using a Minolta calibration plate ( $L^*= 60.5$ ,

a\* = -3.2, and b\* = +6.7).

Water-holding capacity (**WHC**) of the breast meat samples were measured according to the method as described by Lu et al. (2006), with some modifications. A 0.3 g sample of breast muscle was pressed onto an oven-dried Whatman 125 mm filter paper at 2000 psi for 1 min. The WHC values were calculated as the ratio of the area of expressed water to the area of the pressed meat sample, measured with a planimeter. Therefore, a lower ratio indicates a greater WHC. Thawing loss (**TL**) was measured according to Mortensen et al. (2006). Immediately before freezing, samples were weighed. The frozen samples were thawed over a period of 24 h at 4 °C and weighed again. TL was determined as the percentage of BWL after thawing.

Drip loss (**DL**) was conducted according to Berri et al. (2008). The muscle samples were weighed and immediately placed in a plastic bag, hung from a hook, and stored at 4 °C for 48 h. After hanging, the sample was wiped with absorbent paper and weighed again. The difference in weight corresponded to the DL and was expressed as the percentage of the initial muscle weight. For cook loss (**CL**), the individually weighed fillets were placed on stainless steel trays and cooked for 30 min at 98 °C in steam. Upon removal from the oven, the fillets were covered with plastic film and allowed to equilibrate to room temperature (25 °C). Individual fillets were then reweighed to determine CL.

### ***Data Analysis***

Body weight, carcass yield, and meat quality data collected were subjected to one way analysis of variance using the GLM procedure of SAS, with significance reported at  $P < 0.05$ , means were further separated using Duncan's multiple range test (SAS Institute, 2002).

### ***Formulas and Estimated Values***

$$\text{Difference in BWL/chicken} = \frac{\text{BWL of non-treated}}{\text{BWL of treated}}$$

Reduction in BWL of total chickens = (Total treated chickens) X (Difference in BWL of treated chickens)

Value of treatment for total chickens = (Weight gain of total chickens) X (Value of the meat/kg (estimated at USD 1.44/kg))

Total water consumption = (water consumption/chicken) X (total treated chickens)

Total cost of Optimizer = (cost of Optimizer/L (estimated at USD 4.16/L)) X (L of Optimizer used)

$$\text{Benefit to cost ratio} = \frac{\text{Value of treatment}}{\text{Optimizer product cost (expressed as cost:benefit)}}$$

## **RESULTS**

The results of the effect of the OA on BWL during pre-slaughter feed withdrawal and transportation under commercial conditions in different states of Brazil from experiment 1 are summarized in Table 2. In trials 1, 2, and 5, a significant reduction in BWL was observed in the chickens treated with OA when compared with control birds ( $P < 0.05$ ), and numerical reduction in trials 3 and 4. Overall average from all 5 trials, treated birds had a reduction in BWL of 37 g when compared with control non treated chickens. Similar results have been reported previously (Wolfenden et al., 2007a; Pixley et al., 2010).

The results of the effect of the OA product on carcass yield in broiler chickens in Brazil from experiment 2 are summarized in table 3. In this experiment, a numerical reduction of 32.2 g of BWL was observed in treated chickens when compared with control non treated chickens. Remarkably, no differences were observed in carcass weight and carcass yield between treated



and control chickens. Carcass yield (%) in control chickens were 76 % *versus* 75.9 % in OA treated chickens.

The economic analysis from experiments 1 and 2 on chickens treated with the OA product is shown in table 4. From this analysis, the reduction in BWL when converted to a cost benefit ratio suggested that for every one U.S. dollar spent with this OA product, producers were able to recover on average 16 U.S. dollars.

Table 5 summarizes the effect of OA on BWL in broiler chickens during 10 h pre-slaughter feed withdrawal, from experiment 3. As it has been shown previously (Jarquin et al., 2007; Vicente et al., 2007), unlike treatment with lactic or formic acid (Byrd et al., 2001), the OA treatment used in the present study showed a significant increase in water consumption that was associated with a significant minor BWL after 10 h of FW compared with non-treated birds. Table 6 summarizes the results of OA on raw breast meat color, chemical composition, pH, moisture, and water-holding characteristics in broiler chickens after 10 h of FW from broilers in experiment 3. A significant increase in lightness and redness, drip and cooking loss with a significant reduction on meat pH, moisture and WHC were observed in non-treated chickens when compared with OA treated chickens.

## **DISCUSSION**

Prior to slaughter, broiler chickens are exposed to many handlings and conditions such as FW, catching, crating, transport, and shackling that have a profound impact in their welfare (Akşit et al., 2006; Ali et al., 1999; Petracci et al., 2006; Vanderhasselt et al., 2013). Under those stressful circumstances, energy reserves of the birds can be severely affected, modifying their metabolic state at slaughter, which has a negative effect in the final meat quality for the

consumers (Gregory, 1996; Kannan et al., 1997; Ali et al., 1999; Petracci et al., 2001). Feed withdrawal is a common practice that is intended to reduce fecal contamination of carcasses; however, during transport and lairage birds also experience water withdrawal (Northcutt et al., 2003; Corrier et al., 1999). All things considered (FW, crating time, transport, and lairage) could add a minimal of 9 h of feed deprivation, although infrequently, much longer times have been reported (Warriss et al., 1990), which will lead to significant carcass shrinkage (Veerkamp, 1986). Nevertheless, lack of feed and water has been reported to reduce glycogen levels in liver following as little as 3 h of FW (Warriss et al., 1988), which has also correlated with a significant decrease in postmortem liver pH (Warriss et al., 1993).

In the present study, the use of the OA product showed a significant or numerical reduction in BWL during FW period and transportation (tables 2, 3, and 5). The combination of the OA used in the Optimizer™ could have helped to improve the weight loss (Jarquin et al., 2007), even though the use of individual OA alone did not produce such an effect (Byrd et al., 2001). This implies that Optimizer™ had a benefit from the bird welfare point of view that it did not cause much dehydration, in addition to its documented *Salmonella*-recovery reductions in market age broilers when administered during the pre-slaughter FW period (Jarquin et al. 2007; Wolfenden et al. 2007a).

The significant increase in lightness and redness, increase drip and cooking loss as well as significant reduction on meat pH, moisture and WHC observed in control non treated chickens when compared with OA treated chickens (Table 6), suggest that the increased water consumption observed in previous studies (Vicente et al., 2007; Wolfenden et al., 2007a; Jarquin et al., 2007) and confirmed in this study (Table 5), may improve the physiological hydration state of the birds. From the results of carcass yield in experiment 2, where % carcass yield had a slight

difference of 0.1% between treated and control group (yet, numerical difference in BWL of 32.2 g); and meat quality results observed in experiment 3 (Tables 3 and 6), we may infer that perhaps, the increase water consumption induced by this OA product, is retained in the muscle and is not loosed in feces/urine or when blood and viscera are removed. This observation was supported in the present study by a consistent significant or numerical improvement in BWL (Tables 2, 3, and 5).

Several investigators have shown that the distribution and mobility of water in muscle (myowater) and meat have a profound influence on essential meat quality (Bertram et al., 2003; Benibo & Farr, 1985; Castellini et al., 2002; Pearce et al., 2011). During the conversion of the living muscle to meat and during ageing, the myowater content, location and mobility will change as a function of numerous mutual interacting factors of both ante and post mortem biochemistry (Akşit et al., 2006; Ali et al., 1999; Bertram et al., 2003; Bond et al., 2004). After death, oxygen supply is stopped, and energy has to be generated under anaerobic conditions resulting in accumulation of lactic acid, which decrease the pH of the muscle and affects the color of the meat and WHC due to protein breakdown (Warriss & Brown, 1987). When this anaerobic energy supply fails, rigor mortis appears (Maribo et al., 1998), which is directly correlated with the glycogen reserves of the birds and the metabolic state of the muscle before slaughter. The cessation of post mortem energy production in chickens has been reported to happen within 6 h of FW (Grey et al., 1974).

The WHC of chicken meat products is related to final carcass yield which impact both, economics and eating quality such as juiciness and tenderness (Zamorano & Gambaruto, 1997; Dai et al., 2009). Several ante mortem and post mortem factors have been reported to affect the conversion of living muscle to meat as well as the location and content of the myowater (Pearce

et al., 2011). Therefore, any loss of water reduces the weight of the product, which contributes to financial loss through loss of salable product. Most of the water in the muscle fibers is present in the myofibrils, which represent about 80 % of the muscle volume (Cheng & Sun, 2008). When the muscles are cut, a red fluid, called drip, exudes from the cut surfaces. This solution consists primarily of myoglobin and glycolytic enzymes (Cavitt & Sams, 2003). Excessive drip loss not only affects the final yield of the carcass, it also affects the protein concentration of the meat and represent a safety concern because this fluid is an excellent nutrient broth for spoiling and pathogenic bacteria (Pedersen et al., 2003; den Hertog-Meischke et al., 1997; Castellini et al., 2002; Northcutt et al., 2003). This is the first report that demonstrate that this OA induced increased water consumption and reduction of BWL during FW, catching, crating, transport, and shackling of poultry, which are associated with a positive improvement of meat quality attributes - such as color of the meat, pH, moisture and over all, water holding characteristics (higher water holding capacity and lower drip and cook losses). Furthermore, the reduction in BWL when converted to a cost benefit ratio suggested that for every U.S. dollar spent in this OA product, producers may be able to recover on average 16 U.S. dollars.

## TABLES

**Table 1.** Experimental designs for experiments 1 and 2 in Brazil

	State	Date	Age (d)	Time (h)		
				FW period	Transportation period	Total fasting
<b>Experiment 1</b>						
<b>Trial 1</b>	Minas Gerais	March 2012	46	8	1	9
<b>Trial 2</b>	Minas Gerais	April 2012	43	8	1	9
<b>Trial 3</b>	Paraná	March 2012	42	6	5	11
<b>Trial 4</b>	Paraná	March 2012	42	7	3	10
<b>Trial 5</b>	Mato Grosso do Sul	January 2012	44	3	10	13
<b>Experiment 2</b>						
<b>Single Trial</b>	Paraná	November 2012	47	8	2	10

**Table 2.** Effect of the organic acid product (OA) on body weight loss during pre-slaughter feed withdrawal and transportation under commercial conditions in different states of Brazil from experiment 1

<b>Trial</b>	<b>Treatment</b>	<b>Initial BW (g)</b>	<b>BW after transportation (g)</b>	<b>BW change (g)</b>
<b>Trial 1</b>	Control	2761 ± 43 <sup>a</sup>	2578 ± 50 <sup>a</sup>	-183 ± 23 <sup>b</sup>
	OA	2796 ± 57 <sup>a</sup>	2682 ± 57 <sup>a</sup>	-114 ± 12 <sup>a</sup>
<b>BW difference</b>				69
<b>Trial 2</b>	Control	2674 ± 39 <sup>b</sup>	2533 ± 37 <sup>b</sup>	-141 ± 12 <sup>b</sup>
	OA	2797 ± 31 <sup>a</sup>	2702 ± 31 <sup>a</sup>	-95 ± 6 <sup>a</sup>
<b>BW difference</b>				46
<b>Trial 3</b>	Control	3069 ± 68 <sup>a</sup>	2995 ± 74 <sup>a</sup>	-74 ± 37 <sup>a</sup>
	OA	3044 ± 65 <sup>a</sup>	2992 ± 54 <sup>a</sup>	-52 ± 12 <sup>a</sup>
<b>BW difference</b>				22
<b>Trial 4</b>	Control	3202 ± 64 <sup>a</sup>	3158 ± 65 <sup>a</sup>	-44 ± 82 <sup>a</sup>
	OA	3183 ± 63 <sup>a</sup>	3159 ± 62 <sup>a</sup>	-24 ± 79 <sup>a</sup>
<b>BW difference</b>				20
<b>Trial 5</b>	Control	2793 ± 70 <sup>a</sup>	2724 ± 64 <sup>a</sup>	-69 ± 7 <sup>b</sup>
	OA	2757 ± 57 <sup>a</sup>	2736 ± 60 <sup>a</sup>	-21 ± 5 <sup>a</sup>
<b>BW difference</b>				48
<b>Average BW difference</b>				<b>37 g</b>

Different superscripts indicate significant differences  $P < 0.05$ ,  $n = 35$  birds.

BW data is expressed as mean ± SE.

**Table 3.** Effect of the organic acid (OA) product on body weight and carcass yield in broiler chickens in Brazil from experiment 2

	<b>Control</b>	<b>OA</b>
<b>Initial BW (g)</b>	3040 ± 31 <sup>a</sup>	2990 ± 33 <sup>a</sup>
<b>BW after transportation (g)</b>	3004 ± 31 <sup>a</sup>	2986 ± 32 <sup>a</sup>
<b>BW loss (g)</b>	36.8 ± 37 <sup>a</sup>	4.8 ± 32 <sup>a</sup>
<b>Difference (g)</b>		<b>32.2 g</b>
<b>Carcass weight (g)</b>	2967 ± 37 <sup>a</sup>	2981 ± 38 <sup>a</sup>
<b>Carcass yield (%)</b>	76.0 %	75.9 %
<b>Difference (%)</b>		<b>0.1 %</b>

BW data is expressed as mean ± SE. Superscripts indicate significant differences  $P < 0.05$ , n = 40 birds.

**Table 4.** Cost - benefit of organic acid (OA) product from experiments 1 and 2

<b>Trial – State</b>	<b>Cost:benefit ratio* USD</b>
<b>Experiment 1</b>	
<b>Trial 1- Minas Gerais</b>	1:15
<b>Trial 2 - Minas Gerais</b>	1:12
<b>Trial 3 – Paraná</b>	1:14
<b>Trial 4 – Paraná</b>	1:17
<b>Trial 5 - Mato Grosso do Sul</b>	1:17
<b>Experiment 2</b>	
<b>Trial 1 – Paraná</b>	1:20
<b>Overall Average</b>	1:16

\*Estimated according to the numbers of broiler chickens/house treated with OA.



**Table 5.** Effect of organic acids on body weight loss in broiler chickens during ten hours pre-slaughter feed withdrawal, from experiment 3.

	<b>Control</b>	<b>Optimizer</b>
<b>Water consumption mL/bird</b>	38 ± 3 <sup>b</sup>	54 ± 5 <sup>a</sup>
<b>Initial BW (g)</b>	2830 ± 50 <sup>a</sup>	2798 ± 43 <sup>a</sup>
<b>BW after 10 h feed withdraw (g)</b>	2657 ± 35 <sup>b</sup>	2672 ± 54 <sup>a</sup>
<b>BW loss (g)</b>	176 ± 45 <sup>a</sup>	128 ± 57 <sup>b</sup>
<b>Diference</b>		<b>48 g</b>

Data is expressed as mean ± SE. Treatments values within rows with no common superscript differ significantly  $P < 0.05$ . Each group with 4 replicates of n = 30 each replicate.

**Table 6.** Effect of the organic acids on raw breast meat color, pH, moisture, and water-holding characteristics in broiler chickens during pre-slaughter feed withdrawal from experiment 3

Treatment	Breast meat characteristics				Water holding characteristics (%)				
	Lightness (L*)	Redness (a*)	Yellowness (b*)	pH	Moisture	Water Holding capacity	Thawing loss	Drip loss	Cook loss
<b>Control</b>	40.4 ± 0.7 <sup>a</sup>	5.6 ± 0.1 <sup>a</sup>	10.3 ± 0.9 <sup>a</sup>	5.4 ± 0.2 <sup>a</sup>	69.6 ± 0.7 <sup>b</sup>	65.4 ± 0.3 <sup>b</sup>	5.4 ± 0.5 <sup>a</sup>	6.8 ± 0.2 <sup>a</sup>	36.1 ± 0.7 <sup>a</sup>
<b>Organic acids</b>	37.4 ± 0.1 <sup>b</sup>	4.7 ± 0.3 <sup>b</sup>	11.4 ± 0.5 <sup>a</sup>	6.0 ± 0.1 <sup>b</sup>	72.0 ± 0.3 <sup>a</sup>	68.2 ± 0.5 <sup>a</sup>	5.0 ± 0.6 <sup>a</sup>	4.1 ± 0.5 <sup>b</sup>	33.4 ± 0.9 <sup>b</sup>

Data expressed as mean ± SE.

Treatments values within columns with no common superscript differ significantly  $P < 0.05$ , n = 12 birds.

## REFERENCES

- Akşit, M., Yalçın, S., Özkan, S., Metin, K., & Özdemir, D. (2006). Effects of temperature during rearing and crating on stress parameters and meat quality of broilers. *Poultry Science*, 85, 1867-1874.
- Ali, A. S., Harrison, A. P., & Jensen, J. F. (1999). Effects of some ante-mortem stressors on peri-mortem and postmortem biochemical changes and tenderness in broiler breast muscle: A review. *World's Poultry Science Journal*, 55, 403-414.
- Benibo, B., & Farr, A. (1985). The effects of feed and water withdrawal and holding shed treatments on broiler yield parameters. *Poultry Science*, 64, 920-924.
- Berri, C., Besnard, J., & Relandeau, C. (2008). Increasing dietary lysine increases final pH and decreases drip loss of broiler breast meat. *Poultry Science*, 87, 480-484.
- Berkhout, N. (2009). Poultry feed acidifiers - global trends. *WorldPoultry*. Retrieved from <http://www.worldpoultry.net/Home/General/2009/12/Report-Poultry-feed-acidifiers---global-trends-WP004610W/>
- Bertram, H. C., Andersen, H. J., Karlsson, A. H., Horn, P., Hedegaard, J., Nørgaard, L., & Engelsen, S. B. (2003). Prediction of technological quality (cooking loss and Napole Yield) of pork based on fresh meat characteristics. *Meat Science*, 65, 707-712.
- Bond, J., Can, L., & Warner, R. (2004). The effect of exercise stress, adrenaline injection and electrical stimulation on changes in quality attributes and proteins in *Semimembranosus* muscle of lamb. *Meat Science*, 68, 469-477.
- Byrd, J. A., Hargis, B. M., Caldwell, D. J., Bailey, R. H., Herron, K. L., McReynolds, J. L., Brewer, R. L., Anderson, R. C., Bischoff, K. M., Callaway, T. R., & Kubena, L. F. (2001). Effect of lactic acid administration in the drinking water during preslaughter feed withdrawal on *Salmonella* and *Campylobacter* contamination of broilers. *Poultry Science*, 80, 278-283.
- Castellini, C., Mugnai, C., & Dal Bosco, A. (2002). Effect of organic production system on broiler carcass and meat quality. *Meat Science*, 60, 219-225.
- Cavitt, L. C., & Sams, A. (2003). Evaluation of physical dimension changes as nondestructive measurements for monitoring rigor mortis development in broiler muscles. *Poultry Science*, 82, 1198-1204.
- Cheng, Q., & Sun, D. W. (2008). Factors affecting the water holding capacity of red meat products: A review of recent research advances. *Critical Reviews in Food Science and Nutrition*, 48, 137-159.
- Companies and Markets. (2012). Feed acidifiers: A global strategic business report. Ban on animal feed antibiotics throws spotlight on feed acidifiers. Retrieved from

<http://www.companiesandmarkets.com/Market/Chemicals/Market-Research/Feed-Acidifiers-A-Global-Strategic-Business-Report/RPT933926>

- Corrier, D. E., Byrd, J. A., Hargis, B. M., Hume, M. E., Bailey, R. H., & Stanker, L. H. (1999). Presence of *Salmonella* in the crop and ceca of broiler chickens before and after preslaughter feed withdrawal. *Poultry Science*, 78, 45-49.
- Dai, S., Wang, L., Wen, A., Wang, L., & Jin, G. (2009). Dietary glutamine supplementation improves growth performance, meat quality and colour stability of broilers under heat stress. *British Poultry Science*, 50, 333-340.
- den Hertog-Meischke, M. J. A., Van Laack, R. J. L. M., & Smulders, F. J. M. (1997). The water-holding capacity of fresh meat. *Veterinary Quarterly*, 19, 175-181.
- Gregory, N. (1994). Preslaughter handling, stunning and slaughter. *Meat Science*, 36, 45-56.
- Gregory, N. (1996). Welfare and hygiene during preslaughter handling. *Meat Science*, 43, 35-46.
- Grey, T. C., Jones, J. M., & Robinson, D. S. (1974). The influence of death struggle on the rate of glycolysis in chicken breast muscle. *Journal of the Science of Food and Agriculture*, 25, 57-66.
- Jarquín, R. L., Nava, G. M., Wolfenden, A. D., Donoghue, A. M., Hanning, I., Higgins, S. E., & Hargis, B. M. (2007). The evaluation of organic acids and probiotic cultures to reduce *Salmonella* Enteritidis horizontal transmission and crop infection in broiler chickens. *International Journal of Poultry Science*, 6(3), 182-186.
- Kannan, G., Heath, J., Wabeck, C., Souza, M., Howe, J., & Mench, J. (1997). Effects of crating and transport on stress and meat quality characteristics in broilers. *Poultry Science*, 76, 523-529.
- Lua, L., Ji, C., Luo, X. G., Liu, B., Yu, S. X. (2006). The effect of supplemental manganese in broiler diets on abdominal fat deposition and meat quality. *Animal Feed Science and Technology*, 129, 49-59.
- Maribo, H., Olsen, E. V., Barton-Gade, P., Møller, A. J., & Karlsson, A. (1998). Effect of early post-mortem cooling on temperature, pH fall and meat quality in pigs. *Meat Science*, 50, 115-129.
- Mortensen, M., Andersen, H. J., Engelsen, S. B., & Bertram, H. C. (2006). Effect of freezing temperature, thawing and cooking rate on water distribution in two pork qualities. *Meat Science*, 72, 34-42.
- Northcutt, J., Berrang, M., Dickens, J., Fletcher, D., & Cox, N. (2003). Effect of broiler age, feed withdrawal, and transportation on levels of coliforms, *Campylobacter*, *Escherichia coli* and *Salmonella* on carcasses before and after immersion chilling. *Poultry Science*, 82, 169-173.

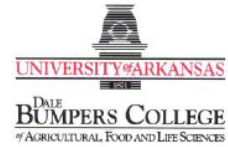
- Pearce, K. L., Rosenvold, K., Andersen, H. J., & Hopkins, D. L. (2011). Water distribution and mobility in meat during the conversion of muscle to meat and ageing and the impacts on fresh meat quality attributes-A review. *Meat Science*, 89, 111-124.
- Pedersen, D. K., Morel, S., Andersen, H. J., & Balling Engelsen, S. (2003). Early prediction of water-holding capacity in meat by multivariate vibrational spectroscopy. *Meat Science*, 65, 581-592.
- Petracci, M., Fletcher, D., & Northcutt, J. (2001). The effect of holding temperature on live shrink, processing yield, and breast meat quality of broiler chickens. *Poultry Science*, 80, 670-675.
- Petracci, M., Bianchi, M., Cavani, C., Gaspari, P., & Lavazza, A. (2006). Preslaughter mortality in broiler chickens, turkeys, and spent hens under commercial slaughtering. *Poultry Science*, 85, 1660-1664.
- Pixley, C., Barton, J., Vicente, J., Wolfenden, A., Hargis, B., & Tellez, G. (2010). Evaluation of a commercially available organic acid product during feed withdrawal and its relation to carcass shrink in commercial turkeys. *International Journal of Poultry Science*, 9, 508-510.
- Qiao, M., Fletcher, D., Smith, D., & Northcutt, J. (2002). Effects of raw broiler breast meat color variation on marination and cooked meat quality. *Poultry Science*, 81, 276-280.
- Rosenvold, K., & Andersen, H. J. (2003). Factors of significance for pork quality-a review. *Meat Science*, 64, 219-237.
- Savenije, B., Lambooi, E., Gerritzen, M., Venema, K., & Korf, J. (2002). Effects of feed deprivation and transport on preslaughter blood metabolites, early postmortem muscle metabolites, and meat quality. *Poultry Science*, 81, 699-708.
- Tung, C. M., & Pettigrew, J. E. (2011). Critical review of acidifiers. Practical ideas to address high feed and production costs. National Pork board web. Board, Des Moines, IA USA. Retrieved from <http://www.pork.org/ResearchDetail/541/CriticalReviewofAcid.aspx>
- Vanderhasselt, R., Buijs, S., Sprenger, M., Goethals, K., Willemsen, H., Duchateau, L., & Tuytens, F. (2013). Dehydration indicators for broiler chickens at slaughter. *Poultry Science*, 92, 612-619.
- Veerkamp, C. (1986). Fasting and yield of broilers. *Poultry Science*, 65, 1299-1304.
- Vicente, J., Higgins, S., Bielke, L., Tellez, G., Donoghue, D., Donoghue, A., & Hargis, B. M. (2007). Effect of probiotic culture candidates on *Salmonella* prevalence in commercial turkey houses. *Journal of Applied Poultry Research*, 16, 471-476.
- Warriss, P., & Brown, S. (1987). The relationships between initial pH, reflectance and exudation in pig muscle. *Meat Science*, 20, 65-74.

- Warriss, P., Kestin, S., Brown, S., & Bevis, E. (1988). Depletion of glycogen reserves in fasting broiler chickens. *British Poultry Science*, 29, 149-154.
- Warriss, P., Bevis, E., & Brown, S. (1990). Time spent by broiler chickens in transit to processing plants. *Veterinary Record*, 127, 617-619.
- Warriss, P., Kestin, S., Brown, S., Knowles, T., Wilkins, L., Edwards, J., & Nicol, C. (1993). The depletion of glycogen stores and indices of dehydration in transported broilers. *British Veterinary Journal*, 149, 391-398.
- Wolfenden, A., Pixley, C., Vicente, J. L., Aviña, L., Hargis, B. M., & Tellez, G. (2007a) Effect of organic acids during feed withdrawal on mortality, shrinkage and carcass condemnation during transportation to the processing plant. *International Journal of Poultry Science*, 6, 497-500.
- Wolfenden, A., Vicente, J., Higgins, J., Andreatti Filho, R., Higgins, S., Hargis, B. M., & Tellez, G. (2007b). Effect of organic acids and probiotics on *Salmonella enteritidis* infection in broiler chickens *International Journal of Poultry Science*, 6, 403-405.
- Yi, G., Allee, G., Knight, C., & Dibner, J. (2005). Impact of glutamine and oasis hatchling supplement on growth performance, small intestinal morphology, and immune response of broilers vaccinated and challenged with *Eimeria maxima*. *Poultry Science*, 84, 283-293.
- Zamorano, J., & Gambaruto, M. (1997). Contribution to improving the meat water holding capacity test by the filter paper press method. A comparison of three methods for measuring areas. *Meat Science*, 46, 129-137.

## APPENDIX



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To Whom It May Concern,

The first author of enclosed paper, “Evaluation of a Commercially Available Organic Acid Product on Body Weight Loss, Carcass Yield, and Meat Quality During Pre-Slaughter Feed Withdrawal in Broiler Chickens: A Poultry Welfare and Economic Perspective”, is Anita Menconi. Anita was primarily responsible for the work and research associated with this paper, and completed greater than 51% of the work.

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B.M. Hargis

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## MEMORANDUM

TO: Lisa R. Bielke

FROM: Craig N. Coon, Chairman  
Institutional Animal Care  
And Use Committee

DATE: June 9, 2011

SUBJECT: IACUC PROTOCOL APPROVAL  
Expiration date : June 2, 2014

The Institutional Animal Care and Use Committee (IACUC) has APPROVED Protocol #11052-  
“USE OF GRAS APPROVED FEED INGREDIENTS FOR INCLUSION IN POULTRY  
DRINKING WATER TO SANITIZE CROP CONTENTS”. You may begin this study  
immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF  
committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has  
components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be  
any changes in the protocol during the research, please notify the IACUC in writing  
[Modification Request form] prior to initiating the changes. If the study period is expected to  
extend beyond 06-02-2014, you must submit a new protocol. By policy the IACUC cannot  
approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines  
for research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian

## CHAPTER III

### **Effect of different concentrations of acetic, citric, and propionic acid dipping solutions on bacterial contamination of raw chicken skin**

A. Menconi\*, S. Shivaramaiah\*, A. Londero†, A. R. Reginatto†, G. R. Huff‡, O. Prado§, J. E. Morales#, N. R. Pumford\*, M. Morgan\*, A. Wolfenden\*, L. R. Bielke\*, B. M. Hargis\*, and G. Tellez\*1

\*Department of Poultry Science, University of Arkansas, Fayetteville AR 72701

†Depto. de Medicina Veterinária – CCR Universidade Federal de Santa Maria, Brazil

‡Poultry Production and Product Safety Research Unit, USDA, Agricultural Research Service, Poultry Science Center, University of Arkansas, Fayetteville 72701

§Laboratorio de Producción Avícola. FMVZ. Universidad de Colima. Tecomán, Colima 28100

#Departamento de Producción Agrícola y Animal, Universidad Autónoma Metropolitana, México D .F. 04960.

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## **ABSTRACT**

Bacterial contamination of raw, processed poultry may include spoilage bacteria and foodborne pathogens. We evaluated different combinations of organic acid (OA) wash solutions for their ability to reduce bacterial contamination of raw chicken skin and to inhibit growth of spoilage bacteria and pathogens on skin during refrigerated storage. In experiment 1, raw chicken skin samples were dipped into a suspension of either  $10^8$  cfu/mL of *Salmonella* Typhimurium (ST), *Escherichia coli* O157:H7 (EC), or *Listeria monocytogenes* (LM) for 30 s and then immersed in either phosphate buffered saline (PBS) or an OA wash solution mixture of 0.8% citric, 0.8% acetic, and 0.8% propionic acid (at equal w/v concentrations) for an additional 30 s. In experiment 2, three different concentrations of the OA wash solution (0.2, 0.4, and 0.6% at equal w/v concentrations) were tested against chicken skin samples contaminated with ST. Viable pathogenic bacteria on each skin sample were enumerated after 1 and 24 h of storage at 4 °C in both experiments. In experiment 3, skin samples were initially treated on day one with either PBS or two concentrations of the OA mixture (0.4% and 0.8%) and total aerobic bacteria were enumerated during a two week storage period. In all experiments, significant ( $p < 0.05$ ) differences were observed when skin samples were treated with the OA wash solution and no spoilage organisms were recovered at any given time-point, while increasing  $\log_{10}$  numbers of spoilage organisms were recovered over time in PBS treated skin samples. These results suggest that 0.2 - 0.8% concentrations of an equal-percentage mixture of this OA combination may reduce pathogens and spoilage organisms and improve food safety properties of raw poultry.

**Key words:** organic acids, foodborne pathogens, skin rinse, chickens, shelf-life

## INTRODUCTION

The poultry and beef industries have the challenge of controlling *Salmonella*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* within processing and manufacturing facilities (Dickson et al., 1992; Harris et al., 2006; Lynch et al., 2006; Laury et al., 2009; Zhao et al., 2009). Poultry and poultry products have been identified by some researchers as the most important source of transmission of *Salmonella* to humans (Lynch et al., 2006). Contamination by *Salmonella* on live animals and carcasses can occur during transportation and processing (Bourassa et al., 2004; Parveen et al., 2007). A 2007 study reported that 88% of chicken carcasses were contaminated with *Salmonella*, and 80% of the isolates were resistant to one or more antibiotics (Parveen et al., 2007).

Chickens contain large numbers of bacteria in their gastrointestinal tract, feathers, and feet; therefore fecal bacteria are present on chicken carcasses immediately after processing (Ramirez et al., 1997; Northcutt et al., 2003). Consequently, acceptable methods of intervention are needed to decrease populations of spoilage bacteria and foodborne enteropathogens. Antimicrobial chemicals are commonly used during processing to reduce pathogen loads on carcasses, and the most common antimicrobial treatment used for decontamination of poultry meat is chlorine (sodium hypochlorite) (Mountney and O'malley, 1965). As reported by Mountney and O'malley (1965), chlorine was effective in reducing *Salmonella* and *Campylobacter* by only as much as 1 to 2 log<sub>10</sub> on poultry carcasses. While this may be enough to eliminate *Salmonella* from most poultry carcasses, chlorine may bind to organic matter, and be ineffective. In fact, the continued lack of decline in rates of foodborne illness (MMWR, 2011; Scallan et al., 2011) indicates that chlorine treatment of carcasses in the processing facility is not effectively reducing the incidence of *Salmonella* contamination. Moreover, failure to optimize

the disinfectant properties of chlorine (improper pH, concentration, or composition of incoming water) may reduce its efficacy. Chlorine treatment may also cause offensive and harmful odors due to the production of chlorine gas and trichloramines (Northcutt et al., 2005; Hinton et al., 2007; Northcutt et al., 2008).

Because of these reasons, alternative methods to disinfect poultry carcass are needed. Studies using organic acids to spray or dip poultry carcasses have shown as much as 3 log<sub>10</sub> of *Salmonella* reduction (Bilgili et al., 1998; Vasseur et al., 1999; Kubena et al., 2001; Hinton and Ingram, 2005; Lu et al., 2005; Harris et al., 2006; Van Immerseel et al., 2006). A specific example was the use of 2 % lactic acid sprayed on chicken carcasses by Yang et al. (1998), which resulted in a 2 log<sub>10</sub> cfu per carcass reduction of *Salmonella*.

In this regard, the use of organic acids may be a viable alternative to avoid hazards associated with chlorine. Therefore, the objectives of these studies were to determine the effects of a mixture of different concentrations of organic acid rinse solutions at reducing foodborne pathogens and spoilage organisms on the surface of contaminated raw chicken skin during storage at 4 °C.

## **MATERIALS AND METHODS**

### ***Chicken skin samples***

Forceps and scissors were used to aseptically remove strips of skin (approximately 2 cm X 2 cm) from chicken thighs (Sarlin et al., 1988) purchased from a local supermarket.

### ***Bacterial strains***

A poultry isolate of *Salmonella enterica* subspecies *enterica* serovar Typhimurium (ST) was used for all experiments. An enterohemorrhagic *Escherichia coli* O157:H7 (EC) strain, negative for sorbitol fermentation, as well as a laboratory strain of *Listeria monocytogenes* (LM) were obtained from the Biomass Research Center and USDA Food Safety Lab (University of Arkansas, Fayetteville, AR). The amplification and enumeration protocol for these isolates has previously been described (Tellez et al., 1993).

### ***ST, EC, and LM culture preparation***

A frozen aliquot of each pathogen was inoculated into 10 mL of brain heart infusion (BHI) broth (Difco, Sparks, MD, USA) and incubated at 37 °C for 24 h in a shaking incubator (New Brunswick Scientific, Edison, N.J., U.S.A.) at 200 rpm. After 24 h, 10 mL of fresh BHI was inoculated with 10 µL of this culture, vortexed, and incubated at 37 °C for 18 h at 200 rpm to ensure that the bacterial culture was in the exponential growth phase. Finally, 10 mL of fresh BHI was inoculated with 20 µL of the 18 h culture to obtain a concentration of approximately 10<sup>8</sup> cfu/mL.

### ***Organic acid (OA) wash solution***

For use in these experiments, mixtures of equal concentrations (w/v) of acetic (Mallinckrodt Chemicals, Phillipsburg, NJ), citric (Sigma, St. Louis, MO), and propionic (Sigma) acids were prepared. All of these acids are considered Generally Recognized as Safe (GRAS) and are commonly employed in the food industry (USDA-FSIS, 2005).

### ***Experimental design***

**Experiment 1.** Chicken skin samples were dipped into a suspension of  $10^8$  cfu/mL of ST (N = 20), EC (N = 20), or LM (N = 20) for 30 seconds. Skin samples were then removed and dipped into a solution of either phosphate buffered saline (PBS) (control; N = 30) or an OA wash solution (N = 30) of 0.8 % final concentration of each of the acids for an additional 30 s. Control and treated samples were placed in individual sample bags and kept in a refrigerator at 4 °C. At 1 h and 24 h, 5 control and 5 treated samples were removed from the refrigerator and cultured separately for each pathogen. Briefly, skin samples were homogenized within sterile sample bags using a rubber mallet. Sterile saline (5 mL) was added to each sample bag and hand stomached. Serial dilutions were spread plated on brilliant green agar (Becton, Dickinson and Co. Sparks, MD) plates containing 25 µg/mL novobiocin (NO; Sigma, St. Louis, MO) and 20 µg/mL nalidixic acid (NA; Sigma, St. Louis, MO) for ST; MacConkey Sorbitol Agar for EC, (Becton, Dickinson and Co. Sparks, MD); or Oxoid *Listeria* selective agar (EMD Chemicals Inc. Gibbstown, NJ) for LM. Each sample was plated in triplicate. The plates were incubated at 37 °C for 24 h, and viable colonies were observed and enumerated.

**Experiment 2.** Skin samples (N= 40) were dipped into a suspension of  $10^6$  cfu/mL of ST for 30 s. Skin samples were then removed and dipped into a solution of either PBS (control; N = 10) or the OA wash solution at 0.2 % (N = 10), 0.4 % (N = 10) or 0.6 % (N = 10) final concentration of each of the acids for an additional 30 s. Samples were placed in individual sample bags and kept in a refrigerator at 4 °C. At 1 h or 24 h, 5 control and 5 treated samples were removed from the refrigerator and cultured separately for ST recovery as described in experiment 1.

**Experiment 3.** Skin samples (N = 105) were dipped into a solution of  $10^6$  cfu/mL of ST for 30 s. Skin samples were then removed and dipped into a solution of either PBS (control; N = 35) or the OA wash solution at 0.4% (N = 35) or 0.8% (N = 35) final concentration of each acid for an additional 30 s. Control and treated samples were placed in individual sample bags and kept in a refrigerator at 4 °C. At 1 h, 24 h, 3 days, 6 days, 9 days, 12 days, and 15 days 5 control and 5 treated samples were removed from the refrigerator and cultured separately for ST recovery as described in experiment 1.

**Experiment 4.** Skin samples were dipped into a solution of either PBS (control; N = 35) or the OA wash solution at 0.4% (N = 35) or 0.8% (N = 35) final concentration of each acid for an additional 30 s. Control and treated samples were placed in individual sample bags and kept in a refrigerator at 4 °C. At 1 h, 24 h, 3 days, 6 days, 9 days, 12 days, and 15 days 5 control and 5 treated skin samples were homogenized within sterile sample bags using a rubber mallet. Sterile saline (5 mL) was added to each sample bag and hand stomached. Serial dilutions were spread plated on tryptic soy agar (TSA) (Becton Dickinson and Co., Sparks, MD) and MacConkey agar (Becton, Dickinson and Co. Sparks, MD). Each sample was plated in triplicate. The plates were incubated at 37 °C for 24 h, and viable colonies were observed and enumerated. Bacterial identification of different morphology colonies that grew on MacConkey agar was determined using the API-20E test kit for the identification of enteric Gram-negative bacteria (bioMerieux, Inc., Hazelwood, MO).



### *Statistical analysis*

In all experiments, for each foodborne pathogen or psychotropic bacteria, cfu/skin section in control or treated group respectively, was analyzed using Analysis of Variance (ANOVA) with further separation of significantly different means using Duncan's Multiple Range test using SAS (SAS Institute, 2002). Significant differences were reported at  $P < 0.05$ .

## **RESULTS**

Table 1 summarizes the effect of 0.8% organic acid (OA) wash solution on chicken skin inoculated with ST, EC or LM in experiment 1. The OA wash solution caused a 3.8 cfu/skin section  $\log_{10}$  and 3.2 cfu/ skin section  $\log_{10}$  reduction in presumptive ST and EC respectively 1 h after cold storage. By 24 h, no ST or EC were recovered from treated samples. For presumptive LM, there was a 1.85 cfu/skin section and 2.87 cfu/skin section  $\log_{10}$  reduction at 1 h and 24 h respectively.

Table 2 summarizes the results of three additional concentrations (0.2%, 0.4%, or 0.6%) of the same OA wash solution used as a sanitizing dip for raw chicken skin samples inoculated with ST. All 3 concentrations were able to significantly reduce presumptive ST at both 1 and 24 h of storage, and no ST were recovered from skin dipped in 0.6% solutions after 24 h of storage. However, 0.6% OA mixture solution showed complete bactericidal activity against ST by 24 h.

Table 3 summarizes the effect of the OA wash solution at a concentration of either 0.4% or 0.8% on ST skin rinse in experiment 3. At 1 h post treatment, the 0.8 % OA wash solution

significantly reduced ( $p < 0.05$ ) presumptive ST cfu by 1.72 cfu/skin section  $\log_{10}$  compared with control skin samples, while at a concentration of 0.4%, there was a numerical decrease in presumptive ST cfu ( $p > 0.05$ ). However, both OA mixtures significantly reduced total

presumptive ST cfu recovered at all other storage times (24 h, 3 d, 6 d, 9 d, 12 d, and 15 d). In all samples treated with either concentration of the OA wash solution, *Salmonella* was not detected at days 9, 12, and 15 post-treatment. In contrast, control skin samples showed a numerical increase in ST cfu at each day of sampling (Table 3).

The results of experiment 4, the effect of 0.4% or 0.8% OA wash solutions on total aerobic bacterial cfu skin section of chicken skin are summarized in tables 4 and 5. On tryptic soy agar, after 1 h of cold storage, the total number of aerobic bacteria detected was low in the control samples. However, in both OA wash solutions, no bacteria were detected at this time of evaluation. At all other times of evaluation, control samples showed an increase in total cfu/skin section of chicken skin with a sharp increase between 3 and 6 d post-storage and was significantly different ( $p < 0.05$ ) from both treated groups. Compared with control samples, the 0.4% OA wash solution showed a significant reduction ( $p < 0.05$ ) in total cfu/skin section at 24 h and 3 d post-storage. At 6, 9, 12, and 15 d, no aerobic bacteria were recovered from skin samples treated with the 0.4 % OA wash solution. Interestingly, at all times of evaluation, no aerobic bacteria were recovered from skin samples treated with the 0.8% OA wash solution (Table 4). Samples from both control and treated bags were plated on MacConkey agar for the detection of Gram-negative bacteria associated with food spoilage (Table 5). Both OA wash solutions inhibited the growth of Gram-negative bacteria at all times of evaluation ( $p < 0.05$ ). However, bacteria were recovered from the 24 h samples and these numbers increased subsequently in the control samples (Table 5). At days 9, 12, and 15, tests determined that *Escherichia* spp., *Enterobacter* spp., and *Pseudomonas* spp. were among the predominant bacterial flora on the broiler skin (data not shown).

## DISCUSSION

In general, carcass rinse applications that decrease *Salmonella* by 2 log<sub>10</sub> cfu/ml are considered effective, since most carcasses are considered to have about 100 *Salmonella* cells (Jetton, et al., 1992). Lactic acid and citric acid at concentrations of 1-3% have been shown to reduce *E. coli* O157:H7, *Salmonella* serotypes, and *Listeria monocytogenes* when sprayed on beef and poultry carcasses by causing intracellular acidification (Vasseur et al., 1999). According to Vasseur et al. (1999), citric acid showed to have the highest inhibitory effect because of its ability to diffuse through the cell membrane. In the same experiment, lactic acid decreased the ionic concentration within the bacterial cell membrane, leading to accumulation of acid within the cell cytoplasm, disruption of the proton motive force, and inhibition of substrate transport (Vasseur et al., 1999).

In these experiments, the blend of OA wash solution showed significant anti-bacterial activity against three foodborne pathogens commonly implicated in meat processing (Table 1). Additionally, we also found that lower concentrations of the OA wash solution are almost as effective as higher concentrations and based on these experiments, we conclude that a concentration of 0.4% demonstrates optimum anti-bacterial/bactericidal activity (Tables 2-5). Furthermore, the OA wash solution, when used at a concentration of 0.4%, was able to prevent recovery of aerobic food-spoilage bacteria up to two weeks of storage at 4 °C, indicating that one wash with this solution may enhance shelf-life of packaged meat significantly. Overall, the results of these experiments suggest that dipping raw chicken skin in an OA wash solution of citric, lactic, and propionic acids can greatly reduce populations of pathogenic bacteria, thus enhancing overall food safety and shelf life of chicken meat. Poultry meat quality is a concern when using different organic acid washes. In an earlier study, the quality effects of acetic, citric,

lactic, malic, mandelic, or tartaric acids at 0.5, 1, 2, 4, and 6% concentrations were tested on broiler carcasses, revealing that in simulated dip application, each of the acids decreased lightness and increased redness and yellowness values in the skin of broiler carcasses with increasing acid concentration (Bilgili et al., 1998). Therefore, future research will be directed at determining the effect of these organic acids on the texture, color, oxidative stability, pH, and consumer acceptance of chicken meat with treatment combinations that exhibited the most effective antibacterial activity.

## TABLES

**Table 1.** Experiment 1: Effect of rinsing chicken skin with an organic acid mixture (OAM)\* on recovery of presumptive *Salmonella* Typhimurium (ST), *Escherichia coli* O: 157:H7 (EC), and *Listeria monocytogenes* (LM).

Time of sampling (h)	Control ST	OAM ST	Control EC	OAM EC	Control LM	OAM LM
1	6.0 ± 0.07 <sup>a</sup>	2.20 ± 0.75 <sup>b</sup>	7.57 ± 0.10 <sup>a</sup>	4.32 ± 0.24 <sup>b</sup>	7.39 ± 0.01 <sup>a</sup>	5.54 ± 0.13 <sup>b</sup>
24	6.90 ± 0.04 <sup>a</sup>	0 ± 0 <sup>b</sup>	7.12 ± 0.09 <sup>a</sup>	0 ± 0 <sup>b</sup>	7.21 ± 0.09 <sup>a</sup>	4.34 ± 0.44 <sup>b</sup>

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\*Organic acid mixture (OAM) = 0.8% acetic acid, 0.8% citric acid, and 0.8% propionic acid

Data expressed as log<sub>10</sub> cfu/ skin section mean ± standard error.

Values within rows for control or treated group for each foodborne pathogen respectively, with different lowercase superscripts differ significantly (P < 0.05).

**Table 2.** Experiment 2: Effect of three different concentrations of an organic acid mixture (OAM)\* rinse solutions on chicken skin inoculated with *Salmonella* Typhimurium (ST).

<b>Treatment</b>	<b>1 hour</b>	<b>24 hours</b>
<b>Control PBS</b>	6.8 ± 0.04 <sup>a</sup>	6.2 ± 0.09 <sup>a</sup>
<b>0.2% OAM</b>	5.5 ± 0.18 <sup>b</sup>	2.08 ± 1.2 <sup>b</sup>
<b>0.3% OAM</b>	4.6 ± 0.09 <sup>c</sup>	1.4 ± 0.87 <sup>b</sup>
<b>0.4% OAM</b>	4.6 ± 0.17 <sup>c</sup>	0.0 ± 0.0 <sup>c</sup>

\* Organic acid mixture (OAM) = acetic acid, citric acid, and propionic acid  
 Data expressed as log<sub>10</sub> cfu/ skin section mean ± standard error. Values within columns with different superscripts differ significantly (P < 0.05).

**Table 3.** Experiment 3: Effect of two different concentrations of an organic acid mixture (OAM)\* rinse solution on chicken skin inoculated with *Salmonella* Typhimurium (ST)

<b>Sample time</b>	<b>Control PBS</b>	<b>0.4% OAM</b>	<b>0.8% OAM</b>
<b>1 hour</b>	3.37 ± 0.20 <sup>a</sup>	2.01 ± 0.83 <sup>ab</sup>	1.65 ± 1.05 <sup>b</sup>
<b>24 hours</b>	3.55 ± 0.30 <sup>a</sup>	1.26 ± 0.77 <sup>bc</sup>	0 ± 0 <sup>c</sup>
<b>3 days</b>	3.31 ± 0.30 <sup>a</sup>	0 ± 0 <sup>b</sup>	0.60 ± 0.60 <sup>b</sup>
<b>6 days</b>	3.40 ± 0.31 <sup>a</sup>	0.60 ± 0.60 <sup>b</sup>	0 ± 0 <sup>b</sup>
<b>9 days</b>	3.49 ± 0.33 <sup>a</sup>	0 ± 0 <sup>b</sup>	0 ± 0 <sup>b</sup>
<b>12 days</b>	4.89 ± 0.32 <sup>a</sup>	0 ± 0 <sup>b</sup>	0 ± 0 <sup>b</sup>
<b>15 days</b>	6.82 ± 0.15 <sup>a</sup>	0 ± 0 <sup>b</sup>	0 ± 0 <sup>b</sup>

\* Organic acid mixture (OAM) = acetic acid, citric acid, and propionic acid  
 Data expressed as log<sub>10</sub> mean ± standard error. Values within treatment rows with different superscripts differ significantly (P < 0.05).

**Table 4.** Experiment 4: Effect of two different concentrations of organic acid mixture (OAM)\* rinse solutions on total cfu/skin section of chicken skin plated on tryptic soy agar plates.

<b>Sample time</b>	<b>Control PBS</b>	<b>0.4% OAM</b>	<b>0.8% OAM</b>
<b>1 hour</b>	0.60 ± 0.60 <sup>a</sup>	0 ± 0 <sup>b</sup>	0 ± 0 <sup>b</sup>
<b>24 hours</b>	1.62 ± 0.66 <sup>a</sup>	0.60 ± 0.60 <sup>ab</sup>	0 ± 0 <sup>b</sup>
<b>3 days</b>	4.49 ± 0.39 <sup>a</sup>	2.45 ± 1.51 <sup>b</sup>	0 ± 0 <sup>c</sup>
<b>6 days</b>	7.03 ± 0.37 <sup>a</sup>	0 ± 0 <sup>b</sup>	0 ± 0 <sup>b</sup>
<b>9 days</b>	7.26 ± 0.19 <sup>a</sup>	0 ± 0 <sup>b</sup>	0 ± 0 <sup>b</sup>
<b>12 days</b>	7.61 ± 0.23 <sup>a</sup>	0 ± 0 <sup>b</sup>	0 ± 0 <sup>b</sup>
<b>15 days</b>	7.99 ± 0.27 <sup>a</sup>	0 ± 0 <sup>b</sup>	0 ± 0 <sup>b</sup>

\* Organic acid mixture (OAM) = acetic acid, citric acid, and propionic acid  
 Data expressed as log<sub>10</sub> mean ± standard error. Values within treatment rows with different superscripts differ significantly (P < 0.05).



**Table 5.** Experiment 4: Effect of two different organic acid mixture (OAM)\* rinse solutions on total cfu/skin of chicken skin plated on MacConkey agar plates.

<b>Sample time</b>	<b>Control PBS</b>	<b>0.4% OAM</b>	<b>0.8% OAM</b>
<b>1 hour</b>	$0 \pm 0^a$	$0 \pm 0^a$	$0 \pm 0^a$
<b>24 hours</b>	$0.60 \pm 0.60^a$	$0 \pm 0^b$	$0 \pm 0^b$
<b>3 days</b>	$5.09 \pm 0.16^a$	$0 \pm 0^b$	$0 \pm 0^b$
<b>6 days</b>	$6.52 \pm 0.26^a$	$0 \pm 0^b$	$0 \pm 0^b$
<b>9 days</b>	TMTC	0	0
<b>12 days</b>	TMTC	0	0
<b>15 days</b>	TMTC	0	0

\* Organic acid mixture (OAM) = acetic acid, citric acid, and propionic acid

TMTC = too many to count at the dilution corresponding to  $10^7$  cfu/skin section.

Data expressed as  $\log_{10}$  cfu/skin section mean  $\pm$  standard error. Values within treatment rows with different lowercase superscripts differ significantly ( $P < 0.05$ ).

## REFERENCES

- Bilgili, S. F., Conner, D. E., Pinion, J. L., & Tamblyn, K. C. (1998). Broiler skin color as affected by organic acids: influence of concentration and method of application. *Poultry Science*, 77, 752-757.
- Bourassa, D. V., Fletcher, D. L., Buhr, R. J., Berrang, M. E., & Cason, J. A. (2004). Recovery of *Salmonellae* from trisodium phosphate-treated commercially processed broiler carcasses after chilling and after seven-day storage. *Poultry Science*, 83, 2079-2082.
- Dickson, J. S., Siragusa, G. R., & Wray Jr, J. E. (1992). Predicting the growth of *Salmonella typhimurium* on beef by using the temperature function integration technique. *Applied and Environmental Microbiology*, 58, 3482-3487.
- Harris, K., Miller, M. F., Loneragan, G. H., & Brashears, M. M. (2006). Validation of the use of organic acids and acidified sodium chlorite to reduce *Escherichia coli* O157 and *Salmonella typhimurium* in beef trim and ground beef in a simulated processing environment. *Journal of Food Protection*, 69, 1802-1807.
- Hinton Jr, A., Northcutt, J. K., Smith, D. P., Musgrove, M. T., & Ingram, K. D. (2007). Spoilage microflora of broiler carcasses washed with electrolyzed oxidizing or chlorinated water using an inside-outside bird washer. *Poultry Science*, 86, 123-127.
- Hinton Jr, A., & Ingram, K. D. (2005). Microbicidal activity of tripotassium phosphate and fatty acids toward spoilage and pathogenic bacteria associated with poultry. *Journal of Food Protection*, 68, 1462-1466.
- Jetton, J. P., Bilgili, S. F., Conner, D. E., Kotrola, J. S., & Reiber, M. A. (1992). Recovery of salmonellae from chilled broiler carcasses as affected by rinse media and enumeration method. *Journal of Food Protection*, 55, 329-332.
- Kubena, L. F., Byrd, J. A., Young, C. R., & Corrier, D. E. (2001). Effects of tannic acid on cecal volatile fatty acids and susceptibility to *Salmonella Typhimurium* colonization in broiler chicks. *Poultry Science*, 80, 1293-1298.
- Laury, A. M., Alvarado, M. V., Nace, G., Alvarado, C. Z., Brooks, J. C., Echeverry, A., & Brashears, M. M. (2009). Validation of a lactic acid- and citric acid-based antimicrobial product for the reduction of *Escherichia coli* O157: H7 and *Salmonella* on beef tips and whole chicken carcasses. *Journal of Food Protection*, 72, 2208-2211.
- Lu, Z., Sebranek, J. G., Dickson, J. S., Mendonca, A. F., & Bailey, T. B. (2005). Application of predictive models to estimate *Listeria monocytogenes* growth on frankfurters treated with organic acid salts. *Journal of Food Protection*, 68, 2326-2332.
- Lynch, M., Painter, J., Woodruff, R., Braden, C., & Centers for Disease Control and Prevention. (2006). Surveillance for foodborne-disease outbreaks-United States, 1998-2002. *Morbidity and Mortality Weekly Report Surveillance Summaries*, 55, 1-42.

- MMWR (2011). Morbidity and Mortality Weekly Report, 60(22). Retrieved from <http://www.cdc.gov/mmwr>
- Mountney, G. J., & O'malley, J. (1965). Acids as poultry meat preservatives. *Poultry Science*, 44, 582-586.
- Northcutt, J. K., Smith, D. P., Musgrove, M. T., Ingram, K. D., & Hinton Jr., A. (2005). Microbiological impact of spray washing broiler carcasses using different chlorine concentrations and water temperatures. *Poultry Science*, 84, 1648-1652.
- Northcutt, J. K., Smith, D., Huezo, R. I., & Ingram, K. D. (2008). Microbiology of broiler carcasses and chemistry of chiller water as affected by water reuse. *Poultry Science*, 87, 1458-1463.
- Northcutt, J., Berrang, M., Dickens, J., Fletcher, D., & Cox, N. (2003). Effect of broiler age, feed withdrawal, and transportation on levels of coliforms, *Campylobacter*, *Escherichia coli* and *Salmonella* on carcasses before and after immersion chilling. *Poultry Science*, 82, 169-173.
- Parveen, S., M. Taabodi, J. G. Schwarz, T. P. Oscar, J. Harter-Dennis, and D. G. White. (2007). Prevalence and antimicrobial resistance of *Salmonella* recovered from processed poultry. *Journal of Food Protection*, 70, 2466-2472.
- Ramirez, G. A., Sarlin, L. L., Caldwell, D. J., Yezak Jr, C. R., Hume, M. E., Corrier, D. E., Deloach, J. R., & Hargis, B. M. (1997). Effect of feed withdrawal on the incidence of *Salmonella* in the crops and ceca of market age broiler chickens. *Poultry Science*, 76, 654-656.
- Sarlin, L. L., Barnhart, E. T., Caldwell, D. J., Moore, R. W., Byrd, J. A., Caldwell, D. Y., Corrier, D. E., Deloach, J. R., & Hargis, B. M. (1998). Evaluation of alternative sampling methods for *Salmonella* critical control point determination at broiler processing. *Poultry Science*, 77, 1253-1257.
- SAS Institute Inc. (2002). SAS user's guide: statistics. SAS Institute Inc., Cary, N.C.
- Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., Jones, J. L., & Griffin, P. M. (2011). Foodborne illness acquired in the United States-major pathogens. *Emerging Infectious Disease*, 17, 7-15.
- Tellez, G., Dean, C. E., Corrier, D. E., Deloach, J. R., Jaeger, L., & Hargis, B. M. (1993). Effect of dietary lactose on cecal morphology, pH, organic acids, and *Salmonella* enteritidis organ invasion in Leghorn chicks. *Poultry Science*, 72, 636-642.
- USDA - United States Department of Agriculture, Food Safety and Inspection Service. (2005). FSIS Directive 7120.1 Amendment 5: Safe and suitable ingredients used in the production of meat and poultry products. Retrieved from [http://www.fsis.usda.gov/OPPDE/rdad/FSISDirectives/7120.1\\_Amend\\_5.pdf](http://www.fsis.usda.gov/OPPDE/rdad/FSISDirectives/7120.1_Amend_5.pdf).

- Van Immerseel, F., Russell, J. B., Flythe, M. D., Gantois, I., Timbermont, L., Pasmans, F., Haesebrouck, F., & Ducatelle, R. (2006). The use of organic acids to combat *Salmonella* in poultry: a mechanistic explanation of the efficacy. *Avian Pathology*, 35, 182-188.
- Vasseur, C., Baverel, L., Hebraud, M., & Labadie, J. (1999). Effect of osmotic, alkaline, acid or thermal stresses on the growth and inhibition of *Listeria monocytogenes*. *Journal of Applied Microbiology*, 86, 469-476.
- Yang, Z., Li, Y., & Slavik, M. (1998). Use of antimicrobial spray applied with an inside-outside birdwasher to reduce bacterial contamination on prechilled chicken carcasses. *Journal of Food Protection*, 61, 829-832.
- Zhao, T., Zhao, P., & Doyle, M. P. (2009). Inactivation of *Salmonella* and *Escherichia coli* O157:H7 on lettuce and poultry skin by combinations of levulinic acid and sodium dodecyl sulfate. *Journal of Food Protection*, 72, 928-936.

## APPENDIX



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To Whom It May Concern,

The first author of enclosed paper, “Effect of Different Concentrations of Acetic, Citric, and Propionic Acid Dipping Solutions on Bacterial Contamination of Raw Chicken Skin”, is Anita Menconi. Anita was primarily responsible for the work and research associated with this paper, and completed greater than 51% of the work.


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**VI. CHAPTER IV**

**Identification and characterization of lactic acid bacteria in a commercial  
probiotic culture**

Anita Menconi, Gopala Kallapura, Juan D. Latorre, Marion J. Morgan, Neil R. Pumford, Billy  
M. Hargis, and Guillermo Tellez\*

*Department of Poultry Science, University of Arkansas, Fayetteville, AR 72701, USA*

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## **ABSTRACT**

The aim of the present study was to describe the identification and characterization (physiological properties) of two strains of lactic acid bacteria (LAB 18 and 48) present in a commercial probiotic culture, FloraMax<sup>®</sup>-B11. Isolates were characterized morphologically, and identified biochemically. In addition, the MIDI System ID, the Biolog ID System, and 16S rRNA sequence analyses for identification of LAB 18 and LAB 48 strains were used to compare the identification results. Tolerance and resistance to acidic pH, high osmotic concentration of NaCl, and bile salts were tested in broth medium. *In vitro* assessment of antimicrobial activity against enteropathogenic bacteria and susceptibility to antibiotics were also tested. The results obtained in this study showed the tolerance of LAB 18 and LAB 48, to pH 3.0, 6.5 % of NaCl, and high bile salts concentration (0.6 %). Both strains evaluated showed *in vitro* antibacterial activity against *Salmonella enterica* serovar Enteritidis, *Escherichia coli* (O157:H7), and *Campylobacter jejuni*. These are important characteristics of lactic acid bacteria that should be evaluated when selecting strains to be used as probiotics. Antimicrobial activity of these effective isolates may contribute to efficacy, possibly by direct antimicrobial activity *in vivo*.

**Keywords:** lactic acid bacteria, probiotic, identification, characterization, poultry



## INTRODUCTION

The use of probiotics in agriculture has increased as potential alternatives to antibiotics used as growth promoters, and in select cases, for control of specific enteric pathogens (Anadón et al., 2006; Tellez et al., 2012). For these reasons, the development of effective probiotic products that can be licensed for animal use continues to receive attention (Patterson & Burkholder, 2003). Some characteristics are important for the selection of a successful probiotic such as being tolerant to gastrointestinal environment, being able to attach to the intestinal mucosa, and being exclusively competitive with enteric pathogens (Fontana et al., 2013). Low pH, gastric enzymes, and bile salts are examples of barriers of the gastrointestinal tract that the probiotic bacteria need to resist after being ingested (Bakari et al., 2011; Fontana et al., 2013). Several years ago, our laboratory worked toward the isolation, evaluation, and combination of lactic acid bacteria (LAB) to control foodborne pathogens in the digestive tract of poultry (Tellez et al., 2006). This defined LAB culture has shown accelerated development of normal microflora in chickens and turkeys, providing increased resistance to *Salmonella* spp. infections under laboratory and field research conditions (Higgins et al., 2007, 2008, 2010; Vicente et al., 2007a, 2007b, 2007c, 2008; Wolfenden et al., 2007a, 2007b). There are several publications regarding the efficacy and success of this LAB culture as a poultry probiotic (Tellez et al., 2012), and the purpose of the present study was to describe preliminary and additional data regarding the identification and characterization (physiological properties) of the strains present in this commercial probiotic product.

## **MATERIAL AND METHODS**

### ***Bacterial strains***

Two lactic acid bacteria present in a commercial probiotic culture identified as LAB 18 and LAB 48 were assessed. This LAB probiotic (FloraMax<sup>®</sup>-B11) was licensed to a commercial company (Pacific Vet Group-USA, Inc., Fayetteville, Arkansas 72704, USA).

### ***Morphological and Biochemical tests***

LAB 18 and LAB 48 were cultured aerobically overnight in Man Rogosa Sharpe (MRS, Catalog no. 288110, Becton Dickinson and Co., Sparks, MD 21152 USA) broth and were tested for Gram stain affinity, catalase and oxidase production. Cell morphology and colonial characteristics were observed on MRS agar.

### ***Comparison between 4 identification schemes***

Isolates were sent out for identification and four identification schemes were carried out by three different laboratories. For the identification of both strains, two private laboratories used the MIDI System ID (Micro Test Lab Inc., Agawam, MA 01001, USA; and Microbial ID Inc., Newark, DE 19713, USA), and one private laboratory used 16S rRNA Sequence Analyses (Microbial ID Inc., Newark, DE 19713, USA). Then, a third laboratory (Department of Poultry Science, University of Arkansas) used the Biolog ID System (Biolog, Inc., Hayward, CA 94545, USA) to compare the identification results obtained.

### ***Resistance to pH, temperature, and sodium chloride***

A basal MRS medium was used in these series of *in vitro* studies. An overnight culture of each isolate was used as the inoculum whereby the cells were centrifuged and re-suspended in 0.9% sterile saline. The suspension (100 µl) was inoculated into 10 mL of MRS broth of each test tube. Two incubation time points, i.e. two and four hours were evaluated for each of the variables (pH, temperature, and sodium chloride - NaCl). The rationale for these two points was mainly based on food matter passage time through the gastrointestinal tract of poultry. The temperatures tested were 15 °C and 45 °C, the concentrations of NaCl tested were 3.5 and 6.5% (w/v). The LAB's were tested for survivability using two different pHs (2.0 and 3.0). The tubes were incubated with reciprocal shaking, at the specific test temperatures or at 37 °C for the tests on pH and concentrations of NaCl. At the time points evaluated, each sample was streaked onto MRS agar for presence or absence of growth, to confirm livability of the strains. The turbidity of each tube was also noted as an indication of growth or no-growth. Each treatment was tested with triplicate tubes.

### ***Bile salts tolerance***

The method of Gilliland et al. (1984), with some modifications, was used to determine bile salt tolerance. MRS broth containing 0%, 0.4%, 0.5%, or 0.6% of bile salts No. 3 (Catalog no. 213010, Becton Dickinson and Co., Sparks, MD 21152 USA) was inoculated with  $10^7$  cfu/mL of each probiotic strain, after being centrifuged at 3000 g for 15 minutes and washed three times from their overnight growth cultures. Samples were incubated for 24 h at 37 °C with shaking at 100 rev./min. Growth in control (no bile salts) and test cultures was evaluated at 2, 4, and 24 hours by streaking samples onto MRS agar for presence or absence of growth.

### ***In vitro* assessment of antimicrobial activity against enteropathogenic bacteria**

The lactic acid isolates were screened for *in vitro* antimicrobial activity against *Salmonella enterica* serovar Enteritidis phage type 13A (SE), *Escherichia coli* (O157:H7) (EC), and *Campylobacter jejuni* (CJ). Ten microliters of lactic acid isolates 18 and 48 in FloraMax<sup>®</sup>-B11 were placed in the centre of MRS plates. After 24 h of incubation at 37 °C, the plated samples were overlaid with TSA (Tryptic Soy Agar, catalog no. 211822, Becton Dickinson, Sparks, MD) containing 10<sup>6</sup> cfu/mL of SE or EC. After 24 h of incubation at 37 °C, plates were evaluated and those colonies that produced zones of inhibition were selected. A similar overlay method as described above was used for CJ, where 10<sup>6</sup> cfu/mL of CJ was inoculated in TSA containing 0.2 g of sodium thioglycolate as a reducing agent, and overlaid over the solid agar. Plates were incubated in a microaerophilic environment for 48 h at 42 °C. Colonies that produced zones of inhibition were selected.

## **RESULTS AND DISCUSSION**

### ***Morphological, biochemical, and genotypic identification***

Both phenotypic and genotypic identifications are part of the first step in the selection of potential probiotic bacteria (Fontana et al., 2013). Table 1 summarizes the morphological and biochemical tests of LAB 18 and 48. Both strains tested Gram-positive and catalase and oxidase negative. However, LAB 18 showed a coccal morphology, whereas LAB 48 showed a rod-shaped morphology. Genotypic systems are becoming valuable tools for use in a wide range of microorganisms (Tellez et al., 2012; Fontana et al., 2013). Genotypic 16S rRNA identification of microorganisms from probiotic cultures may be more consistent than the current standard

microbial techniques (Tellez et al., 2012). On the other hand, this method has shown to have issues and limitations. Speciation relies on the closest match with previously identified species in the database because the identification is based on specific sequence homology compared with a known database generated from previously identified organisms through conventional methodologies (Tellez et al., 2012; Fontana et al., 2013). Because databases have been constantly changing and increasing, the same sequence may match other taxons with greater homology. Therefore, at this moment it is nearly impossible to confidently know the speciation of LAB except with very highly characterized isolates (Tellez et al., 2012). Thus, while 16s RNA sequencing can positively identify one LAB isolate as unique among several, true accuracy of homology comparisons is a somewhat subjective.

Even with many new experimental molecular identification techniques, and with the known problem of database accuracy and consistency over time, sequence analysis of 16S rRNA is the major molecular technology presently available for microbial identification (Wagner et al., 2003). Table 2 shows the identification scheme for LABs 18 and 48 using the MIDI System ID (from two laboratories), the Biolog ID System, and the 16S rRNA Sequence Analyses. These results showed that the identification of these strains is difficult; nevertheless, the use of defined cultures for probiotic use is still safer than undefined cultures.

### ***Resistance to pH, temperature, and sodium chloride***

The first host factors that may affect commercial probiotics are the high acidity in the proventriculus and ventriculus and the high concentration of bile components in the proximal intestine (Bakari et al., 2011; Hyronimus et al., 2000). Therefore, being tolerant to acidic conditions is an important criterion to be considered during the selection of potential probiotic

isolates to assure their viability and functionality. Moreover, probiotic bacteria show variable resistance to acidic conditions, and this characteristic is species and strain dependent (Fontana et al., 2013). LAB 18 and 48 did not survive an incubation period of 2 or 4 h at pH 2.0. However, at a pH of 3.0, both strains were resistant after 2 and 4 h of incubation (table 3). As reported by Fontana et al. (2013), *Lactobacillus* spp. isolates have shown to be very resistant to low pH, with high survival rates at pH 3.0 for 1 h. On the contrary, studies show that *Bifidobacterium* spp. isolates are very sensitive to pH 2.0 and pH 3.0 (Fontana et al., 2013). Lactic acid bacteria are acidophilic, which means they are tolerant to low pH. However, this needs to be differentiated from a condition of high concentration of free acids (H<sup>+</sup>), because the free acids may cause growth inhibition (Amrane & Prigent, 1999). Probiotic bacteria need to survive passage through the stomach, where the pH can be as low as 1.5 to 2.0 (Dunne et al., 2001), and stay alive for 4 h or more (Bakari et al., 2011), before they move to the intestinal tract. However, feed passage rate for birds is faster than for other animals, especially mammals; therefore, bacterial acid tolerance is not as critical in chickens as it is in other animals (Boonkumklao et al., 2006).

Both strains grew at 15 and 45 °C at 2 and 4 h of incubation (table 3). Wouters et al. (2000) demonstrated reduced glycolytic activity leading to reduced production of lactic acid in *Lactococcus lactis* at low temperature. According to Ibourahema et al. (Ibourahema et al., 2008), the bacterial capability to grow at high temperature is a good characteristic as it could be interpreted as indicating an increased rate of growth and lactic acid production. Moreover, a high fermentation temperature decreases contamination by other microorganisms (Ibourahema et al., 2008). Both strains were also able to tolerate high osmotic concentrations of NaCl (table 3). This examination gave an indication of the osmotolerance level of the LAB strains. According to Ibourahema et al. (2008), bacterial cells cultured in a high salt concentration could have a loss of

turgor pressure, which would then affect their physiology, enzyme activity, water activity, and metabolism. According to Adnan and Tan (2007), high osmotolerance would be a requirement of LAB strains to be used as commercial strains, because when lactic acid is produced by the strain, alkali would be pumped into the broth to prevent excessive reduction in pH, and the free acid would be converted to its salt form, increasing the osmotic pressure on the bacterial cells.

### ***Bile salts tolerance***

In general, tolerance to bile salts has been considered a condition for colonization and metabolic activity of bacteria in the host's intestine (Havenaar et al., 1992), bile salts can influence the intestinal microflora by acting as an antimicrobial molecule (Fontana et al., 2013). Consequently, when evaluating the potential use of LAB as a probiotic, it is usually important to evaluate their ability to tolerate bile salts (Lee & Salimen, 1995). Table 4 shows the results of bile tolerance of the strains evaluated. LAB 18 and LAB 48 were able to grow when cultured at 0.4%, 0.5%, and 0.6% bile salts concentration at 2, 4, and 24 h of incubation. The average concentration of bile salts in the small intestine is around 0.2% to 0.3%, and may go up to 2 % (w/v), depending upon the individual and the type and amount of food ingested (Kristoffersen et al., 2007; Bakari et al., 2011). According to Xanthopoulos et al. (1997), the ability to tolerate bile salts vary a lot among the LAB species and between strains themselves. Bile resistance of some isolates is related to the enzyme activity of bile salt hydrolase (BSH) that helps to hydrolyze conjugated bile, reducing its toxic effect (Du Toit et al., 1998). BSH activity has most often been found in microorganisms isolated from animals' intestines or feces (Tanaka et al., 1999).

### ***In vitro assessment of antimicrobial activity against enteropathogenic bacteria***

Both strains evaluated showed *in vitro* antibacterial activity against the three enteropathogenic bacteria (Table 5). The inhibitory activity of LAB has been previously reported and is mainly due to the accumulation of primary metabolites such as lactic acid, ethanol, and carbon dioxide and to the production of other antimicrobial compounds such as bacteriocins (Rattanachaikunsopon & Phumkhachorn, 2010). The production levels and proportions among these compounds depend on the biochemical properties of the strains used and physical and chemical conditions of growth (Tannock, 2004).

## **CONCLUSION**

Characterization and identification of beneficial enteric lactic acid bacterial isolates is highly dependent upon methodology. Bile and salt resistance of enteric resident microflora are high, with tolerances expected from resident microflora. Antimicrobial activity of these effective isolates may contribute to efficacy, possibly by direct antimicrobial activity *in vivo*. Alternatively, localized production of volatile fatty acids, and possibly bacteriocins, may contribute to the colonization ability of these isolates to compete locally and colonize within the gastrointestinal tract. Importantly, previous (Higgins et al., 2011) and unpublished research from our laboratory indicates very rapid induction of specific host-gene expression pathways, temporally associated with reductions in enteric colonization with *Salmonella*. While many mechanisms of action have been proposed for the observed efficacy, precise modalities have not been completely described for this highly effective culture.



## TABLES

**Table 1.** Morphological characteristics of the lactic acid bacteria isolates 18 and 48 present in FloraMax<sup>®</sup>-B11

LAB- ID	Anatomic region isolated	Gram stain	Observation	Catalase	Oxidase
18	Ceca	+	Cocci (clusters)	-	-
48	Ceca	+	Rods	-	-

**Table 2.** MIDI System ID, Biolog ID System, and the 16S rRNA Sequence Analyses identification of the isolates 18 and 48 present in FloraMax<sup>®</sup>-B11

LAB- ID	16S RNA SEQUENCING (FIRST 500 bp) Microbial ID Inc.	MIDI SYSTEM ID Micro Test Lab Inc.	MIDI SYSTEM ID Microbial ID Inc.	Biolog ID Dept. of Poultry Science U. of Arkansas
18	<i>Pediococcus parvulus</i>	<i>Enterococcus cecorum</i>	<i>Lactobacillus gasseri</i>	Unable to identify
48	<i>Lactobacillus salivarius</i>	<i>Lactobacillus helveticus</i>	<i>Lactobacillus gasseri</i>	<i>Lactobacillus salivarius</i>

**Table 3.** Tolerance of the lactic acid bacteria isolates 18 and 48 present in FloraMax®-B11 to pH, temperature, and NaCl

LAB ID	pH2		pH3		15°C		45°C		3.5% NaCl		6.5% NaCl	
	2h	4h	2h	4h	2h	4h	2h	4h	2h	4h	2h	4h
<b>18</b>	-	-	+	+	+	+	+	+	+	+	+	+
<b>48</b>	-	-	+	+	+	+	+	+	+	+	+	+

Symbols: +, tolerant; -, non- tolerant

**Table 4.** Evaluation of FloraMax®-B11 isolates 18 and 48 bile salt tolerance

LAB ID	2 hours				4 hours				24 hours			
	0%	0.4%	0.5%	0.6%	0%	0.4%	0.5%	0.6%	0%	0.4%	0.5%	0.6%
<b>18</b>	+	+	+	+	+	+	+	+	+	+	+	+
<b>48</b>	+	+	+	+	+	+	+	+	+	+	+	+

Symbols: +, tolerant; -, non- tolerant

**Table 5.** *In vitro* assessment of antimicrobial activity of the lactic acid bacteria isolates 18 and 48 present in FloraMax®-B11 against enteropathogenic bacteria

LAB- ID	<i>Salmonella</i> Enteritidis	<i>Escherichia coli</i> (O157:H7)	<i>Campylobacter</i> <i>jejuni</i>
<b>18</b>	+	+	+
<b>48</b>	+	+	+

Symbols: +, inhibition

## REFERENCES

- Adnan, A. F. M., & Tan, I. K. (2007). Isolation of lactic acid bacteria from Malaysian foods and assessment of the isolates for industrial potential. *Bioresource Tech*, 98(7), 1380-1385.
- Amrane, A., & Prigent, Y. (1999). Effect of the main culture parameters on the growth and production coupling of lactic acid bacteria. *Applied Microbiology*, 2, 101-108.
- Anadón, A., Martínez-Larranaga, M. R., & Aranzazu Martínez, M. (2006). Probiotics for animal nutrition in the European Union. Regulation and safety assessment. *Regulatory Toxicology and Pharmacology*, 45(1), 91-95.
- Bakari, D., Tatsadjieu, N. L., Mbawala, A., & Mbofung, C. M. (2011). Assessment of physiological properties of some lactic acid bacteria isolated from the intestine of chickens use as probiotics and antimicrobial agents against enteropathogenic bacteria. *Innovative Romanian Food Biotechnology*, 8, 33-40.
- Boonkumklao, P., Kongthong, P., & Assavanig, A. (2006). Acid and bile tolerance of *Lactobacillus thermotolerans*, a novel species isolated from chicken feces. *Kasetsart Journal: Natural Science*, 40, 13-17.
- Du Toit, M., Franz, C., Schillinger, U., Warles, B., & Holzappel, W. (1998). Characterization and selection of probiotic lactobacilli for a preliminary mini pig-feeding trial and their effect on serum cholesterol level, faeces pH and faeces moisture contents. *International Journal of Food Microbiology*, 40, 93-104.
- Dunne, C., O'Mahony, L., Murphy, L., Thornton, G., Morrissey, D., O'Halloran, S., Feeney, M., Flynn, S., Fitzgerald, G., Daly, C., Kiely, B., O'Sullivan, G. C., Shanahan, F., & Collins, J. K. (2001). *In vitro* selection criteria of probiotic bacteria of human origin: correlation with *in vivo* findings. *American Journal of Clinical Nutrition*, 73, 386S-392S.
- Fontana, L., Brito, M. B., Diaz, J. P., Quezada, S. M., & Gil, A. (2013). Sources, isolation, characterisation and evaluation of probiotics. *British Journal of Nutrition*, 109, S35-S50.
- Gilliland, S. E., Staley, T. E., & Bush, L. J. (1984). Importance in bile tolerance of *Lactobacillus acidophilus* used as a dietary adjunct. *Journal of Dairy Science*, 67, 3045-3051.
- Havenaar, R., Brink, B. T., & Huis Veld, J. H. J. (1992). Selection of strains for probiotic use. In *Probiotics, the scientific basis*, Fuller, R. (ed), Chapman and Hall, London, UK, pp. 209-224.
- Higgins, J., Higgins, S., Vicente, J., Wolfenden, A., Tellez, G., & Hargis, B. M. (2007). Temporal effects of lactic acid bacteria probiotic culture on *Salmonella* in neonatal broilers. *Poultry Science*, 86, 1662-1666.
- Higgins, J. P., Higgins, S. E., Wolfenden, A. D., Henderson, S. N., Torres-Rodriguez, A., Vicente, J. L., Hargis, B. M., & Tellez, G. (2010). Effect of lactic acid bacteria probiotic

- culture treatment timing on *Salmonella* Enteritidis in neonatal broilers. *Poultry Science*, 89, 243-247.
- Higgins, S. E., Higgins, J. P., Wolfenden, A. D., Henderson, S. N., Torres-Rodriguez, A., Tellez, G., & Hargis, B. M. (2008). Evaluation of a lactobacillus-based probiotic culture for the reduction of *Salmonella* Enteritidis in neonatal broiler chicks. *Poultry Science*, 87, 27-31.
- Higgins, S. E., Wolfenden, A. D., Tellez, G., Hargis, B. M., & Porter, T. (2011). Transcriptional profiling of cecal gene expression in probiotic and *Salmonella*-challenged neonatal chicks. *Poultry Science*, 90(1), 901-913.
- Hyronimus, B., Le Marrec, C., Hadj Sassi, A., & Deschamps, A. (2000). Acid and bile tolerance of spore-forming lactic acid bacteria. *International Journal of Food Microbiology*, 61, 193-197.
- Ibourahema, C., Dauphin, R. D., Jacqueline, D., & Thonart, P. (2008). Characterization of lactic acid bacteria isolated from poultry farms in Senegal. *African Journal of Biotechnology*, 7(12), 2006-2012.
- Kristoffersen, S. M., Ravnum, S., Tourasse, N. J., Økstad, O. A., Kolstø, A. B., & Davies, W. (2007). Low concentrations of bile salts induce stress responses and reduce motility in *Bacillus cereus* ATCC 14570. *Journal of Bacteriology*, 189(14), 5302-5313.
- Lee, Y. K., & Salimen, S. (1995). The coming age of probiotics. *Food Science and Technology*, 6, 241-245.
- Patterson, J. A., & Burkholder, K. M. (2003). Application of prebiotics and probiotics in poultry production. *Poultry Science*, 82(4), 627-631.
- Rattanachaikunsopon, P., & Phumkhachorn, P. (2010). Lactic acid bacteria: their antimicrobial compounds and their uses in food production. *Annals of Biological Research*, 1(4), 218-228.
- Tanaka, H., Doesburg, K., Iwasaki, T., & Mireau, I. (1999). Screening of lactic acid bacteria for bile salt hydrolase activity. *Journal of Dairy Science*, 82, 2530-2535.
- Tannock, G. W. (2004). A special fondness for lactobacilli. *Applied Environmental Microbiology*, 70, 3189-3194.
- Tellez, G., Higgins, S., Donoghue, A., & Hargis, B. (2006). Digestive physiology and the role of microorganisms. *The Journal of Applied Poultry Research*, 15, 136-144.
- Tellez G., Pixley C., Wolfenden R. E., Layton S. L., & Hargis B. M. (2012). Probiotics/direct fed microbials for *Salmonella* control in poultry. *Food Research International*, 45, 628-633.

- Vicente, J. L., Torres-Rodriguez, A., Higgins, S. E., Pixley, C., Tellez, G., Donoghue, A. M., & Hargis, B. M. (2008). Effect of a selected *Lactobacillus* spp.-based probiotic on *Salmonella enterica* serovar Enteritidis-infected broiler chicks. *Avian Diseases*, 52, 143-146.
- Vicente, J., Wolfenden, A., Torres-Rodriguez, A., Higgins, S. E., Tellez, G., & Hargis, B. M., (2007c). Effect of a *Lactobacillus*-based probiotic and dietary lactose prebiotic on turkey poult performance with or without *Salmonella* Enteritidis challenge. *Journal of Applied Poultry Research*, 16, 361-364.
- Vicente, J. L., Aviña, L., Torres-Rodriguez, A., Hargis, B., & Tellez, G. (2007b). Effect of a *Lactobacillus* spp.-based probiotic culture product on broiler chicks performance under commercial conditions. *International Journal of Poultry Science*, 6 (3), 154-156.
- Vicente, J., Higgins, S., Bielke, L., Tellez, G., Donoghue, D., Donoghue, A., & Hargis, B. M. (2007a). Effect of probiotic culture candidates on *Salmonella* prevalence in commercial turkey houses. *Journal of Applied of Poultry Research*, 16, 471-476.
- Wagner, R. D., Paine, D. D., & Cerniglia, C. E. (2003). Phenotypic and genotypic characterization of competitive exclusion products for use in poultry. *Journal of Applied Poultry Research*, 94, 1098-1107.
- Wolfenden, A., Pixley, C., Higgins, J., Higgins, S., Vicente, J., Torres-Rodriguez, A., Hargis, B., & Tellez, G. (2007a). Evaluation of spray application of a *Lactobacillus*-based probiotic on *Salmonella* enteritidis colonization in broiler chickens. *International Journal of Poultry Science*, 6, 493-496.
- Wolfenden, A. D., Vicente, J. L., Bielke, L. R., Pixley, C. M., Higgins, S. E., Donoghue, D. J., Donoghue, A. M., Hargis, B. M., & Tellez, G. (2007b). Effect of a defined competitive exclusion culture for prophylaxis and reduction of horizontal transmission of *Salmonella* enteritidis in broiler chickens. *International Journal of Poultry Science*, 6(7), 489-492.
- Wouters, J. A., Kamphuis, H. H., Hugenholtz, J., Kuipers, P., De Vos, W. M., & Abee, T. (2000). Changes in glycolytic activity of *Lactococcus lactis* induced by low temperature. *Applied Environmental Microbiology*, 66, 3686-3691.
- Xanthopoulos, V., Litopoulou-Tzanetaki, E., & Tzanetakis, N. (1997). Lactic acid bacteria. Caen Presses Universitaires de Canada.

## APPENDIX



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0-114 Poultry Science Building, University of Arkansas, Fayetteville, AR 72701-1201  
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To Whom It May Concern,

The first author of enclosed paper, “Identification and Characterization of Lactic Acid Bacteria in a Commercial Probiotic Culture”, is Anita Menconi. Anita was primarily responsible for the work and research associated with this paper, and completed greater than 51% of the work.

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B.M. Hargis

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Bioscience of Microbiota, Food and Health

Editorial Office

IPEC, Inc., Attn: BMFH

1-24-12 Sugamo, Toshima-ku,

Tokyo 170-0002, Japan

TEL +81-3-5978-3329

FAX +81-3-5978-4068

Email: [bmfh@ipcc-pub.co.jp](mailto:bmfh@ipcc-pub.co.jp)

<http://www.bmfh.jp>

## VII. CHAPTER V

### **Physiological properties and *Salmonella* growth inhibition of probiotic *Bacillus* strains isolated from environmental and poultry sources**

Anita Menconi, Marion J. Morgan, Neil R. Pumford, Billy M. Hargis, and Guillermo Tellez\*

Department of Poultry Science, University of Arkansas Fayetteville, AR 72701, USA

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## ABSTRACT

The objective of the present study was to describe the physiological properties of seven potential probiotic strains of *Bacillus* spp. Isolates were characterized morphologically, biochemically, and by 16S rRNA sequence analyses for identification. Tolerance to acidic pH, high osmotic concentrations of NaCl, and bile salts were tested. Isolates were also evaluated for their ability to metabolize different carbohydrates sources. The antimicrobial sensitivity profiles were determined. Inhibition of gastrointestinal *Salmonella* colonization in an avian model was also evaluated. Five strains of *Bacillus* were tolerant to acidic conditions (pH 2.0) and all strains were tolerant to a high osmotic pressure (NaCl at 6.5%). Moreover, all strains were able to tolerate concentration of 0.037 % bile salts after 24 h of incubation. Three strains were able to significantly reduce *Salmonella* Typhimurium levels in the crop and in the ceca of broiler-type chickens. Among the 12 antibiotics tested for antibiotic resistance, all strains were resistant to bacitracin and susceptible to gentamycin, neomycin, ormethoprim, triple sulfa, and spectinomycin. Bacterial spore formers have been shown to prevent gastrointestinal diseases in animals and humans. The results obtained in this study shows important characteristics to be evaluated when selecting *Bacillus* spp. candidates to be used as probiotics.

Key words: *Bacillus*, probiotic, properties

## INTRODUCTION

Probiotics have been commercialized for both animal and human uses. Probiotics for humans use are subject to minimal restrictions and come in many different forms. Probiotics in animal feed have been used for the prevention of gastrointestinal infections, with a wide use in poultry and aquaculture productions (Hong et al., 2005; Jadamu et al., 2002, Kasper, 1998; Sleatorand & Hill, 2008; Rolfe, 2000; Liu et al., 2012).

Diarrhea is one of the major side effects of chemotherapy in cancer treatments, and has been associated with increased morbidity, mortality, increased treatment costs, and restrictions related to the ability to deliver full doses of chemotherapy (Kobayashi, 2003; Savarese et al., 2003). Enterocyte proliferation in the intestinal mucosa and the intestinal microflora can be directly harmed by the effect of chemotherapeutic agents as well as radiation, often causing bacterial translocation, malabsorption, and/or diarrhea (Savarese et al., 2013; McGough et al., 2004). Therefore, in order to reduce systemic bacterial diseases, high doses of broad spectrum antibiotics are usually used in cancer patients undergoing chemotherapy or radiation therapy. The disruption of the beneficial intestinal microflora is a common consequence to this type of treatment, which may lead to the colonization of opportunistic pathogenic bacteria such as *Salmonella* spp. (Noriega et al., 1994; Delaloye et al., 2004) and *Clostridium difficile* (Benchimol & Mack, 2004; Hull & Beck, 2004). Although the most common types of probiotics available are based on lactic acid bacteria (LAB), there are other potentially beneficial microorganisms that are not normally found in the gastrointestinal tract (GIT) such as *Saccharomyces boulardii* or *Bacillus* spp. For example, *Saccharomyces boulardii* has been shown to prevent the recurrence of *Clostridium difficile*-induced pseudomembranous colitis (Czerucka & Rampal, 2002) as well as *Escherichia coli* infections (Czerucka et al., 2000). Spore

forming bacteria such as *Bacillus subtilis*, *B. megaterium*, *B. licheniformis*, *Paenibacillus polymyxa*, and *B. clausii* have also been used as probiotics in humans (Hong et al., 2005).

Many studies have shown that either strains of live bacteria or active spores can efficaciously reach the intestine, preventing colon carcinogenesis (Lee et al., 2007; Malkov et al., 2006). Moreover, they can suppress the development of pre-neoplastic lesions (Park et al., 2007). These microorganisms can also release antimicrobial substances active against Gram-positive bacteria such as *Staphylococcus aureus*, *Enterococcus faecium*, and *Clostridium difficile*, and can induce IFN-gamma production and CD4+ T-cell proliferation (O'Mahony et al., 2002; Urdaci et al., 2004). Products containing *Bacillus* spp. spores are used commercially as probiotics because they have some advantages over the traditional LAB products, for example, the ability to be stored indefinitely in a dry form (Barbosa et al., 2005; Duc et al., 2004; Hong et al., 2005; Sleator and Hill, 2008) and the ability to survive baking processes (Permpoonpattana et al., 2012).

Current research has shown that *Bacillus subtilis* spores, after oral ingestion, are immunogenic and are able to disseminate to the Peyer's patches and mesenteric lymph nodes (Duc et al., 2003a, 2003b; Permpoonpattana et al., 2012). Three main findings have supported the hypothesis that *Bacillus subtilis* spores can germinate in the small intestine. First, following oral ingestion in mice, Hoa et al. (2001) showed that more *Bacillus subtilis* spores were excreted after ingestion than initially given. Second, after administration of spores to mice, expressed mRNA of vegetative cells was detected in the GIT by reverse transcription (RT)-PCR (Casula & Cutting, 2002). Third, after oral administration of spores to mice, systemic immunoglobulin G was produced against vegetative *Bacillus subtilis* cells (Duc et al., 2003a). The above studies indicate that *Bacillus* spp. spores are not merely present in the intestinal tract as transient bacteria,

but they might also have some interaction with the host enterocytes, immunocompetent cells, or with the intestinal microbiota (Duc et al., 2004).

Identifying desirable physiological properties and the ability to inhibit the growth of pathogenic bacteria is very important when selecting potential candidates to be used as probiotics for humans and animals. In the present study, *Bacillus* spp. strains, isolated from poultry and environmental sources, were characterized and evaluated for their ability to metabolize different carbohydrate sources, their antibiotic sensitivity profile, and their tolerance to acidic pH, high osmotic concentrations of sodium chloride (NaCl), and bile salts. In addition, inhibition of *Salmonella* colonization in a well-established avian model was also evaluated.

## **MATERIALS AND METHODS**

### ***Isolation, Biochemical tests, and Identification of selected Bacillus strains***

Strains of *Bacillus* spp., laboratory identified as NP122, AM0904, B2, RW41, AM0902, AM1109A, and AM1109B, were isolated from environmental and poultry sources as described by Wolfenden et al. (2010). Biochemical evaluation tests as well as identification for these seven selected strains were carried out using a bioMerieux API 50 CHB test kit (catalog no. 50430, bioMerieux, Marcy l'Etoile, France). The identification procedure, which followed the manufacturer's instructions, was also important to confirm generally recognized as safe (GRAS) status of the isolates. Besides the biochemical identification, 16S rRNA sequence analyses (Microbial ID Inc., Newark, DE 19713, USA) was carried out.

### ***Bile salts tolerance***

The method of Gilliland et al. (1984), with some modifications, was used to determine bile salt tolerance. Tryptic Soy Broth (TSB) (Becton Dickinson and Co., Sparks, MD) containing 0%, 0.037%, 0.075%, 0.15%, and 0.3% of bile salts No. 3 (Catalog no. 213010, Becton Dickinson and Co., Sparks, MD 21152 USA) was inoculated with  $10^7$  cfu/mL of each potential probiotic strain, after being centrifuged at 3000g for 15 minutes and washed three times from their overnight growth cultures. Samples were incubated for 24 h at 37 °C with shaking at 100 rev./min. Growth in control (no bile salts) and test cultures was evaluated at 2, 4, and 24 hours by streaking samples on Tryptic Soy Agar (TSA) for presence or absence of growth.

### ***Antibiotic resistance***

Selected colonies of NP122, AM0904, B2, RW41, AM0902, AM1109A, and AM1109B on TSA plates were inoculated and cultured overnight in TSB at 37 °C. Strains were then sent to a Veterinary Diagnostic Laboratory (University of Arkansas, Division of Agriculture, Fayetteville, AR, 72703, USA) for antibiotic sensitivity analysis using Kirby-Bauer methodology. The diameter of the inhibition zones and the interpretative zone sizes were reported. Twelve antibiotics were tested and their concentrations were reported as shown on table 6. The results were expressed in terms of resistant, intermediate, and susceptible.

### ***Resistance in conditions of the intestinal tract evaluation: pH, temperature, and sodium chloride***

A basal TSB medium was used in these series of *in vitro* studies. An overnight culture of each isolate was used as the inoculum whereby the cells were spun down and re-suspended in 0.9% sterile saline. Then, 100 µL of the suspension was inoculated into 10 mL of TSB of each

test tube. Two incubation time points, i.e. two and four hours were evaluated for each of the variables (pH, temperature, and NaCl). The rationale for these two points was mainly based on the transit time of food matter in the gastrointestinal tract of poultry. The temperatures tested were 15 and 45 °C. The concentrations of NaCl tested were 3.5 and 6.5% (w/v). The isolates were tested for growth at pH 2 and 3. The tubes were incubated with reciprocal shaking, at the specific test temperatures or at 37 °C for the tests on pH and concentrations of NaCl. At the time points evaluated, each sample was streaked on TSA for presence or absence of growth, to confirm livability of the strains. The turbidity of each tube was also noted as an indication of growth or no-growth. Each treatment was tested with triplicate tubes.

### ***Salmonella Typhimurium in vivo growth inhibition***

A poultry isolate of *Salmonella enterica* subspecies *enterica* serovar Typhimurium (ST), which had previously been selected for resistance to nalidixic acid (NA - Catalog No. N-4382, Sigma, St. Louis, MO 63178), was used in all experiments. The amplification and enumeration protocol for this isolate has been described previously (Tellez et al., 1993). Trials were conducted with day-of-hatch broiler chicks obtained from a local hatchery, with the exception of one trial that was conducted with six to seven weeks old broiler chickens. In all trials, broiler chickens were randomly (n=20) assigned to untreated control diet or dietary treatment of each *Bacillus* spp. isolate at  $10^5$  cfu/g of feed for seven days. Broiler chicks were housed in brooder batteries or floor pens with food and water *ad libitum*. At day four, all birds were challenged with  $2 \times 10^5$  cfu ST/bird. At seven days, birds were humanely killed by CO<sub>2</sub> inhalation and crop, ceca, and cecal tonsils were aseptically harvested. *Salmonella* recovery procedures have been previously described by our laboratory and were followed with some modifications [32]. All

animal handling procedures were in compliance with the Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas.

### ***Statistical Analysis***

Crop and ceca colony forming units (cfu) data were converted to log<sub>10</sub> cfu numbers and then compared using the GLM procedure of SAS (SAS Institute, 2002) with significance reported at P < 0.05. The incidence of ST recovery within experiments was compared using the chi-square test of independence (Zar, 1984) to determine significant (P < 0.05) differences between control and treated group. All values were converted to percent ST reduction comparing treated birds to non-treated birds (control) to be simplified in a single table.

## **RESULTS AND DISCUSSION**

### ***Biochemical tests and Identification of selected Bacillus strains***

As described by Logan and Berkeley (1984), the API 50 CHB system is a rapid and accurate test of *Bacillus* isolate identification, which allows bacterial isolates to be classified according to their ability to ferment 49 different carbohydrates, which are listed in Table 1. Selected *Bacillus* isolates were tested to evaluate their biochemical profile, and the results are presented in Table 1. The carbohydrate fermentation pattern was used to identify each isolates' species. Four isolates were characterized as *Bacillus subtilis/amyloliquefaciens*, and the three remaining isolates were characterized as *Bacillus licheniformis*, *Bacillus pumilus*, and *Bacillus megaterium* (Table 2). Sequence analysis of 16S rRNA is the predominant molecular technology presently available for microbial identification (Wagner et al., 2003). The 16S rRNA analysis (Table 3), matched the biochemical identification results (Table 2).

### ***Bile salts tolerance***

In general, tolerance to bile salts has been considered a prerequisite for colonization and metabolic activity of bacteria in the host's intestine (Havenaar et al., 1992). The average concentration of bile salts in the small intestine is around 0.2% to 0.3%, and may go up to 2% (w/v), depending upon the individual and the type and amount of food ingested (Kristoffersen et al., 2007; Bakari et al., 2011). Nevertheless, bile levels in the intestine are not constant and are relatively low until ingestion of a fatty meal (Begley et al., 2005). The main purpose of bile secretion is to emulsify and dissolve ingested fats (Kristoffersen et al., 2007). However, bile salts also have bactericidal effects; they can disrupt the lipid membrane, get into the bacterial cell, denature proteins, chelate ions, and damage DNA (Kristoffersen et al., 2007; Hernández et al., 2012). According to Begley et al. (2005), many studies have shown that bile tolerance is a strain-specific characteristic and the tolerance of various bacterial species cannot be generalized. Also, Gram-positive bacteria seem to be more sensitive to the harmful effects of bile than Gram-negative bacteria (Begley et al., 2005).

Evaluating bile salts tolerance of the vegetative cells of our selected strains, we found that all strains were able to grow when cultured at 0.037% bile salts concentration at 2 h, 4 h, and 24 h of incubation. Six of the vegetative forms of the *Bacillus* strains tested for bile resistance were not able to survive at the concentrations of 0.075%, 0.15%, and 0.3% of bile salts during the time points evaluated. The isolate B2 was the only one able to survive at 0.075%, 0.15%, and 0.3% at 2 h of incubation (Table 4). These results are in agreement with Barbosa et al. (2005) findings, where vegetative cells of *Bacillus* isolates were very susceptible to bile salts at 0.2%.

Information about the bile tolerance of Gram positive bacteria is limited. It is important to know that bacterial tolerance to bile in broth assays, as with many physiological stresses, may



not reproduce *in vivo*. Because bile salts form micelles with phospholipids, they may not be free to interact with bacterial cells, and the *in vivo* antibacterial activity of bile may be lower than observed in *in vitro* assays (Begley et al., 2005). Exposure to different pH, temperatures, and growth environments may increase bacterial susceptibility to bile or make them more resistant. For example, an exposure of bacteria to low levels of bile salts may increase their tolerance to higher levels (Begley et al., 2005). Also, the presence of food in the intestinal tract can affect survival because bacteria may not be exposed to bile due to the formation of microenvironments by the food particles or food constituents, which may bind to bile components, preventing damage to the bacteria (Begley et al., 2005). Bile resistance of some isolates is related to the enzyme activity of bile salt hydrolase (BSH) that helps to hydrolyze conjugated bile, reducing its toxic effect (Du Toit et al., 1998). BSH activity has most often been found in microorganisms isolated from animals' intestines or feces (Tanaka et al., 1999).

The *Bacillus* spore, which consists of multiple protective layers, has been described to be very resistant to different physical and chemical conditions (Barbosa et al., 2005), and they have been shown to survive at high concentration (usually more than 1%) of bile salts (Barbosa et al., 2005; Kristoffersen et al., 2007). The hypothesis is that *Bacillus* spp. spores, after ingestion, would germinate in distal parts of the small intestine, where the concentration of bile salts would be lower (Casula & Cutting, 2002; Kristoffersen et al., 2007). More physiological analyses are necessary to establish the importance of bile tolerance of bacteria in the intestine (Begley et al., 2005).

***Resistance in conditions of the intestinal tract evaluation: pH, temperature, and sodium chloride***

Probiotic bacteria need to survive the passage through the stomach, where the pH can be as low as 1.5 to 2.0 (Dunne et al., 2001), and stay alive for 4 h or more (Ouwehand et al., 2002), before they move to the intestinal tract. For this reason, the vegetative cells of the isolates were evaluated for conditions similar to that found in the stomach. The isolates AM1109A and B2 were able to survive at pH 2 and pH 3 for 2 h and 4 h of exposure. On the other hand, AM0904 and AM1109B did not survive the harsh pH conditions (Table 5). The remaining isolates (NP122, AM 0902, and RW41) were able to survive at pH 2 and pH 3 at only 2 h of exposure.

According to Ibourahema et al. (2008), the bacterial capability to grow at high temperature is a good characteristic as it could be interpreted as indicating an increased rate of growth. Moreover, a high fermentation temperature reduces contamination by other microorganisms [0]. All strains grew at 15 °C to 44 °C at both times of incubation 2 h and 4 h (Table 5). All strains (vegetative cells) were also able to tolerate high osmotic concentrations of NaCl (Table 5). This examination gave an indication of the osmotolerance level of the *Bacillus* spp. strains. Bacterial cells cultured in a high salt concentration could have a loss of turgor pressure, which would then affect their physiology, enzyme activity, water activity, and metabolism (Ibourahema et al., 2008).

### ***Antibiotic resistance***

The antibiotic resistance and susceptibility of the seven *Bacillus* isolates to twelve antibiotics was analyzed. All isolates were resistant to bacitracin, and sensitive to gentamycin, neomycin, ormethoprim, triple sulfa, and spectinomycin. The isolate AM0902 was also resistant to clindamycin, ceftiofur, novobiocin, penicillin, and tetracycline. The isolate RW 41 also showed resistance to erythromycin, clindamycin, ceftiofur, and novobiocin, to which B2 was

resistant as well. An intermediate susceptibility was observed with AM0902 on erythromycin and with AM0904 on tetracycline (Table 6).

According to Bakari et al. (2011), probiotic bacteria that show resistance to a specific antibiotic can be given at the time of antibiotic treatment. Because antibiotic resistant genes are generally carried on conjugative plasmids, they can be transferred to other bacteria (Bennet, 2008), and could possibly result in antibiotic resistant enteropathogenic bacteria. Therefore, it is also important to determine whether antibiotic resistant genes are present on chromosomes or on plasmids (Bakari et al., 2011).

### ***Salmonella Typhimurium in vivo growth inhibition***

According to Dodgson and Romanov (2004), chickens have been a valuable model for human diseases and genetic analysis. Several spore-forming *Bacillus* spp. have been shown to reduce food-borne pathogens using commercial products available in Europe (Jadamus et al., 2002).

Our results showed that some *Bacillus* isolates, more specifically the isolates NP122 and the combination of the isolates AM1109A with AM1109B, were able to significantly reduce ST levels in the crop and in the ceca of broiler chickens (Table 7). The ability of *Bacillus subtilis* probiotic isolates in reducing *Salmonella* in chickens has been described previously by La Ragione & Woodward (2003) and Vila et al.(2009).

Competitive exclusion of pathogens is a common hypothesis to explain the action of probiotics (Patterson & Burkholder, 2003; Leser et al., 2008). This process has been well demonstrated in *Lactobacillus* spp., and some evidence exists that *Bacillus* spp. may have the same mode of action (Barbosa et al., 2005). Competitive exclusion includes the competition for

receptor sites and nutrients and the production of antimicrobial substances such as bacteriocins, hydrogen peroxide, and volatile fatty acids (Patterson & Burkholder, 2003; Ng et al., 2009). Another potential mechanism of action of probiotics, that has received a lot of attention, is the modulation of the host's immune system (Ng et al., 2009). According to Ng et al. (2009) and Rupa and Mine (2012), the probiotics alter immune functions in humans and animals by interacting with various receptors. An example is in the treatment of inflammatory bowel disease with probiotics in humans. Following probiotic treatment there are improvement of the epithelial and mucosal barrier function, modulation of the intestinal microbiota, and a direct effect on immune cells of both innate and adaptive immune systems. Despite the beneficial effects of the probiotics observed, *in vivo* mechanisms of action have not been clearly elucidated and will be a significant area for future research (Corr et al., 2007). Several studies have shown that either live vegetative cells or spores of some *Bacillus* isolates can prevent colon carcinogenesis (Park et al., 2007) or release antimicrobial substances against bacteria, such as *Staphylococcus aureus*, *Enterococcus faecium*, and *Clostridium difficile* (O'Mahony et al., 2001). These results supported the evidence of colonization and antimicrobial activity of *Bacillus* spp. as probiotic bacteria. Therefore, products containing *Bacillus* spores are used commercially as probiotics (Anadón et al., 2006; Barbosa et al., 2005; Duc et al., 2004; Hong et al., 2005, 2008; McNulty et al., 2007; Osipova et al., 2003; Williams, 2007; Wolken et al., 2003).

## CONCLUSION

Bacterial spore formers, especially of the genus *Bacillus*, are present in current probiotic products that have been shown to prevent gastrointestinal diseases in animals and humans. These probiotic based spores have been shown to have many applications such as treating

immunosuppressive and antibiotic associated diarrhea. The results obtained in this study showed the tolerance of probiotic *Bacillus* spp. strains in different physiological conditions as well as the inhibition of *Salmonella* Typhimurium. Moreover, the methods used to screen isolates may be important in the evaluation of *Bacillus* spp. for use as probiotics for humans and animals.

## TABLES

**Table 1.** Metabolization of different carbohydrates sources by selected isolates of *Bacillus* ssp.\*

	NP122	AM0904	B2	RW41	AM0902	AM1109A	AM1109B
<b>Amidon (starch)</b>	+	+	+	+	-	+	+
<b>Amygdalin</b>	+	+	-	+	+	+	+
<b>Arbutin</b>	+	+	-	-	+	+	+
<b>D-Adonitol</b>	-	-	-	-	-	-	-
<b>D-Arabinose</b>	-	-	-	-	-	-	-
<b>D-Arabitol</b>	-	-	-	-	-	-	-
<b>D-Celiobiose</b>	+	+	+	+	+	+	+
<b>D-Fructose</b>	+	+	+	+	+	+	+
<b>D-Fucose</b>	-	-	-	-	-	-	-
<b>D-Galactose</b>	-	-	-	+	+	-	+
<b>D-Glucose</b>	+	+	+	+	+	+	+
<b>D-Lactose</b>	-	+	+	+	-	+	+
<b>D-Lyxose</b>	-	-	-	-	-	-	-
<b>D-Maltose</b>	+	+	+	+	+	+	+
<b>D-Manitol</b>	+	+	+	+	+	+	+
<b>D-Mannose</b>	+	+	+	+	+	+	+
<b>D-Melezitose</b>	-	-	-	-	-	-	-
<b>D-Melibiose</b>	-	+	+	+	-	+	+
<b>D-Rafinose</b>	-	+	+	+	-	+	+
<b>D-Ribose</b>	+	+	+	+	+	+	+
<b>D-Saccharose</b>	+	+	+	+	+	+	+
<b>D-Sorbitol</b>	+	+	+	+	-	+	+
<b>D-Tagatose</b>	-	-	-	+	+	-	-
<b>D-Trehalose</b>	+	+	+	-	+	+	+
<b>D-Turanose</b>	-	-	-	+	+	-	+
<b>Dulcitol</b>	-	-	-	-	-	-	-
<b>D-Xylose</b>	-	+	+	+	+	+	+
<b>Erythritol</b>	-	-	-	-	-	-	-
<b>Esculin (Ferric Citrate)</b>	+	+	+	+	+	+	+
<b>Gentiobiose</b>	+	-	-	+	-	-	-
<b>Glycerol</b>	+	+	+	+	+	+	+
<b>Glycogen</b>	+	+	+	+	-	+	+
<b>Inositol</b>	+	+	+	+	-	+	+

<b>Inulin</b>	-	-	-	+	-	-	ND
<b>L-Arabinose</b>	+	+	+	+	+	+	+
<b>L-Arabitol</b>	-	-	-	-	-	-	-
<b>L-Fucose</b>	-	-	-	-	-	-	-
<b>L-Rhamnose</b>	-	-	-	+	-	-	-
<b>L-Sorbose</b>	-	-	-	+	-	-	-
<b>L-Xylose</b>	-	-	-	-	-	-	-
<b>Methyl-<math>\alpha</math>D-Glucopyranoside</b>	+	+	+	+	+	+	ND
<b>Methyl-<math>\alpha</math>D-Mannopyranoside</b>	-	-	-	-	+	-	-
<b>Methyl-<math>\beta</math>D-Xylopyranoside</b>	-	-	-	-	-	-	-
<b>N-AcetylGlucosamine</b>	-	-	-	-	+	-	-
<b>Potassium 2-KetoGluconate</b>	-	-	-	-	-	-	-
<b>Potassium 5-KetoGluconate</b>	-	-	-	-	-	-	-
<b>Potassium GlucoNaTe</b>	-	-	-	-	-	-	-
<b>Salicin</b>	+	+	+	+	+	+	+
<b>Xylitol</b>	-	-	-	-	-	-	+

\* BioMerieux API 50 CHB test kit (catalog no. 50430, bioMerieux, Marcy l'Etoile, France)  
Symbols: +, growth; -, no growth. ND: Not Determined.

**Table 2.** Identification (ID) of *Bacillus* spp. isolates by bioMerieux API 50 CHB\*

<b><i>Bacillus</i> isolates</b>	<b>API 50 CHB Identification</b>	<b>% ID</b>
<b>NP122</b>	<i>Bacillus subtilis/amyloliquefaciens</i>	98.2
<b>AM0904</b>	<i>Bacillus subtilis/amyloliquefaciens</i>	96.6
<b>B2</b>	<i>Bacillus subtilis/amyloliquefaciens</i>	99.7
<b>RW41</b>	<i>Bacillus licheniformis</i>	99.9
<b>AM0902</b>	<i>Bacillus pumilus</i>	99.9
<b>AM1109A</b>	<i>Bacillus subtilis/amyloliquefaciens</i>	96.6
<b>AM1109B</b>	<i>Bacillus megaterium</i>	75.3

\* BioMerieux API 50 CHB test kit (catalog no. 50430, bioMerieux, Marcy l'Etoile, France).



**Table 3.** Identification (ID) of *Bacillus* spp. isolates by 16S rRNA sequence analyses\*

<b><i>Bacillus</i> isolates</b>	<b>16 S Identification</b>	<b>% ID</b>
<b>NP122</b>	<i>Bacillus amyloliquefaciens</i>	99.6
<b>AM0904</b>	<i>Bacillus amyloliquefaciens</i>	99.57
<b>B2</b>	<i>Bacillus amyloliquefaciens</i>	99.52
<b>RW41</b>	<i>Bacillus licheniformis</i>	98.66
<b>AM0902</b>	<i>Bacillus pumilus</i>	100
<b>AM1109A</b>	ND	ND
<b>AM1109B</b>	ND	ND

\* 16S rRNA sequence analyses (Microbial ID Inc., Newark, DE 19713, USA).  
ND: Not Determined.

**Table 4.** Evaluation of *Bacillus* spp. isolates bile salt tolerance after 2, 4, and 24 hours of incubation

<i>Bacillus</i> Isolates	0%			0.037%			0.075%			0.15%			0.3%		
	2h	4h	24h	2h	4h	24h	2h	4h	24h	2h	4h	24h	2h	4h	24h
<b>NP122</b>	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
<b>AM0904</b>	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
<b>AM0902</b>	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
<b>AM1109A</b>	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
<b>AM109B</b>	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
<b>RW41</b>	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
<b>B2</b>	+	+	+	+	+	+	+	-	-	+	-	-	+	-	-

Symbols: +, tolerant; -, non- tolerant

**Table 5.** Physiological characteristics of the *Bacillus* spp. isolates to pH, temperature, and sodium chloride (NaCl).

<i>Bacillus</i> isolates	pH2		pH3		15 °C		45 °C		3.5% NaCl		6.5% NaCl	
	2h	4h	2h	4h	2h	4h	2h	4h	2h	4h	2h	4h
<b>NP122</b>	+	-	+	-	+	+	+	+	+	+	+	+
<b>AM0904</b>	-	-	-	-	+	+	+	+	+	+	+	+
<b>AM0902</b>	+	+	+	-	+	+	+	+	+	+	+	+
<b>AM1109A</b>	+	+	+	+	+	+	+	+	+	+	+	+
<b>AM1109B</b>	-	-	-	-	+	+	+	+	+	+	+	+
<b>RW41</b>	+	-	+	-	+	+	+	+	+	+	+	+
<b>B2</b>	+	+	+	+	+	+	+	+	+	+	+	+

Symbols: +, tolerant; -, non- tolerant

**Table 6.** *Bacillus* spp. isolates antibiotic sensitivity test\*

<b>Antibiotics</b>	<b>Concentration</b>	<b>AM0902</b>	<b>AM1109A</b>	<b>AM1109B</b>	<b>AM0904</b>	<b>NP122</b>	<b>RW41</b>	<b>B2</b>
<b>Bacitracin</b>	10 IUI/IE/U	R	R	R	R	R	R	R
<b>Erythromycin</b>	15 ug	I	S	S	S	S	R	S
<b>Gentamycin</b>	10 ug	S	S	S	S	S	S	S
<b>Clindamycin</b>	2 ug	R	S	S	S	S	R	S
<b>Ceftiofur</b>	30 ug	R	S	S	S	S	R	S
<b>Neomycin</b>	30 ug	S	S	S	S	S	S	S
<b>Novobiocin</b>	5 ug	R	S	S	S	S	R	R
<b>Penicillin</b>	10 IUI/ IE/U	R	S	S	S	S	S	S
<b>Ormethoprim</b>	1.25 ug	S	S	S	S	S	S	S
<b>Tetracycline</b>	30 ug	R	S	S	I	S	S	S
<b>Triple Sulfa</b>	1.0 mg	S	S	S	S	S	S	S
<b>Spectinomycin</b>	100 ug	S	S	S	S	S	S	S

\* Veterinary Diagnostic Laboratory (University of Arkansas, Division of Agriculture, Fayetteville, AR, USA)  
R - resistant; I - intermediate; S – susceptible

**Table 7.** Effect of *Bacillus* spp. isolates in reducing *Salmonella* Typhimurium from crop and ceca of broiler chickens in an avian model

<i>Bacillus</i> isolates	Crop % Reduction	Crop log10 Reduction	Cecal Tonsils % Reduction	Ceca log10 Reduction
<b>NP 122</b>	15.8	ND	50	2.5*
<b>AM 0904</b>	0	ND	ND	0
<b>RW 41</b>	0	ND	ND	0
<b>B2</b>	0	ND	ND	0
<b>AM 1109 A and B</b>	8.4	1.62*	ND	ND
<b>AM 1109 A and B (6-7 weeks old broilers)</b>	10	0.63	15.8	1.15*

\*significantly different at  $P < 0.05$   
 ND: Not Determined

## REFERENCES

- Anadón, A., Martínez-Larranaga, M. R., & Aranzazu Martínez, M. (2006). Probiotics for animal nutrition in the European Union. Regulation and safety assessment. *Regulatory Toxicology and Pharmacology*, 45(1), 91-95.
- Bakari, D., Tatsadjieu, N. L., Mbawala, A., & Mbofung, C. M. (2011). Assessment of physiological properties of some lactic acid bacteria isolated from the intestine of chickens use as probiotics and antimicrobial agents against enteropathogenic bacteria. *Innovative Romanian Food Biotechnology*, 8, 33-40.
- Barbosa T.M., Serra, C. R., La Ragione, R. M., Woodward, M. J., & Henriques, A. O. (2005). Screening for *Bacillus* isolates in the broiler gastrointestinal tract. *Applied and Environmental Microbiology*, 71(2), 968-978.
- Begley, M., Gahan, C. G. M., & Hill, C. (2005). The interaction between bacteria and bile. *FEMS Microbiology Reviews*, 29, 625-651.
- Benchimol, E. I., & Mack, D. R. (2004). Probiotics in relapsing and chronic diarrhea. *Journal of Pediatric Hematology/ Oncology*, 26(8), 515-517.
- Bennet, P. M. (2008). Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *British Journal of Pharmacology*, 153, 347-357.
- Casula, G. & Cutting, S. M. (2002). *Bacillus* probiotics: spore germination in the gastrointestinal tract. *Applied and Environmental Microbiology*, 68, 2344-2352.
- Corr, S. C., Li, Y., Riedel, C. U., O'Toole, P. W., Hill, C., & Gahan, C. G. M. (2007). Bacteriocin production as a mechanism for the anti-infective activity of *Lactobacillus salivarius* UCC118. *Proceedings of the National Academy of Sciences*, 104, 7617-7621.
- Czerucka, D., & Rampal, P. (2002). Experimental effects of *Saccharomyces boulardii* on diarrheal pathogens. *Microbes and Infection*, 4, 733-739.
- Czerucka, D., Dahan, S., Mograbi, B., Rossi, B., & Rampal, P. (2000). *Saccharomyces boulardii* preserves the barrier function and modulates the signal transduction pathway induced in enteropathogenic *Escherichia coli*-infected T84 cells. *Infection and Immunity*, 68, 5998-6004.
- Delaloye, J., Merlani, G., Petignat, C., Wenger, A., Zaman, K., Monnerat, C., Matzinger, O., Popovic, M. B., Vuichard, P., Ketterer, N., & Tarr, P. E. (2004). Nosocomial nontyphoidal salmonellosis after antineoplastic chemotherapy: reactivation of asymptomatic colonization. *European Journal of Clinical Microbiology & Infectious Diseases*, 23, 751-758.
- Dodgson, J. B., & Romanov, M. N. (2004). Use of chicken models for the analysis of human disease. *Current Protocols in Human Genetics*, 15(15.5), 15.5.1-15.5.12.

- Du Toit, M., Franz, C., Schillinger, U., Warles, B., & Holzappfel, W. (1998). Characterization and selection of probiotic lactobacilli for a preliminary mini pig-feeding trail and their effect on serum cholesterol level, faeces pH and faeces moisture contents. *International Journal of Food Microbiology*, 40, 93-104.
- Duc le, H., Hong, H. A., Barbosa, T. M., Henriques, A. O., & Cutting, S. M. (2004). Characterization of *Bacillus* probiotics available for human use. *Applied and Environmental Microbiology*, 70(4), 2161-2171.
- Duc, L.H., Hong, H. A., Fairweather, N., Ricca, E., & Cutting, S. M., (2003b). Bacterial spores as vaccine vehicles. *Infection and Immunity*, 71, 2810-2818.
- Duc, L.H., Hong, H.A., & Cutting, S. M. (2003a). Germination of the spore in the gastrointestinal tract provides a novel route for heterologous antigen presentation. *Vaccine*, 21, 4215-4224.
- Dunne, C., O'Mahony, L., Murphy, L., Thornton, G., Morrissey, D., O'Halloran, S., Feeney, M., Flynn, S., Fitzgerald, G., Daly, C., Kiely, B., O'Sullivan, G. C., Shanahan, F., & Collins, J. K. (2001). *In vitro* selection criteria of probiotic bacteria of human origin: correlation with *in vivo* findings. *American Journal of Clinical Nutrition*, 73, 386S-392SA.
- Gilliland, S. E., Staley, T. E., & Bush, L. J. (1984). Importance in bile tolerance of *Lactobacillus acidophilus* used as a dietary adjunct. *Journal of Dairy Science*, 67, 3045-3051.
- Havenaar, R., Brink, B. T., & Huis Veld, J. H. J. (1992). Selection of strains for probiotic use. In *Probiotics, the scientific basis*, Fuller, R. (ed), Chapman and Hall, London, UK, pp. 209-224.
- Hernández, S. B., Cota, I., Ducret, A., Aussel, L., & Casadesús, J. (2012). Adaptation and preadaptation of *Salmonella enterica* to bile. *PLoS Genetics*, 8(1), 1-15.
- Hoa, T. T., Duc, L. H., Isticato, R., Baccigalupi, L., Ricca, E., Van, P. H., & Cutting, S. M. (2001). Fate and dissemination of *Bacillus subtilis* spores in a murine model. *Applied and Environmental Microbiology*, 67, 3819-3823.
- Hong, H. A., Duc, L. H., & Cutting, S. M. (2005). The use of bacterial spores as probiotics. *FEMS Microbiology Reviews*, 29, 813-835.
- Hong, H. A., Huang, J. M., Khaneja, R., Hiep, L. V., Urdaci, M. C., & Cutting, S. M. (2008). The safety of *Bacillus subtilis* and *Bacillus indicus* as food probiotics. *Journal of Applied Microbiology*, 105(2), 510-520.
- Hull, M. W., & Beck, P. L. (2004). Clostridium difficile-associated colitis. *Canadian Family Physician*, 50, 1536-1540, 1543-1545.
- Ibourahema, C., Dauphin, R. D., Jacqueline, D., & Thonart, P. (2008). Characterization of lactic acid bacteria isolated from poultry farms in Senegal. *African Journal of Biotechnology*, 7(12), 2006-2012.

- Jadamus, A., Vahjen, W., Schaè Fer, K., & Simon, O. (2002). Influence of the probiotic strain *Bacillus cereus* var. toyoi on the development of enterobacterial growth and on selected parameters of bacterial metabolism in digesta samples of piglets. *Journal of Animal Physiology and Animal Nutrition*, 86, 42-54.
- Kasper, H. (1998). Protection against gastrointestinal diseases--present facts and future developments. *International Journal of Food Microbiology*, 41(2), 127-131.
- Kobayashi, K. (2003). Chemotherapy-induced diarrhea. *Gan To Kagaku Ryoho*, 30(6), 765-771.
- Kristoffersen, S. M., Ravnum, S., Tourasse, N. J., Økstad, O. A., Kolstø, A. B., & Davies, W. (2007). Low concentrations of bile salts induce stress responses and reduce motility in *Bacillus cereus* ATCC 14570. *Journal of Bacteriology*, 189(14), 5302-5313.
- La Ragione, R. M., & Woodward, M. J. (2003). Competitive exclusion by *Bacillus subtilis* spores of *Salmonella enterica* serotype Enteritidis and *Clostridium perfringens* in young chickens. *Veterinary Microbiology*, 94, 245-256.
- Lee, N. K., Park, J. S., Park, E., & Paik, H. D. (2007). Adherence and anticarcinogenic effects of *Bacillus polyfermenticus* SCD in the large intestine. *Letters in Applied Microbiology*, 44(3), 274-278.
- Leser, T. D., Knarreborg, A., & Worm, V. (2008). Germination and outgrowth of *Bacillus subtilis* and *Bacillus licheniformis* spores in the gastrointestinal tract of pigs. *Journal of Applied Microbiology*, 104, 1025-1033.
- Liu, C. H., Chiu, C. H., Wang, S. W., & Cheng, W. (2012). Dietary administration of the probiotic, *Bacillus subtilis* E20, enhances the growth, innate immune responses, and disease resistance of the grouper, *Epinephelus coioides*. *Fish & Shellfish Immunology*, 33(4), 699-706.
- Logan, N. A., & Berkeley, R. C. W. (1984). Identification of *Bacillus* Strains Using the API System. *Journal of General Microbiology*, 130, 1871-1882.
- Malkov, S. V., Markelov, V. V., Polozov, G. Y., Barabanschikov, B. I., Kozhevnikov, A. Y., & Trushin M. V. (2006). Significant delay of lethal outcome in cancer patients due to peroral administration of *Bacillus oligonitrophilus* KU-1. *Scientific World Journal*, 8(6), 2177-2187.
- McGough, C., Baldwin, C., Frost, G., & Andreyev, H. J. N. (2004). Role of nutritional intervention in patients treated with radiotherapy for pelvic malignancy. *British Journal of Cancer*, 90, 2278-2287.
- McNulty, C. A., Boyle, P., Nichols, T., Clappison, P., & Davey, P. (2007). The public's attitudes to and compliance with antibiotics. *The Journal of Antimicrobial Chemotherapy*, 60(1), i63-68.



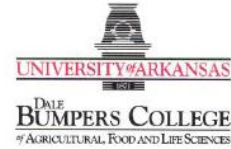
- Ng, S. C., Hart, A. L., Kamm, M. A., Stagg, A. J., & Knight, S. C. (2009). Mechanisms of action of probiotics: recent advances. *Inflammatory Bowel Diseases*, 15, 300-310.
- Noriega, L. M., Van der Auwera, P., & Daneau et al. (1994). *Salmonella* infections in a cancer center. *Support Care Center*, 2, 116-122.
- O'Mahony, L., Feeney, M., O'Halloran, S., Murphy, L., Kiely, B., Fitzgibbon, J., Lee, G., O'Sullivan, G., Shanahan, F., Collins, J. K. (2011). Probiotic impact on microbial flora, inflammation and tumour development in IL-10 knockout mice. *Alimentary Pharmacology & Therapeutics*, 15(8), 1219-1225.
- Osipova, I. G., Makhailova, N. A., Sorokulova, I. B., Vasil'eva, E. A., & Gaidarov, A. A. (2003). Spore probiotics. [Sporovye probiotiki]. *Zhurnal Mikrobiologii, Epidemiologii, i Immunobiologii*, 3(3), 113-119.
- Ouweland, A. C., Salminen, S., & Isolauri, E. (2002). Probiotics: An overview of beneficial effects. *Antonie van Leeuwenhoek*, 82, 279-289.
- Park, E., Jeon, G. I., Park, J. S., & Paik, H. D. (2007). A probiotic strain of *Bacillus polyfermenticus* reduces DMH induced precancerous lesions in F344 male rat. *Biological & Pharmaceutical Bulletin*, 30(3), 569-574.
- Patterson, J. A., & Burkholder, K. M. (2003). Application of prebiotics and probiotics in poultry production. *Poultry Science*, 82(4), 627-631.
- Permpoonpattana P., Hong, H. A., Khaneja, R., & Cutting, S. M. (2012). Evaluation of *Bacillus subtilis* strains as probiotics and their potential as a food ingredient. *Beneficial microbes*, 3(2), 127-135.
- Rolfe, R. D. (2000). The role of probiotic cultures in the control of gastrointestinal health. *Journal of Nutrition*, 130, 396S-402S.
- Rupa, P., & Mine, Y. (2012). Recent advances in the role of probiotics in human inflammation and gut health. *Journal of Agricultural and Food Chemistry*, 60, 8249-8256.
- SAS Institute Inc. (2002). Version 9.1. Carey, NC.
- Savarese, D. M. F., Savy, G., Vahdat, L., Wischmeyer, P. E., & Corey, B. (2003). Prevention of chemotherapy and radiation toxicity with glutamine. *Cancer Treatment Reviews*, 29, 501-513.
- Sleatorand, R. D., & Hill, C. (2008). New frontiers in probiotic research. *Letters in Applied Microbiology*, 46, 143-147.
- Tanaka, H., Doesburg, K., Iwasaki, T., & Mireau, I. (1999). Screening of lactic acid bacteria for bile salt hydrolase activity. *Journal of Dairy Science*, 82, 2530-2535.

- Tellez, G., Dean, C. E., Corrier, D. E., Deloach, J. R., Jaeger, L., & Hargis, B. M. (1993). Effect of dietary lactose on cecal morphology, pH, organic acids, and *Salmonella* Enteritidis organ invasion in leghorn chicks. *Poultry Science*, 72(4), 636-642.
- Urdaci, M.C., Bressollier, P., & Pinchuk, I. (2004). *Bacillus clausii* probiotic strains: antimicrobial and immunomodulatory activities. *Journal of Clinical Gastroenterology*, 38(6) S86-90.
- Vila, B., Fontgibell, A., Badiola, I., Esteve-Garcia, E., Jimenez, G., Castillo, M., & Brufau, J. (2009). Reduction of *Salmonella enterica* var. Enteritidis colonization and invasion by *Bacillus cereus* var. toyoi inclusion in poultry feeds. *Poultry Science*, 88(5), 975-979.
- Wagner, R. D., Paine, D. D., & Cerniglia, C. E. (2003). Phenotypic and genotypic characterization of competitive exclusion products for use in poultry. *Journal of Applied Poultry Research*, 94, 1098-1107.
- Williams, P. (2007). *Bacillus subtilis*: A shocking message from a probiotic". *Cell Host & Microbe*, 1(4), 248-249.
- Wolfenden, R., Pumford, N., Morgan, M., Shivaramaiah, S., Wolfenden, A., Tellez, G., & Hargis, B. (2010). Evaluation of a screening and selection method for *Bacillus* isolates for use as effective direct-fed microbials in commercial poultry. *International Journal of Poultry Science*, 9, 317-323.
- Wolken, W. A., Tramper, J., & van der Werf, M. J. (2003). What can spores do for us? *Trends in Biotechnology*, 21(8), 338-345.
- Zar, J. (1984). In: *Biostatistical Analysis*. 2nd ed. Prentice-Hall, pp. 348-351.

## APPENDIX



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0-114 Poultry Science Building, University of Arkansas, Fayetteville, AR 72701-1201  
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To Whom It May Concern,

The first author of enclosed paper, “Physiological Properties and *Salmonella* Growth Inhibition of Probiotic *Bacillus* Strains Isolated from Environmental and Poultry Sources”, is Anita Menconi. Anita was primarily responsible for the work and research associated with this paper, and completed greater than 51% of the work.

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B.M. Hargis

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<http://dx.doi.org/10.1155/2013/958408>

#### Research Article

### Physiological Properties and *Salmonella* Growth Inhibition of Probiotic *Bacillus* Strains Isolated from Environmental and Poultry Sources

Anita Menconi, Marion J. Morgan, Neil R. Pumford, Billy M. Hargis, and Guillermo Tellez

Department of Poultry Science, University of Arkansas, Fayetteville, AR 72701, USA

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## MEMORANDUM

TO: Lisa R. Bielke

FROM: Craig N. Coon, Chairman  
Institutional Animal Care  
And Use Committee

DATE: June 9, 2011

SUBJECT: IACUC PROTOCOL APPROVAL  
Expiration date: June 2, 2014

The Institutional Animal Care and Use Committee (IACUC) has APPROVED Protocol #11047-  
"EVALUATION OF DIRECT FED MICROBIALS AND PREBIOTICS FOR SALMONELLA  
CONTROL IN POULTRY". You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF  
committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has  
components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be  
any changes in the protocol during the research, please notify the IACUC in writing  
[Modification Request form] prior to initiating the changes. If the study period is expected to  
extend beyond 06-02-2014, you must submit a new protocol. By policy the IACUC cannot  
approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines  
for research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian

## VIII. CHAPTER VI

### **Effect of Glutamine Supplementation Associated with Probiotics on *Salmonella* Typhimurium and Nitric Oxide or Glutamine with Perinatal Supplement on Growth Performance and Intestinal Morphology in Broiler Chickens**

A. Menconi<sup>†</sup>, G. Kallapura<sup>†</sup>, X. Hernandez-Velasco<sup>‡</sup>, J. Latorre<sup>†</sup>, Marion Morgan<sup>†</sup>, Neil R. Pumford<sup>†</sup>, S. Layton<sup>#</sup>, T. Urbano<sup>α</sup>, M. Caseres<sup>\*</sup>, C. Pixley<sup>§</sup>, J. Barton<sup>§</sup>, B. M. Hargis<sup>†</sup>, and G. Tellez<sup>†1</sup>

<sup>\*†</sup>Department of Poultry Science, University of Arkansas, Fayetteville, AR 72701; <sup>‡</sup>Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autonoma de Mexico, 04510

<sup>#</sup>ARGENTINA VETANCO S.A. Chile 33 (B1603CMA) Vicente López. Buenos Aires Argentina; <sup>\*</sup> Nutritionist, Buenos Aires, Argentina; <sup>α</sup> Vetanco do Brasil, Rua Raimundo Zanella, 490D Distrito Industrial Flavio Baldissera, Brazil, CEP 89.801-973; <sup>§</sup>Pacific Vet Group USA Inc., Fayetteville AR 72703.

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## **ABSTRACT**

Glutamine-enriched diets have been linked with favorable intestinal effects including structure maintenance of gut barrier against bacteria attacks and enterocyte differentiation. Although post hatch, immaturity of the GIT in the first week is a limiting factor, early nutrition has shown to be an alternative to alleviate the adverse performance effects of post-hatch starvation. In addition, both live and spore based probiotics have earned tremendous attention as a viable control of enteric pathogens. Present studies were carried out with objectives of evaluating the influence of nutrition and synergistic effects of Gln supplementation in combination with FloraMax-B11 (FM), a defined lactic acid bacteria (LAB) probiotic product; PHL-NP-122, a heat-resistant spore-forming *Bacillus subtilis* (BS); and EarlyBird (EB), a natural hydration and nutrition supplement for neonatal broilers and poults, on *Salmonella Typhimurium* colonization.

Morphometric analysis showed increased ( $P<0.05$ ) villus height, villus width, and villus surface area index in chickens treated with all combination groups. A reduction ( $P<0.05$ ) on nitric oxide (NO) produced was observed in the explant tissues of all the treated groups in comparison with the control group and a synergistic effect ( $P<0.05$ ) in the groups treated with Gln and BS (HPL-NP-122). Reductions in *Salmonella* recovery incidence ( $P<0.05$ ) and colonization ( $P<0.05$  to  $P<0.001$ ) were also observed among the treated groups, suggesting beneficial effects of these combinational feed supplements. Improved gut morphology and *Salmonella* exclusion was very well supported by body weight (BW) data with lower ( $P<0.05$ ) early BW loss and overall BW gains in birds treated with treated groups. Considering the fact that the feed costs represents from 70 to 80% of the poultry production and the integrity of the epithelial cells of the mucosa, hence ensured good performance and production, is dependent of feed and feed supplements, these

studies hold their relevance and importance as beneficial in more than one aspect to the poultry industry.

**Key words:** Glutamine, early feeding, probiotics, *Salmonella* Typhimurium, broiler, gut morphology, performance

## **INTRODUCTION**

The amino acid glutamine (Gln) is traditionally considered as a non-essential amino acid. However, recent researches has shown that Gln may be a conditionally essential amino acid in maintaining gut integrity and reducing inflammation (Reeds & Burrin, 2000; Soltan, 2009; Liu et al., 2002; Bode, 2001; Blikslager et al., 2001). Glutamine-enriched diets have been linked with favorable intestinal effects including maintenance of gut barrier function and enterocyte differentiation (Murakami et al., 2007). Glutamine has also been the focus of many studies in physiology and medicine due to its important pleiotropic roles in metabolism and tissue homeostasis. Glutamine serves as an essential metabolic precursor in nucleotide, glucose and amino sugar biosynthesis, glutathione homeostasis and protein synthesis (Bode, 2001). Presence of two mobilizable Nitrogen (N) groups in its structure, Gln can function as a vehicle for the tissue exchange of N and perform a crucial role in several important metabolic pathways.

Functions of Gln in mucosal barrier function is exclusively studied. It is responsible for mucosa structure maintenance, through mucin synthesis and the maintenance of a barrier against bacteria attacks, in addition to promoting the maturity and integrity of the intestinal flora associated with the immune system. Because glutamine is the main metabolite that nourishes the enterocytes, effect of glutamine supplementation on reconstitution of the intestinal mucosa, after some damage, has been investigated in various studies (Blikslager et al., 2001). In addition, Gln



is an essential substrate in the construction of the passive barrier of mucin to bacteria because it is necessary for the synthesis of N bases and amino sugars of the extracellular matrix, N-acetylglucosamine and N-acetylgalactosamine, and for the glycosylation of mucins (Reeds & Burrin, 2000). Gln has direct action in the elimination of free radicals by being a precursor of glutathione synthesis (Murakami et al., 2007). Glutamine is also considered as an immunonutrient, because of its capability of upregulating or downregulating immune responses to a pathogen or disease condition and may therefore reduce pathogen levels. As an immunonutrient, glutamine is important for promoting the integrity and maturation of intestinal microflora associated with the immune system, for enhancing mucin synthesis to maintain intestinal mucosa structure, and for reinforcing the epithelial barrier against bacterial attacks (Fasina et al., 2010).

From a poultry production perspective, the maintenance, development, and health of GIT is fundamental, since GIT possesses the functions of food content storage, secretion, digestion, and absorption of nutrients. The egg supplies nutrients during embryonic development. These gut functions begin with hatching and has to be maintained throughout the production pyramid. Posthatch the gut maturation process begins and this is a critical point in determining the poultry performance. The first two weeks of post hatch are even critical and represent approximately 30% of the useful life of the bird, considering a 6 week production cycle. Morphological studies by Sell et al. (1991) point out that at the moment of hatching, the weight of the small intestine represents 1.2 to 2.6% of the BW of the bird and 6.2 to 6.6% at maximum development. The development peak of the small intestine is shown to be between d 5 and 7 post hatch (Murakami et al., 2007). Hence the immaturity of the GIT in the first week post hatch is a limiting factor, since major gut transitions like increase in absorption capacity with a relative increase in the area

of absorption through the longitudinal growth of the intestine, and increase in the height of the villi, proper secretion of enzymes, are events yet to happen. In this regard, we suggest and hypothesize that the stimulation of the GIT by different substrates, soon after hatching, can accelerate its development.

On the other hand, delaying access to feed and water has been documented to increase susceptibility to pathogens and cause weight loss, leading to poorly starting flocks with reduced weight gains and mortality (Bigot et al., 2003; Careghi et al., 2005; Casteel et al., 1994). Early nutrition has been widely studied in poultry, and it has been shown that the use of early feeding supplements alleviates the adverse performance effects of post-hatch starvation (Hooshmand, 2006; Pinchasov & Noy, 1993; Uni et al., 2003a; Noy et al., 2001), as well as stimulates yolk utilization (Noy & Sklan, 1998), intestinal maturation (Bigot et al., 2003), development of homeothermy (Meltzer, 1983; van den Brand et al., 2010), and retain passive immunity (Dibner et al., 1998). In this concern, EarlyBird (EB), a natural hydration and nutrition supplement for neonatal broilers and poults, is extensively used to promote instinctive feeding of birds, that leads to a rapid onset and increased early weight gains that will eventually be maintained throughout the bird's lifetime (Henderson et al., 2008).

Alternatively, increasing socio-political concerns with antibiotic usage have led to investigations of potential alternatives for food safety and growth promotion. Both live and spore based probiotics have earned tremendous attention as a viable control of enteric pathogens in this regard. Laboratory and field research conducted by our laboratory with a defined lactic acid bacteria (LAB) probiotic, FloraMax-B11 (FM) have proved extremely influential in accelerated development of normal microflora and reduction in *Salmonella* colonization, in commercial poultry (Tellez et al., 2006; Higgins et al., 2007, 2008, 2011; Farnell et al., 2006; Vicente et al.,

2007, 2008; Wolfenden et al., 2007). More recently, we have confirmed that selected heat-resistant spore-forming *Bacillus* species, PHL-NP-122, can markedly reduce *Salmonella* and *Clostridium* in poultry when administered in very high numbers (Shivaramaiah et al., 2011; Wolfenden et al., 2010, 2011).

The present studies hold it relevance and importance, considering the fact that the feed costs represents 70 to 80% of the poultry production and the integrity of the epithelial cells of the mucosa, hence ensured good performance and production, is dependent of feed and feed supplements. These studies were carried out with multiple objectives of evaluating the influence of nutrition and combinational effects of Gln supplementation in concert with FloraMax-B11 (FM), PHL-NP-122 and EB on *Salmonella* Typhimurium colonization. EB+Gln were used to evaluate their combined effects on neonatals, FM+Gln and PHL-NP-122+Gln were used to evaluate their effects in relatively older birds. These studies also supports numerous trials which have previously tested individual positive effects of Gln, EB, FM and PHL-NP-122, on growth performance by rapid development of intestinal morphology in broiler chickens (Tellez et al., 2006; Higgins et al., 2007, 2008, 2011; Farnell et al., 2006; Vicente et al., 2007, 2008; Wolfenden et al., 2007, 2010, 2011; Shivaramaiah et al., 2011; Henderson et al., 2008). In addition, inflammatory marker like nitric oxide was also measured to know the combinational effects on *Salmonella* Typhimurium induced inflammatory damage.

## **MATERIALS AND METHODS**

### ***Animal Source and diets***

Day-of-hatch, off-sex broiler chickens were obtained from Cobb-Vantress (Siloam Springs, AR, USA) for all the trials mentioned below. All animal handling procedures were in

compliance with Institutional Animal Care and Use Committee at the University of Arkansas. In all experiments, diets were fed in mash form, and were formulated to meet or exceed National Research Council (NRC 1994) estimated nutrient requirements. The common starter diet was a typical corn soy bean meal diet (chemical analysis of nutrients is presented in Table 1). For experiments 2 and 3, the diet with glutamine was similar to the common starter diet but was supplemented with 1% Gln.

### ***Perinatal Supplement and Probiotic Culture***

EarlyBird (EB) is an all-natural hydration and nutrition supplement for young birds. One g of EB contains 64% of water, 22.0% of protein, 10% of fiber, 20% carbohydrate and less than 2.2% of fat (Pacific Vet Group USA Inc., Fayetteville AR 72703). Each bird should be administered 2g of EB according to manufacturer's instructions.

FloraMax B-11 (**FM**) is a probiotic culture derived from poultry, consisting of 2 strains of lactic acid bacterial isolates: *Lactobacillus salivarius* and *Pediococcus parvulus* (Pacific Vet Group USA Inc., Fayetteville AR 72703) was used as drinking water administration. *Bacillus subtilis* (**BS**) spores (PHL-NP122) previously identified as potential probiotic or direct-fed microbial (**DFM**) candidate (Shivaramaiah et al., 2011) was used in the present study.

### ***Bacterial Strain and Culture Conditions***

The challenge organism used in all experiments was poultry isolate of *Salmonella enterica* subspecies *enterica* serovar Typhimurium (ST). This isolate was selected for resistant to 25 µg/mL of novobiocin (**NOV**, catalog no.N-1628, Sigma) and 20 µg/mL of nalidixic acid (**NA**, catalog no.N-4382, Sigma) in our laboratory. For the present studies, 100 µL of SE from a frozen aliquot was added to 10 mL of tryptic soy broth (Catalog no. 22092, Sigma) and incubated at

37°C for 8 h, and passed every 8 h to ensure that all bacteria were in log phase. Post incubation, bacterial cells were washed 3 times in sterile 0.9% saline by centrifugation at  $1,864 \times g$ , quantified with a spectrophotometer (Spectronic 20D+, Spectronic Instruments Thermo Scientific) and diluted in sterile 0.9% saline to a concentration of approximately  $10^8$  cfu/mL. Concentrations of ST were determined retrospectively by serial dilution and further plating on Brilliant Green Agar (BGA, Catalog no. 70134, Sigma) with NOV and NA agar for enumeration of actual colony forming units (cfu) used to challenge the chickens.

### ***Experimental Design***

***Experiment 1.*** This experiment evaluated the effect of L-Glutamine (Catalog no. BDH 4514-1KGP, VWR West Chester, PA 19380) supplementation associated with FM in the drinking water on ST cecal colonization. Day-of hatch off sex broiler chickens were obtained and randomly distributed into 4 separate groups with 25 birds per group: Group 1, Control ST challenged; Group 2, 10 % L-Glutamine (Gln) mixed in the drinking water; Group 3, FM mixed in the drinking water; Group 4, Gln + FM mixed in the drinking water. A small number of chickens (n=20) were humanely killed on arrival, ceca-cecal tonsils and liver and spleen were aseptically removed, cultured in tetrathionate enrichment broth (Tet, Catalog no. 210420, Becton Dickinson, Sparks, MD) and confirmed negative for *Salmonella* by plating the samples on to selective BGA with NOV. All groups were challenged with ST at  $10^5$  cfu/bird. One h post challenge, groups 2, 3 and 4 received their treatment in the drinking water, while group 1 acted as positive control for ST. Twenty chickens from control or treated groups were humanely killed and cultured at 24 h, for ST recovery in ceca-cecal tonsils and enumerated as explained later.

**Experiment 2.** This experiment evaluated the effect of 1% dietary Gln supplementation for 6 days associated with FM in the drinking water on ST cecal colonization and *in vitro* nitric oxide production. Day-of hatch off sex broiler chickens were obtained and randomly distributed into 4 separate groups with 25 birds per group: Group 1, Control ST challenged; Group 2, 1% dietary Gln; Group 3, FM mixed in the drinking water following manufacture instructions; Group 4, 1% dietary Gln + FM mixed in the drinking water following manufacture instructions. A small number of chickens (n=20) were humanely killed on arrival, ceca-cecal tonsils and liver and spleen were aseptically removed, cultured in Tet and confirmed negative for *Salmonella* by plating the samples on to selective BGA with NO. At five days of age, all groups were challenged with ST at  $10^6$  cfu/bird. One h post challenge, groups 3 and 4 received FM in the drinking water, while group 1 acted as positive control for ST. Twelve chickens from control or treated groups were humanly killed and cultured at six days of age (24 h post ST challenge), for ST enumeration and explant samples for nitric oxide determination as explained later.

**Experiment 3.** This experiment evaluated the effect of 1% dietary Gln supplementation for 6 days associated with BS spores (PHL-NP-122) on ST cecal colonization and *in vitro* nitric oxide production. Day-of hatch off sex broiler chickens were obtained and randomly distributed into 4 separate groups with 25 birds per group: Group 1, Control ST challenged; Group 2, 1% dietary Gln; Group 3, DFM with a concentration of  $10^6$  BS spores/g of feed ; Group 4, 1% dietary Gln + DFM with a concentration of  $10^6$  BS spores/g of feed. A small number of chickens (n=20) were humanely killed on arrival, ceca-cecal tonsils and liver and spleen were aseptically removed, cultured in Tet and confirmed negative for *Salmonella* by plating the samples on to selective BGA with NOV. At five days of age, all groups were challenged with ST at  $10^6$  cfu/bird. Twelve chickens from control or treated groups were humanly killed and cultured at six days of

age (24 h post ST challenge), for ST enumeration and explant samples for nitric oxide determination as explained later.

**Experiment 4.** This experiment evaluated the effect of 0.5% Gln supplemented with perinatal supplement on growth performance and intestinal morphology in broiler chickens during 14 days. Three hundred off sex broiler chicks were obtained and transported to the University of Arkansas facility where they were identified through neck tags and randomly distributed in to 3 groups of 100 birds each, into commercial plastic poultry transport crates: Group 1, received no treatment; Group 2, received 200 grams of EB only; and Group 3 received 0.5% Gln supplemented with EB. Perinatal supplement was administered according to manufacturer's instructions. All birds were kept fasted, with no feed or water, for 48 h under simulated shipping conditions, at room temperature (25.5 °C) maintained with constant air flow to ensure the chicks were comfortable. After 48 h under simulated shipping conditions, chicks were placed onto floor pens with fresh wood shavings with a stocking density of 0.15 m<sup>2</sup>/chick. Age appropriate environmental temperatures were maintained and supplemental heat lamps were provided for each pen. Chickens were provided *ad libitum* access to water and a balanced un-medicated corn-soybean diet meeting or exceeding the nutrition requirements of poultry recommended NRC (NRC, 1994). All birds were weighed at 24 h, 48 h, 7 days and 14 days of age. Recorded body weights (BW) were then used to determine either body weight loss (BWL) at 24 h and 48h or body weight gain (BWG) at 7 and 14 days of age. Five ileum and duodenum samples from each group were collected for enteric morphometric analysis of mucosal development at 48 h, 7 and 14 days, processed and analyzed further as explained below.

### ***Salmonella Recovery***

In experiment one, chickens were humanely killed by CO<sub>2</sub> asphyxiation; ceca-ceca tonsils were aseptically removed to culture and enumerate *Salmonella*. Briefly, samples were placed in 10mL of Tet for enrichment and incubated at 37 °C for 24 hours. Samples were then plated on BGA NO and NA plates and incubated at 37 °C for 24 h to confirm presence/absence of typical lactose-negative colonies of *Salmonella*. Ceca were homogenized and diluted with saline (1:4 by wt/vol) and tenfold dilutions were plated on BGA with NO and NA, incubated at 37 °C for 24 h to enumerate total *Salmonella* cfu. Later, the cecal samples were enriched in double strength Tet and further incubated at 37 °C for 24 hours. Following this, ceca enrichment samples were plated on to BGA NO and NA plates and incubated at 37 °C for 24 h to confirm presence/absence of typical lactose-negative colonies of *Salmonella*. This enumeration procedure was also conducted in experiments two and three.

### ***Explant culture***

A novel explant culture method for rapid quantification of nitrite as an inflammatory marker developed in our lab (Kallapura et.al, 2013, Submitted for Publication) was employed here. Briefly, the entire ileum was aseptically removed, cleaned by infusing sterile 0.9% saline through the ileal section to remove all the ingesta. The cleansed ileum section was then incised longitudinally exposing the mucosal surface. Ileal sections (0.5 cm<sup>2</sup>) were made using a sterile surgical blade and placed in a 24 well culture plate. Care was taken in placing the tissue explants, with the serosa facing down and in contact with the well bottom and the mucosa facing up, exposed to the media components. Circular metal meshes measuring approximately 7.5mm in radius with 3mm height were used to keep the floating explants in place. These meshes were made of steel 316L, the same material used in construction of fermenters and bioreactors. The



material is non-reactive, non-additive, non-absorptive and non-corrosive and hence a safe choice to be used with a culture medium. Further care was taken to autoclave these meshes before use, to avoid any contamination. Explants (n=12) for each group were obtained, with one ileal explant per chicken. The ileal explants cultured in 24 well culture plates were then transferred to a laminar airflow hood and 1 mL of RPMI 1640 medium with 5% bovine serum, 1.5 mM L – glutamine, and 1 mL of antibiotic – antimycotic solution (containing 10,000 units of penicillin, 10 mg streptomycin and 25 µg amphotericin - Sigma-aldrich, St. Louis, MO) was added to each well. The cultures were further incubated at 40<sup>0</sup> C, 5% CO<sub>2</sub> and culture supernatants were collected for the nitrite assay at 3, 6 and 12 h post incubation.

### *Nitrite Assay*

The Greiss reaction assay, which colorimetrically quantifies nitrite, was used to measure the nitrite accumulated over time in the culture medium, which served as an indirect measure of NO produced by the explants. The assay was carried out in a 96 well microtitre plate to which 100µL of culture supernatant from the explant culture (at 3-, 6- and 12-h) was added in triplicates, followed by an equal volume of Greiss reagents. First, 50µL of 1% sulfanilamide (Sigma-aldrich, St. Louis, MO) in 5% phosphoric acid, was added and incubated at room temperature for 10min followed by 50µL of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (Sigma-aldrich, St. Louis, MO) in water and incubated further for a visible colored reaction to develop and measured at 540 nm. The Greiss reaction was based on a two-step diazotization reaction in which acidified nitrite (phosphoric acid) produces a nitrosating agent which reacts with sulfanilic acid to produce a diazonium ion. This ion intermediate was then coupled with N-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative

whose absorbance was measured at 540 nm. Optical density (OD) for each explant sample was compared to known amounts of sodium nitrite (1.25, 2.5, 5, 10, 20, 30, 40, 50, 60, 70, 80, and 90  $\mu\text{M}$ ) and extrapolated using a standard curve equation.

### ***Intestinal Morphological Analysis***

For enteric morphometric analysis, birds on the designated evaluation day were euthanized, and ileum and duodenum samples were collected (n=5). A 1-cm segment of the midpoint of the duodenum and the distal end of the lower ileum from each bird was removed and fixed in 10% buffered formaldehyde for 48 h. Each of these intestinal segments was embedded in paraffin, and a 5- $\mu\text{m}$  section of each sample was placed on a glass slide and stained with hematoxylin and eosin for examination under a light microscope. All morphological parameters were measured using the ImageJ software package (<http://rsb.info.nih.gov/ij/>). Ten replicate measurements for each variable studied, were taken from each sample and the average values were used in statistical analysis. Villus length was measured from the top of the villus to the top of the lamina propria. Villus width was measured at the widest area of each villus (Aptekmann et al., 2001). Villus surface area was calculated using the formula  $(2\pi) (VW/2) (VL)$ , where VW = villus width, and VL = villus length (Sakamoto et al., 2000).

### ***Statistical Analysis***

Any statistical differences in BW, BWL, BWG,  $\log_{10}$  SE cfu/g of ceca and morphometric measurements were determined by analysis of variance using the General Linear Models (proc GLM) procedure using commercial SAS<sup>®</sup> statistical software (SAS Institute, 2002). Significant differences, set at  $P < 0.05$ , were further separated using Duncan's multiple range test. The

percent recovery of *Salmonella* was compared using the chi-square test of independence testing all possible group combinations to determine significance for these studies (Zar, 1984).

## RESULTS

The results of the effect of 10% Gln supplementation associated with FM in the drinking water on ST colonization in broiler chickens from experiment 1 are summarized in table 2. A 20% significant reduction ( $P < 0.05$ ) in the rate of intestinal colonization of ST at 24 h were observed in the group that received 10% Gln in the drinking water; and the group that receive 10% Gln + FM had a 35% reduction of ST ( $P < 0.01$ ). However, the group that received just the probiotic in the drinking water showed a 65% ST reduction when compared with control group ( $P < 0.001$ ). A similar trend in the reduction of cfu of ST/g of ceca content was observed in the same groups (Table 2).

The effect of 1% dietary Gln supplementation associated with FM in the drinking water on ST colonization at 6 days of age in broiler chickens from experiment 2 are summarized in table 3. A significant reduction on ST/g of ceca content was observed in both, dietary Gln alone or probiotic alone groups. This reduction was associated with a significant reduction on NO produced in the explant tissues as compared with the control group. However, an even more significant reduction on ST/g of ceca content and synergistic effect in the reduction of NO production was observed in the group that received the inclusion of 1% dietary Gln and FM in the drinking water when compared with the control group (Table 3).

The effect of 1% dietary Gln supplementation associated with PHL-NP-122 on ST colonization at 6 days of age in broiler chickens from experiment 3 is summarized in table 4. A significant reduction on ST/g of ceca content was observed in both, dietary Gln or DFM groups.

As in the previous experiment, this reduction was also associated with a significant reduction on NO produced in the explant tissues as compared with the control group. However, an even more significant reduction on ST/g of ceca content and synergistic effect in the reduction of NO production was observed in the group that received the inclusion of 1% dietary Gln and PHL-NP-122 in the drinking water when compared with the control group (Table 4)

The effect of 0.5% Gln with perinatal supplement on body weight and performance of broiler chickens from experiment 4 are summarized in table 5. At 24 h and 48 h significant BWL were observed with control and EB only groups when compared with the group that received EB + Gln. These differences were maintained at 7 and 14 days of evaluation, with significantly higher BWG seen in the group treated with EB + Gln. It was of significance to mention that by 14 days, the BW of the EB + Gln treated group was, on an average, about 17 g heavier than that of non-treated control group ( $P < 0.05$ ). Although not significant, over all, the EB group had a numerical improvement in performance with about 12 g heavier than that of non-treated control group (Table 1). The effect of 0.5% Gln with perinatal supplement on morphological development of mucosa in duodenum of broiler chickens is summarized in Table 6. Significantly increased villus height, villus width, and villus surface area index were observed in the groups treated with perinatal supplement only or 0.5% Gln and perinatal supplement, when compared to non-treated control group at 24 h. The trend of significant and in some cases numerically, morphometric changes were observed throughout the study in duodenum samples (Table 6). No significant morphometric changes between the three groups were observed in samples from distal ileum (data not shown).

## DISCUSSION

Glutamine is a captivating amino acid that constitutes significant concentrations of muscles and plasma, and represents about 50 to 80% of the total free amino acid in the body (Reeds & Burrin, 2000; Liu et al., 2002; Kim et al., 2004). Since its structure contains two mobilizable N groups Gln is involved in transportation and exchange of N in the cells, as well as participate in important metabolic pathways (Reeds & Burrin, 2000; Soltan, 2009). This amino acid is also a key component in the function and structure of the intestinal mucosa since it is involved in mucin synthesis as well as maintaining the integrity of the gut microbiome (Reeds & Burrin, 2000; Bode, 2001; Liu et al., 2002; Sakamoto et al., 2006; Murakami et al., 2007), which has a profound impact in digestive physiology (Tellez et al., 2006; Fraune & Bosch, 2010; Bäckhed, 2011; Musso et al., 2010), as well as innate and acquire immunity (Neish, 2009; Maslowski & Mackay, 2010; Kau et al., 2011; Salzman, 2011). As if these functions were not important enough, Gln is the principal energetic fuel for cells that has a rapid proliferation such as enterocytes, lymphocytes and other cells involved in inflammation (Blikslager et al., 2001; Bode, 2001; Dai et al., 2009; Fasina et al., 2010).

In the present study, the supplementation of 10% Gln with probiotic culture in the drinking water had a significant reduction on ST colonization in the ceca, but this effect was not synergistic (Table 2). However, dietary supplementation of 1% Gln associated with a lactic acid bacteria probiotic in the drinking water (Table 3), or in the diet through a DFM in form of spores of *Bacillus subtilis* (Table 4) had a significant and synergistic effect on the reduction of *Salmonella* Typhimurium in the ceca. This reduction was associated with a significant reduction of nitric oxide produce in the explant of ileum samples (Tables 3 and 4). Quantifying nitrite, a metabolite of nitric oxide (NO), is a well-established marker for the production of reactive

nitrogen species and an indirect measurement for inflammation. The innate immune response is modulated through the recruitment of various cellular components upon pathogen exposure. Heterophils, monocytes, and macrophages are at the forefront of pathogen recognition, and work in combination with effector leukocytes to initiate an immune response. Studies investigating the role of heterophils, monocytes, and macrophages begin with quantification of reactive nitrogen species (RNS), reactive oxygen species (ROS), along with cytokines and chemokines (Crippen et al., 2003). Though rapid clearance of pathogens has been attributed to ROS (oxidative stress) rather than RNS (nitrosative stress), nitrosative stress is important in chronic and/or prolonged exposure. The sequential progression from a predominant oxidative stress to the production of nitrosative clearance could optimize the reduction in microbial burden along with minimizing immunopathological consequences of host inflammatory response (Vazquez-Torres & Fang, 2001; Chakravorty & Hensel, 2003). Hence, quantifying metabolites of nitric oxide (NO), such as nitrite or expression of inducible nitric oxide synthase (iNOS), have been the principle for investigating the role of RNS during host inflammatory responses. The quantification of NO in the ileal explants from experiments 2 and 3 provided a suitable model for inflammation (Tables 3 and 4), which potentially mimics *in vivo* intestinal conditions that rapidly detected NO (6 hours), at a greater magnitude than other cell culture methods (Qureshi, 2003; He et al., 2008; Setta et al., 2012)

On the other hand, a fasting period of 24 to 72 h after hatch is a common practice in commercial poultry operations (Dibner et al., 1998) due to variation in hatching time and management in the hatchery. This delay in start of feed intake has been shown to negatively affect yolk utilization (Noy & Sklan, 2001), gastrointestinal development (Noy et al., 2001), slaughter weight (Halevy et al., 2000) and breast meat yield (Halevy et al., 2003; Noy & Uni,

2010). In addition, delayed feeding seems to depress immunological development (Juul-Madsen et al., 2004). The immediate post-hatch period is critical for intestinal morphological development in order to digest feed and assimilate nutrients (Uni et al., 1999; Uni et al., 2003b). Decreased intestinal development in chicks fasted for 36 to 48 hours post-hatch have been extensively reported by several investigators (Casteel et al., 1994; Uni et al., 1998; Batal & Parsons, 2002; Bigot et al., 2003; Careghi et al., 2005; Henderson et al., 2008). In the present study, chicks that received 0.5% Gln with a perinatal supplement showed significantly less body weight loss during at 24 h and 48 h under simulated shipping period of 48 h and were significantly heavier at 7 and 14 days (Table 5), and these changes were associated with the significant increased villus height, villus width, and villus surface area index observed in the groups treated with EB only or EB + Gln, when compared to non-treated control group at 24 h. The trend of significant and in some cases numerically, morphometric changes were observed throughout the study in duodenum samples (Table 6). In summary, Gln with probiotics or perinatal supplementation suggest that these nutraceuticals could be a good practical delivery system for this important amino acid during the common fasting conditions, providing several benefits for the poultry industry.

## TABLES

**Table 1.** Composition of the starter diet for broiler chickens from 1 to 14 d (kg)

<b>Ingredient</b>	<b>Glutamine free</b>	<b>Glutamine 1%</b>
<b>Corn</b>	546.389	546.389
<b>Soybean meal</b>	369.359	369.359
<b>Vegetable oil</b>	33.231	33.231
<b>Dicalcium phosphate</b>	15.855	15.855
<b>Calcium carbonate</b>	14.44	14.44
<b>Salt</b>	3.538	3.538
<b>DL-Methionine</b>	2.56	2.56
<b>Vitamin premix<sup>1</sup></b>	1.0	1.0
<b>Solka-floc</b>	10.0	---
<b>L-Lysine HCl</b>	0.977	0.977
<b>L-Glutamine</b>	---	10.00
<b>Choline chloride 60%</b>	1.00	1.00
<b>Mineral premix<sup>2</sup></b>	0.500	0.500
<b>Zinc bacitracin</b>	0.500	0.500
<b>Sodium monensin</b>	0.500	0.500
<b>Antioxidant<sup>3</sup></b>	0.150	0.150
<b>Total</b>	1000	1000
	Calculated analysis	
<b>ME, kcal/ kg</b>	3,035	3,035
<b>CP, %</b>	21.704	21.704
<b>Lysine, %</b>	1.328	1.328
<b>Methionine, %</b>	0.597	0.597
<b>Met + cist, %</b>	0.98	0.98
<b>Threonine, %</b>	0.866	0.866
<b>Tryptophan, %</b>	0.282	0.282
<b>Total calcium, %</b>	0.900	0.900
<b>Available phosphorus, %</b>	0.450	0.450
<b>Sodium, %</b>	0.160	0.160

<sup>1</sup>Vitamin premix supplied the following per kilogram: vitamin A, 20,000,000 IU; vitamin D3, 6,000,000 IU; vitamin E, 75,000 IU; vitamin K3, 9 g; thiamine, 3 g; riboflavin, 8 g; pantothenic acid, 18 g; niacin, 60 g; pyridoxine, 5 g; folic acid, 2 g; biotin, 0.2 g; cyanocobalamin, 16 mg; and ascorbic acid, 200 g.

<sup>2</sup>Mineral premix supplied the following per kilogram: manganese, 120 g; zinc, 100 g; iron, 120 g; copper, 10–15 g; iodine, 0.7 g; selenium, 0.4 g; and cobalt, 0.2 g.

<sup>3</sup>Ethoxyquin, selenium, 0.4; and cobalt, 0.2 g.



**Table 2.** Effect of 10% glutamine (Gln) supplementation associated with FloraMax (FM) in the drinking water on *Salmonella* Typhimurium (ST) colonization in broiler chickens from experiment 1

Treatment	Cecal tonsil	Log <sub>10</sub> <i>S. Typhimurium</i> /gram of ceca content
1. Control ST	20/20 (100 %)	3.12 ± 0.21 <sup>a</sup>
2. Gln 10%	16/20 (80 %) <sup>x</sup>	1.96 ± 0.44 <sup>ab</sup>
3. FM	7/20 (35 %) <sup>z</sup>	0.67 ± 0.35 <sup>c</sup>
4. Gln 10% + FM	13/20 (65 %) <sup>y</sup>	1.72 ± 0.55 <sup>bc</sup>

Chickens were orally gavaged with 10<sup>5</sup> cfu/chicken of *S. Typhimurium* at hatch. One hour later chickens were treated in the drink water. Control chickens received regular water. Twenty chickens from each group were humanly killed and cultured 24 h post challenge, for ST recovery. Data of cecal tonsils is expressed as positive/total chickens (%). <sup>x</sup> P < 0.05 ; <sup>y</sup> P < 0.01; <sup>z</sup> P < 0.001.

Ceca from twelve chickens were enumerated. Log<sub>10</sub> *S. Typhimurium*/ gram of ceca content data is expressed as mean ± standard error. Treatments values with no common superscript differ significantly P<0.05.

**Table 3.** Effect of 1% dietary glutamine (Gln) supplementation associated with with FloraMax (FM) in the drinking water on *Salmonella* Typhimurium (ST) colonization at 6 days of age in broiler chickens from experiment 2

Treatment	Log <sub>10</sub> ST/ gram of ceca content	Nitrite in $\mu$ M at 6 h
1. Control ST	6.1 $\pm$ 0.2 <sup>a</sup>	35.0 $\pm$ 25.1 <sup>a</sup>
2. Glu 1%	6.0 $\pm$ 0.2 <sup>bc</sup>	6.6 $\pm$ 2.0 <sup>b</sup>
3. FM	6.3 $\pm$ 0.3 <sup>ab</sup>	8.4 $\pm$ 2.3 <sup>b</sup>
4. Glu 1% + FM	4.9 $\pm$ 0.11 <sup>d</sup>	4.8 $\pm$ 1.4 <sup>b</sup>

Chickens were orally gavaged with 10<sup>6</sup> cfu/chicken of *S. Typhimurium* at five days of age. One hour later chickens in groups 3 and 4 were treated in the drink water with FM. Twelve chickens from each group were humanly killed and cultured 24 h post challenge, for ST recovery and explant ileal samples. Log<sub>10</sub> *S. Typhimurium*/ gram of ceca content or micro molar ( $\mu$ M) amounts of nitrite, data is expressed as mean  $\pm$  standard error. Treatments values with no common superscript within columns differ significantly P<0.05.

**Table 4.** Effect of 1% dietary glutamine (Gln) supplementation associated with *Bacillus subtilis* spores (PHL-NP-122) on *Salmonella* Typhimurium (ST) colonization at 6 days of age in broiler chickens from experiment 3

<b>Treatment</b>	<b>Log<sub>10</sub> ST/ gram of ceca content</b>	<b>Nitrite in μM at 6 h</b>
<b>1. Control ST</b>	6.9 ± 0.2 <sup>a</sup>	34.8 ± 25.4 <sup>a</sup>
<b>2. Glu 1%</b>	6.1 ± 0.2 <sup>b</sup>	6.6 ± 1.9 <sup>b</sup>
<b>3. NP122</b>	6.0 ± 0.1 <sup>b</sup>	5.1 ± 0.8 <sup>b</sup>
<b>4. Glu 1% + NP122</b>	5.6 ± 0.3 <sup>c</sup>	2.4 ± 0.5 <sup>c</sup>

Chickens were orally gavaged with 10<sup>6</sup> cfu/chicken of *S. Typhimurium*/chicken at five days of age. Twelve chickens from each group were humanly killed and cultured 24 h post challenge, for ST recovery and explant ileal samples. Log<sub>10</sub> *S. Typhimurium*/ gram of ceca content or micromolar (μM) amounts of nitrite, data is expressed as mean ± standard error. Treatments values with no common superscript within columns differ significantly P < 0.05.

**Table 5.** Effect of Glutamine (Gln) with perinatal supplement (EB) on body weight (BWT) and performance of broiler chickens from experiment 4

	<b>Initial body weight</b>	<b>BWT at 24 hours</b>	<b>BWL at 24 hours</b>	<b>BWT at 48 hours</b>	<b>BWL at 48 hours</b>	<b>BWT at 7 days</b>	<b>BWG at 7 days</b>	<b>BWT at 14 days</b>	<b>BWG at 14 days</b>
<b>Control</b>	43.7 ± 0.3 <sup>a</sup>	40.2 ± 0.3 <sup>a</sup>	-3.5 ± 0.1 <sup>b</sup>	38.1 ± 0.3 <sup>a</sup>	-5.6 ± 0.1 <sup>b</sup>	111.3 ± 1.1 <sup>b</sup>	67.7 ± 1.1 <sup>b</sup>	330.1 ± 4.7 <sup>b</sup>	288.7 ± 4.4 <sup>b</sup>
<b>EB</b>	43.0 ± 0.3 <sup>ab</sup>	41.3 ± 0.3 <sup>a</sup>	-1.7 ± 0.1 <sup>b</sup>	38.9 ± 0.3 <sup>a</sup>	-4.1 ± 0.4 <sup>b</sup>	118.6 ± 1.3 <sup>a</sup>	75.4 ± 1.1 <sup>a</sup>	344.0 ± 4.8 <sup>a</sup>	301.8 ± 4.2 <sup>a</sup>
<b>Gln + EB</b>	42.4 ± 0.2 <sup>b</sup>	41.2 ± 0.3 <sup>a</sup>	-1.2 ± 0.1 <sup>a</sup>	39.1 ± 0.3 <sup>a</sup>	-3.3 ± 0.1 <sup>a</sup>	118.5 ± 1.4 <sup>a</sup>	76.1 ± 1.2 <sup>a</sup>	349.6 ± 5.7 <sup>a</sup>	306.3 ± 5.2 <sup>a</sup>

300 day of hatch off sex broiler chickens were obtained and randomly distributed in to 3 separate groups (n=100). Treatments were administered according to groups: Group 1, received no treatment; Group 2, received 200 grams of EB only; and Group3 received EB supplemented with 0.5% L-glutamine. All birds were weighed at 24 h, 48 h, 7 days and 14 days of age. Recorded body weights (BW) were then used to determine either body weight loss (BWL) at 24 h and 48h or body weight gain (BWG) at 7 and 14 days of age. Body weight data were expressed as mean (grams) ± standard error. Values within columns with no common superscript differ significantly P < 0.05.

**Table 6.** Effect of 0.5% Glutamine (Gln) with perinatal supplement (EB) on morphometric analysis of the duodenum mucosa of broiler chickens from experiment 4

<b>Time of evaluation and Treatments</b>	<b>Villus Height (µm)</b>	<b>Villus Width (µm)</b>	<b>Villus Surface Area Index (µm<sup>2</sup>)</b>
<b>48 hours</b>			
<b>1. Control</b>	443.9 ± 46.5 <sup>b</sup>	73.7 ± 6.3 <sup>b</sup>	101,890 ± 11,757 <sup>b</sup>
<b>2. EB</b>	737.4 ± 27.7 <sup>a</sup>	118.3 ± 6.7 <sup>a</sup>	272,083 ± 9,253 <sup>a</sup>
<b>3. 0.5 % Gln + EB</b>	801.7 ± 22.3 <sup>a</sup>	113.7 ± 16.5 <sup>a</sup>	288,554 ± 4,5475 <sup>a</sup>
<b>7 days</b>			
<b>1. Control</b>	1,108.6 ± 30.1 <sup>b</sup>	173.1 ± 10.4 <sup>a</sup>	605,798 ± 51,342 <sup>ab</sup>
<b>2. EB</b>	1,384.7 ± 54.5 <sup>a</sup>	169.7 ± 13.0 <sup>a</sup>	742,877 ± 78,879 <sup>a</sup>
<b>3. 0.5 % Gln + EB</b>	1,333.4 ± 40.3 <sup>a</sup>	126.6 ± 12.1 <sup>b</sup>	528,375 ± 51,378 <sup>b</sup>
<b>14 days</b>			
<b>1. Control</b>	1,318.7 ± 97.9 <sup>b</sup>	144.0 ± 4.4 <sup>b</sup>	599,454 ± 55,244 <sup>b</sup>
<b>2. EB</b>	1,554.9 ± 36.2 <sup>ab</sup>	160.2 ± 19.5 <sup>b</sup>	788,454 ± 109,363 <sup>ab</sup>
<b>3. 0.5 % Gln + EB</b>	1,617.7 ± 120.9 <sup>a</sup>	217.1 ± 19.1 <sup>a</sup>	1,129,650 ± 176,212 <sup>a</sup>

300 day of hatch off sex broiler chickens were obtained and randomly distributed in to 3 separate groups (n=100). Treatments were administered according to groups: Group 1, received no treatment; Group 2, received 200 grams of EB only; and Group3 received EB supplemented with 0.5% L-glutamine. Five duodenum samples/group were collected for enteric morphometric analysis, at all-time points. Values were expressed as means ± SEM representing 5 bird/group and 10 measurements/parameter/bird. Values within columns with no common superscript differ significantly P<0.05.

## REFERENCES

- Bäckhed, F. (2011). Programming of host metabolism by the gut microbiota. *Annals of Nutrition and Metabolism*, 58, 44-52.
- Batal, A., & Parsons, C. (2002). Effect of fasting versus feeding oasis after hatching on nutrient utilization in chicks. *Poultry Science*, 81, 853-859.
- Bigot, K., Mignon-Grasteau, S., Picard, M., & Tesseraud, S. (2003). Effects of delayed feed intake on body, intestine, and muscle development in neonate broilers. *Poultry Science*, 82, 781-788.
- Blikslager, A., Hunt, E., Guerrant, R., Rhoads, M., & Argenzio, R. (2001). Glutamine transporter in crypts compensates for loss of villus absorption in bovine cryptosporidiosis. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 281, G645-G653.
- Bode, B. P. (2001). Recent molecular advances in mammalian glutamine transport. *The Journal of Nutrition*, 131, 2475S-2485S.
- van den Brand, H., Molenaar, R., van der Star, I., & Meijerhof, R. (2010). Early feeding affects resistance against cold exposure in young broiler chickens. *Poultry Science*, 89, 716-720.
- Careghi, C., Tona, K., Onagbesan, O., Buyse, J., Decuyper, E., & Bruggeman, V. (2005). The effects of the spread of hatch and interaction with delayed feed access after hatch on broiler performance until seven days of age. *Poultry Science*, 84, 1314-1320.
- Casteel, E., Wilson, J., Buhr, R., & Sander, J. (1994). The influence of extended posthatch holding time and placement density on broiler performance. *Poultry Science*, 73, 1679-1684.
- Chakravorty, D., & Hensel, M. (2003). Inducible nitric oxide synthase and control of intracellular bacterial pathogens. *Microbes and Infection*, 5, 621-627.
- Crippen, T. L., Sheffield, C. L., He, H., Lowry, V. K., & Kogut, M. H. (2003). Differential nitric oxide production by chicken immune cells. *Developmental & Comparative Immunology*, 27, 603-610.
- Dai, S., Wang, L., Wen, A., Wang, L., & Jin, G. (2009). Dietary glutamine supplementation improves growth performance, meat quality and colour stability of broilers under heat stress. *British Poultry Science*, 50, 333-340.
- Dibner, J., Knight, C., Kitchell, M., Atwell, C., Downs, A., & Ivey, F. (1998). Early feeding and development of the immune system in neonatal poultry. *The Journal of Applied Poultry Research*, 7, 425-436.
- Farnell, M., Donoghue, A., De Los Santos, F. S., Blore, P., Hargis, B., Tellez, G., & Donoghue, D. (2006). Upregulation of oxidative burst and degranulation in chicken heterophils stimulated with probiotic bacteria. *Poultry Science*, 85, 1900-1906.

- Fasina, Y., Bowers, J., Hess, J., & McKee, S. (2010). Effect of dietary glutamine supplementation on *Salmonella* colonization in the ceca of young broiler chicks. *Poultry Science*, 89, 1042-1048.
- Fraune, S., & Bosch, T. C. (2010). Why bacteria matter in animal development and evolution. *Bioessays*, 32, 571-580.
- Halevy, O., Geyra, A., Barak, M., Uni, Z., & Sklan, D. (2000). Early posthatch starvation decreases satellite cell proliferation and skeletal muscle growth in chicks. *The Journal of Nutrition*, 130, 858-864.
- Halevy, O., Nadel, Y., Barak, M., Rozenboim, I., & Sklan, D. (2003). Early posthatch feeding stimulates satellite cell proliferation and skeletal muscle growth in turkey poults. *The Journal of Nutrition*, 133, 1376-1382.
- He, H., Genovese, K. J., Swaggerty, C. L., Nisbet, D. J., & Kogut, M. H. (2008). Differential induction of nitric oxide, degranulation, and oxidative burst activities in response to microbial agonist stimulations in monocytes and heterophils from young commercial turkeys. *Veterinary Immunology and Immunopathology*, 123, 177-185.
- Henderson, S., Vicente, J., Pixley, C., Hargis, B., & Tellez, G. (2008). Effect of an early nutritional supplement on broiler performance. *International Journal of Poultry Science*, 7, 211-214.
- Higgins, J., Higgins, S., Vicente, J., Wolfenden, A., Tellez, G., & Hargis, B. M. (2007). Temporal effects of lactic acid bacteria probiotic culture on *Salmonella* in neonatal broilers. *Poultry Science*, 86, 1662-1666
- Hooshmand, M. (2006). Effect of early feeding programs on broiler performance. *International Journal of Poultry Science*, 5, 1140-1143.
- Juul-Madsen, H. R., Su, G., & Sørensen, P. (2004). Influence of early or late start of first feeding on growth and immune phenotype of broilers. *British Poultry Science*, 45, 210-222.
- Kau, A. L., Ahern, P. P., Griffin, N. W., Goodman, A. L., & Gordon, J. I. (2011). Human nutrition, the gut microbiome and the immune system. *Nature*, 474, 327-336.
- Kim, S. W., McPherson, R. L., & Wu, G. (2004). Dietary arginine supplementation enhances the growth of milk-fed young pigs. *The Journal of Nutrition*, 134, 625-630.
- Liu, T., Peng, J., Xiong, Y., Zhou, S., & Cheng, X. (2002). Effects of dietary glutamine and glutamate supplementation on small intestinal structure, active absorption and DNA, RNA concentrations in skeletal muscle tissue of weaned piglets during d 28 to 42 of age. *Asian Australasian Journal of Animal Sciences*, 15, 238-242.
- Maslowski, K. M., & Mackay, C. R. (2010). Diet, gut microbiota and immune responses. *Nature Immunology*, 12, 5-9.

- Meltzer, A. (1983). Thermoneutral zone and resting metabolic rate of broilers. *British Poultry Science*, 24, 471-476.
- Murakami, A., Sakamoto, M., Natali, M., Souza, L., & Franco, J. (2007). Supplementation of glutamine and vitamin E on the morphometry of the intestinal mucosa in broiler chickens. *Poultry Science*, 86, 488-495.
- Musso, G., Gambino, R., & Cassader, M. (2010). Obesity, diabetes, and gut microbiota the hygiene hypothesis expanded? *Diabetes Care*, 33, 2277-2284.
- Neish, A. S. (2009). Microbes in gastrointestinal health and disease. *Gastroenterology*, 136, 65-80.
- Noy, Y., Geyra, A., & Sklan, D. (2001). The effect of early feeding on growth and small intestinal development in the posthatch poult. *Poultry Science*, 80, 912-919.
- Noy, Y., & Sklan, D. (1998). Metabolic responses to early nutrition. *The Journal of Applied Poultry Research*, 7, 437-451.
- Noy, Y., & Sklan, D. (2001). Yolk and exogenous feed utilization in the posthatch chick. *Poultry Science*, 80, 1490-1495.
- Noy, Y., & Uni, Z. (2010). Early nutritional strategies. *World's Poultry Science Journal*, 66, 639-646.
- Pinchasov, Y., & Noy, Y. (1993). Comparison of post-hatch holding time and subsequent early performance of broiler chicks and Turkey poults. *British Poultry Science*, 34, 111-120.
- Qureshi, M. (2003). Avian macrophage and immune response: An overview. *Poultry Science*, 82, 691-698.
- Reeds, P. J., & Burrin, D. G. (2000). The gut and amino acid homeostasis<sup>1</sup>. *Nutrition*, 16:666-668.
- Sakamoto, M., Murakami, A., Silveira, T., Fernandes, J., & de Oliveira, C. (2006). Influence of glutamine and vitamin E on the performance and the immune responses of broiler chickens. *Revista Brasileira de Ciência Avícola*, 8, 243-249.
- Salzman, N. H. (2011). Microbiota-immune system interaction: an uneasy alliance. *Current Opinion in Microbiology*, 14, 99-105.
- Sell, J. L., Angel, C. R., Piquer, F. J., Mallarino, E. G., & al-Batshan, H. A. (1991). Developmental patterns of selected characteristics of the gastrointestinal tract of young turkeys. *Poultry Science*, 70, 1200-1205.
- Setta, A., Barrow, P. A., Kaiser, P., & Jones, M. A. (2012). Immune dynamics following infection of avian macrophages and epithelial cells with typhoidal and non-typhoidal *Salmonella enterica* serovars; bacterial invasion and persistence, nitric oxide and oxygen



- production, differential host gene expression, NF-kappa signalling and cell cytotoxicity. *Veterinary Immunology and Immunopathology*, 146(3-4), 212-24.
- Shivaramaiah, S., Pumford, N., Morgan, M., Wolfenden, R., Wolfenden, A., Torres-Rodriguez, A., Hargis, B., & Tellez, G. (2011). Evaluation of *Bacillus* species as potential candidates for direct-fed microbials in commercial poultry. *Poultry Science*, 90, 1574-1580.
- Soltan, M. (2009). Influence of dietary glutamine supplementation on growth performance, small intestinal morphology, immune response and some blood parameters of broiler chickens. *International Journal of Poultry Science*, 8, 60-68.
- Tellez, G., Higgins, S., Donoghue, A., & Hargis, B. (2006). Digestive physiology and the role of microorganisms. *The Journal of Applied Poultry Research*, 15, 136-144.
- Uni, Z., Ganot, S., & Sklan, D. (1998). Posthatch development of mucosal function in the broiler small intestine. *Poultry Science*, 77, 75-82.
- Uni, Z., Noy, Y., & Sklan, D. (1999). Posthatch development of small intestinal function in the poul. *Poultry Science*, 78, 215-222.
- Uni, Z., Smirnov, A., & Sklan, D. (2003a). Pre-and posthatch development of goblet cells in the broiler small intestine: effect of delayed access to feed. *Poultry Science*, 82, 320-327.
- Uni, Z., Tako, E., Gal-Garber, O., & Sklan, D. (2003b). Morphological, molecular, and functional changes in the chicken small intestine of the late-term embryo. *Poultry Science*, 82, 1747-1754.
- Vazquez-Torres, A., & Fang, F. C. (2001). Oxygen-dependent anti- *Salmonella* activity of macrophages. *Trends in Microbiology*, 9, 29-33.
- Vicente, J., Higgins, S., Bielke, L., Tellez, G., Donoghue, D., Donoghue, A., & Hargis, B. M. (2007). Effect of probiotic culture candidates on *Salmonella* prevalence in commercial turkey houses. *Journal of Applied of Poultry Research*, 16, 471-476.
- Vicente, J. L., Torres-Rodriguez, A., Higgins, S. E., Pixley, C., Tellez, G., Donoghue, A. M., & Hargis, B. M. (2008). Effect of a selected *Lactobacillus* spp.-based probiotic on *Salmonella enterica* serovar Enteritidis-infected broiler chicks. *Avian Diseases*, 52, 143-146.
- Wolfenden, A., Pixley, C., Higgins, J., Higgins, S., Vicente, J., Torres-Rodriguez, A., Hargis, B., & Tellez, G. (2007). Evaluation of spray application of a *Lactobacillus*-based probiotic on *Salmonella enteritidis* colonization in broiler chickens. *International Journal of Poultry Science*, 6, 493-496.
- Wolfenden, R., Pumford, N., Morgan, M., Shivaramaiah, S., Wolfenden, A., Pixley, C., Green, J., Tellez, G., & Hargis, B. (2011). Evaluation of selected direct-fed microbial candidates on live performance and *Salmonella* reduction in commercial turkey brooding houses. *Poultry Science*, 90, 2627-2631.

Wolfenden, R., Pumford, N., Morgan, M., Shivaramaiah, S., Wolfenden, A., Tellez, G., & Hargis, B. (2010). Evaluation of a screening and selection method for *Bacillus* isolates for use as effective direct-fed microbials in commercial poultry. *International Journal of Poultry Science*, 9, 317-323.

Zar, J. (1984). *Biostatistical Analysis*. 2nd ed. Prentice Hall, Upper Saddle River, NJ.

## APPENDIX



Center of Excellence  
for Poultry Science



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0-114 Poultry Science Building, University of Arkansas, Fayetteville, AR 72701-1201  
479-575-4952 • Fax 479-575-3026 • [www.uark.edu/depts/posc](http://www.uark.edu/depts/posc)

To Whom It May Concern,

The first author of enclosed paper, “Effect of Glutamine Supplementation Associated with Probiotics on *Salmonella* Typhimurium and Nitric Oxide or Glutamine with Perinatal Supplement on Growth Performance and Intestinal Morphology in Broiler Chickens”, is Anita Menconi. Anita was primarily responsible for the work and research associated with this paper, and completed greater than 51% of the work.

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B.M. Hargis



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Editorial office, OMICS Publishing Group, 731 Gull Ave, Foster City, CA 94404, USA,

Tel: +1-650-618-9889, Fax: +1-650-618-1414, USA, E-mail: [contact.omics@omicsonline.org](mailto:contact.omics@omicsonline.org)

### **TO WHOM IT MAY CONCERN**

April 22, 2014

We authorize the reprint of the manuscript entitled "Effect of Glutamine Supplementation Associated with Probiotics on Salmonella Typhimurium and Nitric Oxide or Glutamine with Perinatal Supplement on Growth Performance and Intestinal Morphology in Broiler Chickens", published in *Clinical Microbiology*, 2013, volume 2, issue 5, 120. doi:10.4172/2327-5073.1000120, in the Doctor of Philosophy dissertation of the first author, **Anita Menconi**.

Very truly yours,

**Srinubabu Gedela, Ph.D**

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OMICS Publishing Group

Westlake, Los Angeles, CA 91362-7354, USA

+1-650-353-4497 (Tel), +1-650-618-1417 (Fax)



## MEMORANDUM

TO: Lisa R. Bielke

FROM: Craig N. Coon, Chairman  
Institutional Animal Care  
And Use Committee

DATE: June 9, 2011

SUBJECT: IACUC PROTOCOL APPROVAL  
Expiration date: June 2, 2014

The Institutional Animal Care and Use Committee (IACUC) has APPROVED Protocol #11047-  
"EVALUATION OF DIRECT FED MICROBIALS AND PREBIOTICS FOR SALMONELLA  
CONTROL IN POULTRY". You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF  
committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has  
components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be  
any changes in the protocol during the research, please notify the IACUC in writing  
[Modification Request form] prior to initiating the changes. If the study period is expected to  
extend beyond 06-02-2014, you must submit a new protocol. By policy the IACUC cannot  
approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines  
for research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian

## IX. CONCLUSION

In the series of studies evaluating organic acids, the organic acid mixture and the commercial organic acid product were found to be potential crop sanitizers since they reduced, *in vitro* and *in vivo*, the incidence of *Salmonella* Typhimurium. Moreover, the commercial organic acid product proposed to cause a positive impact on animal welfare and economics concerns by decreasing body weight loss during feed withdrawal and transportation and meat quality improvement of broilers under commercial conditions. Additionally, the organic acid mixtures used in wash solutions demonstrated a reduction of foodborne pathogens and spoilage bacteria from chicken skin, suggesting improvement of raw poultry safety properties.

The lactic acid bacteria characterization showed tolerance to different pHs and high NaCl and bile salts concentrations, which complemented the identification and the *in vitro* reduction of pathogenic bacteria studies regarding these strains. In addition, the identification and characterization of *Bacillus* spp. revealed potential probiotic strains to be used in the poultry industry. This study also reviewed and emphasized the importance of testing probiotic strains to be used in humans and animals. Finally, glutamine association with neonatal nutrition demonstrated intestinal and performance benefits to broiler chickens by increasing body weight gain, villus height, villus width, and villus surface area index compared to control chickens. Moreover, glutamine supplementation in combination with a lactic acid based probiotic or a *Bacillus subtilis* probiotic strain showed reduction in *Salmonella* Typhimurium from the ceca contents of boiler chickens as well as a reduction of nitric oxide from ileal tissues of treated groups, which suggested an interesting anti-inflammatory effect by the treatments.

Taken together, these studies suggest that non-antibiotic treatments can have a significant impact on the quality and safety of poultry meat. Though, one single treatment may not be 100%

effective by itself, combinations of treatments may provide effective means for improved food safety and sustainability of poultry production.