5-2017

The Use of Probiotics to Control the Microbial Load Present in Commercial Broiler Chicken Hatch Cabinets as an Alternative to Formaldehyde Fumigation

Lucas Elzie Graham
University of Arkansas, Fayetteville

The Use of Probiotics to Control the Microbial Load Present in Commercial Broiler Chicken Hatch Cabinets as an Alternative to Formaldehyde Fumigation

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Poultry Science

by

Lucas Graham
University of Arkansas
Bachelor of Science in Poultry Science, 2015

May 2017
University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

Dr. Billy Hargis
Thesis Director

Dr. Lisa Bielke
Committee Member

Dr. Guillermo Tellez
Committee Member

Abstract

Formaldehyde has been used for decades as a disinfectant in the poultry hatchery. Hatch cabinets are treated with formaldehyde to control the microbial bloom that occurs inside of the hatch cabinet as the hatch progresses. Even with formaldehyde being a known human carcinogen and the detriment that it has on living creatures millions of chicks are exposed to formaldehyde in the hatch cabinet. In these experiments we tested a lyophilized probiotic spray inside of the hatch cabinets from day nineteen to day twenty one to control the microbial bloom that occurs. Hatch cabinet environments were sampled in six experiments. Media used allowed for the recovery of Gram-negative bacteria, non-selective bacteria, and presumptive lactic acid bacteria. Intestinal samples were taken on day of hatch in three experiments. The probiotic mixture consisted of *Lactobacilli* and *Bacillus subtilis* isolates. In these trials the probiotic treatment was shown to colonize the gastrointestinal tract of the newly hatched chicks. The probiotic treatment was also able to suppress the early Gram-negative microbial bloom that occurs inside of a hatch cabinet. Later on in the hatch period the probiotic treatment was not able to match formaldehyde for Gram-negative suppression. While the probiotic treatment could not suppress the Gram-negative microbial bloom as well as formaldehyde it did alter the gut microflora on day of hatch. In three separate experiments the probiotic treated chicks had significantly lower levels of Gram-negative bacteria recovered from intestinal samples than the formaldehyde treated chicks. In the third experiment this significant reduction in Gram-negative bacterial recovery by the probiotic continued out to twenty four hours post-hatch as well. When intestinal samples were pasteurized and plated on Tryptic soy agar plates in experiments two and three the only growth was that of the *Bacillus subtilis*. Probiotic hatch cabinet treatment did not have a significant impact on presumptive lactic acid bacteria except for in experiment two where the formaldehyde treated cabinet had significantly higher levels of presumptive lactic acid bacteria recovered.

Acknowledgements

I would like to thank the team at the Poultry Health Lab for their support and help throughout my time with them. Thank you Cheryl, Dennis, Adil, Juan, Amanda, Mikayla, Paula, and Vivek. Any time I needed anything you were all more than happy to help me.

Thank you to Dr. Billy Hargis, Dr. Guillermo Tellez, and Dr. Lisa Bielke for always taking the time to work with me and teach me the proper way to do things. Without your help I would not be where I am today.

Thank you to Kyle and Danielle for everything. I do not think I could have done it without you two by my side. You each showed me that one plus one can equal more than two when you find somebody willing to work with you to achieve your goals. Together we have come far and we will continue to go far.

I would like to thank the Loftis family for having faith in me. Without your support I would never have made it this far in my academic career.

Also, a special thanks to my family. My mom and dad who have encouraged me through it all. My sister who has walked these steps before me so she knows what it takes. Without my family I would not be here today. It makes it easier knowing all of the great people who stand behind me.

Dedication

This thesis is dedicated to my wife Danielle Mahaffey Graham and my sister Malea Frank. Danielle has stood behind me and supported me through everything. She believed in me even when I did not believe in myself. She has been my light at the end of the tunnel. Malea showed me that graduate school was a possibility for me. I had never thought of graduate school as an option until my sister pushed me to do it. Both of you have shaped me as a person. Thank you for everything.

Table of Contents

List of Tables & Figures

List of Papers

Chapter 2. The use of probiotics to control the microbial load present in commercial broiler chickens hatch cabinets as an alternative to formaldehyde fumigation. Submitted to the Journal of Applied Poultry Research.

Chapter 1

Introduction

Formaldehyde, a highly effective disinfectant, has been widely used throughout history. It has commonly been used as a disinfectant in poultry hatcheries even though it is a known human carcinogen (Mackar, 2011). Formaldehyde fumigation is used to control the microbial load present in commercial hatch cabinets while the hatch is in progress (Sander et al., 1995). Proper hatchery sanitation is a must due to the prolonged exposure of chicks to the hatching environment. *Enterobacteriaceae* are commonly found in the hatchery with *Salmonella* being recovered from eggshell fragments and chicken feather dander (Cox et al., 1990; Cox et al., 1991). While formaldehyde has its uses such as inactivating bacteria for vaccine preparation (Petre et al., 1996) it has been shown to cause significant damage to the respiratory system of newly hatched chicks (Zulkifli et al., 1999). Formaldehyde at low levels can cause irritation to the eyes and respiratory tract (Broder et al., 1991). Even though formaldehyde works as a hatchery disinfectant a non-toxic alternative needs to be found. Lactic Acid Bacteria (LAB) have demonstrated the ability to increase the performance parameters of poultry such as average body weight gain (Jin et al., 1996; Kabir et al., 2004). *Bacillus* have been shown to inhibit the colonization of chicks with *Enterobacteriaceae* such as *Salmonella* (Shivaramaiah et al., 2011; Wolfenden et al., 2011). These *Bacilli* and LAB strains are generally recognized as safe (GRAS). If a combination of GRAS *Bacillus* and LAB can control the hatch cabinet microbial bloom while colonizing the gastrointestinal tract with beneficial pioneer colonizers then it may be a viable alternative to formaldehyde.

Literature Review

Formaldehyde

Formaldehyde is a colorless and highly toxic gas with an irritating odor. Formaldehyde is commonly stored in a solution known as formalin. Formalin may contain up 50% formaldehyde by weight with the most common concentration being 37% formaldehyde. Formaldehyde is the active ingredient in formalin. Formalin allows formaldehyde to be used in various applications that may not be suitable for formaldehyde in its gaseous state. Even though formaldehyde has a highly irritating smell, it has been reported that hazardous levels may be possible without any odor (Toxic Substances Portal – Formaldehyde, 2014). Concentrations of formaldehyde at as little as 0.1 ppm may be enough to cause irritation to the eyes and throat (Broder et al., 1991). Formaldehyde concentrations at 100 ppm or more may be immediately dangerous to life (Broder et al., 1991) and ingesting as little as 1 mL has proved fatal (Toxic Substances Portal – Formaldehyde, 2014). Formaldehyde has also been used to disinfect epidemic areas where a disease outbreak has occurred (Tosh et al., 1967), to inactivate bacteria for use in vaccine production (Petre et al., 1996), and to keep feed or feed ingredients *Salmonella* free for up to 21 days (Electronic Code of Federal Regulations, 2017)

Formalin has been classified as a known carcinogen for humans (Mackar, 2011). The lifespan of poultry may not be long enough for the development of cancer to be concern, but formalin has been shown to cause morphological changes in the respiratory tract of chickens (Zulkifli et al., 1999; Di Matteo et al., 2000; Hayretdaug and Kolankaya, 2008). These morphological changes included observable lesions including excessive accumulation of mucus, matted cilia, loss of cilia and sloughing of the epithelium (Zulkifli et al., 1999). Formaldehyde has been shown to cause lung inflammation and oxidative stress in animals (Lino-dos-Santos-

Franco et al., 2011). Formalin treatment in the hatch cabinet reduced feed conversion over a 41 day trial (Zulkifli et al., 1999). In 2015 the United States produced almost nine billion chickens and 57.2 million tons of mixed feed (Broiler Chicken Industry Key Facts, 2016). A difference of multiple points of feed conversion, by ceasing hatch cabinet formaldehyde fumigation, could be worth millions of dollars to the poultry industry.

Alternatives to formaldehyde have been investigated to control the microbial bloom that occurs inside of a hatch cabinet but a more effective alternative has not been found (Maharjan et al., 2016). Ultraviolet light (UV), ozone, or hydrogen peroxide have all been previously tested to determine their ability to suppress *Salmonella* populations present inside of a hatch cabinet (Bailey et al., 1996). None of these three treatments had a negative impact on hatchability. Although all three treatments were able to reduce the *Salmonella* and *Enterobacteriaceae* in the hatch cabinet air samples, these treatments were not all able to reduce *Salmonella* colonization of the chicks. Hydrogen peroxide administered in the hatching environment was able to significantly reduce *Salmonella* colonization in the ceca. While these three methods may not be perfectly applicable to the industry they show that there is hope for finding a formaldehyde replacement. Intestinal microflora that was lyophilized and sprayed over the hatching eggs at day 20 of embryogenesis had shown the ability to significantly reduce *Salmonella* colonization of chicks (Goren et al., 1988). We examined a combined treatment consisting of three *Bacillus subtilis* isolates and two strains of *Pediococcus acidilactici* as a probiotic culture in all experiments. It has shown potential in replacing formalin to control the microbial bloom inside of commercial hatch cabinets. This treatment was applied by a dry spray to the hatch cabinets and it consists of different strains of lyophilized bacteria. This probioitc treatment consisted of generally recognized as safe (GRAS) *bacillus subtilis* that is meant to control the microbial load

inside of the hatch cabinet and GRAS lactic acid bacteria (LAB) that has the ability to colonize the gut of chicks with beneficial bacteria.

Eliminating the use of formalin could save the industry millions of dollars on feed conversion. It would also mean a better quality of life for commercially hatched chicks. Formaldehyde also poses a significant risk to human health with formaldehyde described as a known human carcinogen. A replacement for formalin has to be able to control the bacterial load within the hatch cabinets without having a toxic impact on the newly hatched chicks (Scott and Swetnam, 1993). According to Cadirci, (2009) the physical damage caused to the avian respiratory system by exposure to formalin may cause the chicks to be more susceptible to respiratory disease later on in life.

Hatchery Sanitization

Hatcheries are an important source of pioneer colonizers for the chick soon after hatch, but also a source of opportunistic pathogens. *Salmonella* has been shown to be horizontally transmitted through respiratory transmission (Gast et al., 1998). Oliveira et al., (2006) showed that *Salmonella* can be horizontally transmitted between piglets kept in close proximity. Berchieri Jr et al., (2001) showed that *Salmonella* could also be transmitted vertically when infected laying hens produced infected eggs. Formaldehyde is easy to apply and effective against a large number of microorganisms which resulted in formaldehyde becoming a commercial hatchery disinfectant (Sheldon and Brake, 1991) . Sporulating and nonsporulating bacteria, toxins, and viruses are known to be killed by formaldehyde (Taylor et al., 1969). Due to formaldehyde's ability to reduce bacterial populations it has been used as a method of sanitization for decades (Beesley, 1980; Deeming, 1992; Steinlage et al., 2002). The first

documented testing of formaldehyde to suppress the bacterial levels in a hatch cabinet was published in 1908 (Pernot and others, 1908).

Formalin application to commercial hatch cabinets is common practice to control microbial loads that increase rapidly after pipping begins and humidity within the cabinet rises rapidly. A common application method involves the hatchery personnel to apply 60 mL of 37% formalin every three hours after eggs are transferred to the hatch cabinet at day 18 or day 19 of incubation and continues until 12 hours prior to the chicks being removed from the hatch cabinet. Formaldehyde treatment of hatch cabinets has been shown to have a negative impact if fumigation occurs once the chick has broken through the eggshell (Frank and Wright, 1955). Formaldehyde has been used extensively in history to sanitize the exterior of the egg shell throughout the hatching process (Williams and Siegel, 1969; Williams and Gordon, 1970). Pathogens have been recovered from eggshell fragments present in hatcheries, with rates of *Salmonella* reported above 75% recovery in tested commercial hatcheries (Cox et al., 1990; Cox et al., 1991). Bacteria have also been shown to penetrate the eggshell and infect the embryo (Rathgeber et al., 2013; Rodriguez-Navarro et al., 2013). Hatch cabinet sanitization is a priority to prevent embryos from being infected by *Enterobacteriaceae* circulating in the hatching environment. If there are low levels of bacteria in the hatching environment there is less opportunity for bacteria to penetrate the eggshell. De Reu et al., (2006) showed that there is a significant positive correlation between the amount of external egg shell contamination and internal egg contamination. Sanitization of the eggshell is a necessary step and formaldehyde fumigation has been used extensively in the past (Williams, 1970; Berrang et al., 2000; Cadirci, 2009). Formaldehyde fumigation of the eggs can occur at any point during incubation or pipping but for eggshell disinfection it most commonly occurs prior to incubation (Cadirci, 2009).

While formaldehyde can help reduce the microbial load present on eggshells and the interior of a hatch cabinet, it does not completely inhibit the growth of pathogens such as *Clostridium* and *Salmonella*. Hatcheries have been shown to be contaminated with both bacteria being found on eggshell fragments, chick feather dander, and paper pads used to line the chick boxes (Miura et al., 1964; Cox et al., 1990; Cox et al., 1991; Craven et al., 2001). *Salmonella* can flourish in the warm, moist environment within a hatch cabinet. Cox et al. (1990) demonstrated that these conditions allow for the proliferation of *Enterobacteriaceae*. Due to hatcheries being an ideal breeding ground for bacteria, a hatchery sanitization program must be followed in order to reduce exposure of hatching chicks to undesirable microbes (Magwood, 1964). Producers can suffer significant economic losses if *Enterobacteriaceae* colonize the chicks in the hatchery. Diseases such as *Salmonella* can be spread horizontally throughout the life of the birds so even those not exposed in the hatchery can be exposed during their grow out period. High stocking density present in commercial hatch cabinets lends itself to rapid horizontal transmission of disease.

Previous unpublished research conducted by our laboratory has shown that formalin loses the ability to suppress the growth of Gram-negative bacteria in the hatch cabinet as the hatch period progresses. In our research we have shown that bacterial contamination inside the hatch cabinet increases as the hatch progresses. As the microbial load increases in the hatching environment formaldehyde begins to lose the ability to suppress bacterial growth (Magwood, 1964). Formaldehyde suppressing the growth of Gram-negative *Enterobacteriaceae* is critical for shifting the pioneer colonizers of the newly hatched chicks to a more favorable population. As the hatch nears completion formaldehyde is not only losing its ability to suppress bacterial growth it is also likely causing damage to the newly hatched chicks. Damage to the respiratory

system is likely happening (Zulkifli et al., 1999) along with lung inflammation (Lino-dos-Santos-Franco et al., 2011). Formaldehyde is a powerful tool in controlling the microbial challenge present in a hatch cabinet but a less toxic alternative needs to be found. An alternative that controls the microbial bloom while exposing the chicks to beneficial pioneer colonizers would be ideal.

Pioneer Colonizers

It has been hypothesized that vaginal inoculation of the eggshell during oviposition or coprophagy in the nest soon after hatch are essential for developing gut microflora of newly hatched chicks, but this exposure does not occur in commercial settings. Early microflora development is considered an essential component in development of the gut-associated immune system and overall health of animals ((Biasucci et al., 2008; Biasucci et al., 2010; Jakobsson et al., 2014).

Children delivered through vaginal birth tend to have higher levels of beneficial microflora, such as *Bifidobacteria* (Penders et al., 2006; Biasucci et al., 2010) and lower levels of harmful bacteria, such as *E. coli* when compared to children delivered through cesarean section birth (Penders et al., 2006; Jakobsson et al., 2014). The gut microflora of cesarean delivered children can be altered for up to 24 months when compared to a child delivered by vaginal birth (Grölund et al., 1999; Jakobsson et al., 2014). Vaginally delivered children had significantly higher levels of "Proteobacteria" at 24 months than those delivered via cesarean section birth. According to Shin et al., (2015) neonatal colonization of the GIT by "Proteobacteria" shift the GIT to favor colonization by obligate anaerobes. These obligate anaerobes are then replaced by anaerobic *Firmicutes* and *Bacteroidetes* in the GIT. *Firmicutes* and *Bacteroidetes* are the two most highly populous bacteria in the GIT of adult humans (Clemente et al., 2012). *Bacillus* are

part of the *Firmicutes* phylum. These alterations to the GIT for up to twenty four months show how vital pioneer colonization of the gastrointestinal tract can be for newly hatched or birthed animals. Children without older siblings also had slightly lower levels of *Bifidobacteria* than those with older siblings (Penders et al., 2006; Biasucci et al., 2008). *Bifidobacteria* are believed to be essential in human health (Mitsuoka, 1990). *Bifidobacteria* inhabit the large intestine of humans. Increased exposure to bacteria caused by older siblings may lend credence to the hypothesis that a post hatch source of bacteria, such as coprophagy can add beneficial microflora to the chicks.

While composition of gut microflora changes throughout the life of an animal, it is most susceptible to change immediately following hatch or birth when the gut is essentially sterile (Dibner et al., 2008; Biasucci et al., 2010). Young animals are exposed to a wide range of microbes upon hatching/birth (Dominguez-Bello et al., 2010) that determine the pioneer colonizers of the gastrointestinal tract for the young animal. While the impact of having beneficial bacteria as pioneer colonizers of the gastrointestinal tract is not completely understood, it is believed that it will lead to a higher quality of life for the animal.

Lactic Acid Bacteria

Lactic Acid Bacteria (LAB) are a Gram-positive, non-spore forming bacteria that are either spherical or rod-shaped. LAB can be found in plants, foods, the human gastrointestinal tract, and the vagina (Falagas et al., 2007; Walter, 2008). *Lactobacillus* are among the most important genera of LAB when it comes to use as a probiotic (Klein et al., 1998). They were first described in 1901 by Martinus Willem Beijerinck (Dellaglio et al., 2004). They are considered GRAS because they are found so abundantly in food products and normal microflora and are the most commonly used bacteria to produce probiotics (Naidu et al., 1999). Ewing and Cole (1994)

showed that the optimal daily consumption of a probiotic for animal use is 10^8 to 10^9. When LAB are given as a probiotic treatment have shown the ability to cause a lifelong change in the growth of the animal.

Jin et al., (1996) showed that supplementing a probiotic, such as *Bacillus subtilis* or *Lactobacilli* to broiler diets can cause a significant increase in body weight gain while having a significant impact on feed efficiency. Birds fed *B. subtilis* had an average body weight gain of 1,436.72 grams compared to the average of 1,360.29 grams for the control birds. The birds fed the *Lactobacilli* had an average body weight gain of 1,457.91 grams. At 21 days of age, chickens fed *Lactobacilli* had a decreased level of *E. coli* in the intestine compared to the control. At 21 days of age, the control group had 7.45 +/- 0.36 log CFU per gram of intestinal *E. coli*, while chickens fed *Lactobacillus* had significantly lower levels of intestinal *E. coli* at 6.48 +/- 0.18 log CFU per gram. *B. subtilis* or *Lactobacillus* treatment did not have any impact on cecal *Salmonella* in this study. The results of Jin et al., line up with numerous other research studies (Tortuero, 1973; Cavazzoni et al., 1998; Kabir et al., 2004) that show improved broiler performance parameters through the application of a probiotic treatment.

Torres-Rodriguez et al., (2007) showed that application of a LAB based probiotic could significantly increase body weight and average daily gain in 92 day old turkey hens. The probiotic did not cause a significant difference in the top performing 25% of hens, but it did in the middle 50% and the bottom 25%. In the middle 50% the probiotic treatment treated group had an average body weight (kg) of 6.92 ± 0.06, whereas the non-probiotic treated group had a significantly lower average body weight (kg) of 6.75 ± 0.06. The bottom 25% of hens sampled had an average body weight of 6.85 ± 0.07 when treated with the probiotic. The hens that did not receive probiotic treatment in the bottom 25% had an average body weight of 6.57 ± 0.09. This

significant difference for the bottom 75% of hens sampled suggests that the probiotic is more beneficial to birds that may be lagging behind in production.

Additionally, in other field trials completed by Vicente et al., (2007), application of the same LAB based probiotic resulted in a significant reduction in overall mortality in commercial broilers. The untreated broilers in this experiment consisted of 116,618 birds spread out over six different farms. The probiotic treated farms consisted of 117,497 birds spread out over the same six farms as the untreated broilers. Probiotic treated broilers had a mortality of 5.87% vs 6.72% for the untreated broilers. Put together, these field studies demonstrated the ability of LAB probiotics to improve growth and livability in commercial poultry settings, and suggested that probiotics could provide health benefits to poultry.

Bacillus

Bacillus are a genera of rod-shaped, Gram-positive bacteria, and many species have been used as probiotics. *Bacillus* are able to live as obligate aerobes or facultative anaerobes and can produce endospores which allow the bacteria to remain dormant for long periods of time (Nicholson et al., 2000). *Bacillus* have been recovered from soil at levels as high as 10^8 per gram (Stein, 2005). Wolfenden and co-workers (2010) described isolation techniques for isolating *Bacillus,* from environmental samples, which showed ability to inhibit *Salmonella* growth on agar plates.

The ability to produce endospores allows the *Bacillus* to stay stable with very little impact caused by environmental stressors. Cartman et al., (2008) demonstrated that *Bacillus* given orally can germinate in the gastrointestinal tract of the chicken and has been shown to germinate in the gastrointestinal tract of other species such as mice (Hoa et al., 2001; Casula and Cutting, 2002). Some strains are highly heat resistant to both dry heat and wet heat as shown by

Setlow (2006). This heat stability allows a *Bacillus* based probiotic to survive the pelleting process for poultry feed (Nicholson, 2002; Moeller et al., 2009). Wolfenden et al., (2010) showed that *Bacillus* can be selected for heat resistance and still have an impact on bodyweight and bodyweight gain in poultry.

Shivaramaiah et al., (2011) showed that a *Bacillus* supplemented in the diet can also result in increased bodyweight gain while causing a significant reduction in *Salmonella* colonization of the ceca in broiler chicks and turkey poults. The *Bacillus subtilis* labeled NP122 was provided in the feed to chicks and poults alike. Chicks fed NP122 and challenged with *Salmonella* Typhimurium (ST) had cecal recovery rates at 0.97 ± 0.3 log10 colony forming units (cfu), while untreated positive control chicks had 2.37 ± 0.4 log10 cfu recovered from the ceca. This significant difference in ST recovered from the ceca was also present in turkey poults. Positive control poults had 2.05 ± 0.4 cfu recovered in the ceca while those challenged with ST and given NP122 had 0.75 ± 0.3 cfu recovered. Chicks and poults supplemented with NP122 and challenged with ST both had significantly higher bodyweights at 11 days of age when compared to their counterparts.

Wolfenden et al., (2011) used the same NP122 *Bacillus* in a commercial turkey trial and confirmed the significant increase in bodyweight gain and significant reduction in *Salmonella* recovered from the ceca. *Bacilli* are great candidates for probiotics due to their environmental stability, ease of isolation, ability to germinate in the gastrointestinal tract, and ease of use for *in vitro* screening methods.

Enterobacteriaceae

The *Enterobacteriaceae* family consists of many Gram-negative bacteria including pathogens, such as *Salmonella* and *E. coli*. Members of this family are rod shaped bacteria that

are facultative anaerobes and are commonly found in the gastrointestinal tract. *E. coli* is not always pathogenic and is suggested to be a necessary commensal bacteria in the early days of life (Tenaillon et al., 2010). While this family of bacteria is commonly found in the gut as a commensal bacteria, there are pathogenic strains that can cause disease and even death. Poultry can be exposed to *Enterobacteriaceae* in a hatchery, by vertical or horizontal transmission, or through the feed. Veldman et al., (1995) tested feed samples for *Salmonella* incidence and recovery, and reported that 10% of samples tested positive for *Salmonella*. The highest percentage of *Salmonella* recovery was in mash feeds with 21% testing positive for *Salmonella* recovery. Mash feeds are typically fed to layer-breeders which makes vertical transmission of *Salmonella* from breeder hen to chick a possibility.

In previous work Cox et al., (1983) had found *Enterobacteriaceae* incidence to be the highest in mash feed when compared to samples taken from pelleted feed and meat and bone meal. These mash feeds were collected from ten commercial feed mills. The average *Enterobacteriaceae* per gram of mash feed was 4.1 log10. Pelleted feed had 0.8 log10 of *Enterobacteriaceae* per gram while meat and bone meal had a slightly higher level of *Enterobacteriaceae* recovered at 1.8 log10 per gram. *Enterobacteriaceae* were present in 100% of the mashed feed samples. Compare that to 60% *Enterobacteriaceae* incidence for pelleted feed and 92% incidence for meat and bone meal samples. Mash feed not only has the highest level of incidence of *Enterobacteriaceae*, it also has the highest challenge levels when compared to pelleted feeds and meat and bone meal. The exposure of breeders to *Enterobacteriaceae*, such as *Salmonella* or *E. coli* can prove costly for the industry.

As stated above, when egg shell fragments, belting material and paper pads in three broiler hatcheries were tested, it was reported that 75.4% of samples were positive for

Salmonella (Cox et al., 1990). Separated as individual sample types, 71.4% egg fragments, 80% of belting material, and 74.2% paper pads had detectable levels of *Salmonella*. Cox et al., (1991) performed similar testing with samples being taken from six commercial breeder hatcheries instead of broiler hatcheries. They sampled egg fragments, chick fluff, and paper pads with *Salmonella* being recovered in 22/145 (15.2%), 5/110 (4.5%), and 15/125 (12.0%) samples respectively. *Salmonella* was recovered from 42/380 (11.1%) total samples. *Salmonella* was recovered at much lower levels in this study than the previous study that sampled broiler hatcheries. Lower *Salmonella* incidence may be due to the higher biosecurity standards placed on breeder flocks and breeder hatcheries. Hatchery contamination of breeder flocks with the *Enterobacteriaceae Salmonella* still poses a threat through vertical transmission in the future.

Chickens face many bacterial challenges throughout their life whether it is *Enterobacteriaceae* present in the hatchery or their feed and a *Bacillus* or LAB probiotic may be able to provide protection. Treating the hatch cabinet with a probiotic containing LAB may allow the GIT to be colonized with beneficial pioneer colonizers or treating with specifically screened *Bacillus* isolates may inhibit the growth of *Enterobacteriaceae* such as *Salmonella* and *E. coli* in the hatch cabinet. Tellez et al., (2012) showed that *Bacillus* can be used to inhibit the growth of *Salmonella*. Not only can *Bacillus* inhibit the growth of *Enterobacteriaceae in vitro* they have been shown to inhibit the colonization of chicks by these pathogenic bacteria as well (Vicente et al., 2008).

Conclusion

While formaldehyde use in hatch cabinet and hatching egg sanitization has decreased, it is still being used today. An alternative must be found that can inhibit the growth of *Enterobacteriaceae,* such as *Salmonella,* in the hatch cabinet. This alternative must not be as

toxic to the newly hatched chicks as formaldehyde has been shown to be. While the elimination of formaldehyde is not complete, the willingness to reduce the use of formaldehyde is a step in the right direction. Replacing formaldehyde with a GRAS LAB and *Bacillus* alternative would have far reaching implications that extend beyond just the hatch cabinet. Moving the exposure time to beneficial bacteria to an earlier time point in the life of the chick may allow pioneer colonizers to reduce the colonization of opportunistic pathogens. Early colonization with these beneficial bacteria may lead to decreased colonization by opportunistic pathogens throughout the life of the animal. Not only could they potentially help protect the animal from *Enterobacteriaceae* circulating in the hatching environment but they may also help prevent the animal from being colonized by *Enterobacteriaceae* after leaving the hatchery. In studies probiotics such as LAB and *Bacillus* have reduced mortality while increasing overall bodyweight of poultry. Replacing a known carcinogen such as formaldehyde with a probiotic that consists of GRAS LAB and *Bacillus* may allow the microbial bloom to be controlled without causing any likely harm to the chicks or hatchery personnel.

References

Bailey, J. S., R. J. Buhr, N. A. Cox, and M. E. Berrang. 1996. Effect of hatching cabinet sanitation treatments on *Salmonella* cross-contamination and hatchability of broiler eggs. Poult. Sci. 75:191–6.

Beesley, B. T. 1980. Fumigation of hatching eggs. *Poultry Digest*, 39, pp.570-571.

Berchieri Jr, A., C. Murphy, K. Marston, and P. Barrow. 2001. Observations on the persistence and vertical transmission of *Salmonella* enterica serovars Pullorum and Gallinarum in chickens: effect of bacterial and host genetic background. Avian Pathology 30:221–231.

Berrang, M., N. Cox, J. Frank, R. Burh, and J. Bailey. 2000. Hatching egg sanitization for prevention or reduction of human enteropathogens: A review. The Journal of Applied Poultry Research 9:279–284.

Biasucci, G., B. Benenati, L. Morelli, E. Bessi, and G. Boehm. 2008. Cesarean delivery may affect the early biodiversity of intestinal bacteria. J. Nutr. 138:1796S–1800S.

Biasucci, G., M. Rubini, S. Riboni, L. Morelli, E. Bessi, and C. Retetangos. 2010. Mode of delivery affects the bacterial community in the newborn gut. Early Hum. Dev. 86 Suppl 1:13–5.

Broder, I., P. Corey, P. Brasher, M. Lipa, and P. Cole. 1991. Formaldehyde exposure and health status in households. Environ. Health Perspect. 95:101–4.

Cadirci, S. 2009. Disinfection of hatching eggs by formaldehyde fumigation-a review. Arch. Geflügelk 73:116–123.

Cartman, S. T., R. M. La Ragione, and M. J. Woodward. 2008. *Bacillus subtilis* spores germinate in the chicken gastrointestinal tract. Appl. Environ. Microbiol. 74:5254–8.

Casula, G., and S. M. Cutting. 2002. *Bacillus* probiotics: spore germination in the gastrointestinal tract. Appl. Environ. Microbiol. 68:2344–52.

Cavazzoni, V., A. Adami, and C. Castrovilli. 1998. Performance of broiler chickens supplemented with *Bacillus* coagulans as probiotic. Br. Poult. Sci. 39:526–9.

Clemente, J. C., L. K. Ursell, L. W. Parfrey, and R. Knight. 2012. The impact of the gut microbiota on human health: an integrative view. Cell 148:1258–1270.

Cox, N., J. Bailey, J. Mauldin, and L. Blankenship. 1990. Research note: Presence and impact of *Salmonella* contamination in commercial broiler hatcheries. Poultry science 69:1606–1609.

Cox, N., J. Bailey, J. Mauldin, L. Blankenship, and J. Wilson. 1991. Research note: Extent of *Salmonellae* contamination in breeder hatcheries. Poultry Science 70:416–418.

Cox, N. A., J. S. Bailey, J. E. Thomson, and B. J. Juven. 1983. *Salmonella* and other *Enterobacteriaceae* found in commercial poultry feed. Poult. Sci. 62:2169–75.

Craven, S. E., Cox, N. A., Stern, N. J., and J. M. Mauldin. 2001. Prevalence of Clostridium perfringens in commercial broiler hatcheries. Avian diseases 45(4):1050-1053.

Deeming, C. 1992. Formaldehyde in the hatcher. Poult. Int 30:40-52.

Dellaglio, F., S. Torriani, and G. E. Felis. 2004. Reclassification of *Lactobacillus* cellobiosus Rogosa et al. 1953 as a later synonym of *Lactobacillus* fermentum Beijerinck 1901. Int. J. Syst. Evol. Microbiol. 54:809–12.

Dibner, J. J., Richards, J. D., and C. D. Knight. 2008. Microbial imprinting in gut development and health. The Journal of Applied Poultry Research 17(1):174-188.

Di Matteo, A. M., Sonez, M. C., Plano, C. M., and I. Von Lawzewitsch. 2000. Morphologic observations on respiratory tracts of chickens after hatchery infectious bronchitis vaccination and formaldehyde fumigation. Avian diseases 44(3):507-518.

Dominguez-Bello, M. G., E. K. Costello, M. Contreras, M. Magris, G. Hidalgo, N. Fierer, and R. Knight. 2010. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proceedings of the National Academy of Sciences 107:11971–11975.

Electronic Code of Federal Regulations - Code of Federal Regulations. 2017. ECFR - Code of Federal Regulations. N.p., n.d. Web. 09 Apr. 2017.

Ewing, W. N., and D. J. A. Cole. 1994. Micro-flora of the gastro-intestinal tract. The living gut. An introduction to micro-organisms in nutrition, 45-65.

Falagas, M., G. Betsi, and S. Athanasiou. 2007. Probiotics for the treatment of women with bacterial vaginosis. Clinical Microbiology and Infection 13:657–664.

Frank, J. F., and G. W. Wright. 1955. Susceptibility of *Salmonella* Organisms to Formaldehyde Fumigation. Can J Comp Med Vet Sci 19:71–5.

Gast, R. K., Mitchell, B. W., & P. S. Holt. 1998. Airborne transmission of *Salmonella* enteritidis infection between groups of chicks in controlled-environment isolation cabinets. Avian diseases 42(2):315-320.

Goren, E., W. A. de Jong, P. Doornenbal, N. M. Bolder, R. W. Mulder, and A. Jansen. 1988. Reduction of *salmonella* infection of broilers by spray application of intestinal microflora: a longitudinal study. Vet Q 10:249–55.

Grölund, M. M., Lehtonen, O. P., Eerola, E., and P. Kero. 1999. Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery. Journal of pediatric gastroenterology and nutrition 28(1):19-25.

Hayretdaug, S., and D. Kolankaya. 2008. Investigation of the effects of pre-incubation formaldehyde fumigation on the tracheal epithelium of chicken embryos and chicks. Turkish Journal of Veterinary and Animal Sciences 32:263–267.

Hoa, T. T., L. H. Duc, R. Isticato, L. Baccigalupi, E. Ricca, P. H. Van, and S. M. Cutting. 2001. Fate and dissemination of *Bacillus subtilis* spores in a murine model. Appl. Environ. Microbiol. 67:3819–23.

Jakobsson, H. E., T. R. Abrahamsson, M. C. Jenmalm, K. Harris, C. Quince, C. Jernberg, B. Björkstén, L. Engstrand, and A. F. Andersson. 2014. Decreased gut microbiota diversity, delayed Bacteroidetes colonisation and reduced Th1 responses in infants delivered by caesarean section. Gut 63:559–566.

Jin, L. Z., Y. W. Ho, N. Abdullah, and S. Jalaludin. 1996. Influence of dried *Bacillus subtilis* and *lactobacilli* cultures on intestinal microflora and performance in broilers. Asian Australasian Journal of Animal Sciences 9:397–404.

Kabir, S. L., Rahman, M. M., Rahman, M. B., Rahman, M. M., and S. U. Ahmed. 2004. The dynamics of probiotics on growth performance and immune response in broilers. Int. J. Poult. Sci 3(5):361-364.

Klein, G., A. Pack, C. Bonaparte, and G. Reuter. 1998. Taxonomy and physiology of probiotic lactic acid bacteria. Int. J. Food Microbiol. 41:103–25.

Lino-dos-Santos-Franco, A., M. Correa-Costa, A. C. C. dos S. Durão, A. P. L. de Oliveira, A. C. Breithaupt-Faloppa, J. de A. Bertoni, R. M. Oliveira-Filho, N. O. S. Câmara, T. Marcourakis, and W. Tavares-de-Lima. 2011. Formaldehyde induces lung inflammation by an oxidant and antioxidant enzymes mediated mechanism in the lung tissue. Toxicol. Lett. 207:278–85.

Mackar, R. 2011. New substances added to HHS report on carcinogens. Retrieved 09/30, 2016, from http://www.niehs.nih.gov/news/newsroom/releases/2011/june10/

Maharjan, P., S. Cox, U. Gadde, F. D. Clark, K. Bramwell, and S. E. Watkins. 2016. Evaluation of chlorine dioxide based product as a hatchery sanitizer. Poultry Science 96(3):560-565.

Magwood, S. 1964. Studies in Hatchery Sanitation 1. Fluctuations in Microbial Counts of air in Poultry Hatcheries. Poultry Science 43:441–449.

Mitsuoka, T. 1990. *Bifidobacteria* and their role in human health. Journal of industrial microbiology 6(4):263-267.

Miura, S., Sato, G., and T. Miyamae. 1964. Occurrence and survival of *Salmonella* organisms in hatcher chick fluff from commercial hatcheries. Avian Diseases 8(4):546-554.

Moeller, R., P. Setlow, G. Reitz, and W. L. Nicholson. 2009. Roles of small, acid-soluble spore proteins and core water content in survival of *Bacillus subtilis* spores exposed to environmental solar UV radiation. Applied and environmental microbiology 75:5202–5208.

Naidu, A. S., W. R. Bidlack, and R. A. Clemens. 1999. Probiotic spectra of lactic acid bacteria (LAB). Crit Rev Food Sci Nutr 39:13–126.

"Broiler Chicken Industry Key Facts 2016." The National Chicken Council. Web. 18 Apr. 2017.

Nicholson, W. L. 2002. Roles of *Bacillus* endospores in the environment. Cell. Mol. Life Sci. 59:410–6.

Nicholson, W. L., N. Munakata, G. Horneck, H. J. Melosh, and P. Setlow. 2000. Resistance of Bacillus endospores to extreme terrestrial and extraterrestrial environments. Microbiology and molecular biology reviews 64:548–572.

Oliveira, C. J. B., L. F. O. S. Carvalho, and T. B. Garcia. 2006. Experimental airborne transmission of *Salmonella* Agona and *Salmonella* Typhimurium in weaned pigs. Epidemiol. Infect. 134:199–209.

Penders, J., C. Thijs, C. Vink, F. F. Stelma, B. Snijders, I. Kummeling, P. A. van den Brandt, and E. E. Stobberingh. 2006. Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics 118:511–521.

Pernot, E. F., and others. 1908. An investigation of the mortality of incubator chicks.

Petre, J., Pizza, M., Nencioni, L., Podda, A., De Magistris, M. T., and R. Rappuoli. 1995. The reaction of bacterial toxins with formaldehyde and its use for antigen stabilization. Developments in biological standardization 87:125-134.

Rathgeber, B. M., P. McCarron, and K. L. Budgell. 2013. *Salmonella* penetration through eggshells of chickens of different genetic backgrounds. Poult. Sci. 92:2457–62.

De Reu, K., K. Grijspeerdt, W. Messens, M. Heyndrickx, M. Uyttendaele, J. Debevere, and L. Herman. 2006. Eggshell factors influencing eggshell penetration and whole egg contamination by different bacteria, including *Salmonella* enteritidis. International journal of food microbiology 112:253–260.

Rodriguez-Navarro, A. B., N. Dominguez-Gasca, A. Muñoz, and M. Ortega-Huertas. 2013. Change in the chicken eggshell cuticle with hen age and egg freshness. Poultry science 92:3026–3035.

Samberg, Y., and M. Meroz. 1995. Application of disinfectants in poultry hatcheries. Revue scientifique et technique (International Office of Epizootics) 14(2):365-380.

Scott, T. A., and C. Swetnam. 1993. Screening sanitizing agents and methods of application for hatching eggs I. Environmental and user friendliness. The Journal of Applied Poultry Research 2(1):1-6.

Setlow, P. 2006. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. Journal of applied microbiology 101:514–525.

Sheldon, B., and J. Brake. 1991. Hydrogen peroxide as an alternative hatching egg disinfectant. Poultry science 70:1092–1098.

Shin, N.-R., T. W. Whon, and J.-W. Bae. 2015. Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends in biotechnology 33:496–503.

Shivaramaiah, S., N. R. Pumford, M. J. Morgan, R. E. Wolfenden, A. D. Wolfenden, A. Torres-Rodríguez, B. M. Hargis, and G. Téllez. 2011. Evaluation of *Bacillus* species as potential candidates for direct-fed microbials in commercial poultry. Poult. Sci. 90:1574–80.

Stein, T. 2005. *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Mol. Microbiol. 56:845–57.

Steinlage, S. J. T., Sander, J. E., and J. L. Wilson. 2002. Comparison of Two Formaldehyde Administration Methods of In Ovo–Injected Eggs. Avian diseases 46(4):964-970.

Taylor, L. A., M. S. Barbeito, and G. G. Gremillion. 1969. Paraformaldehyde for surface sterilization and detoxification. Appl Microbiol 17:614–8.

Tellez, G., C. Pixley, R. Wolfenden, S. Layton, and B. Hargis. 2012. Probiotics/direct fed microbials for *Salmonella* control in poultry. Food Research International 45:628–633.

Tenaillon, O., Skurnik, D., Picard, B., and E. Denamur. 2010. The population genetics of commensal *Escherichia coli*. Nature Reviews Microbiology 8(3):207-217.

Torres-Rodriguez, A., A. Donoghue, D. Donoghue, J. Barton, G. Tellez, and B. Hargis. 2007. Performance and condemnation rate analysis of commercial turkey flocks treated with a *Lactobacillus spp.*-based probiotic. Poultry science 86:444–446.

Tortuero, F. 1973. Influence of the implantation of *Lactobacillus* acidophilus in chicks on the growth, feed conversion, malabsorption of fats syndrome and intestinal flora. Poultry Science 52:197–203.

Tosh, F., R. Weeks, F. Pfieffer, S. Hendricks, D. Greer, T. Chin, and others. 1967. The use of formalin to kill Histoplasma capsulatum at an epidemic site. American journal of epidemiology 85:259–65.

Toxic Substances Portal - Formaldehyde. 2014. Centers for Disease Control and Prevention. Web. 09 Apr. 2017.

Veldman, A., Vahl, H. A., Borggreve, G. J., and D. C. Fuller. 1995. A survey of the incidence of *Salmonella* species and *Enterobacteriaceae* in poultry feeds and feed components. The Veterinary Record 136(7):169-172.

Vicente, J., L. Aviña, A. Torres-Rodriguez, B. Hargis, and G. Tellez. 2007. Effect of a *Lactobacillus* spp-based probiotic culture product on broiler chicks performance under commercial conditions. Int. J. Poult. Sci 6:154–156.

Vicente, J. L., A. Torres-Rodriguez, S. E. Higgins, C. Pixley, G. Tellez, A. M. Donoghue, and B. M. Hargis. 2008. Effect of a selected *Lactobacillus spp.*-based probiotic on *Salmonella* enterica serovar enteritidis-infected broiler chicks. Avian Dis. 52:143–6.

Walter, J. 2008. Ecological role of *lactobacilli* in the gastrointestinal tract: implications for fundamental and biomedical research. Applied and Environmental Microbiology 74:4985–4996.

Williams, J. E. 1970. Effect of high-level formaldehyde fumigation on bacterial populations on the surface of chicken hatching eggs. Avian diseases 14(2)386-392.

Williams, J., and C. Gordon. 1970. The hatchability of chicken eggs fumigated with increasing levels of formaldehyde gas before incubation. Poultry Science 49:560–564.

Williams, J., and H. Siegel. 1969. Formaldehyde levels on and in chicken eggs following preincubation fumigation. Poultry science 48:552–558.

Wolfenden, R., N. Pumford, M. Morgan, S. Shivaramaiah, A. Wolfenden, C. Pixley, J. Green, G. Tellez, and B. Hargis. 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., N. Pumford, M. Morgan, S. Shivaramaiah, A. Wolfenden, G. Tellez, B. Hargis, and others. 2010. Evaluation of a screening and selection method for *Bacillus* isolates for use as effective direct-fed microbials in commercial poultry. Int J Poult Sci 9:317–23.

Zulkifli, I., O. Fauziah, A. Omar, S. Shaipullizan, and A. S. Selina. 1999. Respiratory epithelium, production performance and behaviour of formaldehyde-exposed broiler chicks. Veterinary research communications 23:91–99.

Chapter 2

The use of probiotics to control the microbial load present in commercial broiler chickens hatch cabinets as an alternative to formaldehyde fumigation

Lucas E. Graham [1], Kyle D. Teague[1], Yichao Yang[1], Juan D. Latorre[1], Mikayla F. A. Baxter [1], Ruben Merino-Guzman[2], Brittany D. Mahaffey[1], Amanda D. Wolfenden[1], Xochitl Hernandez-Velasco[2], Lisa R. Bielke[3], Billy M. Hargis[1], and Guillermo Tellez[1]*

[1] *Department of Poultry Science, University of Arkansas Fayetteville, USA,* [2]*Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, México,* [3]*Department of Animal Science, Ohio State University, OARDC, Wooster, OH USA*

[*]**Corresponding Author**:
G. Tellez
POSC 0-114
Department of Poultry Science
University of Arkansas
Fayetteville, AR 72701
(479) 575-8495
Email: gtellez@uark.edu

Abstract

Due to health and environmental reasons, it is imperative to find effective alternatives to the use of formaldehyde for bacterial control in commercial poultry hatcheries. The purpose of the present study was to evaluate the use of a spray probiotic formulation as an alternative method to control the bacterial bloom within a broiler hatch cabinet *versus* formaldehyde fumigation. All trials were conducted in a commercial broiler hatchery. Control hatch cabinets were treated using formaldehyde, the current bacterial control method. Probiotic hatch cabinets received a selected mix of *Bacillus subtilis* and *Pediococcus acidilactici* isolates. In Exp 1, two independent trials were conducted to compare hatchery sanitation between the current formaldehyde drip method *versus* spray application of the probiotic. Hatchery sanitation was evaluated using the open-plate method at approximately 20% pip; 30% hatch; and 85% hatch for enumeration of total recovered non-selective aerobic bacteria (TAB); presumptive lactic acid bacteria (LAB); and total recovered Gram-negative bacteria (TGB). In Exp 2, three independent trials were conducted to evaluate the gastrointestinal (GIT) microflora of neonatal chicks from hatch cabinets treated as in Exp 1. Additionally, in trial 3 a sub-group of chicks were held for 24 h to further evaluate GIT microflora. In Exp 1, in both trials, the application of the probiotic increased the number TAB and LAB present in the hatching environment ($P < 0.05$). Additionally, at 20% pip and 30% hatch, in both trials, the probiotic treatment was as effective as formaldehyde in reducing TGB. In Exp. 2, chicks from probiotic treated hatch cabinets also showed a reduction of TGB in the GIT compared to the formaldehyde group ($P < 0.05$). In trial 3, the reduction in TGB persisted 24 h after hatch. The results of the present study suggest that spray application of a probiotic in commercial hatcheries can be as effective as formaldehyde in reducing TGB. More importantly, it decreased the numbers of these bacteria within the GIT at

hatch and 24 h after hatch. Further studies to evaluate this approach using challenge models with enteropathogens and performance field trials are currently being evaluated.

Keywords: chicks, formaldehyde, hatchery, probiotic, microbiota

1. Introduction

From the evolutionary point of view, our related prokaryotic cousins are remarkable living organisms. Under optimal conditions for temperature, nutrients and humidity, some bacteria can divide every 20 minutes. Hence, in 24 hours a single bacterium could divide 72 times becoming 4,700,000,000,000,000,000,000,000 (4.7×10^{24}) organisms. While this theoretical maximum cannot occur in nature, it still means that one bacteria can go from being invisible to the naked eye to a readily visible colony of bacterial cells in less than a day. Interestingly for those microbes, near optimal conditions are present in modern commercial poultry hatcheries. Hatch cabinets, in particular, are where some important groups of pathogens and opportunists, such as *Staphylococcus*, *Pseudomonas*, *Escherichia coli*, *Salmonella*, and *Aspergillus* are able to thrive (Thermote, 2006). Therefore, hatchery sanitation is recognized as an important factor in healthy poultry production (Bailey et al., 1995), since poor sanitization may lead ultimately to a high number or load of pathogenic organisms causing devastating effects on hatchability and health leading to economic losses (Bailey et al., 1998; Kim et al., 2007). In 1908, Pernot was the first investigator to demonstrate the use of formaldehyde fumigation of eggs and incubators as a means of controlling poultry diseases (Pernot and others, 1908). Formaldehyde (H_2CO) is a gas at room temperature that is readily soluble in water and frequently used as a disinfectant or sanitizer; this is due to the fact that it is cheap, noncorrosive (in the gaseous form), and kills most viruses, bacteria (including their spores), and fungi (Salthammer et al., 2010; Swenberg et al., 2013). The biocidal efficacy of formaldehyde is due to

its ability to act on proteins and nucleic acid bases of microorganisms. Formaldehyde also alkylates the nitrogen atoms of purine and pyrimidine bases in DNA and RNA (Fraenkel-Conrat et al., 1947). Because of its widespread use, toxicity, and volatility, formaldehyde poses a significant danger to human health. It is an irritant for the eyes and the nose, and has a persistent noxious odor, making venting of its vapors difficult (Ashford et al., 1983; Kaudla, 1999; Hernandez et al., 1994). In 2011, the US National Toxicology Program described formaldehyde as a "known human carcinogen" (US Department of Health and Human Services, 2010).

An important factor in the effect of formaldehyde on the tracheal mucosa is the dissolution of the gas in mucous secretions producing a pH shift toward acidity. These changes in pH cause damage to the membrane structure and ciliary activity (Sander et al., 1995; Braswell et al., 1970). The excessive mucus production and ciliostasis result in inadequate mucociliary action (McGregor et al., 2006). Transmission electron microscopy has also revealed shortening and loss of cilia in the epithelial cells, vacuolization, and swelling of the mitochondria, in both 18-day-old embryos and 1-day-old chicks. Extending the fumigation period caused an increase in these effects (Hayretdaug and Kolankaya, 2008). Yet, in spite of these adverse effects that are extensively reported in humans and poultry, even today, most commercial hatcheries in the United States still use formaldehyde as a method to control the bacterial bloom within the hatch cabinets (Cadirci, 2009; Kim and Kim, 2010; Lancaster et al., 1960).

Without question, an effective hatchery sanitation program is essential for the successful operation of a poultry hatchery. In an effort to replace the use of formaldehyde, investigations have revealed large microbial populations in many hatch cabinets despite the application of alternative sanitation measures (Wright et al., 1959). The air sampling technique we used has been used to quantitatively measure the degree of contamination by examining the

microbiological loads within the hatch cabinet, and is used extensively in the poultry industry to monitor bacterial and fungal levels circulating in the air of the hatchery and to evaluate the efficiency of the decontamination measures (Wright et al., 1959 ; Berrang et al., 1995; Bailey et al., 1995; Kim and Kim, 2010). The purpose of the present study was to evaluate the use of a spray probiotic formulation as an alternative hatch cabinet bacterial control method *versus* formaldehyde fumigation.

2. Material and methods

2.1 Probiotic

A specifically selected mix of three *Bacillus subtilis* isolates and two strains of *Pediococcus acidilactici* were combined and tested together as a probiotic culture in all experiments. Isolation and selection for the isolates is described below.

2.2 Isolation and selection of lactic acid bacteria candidates

Probiotic candidates were isolated from broiler chickens. Briefly, cecal epithelium, cecal lumen, and ileum epithelium were separated, homogenized, serial diluted in 0.9% sterile saline solution and plated on de Man Rogosa Sharpe (MRS) agar plates (Catalog no. 288110, Becton Dickinson and Co., Sparks, MD 21152 USA). Single colonies were obtained and identified with a number and evaluated for *in vitro* assessment of antimicrobial activity against *Salmonella* Enteritidis as previously described (Menconi et al., 2014). Two candidates were selected based on the zones of inhibition produced against the enteropathogens evaluated. These isolates were identified using API 50 CHL biochemical analysis (bioMerieux, Craponne France) as *Pediococcus acidilactici* and lyophilized individually and mixed during the probiotic preparation.

2.3 Isolation and characterization of *Bacillus spp.*

Previous research conducted in our laboratory focused on isolation of several *Bacillus spp.* from environmental and poultry sources (Shivaramaiah et al., 2011; Wolfenden et al., 2011; Menconi et al., 2013). Identification was completed using API 50 CHB biochemical analysis (bioMerieux, Craponne France). The three strains were identified as *Bacillus subtilis.* The three *B. subtilis* strains selected were grown and sporulated individually and mixed during the probiotic preparation.

2.4 Spore preparation

In an effort to grow high numbers of viable spores, a solid state fermentation media (SS) developed by Zhao et al., (2008) was selected and modified for use in these experiments. Briefly to prepare the SS fermentation media, ammonia broth was added to a mixture of 70% rice straw and 30% wheat bran at a rate of 40% by weight. Then, the SS fermentation media was added to 250 mL Erlenmeyer flasks and sterilized by autoclaving for 30 min at 121°C. Each of the three *B. subtilis* isolates were grown individually overnight at 37°C in test tubes containing 10 mL of tryptic soy broth (TSB, catalog no. 211822, Becton Dickinson, Sparks, MD). Following incubation, 2 mL of each culture was added separately to the previously prepared SS fermentation media flask. The inoculated flasks were incubated for 24 h at 37°C to promote growth of the *B. subtilis* vegetative cells, and then incubated for another 72 h at 30°C to trigger the initiation of the sporulation process. Following this, the inoculated SS fermentation media was removed from the Erlenmeyer flasks, placed onto petri dishes, and dried at 60°C. Then, the SS fermentation media was aseptically ground into a fine powder that contained stable *B. subtilis* spores (~ 10^{10} spores/g). *B. subtilis* spores from each of the three selected strains were combined in equal amounts.

2.5 Separate hatcher hallway setup

All experiments were conducted in a local commercial hatchery with a capacity of 13,000 eggs per hatch cabinet. The commercial hatchery used for these experiments has 48 hatch cabinets divided into 4 separate hallways consisting of 12 hatch cabinets in each hallway. The hatchery hatches 24 hatch cabinets per day, four days a week. To prevent cross contamination by formaldehyde into the probiotic hatch cabinets, or vice versa, the treatment groups were divided into separate hallways in the hatchery. Each hallway was shut off from direct contact with the other hallways. Air was mechanically exhausted out of each hallway through the side of the building and the air intake for each hallway was located on top of the building, and was distant from the exhaust. Using separate hallways for the different treatments should thus prevent any meaningful cross contamination.

2.6 Spray application of the probiotic

The probiotic was applied 4 times through the top of the hatch cabinet using a custom built mechanical applicator. The probiotic was applied once at transfer (19 days of incubation), and then every 10 hours following the initial application until 4 total applications had occurred. Lactic Acid Bacteria were administered at approximately 10^8 total colony forming units (cfu) per application. *B. subtilis* spores were administered at approximately 3×10^{11} spores per application. Control hatch cabinets were treated using the current formaldehyde application method, which consists of drip application of 60 mL of formalin, 37% formaldehyde solution, every 3 hours post transfer from the setter to the hatch cabinet. Formaldehyde treatment stopped 12 hours prior to chicks being removed from the hatch cabinet. Hatch cabinet sampling time points were scheduled to be one or two hours prior to the next application of probiotic. Probiotic treated hatch cabinets were sampled prior to the formaldehyde treated hatch cabinets. This was to

guarantee that all of the probiotic hatch cabinets were sampled prior to the next application of probiotic. All formaldehyde and probiotic treated hatch cabinets were sampled within one hour.

2.7 Experimental design

2.7.1 Experiment 1

2.7.1.1 Hatchery sanitation evaluation

Two independent trials were conducted to compare the hatchery sanitation between the current disinfection method with formaldehyde *versus* spray application of the probiotic. All hatch cabinets sampled contained embryos from the same source flock for the probiotic and the formaldehyde treated hatch cabinets. The only difference was the treatment that the hatch cabinet received. Hatchery sanitation was evaluated using the previously described open-plate method (Berrang et al., 1995). Each hatch cabinet has six carts with fifteen trays of embryos per cart, with a lid covering the top tray of each cart. Each hatch cabinet has two fans against the front wall of the hatch cabinet between the third and fourth carts, pointed at the back of the hatch cabinet. In all experiments, four sampling plates of each selective media were placed into the hatch cabinets on top of the lids of the first, third, fourth, and sixth cart. Previous unpublished results had shown no difference in uniformity of plate growth if the plates were placed in the trays, below the trays, or on top of the lid of the trays.

Bacteriological evaluation was conducted at approximately 20% pip, 30% hatch, and 85% hatch for enumeration of total non-selective aerobic bacteria (TAB) on Tryptic soy agar plates (TSA catalog no. 212081, Becton Dickinson, Sparks, MD); lactic acid bacteria (LAB) on de Man Rogosa Sharpe agar (Difco™ Lactobacilli MRS Agar VWR Cat. No. 90004-084 Suwanee, GA 30024); and total recovered Gram-negative bacteria (TGB) on MacConkey agar. Petri dishes containing each type of media were placed uncovered in the hatching environment

for 5 minutes. Post sampling, all plates were incubated for 18 h at 37ºC before the plates were enumerated. LAB and TGB enumeration was expressed in Log_{10} cfu based on colony counts. Incidence of total pasteurized non-selective aerobic bacteria was performed as described below.

2.7.1.2 Percentage score of plate coverage on TSA

After TSA sampling plates were incubated at 37°C for 18 hours, plates were scored on a percentage of plate coverage. Each individual hatch cabinet had four plates in it that were scored. After scoring all of the TSA sampling plates for all hatch cabinets (6 hatch cabinets per treatment group) the percentages were grouped together by treatment. To remain consistent across all sampling time points and experiments reference pictures were used to determine the percentage of plate coverage. The reference pictures shown in Figure 1 illustrate what was scored as 0%, 20%, 40%, 60%, 80% and 100% plate coverage. If a plate did not fall directly into a category shown by the reference pictures it was scored as a percentage somewhere between the two closest percentages. For instance, if a plate had more than 20% plate coverage but less than 40% plate coverage it would be scored accordingly between 20% and 40%.

2.7.2 Experiment 2

2.7.2.1 Evaluation of intestinal microflora of neonatal broilers

In Experiment 2, three independent trials were conducted to evaluate the intestinal microflora of neonatal chicks from hatch cabinets treated with formaldehyde (current method) *versus* spray application of the probiotic (n = 12/treatment). In addition, in trial 3, a sub-group of chicks (n = 12/treatment) were held for 24 h to further evaluate intestinal microflora. As in Exp. 1, all chicks sampled in Exp. 2 came from the same source flock for both the probiotic and formaldehyde treated hatch cabinets. Whole duodenum, ileum, and ceca were aseptically removed, separated into sterile bags, and homogenized. Samples were weighed and 1:4 w/v

dilutions were made with sterile 0.9% saline. Then, ten-fold dilutions of each sample, from each group were made in a sterile 96 well Bacti flat bottom plate and the diluted samples were plated on three different culture media. TSA plates were utilized for enumeration of TAB in trial 1 and for enumeration of total pasteurized (70° C for 10 min) non-selective aerobic bacteria in trials 2 and 3; MRS agar plates for total LAB; and MacConkey agar for TGB. All plates were incubated for 18 h at 37^{o}C before bacterial count. Bacteria enumeration was expressed as Log_{10} cfu.

2.8 Statistical analysis

In all experiments, data were subjected to one-way ANOVA as a completely randomized design using the GLM procedure of SAS (SAS Institute, 2002). Data are expressed as mean ± SE and a *P*-value of $P < 0.05$ was set as the standard for significance.

2.9 Results

The results of the bacteriological counts recovered from hatching cabinets treated with formaldehyde or following four applications of probiotic are summarized in Table 1 for Experiment 1. In both trials, a significant ($P < 0.05$) increase in the percentage of coverage of TAB was observed in probiotic treated hatch cabinets when compared with formaldehyde treatment at all three times of evaluation, approximately 20% pip; 30% hatch and 85% hatch. Similar results were observed in the total number of Log_{10} cfu of LAB. No significant differences were observed in TGB at 20% pip or 30 % hatch. However, at 85% hatch, a significant increase in TGB was observed in probiotic group when compared with formaldehyde treated hatch cabinets (Table 1).

Table 2 summarizes the results of Log_{10} cfu recovered from intestinal samples on day of hatch following formaldehyde treatment or four applications of probiotic with a 24 h holding period for trial 3 of Experiment 2. No significant differences in the total number of Log_{10} cfu for

the non-pasteurized TAB was observed in trial 1. However, in trial 2 and trial 3, chicks from probiotic treated hatch cabinets showed a significant increase of pasteurized TAB when compared with formaldehyde treated chicks. For total intestinal LAB, no significant differences were observed in trials 1 and 3. Interestingly, in trial 2, a significant reduction in the total number of LAB were observed in the probiotic control group when compared with the formaldehyde group. However, it was remarkable we observed a significant reduction in TGB in the GIT in all three trials in chicks from hatch cabinets treated with the probiotic, when compared with hatch cabinets that were treated with formaldehyde (Table 2). This significant reduction in TGB persisted 24 h post hatch in trial 3. Even though no significant differences were observed in LAB between treatments in the GIT, chicks from hatch cabinets treated with the probiotic and held for 24 h, showed a significant increase in the total number of pasteurized TAB when compared with chicks from formaldehyde treated hatch cabinets (Table 2).

3. Discussion

Microbial contamination of hatching eggs is a major concern for poultry producers as it causes poor hatchability and chick performance, hence high standards of sanitation must be practiced in hatcheries (Thermote, 2006). Methods used include the application of disinfectants by wiping, spraying, and dipping but, arguably, the most effective way of reducing the bacterial load on hatching eggs is fumigation with formaldehyde (Whistler and Sheldon, 1989; Sheldon and Brake, 1991; Kaudla, 1999; Yildirim et al., 2003; Cadirci, 2009). Formaldehyde is still extensively used in commercial hatcheries during the hatching period (during or just after the transfer to the hatcher). Formaldehyde, besides being an excellent anti-microbial agent, is also a toxic chemical and, as such, can seriously damage the embryo (Jasanoff, 1987; Hayretda\ug and Kolankaya, 2008). In experiment 1 of the present study, in both independent trials conducted in a

commercial hatchery, the application of a defined probiotic culture containing a mix of three *Bacillus subtilis* and two *Pediococcus acidilactici,* significantly increased the number of environmental TAB and LAB. However, it is remarkable to observe that probiotic treatment was as effective in reducing TGB in the environment, as formaldehyde treated hatch cabinets evaluated at 20% pip and 30% hatch (Table 1). Equally important, is that chicks that hatch from probiotic treated hatch cabinets, also showed a significant reduction in the total number of Gram-negative bacteria in their GIT, and this significant reduction persisted 24 h post hatch (Table 2). The relevance of these findings is that there is good experimental and epidemiological evidence that primary infection of *Salmonella* is by the oral-fecal route, along with an established infectious dose (Galanis and others, 2006). Nevertheless, recent published results from our laboratory comparing intratracheal *versus* oral administration of *Salmonella enterica* serovars Enteritidis, Typhimurium, or Seftenberg have shown that neonatal chicks, can be infected via the respiratory route at a very low dose (100 cells), with cecal colonization equivalent to that recovered from a higher oral (10,000 cells) challenge (Kallapura et al., 2014b; Kallapura et al., 2014c; Kallapura et al., 2014d; Kallapura et al., 2014a). Understanding the anatomical and immunological defenses of the avian respiratory tract helps to clarify this issue. Architecture of the avian respiratory tract is an important component to susceptibility and resistance to infectious agents. In day old chicks and turkeys, no or very few infiltrating lymphocytes are seen in the primary bronchi region (Fagerland and Arp, 1990; (Smialek et al., 2011) and it is not until 3-4 weeks of age the lymphoid nodules are developed at these locations (Fagerland and Arp, 1993; Drolet et al., 2010). During the following week, the number of IgG, IgA or IgM-producing cells continues to increase, however, the bronchial-associated lymphoid tissue is not mature until chickens are 6–8 weeks old (Bienenstock, 1980; Bienenstock and McDermott, 2005; De Geus,

2012). Hence, commercial neonatal poultry are extremely susceptible to airborne pathogens, regardless of whether or not they are respiratory or enteric bacteria (Arshad et al., 1998). This also supports previous studies demonstrating fan driven spread of *Salmonella* within the hatching cabinet and hatchery incubators (Hashemzadeh et al., 2010). On the other hand, these chicks are deprived of acquiring their natural microflora that colonize their GIT immediately after hatching under natural conditions. Today, the microbiome is recognized as the 'forgotten organ,' operating like an organ within the host and orchestrating numerous physiological and biological functions that have a profound impact on the balance between health and disease (O'Hara and Shanahan, 2006; Tellez, 2014). Early establishment of the microbiome has been reported to improve the assembly of the gut-associated lymphoid tissue (Martin et al., 2010), mediate in the development of the immune system (McFall-Ngai, 2007), maintain mucosal barrier integrity (Duerkop et al., 2009), modulate proliferation of enterocytes (Moran, 2007), adjust blood flow (Sekirov et al., 2010), regulate the enteric nervous system (Tlaskalová-Hogenová et al., 2011), and improve digestion of nutrients (Walter et al., 2011; Qiu et al., 2012; Dass et al., 2007). Essential colonization of these bacterial populations starts at birth/hatch, and is followed by progressive assembly of a complex and dynamic microbial society (Di Mauro et al., 2013). Yet, under commercial conditions, millions of chickens and turkeys hatch in a hostile environment, and are exposed for several hours to heat stress and potential pathogenic bacteria in the hatch cabinet. Increased stress along with the potential abundance of pathogens in the hatch cabinet leads to ideal conditions for pathogen colonization.

3.1 Conclusions and Applications

The spray application of the probiotic used in the present study was clearly able to reduce and exclude Gram-negative bacteria in commercial neonatal broiler chicks. The results of the

present study suggest that the spray application of a probiotic in commercial hatcheries, not only can be as effective as the noxious formaldehyde in reducing Gram-negative bacteria at two critical points of the hatching process, but most importantly, it reduces the number of Gram-negative bacteria present in the chicks at hatch in three commercial trials, and in one commercial trial, 24 h after hatch. Further studies to evaluate competitive exclusion using challenge models with enteropathogens and performance field trials are currently being evaluated.

References

Arshad, M. J., M. Siddique, S. Rehman, and M. S. Aslam. 1998. A Comparative Study of Respiratory Phagocytic Cell Activities in Layer Chicks. Medical Journal of Islamic Academy of Sciences 11:107–110.

Ashford, N. A., C. W. Ryan, and C. C. Caldart. 1983. Law and science policy in federal regulation of formaldehyde. Science (Washington) 222:894–900.

Bailey, J., J. Cason, and N. Cox. 1998. Effect of *Salmonella* in young chicks on competitive exclusion treatment. Poultry science 77:394–399.

Bailey, S., N. Cox, and N. Stern. 1995. Risk Management Factors Associated with Implementation of HACCP in the Poultry Industry.in From an address to the Hazard Analysis and Critical Control Point (HACCP) Symposium in association with the 75th Annual Meeting of the Conference of Research Workers in Animal Diseases, Chicago.

Berrang, M., N. Cox, and J. Bailey. 1995. Measuring air-borne microbial contamination of broiler hatching cabinets. The Journal of Applied Poultry Research 4:83–87.

Bienenstock, J. 1980. Bronchus-associated lymphoid tissue and the source of immunoglobulin-containing cells in the mucosa. Environ. Health Perspect. 35:39–42.

Bienenstock, J., and M. R. McDermott. 2005. Bronchus- and nasal-associated lymphoid tissues. Immunol. Rev. 206:22–31.

Braswell, J. R., D. R. Spiner, and R. K. Hoffman. 1970. Adsorption of formaldehyde by various surfaces during gaseous decontamination. Appl Microbiol 20:765–9.

Cadirci, S. 2009. Disinfection of hatching eggs by formaldehyde fumigation-a review. Arch. Geflügelk 73:116–123.

Dass, N. B., A. K. John, A. K. Bassil, C. W. Crumbley, W. R. Shehee, F. P. Maurio, G. B. T. Moore, C. M. Taylor, and G. J. Sanger. 2007. The relationship between the effects of short-chain fatty acids on intestinal motility in vitro and GPR43 receptor activation. Neurogastroenterol. Motil. 19:66–74.

De Geus, E. 2012. Respiratory immune responses in the chicken; towards development of mucosal avian influenza virus vaccines. Wohrman Print Service, Utrecht, Holland.

Drolet, J.-P., H. Frangie, J. Guay, O. Hajoui, Q. Hamid, and B. D. Mazer. 2010. B lymphocytes in inflammatory airway diseases. Clin. Exp. Allergy 40:841–9.

Duerkop, B. A., S. Vaishnava, and L. V. Hooper. 2009. Immune responses to the microbiota at the intestinal mucosal surface. Immunity 31:368–76.

Fagerland, J. A., and L. H. Arp. 1990. A morphologic study of bronchus-associated lymphoid tissue in turkeys. Developmental Dynamics 189:24–34.

Fagerland, J. A., and L. H. Arp. 1993. Structure and development of bronchus-associated lymphoid tissue in conventionally reared broiler chickens. Avian diseases:10–18.

Fraenkel-Conrat, H., B. A. Brandon, and H. S. Olcott. 1947. The reaction of formaldehyde with proteins IV. Participation of indole groups. Gramicidin. Journal of Biological Chemistry 168:99–118.

Galanis, E., and others. 2006. Web-based Surveillance and Global *Salmonella* Distribution, 2000-2002-Volume 12, Number 3—March 2006-Emerging Infectious Disease journal-CDC.

Hashemzadeh, Z., K. Torshizi, S. Rahimi, V. Razban, and T. Zahraei Salehi. 2010. Prevention of *Salmonella* colonization in neonatal broiler chicks by using different routes of probiotic administration in hatchery evaluated by culture and PCR techniques. Journal of Agricultural Science and Technology 12:425–432.

Hayretdaug, S., and D. Kolankaya. 2008. Investigation of the effects of pre-incubation formaldehyde fumigation on the tracheal epithelium of chicken embryos and chicks. Turkish Journal of Veterinary and Animal Sciences 32:263–267.

Hernandez, O., L. Rhomberg, K. Hogan, C. Siegel-Scott, D. Lai, G. Grindstaff, M. Henry, and J. A. Cotruvo. 1994. Risk assessment of formaldehyde. Journal of hazardous materials 39:161–172.

Jasanoff, S. S. 1987. Contested boundaries in policy-relevant science. Social studies of science 17:195–230.

Kallapura, G., A. Botero, S. Layton, L. Bielke, J. Latorre, A. Menconi, X. Hernández-Velasco, D. Bueno, B. Hargis, and G. Téllez. 2014a. Evaluation of recovery of *Salmonella* from trachea and ceca in commercial poultry. The Journal of Applied Poultry Research 23:132–136.

Kallapura, G., X. Hernandez-Velasco, N. Pumford, L. Bielke, B. Hargis, and G. Tellez. 2014b. evaluation of respiratory route as a viable portal of entry for *Salmonella* in poultry. Veterinary Medicine: Research and Reports 5:59–73.

Kallapura, G., M. Kogut, M. Morgan, N. Pumford, L. Bielke, A. Wolfenden, O. Faulkner, J. Latorre, A. Menconi, X. Hernandez-Velasco, and others. 2014c. Fate of *Salmonella* Senftenberg in broiler chickens evaluated by challenge experiments. Avian Pathology 43:305–309.

Kallapura, G., M. Morgan, N. Pumford, L. Bielke, A. Wolfenden, O. Faulkner, J. Latorre, A. Menconi, X. Hernandez-Velasco, V. Kuttappan, and others. 2014d. Evaluation of the respiratory route as a viable portal of entry for *Salmonella* in poultry via intratracheal challenge of *Salmonella* Enteritidis and *Salmonella* Typhimurium. Poultry science 93:340–

346.

Kaudla, T. 1999. The effect of antibiotic, disinfectant and formaldehyde gas on hatchability of broiler eggs. Journal of Food Technology in Africa 4.

Kim, J. H., and K. S. Kim. 2010. Hatchery hygiene evaluation by microbiological examination of hatchery samples. Poult. Sci. 89:1389–98.

Kim, A., Y. J. Lee, M. S. Kang, S. I. Kwag, and J. K. Cho. 2007. Dissemination and tracking of *Salmonella spp.* in integrated broiler operation. Journal of veterinary science 8:155–161.

Lancaster, J. E., D. M. Cooper, H. Gasperdone, and A. J. Darbey. 1960. Observations on Hatchery Sanitation. Can J Comp Med Vet Sci 24:83–7.

Martin, R., A. J. Nauta, K. Ben Amor, L. M. J. Knippels, J. Knol, and J. Garssen. 2010. Early life: gut microbiota and immune development in infancy. Benef Microbes 1:367–82.

Di Mauro, A., J. Neu, G. Riezzo, F. Raimondi, D. Martinelli, R. Francavilla, and F. Indrio. 2013. Gastrointestinal function development and microbiota. Ital J Pediatr 39:15.

McFall-Ngai, M. 2007. Adaptive immunity: care for the community. Nature 445:153–153.

McGregor, D., H. Bolt, V. Cogliano, and H.-B. Richter-Reichhelm. 2006. Formaldehyde and glutaraldehyde and nasal cytotoxicity: case study within the context of the 2006 IPCS Human Framework for the Analysis of a cancer mode of action for humans. Critical reviews in toxicology 36:821–835.

Menconi, A., G. Kallapura, J. D. Latorre, M. J. Morgan, N. R. Pumford, B. M. Hargis, and G. Tellez. 2014. Identification and characterization of lactic Acid bacteria in a commercial probiotic culture. Biosci Microbiota Food Health 33:25–30.

Menconi, A., M. J. Morgan, N. R. Pumford, B. M. Hargis, and G. Tellez. 2013. Physiological properties and *Salmonella* growth inhibition of probiotic *Bacillus* strains isolated from environmental and poultry sources. International journal of bacteriology 2013.

Moran, N. A. 2007. Symbiosis as an adaptive process and source of phenotypic complexity. Proc. Natl. Acad. Sci. U.S.A. 104 Suppl 1:8627–33.

O'Hara, A. M., and F. Shanahan. 2006. The gut flora as a forgotten organ. EMBO Rep. 7:688–93.

Pernot, E. F., and others. 1908. An investigation of the mortality of incubator chicks.

Qiu, R., J. Croom, R. Ali, A. Ballou, C. Smith, C. Ashwell, H. Hassan, C.-C. Chiang, and M. Koci. 2012. Direct fed microbial supplementation repartitions host energy to the immune

system. Journal of animal science 90:2639–2651.

SAS Institute. 2002. SAS User Guide. Version 9.1. SAS Institute Inc., Cary, NC.

Salthammer, T., S. Mentese, and R. Marutzky. 2010. Formaldehyde in the indoor environment. Chemical reviews 110:2536–2572.

Sander, J., J. Wilson, G. Rowland, and P. Middendorf. 1995. Formaldehyde vaporization in the hatcher and the effect on tracheal epithelium of the chick. Avian diseases :152–157.

Sekirov, I., S. L. Russell, L. C. M. Antunes, and B. B. Finlay. 2010. Gut microbiota in health and disease. Physiological reviews 90:859–904.

Sheldon, B., and J. Brake. 1991. Hydrogen peroxide as an alternative hatching egg disinfectant. Poultry science 70:1092–1098.

Shivaramaiah, S., N. R. Pumford, M. J. Morgan, R. E. Wolfenden, A. D. Wolfenden, A. Torres-Rodríguez, B. M. Hargis, and G. Téllez. 2011. Evaluation of *Bacillus* species as potential candidates for direct-fed microbials in commercial poultry. Poult. Sci. 90:1574–80.

Smialek, M., B. Tykalowski, T. Stenzel, and A. Koncicki. 2011. Local immunity of the respiratory mucosal system in chickens and turkeys. Polish journal of veterinary sciences 14:291–297.

Swenberg, J. A., B. C. Moeller, K. Lu, J. E. Rager, R. C. Fry, and T. B. Starr. 2013. Formaldehyde carcinogenicity research: 30 years and counting for mode of action, epidemiology, and cancer risk assessment. Toxicol Pathol 41:181–9.

Tellez, G. 2014. Prokaryotes versus Eukaryotes: Who is hosting whom? Veterinary Infectious Diseases 1:3.

Thermote, L. 2006. Effective hygiene within the hatchery. International Hatchery Practice 20:18–21.

Tlaskalová-Hogenová, H., R. Stepánková, H. Kozáková, T. Hudcovic, L. Vannucci, L. Tucková, P. Rossmann, T. Hrncir, M. Kverka, Z. Zákostelská, and others. 2011. The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases. Cellular & molecular immunology 8:110–120.

US Department of Health and Human Services. 2010. Report on carcinogens background document for formaldehyde. US Department of Health and Human Services, Research Triangle Park, NC.

Walter, J., R. A. Britton, and S. Roos. 2011. Host-microbial symbiosis in the vertebrate gastrointestinal tract and the *Lactobacillus* reuteri paradigm. Proc. Natl. Acad. Sci. U.S.A. 108

Suppl 1:4645–52.

Whistler, P. E., and B. W. Sheldon. 1989. Biocidal activity of ozone versus formaldehyde against poultry pathogens inoculated in a prototype setter. Poult. Sci. 68:1068–73.

Wolfenden, R., N. Pumford, M. Morgan, S. Shivaramaiah, A. Wolfenden, C. Pixley, J. Green, G. Tellez, and B. Hargis. 2011. Evaluation of selected direct-fed microbial candidates on live performance and *Salmonella* reduction in commercial turkey brooding houses. Poultry science 90:2627–2631.

Wright, M. L., G. W. Anderson, and N. A. Epps. 1959. Hatchery Sanitation. Can J Comp Med Vet Sci 23:288–90.

Yildirim, I., M. Ozsan, and R. Yetisir. 2003. The Use of Oregano (Origanum vulgare L) Essential Oil as Alternative Hatching Egg Disinfectant versus Formaldehyde Fumigation in Quails (Coturnix coturnix japonica). Revue de médecine vétérinaire 154:367–370.

Zhao, S., L. Deng, N. Hu, B. Zhao, and Y. Liang. 2008. Cost-effective production of *Bacillus* licheniformis using simple netting bag solid bioreactor. World Journal of Microbiology and Biotechnology 24:2859–2863.

Table 1. Bacteriological counts recovered from hatching cabinets untreated or following 4 applications of probiotic. Experiment 1.

	20% pip			30% hatch			85% hatch		
	TAB [1] %	LAB [2] Log_{10} cfu	TGB [3] Log_{10} cfu	TAB [1] %	LAB [2] Log_{10} cfu	TGB [3] Log_{10} cfu	TAB [1] %	LAB [2] Log_{10} cfu	TGB [3] Log_{10} cfu
Trial 1									
Formaldehyde	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	19.58 ± 1.12	1.72 ± 0.05	0.54 ± 0.08	37.08 ± 3.73	2.05 ± 0.07	0.77 ± 0.08
Probiotic	51.25 ± 4.14 *	0.94 ± 0.08 *	0.00 ± 0.00	100.00 ± 0.00 *	2.74 ± 0.03 *	0.52 ± 0.08	100.00 ± 0.00 *	2.75 ± 0.03 *	1.31 ± 0.06 *
Trial 2									
Formaldehyde	0.41 ± 0.28	0.27 ± 0.07	0.03 ± 0.02	21.25 ± 2.72	2.03 ± 0.05	1.10 ± 0.06	39.58 ± 4.44	2.16 ± 0.06	1.35 ± 0.06
Probiotic	62.08 ± 5.03 *	1.72 ± 0.06 *	0.01 ± 0.01	92.08 ± 0.84 *	2.46 ± 0.02 *	1.22 ± 0.06	94.58 ± 1.03 *	2.38 ± 0.05 *	1.75 ± 0.02 *

[*] Superscript within columns in each trial between formaldehyde and probiotic treatments indicate significant difference at $P < 0.05$. $n = 12$/group

Data are expressed as mean ± SE.

[1] TAB: Total non-selective aerobic bacteria recovered. Data represents percentage of agar plate coverage

[2] LAB: Total lactic acid bacteria recovered. Data represents Log_{10} cfu/plate recovered

[3] TGB: Total Gram-negative bacteria recovered. Data represents Log_{10} cfu/plate recover

Table 2. Log_{10} cfu/plate recovered from intestinal samples on day-of-hatch following 4 applications of probiotic with a 24 h holding period for Trial 3. Experiment 2.

	TAB[1]	LAB[2]	TGB[3]
Trial 1. Day of hatch			
Formaldehyde	8.95 ± 0.20	7.79 ± 0.17	8.39 ± 0.19
Probiotic	8.22 ± 0.40	6.77 ± 0.56	5.79 ± 0.91 *****
Trial 2. Day of hatch			
Formaldehyde	1.34 ± 0.48	7.26 ± 0.34	6.56 ± 0.83
Probiotic	5.30 ± 0.15 *	2.88 ± 0.79 *****	2.60 ± 0.83 *****
Trial 3. Day of hatch			
Formaldehyde	0.69 ± 0.36	6.26 ± 0.83	3.90 ± 0.93
Probiotic	5.83 ± 0.18 *****	4.64 ± 0.78	1.32 ± 0.70 *****
Trial 3. 24 h Post hatch			
Formaldehyde	0.22 ± 0.22	8.59 ± 0.13	8.16 ± 0.43
Probiotic	5.03 ± 0.17 *****	8.35 ± 0.22	5.81 ± 1.10 *****

[*] Superscript within columns in each trial between formaldehyde and probiotic treatments indicate significant difference at $P < 0.05$. $n = 12$/group
Data are expressed as mean ± SE
[1] TAB: Pasteurized (Trials 2 & 3) non-selective aerobic bacteria recovered (Trial 1 was not pasteurized)
[2] LAB: Total lactic acid bacteria recovered
[3] TGB: Total Gram negative bacteria recovered

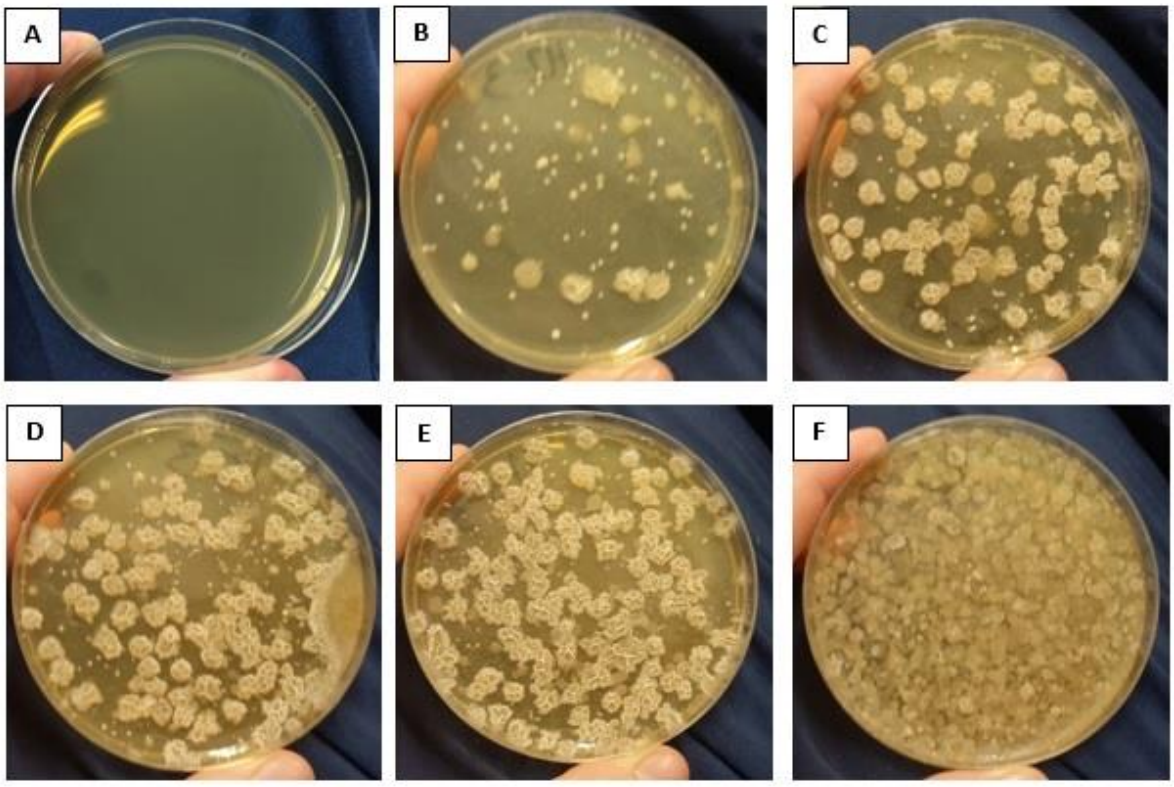

Figure 1. Reference pictures used to determine the percentage of plate coverage on TSA plates placed into the hatch cabinets at all sampling time points. (**A**) 0% plate coverage (**B**) 20% plate coverage (**C**) 40% plate coverage (**D**) 60% plate coverage (E) 80% plate coverage (**F**) 100% plate coverage

Chapter 3 – Conclusion

Formaldehyde use in the poultry hatch cabinet needs to stop. As Broder et al., (1991) showed concentrations as small as 0.1 ppm are enough to cause irritation to the eyes and throat. Exposing millions of day of hatch chicks to formaldehyde is not good for the health of the animals. Formaldehyde has been used for decades as a disinfectant (Beesley, 1980); (Deeming, 1992); (Steinlage et al., 2002). As the hatch progresses and the microbial load inside of the hatch cabinet increases formaldehyde loses its ability to suppress microbial growth (Magwood, 1964). Formaldehyde causes lung inflammation and oxidative stress in animals (Lino-dos-Santos-Franco et al., 2011). A less toxic alternative to formaldehyde hatch cabinet fumigation needs to be found. Not only does formaldehyde cause observable lesions such as matted cilia, loss of cilia, and sloughing of the epithelium. It causes a reduction in feed conversion over a 41 day grow out trial (Zulkifli et al., 1999). Formaldehyde is a known human carcinogen (Mackar, 2011) that causes a detriment to any living organism it comes into contact with. Removing formaldehyde fumigation in the hatchery would have a positive impact on the chicks as well as the humans responsible for handling the formaldehyde.

Pioneer colonization of the gastrointestinal tract is essential because the pioneer colonizers have a lifelong impact on the animal. Newborn children delivered vaginally can have altered microflora for twenty four months when compared to children delivered through a cesarean section birth (Grölund et al., 1999). LAB are present in the vagina (Walter, 2008) which may allow the pioneer colonizers of vaginally birthed children to be beneficial LAB. LAB which have been shown to inhibit *E. coli* colonization while improving performance parameters of poultry (Jin et al., 1996) may be able to provide chicks with a better start to life than those exposed to formaldehyde. *Bacilli* which have been shown to germinate in the gastrointestinal

tract of chickens when given orally (Cartman et al., 2008) can inhibit the colonization of poultry by *Salmonella* (Shivaramaiah et al., 2011); (Wolfenden et al., 2011). The combination of selected strains of *Bacilli* and LAB may be a viable alternative to formaldehyde fumigation while providing beneficial pioneer colonizers to the chicks.

In Experiment 1 we were able to match formaldehyde Gram-negative bacterial suppression earlier on in the hatch period for both experiments. As the hatch neared 85% the probiotic treatment lost its ability to suppress Gram-negative growth when compared to formaldehyde. Probiotic treatment did significantly increase the non-selective bacterial growth recovered from the hatching environment. All non-selective recovery for the probiotic treated group had consistent colony morphology with the *Bacilli* used in the probiotic. Probiotic treated hatch cabinets also had significantly higher levels of LAB circulating in the hatching environment than the formaldehyde treated hatch cabinets at all three time points. Probiotic treatment can inhibit the Gram-negative microbial bloom that occurs inside of a hatch cabinet early on in the hatch period while exposing the chicks to beneficial pioneer colonizers such as *Bacilli* and LAB.

In Experiment 2 chicks from the probiotic treated hatch cabinets did not have significantly higher LAB recovered on day of hatch when compared to the formaldehyde controls. Probiotic treatment did cause a significant reduction in intestinal recovery of Gram-negative bacteria when compared to the formaldehyde control. In Trials 2 and 3 the probiotic treated groups had significantly higher levels of bacteria recovered from pasteurized intestinal samples. Colony morphology was consistent with the *Bacilli* used in the probiotic. This early reduction of intestinal Gram-negative bacteria with an increase in beneficial pioneer colonizers may have a lasting impact on the life of the animal.

Appendix

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO:	Billy Hargis
FROM:	Craig N. Coon, Chairman
DATE:	May 16, 2016
SUBJECT:	IACUC Approval
Expiration Date:	September 14, 2017

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your modification to add Kyle Teague and Lucas Graham to protocol # 15006: "Development of enteric inflammation models for investigation of antibiotic alternatives in poultry".

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond September 14, 2017 you must submit a new protocol prior to that date to avoid any interruption. 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 involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572
Fax: 479-575-3846 • http://vpred.uark.edu/199