ABSTRACT

CHANCE, SOPHIE. The Effects of Age and Applying *M. luteus* to Nest Pads on Hatchability, Fertility, Sperm Motility and Microbiota Composition and Diversity in Cloacas and on Nesting Surfaces for Broiler Breeders (Under the direction of Dr. Aaron Kiess).

This thesis evaluated the microbiota of Ross 708 broiler breeders and their nesting surface in order to determine changes overtime and how changes to the microbiota effect fertility, hatchability and chick parameters. For the trial in which *Micrococcus luteus* was added to the nesting pads in the broiler breeder house, 960 hens and 112 roosters were randomly assigned to one of 16 slat litter pens with 60 hens and 7 rooster per pen. 8 pens had *M. luteus* applied to their nest pads and the other 8 pens had sterile phosphate buffered saline applied as a control. Application took place at the beginning of each period including 26-29 wks (start of lay), 33-36 wks (peak) and 41-44 wks (post peak) of age. 50 eggs were collected weekly during each period and set to evaluated hatchability, fertility, chick weight and 7-day chick mortality. Cloacal swabs and nest pad samples were also collected and sent off for 16S rRNA sequencing to compare differences in microbiota between the *M. luteus* and control groups. There were no significant differences in hatchability or fertility, however there was a significant difference in 7-day chick weight (P=0.009) during the 33-36 wk period favoring the control group and significant differences in beta (Unweighted UniFrac) (FDR=0.0270) and alpha (Shannon) (FDR=0.0286) diversity in the hen's cloaca.

Thirty Ross 708 strain roosters were kept separately from hens and were abdominally massaged on a weekly basis to get them used to human handling. Semen samples were collected at 35 wks, 43 wks, and 45 wks of age and mixed with cultures of *M. luteus*, *Lactobacillus acidophilus*, and *Bacillus subtilis* to evaluate the effect of probiotic and commensal bacteria on sperm motility. High motility samples were mixed with Tryptic Soy Broth (TSB), Man–Rogosa–

Sharpe Broth (MRS), 10^6 and 10^8 *Micrococcus luteus* in TSB, 10^6 and 10^8 *Bacillus subtilis* in TSB, 10^6 and 10^8 *Lactobacillus acidophilus* in MRS, 10^6 *L. acidophilus* in PBS, and Phosphate Buffered Saline (PBS) and turkey semen diluent SQA-Vt as controls. The 10^6 samples were run after 20 minutes in addition to immediate exposure. There were significant differences in sperm motility between the control and MRS ($P=0.0131$), 10^6 and 10^8 *L. acidophilus* in MRS ($P=0.0039$, $P<0.0001$), and 10^6 *L. acidophilus* after 20 min in MRS ($P<0.0001$). Neither *M. luteus* nor *B. subtilis* caused significant differences in sperm motility. Therefore, some nonpathogenic are less detrimental to semen motility than others.

The microbiota samples of four pens of the control birds from the first trial were also evaluated across all three periods of lay to reveal any differences in microbiota diversity and richness. The samples revealed a difference in beta diversity (Unweighted UniFrac) in the hen cloacal samples from the three different time periods (FDR=0.036). No other significant differences in species diversity or richness were found for the other time periods for the hen cloacal swabs, rooster cloacal swabs and nest pad samples. Phylum and genera abundance were also evaluated and showed significant differences for all sample types. *Firmicutes*, *Actinobacteria*, and *Proteobacteria* were the three most abundant phylum across all sample types.

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The Effects of Age and Applying *M. luteus* to Nest Pads on Hatchability, Fertility, Sperm Motility and Microbiota Composition and Diversity in Cloacas and on Nesting Surfaces for Broiler Breeders

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DEDICATION

To my parents who helped me and supported me through every academic challenge and choice I’ve made in college and to my fiancé and friends that have supported and encouraged me through this process.

BIOGRAPHY

Sophie Elizabeth Chance was born and raised in Annapolis, Maryland. She is the daughter of Kimberley and Bruce Chance who raised her to be a hardworking, compassionate, and confident individual. Sophie found her passion for agriculture through FFA during high school. She became confident through public speaking in FFA, 4H and as a representative for the Maryland Farm Bureau, where she was introduced to the many problems that the agricultural industry faces. With a passion to make a difference driving her, Sophie began applying to Land Grant Universities and trying to decide which major offered the most opportunities to make an influence and deepen her passions. That is how Sophie ended up at NC State where she received her Bachelors in Poultry Science with minors in genetics and biology in December of 2021. During her undergraduate, Sophie was a research assistant under Dr. Robert Beakstead and Dr. Sean Chen where she studied parasites in turkey poults and broiler breeders. Through the research she assisted with, Sophie discovered that she enjoyed research and wanted to continue her education, leading her to accept a graduate research position under Dr. Aaron Kiess at North Carolina State University. During her master's she studied the effects of period of lay and applying *M. luteus* to nest pads on hatchability, fertility, sperm motility and microbiota composition and diversity in cloacas and on nesting surfaces for broiler breeders. After graduation, Sophie will be starting a role at Mountaire Farms and is excited to start making a difference out in the industry.

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CHAPTER 1

INTRODUCTION

Hatchability and fertility in broiler breeders must be sustained and improved with every progressing year to supply the growing industry and prevent lag times where broiler houses are empty. Researchers have conducted countless trials working on improving the lighting schedules, feeding, egg storage, and genetics of broiler breeders to achieve optimal hatchability. In the most recent calendar year of 2023, the United States was unable to surpass 81% hatchability during its weekly assessment by the USDA (Albert R. Mann Library). In comparison, hatchability in 2012 reached 85%, with the extra 4% representing millions of chicks hatched (Albert R. Mann Library). A steady decrease in hatchability has been seen since 2012 and the industry's goal is to get back to that level of hatchability or surpass it (Albert R. Mann Library). Hens and roosters must have a 1:10 rooster to hen ratio, healthy body conditioning, high quality feed, good litter management, and sufficient water supply in order for them to perform at the highest caliber. In addition, eggs must be handled and stored appropriately at a temperature between 55- 65° F with limited exposure to further contamination, proper packaging and prevention of sweating. These measures must be taken to avoid damaging or contaminating hatching eggs. The main two factors that contribute to the decrease in hatchability are poor flock and egg management and unfavorable genetic selection, however other factors such as antibiotic removal, nutrition and microbiome dysbiosis have also played a role.

Unfortunately, changing current management practices is difficult, due to labor and money constraints. In addition, changing the species genetic profile takes 4 years to see results and has conflicting consequences on other genetic phenotypes. From this perspective, researchers have been tasked with targeting supplemental ways to improve rooster fertility, including sperm

quality and motility, and increased copulation and libido. Research has also targeted hen reproductive maturity and health, and the reduction of egg contamination. Although many researchers are focused on feed additives as supplements for reproductive health and characterizing the transfer of maternal gastrointestinal tract (GIT) bacteria to eggshell microbiome, few studies have targeted the nest environment. The studies that have been conducted on nest and environmental impacts focus on internal GIT development of chicks and the relationship between wild bird nest microbiome, maternal microbiome and the chicks that are incubated in that environment (Diez-Méndez et al. 2023; Maki et al., 2020). Therefore, there is a gap in research for the impact of the nest and environment microbiota on copulation, fertility and hatchability in broiler breeders. This research works to fill some of these gaps by characterizing broiler breeder nest pad bacteria, observing changes in hatchability and fertility when beneficial bacteria are added to the nest environment and showing the effects of probiotics and skin commensals on sperm motility. Bacteria have been shown to negatively impact semen parameters in studies across multiple species, no matter their gram stain, pathogenicity, or motility (Haines, 2012). However, some bacterial properties have been determined to increase damage to sperm more than others because of certain features they possess. Although many of the detrimental bacteria have been identified, researchers have yet to identify an optimum bacterial community that will cause the least damage. An optimum commensal sperm bacterium is one that lacks all of the detrimental qualities, meaning that the ideal bacterium is nonmotile, aerobic, small, lacking appendages, and nonpathogenic (Haines, 2012). Because very little research has been done on how nonpathogenic bacteria affect fertility and egg contamination, it was valuable to research the possibilities that lie in reproductive probiotics.

The profile of the ideal bacterium mentioned before was applied to bacteria found in healthy and unhealthy sperm samples. One bacterium stood out because of its ubiquitous appearance in healthy sperm samples and its compliance with the profile created for a probiotic. The bacterium is *Micrococcus luteus*. In further investigation it was discovered that very little research had been conducted on this bacterium, but that in scattered studies the bacteria had been shown to inhibit several pathogenic bacterial species and had been shown to increase growth parameters and provide immune protection in tilapia (Abd El-Rhman et al., 2009; Akbar et al., 2014; Sawers, 2012; Majeed, 2017; Umadevi and Krishnaveni, 2013). *M. luteus's* unique and elusive qualities make exploring its usage as a reproductive probiotic in broiler breeders intriguing.

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CHAPTER 2

LITERATURE REVIEW

Broiler Breeders

Broiler breeders are the genetic stock that produce the billions of broilers that are consumed every year around the world. For the purpose of this thesis and in most contexts' broiler breeders are the parents of broilers. However, there is a more complex hierarchical lineage that in ascending order includes the parents, grandparents, great grandparents and pedigree broiler breeders (Paxton et al. 2010). The multistep hierarchy of broiler breeders is used to maintain genetic diversity and variation (Paxton et al. 2010). Today's broiler breeder was created through genetic selection that started as part of the "Chicken of Tomorrow" program, which focused on the creation of the broiler, a strictly meat bird that could feed the families of America post WWII (Gordy, 1974; Siegel, 2023). Since then, genetics, management and nutrition have been continuously improved to achieve the ideal offspring.

Genetic Selection

Genetic selection is the use of phenotypic and genotypic traits to choose which birds should be bred in order to achieve broilers that meet the current or projected needs of the industry and consumers (Hollander et al., 2023). Genetic selection focuses on calculating the most economically valuable traits in a given moment by taking into account past, current, and future problems to decide what the value of each trait is (Hollander et al., 2023). An example of a change in focus for genetic selection was the initial shift from selecting for a dual-purpose bird, that could lay eggs and produce meat, to a broiler that only produces meat (Siegel, 2023). The selection for rapid weight gain and decreased feed intake became the focus of genetic selection and was reduced to the term feed conversion ratio or FCR. FCR is still the most highly selected

for trait today in broiler breeders to achieve low cost, high meat yield offspring (Hollander et al., 2023; Tavárez and de los Santos, 2016). However, genetic selection is made at the pedigree level and therefore the great grandparent, grandparent and parent broiler breeders also inherit these low FCR, high meat yield traits. This is problematic because these birds need to be able to produce eggs and live for up to 60 weeks. Therefore, it is important to also select for traits necessary for the broiler breeders to succeed in their role. Researchers have found that fertility is a heritable trait in both hens and roosters and therefore can be selected for as part of genetic selection (Wilson et al., 1979; Wolc et al., 2009). It is recommended that fertility be considered based on period instead of over the entire lay cycle to avoid overparameterization in the selection model (Makanjuola et al., 2021). In females, mean fertility heritability is estimated to be 13%, but in males it is estimated to be 17% (Wolc et al., 2009). Unfortunately, fertility has an antagonistic relationship with FCR, therefore, it is important that we balance these traits when making genetic selection (Tavárez and de los Santos, 2016; Wilson et al., 1979; Wolc et al., 2009). The selection of traits is a balancing act that poultry geneticists deal with every day, which is why algorithms and selection metrics are constantly improving to give more insight into the decisions that are made (Hollander et al., 2023). Genetic selection is the most important piece separating broiler breeders from other categories of commodity poultry.

Nutrition and Body Conditioning

In addition to genetics, broiler breeder production has been optimized over time through nutrition (Scanes et al., 2020). Nutrition and the process of weight gain in male and female broiler breeders is critical to achieving reproductive success. The feeding of broiler breeders was noticed to have significant effects on reproductive potential over 40 years ago (Wilson and Harms, 1986). Zhang et al. showed that male broiler breeders that had high body weights during

rearing produced less semen once sexually mature, however they also found that body weight gain during sexual maturity was crucial to optimize semen production (Zhang et al., 1999). Broiler breeder hens have been found to be equally difficult to manage and maintain uniformity within the flock. With female broiler breeders it is important to monitor hen body weight, because lack of uniformity can lead to egg production differences, aggression and injuries in the flock (Asensio et al., 2020; Hocking, 1996). When hens have constant access to feed their body weights increase and so do the weights of their eggs. In addition, heavier hens reach peak production faster and decline more quickly, shortening the production period (Wilson and Harms, 1986). As parents of broilers, broiler breeders have a genetic disposition to gain weight and maximize their FCR. Feed restriction for both hens and roosters has become the accepted solution to this problem because it prevents broiler breeders from becoming obese or lame while still sustaining the bird's reproductive health (Widowski and Torrey, 2018).

Environment and Rearing

The environment breeders live in can also impact their reproductive performance and behaviors. The rearing process of pullets in broiler breeder systems is important because the rearing impacts the hen's skeletal health and reproductive success (Widowski and Torrey, 2018). The timing of photo stimulation and lighting intensity both affect the development of reproductive organs in the breeders and control the rate at which the birds come into production (Scanes et al., 2020). The motions that hen's practice as pullets can help them learn to use nest boxes and perches faster in the breeder house and help prevent bone injuries (Widowski and Torrey, 2018; Whitehead, 2004). In a recent study, researchers found that broiler breeders are prone to gregarious nesting or the tendency to occupy a single nest with multiple hens (Van den Oever et al., 2021). This research also discusses the correlation between gregarious nesting and

floor eggs as well as aggressive mating and wound presence that suggests that nest behavior should be part of genetic selection as well as a consideration in nest choice (Van den Oever et al., 2021). Nesting material for hens has been shown in several studies to impact the floor egg numbers and general production parameters (Van den Oever et al., 2020). A study conducted by van den Oever et al. showed a preference to a more natural wooden nest box over a metal or plastic one by broiler breeders (Van den Oever et al., 2020). The environment the flock experiences can explain differences seen between flocks and farms raising broiler breeders. Studies have also focused on determining the impact of environmental circumstances on genetic selection and how that will translate into the produced broilers (Falconer, 1952; Montaldo, 2001). Despite the differences, there are specific standards including feeder and drinker space, nest box ratio to hens and stocking density that are recommended and even mandated, that help reduce the variation in production and health seen in flocks (ROSS an Aviagen Brand, 2018). The progeny of the broiler breeders are reflections of their own production and health, making their environment, management and genetics valuable (Jong and Riel, 2019).

Fertility

Fertility is the most valuable trait in a broiler breeder because their fertility is necessary in order to produce chicks. Due to a combination of factors the United States has seen a decrease in fertility over the past decade, with the weekly agristats hatchability (including infertile eggs) never exceeding 81% for the entire calendar year of 2023 (Albert R. Mann Library). For comparison, in 2012 the United States Agristats reported a weekly hatchability of 85% on a regular basis (Albert R. Mann Library). There are several factors that are contributing to this decline in fertility. One factor that consistently affects fertility is the decline of male and female

reproductive health and production with age (Shaheen et al., 2023). Other components that contribute to poor fertility are more gender specific.

In hens, fertility is based on egg production and quality. Many studies have been conducted measuring egg production rates as well as ways to increase egg production and hatch of fertile (Berry et al., 2003; Fasenko et al., 1992; Hadinia et al., 2019). Research has determined that as hens age their fertility and egg production decrease (Fasenko et al., 1992). In addition, the week that photostimulation starts can significantly impact reproductive tract development, overall egg production and length of time the hen produce eggs for (Shi et al., 2020). This is important because photostimulation that is done to early or to late can result in reduced production. Hen day restriction diets have also become popular because they have been shown to lead to higher egg production because of the ability to control energy allocation (Hadinia, et al., 2019). More specifically, researchers have focused on phytase in laying hens and broiler breeder diets because of its improvement in egg production and quality (Berry et al., 2003).

When it comes to fertility in broiler breeders both the male and the female have important roles, however, the male’s ability to produce quality semen is one of the most important factors. The male's body weight has a significant impact on their ability to produce quality semen, and overweight and underweight males struggle to produce at acceptable levels (Shaheen et al., 2023). Male fertility has a greater impact on overall fertility than female fertility because of the proportion of roosters to hens. Each rooster is responsible for inseminating approximately ten hens on a regular basis and therefore is responsible for those ten hens producing fertile or infertile eggs (Lake, 1967; Wilson et al., 1979). It is recommended to remove poor quality males from the flock and even replace them with spike males to improve fertility (Lake, 1967; Ordas et al., 2015; Wilson et al., 1979). Several researchers studied the effect of light stimulation at

different ages on rooster fertility and mating behavior and found that males that are light stimulated prior to 24 weeks of age resulted in lower fertility (Moyle et al., 2012). The same study found that males that were stimulated at younger ages resulted in reduced sperm production, sperm quality, and mating behaviors translating into less fertile eggs which is not economically beneficial (Moyle et al., 2012).

Spermatogenesis is the process of synthesizing motile sperm cells performed by the testis of the rooster. Adeldust et al. studied the effect of oral herbal additives and aromatase inhibitors on increasing spermatogenesis rates in aging roosters that have low sperm counts in their semen (Adeldust et al., 2017; Ali et al., 2017). Studies have confirmed that both herbal additives and aromatase inhibitor, Letrozole, significantly increase the number of sperm cells produced by the rooster (Adeldust et al., 2017; Ali et al., 2017). Behind sperm production, sperm quality is the most important factor contributing to male fertility. In order to analyze sperm quality, researchers study the semen volume and pH, as well as the sperm concentration, motility, and kinematic parameters (Tesfay et al., 2020). Some researchers have also shown that these observed sperm quality parameters including viability, motility and percent deformity are heritable traits (Hu et al., 2013). Many studies have focused on improving poultry sperm quality characteristics using probiotics and feed additives, however the concept of specializing feed formulation for specifically male fertility is a recent focus for the industry (Asl et al., 2018; Inatomi and Otomaru, 2018; Jafari et al., 2021; Ogbuewu and Anayo, 2022; Raei et al., 2021).

Semen Contamination

Semen contamination is a problem that contributes to poor sperm motility and reduces fertility (Aghazarian et al., 2024; Azenabor et al., 2015). An increase in sperm contamination by pathogens and bacteria is seen more frequently in floor pens and litter environments than cages,

in addition to increases seen with age (shaheen et al., 2022). The reproductive tract of the hen increases in contaminating pathogenic bacteria as the hen ages (shaheen et al., 2022). Urogenital pathogenic bacteria can cause decreased motility and sperm kinematics when contamination occurs in semen. *Ureaplasma Urealyticum* was found to be a harmful bacterium when found in 27 individuals experiencing infertility, resulting in poor sperm kinematics and motility (Aghazarian et al., 2024). In addition, both gram positive and gram-negative cocci and bacillus were found to cause an inflammatory response in semen (Aghazarian et al., 2024). Inflammatory responses in the male reproductive tract limit semen and sperm production because the immune system of the reproductive tract works on a no tolerance policy for infection since immune cells would recognize sperm cell antigens as non-self (Azenabor et al., 2015; Witkin et al., 1996). Inflammation of the reproductive tract can result in destruction of tissues, reduced sperm quality and oxidative stress reducing semen viability (Azenabor et al., 2015). Oxidative stress affects the structural and function components of spermatozoa often resulting in infertility (Cheng and Ko, 2019).

In addition to the bacteria that have been shown to decrease sperm quality, some researchers have focused on determining the effect of bacteria that are often considered probiotics or beneficial bacteria. The research works to develop an understanding of how the industry can improve which bacteria are likely to contaminate semen since maintaining sterility is impractical. In a study conducted by Haines et al. researchers showed that pathogenic bacteria and symbiotic bacteria commonly found in the digestive tract and environment of poultry have negative effectives on sperm motility regardless of bacterial species (Haines et al., 2013). Specifically they looked at *Salmonella enterica, Escherichia coli, Campylobacter jejuni, Clostridium bifermentans, Lactobacillus acidophilus*, and *Bifidobacterium animalis*, and saw a

negative relationship between bacterial exposure and motility, with *L. acidophilis* and *B. animalis* completely immobilizing the sperm (Haines et al., 2013). Future research then characterized the effect of *Lactobacillus* fed orally and determined that it does not affect semen concentration of *Lactobacillus,* however it does increase cloacal *Lactobacillus* which could affect semen if contaminated (Kiess et al., 2016). It should also be noted that the study conducted by Kiess et al. was performed in leghorn breeding roosters held in wire cages which is different from the litter and slat design of most broiler breeder houses (Kiess et al., 2016). Another symbiotic bacteria *Bacillus subtilis* has shown to have no effect on rooster fertility or semen quality when directly added to semen and when orally administered (dos Santos et al., 2018). *B. subtilis* has also been found naturally occurring in healthy tom semen samples with high motility (Lenický et al., 2021). This makes *B. subtilis* a potentially positive bacteria to have present in semen and in the reproductive tract in exchange for more harmful contaminating species when it comes to rooster fertility. Overall, there are many more species to be researched and more development to be done in this area, but care should also be taken moving forward in choosing the specific serotypes and strains of bacteria used because they can all have different effects.

Hatchability

Of the two main measurements of broiler breeder production fertility and hatchability, hatchability is more complex and has more ability to be manipulated or influenced. Therefore, it is important to have control over the laying, storage and incubation process to ensure a quality hatch. In the breeder house, floor eggs and cleaned floor eggs display lower hatchability than clean nest eggs (van den Brand et al., 2016). Bacterial exposure directly after oviposition can significantly affect the hatchability of breeder eggs (Perić et al., 2022). Perić et al. recently researched the effect of broiler breeder age and place of oviposition on egg contamination,

embryo mortality, hatchability and chick weight concluding that increased age and oviposition on the floor instead of in the nest box decreases hatch of fertile, and floor eggs increase egg contamination (Perić et al., 2022). Dirty floor eggs have also been associated with higher early embryonic mortality due to higher contamination and possible hindrances in gas exchange through pores in comparison to clean floor eggs and nest eggs (Perić et al., 2022). This demonstrates that location of oviposition is less important than the cleanliness of the environment the egg is laid in for decreasing embryonic morality and increasing hatchability (Perić et al., 2022).

Once the eggs leave the house their treatment is critical in preventing both early incubation and cell death. Eggs stored for a prolonged period result in increased embryonic mortality and overall poor hatchability (Tona et al., 2003; Yerpes et al., 2020). Many studies compare very drastic differences in storage time using 4-5 days as the control and 14-21 days of storage as the treatment (Dymond et al., 2013; Fasenko et al., 2001; Hamidu et al., 2011; Tona et al., 2003). These extreme prolonged storage periods have significantly negative effects on hatchability however preincubation during storage can improve the effect. It is widely accepted that storing eggs for up to seven days is the maximum storage time that has no impact on the overall hatchability of eggs, but some studies have shown that four days is the ideal storage time (Ayeni et al., 2020; Dymond et al., 2013). All of these studies also kept the storage conditions at a temperature between 55-65°F and an average humidity of 75%, which is the standard in the industry (Dymond et al., 2013; Tona et al., 2003, Brake et al., 1997). Literature from the 1970's by Kirk et al. showed that for egg storage of only two days a higher storage temperature of 65°F is more appropriate for a successful hatch, but for a longer storage time around 7 days a storage temperature of 59°F is more successful (Kirk et al., 1979). The temperature and humidity in the

storage room is important to prevent, early incubation, embryo death, and contamination (Fasenko et al., 2001). Fluctuations in temperature and humidity can result in egg sweating and bacterial contamination, making it important to keep conditions stable and to keep the storage room clean (Fromm and Margolf, 1958).

Incubation is influential on hatchability and chick health. The temperature of the incubator can result in problems with chick weight, dehydration and embryo mortality. The ideal temperature for incubation is between 98.6-100.4 °F (Tesarova et al., 2021). The first 18 days of incubation are different from the following 3 three days that are considered the hatching period (Decuypere et al., 2001; Yalcin et al., 2022). The incubator temperature can be kept lower between 97-99 °F during the hatching period (Decuypere et al., 2001). As embryos develop, they produce heat and receive heat from surrounding eggs making up for the decrease in incubator temperature. Therefore, the eggs themselves are always about 2 °F higher than the incubator temperature (Leksrisompong et al., 2007; Yalcin et al., 2022). High incubator temperatures past day 14 results in an early hatch and lower chick body and organ weights, which can create problems with dehydration and poor chick quality (Leksrisompong et al., 2007; Yalcin et al., 2022). Humidity is also important during incubation, with the ideal moisture loss of hatching eggs being 10-12%. Research has found that humidity can cause detriment to embryonic development and also influence chick weight when it falls above or below the ideal relative humidity of 53% (Bruzual et al., 2000; Kirk et al., 1979).

7 Day Mortality

Although shown to reduce hatchability, floor eggs and cleaned floor eggs have not been shown to cause lower chick weight, FCR or early chick mortality in grow-out in comparison to clean nest eggs (van den Brand et al., 2016). However, eggs shells that have populations of

pathogenic bacteria, like *E. coli* present on them after oviposition that can penetrate the shell and albumin and reach the yolk can cause omphalitis infections in chicks that can cause early mortality (Rezaee et al., 2021). In addition to bacteria, there are other factors such as broiler breeder age that do significantly impact 7-day mortality in chicks (Yerpes et al., 2020). The highest rate of 7-day chick mortality is seen in old broiler breeders that are past peak production and the second highest rate is seen in broiler breeders at the start of lay (Peebles et al., 2004; Suarez et al., 1997; Wilson, 1991; Yassin et al., 2009; Yerpes et al., 2020). It has been inferred that the cause of this mortality could be due to the size of the egg, with young hens laying thick shelled smaller eggs that require longer incubation and older hens producing thin shelled large eggs that require less incubation, due to increased gas exchange, resulting in dehydration before placement (Tona et al., 2004). The diet of broiler breeders is also important to the success of their offspring. Vitamin E supplementation to broiler breeders has been proposed as a supplement in diets because it has been shown to decrease chick mortality and increase hatchability (Yang et al., 2020). Beneficial supplementations, like feed additives, are valuable and can offset involuntary problems with management and storage times. In the hatchery maintaining a clean incubator, vaccination and hatching environment is important for preventing bacterial infections that can easily thrive off the nutrients of the yolk and result in late embryonic and early chick mortality (Rezaee et al., 2021). Additionally, during brooding, drinker choice has been identified as a contributor to 7-day mortality (Yerpes et al., 2020). The nipple system requires learned behavior and can result in early dehydration, making it important to offer supplemental bell drinkers for at least the first couple days (Carpenter et al., 1990). However, no matter controllable factors, it is important to remember mortality can also be influenced by the season, year, and geographic region since there are varying differences in air quality,

temperature, and humidity; making each farm that chicks are placed in a unique experience (Yerpes et al., 2020).

Housing Environment

Most broiler breeders are raised in a house with slats for hen feeding and nest box use, in addition to a scratch area for rooster feeding, breeding, and dust bathing. Wegner et al. showed that the use of two different production environments, one being a slat litter system and the other being solely litter flooring, resulted in conflicting consequences to health and production (Wegner et al., 2022). The slat litter system is the most common system used today, having shown improved livability in comparison to the litter system (Wegner et al., 2022). However, literature has shown that litter systems can produce higher egg weights than slat litter systems (Wegner et al., 2022). Another housing environment factor that was explored by Kaukonen et al. is the effect of poor litter quality in the scratch areas, on broiler breeder foot pad dermatitis and skin burns. Kaukonen et al. found that although poor litter quality is positively correlated to foot and skin lesions it is not the sole cause in broiler breeders (Kaukonen et al., 2016). These researchers revealed that housing environments with large slat sections can also have a negative effect on foot pad lesions (Kaukonen et al., 2016). The concerns associated with foot and skin injuries are welfare related, including morality, infection, and impaired mobility, which all impact fertility and egg production by reducing mating abilities (van den Oever et al., 2020). Other researchers saw contradicting results showing no significant differences in leg health including bone strength and footpad lesions with the use of slats, however they discuss that this could be due to the young age of their birds (Van den Oever et al., 2020; Van der Oever et al., 2021). This study also showed that slats reduced floor eggs and mating behavior (Van der Oever et al., 2021).

The housing environment and management can have significant effects on litter moisture, the house microbiome and nest cleanliness, which can all impact bird health and production. Bedding and litter in the poultry house are capable of containing bacteria and microorganisms that can impact production. Zhen et al. discovered that when ducks are housed in litter that contains large amounts of *Staphylococcus, Corynebacterium*, and *Brevibacterium* egg quality decreases (Zhen et al., 2022). Litter management can affect the intestinal microbiome of the chickens causing changes in pathogen loads and beneficial gut bacteria (Wang et al., 2016). Researchers have heavily focused on the different effects of litter management including moisture levels, pH and number of flock cycles on reused litter, on pathogen load in broiler houses (Oladeinde et al., 2023). The more cycles a house goes through the higher the potential for pathogens like *Salmonella* and *Campylobacter* in the litter (Oladeinde et al., 2023). However, contradicting models have shown that the benefit of recycling litter in broilers can include both lower costs and in healthy flock's competitive exclusion of pathogens by the litter microbiome that is seeded with broiler fecal microbes (Bucher et al., 2020). Litter recycling is less common in broiler breeders, however because of their longer production cycle and higher economic value. Another surface that is important to keep clean particularly in the breeder house is the nest boxes that eggs are laid in and the hens occupy every day. Many companies encourage their growers to maintain clean fecal material free nest boxes, but that is not always managed well. Floor eggs are contaminated by the fecal material and moisture in the litter making it necessary to keep the nesting boxes free of these factors to prevent nest egg contamination in addition to floor egg contamination.

Microbiota

There are microbes everywhere in a broiler breeder house, both in the environment and inside the bird. The microbiota in the environment the birds live in is divided into niches based on the different surfaces and nutrients in different areas such as the litter, slats, nest boxes and water lines. Each niche supports different species of bacteria as well as different growth patterns, like biofilms in the water lines. The microbiota in the house includes both pathogens such as *E. coli*, *Salmonella*, and *Campylobacter* and symbiotic or commensal bacteria such as *Bacillus* and *Lactobacillus* species. There have been many studies performed identifying the microbiota present in chicken litter. Researchers have confirmed that *Bacillus*, *Lactobacillaceae* and *Clostridiaceae* species found is fecal material inhabit the litter, as well as unique species like *Globicatella sulfidofaciens* and *Corynebacterium ammoniagenes* (Lu et al., 2003). Researchers have demonstrated the environmental impact on intestinal microbiota by rearing broilers on both reused litter and fresh litter (Cressman et al., 2010). It was concluded that *Lactobacillus spp.* dominated the intestinal microbiota of chickens raised on fresh litter, while *Clostridiales* dominated the intestinal microbiota of chickens raised of recycled litter showing a significant difference caused by rearing environment (Cressman et al., 2010). Many producers recycle litter from healthy broiler flocks because the established microbiome of the litter can competitively exclude pathogens and reduce *Salmonella* presence (Bucher et al., 2020). Another important niche in the house is the drinker line which although disinfected harbors bacteria on 63% of its surfaces and can facilitate transfer of bacteria to the chickens through the water (Maes et al., 2019). The most common bacteria found on drinker surfaces are *Stenotrophomonas maltophilia*, *Pseudomonas geniculata* and *Pseudomonas aeruginosa* (Maes et al., 2019). In addition, 93% of the bacteria found in and on drinker lines possess biofilm forming capabilities, making them hard

to get rid of (Maes et al., 2019). Despite the many studies on environmental surfaces, the microbiota of broiler breeder nest boxes has yet to be characterized. However, there have been studies conducted in free range hens that evaluate the microbiota present in the litter as well as on the egg belt and in the nest box. Wilson et al. found that the nest box surface has a lower abundance of bacteria than the litter in free range hen houses (Wilson et al., 2021). In the same study it was observed that litter and nest boxes share many species of bacteria, but also that the nest box contains its own unique microbiota (Wilson et al., 2021). All of the microbes present in the environment of the house can impact the microbes present in the bird and vice versa. For example, the microbes in the intestines are shed out into the feces of the chicken that becomes part of the litter. There are multiple pathways including copulation, ingestion, defecation and cloacal drinking in which microbes can be shared between the environment and the bird's microbiota.

The internal microbiota of a chicken is influenced by the environment, feed formulation, and maternal microbes. The microbiota of the oviduct and the cloaca can impact the starting populations of bacteria in the embryo (Lee et al., 2019). Research has shown that the oviduct, cloaca, eggshell, egg white and embryo share bacterial taxonomy, however the egg white lysosomes limit the diversity of bacteria that embryos are exposed to (Lee et al., 2019). The diversity and abundance of bacteria increases along the oviduct, with the majority of bacteria residing in the vagina and cloaca (Wen et al., 2021). At hatch, chicks are exposed to bacteria on their eggshell as well as the eggshells around them that share taxonomy with that of multiple hen's feces (Lee et al., 2019; Trudeau et al., 2020). Different parts of the digestive and reproductive tract harbor different bacteria in the same way different niches in the environment do. However, there are instances in which they seed one another and share species (Andreani et

al., 2020). Researchers have found that the microbiota in the ceca are 59% similar to the microbiota of the cloaca (Andreani et al., 2020). In addition, pairwise analysis has found that the cloacal and cecal microbiota are more closely related in each individual bird than would happen by chance, showing possible interaction between these two niches (Andreani et al., 2020). This could be explained by cloacal drinking or the uptake of bacteria, protozoans and fluid into the cloaca and then into the ceca through antiperistalsis. Although research has been extensively performed on broiler and broiler breeder hen cloacal microbiota, researchers have yet to thoroughly investigate the differences and similarities between broiler breeder rooster and hen cloacal microbiota. The cloacal microbiota of the rooster is also important because it is a possible route of semen contamination. The specific route of semen contamination is yet to be thoroughly investigated in broiler breeders. However, it is speculated that semen microbiota is a combination of reproductive tract, cloacal, skin and environmental bacteria. Semen microbiota is critical because of its impact on sperm health (Aghazarian et al., 2024). Ross 708 broiler breeder semen has been shown to contain high amounts of *Staphylococcus epidermidis, Lactobacillus johnsonii, Escherichia coli, Citrobacter braakii, Enterococcus faecalis and Micrococcus luteus*, but overall low biodiversity (Tvrdá et al., 2023). The Ross 708 broiler breeder's semen was compared to the semen of Lohman Brown egg-laying breeder's in the same breeder house. The Lohman Brown egg-laying breeder semen contained highest amounts of *Escherichia coli, Enterococcus faecalis, Citrobacter braakii, Enterococcus casseliflavus, Pseudomonas putida and Corynebacterium glutamicum* (Tvrdá et al., 2023). Because the only difference in this trial was the breed of bird used it can be claimed that the genetic strain and type of production the bird is bred for cause different microbiota to colonize the bird and contaminate their semen (Tvrdá et al., 2023). The Ross 708 semen was found to have higher motility, better membrane

integrity, and less DNA fragmentation than the Lohman Brown semen (Tvrdá et al., 2023). It was speculated that the worse sperm quality parameters were caused by the uropathogens present in the Lohman Brown Semen, producing harmful metabolites, causing oxidation, and stimulating strong immune responses (Tvrdá et al., 2023). Overall, there have been many studies using 16S rRNA sequencing to characterize and compare the microbiota present in the waste, intestines, semen, eggs, and environment of chickens. These studies of the microbiome are necessary to better understand what other organisms are present both internally and externally of the birds in the house that could be affecting either production.

Bacteria

Although many studies in poultry science have focused on pathogens, there are many other symbiotic bacteria present in the environment and in the bird that impact production and health in unknown ways. Many studies have focused on *Salmonella* and *E. coli* in the litter, gut and even in semen contamination (Bucher et al., 2020; Haines et al., 2013). In addition, researchers have more recently begun to focus research heavily on probiotics and microbiota health (Aalaei et al., 2019; Inatomi and Otomaru, 2018; Lee et al., 2019). Companies have started to feed probiotics while not having full knowledge on how those probiotics at shifting the microbiome or how they are affecting other processes in the bird. For example, *Lactobacillus acidophilus* is a common probiotic and has been shown to improve hatchability and feed conversion when fed orally however, other studies have found that if semen is contaminated with *Lactobacillus*, sperm motility can be severely diminished (Bozkurt et al., 2011; Haines et al., 2013). In addition, so many other bacteria are present in the environment and on the skin of broiler breeders without any investigation. One bacterium that can be found across microbiome studies and in general bacterial characterizations of broiler breeders in *M. luteus*, yet its effects

and purpose in and on the bird have not been explored (Laba et al., 2015; Tvrdá et al., 2023). Throughout this thesis *M. luteus* will be explored in regard to its effect on broiler breeders, in addition to the effects of common probiotics including *L. acidophilus* and *Bacillus subtilis.*

Micrococcus luteus

Micrococcus luteus is a gram positive, aerobic bacterium that is ubiquitous in the environment. This bacterium can withstand high salinity levels and is often found on skin. The ability of *M. luteus* to persist on surfaces and skin makes it a common bacterium found in hospitals (Akbar et al., 2014; Zhu et al., 2021). However, it is not a pathogen and has only caused a handful of opportunistic infections caused by poor aseptic technique during invasive surgeries (Zhu et al., 2021). Certain strains of *M. luteus* have been shown to selectively inhibit other gram-positive bacteria on the skin such as *Staphylococcus aureus* (Akbar et al., 2014; Sawers, 2012). In a study performed in Pakistan, researchers found that *M. luteus* was also capable of competitively inhibiting *Salmonella typhimurium*, *Listeria monocytogenes* and *Escherichia coli* (Akbar al et., 2014). When extracted, the carotenoid pigment produced by *M. luteus* exhibits antimicrobial activity against *Klebsiella spp*. and *Pseudomonas aeruginosa* (Majeed, 2017; Umadevi and Krishnaveni, 2013). In addition to its antimicrobial capabilities, *M. luteus* has been seen to act as a probiotic in tilapia improving feed conversion, body weight, and mortality during infection with *A. hydrophila* (Abd El-Rhman et al., 2009). In regard to poultry, *M. luteus* is found on feathers and has been shown to help break down keratin in by-product feather waste (Laba et al., 2015). *M. luteus* is also extremely prevalent in broiler breeder and human semen and has been shown to have no significant impact on motility in either case (Tvrdá et al., 2022; Tvrdá et al., 2023). *M. luteus* is a promising probiotic in poultry because it is

ubiquitous and persistent in nature, processes antimicrobial properties, lacks negative effects on sperm motility and has positive effects on production parameters at least in aquatic species.

Lactobacillus acidophilus

Lactobacillus acidophilus is considered a probiotic in both humans and animals. It is a gram positive, non-spore forming bacteria that survives well in harsh environments with low pH, acids and bile salts (Gao et al., 2022). *L. acidophilus* is found in anaerobic environments, lacking oxygen, and has the ability to metabolize lactose, glucose and fructose (Gao et al., 2022). The metabolite produced by *L acidophilus* is lactic acid which can neutralize the more basic areas of the digestive tract but can also create slightly acidic environments in some circumstances (Gao et al., 2022). Dietary supplementation of *L. acidophilus* has been shown to counteract the effects of *E. coli* bacterial infections (Wu et al., 2021). *E. coli* and other pathogens can cause decreases in immune responses, increases in inflammation and infection markers, decreases in body weight gain, feed consumption and feed conversion, and decreases in intestinal health with regards to villi:crypt depth ratio and mucosal thickness (Wu et al., 2021). When fed to infected poultry, *L. acidophilus* can significantly improve body weight and weight gain, restore intestinal health parameters and decrease infection markers such as lipopolysaccharides (LPS) (Wu et al., 2021). In another study researchers found that *L. acidophilus* supplemented to the diet in non-infection models did not alter the basal level of *E. coli* in the gut but did appear to reduce coliforms and increase villi height (Forte et al., 2018). With researchers seeing significant effects of *L. acidophilus* in broiler production parameters and intestinal health, it is important that the comprehensive effects of the bacteria are taken into account before using the probiotic in commercial flocks. Broiler breeders for example may benefit from the antimicrobial and intestinal health benefits of *L. acidophilus,* but highly acidic environments that can be created by

the lactic acid metabolite could potentially cause fertility problems in breeders (Kiess et al., 2016).

Bacillus subtilis

Bacillus subtilis is an obligate aerobic bacterium that is found ubiquitously in the environment, having been isolated from land and aquatic environments and found in association with animal gastrointestinal tracts and plant root systems (Earl et al., 2008). The lifecycle of *B. subtilis* allows it to enter a vegetative spore state when in a stressful environment, giving *B. subtilis* the ability to survive in many circumstances (Earl et al., 2008; Hong et al., 2005). In poultry, *B. subtilis* has been found to inhibit the colonization of *E. coli* pathogens in the gut and in internal organs (La Ragione et al., 2001). *B. subtilis* also helps to flush the pathogens out of the body making them incapable of integrating into the microbiome (La Ragione et al., 2001). *B. subtilis*'s effects against pathogens and in restoring the microbiome to a healthy state have been well explored in feed additives (Qiu et al., 2021). Researchers have found that *B. subtilis* promotes the growth of *lactobacillus* and *bifidobacteria* while limiting *coliforms* and *Clostridium perfringens* in the gut (Qiu et al., 2021). *B. subtilis*'s potential as a probiotic is not simply limited to its competitive inhibition of pathogens, the bacteria also promote improved immune responses and increases villi and intestinal integrity (Qiu et al., 2021).

Conclusion

Broiler Breeders are complex and unique birds; therefore, they require a combination of dynamic management, balanced nutrition and a clean environment in order to thrive. Knowing that fertility, hatchability, chick mortality and semen quality are all complex production characteristics helps researchers better evaluate the success of a broiler breeder flock and understand the interrelations between each of these parameters. The microbiota of the broiler

breeder is one more factor that can influence all of these production parameters. It is vital that research continues to explore the effects of specific bacteria, as well as shifts in the community dynamics of the microbiota on the production and health of broiler breeders. With the goal of expanding the knowledge currently available on broiler breeder production this thesis explored the microbiota of broiler breeders over time and determined whether the phase of production and age of the birds affected the microbiota. More specifically, this thesis explores the effects of altering the environmental microbiota that hens are exposed to on the nest pad by adding *M. luteus* to the naturally occurring microbiota on fertility, hatchability and chick parameters. Lastly, we evaluated the impact of *M. luteus* and common probiotics including *L. acidophilus* and *B. subtilis* on sperm motility to better understand how nonpathogenic bacteria can impact a rooster's fertility. This research both fills gaps in knowledge about the relationship between the microbiota and broiler breeder production and identifies areas in which researchers need to continue to conduct further research.

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CHAPTER 3

THE EFFECT OF ADDING *MICROCOCCUS LUTEUS* TO THE NESTING SURFACE OF BROILER BREEDERS ON FERTILITY, HATCHABILITY, AND MICROBIOTA

ABSTRACT

In broiler breeder flocks, hatchability and fertility has decreased over the past decade. Recent studies have focused on addressing problems with low hatchability in the hatchery, but few are looking for ways to address problems in the breeder house. The environmental microbiota of the breeder house is widespread, with opportunistic pathogens and beneficial bacteria that can impact and contaminate the hatching egg. In this study, we explored the impact of a commensal nest pad bacterium on fertility and hatchability of Ross 708 broiler breeder eggs throughout the production cycle. To execute this, we added 10^8 CFU's of *Micrococcus luteus* to the nest pad surfaces of eight replicate breeder pens housing 60 hens and 7 roosters each. Eight additional control pens had their nest pads sprayed with sterile phosphate buffered saline. *M. luteus* was selected because of it's survival conditions, lack of negative effect on sperm quality, and potential to competitively exclude pathogens. Fifty eggs per pen per week were set during each four-week period including: 26-29 wks, 33-36 wks, and 41-44 wks of age. Chick weights were recorded, and 10 randomly selected chicks from each pen were observed for 7-days to obtain body weight and mortality. Microbiota samples of the nest pads, hen cloacas, and rooster cloacas were also taken during each period for 16S rRNA analysis. Hatch and chick data were analyzed via one-way ANOVA with JMP Pro 17, where P<0.05. Microbiota analysis and visualizations were prepared in MicrobiomeAnalyst 2.0 (Lu et al., 2023) using center log transformed (CLR) data to stabilize variance and adjust for compositionality biases. Alpha (Chao1 and Shannon) and beta diversity (Jenson-Shannon, Jaccard, Unweighted UniFrac, and Weighted UniFrac) were considered different at FDR<0.05 using the Benjamini-Hochberg

procedure. Posthoc pairwise comparisons utilized the Wilcoxon Rank Sum test for alpha diversity and PERMANOVA for beta diversity. Differential abundance statistics were performed using MaAsLin2 (Mallick et al., 2021) with age (weeks) as a covariate. Average hatchability and fertility revealed no significant difference between the *M. luteus* and control groups throughout the production cycle. However, results did show a significant difference in average 7-day chick weight (P=0.009) at peak production that favored the control group, but no difference in mortality was detected. The results for fertility and hatchability were expected due to hen age and egg quality, however as production parameters naturally decrease with age, this could change. The microbiota between the control and *M. luteus* groups surfaces revealed that the cloacal samples during 33-36 wks of age had significant differences in species richness and diversity but none of the other sample types displayed changes. In addition to changes in community richness and beta diversity, we saw an increase in *Monoglobus* on *M. luteus* nest pads and a decrease in *Lachnospiraceae* in the cloacas of *M. luteus* hens that may affect food safety and feed conversion parameters. Overall, this study reveals that the application of *M. luteus* to the nest pad does not significantly affect egg fertility or hatchability throughout the production cycle but can reduce 7-day body weights in offspring and alter the hen microbiome. Future research should concentrate on how nest pad bacteria affects fertility, hatchability, and microbiota in late-stage breeder production cycles.

INTRODUCTION

Natural variables like aging affect hatchability and fertility, however there are also voluntary, controllable variables affecting these parameters (Shaheen et al., 2023). In the United States, we have seen a decrease across the past decade, in hatchability, including both infertile and fertile eggs (Albert R. Mann Library). The USDA Agristats reported that in 2023 the

industry did not exceed 81% hatchability for any of the weeks in that calendar year, which is significantly lower than 85% which was achieved regularly in 2012 (Albert R. Mann Library). This low hatchability is caused by a compilation of factors. The first being management, producers have the ability to control the feed and water intake of the birds, as well as the litter and air quality in the house (Wegner et al., 2022; Wilson and Harms, 1986). The way that each of these are managed can significantly impact the health of both the birds and their offspring (Asensio et al., 2020; Hocking, 1996; Oladeinde et al., 2023; Wegner et al., 2022). Management can also affect fertility, specifically when problems with early photostimulation and overfeeding lead to poor reproductive tract development (Scanes et al., 2020; Wilson and Harms, 1986; Zhang et al., 1999). However, management is not the only factor affecting fertility and hatchability. Genetics is also extremely important because fertility is a heritable trait in both hens and roosters (Wilson et al., 1979; Wolc et al., 2009). Unfortunately, fertility is in an antagonistic relationship with feed conversion ratio, or FCR, which is a genetic trait that is highly selected for in broiler breeder offspring (Hollander et al., 2023). Therefore, it is important that we balance these traits when making genetic selection. Another important factor that has affected hatchability in recent years is the removal of antibiotics (Kadykalo et al., 2019). Gentamicin had been a prominent antibiotic used in hatcheries for years, however the removal of antibiotics from hatcheries has been broadcasted from news services since 2014 appealing to antibiotic resistance concerns and consumer preference (Storm, 2014). Antibiotic removal is a problem because contamination of eggs can result in early infections in chicks, and without the use of gentamicin as an antibiotic in the hatchery, contamination can cause increases in early chick mortality (Kadykalo et al., 2019). Lastly, nutrition has a large impact on fertility and hatchability both in roosters and hens. The body conditioning of broiler breeders has a significant impact on their

reproductive success (Asensio et al., 2020; Hocking, 1996; Zhang et al., 1999). Almost all research being done to improve fertility and hatchability falls into one of these areas.

There is, however, one factor that is rarely considered when it comes to fertility and hatchability, the environmental microbiota inside the house. The bacterial population inside the house is rampant with both opportunistic pathogens and symbiotic bacteria (Cressman et al., 2010; Lu et al., 2003). Researchers have focused on pathogen identification and quantification within the housing environment, but few have looked into other symbiotic or beneficial bacteria that are present in the environment of the hens and roosters (Lu et al., 2003; Maes et al., 2019). In the breeder house there are different ecological niches that harbor unique bacterial species. For example, different bacteria thrive in the water lines than in the litter or the nest boxes (Maes et al., 2019; Wilson et al., 2021).

This study concentrated on the effects of changing the microbiota of surfaces that may impact fertility and hatchability. To do this, the microbial niche of the nest pad was disrupted and the cloaca of the hen and the eggs, which make contact with the nest pad surface, also had potential to be impacted. *Micrococcus luteus,* a gram-positive, aerobic, non-pathogenic, bacterium that is ubiquitous in the environment, was chosen as the "disruptant" (Akbar et al., 2014). These bacteria have been found in litter samples, as well as on the skin of poultry (Laba et al., 2015). More significantly, *M. luteus* has been found in healthy semen samples of both humans and broiler breeders (Tvrdá et al., 2022; Tvrdá et al., 2023). *M. luteus's* presence in high-quality healthy semen samples is encouraging for its potential to competitively exclude pathogens and act as a probiotic for fertility and hatchability. We hypothesized that application of *M. luteus* to nest pad surfaces will alter the microbiota, resulting in increased fertility, hatchability, and chick quality. The overall objective of the study was to observe the impact that

adding *M. luteus* to the nest pad microbiota has on hatching egg production parameters of broiler breeders through the production cycle.

MATERIALS AND METHODS

Experimental Design

The project was completed at the North Carolina State University Chicken Education Unit. All methods and procedures used for animal research in this trial were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC #22-435-A). A flock of 960 Ross 708 strain breeder hens and 112 roosters (22 weeks) were randomly assigned to 16 pens holding 60 hens and 7 roosters each. Each pen included a slat area, scratch area and 16 nest boxes. The scratch area was lined with soft pine shaving and was replaced as necessary throughout the trial. Similar to industry standard, the water lines were set on a water clock to shut off during the dark period in the house to prevent water leaks. Water was provided through bell drinkers. Birds were provided feed based on Aviagen recommendations in the Parent Stock Handbook. Eggs were collected by hand twice daily. Gloves and shoe covers were changed between pens to prevent contaminating the control nest pads. The house was blocked by side, leaving eight pens in each block. Four pens from each block were randomly selected to have 10^8 CFU's of *Micrococcus luteus* (4698, ATCC) (probiotic) in phosphate buffered saline (PBS) (Sigma Aldrich, St. Louis, MO) evenly applied onto their nest pads (Hog Slat, Newton Grove, NC, USA). The eight pens with the probiotic applied to their nest pads were used as the treated group. The remaining eight pens became the control group and had sterile PBS (Sigma Aldrich, St. Louis, MO) applied evenly onto their nest pads. *M. luteus* and PBS were applied to the nest pads during three different periods including 26-29 wks of age (pre peak lay), 33-36 wks

of age (peak lay), and 41-44 wks of age (post peak lay). During each period hatchability, fertility, 7-day mortality, chick weights and microbiota changes were evaluated.

Incubation and Hatching

In order to measure fertility and hatchability during each period fifty eggs per week were collected from each pen over a two-day period and stored for 4 days between 55-65°F before being set in large incubators (NatureForm, Jacksonville, FL, USA) at the NCSU chicken education unit. 800 eggs labeled by pen number were set each week for four weeks during each period. The eggs were incubated at 100.2°F dry bulb temperature and slowly decreased to 98.3°F dry bulb temperature over three weeks. The eggs were candled on day 10 and transferred to NatureForm hatching baskets on day 18. During transfer eggs from different pens were separated into red pedigree hatching bags (Amazon, Seattle, WA, USA) made with strong stretchable plastic mesh in order to keep track of experimental units. At hatch (21 days), total pen weight was recorded using a 15kg maximum scale (A and D, Ann Arbor, MI) and divided by number of chicks hatched. All unhatched eggs were broken out and the infertile egg count was totaled by pen.

7 Day Mortality

After each pen weight was recorded for the hatched chicks, ten were randomly selected to be placed for seven-day evaluation. Each chick was tagged, and their individual body weight was recorded at hatch using a 2,200-gram maximum scale (OHAUS, Parsippany, NJ). Chicks were placed in brooder pens with *ad libitum* access to feed and water. Seven days after hatch mortality and individual chick weights were recorded.

Creation of the *Probiotic* Inoculum and application

A pure culture of *Micrococcus luteus* (4698) was purchased from ATCC for this project. The lyophilized culture was reconstituted in tryptic soy broth (TSB) (sigma Aldrich, St. Louis, MO, USA) and incubated at 30°C in an aerating incubator (Benchmark Incu-Shaker 10L, Sayreville, NJ, USA) for 48h. After initial incubation the culture was separated into 1 ml aliquots and frozen at -80°C creating our stock culture. Before freeze back, 1 ml of the purchased culture was used for serial dilution in sterile Phosphate Buffered Saline (PBS) (Sigma Aldrich, St. Louis, MO, USA). One hundred microliters from each serial dilution were spread plated onto tryptic soy agar (TSA) (Sigma Aldrich, St. Louis, MO, USA) in 100mm x 15mm sterile polystyrene petri dishes (Fisherbrand, Waltham, MA, USA) and incubated at 30°C for an additional 48h. After the second incubation, colony formation on the media plates was counted to determine a growth curve for these incubation conditions. The characteristics of *M. luteus* were further evaluated by using an SmartSpec 3000 optical density (OD) machine (BIO-RAD, Hercules, CA, USA) to determine the OD of overnight growth and develop a CFU estimate by diluting and spread-plating the respective growth. An OD of 1 was approximated to equal a concentration of 3.43×10^8 CFU's of *M. luteus* per ml which was used to create the desired concentrations for the project. The desired CFU per ml concentration suspended in PBS (Sigma Aldrich, St. Louis, MO) for this project was 10^8. Once the appropriate concentration for the on-farm project had been acquired, about 1.3ml of the *M. luteus* inoculum was applied to each treated nest pad by misting the inoculum over its entire surface. 24oz plastic spray bottles (Amazon, Seattle, WA, USA) were utilized to apply the inoculum after the difference in weight was calculated per spray to be 1.3ml on average. This same procedure was carried out for our control nest pads, with about 1.3 ml of sterile PBS misted over the entire surface of each control nest pad.

M. luteus Real Time qPCR Evaluation

The presence of *M. luteus* post application was evaluated in order to observe increases in presence in the treatment pens, any cross contamination into the control pens and any increases in presence in the cloaca of the birds. To do this, a week after the hens had an opportunity to use and lay eggs on the treated and control nest pads, one nest pad from each replicate unit was removed and six cloacal swabs from 3 breeder hens and 3 roosters were collected from each pen. Sterile cotton tipped swabs (Amazon, Seattle, WA, USA) were inserted half an inch into the cloaca and swirled three times in each direction. Each nest pad was placed into a sterile 55oz filtered bags (Whirl-pak, Pleasant Prairie, WI, USA) and sealed, and each cloacal swab was swirled in 1 ml of PBS in 15 ml sterile polypropylene tubes (ThermoFisher, Waltham, MA, USA) for 30 seconds. Sealed nest pads and cloacal swab samples were then placed on ice and transported back to the microbiology lab at the Prestage Department of Poultry Science for microbial processing. Cloacal samples from roosters and hens of the same pen were pooled respectively by sex. A 5x5 inch square was cut out of the center of each nest pad and the rest was discarded. 180 ml of PBS was then added to each nest pad bag and shaken for eight minutes. A 5 ml aliquot was then transferred from each of the whirl pack bags into a sterile sample tube. After processing was complete, 1 ml from each pen's hen cloacal sample, 1 ml from each pen's rooster cloacal sample and 3 ml from each pens nest pad sample were transferred into 5 ml sterile Eppendorf tubes (VWR, Radnor, PA, USA) and frozen at -80ºC in a Thermo Scientific TLE series freezer (Waltham, MA, USA) to later be sent off for 16S rRNA analysis, the remainder was used for qPCR. Since there is no commercially available selective agar for *M. luteus* we analyzed the samples using qPCR. qPCR was run on the Applied Biosystems StepOnePlus real time PCR system (Applied Biosystems™ Waltham, Massachusetts, USA). Each reaction was

15ul including 2ul environmental sample as PCR template, 500nM forward and reverse MLUT_RS17275 metal-sulfur cluster assembly factor primer (MLmtScaf) (Gannesen et al., 2021), 2X Power SYBR Green Master mix (Applied Biosystems™ Waltham, Massachusetts, USA), and molecular grade H_2O to a final 15ul volume.

MLmtScaf-F   5' GCT CTA CGG GCT GCA CTA C 3'
MLmtScaf-R   5' CTC GGG GCC CCA CGG GGG 3'

One cycle was completed for initial denaturation at a temperature of 95°C for 10 minutes. 40 cycles were then run, each containing a 30 second denaturation period at 95°C, followed by a 30 second anneal period at 60°C and a 30 sec extension & real time data collection period at 72°C. Standard curves were run on each plate starting at a concentration of 10^8 CFU per ml in order to correlate copy number to concentration. Sterile molecular grade water no-template negative controls were also run on each plate.

Data Analysis

Each pen served as an experimental unit, hatch and chick data were analyzed using SAS, JMP Pro 17 Software. Data were analyzed using a randomized complete block design. Significance was determined using a one-way ANOVA and Tukey's HSD for multiple comparisons with a significance level of $P < 0.05$.

Microbiota Analysis

At the end of the late lay period, frozen samples were sent to the UNC Microbiome Core Facility Center (Chapel Hill, NC) and run for 16S rRNA isolation and sequencing to determine differences in microbiota diversity between the control and treatment sample types. Only the pen replicates that had consistent samples across all periods were sent for analysis. After removing pens whose sample birds experienced mortality or lost tags, we were left with four treatment and

four control pens. The following procedures for DNA isolation and 16S rRNA amplicon sequencing were performed by the UNC Microbiome Core Facility Center in Chapel Hill.

DNA isolation

Samples were transferred into a 2 ml tube containing 200 mg of glass beads sized 106/500 µm (Sigma, St. Louis, MO) along with 0.6 ml of Qiagen ATL buffer (Hilden, Germany). The mixture was then vortexed at 3000 rpm for 10 minutes using a digital vortex mixer and supplemented with 60 mg/ml of lysozyme sourced from ThermoScientific, Rockford, IL. Subsequently, the suspension was incubated at 37 °C for 1 hour and further supplemented with 600 IU of Qiagen proteinase K, followed by another incubation at 70 °C for 1 hour. Upon centrifugation for 3 minutes, 0.5 ml of supernatant was combined with 0.5 ml of Qiagen AL buffer and incubated at 70 °C for 10 minutes. The supernatant was then aspirated and transferred into a new tube containing 0.5 ml of ethanol. DNA purification was performed using a standard on-column purification method employing Qiagen buffers AW1 and AW2 as washing agents, and elution was done in DNase-free water. (Camp, 2022; Marsh et al., 2022; Ribeiro et al., 2022).

16S rRNA amplicon sequencing

A total of 12.5 ng of DNA were subjected to amplification using universal primers designed to target the V4 region of the bacterial 16S rRNA gene. These primers were designed with overhang adapters appended to their 5' ends to ensure compatibility with the Illumina sequencing platform. Specifically, the primers utilized were F515/R806. The master mixes consisted of 12.5 ng of total DNA, 0.5 µM of each primer, and 2x KAPA HiFi HotStart ReadyMix sourced from KAPA Biosystems, Wilmington, MA. The thermal profile for the amplification process involved an initial denaturation step at 95°C for 3 minutes, followed by

cycling with denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds (25 cycles), followed by a 5-minute extension at 72°C, and finally held at 4°C. Subsequently, each 16S amplicon was purified using AMPure XP reagent from Beckman Coulter, Indianapolis, IN. In the subsequent step, each sample underwent amplification using a limited cycle PCR program, incorporating Illumina sequencing adapters and dual-index barcodes (index 1(i7) and index 2(i5)) obtained from Illumina, San Diego, CA. The thermal profile for this amplification involved an initial denaturation step at 95°C for 3 minutes, followed by a cycling process including denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds (8 cycles), followed by a 5-minute extension at 72°C and a final hold at 4°C. The final libraries were purified once again using AMPure XP reagent from Beckman Coulter, quantified, and normalized before pooling. The DNA library pool was then denatured using NaOH, diluted with hybridization buffer, and heat-denatured before being loaded onto the NovaSeq reagent cartridge and the NovaSeq instrument from Illumina. Automated cluster generation and paired–end sequencing with dual reads were conducted following the manufacturer's instructions (Arbeeva et al., 2022; Bertelsen et al., 2022; Haddad et al., 2023).

Bioinformatics analysis

Cassava 1.8 paired in sequences were processed using QIIME2-amplicon version 2023.9 (Bolyen et al. 2019). Samples were demultiplexed and residual primer sequences were screened in CutAdapt (Martin, 2019). Sequence truncation, denoising, and amplicon sequence variant (ASV) generation were performed with DADA2 (Callahan et al., 2016). Remaining features were filtered to those with at least 10 total copies (median frequency 329) that existed in at least 4 samples (median frequency 13). Two nest pad samples from the 27 wk old sample point (03 &

04) were removed from further analysis due to poor sequencing depth (~3.5% of the median frequency 357,773) and a high percentage of chimeric sequences (~20%). ASVs were classified against the SILVA 138.1 (Quast et al., 2013) database in QIIME2 with a V4 classifier generated using the RESCRIPt pipeline (Robeson et al., 2021). An average of 6% of cloacal and 11% of nest pad sequences were unassigned. Sequences mapped to chloroplasts, mitochondria, or eukaryotes were depleted before further processing. Data analysis and visualizations were prepared in MicrobiomeAnalyst 2.0 (Lu et al., 2023) using center log transformed (CLR) data to stabilize variance and adjust for compositionality biases. Alpha (Chao1 and Shannon) and beta diversity (Jenson-Shannon, Jaccard, Unweighted UniFrac, and Weighted UniFrac) were considered significantly different at a false discovery rate (FDR) of $\leq$0.05 using the Benjamini-Hochberg procedure. Posthoc pairwise comparisons utilized the Wilcoxon Rank Sum test for alpha diversity and PERMANOVA for beta diversity. Differential abundance statistics were performed using MaAsLin2 (Mallick et al., 2021) with age (weeks) as a covariate.

RESULTS and DISCUSSION

Comparing hatchability, fertility, and chick weight at hatch demonstrated no significant differences between the *M. luteus* and control group. The results for hatchability and fertility from this trial were expected because the birds were achieving high production parameters in all periods tested. Pervious research has shown that during 25-45 weeks of age rooster fertility remains high (Shaheen et al., 2023). The percent fertility and hatchability during all of the three periods were high for both groups staying between 99-95% fertility and 92-88% hatchability. The difference in fertility between the control and treatment group, although not significant, increased during 26-29 (P=0.34) and 33-36 (P=0.34) weeks of age as seen in table 3.1 and figure 3.1. Hatchability showed no significant differences between the *M. luteus* group and control

group, staying consistent during 26-29 wks (P=0.86), 33-36 wks (P=0.78), and 41-44 wks of age (P=0.77) as shown in figure 3.2. At hatch there was no significant difference between chick weights from the *M. luteus* and control groups as seen in figure 3.3. No other studies to our knowledge have conducted research of broiler breeder production parameters using environmental probiotics. However, previous studies using oral probiotics were able to reveal significant differences in chick weight and hatchability (Bozkurt et al., 2011). Although the first three figures revealed no significant differences, there was a significant difference in seven-day chick weight between the control and treatment groups during 33-36 wks of age (P=0.01) as shown in figure 3.4. This result was unexpected and favored the control group. The average chick weight for the control group was 108.7 grams and the average chick weight from the *M. luteus* group being 103.2 grams as displayed in table 3.2. We can only speculate as to an explanation for this decrease in body weight gain in the treatment chicks. One possibility is that the chicks had a different gastrointestinal tract microbiome that led to gut inflammation as seen in a previous study (Zhang et al., 2022). There was no significant difference in seven-day chick weight between the *M. luteus* and control groups during the other two periods, 26-29 wks of age (P=0.56) and 41-44 wks of age (P=0.08). The results also revealed no significant differences in seven-day chick mortality at any of the given periods (P=0.14, P=0.33, P=0.23). Lastly no significant differences in *M. luteus* presence were detected on the nest pad surfaces or the cloacal swabs between the *M. luteus* and control groups across all periods.

In addition to the production data, we received microbiome results from this trial that provided insight into the changes in microbiota community richness and diversity caused by *M. luteus* application. Chao1 community richness and Shannon richness and evenness alpha diversity metrics were measured. The hen cloacal samples revealed a significant difference in

Shannon alpha diversity (FDR=0.0286) at 34 wks of age between the *M. luteus* and control groups as seen in figure 3.5. This shows an increased species richness and diversity in microbiota in the control group in comparison to the *M. luteus* group during 34 wks of age. There were no other significant differences in alpha diversity for any of the sample types across all three production periods. Beta diversity metrics were also used to measure sample uniqueness based on species presence and absence between sample types. For beta diversity the hen cloacal samples from 34 wks of age were once again the only sample type to reveal a significant difference between *M. luteus* and control groups as seen in figure 3.6. Unweighted UniFrac was significantly different (FDR=0.02) between the *M. luteus* and control groups at 34 wks of age for the cloacal samples, meaning that there are unique ecological environments influenced by species makeup and regardless of species abundance. Overall, the cloacal samples at 34 wks of age displayed significant differences in ASV richness and beta diversity between the *M. luteus* and control groups, but none of the other sample types displayed changes.

At the genus level we found that *Corynebacterium* was the most abundant genera in the hen and rooster cloacal samples, while *Staphylococcus* was the most abundant in the nest pad samples as seen in figure 3.7. However, there were no significant differences detected at the genus level between the *M. luteus* and control groups. The relative abundance of ASVs were also compared within each sample type over each period between *M. luteus* and control groups to identify changes in specific taxa. These comparisons were performed at the ASV level because the higher taxonomic levels displayed no significant differences. AT 26 and 34 weeks of age there was significantly more *Slackia* on the nest pads in the control group than the *M. luteus* group (FDR=0.04). At 34 wks of age samples revealed that there was significantly more *Monoglobus* on the nest pads (FDR=0.02) and more of an unassigned ASV in the hen cloacas

(FDR=0.02) in the *M. luteus* group than the control group. *Monoglobus* has been found in prior research to be more abundant on *campylobacter*-negative farms in studies of the broiler microbiome (Pang et al., 2023). Researchers concluded that this bacterium as well as others may influence the colonization of *Campylobacter* in a positive manner for food safety (Pang et al., 2023). Therefore, the increase in *Monoglobus* seen in the *M. luteus* group could be a positive for food safety. Lastly *Fournierella* was significantly more abundant in the control group than the *M. luteus* in the rooster cloaca (FDR<0.0001) samples at 42 weeks of age. Although no studies have looked at the reproductive effects of *Fournierella* or its value in roosters specifically, it has been correlated to improved feed efficiency in laying hens (Zhou et al., 2023).

Differences in amplicon sequence variant (ASV) abundances across the entire trial between the *M. luteus* and control groups were also analyzed for each sample type, while keeping period of lay as a covariate. One ASV from *Lachnospiraceae* was found to be significantly more abundant in the control group of hen cloacal samples than the *M. luteus* group (FDR=0.01) across the entire trial. This difference in *Lachnospiraceae* is important to note because it is one of the most abundant firmicutes found in cecal content in previous studies, and it has been considered a beneficial bacterium for poultry production with its main role being producing short-chain fatty-acids (SCFA) (Lundberg et al., 2021; Rychlik, 2020). There was also one unassigned ASV in the rooster samples (FDR=0.0334) that was significantly more abundant in the control group than the *M. luteus* group. Lastly there were 5 ASV's including *Aeriscardovia* (FDR=0.01), *Actinobacteria* (FDR=0.02), *Oscillospiraceae* (FDR=0.02), *Isoptericola* (FDR=0.04), and *Bacilli* (RF39) (FDR=0.04) that were all significantly more abundant in the control group of nest pad samples than the *M. luteus* group. There were very few significant differences in abundance between the *M. luteus* and control microbiota overall, with

none seen at the higher taxonomic levels. However, the few that were revealed had interesting effects on bacteria previously correlated to feed conversion, food safety, and SCFA production that could be valuable to the industry.

CONCLUSION

Broiler breeders in this study displayed no significant increases in hatchability or fertility with the application of *M. luteus* to their nest pads. Therefore, the application of this bacteria is not supported as a method of improving reproductive performance in broiler breeders between the 26 and 44 weeks of age. The only significant difference seen in production was a significantly lower 7-day body weight in chicks produced by the broiler breeders exposed to *M. luteus* between 33-36 weeks of age. Broiler breeder production decreases with age and the effects of this trial cannot be applied to breeders post 44 weeks of age. Therefore, further research should be done focusing on the effects of late-stage broiler breeders whose fertility and hatchability are below 80%. As shell quality decreases and hens age the risk of contamination increases making treatments more likely to show a significant impact if one is present. This study also showed that *M. luteus* can cause significant differences in alpha and beta diversity of the microbiota in the cloaca of hens during 34 wks of age that was not characterized across other time periods or sample types. The differences in microbiota richness and diversity at 34 wks of age in the hen may be affecting the microbiota on the eggshell and ultimately the bacteria that colonize the gut of the chick. Differences in microbiota and gut metabolism could explain the differences in weight gain we saw at peak production. Future research should explore the effect of environmental probiotics on hen, eggshell and chick microbiota. In addition to changes in community richness and diversity, individual taxon abundance revealed that *Monoglobus* was increased on *M. luteus* nest pads and *Lachnospiraceae* was decreased in the cloacas of *M. luteus*

hens. These two-taxon showed significant differences at the ASV level along with several others, but higher taxonomic levels showed no significant differences. Evaluating breeder performance with addition of different symbiotic bacteria such as *Bacillus subtilis* is valuable for future research and may show us different changes to the microbiota community that could impact production parameters. Overall, we partially reject our hypothesis because the introduction of *M. luteus* to the nest pad showed no significant differences in egg fertility or hatchability throughout the production cycle but does appear to reduce 7-day body weights at 33-36 weeks of age (peak production) and alter the hen microbiome.

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Table 3.1 The difference in hatchability and fertility between eggs laid on nest pads sprayed with *M. luteus* and control nest pads

	26-29 wks		33-36 wks		41-44 wks	
	Fertility	Hatchability	Fertility	Hatchability	Fertility	Hatchability
Control	96.5%	91.1%	98.3%	91.5%	96.9%	88.5%
Treatment*	96.4%	90.8%	98.6%	91.8%	95.6%	88.0%
SEM	0.0055	0.0110	0.0023	0.0063	0.0090	0.0120
P value	0.9066	0.8632	0.3447	0.7820	0.3428	0.7734

*Treatment represents the production of broiler breeders from the pens with M. luteus applied to their nest pads.

Table 3.2 The difference in chick weight at hatch and 7-day body weight between chicks hatched from eggs laid on nest pads sprayed with *M. luteus* and control nest pads

	26-29 wks		33-36 wks		41-44 wks	
	Chick Weight at Hatch (g)	7-Day Body Weight (g)	Chick Weight at Hatch (g)	7-Day Body Weight (g)	Chick Weight at Hatch (g)	7-Day Body Weight (g)
Control	34.3	105.9	37.8	108.7[a]	41.3	125.3
Treatment*	34.4	104.8	37.9	103.2[b]	41.2	119.6
SEM	0.1820	1.3095	0.2342	1.3009	0.2468	2.1839
P value	0.8516	0.5659	0.8725	0.0097	0.5929	0.0858

*Treatment represents the production of broiler breeders from the pens with M. luteus applied to their nest pads.
[a,b] superscripts are used to signify significant differences

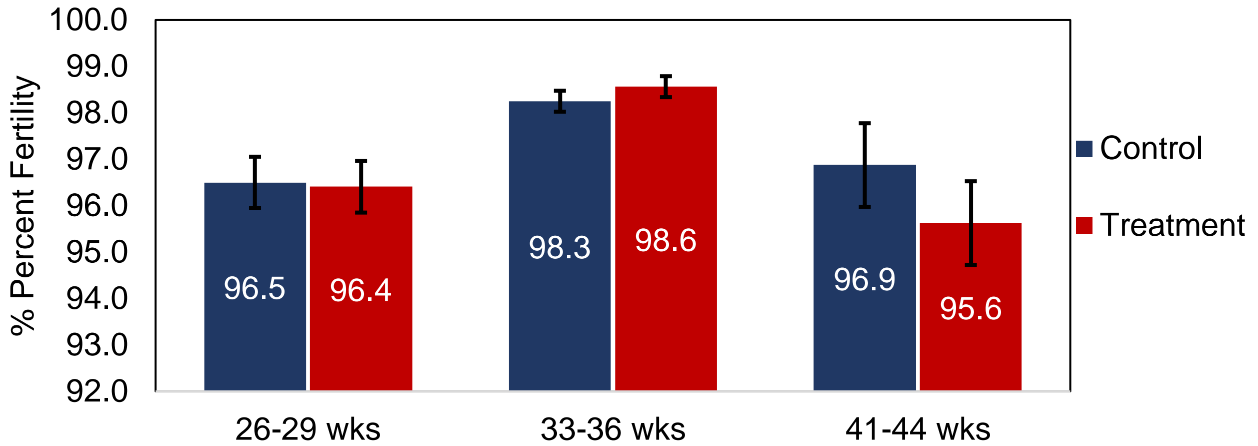

Figure 3.1 This figure's x-axis shows three separate periods including 26-29 wks, 33-36 wks, and 41-44 wks of age. Statistics were run for each individual period not across periods. The y-axis starts at 80% in order to visualize the SEM values. Control is blue and Treatment (*M. luteus*) is Red. The P and SEM values for differences in fertility were P=0.9066 and SEM=0.0055, P=0.3447 and SEM=0.0022, and P=0.3428 and SEM=0.0090 respectively.

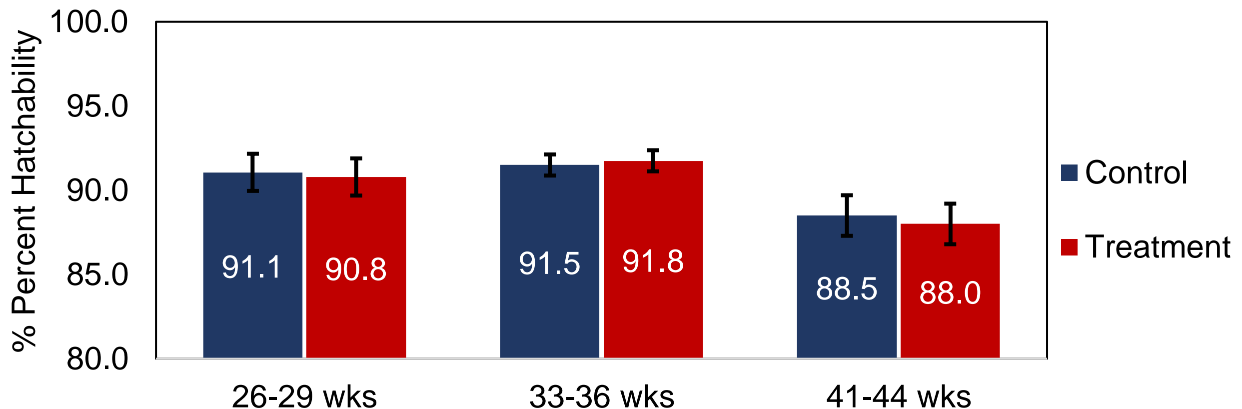

Figure 3.2 This figure's x-axis shows three separate periods including 26-29 wks, 33-36 wks, and 41-44 wks of age. Statistics were run for each individual period not across periods. The y-axis starts at 80% in order to visualize the SEM values. Control is blue and Treatment (*M. luteus*) is Red. The P and SEM values for differences in hatchability were P=0.8632 and SEM=0.0110, P=0.7820 and SEM= 0.0063, and P=0.7734 and SEM=0.0120 respectively.

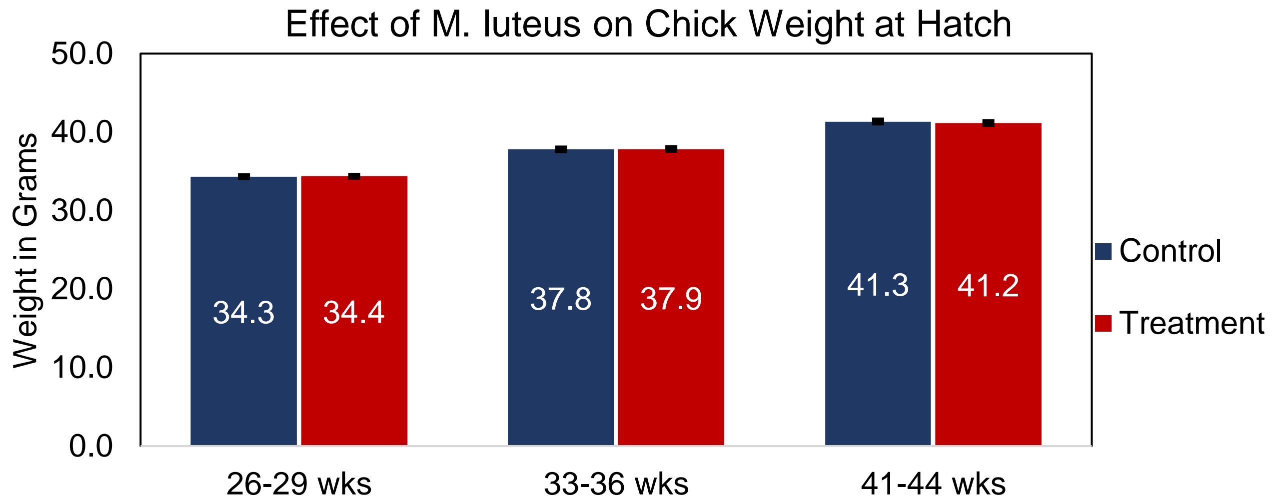

Figure 3.3 This figure's x-axis shows three separate periods including start of lay, peak lay and post peak lay. Statistics were run for each individual period not across periods. The y-axis shows the average body weight in grams. Control is blue and Treatment (*M. luteus*) is Red. The P and SEM values for differences in chick weight at hatch were P=0.8516 and SEM=0.1820, P=0.8725 and SEM=0.2342, and P=0.5929 and SEM=0.2468 respectively.

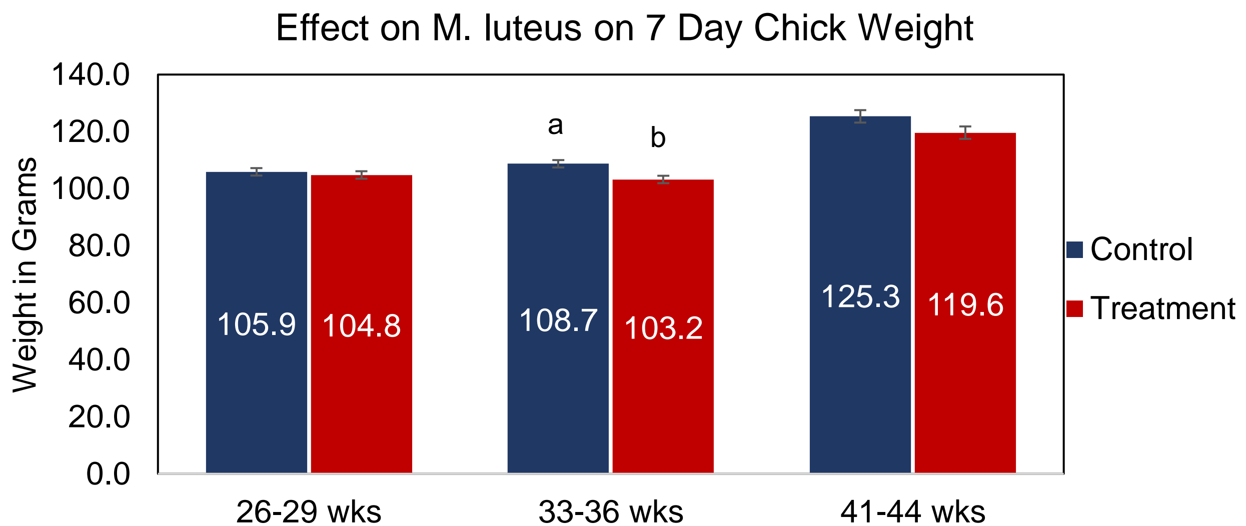

Figure 3.4 This figure's x-axis shows three separate periods including start of lay, peak lay, and post peak lay. Statistics were run for each individual period not across periods. The y-axis shows the average body weight in grams. Control is blue and Treatment (*M. luteus*) is Red. The P and SEM values for differences in 7-day chick weight were P=0.5659 and SEM=1.3094, P=0.0097 and SEM=1.3009, and P=0.0858 and SEM=2.1839 respectively. [a,b] Bar graphs with different superscripts are significantly different.

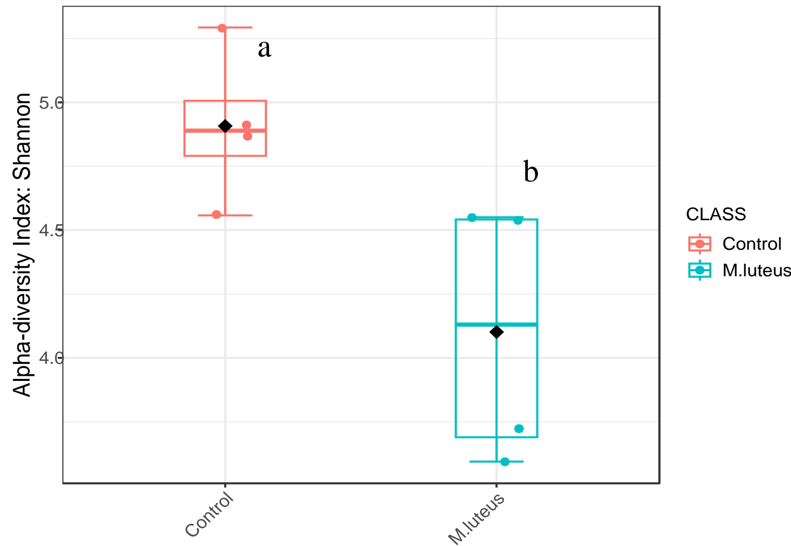

Figure 3.5 Hen Cloacal Alpha Diversity (Shannon) at 34 weeks of age. This figure shows the alpha diversity (Shannon) or the richness and evenness of the microbiota at the ASV level, of the 34-week-old hen cloacal samples in both the Control group and the *M. luteus* group. The hen cloacal samples are represented by their treatment group Control in red and *M. luteus* in blue. The FDR values for measure of significance was (FDR=0.0286). [a,b] Box plots with different superscripts are significantly different.

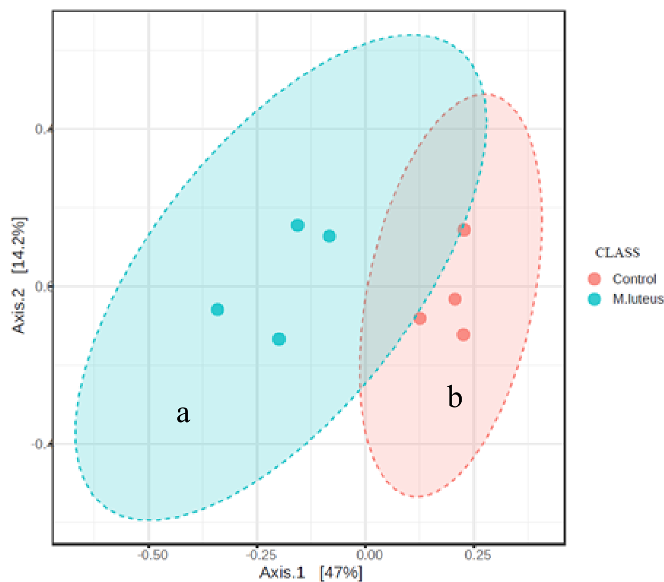

Figure 3.6 Hen Cloacal Beta Diversity (Unweighted UniFrac) At 34 weeks of age. This figure shows the beta diversity (Unweighted UniFrac) or the phylogenetic uniqueness of the microbiota at the ASV level, of the 34-week-old hen cloacal samples in both the Control group and the *M. luteus* group. The hen cloacal samples are represented by their treatment group Control in red and *M. luteus* in blue. The FDR values for measure of significance was (FDR=0.0270). [a,b] ellipses with different superscripts are significantly different.

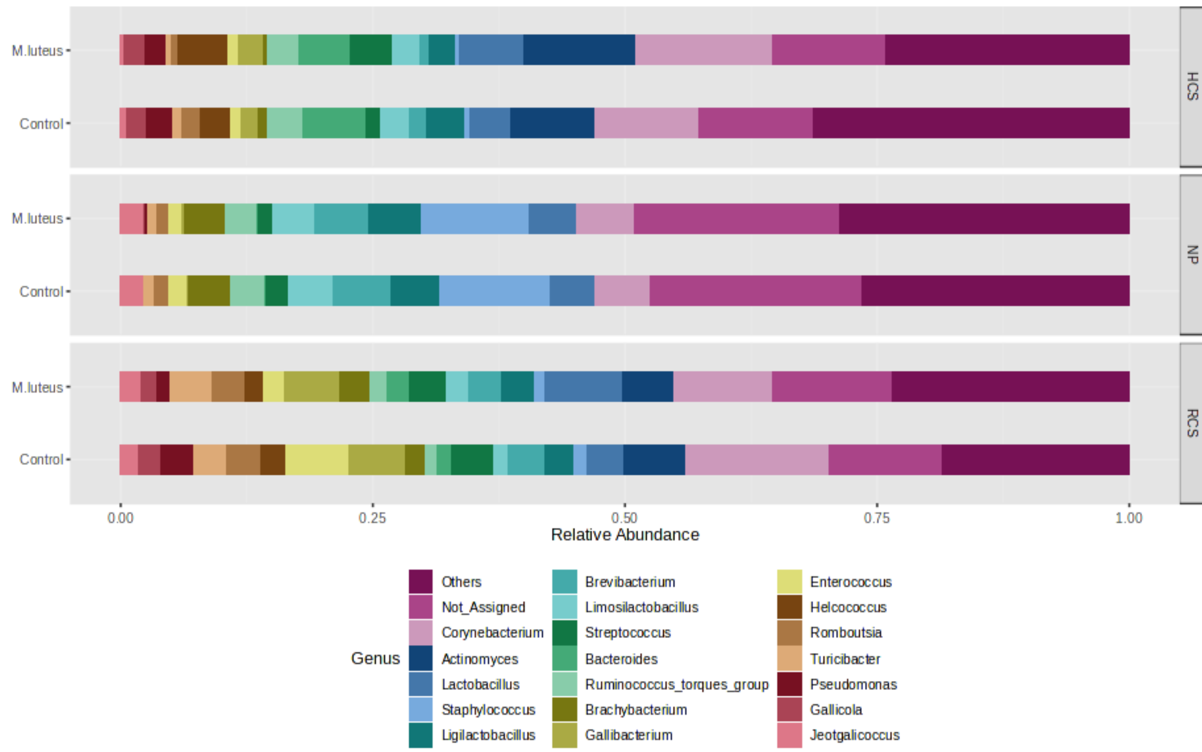

Figure 3.7 Top 20 Genera by Abundance Comparing the *M. luteus* Group to the Control Group. The above figure displays the top 20 genera on a percent abundance scale. The three sample types are represented on the right-hand side including hen cloacal swab (HCS), nest pad (NP), and rooster cloacal swab (RCS). Both the M. luteus and control groups are labeled on the left-hand side for each sample type despite there being no significant differences at this taxonomic level.

CHAPTER 4

THE EFFECT OF EXPOSURE TO *MICROCOCCUS LUTEUS*, *BACILLUS SUBTILIS,* AND *LACTOBACILLUS ACIDOPHILUS* ON ROOSTER SPERM MOTILITY

ABSTRACT

Semen samples with higher bacterial concentrations have problems with oxidation, sperm immobilization, and pH change that can cause infertility. Although semen is never completely sterile, high quality semen samples have less contamination and less harmful species present. In broiler breeders, rooster fertility has been low in recent years. Although bacterial contamination hasn't changed over time, it is something we can try to prevent and change the profile of in today's broiler breeders to optimize fertility. The objective of this study was to test the effect of two probiotic bacteria and a commensal skin bacterium on sperm motility to reveal nonpathogenic bacteria that can harm rooster fertility. We chose to measure motility because of its impact on sperm reaching the germinal disk for fertilization. Thirty semen samples from Ross 708 strain roosters were collected via abdominal massage into microcentrifuge tubes, aerated and kept warm at 40°C until the samples were read using the SQA-Vt-Avian semen analyzer to evaluate semen motility and concentration. The samples with the highest motility were diluted 1:10 in semen diluent, and pooled. 100uL of diluted semen was then used to create 12 treatments and was mixed with 900uL of one of the following medias: Tryptic Soy Broth (TSB), Man–Rogosa–Sharpe Broth (MRS), 10^6 and 10^8 *Micrococcus luteus* in TSB, 10^6 and 10^8 *Bacillus subtilis* in TSB, 10^6 and 10^8 *Lactobacillus acidophilus* in MRS, 10^6 *L. acidophilus* in PBS, and Phosphate Buffered Saline (PBS) and turkey semen diluent as the controls. Each media was mixed with semen directly before using the SQA-Vt-Avian semen analyzer. Additionally, samples were read after 20 min to evaluate the effect of duration of exposure on motility. SQA

readings were analyzed via one-way ANOVA and Tukey's HSD for multiple comparisons in JMP Pro 17, with a significance level of P<0.05. There were significant differences in sperm motility between the control and the following media's: MRS (P=0.01), 10^6 and 10^8 *L. acidophilus* in MRS (P=0.01, P<0.0001), and 10^6 *L. acidophilus* after 20 min in MRS (P<0.0001). The results indicate that MRS has a significant impact on motility likely due to its makeup. When *L. acidophilus* was separated form MRS, the bacteria revealed no significant impacts on sperm motility. In addition, *M. luteus* and *B. subtilis* appear to have no significant impact on sperm motility. Future research should work to model the route of semen contamination, which may help us control semen exposure to bacteria.

INTRODUCTION

Male fertility is a well-studied topic in humans and livestock. In all species the age of the male, body condition, and testosterone levels affect semen production and quality (Shaheen et al., 2023, Wilson et al., 1979). Semen is a combination of sperm cells and seminal plasma, a suspension of sugars and nutrients to help keep the sperm cells viable (Cheng et al. 2019). In roosters the seminal plasma balances the pH the sperm cells are exposed to, keeping the semen between a pH of 6.5 and 8 (Cheng et al. 2019; Wilcox, 1958). It has been shown that pH's that fall out of this range have negative effects on fertility (Wilcox and Shaffner, 1957). One factor that could affect the pH of the semen is bacterial contamination in addition to poor quality seminal plasma (Dhumal et al., 2021). Semen contamination can also impact the sperm's motility which is critical for swimming up the oviduct and competing for the germinal disk (Aghazarian et al., 2024; Azenabor et al., 2015). Researchers have shown that urogenital pathogens negatively impact sperm motility and kinematics (Aghazarian et al., 2024). Therefore, it is necessary to understand the bacterial presence of the cloaca of the rooster since it is a universal exit for the

reproductive, urinary, and digestive tract and can seed the bacteria present in semen. The demographic of bacteria can impact the degree of pH change, oxidation and immune response that will ultimately impact the motility and integrity of the sperm (Azenabor et al., 2015, Cheng and Ko, 2019). The impact bacteria have on contaminated semen is not only a risk to the semen during copulation but poses a risk to stored semen in the hen since the contaminated semen can enter the sperm storage tubules. With all of these risk factors, it is important to maintain a clean breeding environment and consider ways to prevent semen contamination.

Researchers have shown that many bacterial species function as spermicide when incubated with healthy semen (Haines et al., 2013). *Escherichia coli, Clostridium bifermentans*, and *Lactobacillus acidophilus* are examples of bacteria that have all been shown to limit sperm motility (Haines et al., 2013). Haines et al. concluded that all bacterial contamination, whether pathogenic or commensal, can be harmful to rooster semen (Haines et al., 2013). The effect of *L. acidophilus* inspired further research because of its use as a probiotic feed additive in many poultry facilities. Researchers further investigated the pH change caused by *L. acidophilus* and confirmed its negative effects on sperm motility (Kiess et al., 2016). From there Kiess et al. evaluated the transmission of *L. acidophilus* fed orally, to the cloaca and semen, and concluded that cloacal concentrations were increased, but presence in semen were not (Kiess et al., 2016). However, Kiess et al. further discussed that the increased presence of *L. acidophilus* in the cloaca poses a risk for contaminating semen despite not observing a difference in the given study (2016). Another bacteria that has been evaluated for its effect on sperm quality is *Bacillus subtilis*, a typical probiotic used in the poultry industry. Researchers have found that *B. subtilis* can be found in healthy poultry semen samples and that *in vitro* it does not cause damage to sperm (dos Santos et al., 2018; Lenický et al., 2021).

The external method of copulation in birds and common exit with the digestive tract ensures that semen will never be sterile. Therefore, it is important to identify the bacteria that, similar to *L. acidophilus* are considered nonpathogenic, but still negatively impact semen, in addition to determining which bacteria are lower risks to fertility like *B. subtilis*. *Micrococcus luteus* a skin commensal often found in semen, *B. subtilis*, and *L. acidophilus* were the bacteria chosen to be evaluated for this study (dos Santos et al., 2018, La Ragione et al., 2001, Tvrdá et al., 2023). We hypothesized that if broiler breeder semen is exposed to *L. acidophilus, B. subtilis,* and *M. luteus*, then the percentage of motile sperm will significantly decrease with exposure to *L. acidophilus* but will not be affected by *M. luteus* or *B. subtilis*. The overall objective of the study was to observe the impact of nonpathogenic bacteria that may pose a risk to sperm motility due to contamination in order to determine unintentional problems that could be caused by probiotics. The risk that bacteria pose to fertility is reason for further research into the effects of commensal and probiotic bacteria's effects on sperm motility and repercussions on overall fertility and hatchability in broiler breeder flocks.

MATERIALS AND METHODS

Experimental Design

The project was completed at the North Carolina State University Chicken Education Unit. All methods and procedures used for animal research in this trial were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC #22-435-A). A flock of 30 Ross 708 broiler breeder roosters (22 weeks) were randomly assigned to 6 pens. Each pen included male feeders and bell drinkers. The bedding material used was soft pine shavings and it was replaced as necessary throughout the trial. Similar to industry standard, the water lines were set on a water clock to shut off during the dark period in the house to prevent

water leaks. Roosters were provided feed based on Aviagen recommendations in the Parent Stock Handbook. All roosters were abdominally massaged for semen weekly in order to get them use to human touch (Burrows and Quinn, 1937). Semen samples were collected from all thirty roosters via abdominal massage at 35 wks, 43 wks, and 45 wks of age. Each time period sampled represented a replicate, totaling 3 replicates.

Semen Collection and Analysis

Semen samples were collected via abdominal massage into sterile microcentrifuge tubes, regularly aerated and kept warm at 40°C until the samples were read using the SQA-Vt-Avian semen analyzer (Medical Electronic Systems, Los Angeles, CA) produced by Medical Electronic systems to evaluate semen motility and concentration (Burrows and Quinn, 1937). The samples with the highest motility (over 75% percent) and volume were chosen because of their increased ability to show significant effects caused by treatment medias, and lack of confounding additional contamination. The six highest motility samples were diluted 1:10 (100 µL of the neat semen and 900 µL of semen diluent) and pooled. One hundred microliters of the diluted pooled semen was then mixed with 900uL of one of the following medias to create a total of 12 treatments: Tryptic Soy Broth (TSB) (sigma Aldrich, St. Louis, MO), Man–Rogosa–Sharpe Broth (MRS) (sigma Aldrich, St. Louis, MO), 10^6 and 10^8 *Micrococcus luteus* in TSB, 10^6 and 10^8 *Bacillus subtilis* in TSB, 10^6 and 10^8 *Lactobacillus acidophilus* in MRS*,* 10^6 *L. acidophilus* in PBS, and Phosphate Buffered Saline (PBS) (sigma Aldrich, St. Louis, MO) and turkey semen diluent SQA-Vt (Medical Electronic Systems, Los Angeles, CA) as controls. Each media was mixed with semen directly before being evaluated on the SQA-Vt-Avian semen analyzer (Medical Electronic Systems, Los Angeles, CA). The standard 10^6 concentrations of bacteria were additionally read after 20 minutes at room temperature to evaluate the effect of duration of

exposure to the bacteria on motility. Those samples included 10^6 *Micrococcus luteus* in TSB, 10^6 *Bacillus subtilis* in TSB, 10^6 *Lactobacillus acidophilus* in MRS, and PBS. 10^8 concentrations and medias were not included in the duration trial. Prior to adding sperm to the bacterial cultures, an SQA reading was obtained for each cultured media and the sterile broth. The SQA-Vt-Avian semen analyzer (Medical Electronic Systems, Los Angeles, CA) did not detect any movement of bacteria in the media or sterile broth which was determined by a reading of zero, validating that motility readings are the result of sperm motility alone.

Bacterial Cultures

Overnight cultures of the following bacteria were used for analysis: *M. luteus (4698 ATCC), B. subtilis (6051 ATCC)* and *L. acidophilus (NCK 56). M. luteus* and *B. subtilis* cultures were grown overnight in Tryptic soy broth (TSB) (sigma Aldrich, St. Louis, MO) and *L. acidophilus* was grown overnight in Man–Rogosa–Sharpe (MRS) broth (sigma Aldrich, St. Louis, MO). Each bacterium was hydrated in the appropriate broths 1 wk before the start of the experiment. One milliliter of each bacterial culture was aseptically transferred into 9 mL of the respective broth every 24 h to provide optimum growth conditions. *M. luteus* and *B. subtilis* were grown in an aerobic shaking incubator at 30°C. *L. acidophilus* was grown anaerobically in an incubator at 35°C. A standard curve was created and then validated with the SmartSpec 3000 optical density machine (BIO-RAD, Hercules, CA) to ensure our dilutions of 10^6 and 10^8 CFU's per ml were correct for each bacterium.

***L. acidophilus* Testing**

Further testing of *L. acidophilus* was conducted to understand the effects of the bacterium separately from the media, since MRS had such a negative effect on motility on its own. *L. acidophilus* was grown overnight in MRS broth. The culture was run through the OD

machine three times and the average was utilized to calculate the CFUs per ml present. The culture was then diluted to 10^6 CFUs per ml and centrifuged (Eppendorf AG, Hamburg, Germany) at 4000 RPMs for 10 minutes. The supernatant was aspirated, and the remaining *L. acidophilus* pellet was vortexed (Vortex Genie, Bohemia, NY) in Phosphate Buffered Saline (PBS) until dissolved (sigma Aldrich, St. Louis, MO) to achieve a 10^6 CFU per ml concentration. The PBS solution was centrifuged a second time at 4000 RPM for 10 minutes, the supernatant was aspirated, and the pellet was vortex in fresh PBS again. The 10^6 *L. acidophilus* + PBS solution was then mixed with semen and analyzed using the SQA-VT-Avian semen analyzer (Medical Electronic Systems, Los Angeles, CA) as described previously.

Statistical analysis

Each week the experiment was repeated (3 total) which served as an experimental unit and all data was analyzed using SAS, JMP Pro 17 Software. Significance was determined using a one-way ANOVA and Tukey's HSD for multiple comparisons with a significance level of $P< 0.05$.

RESULTS and DISCUSSION

The results for this study were analyzed together and then broken out into four separate figures. Each sterile media and bacteria culture were analyzed using the SQA-Vt-Avian semen analyzer to confirm that none of the samples displayed motility in the absence of semen. All sterile medias and bacteria displayed zero percent motility. The first analysis run with semen compared the sterile growth media that each bacterium was grown in against the control turkey semen diluent. Because the bacteria were grown in different media, we analyzed the different media's effects on motility individually. This allowed for the effect of the bacteria later on to be considered independent of the media type. No significant differences were revealed between the

TSB used to culture *M. luteus* and *B. subtilis,* the PBS used to observe L. acidophilus bacteria, and the control semen diluent (P=1) as seen in figure 4.1. However, the P values for the difference in motility of sperm when diluted with MRS showed that motility was significantly lower than the control (P=0.01), PBS (P=0.03), and TSB (P=0.01). Haines et al. also found that MRS resulted in a significantly reduced Sperm quality index reading (SQI) following these same practices for the bacteria culture broths (Haines et al., 2013). Although the exact ingredient in MRS causing a decrease in motility was not studied it is likely that the significant decreases in sperm motility could be due to one of the ingredients in it's make up (de Man et al., 1960). Unlike MRS, PBS and TSB were not significantly different from using semen diluent to analyze sperm motility. The study by Haines et al. also found a significant decrease in motility with use of TSB that our study does not support (Haines et al., 2013). After the medias were evaluated, each of the bacteria were immediately tested for their effect on sperm motility. The motility of the control was compared to 10^6 *Micrococcus luteus* in TSB (P=1), 10^6 *Bacillus subtilis* in TSB (P=1), 10^6 *Lactobacillus acidophilus* in MRS (P=0.003), and PBS (P=1). Semen exposed to *M. luteus* and *B. subtilis* showed no significant decreases in sperm motility from the controls, but *L. acidophilus* in MRS displayed a significant decrease in sperm motility as seen in table 4.1. *M. luteus* had not been previously studied in vitro, however, it has been found in natural cultures of healthy semen samples in past literature (Tvrdá et al., 2023). Previous studies also showed that *B. subtilis* combined with semen in vitro and feed orally did not affect sperm motility which is supported by our results (dos Santos et al., 2018). The effect of *B. subtilis* was also supported by its natural occurrence in healthy turkey semen (Lenický et al., 2021). The results of the immediately tested 10^6 concentrations were evaluated alongside the motility of sperm with prolonged exposer to 10^6 *Micrococcus luteus* in TSB (P=0.74), 10^6 *Bacillus subtilis* in

TSB(P=0.15), 10^6 *Lactobacillus acidophilus* in MRS (P<0.0001), and PBS (P=0.73) after 20 minutes, seen in figure 4.2. The duration of exposure to each bacterium displayed the same significant effects on motility that were displayed by testing immediately. The results revealed that *L. acidophilus* in MRS was the only culture to significantly decrease sperm motility in both immediate and delayed testing. The significant decreases characterized in this study by *L. acidophilus* in MRS has been supported in previous research (Haines et al., 2013; Kiess et al., 2016). In addition to the significant changes caused by *L. acidophilus*, we saw that time and motility have an inverse relationship which is a trend characterized in previous literature due to the quick deterioration of rooster semen (Shaheen et al., 2023).

Since *M. luteus* and *B. subtilis* had no significant differences from the control at concentrations of 10^6 CFU per mL, we wanted to see if increasing the concentration to 10^8 CFU per mL would reveal a difference. The control was compared to 10^8 *M. luteus* in TSB (P=1), 10^8 *B. subtilis* in TSB (P=1), and 10^8 *L. acidophilus* in MRS (P<0.0001) as shown in figure 4.3. The higher concentrations showed no significant differences between any of the bacteria and the control, except for *L. acidophilus*. Previous literature by Haines et al. showed that the degree of negative effect on sperm motility could be correlated with the concentration of *L. acidophilus* (Haines et al., 2013). These results were therefore expected since it is both supported by prior literature and since we saw a significant decrease at 10^6 CFU's. Due to the significant decreases caused by *L. acidophilus* and MRS we wanted to further explore *L. acidophilus* to better understand whether the bacteria itself is affecting motility. To do this, we compared the MRS media to the *L. acidophilus* bacteria and the entire growth culture. We compared the effect of sterile MRS, 10^6 *L. acidophilus* in MRS, and 10^6 *L. acidophilus* in PBS on sperm motility. MRS and 10^6 *L. acidophilus* in MRS which were both found previously to cause significant decreases

to sperm motility were not found to be significantly different from one another as seen in figure 4.4, however, 10^6 *L. acidophilus* in PBS was found to be significantly different from both with P values of 0.02 and 0.01 respectively. Therefore, the bacteria, isolated away from its own overnight growth products, does not display the same negative effects on sperm motility that are seen by the MRS media and the *L. acidophilus* in MRS media. Pervious literature did not evaluate *L. acidophilus* separately from its growth media and therefore was unable to make these conclusions. However, it is important to remember that *L. acidophilus* does produce lactic acid as a product of fermentation and growth which was neither transferred into the PBS culture nor was it evaluated separately (Gopal., 2011). This may be the reason for Haines et al.'s results showing significant decreases in sperm motility based on *L. acidophilus* abundance and should be evaluated further (Haines et al. 2013).

CONCLUSION

In this study, we observed the effects of *M. luteus*, *L. acidophilus*, and *B. subtilis* on sperm motility. We observed that *M. luteus* and *B. subtilis* had no significant effects on sperm motility independent of concentration and duration of exposure. These results were expected because both *M. luteus* and *B. subtilis* have been found in healthy rooster and tom semen samples with high motility (Lenický et al., 2021; Tvrdá et al., 2023). Therefore, neither bacteria are a major concern for inhibiting the ability of the sperm to swim and reach the germinal disk for fertilization. On the other hand, *L. acidophilus* was revealed to have no significant effect on sperm motility by itself. Therefore, any affect *L. acidophilus* does have on fertility when cultured in MRS is likely due to the products it produces such as lactic acid and not the bacteria physically restraining the sperm from moving. Unfortunately, *L. acidophilus* does not grow *in vivo* without producing lactic acid, therefore the bacteria still poses significant risk to rooster

fertility. Future research should work to model the route of semen contamination, which may help us to limit semen exposure to certain bacteria. Overall, it can be concluded that there are non-pathogenic bacteria that are more preferable in semen contamination than others due to their lack of damage to sperm motility.

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Table 4.1 The difference in motility between each media type and the control

Media Type	Mean Motility	P-Value*	SEM
Control	86.7	----	----
PBS	81.6	1	12.32
TBS	86.7	1	12.32
MRS	33.9	0.01	12.32
10^6 *M. luteus* in TSB	86.4	1	12.32
10^6 *B. Subtilis* in TSB	78.6	1	12.32
10^6 *L. acidophilus* in MRS	28.6	0.003	12.32
10^6 *L. acidophilus* in PBS	84.3	1	12.32
10^8 *M. luteus* in TSB	86.0	1	12.32
10^8 *B. subtilis* in TSB	85.2	1	12.32
10^8 *L. acidophilus* in MRS	0.0	<.0001	12.32
PBS after 20 Mins	59.0	0.73	12.32
10^6 *M. luteus* after 20 Min	59.2	0.74	12.32
10^6 *B. subtilis* after 20 Min	46.3	0.15	12.32
10^6 *L. acidophilus* after 20 Min	7.9	<.0001	12.32

*The P-value represented on this table shows the significance of the difference between the given media type and the control. (n = 3)

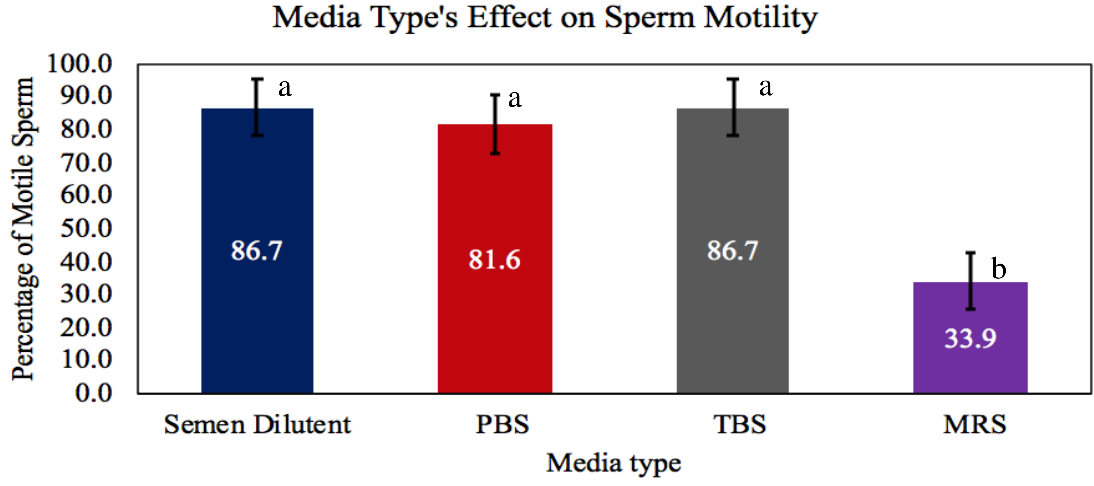

Figure 4.1 This figure's x-axis shows four different media that were used to dilute the semen for analysis by the SQA semen analyzer. Those include the semen diluent as the control (dark blue), PBS (red), TSB (gray) and MRS (purple). Statistics were run comparing each media to the control. The y-axis shows the percentage of motile sperm in the sample. [a,b] Bar graphs with different superscripts are significantly different. (P=1, P=1, P=0.01, SEM was 8.71)

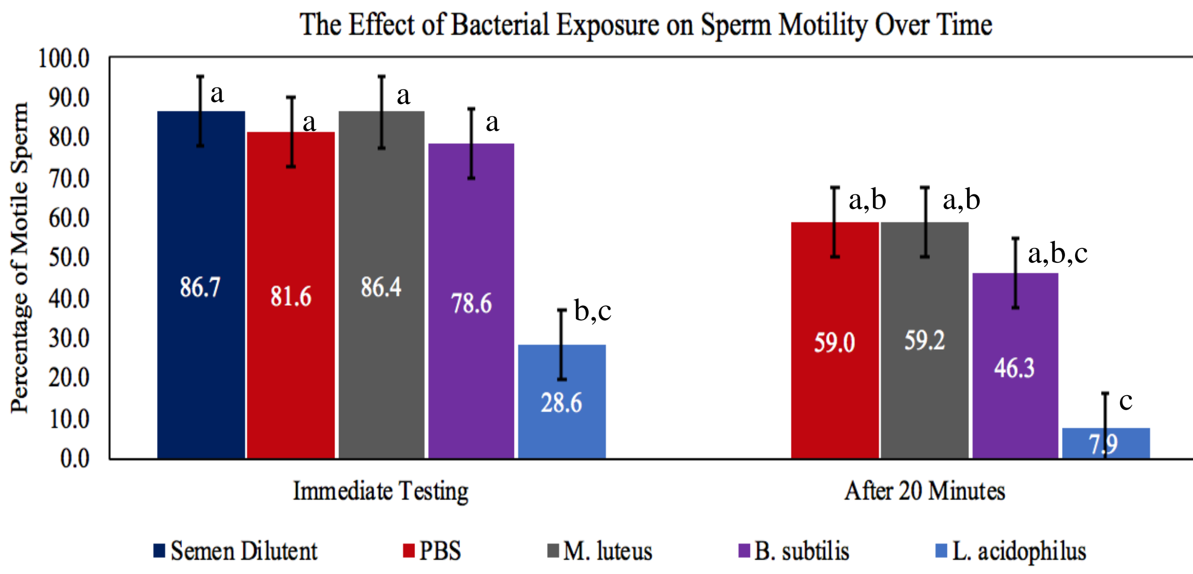

Figure 4.2 This figure's x-axis shows the immediate and delayed testing of PBS, 10^6 *M. luteus* in TSB, 10^6 *B. subtilis* in TSB and 10^6 *L. acidophilus* in MRS. The media are coded as follows; semen diluent as the control (dark blue), PBS (red), *M. luteus* (gray), *B. subtilis* (purple), and *L. acidophilus* (light blue). Statistics were run comparing each media to the control. The y-axis shows the percentage of motile sperm in the sample. [a,b,c] Bar graphs with different superscripts are significantly different. (P=1, P=1, P=1, P=0.003, P=0.73, P=0.74, P=0.15, P<0.0001, SEM was 8.71)

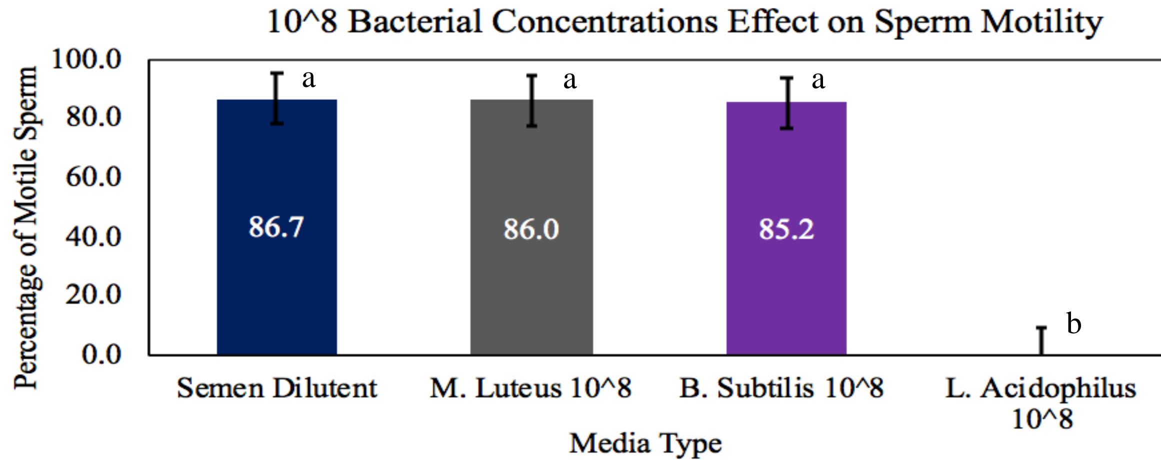

Figure 4.3 This figure's x-axis shows four different media that were used to dilute the semen for analysis by the SQA semen analyzer. Those include the semen diluent as the control (dark blue), 10^8 *M. luteus* in TSB (gray), 10^8 *B. subtilis* in TSB (purple), and 10^8 *L. acidophilus* in MRS (light blue). Statistics were run comparing each media to the control. The y-axis shows the percentage of motile sperm in the sample. [a,b] Bar graphs with different superscripts are significantly different. (P=1, P=1, P<0.0001, SEM was 8.71)

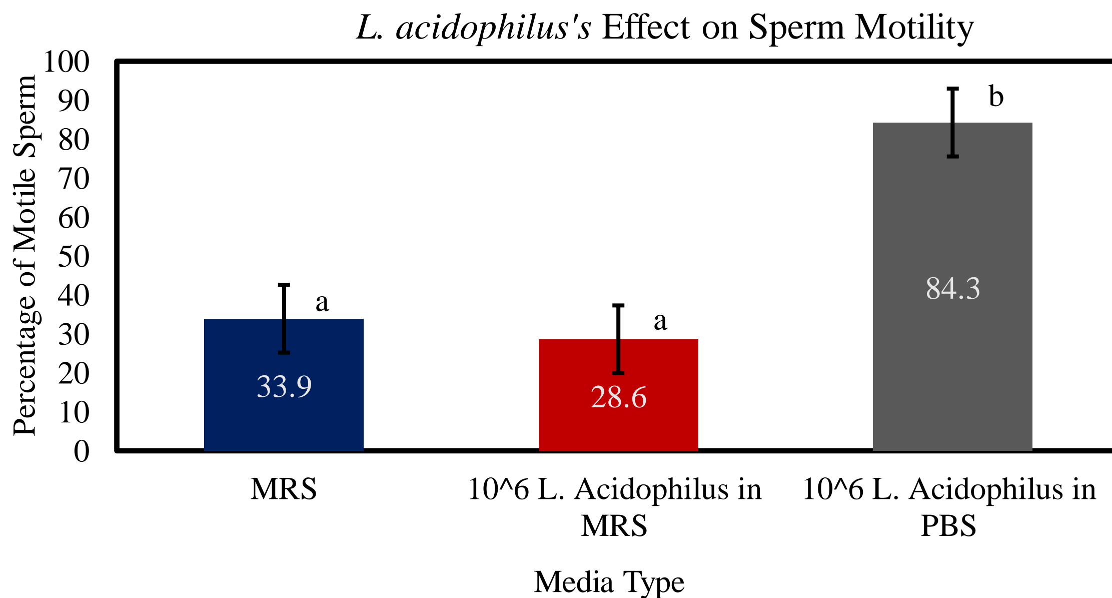

Figure 4.4 This figure's x-axis shows the three components of *L. acidophilus* growth, sterile MRS, the growth media with the bacteria, and the bacteria in PBS. All components were used to dilute the semen for analysis by the SQA semen analyzer. MRS is represented by dark blue, 10^6 *L acidophilus* in MRS is represented by red, and the 10^6 *L. acidophilus* bacteria in PBS is represented in grey. Statistics were run comparing the *L. acidophilus* cultures to the sterile MRS. The y-axis shows the percentage of motile sperm in the sample. [a,b] Bar graphs with different superscripts are significantly different. (P=0.02, P=0.01, SEM was 8.71)

CHAPTER 5

THE EFFECT OF AGE ON THE MICROBIOTA OF THE NESTING SURFACE, HEN'S CLOACA AND ROOSTER'S CLOACA IN BROILER BREEDERS

ABSTRACT

The lifecycle of a broiler breeder is often greater than 60 weeks and consists of multiple life stages including pullet, start of lay, peak production, and post-peak production. These different life stages are defined by different production rates, hormone levels, and body weights. In this study the objective was to characterize the bacteria present in the cloaca and on the nest pads of Ross 708 strain broiler breeders as age and production level changes. We compared the differences in microbiota between production periods and determined the fluctuations in bacterial richness and diversity over time. To accomplish this, 240 hens and 28 roosters (22 weeks) were randomly assigned to 4 pens holding 60 hens and 7 roosters each. Water was provided through bell drinkers and feed was provided based on Aviagen recommendations in the Parent Stock Handbook. The trial measured the changes in microbiota at three different ages including 27 wks (pre-peak lay), 34 wks (peak lay), and 42 wks (post-peak lay). At each time point, nest pad and cloacal swab samples were collected and then sent to UNC Microbiome Center (Chapel Hill, NC) for DNA extraction and 16S rRNA analysis. QIIME2-amplicon version 2023.9 was used to process sequences, followed by sample demultiplexing, sequence truncation, denoising and filtering. Amplicon sequencing variants (ASV) were then analyzed using Marker Data Profiling on microbiomeanalyst.ca. The ASV's were filtered and transformed to normalize the data. Alpha (Chao1 and Shannon) and beta diversity (Jenson-Shannon, Jaccard, Unweighted UniFrac, and Weighted UniFrac) were considered different at $FDR<0.05$ using the Benjamini-Hochberg procedure. Posthoc pairwise comparisons utilized the Wilcoxon Rank Sum test for

alpha diversity and PERMANOVA for beta diversity. Differential abundance statistics were performed using MaAsLin2. The research revealed a significant difference in beta diversity (Unweighted UniFrac) at the genus level for the hen cloacal samples (FDR=0.03). However, there were no other significant differences in beta or alpha diversity across the production periods for either of the other sample types. In comparing phyla abundance over time, there were many significant differences between periods and sample types. *Firmicutes*, *Actinobacteria*, and *Proteobacteria* were the three most abundant phylum across all sample types. We also characterized potentially pathogenic genera abundances with significance to sperm motility and egg contamination between all production periods. The results showed significant increases in known egg contamination pathogens including *Pseudomonas* (FDR<0.0001) and *Streptococcus* (FDR=0.04) between 27 and 34 weeks of age in the hen samples. In addition, *Enterococcus*, *Streptococcus*, *Staphylococcus*, and *Escherichia* were all found in the top 20 most abundant genera present on the nest pad surface across the production periods. Lastly, the results revealed a potential semen contamination pathogen, *Escherichia (FDR=0.04) was significantly decreased between* 27 *and* 34 weeks of age *in rooster samples. Overall, age or* production *period does affect individual microbiota abundances both in the cloaca of the birds and on the nest pad. However, within the production cycles we observed significant differences in microbiota diversity in the hen cloacal samples, and we observed no significant differences in microbiota richness.* Future research should include the egg's microbiota in addition to the cloaca and nest pad and explore the production effects of certain bacterial phylum and genera abundances at different time points.

INTRODUCTION

Broiler breeders are the most valuable of production chickens because of their genetic line and the economic value of the chicks they produce. The economic value of broiler breeders makes understanding the factors that influence their production and health very important. Broiler breeders have complex microbiomes both inside and outside of their bodies (Wilson et al., 2021; Yang et al., 2020). As chickens age their microbiota becomes equally more robust, diverse, and stable (Awad et al., 2016; Cui et al., 2017; Liu et al., 2021; Díaz-Sánchez et al., 2019). In broiler breeders' researchers have studied the differences in fecal, cecal, and ileum microbiota between slow growth and fast growth genetic lines, high and average lay compatibilities, and birds with high and low feed conversion ratios (FCR) (Díaz-Sánchez et al., 2019; Shterzer et al. 2023; Yang et al., 2020). Researchers have also explored the effects of feed additives such as Metformin and Sodium butyrate on the microbiome of broiler breeder hens and how it correlates to production (Van Syoc et al., 2022; *Xiao et al., 2023*). However, only a few studies included the variable of time as a factor affecting microbiota and those that did performed the research using Cobb genetic lines (Díaz-Sánchez et al., 2019; Van Syoc et al., 2022). Research of broiler breeder microbiomes has yet to study the Aviagen genetic lines over time, compare the differences between the hen and rooster microbiome separately, and include the environment in comparison to these sample types in one study.

This study's objective was to characterize the bacteria present in the cloaca and on the nest pads of broiler breeders during the different production periods. During each production period, the hens produce different quantities of eggs and the overall fertility on the flock changes, this led to curiosity into whether or not these periods also affect the microbiome. We wanted to compare the differences in microbiota between production periods and determine the

fluctuations in bacterial richness and diversity over time in both the hens and roosters separately, as well as on the hen nesting surface. The nest pad surface of broiler breeders has yet to be characterized in any previous study. We hypothesized that if microbiome samples of the hen cloaca, rooster cloaca, and nest pad were collected and analyzed at 27, 34, and 42 weeks of age, then there would be significant differences between species richness and abundance between time periods. This foundational research is critical to the expansion of research in regard to understanding species presence patterns that could be influencing egg and semen contamination and the microbiome's contribution to broiler breeder fertility and hatchability.

MATERIALS AND METHODS

Experimental Design

The project was completed at the North Carolina State University Chicken Education Unit. All methods and procedures used for animal research in this trial were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC #22-435-A). A flock of 240 Ross 708 strain breeder hens and 28 roosters (22 weeks) were randomly assigned to 4 pens holding 60 hens and 7 roosters each. Each pen included a slat area, scratch area and 16 nest boxes. The scratch area was filled with soft pine shaving and was replaced as necessary throughout the trial. Similar to industry standard, the water lines were set on a water clock to shut off during the dark period in the house to prevent water leaks. Water was provided through bell drinkers. Birds were provided feed based on Aviagen recommendations in the Parent Stock Handbook. Eggs were collected by hand twice daily. Gloves and shoe covers were changed between pens to prevent contaminating the control nest pads. The house was blocked by side, leaving two pens in each block. The trial measured the changes in microbiome profile of

nest pads and cloacal samples at three different time points including 27 wks, 34 wks, and 42 wks of age. During each time point samples were collected.

Sample Collection and Media Preparation

During each period one nest pad and six cloacal swabs, 3 from breeder hens and 3 from roosters, were collected from each pen. Sterile cotton tipped swabs (Amazon, Seattle, WA, USA) were inserted half an inch into the cloaca and swirled three times in each direction. Each nest pad was placed into a sterile 55oz filter bag (Whirl-pak, Pleasant Prairie, WI, USA) and sealed, and each cloacal swab was swirled in 1 ml of PBS in 15 ml sterile polypropylene tubes (ThermoFisher, Waltham, MA, USA) for 30 seconds. Sealed nest pads and cloacal swab samples were then placed on ice and transported back to the microbiology lab at the Prestage Department of Poultry Science for microbial processing. Cloacal samples from roosters and hens of the same pen were pooled respectively by sex. A 5x5 inch square was cut out of the center of each nest pad and the rest was discarded. 180 ml of PBS was then added to each nest pad bag and shaken for eight minutes. A 5 ml aliquot was then transferred from each of the whirl pack bags into a sterile sample tube. After processing was complete, 1 ml from each pen's hen cloacal sample, 1 ml from each pen's rooster cloacal sample and 3 ml from each pens nest pad sample were transferred into 5 ml sterile Eppendorf tubes (VWR, Radnor, PA, USA) and frozen at -80°C in a Thermoscientific TLE series freezer (Waltham, MA, USA). All samples were then stored until shipped to UNC for further processing. These procedures were performed at 27, 34, and 42 weeks of age. At the end of the 42 weeks, samples were sent to the UNC microbiome center for DNA extraction and 16S rRNA analysis.

Microbiome Analysis

At the end of the 42 weeks, frozen samples were sent to the UNC Microbiome Core Facility Center (Chapel Hill, NC) where 16S rRNA analysis was performed to determine differences in microbiome diversity between samples. The following procedures for DNA isolation and 16srRNA amplicon sequencing were performed by the UNC Microbiome Core Facility Center in Chapel Hill.

DNA isolation

Samples were transferred into a 2 ml tube containing 200 mg of glass beads sized 106/500 µm (Sigma, St. Louis, MO) along with 0.6 ml of Qiagen ATL buffer (Hilden, Germany). The mixture was then vortexed at 3,000 rpm for 10 minutes using a digital vortex mixer and supplemented with 60 mg/ml of lysozyme sourced from ThermoScientific, Rockford, IL. Subsequently, the suspension was incubated at 37°C for 1 hour and further supplemented with 600 IU of Qiagen proteinase K, followed by another incubation at 70°C for 1 hour. Upon centrifugation for 3 minutes, 0.5 ml of supernatant was combined with 0.5 ml of Qiagen AL buffer and incubated at 70°C for 10 minutes. The supernatant was then aspirated and transferred into a new tube containing 0.5 ml of ethanol. DNA purification was performed using a standard on-column purification method employing Qiagen buffers AW1 and AW2 as washing agents, and elution was done in DNase-free water. (Camp, 2022; Marsh et al., 2022; Ribeiro et al., 2022).

16S rRNA amplicon sequencing

A total of 12.5 ng of DNA were subjected to amplification using universal primers designed to target the V4 region of the bacterial 16S rRNA gene. These primers were designed with overhang adapters appended to their 5' ends to ensure compatibility with the Illumina

sequencing platform. Specifically, the primers utilized were F515/R806. The master mixes consisted of 12.5 ng of total DNA, 0.5 μM of each primer, and 2x KAPA HiFi HotStart ReadyMix sourced from KAPA Biosystems, Wilmington, MA. The thermal profile for the amplification process involved an initial denaturation step at 95°C for 3 minutes, followed by cycling with denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds (25 cycles), followed by a 5-minute extension at 72°C, and finally held at 4°C. Subsequently, each 16S amplicon was purified using AMPure XP reagent from Beckman Coulter, Indianapolis, IN. In the subsequent step, each sample underwent amplification using a limited cycle PCR program, incorporating Illumina sequencing adapters and dual-index barcodes (index 1(i7) and index 2(i5)) obtained from Illumina, San Diego, CA. The thermal profile for this amplification involved an initial denaturation step at 95°C for 3 minutes, followed by a cycling process including denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds (8 cycles), followed by a 5-minute extension at 72°C and a final hold at 4°C. The final libraries were purified once again using AMPure XP reagent from Beckman Coulter, quantified, and normalized before pooling. The DNA library pool was then denatured using NaOH, diluted with hybridization buffer, and heat-denatured before being loaded onto the NovaSeq reagent cartridge and the NovaSeq instrument from Illumina. Automated cluster generation and paired–end sequencing with dual reads were conducted following the manufacturer's instructions (Arbeeva et al., 2022; Bertelsen et al., 2022; Haddad et al., 2023).

Bioinformatics Analysis

Cassava 1.8 paired in sequences were processed using QIIME2-amplicon version 2023.9 (Bolyen et al. 2019). Samples were demultiplexed and residual primer sequences were screened

in CutAdapt (Martin, 2019). Sequence truncation, denoising, and amplicon sequenceing variant (ASV) generation were performed with DADA2 (Callahan et al., 2016). Remaining features were filtered to those with at least total 10 copies (median frequency 329) that existed in at least 4 samples (median frequency 13). One nest pad sample from the 27 wk timeframe (03) was removed from further analysis due to poor sequencing depth (~3.5% of the median frequency 357,773) and a high percentage of chimeric sequences (~20%). ASVs were classified against the SILVA 138.1 (Quast et al., 2013) database in QIIME2 with a classifier generated using the RESCRIPt pipeline (Robeson et al., 2021). An average of 6% of cloacal and 11% of nest pad sequences were unassigned. Sequences mapped to chloroplasts, mitochondria, or eukaryotes were depleted before further processing. Data analysis and visualizations were prepared in MicrobiomeAnalyst 2.0 (Lu et al., 2023) using center log transformed (CLR) data to stabilize variance and adjust for compositionality biases. Alpha (Chao1 and Shannon) and beta diversity (Jenson-Shannon, Jaccard, Unweighted UniFrac, and Weighted UniFrac) were considered different at FDR<0.05 using the Benjamini-Hochberg procedure. Posthoc pairwise comparisons utilized the Wilcoxon Rank Sum test for alpha diversity and PERMANOVA for beta diversity. Differential abundance statistics were performed using MaAsLin2 (Mallick et al., 2021).

RESULTS and DISCUSSION

Alpha and beta diversity were compared at the ASV level to achieve an in-depth comparison on species richness and diversity. Bacterial species grouped at the ASV level often have similar biochemical and phenotypic properties, allowing us to gather an understanding of the microbiota present in each sample (Baron, 1996). Alpha diversity describes the richness of the species present within a sample. No significant differences in alpha diversity were found in any of the sample types when compared across periods of lay. Beta diversity measures the

similarity or relatedness between sample types based on genera or ASVs present. The results showed that all three production periods had significantly different beta diversity (Unweighted UniFrac) for the hen cloacal samples (FDR=0.03) as seen in figure 5.1. This result was expected because of the physical changes that are occurring between each sample point. The Unweighted UniFrac beta diversity is more sensitive to the presence of low abundance AVSs than weighted abundance beta diversity metrics. Therefore, the results of beta diversity for the hen cloacal samples indicate that the communities during each time period were unique based on ASV's present regardless of their abundance. No significant differences were found on the nest pad or in rooster cloacal samples in regard to beta diversity. Studies including Awad et al. and Liu et al. analyzed the microbiome of laying pullets and broilers overtime and concluded that the microbiome of these chickens increased in both diversity and abundance over time, which was partially supported by our study (Awad et al., 2016; Liu et al. 2021). The differences between our study and these previously conducted studies is likely the age of the birds, since more changes occur earlier in development and our first measurement was at 27 weeks.

The multivariate factor analysis of time period on sample abundance was performed at the phylum level because of the significant differences we observed down the taxonomic hierarchy. In addition, many other studies comparing the microbiome of chickens performed their analysis at the phylum level and found significant differences that could be correlated to performance parameters (Díaz-Sánchez et al., 2019; *Xiao et al., 2023;* Yang et al., 2020). In the cloacal swab samples from hens, the abundance of *Desulfobacteria* (FDR=0.003), and *Firmicutes* (FDR=0.01) were significantly increased, while *Proteobacteria* (FDR=0.001) was significantly decreased between 27 and 34 weeks of age. A visual representation of the phylum differences can be seen in figure 5.2. Past research that compared the microbiome of broiler

breeders with high and average egg laying abilities at 41 weeks of age found that *Firmicutes* were significantly more abundant in high egg producing hens (Yang et al. 2020). The increase in *Firmicutes* between 27 and 34 weeks of age that we observed in this study could be correlated to the level of egg production, which is the highest at 34 weeks or peak production. In a different study by Díaz-Sánchez et al., researchers saw an increase in abundance of *Firmicutes* overtime in the fecal microbiota of Cobb broiler breeders, showing that changes in phylum abundance in broiler breeders could be based more on age than genetic strain (Díaz-Sánchez et al., 2019). During the transition from the peak period at 34 weeks to the post-peak period at 42 weeks, *Spirochaetota* (FDR=0.01) abundance was significantly increased in the hen samples. A recent study conducted by Yang et al. showed that *Spirochaetes* negatively affect egg production (Yang et al., 2020). In our study we saw an increase in *Spirochaetes* between 34 and 42 weeks of age which could be correlated to the decreased production that comes with age after peak production (Yang et al., 2020). We wanted to highlight differences in genera known to contain pathogens that contaminate hatching eggs to visualize how period of lay affected these parameters (Cortés et al., 2004). The results showed significant increases in *Pseudomonas* (FDR<0.0001) and *Streptococcus* (FDR=0.04) between the 27 and 34 weeks of age in the hen samples. The increase in these potential pathogens over time is important to consider in the sanitation process of eggs and the increased risk of contamination of hatching eggs.

There is limited research available looking at broiler breeder rooster microbiomes outside of semen contamination *(Tvrda et al., 2023)*. In the rooster samples, there were no significant differences at the phylum level between production periods. *Firmicutes*, *Actinobacteria*, and *Proteobacteria* were the three most abundant phylum across all three time periods tested as seen in figure 5.3. With cloacal microbiota being a potential source of semen contamination in

roosters, we choose to look for significant differences in pathogens over time. A recent study concluded that *Salmonella, Campylobacter*, *Bifidobacterium, E. coli, Clostridium*, and *Lactobacillus can all harm sperm motility, therefore inhibiting fertilization (Haines et al. 2013). Of these harmful bacteria, Escherichia (FDR=0.0484) was significantly decreased between* 27 and 34 weeks of age*. Although there were no other significant differences in pathogens over time, monitoring the levels of these pathogenic bacteria with relation to age is still important for understanding potential decreases in fertility caused by semen contamination.*

There is limited if any literature available on the litter microbiota of broiler breeders and the microbiota present on the nesting surface of broiler breeders. For the nest pad samples in this study, *Verrucomicrobiota* (FDR=0.004) was significantly increased, while *Campylobacterota* (FDR=0.004), *Bacteroidota* (FDR=0.008), *Synergistota* (FDR=0.04), and *Elusimicrobiota* (FDR=0.04) phylum abundances were significantly decreased between 27 and 34 weeks of age as shown in figure 5.4. Changes in phylum during this time period could be due to the increased usage of nest boxes as egg production increases. During the transition from the 34 (peak period) to 42 weeks of age (post-peak period), *Thermoplasmatoa* (FDR=0.01) and *Verrucomicrobiota* (FDR=0.04) phylum abundance were significantly decreased, while *Dependentiae* (FDR=0.005) and *Patescibacteria* (FDR=0.02) phylum abundance were significantly increased on the nest pad surface. Only one significant difference was found for the previously described pathogens at the genera level for nest pads. Between the 27 and 34 weeks of age the data revealed a significant decrease in *Staphylococcaceae* (FDR=0.03). *Staphylococcaceae* was also one of the only potential major hatching egg contaminants found in nest shavings in previous literature (Cortés et al., 2004). However, despite there being few differences in contaminants over time, their abundance was persistent. *Enterococcus*, *Streptococcus*, *Staphylococcus*, and *Escherichia* are all

in the top 20 most abundant genera present on the nest pad surface across the production cycle. This information emphasizes the importance of cleaning nesting surfaces and reducing the time eggs are exposed to the bacteria on the nest pad in order to reduce contamination.

Despite the many differences, we observed within and between sample types and time periods, *Firmicutes*, *Actinobacteria*, and *Proteobacteria* were the three most abundant phylum across all sample types which can be seen in figures 5.2, 5.3, and 5.4. Overall, the results characterized many differences over time in both phylum and genera, but only revealed a significant difference in beta diversity in the hen cloacal samples. To view all bacteria associated with female and male cloaca's and nest pads see appendix A.

CONCLUSION

The results from this study revealed a significant difference in taxonomic diversity and unique ASV's between each time point within the production cycle for the hen cloacal samples but revealed no significant differences for the rooster cloacal and nest pad samples. Despite the lack of significant alpha and beta diversity, there were significant differences in phylum and genera found between time periods across all sample types. Production period or age had more of an effect on the hen and nest pad phylum abundances than the rooster. At the genera level, all sample types revealed significant differences between potential pathogen abundances over time, including differences in *Pseudomonas*, *Streptococcus*, *Escherichia*, and *Staphylococcaceae*. Although the abundance of the other semen contamination and hatching egg pathogens did not change, they were still persistent on the surfaces. Therefore, as a persistent source of potential contamination for hatching eggs across all production periods, growers should prioritize keeping the nesting surfaces clean. *Future studies should include an evaluation of egg microbiota in addition to the nest pad microbiota to further understand the nest pads influence in*

contamination. In addition to *monitoring the levels of these pathogenic bacteria on nesting surfaces, paying attention to the impact of age on cloacal pathogen abundances is important as well. One example of this is that researchers should work to compare the bacteria present in rooster semen contamination with the individual rooster's cloacal microbiome over time to determine risk of contamination, which could be affecting fertility. The characterization and comparison over time that this study provides can be used in future studies that may further evaluate feed additives or probiotic effects on pathogen abundance over time. In conclusion, this study provides the poultry industry with the first microbiome characterization of one of the most prominent genetic lines and highlights the effects that* production period and age *has on the microbiome of broiler breeders and their environment.*

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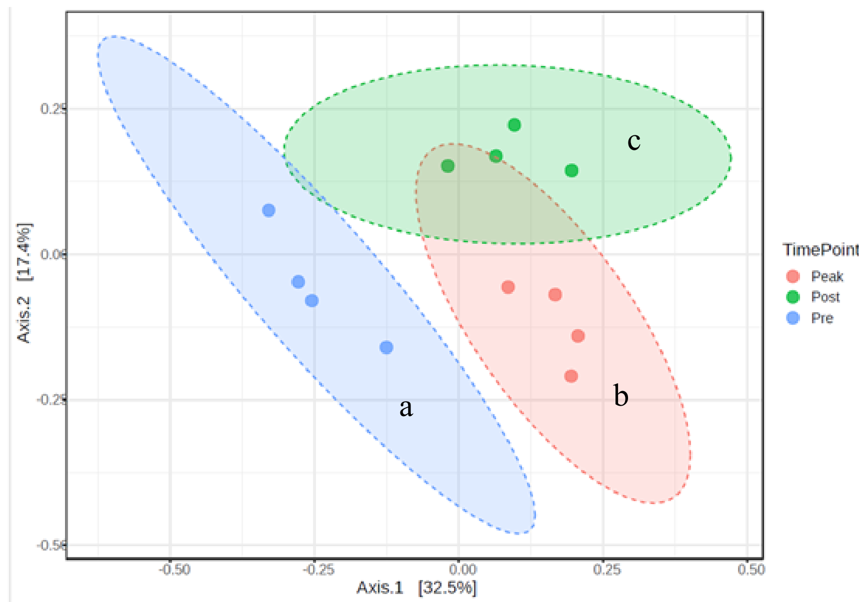

Figure 5.1 Hen Cloaca Beta Diversity (Unweighted UniFrac) by Production Period. This figure shows the beta diversity or the phylogenetic uniqueness of the microbiota at the ASV level, of the hen cloacal samples at 27 weeks or pre-peak (pre), 34 weeks or peak (peak) and 42 weeks or post-peak (post) using Unweighted UniFrac. The hen cloacal samples are represented over time with pre in blue, peak in red, and post in green. The FDR values for measure of significance were as follows pre vs peak (FDR=0.03), pre vs post (FDR=0.03), and peak vs post (FDR=0.03). [a,b,c] ellipses with different superscripts are significantly different.

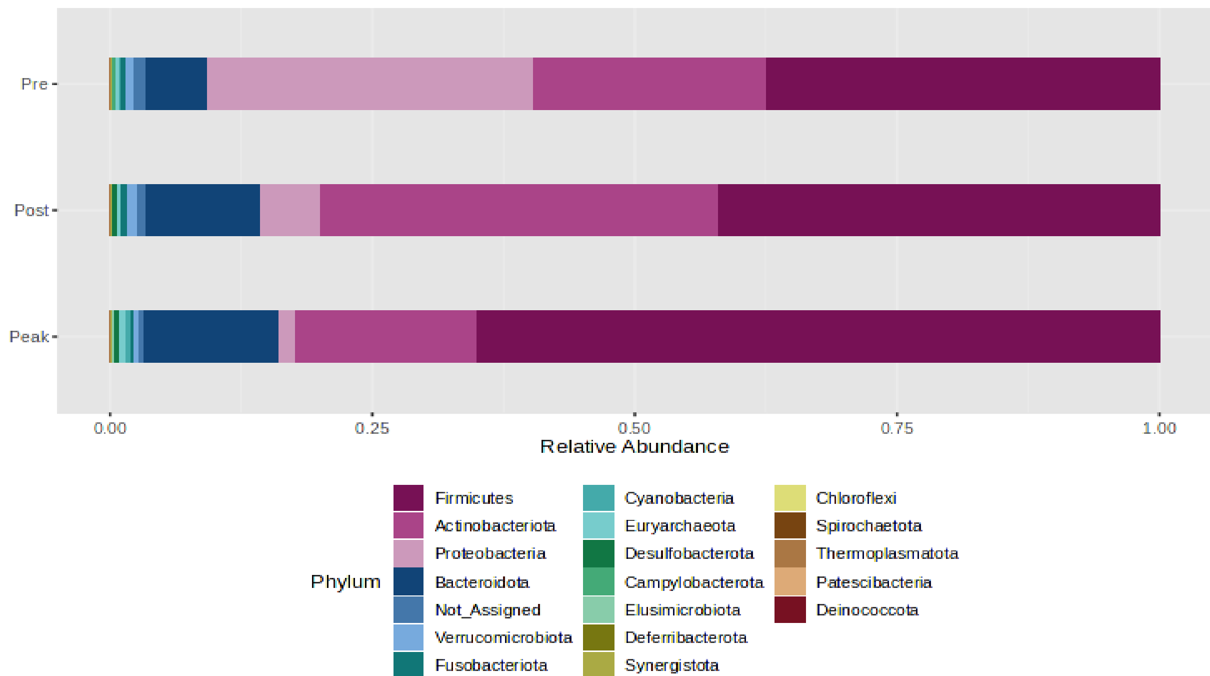

Figure 5.2 Abundance of Phylum in the Hen Cloaca. This figure shows the phylum present in the hen cloacal samples during each production period by percent abundance. The production periods are represented by 27 weeks or pre-peak (pre), 34 weeks or peak (peak), and 42 weeks or post-peak (post).

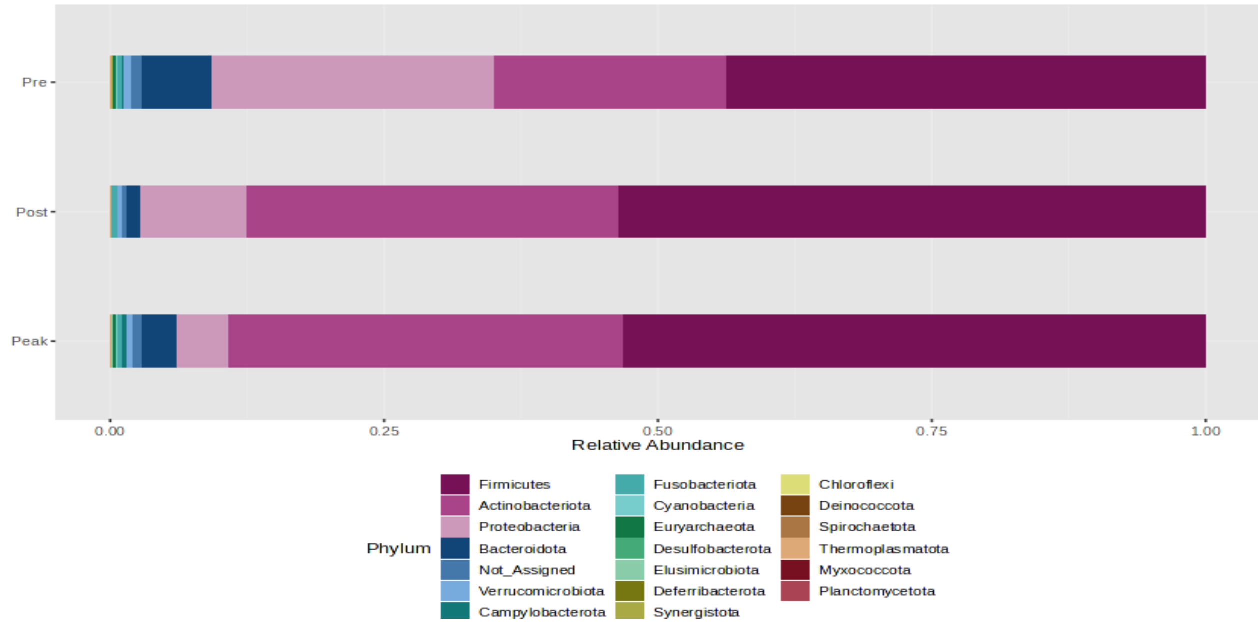

Figure 5.3 Abundance of Phylum in the Rooster Cloaca. This figure shows the phylum present in the rooster cloacal samples during each production period by percent abundance. The production periods are represented by 27 weeks or pre-peak (pre), 34 weeks or peak (peak), and 42 weeks or post-peak (post).

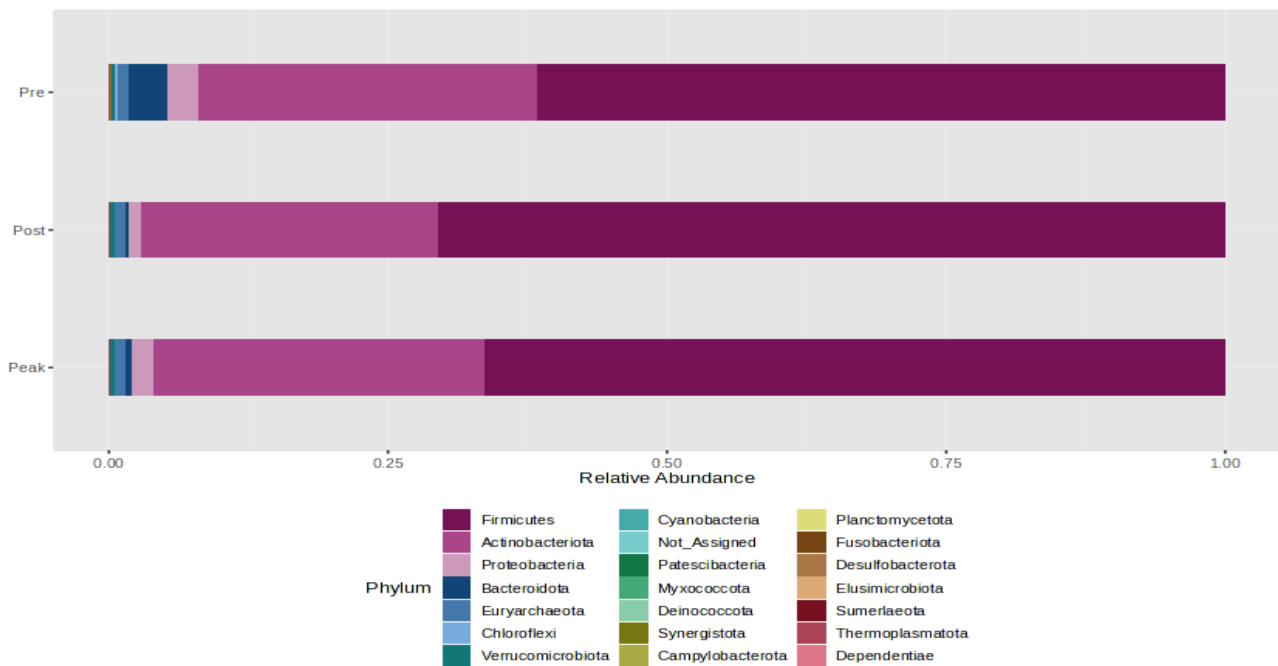

Figure 5.4 Abundance of Phylum on Nest Pads. This figure shows the phylum present in the nest pad samples during each production period by percent abundance. The production periods are represented by 27 weeks or pre-peak (pre), 34 weeks or peak (peak), and 42 weeks or post-peak (post).

CHAPTER 6

FINAL CONCLUSIONS

This thesis aimed to characterize and explore microbial influence on fertility in Ross 708 strain broiler breeders. In the first study we concluded that application of *Micrococcus luteus* to the surface of nest pads had no significant effects on hatchability and fertility, which are the main two production parameters for broiler breeders. However, we did see a significant difference in 7-day chick weight at peak production, with the *M. luteus* treated group having lower body weights. In addition, we saw significant differences in species richness and diversity between the cloacal microbiota of hens laying eggs on the *M. luteus* nest pads versus the control nest pads during peak production (33-36 wks). Therefore, we concluded that the addition of bacteria to the nesting surface of broiler breeders can alter the microbiome of the hens that sit on it. The impact *M. luteus* had although not significantly beneficial for production, does model the potential use of the nest pad for other probiotics and making shifts to the hen's microbiome. In the following study we further explored the effects of *M. luteus* on fertility by directly exposing rooster semen to the bacteria and evaluating any changes in motility. In this study *M. luteus* was studied alongside two probiotic species including *Lactobacillus acidophilus* and *Bacillus subtilis.* Our research revealed that *M. luteus* and *B. subtilis* had no significant effects on sperm motility, while *L. acidophilus* significantly diminished the motility of sperm. Further investigation revealed that diminished motility may have been a result of the media because *L. acidophilus* in PBS did not impact motility. We concluded that certain probiotic and commensal bacteria are of lower risk to fertility than others. This concept is important for the industry to consider when choosing, feed additives and probiotics that may eventually colonize the cloaca of the rooster and contaminate semen. Further research needs to be conducted on this topic to fully understand the

route of semen contamination and ways to manipulate which bacteria are contaminating the rooster semen. The last study that wrapped up this thesis evaluated the effect of period of lay on microbiota richness and diversity as well as pathogen abundance on the nest pad surfaces in broiler breeder pens and in the cloacas of the hens and roosters. The research revealed that there were significantly different communities of microbiota present in the cloaca of hens between each period measured by beta diversity. However, the nest pad and rooster cloacas stayed more consistent across periods showing no significant changes in microbiota richness or diversity. This study also evaluated the taxonomic abundances of each sample type and found significant differences at the phylum level showing that period of lay greatly impacts the abundance of different bacteria. In addition to the phylum level differences, significant differences in the abundance of both egg pathogens and semen contamination pathogens were observed across the production periods. Knowing the pathogens present in higher abundances during different points during the lay cycle allows producers to better evaluate the risks they are taking and cleaning procedures necessary. The last study also helps to wrap up the aim of this thesis and provide characterization that is useful for developing a baseline for the Ross 708 strain microbiota. Overall, this research worked to expand the current knowledge of broiler breeder microbiota, understand the effects that non-pathogenic bacteria have on semen motility, and determine the effects that altering the nest pad surface microbiota has on production parameters and cloacal microbiota.

APPENDIX

Appendix A: Supplemental Tables for Chapter 5

Table A.1: Phylum Percent Abundance within the Hen Cloacal Samples Over Time

	Period of Lay		
Phylum	34 wks	42 wks	27 wks
Actinomyces	0.021	0.195	0.038
Bacteroides	0.071	0.072	0.028
Brevibacterium	0.017	0.027	0.007
Clostridia_UCG_014	0.017	0.006	0.008
Corynebacterium	0.069	0.126	0.156
Enterococcus	0.009	0.008	0.031
Gallibacterium	0.001	0.021	0.046
Gallicola	0.005	0.052	0.002
Helcococcus	0.018	0.045	0.032
Intestinimonas	0.012	0.022	0.006
Lactobacillus	0.061	0.019	0.030
Ligilactobacillus	0.063	0.016	0.013
Limosilactobacillus	0.046	0.014	0.010
Megamonas	0.043	0.004	0.000
Muribaculaceae	0.020	0.007	0.011
Not_Assigned	0.128	0.087	0.128
Others	0.313	0.234	0.235
Pseudomonas	0.000	0.000	0.157
Romboutsia	0.033	0.006	0.004
Ruminococcus_torques_group	0.045	0.026	0.018
Streptococcus	0.007	0.011	0.040

Table A.2: Genus Percent Abundance within the Hen Cloacal Samples Over Time

	Period of Lay		
Genus	34 wks	42 wks	27 wks
A2	0.000062	0.000122	0.000419
Acetanaerobacterium	0.000008	0.000010	0.000004
Acetatifactor	0.000017	0.000042	0.000140
Acholeplasma	0.000112	0.000111	0.000007
Acinetobacter	0.000017	0.000064	0.000164
Actinobacteria	0.000028	0.000000	0.000223
Actinomyces	0.021276	0.195049	0.038233
Adlercreutzia	0.000018	0.000055	0.000031
Aeriscardovia	0.005304	0.000318	0.000201
Aerococcus	0.000000	0.000041	0.000000
Aerosphaera	0.000199	0.000059	0.000000
Akkermansia	0.002569	0.003584	0.006722
Aliicoccus	0.001726	0.000597	0.000440
Alistipes	0.012368	0.002332	0.004414
Alkalibacterium	0.000047	0.000047	0.000000
Alkaliphilus	0.000011	0.000026	0.000239
Alloiococcus	0.000513	0.002255	0.002295
Amphibacillus	0.000006	0.000059	0.000000
Anaerofilum	0.000619	0.000329	0.000176
Anaerofustis	0.000134	0.000020	0.000014
Anaerorhabdus_furcosa_group	0.000034	0.000000	0.000038
Anaerosporobacter	0.000136	0.000197	0.000000
Anaerostipes	0.001040	0.000207	0.000165
Anaerotruncus	0.000063	0.000110	0.000020
Angelakisella	0.000285	0.000071	0.000101
Arcobacter	0.000018	0.000382	0.000025
ASF356	0.000030	0.000030	0.000023
Atopostipes	0.000230	0.000725	0.001357
Bacillus	0.000452	0.000160	0.000248
Bacteroides	0.071467	0.071993	0.028209
Barnesiella	0.000919	0.000131	0.000041
Bifidobacterium	0.001430	0.000777	0.000769
Bilophila	0.000254	0.000682	0.000160
Blautia	0.004047	0.002848	0.000674

Brachybacterium	0.013661	0.008506	0.004322
Brevibacterium	0.016749	0.026610	0.006644
Butyricicoccus	0.004248	0.002846	0.000798
Butyricimonas	0.000605	0.003436	0.000219
CAG_352	0.000110	0.000021	0.000000
CAG_56	0.000391	0.000288	0.000237
Campylobacter	0.000421	0.000141	0.000000
Candidatus_Arthromitus	0.000032	0.000017	0.000000
Candidatus_Saccharimonas	0.000013	0.000013	0.000047
Candidatus_Soleaferrea	0.000008	0.000011	0.000011
Candidatus_Stoquefichus	0.000075	0.000135	0.000000
Caproiciproducens	0.000105	0.000024	0.000025
Catenibacillus	0.000226	0.000127	0.000066
Cellulosilyticum	0.000060	0.000191	0.000043
CHKCI001	0.001212	0.000337	0.000153
CHKCI002	0.000518	0.000188	0.000070
Christensenellaceae_R_7_group	0.006143	0.003320	0.003798
Clostridia_UCG_014	0.017361	0.006211	0.007892
Clostridia_vadinBB60_group	0.007074	0.003120	0.005250
Clostridioides	0.000307	0.000730	0.001211
Clostridium_innocuum_group	0.000120	0.000084	0.000000
Clostridium_sensu_stricto_1	0.006685	0.003377	0.002336
Colidextribacter	0.002568	0.001964	0.001407
Collinsella	0.000721	0.000249	0.000041
Corynebacterium	0.069077	0.126442	0.156268
Defluviitaleaceae_UCG_011	0.000103	0.000070	0.000255
Deinococcus	0.000000	0.000048	0.000000
Desulfovibrio	0.002503	0.003236	0.000000
Dielma	0.000130	0.000042	0.000029
Dietzia	0.001512	0.000728	0.000440
Dorea	0.003209	0.002379	0.001254
DTU089	0.001613	0.000396	0.001042
Eisenbergiella	0.000632	0.000344	0.000124
Elusimicrobium	0.001347	0.000242	0.000699
Enorma	0.011451	0.003527	0.000349
Enterococcus	0.008979	0.007705	0.031220
Enterorhabdus	0.000063	0.000048	0.000000
Epulopiscium	0.000556	0.000044	0.000216

Erysipelatoclostridiaceae	0.000007	0.000021	0.000023
Erysipelatoclostridium	0.004508	0.003352	0.001078
Erysipelothrix	0.000034	0.000087	0.000034
Erysipelotrichaceae	0.000023	0.000020	0.000052
Erysipelotrichaceae_UCG_003	0.001776	0.000532	0.000178
Escherichia_Shigella	0.001950	0.002325	0.058450
Eubacterium	0.000041	0.000081	0.000000
Eubacterium_brachy_group	0.000344	0.000143	0.000018
Eubacterium_coprostanoligenes_group	0.010999	0.001208	0.001720
Eubacterium_fissicatena_group	0.000255	0.000368	0.000696
Eubacterium_hallii_group	0.003672	0.001656	0.000652
Eubacterium_nodatum_group	0.000157	0.000094	0.000099
Eubacterium_ventriosum_group	0.000134	0.000031	0.000049
F082	0.000561	0.000722	0.000000
Facklamia	0.000428	0.000543	0.000016
Faecalibacterium	0.015860	0.008002	0.004362
Faecalicoccus	0.001627	0.001061	0.000248
Faecalitalea	0.000557	0.000164	0.000316
Falsochrobactrum	0.000033	0.000035	0.000000
Family_XIII_AD3011_group	0.000432	0.000170	0.000108
Family_XIII_UCG_001	0.000164	0.000115	0.000047
Fermentimonas	0.000100	0.000506	0.000000
Flaviflexus	0.001345	0.000606	0.000979
Flavobacterium	0.000002	0.000010	0.000018
Flavonifractor	0.000684	0.000399	0.000739
Fournierella	0.004317	0.001916	0.000223
Frisingicoccus	0.002543	0.000648	0.000566
Fusicatenibacter	0.000546	0.000056	0.000101
Fusobacterium	0.002504	0.007634	0.005029
Gallibacterium	0.001314	0.020953	0.045643
Gallicola	0.005350	0.051805	0.001695
Garicola	0.000045	0.000161	0.000000
Gastranaerophilales	0.007158	0.000896	0.002973
GCA_900066575	0.001072	0.000412	0.000581
Georgenia	0.000025	0.000031	0.000000
Globicatella	0.002113	0.004627	0.009326
Glutamicibacter	0.000010	0.000008	0.000079

Glycomyces	0.000087	0.000128	0.000018
Gracilibacillus	0.000015	0.000014	0.000000
Halomonas	0.000009	0.000033	0.000119
Helcococcus	0.017810	0.044675	0.031942
Helicobacter	0.001170	0.000164	0.003668
Holdemania	0.000183	0.000066	0.000049
Idiomarina	0.000006	0.000027	0.000131
Incertae_Sedis	0.003099	0.002058	0.001821
Intestinimonas	0.012336	0.022438	0.005543
Isoptericola	0.000979	0.000439	0.000832
Jeotgalibaca	0.000073	0.000059	0.000056
Jeotgalicoccus	0.006574	0.005302	0.000487
JG30_KF_CM45	0.000196	0.000024	0.000207
Jonesia	0.000051	0.000000	0.000043
Kocuria	0.000022	0.000024	0.000000
Kurthia	0.000017	0.000078	0.000000
Lachnoclostridium	0.002472	0.001726	0.003368
Lachnospiraceae_FE2018_group	0.000131	0.000033	0.000011
Lachnospiraceae_NC2004_group	0.000022	0.000011	0.000000
Lachnospiraceae_NK4A136_group	0.000212	0.000321	0.000545
Lachnospiraceae_UCG_006	0.000032	0.000030	0.000043
Lachnospiraceae_UCG_010	0.000444	0.000191	0.000156
Lactobacillus	0.060539	0.019216	0.030278
Lactococcus	0.001221	0.001820	0.003736
Leucobacter	0.000121	0.000042	0.000128
Ligilactobacillus	0.062746	0.016384	0.013442
Limosilactobacillus	0.046142	0.014374	0.010029
Macrococcus	0.000000	0.000143	0.000000
Mailhella	0.001267	0.000337	0.000000
Marinilutecoccus	0.000055	0.000033	0.000075
Marvinbryantia	0.001567	0.001194	0.000336
Megamonas	0.043267	0.003727	0.000359
Merdibacter	0.000128	0.000039	0.000016
Methanobrevibacter	0.004986	0.002261	0.001779
Methanomassiliicoccus	0.000058	0.000000	0.000025
Microbacterium	0.000170	0.000040	0.000656
Microlunatus	0.000298	0.000042	0.001294
Monoglobus	0.002858	0.001000	0.001021

Moraxella	0.002209	0.017540	0.000058
Mucispirillum	0.000721	0.000727	0.000144
Muribaculaceae	0.020481	0.007332	0.010640
Myceligenerans	0.000020	0.000014	0.000000
Mycetocola	0.000006	0.000047	0.000000
Negativibacillus	0.001990	0.000726	0.000219
Nesterenkonia	0.000065	0.000100	0.000000
Nitrosomonas	0.000010	0.000000	0.000014
NK4A214_group	0.001531	0.001314	0.000559
Nocardioides	0.000110	0.000055	0.000568
Nocardiopsis	0.000181	0.000339	0.000199
Not_Assigned	0.127460	0.087249	0.127931
Oceanisphaera	0.000000	0.000196	0.000000
Oceanobacillus	0.000011	0.000008	0.000038
Odoribacter	0.001846	0.000602	0.000602
Olsenella	0.010863	0.008640	0.001168
Ornithinicoccus	0.000766	0.000111	0.001060
Oscillibacter	0.000831	0.000488	0.000568
Oscillospira	0.000314	0.000298	0.000084
Paenibacillus	0.000297	0.000084	0.000537
Paeniclostridium	0.000068	0.000000	0.000208
Paludicola	0.000494	0.000300	0.000162
Papillibacter	0.000023	0.000021	0.000054
Parabacteroides	0.003408	0.004169	0.005268
Parapusillimonas	0.000035	0.000282	0.000000
Parasutterella	0.002082	0.000791	0.000456
Pedobacter	0.000007	0.000012	0.000153
Peptococcus	0.006195	0.001664	0.001680
Peptoniphilus	0.000920	0.001386	0.000000
Peptostreptococcales_Tissierellales	0.000045	0.000000	0.000058
Phascolarctobacterium	0.005547	0.008337	0.001842
Prevotellaceae_UCG_001	0.000067	0.000089	0.000000
Proteus	0.000215	0.000286	0.003968
Pseudactinotalea	0.000000	0.000046	0.000000
Pseudoalteromonas	0.000124	0.000272	0.000886
Pseudoflavonifractor	0.000444	0.000213	0.000300
Pseudomonas	0.000124	0.000164	0.157093
Pygmaiobacter	0.002105	0.000985	0.000784

RF39	0.003387	0.001091	0.001522
Rikenellaceae	0.000000	0.000025	0.000022
Rikenellaceae_RC9_gut_group	0.008575	0.014024	0.005354
Romboutsia	0.033174	0.006461	0.004037
Rothia	0.000337	0.000265	0.000140
Ruania	0.000343	0.000431	0.000309
Ruminococcus	0.000258	0.000113	0.000212
Ruminococcus_gauvreauii_group	0.000025	0.000011	0.000000
Ruminococcus_torques_group	0.045155	0.026405	0.018147
S5_A14a	0.000060	0.000042	0.000045
Salinicoccus	0.001973	0.004489	0.000041
Sellimonas	0.003778	0.001582	0.000708
Shuttleworthia	0.001391	0.000654	0.000548
Slackia	0.001312	0.000492	0.000115
Solibacillus	0.000258	0.000080	0.000117
Sphingobacterium	0.000059	0.000074	0.000361
Stackebrandtia	0.000176	0.000140	0.000129
Staphylococcaceae	0.000436	0.000285	0.016465
Staphylococcus	0.002351	0.009333	0.000936
Stenotrophomonas	0.000000	0.000000	0.000054
Streptococcus	0.006673	0.010816	0.039563
Streptomyces	0.000047	0.000052	0.000000
Subdoligranulum	0.005392	0.002633	0.000338
Sutterella	0.001380	0.000895	0.000755
Synergistes	0.000508	0.000363	0.000548
Terrisporobacter	0.000471	0.000164	0.000068
Tessaracoccus	0.000030	0.000388	0.000079
Thiopseudomonas	0.000403	0.003364	0.000000
Tissierella	0.000267	0.000097	0.000020
Treponema	0.000000	0.000368	0.000000
Turicibacter	0.011448	0.003543	0.002876
Tuzzerella	0.000584	0.000199	0.000243
Tyzzerella	0.000075	0.000192	0.000000
UBA1819	0.000181	0.000201	0.000000
UCG_002	0.000045	0.000003	0.000014
UCG_005	0.006805	0.002173	0.002743
UCG_007	0.000039	0.000000	0.000000
UCG_008	0.002567	0.000898	0.000776

UCG_009	0.000513	0.000272	0.000385
UCG_010	0.001515	0.000612	0.001280
uncultured	0.009938	0.007008	0.006441
Uruburuella	0.000012	0.000031	0.000160
vadinBE97	0.000037	0.000128	0.000020
Veillonella	0.000023	0.000000	0.000000
Vibrio	0.002137	0.003997	0.016380
Victivallaceae	0.000728	0.000532	0.000149
Victivallis	0.000183	0.000088	0.000034
Virgibacillus	0.000130	0.000379	0.000174
W5053	0.002944	0.005903	0.000047
WCHB1_41	0.000000	0.002288	0.000014
Yaniella	0.000176	0.001009	0.000000

Table A.3: Phylum Percent Abundance within the Rooster Cloacal Samples Over Time

	Period of Lay		
Phylum	34 wks	42 wks	27 wks
Actinomyces	0.024	0.127	0.025
Bacteroides	0.013	0.007	0.031
Brachybacterium	0.022	0.030	0.007
Brevibacterium	0.050	0.040	0.011
Clostridium_sensu_stricto_1	0.013	0.014	0.010
Corynebacterium	0.181	0.109	0.139
Enterococcus	0.117	0.039	0.019
Gallibacterium	0.027	0.070	0.073
Gallicola	0.014	0.044	0.003
Helcococcus	0.022	0.026	0.029
Jeotgalicoccus	0.012	0.029	0.006
Lactobacillus	0.030	0.033	0.053
Ligilactobacillus	0.036	0.030	0.018
Limosilactobacillus	0.019	0.014	0.010
Not_Assigned	0.135	0.090	0.107
Others	0.194	0.120	0.248
Pseudomonas	0.000	0.000	0.140
Romboutsia	0.020	0.061	0.012
Staphylococcus	0.008	0.019	0.005
Streptococcus	0.046	0.035	0.046
Turicibacter	0.017	0.062	0.010

Table A.4: Genus Percent Abundance within the Rooster Cloacal Samples Over Time

	Period of Lay		
Genus	34 wks	42 wks	27 wks
A2	0.000103	0.000140	0.000355
Acinetobacter	0.000029	0.000027	0.000096
Actinobacteria	0.000053	0.000000	0.000267
Actinomyces	0.023696	0.127246	0.024894
Adlercreutzia	0.000062	0.000080	0.000026
Aeriscardovia	0.008327	0.002476	0.003754
Aerococcus	0.000021	0.000016	0.000000
Aerosphaera	0.000313	0.000162	0.000090
Akkermansia	0.002532	0.002615	0.003773
Aliicoccus	0.004924	0.000803	0.002049
Alistipes	0.004051	0.000378	0.004049
Alkalibacterium	0.000046	0.000119	0.000000
Alloiococcus	0.006608	0.001200	0.013100
Anaerofilum	0.000153	0.000068	0.000360
Anaerofustis	0.000011	0.000001	0.000040
Anaerorhabdus_furcosa_group	0.000012	0.000000	0.000019
Anaerosporobacter	0.000638	0.000407	0.000107
Anaerostipes	0.000028	0.000035	0.000236
Angelakisella	0.000075	0.000027	0.000074
Antricoccus	0.000015	0.000000	0.000031
Atopostipes	0.000525	0.000600	0.000439
Bacillus	0.000208	0.000133	0.000224
Bacteroides	0.012801	0.007012	0.030594
Barnesiella	0.000153	0.000076	0.000940
Bifidobacterium	0.007029	0.001061	0.001373
Bilophila	0.000108	0.000017	0.000190
Blautia	0.000324	0.000646	0.001243
Bombiscardovia	0.000918	0.000289	0.000008
Brachybacterium	0.021640	0.029641	0.006560
Brevibacterium	0.050469	0.040469	0.011258
Butyricicoccus	0.000465	0.000198	0.001943
Butyricimonas	0.000080	0.000078	0.000144
CAG_352	0.000062	0.000010	0.000040
CAG_56	0.000112	0.000016	0.000242
Campylobacter	0.004992	0.000003	0.000000
Candidatus_Arthromitus	0.000043	0.000040	0.000006

Candidatus_Soleaferrea	0.000007	0.000007	0.000018
Candidatus_Stoquefichus	0.000016	0.000013	0.000000
Candidatus_Vestibaculum	0.000000	0.000101	0.000000
Caproiciproducens	0.000108	0.000006	0.000145
Caryophanon	0.000095	0.000045	0.000080
Catenibacillus	0.000057	0.000013	0.000082
Cellulosilyticum	0.000376	0.000751	0.000332
Cellulosimicrobium	0.000058	0.000000	0.000018
CHKCI001	0.000147	0.000080	0.000114
CHKCI002	0.000080	0.000071	0.000082
Christensenellaceae_R_7_group	0.003416	0.000620	0.002942
Clostridia_UCG_014	0.003167	0.001038	0.005826
Clostridia_vadinBB60_group	0.003371	0.000307	0.003896
Clostridioides	0.000334	0.000608	0.000772
Clostridium_innocuum_group	0.000123	0.000007	0.000000
Clostridium_sensu_stricto_1	0.013167	0.013956	0.009739
Clostridium_sensu_stricto_13	0.000054	0.000025	0.000056
Colidextribacter	0.000917	0.000361	0.001695
Collinsella	0.000065	0.000139	0.000272
Corynebacterium	0.181140	0.108987	0.139013
Defluviitaleaceae_UCG_011	0.000008	0.000007	0.000074
Deinococcus	0.000039	0.000022	0.000040
Desulfovibrio	0.000930	0.000802	0.000000
Dielma	0.000147	0.000019	0.000133
Dietzia	0.006645	0.003262	0.000688
Dorea	0.001153	0.000825	0.000903
DTU089	0.000339	0.000020	0.000799
Eisenbergiella	0.000112	0.000034	0.000236
Elusimicrobium	0.000772	0.000057	0.000461
Enhydrobacter	0.000007	0.000011	0.000008
Enorma	0.001002	0.000911	0.000799
Enteractinococcus	0.000036	0.000023	0.000000
Enterococcus	0.116711	0.038978	0.019437
Epulopiscium	0.001777	0.001159	0.001521
Erysipelatoclostridium	0.000484	0.000270	0.001864
Erysipelothrix	0.000000	0.000095	0.000004
Erysipelotrichaceae	0.000024	0.000012	0.000031
Erysipelotrichaceae_UCG_003	0.000321	0.000127	0.000184
Escherichia_Shigella	0.002266	0.001380	0.021450
Eubacterium_brachy_group	0.000013	0.000032	0.000037

Eubacterium_coprostanoligenes_group	0.001832	0.000450	0.002468
Eubacterium_fissicatena_group	0.000167	0.000176	0.000192
Eubacterium_hallii_group	0.000624	0.000267	0.000821
Eubacterium_nodatum_group	0.000026	0.000047	0.000000
Eubacterium_ventriosum_group	0.000095	0.000016	0.000087
F082	0.000072	0.000089	0.000000
Facklamia	0.000728	0.001418	0.000049
Faecalibacterium	0.001189	0.000415	0.004714
Faecalicoccus	0.000701	0.000212	0.000230
Faecalitalea	0.000627	0.000031	0.000405
Falsochrobactrum	0.000045	0.000076	0.000000
Family_XIII_AD3011_group	0.000187	0.000031	0.000173
Family_XIII_UCG_001	0.000046	0.000000	0.000058
Flaviflexus	0.000613	0.000448	0.000248
Flavobacterium	0.000004	0.000072	0.000020
Flavonifractor	0.000329	0.000049	0.001325
Fournierella	0.000379	0.000128	0.000627
Frisingicoccus	0.000926	0.000317	0.001457
Fusicatenibacter	0.000283	0.000000	0.000443
Fusobacterium	0.003065	0.004309	0.002353
Gallibacterium	0.027461	0.070466	0.072542
Gallicola	0.013611	0.044395	0.002620
Garicola	0.000073	0.000195	0.000000
Gastranaerophilales	0.001924	0.000142	0.002891
GCA_900066575	0.000710	0.000130	0.000991
Georgenia	0.000106	0.000057	0.000000
Globicatella	0.003404	0.007393	0.007829
Glutamicibacter	0.000022	0.000016	0.000088
Glycomyces	0.000461	0.000220	0.000007
Gordonia	0.000219	0.000048	0.000114
Gracilibacillus	0.000124	0.000043	0.000007
Halomonas	0.000016	0.000000	0.000068
Helcococcus	0.022076	0.025656	0.028657
Helicobacter	0.001094	0.000494	0.004010
Herbinix	0.000066	0.000021	0.000125
Holdemania	0.000053	0.000011	0.000084
Idiomarina	0.000004	0.000000	0.000019
Incertae_Sedis	0.000981	0.000361	0.001903
Intestinimonas	0.006335	0.001118	0.005480
Isoptericola	0.005492	0.001017	0.001961

Jeotgalibaca	0.000102	0.000094	0.000027
Jeotgalicoccus	0.012193	0.028670	0.006365
JG30_KF_CM45	0.000386	0.000086	0.000463
Kocuria	0.000022	0.000062	0.000006
Kurthia	0.000266	0.000071	0.000019
Lachnoclostridium	0.000912	0.000716	0.002278
Lachnospiraceae_FE2018_group	0.000064	0.000000	0.000050
Lachnospiraceae_NK4A136_group	0.000321	0.000351	0.000404
Lachnospiraceae_UCG_006	0.000033	0.000022	0.000051
Lachnospiraceae_UCG_010	0.000112	0.000022	0.000245
Lacticigenium	0.000050	0.000084	0.000000
Lactobacillus	0.030217	0.033231	0.052972
Lactococcus	0.001561	0.001728	0.002603
Leucobacter	0.000370	0.000176	0.000188
Ligilactobacillus	0.036094	0.029837	0.017996
Limosilactobacillus	0.019114	0.013629	0.009749
Listeria	0.000028	0.000030	0.000040
Luteimonas	0.000006	0.000000	0.000032
Macrococcus	0.000068	0.000111	0.000038
Mailhella	0.000211	0.000039	0.000000
Marinilutecoccus	0.000170	0.000081	0.000129
Marvinbryantia	0.000262	0.000133	0.000510
Megamonas	0.000393	0.000211	0.003821
Merdibacter	0.000382	0.000098	0.000183
Methanobrevibacter	0.001042	0.000865	0.001854
Methanomassiliicoccus	0.000042	0.000000	0.000037
Microbacterium	0.000611	0.000178	0.002076
Microlunatus	0.000781	0.000097	0.002185
Monoglobus	0.000344	0.000167	0.001102
Moraxella	0.005774	0.014419	0.000036
Mucispirillum	0.000575	0.000090	0.000461
Muribaculaceae	0.005855	0.001564	0.010781
Myceligenerans	0.000054	0.000035	0.000000
Mycetocola	0.000044	0.000051	0.000012
Mycobacterium	0.000059	0.000007	0.000013
Negativibacillus	0.000382	0.000077	0.000497
Nesterenkonia	0.000427	0.000214	0.000046
NK4A214_group	0.000795	0.000209	0.000833
Nocardioides	0.000339	0.000089	0.000728
Nocardiopsis	0.000803	0.000572	0.000478

Not_Assigned	0.135187	0.090330	0.106692
Oceanisphaera	0.000010	0.000034	0.000000
Oceanobacillus	0.000074	0.000050	0.000239
Odoribacter	0.000567	0.000045	0.000691
Oligella	0.000011	0.000141	0.000000
Olsenella	0.001234	0.001457	0.002564
Ornithinicoccus	0.001249	0.000294	0.001794
Oscillibacter	0.000330	0.000109	0.000445
Oscillospira	0.000260	0.000111	0.000445
Oxalobacter	0.000024	0.000009	0.000027
Paenalcaligenes	0.000002	0.000032	0.000014
Paenibacillus	0.001295	0.000207	0.000850
Paeniclostridium	0.000083	0.000000	0.000141
Paludicola	0.000254	0.000063	0.000419
Papillibacter	0.000022	0.000008	0.000019
Parabacteroides	0.002274	0.001608	0.003010
Parapusillimonas	0.000013	0.002047	0.000000
Parasutterella	0.000134	0.000032	0.000215
Paucisalibacillus	0.000033	0.000013	0.000000
Pedobacter	0.000029	0.000000	0.000129
Peptococcus	0.000824	0.000444	0.002397
Peptoniphilus	0.000466	0.000215	0.000000
Peptostreptococcales_Tissierellales	0.000031	0.000000	0.000089
Phascolarctobacterium	0.001004	0.001366	0.002096
Pisciglobus	0.000028	0.000006	0.000000
Planococcus	0.000056	0.000020	0.000030
Proteiniclasticum	0.000081	0.000000	0.000040
Proteus	0.000400	0.000026	0.001835
Pseudactinotalea	0.000019	0.000095	0.000072
Pseudoalteromonas	0.000375	0.000316	0.000943
Pseudoflavonifractor	0.000214	0.000057	0.000432
Pseudomonas	0.000171	0.000187	0.139979
Pseudonocardia	0.000011	0.000000	0.000011
Pygmaiobacter	0.000589	0.000229	0.002502
RF39	0.001120	0.000227	0.001700
Rhodanobacter	0.000004	0.000004	0.000014
Rhodococcus	0.000018	0.000000	0.000027
Rikenellaceae_RC9_gut_group	0.005599	0.001651	0.009110
Romboutsia	0.019716	0.061061	0.012230
Rothia	0.000658	0.001125	0.000335

Ruania	0.001034	0.000713	0.000519
Ruminococcus	0.000063	0.000047	0.000166
Ruminococcus_torques_group	0.012057	0.003754	0.020662
Saccharopolyspora	0.000017	0.000004	0.000012
Salinicoccus	0.004521	0.014699	0.000013
Sellimonas	0.000443	0.000249	0.000910
Shuttleworthia	0.000161	0.000054	0.000325
Slackia	0.000114	0.000098	0.000196
Solibacillus	0.000589	0.000321	0.000452
Sphingobacterium	0.000077	0.000036	0.000336
Sporobacter	0.000015	0.000000	0.000018
Sporosarcina	0.000023	0.000031	0.000000
Stackebrandtia	0.000747	0.000228	0.000204
Staphylococcaceae	0.001678	0.000765	0.004617
Staphylococcus	0.007945	0.019399	0.004747
Stenotrophomonas	0.000000	0.000000	0.000074
Streptococcus	0.046019	0.034525	0.046030
Streptomyces	0.000342	0.000110	0.000051
Subdoligranulum	0.000207	0.000378	0.001042
Sutterella	0.000228	0.000034	0.001229
Synergistes	0.000472	0.000051	0.000572
Terrisporobacter	0.001859	0.000627	0.001840
Tessaracoccus	0.000047	0.000000	0.000104
Thiopseudomonas	0.000031	0.000743	0.000000
Treponema	0.000000	0.000101	0.000000
Turicibacter	0.016972	0.061839	0.009562
Tuzzerella	0.000551	0.000128	0.000471
UCG_002	0.000068	0.000017	0.000006
UCG_005	0.001905	0.000392	0.002101
UCG_008	0.000131	0.000163	0.000516
UCG_009	0.000173	0.000040	0.000239
UCG_010	0.001292	0.000151	0.001880
uncultured	0.005874	0.001910	0.006018
Uruburuella	0.000035	0.000060	0.000134
vadinBE97	0.000054	0.000004	0.000144
Vagococcus	0.000028	0.000000	0.000014
Veillonella	0.001263	0.000162	0.000355
Vibrio	0.006331	0.004871	0.015760
Victivallaceae	0.000623	0.000100	0.000256
Victivallis	0.000120	0.000022	0.000101

Virgibacillus	0.000266	0.000410	0.000102
W5053	0.002252	0.006442	0.000023
WCHB1_41	0.000000	0.000424	0.000000
Weissella	0.000012	0.000012	0.000015
Yaniella	0.000354	0.002541	0.000000

Table A.5: Phylum Percent Abundance within the Nest Pad Samples Over Time

	Period of Lay		
Phylum	34 wks	42 wks	27 wks
Brachybacterium	0.052	0.026	0.051
Brevibacterium	0.054	0.048	0.077
Clostridia_UCG_014	0.009	0.016	0.015
Corynebacterium	0.073	0.066	0.020
Enterococcus	0.017	0.028	0.013
Escherichia_Shigella	0.010	0.004	0.010
Faecalibacterium	0.004	0.009	0.008
Jeotgalicoccus	0.036	0.019	0.007
Lactobacillus	0.040	0.044	0.055
Ligilactobacillus	0.056	0.057	0.029
Limosilactobacillus	0.049	0.041	0.043
Methanobrevibacter	0.008	0.010	0.009
Not_Assigned	0.211	0.232	0.182
Olsenella	0.009	0.019	0.008
Others	0.179	0.195	0.273
Romboutsia	0.013	0.016	0.014
Ruminococcus_torques_group	0.023	0.035	0.040
Staphylococcaceae	0.005	0.002	0.015
Staphylococcus	0.116	0.106	0.105
Streptococcus	0.026	0.018	0.016
Turicibacter	0.010	0.009	0.010

Table A.6: Genus Percent Abundance within the Nest Pad Samples Over Time

	Period of Lay		
Genus	34 wks	42 wks	27 wks
A2	0.000160	0.000094	0.000074
Acetatifactor	0.000038	0.000004	0.000012
Acinetobacter	0.002892	0.002898	0.006881
Actinobacteria	0.000195	0.000050	0.001782
Actinomyces	0.000278	0.000317	0.000199
Adlercreutzia	0.000056	0.000034	0.000014
Advenella	0.000020	0.000003	0.000263
Aeriscardovia	0.002212	0.001917	0.000617
Aerococcus	0.000473	0.002799	0.000134
Aeromicrobium	0.000044	0.000016	0.000108
Aerosphaera	0.003207	0.000575	0.002321
Agrococcus	0.000046	0.000006	0.000026
AKAU3644	0.000015	0.000003	0.000154
Akkermansia	0.002681	0.001499	0.000950
Alcaligenes	0.000118	0.000000	0.000139
Aliicoccus	0.013920	0.003132	0.002493
Alistipes	0.000044	0.000056	0.000983
Alkalibacterium	0.000060	0.000061	0.000000
Alkaliphilus	0.000020	0.000011	0.000000
Alloiococcus	0.000068	0.000014	0.000020
Altererythrobacter	0.000008	0.000001	0.000080
Aminobacter	0.000022	0.000000	0.000027
Ammoniphilus	0.000027	0.000050	0.000006
Amphibacillus	0.000052	0.000059	0.000005
Anaerofilum	0.000097	0.000166	0.000314
Anaerofustis	0.000057	0.000067	0.000073
Anaerorhabdus_furcosa_group	0.000015	0.000008	0.000040
Anaerosporobacter	0.000200	0.000552	0.000113
Anaerostipes	0.000929	0.000780	0.001721
Anaerotruncus	0.000026	0.000000	0.000067
Angelakisella	0.000110	0.000101	0.000183
Antricoccus	0.000052	0.000010	0.000305
Arcobacter	0.000004	0.000003	0.000024
Arenibacter	0.000008	0.000012	0.000000
Arenimonas	0.000010	0.000006	0.000062
ASF356	0.000003	0.000007	0.000080

Atopostipes	0.000083	0.000041	0.000022
Bacillus	0.000383	0.000219	0.000385
Bacteroides	0.000342	0.000826	0.010822
Barnesiella	0.000000	0.000012	0.000061
Bergeyella	0.000009	0.000002	0.000491
Bifidobacterium	0.001919	0.001926	0.001286
Bilophila	0.000000	0.000000	0.000098
Blautia	0.003092	0.005246	0.003224
Bombiscardovia	0.000056	0.000153	0.000003
Brachybacterium	0.052132	0.026248	0.050829
Bradymonadaceae	0.000028	0.000015	0.000025
Brevibacterium	0.054297	0.048032	0.077436
Brevundimonas	0.000011	0.000000	0.000024
Butyricicoccus	0.002097	0.003843	0.003744
Butyricimonas	0.000000	0.000000	0.000043
CAG_352	0.000064	0.000061	0.000063
CAG_56	0.000092	0.000109	0.000293
Candidatus_Arthromitus	0.000046	0.000052	0.000013
Candidatus_Babela	0.000002	0.000017	0.000000
Candidatus_Berkiella	0.000009	0.000007	0.000006
Candidatus_Saccharimonas	0.000084	0.000667	0.000217
Candidatus_Soleaferrea	0.000014	0.000012	0.000030
Candidatus_Stoquefichus	0.000028	0.000105	0.000000
Caproiciproducens	0.000068	0.000068	0.000108
Caryophanon	0.000014	0.000006	0.000000
Catenibacillus	0.000102	0.000154	0.000228
Cellulosilyticum	0.000088	0.000287	0.000081
Cellulosimicrobium	0.000171	0.000088	0.000284
Cellvibrio	0.000015	0.000020	0.000243
CHKCI001	0.001002	0.000992	0.001189
CHKCI002	0.000442	0.000710	0.000364
Christensenellaceae_R_7_group	0.005152	0.007412	0.007513
Chryseobacterium	0.000003	0.000020	0.000000
Citricoccus	0.000078	0.000000	0.000163
Clostridia_UCG_014	0.009155	0.016362	0.014768
Clostridia_vadinBB60_group	0.000030	0.000033	0.000404
Clostridioides	0.000387	0.000351	0.000544
Clostridium_innocuum_group	0.000033	0.000104	0.000000
Clostridium_sensu_stricto_1	0.005298	0.005273	0.003269
Colidextribacter	0.000290	0.000611	0.002283

Collinsella	0.001488	0.001695	0.000160
Confluentibacter	0.000004	0.000003	0.000033
Coprococcus	0.000008	0.000000	0.000081
Corynebacterium	0.073318	0.065972	0.020483
Cytophaga	0.000016	0.000005	0.000040
Defluviitaleaceae_UCG_011	0.000044	0.000117	0.000260
Deinococcus	0.000266	0.000102	0.000182
Demequina	0.000059	0.000032	0.000024
Desulfovibrio	0.000012	0.000066	0.000000
Devosia	0.000015	0.000022	0.000017
Dielma	0.000050	0.000084	0.000071
Dietzia	0.007436	0.005834	0.005864
Dorea	0.005298	0.005723	0.006211
DTU089	0.000219	0.000303	0.001092
Eisenbergiella	0.000073	0.000211	0.000380
Elusimicrobium	0.000012	0.000005	0.000116
Enhydrobacter	0.000035	0.000022	0.000023
Enorma	0.007291	0.008831	0.002820
Enteractinococcus	0.000105	0.000080	0.000025
Enterococcus	0.016852	0.027893	0.012771
Enterorhabdus	0.000090	0.000188	0.000028
Epulopiscium	0.000212	0.000160	0.000224
Erysipelatoclostridium	0.001182	0.002286	0.002256
Erysipelothrix	0.000043	0.000021	0.000057
Erysipelotrichaceae	0.000025	0.000025	0.000026
Erysipelotrichaceae_UCG_003	0.000961	0.001232	0.000756
Escherichia_Shigella	0.009550	0.004355	0.009657
Eubacterium_brachy_group	0.000195	0.000489	0.000121
Eubacterium_coprostanoligenes_group	0.000992	0.001470	0.002981
Eubacterium_fissicatena_group	0.000247	0.000274	0.000112
Eubacterium_hallii_group	0.002930	0.003372	0.003086
Eubacterium_nodatum_group	0.000029	0.000028	0.000061
Eubacterium_ventriosum_group	0.000093	0.000054	0.000171
Eubacterium_xylanophilum_group	0.000010	0.000008	0.000037
Facklamia	0.007124	0.005571	0.000528
Faecalibacterium	0.003944	0.009099	0.008241
Faecalicoccus	0.000813	0.001545	0.000604
Faecalitalea	0.000160	0.000329	0.000393
Falsochrobactrum	0.000511	0.000150	0.000095
Family_XIII_AD3011_group	0.000195	0.000409	0.000302

Family_XIII_UCG_001	0.000048	0.000109	0.000131
Flaviflexus	0.000103	0.000034	0.000102
Flavobacterium	0.000111	0.000091	0.001081
Flavonifractor	0.000096	0.000110	0.001223
Fournierella	0.000446	0.000799	0.000470
Frisingicoccus	0.001363	0.002822	0.001660
Fusicatenibacter	0.000239	0.000256	0.000322
Fusobacterium	0.000037	0.000083	0.000183
Galbibacter	0.000001	0.000034	0.000000
Gallibacterium	0.000132	0.000111	0.000071
Gallicola	0.000905	0.002420	0.000078
Garicola	0.000177	0.000208	0.000010
Gastranaerophilales	0.000183	0.000560	0.000699
GCA_900066575	0.000088	0.000413	0.001522
Gemmobacter	0.000024	0.000002	0.000184
Georgenia	0.000242	0.000157	0.000036
Globicatella	0.000142	0.000133	0.000036
Glutamicibacter	0.000368	0.000099	0.001684
Glycomyces	0.001030	0.001094	0.000133
Gordonia	0.000279	0.000212	0.000697
Gottschalkia	0.000021	0.000012	0.000027
Gracilibacillus	0.000195	0.000107	0.000040
Gulosibacter	0.000077	0.000031	0.000064
Haloactinopolyspora	0.000009	0.000019	0.000003
Halomonas	0.000347	0.000044	0.000000
HAW_RM37_2	0.000002	0.000006	0.000012
Helcococcus	0.000091	0.000074	0.000070
Helicobacter	0.000004	0.000011	0.000367
Herbinix	0.000039	0.000020	0.000068
Holdemania	0.000061	0.000095	0.000130
Huakuichenia	0.000029	0.000017	0.000022
Incertae_Sedis	0.001866	0.002815	0.003423
Intestinimonas	0.001654	0.002344	0.005191
Isoptericola	0.004558	0.002622	0.008188
Jeotgalibaca	0.001970	0.000288	0.001403
Jeotgalicoccus	0.035876	0.018761	0.006792
JG30_KF_CM45	0.001325	0.000527	0.004167
Jonesia	0.000230	0.000078	0.000782
Kocuria	0.000695	0.000381	0.000199
Kurthia	0.001559	0.003229	0.013567

Lachnoclostridium	0.001228	0.001344	0.002539
Lachnospiraceae_FE2018_group	0.000098	0.000128	0.000114
Lachnospiraceae_NC2004_group	0.000019	0.000072	0.000024
Lachnospiraceae_NK4A136_group	0.000309	0.000176	0.000091
Lachnospiraceae_UCG_001	0.000014	0.000004	0.000026
Lachnospiraceae_UCG_006	0.000031	0.000016	0.000015
Lachnospiraceae_UCG_010	0.000037	0.000115	0.000568
Lacticigenium	0.000033	0.000021	0.000043
Lactobacillus	0.039560	0.044166	0.054636
Lactococcus	0.001670	0.001349	0.001416
Leucobacter	0.001294	0.000609	0.002561
Ligilactobacillus	0.055616	0.056820	0.029012
Limosilactobacillus	0.048934	0.041402	0.043156
Listeria	0.000012	0.000012	0.000026
Luteimonas	0.000076	0.000006	0.000304
Lysinibacillus	0.000126	0.000139	0.000007
Lysobacter	0.000090	0.000006	0.000095
Macrococcus	0.000392	0.000209	0.000386
Marinilutecoccus	0.000181	0.000147	0.000500
Marinimicrobium	0.000031	0.000029	0.000001
Marinobacter	0.000003	0.000007	0.000009
Marinococcus	0.000008	0.000000	0.000009
Marvinbryantia	0.000864	0.001507	0.001004
Megamonas	0.002598	0.004035	0.004792
Merdibacter	0.000134	0.000174	0.000311
Methanobrevibacter	0.008130	0.009903	0.009060
Methanomassiliicoccus	0.000019	0.000001	0.000004
Microbacterium	0.001684	0.000660	0.008589
Microlunatus	0.001762	0.000377	0.014181
Moheibacter	0.000040	0.000017	0.000272
Monoglobus	0.001818	0.003012	0.002774
Moraxella	0.000026	0.000016	0.000000
Muribaculaceae	0.001042	0.000703	0.002009
MWH_CFBk5	0.000044	0.000045	0.000066
Myceligenerans	0.000062	0.000093	0.000013
Mycetocola	0.000050	0.000103	0.000052
Mycobacterium	0.000042	0.000055	0.000023
Myroides	0.000014	0.000017	0.000000
Nakamurella	0.000004	0.000006	0.000044
Negativibacillus	0.000177	0.000407	0.000520

Nesterenkonia	0.000486	0.000548	0.000082
Nitriliruptoraceae	0.000010	0.000023	0.000000
NK4A214_group	0.000766	0.001018	0.001302
Nocardioides	0.000710	0.000223	0.005056
Nocardiopsis	0.002206	0.002297	0.005157
Nosocomiicoccus	0.000000	0.000040	0.000000
Not_Assigned	0.211081	0.231822	0.182016
Oceanimonas	0.000012	0.000006	0.000210
Oceanisphaera	0.000066	0.000095	0.000000
Oceanobacillus	0.000380	0.000248	0.000180
Odoribacter	0.000008	0.000007	0.000114
Oligella	0.000045	0.000031	0.000003
Olsenella	0.008867	0.018641	0.008192
Ornithinicoccus	0.002576	0.000686	0.008984
Oscillibacter	0.000121	0.000232	0.000643
Oscillospira	0.000038	0.000025	0.000243
Paenalcaligenes	0.000314	0.000056	0.000190
Paenibacillus	0.000590	0.000229	0.000840
Paenochrobactrum	0.000097	0.000060	0.000099
Paludicola	0.000231	0.000399	0.000484
Papillibacter	0.000024	0.000023	0.000083
Parabacteroides	0.001936	0.001050	0.001224
Paraclostridium	0.000132	0.000907	0.000000
Paracoccus	0.000026	0.000024	0.000020
Parapedobacter	0.000045	0.000076	0.000066
Parasutterella	0.000014	0.000009	0.000212
Paucisalibacillus	0.000132	0.000064	0.000036
Pedobacter	0.000139	0.000047	0.002639
Pelagibacterium	0.000040	0.000027	0.000129
Peptococcus	0.004867	0.005460	0.007635
Peptostreptococcus	0.000026	0.000008	0.000000
Phascolarctobacterium	0.000618	0.001489	0.002498
Pisciglobus	0.000187	0.001165	0.000099
Planococcus	0.000016	0.000018	0.000040
Propionibacteriaceae	0.000031	0.000014	0.000002
Proteiniclasticum	0.000066	0.000001	0.000075
Proteus	0.000014	0.000005	0.000003
Providencia	0.000000	0.000007	0.000050
Pseudactinotalea	0.000034	0.000139	0.000133
Pseudoalteromonas	0.000029	0.000036	0.000092

Pseudochrobactrum	0.000009	0.000005	0.000054
Pseudoflavonifractor	0.000016	0.000063	0.000384
Pseudomonas	0.000379	0.000192	0.001020
Pseudonocardia	0.000024	0.000007	0.000037
Psychrobacter	0.000701	0.000010	0.000275
Pusillimonas	0.000033	0.000003	0.000010
Pygmaiobacter	0.001224	0.001221	0.000790
RF39	0.002523	0.004497	0.004185
Rhodococcus	0.000044	0.000020	0.000117
Rikenellaceae_RC9_gut_group	0.000060	0.000092	0.001294
Romboutsia	0.013007	0.015526	0.014248
Roseburia	0.000000	0.000005	0.000137
Roseimaritima	0.000050	0.000044	0.000094
Rothia	0.000592	0.000374	0.000233
Ruania	0.001566	0.001276	0.003022
Ruminococcus	0.000006	0.000010	0.000487
Ruminococcus_gauvreauii_group	0.000017	0.000012	0.000036
Ruminococcus_torques_group	0.023465	0.035098	0.039990
S5_A14a	0.000011	0.000027	0.000031
Saccharimonadales	0.000007	0.000077	0.000013
Saccharopolyspora	0.000189	0.000055	0.000143
Salinicoccus	0.007854	0.007633	0.000250
Sandaracinus	0.000011	0.000015	0.000000
Sanguibacter_Flavimobilis	0.000059	0.000011	0.000145
Sellimonas	0.001258	0.001944	0.001460
Shuttleworthia	0.000411	0.000662	0.001082
Slackia	0.000654	0.001042	0.000658
Solibacillus	0.000386	0.000185	0.000914
Sphingobacterium	0.002440	0.000550	0.010998
Sporobacter	0.000002	0.000009	0.000032
Sporosarcina	0.000045	0.000075	0.000007
Stackebrandtia	0.001227	0.000760	0.003780
Staphylococcaceae	0.005166	0.001955	0.015425
Staphylococcus	0.115995	0.105797	0.104806
Stenotrophomonas	0.000023	0.000011	0.000133
Streptococcus	0.026153	0.018195	0.015700
Streptomyces	0.000883	0.000739	0.000912
Subdoligranulum	0.003434	0.007409	0.002313
Sumerlaea	0.000023	0.000017	0.000012
Sutterella	0.000000	0.000008	0.000130

Synergistes	0.000025	0.000043	0.000444
Terrisporobacter	0.000206	0.000410	0.000453
Tessaracoccus	0.000278	0.000056	0.000634
Timonella	0.000014	0.000006	0.000285
Tissierella	0.000059	0.000044	0.000009
TM7a	0.000011	0.000000	0.000017
Tomitella	0.000029	0.000026	0.000000
Truepera	0.000035	0.000013	0.000000
Turicibacter	0.009604	0.009369	0.010196
Tuzzerella	0.000103	0.000222	0.000359
Tyzzerella	0.000014	0.000014	0.000019
UCG_002	0.000019	0.000006	0.000004
UCG_005	0.001156	0.001855	0.002884
UCG_008	0.002631	0.003694	0.003726
UCG_009	0.000136	0.000184	0.000430
UCG_010	0.000085	0.000131	0.001133
uncultured	0.004038	0.004625	0.007452
Uruburuella	0.000006	0.000005	0.000014
V9D2013_group	0.000000	0.000012	0.000014
Vagococcus	0.000087	0.000754	0.000351
Veillonella	0.000085	0.000096	0.000156
Vibrio	0.000371	0.000594	0.001363
Virgibacillus	0.000301	0.000773	0.000404
W5053	0.000109	0.000106	0.000005
WCHB1_41	0.000008	0.000092	0.000002
Weeksella	0.000005	0.000000	0.000091
Weissella	0.000933	0.009139	0.000054
Yaniella	0.000639	0.002254	0.000013