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Bacteria and their Effects on Fertility in the Chicken

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By

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Pathogenic and non-pathogenic bacteria that are commonly associated with Poultry include: *Salmonella*, *E. coli*, *Campylobacter*, *Clostridium, Lactobacillus,* and *Bifidobacterium.* These bacteria are often transmitted to the bird from the environment and sometimes to the offspring which may affect bird performance and health. Therefore, the objective of our first experiment was to determine if rooster semen exposed to these bacteria had an effect on sperm motility. Our results indicated that all 6 bacteria lowered sperm motility but the 2 non-pathogenic bacteria, eliminated sperm motility immediately after exposure. These results led to the second experiment which was to determine if semen exposed to different concentrations of *Lactobacillus* (non-pathogenic bacteria) affected overall hen fertility. The results of the second experiment indicated that hens inseminated with semen exposed to a high dose of *Lactobacillus* produced infertile eggs. In conclusion, pathogenic and non-pathogenic bacteria affect male fertility and overall fertility leading to reduced bird performance.

DEDICATION

I would like to dedicate this work to my loving and supportive mother, Helma Haines and my husband, Trey Triplett.

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CHAPTER I

INTRODUCTION

Transmission and contamination of pathogenic bacteria has increased over the decades increasing the probability of foodborne outbreaks worldwide. Foodborne illness costs the United States approximately $77.7 billion annually (Scharff, 2011). In a report by Scallan et al. (2011), foodborne illnesses were found to result in approximately 48 million cases, 3,000 deaths, and 128,000 hospitalizations annually. Due to these foodborne outbreaks, more research is needed to better understand how contamination occurs and how to control outbreaks starting at the source through the final product.

There are many different species of bacteria that are beneficial; however others are potentially harmful to the host's body. The most common bacteria to cause illnesses are described as pathogenic. Examples of these bacteria include *Salmonella* spp., *Escherichia coli (E. coli)*, *Campylobacter* spp., and *Clostridium* spp. The more common non-pathogenic bacteria include *Lactobacillus* spp. and *Bifidobacterium* spp which are known to have beneficial effects to the host's body.

Within the Poultry industry, pathogenic bacteria are frequently thought to be transmitted from the live bird to the carcass, which can lead to contamination of food that is consumed by the public. There are two main pathways that bacteria can be transmitted. These pathways are described as horizontal, environment to bird, or vertical, parents to offspring. Examples of horizontal transmission include when a bird ingests

excreta from another bird, from feeders, nipple drinkers, or farm workers (Corrier et al., 1999; Cox et al., 1990). The other transmission pathway by which birds become colonized by bacteria is referred to as vertical transmission. Baker et al. (1980) and McGarr et al. (1980) demonstrated vertical transmission through their work with *Salmonella*. They found that *Salmonella* was present in the hen's ovary as well as in the offspring from those hens.

Bacteria can also be found within the reproductive tract of many species. It has also been determined that the reproductive tracts of female chickens are positive for *Campylobacter* in the mature ovarian follicles (Cox et al., 2005) as well as in the shell gland, vagina, and cloaca (Buhr et al., 2002). Pathogenic bacteria have also been found to be naturally occurring in mammal and avian semen. *Escherichia coli* have been detected in boar (Bussalleu et al., 2005) and human semen (Auroux et al., 1991; Diemer et al., 1996), and due to its presence, a negative effect has been found on the overall motility of the sperm. Vizzier-Thaxton et al. (2006) determined that *Campylobacter* and *Salmonella* can attach to the roosters' sperm head, acrosome and tail. Because pathogenic bacteria are naturally occurring in the male and female reproductive tracts, precautionary steps should be considered during breeding programs to reduce the potential of horizontal or vertical transmission. By doing this contamination throughout production and processing can be reduced leading to safer products being consumed by the public.

Non-pathogenic bacteria such as *Lactobacillus* spp. and *Bifidobacterium* spp. are also known as probiotics. Probiotics are defined as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance"

(Fuller, 1989). *Lactobacillus* begins to naturally colonize the chick's small intestine just one week after hatch (Mead, 1997). *Lactobacillus* when fed as a supplement has been shown to improve shell strength, shell weight, and shell thickness (Panda et al., 2008). It has also been found to increase egg production, egg weight, and egg size (Kalavathy et al., 2005). Jin et al. (1996) and Zamanzad-Ghavidel et al. (2011) found that Lactobacilli fed to broiler chicks increased their feed efficiency, weight gain, and meat yield.

However, little information is available on the potential negative effects pathogenic and non-pathogenic organisms may have on poultry fertility. Because bacteria can be found ubiquitously in the poultry industry, bacteria can be transferred, and precautionary actions are imperative to control foodborne outbreaks or loss of production. Pathogenic bacteria cost the United States billions of dollars annually in food related illnesses, and they also affect poultry production, especially from the breeder's standpoint. Although not all bacteria are thought to be harmful to the host's body, such as *Lactobacillus*, it is important to fully investigate the impact these organisms may have on the bird's overall health. Because there is limited research demonstrating the effect beneficial bacteria as well as pathogenic bacteria have on avian fertility, further research is necessary to obtain a better understanding of the possible consequences that could affect flock performance.

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CHAPTER II

LITERATURE REVIEW

History of the Domestic Chicken

The first known domesticated chicken in history was the Red Junglefowl (*Gallus Gallus*) which originated in Southeast Asia over 8,000 years ago (Zeuner, 1963). Over 4,000 years ago, *Gallus Gallus* was domesticated and is representative of the modern day chicken (*Gallus Domesticus)* which is known for meat and egg production (Crawford, 1984; Zeuner, 1963). Today's domesticated chicken is far less aggressive and less active than their ancestors, due to the lack of territorial circumstances. On the production side, *Gallus Domesticus* produces more meat (Sawyer, 1971), starts lay earlier, lays eggs more often, and lays larger eggs than *Gallus Gallus* (Lerner and Hazel, 1947).

In the early 20th century, farmers reared chickens from hatch to provide food for their family. Those birds were used for egg production and producing offspring until they reached their peak production, then after peak production the birds were consumed for meat (Martinez, 1999). Sawyer (1971) reported that farmers would sell the cockerels for meat (broilers) and keep the hens for egg production. Since that time period, the demand for poultry has steadily increased and farmers had to keep up production while also trying to maintain economic stability. Broilers were sold at market weighing approximately 2.5 pounds at 12-16 weeks of age, whereas today birds are processed at 6-8 weeks and weigh approximately 5-6 pounds (Martinez, 1999).

After many years of trial and error of raising birds, a new concept was adopted in the 1950's which was referred to as vertical integration. Vertical integration involves the formation of a company. Each company then built their operations to include breeder farms, hatcheries, feed mills, and processing plants (Connor, 2008). Once the companies were operational, they would then contract farmers to grow their birds. The growers were responsible for providing housing, equipment, labor, and fuel while the company would provide the grower with the chicks and feed thus laying the foundation for production (Martinez, 1999). By forming agreements between both entities it allowed for better control over production, so the industry as a whole could keep up with the demand for poultry products by the consumers.

Genetics

As the poultry industry moved to the concept of vertical integration, it became obvious that for them to meet consumer demand the birds themselves had to evolve. To evolve, genetics was a major factor and it involved the process of selecting for desirable characteristics. There are different types of selection among species such as natural and artificial selection. Natural selection is a slower process where the animals themselves naturally select for the best traits such as livability, reproduction, and body size for their specific environment so they may procreate and prosper (Siegel and Dunnington, 1997). However, artificial selection is where geneticists select for certain characteristics that are desired in an animal for production purposes (Siegel and Dunnington, 1997). For example, the traits most important for poultry are high meat yield or high egg production. The environment, overall population, and the individual are all intertwined as key factors when examining changes across generations (Siegel and Dunnington, 1997). Sometimes

the genetic selection process can take numerous reproductive cycles or very few cycles due to fluctuations such as natural fitness, differences between natural or artificial selection, and the variability with artificial selection (Siegel and Dunnington, 1997). This is important so that certain traits are maintained or enhanced so that production can meet consumer demand.

Before 1950, the genetic developments of laying hens were based on individual egg production records and farmer information about the different breeds used for laying eggs (Sawyer, 1971). Today, the most desired hereditary characteristics of the laying hen are body size, egg weight, egg size, shell strength, and egg production. Chickens were also genetically selected for meat production to increase meat yield for consumer demand (Sawyer, 1971; Pym, 1990). Due to the fierce competition of primary breeder companies, mating schemes are kept confidential so that the knowledge gained from inbreeding and crossbreeding experiments provide a slight advantage to the company (Tixier-Boichard, 2012). Once desired traits were established, breeding of those selected birds occurred and those traits were adapted for each breeder company where it became competitive to maintain tight genetic selection. As a result, the great-grandparent and grandparent flocks for each primary breeding company are the most important because of the genetic traits they carry. Both, males and females play a large role when it comes to selecting desirable traits that will provide better meat and egg production. This is due to certain physiological qualities each sex carries.

Male Reproductive Tract

Roosters are known for protecting the flock and fertilizing the hen's ova upon copulation. Roosters have two testes which are located within the body cavity, and they

sit on either side of the vertebra. The testes can vary in size due to their reproductive maturity and/or activity. The testes are responsible for the production of sperm cells and the hormone testosterone. Spermatogenesis is the process in which germ cells are multiplied, and in which primary spermatocytes are enlarged and maturate (Witschi, 1961). Spermiogenesis is the process when spermatids become spermatozoa through morphological changes. Once the spermatozoa are mature, they are released from the mucosal lining and travel down the seminiferous tubules to the epididymis. The epididymis is located on the dorsal side of the testis and is responsible for carrying spermatozoa from the testes to the ductus deferens. The ductus deferens are tubules with a zigzag appearance and are attached at the testes and cloaca. Spermatozoa take 4-5 days to travel down the ductus deferens due to the density of the tubule (King et al., 1984). At the distal end of the ductus deferens, there is a storage site for sperm called the glomula. During copulation, semen travels down the seminal groove of the rooster's phallus (which only barely protrudes outside the body cavity). Sperm then exit through the rooster's cloaca and enter the female's vagina which is also located in her cloaca.

Individual rooster spermatozoa are very long and thin and consist of an acrosome, head, and tail. The acrosome is approximately 2.5 μm long, the head is 12.5 μm long, the mid-piece is 4.3 μm and the remaining tail is approximately 90 μm in length (Lake et al., 1978). The acrosome is a cap that covers the sperm head and is responsible for breaking the outer perivitelline layer of the ova. The sperm head contains the DNA material that will potentially fertilize an egg to produce offspring. The tail is responsible for motility of sperm to travel through the hens oviduct. Chicken sperm are longer than mammalian sperm. For example, bull sperm is 50-60 μm in length, but the larger the size of the avian

sperm makes it more fragile when put through physical challenges such as centrifugation or dilution with other mediums (Saacke and Almquist, 1964). By volume, rooster semen ranges from 0.1-0.3 mL and is very concentrated at 3.5 billion sperm/ mL. Also, avian sperm thrive in a neutral environment with a pH of 7.0 ± 1, but survival is optimum at a pH of 7.25 (Lardy and Phillips, 1943). However, depending on incubational temperatures the range of pH can change. Barna and Boldizsar (1996) reported that chicken spermatozoa incubated at 39°C reached maximum motility when the pH range was between 7.4 and 7.5. Also, Ashizawa et al. (2000) found that chicken spermatozoa incubated at 30°C were motile when the pH was between 7.0 and 9.0.

Semen Collection for Roosters

The collection of semen from individual males allows the primary breeding companies to utilize artificial insemination (AI) to breed specific hens. For example, semen collection allows primary breeding companies to control and maintain specific genetic traits such as meat yield, skin color, egg production, and egg size. The abdominal massage technique is one method which is a used to collect semen from roosters (Burrows and Quinn, 1937). Collecting semen from roosters, in this manner allows for controlled sperm usage during AI and allows individual roosters to be used for their specific genetic traits in a number of different hens. Semen collection begins when the rooster is held by one person while another person massages the bird's lower back (pygostyle) as well as the lower abdomen with the thumb and forefinger. The reason the back is massaged is because the rooster's testes and phallus are located in this region. The rooster's copulatory organ is a tissue that lies on either side of the cloaca (Kaupp, 1915) and when it is stimulated or pressure is applied, the bird will ejaculate. The

ejaculate is then collected into a beaker or funnel to be aliquoted to the hens. The average ejaculate has a sperm concentration of approximately 3.5 billion spermatozoa/ mL (Lake, 1957). It is imperative for the handler to collect rooster semen accurately to insure the sample is as pure as possible and free from contaminates.

Semen Evaluation Techniques

After semen collection and before AI, it is important to determine the quality of sperm in the ejaculate. Concentration, viability, and motility are all characteristics used in determining sperm quality. All three characteristics are imperative when determining the total number of sperm that are capable of fertilizing an egg because only viable motile sperm are capable of fertilization (King et al., 2000). After semen collection, the sample is kept in an aerobic environment and is constantly being rotated for aeration to ensure that the sperm stay viable as well as motile. There are many tests to determine rooster semen quality but a few common methods include the Sperm Quality Index (SQI), a photometric assay, and a fluorometric assay.

The first method of semen testing is the Sperm Quality Index (SQI) which is obtained with a Sperm Quality Analyzer (SQA). An SQI, which is a measurement of overall semen quality, can be obtained by monitoring the number of times motile sperm cross a beam of light in 20 seconds (Bartoov et al., 1991). Baskt and Cecil (1997) reported that the SQI was a quick assessment of an avian semen sample and could be used for research purposes or AI for either chickens or turkeys. The SQI considers three main semen characteristics which are sperm concentration, viability, and motility (McDaniel et al, 1998). Prior to testing for the SQI, neat or undiluted semen samples must be diluted 10-fold. The diluted sample is then drawn into a capillary tube, which

will be used during testing. By diluting the semen 10-fold, it allows for proper sperm movement within the capillary tube. Once the sample is drawn into the capillary tube the tip is wiped off and then placed into the SQA to be read. Generally, three separate capillary tubes are used to obtain an average of viable motile sperm.

The SQI measures overall semen quality but does not directly measure sperm concentration. The IMV II micro-reader (Minneapolis, MN) determines actual sperm concentration. To determine sperm concentration, the micro-reader measures how much light is absorbed by a diluted semen sample. This method for determining sperm concentration was adapted for avian species by King and Donoghue (2000). The neat semen sample collected from avian males must be diluted with 33% sodium citrate to keep sperm from agglutinating. Neat semen is diluted in a cuvette containing sodium citrate and mixed thoroughly prior to obtaining an absorbance reading from the micro-reader. After obtaining an absorbance reading, the sperm concentration is obtained using the following linear standard curve: 10.99 x absorbance average + 0.18. This formula is established by the manufacturer and determines how many billion sperm are in a milliliter of semen. To determine sperm concentration, this standard curve is generated by using both the IMV II spectrophotometer and microscopic sperm counts. This will determine if the semen samples from the roosters are near the normal semen concentration of 3.5 billion sperm/ mL (Sexton, 1977). Knowing an SQI and the number of sperm per mL provides important information that will allow individuals performing AI to control the number of sperm needed for a specific AI dose.

However, the IMV II method does not distinguish between live and dead sperm, which is important when trying to determine viability. Lake and Stewart (1978) reported

that the first ejaculate of a rooster has a higher concentration than the second ejaculate because the second ejaculate contains more fluid, leading to a lower sperm concentration. If an ejaculate has a large concentration of sperm, this does not necessarily mean that the sperm are capable of fertilizing an ovum as only live sperm have the capacity to fertilize an egg. Therefore, in order to know the percentage of sperm that are viable, the presence of dead sperm in each semen sample must be determined. One method used to determine the amount of dead sperm in a semen sample is the fluorometric method of Bilgili and Renden (1984). For this method, the fluorometer uses a mercury lamp which provides an excitatory light of 365 nm and the emissions are measured above 560 nm using a filter. Assay tubes are filled with phosphate buffered saline and Ethidium Bromide (EtBr). One tube is placed in the fluorometer prior to the addition of semen to zero the fluorometer to ensure the EtBr will not interfere with the measurements. The neat semen samples are aliquoted using 10 µl of neat semen which is placed into the EtBr solution and the reading is recorded (pre-reading). This first reading is a result of EtBr staining the DNA of dead sperm cells. After recording the first reading, digitonin is added to the diluted sample to disrupt all sperm cell membranes exposing each sperm cell's DNA. After cell membranes are ruptured and the DNA is stained, a second reading is obtained (post-reading). After the first and second readings are obtained, the following calculation is used: pre-reading average / post-reading average x 100. This is used to determine the average percentage of dead sperm. After the rooster's semen is properly evaluated and is determined to be within the normal range, it is then ready to be inseminated into the hens.

Female Reproductive Tract

On a flock basis, the male contributes more genetically than the female due to the fact that in breeder houses there is only 1 rooster per 10 hens. Although males are a major contributor to the gene pool, the female and her reproductive organs also play a role in maintaining fertility. Each hen is responsible for being bred as well as laying fertile eggs. Once the hen is bred, there is a lengthy process in which the hen must lay fertile eggs to maintain overall production. The female's reproductive anatomy is unique in that it has both a left and a right ovary at hatch but only the left ovary is functional in the adult hen, whereas the right ovary is considered as residual by the fourth day of incubation (Hutson et al., 1985). At 16-24 weeks of age, the hen's left oviduct is completely functional prior to the start of egg production. The ovary produces approximately 28,000 oocytes by the 9^{th} day of incubation, and by the 17^{th} day, the embryo has 680,000 oocytes, but this number decreases to approximately 200,000 on the day of hatch (Johnson, 2000). Throughout the females lifetime a mere 250-500 ova reach maturity and are ovulated. Interestingly, hens have what is known as a follicular hierarchy where the largest and most mature ovum is considered as the next to be ovulated and it is referred to as the F1 follicle. The follicular hierarchy contains both white and yellow colored yolks or ova and the five yellow largest follicles are approximately 6-12 mm in diameter whereas the white follicles are less than 6 mm (Johnson, 1990). The mature hens' follicular hierarchy of both the white and yellow follicles has a total weight anywhere from 20 to 30 g. There are many layers to a single follicle, but the outer layers include the plasma membrane, perivitelline layer, granulose

cells, and the basal lamina. Every follicle has many veins and arteries, except on the stigma, which is ruptured at the start of ovulation (Nalbandov and James, 1949).

Once an ovum is ovulated, it enters the female's reproductive tract or the oviduct. The oviduct contains five major sections starting at the highest dorsal segment which is known as the infundibulum, followed by the magnum, isthmus, uterus and vagina. Upon release from the ovary, the ovum enters into the infundibulum which envelops it. The ovum remains in the infundibulum for about 15 minutes so that fertilization can occur. The primary site for sperm to attach and fertilize the ovum is at the germinal disc which is approximately 250 μm thick and 4 to 5 mm in diameter. Sperm only have 15 minutes to penetrate the ovum multiple times to ensure fertilization. After 15 minutes the albumen is placed on the ovum which stops sperm penetration (King et al., 1984; Baskt and Howarth, 1977). After fertilization and the preliminary addition of albumen, the ovum progress to the next segment of the oviduct or the magnum. This is where the majority of the albumen is applied over the entire ovum. The magnum is the largest segment in the oviduct measuring 33 cm long, and the ovum will remain in the magnum for approximately 2-3 hours. After being held in the magnum, the ovum travels to the third segment which is the isthmus. While the ovum is in the isthmus, the inner and outer shell membranes are formed which takes approximately 1-2 hours. Once the membranes are formed, the ovum travels to the next segment which is the uterus. The uterus is where calcification of the egg shell occurs, and this happens over a period of approximately 20 hours (Johnson, 1990). After the egg shell is complete, the egg is then positioned correctly for oviposition which takes an additional 3 hours. The final segment of the oviduct is the vagina. This is where muscles are responsible for ejecting the egg.

Beginning where the ovum enters the infundibulum until oviposition, the total process takes approximately 24-28 hours (King and McLelland, 1984). Hens are designed to ovulate essentially every day without a rooster present, and if hens are naturally mated or artificially inseminated they are then able to produce fertile eggs.

Artificial Insemination and Sperm Storage

Artificial insemination is a convenient technique to insure every hen in the house will potentially lay a fertile egg when roosters are not used to naturally inseminate the hens. Once the semen is collected from the roosters, the sperm concentration is determined as to properly distribute semen to each inseminated hen. When a hen undergoes AI, the inseminator must be cautious of how deep to insert the semen. Wentworth et al. (1975) found that only 2 cm is sufficient for insemination due to the probability of injuring the vaginal tissue when artificially inseminating. However, penetration up to 7 cm could cause serious damage to the reproductive tissue. For ease, AI should be performed when breeding hens and roosters are in cages rather than housed on the floor due to physical exertion. Also, labor costs are decreased as well as disturbance to the birds when they are housed in cages (Moultrie, 1956). Once copulation or AI occurs, the hens are able to store sperm in their sperm storage tubules (SST) which are located at the utero-vaginal junction (Bobr et al., 1964). Sperm can be stored in the SST anywhere from a couple of days up to three weeks in the chicken. The sperm stored in the SST is released for fertilization when the next ovum in the hierarchy is released. Females that store sperm in the SST for longer periods of time have a higher fertility rate whereas hens that cannot store sperm for long periods of time have lower

fertility rates. In order to reach the SST, rooster sperm must be motile and competitive when introduced into the hen's oviduct for fertilization of the ova (Parker, 1970).

When collecting semen from roosters there are many possibilities for contamination to occur such as fecal material being deposited into the pooled semen sample, which may then be inseminated into the hen's vagina. Another possibility for contamination of the hen is the equipment used such as collection tubes or pipettes which may be contaminated with bacteria. When natural breeding occurs, the male's cloaca touches the female's cloaca which is also a potential mode of bacterial transmission from one bird to another or to future offspring. The excreta, semen, and egg are all expelled through the rooster or hen's cloaca respectively which significantly increases the possibility of bacterial contamination.

Bacteria in General

Millions of different species of bacteria are found ubiquitously. Bacteria are one of the smallest living cells in size measuring from 0.1 to 10 μm (Ryan, 2004). Bacteria are in the domain known as prokaryotes due to the fact that they do not possess a nucleus. Even though bacteria do not have a nucleus, they still contain single and double stranded DNA (Ryan, 2004). They are found in many different shapes and sizes such as rods/curves, spirals, or spheres. Spherical bacteria measure 0.5 μm in width to 2.0 μm in length whereas rod-shaped are 0.2 μm in width to 2.0 μm in length (Neidhardt, 2004). Bacterial cells are also classified by their cell wall structure as being either gram-positive or gram-negative. Gram-positive have a thick peptidoglycan layer that when stained with crystal violet can retain the color whereas gram-negative have a thin peptidoglycan layer and are not able to retain that intense color. Attached to the outside of the cell wall at one

or both ends are organelles called flagella which are responsible for motility of many species of bacteria. Flagella are important for colonization within the host's body and contribute to virulence of the microorganism. Bacteria require certain environmental conditions to proliferate such as the temperature and certain gas requirements. Temperatures of bacteria range from 10-65°C but the most common is 37-42°C which is the average body temperature of mammals and avian species. Bacteria also require certain gas mixtures such as aerobic (oxygen), anaerobic (no oxygen), microaerophilic (low oxygen), or facultative anaerobic (bacteria can survive in either aerobic or anaerobic conditions). Bacteria can proliferate in the host such as in mammals, avian species, reptiles, or insects. If the host is introduced or becomes colonized with bacteria the method of introduction is referred to as a transmission pathway.

Transmission of Bacteria

Bacteria can be transmitted to a host by 2 pathways, the horizontal or vertical pathways. Horizontal transmission occurs when bacteria are passed from the environment to a host. Vertical transmission of bacteria is defined as the transmission of bacteria from parents to their offspring. Horizontal and vertical transmission can coincide with each other. For example, Reiber et al. (1995) provided evidence that semen might serve as a vehicle for vertical transmission of bacteria such as *Salmonella,* but it can also be transmitted from the environment or feed, which are horizontal transmission pathways. By limiting the possibility of bacterial transmission, the risk of future contamination of poultry products as well as flock health is decreased.

Horizontal Transmission

When discussing horizontal transmission pathways within the poultry industry, the possibilities are immense. This means there are several areas that could be potential routes by which bacteria can colonize a bird. Potential horizontal transmission sources within a poultry environment include; the poultry house, hatchery pads, litter, feed, water, farm workers, rodents, small birds, and flies (Genigeorgis et al., 1986; Kazwala et al., 1990; Lindblom et al., 1986; Pearson et al., 1993). The shedding of bacteria is a possible route of transmission from one bird to another. This can occur through the ingestion of fecal droppings (Corrier et al., 1999; Cox et al., 1990, 2002a; Cox and Bailey, 1991), saliva from the feeder, water nipple drinkers, farm employees transferring equipment or birds, employees clothing or shoes, trucks driving from farm to farm, airborne dust, and the eggshell (Cox et al., 2000; Henzler et al., 1994; Hoover et al., 1997; Smeltzer et al., 1979). Bacteria can also be transmitted horizontally from bird to bird by cuts in the skin or through mucous membranes from fighting, pecking, or vaccine needles (Stuart, 1990).

Bacteria can be transmitted from breeder farms to hatcheries from hatcheries to grow-out farms, and from grow-out farms to the processing plants. However, the most common mode of horizontal transmission is from the grow-out farm to the processing plant. Doyle et al. (1984) tested hatching eggs for *Campylobacter* but they discovered all samples were negative. At 1 or 4 weeks of age it has been discovered that broiler flocks can be 100% positive for *Campylobacter* (Neill et al., 1984; Pokamunski et al.,1984). Pokamunski et al. (1986) reported that at 8 weeks of age when birds were slaughtered, isolation of *Campylobacter* was lower than at four weeks of age. When birds are brought to the processing plant, they undergo stress from transport and handling. These birds can

shed bacteria through excreta where another bird can ingest that excreta and possible contamination can occur. These pathways of transmission are not the only possibilities of transmission. Another pathway bacteria can be transmitted is known as vertical transmission.

Vertical Transmission

Vertical transmission is not widely accepted within the poultry industry although there is limited research that provides some evidence. Research has shown that *Salmonella* and *E. coli* are vertically transmitted from the parents to the chick (Cox et al., 2005b). In theory, if the hen's reproductive system is contaminated, the bacteria should be able to proliferate on the reproductive tissues and thereby enter the egg prior to egg shell formation. For example, Cox et al. (2005b) determined that *Campylobacter* was found naturally in mature ovarian follicles from broiler breeder hens. Buhr et al. (2002) conducted a study were hens were divided into three different groups, with each group being raised in different environments. In group one, hens were found positive for *Campylobacter* on the shell gland, vagina, and cloaca. Whereas in group two, hens were found positive only in the cloaca and group three hens were positive in the magnum, isthmus, and cloaca. For all groups, the cloacal samples were 100% positive for *Campylobacter*. This demonstrates that bacteria are colonizing on the hens reproductive tracts which could be transferred to the ovum before shell formation. *Salmonella* was also sampled in the mature and immature follicles and only 1 out of 47 samples were positive for the mature follicles, and no *Salmonella* was found in the immature follicles (Buhr et al., 2002), but in other studies, *Salmonella* has been found to be vertically

transmitted from the parents to their offspring via the ovaries (Baker et al., 1980; McGarr et al., 1980; Guthrie, 1992).

Other researchers have stated that *Campylobacter* cannot be transferred from breeder flocks to chicks because the bacterium was not cultured from the hatched chicks (Doyle, 1984; Jones et al., 1991). Clark and Bueschkens (1985) even inoculated chicks with *Campylobacter jejuni* and determined that only 11% of the chicks inoculated tested positive for *Campylobacter* in their intestinal tract. However, Lindblom et al. (1986) revealed that *Campylobacter jejuni* was present in chickens that were raised in a sterile laboratory setting where birds did not have exposure to a farm environment which ruled out potential horizontal transmission. What they found was that possible vertical transmission of *Campylobacter* could occur from the parents to the egg. Wilcox and Shorb (1958) also determined that semen may contain bacteria such as *Escherichia*, *Staphylococcus*, *Bacillus*, and *Enterococcus* at 2.2 x 10^6 cfu/mL. Because semen comes in contact with the cloaca in the male and female, it can become contaminated with bacteria at that location, which may be responsible for transmitting bacteria into the hens reproductive tract (Smith, 1949; Lake, 1956).

Transmission of bacteria is of great concern to the Poultry industry due to the fact that foodborne disease outbreaks are significantly increasing and the different modes of transmission must contribute to the contamination. Not only can pathogenic bacteria cause foodborne outbreaks but they can also cause infections in the bird, leading to lower levels of performance. Therefore, methods that reveal the true pathway by which Poultry become colonized by bacteria and more specifically pathogens are essential.

Pathogenic Bacteria

Pathogenic bacteria are the major cause of foodborne illness outbreaks in the United States. Scharff (2011) reported that foodborne illness costs the United States approximately $77.7 billion annually. Scallan et al. (2011) reported that food-related illnesses result in approximately 48 million illness cases, 3,000 deaths, and 128,000 hospitalizations, annually. Pathogenic bacteria are bacteria commonly known to cause infections internally and/or externally of the host's body. Internal infections usually start in the gastrointestinal tract whereas external infections can occur on the skin, and within the skin layers, of the eyes, feet, hands, and ears. Although many pathogens are associated with human illness, the following are the most common associated with Poultry; *Salmonella spp.*, *Escherichia coli species*, *Campylobacter* spp., and *Clostridium* spp.

Pathogenic bacteria are found ubiquitously and can cause numerous types of infections to all different species of humans and animals. One of the most consistent places to find pathogenic bacteria contamination is in food and food products consumed by the public. Contamination of food products can result in human infections, which display symptoms that include fever, diarrhea (often bloody), vomiting, dehydration, nausea, and abdominal cramps. After consumption of the bacterium it can take 12-72 hours for the illness to initiate.

Avian species can also be infected from pathogenic bacteria. The infection typically occurs in the intestinal tract and can lead to a decrease in feed efficiency and flock livability. There are three common bacterial diseases that affect intestinal health and flock livability they include necrotic enteritis, ulcerative enteritis, and spirochetosis

(Van Immerseel et al., 2004). Other bacterial diseases that affect different organs are known as salmonellosis, *coli*bacillosis, myobacteriosis, erysipelas, and fowl cholera (Porter, 1998). When a bird becomes infected by pathogenic bacteria, symptoms include inflammation or ulcers in the intestinal tract (duodenum, liver, and ceca), comb or wattle scabbing, ruffled feathers, footpad infection, or diarrhea.

Poultry and Poultry products are a common food source found to be contaminated with pathogenic bacteria. Bacterial contamination in poultry products can occur during the processing phase due to bleeding, skinning, evisceration, and handling of the birds (Patterson, 1969). The illnesses associated with poultry are a major concern in the poultry industry due to the loss of productivity, increased mortality, and the possibility of humans consuming contaminated poultry and/or products. As stated previously, pathogenic bacteria commonly associated with poultry and their products include *Salmonella* sp., *Escherichia coli*, *Campylobacter* spp. and *Clostridium* spp.

Salmonella spp.

Salmonella spp. are gram-negative, rod-shaped, non-sporeforming bacterium with many flagella. They are facultatively anaerobic and thrive at an optimum temperature of 37°C. *Salmonella* spp. are relatively small measuring approximately 0.7 to 1.5 μm in width and 2 to 5 μm in length (Holt et al., 2000). The optimum pH for *Salmonella* is 6.5 to 7.5. These organisms are associated with cold and warm blooded animals, as well as in environments such as soil and plants. Certain *Salmonella* species can cause illnesses such as typhoid fever, paratyphoid fever, and *Salmonellosis*.

A specific pathogenic strain of *Salmonella*, *Salmonella enteritidis* (S.E.), is often associated with Poultry and Poultry products. One of the most common incidences of

foodborne illness in Poultry occurs in eggs contaminated with S.E.. *Salmonella enteritidis* outbreaks have significantly increased around the world since the 1980's (Hogue et al., 1997). Some species can survive in temperatures as low as -18°C for 4 months (D'Aoust, 1989). In the Poultry industry, *Salmonella* in eggs is often a main focus at the great-grandparent, grandparent, and parent breeder bird as well as table egg layer levels. To reduce the possibility of *Salmonella* infection, the Poultry industry is required to maintain overall health of the flock, vaccinations, biosecurity, and waste management, as well as through cleaning, and the disinfection of surfaces and work areas (CDC, 2011).

The main source of *Salmonella* contaminated eggs occurs when the bacteria from the feces penetrates the egg shell during or after oviposition (Gast and Beard, 1990). Bacterial contamination can also occur in the yolk, albumen, and eggshell membranes due to an infection in the hen's reproductive organs (Shivaprasad et al. 1990). For example, Barnhart et al, (1993) found 3 flocks to be positive for *Salmonella* in their ovaries as well as 2 samples that were positive in the oviduct. Miyamoto et al. (1998) reported that *Salmonella* can penetrate the eggshell via horizontal transmission; however the egg does provide physical restraints which include the albumen, the cuticle, and inner and outer membranes of the eggshell (Haigh and Betts, 1991). There are numerous studies that have shown *Salmonella* positive eggs but limited research showing contamination can be contributed from roosters.

Salmonella is found not only in hens but males as well. For example, *Salmonella* has been found in turkey semen (Donaghue et al, 2004). In their study, it was reported that the total count of bacteria (*in vitro* challenge) was not reduced in pooled semen

samples in four of the five semen extenders examined. The extender, with the most antibiotics, showed the greatest reduction of bacterial counts in the semen sample.

Escherichia coli spp.

Escherichia coli (*E. coli*) are gram-negative, rod-shaped, non-sporeforming bacteria that are highly motile due to their flagellum. They are facultatively anaerobic and thrive at an optimum temperature of 37°C (Holt et al., 2000). *Escherichia* spp. measure approximately 1.1-1.5 μm in width and 2.0-6.0 μm in length and can grow singly or in pairs. *E. coli* spp. require a neutral pH between 6 and 7. They are naturally occurring in warm-blooded mammal intestines as a part of their normal microflora. *E. coli* cells can survive outside of the host's body for short periods of time yet truly thrive within the host. Certain serotypes of *E. coli* are known to cause foodborne diseases and are also a causative agent of travelers' diarrhea, often referred to as Montezuma's revenge. The strains of *E. coli* that cause foodborne illnesses are enterohemorrhagic *E. coli* (EHEC), Shiga toxin-producing *E.coli* (STEC), enteropathogenic *E. coli* (EPEC) or enteroadherent *E. coli* (EAEC). Symptoms of *E. coli* infections in humans include stomach pain, nausea, vomiting and diarrhea (Riley, 1987; Riley et al., 1983). *E. coli* can also be transmitted through oral ingestion of feces because cells are able to survive outside of the host for a limited amount of time. Transmission of feces can occur by not properly sanitizing equipment, utensils, and surroundings properly and/or regularly in the processing plant (Barros et al., 2006)

The presence of *E. coli* is not limited to food sources. For example, Auroux et al., (1991) and Diemer et al., (1996) reported that when *E. coli* is present in human semen, that sperm motility is decreased and causes agglutination of sperm leading to infertility.

E. coli has been found to produce a spermicidal effect but it does not produce an acidic environment (Althouse et al., 2000). In boars, Bussalleu et al. (2005) reported that different concentrations of *E. coli* decreased boar sperm motility over time. Although *E. coli* decreased sperm motility, there was no anatomical difference in individual sperm. All sections of the sperm cells were intact. Martin et al, (2010) found that *E. coli* was the main contaminate of boar semen, (79%), and that litter size was significantly reduced when concentrations rose above 3.5 x 10^3 cfu/ mL.

Campylobacter spp.

Campylobacter are gram-negative, helical-shaped, non-sporeforming bacteria that contain 1-2 flagella on either end for motility. *Campylobacter* spp. prefer a microaerophilic environment that provides 3-5% oxygen and 2-10% carbon dioxide and a temperature of 37°C to 42°C (Holt et al., 2000). They are approximately 0.2-0.5 µm wide by 0.5-5.0 µm long (Holt et al., 2000). *Campylobacter* survives at an optimum pH of 6.5 to 7.5. *Campylobacter* was not recognized until the 1970's as a human pathogen. Once infected, the symptoms in humans will include fever, diarrhea, abdominal pain, nausea, and muscle pain that can last 3-10 days (Keener et al., 2004). *Campylobacter* is known around the world to cause illness due to food contamination by raw or undercooked chicken (Friedman et al., 2006) or by fecal transmission on layer eggs (Doyle, 1984).

Campylobacter has been found to be naturally occurring in broiler and layer hens. *Campylobacter* has been found to occur naturally in the shell gland, vagina, and cloaca of broiler breeder hens (Buhr et al., 2002). Cox et al. (2005) found *Campylobacter* in mature and immature follicles. Camarda et al. (2000) discovered *Campylobacter* in layer

hens' oviducts and mature and immature follicles. Cox et al. (2009) also isolated *Campylobacter* in laying hens' ovarian follicles, upper and lower reproductive tract, and ceca.

Even though *Campylobacter* has been detected in the female reproductive tract, they have also been reported to be associated with rooster semen and their reproductive tissues. For example, Cox et al. (2002b) and Buhr et al. (2002) found *Campylobacter* in commercial broiler breeder rooster semen and also in their vas deferens. Buhr et al. (2002) also determined that *Campylobacter* is naturally occurring in the entire broiler breeder hen's reproductive tract. Donoghue et al. (2004) found *Campylobacter* to be naturally occurring in turkey semen at 1.2×10^3 cfu/ mL. Because *Campylobacter* has been found in semen samples and in the reproductive tract of males, there is a potential chance for contamination of the hen and the eggs they are laying if semen is infected with *Campylobacter*. Vizzier-Thaxton et al. (2006) reported that *Campylobacter* (and *Salmonella*) were attached to individual sperm and were observed on the three segments of the sperm (head, midpiece, and tail). If *Campylobacter* and *Salmonella* are actually attached to the sperm it is possible these bacteria can be potentially transmitted via the vertical transmission pathway.

Other research has revealed that *Campylobacter fetus* subsp. *fetus* attaches to ram sperm on the tail and the acrosome (Bar et al., 2008). This experiment showed that bacteria-infected sperm displayed a decrease in sperm motility because the sperm head was separated from the tail when *C. fetus* subsp. *fetus* was present, also causing an increase in acrosomal damage. If *Campylobacter* spp. in rooster semen damages the

structure of the sperm, it may raise major concerns for the Poultry industry when discussing the fertilizing capability of the male breeders.

Clostridium spp.

Clostridium spp. are gram-positive, rod-shaped (often arranged in pairs or short chains), sporeforming organism, which are typically motile. They are obligate anaerobes and their optimum temperature is between 10 and 65°C (Holt et al., 2000). *Clostridium* spp. are approximately 0.3 -2.0 μm wide by 1.5-20.0 μm in length (Holt et al., 2000). They survive at an optimum pH of 6-8 where sporulation occurs. *Clostridium* spp. are ubiquitous and are frequently found in human and animal intestines. The spores of *Clostridium* can survive in water, soil, and sediments (Davies and Wray, 1996). *Clostridium* infection can cause gastroenteritis that is frequently mild and self-limiting but this organism replicates quicker than most bacteria. The disease begins by oral ingestion and lasts from 12-24 hours up to 2 weeks in infants or the elderly (Anand et al., 1994). Scallan et al. (2011) reported a 20% increase of foodborne outbreaks from *Clostridium perfringens* than Mead et al. 12 years earlier in 1999.

Clostridium spp. have been found to occur naturally in Poultry and are considered to be part of the normal gut flora. But if chickens are infected with *Clostridium* spp., and predisposing factors are present such as coccidiosis, the bird could develop necrotic enteritis (NE). Characteristics of NE include lesions in the intestines, increased mortality, reduced digestion and absorption, reduced weight gain, and increased feed conversion (Kaldhusdal and Hofshagen, 1992; Lovland and Kaldhusdal, 2001). Other signs of infection in birds include dehydration, ruffled feathers, and diarrhea (Helmboldt and Bryant, 1971; Gadzinski and Julian, 1992).

Craven et al. (2001, 2003) found contamination of *C. perfringens* at breeder farms, hatcheries, grow-out houses, and processing plants. Cravens et al. (2003) discovered *Clostridium perfringens* in fecal samples from 2 commercial broiler breeder flocks. In one house, 32% of the fecal samples examined were positive for *Clostridium* and 44% of the fecal samples from the second house were found to be positive. Also in Poultry, Cox et al. (2005b) also found *C. perfringens* in 14 out of 15 ceca samples. *Clostridium* spp. can cause intestinal mucosal lining damage which causes decreased absorption of feed and a decline in weight gain (Elwinger et al., 1992). *Clostridium* has also been found in the male reproductive tract. Cox et al. (2005b) reported that 1 out of 15 samples from the ductus deferens were positive for *C. perfringens*. Infection in broiler flocks from *Clostridium* can increase flock mortality. Because *Clostridium* has been reported in Poultry and Poultry products this could lead to the potential of foodborne illnesses in humans.

Non-Pathogenic Bacteria

Although there are numerous bacteria that can cause illness, there are many that are known to be beneficial. They are often referred to as non-pathogenic bacteria. They are naturally occurring in the host's gastrointestinal tract or can be added as a feed supplement. Non-pathogenic bacteria are not typical infectious agents in their hosts. There are many non-pathogenic bacteria used as probiotics but two of the most common include *Bifidobacterium* spp. and *Lactobacillus* spp.

Bifidobacterium spp.

Bifidobacterium are gram-positive, non-motile, rod shaped, non-sporeforming bacteria. Their optimum environment is anaerobic at an ambient temperature of 37-41°C. They are approximately 0.5-1.3 μm wide x 1.5-8 μm long (Holt et al., 2000). This bacterium can grow at an environmental pH ranging from 4.5 to 8.5. *Bifidobacterium* are also lactic acid producing bacteria.

Sewage, the mouth, and the intestinal tracts of warm-blooded vertebrates, and insects are the most common locations where *Bifidobacterium* are found. *Bifidobacteria* are highly represented in human and animal gastrointestinal microbiota (Mitsuoka, 1992) and are frequently applied in probiotics to promote human and animal health (Fuller, 1989). This bacterium has been shown to benefit the host throughout the host's life in any age group by improving the balance of microbes (Fuller, 1989). In humans, the benefits include decreases in colon cancer, the inhibition of intestine inflammation (Hiromi et al., 2003) and prevention of the growth of numerous pathogenic bacteria such as *Salmonella* (Henriksson and Conway, 2001). For *Bifidobacterium* to be effective in the gastrointestinal tract, they must be able to withstand gastric juices, hydrolytic enzymes, and bile salts (Liu et al., 2006). If they overcome these obstacles they will then be able to attach to the epithelial lining and proliferate in the gastrointestinal tract.

Because *Bifidobacteria* is associated with the intestines, it is no surprise that it is present in Poultry ceca and can reach levels as high as 10^9-10^{10} cfu/g of digesta (Mead, 1997). According to Rada and Petri (2000) it was determined that *Bifidobacteria* could be higher than 10^{10} cfu/g in the ceca of hens. Birds that are naturally colonized by

Bifidobacterium increase their overall performance and microbial balance without supplementation (Fuller, 1989).

Lactobacillus

Lactobacillus spp. are gram-positive, rod-shaped, non-sporeforming, non-motile bacteria. Lactobacilli range from 0.5 to 1.2 μm in width and 1.0 to 10.0 μm in length. They are facultative anaerobes but can grow in a microaerophilic environment at 30-40°C (Holt et al., 2000). Lactobacilli survive in acidic conditions at a pH between 4 and 5 or even lower. Gu et al. (1988) found that lactobacilli have a rapid growth period that is between 6 and 16 hours in length when cultured and replicates in approximately 54 minutes. They are found to be ubiquitous in the environment and are not harmful to the host. Lactobacilli, a microorganism in humans and animals, benefit the intestinal microflora of the host (Fuller, 1989) and consist of a large part of the lactic acid bacteria group.

The non-pathogenic species researched the most are; *Lactobacillus acidophilis*, *Lactobacillus bulgaricus,* and *Lactobacillus planatarum* (Fuller, 1989). The most commonly colonized in the gastrointestinal tracts of mammals, are *Lactobacillus brevis, L. casei, L. acidophilus, L. plantarum, L. fermentum*, and *L. salivarius* (Kononen and Wade, 2007). It has also been reported that Lactobacilli spp. are prevalent in the human vagina and are deemed as imperative for the maintenance of vaginal microflora and for ecological balance (Reid et al., 1990; Hudault et al., 1997).

Pivnick and Nurmi (1982) determined that lactobacilli dominate the crop and lower regions of the intestine in the chicken (Shapiro and Sarles, 1949). It has been shown that lactobacilli naturally colonize the chick's small intestine and caeca, 1 week

after hatch (Mead, 1997). Yet *Lactobacillus* spp. are also fed to chickens to improve their overall gut microflora and potential performance. Panda et al. (2008) showed that by feeding *Lactobacillus sporogenes* to White Leghorn layers, an increase in egg production and feed efficiency was obtained. Shell breaking strength, shell weight, and shell thickness were also significantly increased. This increase in performance could be due to the probiotic bacteria contributing to better intestinal absorptive efficiency which provides more nutrients for shell production. Kalavathy et al. (2005) evaluated the effects of adding 12 *Lactobacillus* strains to laying hens to determine if their overall performance and egg quality would differ between the 12 strains of *Lactobacillus*. They found that feed efficiency as well as egg production, egg weight, and egg size was improved. This research has been further supported by the research by Nahashon et al. (1994a,b, 1996).

Probiotics

Probiotics are identified as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 1989). They are organisms typically from the bacilli class which include the following species: *Lactobacillus, Bifidobacterium, Bacillus, Enterococcus,* and *Streptococcus*. All of these organisms have been investigated since the 1900's. The most common bacteria used in probiotics for humans are *Lactobacillus* and *Bifidobacterium,* whereas *Bacillus, Enterococcus and Saccharomyces* yeast are also used in formulating probiotics supplements for livestock production. The benefit of multiple strains in a probiotic supplement is their broad range of activities which allow them to withstand more challenges (Fuller, 1989). Probiotics adapt to beneficially assist several host cell

functions, yet the main benefits they provide to their hosts include an improved immune system as well as intestinal protection. An important factor to consider when discussing the use of bacteria as a probiotics, is that they need to be able to survive in large amounts of medium and remain durable in different situations as well as being able to continue to be viable throughout the desired time frame.

If the host is experiencing stress, the body's defense mechanism can be out of balance, resulting in poor performance and increases in infectious diseases (Fuller, 1999). For probiotics to be considered beneficial, they must be able to tolerate gastric juices, a low pH and bile salts (Salminen et al., 1999). Several bacteria species have adapted and evolved enabling them to thrive and grow in the human intestine. For example an individual's gastrointestinal tract can have 300-500 different species of bacteria (Simon et al., 1984; Borriello, 1986). These species of bacteria can withstand many physical variations in food products such as storage temperatures, processing, and resistant to acidic conditions and then after consumption, they resume activity in the host's gastrointestinal tract (Chateau et al., 1993). When probiotics are supplemented, predisposing circumstances such as intestinal infections are known to not cause an increase in diseases symptoms in people. Once beneficial bacteria have attached to the intestinal lining, they are capable of changing the pH in the gut, this change in pH prevents other bacteria from attaching and proliferating in the gut reducing the potential for foodborne outbreaks.

In the intestinal tract, there are many species of bacteria that can proliferate and flourish, but not all can colonize in the gastrointestinal tract. The inability to colonize would lead to a diminishment of their numbers in the host. Another way bacteria can be

expelled from the body is by being flushed out with excrement through a process called peristalsis. Bacteria such as *Lactobacillus* or *Bifidobacteria* colonize in the gastrointestinal tract and become the dominate bacteria by not allowing other potential pathogenic bacteria to colonize. Fuller (1977) showed that once lactobacilli established a population within the gut, they produce an acidic environment with a pH of 4.5. Miyamoto et al. (2000) reported that increasing *Lactobacillus* in laying hens may prevent colonization of pathogenic bacteria and improve production.

Probiotics were first used as an alternative to antibiotics and became of interest to many different industries such as Poultry, livestock, and human product industries after it was discovered that they prevented pathogen colonization. Probiotic supplements can be added to an animal's diet by capsules, paste, powder or granules. It can also be administered orally in a liquid form. Several Poultry studies have shown when probiotics are supplemented into layer hen feed; it not only decreases pathogens in the GI tract but also increased egg weight, egg production, and feed conversion (Nahashon, et al., 1994, 1996; Mohan, et al., 1995; Abdulrahim, et al., 1996). Conversely, several other studies have shown there was no significant difference in layer overall performance by the addition of probiotics (Goodling, et al., 1987; Balevi, et al., 2001). After extensive research of feeding probiotics, there is still debate whether or not a negative effect occurs in the host's gastrointestinal tract. The effect these bacteria may have on other anatomical systems will need to be further investigated to better understand the possible negative effects they could cause to the host.

Conclusion

Poultry production has vastly advanced over the past hundred years in the process of domesticating the Red Jungle fowl to meat and egg production today. Many factors in this process are taken into consideration from genetics to general maintenance of flocks. A few factors include genetic selection between males and females, reproductive traits, and flock health. Pathogenic bacteria are a major concern within the Poultry industry due to the possibility of horizontal transmission between birds, such as male and female contact in the breeder houses, to vertical transmission where those very same parents contaminate their offspring. Pathogenic and non-pathogenic bacteria are ubiquitous in the Poultry environment as well as in the bird itself (crop, gastrointestinal tract, and/or reproductive tract). Some bacteria are naturally occurring at a young age in the bird and others are beneficial to the gastrointestinal tract, and still others in large doses can cause illness. Many factors arise when discussing transmission of bacteria from parents to offspring due to the probability of chicks being colonized by pathogenic bacteria. Continuous concern, control, and maintenance of the transmission of any bacteria can always be beneficial within the Poultry industry. To better understand why and how bacteria are affecting the flock will be beneficial when determining where the bacteria originated and how it is detrimental to the flock. One main focus that is under continuous investigation within the Poultry industry is the concern that bacteria are being transmitted from parents to their offspring by way of the reproductive tract which may not only affect human health but also bird health and overall performance.

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CHAPTER III

IMPACT OF SIX DIFFERENT INTESTINAL BACERIA ON BROILER BREEDER SPERM MOTILITY IN VITRO

Abstract

Male fertility is most often evaluated through determination of sperm concentration, viability, and motility. In some mammalian species, including humans, sperm samples have been shown to have reduced motility when bacteria are present. In male broiler breeders, bacteria have been shown to be associated with spermatozoa, but their effect on motility has not been thoroughly investigated. Additionally, the sperm quality index (SQI) is a modern rapid method of evaluating avian sperm motility. Therefore, the objective of this study was to use the SQI and determine if broiler breeder sperm motility is reduced when various bacteria are introduced to the ejaculate. In this experiment, semen was collected from 20 Cobb MX broiler breeders by the abdominal massage method. Individual semen samples were pooled and stored at room temperature on a rotary shaker to provide aeration. Six different intestinal bacteria, *Salmonella enterica, Escherichia coli, Campylobacter jejuni, Clostridium bifermentans, Lactobacillus acidophilus and Bifidobacterium animalis* were cultured overnight and used to determine if they inhibited the SQI of broiler breeder sperm. For each bacterium, 50 µl of semen was diluted in 450 µl of either saline, sterile broth, or a broth containing bacteria, which subsequently created a saline control, broth control, or broth containing

bacteria treatments, respectively. The entire experiment was repeated twice. In each treatment, 3 replicates were evaluated at 0 and 10 min post inoculation creating a completely randomized design with a split plot over time. pH was taken of each treatment at 0 min and at 10 min. The results indicated that all broths containing bacteria immediately reduced broiler sperm motility when compared to controls ($P < 0.0001$). Broths containing *B. animalis* or *L. acidophilus* completely and immediately eliminated sperm motility. Although broth containing *S. enteric* immediately reduced sperm motility, the reduction did not change over time. On the other hand, broths containing either *C. jejuni, C. bifermentans,* or *E. coli* reduced sperm motility immediately, but as time passed the motility continued to decrease ($P = 0.0043$, 0.0001, and 0.0002, respectively). For pH, there was a difference when semen was exposed to the each bacterium. The *L. acidophilus* and *B. animalis* treatment had the lowest pH as well as the lowest SQI. In conclusion, bacteria are capable of reducing the motility of broiler breeder sperm. pH may be a factor that bacteria are using to lower rooster sperm motility. It is also apparent that the degree to which motility is affected is dependent upon the bacteria and in some cases time.

Key words: sperm motility, sperm quality index, bacteria, fertility

Introduction

Bacteria can be transmitted numerous ways but the 2 main pathways are known as horizontal and vertical. Horizontal transmission is when bacteria (pathogenic or non-pathogenic) are transmitted from the environment to the bird. Vertical transmission is when bacteria are transferred from the parents to their offspring. These transmission pathways are constantly under investigation to better understand and control potential

contamination within the Poultry industry. When investigating vertical transmission, numerous studies have found that mammal semen is contaminated with pathogenic bacteria such as *E. coli* or *Campylobacter*. In mammals, research has shown that pathogenic bacteria affect mammalian sperm structure and motility which leads to a decrease in male fertility. For example, *E. coli* has been shown to reduce sperm motility in ram semen (Yaniz et al., 2010) as well as boar semen (Martin et al., 2010; Bussalleu et al., 2011). *Campylobacter* has also been determined to reduce semen quality in the ram (Bar et al., 2008). In this study, Bar and colleagues found that *Campylobacter* damaged the acrosome of ram sperm and caused the sperm head to separate from the tail.

In Poultry, Cox et al. (2002a) found *Campylobacter* to be naturally occurring in broiler breeder male semen and in the vas deferens. Donoghue et al. (2004) discovered *Salmonella* and *Campylobacter* to be naturally occurring in turkey semen, and Gale and Brown (1960) showed that turkey semen was contaminated with *Staphylococcus* spp., *coli*forms, *Streptococci* spp, and *Bacillus* spp. Wilcox and Shorb (1958) discovered that concentrations of bacteria in rooster semen was approximately 2.2 x 10□ cfu/ mL, and included *Escherichia, Staphylococcus, Bacillus*, and *Enterococcus* species. Many studies have isolated bacteria from avian semen, but these studies have not demonstrated these bacteria have an effect on sperm quality.

The previous research raises a couple of concerns in the Poultry industry about broiler breeders, mainly food safety and overall fertility. The first major concern is the probability of pathogenic bacteria being vertically transmitted from the parents to their respective offspring and ultimately to the consumer. Pathogenic bacteria such as *Salmonella, E. coli, Campylobacter* and *Clostridium* are most commonly found in the

digestive tract of Poultry. *Salmonella* has been shown to be vertically transmitted through the trans-ovarian route (McGarr et al., 1980; Ranta and Maijala, 2002). Vizzier-Thaxton et al. (2006) demonstrated that *Campylobacter* has the ability to attach to rooster sperm, which can then be vertically transmitted from the parents to their respective offspring. Having a flock that is infected with pathogenic bacteria can lead to a larger concern about the bacteria's contamination of food for human consumption. These pathogenic bacteria are also a health concern worldwide and cost the United States approximately $77.7 billion per year (CDC, 2011). *Salmonella* and *Campylobacter* are the most common pathogens reported that can cause foodborne infections that alone cost $2.4 billion every year (CDC, 2011).

Non-pathogenic bacteria are used within the Poultry industry as feed supplements also known as probiotics. Two of the most commonly used bacteria in feed supplements are *Lactobacillus* spp. and *Bifidobacterium*. These bacteria have been found to be beneficial to the gastrointestinal microflora of mammals and avian species (Fuller, 1989). On the production side, research has been shown that when layer hens are feed probiotics, an increase in egg production, egg weight, and feed conversion occurs (Nashashon et al., 1994, 1996). There is limited research indicating non-pathogenic bacteria are detrimental flock fertility but if semen comes in contact with these bacteria in the cloaca, the potential for vertical transmission arises as well as their potential to affect male fertility.

Rooster fertility is also a major concern of the Poultry industry. Male fertility is commonly evaluated by three parameters, which include sperm concentration, viability, and motility. The ability of pathogens to affect sperm quality is a concern that needs to be addressed. One way to determine the overall sperm quality is by using the Sperm

Quality Analyzer (McDaniel et al., 1998). This machine measures how many times the sperm pass over a light beam, which then provides a single reading known as the Sperm Quality Index (SQI). McDaniel et al. (1998) determined that any changes to the sperm or semen dilution are detected by the SQI reading. Because the SQA can provide an accurate reading which evaluates the quality of the semen, different alterations like concentration or addition of materials can determine if there is an effect on sperm quality. Since there is little research examining the effect that bacteria have on the quality of rooster sperm, the objective of this study was to determine if different pathogenic and nonpathogenic bacteria associated with male broiler breeders have an effect on sperm quality.

Materials and Methods

Semen Collection and Analysis

Twenty White Rock roosters were housed in individual cages, provided food and water ad libitum, and they received 16 h of light per day. Semen was collected from the White Rock roosters by the abdominal massage method of Burrows and Quinn (1937). The neat semen was pooled into a sterile scintillation vile and kept aerated on a rotary shaker for the duration of the experiment to maintain viability of the sample. To insure the pooled neat semen sample was within the normal range it was analyzed by the fluorometric method of Bilgili and Renden (1984) for sperm viability and the photometric method of King and Donoghue for sperm concentration (2000; IMV microreader, IMV International, Maple Grove, MN).

Bacterial Cultures

Overnight cultures of the following bacteria were used for analysis: *Salmonella enterica* (American Type Culture Collection; ATCC 4931) *Escherichia coli* (ATCC 8739) , *Campylobacter jejuni* (ATCC 33291), *Clostridium bifermentans* (ATCC 17839), *Bifidobacterium animalis* (ATCC 27536) and *Lactobacillus acidophilus* (ATCC 314). *S. enterica, E. coli* and *C. jejuni* were cultured in Brucella broth (Acumedia, Neogen, Lansing, MI), *C. bifermentans* was cultured in Tryptic Soy broth (Bacto, Sparks, MD), *B. animalis* was cultured in Reinforced *Clostridium* broth (Difco, Sparks, MD), and *L. acidophilus* was cultured in Lactobacilli deMan, Rogosa Sharpe broth (MRS; Difco, Sparks, MD). Each bacterium was hydrated in their appropriate broths one week prior to the experiment. One milliliter of each bacterial culture was aseptically transferred into 9 mL of their respective broth every 24 hours to provide optimum growth conditions. *S. enteric, E. coli*, and *L. acidophilus* were grown aerobically, *C. jejuni* was grown in a microaerophilic environment (80% N_2, 10% CO_2, 5% H_2, and 5% O_2), and *C. bifermentans* and *B. animalis* were grown in an anaerobic environment. All cultures were incubated in an incubator (VWR, Model 1535, Cornelius, OR) at 37°C while on a constant orbit junior shaker (Model 3520, Pittsburgh, PA).

Treatments

The neat semen sample had a total of 3 different treatments tested. All 6 bacteria were grown to a concentration of 106 cfu/ mL. The treatments included; 1) pooled neat semen diluted in saline, 2) sterile broth in which each bacteria was cultured in, or 3) one of the 6 bacterial cultures. Each treatment was mixed in a microcentrifuge tube to make a 1:10 dilution (50 µl of the neat semen and 450 µl of the treatment medium) in triplicate.

Immediately after the semen was exposed to the respective treatment medium, 3 simultaneous readings were obtained using the Sperm Quality Analyzer (SQA; McDaniel et al. 1998) from each microcentrifuge tube. Samples in the microcentrifuge tubes were then kept open to aerate the sperm so an additional 3 readings could be obtained after 10 minutes of incubation. Because sperm concentration was identical across treatments, the SQI was only influenced by sperm motility and not sperm concentration.

pH

After the first SQA readings, a pH indicator strip (VWR, West Chester, PA) was placed into each microcentrifuge sample tube containing the sample to obtain a pH reading. A second pH reading was obtained after the 10 min incubation period. For samples with a pH falling below the detectable limits of the pH indicator strip (< 6.0), a pH meter (Fisher Scientific, XL60, Accumet excel) with a micro pH electrode (Lazar research lab, LA, CA model, PHR-146B) was used to obtain an accurate pH reading.

Statistical Analysis

Data were analyzed using a completely randomized split plot design. Treatment represented the whole plot split over time. The GLM statistical procedure of SAS was used (Steel and Torrie, 1980). Means were separated with Fishers protected LSD and where considered significant at $p \leq 0.05$.

Results

Prior to the start of the experiment, the pooled neat semen sample was analyzed to determine if it was within the normal range. Semen that is within the normal range contain approximately 3.5 billion sperm/ mL and has a dead sperm percentage around

10% (King and Donoghue, 2000; Bilgili and Renden, 1984). The neat semen sample in our study was determined to have approximately 3.6 billion sperm/ mL. The percentage of dead sperm was 11.2% for the pooled neat semen sample. Therefore, our semen sample was within the normal range, giving us viable semen to conduct the experiment.

When semen was exposed to *Salmonella enterica* there was a significant decrease in SQI (385.8; Fig. 3.1). However, there was no significant difference in the SQI between exposed to saline (385.7) and semen exposed to Brucella broth (377.6), at 0 min or after 10 min of incubation. There were no differences detected between the 3 treatments for pH at 0 min or at 10 min (Fig. 3.2).

When semen was exposed to *E. coli*, a time by treatment interaction was detected for sperm motility (Figs. 3.3, A & B). This resulted from a reduction in sperm motility for semen exposed to *E. coli* between 0 minutes (151) and 10 minutes (111.5) of incubation. No difference was detected between the saline (397.6) and broth (378.9) treatments. Semen exposed to saline had the highest pH reading (7.3), whereas semen exposed to the broth treatment had a reduced pH of 6.9 and when semen was exposed to the *E. coli* treatment the pH was further reduced to 6.5 (Fig. 3.4).

For *Campylobacter,* a significant decrease was detected in the SQI when semen was exposed to *C. jejuni* (115.08) compared to semen exposed to saline (408.9) or the broth treatments (382.4; Fig. 3.5). No difference was detected between the saline or broth treatments for SQI. For pH, no significant difference was observed between the saline (7.4) and broth (6.9) treatments and there was no observed difference in pH between the broth (6.9) and bacteria treatments pH. However, the pH of the *C. jejuni* treatment was different when compared to the saline treatment (7.4; Fig. 3.6).

Semen exposed to *Clostridium* showed a time by treatment interaction where the SQI significantly decreased immediately after semen was exposed to the *Clostridium* culture (221.3), but decreased even further after 10 minutes of incubation (121.6; Fig. 3.7). No significant difference between the saline (423.17) and broth (365.5) treatments over time was observed. The mean SQI for the *Clostridium* treatment was 221.3 at 0 min and it was then reduced to 171.5 after 10 min of incubation. For saline control treatment (7.3) had the highest pH value when compared to the broth (6.6) and the *Clostridium* treatments (6.4). No difference in pH was detected between the broth or *Clostridium* treatments (Fig. 3.8).

Bifidobacterium treatment resulted in a significant decrease in SQI when compared to all other treatments (Fig. 3.9). Mean SQI for the broth treatment group (283.8) was reduced when compared to the saline treatment (417.8), but when semen was exposed to *Bifidobacterium*, mean SQI was reduced further to 38.8. There was no difference between treatments after 10 minutes of incubation. The pH for the *Bifidobacterium* treatment was not different between the saline (6.9) and broth (6.5) treatments but when semen was exposed to *Bifidobacterium*, the pH was significantly reduced (4.9; 3.10).

When semen was exposed to *Lactobacillus*, a time by treatment interaction was detected for sperm motility (Figs 3.11 A & B). When semen was exposed to broth at 0 minutes, SQI was 178.8 but after 10 minutes of incubation, SQI was further reduced to 90.1. When semen was exposed to *Lactobacillus*, mean SQI at 0 min was 35.8 and after 10 minutes of incubation, mean SQI was reduced to zero (Fig. 3.1). When semen was exposed to the saline treatment, an SQI mean of 420.3 was detected and when semen was

exposed to broth a reduction was determined at 134.5. When semen was exposed to *Lactobacillus,* a further reduction occurred (17.9) as compared to the saline and broth control treatments. For pH, there was no significant difference between saline (6.6) and broth (6.4) treatments but when semen was exposed to *Lactobacillus*, the pH was reduced to 4.3 (3.12).

Discussion

Pathogenic and non-pathogenic bacteria have been found to be ubiquitous and more specifically to be naturally occurring in Poultry semen. Pathogenic bacteria such as *Salmonella*, *E. coli*, *Campylobacter* and *Clostridium* are all of concern when it comes to bird health and food safety. Non-pathogenic bacteria, Lactobacilli spp. and *Bifidobacterium* spp., are not known to cause harm to the bird when ingested as a feed supplement. Numerous studies have been conducted on mammalian species to determine if bacteria have an effect on sperm motility. *E. coli* has been shown to have a negative effect on ram semen (Yaniz et al., 2010) and boar semen (Martin et al., 2010; Bussalleu et al., 2011). *Campylobacter* has been shown to actually damage the structure of ram semen. The results from this experiment demonstrate that these pathogenic and non-pathogenic bacteria have a negative effect on avian sperm motility as well.

Semen contaminated with bacteria is a major concern with regards to flock health, as well as the transmission of bacteria from parents to offspring. Since sperm are analyzed by 3 parameters, which are sperm concentration, viability, and motility, they must be able to overcome many barriers, some of which bacteria may influence. If there are obstructions blocking the sperms' ability to swim properly then the sperm's ability to fertilize the ovum efficiently is negatively affected.

Salmonella is one of the most common pathogenic bacteria found in Poultry and Poultry products. *Salmonella* has been found to be naturally occurring in turkey sperm but differences in the sperm structure due to the presence of *Salmonella* were not reported (Donaghue et al., 2004). *Salmonella* contamination has also been found in the cloaca (Smith, 1949), uterus, and vagina (Harry, 1963; Jacobs et al., 1989) of the hens. Since, *Salmonella* has been found to occur naturally in rooster semen (Reiber et al., 1995), transmission of *Salmonella* from the male to the female's reproductive tissues is also possible due to the passage of excreta from the cloaca where semen is released. There is minimal research on *Salmonella* effecting sperm motility in mammals and avian species. Nevertheless, our results show that *Salmonella* at a concentration of 1,000,000 cells decreases rooster sperm motility immediately upon exposure, which could be detrimental in the breeder industry if *Salmonella* is at that concentration in the cloaca or reproductive tissues of the breeders. Although *Salmonella* thrives in a neutral pH, the results in this experiment show when rooster semen is exposed to *Salmonella* a numerical difference is detected as well as a decrease in sperm motility. These detrimental effects involve not only decreases in sperm motility but also the possibility that this organism can be transmitted through horizontal and vertical pathways which could lead to foodborne outbreaks.

Escherichia coli are another pathogenic bacteria to consider, when evaluating transmission from one bird to another. If this occurs it may ultimately cause illness to humans by the consumption of contaminated food products. More specifically, *E. coli* has been reported to be naturally occurring in human semen causing a decrease in motility and subsequent infertility (Auroux et al., 1991; Diemer et al., 1996). Bussalleu

et al. (2005) reported that even though boar semen contaminated with *E. coli* decreased sperm motility they did not affect the structure of the sperm. In our study, when rooster semen was exposed to *E. coli*, semen motility was significantly decreased supporting the previous work conducted in mammals. Another factor to consider is pH, when discussing rooster sperm motility. As pH was reduced upon exposure to *E. coli*, semen motility was also reduced in this experiment.

Because Poultry products can be a major reservoir for *Campylobacter* there is a major concern when evaluating its mode of transmission. Bacteria can be transmitted horizontally by the ingestion of fecal droppings (Cox et al., 1990; Cox and Bailey, 1991). *Campylobacter* has also been found to be naturally occurring in the vas deferens and semen of rooster's (Cox et al., 2002b; Buhr et al., 2002). More specifically, C. *jejuni* has been shown to be even attached to rooster sperm (Vizzier-Thaxton et al., 2006). This is not only important for understanding the horizontal transmission pathway but demonstrates the potential for vertical transmission. This may not only be an area of focus for reducing foodborne illness but additionally may impact chick production. In our study, C. *jejuni* reduced rooster sperm motility immediately after exposure. The reason for this immediate reduction may be due to the attachment of *Campylobacter* to the sperm which Vizzier-Thaxton et al. (2006) demonstrated. *Campylobacter* in ram semen has also been shown to attach on the tail and acrosome resulting in a decrease in sperm motility (Bar et al., 2008) which demonstrates a mechanism by which *Campylobacter* may reduce offspring production. When semen was exposed to *Campylobacter* in this experiment, pH was reduced which could be a contributing factor when examining sperm motility due to the fact they prefer a neutral pH environment to

swim properly. To determine if *Campylobacter* affects the rooster sperm structure, further research will be required.

Clostridium perfringens, another pathogenic bacterium responsible for foodborne illness outbreaks is also located in Poultry fecal droppings (Craven et al., 2003). *Clostridium* has been found to be naturally occurring in chicken ceca (Cox 2005). Although *Clostridium* has not been found in the entire reproductive tract of roosters, they have been in found in the vas deferens and ceca (Cox et al., 2005). The chance of transmission increases significantly when found in the reproductive tract, especially when natural mating occurs. The results of our study showed a significant decrease in sperm motility immediately after exposure to *Clostridium* as well as a reduction in pH. Due to pH being further reduced after semen was exposed to *Clostridium*, could indicate the reduction in sperm motility may have been discovered because sperm thrive in a neutral pH.

Bifdobacterium and *Lactobacillus* are considered non-pathogenic bacterium that reside in the gastrointestinal tract, and are likewise used in formulation of probiotic supplements. *Bifidobacterium* are naturally occurring at high concentrations in the gastrointestinal tracts of humans and animals (Mitsuoka, 1992). Rada and Petr (2000) found *Bifidobacterium* at concentrations as high as 1010 cfu/ g in the cecum of hens. Lactobacilli and Bifidobacteria are also known as microorganisms which benefit the intestinal microflora of the host (Fuller, 1989). To our knowledge, no research has been conducted demonstrating the effects that these bacteria have on sperm motility. Interestingly, our study showed *Lactobacillus* and *Bifidobacterium* provided the greatest decrease in rooster sperm motility immediately after exposure when compared to the

more familiar pathogenic bacteria. *Lactobacillus* and *Bifidobacterium* are also non-motile microorganisms. These non-motile bacteria could be either inhibiting sperm motility by attaching directly to the sperm or by obstructing the pathway for sperm movement. Further investigation is necessary in order to better understand why semen becomes immotile by these organisms. The production of lactic acid from *Lactobacillus* or *Bifidobacterium* could be a causative agent which reduces sperm motility, because sperm require a neutral pH balance leading to a decrease in sperm motility. The decrease in pH from *L. acidophilus* and *B. animalis* treatments in this study could be an indicator for why sperm motility was significantly decreased because they reduced the pH of the semen sample lower than the pathogenic bacteria. However, further research is needed before this suggestion can be confirmed.

In conclusion, pathogenic bacteria such as *E. coli* and *Campylobacter* spp. have a negative effect on mammalian semen motility as well as avian rooster semen. Pathogenic bacteria can cause harmful effects to birds and to individuals' consuming their meat, but the current research demonstrates that all the bacteria tested in this study also affect male broiler breeder sperm motility. The pH is also a major factor when discussing sperm motility due to the sperms requirement for a neutral pH environment. Further investigation is needed to determine what the major contributor to sperm immotility actually is. Although these non-pathogenic bacteria have been shown to be beneficial, these organisms have been shown to have a significant negative effect on rooster sperm motility when artificially exposed. Which raises the questions of whether probiotic supplementation should be monitored when fed to male broiler breeders.

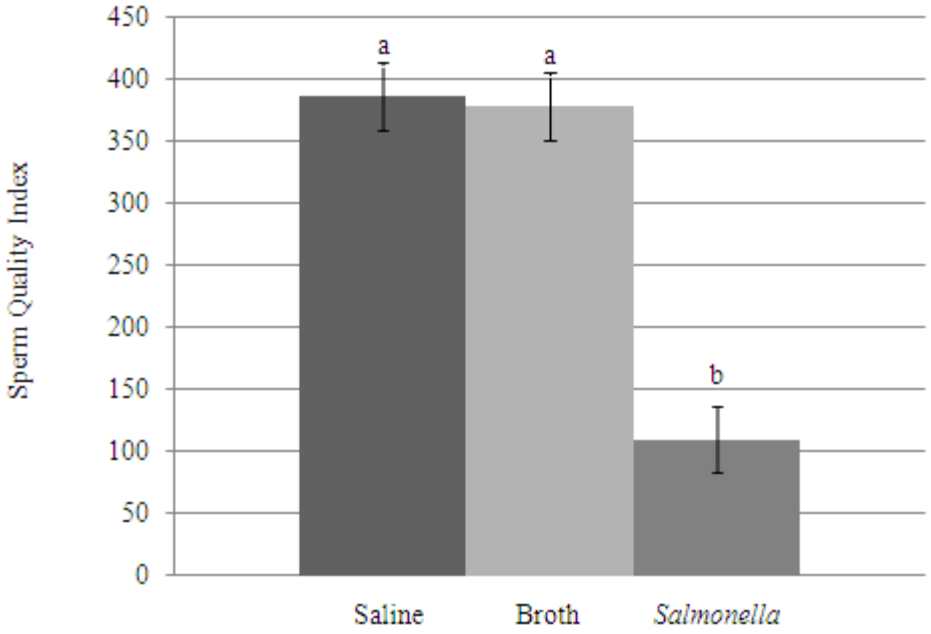

Figure 3.1 *Salmonella* Sperm Quality Index

Mean Sperm Quality Index (SQI) when sperm was diluted in either saline, broth or *salmonella*, respectively Means with different superscripts indicate differences in the SQI due to diluent type ($p<0.0285$; SEM=26.87; n=12)

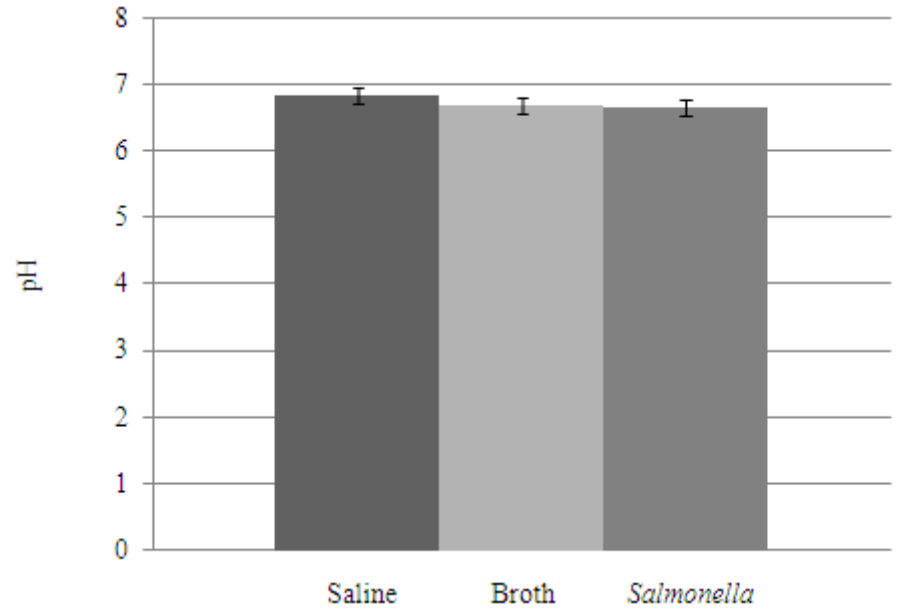

Figure 3.2 *Salmonella* pH

Mean pH when sperm diluted in saline, broth or Salmonella, respectively. No differences were found due to diluents type. ($p<0.5467$; SEM= 0.118; n=4)

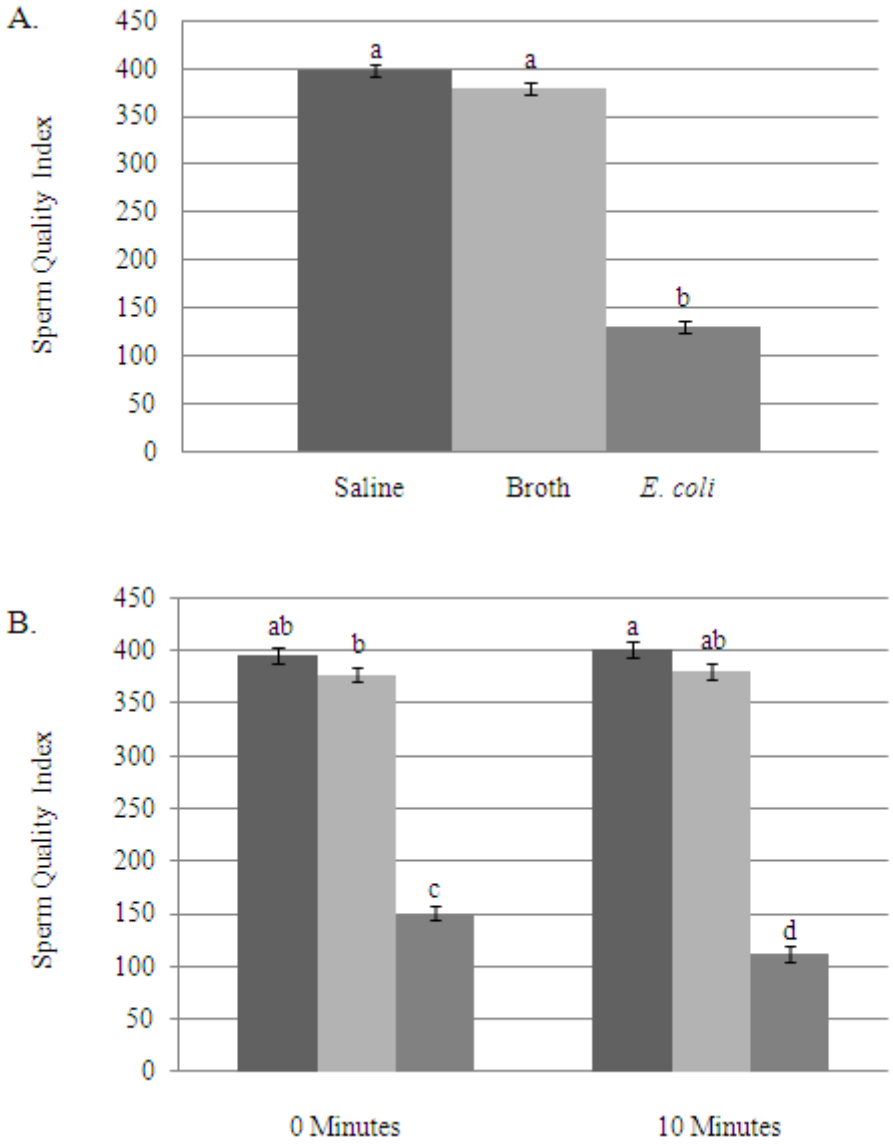

Figure 3.3 *E. coli* Sperm Quality Index (Main effects and Interaction)

Sperm quality index (SQI) for sperm diluted in saline, broth or *E. coli* with A) mean SQI values and B) SQI interaction means for the SQI between 0 minutes and 10 minutes. The dark gray bar represents saline, light gray represents broth, and medium gray represents *E. coli*. Means with different superscripts are significantly different at $p < 0.0084$; SEM= 6.625; n=12. B.) a-d superscripts represent interaction means that are significantly different for the SQI ($p<0.0084$; SEM= 7.52; n=6)

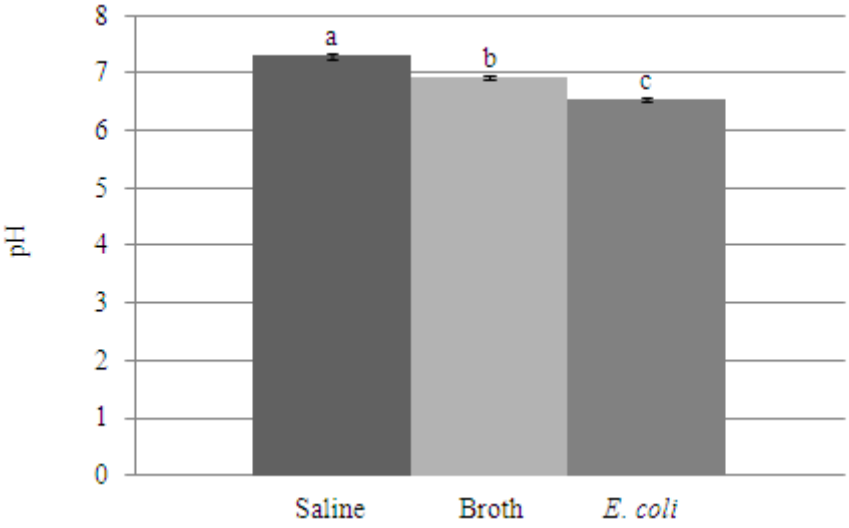

Figure 3.4 *E. coli* pH

Mean pH when sperm was diluted in saline, broth or *E. coli,* respectively. Means with different superscripts indicate differences in the SQI means ($p < 0.0132$; SEM=0.0433; n=4)

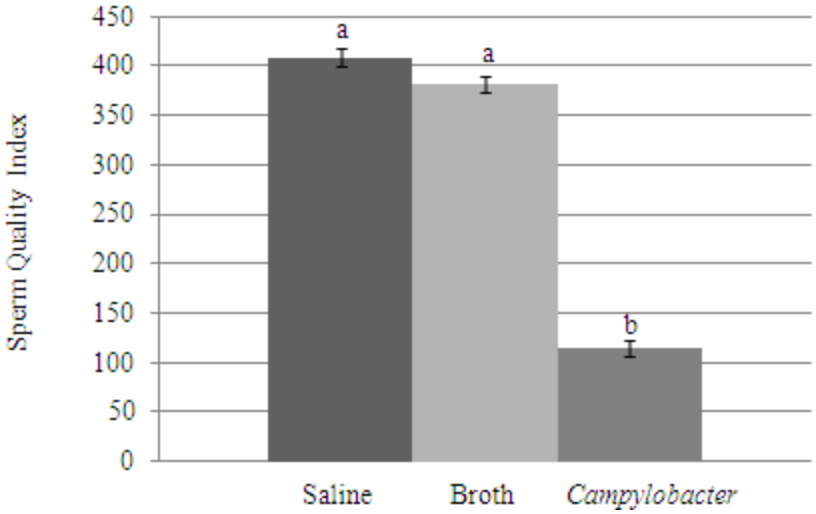

Figure 3.5 *Campylobacter* Sperm Quality Index

Mean Sperm Quality Index (SQI) when sperm was diluted in saline, broth or *Campylobacter,* respectively. Means with different superscripts indicate differences in the SQI means ($p < 0.0025$; SEM=8.13; n=12).

Figure 3.6 *Campylobacter* pH

Mean pH when sperm was diluted in saline, broth or *Campylobacter*, respectively. Means with different superscripts indicate differences in the SQI means. ($p<0.07$; SEM=0.115; n=4).

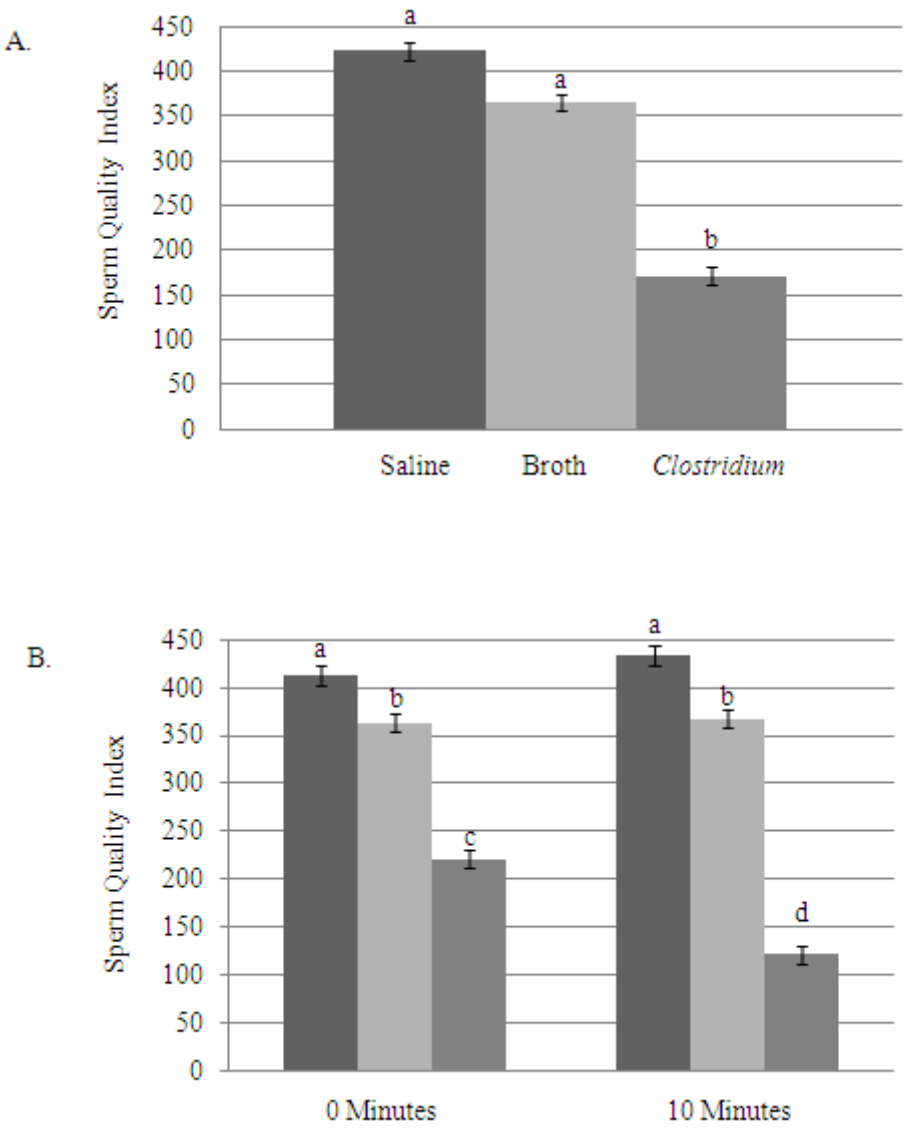

Figure 3.7 *Clostridium* Sperm Quality Index (Main Effects and Interaction)

Sperm quality index (SQI) for sperm diluted in saline, broth or *Clostridium* with A) mean SQI values and B) SQI interaction means for the SQI between 0 minutes and 10 minutes. The dark gray bar represents saline, light gray represents broth, and medium gray represents *Clostridium*. Means with different superscripts are significantly different at $p< 0.0001$; SEM= 9.925; n=6. B.) a-d superscripts represents interaction means that are significantly different for the SQI ($p<0.0001$; SEM= 9.85; n=12)

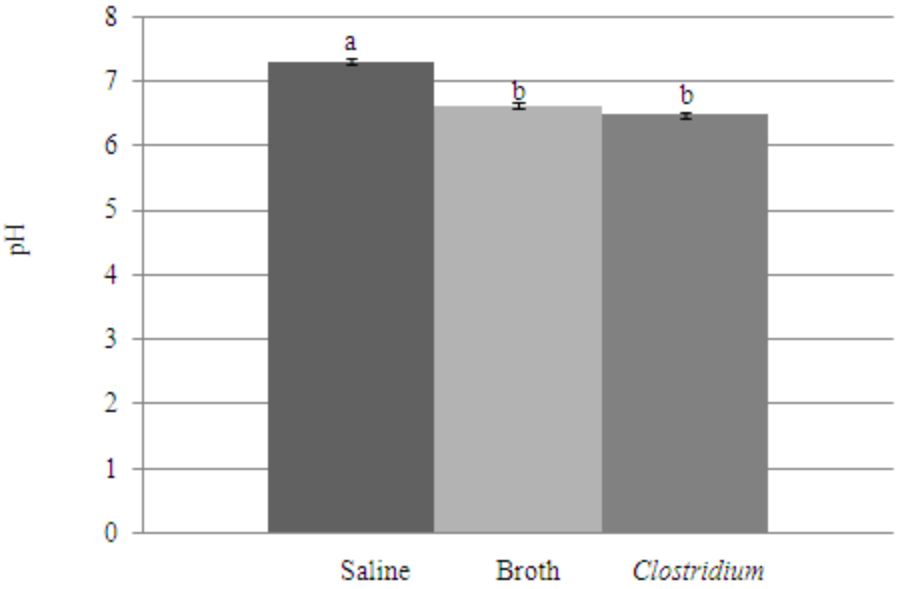

Figure 3.8 *Clostridium* pH

Mean pH when sperm was diluted in saline, broth or *Clostridium,* respectively. Means with different superscripts indicate differences in the SQI means. ($p<0.0096$; SEM=0.043; n=4).

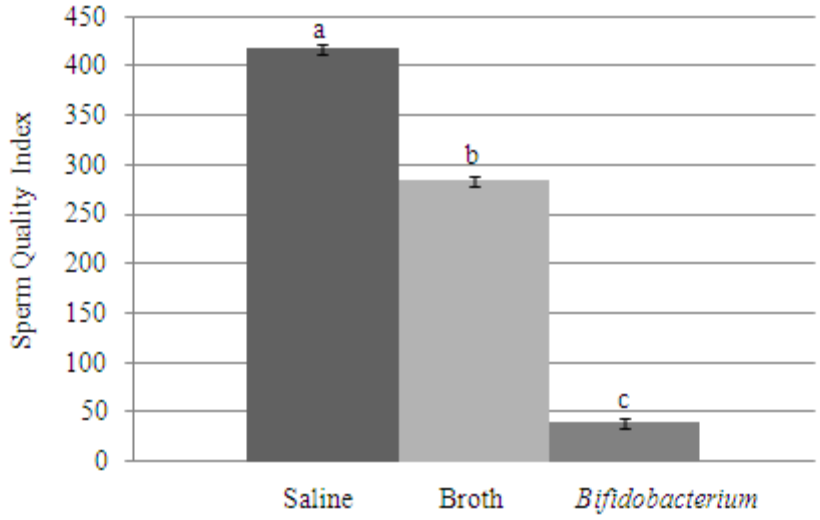

Figure 3.9 *Bifidobacterium* Sperm Quality Index

Mean Sperm Quality Index (SQI) when sperm was diluted in saline, broth or *Bifidobacterium,* respectively. Means with different superscripts indicate differences in the SQI means ($p<0.0008$; SEM=5.34; n=12).

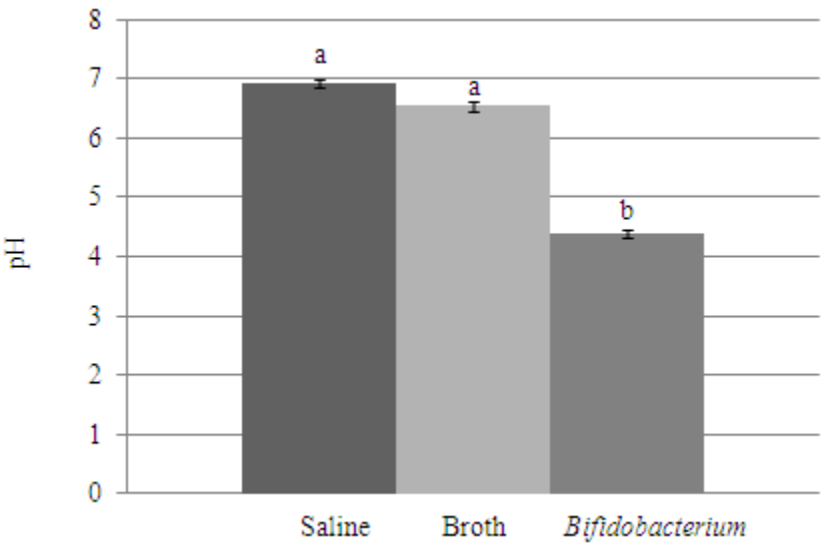

Figure 3.10 *Bifidobacterium* pH

Mean pH when sperm was diluted in saline, broth or *Bifidobacterium,* respectively. Means with different superscripts indicate differences in the SQI means. ($p<0.0036$; SEM=0.083; n=4).

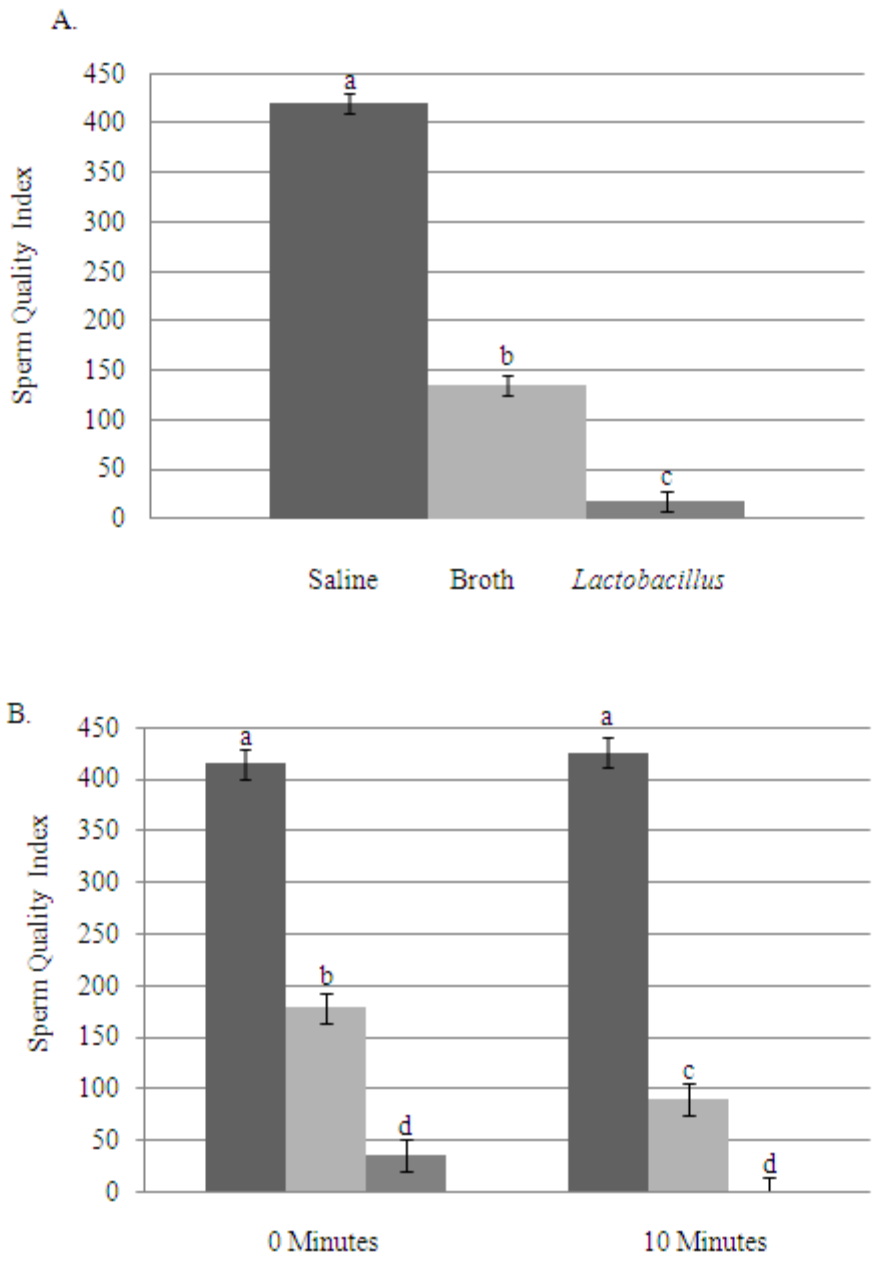

Figure 3.11 *Lactobacillus* Sperm Quality Index (Main Effects and Interaction)

Sperm quality index (SQI) for sperm diluted in saline, broth or *Lactobacillus* with A) mean SQI values and B) SQI interaction means for the SQI between 0 minutes and 10 minutes. The dark gray bar represents saline, light gray represents broth, and medium gray represents *Lactobacillus*. Means with different superscripts are significantly different at over each time period. A-B. SQI means in graph A with different superscripts are significantly different at $p< 0.0090$; SEM= 10.35; n=12. B.) Whereas a-d represents interaction means that are significantly different for the SQI ($p<0.0090$; SEM= 14.81; n=6)

Figure 3.12 *Lactobacillus* pH

Mean pH when sperm was diluted in saline, broth or *Lactobacillus,* respectively. Means with different superscripts indicate differences in the SQI means. ($p<0.0106$; SEM=0.131; n=4).

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CHAPTER IV

THE EFFECT OF EXPOSING ROOSTER SEMEN TO *LACTOBACILLUS* ON FERTILITY

Abstract

It has been proven that *Lactobacillus* resides in the gastrointestinal tract, vagina and cloaca of hens. From a previous experiment, semen exposed to *Lactobacillus in vitro* immediately reduced sperm motility. Therefore, the objective of this study was to determine if semen exposed to *Lactobacillus* immediately prior to artificial insemination affects the overall fertility of White Leghorn hens. For the experiment, at least 20 mL of semen was collected from White Rock roosters. The semen was used to create 4 different treatments: 1) semen with saline, 2) semen with deMan, Rogosa, Sharpe (MRS) broth, 3) semen with a low dose of *Lactobacillus* and 4) semen with a high dose of *Lactobacillus*. Each sample was diluted to a 1:1 ratio of 275 µl of semen to 275 µl of the respective medium, and a Sperm Quality Index (SQI) reading was obtained. Once the samples were diluted, 80 hens in each treatment were equally divided among 10 blocks and were inseminated with 50 µl of diluted semen. Eggs were collected from 2 to 7 days post insemination (DPI). Artificial insemination occurred again on day 8 and eggs were collected for another 7 days. Eggs were incubated for 10 d. After incubation, all eggs were examined for fertility, and a portion of the eggs were examined for *Lactobacillus*. The entire experiment was repeated twice. The results showed that semen quality was

within the normal range for treatments 1, 2, and 3, but semen from treatment 4 was entirely immotile before insemination. Additionally, 84% of the eggs from treatments 1, 2 and 3 were fertile; however, all eggs from treatment 4 were infertile. There was no difference between treatments for *Lactobacillus* counts in the yolk or on the shell. However, fertility and *Lactobacillus* shell counts declined over DPI. In fact a negative correlation (r = -0.28) between fertility and *Lactobacillus* shell counts was observed for DPI averages from each block. In conclusion, the high dose of *Lactobacillus* exposed to rooster semen resulted in completely infertile eggs most likely because sperm were immotile at the time of insemination.

Introduction

Understanding male and female fertility is imperative in order to control Poultry production and to preserve genetic selection among flocks. Artificial insemination is commonly used within the Poultry industry to maintain certain genetic characteristics and produce breeding stock. Rooster sperm for artificial insemination must meet three criteria. The criteria that are necessary to optimize egg fertilization are sperm viability, concentration, and motility. The ovum is fertilized in the hen's infundibulum, but the sperm must overcome many barriers and travel a long distance in the oviduct to be able to penetrate the germinal disc of the egg (King and McLelland, 1984). Not only is fertility important, but hatchability is also important when determining flock performance. If either the rooster or hen is not performing efficiently, fertility will decrease, which affects overall hatchability.

Also, many species of bacteria are naturally occurring within Poultry and mammalian reproductive tracts. Pathogenic bacteria such as *E. coli* have been found in

mammal semen such as the boar (Martin et al., 2010) and ram (Yaniz et al., 2010). Bussalleu and others (2011) determined that different types of *E. coli* as well as different concentrations have an effect on sperm motility even though sperm structural alterations due to *E. coli* were not found. Bar et al. (2008) found that *Campylobacter* also has detrimental effects on ram semen. These organisms actually attach to the sperm's tail and acrosome causing structural damage.

Research has shown that bacteria in avian semen such as *Campylobacter*, *Escherichia coli*, *Staphylococcus*, *Bacillus*, and *Enterococcus,* are also naturally occurring (Donoghue et al., 2004; Wilcox and Shorb, 1958). Other pathogenic bacteria such as *Salmonella* have been shown to be vertically transmitted from parents to their offspring, which raises health concerns when it comes to the human consumption of Poultry (Baker et al., 1980; Guthrie, 1992). Vizzier-Thaxton et al. (2006) demonstrated that *Campylobacter* and *Salmonella* can both attach to rooster sperm, which could be a mode of transmission from one bird to another as well as from the parents to their offspring.

Contaminated eggs are a major concern in the Poultry industry due to the possibility of pathogenic bacteria infecting chicks or entering the food chain thereby causing foodborne illness. Contamination of the egg has been shown to occur in the shell gland, vagina, and cloaca while the egg travels through the reproductive tract during oviposition (Smith, 1949). Another factor to consider is the possibility of contaminated semen being transferred to the hen during copulation. Sexton et al. (1980) showed that semen in the vas deferens are typically free of bacteria, but during ejaculation, semen can become contaminated once the ejaculate touches the surface of the cloaca (Smith, 1949).

If bacteria are found naturally in the rooster and hen reproductive tracts, there is an increase for possible transmission of harmful bacteria leading to contamination of Poultry products or the possible negative effect on fertility within the breeder flock.

Previous research conducted in our lab tested 4 pathogenic bacteria (*Salmonella*, *E. coli*, *Campylobacter* and *Clostridium*) and 2 non-pathogenic bacteria (*Lactobacillus* and *Bifidobacterium*) in order to determine if semen exposed to these bacteria yielded poor sperm motility as measured by the sperm quality index. In that research semen exposed to *Lactobacillus* gave an index of zero after 10 minutes of incubation, revealing a total lack of movement.

Lactobacilli are non-pathogenic and non-toxic, but are more commonly used when formulating probiotic supplements which are considered beneficial to the gastrointestinal microflora of animals and humans as well as an antagonist to certain pathogens. As defined by Fuller (1989), probiotics are "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". Parker (1974) found that probiotic supplements fed to animals are beneficial to their intestinal microflora. Another study demonstrated that when *Lactobacillus sporogenes* was fed as a supplement to White Leghorn hens, overall egg production, feed efficiency, and egg characteristics such as shell strength, weight, and thickness were significantly improved (Panda et al., 2008). Lactobacilli have also been found to be naturally occurring in the avian cloaca and vagina of laying hens (Van Coillie et al., 2007). In one week old chicks, Lactobacilli have been found to naturally colonize in the small intestine and cecum (Mead, 1997). Although *Lactobacillus* is beneficial to the host, because it is

located in the hen's reproductive tract, there is concern that rooster semen is exposed to *Lactobacillus* resulting in reduced sperm motility and ultimately infertility.

There has been minimal research demonstrating the effects that *Lactobacillus* has on rooster sperm motility, fertility, or the transmission of bacteria from parents to their respective offspring. In our previous research, it was shown that sperm motility was immediately eliminated when semen was exposed to a high dose of *Lactobacillus*. Therefore, the objective of this study was to determine if semen exposed to different concentrations of *Lactobacillus* inhibits fertility after artificial insemination.

Materials and Methods

Housing and Environment

Three hundred and eighty four, 45 week-old White Leghorn hens were housed in commercial type cages. Also, 40 White Rock roosters were individually caged for semen collection. Both hens and roosters were fed a common layer diet and provided water *ad libitum* and exposed to 16 hours of light per day. Females and males were caged in a house with conventional environmental controls.

House Layout

Prior to the experimental period, 320 White Leghorn hens were arranged equally in 10 blocks where in each of the 4 treatments were randomly represented. For each treatment, 8 hens were placed into 2 side by side cages (4 hens per cage), to represent one treatment in each block. Each block contained a total of 32 hens. Also, an additional 64 hens were used as negative-negative controls. The negative-negative hens were not

inseminated with any treatment (semen or *Lactobacillus*) so that baseline *Lactobacillus* levels could be obtained on hens never inseminated.

Semen Analysis

Semen was collected from 40 White Rock roosters using the abdominal massage method of Burrows and Quinn (1937). Neat semen samples were pooled prior to analysis. The neat semen sample was analyzed in duplicate for sperm viability by using the fluorometric method from Bilgili and Renden (1984). Sperm concentration was determined using 2 readings from a microreader (IMV, International, Maple Grove, MN) using the method of King and Donoghue, (2000). Sperm motility was determined in triplicate by the SQA procedure of McDaniel et al., (1998). All analyses were performed before treatments were mixed.

Treatments

Lactobacillus acidophilus (American Type Culture Collection; ATCC #314) was received in a lyophilized form and was hydrated in de Man, Rogosa, Sharpe (MRS) broth (Difco, Sparks, MD) and placed on a shaker in an incubator at 37°C for 48 hours. The culture was aseptically transferred to fresh MRS broth every 24 hours prior to the start of the experiment. *L. acidophilus* was cultured in MRS broth for 12 hours prior to artificial insemination providing approximately 10^6 cfu/ mL of *L. acidophilus*. Five treatments were used for this study. The first treatment was represented as a negative-negative control where hens were not inseminated with rooster sperm, sterile broth, or concentrations of *Lactobacillus*. The following 4 semen diluent treatments were used for insemination: saline, sterile MRS broth, low dose of *Lactobacillus* (3.0 x 10^3 cfu/ml, prior

to diluting), and a high dose of *Lactobacillus* (3.0 x 10^6 cfu/ml, prior to diluting). Each treatment was diluted in a 1:1 ratio (275 µl of the pooled neat semen sample and 275 µl of the respective diluent). Each treatment was thoroughly mixed immediately before an additional SQI reading was obtained followed by insemination.

Sperm Quality Index

The Sperm Quality Index (SQI) is a measurement of overall semen quality. This is achieved by determining how many times motile sperm cross a light beam over a 40 second period of time. After each of the 4 treatment samples were prepared, an additional 1:1 dilution was made, and a portion was drawn into a capillary tube and placed into the SQA (McDaniel et al, 1998). A total of 3 separate readings were taken for each treatment and the values recorded. All readings were obtained immediately prior to insemination of the hens.

Artificial Insemination

After each treatment was diluted and the SQI readings were obtained, each hen for that treatment was inseminated with approximately 87 million sperm/50 µl of respective treatment. Hens were artificially inseminated a second time on day 8 with the same treatments administered as in the first artificial insemination.

Egg Collection and Incubation

Eggs were collected at the same time every day, mid-afternoon. Collection started 2 days post-insemination (DPI) from the first artificial insemination. Collection of eggs was consecutive for a 2 week period of time. Eggs were collected with new rubber gloves for each treatment (4) in all 10 blocks. They were labeled with the block

number, treatment number, date collected, and individual egg number in each treatment in each block. Eggs were stored in a cooler for 0 to 3 days, respectively, and then set in a Jamesway incubator for 10 days at 37.5°C and 86˚C dry and wet bulb temperatures, respectively. After 10 days of incubation, eggs were removed and broken out to determine overall fertility of each treatment, including infertile, early dead, and fertile categories.

Microbial Analysis

An individual egg was removed from each treatment, within each block on each day of collection prior to initial breakout. These eggs were examined for the presence of *Lactobacillus* on the shell, in the yolk, or on the embryo. Each egg was placed in a sterile Whirl Pak ™ bag with 10 mL of buffered peptone water (BPW; Difco, Sparks, MD) and gently massaged for 2 minutes. After 2 minutes, eggs were aseptically removed from BPW and placed in 70% ethanol for 30 seconds. They were then removed and placed on egg trays to air dry. After an egg was removed from the Whirl Pak™ bag containing BPW, the BPW was serial diluted. Once ethanol had evaporated, the remaining eggs were first broken out to determine fertility. After breakout, a sterile swab was placed into the yolk of the egg or on the embryo and placed into a sterile glass tube containing 9 mL of BPW, which was then serially diluted. Each dilution from the shell, embryo, or yolk sampling was thoroughly mixed, and 100 µl was spread onto MRS agar plates. Plates were placed in a low temperature Thermo Scientific incubator (Model 315) at 37°C for 48 hours under aerobic conditions. After 48 hours, plates were examined for smooth white/opaque colonies and each colony was counted and recorded.

Statistical Analysis

Data from the experiment were analyzed by using a completely randomized block design with a split split plot over 2 artificial inseminations and over 7 days post insemination. Replicates were represented within the 10 blocks. The GLM statistical procedure of SAS was used (Steel and Torrie, 1980). Means were separated by using Fisher's protected least significant difference at $p \leq 0.05$.

Results

Once the roosters were abdominally massaged, the pooled neat semen sample was analyzed to determine if the sample was within the normal range prior to artificial insemination as well as after artificial insemination. Semen that is within the normal range contains approximately 3.5 billion sperm/ mL, a percentage of dead sperm around 10%, and SQI reading of ≥ 350 (King and Donoghue, 2000; Bilgili and Renden, 1984; McDaniel et al., 1998). The average sperm concentration was 3.48 billion sperm/ mL, average percentage dead sperm was 9.3%, and the mean SQI was 383. Therefore, the neat semen sample was within the normal range prior to addition of treatments.

After each treatment was mixed prior to artificial insemination of the hens, an additional SQI was determined using the SQA. There was no significant difference between the control (387.5), broth (387.8), or low dose (379.5) treatments, but when the neat semen sample was exposed to the high dose treatment, a value of zero was obtained immediately on the SQA for this treatment in each block ($p<0.0001$; Fig. 4.1). Overall fertility was similar for the control (83.7%), broth (84.6%), and low dose (84.2%) treatments. However, all eggs given the high dose were infertile (0%),($p<0.0001$; Fig. 4.2). Early dead numbers mirrored overall fertility, where the control (2.4%), broth

(2.4%), and low dose (2.6%) treatments were not significantly different, but the high dose treatment yielded no early dead embryonic mortality because all eggs were infertile ($p<0.0144$; Fig. 4.3).

Days post insemination, showed overall fertility following a typical dosimetric curve ($p<0.0001$; Fig. 4.4). At 2 days post insemination, average fertility was 66.8% but on days 3 and 4, fertility peaked at 70 and 69.9% respectively. On day 5, percent fertile decreased to 65.4% and continued to decrease through days 6 (61.9%) and 7 (57.5%). On day 8 fertility was lowest at 51.1%.

Shell samples positive for *Lactobacillus* mirrored the DPI curve for fertility ($p<0.0001$; Fig. 4.5). During days 2, 3, 4 the percentage of shell samples positive for *Lactobacillus* were not significantly different. On those days the percentage of shells positive for *Lactobacillus* was 70, 72.5, and 67.5%, respectively. At day five, the percent positive for *Lactobacillus* peaked at 95.6% but numerically decreased over days 6, 7, and 8 (87.5, 45, 38.5%, respectively).

After 48 hours of incubation, there was no colony growth for the yolk samples on MRS agar plates. The egg shell samples exhibited positive *Lactobacillus* growth after incubation, but no significant difference in colony counts occurred between the control (mean colonies 1.95 cfu/ mL), broth (mean colonies 1.9 cfu/ mL), low dose (mean colonies 1.9 cfu/ mL), high dose (mean colonies 1.8 cfu/ mL), or negative-negative control (mean colonies 1.4 cfu/ mL) groups ($p<0.488$; Fig. 4.6).

Discussion

Both the hen and rooster play an important role when managing fertility for optimum breeder flock performance. Breeder roosters are responsible for fertilizing the

hen's ova upon copulation. Rooster semen quality is often determined by 3 parameters: sperm viability, concentration and motility. A quick procedure for the determination of semen quality involves use of the SQA which measures sperm movement by how many times the sperm cross a light beam (McDaniel et al., 1998). The rooster's sperm must be motile and competitive in order to survive in the hen's oviduct for ovum fertilization (Parker, 1970). If sperm are immotile while in the female's reproductive tract, immediately after copulation, the egg cannot be fertilized. This experiment supports our previous experiment in which semen exposed to *Lactobacillus* at a 10^6 cfu/ mL was immediately immotile. Artificially inseminating hens with a high dose of *Lactobacillus* in the current study resulted in egg infertility. Egg infertility is detrimental to the breeder, subsequently leading to a loss in profit and production.

Although bacteria are found ubiquitously, some are known as pathogenic or non-pathogenic. Pathogenic bacteria such as *E. coli* have been found to decrease sperm motility in humans (Auroux et al., 1991; Diemer et al., 1996) and boars (Bussalleau et al., 2005). *Campylobacter* has been found to decrease ram semen motility by attaching to the sperm acrosome and tail, thereby causing damage to its structure (Bar et al., 2008). There are many studies demonstrating that pathogenic bacteria affect mammalian sperm motility. However, research is limited regarding bacterial effects on avian sperm motility. Lombardo and Thrope (2000) discovered *E. coli, Salmonella* spp., *Shigella* spp., and *Yersina* spp. in wild tree swallow semen. Furthermore, one out of 19 samples were found to be positive for Lactobacilli spp. However, their study did not determine if these bacteria are transmitted to their offspring causing structural damage to the sperm or altering sperm motility. There is minimal research discussing the effects that non-

pathogenic bacteria have on mammalian or avian sperm. This current research demonstrates that non-pathogenic bacteria (i.e. *Lactobacillus*) have a negative effect on rooster semen. In this study, non-pathogenic bacteria clearly demonstrate a negative effect on fertility, particularly when rooster semen is exposed to a high concentration of *Lactobacillus* prior to insemination

This study supported our previous experiment and current hypothesis that neat semen exposed to a high dose of *Lactobacillus* lowers sperm motility, subsequently resulting in egg infertility. Interestingly, Lactobacilli spp. have been found to be naturally occurring in the hen's cloaca (7.7 cfu/ g) and vagina (5.7 cfu/ g; Miyamoto et al., 1998, 2000). Although Lactobacilli are naturally occurring at the site where semen enter the hen, there is no evidence indicating that naturally occurring Lactobacilli are affecting sperm motility. Reiber et al. (1995) discovered bacteria naturally occurring in rooster semen at an average concentration of 3.2×10^5 cfu/ mL. The current study used concentrations of bacteria at approximately 10^6 cfu/ mL, which is similar to that naturally occurring in rooster semen. However, in the current study, the motility of sperm is decreased when they are exposed to this concentration of *Lactobacillus* prior to insemination.

There are many possibilities when considering the inhibition of sperm motility by *Lactobacillus*. One concern could be the possibility of the hen's defensive mechanisms such as her body recognizing this higher concentration of *Lactobacillus* in her oviduct resulting in rejection of the insemination (Lombardo et al., 1999). The results of this study also suggest that when hens are inseminated with a high dose of *L. acidophilus*, sperm are unable to travel to the infundibulum to fertilize the ova. This could be due to

the fact that *Lactobacillus* is non-motile, which could block the pathway for sperm to swim properly. Also, *Lactobacillus* produces lactic acid and a pH between 4 and 5 (Holt et al., 2000), not a neutral environment that sperm thrive in hence not allowing them to swim properly. Another consideration would be that Lactobacilli are attaching to the sperm themselves, thereby damaging the sperm's structure and its ability to swim properly. Further investigations are needed to determine how *Lactobacillus* inhibit rooster sperm motility. Another question of importance is if flock health, fertility, and overall production can be improved as well as balanced from feeding probiotics to breeder flocks.

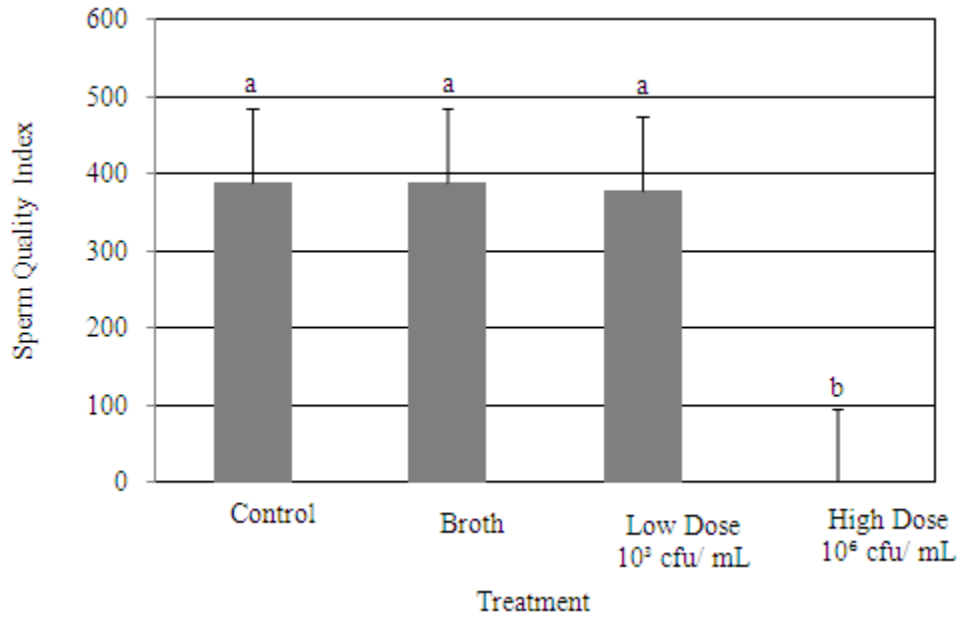


Figure 4.1 Sperm Quality Index before Artificial Insemination

Mean Sperm Quality Index (SQI) before artificial insemination when sperm was diluted in saline, broth, low dose or high dose of *L.acidophillus,* respectively. Means with different superscripts indicate differences in the SQI due to diluent type. ($p<0.0001$; SEM=7.03; n=20)

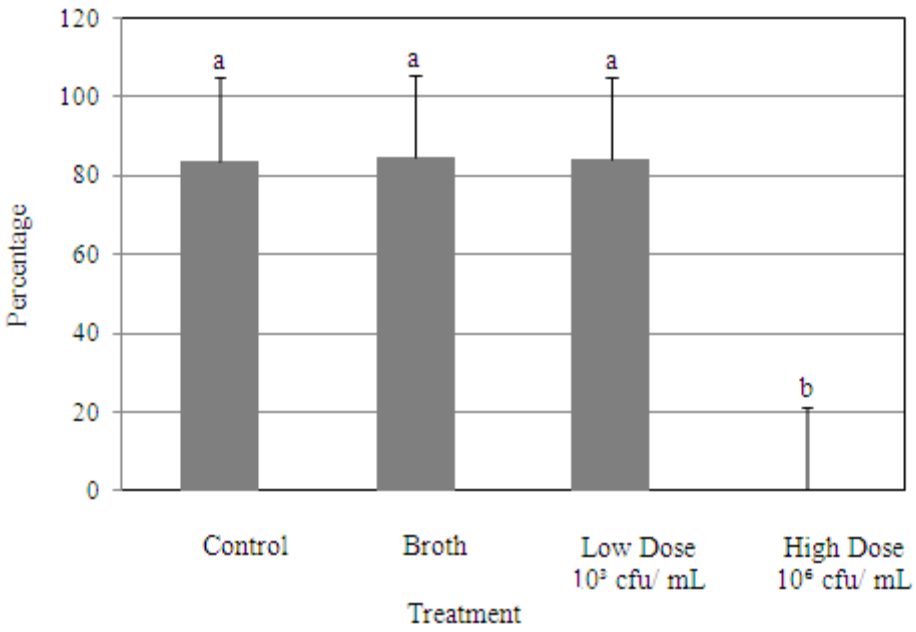
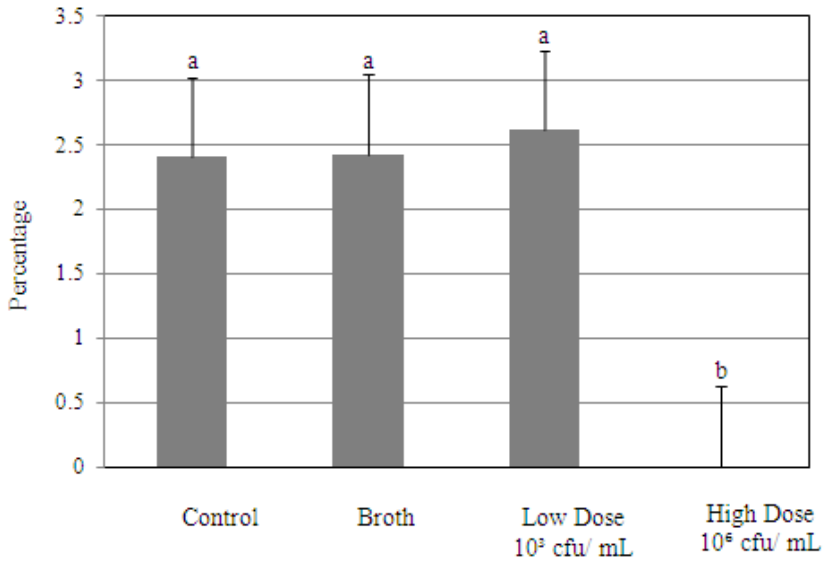


Figure 4.2 Overall Fertility

Percentage of overall fertility when sperm was diluted in saline, broth, low dose or high dose of *L.acidophillus,* respectively. Means with different superscripts indicate differences in the percentage fertile eggs among treatment type. ($p<0.0001$; SEM=0.019; n=133)



35

Figure 4.3 Early Dead

Percentage of early dead when sperm was diluted in saline, broth, low dose or high dose of *L.acidophillus,* respectively. Means with different superscripts indicate differences in percentage of early dead embryos. ($p<0.0144$; SEM=0.004; n=133)

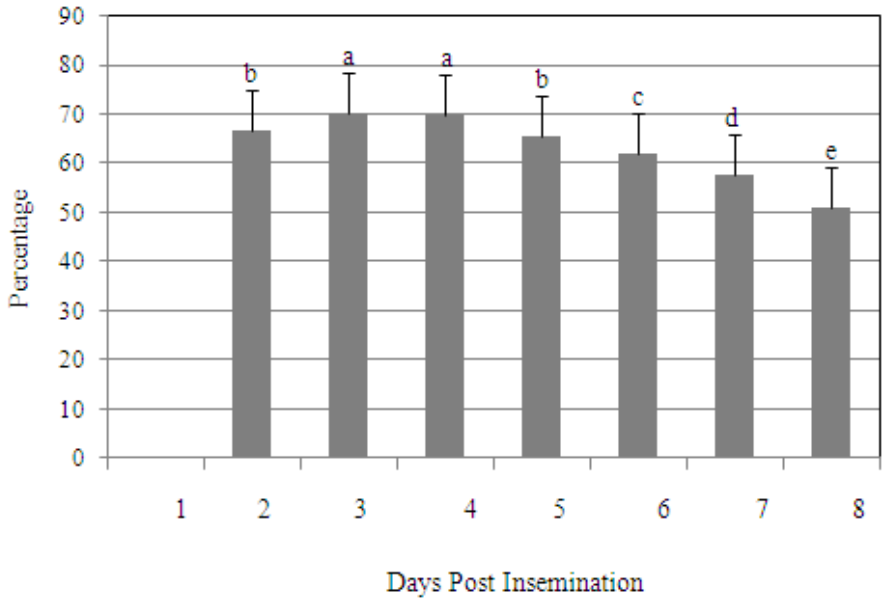


Figure 4.4 DPI for Fertility

Percentage of total fertile eggs among days post insemination, representing all four treatments. Means with different superscripts indicate differences in percentage of total fertile eggs among days. ($p<0.0001$; SEM=0.01; n=76)

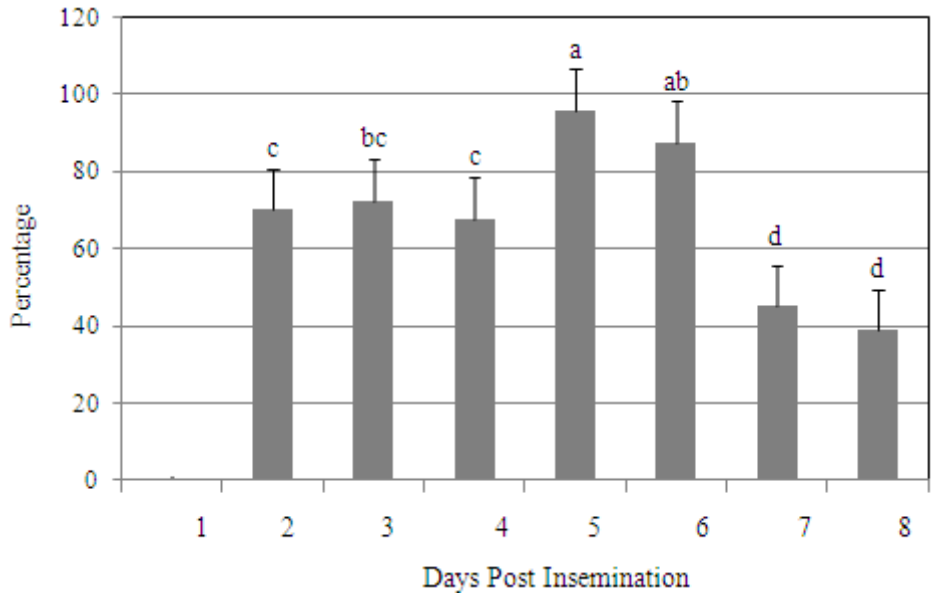


Figure 4.5 Shell *Lactobacillus* by DPI

Percentage of shell samples positive for *Lactobacillus* among days post insemination, representing all four treatments. Means with different superscripts indicate differences in percentage of total positive shell samples. ($p<0.0001$; SEM=0.024; n=19)

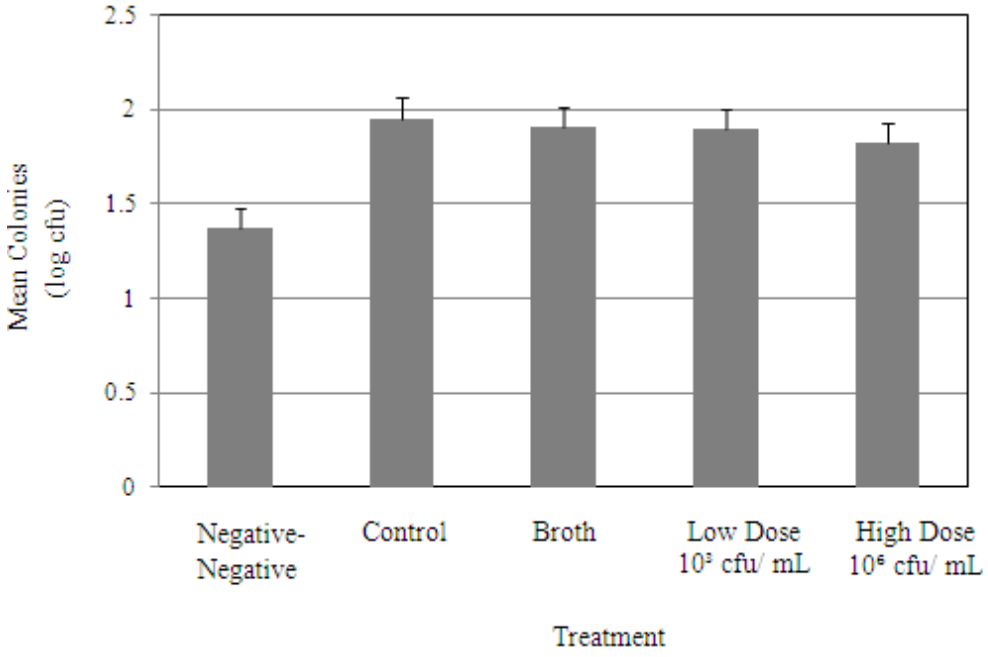


Figure 4.6 *Lactobacillus* counts on shell

Mean colonies among treatments are represented. No significant differences were found due to diluent type. ($p<0.4879$; SEM=0.086; n=133)

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CHAPTER V

CONCLUSION

The main objective of this thesis was to examine the effects different pathogenic and non-pathogenic bacteria have on overall chicken fertility. There are many studies investigating the effects bacteria have on mammalian sperm motility, but there is limited research on the effects that bacteria have on avian semen. Pathogenic bacteria such as *E. coli* have been shown to have an effect on mammalian sperm. These effects include decreases in human and boar sperm motility as well as the ability of *Campylobacter* to decrease ram semen motility. Also, bacterial transmission pathways are constantly under investigation to better understand how bacteria are transferred from environment to bird or from parents to their offspring. By controlling possible routes of bacterial transmission from the beginning, Poultry producers can prevent contamination causing foodborne illness in humans as well as preventing possible negative effects of bacteria on avian reproduction.

The rooster and hen are both responsible for properly fertilizing eggs and maintaining production. Avian semen quality is analyzed by three parameters. These parameters are sperm concentration, viability and motility. Sperm must overcome many barriers in order to travel through the oviduct to fertilize the ovum. If one of the aforementioned parameters is compromised, such as sperm motility, they cannot traverse the hen's oviduct to fertilize the ovum.

There is minimal research investigating the possibility of bacterial attaching to avian sperm, thereby inhibiting their motility. Such attachment would distort sperm structure or block the paths they travel through the oviduct. *Campylobacter* and *Salmonella* have been shown to attach to rooster sperm, without causing structural damage to the sperm. There are also many different species of bacteria that are naturally occurring in the male and female reproductive tract of Poultry. If bacteria naturally colonize the digestive and reproductive tract of birds, vertical transmission of bacteria could occur during mating in association with ejaculation of semen through the cloaca.

In the first study conducted, six different bacteria were exposed to neat rooster semen to determine their effect on motility. Pure cultures of *Salmonella enterica, Escherichia coli, Campylobacter jejuni, Clostridium bifermentans, Bifidobacterium animalis*, and *Lactobacillus acidophilus* were used. Rooster semen was exposed to each bacteria and immediately after exposure as well as after 10 minutes of incubation sperm quality was measured by using the SQA. When each bacterium was exposed to neat semen, sperm motility was immediately decreased. The non-pathogenic bacteria, *Lactobacillus* and *Bifidobacterium* at a concentration of 10^6 cfu/ mL caused the greatest reduction in rooster sperm motility immediately after exposure. This led to the creation of a second hypothesis in this study. This hypothesis was that if sperm motility was immediately decreased after exposure to these non-pathogenic bacteria, would bacteria have an overall effect on fertility?

In the second experiment, 2 different concentrations of *Lactobacillus* were exposed to neat rooster semen, which was subsequently used to inseminate layer hens. When the rooster semen was exposed to 10^6 cfu *Lactobacillus* / mL of prior to hen

insemination, all subsequent eggs were infertile. These results supported results in the first study as well as confirmed the hypothesis which demonstrated *Lactobacillus* eliminating sperm motility which in turn affected overall fertility.

Further research should be conducted to take a closer look at how these bacteria are affecting rooster sperm motility. As noted in this thesis, bacteria may modify sperm structure, directly attach to sperm, and be affected by lactic acid production by *L. acidophilus* and *B. animalis*. The attachment of *L. acidophilus* or *B. animalis* to sperm may be damaging the sperm structure, or the bacteria may be simply clumping together to obstruct the course of sperm movement. Although studies have shown bacterial attachment to the sperm by *Salmonella* and *Campylobacter* there is limited research determining if other bacteria in Poultry attach to avian sperm or damage their structure. Possible pH changes from the production of lactic acid by *L. acidophilus* and *B. animalis* should also be inspected. This effect is very possible because sperm thrive in a neutral environment and these bacteria could cause decreases in pH. A repeat study could be performed using more concentrations of *Lactobacillus* to determine if there is a linear dosimetric effect on fertile eggs produced.

There is minimal research demonstrating the effects that bacteria have on hen fertility or rooster sperm motility. There is evidence that bacteria can be transmitted horizontally or vertically but there is a lack of evidence on the impact that bacteria may have on overall fertility in Poultry. Although each bacterium (*S. enterica, E. coli, C. jejuni, C. bifermentans, B. animalis*, and *L. acidophilus)* yielded an immediate decreased rooster sperm motility, the non-pathogenic bacteria had the greatest effect on rooster sperm as well as fertility. If different concentrations of non-pathogenic bacteria are

affecting sperm motility which also affects fertility, profits will decrease. These current studies as well as previous studies, validate the importance of understanding the possibilities of modes of bacterial transmission, overall fertility in the male and female exposed to bacteria, and sperm structure and motility after exposure to bacteria. Further investigation is essential to determine how these pathogenic and non-pathogenic bacteria are altering rooster sperm motility and fertility.

APPENDIX A

LIST OF AGARS AND BROTHS

1. ANAEROBIC AGAR
2. BLOOD AGAR
3. BRUCELLA AGAR
4. CAMPY-CEFEX AGAR
5. EOSIN METHYLENE BLUE AGAR, LEVINE
6. LACTOBACILLI MRS AGAR
7. PERFRINGENS AGAR BASE (TSC)
8. TRYPTICASE SOY AGAR
9. XLT4 AGAR
10. BRUCELLA BROTH
11. BUFFERED PEPTONE WATER
12. LACTOBACILLI MRS BROTH
13. REINFORCED CLOSTRIDIAL MEDIUM BROTH
14. TRYPTIC SOY BROTH

APPENDIX B

SCIMEDX®-CAMPY (JCL)™ CULTURE CONFORMATION TEST FOR *CAMPYLOBACTER JEJUNI, C. COLI* AND *C. LARIDIS*

SCIMEDX®-CAMPY(JCL)™ CULTURE CONFORMATION TEST FOR

CAMPYLOBACTER JEJUNI, C. COLI AND *C. LARIDIS*

MATERIALS

Scimedx®-campy (jcl)™ **Latex Detection Reagent** (2 x 3.5 mL)-consists of rabbit antiserum to common antigens of selected *Campylobacter* species bound to latex particles suspended in a buffer containing a preservative.

Scimedx®-campy (jcl)™ **Extraction Reagent** (2.8 mL)- consists of a dilute solution of hydrochloric acid

Scimedx®-campy (jcl)™ **Neutralization Reagent** (2.8 ml)- consists of glycine buffer containing a preservative

Scimedx®-campy (jcl)™ **Positive Antigen Control Reagent** (2.7 ml)- consists of neutralized acid extract of appropriate *Campylobacter* organisms in buffer containing a preservative.

Test Slide
Applicator sticks
High intensity lamp
Slide Rotator

PROCEDURE

1. Remove reagents from refrigerator and allow warming to room temperature before use.
2. Label one circle on the test slide for each specimen to be tested.
3. Identify one circle for the positive control and another for the negative control reactions.
4. Remove the cap and tip protector from the vial of Extraction Reagent. While holding the vial vertical position, dispense one free-falling drop of Extraction Reagent into each specimen circle and the negative control circle. Replace the tip protector and cap.
5. Touch one isolated colony with the end of a wooden applicator stick to remove it from the agar surface. Generally, one colony with a diameter of 2mm (about the diameter of the applicator stick) will provide an adequate inoculum. If colonies are small, yet distinct from the surface of the agar, it may be necessary to pick 2-6 colonies. However, care must be taken as too much inoculum may contribute to poor readability.
6. Make homogenous suspension by rotating the inoculum containing stick in the Extraction Reagent within the appropriate specimen circle. It is very important to dissociate all visible clumps of the inoculums and distribute the suspension over the entire area within the circle. Repeat steps 5 and 6 for each specimen to be tested. No incubation time is required for this step. Proceed to step 7.
7. Remove the cap and tip protector from the vial of Neutralization Reagent. While holding the vial in a vertical position, dispense one free-falling drop of Neutralization Reagent into the fluid spread in each specimen circle and the negative control circle. Replace tip protector and cap.
8. Remove the cap from the Positive Control Reagent and wipe the tip with a clean-lint free tissue. While holding the vial in a vertical position, dispense one free falling drop into the positive control circle. Replace the cap.

9. Gently re-suspend the Latex Detection Reagent to assure a homogenous suspension. Do not shake the reagent and avoid the formation of foam or bubbles.
10. Remove the cap from the Latex Detection Reagent and wipe the tip with a clean lint-free tissue. While holding the vial in a vertical position, dispense one free-falling drop Latex Detection Reagent into each circle, as appropriate, on the slide. Avoid forming bubbles on the dropper tip as the latex reagent is dispensed. Do not touch the tip of the dropper vial to the material on the slide. Replace the cap.
11. At this point each circle with have received the following:

Specimen: 1. Extraction Reagent, 2. Bacterial colony(ies), 3. Neutralization Reagent and 4. Latex Detection Reagent

Negative control: 1.Extraction Reagent, 2. Neutralization Reagent and 3. Latex Reagent

Positive control: 1. Positive control Reagent and 2. Latex Detection Reagent

12. Use a separate applicator stick to mix the contents of each circle thoroughly.
13. Place slide on a rotator and rotate at 100-110 rmp for 5 minutes at room temperature.
14. After rotation is completed, immediate observe the reactions for visible agglutination under a high intensity light.
15. A positive test is indicated when the Latex Detection Reagent clearly agglutinates with the test specimen and no agglutination occurs in the negative control circle. The presence of agglutination in the negative control circle renders the test invalid.
16. A negative test is indicated by the absence of agglutination of the Latex Detection Reagent with the test specimen.

Scimdex Corporation Denville, NJ 07834 USA.

APPENDIX C

L-LACTATE AND D-LACTATE KIT

Assay Protocol

1. Cell culture supernatant measured directly by a series of dilutions of the sample (1/2, ¼, 1/8 etc)
2. All standards and samples be duplicated.

2a. thaw L-Lactate assay solution and L-Lactate standards on ice. Add 50µl of each standard per well to the designated wells on the 96-well flat bottom plate.

2b. Prepare test samples to a final volume of the 50µl per well on the 96-well flat bottom plate

3. A. add 50µl of L-Lactate assay solution to each well containing L-Lactate standards and test samples.

B. Incubate for 30 minutes at 37°C incubator

C. Stop the reaction by adding 50µl of 0.5 M acetic acid per well followed by brief gentle agitation. (if bubbles are present in well, can use a needle prior to measurement)

D. Measure the absorbance at 490nM using a microplate reader.

4. Calculations

A. Average the OD 490 nm values of replicate wells of each L-Lactate standard, test samples, and blank. In order to get the corrected absorbance, subtract the average OD490 nm value of the blank (L-Lactate standard #8) from the average OD490 nm values from all standards and samples.

B. Make a standard curve by plotting OD490 nm values from each L-Lactate standards as a function of L-Lactate concentration. Calculate the value of L-Lactate in samples using the equation obtained from the linear regression of the standard curve.

$$\text{L-Lactate(uM)} = \frac{[(\text{corrected absorbance}) - (\text{y-intercept})]}{\text{Slope}}$$

APPENDIX D

IMV MICRO-READER

Materials:

IMV micro-reader

Clear cuvettes

Pipettes and tips

Petri film

Kim wipes

Methods:

1. Plug in the IMV micro-reader and let warm up for approximately 20 minutes prior to use.
2. Micro-reader will ask if you want to “Standardize” press “no”
3. Read blank press “yes”
4. Pipette diluent in clean cuvette (new cuvette for every sample)
5. Pipette sample into cuvette and place a piece of petri film over top and mix thoroughly (Note: do NOT mix vigorously where bubbles appear)
6. Wipe all 4 sides with kim wipes of cuvette prior to placing in micro-reader
7. Place mixed cuvette in micro-reader and make sure triangle is facing you.
8. Place the black cap on top. (Note: insure cap is secure and flush with the slot)
9. Press the “read” button to obtain reading (Note: take at least 2 samples using different cuvettes)
10. Unplug once you are finished taking readings

Calculation:

$$10.99 \times \text{absorbance mean} + 0.18 = \text{Billion sperm/ mL}$$

APPENDIX E

FLUOROMETER

Materials:

Fluorometer machine (St. Johns Associates Inc, 2001A Fluoro-Tec
Pipettes and tips
Kim wipes
Petri film
Ethidium Bromide (EtBr)
Digitonin
Latex gloves
Micro glass tubes (10x75mm)
Tube rack
Methods:

1. Plug in Fluorometer
2. Press the "power" button then "PMT" (Note: do NOT press or move any of the other knobs)
3. Let machine warm up for approximately 20-30 minutes
4. Fill micro glass tubes with 1.99 mL EtBr (Amount is set on bottle)
5. Once machine is warmed up, pipette 10 µl of the neat semen sample in tubes containing EtBr and place petri film over top and mix thoroughly.
6. Place mixed tube in slot in the back section of the fluorometer and place black cap on top.
7. Turn the knob to the "on" position.
8. Wait until the number on the display stops and record (Note: this is your "pre" reading.)
9. After first readings are taken place 25µl of Digitonin in the glass tube containing EtBr and neat semen (Note: MUST WEAR GLOVES!)
10. Place petri film on top and mix thoroughly and place back into fluorometer for a second reading. (Note: this is your "post" reading)
11. Properly discard chemicals used

Calculation:

Divide pre reading average by the post reading average multiplied by 100=

percentage of dead sperm per sample

APPENDIX F

SPERM QUALITY ANALYZER

Materials:

Sperm Quality Analyzer (SQA)
Capillary tubes
Saline (0.85%)
Kim wipes
Test kit button
Methods:

1. Turn on SQA
2. Press option “measurements”
3. Choose “pass/review” option
4. Mix diluted samples in a micro centrifuge tube
5. Take the capillary tube and squeeze and bend the green rubber end to draw a portion of the sample inside
6. Sample must cover the 3 circles on the capillary tube without any bubbles.
7. Wipe sides off of the capillary tube before placing in the SQA
8. Press “test” to obtain first samples reading
9. After approximately 40 seconds a reading will be displayed on the screen and record (SQI)
10. After reading is displayed, press “clear” button and press the “test” button to continue sample readings
11. Repeat for each sample.
12. Once all samples are read, press the “clear” button until back to the main screen and select the “power off” option.

To add new test:

1. Press the “clear” button until on the screen says “ Utilities”
2. Press “enter” and select option “read new test kit button”
3. Place button on the SQA on the right side towards the back.
4. Machine will let you know that the tests were loaded.

APPENDIX G

SEMEN COLLECTION AND ARTIFICIAL INSEMINATION

Materials:

Small funnel with plug in stem
Wipes
Sterile scintillation vial

Methods:

1. Two-three people are needed for semen collection
2. One person will handle and hold the rooster
3. The semen collector will massage the roosters lower back with 1-3 hand strokes and firmly pinch the outer sides of the vent
4. Place the funnel at the bottom of the vent
5. Collect semen in funnel (Semen will drip through and down the vent)
6. Use wipe to remove any fecal material
7. Pour ejaculate into the sterile scintillation vial
8. Tap edge of vial to get as much semen as possible
9. Keep semen aerated without exposing to direct sunlight
    a. Swirl vial or waft air into vial
10. Once all roosters are collected, semen is ready for analysis, artificial insemination and/or addition of treatments

<u>Artificial insemination</u>

Materials:

Pipette
Pipette tips
Semen sample (with or without treatment)
Person to break hens (breaker)
Person to inseminate hens
Methods:

1. Breaker holds hen over leg or cage
2. Inseminator gently pipettes semen dosage (~50µl)
3. Place hen back in cage

APPENDIX H

EGG INCUBATION

Materials:

Incubator (Jamesway)
De-ionized water
Wick for wet bulb
Egg trays
Egg carts
Record sheet for temperatures

Methods:

1. Turn incubator on 1-2 days prior to setting eggs
2. Temperature should be set at 99.5°F and 86 wet bulb
3. Fill wet bulb reservoir with de-ionized water which is located at the top left corner inside the incubator. Place wick inside hole in the DI water reservoir. Will need to add DI water every couple of days.
4. If eggs were kept in cooler from 1+ days, remove and let warm to room temperature before placing into incubator
5. If randomizing in incubator, can prep eggs prior to setting in egg trays
6. Before setting eggs in incubator, make sure racks are horizontal to ease of egg placement. This can be done by connecting the electrical wiring at the top right until racks are horizontal. Disconnect quickly once racks are horizontal
7. Slide egg trays into the rows in incubator cart
8. Once all eggs are set, connect the electrical wiring and shut door.
9. Temperature gauges can be placed within the racks to insure proper temperature.
10. Incubator should be checked for proper temperature and turning at least once every day.

APPENDIX I

BREAKOUT OF EGGS

Materials:

Egg trays
Butter knife or metal stick
Record sheet
Incubated eggs

Methods:

After 10 days of incubation, egg can be candled to determine if fertile or infertile.

Breakout is necessary to determine what stage the embryo is currently at.

1. Place eggs in trays to be broken out
2. Record eggs to be broken out
3. Tap eggs with butter knife end to crack eggs
4. Break apart egg shell with fingers
5. Determine if egg is infertile, fertile (early dead, mid-dead, late dead)
6. Infertile egg will not have any blood vessels or embryo
7. Dead embryos will have a ring or smear of blood in the egg or a dried spot on the inside of the shell
8. Early dead-embryos died within the first 7 days of incubation, blood is apparent and vital organs begin to form
9. Middle dead-embryos died between 8 and 14 days of incubation, pip tooth is apparent, scales and feathers are present
10. Late dead-embryos died between 15 and 21 days of incubation-yolk sack is drawn into embryo
11. Record embryos as they are broken out for analysis

APPENDIX J

TRANSFERRING PURE CULTURES OF BACTERIA

Transferring Cultures

1. Clean work bench with alcohol. Remove the 14ml tubes from the refrigerator with broth and label with today's date & your initials.

2. Wait about 15-20 minutes to let broth warm up to room temperature.

3. After the broth is warmed to room temperature remove the anoxomat chambers and beaker with culture tubes out of the gray incubator from the shaker. The temperature should be approx. 37°C. Place inoculated tubes into the metal rack.

4. Then wipe the 1ml (1000µl) pipette with an alcohol swab before placing into pipette tip. Vortex the 'brucella' for approx 2 seconds or until well homogenized. Pipette 1ml of the negative control to the new labeled 'brucella' tube, cover with cap and place into rack.

5. Then vortex sample 'Campy' for approx 2 seconds and Pipette 1ml of the old 'campy' to the new labeled 'campy' tube, cover with cap loosely and place into rack.

6. Vortex sample '*Salmonella*' for approx 2 seconds and Pipette 1ml of the old '*Salmonella*' to the new labeled tube, cover with cap loosely and place into rack.

7. Vortex sample 'E.*coli*' for approx 2 seconds and Pipette 1ml of the old 'E.*coli*' to the new labeled tube, cover with cap loosely and place into rack.

8. Vortex the 'brucella' for approx 2 seconds or until well homogenized. Pipette 1ml of the negative control to the new labeled 'brucella' tube, cover with cap loosely and place into rack.

9. Vortex sample 'Lacto' for approx 2 seconds and Pipette 1ml of the old 'Lacto' to the new labeled tube, cover with cap loosely and place into rack.

10. Vortex the ‘MRS’ for approx 2 seconds or until well homogenized. Pipette 1ml of the negative control to the new labeled ‘MRS’ tube, cover with cap loosely and place into rack.

11. Vortex sample ‘*Clostridium*’ for approx 2 seconds and Pipette 1ml of the old ‘*Clostridium*’ to the new labeled tube, cover with cap loosely and place into rack.

12. Vortex the ‘TSB’ for approx 2 seconds or until well homogenized. Pipette 1ml of the negative control to the new labeled ‘TSB’ tube, cover with cap loosely and place into rack.

13. Vortex sample ‘Bifido’ for approx 2 seconds and Pipette 1ml of the old ‘Bifido’ to the new labeled tube, cover with cap loosely and place into rack.

14. Vortex the ‘rein. Clost.’ for approx 2 seconds or until well homogenized. Pipette 1ml of the negative control to the new labeled ‘rein. Clost.’ tube, cover with cap loosely and place into rack.

15. Place the 2 new inoculated Brucella & campy tubes into the anoxomat chamber spaced evenly apart. Put lid back onto the canister and tighten down with bracket hand tight. Connect to the anoxomat tank and chose the microaerophilic option and start process.

16. Place the ‘*Salmonella*, E.*coli*, brucella, Lacto & MRS’ inoculated tubes into the beaker with aluminum pan over with holes.

17. Place the ‘*Clostridium*, TSB, Bifido and Rein. Clost.’ in their respective anoxomat container and replace the catalyst sash. Put the lid back onto the canister and tighten down with bracket hand tight. Connect to the anoxomat tank and choose the anaerobic option and start process once campy canister is complete.

18. Place old tubes into the dirty fridge in the designated racks.

19. Once anoxomat system is complete please place back onto the shaker in the gray incubator.