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When Rooster Semen is Exposed to *Lactobacillus* Fertility is Reduced¹

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Abstract: *Lactobacillus* resides in the hen's gastrointestinal tract, vagina and cloaca. When fed to hens, *Lactobacillus* has improved egg weight, size and shell strength. However, data from our lab suggests *Lactobacillus* may be detrimental to semen and possibly fertility. Therefore, the objective of this study was to determine if semen exposed to *Lactobacillus* affected fertility in hens. For the experiment, semen was collected from 40 roosters and used to create 4 treatments: saline, broth, 10^3 cfu/mL of *Lactobacillus* and 10^8 cfu/mL of *Lactobacillus*. Each treatment was diluted 1:1 with pooled semen and sperm quality index readings were obtained prior to insemination. Eighty hens in each treatment were equally divided among 10 blocks and inseminated. Eggs were collected from 2 to 7 d post-insemination (DPI) and hens were artificial insemination again on DPI 8, eggs were collected for another 7 d. Eggs were incubated for 10 d, candled for fertility and a portion of the eggs were examined for *Lactobacillus*. The entire experiment was repeated twice. Semen quality was within the normal range for treatments 1, 2 and 3, but semen from treatment 4 was 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 conclusion, if *Lactobacillus* exceeds 10^8 cfu/mL in the hen or roosters reproductive tract, fertility could be impacted.

Key words: *Lactobacillus*, rooster, hen, sperm motility, fertility

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. In order to optimize egg fertilization, rooster sperm for artificial insemination must excel in three semen quality traits: sperm viability, concentration and motility. The sperm must utilize motility to overcome many barriers such as mucus and peristalsis as well as travel through the oviduct in order to penetrate the germinal disc of the egg (King and McLelland, 1984). If sperm cannot fertilize the ovum, not only is overall fertility affected, but sequentially hatchability will be negatively affected. Both the hen and rooster are responsible for maintaining fertility and if one is not properly contributing, chick production is decreased.

Bacteria can impact reproduction, because many species of bacteria are naturally occurring within poultry and mammalian reproductive tracts. 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.* (1977) suggested that semen in the vas deferens is typically free of bacteria, but during

ejaculation, semen can become contaminated once the ejaculate touches the surface of the cloaca (Smith, 1949).

Pathogenic bacteria such as *E. coli* have been found in mammalian semen such as the boar (Martin *et al.*, 2010) and ram (Yaniz *et al.*, 2010). Bussalleu *et al.* (2011) determined that different types of *E. coli* as well as different concentrations have an effect on sperm motility even though no structural alterations, due to *E. coli*, were found on the sperm. 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 such as *Campylobacter*, *Escherichia coli*, *Staphylococcus*, *Bacillus* and *Enterococcus*, are also naturally occurring in avian semen (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 (Baker *et al.*, 1980; Guthrie, 1992). Vizzier-Thaxton *et al.* (2006) demonstrated that both *Campylobacter* and *Salmonella* can 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. Because bacteria are found naturally in the rooster and hen reproductive tracts, the possibility for transmission of bacteria could lead to a decrease in

fertility within the breeder flock. However, research in avian species that evaluates the effect of bacteria on sperm quality is limited.

In 2013, a study by Haines and colleagues demonstrated that *in vitro* exposure of semen to pathogenic as well as non-pathogenic bacteria negatively impacted semen quality in breeders. The results were surprising due to the fact that beneficial, probiotic bacteria, like *Lactobacillus*, are normally associated with an improved intestinal microflora and production characteristics in poultry (Park, 1974; Panda *et al.*, 2008). However, it was determined that beneficial bacteria decreased sperm quality rapidly even when compared to the pathogenic bacteria (Haines *et al.*, 2013). *Lactobacilli* have also been found to be natural residents of the cloaca and vagina of laying hens at concentration as high as \log_{10} 5.5 and 2.5 cfu/g, respectively (Miyamoto *et al.*, 2000; Van Coillie *et al.*, 2007). Other research has demonstrated that *Lactobacillus* is a natural resident of the cloaca and semen of male breeders at concentrations as high as \log_{10} 7 and 5 cfu/g, respectively (Hirai *et al.*, 2015; unpublished data). This is of concern because the research of Haines and colleagues demonstrated that a concentration of \log_{10} 6 cfu/ml of *Lactobacillus* reduced semen quality immediately upon exposure. With concentrations of *Lactobacillus* in the cloaca, vagina and semen approaching those levels naturally, overall fertility, could be impacted if a probiotic supplement containing *Lactobacillus* is used in the diet formulation. Therefore, the objective of this study was to determine if semen exposed to different concentrations of *Lactobacillus in vitro* inhibits fertility after artificial insemination.

MATERIALS AND METHODS

Housing and environment: Three hundred and eighty four, 45 wk 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 h of light per day. Females and males were caged in a house with conventional environmental controls. All birds were treated in accordance with the Guide for the Care and Use of Laboratory animals.

House layout: Prior to the experimental period, 320 White Leghorn hens were arranged equally in 10 blocks where each of the 4 treatments was randomly represented in each block. For each treatment, 8 randomly selected 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 *L. acidophilus*) so that baseline *L. acidophilus* levels could be obtained from virgin hens.

Semen analysis prior to treatment: Semen was collected from 40 White Rock roosters using the abdominal massage method of Burrows and Quinn (1937) and the neat semen samples were pooled prior to treatment application. Semen was then analyzed to insure that the pooled neat semen sample was acceptable for experimentation. The sample was analyzed in duplicate for sperm viability by using the fluorometric method of Bilgili and Renden (1984). Sperm concentration was determined using 2 readings from a microreader (IMV, International, Maple Grove, MN) by the method of King and Donoghue (2000) and sperm motility was determined in triplicate by the Sperm quality index (SQI) procedure of McDaniel *et al.* (1998).

Treatments: Five treatments were used for this study. The first treatment was represented as the negative-negative control. The following 4 semen diluent treatments were used for insemination: saline, sterile de Man, Rogosa, Sharpe (MRS) broth, low dose of *L. acidophilus* (3.0×10^3 cfu/ml, prior to diluting) and a high dose of *L. acidophilus* (3.0×10^6 cfu/ml, prior to diluting). Each treatment was diluted in a 1:1 ratio (275 μ l of the pooled neat semen sample and 275 μ l of the respective diluent). The 1:1 dilution was used as the standard for insemination of hens. Each treatment was thoroughly mixed immediately before semen analysis and insemination.

***Lactobacillus* culture:** *Lactobacillus acidophilus* (American Type Culture Collection; #314) was received in a lyophilized form, hydrated in MRS broth (Difco, Sparks, MD) and placed on a shaker in an incubator at 37°C for 48 h. The culture was aseptically transferred to fresh MRS broth every 24 h prior to the start of the experiment. *L. acidophilus* was cultured in MRS broth for 12 h prior to artificial insemination providing approximately 3.0×10^6 cfu/mL of *L. acidophilus*. The low dose was serially diluted in MRS broth from the overnight culture to make approximately 3.0×10^3 cfu/mL.

Semen analysis and artificial insemination after treatment: Each of the 4 treatment samples was prepared making a 1:1 dilution and a portion was drawn into a capillary tube and placed into a Sperm Quality Analyzer (SQA; McDaniel *et al.*, 1998). A total of 3 separate readings were taken for each treatment and the SQI values were recorded. All readings were obtained immediately prior to insemination of the hens. After each treatment was diluted and the SQI readings were obtained, each hen for that treatment was inseminated with approximately 87 million sperm/50 μ l

of respective treatment. Hens were artificially inseminated again a second time on day post-insemination (DPI) 8 using the same treatments and house layout utilized in the first insemination.

Egg collection and incubation: Eggs were collected at the same time every day, mid-afternoon. Collection started on DPI 2 from the first artificial insemination and eggs were collected daily for 2 wk. Eggs were collected with new rubber gloves for each treatment (4) in all 10 blocks and labeled with the block, treatment and individual egg number (total eggs laid/day) as well as date collected. Eggs were stored in a cooler for 0 to 3 d at 20°C and then set in a Jamesway Model PS 500 incubator (Jamesway Incubator Company Inc., Cambridge, Ontario, Canada) for 10 d at 37.5°C and 30°C dry and wet bulb temperatures, respectively. After 10 d of incubation, eggs were removed and broken out to determine overall fertility of each treatment, including infertile and dead embryos.

Microbial analysis: An individual egg was removed from each treatment, within each block on each day of collection immediately prior to the initial fertility 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 min. After 2 min, eggs were aseptically removed from BPW and placed in 70% ethanol for 30 sec. Eggs 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 serially diluted. Once ethanol had evaporated, the remaining eggs were 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 in duplicate. Plates were placed in a low temperature Thermo Scientific incubator Model 815 (Thermo Scientific, Marietta, OH) at 37°C for 48 h under aerobic conditions. After 48 h, plates were examined for smooth white/opaque colonies and each colony was counted and recorded.

Statistical analysis: Data from the experiment were analyzed using a completely randomized block design with a split plot over 2 artificial inseminations and over 7 DPI (2-8). 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 neat semen was collected from each male, the samples were pooled. The pooled neat semen sample

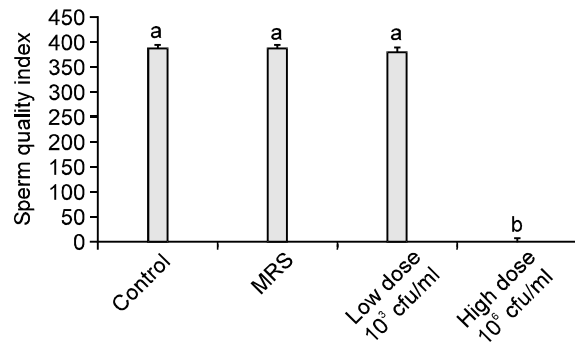


Fig. 1: Mean Sperm Quality Index (SQI) prior to artificial insemination when sperm was diluted in saline, broth, low dose or high dose of *L. acidophilus*, respectively. Means with different superscripts indicate differences in the SQI due to diluent type. ($p < 0.0001$; SEM = 7.03; n = 20)

was then analyzed and determined to be acceptable prior to artificial insemination. In this study, the average sperm concentration was 3.48 billion sperm/mL, with 9.3% dead sperm and a mean SQI of 383.

After each treatment was mixed and prior to artificial insemination of the hens, additional SQI readings were taken. There was no significant difference in the SQI between the control (387.5), broth (387.8), or low dose *Lactobacillus* (379.5) treatments. However, when the neat semen sample was exposed to the high dose *Lactobacillus* treatment, a value of zero was obtained immediately for the SQI ($p = 0.0001$; Fig. 1). The negative-negative control had no results because those hens were not inseminated with any treatment (data not shown).

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 *Lactobacillus* or negative-negative treatment were infertile (0%), ($p = 0.0001$; Fig. 2). Early dead embryos mirrored overall fertility, where the control (2.4%), broth (2.4%) and low dose *Lactobacillus* (2.6%) treatments were not significantly different, but the high dose *Lactobacillus* and negative-negative treatment yielded no early dead embryonic mortality because all eggs were infertile ($p < 0.0144$; Fig. 3).

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

Eggshell samples positive for *Lactobacillus* followed a curve similar to the DPI curve for fertility ($p = 0.0001$; Fig. 5). During DPI 2, 3 and 4 the percentage of eggshell samples positive for *Lactobacillus* were not significantly different from each other. On those days, the percentage

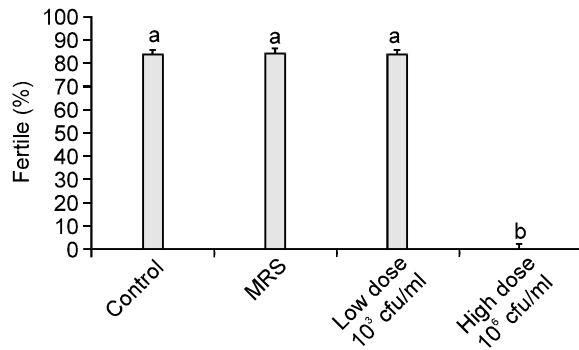


Fig. 2: Percentage of overall fertility when sperm was diluted in saline, broth, low dose or high dose of *L. acidophilus*, respectively. Means with different superscripts indicate differences in the percentage fertile eggs among treatment type. ($p < 0.0001$; SEM = 1.9; n = 133)

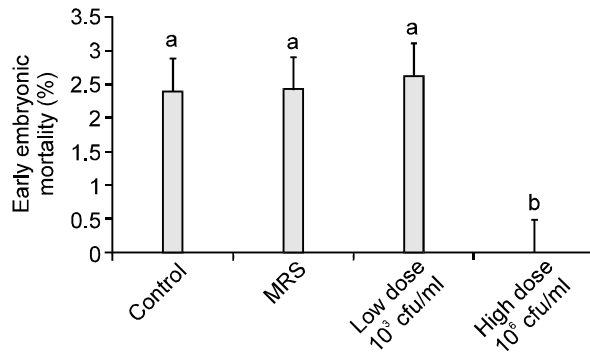


Fig. 3: Percentage of early dead embryos when sperm was diluted in saline, broth, low dose or high dose of *L. acidophilus*, respectively. Means with different superscripts indicate differences in percentage of early dead embryos. ($p < 0.0144$; SEM = 0.48; n = 133)

of eggshells positive for *Lactobacillus* was 70, 72.5 and 67.5%, respectively. At DPI 5, the percent of eggshells positive for *Lactobacillus* peaked at 95.6% but decreased by d 7 and 8 (45 and 38.5%, respectively). After 48 h of incubation, there was no colony growth for the egg yolk or embryo samples plated on MRS agar. The egg shell samples exhibited positive *Lactobacillus* growth after incubation, but no differences in colony counts occurred between the control (mean colonies 1.95 cfu/mL), broth (mean colonies 1.9 cfu/mL), low dose *Lactobacillus* (mean colonies 1.9 cfu/mL), high dose *Lactobacillus* (mean colonies 1.8 cfu/mL), or negative-negative control (mean colonies 1.4 cfu/mL) groups ($p = 0.488$; Fig. 6).

DISCUSSION

Lactobacilli spp. are known to be beneficial to avian gastrointestinal tracts and improve egg production when

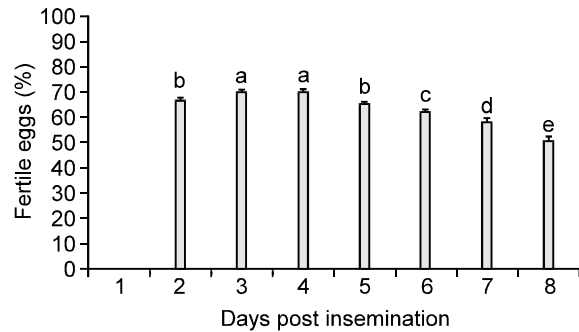


Fig. 4: Percentage of total fertile eggs over days post insemination, representing all four treatments. Means with different superscripts indicate differences in percentage of total fertile eggs among days. ($p < 0.0001$; SEM = 1.02; n = 76)

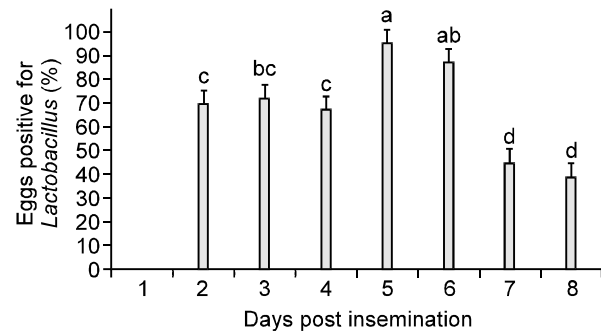


Fig. 5: Percentage of eggshell samples positive for *Lactobacillus* over days post insemination, representing all four treatments. Means with different superscripts indicate differences in percentage of total positive shell samples. ($p < 0.0001$; SEM = 6.007; n = 19)

provided in the diet (Panda *et al.*, 2008); however, there is limited research demonstrating the effects *Lactobacilli* may have on avian fertility. Miyamoto *et al.* (2000) found *Lactobacilli* naturally occurring in the laying hens vagina at 10^2 cfu/ mL. The current study used concentrations of *L. acidophilus* at approximately 10^6 cfu/mL, which decreased sperm motility and resulted in complete infertility. Therefore, during copulation (natural or artificial insemination) if the rooster and hen both contribute *Lactobacilli*, fertility could be decreased leading to poor reproductive performance in the breeder flock.

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 (King *et al.*, 2000). A quick procedure for the determination of overall semen quality involves use of the SQI (McDaniel *et al.*, 1998). Excellent fertility is obtained if the SQI is ≥ 350 (Parker and

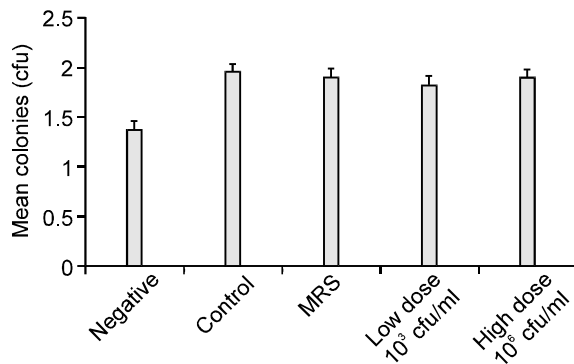


Fig. 6: Mean colonies of *Lactobacillus* on eggshells among treatments. No significant differences were found due to diluents type. ($p < 0.4879$; SEM = 0.086; $n = 133$)

McDaniel, 2002, 2003). However, previous and current research conducted in our lab yielded SQI values of 36 (Haines *et al.*, 2013) and 0, respectively, immediately upon exposure of rooster sperm to 10^6 cfu *Lactobacillus*/mL. It has also been established that a 1:10 dilution of chicken semen yields the greatest sensitivity for a SQI reading (McDaniel *et al.*, 1998). However, in the current study, the industry standard (a 1:1 dilution) for artificial insemination was used (Baskt and Dymond, 2013). This same 1:1 dilution of semen in the present study may have reduced the sensitivity of the SQI due to the higher concentration of sperm in the capillary tube (McDaniel *et al.*, 1998). Although, a complete reduction in the SQI was obtained for the highest dose of *Lactobacillus*, the lowest *Lactobacillus* dose may have also reduced the SQI if, the more sensitive, 1:10 semen dilution had been utilized. Additionally, even though the sperm to *Lactobacillus* ratio was high (3,480 sperm per 1 bacteria for each insemination) at the 1:1 dilution, sperm motility was still eliminated immediately upon exposure which could be due to a chemical or pH change produced by *Lactobacillus*.

The SQI readings obtained in this study prior to insemination provided an inclination to the final results for fertility after insemination. The control treatments (saline and MRS broth) and the low dose of *Lactobacillus* (10^3 cfu/mL) did not show an effect on fertility or early embryonic mortality; however the highest dose of *Lactobacillus* resulted in total infertility. These results coincided with the SQI readings prior to insemination where the high dose of *Lactobacillus* (10^6 cfu/ mL) provided a zero reading indicating no sperm movement. If sperm are unable to swim they cannot reach the utero-vaginal junction (Parker, 1970). Therefore if they are unable to swim prior to insemination, then the expected results should lead to infertility as shown in this study.

Also, in the current study, there was no *Lactobacillus* growth for yolk samples but the egg shell samples were positive for *Lactobacilli* at approximately log 1.9 cfu/mL. Although there were no differences among treatments for positive egg shells, *Lactobacillus* was still present even in the negative-negative treatment. The amount of *Lactobacillus* found on the egg shell also diminished over time following a typical dosimetric curve similar to that seen in fertility over days post insemination (Kirby and Froman, 1990; Kirby *et al.*, 1998). The decrease of positive *Lactobacillus* on the egg shells could be due to *Lactobacillus* from insemination being expelled onto the egg shell surface prior to the egg being laid. Interestingly, *Lactobacilli* spp. are also naturally occurring in the hen's cloaca at approximately 10^5 cfu/mL and vagina at 10^2 cfu/mL, (Miyamoto *et al.*, 2000). Although *Lactobacilli* are naturally occurring at the site where semen enters the hen, there is no previous evidence indicating that naturally occurring *Lactobacilli* affect fertility. Because *Lactobacilli* spp. are naturally occurring in the male and female reproductive tract, further difficulties could occur such as sperm immotility upon exposure of semen to the hen's cloaca. If both the male and female's combined reproductive tract contribute *Lactobacillus* at a high enough concentration of bacteria, the possibility of contaminated semen increases and fertility may decrease.

There are many possibilities when considering potential reduction in fertility by *Lactobacillus*. One concern could be the possibility of the hen's defensive mechanisms such as her body's immune system recognizing this higher concentration of *Lactobacillus* in her oviduct resulting in rejection of the ejaculate. Lombardo *et al.* (1999) found numerous bacteria in wild tree swallow semen which could result in lower fertility. The results of this current 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 ova due to immotility. Although there are many sperm per each bacteria, *Lactobacilli* could be attaching to multiple sperm at once, thereby damaging the sperm's structure and its ability to swim properly. Vizzier-Thaxton *et al.* (2006) found that pathogenic bacteria (i.e., *Salmonella* and *Campylobacter*) can attach to broiler breeder rooster sperm which could lead to decreased fertility. Also, *Lactobacillus* produces lactic acid which results in a lower pH (4 to 5; Holt *et al.*, 2000). A pH in this range does not provide a neutral environment which sperm need to survive (Bogdonoff and Shaffner, 1954; Lardy and Phillips, 1943). The pH in the hen's oviduct ranges from 6.8-7.8 (Baskt, 1980) which is neutral allowing sperm to survive and later fertilize the ovum. If *Lactobacillus* is introduced into the hen's oviduct and is able to proliferate to high enough concentrations then it is possible that they may change the hen's oviductal pH therefore affecting the sperm's motility during copulation or insemination.

Conclusion: In conclusion, the exposure of sperm to a high concentration of *Lactobacillus* does affect sperm motility and subsequent fertility upon insemination. Therefore, additional research is necessary to determine how *Lactobacillus* inhibits rooster sperm motility (i.e., chemical variations or lactic acid production) leading to infertility. Because fertility plays a large role in the poultry industry, every aspect needs to be completely investigated to ensure the highest breeder production.

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