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The Impacts of Yeast Fermentation and Bacillus Subtilis Dietary Products on Sperm Quality and Semen Microbiota of Aged White Leghorn Roosters

Midian Nascimento dos Santos

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By

Midian Nascimento dos Santos

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By

Midian Nascimento dos Santos

Approved:

_____

Christopher D. McDaniel
(Major Professor / Graduate Coordinator)

_____

Kelley G.S. Wamsley
(Committee Member)

_____

Aaron S. Kiess
(Committee Member)

_____

George M. Hopper
Dean
College of Agriculture and Life Sciences

Name: Midian Nascimento dos Santos

Date of Degree: August 11, 2017

Institution: Mississippi State University

Major Field: Agriculture

Major Professor: Christopher D. McDaniel

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Candidate for Degree of Master of Science

Alternatives to antibiotic growth promoters have been widely exploited due to concerns about antimicrobial resistance. These feed additives improve growth, in part, by modulating intestinal microbiota. However, their impact on male reproductive performance is not well elucidated. Therefore, the objective of this study was to evaluate the impacts of a yeast fermentation product (YP) and *Bacillus subtilis* on rooster semen quality and microbiota. Dietary supplementation of YP linearly increased the concentration of yeast and bacteria in semen, whereas it linearly decreased sperm motility, suggesting that bacteria attached to yeast were excreted from the gut, contaminated semen at the cloaca and then decreased sperm movement. However, direct *in vitro* exposure of semen or dietary supplementation with *B. subtilis* did not affect semen quality or seminal concentration of this bacterium, likely because *Bacillu*s naturally occur in semen. In conclusion, unlike *B. subtilis*, dietary YP can alter semen quality by altering semen microbiota.

Keywords: yeast fermentation product, *Bacillus subtilis*, sperm, semen quality, bacteria.

DEDICATION

This thesis is dedicated to God, my parents, family and friends for all the support, help, encouragement and unconditional love. I also would like to dedicate this research to my lovely nephews Gustavo and Gabriel, with whom I could not spend much time but are always present in memories and thoughts.

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CHAPTER I

INTRODUCTION

Broiler growth performance is closely associated with gut microbiota composition. In fact, gut microbiota plays a crucial role in maintaining animal health due to its interaction with the host immune system, as well as with intestinal morphology and physiology (Round and Mazmanian, 2009, Pan and Yu, 2014). Previous studies have demonstrated that many factors affect the microorganisms that inhabit the broiler gastrointestinal tract, including age, diet and environmental conditions (Lu et al., 2003; Torok et al., 2008; Round and Mazmanian, 2009; Torok et al., 2009). Moreover, dietary supplementation with antibiotic growth promoters (AGP) to livestock and poultry are known to affect intestinal microbiota (Pan et al., 2014).

AGP are provided in the diet at sub-therapeutic levels to improve animal performance; and although their mechanisms of action have not been completely elucidated, previous findings suggest that these feed additives improve animal performance through the modulation of intestinal microbiota (Niewold et al., 2007, Pedroso et al., 2006; Torok et al., 2011). In fact, AGP improve broiler performance by increasing growth, improving feed efficiency and inhibiting the population of adverse and pathogenic bacteria commonly associated with enteric diseases, morbidity and mortality in poultry production (Pan and Yu, 2014). However, growing public concern about antimicrobial resistance has led to the abolishment or reduction in use of AGP in

livestock and poultry feed (Edens, 2003; Pan and Yu, 2014). For example, in 2006 the European Union completely banned the use of antibiotics as AGP, and in the United States and other countries there is an increasing demand for antibiotic-free products (Van Immerseel et al., 2009; Pan and Yu, 2014). Hence, alternatives to AGP have been exploited to meet consumer requirements, prevent human health issues and alleviate the reduction in animal performance associated with the removal of conventional AGP in the feed (Edens, 2003).

Bacillus subtilis, live yeast cells and yeast fermentation products (YP) derived from *Saccharomyces cerevisiae* are examples of alternatives to AGP that are supplemented in livestock and poultry feed (Martin et al., 1989*;* Opalinski et al., 2007; Gaggia et al., 2010; Vohra et al., 2016). Their supplementation in broiler diets has been associated with immunostimulation and improvements in body weight and feed conversion (Gaggia et al., 2010; Vohra et al., 2016). Even though their mechanisms of action are very diverse and complex, they are known to increase animal performance by modulating intestinal microbiota, which in turn increases animal resistance to common stress factors such as transportation, heat, and bacterial infection (O'dea et al., 2006; Huff et al., 2013). In fact, the supplementation of these feed additives has been reported to decrease the population of harmful bacteria (Vohra, 2016). For example, a reduction in the gastrointestinal population of *Salmonella* and *E. coli* have been described in response to the addition of *B. subtilis* in broiler diets (Molnar et al., 2011; Manafi et al., 2016). Similarly, the supplementation of yeast and YP in poultry feed inhibits the growth of pathogenic bacteria due to their specific binding site for mannose, present in the outer yeast cell wall (Vohra et al., 2016)

Besides the effects on growth and meat production traits, dietary supplementation of AGP alternatives have been reported to impact animal reproductive performance. For example, the use of yeast and YP for broiler breeder hens has been shown to increase egg specific gravity, egg production, fertility, and hatchability, while decreasing egg contamination (Shashidhara, and Devegowda, 2003). Similarly, when fed as a supplement to breeder hens, *B. subtilis* has also increased egg fertility and hatchability (Xu et al., 2006; Nietfeld et al., 2016). However, research is scarce concerning the impact of these feed additives on rooster reproductive performance and their ability to sire offspring.

Even though the production of fertile eggs relies on both sexes, the contribution of the rooster is more critical due to the lower number of males compared to females in natural mated and artificially inseminated flocks (Ommati et al., 2013). The determination of semen quality is an important tool to evaluate the reproductive ability of roosters (Parker and McDaniel, 2002). In fact, the selection of broiler breeder roosters based on their semen quality improves fertility and hatchability (Pollock, 1999; Parker and McDaniel, 2002). Parameters commonly used to estimate semen quality include semen volume as well as sperm concentration, viability and motility. By determining these characteristics, it is possible to estimate the number of viable and motile sperm, capable of fertilizing the egg (King and Donogue, 2000).

Semen quality may be affected by several factors such as genetic selection (Hocking et al., 2003), age (Tabatabaei et al. 2010), photoperiod (Floyd and Tyler, 2011) and nutrition (Olubowale et al. 2014). Additionally, the detrimental effect of some species of bacteria on semen quality has been reported in poultry. For example, Vizzier-

Thaxton (2006) suggested apparent attachment of *Salmonella* and *Campylobacter* to different parts of the spermatozoa *in vitro*, this could be a potential source of horizontal and vertical transmission of diseases if the attachment of these pathogenic bacteria occurs under *in vivo* conditions. Additionally, Haines and cohorts (2013) described a decline in sperm motility when rooster semen was exposed *in vitro* to pathogenic bacteria, including *E. coli*, *Salmonella*, *Campylobacter* and *Clostridium,* whereas *in vitro* exposure of semen to *Lactobacillus* and *Bifidobacterium*, commonly used as probiotics in animal feed, eliminated sperm motility (Haines et al., 2013).

Because the ejaculate and excreta of the rooster empty into the cloaca, semen is exposed to microbiota released from the digestive tract through the cloaca (Smith, 1949, Haines et al., 2013). In fact, a diverse microbiota has been described to naturally occur in rooster ejaculates. Examples of bacteria that have been isolated in rooster semen include *Bacillus, Enterococcus, Escherichia*, *Staphylococcus, Micrococcus, Campylobacter*, and *Salmonella* (Donoghue et al., 2004; Reiber et al., 1995). However, there is sparse literature about effects of AGP alternatives on bacteria present in the roosters' reproductive tract and semen as well as the effects of AGP alternatives on semen quality. Therefore, this thesis' research was conducted to elucidate the impacts of *B. subtilis* and YP on semen microbiota and semen quality that will ultimately affect fertility.

References

Donoghue, A.M., P.J. Blore, K. Cole, N.M Loskutoff, and D.J Donoghue. 2004. Detection of Campylobacter or Salmonella in turkey semen and the ability of poultry semen extenders to reduce their concentrations. *Poultry Science* 83:1728-1733.

Edens, F. W. 2003. An alternative for antibiotic use in poultry: probiotics. *Revista Brasileira de Ciência Avícola*, *5*(2), 75-97.

Floyd, M.H., and N.C. Tyler. 2011. Photostimulation of male broiler breeders to different photoperiods. *South African Journal of Animal Science 41*.2 (2011): 146-155

Gaggìa, F., P. Mattarelli, B. Biavati. 2010. Probiotics and prebiotics in animal feeding for safe food production. *International journal of food microbiology*, *141*, S15-S28.

Haines, M. D., H. M. Parker, C. D. McDaniel, and A. S. Kiess. 2013. Impact of 6 different intestinal bacteria on broiler breeder sperm motility in vitro. *Poultry science 92*, 8, 2174-2181.

Hocking, P. 2003. Maximizing productivity in broiler breeders. In *Proceedings Australian Poultry Science Symposium* (p. 15).

Huff, G.R., W.E Huff, S. Jalukar, J. Oppy, N.C. Rath, and B. Packialakshmi. 2013. The effects of yeast feed supplementation on turkey performance and pathogen colonization in a transport stress/Escherichia coli challenge. *Poultry science*, *92*(3), pp.655-662.

King, L.M., and A.M. Donoghue. 2000. Adaptation of the sperm mobility test for the identification of turkey toms with low fertilizing potential. *Journal of Applied Poultry Research 9*:66-73.

Lu, J., U. Idris, B. Harmon, C. Hofacre, J.J. Maurer, and M.D. Lee, M. D. 2003. Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. *Applied and environmental microbiology*, *69*(11), 6816-6824.

Manafi, M., S. Khalaji, M. Hedayati, and N. Pirany. 2016. Efficacy of Bacillus subtilis and bacitracin methylene disalicylate on growth performance, digestibility, blood metabolites, immunity, and intestinal microbiota after intramuscular inoculation with Escherichia coli in broilers. *Poultry Science* (2016): 347.

Martin, S. A., Nisbet, D. J., & Dean, R. G. 1989. Influence of a commerical yeast supplement on the in vitro ruminal fermentation. *Nutrition reports international (USA)* Reprod. Int. 40, 395–403.

Molnár AK, B. Podmaniczk, P. Kürti, I.Tenk, R. Glávits, G. Virág and Z. Szabó . 2011. Effect of different concentrationsof *Bacillus subtilis* on growth performance, carcase quality, gut microflora and immune responseof broiler chickens. Br *Poultry Science*; 52:658-65.

Nietfeld, R, D.M Hooge and J. Schleifer. 2016. Effect of diets with or without BacPack (*Bacillus subtilis* C-3102 plus yeast cell wall) on hatchery parameters in three commercial broiler breeder field trials at the same company. Abstract: Poult. Sci. 95(E-Suppl. 1)

Niewold, T.A., 2007. The nonantibiotic anti-inflammatory effect of antimicrobial growth promoters, the real mode of action? A hypothesis. *Poultry Science*, *86*(4), pp.605-609

O'dea, E.E., G.M. Fasenko, G.E. Allison, D.R. Korver, G.W. Tannock, and L.L Guan. 2006. Investigating the effects of commercial probiotics on broiler chick quality and production efficiency. *Poultry science*, *85*(10), pp.1855-1863.

Olubowale, O. S., J.P.C. Greyling, F.H. deWitt, A. Hugo, and M.B. Raito. 2014. Effects of dietary lipid sources on the semen quality of Hy-line Silver-Brown cockerels. *JAPS: Journal of Animal & Plant Sciences*, *24*(4).

Opalinski, M., A. Maiorka, F. Dahlke, F.Cunha, F.S.C. Vargas, and E. Cardozo. 2007. On the use of a probiotic (Bacillus subtilis-strain DSM 17299) as growth promoter in broiler diets. *Revista Brasileira de Ciência Avícola*, *9*(2), pp.99-103.

Pan, D. and Yu, Z., 2014. Intestinal microbiome of poultry and its interaction with host and diet. *Gut Microbes*, *5*(1), pp.108-119.

Parker, H. M., & McDaniel, C. D. 2002. Selection of young broiler breeders for semen quality improves hatchability in an industry field trial. *The Journal of Applied Poultry Research*, *11*(3), 250-259.

Pedroso, A.A., J.F. Menten, M.R. Lambais, A.M.C. Racanicci, F.A, Longo, and J.O.B. Sorbara. 2006. Intestinal bacterial community and growth performance of chickens fed diets containing antibiotics. *Poultry Science*, *85*(4), pp.747-752.

Reiber, M. A., J. A. McInroy, and D. E. Conner. 1995. "Enumeration and identification of bacteria in chicken semen." *Poultry science* 74, 5: 795-799.

Round, J.L. and S.K Mazmanian. 2009. The gut microbiota shapes intestinal immune responses during health and disease. *Nature Reviews Immunology*, 9(5), pp.313-323.

Shashidhara, R. G., and G. Devegowda. 2003. Effect of dietary mannan oligosaccharide on broiler breeder production traits and immunity." *Poultry science* 82, 8: 1319-1325.

Smith, A.U. 1949. The control of bacterial growth in fowl semen. *Journal of Agricultural Science. 39*, 194-200.

Tabatabaei, S., M. Chaji, and T. Mohammadabad. 2010. Correlation between age of rooster and semen quality in Iranian indigenous broiler breeder Chickens. *Journal of Animal and Veterinary Advances*, *9*(1), 195-198.

Torok, V.A., Hughes, R.J., Mikkelsen, L.L., Perez-Maldonado, R., Balding, K., MacAlpine, R., Percy, N.J. and K. Ophel-Keller. 2011. Identification and characterization of potential performance-related gut microbiotas in broiler chickens across various feeding trials. *Applied and Environmental Microbiology*, 77(17), pp.5868-5878.

Torok, V.A., K. Ophel-Keller, M. Loo, R.J. and Hughes. 2008. Application of methods for identifying broiler chicken gut bacterial species linked with increased energy metabolism. *Applied and Environmental Microbiology*, *74*(3), pp.783-791.

Torok, V.A., R.J. Hughes, K. Ophel-Keller, K., M. Ali and R. MacAlpine. 2009. Influence of different litter materials on cecal microbiota colonization in broiler chickens. *Poultry Science*, *88*(12), pp.2474-2481.

Van Immerseel, F., J.I. Rood, R.J. Moore, and R.W. Titball. 2009. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. *Trends in microbiology*, *17*(1), pp.32-36.

Vizzier-Thaxton, Y., N.A. Cox, L.J. Richardson, R.J. Buhr, C.D. McDaniel, D.E. Cosby, J.L. Wilson, D.V. Bourassa, and M.B. Ard. 2006. Apparent Attachment of *Campylobacter* and *Salmonella* to Broiler Breeder Rooster Spermatozoa. *Poultry Science. 85*:619-624.

Vohra, A., P. Syal, and A. Madan, A. 2016. Probiotic yeasts in livestock sector. *Animal Feed Science and Technology*, *219*, 31-47.

Xu, C-L., C. Ji, Q. Ma, K. Hao, Z-Y. Jin, and K. Li. 2006. Effects of a dried Bacillus subtilis culture on egg quality. *Poultry Science 85*, no. 2: 364-368.

CHAPTER II

LITERATURE REVIEW

Importance of the poultry industry

The poultry industry is one of the most dynamic sectors of agriculture in the world. It continues to provide high quality and affordable products, including chicken meat, eggs, and poultry by products. During the past 60 years, population growth and high consumer demand for animal products have contributed to the evolution of this sector, from a locally oriented business of 'backyard' producers and larger family operations, into a vertically integrated, highly efficient and competitive sector (Martinez, 1999).

The United States plays a prominent role in the world poultry industry with the greatest poultry consumption in 2016 (15,379 million tons), followed by China (12,715 million tons), and the European Union (10,570 million tons). In the same year, the United States was also the greatest poultry producer (18,283 million tons), followed by Brazil (13,605 million tons) and China (12,700 million tons; Foreign Agricultural Service, 2016). In terms of egg production, the United States occupies the second position (5.6 billion kg unprocessed in shell eggs) after China (24.8 billion kg unprocessed in shell eggs; Poultry trends, 2016).

Success achieved in the poultry industry is a result of different factors like increased efficiency of production, processing technologies, structural organization,

improved nutrition, and especially, intense genetic selection carried out for many years by the primary breeding companies. This genetic selection has led to tremendous progress in productive traits such as growth and feed conversion. However, as a result of this intense genetic selection, reproductive performance, especially fertility in naturally mated flocks, has been negatively affected. In fact, selection for growth over several generations has been related to a decline in fertility or in the efficiency of mating (Chambers, 1990).

Fertility

Fertility in avian species refers to the percentage of incubated eggs that are fertilized. It is an important parameter of reproductive performance that is influenced by genetic and non-genetic factors originating from both males and females (Brillard, 2003).

Fertilization occurs in the infundibulum of the oviduct within 15 min of ovulation (Olsen, 1942). During fertilization, the spermatozoa undergo an acrosome reaction in order to penetrate the perivitelline layer of the ova in the region of the germinal disc, which contains the female pronucleus (Okamura and Nishiyama, 1978). Unlike mammals, physiological polyspermy (or presence of multiple spermatozoa) has been reported in avian species. Because the germinal disc occupies a small area in relation to the entire ovum, it is believed that several spermatozoa must penetrate the oocyte to ensure syngamy. In fact, to assure maximum fertility in the chicken, a minimum of 30 spermatozoa must penetrate the oocyte around the germinal disc (Bramwell et al., 1995). Although, several spermatozoa enter the egg, the DNA of the oocyte will combine with the DNA of only one spermatozoa. The presence of supernumerary sperm penetrating the

ovum seems to increase the likelihood of a single sperm entering the ovum and its chance to fertilize the egg at the right place and time (Bramwell et al., 1995).

Successful fertilization of the avian egg depends on some specific conditions such as: viability of the ova and the sperm, synchronization of the sperm presence in the infundibulum with ovulation, and frequent copulation to provide viable sperm at the time of ovulation (Bramwell et al., 1995). Although, the female contribution is essential to the production of fertile eggs, the male contribution is more critical due to the lower number of males to females in natural mated flocks and especially when artificial insemination is practiced. Therefore, maintaining male fertility is crucial to achieve high reproductive performance in the flock (Ommati et al., 2013).

Male anatomy and physiology

Due to the impact of the male on flock fertility, it is important to understand the rooster reproductive tract and factors that may affect a successful mating and fertilization. Unlike mammals, in which the testes are outside the body, both testes of the rooster are located within the abdominal cavity on either side of their backbone, near the upper kidney. Therefore, avian sperm are viable at body temperature. In addition, the penile structure (phallus) is practically absent in many species of birds (Lake, 1957).

Usually, the testes are either oblong, cylindrical or bean-shaped with a smooth surface and light pigmentation in sexually mature roosters. The size of testes varies with the breeding cycle, sexual activity, and reproductive maturity. In general, they constitute about 1% of the total body weight and are responsible for the production of sperm and testosterone. Seminiferous tubules are prominent tubular structures within the testes, consisting of two type of cells: spermatogonia and Sertoli cells (Lake, 1957).

During the process of sperm formation, spermatogonia undergo proliferation and differentiation through distinct stages of development. In the first stage, spermatogonia undergo multiplication and growth to form primary spermatocytes (2n). Then, the primary spermatocytes undergo Meiosis I to form two secondary spermatocytes (n). These haploid cells then undergo Meiosis II to form spermatids (n). In this process, the number of chromosomes in the parent cell is reduced by half, giving rise to four spermatids, with half the normal number of chromosomes found in a diploid cell. The spermatids will grow and differentiate to form four spermatozoa. Therefore, one spermatozoa will be formed from each spermatid (Witschi, 1961; Alberts et al., 2002).

Sertoli cells are found within the seminiferous tubules between spermatogonia, from the base of the seminiferous epithelium to the interior of the tubules. Primarily, they regulate spermatogenesis and alter the rate of production of spermatozoa. Also known as 'nurse cells', Sertoli cells create the blood-testis barrier and provide required nutrients to the germ cells by transferring nutrients from nearby capillaries in order to ensure complete spermatogenesis. They also act as phagocytes, consuming excess spermatid cytoplasm not required for the formation of spermatozoa (Steinberger et al., 1979; Griswold, 1998; Barrionuevo et al., 2011).

Following spermatogenesis, the sperm leave the testes and travel through the epididymis into the ductus deferens. Unlike mammals, the epididymis is a very short structure in birds. In the epididymis, networks of seminiferous tubules from the testis unite and empty their contents into the ductules. Ultimately, the sperm flow through the ductules into the ductus deferens, a long and narrow tube located next to the ureter that ejects sperm into the cloaca during ejaculation.

At the moment of ejaculation, avian sperm are immediately capable of fertilization. In contrast to mammalian sperm, avian spermatozoa do not require capacitation in the female reproductive tract to ensure fertilization. In fact, a previous *in vitro* study proposed that avian sperm collected from the testis are able to bind to the perivitelline layer and undergo the acrosome reaction (Nixton et al., 2013). However, semen analysis revealed that only 20% of testicular sperm were motile and only 12% of the motile sperm showed a velocity greater than the sperm velocity found in the epididymis. Similarly, Howarth (1983) described that when hens were inseminated with testicular spermatozoa, a total absence of fertility was observed. These findings suggest that the transit of the sperm in the epididymis plays an important role in post- testicular maturation, by increasing the number of motile sperm and the sperm velocity (Nixton et al., 2013).

The lack of accessory sex glands, such as prostate, seminal vesicle, and bulbourethral glands is another remarkable characteristic of the rooster's reproductive tract when compared to mammals (Lake, 1957). In addition, avian sperm are longer, elongated, and more fragile than mammalian sperm. For example, the length of the rooster's sperm is about 100 μm, whereas for mammalian species such as the goat and bull, the length of sperm was found to be about 60 μm and 53 μm, respectively (Cummins and Woodall, 1985; Jamieson, 2007).

During ejaculation, about one half to two thirds of the contents of both ductus deferens is released. Due to the cell density within the tubule of the ductus deferens, spermatozoa take about 4-5 days to pass down this structure and reach the glomula located at the distal end of the ductus deferens where the sperm are stored. During

copulation, semen descends the seminal groove of the rooster's rudimentary phallus, and passes through the rooster's cloaca, the common cavity for the digestive, urinary and reproductive tract. The sperm then enter the female vagina, located within the female cloaca (King and McLelland, 1984).

Avian sperm can be divided into four different parts: acrosome, head, mid-piece, and tail, which are 2.5, 12.5, 4.3, and 90 μm in length, respectively (Lake et al., 1978). Each part of the sperm plays an important role in achieving successful fertilization. For example, the tail provides motility required for the sperm to migrate to the ovum. The midpiece contains the mitochondria that generate energy required for sperm movement. The acrosome protects the sperm head and contains hydrolytic enzymes that help the sperm penetrate the outer perivitelline layer of the ovum during fertilization. The sperm DNA is located within the head and will fuse with the female DNA located within the ovum to produce the zygote, which will undergo several divisions leading to the formation of progeny (Alberts et al., 2002).

Avian semen

Due to the lack of accessory glands, roosters have a concentrated ejaculate composed of spermatozoa and a natural biological fluid called seminal plasma. The total volume of the rooster's ejaculate ranges from 0.01 to 0.9 mL, of which approximately 83-90% is seminal plasma (Marks, 1981; Al-Aghbari, 1992; McDaniel et al., 1995). The functions and components of seminal plasma are broad and complex, and they ensure the livability of spermatozoa and their survival in the female reproductive tract (Lake, 1971; Al-Aghbari, 1992).

Buffer activity is an important function of seminal plasma because the modification of semen pH may be detrimental to sperm. In fact, the optimum pH for avian semen is about 7.25, although incubation temperature may affect this parameter. For example, when incubated at 30°C and 39°C, semen pH for maximum motility ranges from 7.0 to 9.0 and 7.4 to 7.5, respectively. In addition, pH may also be affected by the level of uric acid contamination and lactic acid concentration present in semen (Barna and Boldizsar 1996; Ashizawa et al., 2000; Hildebrandt, 2001) In fact, uric acid is present in bird excrement at high concentrations because it is the main end- product of protein metabolism in avian species (Shannon, 1938; Donsbough et al., 2010). Additionally, lactic acid can be produced in the bird gastrointestinal tract and found in excreta along with other organic acids, such as propionic and butyric acid (Carre and Gomez, 1994). Therefore, both uric acid and lactic acid can be excreted through the cloaca and contaminate rooster semen during ejaculation.

Semen contains several inorganic elements, such as Na^+, K^+, Cl^-, Ca^{2+}, Mg, Cu, HCO_{3-} and Zn, which surround spermatozoa and affect cellular functions either by regulating osmolality or by participating as cofactors for some proteolytic and hydrolytic enzymes. The concentration of these electrolytes is different than those found in blood plasma and is possibly regulated by the epithelia of the male reproductive tract (Al-Aghbari, 1992). The most common electrolyte in seminal plasma is Na^+, followed by Cl^-. The concentrations of O_2, CO_2, Ca^{2+}, Na^+, K^+, and Cl^- in undiluted rooster semen were found to be 0, 0.10, 0.64, 121, 10.4, and 65 mmol/mL, respectively (Parker and McDaniel, 2006). However, the concentration of these electrolytes change in response to spermatozoa passage and location in the male reproductive tract (Al-Aghbari, 1992). In

addition, these ions can also change in response to heat stress. For example, Karaca et al. (2002a) reported that the semen concentrations of Ca^{2+}, Na^{+}, and Cl^{-} were lower in males exposed to heat stress as compared to controls. In addition, there was also a decline in sperm motility of males under heat stress. These results suggest that ions play an important role in sperm function as well as male fertility.

Semen collection procedure

Semen collection is a procedure practiced by avian reproductive physiologists and poultry breeding companies to artificially inseminate hens. In turkeys, low fertility combined with unsuccessful and incomplete mating due to the large size of males, justify the use of artificial insemination at the parent level. Whereas in broiler breeders, natural mating is the predominant breeding method at the parent level in the United States. However, semen collection followed by artificial insemination may be carried out in broiler breeders under special breeding programs. For example, when selecting desirable genetic traits, such as egg production, egg size, and meat quality at the grandparent level and above (Dhama et al., 2014).

For semen collection, one person usually holds the rooster, while the second person massages the bird's lower abdomen and lower back. The testes and phallus are located in this region and release semen during massage. During this procedure of abdominal massage, described by Burrows and Quinn (1937), arterial blood moves to the paracloacal vascular bodies, resulting in sexual excitement and contraction of structures of the ductus deferens leading to ejaculation through the cloaca (King and Millar, 1982). Although, this procedure is labor intensive compared to natural mating, it allows the use

of semen collected from a single male selected for superior productive/reproductive genetic traits to be transmitted to several hens (Haines, 2012).

The ejaculate is collected into a beaker or funnel and is either diluted or directly used to inseminate hens. Because in birds, fluids from the digestive, reproductive and urinary tract are released through the cloaca, it is important to avoid any source of contamination of semen samples to prevent the deterioration of spermatozoa and the horizontal and vertical transmission of pathogenic bacteria to hens and their progenies, respectively (Dhama et al., 2014).

Semen parameters and analysis

Even though flock fertility relies on several different factors, such as ability of mating, strain, management and health of the breeder flock, semen quality is an extremely important parameter to estimate male reproductive performance and fertilizing ability (Parker and McDaniel, 2002). The parameters commonly evaluated to analyze avian semen quality include sperm concentration, viability, volume, and motility (McDaniel et al., 1998). By determining these characteristics, it is possible to predict the number of sperm capable of fertilizing an egg based on the number of viable and motile sperm, which allows for the selection of males capable of producing offspring (King and Donoghue, 2000).

Sperm concentration indicates the total number of sperm present per ejaculate. Chicken semen contains a high concentration of sperm, ranging from 3-8 billion spermatozoa/mL (Etches, 1996). This parameter can be estimated using a photometer, which determines the total amount of light absorbed by the semen sample previously diluted with 3.3% sodium citrate. The addition of sodium citrate prevents sperm

agglutination, especially due to the high number of spermatozoa in the ejaculate. In order to obtain total sperm concentration, the semen and sodium citrate solution must be mixed in a cuvette and placed in the photometer to provide an absorbance reading. Based on the reading, the total amount of spermatozoa/ mL of ejaculate can be predicted from a standard curve. This standard curve is developed by regression of microscopic sperm counts from a hemocytometer against absorbance readings of the same semen sample from a photometer (Donoghue et al., 1996). Although fertilization is more likely to occur if sperm concentration is adequate, it is also important to evaluate the viability and motility of sperm, which will ultimately be required to fertilize the egg (Bakst and Cecil, 1997)

Several procedures are used to determine sperm viability, including a nigrosin/eosin (N/E) staining method (Bakst and Cecil, 1997) and a fluorometric method (Bilgili and Renden, 1984). In the first procedure, only damaged sperm, containing a permeable cell membrane are stained by the eosin. The nigrosin is added to provide a blue background in the microscope to distinguish the eosin (pink) stained dead and unstained live sperm. For the fluorometric method the semen is added in a tube containing phosphate buffered saline and ethidium bromide (EtBr). The first reading is obtained by the nuclear fluorescence emitted when EtBr crosses the damaged cell membrane of dead spermatozoa and binds the DNA. After obtaining the first reading, digitonin is added to the sample to disrupt all the remaining intact membranes and expose the DNA of all spermatozoa in the sample to EtBr. The second reading is obtained by the nuclear fluorescence light emitted by the stained DNA from all sperm cells after exposure to digitonin. The percentage of dead sperm is determined by dividing the first reading by

the second reading and multiplying by 100. The measurement of sperm viability is important to determine male fertility because there is a negative correlation between percentage dead sperm and fertility (r= - 0.27; Wilson et al., 1979). In addition, the determination of sperm viability is a compliment to sperm concentration, because a high concentration of sperm may also have a high number of dead sperm, which are not capable of fertilizing the egg (Haines, 2012).

Semen volume is another important variable when analyzing semen quality. For example, increased ejaculate volumes containing an adequate number of spermatozoa can be used to inseminate several females and are also important to fertilization efficacy (Stratman et al., 1960). Additionally, a previous study conducted in boars suggested that higher ejaculate volumes positively impact sperm shape, by increasing the amount of well-shaped spermatozoa (Gorsk, 2016). However, Wilson et al (1979) found a negative correlation between avian semen volume and fertility, probably due to the presence of other components in the semen sample, such as feces and uric acid that can be detrimental to semen quality. Semen volume can be easily obtained using a graduated microtube to estimate the approximate amount of semen per ejaculate (Zhang et al., 2011). Ejaculate volume can also be obtained by weighing the semen sample using a precision scale (Gorsk, 2016).

Although sperm concentration, viability, and volume are important characteristics that must be considered when analyzing male reproductive performance, sperm motility is a determinant factor of fertility because only motile sperm are capable of passing through the vagina. Once motile sperm reach the uterovaginal junction, where the sperm storage tubules are located, they are released to ensure fertilization (Bakst et al., 1994).

Sperm motility has been assessed by different methods and procedures (Rurangwa et al., 2003). In fact, subjective estimates of sperm motility have been practiced for many years to determine the ratio of motile sperm, duration of movement, swimming vigor, or the combination of these variables. In this method, the sperm movement is analyzed using a microscope to generate a motility score that ranges from 0 (immotile sperm in the semen sample) to 5 (all sperm in the sample are vigorously moving; Guest et al., 1976; Rurangwa et al., 2003). A non-subjective measure of avian sperm motility can also be obtained using a sperm quality analyzer (SQA), which provides the sperm quality index (SQI). This parameter provides an overall estimative of sperm quality, and quantity, because sperm concentration, viability and motility collectively influence the SQI (McDaniel et al., 1998; Parker et al., 2002). However, when sperm concentration and viability are known, then sperm motility can be directly deduced from the SQI. The SQA measures the number of times the sperm present in a diluted semen sample cross a light beam in 20 seconds. Prior to the test, a 10-fold dilution of the semen sample is required due to the high concentration of sperm in avian semen and to allow the normal movement of sperm within the capillary tube in which the diluted semen is drawn. The capillary tube containing the sample is then placed on the SQA to obtain the reading.

Selecting males based on semen quality plays an important role in maximizing fertility (Parker et al., 2000). Therefore, it is crucial to understand the different factors that may affect semen quality and ultimately impact overall flock fertility.

Factors affecting rooster semen quality and fertility

Genetic

Genetic selection for improvements in performance plays a crucial role in the competitiveness and efficiency of the poultry industry. However, selection for meat production traits negatively effects reproductive parameters, such as decreasing fertility and hatchability and delaying sexual maturity (Pollock, 1999). For example, Hocking (2003) reported that excessive breast muscle development has a negative effect on fertility due to the inability of males to mate hens adequately. In fact, uncontrolled body weight gain in males is associated with incomplete and unsuccessful mating due to intense activity required from males to naturally mate the hens. Specifically, the author reported that low fertility (less than 80%) was associated with males over 5 kg. Also, this failure in mating may be a result of male leg problems due to uncontrolled growth (Brillard, 2003). Besides the inefficiency of mating, genetic selection for meat production variables may also affect semen quality. For example, Nestor (1977) reported that turkey males selected for increased body weight showed a reduction in sperm concentration and total sperm per ejaculate as compared to males from a control population.

Previous studies have shown that genetic selection for reproductive fitness traits, such as fertility, hatchability, and other characteristics that contribute to reproductive success, results in slow improvements due to their low heritability, ranging from 0 to 15%. However, fertility improves due to genetic selection for semen quality (Pollock, 1999). In addition, Jones and Lamoreux (1942) reported that males from a high fertility line exhibited better semen quality, earlier sexual maturity and greater fertilizing rates and testes weights compared to males from a line selected for low fertility.

Age

Flock age has also been reported to affect fertility. Usually, a decline in fertility is observed after peak production, although the effect of age is more evident in females than in males (Bramwell et al., 1996). A decline in egg production, fertility, and hatchability has been associated with a greater proportion of short clutches for hens in the latter half of the laying phase (Lerner et al., 1993). The decline in fertility in older hens has been attributed to the inefficiency in storage and release of spermatozoa from sperm storage tubules (Fasenko et al., 1992).

In order to evaluate the relationship between semen quality and age, Cherms (1968) collected and analyzed the semen from toms that ranged from 36-41 wk old for 20 consecutive weeks. The author found a decrease in sperm concentration after 11 weeks of semen production, when the toms were 47-52 wk old, although the motility was not affected. When analyzing sperm quality in Iranian indigenous roosters between 26 and 34 weeks, Tabatabaei et al. (2010) described a decrease in sperm motility and viability associated with an increase in morphological defects in spermatozoa due to ageing of roosters. However, another study has suggested an increase in fertility and sperm penetration of the perivitelline layer by old (69-73 wk old) males compared to young (39-43 wk old) broiler breeder males (Bramwell et al., 1996).

Temperature

Because domestic birds are homoeothermic, even a minor change in temperature can affect their homeostasis and negatively impact productive and reproductive performance. For example, in hens, a decline in fertility has been reported as a consequence of high environmental temperature (Kiers, 1982). Although heat stress has a

negative impact on reproductive performance of both males and females, male breeders are more susceptible to high temperature than females. In fact, rooster reproductive performance is very sensitive to environmental stress (McDaniel et al., 1995). Heat stress has been directly associated with detrimental effects on semen quality by decreasing sperm viability and SQI. Furthermore, a decline in semen quality in response to high temperature was more evident in males from a population that exhibited a high SQI as compared to males having a poor SQI (Karaca et al., 2002b).

Photoperiod

Previous research has also revealed the effect of photoperiod on reproductive performance of domestic birds. In fact, the length of light exposure per day plays an important role in the reproductive processes in both female and male birds. For example, a longer photoperiod results in hormonal changes in female birds, leading to egg production and mating behavior (Sharp, 2005).

As duration of light per day increases, GnRH (gonadotropin releasing hormone) is released from the hypothalamus stimulating the anterior pituitary to secrete follicle-stimulating hormone (FSH) and luteinizing hormone (LH). In males, FSH and LH are associated with sperm-production structures in the testis and secretion of the steroid hormone, testosterone, respectively (Husvéth, 2011). The length of light exposure has also been reported to affect semen parameters. In an experiment conducted to test the effect of photostimulation on broiler breeder males, Cobb roosters from 20-51 wk old were exposed to different photoperiods, including 8, 9, 9.5, 10, 10.5, 11, 11.5, 12, 12.5, 13, 14 and 18 h. The authors observed that higher sperm concentration was reached when the birds were exposed to 8-11 hours of photoperiod, and a decrease in this parameter

was reported as the exposure to light increased above this period (Floyd and Tayler, 2011)

Nutrition

Although reproductive performance relies on several different factors, nutrition is one of the most determinant factors of poultry fertility due to its direct and indirect effects on physiological processes, especially during the growth stage. During the growing stage, meeting the bird's nutrient requirement is crucial not only for growth, but ultimately for successful functioning of the reproductive tract (Waldroup et al., 1976). It has been established that nutrient density in a rooster's diet during the developmental stages (including pre-puberty, puberty and post puberty) may drastically impact semen quality (Wilson et al., 1979). On the other hand, at sexual maturity, feed restriction is practiced for broiler breeder males in order to prevent obesity, which is commonly associated with low fertilizing capacity (McDaniel, 1983).

In the bird's diet formulation, protein is an important nutrient that affects male performance. For example, when turkeys were fed 11,13, 15, or 17 % protein, the age at which 85% of males were producing semen was found to be 43, 39, 37 and 28 wk old, respectively. These results suggest that a low inclusion of protein in the turkey tom diet was associated with a delay in sexual maturity (Cecil, 1981). Also, due to the participation of micronutrients such as Se, Mn, and Zn, in several physiological processes, their excess or deficiency impairs reproductive traits, such as spermatogenesis, libido, embryonic development, and fertility (Barber et al., 2005).

Besides the amount of nutrients included in the diet, diverse feed ingredients have been tested to analyze their impact on reproductive performance due to their impact on

fertility and also semen quality. For example, in order to analyze the effect of lipid composition on semen quality, Olubowale and colleagues (2014) included different sources of lipids in isocaloric and isonitrogeous diets of Hy-Line Silver cockerels. The authors reported that fish oil had a negative impact on sperm motility and frequency of ejaculation, whereas an increase in semen volume was observed in cockerels fed tallow. These results suggest that the source of dietary fatty acid may affect semen quality and male reproductive performance.

Previous research has also demonstrated modification in reproductive performance of domestic birds in response to inclusion of antibiotics. For instance, Dean et al. (1958) reported an increase in the percentage of fertile eggs in hens supplemented with furazolidone. Also, the addition of bacitracin methylene disalicylate (BMD) in breeder pullet diets has been described to improve egg fertility and total hatchability (Damron and Wilson, 1985). However, in another study, McCracken and cohorts (2005) found that hens fed nitrofurans transfer the residue of this antibiotic to their progeny, which may present a threat to human health due to the risk of antimicrobial resistance.

Feed additives: the use of antibiotics and antibiotic alternatives

Antibiotics have been added to the feed extensively throughout the poultry industry to treat and control harmful bacteria associated with minimization of broiler performance and, more recently as antimicrobial growth promoters (AGP). However, many antibiotics that have been supplemented in poultry and livestock feed have also been used in human medicine, which has led to an increased worldwide concern associated with the development of antibiotic resistance (Nunes et al., 2012). In fact, antibiotic resistance has been shown to pass from one bacterial species to another,

unrelated bacteria. Resistance occurs when a bacterium survives upon exposure to a level and type of antibiotic that normally kills susceptible bacteria (Edens, 2003).

The restrictions imposed concerning the use of antibiotics in livestock and poultry coupled with increasing demand for antibiotic-free products have stimulated the use of antibiotic growth promoter (AGP) alternatives, to maintain high animal performance, meet consumer requirements, and to prevent risk associated with human health (Nunes et al., 2012). The addition of AGP alternative products in the diet have been utilized to improve animal health, immune function and overall performance (Edens, 2003). In fact, changes in management procedures and inclusion of different AGP alternatives are examples of important measures and approaches that have been adopted to maintain or improve growth, meat production and reproductive performance in domestic birds (Huyghebaert et al., 2005).

Even though their exact mode of action is still unclear, modulation of intestinal microbiota is the most accepted mechanism of AGP to prevent diseases and enhance growth and animal performance. Dietary supplementation of AGP alternatives have been found to prevent proliferation of harmful bacteria and modulation of indigenous bacteria in the gut (Dibner and Richards, 2005). In this context, several studies have tested many potential AGP alternatives and their impacts on animal performance (Edens, 2003; Huyghebaert et al., 2005; Kabir, 2009; Nunes et al., 2012). Prebiotics and probiotics are examples of antibiotic alternatives that are exploited in livestock and poultry.

Prebiotics

Prebiotics are non-digestible dietary constituents that selectively enhance the growth and activity of a limited number of species of non-pathogenic microorganisms in

the gut, which in turn benefit host health. The most common prebiotics include oligosaccharides (mannanoligosaccharides (MOS), galactooligosaccharides, fructooligosaccharides (FOS), soy oligosaccharides, isomalto-oligosaccharides, xylooligosaccharides, and lactulose) and polysaccharides. Although the research has been inconsistent, the addition of prebiotics in the animal diet have been reported to decrease the colonization of harmful bacteria, stimulate the immune system, and neutralize toxins (Papatsiros et al., 2013).

In poultry, Futaka et al. (1999) reported that supplementation of FOS in broilers inoculated with *Salmonella enteritidis* decreased the colonization of this bacteria in the ceca and improved growth performance. Moreover, improvements in intestinal morphology, intestinal enzyme activity and growth performance were observed in birds fed MOS (McCann et al., 2006). In addition, birds fed oligosaccharide beta-glucans derived from the yeast cell wall also showed improvements in performance due to the immunomodulatory activity of this compound (Novak and Vetvicka, 2008).

Probiotics

The dietary supplementation of probiotics has also been investigated in livestock and poultry. The definition and concept of probiotics have changed over the years, and currently, FAO/WHO redefined probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host”. In other words, probiotics are microorganisms, including bacteria, yeast, and fungi, which when adequately administered and consumed, favor host health (Guillot, 1998). Common probiotics used in animal diets include *Lactobacilli*, *Bacilli*, *Streptococci*, *Bifidobacterium,* and yeast (*Saccharomyces)* varieties. Research conducted on the effects

of probiotics in poultry does not always yield positive results. However, data in favor of probiotic supplementation suggest improvements in animal performance due to their mechanisms of action such as competitive exclusion, increased feed utilization, production of specific metabolites with antimicrobial effects, reduction of gut pH and stimulation of the immune system (Grashorn, 2010). Specifically, in poultry, dietary supplementation of probiotics increases digestive enzyme activity, improves feed intake and utilization, reduces pathogenic bacteria, modulates intestinal microbiota, and prevents or alleviates the negative effects and injuries associated with dysbacteriosis, especially necrotic enteritis (Patterson and Burkholder, 2003; Kabir, 2009).

Effect of antibiotic alternatives on meat production and reproductive performance

Although growth performance traits and meat production are most often the main focus of studies testing the effects of antibiotic alternatives, the impacts of these compounds on reproductive performance of avian species has also been documented. For example, Akhlaghi et al. (2014) described that roosters supplemented with dried ginger rhizome showed improved sperm forward motility, live sperm percentage, sperm plasma membrane integrity, and a decreased percentage of abnormal sperm as compared to non-supplemented control birds.

In order to examine the effect of different feed additives on productive performance, serum components, digestibility, semen quality, fertility, hatchability, and economic efficiency, Abaza et al. (2006) supplemented layer breeder diets with different additives, including Dinaferm (*Saccharomyces cerevisiae*), biotope (*Bacillus subtilis* and *Bacillus licheniformis*) and black seed oil versus antibiotics (amoxicillin and zinc bacitracin). The authors reported that the addition of all feed additives improved overall

hen performance by increasing egg number and egg mass and by improving feed conversion, while no difference was observed for egg weight compared to the control group. In addition, the highest egg number and lowest feed conversion was obtained for the group fed *Saccharomyces cerevisiae*. Furthermore, the addition of feed additives improved semen quality by increasing semen ejaculate volume, sperm cell concentration, and sperm motility, while dead spermatozoa and sperm abnormalities were decreased as compared to the control group.

Similarly, previous research conducted in diabetic rats revealed a significant reduction in genetic alteration and sperm abnormalities in a group fed yeast, with or without chromium as opposed to the non-supplemented group. The improvement in these variables was attributed to the antioxidant capacity of yeast followed by a decrease in the generation of reactive oxygen species that are detrimental to sperm function and viability (Ahmed et al., 2012; Guthrie and Welch, 2012). Because mammals and birds have evident differences in their reproductive tract, the dietary supplementation of yeast to poultry could have distinct effects and modes of action as compared to mammals. Therefore, it is important to specifically understand the role and mechanism of action of feed additives, such as yeast, yeast fermentation products (YP) and *Bacillus subtilis* on poultry reproductive performance.

Yeast and yeast fermentation products (*Saccharomyces cerevisiae*) as prebiotics and probiotics

Yeasts belong to the kingdom fungi, are unicellular and eukaryotic microorganisms and reproduce both sexually and asexually. Although variations in shape and size has been described among species, yeast cells are typically globular, oval or

spherical in shape, measuring 4-6 μm in diameter, (Walker et al., 2002). Unlike plants, yeasts lack chlorophyll and are unable to obtain their organic needs by photosynthesis (Hayat, 1992). Also, yeast species can be aerobic or facultative anaerobic and grow at low pH (4-4.5). Yeasts are active in a broad temperature range from 0 to 50°C, although they prefer a temperature range of 20°C to 30°C (Mountney and Gouldi, 1988). Yeasts can be found in many natural environments, such as water, plants, microflora of humans, food products, and in different ecological niches (Rima et al., 2012). Additionally, most species of yeast can be produced in large scale without threat to public health (Barnett, 1990). In fact, yeasts and YP, are classified as generally recognized as safe (GRAS) by the US Food and Drug Administration (FDA, 2015).

Due to the ease of gene manipulation, yeast is a common experimental organism for scientific studies. Furthermore, in 1992 the first eukaryotic genome completely sequenced was that of a strain of *Saccharomyces cerevisiae* (Goffeau et al., 1996). Currently, about 1,500 species of yeast have been identified; however the yeast *Saccharomyces cerevisiae*, also known as brewer's or baker's yeast, is one of the most commercially exploited and well known species. In fact, this specie has been used for several years for fermentation purposes and, more recently, for biomolecular studies and pharmaceutical purposes (Kurtzman, and Fell, 2006; Moyad, 2007). They are also commonly used as a probiotic for humans, but within the last three decades there has been increased interest in adding yeast and its derivatives as potential AGP alternatives for livestock and poultry (Martin et al., 1989; Vohra et al., 2016).

Yeast products commonly used as feed supplements include active dry yeast, yeast cell wall, yeast extract, and yeast culture (Xu, 2014). Yeast and YP are naturally

produced, non-antibiotic, and non-chemical feed additives. Their inclusion in the diets for ruminants, swine, aquatic species, and poultry have been known to improve animal performance by enhancing the nutritive quality of feed and feed utilization (Xu, 2014). The live yeast and YP are commonly included in the diet as microbial supplements and a natural nutrient source, respectively. They contain biologically valuable proteins, vitamin B complex, trace minerals, and extracellular digestive enzymes, such as phytase and amylase (Thayer et al., 1978; Moore et al., 1994).

The mechanisms of action of yeast and YP that lead to beneficial effects on animal performance are not completely elucidated and are still controversial due to the lack of extensive scientific evidence to support these claims (Hayat, 1992; Madriqal et al., 1993; Kidd et al., 2013). However, these feed additives have been associated with immune system stimulation, production of antagonist compounds against pathogenic bacteria, increased digestive function of the gastrointestinal tract and especially modulation of the intestinal microbiota (Roto et al., 2015). In fact, the inhibition of harmful microorganisms in response to the supplementation of yeast and YP has been attributed to their antagonistic properties, such as competition for nutrients, production of ethanol and killer toxins, and pH alteration (Rima et al., 2012).

In mammals, Hristov et al. (2010) reported that supplementation of YP had little impact on ruminal fermentation, digestibility and nitrogen losses. However, in a study conducted to examine the dietary effects of YP on rumen fermentation and performance in growing and lactating ruminants, Robinson et al. (2010) reported an average increase in ruminal pH, an overall decrease in lactate concentration and overall increase in total rumen volatile fatty acids.

In an *in vitro* study, supplementation of yeast increased fiber digestion by stimulating growth and activities of the fibrolytic bacteria community (Marden et al., 2008). Jouany et al. (2006) proposed that in feed, aerobic yeast cells maintain an anaerobic environment by utilizing the oxygen present in the feed, this could possibly stimulate the growth of anaerobic microorganisms inhabiting the rumen.

Shen and cohorts (2009) reported that supplementation with 5g/kg of YP has a positive effect on the average daily gain of nursery pigs, whereas no difference was found between a conventional AGP and YP supplementation. Further, the digestibility of dry matter, crude protein, and gross energy was also improved in pigs fed YP. This improvement in pig performance was attributed to the modulation of the gut immune response, and increased jejunal villus height and villus height: crypt depth ratio.

In avian species, yeast and YP have been used as inexpensive feed additives and as potential alternatives to AGP. It has been reported that dietary yeast improves live performance in broilers, although the results are not always consistent. This variability in the results may be due to differences in the feed composition, nutrient digestibility, experimental conditions, flock health, breed, level of inclusion, and yeast product (Madriqal et al., 1993). However, several researches have shown that the addition of these preparations in the feed can have positive effects in poultry, by controlling the composition of the microbial population in the gastrointestinal tract, binding to toxins and modulating the immune system (Line et al., 1998; Javadi et al., 2012; Saadia and Hassanein, 2010). These effects may improve animal performance and provide some activities comparable to AGP (Roto et al., 2015).

Gao and cohorts (2008) reported that the addition of yeast culture to broiler diets improved overall performance by modulating the intestinal mucosal morphology and by increasing the absorption of calcium and phosphorus. In addition, antibody titers to Newcastle disease virus, and IgM and IgA concentrations in the duodenum were increased suggesting an enhancement in the immune system in response to YP supplementation.

Besides numerous studies on meat production traits, the impact of yeast and YP on poultry reproductive performance has also been examined to but to a lesser extent. For example, Hayat (1991) reported that hen fertility and hatch of fertilized eggs were increased in hens fed yeast culture as compared to non-supplemented hens. On the other hand, Brake (1990) reported that egg production, feed conversion, mortality, hatchability of fertile eggs, egg weight and percentage of shell were not affected when broiler breeders were fed 0, 0.1, 0.3, or 0.5% of YP. However, 0.3% yeast culture resulted in a reduction of fertility and hatchability of all eggs set when compared to other treatments.

In an experiment conducted to investigate the effect of YP on reproductive parameters and progeny performance, Kidd et al. (2013) reported a reduction in hatching egg contamination from hens at 32 wk of age but not by 39 wk of age. Furthermore, in both 32 and 39 wk hatches, hatchability of fertile eggs was improved in hens fed YP. The other egg parameters were not affected by the addition of YP, but an improvement in feed conversion, and breast meat yield were reported in the progeny from hens fed YP.

The reduction in egg contamination in response to YP supplementation might be associated with the ability of yeast and YP to modulate intestinal microbiota. In fact, Line et al. (1998) reported that the supplementation of live yeast inhibits the colonization of

harmful bacteria in the intestine. Possibly, the harmful bacteria are bound to mannose that is present in the outer cell wall of yeast (*Saccharomyces cerevisiae*). Similarly, Baurhoo et al. (2007) stated that the supplementation of mannanoligosaccharides may decrease the colonization of pathogenic bacteria due to their ability to bind to mannose-specific lectin present in gram negative bacteria, such as *Salmonella* and *E. coli*, with type I fimbriae.

The presence of yeast- bound pathogens in the intestinal tract is not permanent. Therefore, yeast and any yeast- bound pathogens are likely released through excretion, which would ultimately decrease bacteria colonization (Javadi et al., 2012). Similarly, Huff et al. (2013) reported that isolation of *Salmonella* spp. and *Campylobacter* spp. from the ceca declined in response to continuous supplementation of YP to turkeys challenged with *E. Coli* and under transportation stress.

Although the contribution of yeast and YP in establishing a healthy gut microbiota has been well studied, it is not completely understood whether modulation of bacteria in response to yeast supplementation will affect semen quality that will ultimately impact fertility in domestic birds. In addition, the direct effects and mechanism of action of yeast and YP levels on avian semen parameters are still unclear.

Bacillus subtilis **as a probiotic**

Bacillus species comprise rod-shaped, gram-positive, endospore-forming, and aerobic or facultative anaerobic bacteria. They are widely distributed in nature and are commonly found in soil, water, and air (Priest, 1989). Due to their physiological abilities, *Bacillus* spp. can withstand a variety of environmental conditions. Moreover, many species have been commercially exploited for different purposes, such as production of enzymes, antibiotics, and insecticides. In addition, most of the species are non-pathogenic

for humans and animals. The pathogenic species of Bacillus include *B. anthracis* and *B. cereus* which are associated with anthrax and food poisoning, respectively (Harwood, 1992).

B. subtilis is an example of a beneficial bacteria generally recognized as safe (GRAS) by the Food and Drug Administration (FDA, 2015). The complete sequencing of its genome has led to a variety of biomolecular and genetic studies, as well as a deep understanding of this species (Harwood and Cutting, 1990). Additionally, this bacterium will grow efficiently with low cost ingredients, due to its ability to produce enzymes capable of breaking down nutrients such as carbohydrates, proteins, and lipids from both animal and vegetable sources (Sonnenschein et al., 1992). The production of antibiotics, especially of peptide origin, plays an important role in the antimicrobial activity of *Bacillus* spp. In fact, 795 antibiotics were identified from *Bacill*us species, and *B. subtilis* is known to be the most productive species of the genus with 66 antibiotics (Stein, 2005).

Due to its beneficial properties and safety, *B. subtilis* has been considered a potential probiotic for different species including: humans, livestock, and poultry. The capacity of *B. subtilis* spores to resist harsh environmental conditions, such as heat, cold, dehydration and UV radiation, allows them to survive during feed preparation and storage. In addition, the supplementation of *B. subtilis* spores as compared to vegetative cells is preferred because they can tolerate low pH and bile salts present in the gastrointestinal tract and yet maintain their viability during digestion. Because there is a gradual decline after supplementation, *B. subtilis* spores must be continuously supplemented in the diet (Casula and Cutting, 2002).

For humans, *B. subtilis* spores are provided to prevent mild gastrointestinal disorders or as a nutritional supplement. In agriculture, *B. subtilis* has been exploited as an alternative to AGP (Casula and Cutting, 2002). Dietary supplementation of *B. subtilis* has been reported to improve animal performance by several mechanisms, such as modulation of intestinal microbiota, competitive exclusion, stimulation of the immune system, and alleviation of intestinal disorders (Gaggia et al., 2010). However, these proposed mechanisms are still controversial and the efficacy of *B. subtilis* has not been completely proven and understood.

For example, when piglets were supplemented with *B. subtilis* for 28 days, Hu et al. (2004) reported an increase in average daily gain and feed efficiency and a decrease in diarrhea index as compared to the control group. In addition, there was a change in the bacterial communities due to *B. subtilis* supplementation resulting in a higher number of *Lactobacillus* spp. and a lower number of *E. coli*. However, no improvement in performance, change in gut microbiota, or alteration of the diarrhea index were found by Utiyama et al., (2006) when newly weaned piglets were fed *B. subtilis*.

In an *in vitro* study conducted to analyze the inhibitory effect of *B. subtilis* against pathogenic bacteria in the performance of calves, Garcia (2008) reported a higher efficacy of this probiotic against *Clostridium perfringens* as compared to *Salmonella* spp. and *E. coli*. In the same study, the author also stated an increase in feed intake, body weight gain and thoracic perimeter in calves supplemented with different levels (1, 2, and 4g/day) of *B. subtilis* as compared to the control group. However, Qiao et al. (2010) reported that the addition of *B. subtilis*, twice a day, did not affect feed intake, feed efficiency, and body weight or the production and composition of milk in Holstein cows.

Further, rumen pH and concentration of propionate, acetate and butyrate were also not affected by the addition of *B. subtilis*.

Similar to the supplementation of yeast and YP, the addition of *B. subtilis* in poultry diets has been shown to modulate intestinal microbiota. For instance, the oral supplementation of *B. subtilis* spores have been reported to reduce the infection caused by pathogenic bacteria, such as *Salmonella* spp., *Clostridium perfringens,* and *E. coli* (La Ragione and Wooward, 2003). According to Maruta et al. (1996), the supplementation of *B. subtilis* resulted in a decrease in harmful bacteria followed by an increase in the number of *Lactobacilli*, suggesting that *B. subtilis* stimulates the growth of other probiotics in the gut. Increases in body weight, feed conversion, and reduction in the number of coliform and *Campylobacter* spp. were also reported when broilers were fed 30g/ton of *B. subtilis* (Fritts et al., 2000). In laying hens, improvements in feed conversion ratio and egg shell quality were also reported in a supplemented group (Pedroso et al., 1999).

The use of *B. subtilis* also demonstrated an inhibitory effect against enterobacteria in broiler breeder litter. For example, Brito and Tagliari (2007) found that the addition of *B. subtilis* in the litter reduced the number of *E. coli*. Furthermore, this probiotic was efficient in preventing cellulitis in broilers exposed to pathogenic strains of *E. coli*. Similar findings were reported by Roll et al. (2008), in which litter treated with 5.0g/m^2 of a commercial product containing *B. subtilis* and its protease enzymes showed a 13% reduction in log CFU counts of enterobacterium as compared to the untreated group. These findings suggest that the role of *B. subtilis* in modulating microbiota is not restricted to the gastrointestinal tract, and it can indirectly affect animal performance by

reducing pathogenic bacteria in various conditions and environments. Therefore, it is possible that dietary supplementation with *B. subtilis* could also modulate microbiota of the avian male reproductive tract and hence semen quality, because the presence of bacteria in the ejaculate can influence semen quality.

Presence of bacteria in the poultry reproductive tract and semen

The presence of bacteria in the gastrointestinal tract and their negative impact on animal performance has been widely researched. Additionally, previous findings also provide evidence that bacteria are routinely found in the reproductive tract of several species, including poultry. Buhr and cohorts. (2002) conducted a study to determine the presence of bacteria in the female reproductive tract of broiler breeder hens sourced from a research flock and a commercial farm. Regardless of source, hens were positive for *Campylobacter* spp.in the cloaca. In addition, these bacteria were found in the shell gland and vagina and in the magnum and isthmus from hens sourced from research and commercial flocks, respectively. Further, *Salmonella* spp. have also been isolated from the ovaries and oviduct of the hen's reproductive tract at rate of 1.47 and 0.5 %, respectively. Moreover, in the ovaries, single and multiple serotypes of *Salmonella* have been detected (Barnhat, 1993).

The presence of bacteria in semen and male reproductive tissues has also been documented. In human semen from patients with urogenital tract infections, Moretti et al. (2009) found that *E. coli* is the most common microorganism detected, and it is associated with a detrimental effect on sperm motility. Whereas, from infertile couples, aerobic cocci were detected in about 50% of semen samples. Moreover, the authors stated

that the presence of bacteria such as *Ureaplasma* and *Mycoplasma* may contaminate the semen by their colonization in the male urethra.

According to Donoghue et al. (2004), *Campylobacter* is indigenous in turkey semen at about 1.2 x 10^3 CFU/mL. In addition, Vizzier-Thaxton and cohorts (2006) found in an *in vitro* study (2006) that *Salmonella* spp. were attached to all the segments of the sperm (head, midpiece and tail), whereas *Campylobacter* spp. were mainly limited to the midpiece and tail. These findings suggest that semen can be a source of transmission of pathogenic bacteria to broiler breeder flocks if *Salmonella* spp. and *Campylobacter* spp. attach to spermatozoa under *in vivo* conditions.

Additionally, the presence of bacteria has been described to be higher in poultry species as compared to mammals. For example, in bulls, Myers and Almquist (1951) reported a concentration of 85,000 bacteria per mL of semen; whereas in rooster and turkey semen the concentration was determined at 2.2 million and 1.3 billion bacteria per mL, respectively (Wilcox and Shorb, 1958; Gale and Brown, 1961). The most common bacteria isolated from the semen samples included *Escherichia, Staphylococcus, Bacillus, and Enterococcus* (Gale and Brown; Donoghue et al., 2004).

Ahmed and cohorts (2015) investigated the presence of bacteria in mature Vanaraja cockerels (a dual-purpose chicken strain from India) and the antibiotic sensitivity when these bacteria were exposed to different antibiotics. The authors revealed that all the semen samples were positive for one or more bacteria. The microorganisms isolated from the samples include *E. coli, Kluyvera ascorbata, Salmonella enteritidis, Pseudomonas, Serratia plymuthica* and *Klebsiella,* which were all highly sensitive to norfloxacin, ciprofloxacin, and ceftriaxone.

The direct impact of pathogenic and non- pathogenic bacteria on avian sperm motility has been examined. In an *in vitro* study, Haines and cohorts (2013) exposed rooster semen to pathogenic bacteria (*Salmonella*, *E. coli*, *Campylobacter*, and *Clostridium)* and non- pathogenic bacteria commonly used as probiotics *(Lactobacillus,* and *Bifidobacterium).* It was found that sperm motility was negatively affected by all the bacteria tested, and sperm motility was eliminated by the non-pathogenic bacteria. Furthermore, when artificial insemination was performed using semen exposed to high levels of *Lactobacillus,* hens produced only infertile eggs.

Even though previous research suggests that antibiotic alternatives (e.g yeast, YP, and *B. subtilis*), modulate pathogenic and non- pathogenic bacteria in the gut, the effects of these specific alternative to antibiotics on semen quality is not completely understood (Vohra et al., 2016; La Ragione and Wooward, 2003). Therefore, additional research is needed to determine if yeast, YP, or *B. subtilis* impact avian semen quality.

Conclusion

The poultry industry has tremendously evolved from backyard production and family consumption into a highly successful and prominent business of global importance. The evolution of this sector may be attributed to several factors, such as advances in nutrition, management, research, and genetics. However, in part as a consequence of intense genetic selection for meat production traits in domestic birds, reproductive performance has been negatively affected, especially fertility. Because fertility plays a crucial role in supplying chicks, it is important to evaluate the different parameters that may impact fertility and ultimately poultry production. In order to achieve high fertility, semen quality is an important factor that must be considered due to

the essential contribution of the male towards flock fertility. Nutrition has been shown to be a determinant aspect of semen quality. Previous work has shown the impact of nutrients, energy, ingredient source and feed additives on fertility and semen quality. Alternatives to AGP, including probiotics and prebiotics, are feed additives that have been broadly studied to replace antibiotics used in livestock and poultry. Although their mechanism of action is very diverse, they have been found to improve animal performance by modulating intestinal microbiota. Besides their presence in the gut, bacteria have also been found in the reproductive tract where they impact sperm motility in several species, including poultry. Evaluation of poultry semen quality and semen microbiota in response to the supplementation of AGP alternatives (YP and *Bacillus*) in this thesis research will provide useful information on rooster fertility as more poultry companies eliminate the use of AGP .

References

Abaza, I. M., M.A.Shehata, and M.S. Shoieb.2006. Evaluation of some natural feed additive in layer diets. Egyptian *Poultry Science, 26*, 891-909.

Ahmed, E.S., H.F. Booles, I.M. Farag, S.A. Nada. and M.Fadel. 2012. Study of Therapeutical Role of Chromium-Enriched Yeast (Saccharomyces cerevisiae) on Genetic Alterations and Sperm Abnormalities in Streptozotocin-Induced Hyperglycemic Rats. *World Applied Sciences Journal, 16*(1), pp.39-51.

Ahmed, K.; K. Das, & S. George. 2015. Bacterial flora of poultry semen and their antibiotic sensitivity pattern. *International Journal of Applied and Pure Science and Agriculture*. 1: 1-4

Akhlaghi, A., Y. J. Ahangari, B Navidshad, Z. A Pirsaraei, M., Zhandi, H. Deldar, & E. D. Peebles. 2014. Improvements in semen quality, sperm fatty acids, and reproductive performance in aged Cobb 500 breeder roosters fed diets containing dried ginger rhizomes (Zingiber officinale). *Poultry science*, *93*(5), 1236-1244.

Alberts B., A. Johnson, J Lewis. 2002. Sperm. In: Molecular Biology of the Cell. 4th edition. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK26914/. Acessed 2/3/2017

Al-Aghbari, A. M. 1992. Demonstration of a link between seminal plasma proteins and male fertility in the domestic fowl *(Gallus domesticus)*. Oregon State (Doctoral dissertation).

Ashizawa K, G.J. Wishart & Y Tsuzuki. 2000. Avian sperm motility: environmental and intracellular regulation. *Avian and Poultry Biology Reviews 11*:161–172.

Bakst, M. R., G. Wishart, and J. P. Brillard, 1994. Oviductal sperm selection, transport, and storage in poultry. *Poultry Science* Rev. 5:117–143.

Bakst, M.R., and H.C. Cecil. 1997. Techniques for semen evaluation, semen storage, and fertility determination. Poultry science association, inc., Savoy, IL.

Barber, S. J., H. M. Parker, & C. D. McDaniel, 2005. Broiler breeder semen quality as affected by trace minerals in vitro. *Poultry science*, *84*(1), 100-105.

Barna, J., and H. Boldizsar. 1996. Motility and agglutination of fowl spermatozoa in media of different amino acid content and pH value in vitro. *Acta Veterinaria Hungarica 44*: 221-232.

Barnett, J. A, R. W Payne, and D. Yarrow. 1990. Yeasts, characteristics and identification. 2nd ed. Cambridge University Press, New York, NY.

Barnhart, H.M., D.W. Dreesen, and Julia L. Burke, 1993. Isolation of *Salmonella* from ovaries and oviducts from whole carcasses of spent hens. *Avian Diseases. 37*:977-980

Barrionuevo, F., M. Burgos& R. Jiménez. 2011. Origin and function of embryonic Sertoli cells. *Biomolecular concepts*, *2*(6), 537-547.

Baurhoo, B., A. LetellierZhao, X., C.A. Ruiz-Feria. 2007. Cecal populations of Lactobacilli and Bifidobacteria and Escherichia coli after in vivo Escherichia coli challenge in birds fed diets with purified lignin or mannanoligo-saccharides. *Poultry Science 86,* 2509–2516.

Bilgili, S.F., and J.A. Renden, 1984. Fluorometric determination of avian sperm viability and concentration. *Poultry Science. 63:*2275-2277.

Brake, J. 1991. Research note: Lack of effect of a live yeast culture on broiler breeder and progeny performance. *Poultry science*, *70*(4), 1037-1039.

Bramwell, R. K., H. L. Marks and B. B. Howarth, 1995. Quantitative determination of spermatozoa penetration of the perivitelline layer of the hen's ovum as assessed on oviposited eggs. *Poultry Science*, *74*(11), 1875-1883

Bramwell, R. K., C. D. McDaniel., J. L Wilson, and B. Howarth. 1996. Age effect of male and female broiler breeders on sperm penetration of the perivitelline layer overlying the germinal disc. *Poultry science*, *75*(6), 755-762.

Brillard, J. P. 2003. Practical aspects of fertility in poultry. *World's Poultry Science Journal*, *59*(04), 441-446.

Brito, B. G., & K. C. Tagliari, 2007. Efeito da utilização de Impact-p na ocorrência de celulite em frangos de corte. *A Hora Veterinária, Porto Alegre*, *26*(155), 13-20.

Buhr, R.J., N.A. Cox, N.J. Stern, M.T. Musgrove, J.L. Wilson, and K.L. Hiett. 2002. Recovery of *Campylobacter* from segments of the reproductive tract of broiler breeder hens. Avian *Diseases 46*:919-924

Burrows, W.H., and J.P. Quinn, 1937. The collection of spermatozoa from the domestic fowl and turkey. *Poultry Science 16*:19-24.

Carre, B., and J. Gomez. 1994. Digestibility of water-soluble pectin and organic acid losses in intact or cecectomized adult cockerels." *Poultry science 73, no. 12* : 1881-1886.

Casula, G., and S. M. Cutting, 2002. Bacillus probiotics: spore germination in the gastrointestinal tract. *Applied and environmental microbiology*, *68*(5), 2344-2352.

Cecil, H.C., 1981. Effects of dietary protein on body weight and reproductive performance of male turkeys. *Poultry Science, 60*(5), pp.1049-1055.

Chambers, J. R. 1990. Genetics of growth and meat production in chickens. *Developments in Animal and Veterinary Sciences (Netherlands)*.

Cherms, F. L. 1968. Variations in semen quality and the relationship of semen quality to fertility in turkeys. *Poultry science*, *47*(3), 746-754.

Cummins, J. M., and P. F. Woodall. 1985. On mammalian sperm dimensions. *Journal of Reproduction and Fertility*, *75*(1), 153-175.

Damron, B. L., & H. R. Wilson. 1985. Bacitracin methylene disalicylate in broiler breeder feeds. *Poultry science*, *64*(8), 1451-1454.

Dean, W. F., and E. L. Stephenson. 1958. The influence of dietary furazolidone on egg production, hatchability, fertility, and feed efficiency of laying and breeding hens. *Poultry Science*, *37*(1), 124-128.

Dhama, K., R. P. Singh, K. Karthik, S. Chakraborty, R. Tiwari, M. Y Wani, & J Mohan. 2014. Artificial insemination in poultry and possible transmission of infectious pathogens: A review. *Asian Journal of Animal and Veterinary Advances*, *9*(4), 211-228.

Dibner, J. J., & J. D. Richards. 2005. Antibiotic growth promoters in agriculture: history and mode of action. *Poultry science*, *84*(4), 634-643.

Donsbough, A. L., S. Powell, A. Waguespack, T. D. Bidner, and L. L. Southern. 2010. Uric acid, urea, and ammonia concentrations in serum and uric acid concentration in excreta as indicators of amino acid utilization in diets for broilers." *Poultry science 89*, no. 2 : 287-294.

Donoghue, A.M., D. Thistlethwaite, D.J Donoghue, and J.D Kirby. 1996. A new method for rapid determination of sperm concentration in turkey semen. *Poultry science, 75*(6), pp.785-789.

Donoghue, A.M., P.J. Blore, K. Cole, N.M Loskutoff, and D.J Donoghue. 2004. Detection of *Campylobacter* or *Salmonella* in turkey semen and the ability of poultry semen extenders to reduce their concentrations. *Poultry Science. 83*:1728-1733.

Edens, F. W. 2003. An alternative for antibiotic se in poultry: probiotics. *Revista Brasileira de Ciência Avícola*, *5*(2), 75-97.

Etches, R. 1996. Reproduction in poultry. CAB International. Wallingford.UK.

FAO/WHO. 2012. Guidelines on Probiotics: 10 years later. Accessed 2/20/2017. Retrieved from http://journals.lww.com/jcge/Citation/2012/10001/FAO_WHO_Guidelines_on_Probiotics__10_Years_Later.2.aspx

Fasenko, G. M., R. T. Hardin, and F. E. Robinson, 1992. Relationship of hen age and sequence position with fertility, hatchability, viability, and preincubation embryonic development in broiler breeders. *Poultry Science 71*: 1374-1383.

Floyd, M.H., and N.C. Tyler. 2011. Photostimulation of male broiler breeders to different photoperiods. *South African Journal of Animal Science 41*.2 (2011): 146-155.

Food and Drug Administration. 2015. Microorganisms & Microbial-Derived Ingredients Used in Food (Partial List). Access: February 2017. Retrieved from: https://www.fda.gov/food/ingredientspackaginglabeling/gras/microorganismsmicrobialderivedingredients/default.htm.

Foreign Agricultural Service/USDA. October 2016. Livestock and Poultry: World Markets and Trade. Accessed February 2017. Retrieved from http://apps.fas.usda.gov/psdonline/circulars/livestock_poultry.PDF.

Fritts, C. A., J. H., Kersey, M. A. Motl, E. C. Kroger, F Yan, J.Si and P. W Waldroup,. 2000. Bacillus subtilis C-3102 (Calsporin) improves live performance and microbiological status of broiler chickens. *The Journal of Applied Poultry Research*, *9*(2), 149-155.

Fukata T, K. Sasai, T. Miyamoto, E. Baba 1999. Inhibitory effects of competitive exclusion and fructooligosaccharide, singly, and in combination, on Salmonella colonization of chicks. *Journal of Food Protection, 62*: 229-33.

Gaggìa, F., P. Mattarelli, and B. Biavati, 2010. Probiotics and prebiotics in animal feeding for safe food production. *International journal of food microbiology*, *141*, S15-S28.

Gao, J., H.J. Zhang, S.H. Yu, S.G. Wu, I. Yoon, J. Quigley, Y.P Gao and G.H., Qi, 2008. Effects of yeast culture in broiler diets on performance and immunomodulatory functions. *Poultry Science*, *87*(7), pp.1377-1384.

Garcia, G. 2008. Caracterização microbiológica e avaliação de uma cepa de *Bacillus subtilis* no desempenho de bezerros da raça holandesa. *68f* (Doctoral dissertation, Tese (Doutorado em Microbiologia Agropecuária)-Faculdade de Ciências Agrárias e Veterinária–Universidade Estadual Paulista Julio de Mesquita, Jaboticabal).

Goffeau A, B.G. Barrell, H. Bussey, R.W. Davis, B. Dujun, H. Feldmann, et al. 1996 Life with 6000 genes. *Science.* 274 (5287), 546.

Grashorn, M. A. 2010. Use of phytobiotics in broiler nutrition–an alternative to infeed antibiotics. *Journal of Animal and Feed Sciences*, *19*(3), 338-347.

Griswold, M.D., 1998. The central role of Sertoli cells in spermatogenesis. *Seminars in Cell and Developmental Biology 9(4)*:411-6.

Guest, W.C., J.W Avault Jr, and J.D Roussel.1976. Preservation of channel catfish sperm. *Transactions of the American Fisheries Society*, 105(3), pp.469-474.

Guillot, J. F. 1998. Les probiotiques en alimentation animale. *Cahiers Agricultures*, *7*(1), 49-54.

Guthrie, H.D. and G.R Welch. 2012. Effects of reactive oxygen species on sperm function. *Theriogenology*, 78(8), pp.1700-1708.

Haines, M. D. 2012. *Bacteria and their effects on fertility in the chicken* (Master thesis, Mississippi State University).

Haines, M. D., H. M. Parker, C. D. McDaniel, and A. S. Kiess. 2013. Impact of 6 different intestinal bacteria on broiler breeder sperm motility in vitro. *Poultry science 92*, 8: 2174-2181.

Harwood C.R., S.M. Cutting 1990. Molecular Biological Methods for *Bacillus*. Wiley, Chichester, England, pp. 1-581.

Harwood, C.R. 1992. *Bacillus subtilis* and its relatives: molecular biological and industrial workhorses. *Trends Biotechnology 10:*247-256.

Hayat, J. 1992. *Reproductive performance of Wrolstad Medium White turkey hens fed a breeder diet containing a yeast culture* (Master thesis, Oregon University).

Hildebrandt, J.-P. 2001. Coping with excess salt: adaptive functions of extrarenal osmoregulatory organs in vertebrates. *Zoology 104*:209-220

Hocking, P. 2003. Maximizing productivity in broiler breeders. In *Proceedings Australian Poultry Science Symposium* (p. 15).

Howarth, B. 1983. Fertilizing ability of cock spermatozoa from the testis epididymis and vas deferens following intramagnal insemination. *Biology of reproduction*. Apr 1; 28 (3):586-90.

Hristov, A. N., G. Varga, T. Cassidy, M Long, K. Heyler, S. K. R Karnati and I.Yoon. 2010. Effect of *Saccharomyces cerevisiae* fermentation product on ruminal fermentation and nutrient utilization in dairy cows. *Journal of Dairy Science*, *93*(2), 682-692

Hu, Y., Y. Dun, S.Li, S. Zhao, N. Peng and Y. Liang. 2014. Effects of Bacillus subtilis KN-42 on growth performance, diarrhea and fecal bacterial flora of weaned piglets. *Asian-Australasian journal of animal sciences*, *27*(8), 1131-1140.

Huff, G. R., W. E. Huff, S. Jalukar, J. Oppy, N. C Rath and B. Packialakshmi. 2013. The effects of yeast feed supplementation on turkey performance and pathogen colonization in a transport stress/Escherichia coli challenge. *Poultry science*, *92*(3), 655-662.

Husvéth, F. 2011. Physiological and reproductional aspects of animal production. *Debrecen University, University of West Hungary, Pannon University. p3*.

Huyghebaert, G., R. Ducatelle, and F. Van Immerseel. 2011. An update on alternatives to antimicrobial growth promoters for broilers. *The Veterinary Journal*, *187*(2), 182-188.

Jamieson, B. G. M. 2007. Avian spermatozoa: structure and phylogeny. In Jamieson, BGM (Ed.), Reproductive Biology and Phylogeny of Birds. P- 349-398

Javadi, A., H. Mirzaei, S. Safarmashaei, S.Vahdatpour. 2012. Effects of probiotic (live and inactive Saccharomyces cerevisiae) on meat and intestinalmicrobial properties of Japanese quails. *African Journal of Biotechnology* 11, no. 57 (2012): 12083-12087.

Jones, D. G., and W. F. Lamoreux, 1942. Semen production of White Leghorn males from strains selected for high and low fecundity. *Poultry Science*. *21*: 173-184.

Jouany, J.P., 2006. Optimizing rumen functions in the close-up transition period and early lactation to drive dry matter intake and energy balance in cows. *Animal Reproduction. Science. 96*, 250–264.

Kabir, S. M. 2009. The role of probiotics in the poultry industry. *International Journal of Molecular Sciences*, *10*(8), 3531-3546.

Karaca, A. G., H. M. Parker, J. B. Yeatman, and C.D. McDaniel. 2002a. Role of seminal plasma in heat stress infertility of broiler breeder males. *Poultry science*, *81*(12), 1904-1909.

Karaca, A. G., H. M. Parker, J. B Yeatman, and C. D McDaniel.2002b. The effects of heat stress and sperm quality classification on broiler breeder male fertility and semen ion concentrations. *British poultry science*, *43*(4), 621-628.

Kidd, M. T., L. Araujo, C. Araujo, C. D. McDaniel and D. McIntyre, 2013. A study assessing hen and progeny performance through dam diet fortification with a Saccharomyces cerevisiae fermentation product. *The Journal of Applied Poultry Research*, *22*(4), 872-877.

Kiers, B. 1982. Summer heat loss of fertility in hatching eggs. *Poultry Digest* 41:352-355.

King A.S. and J. McLelland, 1984. Female reproductive system. In: King, A.S. and J. McLelland, editors. Birds: their structure and function. 2nd edition. Philadelphia: Bailliere Tindal. P. 145-165.

King A.S. and J. McLelland, 1984. Male reproductive system. In: King, A.S. and J. J. McLelland, editors. Birds: their structure and function. 2nd edition. Philadelphia: Bailliere Tindal. P. P.166-174.

King, J.A., and R.P. Millar. 1982. Structure of chicken hypothalamic luteinizing hormone- releasing hormone. II. Isolation and characterization. *Journal of Biological Chemistry 257*: 10729-10732.

King, L.M., and A.M. Donoghue. 2000. Adaptation of the sperm mobility test for the identification of turkey toms with low fertilizing potential. *Journal of Applied Poultry Research 9*:66-73.

Kurtzman, C.P., and J. W. Fell. (2006). Yeast systematics and phylogeny: Implications of molecular identification methods for studies in ecology. In C. A. Rosa and G. Peter, eds., *The Yeast Handbook.* Germany: Springer-Verlag Berlin Herdelberg.

La Ragione, R.M., M.J. Woodward, 2003. Competitive exclusion by Bacillus subtilis spores of Salmonella enterica serotype Enteritidis and Clostridium perfringens in young chickens. *Veterinary Microbiology. 94*, 245e256.

Lake P.E. 1971. The male in reproduction. In: Physiology and Biochemistry of the Domestic Fowl. DJ Bell, BM Freeman, editors. Academic Press, London, Vol 3, pp. 1411-1442.

Lake, P. E. 1957. The male reproductive tract of the fowl. *Journal of Anatomy*, *91*(Pt 1), 116.

Lake, P.E., and J.M. Stewart, 1978. Artificial insemination in Poultry. Bull. 213. ARC Poultry Res. Ctr. Edinburgh.

Lerner, S. P., N. French, D. McIntyre and C. Baxter-Jones. 1993. Age-related changes in egg production, fertility, embryonic mortality, and hatchability in commercial turkey flocks. *Poultry science*, *72*(6), 1025-1039.

Line, J. E., J. S Bailey, N. A. Cox, N. J. Stern and T. Tompkins. 1998. Effect of yeast-supplemented feed on Salmonella and Campylobacter populations in broilers. *Poultry science*, *77*(3), 405-410.

Madriqal, S. A., S. E. Watkins, M. H. Adams, A. L. Waldroup, and P. W. Waldroup. 1993. Effect of an active yeast culture on performance of broilers. *Poultry Science 7*2(Suppl.1):87. (Abstr.)

Marden, J.P., C. Julien, V. Monteils, E. Auclair, R. Moncoulon, C. Bayourthe. 2008. How does live yeast differ from sodium bicarbonate to stabilize ruminal pH in high yielding dairy cows. *Journal Dairy Science 91*, 3528–3535.

Marks, H.L., 1981. Selection for egg mass in the domestic fowl. 2. Packed sperm volume; fertility and hatchability responses. *Poultry Science, 60*(8), pp.1782-1787.

Martin, S. A., D. J. Nisbet, and R. G Dean. 1989. Influence of a commerical yeast supplement on the in vitro ruminal fermentation. *Nutrition reports international (USA)* Reprod. Int. 40, 395–403.

Martinez, S.W. 1999. The role of changing vertical coordination in the broiler industry. ERS-USDA.

Maruta, K., H. Miyazaki, S. Masuda, M. Takahashi, T. Marubashi, Y. Tadano, and H.Takahashi. 1996. Exclusion of intestinal pathogens by continuous feeding with Bacillus subtilis C-3102 and its influence on the intestinal microflora in broilers. *Animal Science and Technology (Japan)*.

McCann, M. E. E., E. Newell, C. Preston, and K.Forbes. 2006. The use of mannan-oligosaccharides and/or tannin in broiler diets. *International Journal of Poultry Science*, *5*(9), 873-879.

McCracken, R. J., J. A. Van Rhijn, and D. G Kennedy. 2005. Transfer of nitrofuran residues from parent broiler breeder chickens to broiler progeny. *British poultry science*, *46*(3), 287-292.

McDaniel, C.D., R.K. Bramwell, J.L. Wilson, and B.Howarth. 1995. Fertility of male and female broiler breeders following exposure to elevated ambient temperatures. *Poultry Science, 74*(6), pp.1029-1038.

McDaniel, C. D., J. L. Hannah, H. M. Parker, T. W. Smith, C. D. Schultz, and C. D. Zumwalt. 1998. Use of a sperm analyzer for evaluating broiler breeder males. 1. Effects of altering sperm quality and quantity on the sperm motility index. *Poultry science*, *77*(6), 888-893.

McDaniel, C. D., R. K. Bramwell, J. L. Wilson, B. Howarth, Jr., 1995. Fertility of male and female broiler breeders following exposure to elevated ambient temperatures. *Poultry Science*. 74:1029-1038.

McDaniel, G.R. 1983. Factors affecting broiler breeder performance. 5. Effect of preproduction feeding regimens on reproductive performance. *Poultry. Science*. 62: 1949-1953.

Moore, B.E., K. E. Newman, P. Spring, F.E .Chandler. 1994. The effect of yeast culture (Yea Sacc 1026) in microbial populations digestion in the cecum and colon of the equine. *Journal of Animal Science. 72*, 1.

Moretti, E., S. Capitani, N.Figura, A .Pammolli, M. G. Federico, V .Giannerini, and G. Collodel. 2009. The presence of bacteria species in semen and sperm quality. *Journal of assisted reproduction and genetics*, *26*(1), 47.

Mor-Mur, M., & J. Yuste. 2010. Emerging bacterial pathogens in meat and poultry: an overview. *Food and Bioprocess Technology*, *3*(1), 24.

Mountney, G. J. and W.A Gould. 1988. *Practical Food Microbiology and Technology*. AVI Books, Van Nostrand Reinhold Company, New York, USA.

Moyad, M. A. 2007. Brewer's/baker's yeast (*Saccharomyces cerevisiae*) and preventive medicine: part I. *Urologic nursing*, *27*(6), 560.

Myers, R. M., and J. O. Almquist. 1951. A comparison of the effects of aureomycin, penicillin and streptomycin upon spermatozoan livability and control of bacteria in bovine semen. *Journal of animal science* 10, 322-330.

Nixon, B., K.A. Ewen, K.M. Krivanek, J. Clulow, G. Kidd, H. Ecroyd, and R.C. Jones. 2014. Post-testicular sperm maturation and identification of an epididymal protein in the Japanese quail (Coturnix coturnix japonica). *Reproduction*, *147*(3), pp.265-277.

Nestor, K. E. 1977. The influence of a genetic change in egg production, body weight, fertility or response to cold stress on semen yield in the turkey. *Poultry science*, *56*(2), 421-425.

Novak, M., & V. Vetvicka. 2008. β-glucans, history, and the present: immunomodulatory aspects and mechanisms of action. *Journal of immunotoxicology*, *5*(1), 47-57.

Nunes, R. V., C. Scherer, P. C. Pozza, C. Eyng, L. D. G. Bruno and F. M. Vieites, 2012. Use of probiotics to replace antibiotics for broilers. *Revista Brasileira de Zootecnia*, *41*(10), 2219-2224.

Okamura F. and Nishiyama, .1978. The passage of Spermatozoa through the vitelline membrane in the domestic fowl, Gallus. *Cell and Tissue Research, 188*(3):497-508

Olsen, M. W. 1942. Maturation, fertilization, and early cleavage in the hen's egg. *Journal of Morphology,* 70:523-533.

Olubowale, O. S., J.P.C. Greyling, F.H. deWitt, A. Hugo, and M.B. Raito. 2014. Effects of dietary lipid sources on the semen quality of Hy-line Silver-Brown cockerels. *JAPS: Journal of Animal & Plant Sciences*, *24*(4).

Ommati, M. M., M. J. Zamiri, A. Akhlaghi, H. Atashi, M. R. Jafarzadeh, R. Rezvani, and F. Saemi. 2013. Seminal characteristics, sperm fatty acids, and blood biochemical attributes in breeder roosters orally administered with sage (*Salvia officinalis*) extract. *Animal Production Science 53*:548–554.

Papatsiros, V. G., P. D. Katsoulos, K. C. Koutoulis, M. Karatzia, A. Dedousi, and G. Christodoulopoulos. 2013. Alternatives to antibiotics for farm animals. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, *8*, 1-15.

Parker, H. M., & C. D. McDaniel. 2002. Selection of young broiler breeders for semen quality improves hatchability in an industry field trial. *The Journal of Applied Poultry Research*, *11*(3), 250-259.

Parker, H. M., A. G. Karaca, J. B. Yeatman, L. R. Frank, and C. D. McDaniel. 2002. Fertility of broiler breeders following categorization by the Optibreed sperm quality index when hens are inseminated with a constant number of sperm. *Poultry Science* 81:239–245.

Parker, H. M., & C. D. McDaniel. 2006. The immediate impact of semen diluent and rate of dilution on the sperm quality index, ATP utilization, gas exchange, and ionic balance of broiler breeder sperm. *Poultry science*, *85*(1), 106-116.

Parker, H. M., J. B. Yeatman, C. D. Schultz, C. D. Zumwalt, and C. D. McDaniel. 2000. Use of a sperm analyzer for evaluating broiler breeder males. 2. Selection of young broiler breeder roosters for the sperm quality index increases fertile egg production. *Poultry science*, *79*(5), 771-777.

Patterson, J. A., and K. M. Burkholder. 2003. Application of prebiotics and probiotics in poultry production. *Poultry science*, *82*(4), 627-63.

Pedroso AA, V.M.B. Moraes, J.Ariki. 1999. Effects of protein and probiótico (Bacillus subtilis) levels in pullet and laying hen diets. *Brazilian Poultry Science*, 1(1):49-54.

Pollock, D. L. 1999. A geneticist's perspective from within a broiler primary breeder company. *Poultry science*, *78*(3), 414-418.

Poultry trends. Egg production. 2016. Accessed February 2017. Retrieved from http://www.poultrytrends.com/2016/#/38

Priest, F.G. 1989. Isolation and identification of aerobic endospore-forming bacteria. p. 27-56. *In* C.R. Harwood (ed.), *Biotechnology Handbooks 2: Bacillus*. Plenum Publishing Corp., New York.

Qiao, G. H., A. S. Shan, N. Ma, Q. Q. Ma, and Z. W Sun. 2010. Effect of supplemental Bacillus cultures on rumen fermentation and milk yield in Chinese Holstein cows. *Journal of animal physiology and animal nutrition*, *94*(4), 429-436.

Rima, H. and Ismail, F. (2012). Antimicrobial and probiotic properties of yeasts: from fundamental to novel applications. *Frontiers in microbiology*, *3*, 421.

Robinson, P.H., 2010. Yeast Products for Growing and Lactating Ruminants: A Literature Summary of Impacts on Rumen Fermentation and Performance. Cooperative Extension University of California, Davis.

Roll, V. F. B., L. L Lopes, F. M. Gonçalves, M. Anciuti, F. L. Leite, E. K Corrêa, and E.G. Xavier. 2008. Condição microbiológica de cama tratada com Impact P® em matrizes de frangos de corte. *Ciência Rural*, *38*(9), 2650-2653.

Roto, S. M., P. M. Rubinelli and S. C Ricke. 2015. An introduction to the avian gut microbiota and the effects of yeast-based prebiotic-type compounds as potential feed additives. *Frontiers in Veterinary Science*, *2*, 28.

Rurangwa, E., D. E. Kime, F. Ollevier, and J.P. Nash.2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. *Aquaculture*, 234(1), pp.1-28.

Saadia, M., Hassanein, N.K.S., 2010. Effect of probiotic (Saccharomyces cerevisiae) adding to diets on intestinal microflora and performance of hy-line layer hens. J. Am. Sci. 6, 159–169.

Sexton, T.J., 1977. A new Poultry semen extender 1. Effect of extension on the fertility of chicken semen. *Poultry Science 56*:1443-1446.

Shannon, J. A. 1938. The excretion of uric acid by the chicken. Journal of Cellular and Comparative Physiology, 11(1), 135-148

Sharp, Peter J. "Photoperiodic regulation of seasonal breeding in birds." *Annals of the New York Academy of Sciences* 1040.1 (2005): 189-199.

Shen, Y.B., X.S. Piao, S.W. Kim, L. Wang., P. Liu, I. Yoon, Y.G. Zhen. 2009. Effects of yeast culture supplementation on growth performance, intestinal health, and immune response of nursery pigs. *Journal of Animal Science 87*, 2614–2624.

Sonnenschein AL, R. Losick, J.A Hoch .1993. *Bacillus subtilis* and Others Gram-Positive Bacteria: Biochemistry, Physiology and Molecular Genetics. American Society for Microbiology, Washington, DC.

Stein T .2005. *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. *Molecular Microbiology 56*: 845-857.

Steinberger, E., B. Sanborn, and A. Steinberger. 1979. Sertoli cell function. In n: Talwar GP ed. Recent advances in reproduction and regulation of fertility. New York, Elsevier/North Holland Biomedical Press, 219-31.

Stratman F.W, H.L Self. 1960. Effect of semen volume and number of sperm on fertility and embryo survival in artificially inseminated gilts. *Journal of Animal Science*, 19, 1081-1088.

Tabatabaei, S., M. Chaji, and T. Mohammadabadi. 2010. Correlation between age of rooster and semen quality in Iranian indigenous broiler breeder Chickens. *Journal of Animal and Veterinary Advances*, *9*(1), 195-198.

Thayer, R.H., R.F. Burkitt, R.D. Morrison, E.E. Murray. 1978. Efficiency of utilization of dietary phosphorus by caged turkey breeder hens fed ration supplemented with live yeast culture. In: Animal Science Research Report, Oklahoma. Agricultural Experiment Station, Oklahoma State University,Stillwater, OK, pp. 173–181.

Utiyama, C. E., L. L . Oetting, P. A. Giani, U. D. S. Ruiz and V. S. Miyada. 2006. Efeitos de antimicrobianos, prebióticos, probióticos e extratos vegetais sobre a microbiota intestinal, a freqüência de diarréia e o desempenho de leitões recém-desmamados. *Revista Brasileira de Zootecnia*, *35*(6), 2359-2367.

Vizzier-Thaxton, Y., N.A. Cox, L.J. Richardson, R.J. Buhr, C.D. McDaniel, D.E. Cosby, J.L. Wilson, D.V. Bourassa, and M.B. Ard. 2006. Apparent Attachment of *Campylobacter* and *Salmonella* to Broiler Breeder Rooster Spermatozoa. *Poultry Science. 85*:619-624.

Vohra, A., P. Syal, and A. Madan, 2016. Probiotic yeasts in livestock sector. *Animal Feed Science and Technology*, *219*, 31-47.

Waldroup, P.W., K. Hazen, W.D. Bussell, and Z.B. Johnson. 1976. Studies on the daily protein and amino acid needs of broiler breeder hens. *Poultry Science 55*: 2342-2347.

Walker, K., H. Skelton and K. Smith. 2002. Cutaneous lesions showing giant yeast forms of Blastomyces dermatitidis. *Journal of cutaneous pathology, 29*(10), 616-618.

Wilcox, F.H., and M.S. Shorb, 1958. The effect of antibiotics on bacteria in semen and on the motility and fertilizing ability of chicken spermatozoa. A. *Journal of Veterinary Research*. 19:945-949.

Wilson, H.R., N.P. Piesco, E.R. Miller, and W.G. Nesbeth. 1979. Prediction of fertility potential of broiler breeder males. *World's Poultry Science 35*: 95-118.

Witschi E., 1961. Sex and secondary sexual characters. In: Marshal AJ, editor. Biology and comparative physiology, vol. II. New York: Academic Press. p. 115–68.

Xu, Liping. 2014. Yeast Culture and Its Application in Feed. *Agricultural Science & Technology* 15.12: 2183.

Zhang, X.Z., K. S. Yao, and C.L. Xiong. 2011. A combined method for weighing human semen volume. *National journal of andrology*, *17*(10), 902-904.

CHAPTER III

THE IMPACT OF DIETARY YEAST FERMENTATION PRODUCT DERIVED FROM *SACCHAROMYCES CEREVISIAE* ON SEMEN QUALITY AND SEMEN MICROBIOTA OF AGED WHITE LEGHORN ROOSTERS

Abstract

Dietary supplementation of yeast fermentation products (YP) derived from *Saccharomyces cerevisiae* has been examined in broilers and laying hens. However, limited information is available about the impact of YP on rooster reproductive performance. Thus, the objective of this study was to determine the effects of feeding different levels of YP on rooster semen quality and semen microbiota (yeast and bacteria). A common basal diet was formulated to meet or exceed NRC recommendations. A commercially available YP was included at either 0, 0.5 (manufacturer recommendation), or 1.0% of the diet. Sand was included in these diets at either 1, 0.5, or 0%, respectively, to keep nutrients provided by the basal diet consistent. Individually caged White Leghorn roosters (n = 63), 60 wk of age, were divided equally among the 3 diets. Feed intake and individual semen samples were obtained weekly (8 wk). Semen samples were analyzed for the sperm quality index (SQI), semen volume, sperm concentration, and sperm viability. Biweekly, body weight and body weight gain were determined, and semen samples were serially diluted and spread plated to detect yeast as well as total aerobic bacteria. Regression analyses were performed to evaluate

the impacts of different dietary levels of YP on semen characteristics, semen microbiota and rooster growth performance, whereas correlation analyses were conducted to evaluate the relationship between semen quality variables and semen microbiota. Effects were considered significant at $P \leq 0.10$. Dietary YP did not impact feed intake (P=0.486), body weight (P= 0.419), or body weight gain (P=0.684). However, as the dietary levels of YP increased, there was a linear decrease in the SQI (P = 0.068, R^2= 0.054) but a linear increase in bacteria per billion sperm (P =0.10, R^2=0.043) and yeast per billion sperm (P = 0.081, R^2= 0.049). Additionally, yeast per billion sperm was positively correlated with bacteria per billion sperm (P<0.0001, r =0.5003). The decrease in SQI may be a result of the increase in bacteria per billion sperm and yeast per billion sperm with dietary supplementation of YP, because the SQI was negatively correlated with bacteria per billion sperm (P<0.0001, r = -0.577) and yeast per billion sperm (P= 0.012, r = -0.404). Additionally, the SQI is a measurement of overall sperm movement, and because total sperm concentration (P=0.946) and sperm viability (P=0.115) were unaffected by dietary treatments, YP may reduce the SQI by reducing sperm motility. Also, total sperm concentration (P<0.0001, r = -0.684; P=0.042, r = -0.258) and live sperm concentration (P<0.0001, r= -0.688; P=0.0165, r = -0.303) were negatively correlated with bacteria and yeast per billion sperm, respectively, whereas positive correlations were found between percentage of dead sperm with bacteria (P= 0.004, r= 0.362) and yeast per billion sperm (P<0.0001, r= 0.521). Possibly the increased number of bacteria and yeast per sperm, as a result of YP supplementation, is detrimental to semen quality due to the bacteria and yeast directly or due to their toxins and products.

Introduction

The use of antibiotics has been widely practiced for decades in poultry production. Besides therapeutic use aimed at improving animal health, antibiotics are also supplemented for prophylactic purposes and as antimicrobial growth promoters (AGP) to improve feed efficiency and growth rate and increase or maintain high broiler performance (Edens, 2003; Huyghebaert et al, 2011). However, global concern about the emergence of antimicrobial resistant bacteria has led to the prohibition or reduction of the use of antibiotics in livestock and poultry (Edens, 2003; Kabir, 2009).

In 2006, the European Union banned antibiotics used for growth promotion purposes in poultry and livestock due to the risk to human health. In other countries, such as the United States, there has been an increasing consumer demand for antibiotic-free products (Huyghebaert et al, 2011). However, antibiotic removal has increased the incidence of diseases and disorders, thus decreasing broiler performance. Therefore, AGP alternatives have been used in the poultry industry to provide antibiotic-free chicken to consumers, to decrease the risk associated with antimicrobial resistance and to maintain high growth performance (Huyghebaert et al, 2011). Prebiotics and probiotics are examples of alternatives to AGP that have been widely used in poultry and livestock due to their established benefits to animal health. Prebiotics are non-digestible dietary ingredients that selectively promote the growth of one or more beneficial bacteria (Papatsiros et al., 2013; Huyghebaert et al., 2010). Probiotics are live and non- harmful-microorganisms that when adequately included in the diet, improve host health (Guillot, 1998)

Additionally, the dietary supplementation of yeast and YP, especially those derived from *Saccharomyces cerevisiae*, have been shown to improve the nutritive quality of feed and feed utilization, leading to improved animal performance (Yalcin et al., 2015). Active dried yeast, yeast cell wall, yeast culture and yeast extract are examples of different forms of yeasts commonly included in animal diets. Inactive yeasts are regarded as a prebiotic, whereas live yeasts are classified as probiotics (Yalcin et al., 2015). In fact, the fermentation products derived from different strains of *Saccharomyces cerevisiae* have characteristics of both prebiotics and probiotics. As such, these products contain yeast cell wall components (eg. mannooligosaccharides and betaglucans), yeast metabolites, the media used for growth and maintenance of yeast fermentation activity, and yeast cell wall components as well as live yeast cells (Shen et al., 2011). Other names, such as yeast culture (Kidd et al., 2013) and prebiotic-like substances (Roto et al., 2015) have been used to describe YP included in the animal diet. However, in order to maintain consistency, the term YP will be used in this research.

The dietary inclusion of yeast and YP in poultry diets has yielded improvements in body weight gain, feed efficiency and egg production (Roto et al., 2016; Yacin et al., 2015). However, the results are still controversial, mainly due to different types and levels of yeast or YP inclusion in the diet, flock heath, strain, age and variability in experimental conditions (Madriqal et al., 1993). For example, Hassanein and Soliman (2010) reported an increase in egg mass and egg production in layer hens fed 0.4% or 0.8% live yeast as compared to the non-supplemented group. However, in a study conducted to evaluate the effects of YP on broiler breeder performance, the supplemented birds exhibited similar egg production, egg weight, mortality, hatchability of fertile eggs,

percentage of shell, and shell weight when compared to the control group. (Brake et al., 1991). In a study conducted to evaluate the effects of YP on hen and progeny performance from hatches at 32 and 39 wk of age, a reduction in egg contamination was reported in the 32 wk hatch (Kidd et al., 2013). This may be due to the ability of yeast to modulate intestinal microbiota in poultry by decreasing the population of harmful bacteria. In fact, the inclusion of yeast and YP in the diet prevent several harmful bacteria from binding to the intestinal epithelia, as these microorganisms have a specific binding site for mannose present in the yeast cell wall (Roto et al., 2015).

Because yeast and YP modulate intestinal microbiota, it is possible they can also alter bacteria present in the male and female reproductive tract of poultry, which could affect overall fertility. In fact, both yeast and bacteria alter semen quality in rats (Ahmed et al., 2012) and humans (Sikka et al., 2004; Purvis et al., 1993) and hence alter male fertility. In diabetic rats, the addition of yeast in the diet decreased sperm abnormalities as compared to untreated diabetic rats. This improvement in semen quality was attributed to the antioxidant activity of yeast, achieved by reducing the reactive oxygen species and other aqueous peroxyl radicals detrimental to sperm function and quality (Ahmed et al., 2012). On the other hand, the presence of bacteria in human semen has been associated with poor semen quality, due to negative effects of these microorganisms in the ejaculate, including decreased motility, agglutination and production of toxins detrimental to sperm function movement (Sikka et al., 2004).

The bacteria present in human semen generally originates from the urinary tract or is sexually transmitted (Purvis et al., 1993). However, in avian species, the cloaca is a common opening for the digestive, reproductive and urinary tracts. Therefore, it is

possible that the microorganisms present in this region can contaminate semen (Smith, 1949). Moreover, it has been established that yeast and YP do not permanently colonize the gastrointestinal tract. Therefore, any yeast and microorganisms attached to yeast (especially pathogenic bacteria) are excreted through the cloaca (Vohra et al., 2016), which could potentially contaminate semen. In fact, several different species of bacteria, such as *Salmonella* spp., *Campylobacter* spp., *Staphylococcus* spp., *coliforms, Streptococci* spp., and *Bacillus* spp. have been found in poultry semen (Gale and Brown, 1961).

Additionally, some species of bacteria have a negative effect on poultry semen quality. For example, Haines (2012) found that *in vitro*, when semen was directly exposed to *Salmonella, E. coli, Campylobacter,* and *Clostridium,* classified as pathogenic bacteria, sperm motility decreased. Also, the direct exposure of semen to *Lactobacillus* and *Bifidobacterium,* classified as non-pathogenic bacteria, eliminated sperm motility. Although the effect of yeast and YP on modulating intestinal microbiota and improving growth performance has been well studied, the impact of these feed additives on rooster semen microbiota and reproductive performance are not well understood. Hence, the objective of this study was to determine if dietary supplementation of YP impacts semen quality and semen microbiota. Further, the relationships between semen quality parameters and semen microbiota were evaluated.

Materials and methods

Housing and care

In this study, 63 individually caged White Leghorn roosters, 60 wk old, were used. Feed and water were provided *ad libitum*, and the birds received 16 h of light per

day. Prior to the study, all the roosters were fed the same basal diet. The birds were caged in raised wire cages and treated according to the Guide for Care and Use of Laboratory animals in Agricultural Research and Teaching (1996).

Treatments and preparation of experimental diets

The YP used in this study was a fermentation product derived from *Saccharomyces cerevisiae* (XP, Diamond V, Cedar Rapids, IA). To determine the concentration of live yeast cell, the YP was diluted 10-fold with phosphate buffered saline and spread plated in duplicate on sabouraud dextrose agar (SDA, Catalogue no. 210950, Beckton Dickinson, Sparks, MD) plates for each dilution. Plates were incubated at 30°C for 48 h. The determination of live yeast cells was replicated 3 times. The concentration of live yeast was determined to be 10^6 CFU/ g of YP, indicating that in addition to the cell components present in the product, live yeast cells should also be considered when determining the impact of this dietary product on parameters evaluated in this trial.

Prior to the study, semen samples were collected from all the birds to remove roosters that did not produce semen or yielded clear/ transparent samples, commonly associated with low semen quality. The roosters were equally divided into three groups according to dietary treatments, which all contained the same basal diet but different levels of inclusion of YP (Table 3.1). The diets were formulated to meet or exceed NRC recommendations (NRC, 1994). During the 8 wk experimental period, the following 3 dietary treatments were provided: 0% YP or control - conventional rooster basal diet (corn, soybean meal based diet) without any inclusion of YP; 0.5 % YP inclusion in the basal diet (as per manufacturer's recommendation); and 1% YP inclusion in the basal

diet. In order to keep the nutrients provided by the basal diet consistent, an inert filler (sand) was included at either 1, 0.5, or 0% for the control, 0.5 and 1% treatments, respectively. Ingredients included at less than 0.5 % were considered a premix and were mixed in a small mixer (capacity of 11 kg) for approximately 5 min, separated from the remaining ingredients. The basal diet was mixed in a vertical screw mixer (capacity of 0.907 tonne) for 20 min, 10 min before and 10 min after the inclusion of fat. Next, the basal diet was divided into 3 equal parts, and the appropriate concentration of YP or sand was added to each respective experimental diet and mixed for 5 min in a horizontal mixer (capacity of 225 kg). Each dietary treatment was kept in a closed barrel and fed within 1-3 wk of mixing.

Semen collection and analysis

Each week, for 8 wk, semen samples were individually collected from all the roosters by the abdominal massage method (Burrows and Quinn, 1937). Samples were collected in graduated microcentrifuge tubes and were analyzed immediately after collection to prevent deterioration of semen. Each sample was individually analyzed for semen volume, sperm viability, sperm quality index (SQI) and sperm concentration. Semen volume was obtained by using a graduated microcentrifuge tube (Zhang et al., 2011; Thermo scientific QSP, San Diego, CA). Sperm viability was determined by the fluorometric method of Bilgili and Renden (1984) using a fluorometer (2001 A Fluorotec, St. Johns Associates, Beltsville, MD). To determine SQI, semen was diluted 10-fold in 0.85% saline (McDaniel et al., 1998) and then immediately analyzed in a Sperm Quality Analyzer (Medical Electronic Systems, Rochester, MI). The sperm

concentration was measured by the photometric method of King and Donoghue (2000) using a microreader (IMV International, Maple Grove, MN).

Microbial analyses

Every 2 wk immediately following semen analysis, the semen samples were kept on ice for a maximum of 2 h until microbial analyses were performed. The samples were serially diluted and plated on tryptic soy agar plates (TSA, Catalog no. 236950, Beckton Dickinson, Sparks, MD) and SDA to determine total aerobic bacteria and yeast concentrations, respectively. From each bird, semen samples were serially diluted in 10-fold increments in phosphate buffered saline. Two plates were utilized for each serial dilution. The plates were incubated at 37°C for 24 h and at 30°C for 48 h for TSA and SDA, respectively.

Plates with more than 30 and less than 300 CFU (Breed and Dotterrer, 1916), were counted for each dilution and averaged for each rooster to estimate the concentration of total aerobic bacteria and yeast cells per ejaculate. Microbiological data (log CFU of bacteria and log CFU of yeast) were expressed both in per mL of semen and per billion sperm in the ejaculate basis.

Live performance

Feed intake was measured weekly for each rooster. Because all the birds used in this trial were over 60 wk and no longer in their growth stage, a rapid body weight change was not expected. Therefore, body weight and body weight gain were individually obtained only three times throughout the experiment period at 60, 64 and 68 wk of age.

Statistical analysis

Data were analyzed as a split plot design, in which the treatments were represented in the whole plots split over time (8 wk), with 21 roosters per treatment (n=21). Data were analyzed by the GLM statistical procedure of SAS; however, no significant differences due to treatments and no treatment by time interactions were detected ($P > 0.10$). Therefore, regression analyses were performed to evaluate the relationships between the level of inclusion of YP and the semen parameters, and correlation analyses were used to study the relationships between semen quality variables and semen microbiota (Steel and Torrie, 1980).

Results and Discussion

In the current study, dietary supplementation of YP lead to a linear decrease in the SQI ($P= 0.068$, $R^{2=} 0.054$), which is indicative of overall semen quality and is affected by sperm viability, concentration and motility (McDaniel et al.; 1998). However, because sperm viability ($P= 0.115$) and sperm concentration ($P= 0.946$; Table 3.2) were not significantly affected by the addition of YP, the effect of YP on the SQI was likely due to a reduction in sperm motility. Sperm motility is essential to ensure fertilization, therefore inclusion of YP in the rooster's diet could negatively impact fertility as well. The other semen variables evaluated, including live sperm concentration ($P= 0.794$), semen volume ($P= 0.909$), sperm per ejaculate ($P= 0.782$) and live sperm per ejaculate ($P= 0.924$; Table 3.2), were not altered by dietary supplementation of YP. Conversely, Ahmed et al. (2012) stated that when diabetic rats were fed yeast, semen quality improved due to a reduction in genetic alterations and sperm abnormalities as compared to untreated diabetic rats. However, mammals and birds exhibit remarkable differences in their reproductive

systems, so it is possible that the yeast benefits reported in rats would not apply to avian species. Yet, Abaza et al. (2006) reported that dietary supplementation with *Saccharomyces cerevisiae* to layer breeders improved semen quality by increasing semen volume, sperm concentration and motility and by reducing dead and abnormal sperm as opposed to the untreated group. Nevertheless, the opposing results reported in their study compared to the current study could be partially attributed to the differences in both experiments in terms of breed, age, frequency of semen collection and product. For example, in the study conducted by Abaza et al. (2006), semen samples were collected only once from 43 wk old local Egyptian breed males, fed Dinaferm (*Saccharomyces cerevisiae*). However, in the current study, ejaculates were collected weekly for 8 wk from 60-68 wk old White Leghorn roosters supplemented with a commercial YP, that contained live yeast cells and its fermentation products derived from *Saccharomyces cerevisiae*. Additionally, in this past research the roosters were fed only 0.1% of Dinaferm (*Saccharomyces cerevisiae*), whereas in this current study higher levels of 0.5 and 1% of YP were included in the diet.

The product used in the current research is a yeast fermentation product, which has been reported to improve growth performance in livestock and poultry, especially by stimulating the immune system and decreasing the population of pathogenic bacteria in the gut (Price et al., 2003; Gao et al., 2008; Feye et al., 2016.; Rubinelli et al., 2016). However, because the highest level of YP used in the current study is twice the dose recommended by the manufacturer, it is possible that the detrimental effect of YP on sperm motility was due to the excess inclusion of this product in the rooster's diet that might have modulated semen microbiota and altered semen quality.

Interestingly, bacteria (P= 0.59, Table 3.3) and yeast (P= 0.472, Table 3.4) per mL of semen were not affected by treatments. However, there was a linear increase in the amount of bacteria per billion sperm (P=0.10, R^2= 0.043, Table 3.3) and yeast cells per billion sperm (P= 0.081, R^2= 0.049, Table 3.4) as the level of YP in the diet increased. Additionally, there was a positive correlation between bacteria per billion sperm and yeast per billion sperm (P<0.0001; r= 0.5003; Table 3.5). These data indicate that the bacteria present in the gut may have attached to the yeast and then excreted from the gastrointestinal tract, through the cloaca. In fact, previous research has reported the attachment of pathogenic bacteria to yeast and YP. For example, Line et al. (1998) demonstrated that harmful bacteria contain a specific binding site for mannose, which is present in the yeast cell wall. This structure allows the bacteria to attach to the yeast, inhibiting bacterial colonization in the gut due to excretion of both yeast and yeast bound-pathogens, as yeast do not colonize the gastrointestinal tract. Since fluids from the avian digestive, reproductive and urinary tracts are all released through the cloaca, the bacteria present in this region could contaminate rooster semen (Smith, 1949). In fact, semen microflora are of similar composition to the microorganisms found in the cloaca, whereas the vas deferens contain sterile semen (Smith, 1949). In broiler breeder hens, Kidd and cohort (2013) reported that the supplementation of YP significantly decreased egg contamination at 32 wk of age. This improvement may have been due to a reduction in bacteria in the female reproductive tract or cloaca, in response to the addition of YP. However, due to inclusions YP in the current study being 2 to 4 times higher than that of Kidd et al. (2013), it is possible that the high inclusion of YP led to greater cloacal excretion of yeast and bacteria attached to yeast. Therefore, in the current study even

though the semen samples were collected by abdominal massage and all the possible sources of contaminations were avoided, it is likely that bacteria and other microorganisms present in the cloaca contaminated the semen samples. In fact, Ahmed et al. (2015) described the presence of several bacteria species in semen samples from roosters, including *E. coli, Kluyvera ascorbata, Salmonella enteritidis, Pseudomonas, Serratia plymuthica,* and *Klebsiella.* Because in this current study only the bacteria present in the ejaculate were evaluated, it is not possible to know for certain if the increase in bacteria with supplementation of YP was also found in feces or the cloaca, due to excretion from the gastrointestinal tract.

Additionally, in the current study, the SQI was negatively correlated with bacteria per billion sperm ($P < 0.0001$, $r = -0.577$), as well as with yeast per billion sperm ($P = 0.0012$, $r = -0.404$). Moreover, yeast per mL of semen ($P = 0.097$, $r = 0.2112$), bacteria per billion sperm ($P = 0.0038$, $r = 0.362$), and yeast per billion sperm ($P < 0.0001$ $r = 0.521$) were positively correlated with percentage of dead sperm. However, negative correlations were found for total sperm concentration with bacteria per billion sperm ($P < 0.0001$, $r = -0.684$) and with yeast per billion sperm ($P = 0.042$, $r = -0.258$). Similarly, live sperm concentration was negatively correlated with bacteria ($P < 0.0001$, $r = -0.688$) and yeast per billion sperm ($P = 0.0165$, $r = -0.303$, Table 3.6). Semen volume was negatively correlated with bacteria per mL of semen ($P = 0.019$, $r = -0.296$) and bacteria per billion sperm ($P = 0.0146$, $r = -0.309$, Table 3.6), possibly due to the contamination of a small volume of semen with a high concentration of bacteria already present in the cloaca during ejaculation. Also, total sperm per ejaculate ($P < 0.0001$, $r = -0.594$) and live sperm per ejaculate ($P < 0.0001$, $r = -0.608$) were negatively correlated with bacteria per billion

sperm. Lastly, total sperm per ejaculate (P=0.064, r= -0.236) and live sperm per ejaculate (P=0.0354, r= -0.2677, Table 3.6) were also negatively correlated with yeast per billion sperm. Collectively, these results indicate that higher concentrations of yeast and bacteria in the ejaculate, due to YP supplementation, have a detrimental effect on semen quality. In agreement with these data, Haines et al. (2013) reported that under *in vitro* conditions, the direct exposure of rooster semen to pathogenic bacteria (*E. coli*, *Campylobacter*, *Clostridium* and *Salmonella*) reduced sperm motility. However, the greatest reduction in sperm movement occurred when rooster semen was exposed to the non-pathogenic bacteria (*Lactobacillus* and *Bifidobacterium*), commonly used as AGP alternatives supplements. In addition, hens inseminated with semen samples treated with a high concentration of *Lactobacillus*, produced only infertile eggs, probably due to the inefficiency of immotile sperm in passing through the vagina and penetrating the egg.

The negative correlation between semen quality and bacteria in the ejaculate has also been described in other species. For instance, in humans, *E. coli* is the most common microorganism present in patients with contaminated semen or urogenital tract infection. This bacterium in turn has a negative impact on sperm quality, in part by decreasing sperm motility (Diemer et al., 2003). According to Auroux et al. (1991), *E. coli* is associated with reducing sperm motility, followed by clustering of sperm and infertility. Moreover, Mehta et al. (2002) reported that 50% of semen samples from infertile male patients contained aerobic cocci. In order to study the effects of different bacteria on human semen quality, Moretti et al. (2009) evaluated different bacteria present in semen of infertile and fertile patients. Of the seven bacteria examined, five were associated with decreasing sperm motility, including *E. coli*, which is frequently isolated from birds and

commercial poultry houses (Baurhoo et al., 2007; Manafi et al., 2016). In fact, the presence of *E. coli* in boar semen has also been associated with a negative impact on sperm motility (Yaniz et al., 2010). The same effect was observed with *Campylobacter fetus* subsp. *fetus* in ram semen due to the attachment of the bacterium to the tail and acrosome of sperm, resulting in separation of the sperm head from the tail (Bar et al., 2008). However, the exact mechanisms by which various bacteria species negatively impact semen quality and specifically sperm motility, are still unclear. Previous research has demonstrated that the harmfulness of bacteria in semen depends on the species of microorganisms present in the ejaculate. Therefore, it is possible that different bacterial species use distinct mechanisms of action, ultimately affecting or having no effect on semen quality. For example, in an *in vitro* study, Qiang et al. (2007) stated that enterococci had a detrimental impact on the membrane integrity of the human sperm head, neck and mid piece. When human semen was incubated with *E. fecalis*, *E. coli* and *S. aureus*, Villegas and cohorts, (2005) reported induced apoptosis, possibly due to the direct cytotoxic activity of bacterial toxins as well as contact with pili and flagella. The presence of *U. urealyticum* in human semen decreases the number of microelements, such as zinc and selenium, which play an important role in the integrity of semen by maintaining its antioxidative defensive properties (Fraczek et al., 2007). Moreover, bacteria can also decrease semen quality by agglutinating motile sperm and altering cell morphology (Sikka et al., 2004). In fact, the production of reactive oxygen species as a result of the inflammatory response to infection negatively impacts semen quality (Tremellen, 2008). The production of toxins and metabolic products as a result of

bacterial proliferation in the ejaculate could also damage sperm function and decrease semen quality (Moretti et al., 2009).

In the current study, the species of bacteria present in the ejaculate were not investigated. However, because various bacteria have been described in poultry semen, it is possible that the bacteria present in the rooster semen employed different mechanisms, ultimately decreasing sperm motility. This theory is supported by an *in vitro* study, conducted by Vizzier and cohorts (2005), where rooster semen was inoculated with *Salmonella* and *Campylobacte*r. *Salmonella* was found to be associated with all the segments of the sperm (head, midpiece and tail), whereas *Campylobacter* was mainly found on the midpiece and tail segments of spermatozoa. Also in this research, often more than one bacterium was found attached to the sperm. However, the authors suggested that in natural semen samples a lower bacterium: spermatozoa ratio could occur, resulting in a different site of attachment to sperm. Therefore, since semen in the current study were not inoculated with bacteria *in vitro*, it is possible that attachment was not the main mechanism responsible for decreasing sperm motility.

Additionally, semen pH also plays a crucial role in sperm function and viability. In fact, the pH change in response to the presence of bacteria in the ejaculate could be detrimental to semen quality. For example, Haines et al. (2013) described a decline in rooster semen pH when semen was exposed to *Lactobacillu*s or *Bifidobacterium* under *in vitro* conditions. Furthermore, this decrease in pH was probably due to the production of lactic acid by these bacteria, leading to a negative impact on sperm movement. In fact, Haines and cohorts (2013) reported that sperm motility was entirely eliminated when semen was incubated with these bacteria. Therefore, it is possible that the linear increase

in bacteria per sperm in response to dietary YP reported in the current study altered pH and ultimately decreased sperm motility. However, in the present study, bacteria were grown in a non-selective media, so it is unknown if *Lactobacillus* or *Bifidobacterium* levels increased.

Additionally, it is also possible that the presence of live as well as dead bacteria in the ejaculate decreased sperm motility. For example, in an *in vitro* study conducted to evaluate the effect of bacteria and their metabolites on rooster semen quality, Triplett and cohorts (2015) exposed rooster semen to both living and heat killed overnight cultures of *Bifidobacterium* and *Lactobacillu*s. The authors stated that rooster semen exposed to both living and killed cultures exhibited similar sperm quality, which was significantly lower as compared to the saline control. Because the heating process likely denatured proteins and components of *Lactobacillus* and *Bifidobacterium*, the authors suggested the presence of heat resistant inorganic compounds produced by these bacteria ultimately reduced sperm quality. Therefore, further research is needed to determine which potential substances and bacterial species might increase in the ejaculate following dietary supplementation of YP in order to study their mechanism of action or relationship on semen quality.

Although the impacts of yeast and other fungi on semen quality have not been thoroughly investigated as compared to bacteria, the current study and previous research suggest that these microorganisms can alter semen quality using mechanisms similar to modes of action described for bacteria (Tian et al., 2007; Ngu et al., 2014). In fact, Watson and cohorts (1990) described that both fungi and bacteria, can decrease sperm viability and overall semen quality by utilizing nutrients present in the seminal plasma

and by producing metabolic products and toxins, ultimately impairing sperm function, motility and viability. This is verified by Fapahunda and cohorts (2008) who reported that mice continuously fed aflatoxin-contaminated corn demonstrated a higher frequency of morphologically abnormal sperm cells.

Even though the direct effects of *Saccharomyces cerevisiae* on semen quality has not been elucidated, other yeast species and microorganisms other than bacteria have been found to impact semen quality. For example, Tian et al. (2007) demonstrated that the *in vitro* exposure of human spermatozoa to *Candida albicans*, an opportunistic fungus that can grow as yeast cells, reduced sperm motility and impaired membrane structure. The authors also described sperm agglutination and attachment to spermatozoa, especially to the head, by *C. albicans*. Additionally, multiple ultrastructural lesions were reported in response to the exposure of semen to *C. albicans*, suggesting an inhibitory effect of this microorganism on sperm movement and ultrastucture, which may negatively impact male fertility. In fact, previous work revealed that *C. albicans* increases spermatozoa DNA fragmentation and inhibits oocyte fertilization (Burruoelo et al., 2002). However, due to the remarkable differences found between chicken and human spermatozoa, it is not possible to estimate if similar effects would be obtained if avian spermatozoa were exposed to yeast cells. Additionally, because *C. albicans* and *Saccharomyces cerevisiae* are different species, different results could be observed in the present study. However, both *Saccharomyces* and *Candida*, have strains classified as killer yeasts, due to the production of toxins, proteins, and glycoproteins that have antimicrobial activity against susceptible microorganisms, such as other yeast, fungi and bacteria strains (El-Banna et al., 2011). These strains have been found in different

environments and conditions (Woods et al., 1974). Therefore, it is possible that the high level of YP included in the rooster's diet in the current study contained killer yeast strains that impaired sperm motility due to the high production of toxins.

Despite the effects on semen quality and microbiota, the supplementation of YP in the current study did not affect feed intake (P= 0.486), body weight (P= 0.419), or body weight gain (P= 0.684, Table 3.7). Similarly, Brake (1991) reported that broiler breeders fed different levels of YP did not exhibit any change in feed conversion, egg production, mortality or body weight gain as opposed to the untreated group. However, a reduction in fertility was observed at the level of 0.3% as compared to the other treatments. Because both males and females were fed YP, it is possible that the decline in fertility was caused by a reduction in semen quality, in response to the dietary supplementation of YP as was seen in the present study. However, Brake (1991) did not examine semen quality and semen microbiota. Therefore, either or both sexes could be responsible for the decline in fertility due to YP.

In conclusion, this study suggests that although YP has been reported to increase broiler growth performance, the dietary supplementation of YP to roosters linearly decreased sperm motility possibly due to the linear increase in the number of bacteria per sperm and yeast per sperm. Because bacteria per sperm and yeast per sperm were positively correlated, it is also possible that some species of bacteria attached to the mannose, present on the yeast cell wall; and bacteria bound to the yeast contaminated semen during fecal excretion. As previously mentioned, past research has found that the presence of some pathogenic and non- pathogenic bacteria in semen can negatively impact sperm motility. Therefore, further research is required to elucidate which bacteria

are present in the ejaculate following dietary supplementation of YP and to determine the mechanism of action that decreases semen quality. In addition, research should also be conducted to determine whether the inclusion of YP in the rooster's diet will affect the number of pathogenic bacteria that can be transmitted from the roosters and hens to their progeny, and ultimately pose a threat to human health.

Table 3.1 Experimental diet and composition

Diet formulation	
Ingredient name	Percent inclusion
Corn	60.02
SBM	14.96
Wheat Midds	20.00
Poultry fat	0.50
Dicalcium Phosphate	1.42
Sand or yeast fermentation product[1]	1.00
Limestone: Calcium Carbonate	0.97
Salt(NaCl)	0.15
Sodium Bicarbonate	0.36
L- Lysine HCL	0.23
DL- Methionine	0.07
Choline- Cl	0.07
Nutrablend Vit TM Premix[2]	0.25
Calculated composition	
Crude protein, CP (%)	14.89
AME Poultry (Kcal/Kg)	2,865.99
Lys, digestible poultry (%)	0.79
Met, digestible poultry (%)	0.26
TSAA, digestible poultry (%)	0.47
Thr, digestible poultry (%)	0.44
Calcium (%)	0.75
Phosphorus, total (%)	0.67
Phosphorus, available (%)	0.35
Sodium (%)	0.18

[1] Sand was provided in the absence of yeast to maintain percentage inclusion levels for remaining ingredients of the diet.

[2] The vitamin and mineral premix provided the following per kg diet: vitamin A, 7,717 IU; vitamin D_3, 2,756 UI; vitamin E, 17 UI; vitamin B_{12}, 0.01 mg, vitamin B6, 1.38 mg; niacin 28 mg; d- pantothenic acid, 6.6 mg; menadione, 0.83 mg; folic acid,0.69 mg; thiamine,1.1 mg; biotin 0.007 mg; choline, 386 mg; riboflavin, 6.61; zinc; 4%; iron, 2%; manganese, 4%; copper, 4,500 ppm; iodine, 500ppm; selenium, 60 ppm.

Table 3.2 Semen quality parameters[1] from 60-68 wk old White Leghorn roosters fed different levels of yeast fermentation product (YP).

			Sperm Concentration			Ejaculated Sperm	
Inclusion of YP	SQI[2]	Dead sperm	Total	Live	Volume	Total	Live
----%-----		-%-	-billion sperm/mL-		-mL-	Billion sperm/ejaculate	
0	418	9	2.6	2.3	0.39	1.02	0.93
0.5	413	10	2.6	2.3	0.36	0.91	0.82
1	398	12	2.5	2.3	0.39	1.04	0.94
SEM	7.6	1.4	0.13	0.12	0.015	0.063	0.059
ANOVA P	0.171	0.292	0.994	0.964	0.220	0.310	0.295
Linear Equation	Y= -20x +420	-	-	-	-	-	-
P (slope=0)	0.068	0.115	0.946	0.794	0.909	0.782	0.924
R^2	0.054	-	-	-	-	-	-

[1]n=63 (21 roosters per treatment)
[2] Sperm quality index

Table 3.3 Presence of bacteria in semen[1] from 60-68 wk old White Leghorn roosters feed different levels of yeast fermentation product (YP)

	Bacteria	
Inclusion of YP	Log CFU/mL of semen	Log CFU/billion sperm
--%--		
0	2.8	1.3
0.5	2.9	1.4
1	2.8	2.3
SEM	0.13	0.37
ANOVA P	0.59	0.14
Linear Equation	-	Y= 1.032X+ 1.181
P (slope=0)	0.59	0.10
R^2	-	0.043

[1]n=63 (21 roosters per treatment)

Table 3.4 Presence of yeast in semen[1] from 60-68 wk old White Leghorn roosters fed different levels of yeast fermentation product (YP)

	Yeast	
Inclusion of YP	Log CFU mL of semen	Log CFU/billion sperm
--%--		
0	0.20	0.08
0.5	0.23	0.15
1	0.26	0.38
SEM	0.055	0.127
ANOVA P	0.773	0.230
Linear Equation	-	Y= 0.315X+0.053
P (slope=0)	0.472	0.081
R^2	-	0.049

[1]n=63 (21 roosters per treatment)

Table 3.5 Correlation analysis[1] between bacteria and yeast present in semen samples from 60-68 wk old White Leghorn roosters fed different levels of yeast fermentation product (YP).

		Yeast cell	
Bacteria	Corr. coeff. and P[2]	Log CFU per mL semen	Log CFU per billion sperm
Log CFU per mL semen	r	0.188	0.049
	P	0.143	0.699
Log CFU per billion sperm	r	0.056	0.500
	P	0.664	<0.0001

[1] n=63 (21 roosters per treatment)
[2]Correlation coefficient and P-value

Table 3.6 Correlation analysis[1] between semen microbiota and semen quality parameters from 60-68 wk old White Leghorn roosters fed different levels of yeast fermentation product (YP).

		Semen quality variable						
				Sperm Concentration			Ejaculate sperm	
Semen microbiota parameter	**Corr. coeff. and P[2]**	SQI[3]	Dead sperm	Total	Live	Volume	Total	Live
Log CFU per mL Semen (Bacteria)	r	-0.152	0.052	-0.011	-0.010	-0.296	-0.181	-0.184
	P	0.237	0.688	0.934	0.936	0.019	0.161	0.151
Log CFU per mL semen (Yeast)	r	-0.1686	0.212	0.011	0.005	-0.132	-0.05	-0.058
	P	0.190	0.097	0.929	0.968	0.307	0.699	0.656
Log CFU per billion sperm (Bacteria)	r	-0.577	0.362	-0.684	-0.688	-0.3089	-0.594	-0.608
	P	<0.0001	0.004	<0.0001	<0.0001	0.0146	<0.0001	<0.0001
Log CFU per billion sperm (Yeast)	r	-0.404	0.521	-0.258	-0.303	-0.077	-0.236	-0.2677
	P	0.0012	<0.0001	0.042	0.0165	0.551	0.064	0.0354

[1] n=63 (21 roosters per treatment)
[2]Correlation coefficient and P-value
[3] Sperm quality index

Table 3.7 Feed intake, body weight and body weight gain [1] from 60-68 wk old White Leghorn roosters fed different levels of yeast fermentation product (YP)

Inclusion of YP	Feed intake/day	Body weight	Body weight gain
---%---	----------Kg----------		
0	0.082	2.14	0.01
0.5	0.079	2.16	0.02
1	0.080	2.19	0.02
SEM	0.0021	0.042	0.019
ANOVA P	0.656	0.719	0.912
Linear Equation	-	-	-
P (slope=0)	0.486	0.419	0.684
R^2	-	-	-

[1]n=63 (21 roosters per treatment)

References

Abaza, I. M., M.A Shehata, & M.S Shoieb. 2006. Evaluation of some natural feed additive in layer diets. *Egyptian Poultry Science*, *26*, 891-909.

Ahmed, E.S., H.F Booles, I.M Farag, S.A Nada, and M. Fadel. 2012. Study of Therapeutical Role of Chromium-Enriched Yeast (Saccharomyces cerevisiae) on Genetic Alterations and Sperm Abnormalities in Streptozotocin-Induced Hyperglycemic Rats. *World Applied Sciences Journal*, *16*(1), pp.39-51.

Auroux, M.R., L. Jacques, D. Mathieu, J. Auer. 1991. Is the sperm bacterial ratio a determining factor in impairment of sperm motility: an in-vitro study in man with *Escherichia coli*. *International Journal of Andrology*, 14, 264-270.

Bar, T.Z., R. Yahuda, T. Hacham, S. Krupnik and B. Bartoov. 2008. Influence of *Campylobacter* fetus subsp. fetus on ram sperm cell quality. *Journal of Medical Microbiology, 57*, 1405 1410.

Burrello, N., A.E. Calogero, A. Perdichizzi, M.Salmeri, R.. D'Agata, and E.Vicari. 2004. Inhibition of oocyte fertilization by assisted reproductive techniques and increased sperm DNA fragmentation in the presence of Candida albicans: a case report. *Reproductive biomedicine online*, 8(5), 569-573.

Bilgili, S.F., and J.A. Renden. 1984. Fluorometric determination of avian sperm viability and concentration. *Poultry Science* 63, 2275-2277.

Baurhoo, B., A. Letellier, X. Zhao, and C. A. Ruiz-Feria. 2007. Cecal populations of lactobacilli and bifidobacteria and Escherichia coli populations after in vivo Escherichia coli challenge in birds fed diets with purified lignin or mannanoligosaccharides. Poultry Science, 86(12), 2509-2516.

Brake, J. 1991. Research note: Lack of effect of a live yeast culture on broiler breeder and progeny performance. *Poultry science*, *70*(4), 1037-1039.

Breed, R and WD Dotterrer. 1916. The Number of Colonies Allowable On Satisfactory Agar Plates. *Journal of Bacteriology*. 1, 321-331.

Burrows, W.H., and J.P. Quinn. 1937. The collection of spermatozoa from the domestic fowl and turkey. *Poultry Science* 16:19-24.

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

Diemer, T., P. Huwe, M. Ludwig, I. Schroeder-Printzen, H.W Michelmann, H.G Schiefer & W. Weidner. 2003. Influence of autogenous leucocytes and Escherichia coli on sperm motility parameters in vitro. *Andrologia, 35*(2), 100-105.

El-Banna, A., A. Malak; M.G. Shehata. 2011. Yeasts Producing Killer Toxins: An Overview. Alexandria Journal of Food Science and Technology, 8 (2), 41-53.

Edens, F.W. 2003. An alternative for antibiotic se in poultry: probiotics. *Revista Brasileira de Ciência Avícola*, 5(2), 75-97.

Fapohunda, S. O., C.N Ezekiel, O.A. Alabi, A. Omole, & S.O Chioma. 2008. Aflatoxin-mediated sperm and blood cell abnormalities in mice fed with contaminated corn. *Mycobiology, 36*(4), 255-259.

Feye, K. M., K. L. Anderson, M. F. Scott, D. R. McIntyre, and S. A. Carlson. "Inhibition of the virulence, antibiotic resistance, and fecal shedding of multiple antibiotic-resistant Salmonella Typhimurium in broilers fed Original XPC™." Poultry Science 95, no. 12 (2016): 2902-2910.

Fraczek, M., & M. Kurpisz. 2007. Inflammatory mediators exert toxic effects of oxidative stress on human spermatozoa. *Journal of andrology, 28*(2), 325-333.

Gale, C., and K.I. Brown. 1961. The identification of bacteria contaminating collected semen and the use of antibiotics in their control. *Poultry Science*, 40: 50-55.

Gao, J., H.J. Zhang, S.H. Yu, S.G. Wu, I. Yoon, J. Quigley, Y.P Gao and G.H., Qi, 2008. Effects of yeast culture in broiler diets on performance and immunomodulatory functions. *Poultry Science*, *87*(7), pp.1377-1384.

Guillot, J. F. 1998. Les probiotiques en alimentation animale. *Cahiers Agricultures*, *7*(1), 49-54.

Haines, M. D. 2012. *Bacteria and their effects on fertility in the chicken* (Master thesis, Mississippi State University).

Haines, M. D., H. M. Parker, C. D. McDaniel, and A. S. Kiess. 2013. Impact of 6 different intestinal bacteria on broiler breeder sperm motility in vitro. *Poultry science 92*, 8: 2174-2181.

Hassanein, S.M., K.N Soliman. 2010. Effect of probiotic (*Saccharomyces cerevisiae*) adding to diets on intestinal microflora and performance of hy-linelayers hens. *Journal of American Science*. 6, 159–169.

Hayat, J. 1992. Reproductive performance of Wrolstad Medium White turkey hens fed a breeder diet containing a yeast culture (Doctoral dissertation, University of Oregon).

Huyghebaert, G., R. Ducatelle, and F. Van Immerseel. 2011. An update on alternatives to antimicrobial growth promoters for broilers. *The Veterinary Journal*, *187*(2), 182 188.

Kabir, S.M. 2009. The role of probiotics in the poultry industry. *International Journal of Molecular Sciences*, *10*(8), 3531-3546.

Kidd, M.T., L. Araujo, C.D McDaniel, and D. McIntyre. 2013. A study assessing hen and progeny performance through dam diet fortification with a Saccharomyces cerevisiae fermentation product. *The Journal of Applied Poultry Research*, *22*(4), 872-877.

King, L.M., and A.M. Donoghue. 2000. Adaptation of the sperm mobility test to the identification of turkey toms with low fertilizing potential. *Journal of Applied Poultry Research*. 9, 66-73.

Line, J. E., Bailey, J. S., Cox, N. A., Stern, N. J., & Tompkins, T. 1998. Effect of yeast supplemented feed on Salmonella and Campylobacter populations in broilers. *Poultry science*, *77*(3), 405-410.

Madriqal, S. A., S. E. Watkins, M. H. Adams, A. L. Waldroup, and P. W. Waldroup. 1993. Effect of an active yeast culture on performance of broilers. *Poultry Science* 72(Suppl.1):87. (Abstr.)

Manafi, M., S. Khalaji, M. Hedayati and N. Pirany. 2016. Efficacy of Bacillus subtilis and bacitracin methylene disalicylate on growth performance, digestibility, blood metabolites, immunity, and intestinal microbiota after intramuscular inoculation with Escherichia coli in broilers. *Poultry Science*, pew347.

McDaniel, C. D., J.L.Hannah, H.M. Parker, T.W.Smith, C.DSchultz, & C.D.Zumwalt.. 1998. Use of a sperm analyzer for evaluating broiler breeder males. 1. Effects of altering sperm quality and quantity on the sperm motility index. *Poultry science*, *77*(6), 888-893.

Mehta, R. H., H. Sridhar, B.V. Kumar, & T.A. Kumar. 2002. High incidence of oligozoospermia and teratozoospermia in human semen infected with the aerobic bacterium Streptococcus faecalis. *Reproductive biomedicine online, 5*(1), 17-21.

Moretti, E., S.Capitani, N. Figura, A.Pammolli, A., M.G Federico, V. Giannerini and G. Collodel 2009.. The presence of bacteria species in semen and sperm quality. *Journal of assisted reproduction and genetics, 26*(1), p.47.

Ngu, G. T., K.A. Etchu, I.S.R. Butswat, & I.N. Woogeng. 2014. Semen and microbial characteristics of two breeds of turkeys in an arid tropical environment of Bauchi State, Nigeria. *African Journal of Microbiology Research*, 8(21), 2174-2182.

NRC. 1994. Nutrient Requirements of Poultry. 9th rev. ed. Natl. Acad. Press, Washington, DC.

NRC. 1996. Guide for the care and Use of Laboratory Animals. National Academy Press Washington, DC.

Papatsiros, V. G, P. D.Katsoulos, K. C. Koutoulis, M. Karatzia, ,A. Dedousi and, G .Christodoulopoulos. 2013. Alternatives to antibiotics for farm animals. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, *8*, 1-15.

Price, K. L., H. R. Totty, H. B. Lee, M. D. Utt, G. E. Fitzner, I. Yoon, M. A. Ponder, and J. Escobar. 2010. "Use of fermentation product on growth performance and microbiota of weaned pigs during infection." *Journal of animal science 88*, no. 12: 3896-3908.

Purvis K, E. Christiansen. Infection in the male reproductive tract. 1993. Impact, diagnosis and treatment in relation to male infertility. International *Journal of Andrology*.*16*:1–13.

Qiang, H., M.S Jiang, J.Y Lin, & W.M. He. 2007. Influence of enterococci on human sperm membrane in vitro. *Asian journal of andrology, 9*(1), 77-81.

Roto, S.M., P.M. Rubinelli and S.C Ricke. 2015. An introduction to the avian gut microbiota and the effects of yeast-based prebiotic-type compounds as potential feed additives. *Frontiers in Veterinary Science*, *2*, 28.

Rubinelli, P., S. Roto, S.A Kim, S.H. Park, H.O. Pavlidis, D. McIntyre, and S.C. Ricke. 2016. Reduction of Salmonella Typhimurium by Fermentation Metabolites of Diamond V Original XPC in an In Vitro Anaerobic Mixed Chicken Cecal Culture. Frontiers in Veterinary Science, 3.

Shen Y. B, J.A. Carroll, I. Yoon, R.D. Mateo, S.W. Kim. 2011. Effects of supplementing *Saccharomyces cerevisiae* fermentation product in sow diets on performance of sows and nursing piglets. *Journal of Animal Science 89*:2462–71. doi:10.2527/ jas.2010-3642.

Shen, Y.B., X.S. Piao, S.W. Kim, L. Wang, P. Liu, I. Yoon, Y.G. Zhen .2009. Effects of yeast culture supplementation on growth performance, intestinal health, and immune response of nursery pigs. *Journal of Animal Science*, 87, 2614–2624.

Sikka S.C. 2004. Role of oxidative stress and antioxidant in andrology and assisted reproductive technology. *Journal of Andrology 25(1)*:5–19.

Smith, A.U. 1949. The control of bacterial growth in fowl semen. *Journal of Agricultural Science. 39*, 194-200.

Steel, R. G. D. and J. H. Torrie. 1980. Principles and Procedures of Statistics: A Biometrical Approach. McGraw-Hill, Inc., New York, NY.

Tian, Y. H., J.W. Xiong, L. Hu, D.H Huang, & C.L. Xiong. 2007. Candida albicans and filtrates interfere with human spermatozoal motility and alter the ultrastructure of spermatozoa: an in vitro study. *International journal of andrology, 30*(5), 421-429.

Tremellen, K. 2008. Oxidative stress and male infertility—a clinical perspective. *Human reproduction update*, 14(3), 243-258.

Tripplet, M; C.D. McDaniel, A.S. Kiess. 2015. Killed vs. living cultures of Bifidobacterium and Lactobacillus on broiler breeder semen. 94(E- Supplement-1) Abstract.http://www.poultryscience.org/psa15/abstracts/2015-PSA-Abstracts.pdf

Villegas, J., M. Schulz, L. Soto, & R. Sanchez. 2005. Bacteria induce expression of apoptosis in human spermatozoa. *Apoptosis*, 10(1), 105-110.

Vizzier-Thaxton, Y., N.A. Cox, L.J. Richardson, R.J. Buhr, C.D. McDaniel, D.E. Cosby, J.L. Wilson, D.V. Bourassa, and M.B. Ard. 2006. Apparent Attachment of *Campylobacter* and *Salmonella* to Broiler Breeder Rooster Spermatozoa. *Poultry Science. 85*:619-624.

Vohra, A., P. Syal, and Madan, A. 2016. Probiotic yeasts in livestock sector. *Animal Feed Science and Technology*, *219*, 31-47.

Watson P. F .1990. Artificial insemination and the preservation of semen, In: Lamming GE (ed). Marshall‟s Physiology of Reproduction, Vol.2. Reproduction in the Male, 4th Edition, Longman Group U.K. pp. 747-869.

Woods, D. R., I.W. Ross, D.A Hendry. 1974. A new killer factor produced by a killer/sensitive yeast strain. Journal of General Microbiology, 81: 285-289.

Yalcin, S., A. Sahin .2015. Effects of dietary inactive yeast and live yeast on performance, egg quality traits, some blood parameters and antibody production to SRBC of laying hens. *Kafkas Universitesi Veteriner Fakultesi Dergisi*, *21*(3), 345-350.

Yaniz, J.L., M.A. Marco-Aguado, J.A. Mateos, and P. Santolaria. 2010. Bacterial contamination of ram semen, antibiotic sensitivities, and effects on sperm quality during storage at 15°C. *Animal Reproduction Science* 22:142-149.

Zhang, X.Z., K.S. Yao, C.L Xiong. 2011. A combined method for weighing human semen volume. *National journal of andrology*, *17*(10), 902-904.

CHAPTER IV

IMPACT OF *IN VITRO* INOCULATION AND DIETARY SUPPLEMENTATION WITH *BACILLUS SUBTILIS* ON SPERM QUALITY OF AGED WHITE LEGHORN ROOSTERS

Abstract

Bacillus subtilis has been fed to livestock and poultry as an alternative to antibiotic growth promoters due to the risk of antimicrobial resistance. The inclusion of this probiotic in the diet has been shown to increase animal performance by several modes of action, including modulation of intestinal microbiota. Previous research has demonstrated that some bacterial species negatively affect sperm motility. However, information is scarce concerning the effects of *B. subtilis* on semen quality. As a result, two experiments were conducted. The objective of the first study was to evaluate if sperm motility is altered when rooster semen is directly exposed *in vitro* to *B. subtilis* or its metabolites. The second objective was to determine the impact of supplementation with *B. subtilis* on rooster feed intake, body weight, body weight gain, sperm quality and the concentration of *Bacillus* spp. in semen. In Exp. 1, *B. subtilis* was cultured for 48 h to a concentration of 10^8 CFU/mL. In order to examine the effect of *B. subtilis*, its metabolites and also the broth where this bacterium was grown on rooster semen quality, the pooled semen from 30, 72 wk old, White Leghorn roosters, was diluted 10-fold with the following treatments: 1) saline control, 2) sterile broth, 3) culture of *B. subtilis*, 4)

supernatant from the culture and 5) bacterial pellet from the culture. Semen pH and the sperm quality index (SQI) were obtained at 0 and 10 min post dilution to analyze the effect of exposure length to each treatment. The entire experiment was replicated three times. Semen pH and SQI were not affected by the *B. subtilis* pellet as compared to saline control. However, pH and SQI for every treatment containing broth was lower than the saline control and *B. subtilis* pellet treatments. Over time, pH of the saline control and culture of *B. subtilis* declined and increased, respectively. The SQI increased 10 min post dilution with the saline control and the *B. subtilis* pellet, but decreased for all the other treatments. For Exp. 2, 42 individually caged White Leghorn roosters, 74 wk old, were fed either 0 or 0.045 % Opti - Bac S (manufacturer's recommended level). Each week, for 4 wk, individual semen samples were analyzed for pH, semen volume, sperm concentration, sperm viability and SQI. Additionally, semen concentrations of $Na^{+,}$ $Ca^{2+,}$ K^{+}, Cl^{-}, CO_2, and O_2 were measured (n=4). Feed intake was individually obtained weekly (n=4), and body weight and body weight gain were measured every 2 wk (n=2). In the last week, after the semen analyses were performed, the remaining ejaculates were serially diluted and plated to determine *Bacillus* spp. counts. The inclusion of *B. subtilis* into feed did not alter any sperm quality characteristics, pH, seminal ion concentrations, or *Bacillus* spp. counts in semen. Feed intake, body weight and body weight gain were also not affected by the supplementation of *B. subtilis*. In conclusion, these data demonstrated that neither direct *in vitro* exposure to *B. subtilis*, nor dietary inclusion of 4.5×10^4 CFU of *B. subtilis* / g of feed to roosters alters sperm quality, possibly due to this bacterium being indigenous to the rooster's reproductive tract and semen.

Introduction

The increasing demand for poultry products contributed to the evolution of the poultry industry from a backyard flock into a competitive and sophisticated sector. The United States is a distinguished producer and consumer of chicken meat and eggs, and its prominence in the poultry industry is a result of several factors, including improvements in nutrition, management and genetic selection (Barbato, 1999**)**. Additionally, the use of antibiotics in broiler production has been widely practiced for decades to improve feed efficiency, body weight gain, and growth and to reduce mortality. In fact, apart from their therapeutic and prophylactic use, antibiotics have been supplemented into animal diets as antimicrobial growth promoters (AGP) for years (Castanon et al., 2007).

The addition of antibiotics as AGP to livestock and poultry feed has been reported to improve animal performance by interacting with intestinal microbiota and by decreasing the population of pathogenic bacteria (Castanon et al., 2007). However, previous research suggests that inclusion of AGP to animal diets may result in antibiotic resistance of several bacterial species (Van Immerseel et al., 2004). These bacteria include *Salmonella, Campylobacter, and E. coli*, which are pathogenic and frequently associated with foodborne outbreaks (Van Immerseel et al., 2004). Therefore, the risk associated with antimicrobial resistance has led to the use of alternatives to AGP, such as probiotics. Probiotics are live microorganisms, including bacteria and fungi, that when adequately supplemented in the diet benefit host health (Miles et at., 1991; FAO, 2001). Supplementation with these feed additives helps to meet the consumer demand for antibiotic free livestock and poultry products, decreases the risk to human health, and

potentially alleviates the reduction in animal performance caused by the removal of antibiotics from animal feed (Park et al., 2016).

Bacillus spp. are examples of microorganisms commonly exploited as probiotics for livestock and poultry (Gaggia et al., 2010; Huyghebaert et al., 2011). *Bacillus* spp. are gram positive, aerobic or facultative anaerobic and endospore-forming bacteria (Turnbull et al., 1992). The genus encompasses a few pathogenic species and especially non-pathogenic bacteria, such as *B. subtilis*. This bacterium is commonly used as a dietary supplement to prevent gastrointestinal disorders and enhance growth performance (Turnbull et al., 1990; Gaggia et al., 2010). Because the population of *B. subtilis* gradually decreases after supplementation, its constant addition in the diet is required (Souza, 2012). Unlike other non-pathogenic and gram-positive bacteria such as *Lactobacillus*, *Streptococcus* and *Bifidobacterium*, *B. subtilis* can form spores (dormant life forms). In fact, these spores are predominantly provided in feed (rather than vegetative cells) due to their ability to resist heat, dehydration, and storage prior to consumption as well as the low pH and bile salts found in the gastrointestinal tract (Hoal et al., 2000).

B. subtilis produce bacteriocins, antimicrobial peptides that disrupt the bacterial cytoplasmic membrane, causing the release of cell components followed by the loss of cell viability and function (Moll et al., 1999; Garcia, 2008). Bacteriocins are structurally similar to conventional antibiotics with antagonist effects toward several microorganisms (Moll et al., 1999). In fact, bacteriocins facilitate the introduction or growth of bacteriocin producers into an established microbiota, such as that of the intestine, by altering the composition of the resident microbial population (Dobson et

al., 2012). Furthermore, *B. subtilis* is also associated with secretion of several enzymes that aide in digestion, such as proteases, amylase, and cellulase (Garcia, 2008).

Despite its complex and diverse effects, the modulation of intestinal microbiota by *B. subtilis* is an important mechanism of action to improve animal performance. For example, in an *in vitro* study that examined the antimicrobial activity of cultured *B. subtilis*, Garcia (2008) revealed a higher efficiency of this bacterium against *Clostridium perfringens* as compared to *Salmonella* spp. and *E. coli*. The author also reported that calves supplemented with any level of inclusion of *B. subtilis* (1, 2, and 4g/day) showed higher feed intake, body weight gain, and thoracic perimeter in comparison with the untreated group. In 22-42d old broilers, Wu et al. (2011) reported that supplementation of cultured *B. subtilis* improved broiler intestinal microbiota by increasing the population of *Lactobacilli* and decreasing the population of *E. coli* as compared to the control group. Furthermore, improvements in average daily gain and feed conversion rate were reported. Similar results were reported by Knap et al. (2011) in broilers fed cultured *B. subtilis*, which showed a reduction of 58% and 3 log units in *Salmonella* positive drag swabs and ceca counts, respectively, as opposed to the untreated group. Furthermore, a numerical improvement was reported in feed conversion rate and body weight gain at 42 d. In layer hens, the supplementation of a commercial probiotic containing *B. subtilis* was associated with improvements in egg quality by enhancing yolk color, albumen quality, shell strength and shell thickness (Sobczak et al., 2015).

Although research concerning the effects of *B. subtilis* on poultry growth performance have been well documented, scarce information is available concerning the effect of this probiotic on rooster reproductive performance.

Bacillus spp. have been found in contaminated turkey semen, along with other bacteria species, such as *Staphylococcus* spp., coliforms, and *Streptococcus* spp. (Gale and Brown, 1961). Wilcox and Shorb (1958) also described the presence of different bacteria in rooster semen at a concentration of 2.2 x 10^6 CFU/mL. These findings suggest that semen contains several species of bacteria, however their impacts on semen quality and fertility were not elucidated.

Alternatively, research has demonstrated the direct effect of some species of bacteria on semen quality. For example, in an *in vitro* study, Vizzier–Thaxton and cohorts (2006) revealed that *Salmonella* and *Campylobacter* were apparently attached to different parts of spermatozoa when semen was exposed to these bacteria. Haines and cohorts. (2013) studied the effects of pathogenic (*Salmonella, E. coli, Campylobacter,* and *Clostridium*) and non-pathogenic bacteria (*Lactobacillus* and *Bifidobacterium*) on sperm motility. The author described a decrease in sperm motility when semen was exposed to harmful bacteria, but the detrimental effect of bacteria on sperm motility was even more evident in the presence of non-pathogenic bacteria that were commonly used as probiotics. However, research analyzing the effects of *B. subtilis* on semen quality is scarce. As a result, two experiments were conducted. The first objective was to evaluate if sperm motility was altered when rooster semen was directly exposed to *B. subtilis* or its metabolites, *in vitro*. The second objective was to determine the impact of dietary supplementation of *B. subtilis* on sperm quality as well as on semen pH, ionic composition and *Bacillus* concentration.

Materials and methods

Experiment 1

Housing and care

In this experiment, semen from 30 White Leghorn roosters, 72 wk old was obtained. Feed and water were provided *ad libitum,* and the birds received 16 h of light per day. The birds were fed a common basal diet (Table 4.1) for 4 weeks before and also during the experiment period. Each rooster was caged in raised-wire cages and treated in accordance with the Guide for Care and Use of Laboratory animals in Agricultural Research and Teaching (1996).

Semen collection and analysis prior to treatment

On each of 3 alternated days, ejaculates from 10 White Leghorn roosters (30 roosters total), 72 wk old, were collected by the abdominal massage method of Burrows and Quinn (1937) and pooled into a sterile scintillation vile. Before the addition of treatment solutions, semen was examined to determine if sperm concentration and viability were within the normal range. Sperm concentration was estimated by the photometric method of King and Donoghue (2000) utilizing a microreader (IMV microreader, IMV International, Maple Grove, MN). Sperm viability was determined using a fluorometer (2001 A Fluorotec, St. Johns Associates, Beltsville, MD) according to the fluorometric method of Bilgili and Renden (1984).

B. subtilis culture

One week prior to the experiment, 1 g of *B. subtilis* probiotic product (QST 713; Opti Bac, Huvepharma, Peachtree City, GA) was cultured in 9 mL of sterile fresh

nutrient broth (Catalog no.234000, Beckton Dickinson, Sparks, MD). To provide appropriate growth conditions, 1 mL of the culture was aseptically transferred to 9 mL of sterile fresh nutrient broth every 48 h. The culture was incubated under aerobic conditions at 37°C (VWR, Model 1535, Cornelius, OR) and simultaneously kept in constant motion on an orbit junior shaker (Model 3520, Pittsburgh, PA). Immediately before inoculation of semen samples, *B. subtilis* counts for the product were found to be 10^8 CFU/mL after 24 h of incubation on mannitol egg yolk polymyxin agar (MYP, Catalog no. 2281010, Beckton Dickinson, Sparks, MD).

Treatments

The pooled semen samples were exposed to the following 5 treatments: phosphate buffered saline (PBS) control, sterile nutrient broth, *B. subtilis* culture of 10^8 CFU/mL, supernatant from the *B. subtilis* culture, and pellet from the *B. subtilis* culture. *B. subtilis* culture was derived from Opti Bac S, a commercially available probiotic. To create the supernatant and bacterial pellet treatments, 1 mL of the *B. subtilis* culture was placed in a microcentrifuge tube and centrifuged for 5 min in a microcentrifuge (Eppendorf minispin, Hamburg, Germany) at 8,400 rpm (4,700 x g). After centrifugation, the supernatant was aspirated and used for the supernatant treatment. The pellet in the bottom of the microtube after centrifugation was reconstituted with PBS to the original volume and then added to the neat semen. For all treatments, semen was diluted 10-fold (50 µl of semen and 450 µl of treatment solution) and thoroughly mixed in a microcentrifuge tube before the tests were performed.

Semen analysis after treatment

After the addition of treatments, diluted semen was analyzed for the sperm quality index (SQI) and pH. Two readings for SQI and pH were obtained for each treatment at both 0 and 10 min after exposure of semen to each treatment under aerobic conditions. Semen was analyzed for the SQI (McDaniel et al., 1998) using a Sperm Quality Analyzer (Medical Electronic Systems, Rochester, MI). Seminal pH was obtained with pH indicator strips (VWR, West Chester, PA). The experiment was replicated three times, on alternate days.

Experiment 2

Housing and care

A total of 42, White Leghorn roosters were used in this experiment. Feed and water were provided *ad libitum,* and the birds received 16 h of light per day. All the roosters were fed a basal diet (Table 4.1) for an adaptation period of 5 weeks. Roosters were individually caged in raised-wire cages and treated in accordance with the Guide for Care and Use of Laboratory animals in Agricultural Research and Teaching (1996).

Experimental diets and procedures

The concentration of *B. subtilis* (QST 713) in the commercially available product used in this current study was previously evaluated in the first experiment and determined to be 10^8 CFU/g. One week before the beginning of the study, 42 White Leghorn roosters were divided into two equal groups, with 21 males per group. For 4 wk, males were fed, *ad libitum*, the following experimental diets: a control conventional rooster basal diet with no inclusion of *B. subtilis* or a *Bacillus* diet with inclusion of 4.5×10^4 CFU of *B.*

*subtilis/*g of feed (0.045 % of Opti bac S- manufacturer recommendation). Both diets were formulated to meet or exceed the NRC recommendations.

In the control diet an inert filler (sand) was added in place of *Bacillus* to ensure that nutrients provided by the basal diet remained consistent (Table 4.1). The premixes were placed in a small mixer (capacity of 11 kg) and mixed for 5 min separately from the other basal ingredients, including macro ingredients, corn and soybean meal. Any ingredients with inclusion less than 0.5% were considered a premix, including vitamins, minerals, and amino acids such as methionine and lysine. The basal diet was mixed in a horizontal mixer (approximately 230 kg) for 10 min prior and 10 min after the addition of fat. The feed was divided into 2 equal parts, and *B. subtilis* or sand was added to each respective dietary treatment before mixing for 5 min in the horizontal mixer to provide a homogenous mixture.

Semen collection and analysis

Individual semen samples from 42, White Leghorn roosters, 74 weeks old, were collected by abdominal massage (Burrows and Quinn 1937) weekly, for 4 wk. Immediately after semen collection, semen analysis was performed. Semen volume was obtained with a graduated microcentrifuge tube (Thermo scientific QSP, San Diego, CA). The SQI, sperm concentration and sperm viability were also obtained by using the same equipment and methods described in Exp 1. Two readings were obtained for each parameter. Additionally, pH and semen concentrations of Na^+, $Ca^{2+,}$ K^+, Cl^-, CO_2, and O_2 were measured using an ABL77 gas and electrolyte analyzer (Parker and McDaniel, 2006; Radiometer, Copenhagen, Denmark).

Live performance

Every week, unconsumed feed was weighed for each rooster to determine feed intake. Because all the roosters were over 70 wk old and no longer in the growth stage, body weight and body weight gain were individually obtained only every 2 wk, at 74, 76 and 78 wk of age.

Seminal microbial analysis

During the last week (wk 4) of semen collection and immediately after the semen parameters were estimated, semen samples were kept on ice for a maximum of 2 h and analyzed to determine *Bacillus* concentrations. From each sample, 100 µL of semen was serially diluted in 900 µL of PBS and mixed using a vortex to provide a homogenous mixture. For each serial dilution, 100 µL was aspirated and spread plated on petri dishes containing MYP agar. All samples were plated within 2-5 h after semen collection. Two agar-plates were incubated for each dilution at 37°C for 48 h. After the plates were removed from the incubator, CFU were determined on plates that contained between 30 and 300 CFU. The variables measured to determine the concentration of *Bacillus* in semen samples included Log CFU of *Bacillus* per mL of semen and per billion sperm in the ejaculate.

Statistical Analysis

Data from Experiment 1 were analyzed using a randomized complete block design with a split plot in time. Days (n=3) represented the blocks, and split plots were the 2 lengths of incubation (0 or 10 min). The measured variables were analyzed using

the GLM statistical procedure of SAS. When global $P \leq 0.10$, means were separated by Fisher's protected least significant difference with $\alpha = 0.05$ (Steel and Torrie, 1980).

In Experiment 2, data were analyzed using a split plot design, with individually caged roosters serving as the experimental units and dietary treatments split over weeks of the study. All variables were analyzed with the GLM statistical procedure of SAS. Differences were considered significant when global $P \leq 0.10$ (Steel and Torrie, 1980).

Results and discussion

Experiment 1

Semen analysis is a useful tool to predict rooster reproductive performance, by determining the number of viable and motile sperm in the ejaculate that is capable of fertilizing the egg and ultimately producing offspring (Parker and McDaniel, 2002). In this current study, neat semen analysis performed before addition of any treatments revealed that the semen samples contained 3.3 billion sperm/mL and 7.4% dead sperm, which were similar to values reported in previous studies (Davila et al., 2015; Bilgili and Renden, 1984). Due to semen being collected from old roosters, it was expected that these parameters could be slightly worse as compared to younger roosters (Tabatabaei et al., 2010).

When the different treatments were added to semen, the overall main effect revealed that all treatments containing broth (sterile broth, *Bacillus* culture, and supernatant from the culture) had similar SQI values that were all drastically lower than those of the saline control or bacterial pellet treatments ($P= 0.0001$; Figure 4.1 A). However, a time by treatment interaction was found for the SQI ($P= 0.0007$; Figure 4.1 B). The interaction was due to an increase over incubation in the SQI of the saline control

and pellet of *B. subtilis* treatments. However, a reduction in the SQI was observed in all remaining treatments between 0 and 10 min of exposure of semen to the treatments. During both 0 and 10 min of incubation, no difference was detected between the saline control and pellet of *B. subtilis*. However, at each of these time periods, the SQI was reduced in all the remaining treatments ($P < 0.0001$).

The SQI is a measure of general sperm movement that is influenced by how often and how many sperm move across a light path (McDaniel et al., 1998). Because the same original pool of semen, with a constant sperm concentration, was utilized to create all *in vitro* treatments in the present study, the SQI could only have been affected by sperm motility changes among treatments. The lack of a detrimental effect on sperm motility when semen was exposed *in vitro* to the reconstituted bacterial pellet suggests that *B. subtilis* does not directly have a negative effect on sperm movement.

Additionally, because the SQI of the supernatant was actually greater than that of the broth, it is unlikely that *B. subtilis* metabolites negatively affect sperm motility. The detrimental effect on sperm motility of the treatments containing broth was possibly due to components of nutrient broth that could inhibit sperm motility. Similarly, Haines and cohorts (2013) described a decline in SQI when rooster semen was exposed *in vitro* to tryptic soy broth. The high content of amino acids in these treatment solutions, due to the presence of peptone and beef extract in nutrient broth and soytone and tryptone in tryptic soy broth, might have inhibited sperm motility. For example, Sliwa et al. (1990) described a decreased in motility when mouse sperm was exposed *in vitro* to different amino acids. Additionally, Haines et al. (2013) revealed a decline in pH as compared to the saline control when rooster semen was incubated with tryptic soy broth at both 0 and

10 min. This decline in pH possibly contributed to the reduction observed in the SQI. In fact, similar to the SQI, the main effect for *in vitro* treatments revealed that all treatments containing broth yielded lower pH (P= 0.0013; Figure 4.2 A) values as compared to the saline control and pellet treatments. However, a time by treatment interaction also occurred due to a decrease in pH over incubation when semen was exposed to the saline control but an increase in pH over incubation when semen was diluted in bacterial culture. At 0 min, semen exposed to the saline control exhibited the highest pH compared to the other treatments, whereas the bacterial pellet exhibited a higher pH than sterile broth, bacterial culture or the supernatant. By 10 min of incubation, no significant difference in pH was found between the saline control and the bacterial pellet, whereas semen pH was lower in all the remaining treatments, with the broth diluent exhibiting the lowest pH. These data suggest that the nutrient broth used to culture *B. subtilis* is mostly responsible for not only the reduction in sperm motility, but also a reduction in pH, whereas the direct exposure of semen to *B. subtilis* cells, only, does not alter the SQI or semen pH.

However, the presence of other species of bacteria have been described to have a negative effect on sperm motility and semen pH. Haines and cohorts (2013) discovered that sperm motility is reduced when rooster semen is directly exposed *in vitro* to *Salmonella*, *E. coli*, *Campylobacter* and *Clostridium*. However, in the same study, sperm motility was eliminated with exposure of rooster semen to *Lactobacillus* and *Bifidobacterium,* which, similar to B. *subtilis,* are gram positive bacteria commonly supplemented as probiotics in animal feed. Furthermore, the direct exposure of rooster semen to all bacteria, except *Salmonella*, significantly lowered pH as compared to the

saline control, and the greatest reduction in pH was again observed in semen exposed to *Lactobacillus* and *Bifidobacterium* as compared to the pathogenic bacteria. The reduction in pH upon exposure to *Bifidobacterium* and *Lactobacillus* was probably due to the production of lactic acid by these bacteria (Ljungh and Wadstrom, 2006). Because semen pH plays an important role in sperm function and movement, it is possible that this reduction in sperm motility was partially attributed to the reduction in pH (Al- Aghbari, 1992). In fact, in our study, the sterile broth treatment showed the lowest SQI and pH after 10 min of incubation, suggesting that the decrease in pH negatively affected sperm motility. Alternatively, the direct exposure to the pellet from the culture of *B. subtilis* did not alter sperm movement and pH after 10 min of incubation, as compared to the saline control.

Because in the present study, the direct *in vitro* exposure of rooster semen to *B. subtilis* did not alter pH and motility, it is possible that *B. subtilis* do not use the damaging mechanisms described in other species of bacteria to reduce sperm function and semen quality. Similar to this current study, the presence of other gram-positive bacteria, such as Micrococci and alpha-haemolytic Streptococci, in the ejaculate also did not alter human sperm movement and semen quality (Mehta et al., 2002). Perhaps *B. subtilis* does not have any detrimental effect on sperm quality, because *Bacillus* naturally occurs in the rooster reproductive tract and semen (Gale and Brown, 1961; Donoghue et al., 2004).

Experiment 2

Throughout the study, no significant interactions were observed between dietary treatments and time (week) for any parameter evaluated, therefore only results for the

main effect of diet will be discussed. Dietary supplementation of *B. subtilis* did not significantly alter SQI (P= 0.320), percentage dead sperm (P= 0.609), total sperm concentration (P= 0.929), live sperm concentration (P=0.918), semen volume (P=0.657), total sperm concentration per ejaculate (P= 0.727), and live sperm per ejaculate (P= 0.740; Table 4.2). These data suggest that the manufacturer recommended inclusion of *B. subtilis* (0.045% of Opti Bac S) does not alter rooster semen quality. Although the manufacturer claims that this probiotic contains 10^9 CFU of *B. subtilis* /g of product, in the current study the concentration of this bacterium was determined to be 10^8 CFU/g of product. Therefore, the concentration of *B. subtilis* added in the feed was about 4.5 x 10^4 CFU/g of feed. In contrast to this study, previous research suggests that the addition of *B. subtilis* and *B. licheniformis* in the rooster's diet improves semen volume, sperm concentration, and sperm motility, and decreases the percentage of abnormal and dead spermatozoa in comparison to a control group (Abaza et al., 2016). However, in that work both *B. subtilis* and *B. licheniformis* were supplemented together in the rooster's diet. Hence, it is unknown if an individual bacteria species or the interaction between both bacteria species improved semen quality. Additionally, semen samples were collected only once from 43 wk old Al – Salam roosters (a local Egyptian strain), whereas in this present research, ejaculates were obtained weekly from 74-78 wk old White Leghorn roosters.

Similarly, pH (P=0.548) as well as gas concentrations of O_2 (P= 0.159) and CO_2 (P=0.189) and electrolyte concentrations of Na^+ (P=0.849), K^+ (P=0.315), Ca^{2+} (P= 0.654) and Cl^- (P= 0.928, Table 4.3) were not significantly affected by the dietary supplementation of *B. subtilis*. Avian semen pH ranges from 6.9 to 7.1, and seminal

buffer activity plays an important role in maintaining sperm livability because pH changes can be detrimental to spermatozoa. In fact, temperature, as well as concentrations of urine and lactic acid have been shown to affect semen pH (Barna and Boldizsar, 1996). Semen also contains several elements that surround sperm and ensure viability by controlling osmolality and participating in enzymatic activity (Al-Aghbari, 1992). Research suggests that the concentration of various semen components may be affected by different factors, such as location of semen in the male reproductive tract and temperature to which roosters are exposed (Al-Aghbari, 1992). Additionally, other species of bacteria have been known to alter semen composition and pH and, ultimately, decrease semen quality. For example, in humans, the presence of *U. urealyticum* is associated with poor semen quality due to the utilization of microelements in the ejaculate by this bacterium (Fraczek et al., 2007). Moreover, in avian species, the *in vitro* inoculation of semen with *Lactobacillus* and *Bifidobacterium*, commonly used as probiotics in animal feed, decrease sperm motility probably due to the reduction in pH caused by the production of lactic acid (Haines et al., 2013). However, in the current study, the results indicate that dietary addition of *B. subtilis* does not alter semen pH and composition probably because *B. subtilis* is a natural inhabitant of the male reproductive tract and semen.

Additionally, *B. subtilis* supplemented roosters in the current study showed similar feed intake (P=0.636), body weight (P=0.515) and body weight gain (P=0.825, Table 4.4) as compared to untreated birds. Although improvements in feed conversion, body weight and other meat production parameters have been observed in response to the addition of dietary *Bacillus* spp. (Opalinsk et al., 2007; Li et al., 2016), there are studies,

which report no improvement in growth performance with supplementation. For example, in a commercial trial, the addition of *Bacillus* spp. in broiler diets did not significantly affect body weight, body weight gain, feed intake, or feed conversion ratio when compared to bacitracin methylene disalicylate (BMD*)* and control treatments (Dersjant et al., 2013). Furthermore, the previous studies on *B. subtilis* supplementation were focused mainly on broiler chicken performance. Therefore, because the current study tested this probiotic in mature male layer breeders that are no longer in the growth stage, a rapid body weight change was not expected. Thus, results obtained in this study might be different from the broiler research with *B. subtilis*.

Additionally, supplementation of *B. subtilis* in the feed did not alter *Bacillus* spp. counts per mL semen (P= 0.199) or *Bacillus* spp. counts per billion sperm (P=0.381, Table 4.5). Previous studies suggest that some direct fed microorganisms, including *B. subtilis* must be continuously supplemented in the diet because they are partially excreted from the gastrointestinal tract through the cloaca (Sousa, 2012). Because the semen is in direct contact with the cloaca during ejaculation, the bacteria present in this region might be a source of contamination in both natural mating and artificial inseminated flocks (Smith, 1949; Haines, 2012). However, in our study the presence of *Bacillus* was also observed in seminal samples of non-treated birds, likely because these bacteria naturally occur in the rooster's reproductive tract and semen. Different species of bacteria, such as *Bacillus*, *Staphylococcus*, *Escherichia,* and *Enterococcus* were previously described in turkey semen at a concentration of approximately 9 log CFU/ mL (Gale and Brown, 1961). Additionally, Wilcox et al. (1958) also revealed the presence of bacteria in roosters'semen at concentration of 6 log CFU/mL. Similarly, in the present study, the

concentration of *Bacillus* spp. found in semen from control and treated roosters were found to be 6.9 and 6.6 log CFU/mL, respectively (Table 4.5).

In conclusion, this study suggests that direct *in vitro* exposure to semen or supplementation in the diet with *B. subtilis* does not have any detrimental impact on rooster semen volume, pH, ion and gas composition or sperm motility, concentration and viability. Additionally, supplementation of this probiotic in the feed did not alter the concentration of *Bacillus* spp. in semen, possibly because this bacterium is naturally found in rooster semen. However, due to the ability of *B. subtilis* to modulate intestinal microbiota and decrease the population of harmful bacteria, future research should investigate the impact of this bacterium on bacterial pathogens in semen. Its interaction with harmful bacteria present in the ejaculate, that could be vertically and horizontally transmitted to the offspring, could impact the incidence of foodborne diseases.

Table 4.1 Experimental diet composition provided to 74-78 wk old White Leghorn roosters in Exp. 2

Diet formulation	
Ingredient name	Percent inclusion
Corn	60.973
SBM	14.958
Wheat Midds	20.000
Poultry fat	0.500
Dicalcium Phosphate	1.419
Sand or *B. subtilis*	0.045
Limestone: Calcium Carbonate	0.971
Salt(NaCl)	0.155
Sodium Bicarbonate	0.358
L- Lysine HCL	0.232
DL- Methionine	0.071
Choline- Cl	0.069
Nutra blend Vit TM Premix[2]	0.250
Calculated composition	
Crude Protein, CP (%)	15.261
AME Poultry (Kcal/Kg)	2825.690
Lys, digestible (%)	0.777
Met, digestible (%)	0.265
TSAA, digestible (%)	0.459
Thr, digestible (%)	0.452
Calcium (%)	0.750
Phosphorus, Total (%)	0.694
Phosphorus, Available (%)	0.376
Sodium (%)	0.180

[1] Sand was included to replace *B. subtilis* and maintain the inclusion level for remaining ingredients provided in the basal diet consistent.

[2] The vitamin and mineral premix provided the following per kg diet: vitamin A, 7,717 IU; vitamin D_3, 2,756 UI; vitamin E, 17 UI; vitamin B_{12}, 0.01 mg; vitamin B6, 1.38 mg; niacin 28 mg; d- pantothenic acid, 6.6 mg; menadione, 0.83 mg; folic acid,0.69 mg; thiamine,1.1 mg; biotin 0.007 mg; choline, 386 mg; riboflavin, 6.61; zinc; 4%; iron, 2%; manganese, 4%; copper, 4,500 ppm; iodine, 500ppm; selenium, 60 ppm.

Table 4.2 Semen quality parameters from 74-78 wk old White Leghorn roosters[1] in Exp 2

Treatment	SQI[2]	Dead sperm	Sperm concentration		Volume	Ejaculated Sperm	
			Total	Live		Total	Live
		--%--	billion sperm/mL		mL	billion sperm/ejaculate	
Control	453	8.1	2.7	2.4	0.44	1.18	1.08
B. subtilis	439	8.5	2.6	2.4	0.45	1.21	1.11
SEM	10.3	0.67	0.13	0.12	0.021	0.076	0.071
P-value	0.320	0.609	0.929	0.918	0.657	0.727	0.740

The roosters were fed two experimental diets, varying in the inclusion of B. subtilis.
[1] n=42 (21 roosters per treatment)
[2] Sperm quality index

Table 4.3 Semen pH and ionic concentrations from 74-78 wk old White Leghorn roosters in Exp 2.

Treatment	pH	O_2	CO_2	Na^+	K^+	Ca^{2+}	Cl^-
		nmol/mL	----------µmol/mL----------				
Control	6.98	1.4	104	132.3	9.3	1.48	78
B. subtilis	7.01	2.1	95	132.1	8.8	1.45	78
SEM	0.033	0.34	4.5	1.05	0.32	0.051	2.2
P-value	0.548	0.159	0.189	0.849	0.315	0.654	0.928

The roosters were fed two experimental diets, varying in the inclusion of B. subtilis.
[1] n=42 (21 roosters per treatment)

Table 4.4 Rooster growth performance from 74-78 wk old White Leghorn roosters in Exp 2.

Treatment	Feed intake	Body weight	Body weight gain
	----------kg----------		
Control	0.10	2.19	0.003
B. subtilis	0.10	2.15	0.009
SEM	0.004	0.041	0.019
P-value	0.636	0.515	0.825

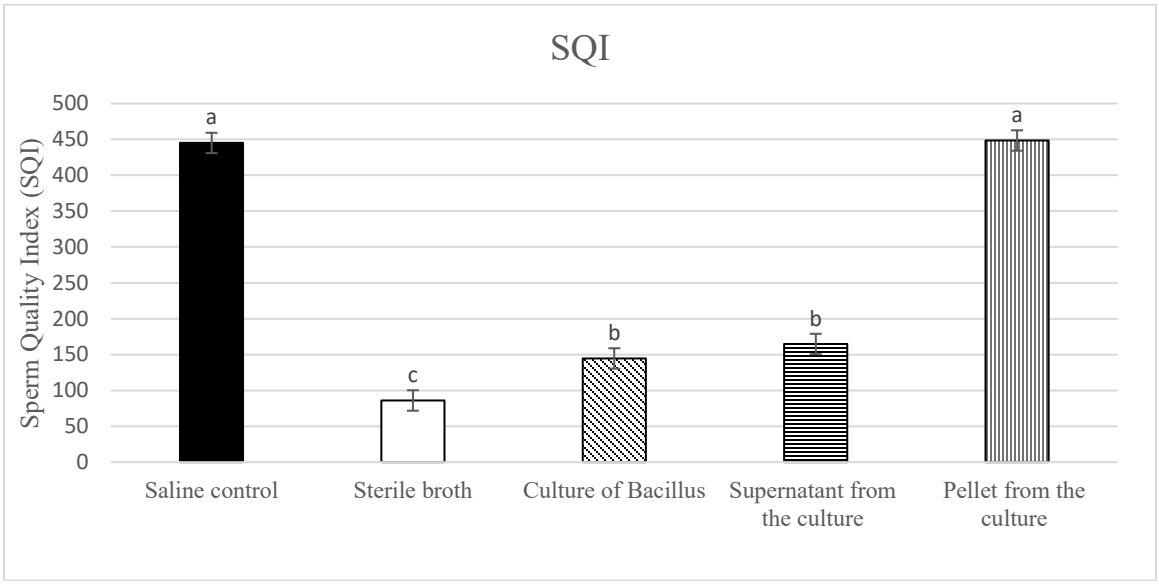
The roosters were fed two experimental diets, varying in the inclusion of B. subtilis.

Table 4.5 *Bacillus* spp. concentration in semen from 74-78 wk old White Leghorn roosters in Exp 2.

	Bacillus spp.	
Treatment	Log CFU/mL of semen	Log CFU/billion sperm
Control	6.9	2.8
B. subtilis	6.6	4.2
SEM	0.14	1.13
P-value	0.199	0.381

The roosters were fed two experimental diets, varying in the inclusion of B. subtilis.
[1]n=39 (20 roosters for control and 19 roosters for birds supplemented with *B. subtilis*)

A.



.

B

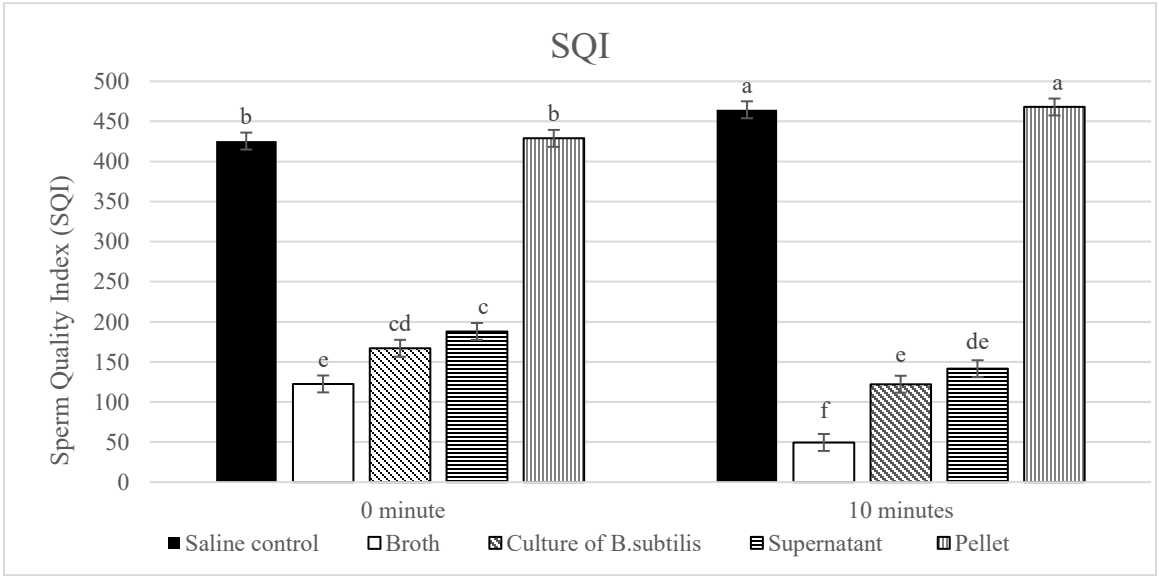
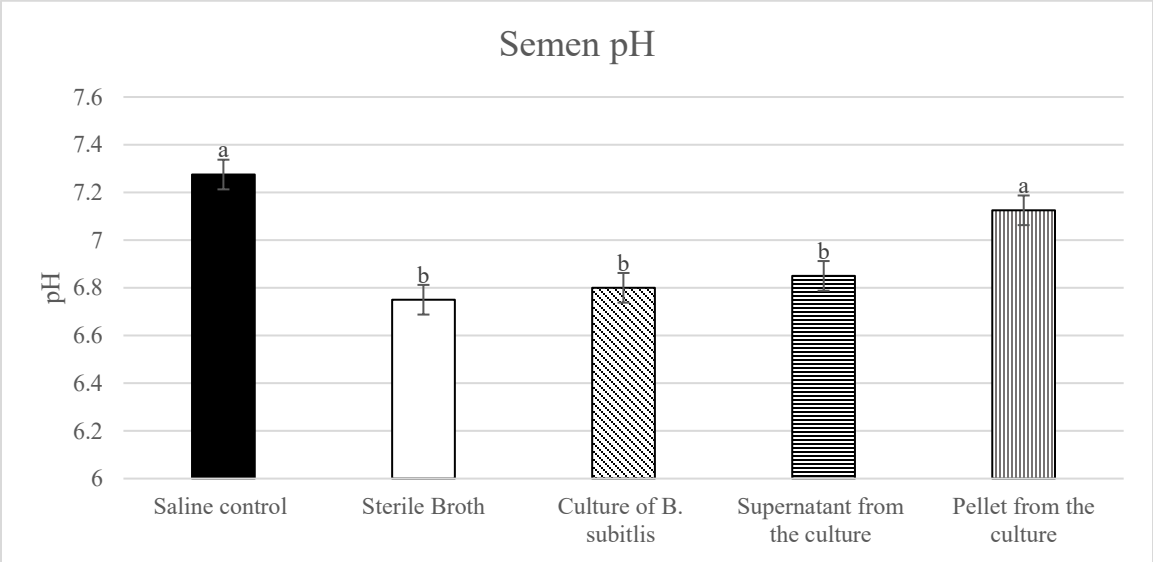


Figure 4.1 Sperm quality index (SQI) for rooster semen exposed to *B. subtilis* and diluents in Exp 1.

A) Main effect of treatment on SQI. Means with no common superscript are significantly different at $P < 0.0001$; SEM= 14.22; n=6 per treatment (3 blocks * 2 incubation times). B) SQI interaction between treatment and time. Means with no common superscript are significantly different at $P < 0.0001$; SEM= 10.552; n=3 blocks.

A.



B.

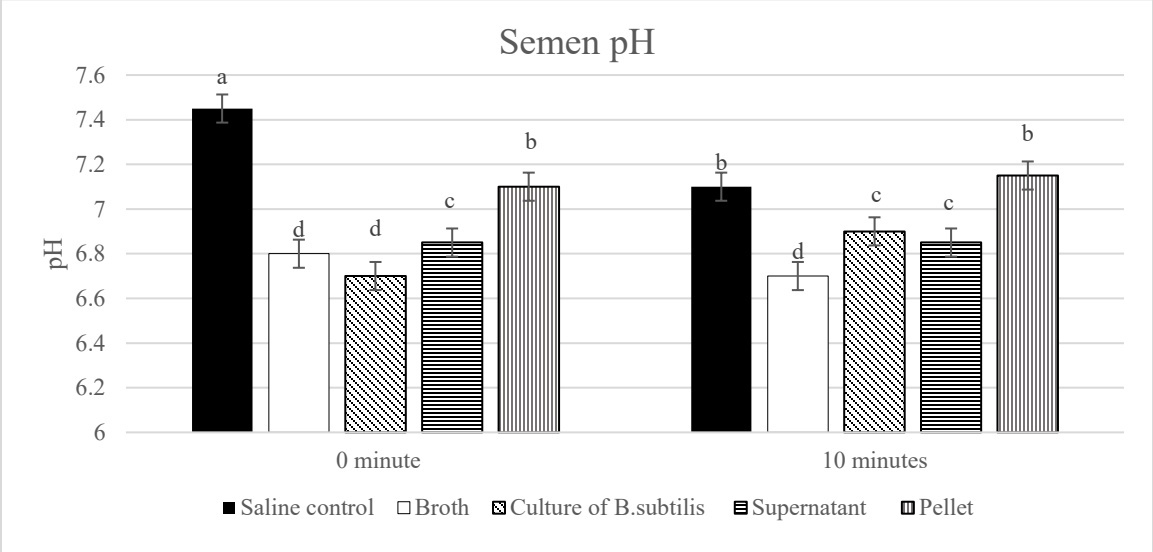


Figure 4.2 pH for rooster semen exposed to *B. subtilis* and diluents in Exp 1.

A) Main effect of treatment on pH. Means with no common superscript are significantly different at $P < 0.0013$; SEM= 0.062; n=6 per treatment (3 blocks * 2 incubation times). B) pH interaction between treatment and time. Means with no common superscript are significantly different at $P < 0.0013$; SEM= 0.063; n=3 blocks.

References

Abaza, I. M., Shehata, M. A., & Shoieb, M. S. 2006. Evaluation of some natural feed additive in layer diets. *Egyptian Pultry Science*, *26*, 891-909.

Al-Aghbari, A. M. 1992. Demonstration of a link between seminal plasma proteins and male fertility in the domestic fowl *(Gallus domesticus)*. Oregon State. (Doctoral dissertation).

Barbato, G. F. 1999. Genetic relationships between selection for growth and reproductive effectiveness. *Poultry Science*, *78*(3), 444-452.

Barna, J., and H. Boldizsar. 1996. Motility and agglutination of fowl spermatozoa in media of different amino acid content and pH value in vitro. *Acta Veterinaria Hungarica. 44*: 221-232.

Bilgili, S.F., and J.A. Renden. 1984. Fluorometric determination of avian sperm viability and concentration. *Poultry Science*, 63:2275-2277.

Burrows, W.H., and J.P. Quinn. 1937. The collection of spermatozoa from the domestic fowl and turkey. *Poultry Science. 16*:19-24.

Castanon, J. I. R. 2007. History of the use of antibiotic as growth promoters in European poultry feeds. *Poultry Science. 86*, 2466-2471.

Dávila, S. G., J. L. Campo, M. G. Gil, C. Castaño, and J. Santiago-Moreno. 2015. Effect of the presence of hens on roosters sperm variables. *Poultry science 94*: 1645-1649

Dersjant-Li, Y., A. Awati, C. Kromm, and C. Evans. 2013. A direct fed microbial containing a combination of three-strain Bacillus sp. can be used as an alternative to feed antibiotic growth promoters in broiler production. *Journal of Applied Animal Nutrition*, *2*, e11.

Dobson, Alleson, D. Paul, R. Cotter, H. Colin. 2012. Bacteriocin production: a probiotic trait? *Applied and environmental microbiology 78*, 1-6

FAO/WHO. 2001. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. *Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria*; FAO/WHO: Amerian Córdoba Park Hotel, Córdoba, Argentina, 1-34.

Fraczek, M, and M. Kurpisz. 2007. Inflammatory mediators exert toxic effects of oxidative stress in human spermatozoa. *Journal of andrology, 28*(2), 325-333.

Gaggìa, F., P. Mattarelli, and B. Biavati. 2010. Probiotics and prebiotics in animal feeding for safe food production. *International journal of food microbiology, 141*, S15-S28.

Gale, C., and K.I. Brown. 1961. The identification of bacteria contaminating collected semen and the use of antibiotics in their control. *Poultry Science*. 40, 50-55.

Garcia, G. 2008.Caracterização microbiológica e avaliação de uma cepa de Bacillus subtilis no desempenho de bezerros da raça holandesa. 68f (Doctoral dissertation, Faculdade de Ciências Agrárias e Veterinária–Universidade Estadual Paulista Julio de Mesquita, Jaboticabal).

Haines, M. D. 2012. *Bacteria and their effects on fertility in the chicken* (Master thesis, Mississippi State University).

Haines, M. D., H. M. Parker, C. D. McDaniel, and A. S. Kiess. 2013. Impact of 6 different intestinal bacteria on broiler breeder sperm motility in vitro. *Poultry science 92*, 8: 2174-2181.

Hoal, N.T.; L. Baccigalupi, A. Huxham. 2000. Characterization of *Bacillus* species used of oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders. *Applied and Environmental Microbiology*, v.66, n.12, 5241-5247.

Huyghebaert, G., R. Ducatelle, and F. Van Immerseel. 2011. An update on alternatives to antimicrobial growth promoters for broilers. *The Veterinary Journal, 187*(2), 182-188.

King, L.M., and A.M. Donoghue. 2000. Adaptation of the sperm mobility test for the identification of turkey toms with low fertilizing potential. *Journal of Applied Poultry Research* 9:66-73.

Knap, I., Kehlet, A. B., Bennedsen, M., Mathis, G. F., Hofacre, C. L., Lumpkins, B. S., ... & Lay, A. 2011. Bacillus subtilis (DSM17299) significantly reduces Salmonella in broilers. *Poultry science*, *90*(8), 1690-1694.

Li, Y., Q. Xu, Z. Huang, L. Lv, X. Liu, C. Yin, H. Yan, and J. Yuan. 2016. Effect of Bacillus subtilis CGMCC 1.1086 on the growth performance and intestinal microbiota of broilers. *Journal of applied microbiology* 120, no. 1: 195-204.

Ljungh, A., & Wadstrom, T. 2006. Lactic acid bacteria as probiotics. *Current issues in intestinal microbiology, 7*(2), 73-90.

McDaniel, C. D., J.L. Hannah, H.M. Parker, T.W. Smith, C.D. Schultz, and C.D Zumwalt. 1998. Use of a sperm analyzer for evaluating broiler breeder males. 1. Effects of altering sperm quality and quantity on the sperm motility index. *Poultry science*, *77*(6), 888-893.

Miles, R.D.; Bootwalla, S.M. Direct-fed microbials in animal production. 1991.In *Direct-Fed Microbials in Animal Production. A Review*; National Food Ingredient Association: West Des Monies. Iowa, USA, 117-132.

Mehta RH, H. Sridhar, B.R. Vijay Kumar, T.C.Anand Kumar .2002. High incidence of oligozoospermia and teratozoospermia in human semen infected with the aerobic bacterium Streptococcus faecalis. *RBM Online*, 5, 17–21.

Moll, G. N.; Konings, W. N.; Driessen, A. J. 1999. Bacteriocins: mechanism of membrane insertion and pore formation. 1999. *Antonie van Leeuwenhoek*, 76,185-98.

NRC. 1994. Nutrient Requirements of Poultry. 9th rev. ed. Natl. Acad. Press, Washington, DC

NRC. 1996. Guide for the care and Use of Laboratory Animals. National Academy Press Washington, DC.

Opalinski, M., A. Maiorka, F. Dahlke, F. Cunha, F. S. C. Vargas, and E. Cardozo. 2007.On the use of a probiotic (Bacillus subtilis-strain DSM 17299) as growth promoter in broiler diets. *Revista Brasileira de Ciência Avícola 9*, 2: 99-103.

Park, Y. H., F.Hamidon, C. Rajangan, K.P. Soh, C.Y. Gan, T.S. Lim, and M.T. Liong, 2016. Application of Probiotics for the Production of Safe and High-quality Poultry Meat. *Korean Journal for Food Science of Animal Resources*, *36*(5), 567.

Parker, H. M., & McDaniel, C. D. 2002. Selection of young broiler breeders for semen quality improves hatchability in an industry field trial. *The Journal of Applied Poultry Research*, *11*(3), 250-259.

Parker, H. M., and C. D. McDaniel. 2006. The immediate impact of semen diluent and rate of dilution on the sperm quality index, ATP utilization, gas exchange, and ionic balance of broiler breeder sperm. *Poultry science* 85, 1 (2006): 106-116.

Sliwa, L. 1990. Effect of selected aminoacids on in vitro motility of mouse sperm. Centralna Biblioteka Rolnicza/Central Agricultural Library.

Smith, A.U. 1949. The control of bacterial growth in fowl semen. *Journal of Agricultural Science. 39*, 194-200.

Sobczak, A., & Kozłowski, K. 2015. The effect of a probiotic preparation containing *Bacillus subtilis* ATCC PTA-6737 on egg production and physiological parameters of laying hens. *Annals of Animal Science*, *15*(3), 711-723.

Souza, V. L. D. 2012. Desempenho e utilização de nutrientes por vacas leiteiras suplementadas com *Bacillus Subtilis* (PhD dissertation, Universidade Federal do Parana).

Steel, R. G. D. and J. H. Torrie. 1980. Principles and Procedures of Statistics: A Biometrical Approach. McGraw-Hill, Inc., New York, NY.

Tabatabaei, S., M. Chaji, and T. Mohammadabadi. 2010. Correlation between age of rooster and semen quality in Iranian indigenous broiler breeder Chickens. *Journal of Animal and Veterinary Advances*, *9*(1), 195-198.

Turnbull P, J. Kramer, J. Melling. *Bacillus* In: Topley and Wilson Principles of Bacteriology. 1990. Virology and Immunity 8th ed Edward Arnold, London 185-210.

Turnbull, P. C. B., Hutson, R. A., Ward, M. J., Jones, M. N., Quinn, C. P., Finnie, N. J., ... & Melling, J. 1992. Bacillus anthracis but not always anthrax. *Journal of Applied Bacteriology, 72*(1), 21-28.

Van Immerseel, F., V. Fievez, J. De Buck, F. Pasmans, A., Martel, F. Haesebrouck, and R. Ducatelle. 2004. Microencapsulated short-chain fatty acids in feed modify colonization and invasion early after infection with Salmonella enteritidis in young chickens. *Poultry Science. 83*, 69-74.

Vizzier-Thaxton, Y., N.A. Cox, L.J. Richardson, R.J. Buhr, C.D. McDaniel, D.E. Cosby, J.L. Wilson, D.V. Bourassa, and M.B. Ard. 2006. Apparent Attachment of *Campylobacter* and *Salmonella* to Broiler Breeder Rooster Spermatozoa. *Poultry Science 85*:619-624.

Wilcox, F.H., and M.S. Shorb. (1958). The effect of antibiotics on bacteria in semen and on the motility and fertility ability of chicken spermatozoa. *American Journal of Veterinary. Research, 19*, 945-949.

Wu, B. Q., Zhang, T., Guo, L. Q., & Lin, J. F. (2011). Effects of Bacillus subtilis KD1 on broiler intestinal flora. *Poultry science*, *90*(11), 2493-2499.

CHAPTER V

CONCLUSION

Research conducted in fulfillment of this thesis had the overall objective to evaluate the effects of alternatives to antibiotic growth promoters (AGP) on rooster semen quality and microbiota. The effect of these feed additives on growth performance has been well exploited in livestock and poultry due to the worldwide concern associated with antimicrobial resistance to antibiotics. However, the effects of alternatives to AGP on poultry reproductive performance has been sparsely investigated. Even though flock fertility depends on both male and female, a decrease in male reproductive performance due to these alternatives could severely reduce the production of progeny due to the low number of males compared to females in the flock.

The evaluation of semen quality is an important tool to determine the capacity of the male to fertilize the egg. Semen quality is affected by several factors, including the inclusion of feed additives in the diet and bacteria. Also, the feed additives used as alternatives to AGP have been reported to modulate intestinal microbiota and decrease the population of harmful bacteria in the gut. These bacteria are excreted from the gastrointestinal tract through the cloaca, where semen is also released during ejaculation. Therefore, semen contamination can occur when the ejaculate comes into contact with pathogenic and non-pathogenic bacteria present in the cloaca.

Different species of bacteria have been described to negatively affect semen quality and especially sperm motility by using different mechanisms, such as altering pH, producing toxins and reactive oxygen, and direct attachment to spermatozoa. Additionally, previous research has suggested that both fungi and bacteria present in the ejaculate can use similar modes of action to alter sperm function and decrease semen quality.

In the first study of this thesis, roosters were fed different levels of a commercially available yeast fermentation product (YP) and evaluated for live performance, semen quality and semen microbiota. As expected with the use of mature roosters, the dietary supplementation of YP did not significantly alter feed intake, body weight or body weight gain. However, as YP inclusion increased a linear increase in yeast and bacteria per billion sperm and a linear decrease in the sperm quality index (SQI) was found.

The SQI is affected by sperm concentration, viability and motility. However, because sperm concentration and viability were not affected by the inclusion of YP, the linear decrease in SQI was most likely caused by a decrease in sperm motility. Moreover, a positive correlation was observed between yeast and bacteria per billion sperm. Previous research has shown that pathogenic bacteria can bind to mannose, present on the yeast cell wall. Therefore, it is possible that bacteria attached to the yeast was released through excretion at the cloaca and then contaminated semen samples during ejaculation. Therefore, the linear increase in bacteria and yeast present in semen, as a result of the addition of YP in the diet, most likely decreased sperm motility due to the detrimental effect of these microorganisms on semen quality and sperm movement.

In the second study of this thesis, two experiments were conducted. In the first experiment, rooster semen was directly exposed *in vitro* to a commercially available product containing *B. subtilis* or its metabolites, and sperm motility and pH were determined at 0 and 10 min of incubation. The results revealed that unlike the incubation with some pathogenic and non-pathogenic bacteria described in other studies, the direct exposure of rooster semen to *B. subtilis* cells does not alter sperm motility or pH. In fact, the reduction in pH caused by some species of bacteria in semen can be detrimental to sperm function and movement. Because different results can be obtained under *in vivo* vs. *in vitro* conditions, a second study was conducted to evaluate the effects of dietary supplementation of *B. subtilis* on semen quality, rooster live performance, semen ionic composition, semen pH and the concentration of *Bacillus* spp. per mL of semen and per sperm. As expected, body weight, body weight gain, and feed intake were not affected by the supplementation with *B. subtilis* , since the roosters evaluated in this experiment were 74 wk of age and no longer in the growth stage. Similar to the results obtained *in vitro*, the dietary inclusion of *B. subtilis* did not alter any semen parameter evaluated, including the concentration of *Bacillus* in the ejaculate, possibly because these bacteria are already naturally present in rooster semen.

Although alternatives to AGP have been reported to improve growth performance in broilers, the supplementation of YP and *Bacillus subtilis* in the present research did not improve any of the semen quality parameters, which are crucial in determining flock fertility. In fact, the inclusion of YP linearly decreased sperm motility, likely due to a linear increase in bacteria and yeast per billion sperm because these microorganisms can negatively affect semen quality. However, *in vitro* exposure and supplementation with

Bacillus subtilis in the feed did not alter any semen parameter. Due to the ability of YP, *B. subtilis* and other alternatives to AGP to modulate intestinal microbiota and reduce the population of pathogenic bacteria in the gut, further research must be conducted to investigate if these feed additives will also alter the concentration of harmful bacteria present in the ejaculate or if they will affect overall flock fertility. It is also important to investigate the mechanisms of action of bacteria and yeast in semen that ultimately decrease semen quality. Additionally, because some pathogenic bacteria are commonly associated with foodborne diseases, assessment of the specie of bacteria present in semen in response to dietary supplementation of AGP alternatives is needed to evaluate the potential risk of bacterial transmission to hens, broiler chicks and, ultimately, humans.