12-2016

The effects of dietary probiotic inclusion on skeletal health of poultry and its possible mechanisms

Feifei Yan
Purdue University

THE EFFECTS OF DIETARY PROBIOTIC INCLUSION ON SKELETAL HEALTH OF POULTRY AND ITS POSSIBLE MECHANISMS

by

Feifei Yan

A Dissertation

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Doctor of Philosophy

Department of Animal Sciences

West Lafayette, Indiana

December 2016

THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF DISSERTATION APPROVAL

Dr. Heng-wei Cheng, Co-chair
Department of Animal Sciences
Dr. Patricia Y. Hester, Co-chair
Department of Animal Sciences
Dr. Todd J. Applegate
Department of Animal Sciences
Dr. John A. Patterson
Department of Animal Sciences
Dr. Tsang-long Lin
Department of Comparative Pathobiology

Approved by:
Dr. Ryan A. Cabot
Head of the Departmental Graduate Program

For my parents

ACKNOWLEDGMENTS

I wish to thank, first and foremost, my advisors Dr. Heng-wei Cheng and Dr. Patricia Y. Hester. Without their guidance and persistent help, this dissertation would not be possible. Also, big thanks to my committee members, Dr. Todd J. Applegate, Dr. Tsang-long Lin, and Dr. John A. Patterson, who provided good suggestions and great help in my study.

I would like to express my deep appreciate to the scientists, technicians, post doctorates, (under)graduate students, and visiting scholars in the USDA-Agricultural Research Services research unit.

In addition, it gives me great pleasure in acknowledging the support and help of the staff of the Purdue University Poultry Farm (Barry Little and Jason Fields), Dr. Richard Stroshine's lab in the College of Engineering, and Blaine Brown from the Meats Lab.

Last but not least, I am indebted to my friends here in the United States, who share their happiness and sadness with me and accompany me on my way to be a PhD.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5-HIAA	5-hydroxyindoleacetic acid
5-HT	serotonin or 5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
ACTH	adrenocorticotropic hormone
B cells	B lymphocytes
BCO	bacterial chondronecrosis with osteomyelitis
BDNF	brain derived neurotrophic factor
BMC	bone mineral content
BMD	bone mineral density
BSM	bifidus selective medium
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CORT	corticosterone
CREB	cAMP response element-binding protein
CRH	corticotropin-releasing hormone
CTX	c-terminal telopeptide of type I collagen
DA	dopamine
DEXA	dual energy x-ray absorptiometry
dNTP	deoxynucleoside triphosphates
DOPAC	3,4-Dihydroxyphenylacetic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EP	epinephrine
FGF	fibroblast growth factor
GF	germ free
H:L	heterophil to lymphocyte
HPA axis	hypothalamic-pituitary-adrenal axis
HPLC	high performance liquid chromatography

HVA	homovanillic acid
IDO	indoleamine-2,3-dioxygenase
IFN	interferons
IGF	insulin-like growth factor
LITAF	lipopolysaccharide induced TNF factor
NE	norepinephrine
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NSAIDs	non-steroidal anti-inflammatory drugs
OC	osteocalcin
OPG	osteoprotegerin
OVX	ovariectomy
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
Pi	phosphate
RANK	receptor activator of nuclear factor kappa-B
RANKL	receptor activator of nuclear factor kappa-B ligand
RIA	radioimmunoassay
RNA	ribonucleic acid
RPM	revolutions per minute
SEM	standard error of the mean
SERT	5-HT transporter
TD	tibial dyschondroplasia
TDO	tryptophan-2,3-dioxygenase
TGF	transforming growth factor
TLR	toll-like receptor
TNF	tumor necrosis factor
TPH	tryptophan hydroxylase
Treg	regulatory T cells
TRP	tryptophan

ABSTRACT

Author: Yan, Feifei. Ph.D.
Institution: Purdue University
Degree Received: December 2016
Title: The Effects of Dietary Probiotic Inclusion on Skeletal Health of Poultry and Its Possible Mechanisms.
Major Professor: Heng-wei Cheng and Patricia Hester

Probiotics are live microorganisms which may confer health benefits on the host when administered in appropriate amounts. Numerous studies have shown that probiotics improve bone health in humans and rodents with less information available on the skeletal health of avians given probiotics. The objective of this study was to determine the effects of probiotic supplementation on bone health of egg-laying and meat-type chickens.

Dietary supplementation of a multi-species based probiotic reduced the percentage of shell-less eggs beginning at 4 wk following treatment and increased tibial and femoral bone mineral density in egg-laying hens at 7 wk post-treatment. Similarly, bone mineralization accrual of the tibia and femur was improved in broilers subjected to daily cycling heating episodes fed the same probiotic for 6 wk. Broilers consuming the probiotic also had a lower incidence of lameness as characterized by an improved gait score and longer latency to lie.

The effects of a single-species based probiotic fed to broilers beginning at 1 d of age on bone health were investigated. Bone mineral densities of the tibia and femur were increased at 43 d of age. Concomitantly, serum calcium concentrations were increased at 14 d of age, serotonin levels were up-regulated in the raphe nuclei, and the catecholamine concentrations of NE and DA were down-regulated in the hypothalamus at 43 d of age. With the exception of bone mineral density, the bone health of probiotic-fed broilers was maintained under elevated temperature as indicated by larger bones and higher bone mineral content of the tibia and femur at 43 d of age accompanied by reduced plasma concentrations of TNF-α, a pro-inflammatory cytokine.

Dietary inclusion of probiotics is a useful strategy for improving skeletal health in chickens under both normal and elevated ambient temperature. Probiotics enhance intestinal absorption of nutrients like calcium and may reduce sympathetic activity, thus improving mineralization of bone. Under the condition of daily cycling heating episodes, probiotics increased bone growth and bone size of broilers perhaps via inhibition of bone resorption resulting from the down-regulation of TNF-α, thereby reducing systemic inflammation.

CHAPTER 1. LITERATURE REVIEW

1.1 Avian Bone Biology

1.1.1 Osteoporosis in egg-laying hens

1.1.1.1 Egg formation

The formation of an egg is a remarkable process, from ovulation to oviposition that takes just over 1 d. The reproductive system of a hen consists of the left ovary and oviduct, with the right side organs shrunken without functions. The ovary contains a cluster of tiny ova, each ovum within its own follicle. When the hen reaches sexual maturity, one or a few of these ova are recruited at a time into the growing pool of follicles that begin to accumulate yellow yolk (Johnson et al., 2015). The selected ovum grows rapidly and is released from the ruptured follicle into the oviduct when it reaches the right size and stage, a process called ovulation. The ovum travels down the oviduct to acquire albumen (egg white), shell membranes, and shell. The hen's oviduct can be divided into 5 sections, each of them has a specific function (Table 1.1). After a total of 24 to 26 h, an egg is ultimately formed and laid through the vent, which is called oviposition. Approximately 30 min later, the hen starts the process all over again.

Due to the daily egg laying cycle, there is an unusually high demand for Ca in laying hens. Each egg contains up to 3 g of Ca in the form of Ca carbonate (Perry and Yousef, 2013), which is nearly 10% of the total Ca of a hen (Stanford, 2006). Diet is the main source of Ca for the eggshell (Wistedt et al., 2014). Dietary Ca enters the blood stream via duodenal absorption and is taken up by the uterus; uterine absorption is increased several fold during formation of the shell (Bar, 2009; Jonchere et al., 2012). However, hens stop eating after switching off of the artificial lights, and the duodenum absorption of Ca ceases approximately 4 h afterwards according to the average gastro-intestinal transit rate (Pan and Yu, 2014). A great proportion of the eggshell Ca is deposited while the intestine lacks dietary Ca, indicating that there is a secondary Ca source for eggshell formation in laying hen - the bone. During the later hours of darkness bone, as the primary Ca source, comprises as much as 20 to 40% of the shell Ca (Johnson, 2015).

Table 1.1. Functions of different sections of a hen's oviduct

The sections of the oviduct	Approximate time an egg spends in each section	Functions of each section of the oviduct
Funnel (Infundibulum)	15 min	Receives ovum (yolk) from the ovary. If living sperm are present, fertilization occurs here (commercially produced table eggs are generally not fertilized)
Magnum	3 h	Albumen is secreted and layered around the yolk
Isthmus	1 h 15 min	Inner and outer shell membranes are added along with some water and mineral salts
Shell gland (Uterus)	19 to 21 h	Initially add some water to make the outer white thinner; then the shell material (mainly Ca carbonate) is added. Pigments may also be added followed by cuticle.
Vagina/cloaca	Few min	The egg passes through this section before laying

1.1.1.2 Bone turnover

Osteoblasts, osteoclasts and osteocytes are the most well-known bone cells. Osteoblasts, largely known for their bone forming function, are located along the bone surface and comprise 4 to 6% of the total bone cells (Capulli et al., 2014). Osteoclasts are multinucleated cells that are responsible for bone resorption. Osteocytes comprise 90 to 95% of the total bone cells. Unlike osteoblasts and osteoclasts, it is not until recently that osteocytes have been recognized for playing important functions in bone health rather than serving as passive placeholders (Rochefort et al., 2010; Bonewald, 2011; Chen et al., 2015; Goldring, 2015b). Distributed throughout the mineralized bone matrix, osteocytes form an interconnected network, where osteocytes sense and respond to local and systemic stimuli, then secrete several molecules, including sclerostin, RANKL and FGF23, to regulate osteoblastic bone formation, osteoclastic bone resorption, and mineral homeostasis.

There are four types of bone in a laying hen: cortical bone, trabecular bone, medullary bone, and pneumatic bone. Cortical and trabecular bones, also called lamellar bone, provide the structural support. Medullary bone, also called woven bone, is unique to an adult avian female. Pneumatic bone is hollow and acts functionally as a part of the avian respiratory system.

Bone growth of laying hens, similar to mammals, during the rearing period (pullet phase) relies on 2 processes: growing longitudinally by endochondral ossification and widely by intramembranous ossification (Whitehead, 2004). Upon reaching sexual maturity, a dramatic change takes place in the physiology and bone biology of laying hens. The concentrations of estrogen markedly increase and shift the function of osteoblasts from forming lamellar structural bone to medullary bone (Whitehead and Fleming, 2000), accompanying with the maturation of the ovarian follicles. Unique to birds (Schweitzer et al., 2007) and dinosaurs, the medullary bone is intended to become a labile source of Ca for eggshell formation (Whitehead, 2004). Medullary bone is laid down along the inner surfaces of structural bone and formed in spicules within the marrow cavities. The highest content of medullary bone is found in the femur and tibia bones. Characterized by the haphazard organization of collagen fibers in the matrix, medullary bone may provide some contributions to the overall bone strength (Fleming et

al., 1998), but is mechanically weaker than structural bone, resulting from its mainly existing form as isolated spicules (Whitehead, 2004).

With a labile nature, medullary bone undergoes a dynamic remodeling after formation. Rapid and sequential changes, from resorption to formation, occur according to egg-formation cycles (Thorp, 1994). At night, when dietary Ca is not sufficient for eggshell formation, activated osteoclasts resorb medullary bone to release stored Ca. The resorbed medullary bone is soon replaced by osteoblasts formed organic matrix with low Ca. During daytime, when adequate Ca is ingested and absorbed from the intestine, medullary bone is further calcified, with a low bone resorption and formation rate (Dacke et al., 1993). When hens go out of lay, such as during the period of molting, medullary bone is gradually resorbed and structural bone formation is recommenced. Post molting, medullary bone is rapidly regenerated after hens regain capability to lay eggs, and the daily bone cycling is restarted.

1.1.1.3 Osteoporosis and bone fracture

Osteoporosis is a widespread welfare issue in laying hens. As in humans, osteoporosis in laying hen is characterized by a progressive loss of structural bone, leading to skeletal fragility and susceptibility to fracture (Whitehead, 2004).

Osteoporosis is caused by an imbalance in bone remodeling between bone formation (osteoblasts) and resorption (osteoclasts) under the influence of estrogen. Structural bone formation ceases as estrogens favor medullary bone deposition, which means the peak structural bone mass is reached at the onset of follicular activity. The hypothesis is supported by the fact that the initial formation of medullary bone coincides with a marked reduction in structural bone volume. On the other hand, when medullary bone undergoes high rate of bone resorption as a source of eggshell Ca, at the same time, structural bone resorption also occurs as activated osteoclasts is not targeting medullary bone only (Whitehead, 2004). The turnover of structural bone is slow but continuous. Consequently, the negative balance between the processes of bone formation and resorption of structural bone over the course of the production cycle eventually culminates into osteoporosis and skeletal weakness (Whitehead and Fleming, 2000). The

bone damage becomes most severe at the end of lay at about 68 to 72 wk of age (Whitehead and Fleming, 2000; Beck and Hansen, 2004).

Fractures are common in laying hens regardless of the housing system (Wilkins et al., 2011). High rates of fracture prevalence have been reported worldwide, with a pattern of increase by age (Gebhardt-Henrich and Frohlich, 2015; Petrik et al., 2015; Toscano et al., 2015). Referred to as either old (those occurred during the laying period) or new (those occurred during depopulation, transportation, or slaughter), fracture can occur at the any location of the whole skeleton but the highest incidences are at ischium, humerus, keel, and furcular (Bishop et al., 2000; Whitehead, 2002). Osteoporosis induced bone weakness is one of the main factors that determine fracture incidence. The other two are the housing design and handling at depopulation. As osteoporosis in hens is gradually developed and is worse at the end of their laying cycle, new fractures may be a better example of osteoporosis-associated bone damage. Improper handling also contributes to new bone fracture, as the number of fracture increased with sequential handling events (Gregory and Wilkins, 1989). However, high incidence of broken bones is still reported when better procedures for handling are applied (Gregory et al., 1992; Gregory et al., 1993), indicating bone weakness is an important contributor.

1.1.1.4 Animal welfare and economic effects

Osteoporosis-associated animal welfare issues have drawn great awareness by the public. Osteoporosis induces a high incidence of bone fracture, which is painful in mammals (Koewler et al., 2007). It is generally accepted that birds perceive pain similarly to mammals, as various nociceptors have been identified and characterized in many body parts of a chicken such as the beak, mouth, nose, joint capsule and scaly skin (Gentle, 2011). For instance, A-delta mechanothermal nociceptors, responding to both thermal and mechanical stimulations, have been identified in the scaly skin of adult chickens' legs (Gentle et al., 2001). Hens with keel bone fractures exhibit reduced mobility (Nasr et al., 2012b; Nasr et al., 2015), which resulted from either physical impairment or the feeling of pain. Administration of butorphanol, a kappa opioid agonist that works as an analgesic, improves mobility in hens with keel fracture as indicated by reduced landing time from perches with multiple heights (Nasr et al., 2012a). In a

preference test, hens previously treated with butorphanol or saline in environments marked with different colors, hens preferred the environment with the same color where they experienced butorphanol. The positive correlations were received from the fractured birds only (Nasr et al., 2013). These results support the concept that hens with fractures have the capacity to feel pain and the painful feeling in turn reduces their mobility. To be noted, pathological examinations conducted in these two studies indicated that the keel fractures had healed already, which indicates that old keel fracture is still a source of chronic pain in hens.

Another welfare problem associated with osteoporosis in laying hens is that hens die from this non-infectious disease. It is estimated that osteoporosis contributes to approximately 20 to 35% of all mortality during the egg production cycle of caged hens (McCoy et al., 1996; Anderson, 2002).

Osteoporosis and its related bone fracture cause considerable economic loss to the poultry industry. Spent hens, i.e. hens who are past their prime egg laying years, were usually slaughtered and used in processed foods (such as chicken soup) or rendered as pet foods. But, bone fragments from fractures can penetrate the meat and induce quality and safety problems. As a result, most soup-based companies have stopped using meat from spent hens and use broiler meat instead. The USDA also reduced the purchase of spent hen as a food resource, from 30% of all spent hens processed nationwide in 2006 to less than 10% in 2009. The value of spend hens could be raised if the market could be regained or partly regained due to decreased bone splinters.

1.1.1.5 Current methods to improve skeletal health

1.1.1.5.1 Housing systems

Osteoporosis is most prevalent in caged layers (Whitehead and Fleming, 2000), indicating that osteoporosis is affected by housing environment. Compared to conventional cages, furnished cages with perches, nest boxes, and/or a raised dust bath increased tibial and femoral cortical bone mass and reduced trabecular space in 65-wk old hens (Jendral et al., 2008). Perch enrichment alone also provides benefits to bone health, increasing tibial trabecular bone volume (Hughes et al., 1993) and mineral density of wing bones, leg bones, and keel bone (Hester et al., 2013). In addition, a number of

studies suggest cage free systems, such as floor pen and aviary, contribute to higher cortical bone density and bone strength of hens when compared to cage systems (Silversides et al., 2012; Regmi et al., 2015; Regmi et al., 2016). It has become increasingly clear that the enhanced activity or movement of laying hen is the key component that improves bone quality in the alternative housing systems (Lanyon, 1992). The improvement of humerus strength, for example, is particularly apparent in the systems that allow hens to fly. Thus, alternative housing systems are widely considered as a potential means of alleviating osteoporosis.

However, improper design of the extensive housing facility may induce novel welfare problems, such as keel fracture, resulting from collisions with internal housing structures (Toscano et al., 2013). For instance, 73% of hens in a perch system were reported to have incurred old breaks (Freire et al., 2003), and the incidence was positively correlated with perch height (Wilkins et al., 2011). Therefore, further studies are needed to investigate the appropriate design of alternative systems to improve hens' bone quality as well as their welfare. A recent study indicated soft perches might be a promising tool for reducing keel bone damage in these loose systems while maintaining activity levels (Stratmann et al., 2015).

1.1.1.5.2 Nutrition

Nutritional intervention has been considered to play a limited role in minimizing the deleterious effects of osteoporosis in laying hens.

Results from a previous study that investigated the effects of various dietary factors (including oystershell, fluoride, 1,25-dihydroxycholecalciferol, ascorbic acid, a lower concentration of P, and a combination of a lower concentration of crude protein and higher concentration of vitamin K) on bone composition during the laying period showed that nutritional supply did not prevent the development of osteoporosis, except oystershell with Ca in large particle form had some beneficial effects on eggshell quality and medullary bone formation (Rennie et al., 1997). The results from another study indicated that addition of vitamin K3 alone or together with sodium fluoride and limestone promoted proximal tarsometatarsus cancellous bone volumes at 15, 25, and 70 wk of age (Fleming et al., 2003). These different results suggest that timing of dietary

administration is one of the key factors of nutritional intervention, as the initiated dietary supplementation of the former study was started when hens were 16-wk-old, whereas in the latter study it was started with 1-d-old chicks. Adequate dietary inclusion to meet nutrient requirement for bone growth during rearing and pre-lay period is critical to maximize the peak structural bone mass. Increased Ca content in pre-lay and laying diet has been showed to reduce the osteoporosis incidence in caged hens (Mayeda and Ernst, 2008).

Some pharmacological intervention used in humans to combat osteoporosis were also tested in laying hens. Bisphosphonate and strontium, for example, acting by inhibiting bone resorption and/or enhancing bone formation, have been shown to increase structural bone volume in hens (Thorp et al., 1993; Shahnazari et al., 2006). However, the use of treating human osteoporosis drugs is unlikely to be a practical solution for reducing or preventing osteoporosis in laying hens.

1.1.1.5.3 Genetic selection

Bone development has strong genetic components; and bone traits are inheritable (Sparke et al., 2002). Genetic selection based on bone strength index exhibited divergent bone characteristics over the first 3 generations and resulted in lines of hens with high or low bone strength; between the lines the difference was 2 fold (Bishop et al., 2000). Meanwhile, no difference in BW was reported between the 2 lines. The difference of bone strength was mostly attributed to higher structural bone mass, resulting from greater bone formation during the pullet rearing and less bone resorption during egg laying. Greater crosslinking in the collagen matrix, an indicator of a better bone quality, may also contribute to the improved bone strength in the high line (Sparke et al., 2002). Compared to low line hens, the high line hens had fewer of osteoclasts (Whitehead, 2004), higher bone mineral density (Fleming et al., 2006; Stratmann et al., 2016), and lower prevalence of keel bone deviations and fractures (Fleming et al., 2004, 2006). The effect of selection on egg production and eggshell quality varied among studies, either impaired (Whitehead, 2004; Stratmann et al., 2016) or unaffected (Fleming et al., 2006).

In summary, environment (housing facility), nutrition, and genetics all have independent effects on bone health in laying hen with genetic manipulation being most

effective, environment secondary, and nutrition last (Fleming et al., 2006). An approach that considers all 3 components may be the best potential solution for reducing osteoporosis in laying hens.

1.1.2 Lameness in meat-type broilers

1.1.2.1 Rapid growth

Poultry is one of the most popular animals as a food source over the world. In the U.S. alone, more than 9 billion broilers were hatched and reared in 2015 (NASS, 2016). In the past century and especially the last several decades, broilers have been successfully selected for a short growth cycle plus high meat yield in particular breast muscle. Studies indicated that within approximately 50 years (from 1957 to 2001), broiler growth rate increased by about 400% during an 84 d rearing period (Figure 1.1). With the continued selection for increase market BW, the rearing period was also significantly reduced. For example, the time required to reach a 2.5 lb. live weight was 112 d in 1925, whereas the time required for a 6.2 lb. live weight was 48 d in 2015 (Figure 1.2). However, the selection program also makes the broilers more susceptible to metabolic disorders and leg abnormalities that lead to poor locomotion.

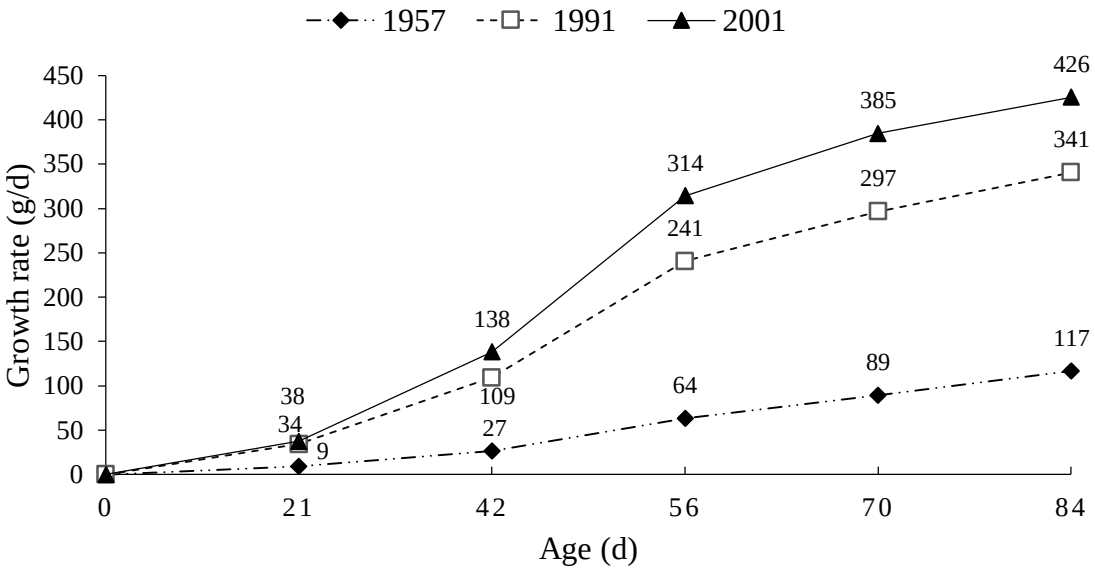


Figure 1.1 A comparison of male broiler BW at 84 d of age in 1957, 1991, and 2001 (Havenstein et al., 1994, 2003)

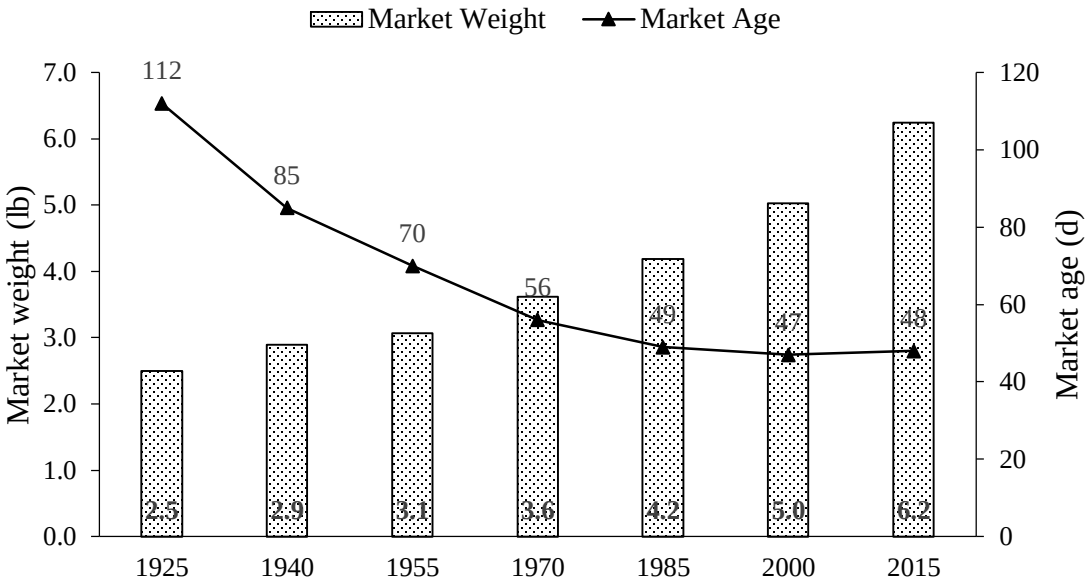


Figure 1.2 United States broiler market BW and market age from 1925 to 2015

Data source: http://www.nationalchickencouncil.org/about-the-industry/statistics/u-s-broiler-performance/

1.1.2.2 Lameness

Predominantly affecting the locomotor system and resulting in impaired mobility or lameness, leg disorders have become a considerable problem in commercial poultry meat production. Leg disorders in broilers could be cause by multiple factors such as abnormal bone development, infections, and degenerative diseases (European Commission, 2000). Developmental disorders of the skeletal system include 1) bone deformity, such as angular deformity, as either outward (more common but rarely severe) or inward (less common but more severe) angulation of the limbs, and rotational deformity (rotation of the shaft of the tibia); and 2) TD, a common lesion consisting of a cartilaginous plug in the growth plate of broiler leg bones that can lead to angular deformity when severe. Infectious disorders include 1) BCO, a severe degenerative disorder in which the cartilaginous epiphysis separates from the metaphysis; 2) synovitis, characterized by inflammation of joints; and 3) tenosynovitis that involves inflammation of the tendons. Degenerative disorders, such as osteoarthrosis in the hip joints, are more prevalent in old broiler breeders.

A common method for evaluating leg problems is assessing lameness. Two gait scoring systems have been developed to rate the lameness of broilers, either the 0 to 5 scale (Kestin et al., 1992; Garner et al., 2002) or the 0 to 2 scale gait score (Webster et al., 2008), with the lower score indicating better leg health. For example, over 27.6% of broilers are estimated to exhibit poor locomotion (gait score > 2 in the 5 point score system) in the United Kingdom (Knowles et al., 2008), with a range between 14.1% to 30.1% in other European countries (Sanotra et al., 2001; Sanotra et al., 2003). Latency to lie test is another method commonly used to assess lameness. This test was developed by Weeks et al. (2002) based on the fact that broilers are aversive to contact with water and was modified to be simpler and more effective by Berg and Sanotra (2003). Latencies to lie test is highly and negatively correlated to gait score (Weeks et al., 2002; Berg and Sanotra, 2003).

A relationship exists between the degree of lameness and the type of leg disorder. Pathohistological studies reported that broilers with gait scores of 4 or 5 had a high possibility of being diagnosed with BCO, whereas broilers with lower scores had TD or tibial angulation (McNamee et al., 1998; Vestergaard and Sanotra, 1999). In a survey

conducted in the United Kingdom, 67.8% and 3.3% of broilers had a gait score 2 to 3 and 4 to 5, respectively (Knowles et al., 2008), suggesting that developmental, non-infectious disorders of TD and bone angulation may be the leading cause of lameness in commercial broiler flocks (Dinev et al., 2012). Similarly, high incidences of TD have been reported in Denmark (57%) and Sweden (Sanotra et al., 2003), with a small variation among commercial broiler lines (Dinev et al., 2012).

Evidence suggests that rapid growth is a key factor contributing to lameness (Rath et al., 2000; Talaty et al., 2009). Supporting data came from a study examining 13 broiler strains (Kestin et al., 2001). They reported that slower-growing broilers had a lower incidence of lameness compared to fast-growing strains, as the mean difference of gait score was over 1 using the 5-point gait score system. In addition, when adjusted to BW, fast-growing broilers had lower tibia density and percentage of bone ash than slow-growing broilers (Shim et al., 2012) as a consequence of less bone mineralization and higher porosity (Williams et al., 2004). Importantly, leg abnormalities could be reduced in the fast-growing broilers via restricting growth to the level similar to slow-growing broilers (Williams et al., 2004). A fast growth rate generally comes with reduced locomotor activity and prolonged sitting or lying (Bessei, 2006). Behavioral studies proved that slow-growing broilers were more active than fast-growing broilers. The slow-growing broilers exhibited more pecking, perching, scratching, and walking and were able to walk much longer distances (Bokkers and Koene, 2003; Reiter and Bessei, 2009). The lack of activity may further worsen the condition of legs as mechanical loading is essential for normal bone formation.

Selection for increased breast meat yield in broilers and turkeys has resulted in uneven distribution of skeletal muscle. Redistribution of BW towards more breast muscle deposition relative to leg muscle may impair the walking ability of heavy strains of turkeys (Nestor et al., 1985, 1987; Nestor and Emmerson, 1990) and broilers. Less muscle on leg bones may decrease mechanical load making bones weaker and less dense.

1.1.2.3 Animal welfare and economic effects

The welfare impacts of lameness in broilers include pain, discomfort and poor locomotion. Do lame broilers experience pain? In a preference test, lame broilers

selectively consumed more feed mixed with carprofen, a non-steroidal anti-inflammatory drug, than control broilers, and the amount of carprofen-spiked feed they consumed was parallel with the severity of lameness (Danbury et al., 2000). In addition, the administration of carprofen caused lame broilers to complete a mobility test faster but not control broilers (McGeown et al., 1999). The administration of carprofen or meloxicam, also a non-steroidal anti-inflammatory drug, improved gait function in moderately lame broilers as measured through kinematic analysis using a commercial motion-capturing system (Caplen et al., 2013b). However, Siegel et al. (2011) failed to observe that lamed broilers would self-select a carprofen-mixed diet, questioning the suitability of a broiler self-selection paradigm. To corroborate the analgesic effect of non-steroidal anti-inflammatory drugs, Hothersall et al. (2011) developed a method to measure thermal nociceptive threshold, a useful indicator of the perception and processing of noxious stimuli underlying pain status. Thermal nociceptive thresholds were decreased in broilers with experimentally induced articular pain and naturally obtained lameness; the threshold was reversed following the administration of carprofen or meloxicam (Caplen et al., 2013a; Hothersall et al., 2014).

Immobility has obvious welfare implications. Lame broilers, failing to reach bell drinkers that were raised 400 mm above the litter, became dehydrated as indicated by increased plasma osmolality (Butterworth et al., 2002). Reduced BW was also commonly related to the presence of severe lameness in broilers due to their inability to reach the feeders. Moreover, broilers with lameness are more susceptible to developing breast blisters, footpad dermatitis, hock burns, or combinations thereof, most likely because they spent more time lying in the litter and they can be stepped on by other broilers.

Lameness is not only a welfare issue but also an economic issue. It is costly to the poultry industry. Parallel with the high morbidity, lameness-associated starvation and dehydration is one of the leading causes of mortality in broilers. Broilers with severe lameness are culled.

1.1.2.4 Current methods to reduce lameness

1.1.2.4.1 Management

Numerous studies have demonstrated that leg health in broilers can be improved by management. For example, the early application of UV lights improves tibia bone strength, radiographic density, and ash percentage in broilers fed a diet with a Ca and P imbalance and low in vitamin D (Fleming, 2008). Whether or not broilers consuming normal diets would benefit from UV exposure to stimulate vitamin D synthesis was not evaluated (Fleming, 2008).

Prenatal management may also play a role as broiler leg health is affected by the temperature of the incubator during embryonic development. For example, either cooling or overheating hatching eggs during early embryonic development (0 to 8 d) induced a high prevalence of TD in broilers at 49 d post-hatch (Yalcin et al., 2007). A meta-analysis of 8 incubation experiments developed a model showing benefits to delaying hatch so as to improve broiler leg strength. Rather than using the standard egg shell temperature of 37.8 C, the model recommended lowering and raising incubation temperature during early to mid (1 t o15 d) and late (16-18 d) embryogenesis, respectively, because later hatching chicks were able to stand longer at 6 wk of age as measured through the latency to lie test (Groves and Muir, 2014).

1.1.2.4.2 Nutrition

Recent studies have shown the importance of nutrition on broiler leg health. Proper supplementation of Ca, P, and vitamin D are effective in the prevention or alleviation of TD (Parkinson and Cransberg, 2004; Khan et al., 2010; Bachmann et al., 2013) and BCO (Wideman et al., 2015b). The essential fatty acids, α-linolenic acid (ω -3) and linoleic acid (ω -6), have beneficial effects on various bone characteristics (McCormack et al., 2006). Probiotics reduce BCO induced lameness in broilers reared in pens with wire floors (Wideman et al., 2012; Wideman et al., 2015a).

1.1.2.4.3 Genetic influence

Breeding companies top selection criteria for broilers are rapid growth and efficient feed conversion. Of the 12 traits used by breeding companies in their selection

criteria, leg disorders are ranked 9th (Hardiman, 1996). Leg health is a heritable trait (Sheridan et al., 1978; Mercer and Hill, 1984; Bihan-Duval et al., 1997). The prevalence of long bone deformities and TD in broilers was reduced by 0.6 to 0.9% and 0.4 to 1.2% per yr, respectively, after 25 yr of selection (Kapell et al., 2012). Although a negative correlation exists between growth and leg health, it is of low magnitude (Rekaya et al., 2013; Gonzalez-Ceron et al., 2015). Therefore, breeding companies could place a higher priority on selecting broilers with stronger legs with minimal effects on market BW and carcass traits.

In summary, simultaneous genetic improvement in leg soundness, innovative incubation and husbandry practices, and proper nutrition including dietary supplementation will improve broiler leg health and welfare with little impact on production efficiency.

1.2 Probiotics Regulate Bone Health

1.2.1 Gut microbiota

Gut microbiota, the ecological community of commensal microorganisms, lives in host's body (Rosenbaum et al., 2015). The term "microbiome" means the combined genetic material of the microbiotas in a particular environment like the gut (Giorgetti et al., 2015), which is about 150 times larger than the entire human genome (Rosenbaum et al., 2015). Gut microbiota is primarily composed of 5 microbial phyla of *Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria*, and *Fusobacteria* with the first 2 representing over 90% of the total adult human gut microbiota (Rajilic-Stojanovic et al., 2007). The top 5 bacterial phyla in chicken cecum are *Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria* and *Verrucomicrobi* with the first 3 representing approximately 97% of the total environmental gene tags (Qu et al., 2008). Gut microbiota varies by age in animals, not only in the total number of bacteria, but also in the diversity of microbial taxa (Eckburg et al., 2005; Wise and Siragusa, 2007).

Expanding evidence supports the view that the gut microbiota contributes to host health (Marchesi et al., 2016). Microbiota, dietary nutrients, and host cells interact extensively, comprising an extremely complex ecosystem. Symbiosis of the gut

microbiota can maintain a normal physiological homeostasis in the host, whereas any bacterial unbalance, called dysbiosis, may cause disease. As a result, enthusiasm to modify the gut microbiota in a beneficial direction has grown substantially.

Gut microbiota is regulated by multiple factors such as diet, environment, and the health status of the host with diet in the predominate role (David et al., 2014). For example, humans consuming either a plant- or meat-based diet exhibited distinct profiles of the gut microbiota composition (David et al., 2014). Dietary fiber, as a specific diet component, can modify gut composition via promoting the growth of a small number of taxa (Simpson and Campbell, 2015). A number of dietary interventions can modulate either the composition or activity of bacteria. Probiotics, prebiotics, synbiotics, and antibiotics are among the most well established dietary supplements influencing gut microbiota. Moreover, gut microbiota is regulated by hosts, such that human genetics shape the gut microbiome (Goodrich et al., 2014). The environment can also influence gut microbiota. An example in poultry showed that broilers had different dominant ileal mucosal microbiome when reared on fresh litter as compared to reused litter (Cressman et al., 2010).

1.2.2 Probiotics

The word “probiotic,” derived from the Greek word for life “bios”, was firstly used in the middle of the last century as a result of observing beneficial influence of certain microorganisms on the intestinal flora (Lilly and Stillwell, 1965). Thereafter, the term was better specified due to scientific discoveries (Schrezenmeir and de Vrese, 2001) and is now defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2001). Unfortunately, many products in the market place use the term "probiotic" without meeting the specified criteria (Hill et al., 2014).

Prebiotics are a non-digestible food ingredient that are often combined with probiotics as they facilitate the growth of beneficial microorganisms in the gut. When prebiotics and probiotics are used in combination, they are often referred to as synbiotics because of their synergism. In contrast, antibiotics when given to animals destroy or inhibit the growth of bacteria. Because antibiotic resistance has become a public health

issue, the broiler and turkey industries are gradually eliminating the use of low subtherapeutic dosages of antibiotics as a growth promotor and safety net for infection. Probiotics may serve as a possible alternative.

Probiotics modulate microbiota (Uchiyama-Tanaka, 2014) by promoting symbiotic and inhibiting pathogenic microorganisms. Benefits of using probiotics in humans include alleviation of allergies due to food or inhalants (Cuello-Garcia et al., 2015), prevention of diarrhea (Guarino et al., 2015) and infection (Araujo et al., 2015; Schwenger et al., 2015), reduction of blood pressure (Khalesi et al., 2014), modification of metabolic diseases (Le Barz et al., 2015) and cancer (Redman et al., 2014), and promoting weight loss in obese patients (Park and Bae, 2015).

The wide array of mechanisms underlying probiotic effects are summarized in Figure 1.3 (Hill et al., 2014). One widespread function of probiotics is pathogen inhibition due to the synthesis and release of 1) broad-spectrum inhibitors known as bacteriocins (Messaoudi et al., 2012); 2) metabolites, such as SCFA, that decreases pH inhibiting bacterial growth (Van Immerseel et al., 2006); and 3) biosurfactants that possess antimicrobial activity (Madhu and Prapulla, 2014) and inhibit pathogen adhesion (Chapman et al., 2014). Through competitive exclusion, probiotics compete with gastrointestinal pathogens for binding sites and nutrients (Gielda and DiRita, 2012; Lawley and Walker, 2013). Less common strain-specific effects of probiotics on host include modulation of the immune and central nervous system and antioxidant effects (Giorgetti et al., 2015; Mishra et al., 2015; Wang et al., 2016).

The most commonly used probiotics in humans are dairy lactic acid bacteria, including *Lactobacillus* and *Bifidobacterium* (Soccol et al., 2010). Other common species are dairy *Propionibacteria*, yeasts (*Saccharomyces boulardii*), *Bacillus*, and the gram-negative *Escherichia coli* strain Nissle 1917 (Gareau et al., 2010). In livestock and poultry, species of *Bacillus*, *Enterococcus*, and *Saccharomyces* yeast are the most common microorganisms used, with an increasing use of *Lactobacillus* strains in the last 2 decades (Patterson and Burkholder, 2003; Sornplang and Piyadeatsoontorn, 2016).

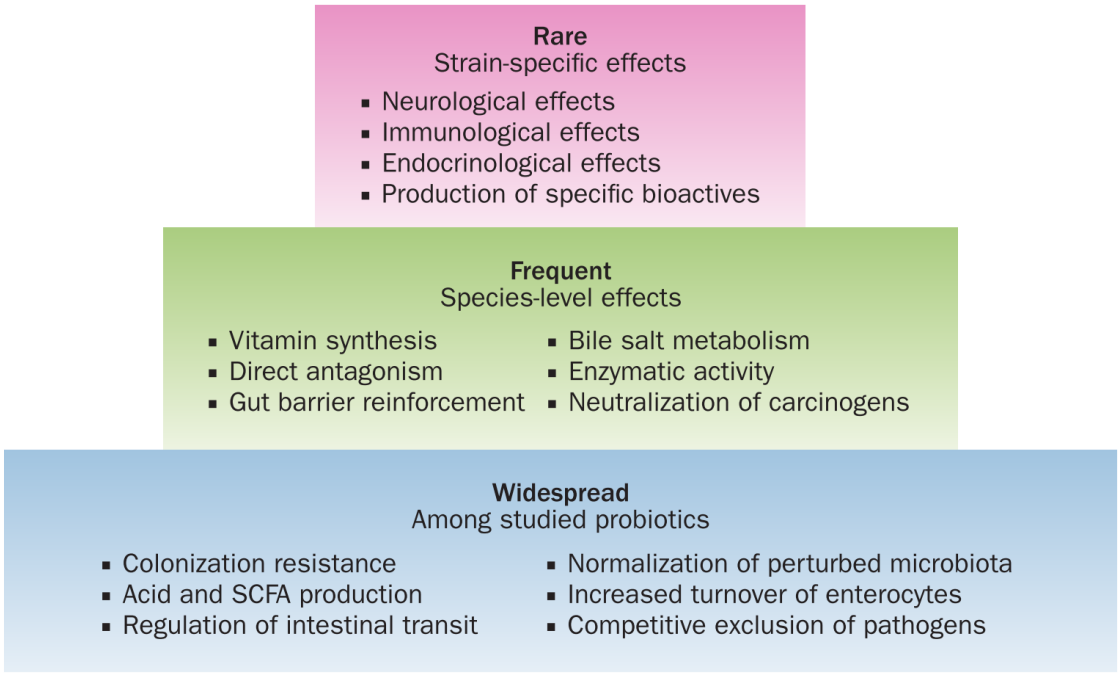


Figure 1.3. Mechanisms of action of probiotics (Hill et al., 2014)

Starting from the base of the diagram, many effects of probiotics are common and widespread among strains. Other mechanisms of action are less common, but occur frequently within species of probiotic. Other modes of action of probiotics are very strain specific and are therefore categorized as rare effects.

1.2.3 Probiotics effects on bone health

1.2.3.1 In various animals

1.2.3.1.1 Rodents

The beneficial effects of probiotics on bone health have been extensively investigated in rats and mice. Various strains of probiotics increase bone mass density in both healthy and experimentally induced diseased rodents.

Providing infant formula containing *Bifidobacterirum bifidum* and *Bifidobacterium longum* to 3-wk-old weaning rats for 30 d increased the Ca content in both the tibia and femur, but there were no effects on bone ash weight and P content (Pérez-Conesa et al., 2007). Daily intake of *Bifidobacterium longum* for 28 d increased Ca, P, and Mg content in the tibia of rats of unknown age (Rodrigues et al., 2012). Growing mice consuming *Lactobacillus helveticus*-fermented milk had increased BMD (Narva et al., 2004a). The effect of probiotic on bone can be gender dependent. After receiving *Lactobacillus reuteri* orally by gavage for 4 wk, male mice, but not females, had enhanced trabecular bone traits in both distal femur and lumbar vertebrae. In addition, higher serum OC concentrations and greater bone formation occurred in male but not female mice. The authors proposed that *Lactobacillus reuteri* may have activated sex hormones related pathways to regulate bone status in male mice but not in females as the pathways in the later ones may have been activated already (McCabe et al., 2013).

Ovariectomy (OVX) is surgical removal 1 or both ovaries culminating in loss of estrogen and progesterone production, ultimately inducing bone loss. Thus OVX, as an animal model, resembles post-menopausal osteoporosis in humans. Consumption of single *Lactobacillus* strain or a mixture of 3 strains of *Lactobacillus* starting 2 wk before OVX for a total of 6 wk protected mice from cortical bone loss and bone resorption (Ohlsson et al., 2014). Dietary supplementation with *Lactobacillus rhamnosus* GG or VSL#3 (a commercial probiotic for humans with 8 species of live bacteria) in OVX mice for 4 wk post-surgery improved trabecular femur and spine traits as compared to vehicle-treated OVX mice, and the outcomes were even comparable to those of vehicle-treated sham mice (Li et al., 2016). Serum concentrations of CTX and OC were further measured to assess bone turnover. Consumption of *Lactobacillus rhamnosus* GG or VSL#3 not

only inhibited OVX induced bone resorption, but also promoted bone formation, indicating both anti-catabolic effects and anabolic effects of probiotics on bone, respectively. Moreover, the bone formation promoting role by either *Lactobacillus rhamnosus* GG or VSL#3 was maintained in sham-operate mice, which was opposite the results from McCabe et al. (2013). Similar results were reported by others when probiotics were administered to rodents following OVX (Kim et al., 2009a; Chiang and Pan, 2011; Britton et al., 2014; Parvaneh et al., 2015).

Bone is adversely affected by inflammation related diseases due to the close relationship between the skeletal and the immune systems (Crotti et al., 2015; Goldring, 2015a). Loss of bone mass is commonly reported with inflammatory diseases regardless of cause (Hardy and Cooper, 2009), such as inflammatory bowel disease (Hisamatsu et al., 2015; Straub et al., 2015), rheumatoid arthritis (Engdahl et al., 2013; Li et al., 2015; Ornbjerg et al., 2015; Krishnamurthy et al., 2016), and periodontal disease (Izawa et al., 2014; Lin et al., 2015). Probiotic treatment might be a promising intervention for preventing or reducing inflammation-induced bone loss. For example, a *Bacillus* species based probiotic was used in a study dealing with experimentally induced periodontitis, either as a mono-treatment or as an adjunct to the standard therapy of scaling and root planing (Messora et al., 2016). Scaling and root planing remove dental plaque that can cause inflammation. When used alone, the probiotic treatment effectively inhibited periodontitis-induced bone loss, as indicated by increased bone volume and decreased bone porosity as compared to controls. In addition, probiotic treatment reduced the production of RANK and promoted OPG synthesis in rats with experimental periodontitis, resulting in a moderate RANKL/OPG ratio that was comparable to the control. These results were in line with previous studies that demonstrated the bone protective effects of probiotics in the periodontitis model by using the same or different probiotic strains (Messora et al., 2013; Foureaux Rde et al., 2014; Maekawa and Hajishengallis, 2014). When combined with scaling and root planing, providing probiotics reduced the number of active osteoclasts compared to rats with standard therapy alone, concomitant with decreased immunolabeling of IL-1β, a pro-inflammatory cytokine, and increased immunolabeling of IL-10, an anti-inflammatory cytokine, in the hemimandibles. The adjunct role of probiotics was confirmed in another periodontal

study (Garcia et al., 2016). Compared to negative controls, probiotic plus the standard therapy showed reduction in alveolar bone loss as well as levels of pro-inflammatory cytokines of TNF-α and IL-1β. However, these results were not evident when the comparison was made between the negative control group and the standard therapy of scaling and root planing.

In the previously mentioned studies, McCabe et al. (2013) failed to find a probiotic effect on bone of healthy female mice but did find a positive effect of probiotics in the bones of OVX mice (Britton et al., 2014). These same investigators further conducted an additional study in intact female mice with a mild inflammatory state induced by dorsal surgical incision (Collins et al., 2016). Again, oral supplementation of *Lactobacillus reuteri* did not show an improvement of bone in non-inflammatory mice. However, an increase in trabecular bone volume fraction and trabecular number, together with cortical bone width and area, were identified in the femur of inflammatory mice treated with *Lactobacillus reuteri* compared to both inflammatory mice without probiotic treatment and control mice. These results indicated that *Lactobacillus reuteri* may only show beneficial effects on bone health in female mice with an elevated inflammatory status, probably due to the outstanding anti-inflammatory characteristics of *Lactobacillus reuteri*. As expected, *Lactobacillus reuteri* supplementation altered intestinal cytokine gene expression in a region-dependent manner. However, this study did not measure cytokine levels in the systemic circulation and bone marrow nor were changes of bone remodeling markers reported, which may have facilitated interpretation of probiotic-based mechanisms.

1.2.3.1.2 Poultry

There are several studies conducted in poultry indicating the bone promoting effects of probiotics (Table 1.2). Most studies were in broilers, with one study in laying hens. Interestingly, all of these studies focused on the tibia, which is probably due to the fact that the tibia is one of the bones that are prone to skeletal diseases in both broilers and laying hens.

In broilers, the improved tibia indexes post probiotics supplementation included tibial weight, size, wall thickness, tibiotarsal index, ash content, ash Ca and P percentage,

and breaking strength. The probiotics used in these studies ranged from *Lactobacillus* to *Bacillus,* from single strain to mixed strains. Furthermore, probiotics also showed their bone protecting benefits under various challenging conditions. A *Salmonella enteritidis* challenge was known to not only reduce broiler performance and survivability, but also decrease bone health (Sadeghi, 2014). The supplementation of *Bacillus subtilis* in the basal diet protected tibia bone ash content and ash Ca content in broilers at 21 but not at 42 d of age (Sadeghi, 2014). Dietary inclusion of *Bacillus subtilis* and *Clostridium butyricum* for 21 d completely overcame the negative effects of a low Ca diet on bone as indicated by tibial density and breaking strength (Houshmand et al., 2011). Interestingly, broiler tibia ash content and ash minerals were also improved by a multifunctional transgenic *Lactobacillus,* which is functionally able to degrade β-glucan and phytic acid (Wang et al., 2014).

The only study in laying hens that evaluated bone health used Lohman Selected Leghorns from 64 to 73 wk of age that consumed either 0.5g or 1.0g of *Bacillus subtilis*/kg of feed for 10 wk. These hens experienced enhanced tibial traits including weight, length, volume, density, and ash percentage. The 2 probiotic doses showed similar beneficial effects, with the higher dose demonstrating superiority in tibia weight and density. Concomitant improvements were exhibited in egg related measures such as egg production, eggshell weight, and eggshell thickness. Moreover, the authors pointed out that the average time required for *Bacillus subtilis* to show a significant improvement on egg production was 3 and 6 wk for the higher and lower dose of probiotic, respectively. A reduction in unmarketable eggs also occurred as result of consuming the *Bacillus subtilis* probiotic (Abdelqader et al., 2013b). Similar decreases in unmarketable and shell-less eggs using a wide array of probiotics fed at different ages with multiple strains of laying hens have been reported (Kurtoglu et al., 2004; Mikulski et al., 2012; Zhang et al., 2012; Abdelqader et al., 2013a).

Several studies have reported improvements in shell traits such as thickness, strength, density, weight, etc., as a result of feeding probiotics for 47 wk (Panda et al., 2003), 16 wk (Panda et al., 2008), 39 wk (Gallazzi et al., 2008), and 8 wk (Lei et al., 2013). However, there are many studies reporting that probiotics have no effect on shell quality traits in laying hens (Nahashon et al., 1994; Haddadin et al., 1996; Yoruk et al.,

2004; Hayirli et al., 2005; Mahdavi et al., 2005; Li et al., 2006; Applegate et al., 2009; Aghaii et al., 2010; Yalcin et al., 2010; Ribeiro et al., 2014).

Some studies did not provide direct supports for probiotic's role in bone health but reported changes in blood Ca concentrations. Upregulated serum Ca concentrations were reported, along with some other alterations such as eggshell quality and blood cholesterol concentrations, after dietary probiotic inclusion in laying hens (Panda et al., 2003; Capcarova et al., 2010a). Similarly, broilers exhibited higher serum Ca concentrations following various probiotic treatments (Capcarova et al., 2010b, 2011).

Table 1.2 The effects of probiotic supplementation on bone health in poultry

Subject	Duration	Probiotic strains	Effects on bone	References
Hens				
Lohmann Leghorn layer	10 wk	*Bacillus subtilis*	↑tibia weight, density, and ash%	Abdelqader et al., 2013
Broilers				
Ross 308 broiler	42 d	*Bacillus subtilis* (challenged with *Salmonella enteritidis*)	↑ tibia ash% and ash Ca% at d 21 but not d 42	Sadeghi, 2014
Ross 308 broiler	42 d	Lactic acid bacteria-based and *Enterococcus faecium*	↑tibia weight, length, tibiotarsi index, wall thickness, tibiotarsal index, ash%, ash Ca% and P%, modulus elasticity, and yield stress; ↓Canal diameter	Ziaie et al., 2011
Cobb 500 broiler	Study1: 30 d Study2: 49 d	Lactic acid bacteria-based probiotic	↑tibia weight, strength, diameter, ash%, ash Ca% and P%	Fuentes et al., 2013
Avian x Avian broiler	42 d	*Bacillus licheniformis* and *Bacillus subtilis*	↑tibia wall thickness, tibiotarsal index, ash%, and ash P%; ↓Canal diameter	Mutus et al., 2006
Krishibro broiler	42 d	*Lactobacillus sporogenes*	↑tibia breaking strength and ash%	Panda et al., 2006
Cobb 500 broiler	21 d	*Bacillus subtilis* and *Clostridium butyricum*	↑tibia length, weight, weight/length index, ash%, and breaking strength	Houshmand et al., 2010

1.2.3.1.3 Zebrafish

Interestingly, *Lactobacillus rhamnosus* administration promoted zebrafish development, with earlier onset of backbone calcification compared to controls (Avella et al., 2012). In addition, the authors detected higher gene expression levels of IGF-I and -II, which are important local regulators of bone formation (Lindsey and Mohan, 2016). A further study was conducted by the same group to identify the pathways affected by *Lactobacillus rhamnosus* (Maradonna et al., 2013). Consistently, they noticed faster and greater skeletal calcification which resulted from stimulation of the expression of key genes involved in ossification. Following *Lactobacillus rhamnosus* treatment, upregulation of the expression of *runx2* and *sp7* mRNA were revealed, both of which are involved in early osteoblast differentiation and bone formation. A similar change was also detected in the expression of *bglap* as to *runx2* in the *Lactobacillus rhamnosus* treated zebrafish, a gene codes for OC whi*ch* is a biomarker of bone formation. In addition, the expression of *sost*, a gene that encodes sclerostin that down-regulates osteoblast formation, was inhibited by *Lactobacillus rhamnosus* supplementation.

1.2.3.2 Humans

In human, limited studies have been conducted to examine the effects of probiotics on skeletal health. Several studies have revealed probiotic roles in increasing blood Ca concentrations. A double blind randomized controlled experiment in geriatrics reported that probiotic fermented milk with at least 10^8 cfu/mL of viable *Lactobacillus helveticu*s MTCC 5463 increased serum Ca concentrations in participants when compared to those that received a similar product but without the tested strain (Gohel et al., 2016). Similar results occurred in healthy adults (Cox et al., 2014) and postmenopausal women (Narva et al., 2004b) after probiotic supplementation. Moreover, consumption of a probiotic yogurt containing *Lactobacillus acidophilus* and *Bifidobacterium lactis* maintained serum Ca concentrations in pregnant women during their third trimester, whereas a reduction in Ca concentrations occurred in pregnant women consuming conventional yogurt (Asemi and Esmaillzadeh, 2013).

1.3 The Possible Mode of Actions of Probiotics

1.3.1 Nutrient acquisition

It is apparent that Ca availability plays an important role in influencing bone mass throughout an animal's lifespan. Improved Ca absorption by probiotics has been regarded as one of the basic mechanisms underlying probiotic's ability to enhance bone mass. As an example, the probiotic of *Lactobacillus salivarius* stimulated transepithelial Ca transport in fully differentiated human intestinal-like Caco-2 cells (Gilman and Cashman, 2006). In vivo studies provide additional support. For example, the absorption of Ca was higher in rats fed probiotic yogurt than controls (Ghanem et al., 2004), and serum Ca and P concentrations were about 2-fold higher than that of controls (El-Gawad et al., 2014).

Probiotics enhance Ca bioavailability using several mechanisms. Firstly, probiotics increase Ca bioavailability in the digesta. Phytase, for example, is an enzyme that can catalyze the hydrolysis of phytic acid in many plants releasing bound minerals like Ca and P. Some probiotics species, such as *Lactobacillus, Bifidobacteria,* and *Bacilus,* have the ability to produce phyatse and degrade phytate (Cho et al., 2011; Tamayo-Ramos et al., 2012; Ghosh et al., 2015). Secondly, probiotics favor Ca absorption by increasing the intestinal epithelial absorption area. The small intestine is the major site for nutrient absorption. Increased intestinal surface area resulting from higher villi promotes absorption of Ca and other minerals. Probiotics have positive effects on intestinal morphology. Broilers fed probiotics beginning at 1 d of age had longer villus height and higher villus:crypt ratios in the duodenum, jejunum, and ileum in 21 and 42 d-old chickens (Min et al., 2016). Similar results were found in turkey poults at 9 and 11 d of age fed probiotics (Hutsko et al., 2016). In rats, probiotics restored the damaged intestinal mucosa induced by 5-fluorouracil, resulting in longer jejunal villi and a higher villus:crypt ratio (Yeung et al., 2015). The improved intestinal epithelial structure promotes the production of SCFA. It is reported that SCFA can enhance growth and proliferation of enterocytes via releasing growth factors or gastrointestinal peptides such as gastrin, regulating intestinal blood flow and acting directly on genes related to cell proliferation (Blottiere et al., 2003). Third, probiotics may enhance Ca absorption by lowering intestinal microenvironment pH, resulting in an acidic condition, which is in favor of Ca absorption (Suvarna and Boby, 2005).

1.3.2 Immune regulation

Probiotics stimulate humoral immunity. Probiotics induce production of activating factors, such as TGF-β, from the intestinal epithelial cells and dendritic cells located in the intestines. These activating factors promote the differentiation of B cells into IgA producing plasma cells. In mammals, the IgA producing plasma cells can traffic from the intestinal lymphoid tissue to the bloodstream through the lymphatics (Praharaj et al., 2015). For example, increased antibody concentrations in both intestinal and blood circulation were reported in chickens receiving different probiotics (Koenen et al., 2004; Haghighi et al., 2005; Haghighi et al., 2006; Haghighi et al., 2008; Brisbin et al., 2011). Besides antibody-mediated responses, cell-mediated immunity is regulated by probiotics as well. Various *Lactobacillus* species induce cytokine expression in T cells in chicken's cecal tonsils, including IL-10, TNF-α, and IFN-γ, facilitating intestinal homeostasis (Brisbin et al., 2012). A probiotic containing *Lactobacillus acidophilus, Bifidobacterium bifidum, and Streptococcus faecalis* reduced IL-12 and IFN-γ mRNA expression in Salmonella challenged broilers (Haghighi et al., 2008). In addition, *Lactobacillus reuteri* activates Treg (Livingston et al., 2010), a subpopulation of suppressor T cells responsible for regulating tolerance to self-antigens and averting autoimmune disease.

Probiotics also play an important role in the innate immune response. The NF-κB is a key transcription factor for pro-inflammatory cytokines. Several probiotic strains can prevent degradation of IκB, the inhibitor of NF-κB, therefore preventing the expression of pro-inflammatory cytokines. Intestinal epithelial cells synthesizing IL-8, for example, are regulated by probiotics via the NF-κB pathway under both normal (Zhang et al., 2005; Thomas and Versalovic, 2010) and challenged conditions (Tien et al., 2006). Probiotics decrease TLR2 and TLR4 mRNA expression in immature enterocytes in humans (Ganguli et al., 2013). Cellular components of the innate system, such as macrophages and neutrophils (or heterophils, the avian equivalent) were influenced by probiotics. Improved mucus lysozyme activity and phagocytic activity of innate immune cells were revealed in olive flounder (Beck et al., 2015), whereas upregulated oxidative burst and degranulation of heterophils were reported in chickens (Farnell et al., 2006).

As previously discussed, McCabe et al. (2013) reported that *Lactobacillus reuteri* increased trabecular bone mass of the femur and vertebrae of male mice. These

improvements were accompanied by a suppression of mRNA levels of TNF-α (pro-inflammatory cytokine) in the jejunum and ileum. The same lab reported that *Lactobacillus reuteri* protected OVX mice from bone loss, but also noted a suppression of CD4+ T- helper cells in bone marrow (Britton et al., 2014). The CD4+ T-lymphocytes are known to regulate osteoclastogenesis (Li et al., 2011). Consistently, OVX-induced bone loss and bone resorption were also suppressed by *Lactobacillus paracasei* or a mixture of 3 *Lactobacillus* strains (Ohlsson et al., 2014). The probiotics increased cortical bone mass, bone area, and bone thickness of the femur and maintained serum CTX (a biomarker in blood that indicates bone turnover rate) levels similar to that in sham operated controls. The mRNA expressions of pro-inflammatory cytokines of TNF-α and IL-1β in bone marrow were reduced by probiotics, whereas Treg in bone marrow were comparable to sham operated controls. In contrast, OVX mice without probiotic treatments had lower Treg. Similar protective effects of probiotics were reported in mice with periodontal inflammation-induced bone loss. *Lactobacillus brevis* not only inhibited bone loss but also reduced mRNA expression of pro-inflammatory cytokines in gingival tissue, including TNF, IL-1β, IL-6, and IL-17A (Maekawa and Hajishengallis, 2014).

In light of these findings, probiotic-derived factors can activate a multitude of different pathways that control innate and adaptive immunity in the gut (mucosal immunity) which facilitates maintenance of intestinal mucosal integrity to ensure nutrients absorption. Moreover, mucosal immunity subsequently influences systemic immunity, which in turn affects local organ immunity, including bone. Extensive research has been conducted and reviews written on the regulatory role of the immune system on bone (Lorenzo et al., 2008; D'Amelio et al., 2011; Guerrini and Takayanagi, 2014; Humphrey and Nakamura, 2015).

1.3.3 Hormonal regulation

Sex steroid hormones exert potent influences on bone development during growth and contribute to bone homeostasis during adulthood. They act on their target bone cells by binding to their receptors, inducing inhibition of pro-osteoclastogenic cytokine production (IL-1, IL-6, and TNF-α), promoting osteoclast apoptosis, and inhibiting osteoblast and osteocyte apoptosis, thus favoring bone formation and suppressing bone

resorption (Jilka et al., 1992; Hughes et al., 1996; Kameda et al., 1997; Di Gregorio et al., 2001; Kousteni et al., 2001; Almeida et al., 2010; Manolagas et al., 2013; Sinnesael et al., 2013). Colonization by commensal microbes elevated serum testosterone concentrations in both GF male and female mice. In addition, wild-type female mice exhibited increased circulating concentrations of testosterone after colonization with feces from male mice (Markle et al., 2013). These studies suggested that probiotics could regulate bone through sex steroid hormones.

In contrast, glucocorticoids decrease bone mass and strength at least in part by acting directly on osteoblasts and osteocytes. Excessive glucocorticoids inhibit osteoblastogenesis, increase osteoblast and osteocyte apoptosis, and transiently promote osteoclast survival (O'Brien et al., 2004; Jia et al., 2006; Rauch et al., 2010). Excessive glucocorticoids reduce Ca absorption in the duodenum and reabsorption in the kidney (Reid, 1997; Lee et al., 2006; Kim et al., 2009b). Probiotics are able to modify the HPA axis at multiple points. For example, probiotics attenuated HPA response to psychological stress in rats and humans as indicated by decreased plasma concentrations of CORT/cortisol, ACTH, and CRF concentrations or hypothalamic CRF mRNA expression (Ait-Belgnaoui et al., 2012; Ait-Belgnaoui et al., 2014; Yang et al., 2016). Similar results were also found in animal studies with heat stress (Sohail et al., 2010; Deng et al., 2012; Sohail et al., 2012) or under regular management, without artificial stress (Zhang et al., 2016). Thus, probiotics may regulate bone through perturbation of the HPA axis.

1.3.4 Neurotransmitter regulation

1.3.4.1 The 5-HT system

Serotonin, a well-known neurotransmitter, plays a key role in modulating central and peripheral functions of both neurons and non-neuronal cells (Adayev et al., 2005). Several 5-HT receptors have been found in bone cells implicating serotonin's involvement in bone metabolism. For example, osteoblast contains 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors (Bliziotes et al., 2001; Westbroek et al., 2001; Bliziotes et al., 2006; Yadav et al., 2008); osteoclast has 5-HT_{1B}, 5-HT_{2B}, and 5-

HT_4 receptors (Battaglino et al., 2004); and osteocyte possesses 5-HT_{1A} and 5-HT_{2A} receptors (Bliziotes et al., 2006).

Tryptophan is an essential amino acid that animals cannot synthesize on their own; therefore, they must retrieve this nutrient from the diet (Le Floc'h et al., 2011). Once absorbed from the gut and made available in the systemic circulation, tryptophan exists in both a free and albumin-bound fraction (Fernstrom and Fernstrom, 2006). Only the free form of tryptophan can cross the blood-brain-barrier via the large amino acid transporters to participate in 5-HT synthesis in the serotonergic neurons of the CNS (Ruddick et al., 2006). Most serotonergic neurons are located in the raphe nuclei of the brainstem (Janusonis, 2014). However, the vast majority of 5-HT (95%) is synthesized from tryptophan in the enterochromaffin cells of the gastrointestinal tract (Gershon and Tack, 2007; Mawe and Hoffman, 2013).

The synthetic cascade of serotonin is similar irrespective of the location of where it is made, i.e., the brain or gut. Tryptophan is first converted to 5-HTP by the rate-limiting enzyme of TPH: TPH1 is found in non-neuronal cells and TPH2 is located in neurons. The intermediate metabolite of 5-HTP is short-lived, catalyzed by aromatic amino acid decarboxylase to 5-HT. Extracellular 5-HT is pumped by the serotonin transporter, referred to as SERT, back into serotonergic neurons in the brain or epithelial cells in the gut, where the 5-HT is metabolized mainly to 5-HIAA. Metabolism involves first oxidation by monoamine oxidase to the corresponding aldehyde, followed by oxidation by aldehyde dehydrogenase to 5-HIAA. However, the dominant physiological pathway for tryptophan is actually along the kynurenine pathway. Kynurenine is produced from the degradation of tryptophan by the action of the largely hepatic based enzyme, TDO or IDO (Stone et al., 2012). The TDO enzyme can be induced by glucocorticoids or tryptophan itself, whereas IDO is influenced by certain inflammatory stimuli, IFN-γ being the most potent inducer (Ruddick et al., 2006).

1.3.4.1.1 Gut-derived 5-HT

Gut-derived 5-HT directly regulates bone osteoblast via its receptors. Gut derived serotonin can inhibit and stimulate bone formation depending on which serotonin receptors are activated. The inhibitory effect of 5-HT on osteoblast proliferation is

through 5-HT_{1B} receptor, as 5-HT_{1B} receptor deleted mice developed high bone mass with increased bone osteoblast number (Yadav et al., 2008). In contrast, serotonin has a positive effect on osteoblast recruitment, differentiation, and proliferation through other receptors of 5-HT_{2A} and 5-HT_{2B}. Expression of 5-HT_{2B} receptor increased during osteoblast differentiation, and 5-HT_{2B} receptor knockout induced a remarkable decrease in osteoblast recruitment and proliferation, but not differentiation (Collet et al., 2008). In addition, 5-HT_{2B} receptor knockout female mice exhibited an osteopenic phenotype with reduced BMD and volumes of trabecular and cortical bone. The involvement of the 5-HT_{2A} receptor, but not 5-HT_{2B} receptor, in stimulating osteoblasts was documented in another study using anaplastic osteoblasts (Hirai et al., 2009). The same research group provided evidence of the role that the 5-HT_{2A} receptor plays in stimulating osteoblast proliferation in vitro (Hirai et al., 2010) and differentiation in vivo (Tanaka et al., 2015).

Peripheral serotonin is essential for the formation of osteoclasts. In the presence of RANKL, osteoclast precursors positively express TPH1 and synthesize 5-HT. Pharmacological inhibition of 5-HT_{1B} and 5-HT_{2A} receptors reduced the number of osteoclasts, indicating 5-HT's role in osteoclastogenesis may be mediated through 5-HT_{1B} and 5-HT_{2A} receptors (Chabbi-Achengli et al., 2012). Furthermore, it is the intracellular 5-HT that promotes osteoclast differentiation, as an inhibitor of intracellular SERT increased osteoclast differentiation whereas an inhibitor of extracellular SERT showed the opposite effect (Battaglino et al., 2004).

Despite the diverse effects of peripheral 5-HT on osteoblast, the overall effect of gut-derived 5-HT on bone remodeling is to inhibit bone formation. Evidence came from TPH1 knockout mice as they developed a severe high bone mass with an increase in osteoblast numbers and bone formation rate (Yadav et al., 2008). Bone resorption was also reported to be markedly decreased in both growing and mature TPH1 knockout mice, as assessed by biochemical markers and bone histomorphometry (Chabbi-Achengli et al., 2012). These results may explain, at least partially, the low bone mass of the hip and a high risk of osteoporotic fractures in patients treated with some antipsychotic drugs, e.g. serotonin reuptake inhibitors (Gebara et al., 2014).

Certain bacterial strains are able to utilize (Lee and Lee, 2010; Li and Young, 2013) and synthesize (Yanofsky, 2007; Raboni et al., 2009) tryptophan and even produce

5-HT (Lyte, 2011; Clarke et al., 2014). Some bacteria are able to regulate gut-derived 5-HT formation and metabolism. *Bacillus licheniformis* strains isolated from traditional Korean food sources were found to upregulate serotoninergic signaling genes in nematodes of *Caenorhabditis elegans*, including *TPH1*, *HTR1*, and *HTR7* (Park et al., 2015). Spore-forming bacteria isolated from the mouse and human microbiota promotes 5-HT biosynthesis in colonic enterochromaffin cells of GF mice by increasing TPH1 expression (Yano et al., 2015). An ex-vivo study additionally showed that *Escherichia coli* Nissle 1917 could increase concentrations of extracellular 5-HT, increase intracellular concentrations of 5-HTP, and reduce intracellular concentrations of 5-HIAA of mouse ileal tissue, pointing to the modulation of TPH1 and SERT (Nzakizwanayo et al., 2015).

1.3.4.1.2 Brain-derived 5-HT

Brain-derived 5-HT acts as a neurotransmitter to exert a positive and dominant effect on bone mass accrual by enhancing bone formation and limiting bone resorption (Ducy and Karsenty, 2010). Binding to 5-HT_{2C} receptor on ventromedial hypothalamic neurons, 5-HT regulates both arms of remodeling via sympathetic tone as well as through molecular regulation of food intake (Yadav et al., 2009). A possible molecular pathway is that 5-HT regulates a calmodulin kinase-dependent signaling cascade via CREB to decrease the negative effects of sympathetic tone on bone remodeling, thus increasing bone mass accrual (Oury et al., 2010).

Several probiotics are able to regulate central 5-HT metabolism through multiple pathways. One path is via regulation of tryptophan metabolism. The free tryptophan that enters the brain for central serotonin synthesis is mainly dependent on the tryptophan-kynurenine pathway. *Bifidobacterium infantis* 35624 was able to induce an elevation of plasma concentrations of kynurenic acid and tryptophan in Sprague-Dawley rats (Desbonnet et al., 2008), which might be due to the reduced enzyme activities that are responsible for tryptophan degradation along the kynurenine pathway (Clarke et al., 2009), resulting from alteration of inflammatory cytokine or CORT concentrations (Bravo et al., 2011; Gareau et al., 2011). On the other hand, tryptophan concentrations may also be directly affected by probiotics as discussed in 1.3.4.1.1. The second path

may be via regulation of BDNF. One of the functions of BDNF is to promote the growth and differentiation of new neurons and synapses that are involved in the central serotonin system (Mamounas et al., 1995; Mamounas et al., 2000). Another function of BDNF is to activate SERT (Mossner et al., 2000; Benmansour et al., 2008). Both probiotics and prebiotics as well as microbiota increase central concentrations of BDNF via regulation of systemic inflammatory cytokines ((Logan and Katzman, 2005; Savignac et al., 2013). These results provide some possibility that probiotics regulate brain serotonin.

1.4 Summary

Studies conducted to date provide evidence that probiotics improve skeletal health in poultry. Probiotics improve gut health allowing for increased intestinal absorption and bioavailability of minerals such as Ca and P for bone mineralization. Besides enhanced bioavailability of nutrients, other modes of action of probiotics that may include neuroendocrine mechanisms have not been fully investigated. The objectives of the current study were to examine the effects of probiotics on skeletal health and underlying cellular mechanisms under different circumstances, including using single or multiple species based probiotics at different dosages, laying hens or broilers, as well as thermoneutral or elevated temperatures. Probiotic mediated mechanisms that will be investigated include immune cytokines, glucocorticoids, and the serotonin system.

1.5 References

Abdelqader, A., Al-Fataftah, A. R., and Das, G. (2013a). Effects of dietary Bacillus subtilis and inulin supplementation on performance, eggshell quality, intestinal morphology and microflora composition of laying hens in the late phase of production. *Anim Feed Sci Tech,* 179(1-4), 103-111.

Abdelqader, A., Irshaid, R., and Al-Fataftah, A. R. (2013b). Effects of dietary probiotic inclusion on performance, eggshell quality, cecal microflora composition, and tibia traits of laying hens in the late phase of production. *Trop Anim Health Prod,* 45(4), 1017-1024.

Adayev, T., Ranasinghe, B., and Banerjee, P. (2005). Transmembrane signaling in the brain by serotonin, a key regulator of physiology and emotion. *Biosci Rep,* 25(5-6), 363-385.

Aghaii, A., Chaji, M., Mohammadabadi, T., and Sari, M. (2010). The effect of probiotic supplementation on production performance, egg quality and serum and egg chemical composition of laying hens. *J Anim Vet Adv,* 9, 2774-2777.

Ait-Belgnaoui, A., Durand, H., Cartier, C., Chaumaz, G., Eutamene, H., Ferrier, L., Houdeau, E., Fioramonti, J., Bueno, L., and Theodorou, V. (2012). Prevention of gut leakiness by a probiotic treatment leads to attenuated HPA response to an acute psychological stress in rats. *Psychoneuroendocrinology,* 37(11), 1885-1895.

Ait-Belgnaoui, A., Colom, A., Braniste, V., Ramalho, L., Marrot, A., Cartier, C., Houdeau, E., Theodorou, V., and Tompkins, T. (2014). Probiotic gut effect prevents the chronic psychological stress-induced brain activity abnormality in mice. *Neurogastroenterol Motil,* 26(4), 510-520.

Almeida, M., Martin-Millan, M., Ambrogini, E., Bradsher, R., 3rd, Han, L., Chen, X. D., Roberson, P. K., Weinstein, R. S., O'Brien, C. A., Jilka, R. L., and Manolagas, S. C. (2010). Estrogens attenuate oxidative stress and the differentiation and apoptosis of osteoblasts by DNA-binding-independent actions of the ERalpha. *J Bone Miner Res,* 25(4), 769-781.

Anderson, K. E. (2002). *Final report of the thirty fourth North Carolina layer performance and management test*. Cooperative Extension Service, North Carolina State University, Raleigh.

Applegate, T., Onyango, E., Angel, R., and Powers, W. (2009). Effect of amino acid formulation and dietary direct-fed microbial supplementation on egg production and egg characteristics in laying hens. *J Appl Poult Res,* 18(3), 552-561.

Araujo, G. V., Oliveira Junior, M. H., Peixoto, D. M., and Sarinho, E. S. (2015). Probiotics for the treatment of upper and lower respiratory-tract infections in children: systematic review based on randomized clinical trials. *J Pediatr (Rio J),* 91(5), 413-427.

Asemi, Z. and Esmaillzadeh, A. (2013). Effect of daily consumption of probiotic yoghurt on serum levels of calcium, iron and liver enzymes in pregnant women. *Int J Prev Med,* 4(8), 949-955.

Avella, M. A., Place, A., Du, S. J., Williams, E., Silvi, S., Zohar, Y., and Carnevali, O. (2012). Lactobacillus rhamnosus accelerates zebrafish backbone calcification and gonadal differentiation through effects on the GnRH and IGF systems. *PLoS One,* 7(9), e45572.

Bachmann, H., Autzen, S., Frey, U., Wehr, U., Rambeck, W., McCormack, H., and Whitehead, C. C. (2013). The efficacy of a standardised product from dried leaves of Solanum glaucophyllum as source of 1,25-dihydroxycholecalciferol for poultry. *Br Poult Sci,* 54(5), 642-652.

Bar, A. (2009). Calcium transport in strongly calcifying laying birds: mechanisms and regulation. *Comp Biochem Physiol A Mol Integr Physiol,* 152(4), 447-469.

Battaglino, R., Fu, J., Spate, U., Ersoy, U., Joe, M., Sedaghat, L., and Stashenko, P. (2004). Serotonin regulates osteoclast differentiation through its transporter. *J Bone Miner Res,* 19(9), 1420-1431.

Beck, B. R., Kim, D., Jeon, J., Lee, S. M., Kim, H. K., Kim, O. J., Lee, J. I., Suh, B. S., Do, H. K., Lee, K. H., Holzapfel, W. H., Hwang, J. Y., Kwon, M. G., and Song, S. K. (2015). The effects of combined dietary probiotics Lactococcus lactis BFE920 and Lactobacillus plantarum FGL0001 on innate immunity and disease resistance in olive flounder (Paralichthys olivaceus). *Fish Shellfish Immunol,* 42(1), 177-183.

Beck, M. M. and Hansen, K. K. (2004). Role of estrogen in avian osteoporosis. *Poult Sci,* 83(2), 200-206.

Benmansour, S., Deltheil, T., Piotrowski, J., Nicolas, L., Reperant, C., Gardier, A. M., Frazer, A., and David, D. J. (2008). Influence of brain-derived neurotrophic factor (BDNF) on serotonin neurotransmission in the hippocampus of adult rodents. *Eur J Pharmacol,* 587(1-3), 90-98.

Berg, C. and Sanotra, G. (2003). Can a modified latency-to-lie test be used to validate gait-scoring results in commercial broiler flocks? *Anim Welfare* 12(4), 655-659.

Bessei, W. (2006). Welfare of broilers: a review. *World Poultry Sci J* 62(3), 455-466.

Bihan-Duval, E. L., Beaumont, C., and Colleau, J. J. (1997). Estimation of the genetic correlations between twisted legs and growth or conformation traits in broiler chickens. *J Anim Breed Genet,* 114(1-6), 239-259.

Bishop, S. C., Fleming, R. H., McCormack, H. A., Flock, D. K., and Whitehead, C. C. (2000). Inheritance of bone characteristics affecting osteoporosis in laying hens. *Br Poult Sci,* 41(1), 33-40.

Bliziotes, M. M., Eshleman, A. J., Zhang, X. W., and Wiren, K. M. (2001). Neurotransmitter action in osteoblasts: expression of a functional system for serotonin receptor activation and reuptake. *Bone,* 29(5), 477-486.

Bliziotes, M. M., Eshleman, A. J., Burt-Pichat, B., Zhang, X. W., Hashimoto, J., Wiren, K. M., and Chenu, C. (2006). Serotonin transporter and receptor expression in osteocytic MLO-Y4 cells. *Bone,* 39(6), 1313-1321.

Blottiere, H. M., Buecher, B., Galmiche, J. P., and Cherbut, C. (2003). Molecular analysis of the effect of short-chain fatty acids on intestinal cell proliferation. *Proc Nutr Soc,* 62(1), 101-106.

Bokkers, E. A. M. and Koene, P. (2003). Behaviour of fast- and slow growing broilers to 12 weeks of age and the physical consequences. *Appl Anim Behav Sci* 81(1), 59-72.

Bonewald, L. F. (2011). The amazing osteocyte. *J Bone Miner Res,* 26(2), 229-238.

Bravo, J. A., Forsythe, P., Chew, M. V., Escaravage, E., Savignac, H. M., Dinan, T. G., Bienenstock, J., and Cryan, J. F. (2011). Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc Natl Acad Sci U S A,* 108(38), 16050-16055.

Brisbin, J. T., Gong, J., Orouji, S., Esufali, J., Mallick, A. I., Parvizi, P., Shewen, P. E., and Sharif, S. (2011). Oral treatment of chickens with lactobacilli influences elicitation of immune responses. *Clin Vaccine Immunol,* 18(9), 1447-1455.

Brisbin, J. T., Parvizi, P., and Sharif, S. (2012). Differential cytokine expression in T-cell subsets of chicken caecal tonsils co-cultured with three species of Lactobacillus. *Benef Microbes,* 3(3), 205-210.

Britton, R. A., Irwin, R., Quach, D., Schaefer, L., Zhang, J., Lee, T., Parameswaran, N., and McCabe, L. R. (2014). Probiotic L. reuteri treatment prevents bone loss in a menopausal ovariectomized mouse model. *J Cell Physiol,* 229(11), 1822-1830.

Butterworth, A., Weeks, C. A., Crea, P. R., and Kestin, S. C. (2002). Dehydration and lameness in a broiler flock. *Anim Welfare* 11(1), 89-94.

Capcarova, M., Chmelnicna, L., Kolesarova, A., Massanyi, P., and Kovacik, J. (2010a). Effects of Enterococcus faecium M 74 strain on selected blood and production parameters of laying hens. *Br Poult Sci,* 51(5), 614-620.

Capcarova, M., Weiss, J., Hrncar, C., Kolesarova, A., and Pal, G. (2010b). Effect of Lactobacillus fermentum and Enterococcus faecium strains on internal milieu, antioxidant status and body weight of broiler chickens. *J Anim Physiol Anim Nutr (Berl),* 94(5), e215-224.

Capcarova, M., Hascik, P., Kolesarova, A., Kacaniova, M., Mihok, M., and Pal, G. (2011). The effect of selected microbial strains on internal milieu of broiler chickens after peroral administration. *Res Vet Sci,* 91(1), 132-137.

Caplen, G., Baker, L., Hothersall, B., McKeegan, D. E., Sandilands, V., Sparks, N. H., Waterman-Pearson, A. E., and Murrell, J. C. (2013a). Thermal nociception as a measure of non-steroidal anti-inflammatory drug effectiveness in broiler chickens with articular pain. *Vet J,* 198(3), 616-619.

Caplen, G., Colborne, G. R., Hothersall, B., Nicol, C. J., Waterman-Pearson, A. E., Weeks, C. A., and Murrell, J. C. (2013b). Lame broiler chickens respond to non-steroidal anti-inflammatory drugs with objective changes in gait function: a controlled clinical trial. *Vet J,* 196(3), 477-482.

Capulli, M., Paone, R., and Rucci, N. (2014). Osteoblast and osteocyte: games without frontiers. *Arch Biochem Biophys,* 561, 3-12.

Chabbi-Achengli, Y., Coudert, A. E., Callebert, J., Geoffroy, V., Cote, F., Collet, C., and de Vernejoul, M. C. (2012). Decreased osteoclastogenesis in serotonin-deficient mice. *Proc Natl Acad Sci U S A,* 109(7), 2567-2572.

Chapman, C. M., Gibson, G. R., and Rowland, I. (2014). Effects of single- and multi-strain probiotics on biofilm formation and in vitro adhesion to bladder cells by urinary tract pathogens. *Anaerobe,* 27, 71-76.

Chen, H., Senda, T., and Kubo, K. Y. (2015). The osteocyte plays multiple roles in bone remodeling and mineral homeostasis. *Med Mol Morphol,* 48(2), 61-68.

Chiang, S. S. and Pan, T. M. (2011). Antiosteoporotic effects of Lactobacillus -fermented soy skim milk on bone mineral density and the microstructure of femoral bone in ovariectomized mice. *J Agric Food Chem,* 59(14), 7734-7742.

Cho, E. A., Kim, E. J., and Pan, J. G. (2011). Adsorption immobilization of Escherichia coli phytase on probiotic Bacillus polyfermenticus spores. *Enzyme Microb Technol,* 49(1), 66-71.

Clarke, G., Fitzgerald, P., Cryan, J. F., Cassidy, E. M., Quigley, E. M., and Dinan, T. G. (2009). Tryptophan degradation in irritable bowel syndrome: evidence of indoleamine 2,3-dioxygenase activation in a male cohort. *BMC Gastroenterol,* 9, 6.

Clarke, G., Stilling, R. M., Kennedy, P. J., Stanton, C., Cryan, J. F., and Dinan, T. G. (2014). Minireview: Gut microbiota: the neglected endocrine organ. *Mol Endocrinol,* 28(8), 1221-1238.

Collet, C., Schiltz, C., Geoffroy, V., Maroteaux, L., Launay, J. M., and de Vernejoul, M. C. (2008). The serotonin 5-HT2B receptor controls bone mass via osteoblast recruitment and proliferation. *FASEB J,* 22(2), 418-427.

Collins, F. L., Irwin, R., Bierhalter, H., Schepper, J., Britton, R. A., Parameswaran, N., and McCabe, L. R. (2016). Lactobacillus reuteri 6475 Increases Bone Density in Intact Females Only under an Inflammatory Setting. *PLoS One,* 11(4), e0153180.

Cox, A. J., West, N. P., Horn, P. L., Lehtinen, M. J., Koerbin, G., Pyne, D. B., Lahtinen, S. J., Fricker, P. A., and Cripps, A. W. (2014). Effects of probiotic supplementation over 5 months on routine haematology and clinical chemistry measures in healthy active adults. *Eur J Clin Nutr,* 68(11), 1255-1257.

Cressman, M. D., Yu, Z., Nelson, M. C., Moeller, S. J., Lilburn, M. S., and Zerby, H. N. (2010). Interrelations between the microbiotas in the litter and in the intestines of commercial broiler chickens. *Appl Environ Microbiol,* 76(19), 6572-6582.

Crotti, T. N., Dharmapatni, A. A., Alias, E., and Haynes, D. R. (2015). Osteoimmunology: Major and Costimulatory Pathway Expression Associated with Chronic Inflammatory Induced Bone Loss. *J Immunol Res,* 2015, 281287.

Cuello-Garcia, C. A., Brozek, J. L., Fiocchi, A., Pawankar, R., Yepes-Nunez, J. J., Terracciano, L., Gandhi, S., Agarwal, A., Zhang, Y., and Schunemann, H. J. (2015). Probiotics for the prevention of allergy: A systematic review and meta-analysis of randomized controlled trials. *J Allergy Clin Immunol,* 136(4), 952-961.

D'Amelio, P., Fornelli, G., Roato, I., and Isaia, G. C. (2011). Interactions between the immune system and bone. *World J Orthop,* 2(3), 25-30.

Dacke, C., Arkle, S., Cook, D., Wormstone, I., Jones, S., Zaidi, M., and Bascal, Z. (1993). Medullary bone and avian calcium regulation. *The Journal of Experimental Biology,* 184(1), 63-88.

Danbury, T. C., Weeks, C. A., Chambers, J. P., Waterman-Pearson, A. E., and Kestin, S. C. (2000). Self-selection of the analgesic drug carprofen by lame broiler chickens. *Vet Rec,* 146(11), 307-311.

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., Ling, A. V., Devlin, A. S., Varma, Y., Fischbach, M. A., Biddinger, S. B., Dutton, R. J., and Turnbaugh, P. J. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature,* 505(7484), 559-563.

Deng, W., Dong, X. F., Tong, J. M., and Zhang, Q. (2012). The probiotic Bacillus licheniformis ameliorates heat stress-induced impairment of egg production, gut morphology, and intestinal mucosal immunity in laying hens. *Poult Sci,* 91(3), 575-582.

Desbonnet, L., Garrett, L., Clarke, G., Bienenstock, J., and Dinan, T. G. (2008). The probiotic Bifidobacteria infantis: An assessment of potential antidepressant properties in the rat. *J Psychiatr Res,* 43(2), 164-174.

Di Gregorio, G. B., Yamamoto, M., Ali, A. A., Abe, E., Roberson, P., Manolagas, S. C., and Jilka, R. L. (2001). Attenuation of the self-renewal of transit-amplifying osteoblast progenitors in the murine bone marrow by 17 beta-estradiol. *J Clin Invest*, 107(7), 803-812.

Dinev, I., Denev, S. A., and Edens, F. W. (2012). Comparative clinical and morphological studies on the incidence of tibial dyschondroplasia as a cause of lameness in three commercial lines of broiler chickens. *J Appl Poultry Res* 21(3), 637-644.

Ducy, P. and Karsenty, G. (2010). The two faces of serotonin in bone biology. *J Cell Biol*, 191(1), 7-13.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E., and Relman, D. A. (2005). Diversity of the human intestinal microbial flora. *Science,* 308(5728), 1635-1638.

El-Gawad, I. A. A., Mehriz, A. E. M., Saleh, F. A., and Rayan, E. A. (2014). Bioavailability of Ca, P and Zn and Bone Mineralization in Rats Fed Yoghurt and Soy-yoghurt Containing Bifidobacteria. *European J Nutr Food Saf*, 4(2), 110.

Engdahl, C., Lindholm, C., Stubelius, A., Ohlsson, C., Carlsten, H., and Lagerquist, M. K. (2013). Periarticular bone loss in antigen-induced arthritis. *Arthritis Rheum*, 65(11), 2857-2865.

European Commission - Scientific Committee on Animal Health and Animal Welfare. (2000). The Welfare of Chickens Kept for Meat Production (Broilers). Retrieved from https://ec.europa.eu/food/sites/food/files/animals/docs/aw_arch_2005_broilers_scientific_opinion_en.pdf Accessed on Dec 3rd.

FAO/WHO. (2001). *Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria*. Paper presented at the Joint FAO/WHO Expert Consultation.

Farnell, M. B., Donoghue, A. M., de Los Santos, F. S., Blore, P. J., Hargis, B. M., Tellez, G., and Donoghue, D. J. (2006). Upregulation of oxidative burst and degranulation in chicken heterophils stimulated with probiotic bacteria. *Poult Sci*, 85(11), 1900-1906.

Fernstrom, J. D. and Fernstrom, M. H. (2006). Exercise, serum free tryptophan, and central fatigue. *J Nutr,* 136(2), 553s-559s.

Fleming, R. H., McCormack, H. A., McTeir, L., and Whitehead, C. C. (1998). Medullary bone and humeral breaking strength in laying hens. *Res Vet Sci,* 64(1), 63-67.

Fleming, R. H., McCormack, H. A., McTeir, L., and Whitehead, C. C. (2003). Effects of dietary particulate limestone, vitamin K3 and fluoride and photostimulation on skeletal morphology and osteoporosis in laying hens. *Br Poult Sci,* 44(5), 683-689.

Fleming, R. H., McCormack, H. A., McTeir, L., and Whitehead, C. C. (2004). Incidence, pathology and prevention of keel bone deformities in the laying hen. *Br Poult Sci,* 45(3), 320-330.

Fleming, R. H., McCormack, H. A., McTeir, L., and Whitehead, C. C. (2006). Relationships between genetic, environmental and nutritional factors influencing osteoporosis in laying hens. *Br Poult Sci,* 47(6), 742-755.

Fleming, R. H. (2008). Nutritional factors affecting poultry bone health. *Proc Nutr Soc,* 67(2), 177-183.

Foureaux Rde, C., Messora, M. R., de Oliveira, L. F., Napimoga, M. H., Pereira, A. N., Ferreira, M. S., and Pereira, L. J. (2014). Effects of probiotic therapy on metabolic and inflammatory parameters of rats with ligature-induced periodontitis associated with restraint stress. *J Periodontol,* 85(7), 975-983.

Freire, R., Wilkins, L. J., Short, F., and Nicol, C. J. (2003). Behaviour and welfare of individual laying hens in a non-cage system. *Br Poult Sci,* 44(1), 22-29.

Gallazzi, D., Giardini, A., Mangiagalli, G. M., Marelli, S., Ferrazzi, V., Orsi, C., and Cavalchini, G. L. (2008). Effects of Lactobacillus acidophilus D2/CSL on laying hen performance. *Ital J Anim. Sci,* 7(1), 27-37.

Ganguli, K., Meng, D., Rautava, S., Lu, L., Walker, W. A., and Nanthakumar, N. (2013). Probiotics prevent necrotizing enterocolitis by modulating enterocyte genes that regulate innate immune-mediated inflammation. *Am J Physiol Gastrointest Liver Physiol,* 304(2), G132-141.

Garcia, V. G., Knoll, L. R., Longo, M., Novaes, V. C., Assem, N. Z., Ervolino, E., de Toledo, B. E., and Theodoro, L. H. (2016). Effect of the probiotic Saccharomyces cerevisiae on ligature-induced periodontitis in rats. *J Periodontal Res*, 51(1), 26-37.

Gareau, M. G., Sherman, P. M., and Walker, W. A. (2010). Probiotics and the gut microbiota in intestinal health and disease. *Nat Rev Gastroenterol Hepatol,* 7(9), 503-514.

Gareau, M. G., Wine, E., Rodrigues, D. M., Cho, J. H., Whary, M. T., Philpott, D. J., Macqueen, G., and Sherman, P. M. (2011). Bacterial infection causes stress-induced memory dysfunction in mice. *Gut,* 60(3), 307-317.

Garner, J. P., Falcone, C., Wakenell, P., Martin, M., and Mench, J. A. (2002). Reliability and validity of a modified gait scoring system and its use in assessing tibial dyschondroplasia in broilers. *Br Poult Sci,* 43(3), 355-363.

Gebara, M. A., Shea, M. L., Lipsey, K. L., Teitelbaum, S. L., Civitelli, R., Muller, D. J., Reynolds, C. F., 3rd, Mulsant, B. H., and Lenze, E. J. (2014). Depression, antidepressants, and bone health in older adults: a systematic review. *J Am Geriatr Soc,* 62(8), 1434-1441.

Gebhardt-Henrich, S. G. and Frohlich, E. K. (2015). Early Onset of Laying and Bumblefoot Favor Keel Bone Fractures. *Animals (Basel),* 5(4), 1192-1206.

Gentle, M. J., Tilston, V., and McKeegan, D. E. (2001). Mechanothermal nociceptors in the scaly skin of the chicken leg. *Neuroscience,* 106(3), 643-652.

Gentle, M. J. (2011). Pain issues in poultry. *Appl Anim Behav Sci* 135(3), 252-258.

Gershon, M. D. and Tack, J. (2007). The serotonin signaling system: from basic understanding to drug development for functional GI disorders. *Gastroenterology,* 132(1), 397-414.

Ghanem, K., Badawy, I., and Abdel-Salam, A. (2004). Influence of yoghurt and probiotic yoghurt on the absorption of calcium, magnesium, iron and bone mineralization in rats. *Milchwissenschaft,* 59(9-10), 472-475.

Ghosh, K., Ray, M., Adak, A., Halder, S. K., Das, A., Jana, A., Parua Mondal, S., Vagvolgyi, C., Das Mohapatra, P. K., Pati, B. R., and Mondal, K. C. (2015). Role of probiotic Lactobacillus fermentum KKL1 in the preparation of a rice based fermented beverage. *Bioresour Technol,* 188, 161-168.

Gielda, L. M. and DiRita, V. J. (2012). Zinc competition among the intestinal microbiota. *MBio,* 3(4), e00171-00112.

Gilman, J. and Cashman, K. D. (2006). The effect of probiotic bacteria on transepithelial calcium transport and calcium uptake in human intestinal-like Caco-2 cells. *Curr Issues Intest Microbiol,* 7(1), 1-5.

Giorgetti, G., Brandimarte, G., Fabiocchi, F., Ricci, S., Flamini, P., Sandri, G., Trotta, M. C., Elisei, W., Penna, A., Lecca, P. G., Picchio, M., and Tursi, A. (2015). Interactions between Innate Immunity, Microbiota, and Probiotics. *J Immunol Res,* 2015, 501361.

Gohel, M. K., Prajapati, J. B., Mudgal, S. V., Pandya, H. V., Singh, U. S., Trivedi, S. S., Phatak, A. G., and Patel, R. M. (2016). Effect of Probiotic Dietary Intervention on Calcium and Haematological Parameters in Geriatrics. *J Clin Diagn Res,* 10(4), Lc05-09.

Goldring, S. R. (2015a). Inflammatory signaling induced bone loss. *Bone,* 80, 143-149.

Goldring, S. R. (2015b). The osteocyte: key player in regulating bone turnover. *RMD Open,* 1(Suppl 1), e000049.

Gonzalez-Ceron, F., Rekaya, R., Anthony, N. B., and Aggrey, S. E. (2015). Genetic analysis of leg problems and growth in a random mating broiler population. *Poult Sci,* 94(2), 162-168.

Goodrich, J. K., Waters, J. L., Poole, A. C., Sutter, J. L., Koren, O., Blekhman, R., Beaumont, M., Van Treuren, W., Knight, R., Bell, J. T., Spector, T. D., Clark, A. G., and Ley, R. E. (2014). Human genetics shape the gut microbiome. *Cell,* 159(4), 789-799.

Gregory, N. G. and Wilkins, L. J. (1989). Broken bones in domestic fowl: handling and processing damage in end-of-lay battery hens. *Br Poult Sci,* 30(3), 555-562.

Gregory, N. G., Wilkins, L. J., Austin, S. D., Belyavin, C. G., Alvey, D. M., and Tucker, S. A. (1992). Effect of catching method on the prevalence of broken bones in end of lay hens. *Avian Pathol,* 21(4), 717-722.

Gregory, N. G., Wilkins, L. J., Alvey, D. M., and Tucker, S. A. (1993). Effect of catching method and lighting intensity on the prevalence of broken bones and on the ease of handling of end-of-lay hens. *Vet Rec,* 132(6), 127-129.

Groves, P. J. and Muir, W. I. (2014). A meta-analysis of experiments linking incubation conditions with subsequent leg weakness in broiler chickens. *PLoS One,* 9(7), e102682.

Guarino, A., Guandalini, S., and Lo Vecchio, A. (2015). Probiotics for Prevention and Treatment of Diarrhea. *J Clin Gastroenterol,* 49 Suppl 1, S37-45.

Guerrini, M. M. and Takayanagi, H. (2014). The immune system, bone and RANKL. *Arch Biochem Biophys,* 561, 118-123.

Haddadin, M. S., Abdulrahim, S. M., Hashlamoun, E. A., and Robinson, R. K. (1996). The effect of Lactobacillus acidophilus on the production and chemical composition of hen's eggs. *Poult Sci,* 75(4), 491-494.

Haghighi, H. R., Gong, J., Gyles, C. L., Hayes, M. A., Sanei, B., Parvizi, P., Gisavi, H., Chambers, J. R., and Sharif, S. (2005). Modulation of antibody-mediated immune response by probiotics in chickens. *Clin Diagn Lab Immunol,* 12(12), 1387-1392.

Haghighi, H. R., Gong, J., Gyles, C. L., Hayes, M. A., Zhou, H., Sanei, B., Chambers, J. R., and Sharif, S. (2006). Probiotics stimulate production of natural antibodies in chickens. *Clin Vaccine Immunol,* 13(9), 975-980.

Haghighi, H. R., Abdul-Careem, M. F., Dara, R. A., Chambers, J. R., and Sharif, S. (2008). Cytokine gene expression in chicken cecal tonsils following treatment with probiotics and Salmonella infection. *Vet Microbiol,* 126(1-3), 225-233.

Hardiman, J. W. (1996). *Broiler breeding by the year 2006.* Page 461-467 in: Proceedings of the XX World's Poultry Congress. New Delhi, India.

Hardy, R. and Cooper, M. S. (2009). Bone loss in inflammatory disorders. *J Endocrinol,* 201(3), 309-320.

Havenstein, G. B., Ferket, P. R., Scheideler, S. E., and Larson, B. T. (1994). Growth, livability, and feed conversion of 1957 vs 1991 broilers when fed "typical" 1957 and 1991 broiler diets. *Poult Sci,* 73(12), 1785-1794.

Havenstein, G. B., Ferket, P. R., and Qureshi, M. A. (2003). Growth, livability, and feed conversion of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. *Poult Sci,* 82(10), 1500-1508.

Hayirli, A., Esenbuga, N., Macit, M., Yoruk, M. A., Yildiz, A., and Karaca, H. (2005). Nutrition practice to alleviate the adverse effects of stress on laying performance, metabolic profile and egg quality in peak producing hens: II. The probiotic supplementation. *Asian Austral J Anim,* 18(12), 1752-1760.

Hester, P. Y., Enneking, S. A., Haley, B. K., Cheng, H. W., Einstein, M. E., and Rubin, D. A. (2013). The effect of perch availability during pullet rearing and egg laying on musculoskeletal health of caged White Leghorn hens. *Poult Sci,* 92(8), 1972-1980.

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R. B., Flint, H. J., Salminen, S., Calder, P. C., and Sanders, M. E. (2014). Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol,* 11(8), 506-514.

Hirai, T., Tokumo, K., Tsuchiya, D., and Nishio, H. (2009). Expression of mRNA for 5-HT2 receptors and proteins related to inactivation of 5-HT in mouse osteoblasts. *J Pharmacol Sci,* 109(2), 319-323.

Hirai, T., Kaneshige, K., Kurosaki, T., and Nishio, H. (2010). Functional expression of 5-HT2A receptor in osteoblastic MC3T3-E1 cells. *Biochem Biophys Res Commun,* 396(2), 278-282.

Hisamatsu, T., Wada, Y., and Kanai, T. (2015). Inflammatory bowel disease and bone decreased bone mineral density. *Clin Calcium,* 25(11), 1639-1644.

Hothersall, B., Caplen, G., Nicol, C. J., Taylor, P. M., Waterman-Pearson, A. E., Weeks, C. A., and Murrell, J. C. (2011). Development of mechanical and thermal nociceptive threshold testing devices in unrestrained birds (broiler chickens). *J Neurosci Methods,* 201(1), 220-227.

Hothersall, B., Caplen, G., Parker, R. M., Nicol, C. J., Waterman-Pearson, A. E., Weeks, C. A., and Murrell, J. C. (2014). Thermal nociceptive threshold testing detects altered sensory processing in broiler chickens with spontaneous lameness. *PLoS One*, 9(5), e97883.

Houshmand, M., Azhar, K., Zulkifli, I., Bejo, M. H., Meimandipour, A., and Kamyab, A. (2011). Effects of non-antibiotic feed additives on performance, tibial dyschondroplasia incidence and tibia characteristics of broilers fed low-calcium diets. *J Anim Physiol Anim Nutr (Berl)*, 95(3), 351-358.

Hughes, B. O., Wilson, S., Appleby, M. C., and Smith, S. F. (1993). Comparison of bone volume and strength as measures of skeletal integrity in caged laying hens with access to perches. *Res Vet Sci*, 54(2), 202-206.

Hughes, D. E., Dai, A., Tiffee, J. C., Li, H. H., Mundy, G. R., and Boyce, B. F. (1996). Estrogen promotes apoptosis of murine osteoclasts mediated by TGF-beta. *Nat Med*, 2(10), 1132-1136.

Humphrey, M. B. and Nakamura, M. C. (2015). A Comprehensive Review of Immunoreceptor Regulation of Osteoclasts. *Clin Rev Allergy Immunol*.

Hutsko, S. L., Meizlisch, K., Wick, M., and Lilburn, M. S. (2016). Early intestinal development and mucin transcription in the young poult with probiotic and mannan oligosaccharide prebiotic supplementation. *Poult Sci*, 95(5), 1173-1178.

Izawa, A., Ishihara, Y., Mizutani, H., Kobayashi, S., Goto, H., Okabe, E., Takeda, H., Ozawa, Y., Kamiya, Y., Sugita, Y., Kubo, K., Kamei, H., Kikuchi, T., Mitani, A., Hayashi, J., Nishihara, T., Maeda, H., and Noguchi, T. (2014). Inflammatory bone loss in experimental periodontitis induced by Aggregatibacter actinomycetemcomitans in interleukin-1 receptor antagonist knockout mice. *Infect Immun*, 82(5), 1904-1913.

Janusonis, S. (2014). Serotonin dynamics in and around the central nervous system: is autism solvable without fundamental insights? *Int J Dev Neurosci*, 39, 9-15.

Jendral, M. J., Korver, D. R., Church, J. S., and Feddes, J. J. (2008). Bone mineral density and breaking strength of White Leghorns housed in conventional, modified, and commercially available colony battery cages. *Poult Sci*, 87(5), 828-837.

Jia, D., O'Brien, C. A., Stewart, S. A., Manolagas, S. C., and Weinstein, R. S. (2006). Glucocorticoids act directly on osteoclasts to increase their life span and reduce bone density. *Endocrinology,* 147(12), 5592-5599.

Jilka, R. L., Hangoc, G., Girasole, G., Passeri, G., Williams, D. C., Abrams, J. S., Boyce, B., Broxmeyer, H., and Manolagas, S. C. (1992). Increased osteoclast development after estrogen loss: mediation by interleukin-6. *Science,* 257(5066), 88-91.

Johnson, A. L. (2015). Chapter 28: Reproduction in the Female. Page 635-665 in: Scanes, C. G. (Ed.) *Sturkie's Avian Physiology (Sixth Edition)*. San Diego, Academic Press.

Johnson, P. A., Stephens, C. S., and Giles, J. R. (2015). The domestic chicken: Causes and consequences of an egg a day. *Poult Sci,* 94(4), 816-820.

Jonchere, V., Brionne, A., Gautron, J., and Nys, Y. (2012). Identification of uterine ion transporters for mineralisation precursors of the avian eggshell. *BMC Physiol,* 12, 10.

Kameda, T., Mano, H., Yuasa, T., Mori, Y., Miyazawa, K., Shiokawa, M., Nakamaru, Y., Hiroi, E., Hiura, K., Kameda, A., Yang, N. N., Hakeda, Y., and Kumegawa, M. (1997). Estrogen inhibits bone resorption by directly inducing apoptosis of the bone-resorbing osteoclasts. *J Exp Med,* 186(4), 489-495.

Kapell, D. N., Hill, W. G., Neeteson, A. M., McAdam, J., Koerhuis, A. N., and Avendano, S. (2012). Twenty-five years of selection for improved leg health in purebred broiler lines and underlying genetic parameters. *Poult Sci,* 91(12), 3032-3043.

Kestin, S. C., Knowles, T. G., Tinch, A. E., and Gregory, N. G. (1992). Prevalence of leg weakness in broiler chickens and its relationship with genotype. *Vet Rec,* 131(9), 190-194.

Kestin, S. C., Gordon, S., Su, G., and Sorensen, P. (2001). Relationships in broiler chickens between lameness, liveweight, growth rate and age. *Vet Rec,* 148(7), 195-197.

Khalesi, S., Sun, J., Buys, N., and Jayasinghe, R. (2014). Effect of probiotics on blood pressure: a systematic review and meta-analysis of randomized, controlled trials. *Hypertension,* 64(4), 897-903.

Khan, S. H., Shahid, R., Mian, A. A., Sardar, R., and Anjum, M. A. (2010). Effect of the level of cholecalciferol supplementation of broiler diets on the performance and tibial dyschondroplasia. *J Anim Physiol Anim Nutr (Berl),* 94(5), 584-593.

Kim, J. G., Lee, E., Kim, S. H., Whang, K. Y., Oh, S., and Imm, J. Y. (2009a). Effects of a Lactobacillus casei 393 fermented milk product on bone metabolism in ovariectomised rats. *Int Dairy J* 19(11), 690-695.

Kim, M. H., Lee, G. S., Jung, E. M., Choi, K. C., and Jeung, E. B. (2009b). The negative effect of dexamethasone on calcium-processing gene expressions is associated with a glucocorticoid-induced calcium-absorbing disorder. *Life Sci,* 85(3-4), 146-152.

Knowles, T. G., Kestin, S. C., Haslam, S. M., Brown, S. N., Green, L. E., Butterworth, A., Pope, S. J., Pfeiffer, D., and Nicol, C. J. (2008). Leg disorders in broiler chickens: prevalence, risk factors and prevention. *PLoS One,* 3(2), e1545.

Koenen, M. E., Kramer, J., van der Hulst, R., Heres, L., Jeurissen, S. H. M., and Boersma, W. J. A. (2004). Immunomodulation by probiotic lactobacilli in layer- and meat-type chickens. *Brit Poultry Sci,* 45(3), 355-366.

Koewler, N. J., Freeman, K. T., Buus, R. J., Herrera, M. B., Jimenez-Andrade, J. M., Ghilardi, J. R., Peters, C. M., Sullivan, L. J., Kuskowski, M. A., Lewis, J. L., and Mantyh, P. W. (2007). Effects of a monoclonal antibody raised against nerve growth factor on skeletal pain and bone healing after fracture of the C57BL/6J mouse femur. *J Bone Miner Res,* 22(11), 1732-1742.

Kousteni, S., Bellido, T., Plotkin, L. I., O'Brien, C. A., Bodenner, D. L., Han, L., Han, K., DiGregorio, G. B., Katzenellenbogen, J. A., Katzenellenbogen, B. S., Roberson, P. K., Weinstein, R. S., Jilka, R. L., and Manolagas, S. C. (2001). Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. *Cell,* 104(5), 719-730.

Krishnamurthy, A., Joshua, V., Haj Hensvold, A., Jin, T., Sun, M., Vivar, N., Ytterberg, A. J., Engstrom, M., Fernandes-Cerqueira, C., Amara, K., Magnusson, M., Wigerblad, G., Kato, J., Jimenez-Andrade, J. M., Tyson, K., Rapecki, S., Lundberg, K., Catrina, S. B., Jakobsson, P. J., Svensson, C., Malmstrom, V., Klareskog, L., Wahamaa, H., and Catrina, A. I. (2016). Identification of a novel chemokine-dependent molecular mechanism underlying rheumatoid arthritis-associated autoantibody-mediated bone loss. *Ann Rheum Dis,* 75(4), 721-729.

Kurtoglu, V., Kurtoglu, F., Seker, E., Coskun, B., Balevi, T., and Polat, E. S. (2004). Effect of probiotic supplementation on laying hen diets on yield performance and serum and egg yolk cholesterol. *Food Addit Contam,* 21(9), 817-823.

Lanyon, L. E. (1992). Control of bone architecture by functional load bearing. *J Bone Miner Res,* 7 Suppl 2, S369-375.

Lawley, T. D. and Walker, A. W. (2013). Intestinal colonization resistance. *Immunology,* 138(1), 1-11.

Le Barz, M., Anhe, F. F., Varin, T. V., Desjardins, Y., Levy, E., Roy, D., Urdaci, M. C., and Marette, A. (2015). Probiotics as Complementary Treatment for Metabolic Disorders. *Diabetes Metab J,* 39(4), 291-303.

Le Floc'h, N., Otten, W., and Merlot, E. (2011). Tryptophan metabolism, from nutrition to potential therapeutic applications. *Amino Acids,* 41(5), 1195-1205.

Lee, G. S., Choi, K. C., and Jeung, E. B. (2006). Glucocorticoids differentially regulate expression of duodenal and renal calbindin-D9k through glucocorticoid receptor-mediated pathway in mouse model. *Am J Physiol Endocrinol Metab,* 290(2), E299-307.

Lee, J. H. and Lee, J. (2010). Indole as an intercellular signal in microbial communities. *FEMS Microbiol Rev,* 34(4), 426-444.

Lei, K., Li, Y. L., Yu, D. Y., Rajput, I. R., and Li, W. F. (2013). Influence of dietary inclusion of Bacillus licheniformis on laying performance, egg quality, antioxidant enzyme activities, and intestinal barrier function of laying hens. *Poult Sci,* 92(9), 2389-2395.

Li, G. and Young, K. D. (2013). Indole production by the tryptophanase TnaA in Escherichia coli is determined by the amount of exogenous tryptophan. *Microbiology*, 159(Pt 2), 402-410.

Li, H., Liu, R., Chi, S., Lu, Z., and Yang, L. (2015). Adalimumab arrests bone loss in the spine and hip of active rheumatoid arthritis patients with osteopenia. *Clin Exp Rheumatol*, 33(1), 134-135.

Li, J. Y., Tawfeek, H., Bedi, B., Yang, X., Adams, J., Gao, K. Y., Zayzafoon, M., Weitzmann, M. N., and Pacifici, R. (2011). Ovariectomy disregulates osteoblast and osteoclast formation through the T-cell receptor CD40 ligand. *Proc Natl Acad Sci U S A*, 108(2), 768-773.

Li, J. Y., Chassaing, B., Tyagi, A. M., Vaccaro, C., Luo, T., Adams, J., Darby, T. M., Weitzmann, M. N., Mulle, J. G., Gewirtz, A. T., Jones, R. M., and Pacifici, R. (2016). Sex steroid deficiency-associated bone loss is microbiota dependent and prevented by probiotics. *J Clin Invest*, 126(6), 2049-2063.

Li, L., Xu, C. L., Ji, C., Ma, Q., Hao, K., Jin, Z. Y., and Li, K. (2006). Effects of a dried Bacillus subtilis culture on egg quality. *Poult Sci*, 85(2), 364-368.

Lilly, D. M. and Stillwell, R. H. (1965). Probiotics: Growth-Promoting Factors Produced by Microorganisms. *Science*, 147(3659), 747-748.

Lin, P. L., Huang, P. W., Huang, P. Y., and Hsu, H. C. (2015). Alveolar bone-loss area localization in periodontitis radiographs based on threshold segmentation with a hybrid feature fused of intensity and the H-value of fractional Brownian motion model. *Comput Methods Programs Biomed*, 121(3), 117-126.

Lindsey, R. C. and Mohan, S. (2016). Skeletal effects of growth hormone and insulin-like growth factor-I therapy. *Mol Cell Endocrinol*, 432, 44-55.

Livingston, M., Loach, D., Wilson, M., Tannock, G. W., and Baird, M. (2010). Gut commensal Lactobacillus reuteri 100-23 stimulates an immunoregulatory response. *Immunol Cell Biol*, 88(1), 99-102.

Logan, A. C. and Katzman, M. (2005). Major depressive disorder: probiotics may be an adjuvant therapy. *Med Hypotheses*, 64(3), 533-538.

Lorenzo, J., Horowitz, M., and Choi, Y. (2008). Osteoimmunology: interactions of the bone and immune system. *Endocr Rev*, 29(4), 403-440.

Lyte, M. (2011). Probiotics function mechanistically as delivery vehicles for neuroactive compounds: Microbial endocrinology in the design and use of probiotics. *Bioessays,* 33(8), 574-581.

Madhu, A. N. and Prapulla, S. G. (2014). Evaluation and functional characterization of a biosurfactant produced by Lactobacillus plantarum CFR 2194. *Appl Biochem Biotechnol,* 172(4), 1777-1789.

Maekawa, T. and Hajishengallis, G. (2014). Topical treatment with probiotic Lactobacillus brevis CD2 inhibits experimental periodontal inflammation and bone loss. *J Periodontal Res,* 49(6), 785-791.

Mahdavi, A. H., Rahmani, H. R., and Pourreza, J. (2005). Effect of probiotic supplements on egg quality and laying hen's performance. *Int J Poult Sci,* 4(4), 488-492.

Mamounas, L. A., Blue, M. E., Siuciak, J. A., and Altar, C. A. (1995). Brain-derived neurotrophic factor promotes the survival and sprouting of serotonergic axons in rat brain. *J Neurosci,* 15(12), 7929-7939.

Mamounas, L. A., Altar, C. A., Blue, M. E., Kaplan, D. R., Tessarollo, L., and Lyons, W. E. (2000). BDNF promotes the regenerative sprouting, but not survival, of injured serotonergic axons in the adult rat brain. *J Neurosci,* 20(2), 771-782.

Manolagas, S. C., O'Brien, C. A., and Almeida, M. (2013). The role of estrogen and androgen receptors in bone health and disease. *Nat Rev Endocrinol,* 9(12), 699-712.

Maradonna, F., Gioacchini, G., Falcinelli, S., Bertotto, D., Radaelli, G., Olivotto, I., and Carnevali, O. (2013). Probiotic supplementation promotes calcification in Danio rerio larvae: a molecular study. *PLoS One,* 8(12), e83155.

Marchesi, J. R., Adams, D. H., Fava, F., Hermes, G. D., Hirschfield, G. M., Hold, G., Quraishi, M. N., Kinross, J., Smidt, H., Tuohy, K. M., Thomas, L. V., Zoetendal, E. G., and Hart, A. (2016). The gut microbiota and host health: a new clinical frontier. *Gut,* 65(2), 330-339.

Markle, J. G., Frank, D. N., Mortin-Toth, S., Robertson, C. E., Feazel, L. M., Rolle-Kampczyk, U., von Bergen, M., McCoy, K. D., Macpherson, A. J., and Danska, J. S. (2013). Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. *Science,* 339(6123), 1084-1088.

Mawe, G. M. and Hoffman, J. M. (2013). Serotonin signalling in the gut--functions, dysfunctions and therapeutic targets. *Nat Rev Gastroenterol Hepatol,* 10(8), 473-486.

Mayeda, B. and Ernst, R. A. (2008). Prevention of fatal cage-layer osteoporosis. *Avian Dis,* 52(3), 544-545.

McCabe, L. R., Irwin, R., Schaefer, L., and Britton, R. A. (2013). Probiotic use decreases intestinal inflammation and increases bone density in healthy male but not female mice. *J Cell Physiol,* 228(8), 1793-1798.

McCormack, H. A., Fleming, R. H., McTier, L., and Whitehead, C. C. (2006). Influence of dietary fatty acid n-3/n-6 balance on bone characteristics in young broilers. *British Poultry Abstracts,* 3, 26-26.

McCoy, M. A., Reilly, G. A., and Kilpatrick, D. J. (1996). Density and breaking strength of bones of mortalities among caged layers. *Res Vet Sci,* 60(2), 185-186.

McGeown, D., Danbury, T. C., Waterman-Pearson, A. E., and Kestin, S. C. (1999). Effect of carprofen on lameness in broiler chickens. *Vet Rec,* 144(24), 668-671.

McNamee, P. T., McCullagh, J. J., Thorp, B. H., Ball, H. J., Graham, D., McCullough, S. J., McConaghy, D., and Smyth, J. A. (1998). Study of leg weakness in two commercial broiler flocks. *Vet Rec,* 143(5), 131-135.

Mercer, J. and Hill, W. (1984). Estimation of genetic parameters for skeletal defects in broiler chickens. *Heredity,* 53(1), 193-203.

Messaoudi, S., Kergourlay, G., Dalgalarrondo, M., Choiset, Y., Ferchichi, M., Prevost, H., Pilet, M. F., Chobert, J. M., Manai, M., and Dousset, X. (2012). Purification and characterization of a new bacteriocin active against Campylobacter produced by Lactobacillus salivarius SMXD51. *Food Microbiol,* 32(1), 129-134.

Messora, M. R., Oliveira, L. F., Foureaux, R. C., Taba, M., Jr., Zangeronimo, M. G., Furlaneto, F. A., and Pereira, L. J. (2013). Probiotic therapy reduces periodontal tissue destruction and improves the intestinal morphology in rats with ligature-induced periodontitis. *J Periodontol,* 84(12), 1818-1826.

Messora, M. R., Pereira, L. J., Foureaux, R., Oliveira, L. F., Sordi, C. G., Alves, A. J., Napimoga, M. H., Nagata, M. J., Ervolino, E., and Furlaneto, F. A. (2016). Favourable effects of Bacillus subtilis and Bacillus licheniformis on experimental periodontitis in rats. *Arch Oral Biol,* 66, 108-119.

Mikulski, D., Jankowski, J., Naczmanski, J., Mikulska, M., and Demey, V. (2012). Effects of dietary probiotic (Pediococcus acidilactici) supplementation on performance, nutrient digestibility, egg traits, egg yolk cholesterol, and fatty acid profile in laying hens. *Poult Sci,* 91(10), 2691-2700.

Min, Y. N., Yang, H. L., Xu, Y. X., and Gao, Y. P. (2016). Effects of dietary supplementation of synbiotics on growth performance, intestinal morphology, sIgA content and antioxidant capacities of broilers. *J Anim Physiol Anim Nutr (Berl)*.

Mishra, V., Shah, C., Mokashe, N., Chavan, R., Yadav, H., and Prajapati, J. (2015). Probiotics as potential antioxidants: a systematic review. *J Agric Food Chem,* 63(14), 3615-3626.

Mossner, R., Daniel, S., Albert, D., Heils, A., Okladnova, O., Schmitt, A., and Lesch, K. P. (2000). Serotonin transporter function is modulated by brain-derived neurotrophic factor (BDNF) but not nerve growth factor (NGF). *Neurochem Int,* 36(3), 197-202.

Nahashon, S. N., Nakaue, H. S., and Mirosh, L. W. (1994). Production variables and nutrient retention in single comb White Leghorn laying pullets fed diets supplemented with direct-fed microbials. *Poult Sci,* 73(11), 1699-1711.

Narva, M., Collin, M., Lamberg-Allardt, C., Karkkainen, M., Poussa, T., Vapaatalo, H., and Korpela, R. (2004a). Effects of long-term intervention with Lactobacillus helveticus-fermented milk on bone mineral density and bone mineral content in growing rats. *Ann Nutr Metab,* 48(4), 228-234.

Narva, M., Nevala, R., Poussa, T., and Korpela, R. (2004b). The effect of Lactobacillus helveticus fermented milk on acute changes in calcium metabolism in postmenopausal women. *Eur J Nutr,* 43(2), 61-68.

Nasr, M. A., Nicol, C. J., and Murrell, J. C. (2012a). Do laying hens with keel bone fractures experience pain? *PLoS One,* 7(8), e42420.

Nasr, M. A. F., Murrell, J., Wilkins, L. J., and Nicol, C. J. (2012b). The effect of keel fractures on egg-production parameters, mobility and behaviour in individual laying hens. *Anim Welfare* 21(1), 127-135.

Nasr, M. A. F., Browne, W. J., Caplen, G., Hothersall, B., Murrell, J. C., and Nicol, C. J. (2013). Positive affective state induced by opioid analgesia in laying hens with bone fractures. *Appl Anim Behav Sci* 147(1-2), 127-131.

Nasr, M. A. F., Nicol, C. J., Wilkins, L., and Murrell, J. C. (2015). The effects of two non-steroidal anti-inflammatory drugs on the mobility of laying hens with keel bone fractures. *Vet Anaesth Analg,* 42(2), 197-204.

NASS. (2016). *Chickens and Eggs Annual Summary*. Retrieved from http://usda.mannlib.cornell.edu/usda/current/ChickEgg/ChickEgg-02-25-2016.pdf.

Nestor, K. E., Bacon, W. L., Saif, Y. M., and Renner, P. A. (1985). The influence of genetic increases in shank width on body weight, walking ability, and reproduction of turkeys. *Poult Sci,* 64(12), 2248-2255.

Nestor, K. E., Bacon, W. L., Moorhead, P. D., Saif, Y. M., Havenstein, G. B., and Renner, P. A. (1987). Comparison of bone and muscle growth in turkey lines selected for increased body weight and increased shank width. *Poult Sci,* 66(9), 1421-1428.

Nestor, K. E., and Emmerson D. A.. 1990. Role of genetics in expression and prevention of leg weakness. Pages 14-21 in: Proceedings of the Avian Skeletal Disease Symposium, American Association of Avian Pathologists, San Antonio, TX.

Nzakizwanayo, J., Dedi, C., Standen, G., Macfarlane, W. M., Patel, B. A., and Jones, B. V. (2015). Escherichia coli Nissle 1917 enhances bioavailability of serotonin in gut tissues through modulation of synthesis and clearance. *Sci Rep,* 5, 17324.

O'Brien, C. A., Jia, D., Plotkin, L. I., Bellido, T., Powers, C. C., Stewart, S. A., Manolagas, S. C., and Weinstein, R. S. (2004). Glucocorticoids act directly on osteoblasts and osteocytes to induce their apoptosis and reduce bone formation and strength. *Endocrinology,* 145(4), 1835-1841.

Ohlsson, C., Engdahl, C., Fak, F., Andersson, A., Windahl, S. H., Farman, H. H., Moverare-Skrtic, S., Islander, U., and Sjogren, K. (2014). Probiotics protect mice from ovariectomy-induced cortical bone loss. *PLoS One*, 9(3), e92368.

Ornbjerg, L. M., Ostergaard, M., Jensen, T., Hyldstrup, L., Bach-Mortensen, P., Boyesen, P., Thormann, A., Tarp, U., Bohme, W. P., Lindegaard, H., Poulsen, U. E., Schlemmer, A., Graudal, N., Rodgaard, A., Espesen, J., Kollerup, G. B., Glintborg, B., Madsen, O. R., Jensen, D. V., and Hetland, M. L. (2015). Establishment of age- and sex-adjusted reference data for hand bone mass and investigation of hand bone loss in patients with rheumatoid arthritis treated in clinical practice: an observational study from the DANBIO registry and the Copenhagen Osteoarthritis Study. *Arthritis Res Ther,* 18, 53.

Oury, F., Yadav, V. K., Wang, Y., Zhou, B., Liu, X. S., Guo, X. E., Tecott, L. H., Schutz, G., Means, A. R., and Karsenty, G. (2010). CREB mediates brain serotonin regulation of bone mass through its expression in ventromedial hypothalamic neurons. *Genes Dev*, 24(20), 2330-2342.

Pérez-Conesa, D., López, G., and Ros, G. (2007). Effects of probiotic, prebiotic and synbiotic follow-up infant formulas on large intestine morphology and bone mineralisation in rats. *J Sci Food Agric*, 87(6), 1059-1068.

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

Panda, A. K., Reddy, M. R., Rama Rao, S. V., and Praharaj, N. K. (2003). Production performance, serum/yolk cholesterol and immune competence of white leghorn layers as influenced by dietary supplementation with probiotic. *Trop Anim Health Prod*, 35(1), 85-94.

Panda, A. K., Rao, S. S. R., Raju, M. V., and Sharma, S. S. (2008). Effect of probiotic (Lactobacillus sporogenes) feeding on egg production and quality, yolk cholesterol and humoral immune response of White Leghorn layer breeders. *J Sci Food Agric*, 88(1), 43-47.

Park, M. R., Oh, S., Son, S. J., Park, D. J., Oh, S., Kim, S. H., Jeong, D. Y., Oh, N. S., Lee, Y., Song, M., and Kim, Y. (2015). Bacillus licheniformis Isolated from Traditional Korean Food Resources Enhances the Longevity of Caenorhabditis elegans through Serotonin Signaling. *J Agric Food Chem*, 63(47), 10227-10233.

Park, S. and Bae, J. H. (2015). Probiotics for weight loss: a systematic review and meta-analysis. *Nutr Res*, 35(7), 566-575.

Parkinson, G. B. and Cransberg, P. H. (2004). Effect of casein phosphopeptide and 25-hydroxycholecalciferol on tibial dyschondroplasia in growing broiler chickens. *Br Poult Sci*, 45(6), 802-806.

Parvaneh, K., Ebrahimi, M., Sabran, M. R., Karimi, G., Hwei, A. N., Abdul-Majeed, S., Ahmad, Z., Ibrahim, Z., and Jamaluddin, R. (2015). Probiotics (Bifidobacterium longum) Increase Bone Mass Density and Upregulate Sparc and Bmp-2 Genes in Rats with Bone Loss Resulting from Ovariectomy. *Biomed Res Int,* 2015, 897639.

Patterson, J. A. and Burkholder, K. M. (2003). Application of prebiotics and probiotics in poultry production. *Poult Sci,* 82(4), 627-631.

Perry, J. J. and Yousef, A. E. (2013). Factors affecting thermal resistance of Salmonella enterica serovar enteritidis ODA 99-30581-13 in shell egg contents and use of heat-ozone combinations for egg pasteurization. *J Food Prot*, 76(2), 213-219.

Petrik, M. T., Guerin, M. T., and Widowski, T. M. (2015). On-farm comparison of keel fracture prevalence and other welfare indicators in conventional cage and floor-housed laying hens in Ontario, Canada. *Poult Sci*, 94(4), 579-585.

Praharaj, I., John, S. M., Bandyopadhyay, R., and Kang, G. (2015). Probiotics, antibiotics and the immune responses to vaccines. *Philos Trans R Soc Lond B Biol Sci*, 370(1671).

Qu, A., Brulc, J. M., Wilson, M. K., Law, B. F., Theoret, J. R., Joens, L. A., Konkel, M. E., Angly, F., Dinsdale, E. A., Edwards, R. A., Nelson, K. E., and White, B. A. (2008). Comparative metagenomics reveals host specific metavirulomes and horizontal gene transfer elements in the chicken cecum microbiome. *PLoS One*, 3(8), e2945.

Raboni, S., Bettati, S., and Mozzarelli, A. (2009). Tryptophan synthase: a mine for enzymologists. *Cell Mol Life Sci,* 66(14), 2391-2403.

Rajilic-Stojanovic, M., Smidt, H., and de Vos, W. M. (2007). Diversity of the human gastrointestinal tract microbiota revisited. *Environ Microbiol,* 9(9), 2125-2136.

Rath, N. C., Huff, G. R., Huff, W. E., and Balog, J. M. (2000). Factors regulating bone maturity and strength in poultry. *Poult Sci,* 79(7), 1024-1032.

Rauch, A., Seitz, S., Baschant, U., Schilling, A. F., Illing, A., Stride, B., Kirilov, M., Mandic, V., Takacz, A., Schmidt-Ullrich, R., Ostermay, S., Schinke, T., Spanbroek, R., Zaiss, M. M., Angel, P. E., Lerner, U. H., David, J. P., Reichardt, H. M., Amling, M., Schutz, G., and Tuckermann, J. P. (2010). Glucocorticoids suppress bone formation by attenuating osteoblast differentiation via the monomeric glucocorticoid receptor. *Cell Metab,* 11(6), 517-531.

Redman, M. G., Ward, E. J., and Phillips, R. S. (2014). The efficacy and safety of probiotics in people with cancer: a systematic review. *Ann Oncol,* 25(10), 1919-1929.

Regmi, P., Deland, T. S., Steibel, J. P., Robison, C. I., Haut, R. C., Orth, M. W., and Karcher, D. M. (2015). Effect of rearing environment on bone growth of pullets. *Poult Sci,* 94(3), 502-511.

Regmi, P., Smith, N., Nelson, N., Haut, R. C., Orth, M. W., and Karcher, D. M. (2016). Housing conditions alter properties of the tibia and humerus during the laying phase in Lohmann white Leghorn hens. *Poult Sci,* 95(1), 198-206.

Reid, I. R. (1997). Glucocorticoid osteoporosis--mechanisms and management. *Eur J Endocrinol,* 137(3), 209-217.

Reiter, K. and Bessei, W. (2009). Effect of locomotor activity on leg disorder in fattening chicken. *Berl Munch Tierarztl Wochenschr,* 122(7-8), 264-270.

Rekaya, R., Sapp, R. L., Wing, T., and Aggrey, S. E. (2013). Genetic evaluation for growth, body composition, feed efficiency, and leg soundness. *Poult Sci,* 92(4), 923-929.

Rennie, J. S., Fleming, R. H., McCormack, H. A., McCorquodale, C. C., and Whitehead, C. C. (1997). Studies on effects of nutritional factors on bone structure and osteoporosis in laying hens. *Br Poult Sci,* 38(4), 417-424.

Ribeiro, V., Albino, L. F. T., Rostagno, H. S., Barreto, S. L. T., Hannas, M. I., Harrington, D., de Araujo, F. A., Ferreira, H. C., and Ferreira, M. A. (2014). Effects of the dietary supplementation of Bacillus subtilis levels on performance, egg quality and excreta moisture of layers. *Anim Feed Sci Tech,* 195, 142-146.

Rochefort, G. Y., Pallu, S., and Benhamou, C. L. (2010). Osteocyte: the unrecognized side of bone tissue. *Osteoporos Int,* 21(9), 1457-1469.

Rodrigues, F. C., Castro, A. S., Rodrigues, V. C., Fernandes, S. A., Fontes, E. A., de Oliveira, T. T., Martino, H. S., and de Luces Fortes Ferreira, C. L. (2012). Yacon flour and Bifidobacterium longum modulate bone health in rats. *J Med Food,* 15(7), 664-670.

Rosenbaum, M., Knight, R., and Leibel, R. L. (2015). The gut microbiota in human energy homeostasis and obesity. *Trends Endocrinol Metab,* 26(9), 493-501.

Ruddick, J. P., Evans, A. K., Nutt, D. J., Lightman, S. L., Rook, G. A., and Lowry, C. A. (2006). Tryptophan metabolism in the central nervous system: medical implications. *Expert Rev Mol Med,* 8(20), 1-27.

Sadeghi, A. A. (2014). Bone Mineralization of Broiler Chicks Challenged with Salmonella enteritidis Fed Diet Containing Probiotic (Bacillus subtilis). *Probiotics Antimicrob Proteins,* 6(3-4), 136-140.

Sanotra, G. S., Lund, J. D., Ersboll, A. K., Petersen, J. S., and Vestergaard, K. S. (2001). Monitoring leg problems in broilers: a survey of commercial broiler production in Denmark. *World Poultry Sci J* 57(1), 55-69.

Sanotra, G. S., Berg, C., and Lund, J. D. (2003). A comparison between leg problems in Danish and Swedish broiler production. *Anim Welfare* 12(4), 677-683.

Savignac, H. M., Corona, G., Mills, H., Chen, L., Spencer, J. P., Tzortzis, G., and Burnet, P. W. (2013). Prebiotic feeding elevates central brain derived neurotrophic factor, N-methyl-D-aspartate receptor subunits and D-serine. *Neurochem Int,* 63(8), 756-764.

Schrezenmeir, J. and de Vrese, M. (2001). Probiotics, prebiotics, and synbiotics - approaching a definition. *Am J Clin Nutr* 73(2), 361s-364s.

Schweitzer, M. H., Elsey, R. M., Dacke, C. G., Horner, J. R., and Lamm, E. T. (2007). Do egg-laying crocodilian (Alligator mississippiensis) archosaurs form medullary bone? *Bone,* 40(4), 1152-1158.

Schwenger, E. M., Tejani, A. M., and Loewen, P. S. (2015). Probiotics for preventing urinary tract infections in adults and children. *Cochrane Database Syst Rev*(12), Cd008772.

Shahnazari, M., Sharkey, N. A., Fosmire, G. J., and Leach, R. M. (2006). Effects of strontium on bone strength, density, volume, and microarchitecture in laying hens. *J Bone Miner Res,* 21(11), 1696-1703.

Sheridan, A. K., Howlett, C. R., and Burton, R. W. (1978). The inheritance of tibial dyschondroplasia in broilers. *Br Poult Sci,* 19(4), 491-499.

Shim, M. Y., Karnuah, A. B., Mitchell, A. D., Anthony, N. B., Pesti, G. M., and Aggrey, S. E. (2012). The effects of growth rate on leg morphology and tibia breaking strength, mineral density, mineral content, and bone ash in broilers. *Poult Sci,* 91(8), 1790-1795.

Siegel, P., Gustin, S., and Katanbaf, M. (2011). Motor ability and self-selection of an analgesic drug by fast-growing chickens. *J Appl Poult Res,* 20(3), 249-252.

Silversides, F. G., Singh, R., Cheng, K. M., and Korver, D. R. (2012). Comparison of bones of 4 strains of laying hens kept in conventional cages and floor pens. *Poult Sci,* 91(1), 1-7.

Simpson, H. L. and Campbell, B. J. (2015). Review article: dietary fibre-microbiota interactions. *Aliment Pharmacol Ther,* 42(2), 158-179.

Sinnesael, M., Claessens, F., Boonen, S., and Vanderschueren, D. (2013). Novel insights in the regulation and mechanism of androgen action on bone. *Curr Opin Endocrinol Diabetes Obes,* 20(3), 240-244.

Soccol, C. R., Vandenberghe, L. P. d. S., Spier, M. R., Medeiros, A. B. P., Yamaguishi, C. T., Lindner, J. D. D., Pandey, A., and Thomaz-Soccol, V. (2010). The potential of probiotics: a review. *Food Technology and Biotechnology,* 48(4), 413-434.

Sohail, M. U., Ijaz, A., Yousaf, M. S., Ashraf, K., Zaneb, H., Aleem, M., and Rehman, H. (2010). Alleviation of cyclic heat stress in broilers by dietary supplementation of mannan-oligosaccharide and Lactobacillus-based probiotic: dynamics of cortisol, thyroid hormones, cholesterol, C-reactive protein, and humoral immunity. *Poult Sci,* 89(9), 1934-1938.

Sohail, M. U., Hume, M. E., Byrd, J. A., Nisbet, D. J., Ijaz, A., Sohail, A., Shabbir, M. Z., and Rehman, H. (2012). Effect of supplementation of prebiotic mannan-oligosaccharides and probiotic mixture on growth performance of broilers subjected to chronic heat stress. *Poult Sci,* 91(9), 2235-2240.

Sornplang, P. and Piyadeatsoontorn, S. (2016). Probiotic isolates from unconventional sources: a review. *J Anim Sci Technol,* 58, 26.

Sparke, A. J., Sims, T. J., Avery, N. C., Bailey, A. J., Fleming, R. H., and Whitehead, C. C. (2002). Differences in composition of avian bone collagen following genetic selection for resistance to osteoporosis. *Br Poult Sci,* 43(1), 127-134.

Stanford, M. (2006). Effects of UVB radiation on calcium metabolism in psittacine birds. *Vet Rec,* 159(8), 236-241.

Stone, T. W., Forrest, C. M., and Darlington, L. G. (2012). Kynurenine pathway inhibition as a therapeutic strategy for neuroprotection. *Febs j,* 279(8), 1386-1397.

Stratmann, A., Frohlich, E. K., Harlander-Matauschek, A., Schrader, L., Toscano, M. J., Wurbel, H., and Gebhardt-Henrich, S. G. (2015). Soft perches in an aviary system reduce incidence of keel bone damage in laying hens. *PLoS One,* 10(3), e0122568.

Stratmann, A., Frohlich, E. K., Gebhardt-Henrich, S. G., Harlander-Matauschek, A., Wurbel, H., and Toscano, M. J. (2016). Genetic selection to increase bone strength affects prevalence of keel bone damage and egg parameters in commercially housed laying hens. *Poult Sci,* 95(5), 975-984.

Straub, R. H., Cutolo, M., and Pacifici, R. (2015). Evolutionary medicine and bone loss in chronic inflammatory diseases--A theory of inflammation-related osteopenia. *Semin Arthritis Rheum,* 45(2), 220-228.

Suvarna, V. and Boby, V. (2005). Probiotics in human health: A current assessment. *Current science,* 88(11), 1744-1748.

Talaty, P. N., Katanbaf, M. N., and Hester, P. Y. (2009). Variability in bone mineralization among purebred lines of meat-type chickens. *Poult Sci,* 88(9), 1963-1974.

Tamayo-Ramos, J. A., Sanz-Penella, J. M., Yebra, M. J., Monedero, V., and Haros, M. (2012). Novel phytases from Bifidobacterium pseudocatenulatum ATCC 27919 and Bifidobacterium longum subsp. infantis ATCC 15697. *Appl Environ Microbiol,* 78(14), 5013-5015.

Tanaka, K., Hirai, T., Ishibashi, Y., Izumo, N., and Togari, A. (2015). Modulation of osteoblast differentiation and bone mass by 5-HT2A receptor signaling in mice. *Eur J Pharmacol,* 762, 150-157.

Thomas, C. M. and Versalovic, J. (2010). Probiotics-host communication: Modulation of signaling pathways in the intestine. *Gut Microbes,* 1(3), 148-163.

Thorp, B. H., Wilson, S., Rennie, S., and Solomon, S. E. (1993). The effect of a bisphosphonate on bone volume and eggshell structure in the hen. *Avian Pathol,* 22(4), 671-682.

Thorp, B. H. (1994). Skeletal disorders in the fowl: a review. *Avian Pathol,* 23(2), 203-236.

Tien, M. T., Girardin, S. E., Regnault, B., Le Bourhis, L., Dillies, M. A., Coppee, J. Y., Bourdet-Sicard, R., Sansonetti, P. J., and Pedron, T. (2006). Anti-inflammatory effect of Lactobacillus casei on Shigella-infected human intestinal epithelial cells. *J Immunol,* 176(2), 1228-1237.

Toscano, M. J., Wilkins, L. J., Millburn, G., Thorpe, K., and Tarlton, J. F. (2013). Development of an ex vivo protocol to model bone fracture in laying hens resulting from collisions. *PLoS One,* 8(6), e66215.

Toscano, M. J., Booth, F., Wilkins, L. J., Avery, N. C., Brown, S. B., Richards, G., and Tarlton, J. F. (2015). The effects of long (C20/22) and short (C18) chain omega-3 fatty acids on keel bone fractures, bone biomechanics, behavior, and egg production in free-range laying hens. *Poult Sci,* 94(5), 823-835.

Uchiyama-Tanaka, Y. (2014). A 10-patient case study on the influence of two different probiotics on individual intestinal microbiota. *J Altern Complement Med,* 20(10), 800-803.

Van Immerseel, F., Russell, J. B., Flythe, M. D., Gantois, I., Timbermont, L., Pasmans, F., Haesebrouck, F., and Ducatelle, R. (2006). The use of organic acids to combat Salmonella in poultry: a mechanistic explanation of the efficacy. *Avian Pathol,* 35(3), 182-188.

Vestergaard, K. S. and Sanotra, G. S. (1999). Relationships between leg disorders and changes in the behaviour of broiler chickens. *Vet Rec,* 144(8), 205-209.

Wang, H., Lee, I. S., Braun, C., and Enck, P. (2016). Effect of probiotics on central nervous system functions in animals and humans - a systematic review. *J Neurogastroenterol Motil*, 22(4):589-605.

Wang, L., Yang, Y., Cai, B., Cao, P., Yang, M., and Chen, Y. (2014). Coexpression and secretion of endoglucanase and phytase genes in Lactobacillus reuteri. *Int J Mol Sci,* 15(7), 12842-12860.

Webster, A. B., Fairchild, B. D., Cummings, T. S., and Stayer, P. A. (2008). Validation of a three-point gait-scoring system for field assessment of walking ability of commercial broilers. *J Appl Poultry Res,* 17(4), 529-539.

Weeks, C. A., Knowles, T. G., Gordon, R. G., Kerr, A. E., Peyton, S. T., and Tilbrook, N. T. (2002). New method for objectively assessing lameness in broiler chickens. *Vet Rec,* 151(25), 762-764.

Westbroek, I., van der Plas, A., de Rooij, K. E., Klein-Nulend, J., and Nijweide, P. J. (2001). Expression of serotonin receptors in bone. *J Biol Chem,* 276(31), 28961-28968.

Whitehead, C. C. and Fleming, R. H. (2000). Osteoporosis in cage layers. *Poult Sci,* 79(7), 1033-1041.

Whitehead, C. C. (2002). *Bone breakage and osteoporosis in laying hens: causes and solutions.* Page 61-68 in: Proceedings of the Australian Poultry Science Symposium.

Whitehead, C. C. (2004). Overview of bone biology in the egg-laying hen. *Poult Sci,* 83(2), 193-199.

Wideman, R. F., Jr., Hamal, K. R., Stark, J. M., Blankenship, J., Lester, H., Mitchell, K. N., Lorenzoni, G., and Pevzner, I. (2012). A wire-flooring model for inducing lameness in broilers: evaluation of probiotics as a prophylactic treatment. *Poult Sci,* 91(4), 870-883.

Wideman, R. F., Jr., Al-Rubaye, A., Kwon, Y. M., Blankenship, J., Lester, H., Mitchell, K. N., Pevzner, I. Y., Lohrmann, T., and Schleifer, J. (2015a). Prophylactic administration of a combined prebiotic and probiotic, or therapeutic administration of enrofloxacin, to reduce the incidence of bacterial chondronecrosis with osteomyelitis in broilers. *Poult Sci,* 94(1), 25-36.

Wideman, R. F., Jr., Blankenship, J., Pevzner, I. Y., and Turner, B. J. (2015b). Efficacy of 25-OH Vitamin D3 prophylactic administration for reducing lameness in broilers grown on wire flooring. *Poult Sci,* 94(8), 1821-1827.

Wilkins, L. J., McKinstry, J. L., Avery, N. C., Knowles, T. G., Brown, S. N., Tarlton, J., and Nicol, C. J. (2011). Influence of housing system and design on bone strength and keel bone fractures in laying hens. *Vet Rec,* 169(16), 414.

Williams, B., Waddington, D., Murray, D. H., and Farquharson, C. (2004). Bone strength during growth: influence of growth rate on cortical porosity and mineralization. *Calcif Tissue Int,* 74(3), 236-245.

Wise, M. G. and Siragusa, G. R. (2007). Quantitative analysis of the intestinal bacterial community in one- to three-week-old commercially reared broiler chickens fed conventional or antibiotic-free vegetable-based diets. *J Appl Microbiol,* 102(4), 1138-1149.

Wistedt, A., Ridderstrale, Y., Wall, H., and Holm, L. (2014). Exogenous estradiol improves shell strength in laying hens at the end of the laying period. *Acta Vet Scand,* 56, 34.

Yadav, V. K., Ryu, J. H., Suda, N., Tanaka, K. F., Gingrich, J. A., Schutz, G., Glorieux, F. H., Chiang, C. Y., Zajac, J. D., Insogna, K. L., Mann, J. J., Hen, R., Ducy, P., and Karsenty, G. (2008). Lrp5 controls bone formation by inhibiting serotonin synthesis in the duodenum. *Cell,* 135(5), 825-837.

Yadav, V. K., Oury, F., Suda, N., Liu, Z. W., Gao, X. B., Confavreux, C., Klemenhagen, K. C., Tanaka, K. F., Gingrich, J. A., Guo, X. E., Tecott, L. H., Mann, J. J., Hen, R., Horvath, T. L., and Karsenty, G. (2009). A serotonin-dependent mechanism explains the leptin regulation of bone mass, appetite, and energy expenditure. *Cell,* 138(5), 976-989.

Yalcin, S., Molayoglu, H. B., Baka, M., Genin, O., and Pines, M. (2007). Effect of temperature during the incubation period on tibial growth plate chondrocyte differentiation and the incidence of tibial dyschondroplasia. *Poult Sci,* 86(8), 1772-1783.

Yalcin, S., Yalcin, S., Cakin, K., Eltan, O., and Dagasan, L. (2010). Effects of dietary yeast autolysate (Saccharomyces cerevisiae) on performance, egg traits, egg cholesterol content, egg yolk fatty acid composition and humoral immune response of laying hens. *J Sci Food Agric,* 90(10), 1695-1701.

Yang, H., Zhao, X., Tang, S., Huang, H., Zhao, X., Ning, Z., Fu, X., and Zhang, C. (2016). Probiotics reduce psychological stress in patients before laryngeal cancer surgery. *Asia Pac J Clin Oncol,* 12(1), e92-96.

Yano, J. M., Yu, K., Donaldson, G. P., Shastri, G. G., Ann, P., Ma, L., Nagler, C. R., Ismagilov, R. F., Mazmanian, S. K., and Hsiao, E. Y. (2015). Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell,* 161(2), 264-276.

Yanofsky, C. (2007). RNA-based regulation of genes of tryptophan synthesis and degradation, in bacteria. *RNA,* 13(8), 1141-1154.

Yeung, C. Y., Chan, W. T., Jiang, C. B., Cheng, M. L., Liu, C. Y., Chang, S. W., Chiang Chiau, J. S., and Lee, H. C. (2015). Amelioration of Chemotherapy-Induced Intestinal Mucositis by Orally Administered Probiotics in a Mouse Model. *PLoS One,* 10(9), e0138746.

Yoruk, M. A., Gul, M., Hayirli, A., and Macit, M. (2004). The effects of supplementation of humate and probiotic on egg production and quality parameters during the late laying period in hens. *Poult Sci,* 83(1), 84-88.

Zhang, J. L., Xie, Q. M., Ji, J., Yang, W. H., Wu, Y. B., Li, C., Ma, J. Y., and Bi, Y. Z. (2012). Different combinations of probiotics improve the production performance, egg quality, and immune response of layer hens. *Poult Sci*, 91(11), 2755-2760.

Zhang, L., Li, N., Caicedo, R., and Neu, J. (2005). Alive and dead Lactobacillus rhamnosus GG decrease tumor necrosis factor-alpha-induced interleukin-8 production in Caco-2 cells. *J Nutr*, 135(7), 1752-1756.

Zhang, R., Zhou, M., Tu, Y., Zhang, N. F., Deng, K. D., Ma, T., and Diao, Q. Y. (2016). Effect of oral administration of probiotics on growth performance, apparent nutrient digestibility and stress-related indicators in Holstein calves. *J Anim Physiol Anim Nutr (Berl)*, 100(1), 33-38.

CHAPTER 2. EFFECTS OF A MULTI-SPECIES BASED PROBIOTIC SUPPLEMENT ON PERFORMANCE TRAITS AND BONE MINERALIZATION IN LAYING HENS

2.1 Abstract

The objective of this study was to determine the effects of a multi-species based probiotic supplement on performance traits and bone health of laying hens. Ninety-six 60-wk-old White Leghorn hens were assigned to 4-hen cages based on their BW. The cages were randomly assigned to 1 of 4 treatments: a layer diet mixed with a commercial probiotic product at 0, 0.5, 1.0 or 2.0 g/kg (Control, 0.5X, 1.0X, and 2.0X) for 7 wk. Cecal *Bifidobacterium* spp. counts were higher in all treatment groups ($P < 0.001$). The percent of unmarketable eggs (cracked and shell-less eggs) was decreased in both 0.5X and 2.0X groups compared to the control ($P = 0.02$), mainly due to the reduction of shell-less eggs ($P = 0.05$). Increases in tibial and femoral mineral density and femoral mineral content ($P = 0.04$, 0.03, and 0.02, respectively), with a concomitant trend for increases in humerus mineral density and tibial mineral content ($P = 0.07$ and 0.08, respectively), occurred in the 2.0X group. However, the bone remodeling indicators of circulating OC, CTX, and Pi were similar among groups ($P > 0.05$). Further measures comparing 2.0X and control groups indicated that probiotic administration did not affect the concentrations of 5-HT in plasma and the ceca, and the TRP concentrations in plasma ($P > 0.05$). In addition, no differences in 5-HT, DA, and their metabolites occurred in the raphe nuclei and hypothalamus ($P > 0.05$). Cytokine concentrations, both pro-inflammatory (IL-1β, IL-6, IFN-γ, and TNF-α) and anti-inflammatory (IL-10) as well as CORT were similar in plasma between the 2.0X group and the control group ($P > 0.05$). In line with these findings, no differences of mRNA expression of IL-1β, IL-6, and LITAF were detected in the ceca tonsil between the two groups ($P > 0.05$). In conclusion, dietary probiotic supplementation altered cecal microbiota composition resulting in reduced shell-less egg production and improved bone mineralization in laying hens. These results suggest that the immune cytokines, 5-HT, CORT, as well as the bone remodeling indicators of OC, CTX, and Pi are not involved in probiotic's effect of improving shell and bone mineralization.

2.2 Introduction

Osteoporosis is a widespread health and welfare issue in laying hens. It contributes to approximately 20 to 35% of all mortality during the egg production cycle of caged hens (Anderson, 2002). As in humans, osteoporosis in laying hen is characterized by progressive loss of structural bone, leading to skeletal fragility and increasing susceptibility to fracture (Whitehead, 2004). Osteoporosis is caused by an imbalance in bone remodeling between bone formation (osteoblasts) and resorption (osteoclasts) under the influence of estrogen. At the onset of sexual maturity, the level of estrogen markedly increases and is in favor of medullary bone deposition, providing a labile source of calcium for eggshell formation (Whitehead and Fleming, 2000). However, continuous deposition of medullary bone with age results in deterioration of structural bone, namely cancellous and cortical bone (Dacke et al., 1993). Consequently, age-related loss of structural bone over the course of the egg production cycle eventually leads to osteoporosis (Whitehead and Fleming, 2000), being most severe at the end of lay at about 68 to 72 wk of age (Whitehead and Fleming, 2000; Beck and Hansen, 2004).

Osteoporosis is most prevalent in caged layers (Whitehead and Fleming, 2000). Although a modified laying hen cage system (named the enriched or furnished cage) has been developed for promotion of activity and behavioral repertoires with the aim to benefit bone health (Jendral et al., 2008; Hester et al., 2013), currently about 94% of all eggs produced in the United States (and 90% around the world) are laid by hens kept in the conventional cage system (UEP, 2016). Osteoporosis causes considerable economic losses to the poultry industry. In addition, osteoporosis-associated animal welfare issues, such as increased fractures that subject hens to chronic pain (Nasr et al., 2012), have drawn great awareness by the public.

Supplementations of probiotics are common management practices in the poultry industry as an alternative to antibiotics. Probiotics are live microorganisms that after ingestion confer beneficial effects on the health of the host. Recent studies demonstrate that probiotics also contribute to bone health (Scholz-Ahrens et al., 2007; McCabe et al., 2015). Skeletal benefits of certain probiotics, for example, have been observed under various pathological conditions. Administration of *Bacillus subtilis* for 44 d (Messora et al., 2013) or *Lactobacillus brevis* for 5 d (Maekawa and Hajishengallis, 2014) prevented

experimental periodontitis induced bone loss in rats and mice. Ovariectomy induced bone loss in mice was prevented by feeding a diet mixed with *Lactobacillus reuteri* for 4 wk (Britton et al., 2014) or a mixture of multiple *Lactobacillus* strains for 6 wk (Ohlsson et al., 2014). Bone loss in type 1 diabetic mice was also blocked by administering *Lactobacillus reuteri* for 4 wk (Zhang et al., 2015).

Limited studies have been conducted in poultry to evaluate the effect of probiotics on skeletal health. An increase in tibial weight, length, wall thickness, ash content, strength as well as tibiotarsal index occurred in broiler chickens fed various probiotics such as lactic acid bacteria and *Bacillus spp.* (Mutus et al., 2006; Panda et al., 2006; Houshmand et al., 2011; Ziaie et al., 2011; Fuentes et al., 2013; Sadeghi, 2014). In laying hens, tibia weight, density, and ash content increased after feeding a diet containing *Bacillus subtilis* (Abdelqader et al., 2013b). The objective of the present experiment was to investigate the effects of dietary probiotic inclusion on 1) the performance and bone mineralization of laying hens and 2) the expression of the 5-HT system, stress indicators, and immune cytokines. We hypothesized that the probiotic supplementation will improve egg production performance traits and bone mineralization in aging laying hens through regulating 5-HT, immune cytokines, and/or CORT.

2.3 Materials and Methods

2.3.1 Birds, Management, and Sample Collection

Ninety-six 60-wk-old White Leghorn laying hens of the Hy-Line W-36 strain were provided by Creighton Brothers Farm, Atwood, IN. Hens were assigned to 24 cages with 4 hens per cage based on their BW so that each cage had similar mean BW. The cage dimensions were 38 x 51 x 48 cm (length x width x height) providing 484.5 cm^2 of floor space per hen. Each cage was equipped with a lined under tray for collecting manure. Liners were replaced daily with clean new ones. Each cage contained 2 nipple drinkers and 1 feeder providing 10.3 cm feeder space per hen. A piece of cardboard was installed between every 2 feeders to ensure that hens were not able to consume feed from the adjacent feeders. Hens were housed in one room; and average room temperature was 20° C throughout the experimental period. The photoperiod was 16 light (0400h to

2000h):8 dark. The Purdue Animal Use and Care Committee approved the experimental protocol (PACUC Number: 1111000262).

Prior to the start of experiment, all hens were given 4 wk (56 to 59 wk of age) to adapt to their housing environment and fed a layer diet (Table 2.1). Egg production and BW were monitored during the pre-trail period. Hens were transferred among the cages as necessary to ensure egg production and BW were evenly distributed among the cages by 59 wk of age. At 60 wk of age, the 24 cages were randomly assigned to 1 of 4 dietary treatments consisting of a layer diet mixed with a commercial probiotic product (PoultryStar®, BIOMIN America, Inc., San Antonio, TX) at 0, 0.5, 1.0, or 2.0 g/kg (Control; 0.5X, 10^6 cfu/g; 1.0X, 2 x 10^6 cfu/g; and 2.0X, 4 x 10^6 cfu/g of feed, respectively) for 7 wk. The probiotic consisted of 4 microbial strains (*Enterococcus faecium, Pediococcus acidilactici, Bifidobacterium animalis,* and *Lactobacillus reuteri*). Feed and water were provided ad libitum. Diets were mixed at the Purdue University feed mill. Feed samples were collected at the beginning and end of the study for analysis of probiotic recovery by the manufacturer's proprietary assay (Biomin America Inc., San Antonio, TX).

At the end of this study, starting from 0900h, all hens were injected intravenously with sodium pentobarbital (30 mg/kg BW). A 5-mL blood sample was collected from each hen via cardiac puncture within 2 min after removal from her cage. Each sample was placed into an ice cooled EDTA-coated tube. Duplicate blood smears on glass slides were made per hen. Plasma was collected by centrifuging the whole blood at 700 x g for 15 min and frozen at -80° C until assayed.

Following blood collection, hens were euthanized by cervical dislocation. A 1 cm cecal tissue section that included the cecal tonsil, the hypothalamus, and raphe nuclei were collected from 1 hen per cage. Tissue samples were immediately frozen with dry ice and kept at -80° C until assayed. Cecal content was collected from the lumen of both ceca of all hen, and stored at -80° C. The left wing, thigh, drum, and breast were collected from all hens in the study and frozen at -20° C.

2.3.2 Production Performance

Eggs were collected daily and classified as normal (intact egg with clean shell and without visual cracks), dirty (intact egg with blood spots or feces), and unmarketable (visually cracked or shell-less egg). The egg collection area, inside the cages, and the trays under the cages were checked carefully for shell-less eggs. Hen-d egg production and % dirty eggs were calculated on a weekly basis. The productions of cracked, shell-less, and unmarketable eggs were calculated on a weekly (Table 2.4) as well as on a cumulative basis (Figure 2.1). Within a treatment, calculations used for weekly cumulative production involved adding the number of eggs produced in a given wk to the number of eggs laid in prior wk divided by the number of total d in these wk and multiplying the quotient by 100 to express the data as a percentage.

Twenty-four intact hard-shelled eggs per treatment were collected randomly over a 2-d period biweekly. Individual egg and shell weights were determined as described by Klingensmith and Hester (1985). The proportion of shell was calculated by dividing shell weight by egg weight and multiplying the quotient by 100. Eggshell strength was measured using an egg force reader (Orka Food Technology, Bountiful, UT). Eggshell thickness was determined at 3 different places along the egg's equator using a digital micrometer (Coolant Proof Micrometer Series 293, Mitutoyo America Corp., Aurora, IL) as previously introduced (Poggenpoel, 1986).

Hens were weighed individually at 60, 63, and 66 wk of age. Mortality was recorded daily. Feed intake and total egg weight during a 3-d period were determined by cage at 60, 62, 64, and 66 wk of age. Feed conversion was calculated as kg of feed per kg of eggs laid.

2.3.3 Bone Mineralization and Breaking Force

The left wing, thigh, drum, and breast were thawed and then scanned using DEXA (Norland Medical Systems Inc., Fort Atkinson, WI) with muscle, skin, and feathers intact (except the keel) to quantify BMD, BMC, and bone area of the humerus, femur, tibia/fibula, and keel. The muscles were removed from the keel to allow the bone to be oriented laterally in a similar manner among all samples (Hester et al., 2013). After

scanning, soft tissue was removed from the tibia. The tibia bones were placed in plastic bags and refrozen until bone-breaking strength analysis.

Bone-breaking force was determined using a shear testing method that entailed a load frame (MTS Criterion Model 43, MTS Systems Corp., Eden Prairie, MN) with the MTS TestSuite TW Elite Software. Before the test, bones were removed from the freezer and brought to room temperature. Bones were sheared at midshaft using a crosshead speed of 5.0 mm/min to minimize splintering (Onyango et al., 2003; ASABE, 2007).

2.3.4 Parameters of Bone Turnover

Plasma samples collected from hens of the same cage were pooled. Commercial ELISA kits (MyBioSource, San Diego, CA) were used for detecting plasma concentrations of OC and CTX. The Pi concentrations were determined using a QuantiChrom kit (BioAssay Systems, Hayward, CA) following manufacturer's instructions.

2.3.5 Cecal Microbial Analysis

Microbial analysis of the cecal content was conducted the next d following collection. One gram of the cecal content that was collected from each hen and diluted with 9 mL of buffered peptone water (Neogen Corp., Lansing, MI) and homogenized in a snap-cap tube. Each homogenized sample was serially diluted from 10^{-1} to 10^{-5}. Ten microliters of each diluted sample were plated on Rogosa agar and BSM agar to determine if *Lactobacillus* spp. and *Bifidobacterium* spp. were present, respectively. Both plates were incubated anaerobically at 37° C and counted for bacterial colonies after 48 h of incubation. The results were expressed as $\log_{10}$ cfu per gram of fresh sample (Salim et al., 2013).

2.3.6 Serotonin System

Plasma samples were measured for 5-HT and its precursor TRP by using commercial ELISA kits (MyBioSource, San Diego, CA). Cecal tissue without lumen contents was analyzed for 5-HT. Briefly, a small piece of cecum was weighed and homogenized (10 mg tissue to 100 µl PBS) using a tissue homogenizer. Homogenates

were centrifuged for 15 min at 1500 x *g*. The supernatants were collected and assayed immediately following manufacturer's instruction.

The hypothalamus and raphe nuclei from the left hemisphere of the brain were analyzed using HPLC (UltiMate™ 3000 RSLCnano System, Thermo Fisher Scientific Inc., Waltham, MA). The brain regions were weighed and homogenized in ice-cold 0.2 M perchloric acid, at a 10:1 ratio (4 µL of perchloric acid:mg of sample). The homogenized mixture was centrifuged at 18,187g for 15 min at 4° C. The resultant supernatant was drawn into a microcentrifuge tube and diluted 1:1 with mobile phase. The mixture was centrifuged at 18,187g for 10 min at 4° C. The supernatant was draw off and filtered through a 0.2-µm polyvinylidene fluoride filter into an HPLC sample vial. The mobile phase flow rate was 0.8 mL/min. The concentrations of NE, EP, DOPAC, DA, 5-HIAA, HVA, 5-HT, and TRP were calculated from a reference curve made using relative standards.

2.3.7 Cytokines and Immunoglobulin Analyses

Commercial ELISA kits were used for measuring plasma cytokine concentrations of IL-1β (Lifeome BioLabs, Oceanside, CA), IL-6 (MyBioSource, San Diego, CA), IL-10 (MyBioSource, San Diego, CA), and IFN-γ (MyBioSource, San Diego, CA) as well as immunoglobulin concentrations of IgA, IgG, and IgM (Bethyl Laboratories Inc., Montgomery, TX).

Cecal tonsil mRNA expression of IL-6, IL-1β, and LITAF was detected by real-time PCR using primers and probes (Table 2.2) developed elsewhere (Strong et al., 2015). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. Cecal tonsils were homogenized using a tissue homogenizer, and RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA). After quantifying using the GeneQuant™ 100 Spectrophotometer (GE Healthcare, UK), RNA sample and RNase-free water (Ambion Inc.) were added to 61.5 µL of master mix for a total of 100 µL. The master mix consisted of 2.5µL of Multi-Scribe reverse transcriptase, 22 µL of 25 mM MgCl, 5 µl random hexamers, 2µL RNase inhibitor, 20 µl dNTPs, and 10 µL of TaqMan reverse transcription buffer provided in the TaqMan Reverse Transcription Reagent Pack (Applied Biosystems, Foster City, CA). It was followed by

reverse transcription using Techne TC-3000G PCR Thermal Cycler (Bibby Scientific Limited, UK) and amplification using StepOnePlus™ System (Applied Biosystems, Foster City, CA). The PCR mixture contained 1.625 µL of TaqMan probe, 2.25 µL of gene- specific TaqMan forward and reverse primers each, 12.5 µL of PCR Mastermix (Applied Biosystems), 3.875 µL RNase-free water, and 2.5 µL of sample cDNA. The cycling conditions were 50° C for 2 min and 95° C for 10 min of the holding stage, followed by 40 cycles of 95° C for 15 s, 60° C for 1 min. Results were quantitated by standard curve method. Standards were measured in triplicates unless duplicates had a standard deviation of less than 2.0 and a coefficient of variation less than 2.0%.

2.3.8 CORT and H:L ratio

Plasma samples were analyzed for CORT concentrations using a commercial kit (Arbor Assays LLC, MI). Blood smears were stained with Camco 3 step staining reagents (Cambridge Diagnostic, Inc., Fort Lauderdale, FL). The heterophils and lymphocytes were counted at 1,000× (oil immersion lens) until a total of 100 cells per slide was reached. The H:L ratio was calculated by dividing the number of heterophils by the number of lymphocytes (Cheng et al., 2001). The ratios from the 2 slides per pullet were averaged and the mean was used for the statistical analysis.

2.3.9 Statistical Analysis

The BW, egg production, % unmarketable egg, % cracked egg, % shell-less egg, % dirty egg, egg weight, eggshell strength, % shell, shell thickness, feed intake, and feed conversion were analyzed using a 2-way ANOVA with repeated measures over the age of the hen. The mixed model procedure of SAS 9.4 software was used (SAS Institute Inc., Cary, NC). Fixed effects included probiotic treatment and the hen's age. A one-way ANOVA was used for all other measures including the weekly cumulative cracked, shell-less, and unmarketable eggs. BW was used as a covariate for bone mineralization and bone area (Steel et al., 1997). Transformation of data was performed for normality when variances were not homogeneous (Steel et al., 1997). Logarithmic transformation was performed for cecal microbial count, and arcsine square root transformation was performed for % unmarketable egg, % cracked egg, % shell-less egg, and % dirty egg.

Statistical trends were similar for both transformed and untransformed data; therefore, the untransformed least square means and SEM were presented, except for microbial analysis. The Tukey-Kramer test was used to partition differences among means. Statistical significance was set at $P < 0.05$.

2.4 Results

The concentrations of probiotic microorganisms in the probiotic-supplemented diets at the start and end of the experiment were similar to the targeted levels (Table 2.3).

There was no probiotic treatment effect on BW, feed intake, feed conversion, egg weight, egg production, % dirty eggs, % cracked eggs, and shell quality traits (Table 2.4). No mortality occurred in the study. The percentage of unmarketable eggs (sum of cracked and shell-less eggs) was decreased in hens from both the 0.5X and 2.0X groups as compared to the controls (P = 0.02) with the reduction in % shell-less eggs, and uncracked eggs, being the main reason for this reduction (P = 0.05, Table 2.4). The % of unmarketable eggs laid by hens given the 1.0X probiotic treatment was intermediate in value, not differing from the other groups (Table 2.4). There was no probiotic treatment by age interaction for any parameter measured in the study. The percentages for the weekly cumulative shell-less eggs (Figure 2.1b) and unmarketable eggs (Figure 2.1c) were reduced by at least one-half in hens given probiotics as compared to control hens at 64, 65, and 66 wk of age with no differences among treatments prior to 64 wk of age. An age effect occurred only with BW. As expected, hens weighed more as they aged (1.47, 1.48, and 1.51 kg at 60, 63, and 66 wk of age, respectively; $P < 0.0001$).

In comparison to the control group, cecal *Bifidobacterium* spp. counts, but not *Lactobacillus* spp., were increased in all probiotic groups (P < 0.001, Figure 2.2) at 66 wk of age.

Increases in tibial and femoral mineral density and femoral mineral content (P = 0.04, 0.03, and 0.02, respectively), with a concomitant trend for increases in humerus mineral density and tibial mineral content (P = 0.07 and 0.08, respectively), occurred in the 2.0X group as compared to the control group. Bone mineralization values for the 0.5X and 1.0X groups were intermediate between the control and 2.0X groups. Keel

mineralization, bone area, bone breaking force (Table 2.5), and the bone remodeling indicators of circulating OC, CTX, and Pi were similar among treatments (Figure 2.3).

As the 2.0X group exhibited the greatest bone accrual effect as compared to the controls, it was chosen to further investigate the effects of the probiotic treatment on the serotonin system, immune cytokines, and indicators of stress. The cecal and plasma 5-HT and plasma TRP concentrations (Figure 2.4), monoamines and their metabolites of the raphe nuclei (Table 2.6) and the hypothalamus (Table 2.7), including DA, NE, EP, DOPAC, HVA, TRP, 5-HT, and 5HIAA were unaffected by treatment. Moreover, no differences were observed for plasma concentrations of cytokines, including pro-inflammatory IL-1β, IL-6, IFN-γ and TNF-α as well as anti-inflammatory IL-10 (Figure 2.5). In line with the plasma cytokine concentrations, similar mRNA expression of IL-1β, IL-6, and LITAF in the ceca tonsil occurred between the 2.0X group and controls (Figure 2.6). Plasma concentrations of IgM, IgY, and IgA in the probiotic group were also comparable to the control group (Figure 2.7). With respect to the stress response, the probiotic supplementation did not affect the H:L ratio and plasma CORT concentrations (Figure 2.8).

2.5 Discussion

2.5.1 Performance

Dietary probiotic inclusion was beneficial in decreasing unmarketable eggs (sum of cracked eggs and shell-less eggs) in aging hens of the present study. Similar decreases in unmarketable and shell-less eggs using a wide array of probiotics fed at different ages with multiple strains of laying hens have been reported (Balevi et al., 2001; Kurtoglu et al., 2004; Mikulski et al., 2012; Zhang et al., 2012; Abdelqader et al., 2013a, 2013b). For example, laying hens fed diets mixed with *Pediococcus acidilactici* (Mikulski et al., 2012) or *Bacillus subtilis* (Abdelqader et al., 2013a, 2013b) or a multiple strain based probiotic (Mikulski et al., 2012; Zhang et al., 2012) showed reduced production of unmarketable eggs as compared to control hens fed no probiotic. Among these studies, Mikulski et al. (2012) additionally found an increase in shell thickness, specific gravity, and % shell in hens fed probiotic for 24 wk, whereas Zhang et al. (2012) failed to find

any difference in eggshell thickness when probiotic was fed to Lohman Pink hens for 8.6 wk, similar to the results of the current study. The short duration of the current study (7 wk) along with the study of Zhang et al. (2012) could be one reason for a lack of an effect on shell traits. The cumulative production of unmarketable eggs of the current study did not show a reduction until the last 3 wk of the 7 wk study (Figure 2.1c) suggesting that the beneficial bacteria in the probiotic supplement required time to establish themselves in the intestinal lumen before beneficial effects occurred. Other studies have reported improvements in shell traits such as thickness, strength, density, weight, etc., as a result of feeding probiotics for 47 wk (Panda et al., 2003), 16 wk (Panda et al., 2008), 39 wk (Gallazzi et al., 2008), and 8 wk (Lei et al., 2013). However, there are many studies reporting that probiotics have no effect on shell quality traits in laying hens (Nahashon et al., 1994; Haddadin et al., 1996; Yoruk et al., 2004; Hayirli et al., 2005; Mahdavi et al., 2005; Li et al., 2006; Applegate et al., 2009; Aghaii et al., 2010; Yalcin et al., 2010; Ribeiro et al., 2014). In addition to length of time the probiotic supplement is fed, variations in microbial strain content and dose, the genetics and age of the hens, and environmental conditions such as ambient temperature are factors that can influence the outcome of experiments.

An age-induced increase of unmarketable eggs in laying flocks is common due to the reduction in eggshell quality (Zita et al., 2012; Roberts et al., 2013) resulting from age-associated changes in hormone profile (Bar et al., 1999; Wistedt et al., 2014), including decreased sensitivity to reproductive hormones and diminished calcium absorption efficiency (Bar et al., 1999; Hansen, 2002). Therefore, it is reasonable to hypothesize that aging hens with poor shell quality may be more responsive to probiotics in improving shell traits than younger hens. For example, Abdelqader et al. (2013b) reported a decrease in unmarketable eggs accompanied by improved eggshell weight, thickness, and density in aged Lohmann Leghorns fed *Bacillus subtilis*. Aging laying hens can respond to increased levels of dietary calcium by placing more Ca in the shell as indicated by increased eggshell thickness as well as reduced broken and shell-less egg percentage (Safaa et al., 2008). The production of short chain fatty acids through fermentation by intestinal microbiota decreases the pH of the intestinal lumen creating an acidic environment that facilitates the ionization of minerals such as Ca leading to

improved absorption (Haddadin et al., 1996). Specifically, the reduction in the pH of the intestines caused by enhanced microbial fermentation converts water-insoluble calcium to its soluble, ionic form (Remesy et al., 1993) increasing calcium bioavailability and retention in egg laying strains of chickens (Abdelqader et al., 2013a). A variety of microbial species fed for varying lengths of time to a wide array of egg laying strains of chickens of different ages have shown improvements in egg production (Haddadin et al., 1996; Panda et al., 2003; Kurtoglu et al., 2004; Yoruk et al., 2004; Gallazzi et al., 2008; Panda et al., 2008; Aghaii et al., 2010; Yalcin et al., 2010; Zhang et al., 2012; Abdelqader et al., 2013a, 2013b; Lei et al., 2013; Zhang and Kim, 2013; Ribeiro et al., 2014). As an example, Lohman white hens consuming *Bacillus subtilis* at doses of 0.5 and 1 g/kg of feed from 64 to 73 wk of age exhibited an increase in egg production in a dose dependent matter. Hens responded with increased egg production as early as the 3rd and 6th wk of feeding the high and low dose of *Bacillus subtilis*, respectively (Abdelqader et al., 2013b). However, for reason already stated with respect to shell traits, there are several studies, including the current one, reporting no improvement in egg production in hens supplemented with probiotics (Balevi et al., 2001; Davis and Anderson, 2002; Mahdavi et al., 2005; Applegate et al., 2009; Ramasamy et al., 2009; Capcarova et al., 2010; Mikulski et al., 2012; Salma et al., 2012).

Numerous studies have reported an increase in egg weight when hens are given probiotics in their feed (Nahashon et al., 1994; Davis and Anderson, 2002; Ramasamy et al., 2009; Yalcin et al., 2010; Mikulski et al., 2012; Abdelqader et al., 2013a; Ribeiro et al., 2014). For example, hens consuming a mixture of different microbial species laid eggs that were 0.6 g heavier than that of control laying hens causing the weight grade to shift from medium to extra-large eggs (Davis and Anderson, 2002). However, there are many studies, similar to the current one, where egg weight was not affected by probiotic supplementation (Haddadin et al., 1996; Balevi et al., 2001; Panda et al., 2003; Kurtoglu et al., 2004; Yoruk et al., 2004; Mahdavi et al., 2005; Li et al., 2006; Gallazzi et al., 2008; Panda et al., 2008; Applegate et al., 2009; Aghaii et al., 2010; Lei et al., 2013). Egg production improved in all but 4 of the above studies (Balevi et al., 2001; Mahdavi et al., 2005; Li et al., 2006; Applegate et al., 2009). An explanation for these results may be due

to the negative correlation between egg production and egg weight (Du Plessis and Erasmus, 1972; Zeidler, 2002).

Feed efficiency is often improved when hens consume probiotics (Haddadin et al., 1996; Balevi et al., 2001; Kurtoglu et al., 2004; Yoruk et al., 2004; Hayirli et al., 2005; Li et al., 2006; Gallazzi et al., 2008; Panda et al., 2008; Aghaii et al., 2010; Yalcin et al., 2010; Mikulski et al., 2012; Zhang et al., 2012; Abdelqader et al., 2013a, 2013b). Feed efficiencies in other studies, like the current one, were similar (Davis and Anderson, 2002; Panda et al., 2003; Mahdavi et al., 2005; Lei et al., 2013; Ribeiro et al., 2014) or worse (Nahashon et al., 1994) in hens due to the consumption of supplemental probiotics as compared to hens fed a control diet.

Inhibiting the growth of pathogens within the intestines (Jin et al., 1996; Fulton et al., 2002) and altering the intestinal flora (Netherwood et al., 1999; Jadamus et al., 2001) towards non-pathogenic facultative anaerobic and Gram positive bacteria that generate hydrogen peroxide and lactic acid and may contribute to improvements in feed efficiency (Ehrmann et al., 2002). As mentioned with Ca, improved nutrient availability and utilization can contribute to improved feed conversion (Schneitz et al., 1998; Jin et al., 2000).

2.5.2 Bone health

Previous studies, including the current study, reported that probiotics with various strains of bacteria have positive effects on skeletal health in both broiler chickens and laying hens (Mutus et al., 2006; Panda et al., 2006; Houshmand et al., 2011; Ziaie et al., 2011; Wideman et al., 2012; Abdelqader et al., 2013b; Fuentes et al., 2013; Sadeghi, 2014). A possible mode of action could involve modulation of calcium metabolism and bone remodeling cells. For example, short chain fatty acids, a major product of anaerobic bacterial fermentation in the intestine, regulate bone remodeling directly through inhibiting osteoclast formation and activating the bone-forming osteoblasts (Iwami and Moriyama, 1993) and indirectly through increased intestinal absorption of minerals (Scholz-Ahrens et al., 2007; Yonezawa et al., 2007; Legette et al., 2012). Although cecal short chain fatty acids concentrations were not measured in the current study, increased cecal propionate, butyrate, and total short chain fatty acids concentrations occurred in

broilers using the same probiotic product of the current study (Murugesan and Persia, 2015). However, the lack of a probiotic effect on the bone remodeling indicators of circulating OC, CTX, and Pi in hens of the current study provided no indication of possible modes of action of probiotics in improving bone mineralization. Because EDTA was used as the anticoagulant when collecting blood samples, Ca was not measured as EDTA interferes with the assay.

2.5.3 Cecal microbial composition

Cecal microbiota composition was modified in hens of the present study as indicated by the expected increase in *Bifidobacterium* spp. population in all of the probiotic treatment groups compared with the control group (Figure 2.2). However, the concentrations of *Lactobacillus* spp. were not affected by probiotic supplementation. Our results are comparable to studies on broilers at 42 d of age using the same probiotic product (Giannenas et al., 2012; Mountzouris et al., 2015); although some studies reported an increase in the counts of both *Bifidobacterium* spp. and *Lactobacillus* spp. (Mountzouris et al., 2007; Mountzouris et al., 2010).

Bifidobacterium spp. produce a wide range of metabolites in the intestines including vitamins (Deguchi et al., 1985; Hou et al., 2000; Crittenden et al., 2003), short chain fatty acids (Wang et al., 2007), and conjugated linoleic acid (Coakley et al., 2003; Coakley et al., 2006; Barrett et al., 2007). *Bifidobacterium* also inhibits the secretion of pro-inflammatory cytokines (Drago et al., 2015). Similar to short chain fatty acids, conjugated linoleic acid (Drago et al., 2015), vitamins (Weber, 1999), and folate (Hancock and Viola, 2001) are involved in calcium metabolism and are required for bone matrix formation and bone accretion. In the current study, increased *Bifidobacterium* spp. may lead to increased calcium absorption, which may in turn lead to the improvement in bone health.

2.5.4 The possible mechanisms

To further elucidate the possible mechanisms of how probiotics promotes bone health, we measured metabolites involved in the 5-HT system, the stress response, and immune cytokines, all of which regulate bone health (Charles et al., 2015). Only the 2.0X

group among the probiotic treatments was selected for further investigation as it showed the greatest bone benefits. Our hypothesis was that dietary probiotics fed to laying hens would reduce cecal 5-HT, pro-inflammatory cytokines and indicators of stress and enhance brain 5-HT. Our hypothesis was rejected as none of these parameters were affected by probiotic treatment.

2.5.4.1 The 5-HT system

In the intestinal tract, enterocytes together with goblet cells, Paneth cells, and enteroendocrine cells (including 5-HT secreting enterochromaffin cells) constitute the intestinal mucosal epithelia that directly or indirectly interacts with gut microbes (Kim and Ho, 2010). Therefore, it is reasonable to explore whether gut-derived 5-HT is affected by supplementary probiotics. To determine if peripheral serotonergic system plays a role in the probiotic related upregulation of bone mineralization in White Leghorn laying hens, we first determined the 5-HT level in plasma, which functions directly to reduce bone mass via binding to its receptors in bone (Yadav et al., 2010). We found probiotic fed laying hens had similar concentrations of plasma 5-HT compared to the hens from the control group. We further measured 5-HT concentrations in the ceca tissue, where 5-HT is synthesized and released into the systemic circulation. Again, no differences in cecal 5-HT concentrations were found between these 2 groups. The source of blood 5-HT is the ceca, so a similar pattern in 5-HT concentrations in circulation and the cecal tissue were expected. These data suggest that the increase in bone mineralization in probiotic fed hens was not caused by the peripheral 5-HT system. In line with the current study, altered serum 5-HT level was also excluded as a main cause of high bone mass in a study conducted in GF mice (Sjogren et al., 2012). Because a lack of intestinal microbiota causes an increase in bone mass in GF mice, it was used as a model to elucidate the mechanisms of action of gut microbiota on bone. Compared to conventional mice, GF mice exhibited higher bone mass with a concomitant reduction in both serum 5-HT concentrations and colon mRNA expression of TPH1, an enzyme needed for 5-HT synthesis. In addition, the expression of SERT was also increased in the colon. However, when bone mass of GF mice was normalized by colonization with gut microbiota from donor mice, serum 5-HT concentrations did not change.

Recent advances in understanding how gut microbiota regulates brain function through the gut-brain axis have pointed to a critical role of 5-HT in this regulation (O'Mahony et al., 2015). Brain-derived 5-HT can stimulate bone mass accrual via binding to 5-HT_{2C} receptor on ventromedial hypothalamic neurons, which modulates sympathetic tone and in turn regulates both bone formation and bone resorption (Yadav et al., 2009). Dietary probiotic supplementation may regulate the central 5-HT system via the gut-brain axis, thus promoting bone mass in laying hens. To investigate if the central 5-HT was affected in probiotic fed laying hens, we first measured plasma TRP concentrations. Free TRP, as the precursor of 5-HT, can cross the blood-brain barrier and be metabolized to 5-HT in the brain neurons via TPH2. In the current study, group 2.0X hens fed probiotics exhibited similar plasma TRP concentrations to the control hens. The concentrations of monoamines and their metabolites were further measured in the raphe nuclei and the hypothalamus. The raphe nuclei is the major site of 5-HT synthesis in the brain, whereas the hypothalamus is the site where 5-HT binds to its receptors to initiate its regulation of bone via the sympathetic system. There were no difference of 5-HT and 5-HT turnover at both brain areas. In addition, catecholamine concentrations of NE, EP, and DA were not affected by probiotic supplementation, indicating similar sympathetic activities between treatment groups. In contrast to our findings, alteration of brain 5-HT metabolism were reported in GF animals and animals fed probiotics (Desbonnet et al., 2010; Diaz Heijtz et al., 2011; Liu et al., 2016). These studies, however, were not designed to investigate the effect of microorganisms on bone health but on behavior and stress response instead. For example, GF mice displayed increased motor activity and reduced anxiety, associated with increased NE, DA, and 5-HT turnover in the striatum (Diaz Heijtz et al., 2011). Rats with maternal separation showed an increase in immobility and a decrease in swimming behavior in forced swimming test. Supplementation of *Bifidobacteria infantis* to these rats attenuated the behavioral changes and reduced 5-HIAA concentration in the frontal cortex (Desbonnet et al., 2010). A recent study also reported that *Lactobacillus plantarum* normalized both stress-like and anxiety-like behaviors, with elevated 5-HT concentrations and decreased 5-HIAA concentrations in the prefrontal cortex, in both normal mice and mice subjected to early life stress (Liu et al., 2016).

2.5.4.2 The immune system

Numerous evidence has demonstrated the important role of cytokines in regulating bone mass (Lorenzo et al., 2008), making it one of the major themes in pursuing the proposed mechanisms of how the intestinal microbiome influences bone biology. Our results showed that dietary probiotic inclusion in hens did not lead to changes in immune cytokines in both blood and the ceca tonsil. Whether cytokines were altered in the bone of probiotic fed hens remains to be determined. Most studies on the effect of probiotics on bone physiology and health have been conducted in rats and mice under different pathophysiological conditions. Among them, a number of research groups have attributed the bone accrual function of probiotics to cytokine regulation. For example, oral *Saccharomyces cerevisiae* administration, alone or with standard therapy, led to reduced alveolar bone loss, associated with decreased pro-inflammatory cytokines concentrations of TNF-α and IL-1β as well as increased anti-inflammatory cytokine IL-10 concentrations (Garcia et al., 2016). In a type 1 diabetes-mediated bone loss model, *Lactobacillus reuteri* inhibited bone loss and rescued the TNF-α induced down-regulation of Wnt10b in whole bone and osteoblasts (Zhang et al., 2015). Moreover, the anti-TNF-α activity of *Lactobacillus reuteri* given to healthy mice reduced bone resorption (McCabe et al., 2013). Going back to the model of GF mice with high bone mass that lack gut microbiota, these mice also have reduced CD4+ T helper cells as well as expression of the osteolytic cytokine of TNF-α in the colon and bone. It was hypothesized that a decrease in the expression of inflammatory cytokines such as TNF-α leads to fewer osteoclasts causing bone mass to increase in GF mice. This hypothesis was supported by the fact that colonizing GF mice with gut microbiota from donor mice caused bone mass to normalize and increased the CD4+ T cells and expression of TNF-α in bone and the colon (Sjogren et al., 2012).

Probiotic inclusion in the diet stimulates humoral immunity. Probiotics induce production of B-cell activating factor, such as TGF-β, in the intestinal epithelial cells and dendritic cells, which in turn promotes the differentiation of B cells into IgA producing plasma cells. Furthermore, IgA producing plasma cells can traffic from the intestinal lymphoid tissue to the bloodstream through the lymphatics in mammals (Praharaj et al., 2015). Plasma immunoglobulin concentrations of IgM, IgY, and IgA of the current study

were unaffected by dietary probiotics in unchallenged hens, similar to results in humans (Wen et al., 2014; Mansouri-Tehrani et al., 2015). Other studies reported an increase in antibody concentrations regardless of vaccination or disease in chickens (Brisbin et al., 2011; Surono et al., 2014; Salehimanesh et al., 2016) consuming probiotics.

2.5.4.3 The stress response

Excessive CORT is well known to decrease bone mass through inhibition of osteoblastogensis, increasing osteoblast and osteocyte apoptosis, and promoting osteoclast survival (O'Brien et al., 2004; Jia et al., 2006; Rauch et al., 2010). Probiotics alleviate the stress response along the HPA axis by reducing plasma or brain concentrations of CRH, ACTH, and CORT (Sohail et al., 2010; Ait-Belgnaoui et al., 2012; Sohail et al., 2012; Ait-Belgnaoui et al., 2014; Yang et al., 2016). In these studies, stressors were applied such as heat or psychological factors. The laying hens in the current study were raised under normal management and were not subjected to stressors, so it is not surprising that plasma CORT concentrations and H:L ratios were unaffected by the probiotic treatment. Under stressful conditions, the hens consuming probiotics may have responded with reduced circulating concentrations of CORT as well as H: L ratios.

2.6 Conclusions

The present study demonstrated that dietary probiotic inclusion that increased the cecal population of *Bifidobacterium* spp. provided beneficial effects to aging White Leghorn laying hens that included a reduction in the production of unmarketable eggs and improved bone mineralization. The possible modes of action of improving bone mineralization and increasing saleable eggs do not appear to be through the modulation of the peripheral and central 5-HT system, immune cytokines, or CORT. Because the probiotic supplement used in the current study was multi-species, investigating the mechanisms of probiotic on bone biology may be more difficult as compared to using a single strain of beneficial bacteria. Therefore, future studies evaluating the role of 5-HT in probiotic associated bone improvement should use a single-species probiotic. In

addition, extending the length of time the probiotic is fed to laying hens may facilitate detecting a bone remodeling response.

Table 2.1 Composition and nutrient analysis of the layer diet

Item	Amount
Ingredient (%)	
Corn	54.27
Soybean Meal (48% crude protein)	29.54
Soybean Oil	3.91
Salt	0.41
DL Methionine	0.19
Limestone	10.42
Monocalcium phosphorus	0.83
Mold inhibitor [1]	0.05
Antioxidant[2]	0.03
Vitamin and mineral premix[3]	0.35
Calculated analysis	
Crude protein (%)	18.30
ME (MJ/kg)	12.09
Calcium (%)	4.20
Phosphorus (%)	0.53
Lys (%)	1.01
Met (%)	0.48

[1]Myco curb Dry: propionic acid, sodium hydroxide, calcium hydroxide, amorphous silicon dioxide, sorbic acid, benzoic acid, propylparaben, methylparaben, and BHA.
[2]Ethoxyquin.
[3]The premix supplied per kg of diet: vitamin A, 12,320 IU; vitamin D_3, 4,620 IU; vitamin E, 15.4 IU; vitamin K, 3.08 mg; riboflavin, 6.16 mg; niacin, 46.2 mg; vitamin B_{12}, 23.1 mg; pantothenic acid, 15.4 mg; folic acid, 0.31 mg; choline, 401 mg; iron, 50.4 mg; zinc, 71 mg; manganese, 90 mg; copper, 7 mg; iodine, 0.7 mg; and selenium, 0.25 mg.

Table 2.2 Taqman primers and probes used

Gene[1]	Primers and Probe (5'-3')[2]	Application Efficiencies (%)	Product Length (bp)	Reference/ Accession no.
IL-1β	(f)TGCTGGTTTCCATCTCGTATGTAC (r)CCCAGAGCGGCTATTCCA (p)AGTACAACCCCTGCTGCCCCGC (VIC/MGB)	95	80	NC_006096.3
IL-6	(f)CCCGCTTCTGACTGTGTTT (r)GCCGGTTTTGAAGTTAATCTTTT (p)TGTGTTTCGGAGTGCTTT (VIC/MGB)	86	139	NC_006089.3
LITAF	(f)CCCCTACCCTGTCCCACAA (r)ACTGCGGAGGGTTCATTCC (p)CTGGCCTCAGACCAG (VIC/MGB)	75	62	NC_006101.3

IL-1β = interleukin 1 beta mRNA; IL-6 = interleukin 6 mRNA; LITAF = lipopolysaccharide-induced TNF factor mRNA.

[1]Gene expression reported in relative abundance to GAPDH.

[2]f = forward primer; r = reverse primer; p = probe

Table 2.3 The targeted concentrations of probiotic microorganisms and the actual concentrations in feed samples collected at the beginning and end of the experiment

Treatment	Targeted (cfu/g feed)	Beginning (cfu/g feed)	End (cfu/g feed)
Control	0	0.02×10^5	0.04×10^5
0.5X	1.0×10^5	1.11×10^5	0.95×10^5
1.0X	2.0×10^5	2.04×10^5	1.87×10^5
2.0X	4.0×10^5	4.05×10^5	3.80×10^5

The duration between beginning and end was 7 wk.

Table 2.4 The effects of dietary supplementation of a probiotic on the performance traits of White Leghorn hens

Parameter	Treatment[5]				SEM	P
	Control	0.5X	1.0X	2.0X		
BW (kg)[1]	1.52	1.48	1.48	1.47	0.01	0.36
Feed intake (g)[2]	104.92	101.26	106.30	102.61	0.72	0.21
Feed conversion (kg of feed/kg of eggs)[2]	1.85	1.89	1.76	1.87	0.03	0.59
Egg weight (g)[2]	65.19	62.71	64.69	63.90	0.33	0.18
Egg production (%)[3]	91.44	88.12	93.26	88.21	0.91	0.30
Dirty eggs (%)[3]	1.13	0.85	1.59	1.78	0.17	0.37
Cracked eggs (%)[3]	2.24	0.48	1.53	0.82	0.28	0.30
Shell-less eggs (%)[3]	3.49[a]	1.51[ab]	1.35[ab]	1.09[b]	0.26	**0.05**
Unmarketable eggs (%)[3]	5.73[a]	2.00[b]	2.87[ab]	1.92[b]	0.37	**0.02**
% Shell[4]	8.12	8.29	7.96	8.19	0.06	0.45
Eggshell thickness (mm)[4]	0.33	0.33	0.32	0.33	0.002	0.71
Eggshell strength (kg)[4]	3.24	3.23	3.05	3.28	0.05	0.53

[a,b] Least square means within a row lacking a common superscript differ ($P < 0.05$).
[1]Values for BW represent the least square means averaged over 3 ages of the hen at 60, 63, and 66 wk of age. The number of observations per least square mean is 72.
[2]Values for feed intake, feed conversion rate, and egg weight were determined over a 3-d period and averaged by cage over 4 ages of the hen at 60, 62, 64, and 66 wk of age. The number of observations per least square mean is 24.
[3]Values for hen-d egg production and the proportion of dirty egg, shell-less egg, and cracked egg were calculated on a weekly basis. Unmarketable eggs are the sum of cracked and shell-less eggs. The number of observations per least square mean is 42.
[4]Values for % shell, eggshell thickness and strength were determined over 4 ages of the hen at 60, 62, 64, and 66 wk of age. The number of observations per least square mean is 96.
[5] The probiotic dosage was 0 (Control), 0.5 (0.5X), 1.0 (1.0X), or 2.0 (2.0X) g/kg of feed.

Table 2.5 The effects of dietary supplementation of a probiotic on the bone mineral density, mineral content, area, and breaking force of bones retrieved from 66-wk-old White Leghorns[1]

Parameter	Treatment[2]				SEM	P[3]
	Control	0.5X	1.0X	2.0X		
Bone mineral density						
Tibia (g/cm^2)	0.1912^{b}	0.2018^{ab}	0.1978^{ab}	0.2034^{a}	0.001	**0.04**
Femur (g/cm^2)	0.1931^{b}	0.2048^{ab}	0.2023^{ab}	0.2100^{a}	0.002	**0.03**
Humerus (g/cm^2)	0.1069^{B}	0.1102^{AB}	0.1124^{AB}	0.1136^{A}	0.001	0.07
Keel (g/cm^2)	0.1109	0.1122	0.1164	0.1138	0.001	0.30
Bone mineral content						
Tibia (g)	2.2463^{B}	2.3495^{AB}	2.3498^{AB}	2.3556^{A}	0.02	0.08
Femur (g)	1.7068^{b}	1.8304^{ab}	1.8259^{ab}	1.8597^{a}	0.02	**0.02**
Humerus (g)	1.0441	1.0698	1.0966	1.1048	0.008	0.11
Keel (g)	0.6880	0.7067	0.7312	0.7178	0.008	0.50
Bone area						
Tibia (cm^2)	11.75	11.65	11.90	11.58	0.04	0.16
Femur (cm^2)	8.72	8.97	8.99	8.84	0.04	0.16
Humerus (cm^2)	9.77	9.69	9.77	9.72	0.04	0.92
Keel (cm^2)	6.37	6.32	6.26	6.29	0.06	0.88
Bone breaking force						
Tibia (N)	394.60	398.11	347.35	412.03	9.30	0.21

[A, B] Least square means within a row lacking a common superscript tend to differ ($P < 0.1$).

[a,b] Least square means within a row lacking a common superscript differ ($P < 0.05$).

[1]The number of observations per least square mean is 24.

[2] The probiotic dosage was 0 (Control), 0.5 (0.5X), 1.0 (1.0X), or 2.0 (2.0X) g/kg of feed.

[3]BW was used as a covariate except for keel bone area and tibia breaking force.

Table 2.6 The effects of dietary supplementation of a probiotic on catecholamine, 5-HT, metabolites, and turnover in the raphe nuclei of 66-wk-old White Leghorns[1]

Parameter	Treatment[2]		SEM	P
	Control	2.0X		
Catecholamine system				
DA (ng/g)	250.78	268.25	15.76	0.76
NE (ng/g)	1075.45	1214.42	60.21	0.52
EP (ng/g)	240.64	258.28	12.64	0.70
DOPAC (ng/g)	43.36	47.32	2.04	0.59
HVA (ng/g)	94.74	101.88	7.54	0.79
DOPAC/DA	0.20	0.21	0.02	0.94
HVA/DOPAC	1.68	1.76	0.04	0.54
5-HT system				
TRP (ng/g)	3272.42	3839.35	109.89	0.17
5HT (ng/g)	618.95	763.60	49.44	0.42
5HIAA (ng/g)	171.58	207.42	6.86	0.16
5HIAA/5HT	0.29	0.30	0.01	0.92

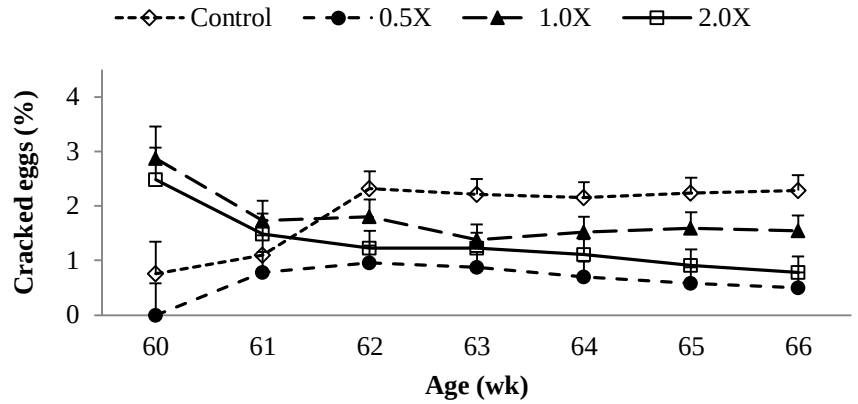
[1]The number of observations per least square mean is 6.
[2]The probiotic dosage was 0 (Control) or 2.0 (2.0X) g/kg of feed.

Table 2.7 The effects of dietary supplementation of a probiotic on catecholamine, 5-HT, metabolites, and turnover in the hypothalamus of 66-wk-old White Leghorns[1]

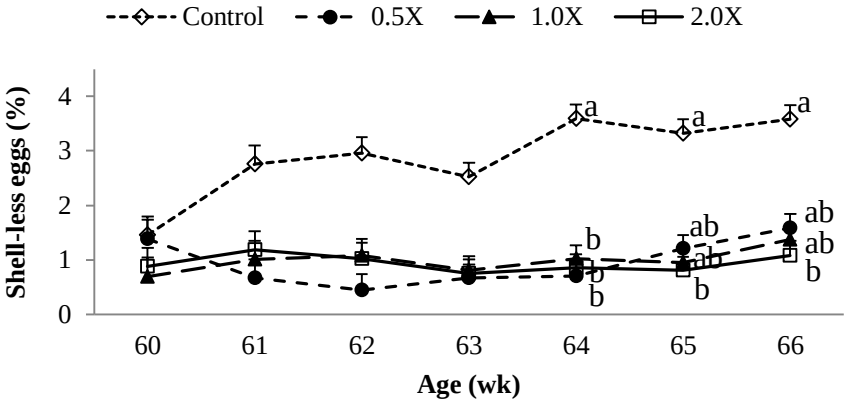
Parameter	Treatment[2]		SEM	P
	Control	2.0X		
Catecholamine system				
DA (ng/g)	376.45	335.41	11.83	0.34
NE (ng/g)	2520.88	2163.81	64.18	0.14
EP (ng/g)	654.42	548.75	30.68	0.34
DOPAC (ng/g)	115.50	107.59	2.39	0.36
HVA (ng/g)	192.75	185.84	2.22	0.39
DOPAC/DA	0.31	0.33	0.008	0.56
HVA/DOPAC	2.11	2.10	0.09	0.97
5-HT system				
TRP (ng/g)	3524.07	3487.84	76.60	0.89
5HT (ng/g)	1329.86	1219.37	32.31	0.35
5HIAA (ng/g)	268.94	257.27	5.58	0.56
5HIAA/5HT	0.21	0.21	0.004	0.78

[1]The number of observations per least square mean is 6.
[2]The probiotic dosage was 0 (Control) or 2.0 (2.0X) g/kg of feed.

a)



b)



c)

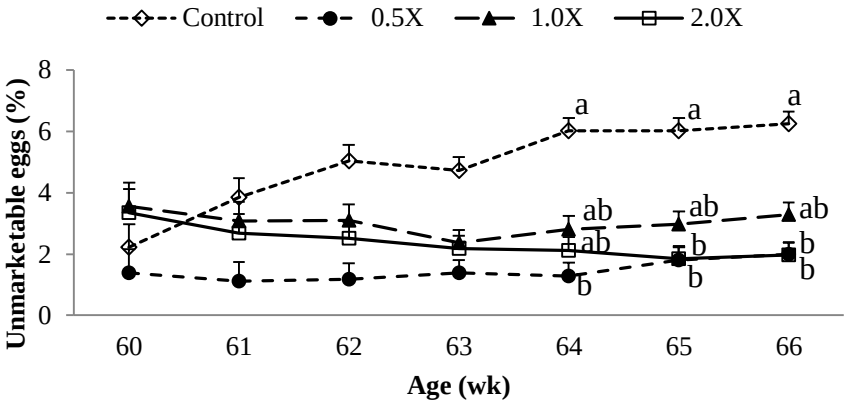


Figure 2.1 The weekly production of unmarketable eggs (c) which is the sum of shell-less (b) and cracked eggs(a) in hens fed with probiotic from 60 to 66 wk of age

The probiotic dosage was 0 (Control), 0.5 (0.5X), 1.0 (1.0X), or 2.0 (2.0X) g/kg of feed. Least square means ± the SEM within the age of a hen lacking common superscripts differ ($P < 0.05$). The average number of observations per least square mean is 6.

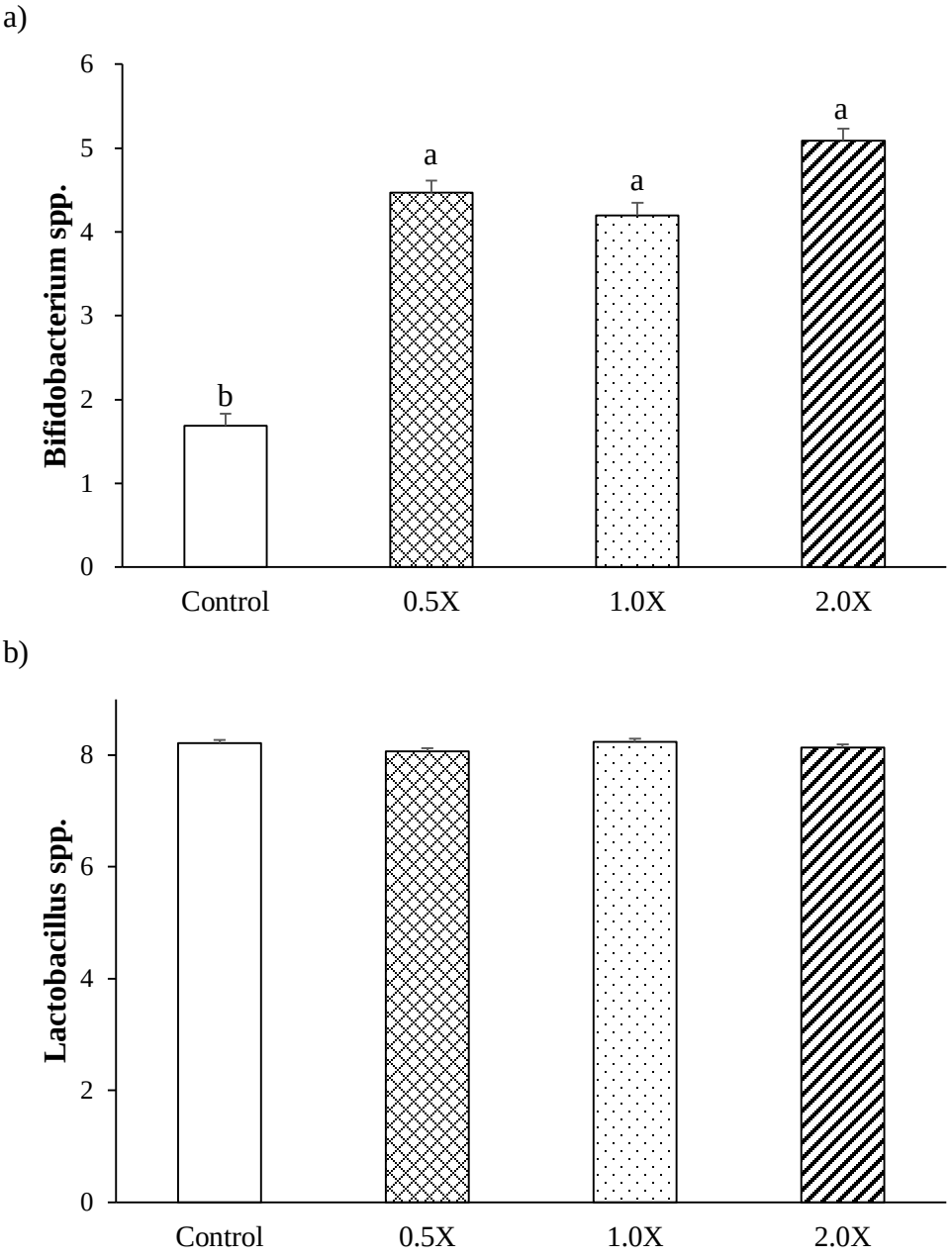


Figure 2.2 The cecal microbial count of *Bifidobacterium* (a) and *Lactobacillus* (b) in hens supplied with a probiotic at 66 wk of age

The probiotic dosage was 0 (Control), 0.5 (0.5X), 1.0 (1.0X), or 2.0 (2.0X) g/kg of feed. Least square means ± the SEM lacking common superscripts differ ($P < 0.05$). The number of observations per least square mean is 24.

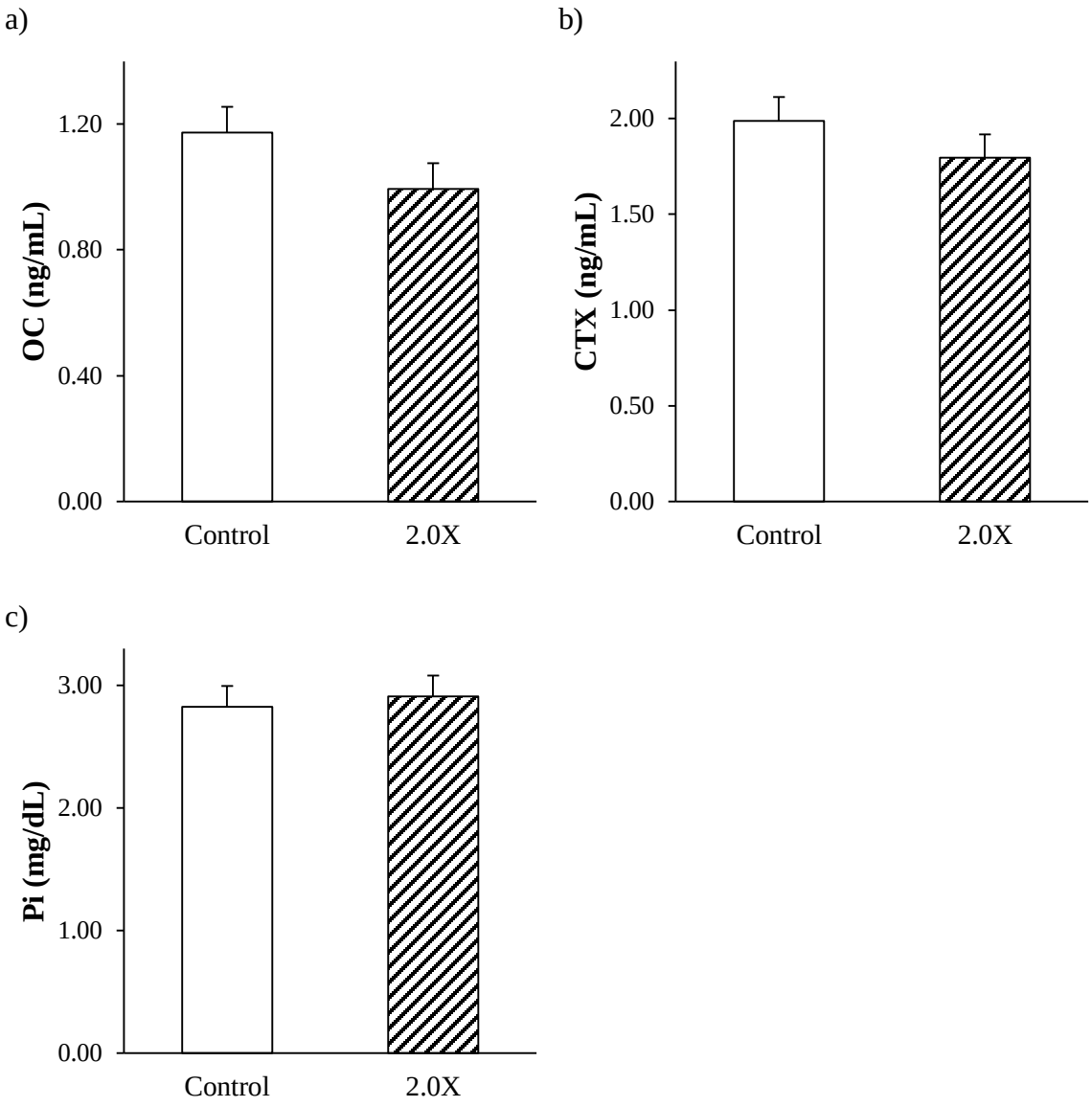


Figure 2.3 The plasma bone remodeling indicator OC (a) and CTX (b) and phosphate (c) concentrations in hens supplied with a probiotic at 66 wk of age

The probiotic dosage was 0 (Control) or 2.0 (2.0X) g/kg of feed. The average number of observations per least square mean is 6.

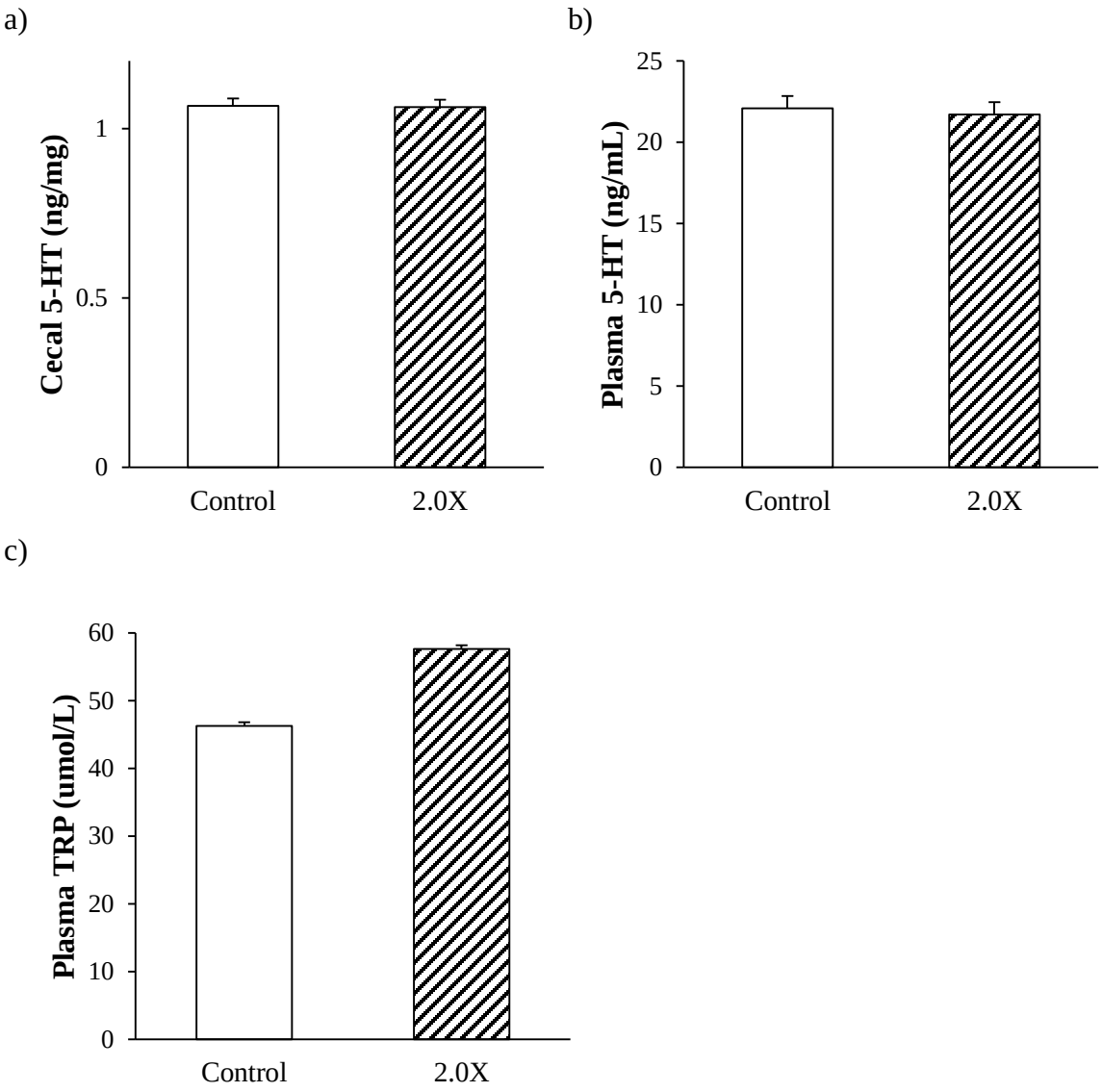


Figure 2.4 The cecal (a) and plasma (b) 5-HT and plasma TRP (c) in hens supplied with a probiotic at 66 wk of age

The probiotic dosage was 0 (Control) or 2.0 (2.0X) g/kg of feed. The average number of observations per least square mean is 6.

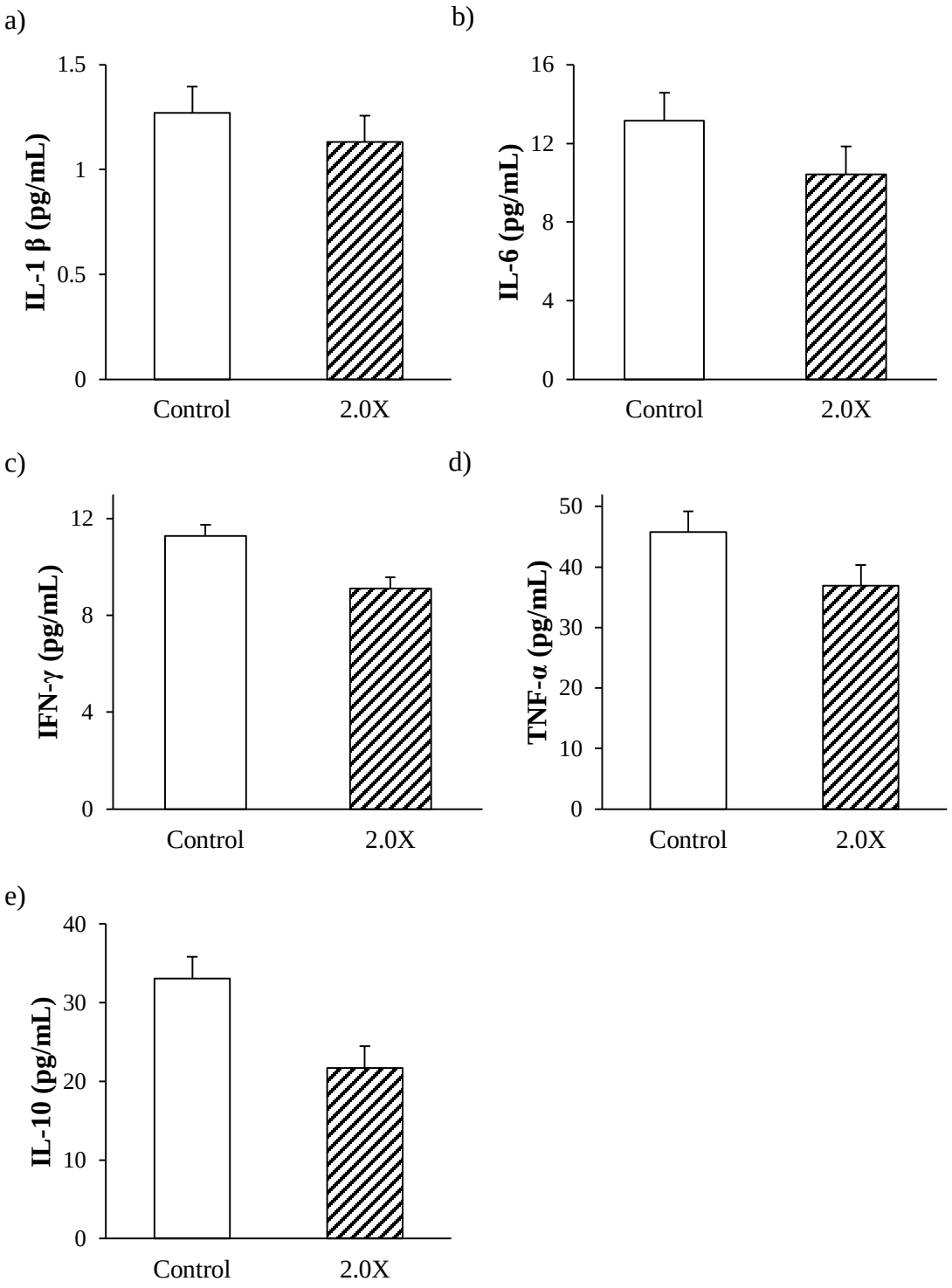


Figure 2.5 The plasma concentrations of immune cytokines in hens supplied with a probiotic at 66 wk of age

The probiotic dosage was 0 (Control) or 2.0 (2.0X) g/kg of feed. The average number of observations per least square mean is 6.

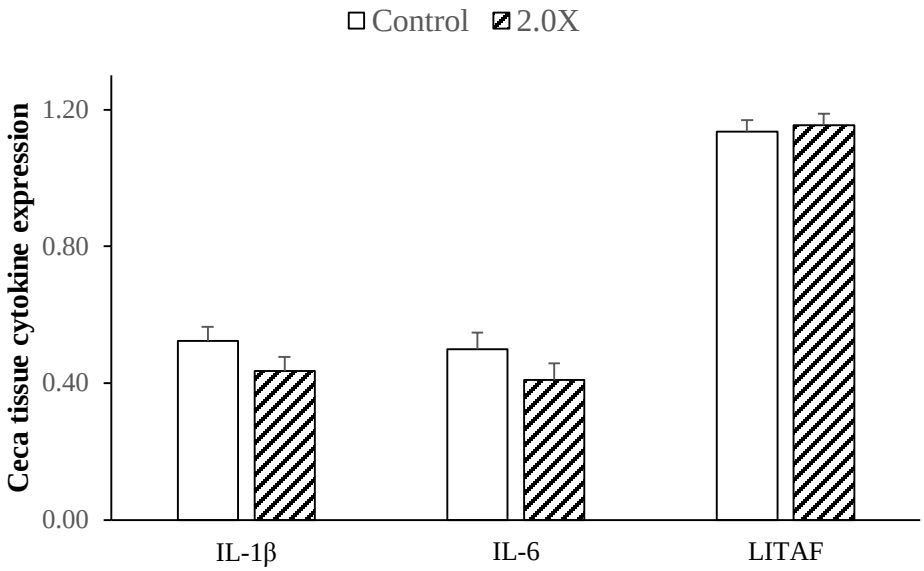


Figure 2.6 The ceca tissue immune cytokines expression in hens supplied with a probiotic at 66 wk of age

The probiotic dosage was 0 (Control) or 2.0 (2.0X) g/kg of feed. The average number of observations per least square mean is 6.

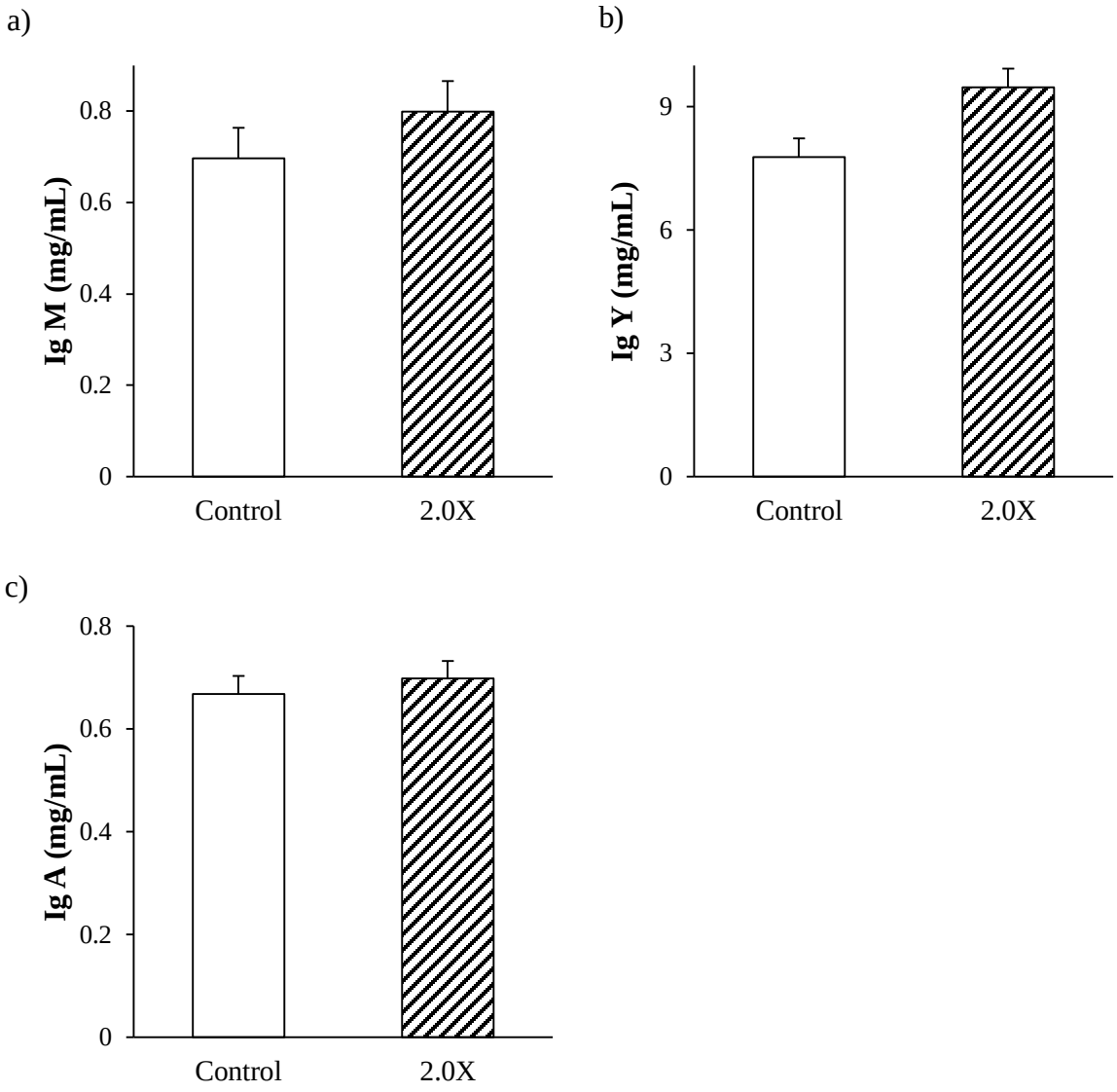


Figure 2.7 The plasma immunoglobulin concentrations of Ig M (a), Ig Y (b), and Ig A (c) in hens supplied with a probiotic at 66 wk of age

The probiotic dosage was 0 (Control) or 2.0 (2.0X) g/kg of feed. The average number of observations per least square mean is 6.

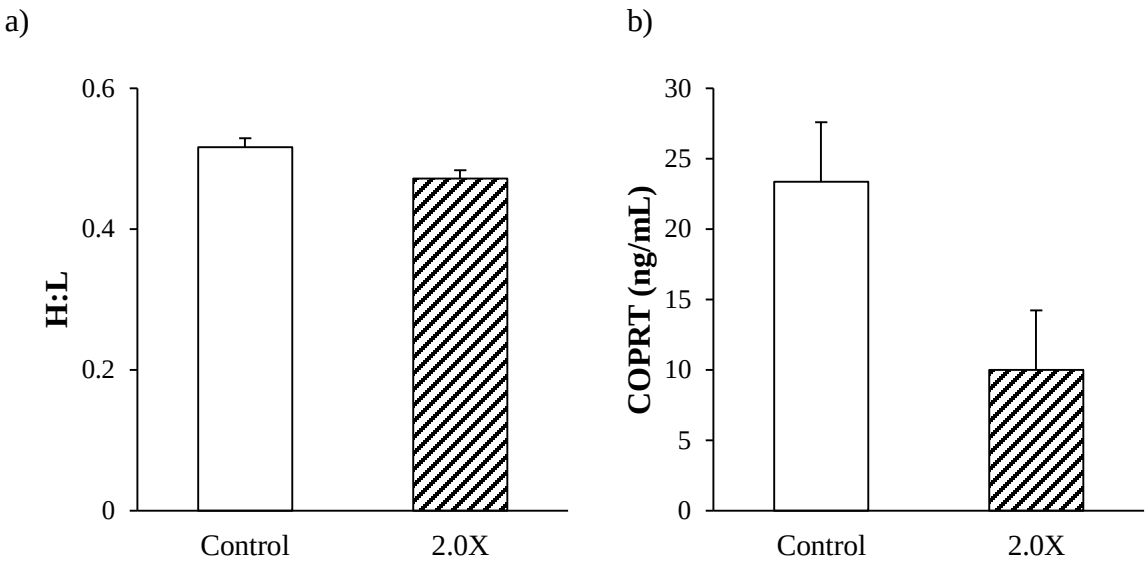


Figure 2.8 The H:L ratio (a) and plasma CORT (b) concentrations in hens supplied with a probiotic at 66 wk of age

The probiotic dosage was 0 (Control) or 2.0 (2.0X) g/kg of feed. The average number of observations per least square mean is 6.

2.7 References

Abdelqader, A., Al-Fataftah, A. R., and Das, G. (2013a). Effects of dietary Bacillus subtilis and inulin supplementation on performance, eggshell quality, intestinal morphology and microflora composition of laying hens in the late phase of production. *Anim Feed Sci Tech,* 179(1-4), 103-111.

Abdelqader, A., Irshaid, R., and Al-Fataftah, A. R. (2013b). Effects of dietary probiotic inclusion on performance, eggshell quality, cecal microflora composition, and tibia traits of laying hens in the late phase of production. *Trop Anim Health Prod,* 45(4), 1017-1024.

Aghaii, A., Chaji, M., Mohammadabadi, T., and Sari, M. (2010). The effect of probiotic supplementation on production performance, egg quality and serum and egg chemical composition of laying hens. *J Anim Vet Adv,* 9, 2774-2777.

Ait-Belgnaoui, A., Durand, H., Cartier, C., Chaumaz, G., Eutamene, H., Ferrier, L., Houdeau, E., Fioramonti, J., Bueno, L., and Theodorou, V. (2012). Prevention of gut leakiness by a probiotic treatment leads to attenuated HPA response to an acute psychological stress in rats. *Psychoneuroendocrinology,* 37(11), 1885-1895.

Ait-Belgnaoui, A., Colom, A., Braniste, V., Ramalho, L., Marrot, A., Cartier, C., Houdeau, E., Theodorou, V., and Tompkins, T. (2014). Probiotic gut effect prevents the chronic psychological stress-induced brain activity abnormality in mice. *Neurogastroenterol Motil,* 26(4), 510-520.

Anderson, K. E. (2002). *Final report of the thirty fourth North Carolina layer performance and management test*. Cooperative Extension Service, North Carolina State University, Raleigh.

Applegate, T., Onyango, E., Angel, R., and Powers, W. (2009). Effect of amino acid formulation and dietary direct-fed microbial supplementation on egg production and egg characteristics in laying hens. *J Appl Poult Res,* 18(3), 552-561.

ASABE. (2007). *Shear and Three-Point Bending Test of Animal Bone*. Retrieved from http://elibrary.asabe.org/azdez.asp?search=0&JID=2&AID=23585&CID=s2000&v=&i=&T=2.

Balevi, T., Ucan, U. S., Coskun, B., Kurtoglu, V., and Cetingul, I. S. (2001). Effect of dietary probiotic on performance and humoral immune response in layer hens. *Br Poult Sci*, 42(4), 456-461.

Bar, A., Vax, E., and Striem, S. (1999). Relationships among age, eggshell thickness and vitamin D metabolism and its expression in the laying hen. *Comp Biochem Phys A*, 123(2), 147-154.

Barrett, E., Ross, R. P., Fitzgerald, G. F., and Stanton, C. (2007). Rapid screening method for analyzing the conjugated linoleic acid production capabilities of bacterial cultures. *Appl Environ Microbiol*, 73(7), 2333-2337.

Beck, M. M. and Hansen, K. K. (2004). Role of estrogen in avian osteoporosis. *Poult Sci*, 83(2), 200-206.

Brisbin, J. T., Gong, J., Orouji, S., Esufali, J., Mallick, A. I., Parvizi, P., Shewen, P. E., and Sharif, S. (2011). Oral treatment of chickens with lactobacilli influences elicitation of immune responses. *Clin Vaccine Immunol*, 18(9), 1447-1455.

Britton, R. A., Irwin, R., Quach, D., Schaefer, L., Zhang, J., Lee, T., Parameswaran, N., and McCabe, L. R. (2014). Probiotic L. reuteri treatment prevents bone loss in a menopausal ovariectomized mouse model. *J Cell Physiol*, 229(11), 1822-1830.

Capcarova, M., Chmelnicna, L., Kolesarova, A., Massanyi, P., and Kovacik, J. (2010). Effects of Enterococcus faecium M 74 strain on selected blood and production parameters of laying hens. *Br Poult Sci*, 51(5), 614-620.

Charles, J. F., Ermann, J., and Aliprantis, A. O. (2015). The intestinal microbiome and skeletal fitness: Connecting bugs and bones. *Clin Immunol*, 159(2), 163-169.

Coakley, M., Ross, R. P., Nordgren, M., Fitzgerald, G., Devery, R., and Stanton, C. (2003). Conjugated linoleic acid biosynthesis by human-derived Bifidobacterium species. *J Appl Microbiol*, 94(1), 138-145.

Coakley, M., Johnson, M. C., McGrath, E., Rahman, S., Ross, R. P., Fitzgerald, G. F., Devery, R., and Stanton, C. (2006). Intestinal bifidobacteria that produce trans-9, trans-11 conjugated linoleic acid: A fatty acid with antiproliferative activity against human colon SW480 and HT-29 cancer cells. *Nutr Cancer* 56(1), 95-102.

Crittenden, R. G., Martinez, N. R., and Playne, M. J. (2003). Synthesis and utilisation of folate by yoghurt starter cultures and probiotic bacteria. *Int J Food Microbiol,* 80(3), 217-222.

Dacke, C. G., Arkle, S., Cook, D. J., Wormstone, I. M., Jones, S., Zaidi, M., and Bascal, Z. A. (1993). Medullary Bone and Avian Calcium Regulation. *J Exp Biol,* 184(1), 63-88.

Davis, G. S. and Anderson, K. E. (2002). The effects of feeding the direct-fed microbial, primalac, on growth parameters and egg production in single comb white leghorn hens. *Poult Sci,* 81(6), 755-759.

Deguchi, Y., Morishita, T., and Mutai, M. (1985). Comparative Studies on Synthesis of Water-Soluble Vitamins among Human Species of Bifidobacteria. *Agric Biol Chem,* 49(1), 13-19.

Desbonnet, L., Garrett, L., Clarke, G., Kiely, B., Cryan, J. F., and Dinan, T. G. (2010). Effects of the probiotic Bifidobacterium infantis in the maternal separation model of depression. *Neuroscience,* 170(4), 1179-1188.

Diaz Heijtz, R., Wang, S., Anuar, F., Qian, Y., Bjorkholm, B., Samuelsson, A., Hibberd, M. L., Forssberg, H., and Pettersson, S. (2011). Normal gut microbiota modulates brain development and behavior. *Proc Natl Acad Sci U S A,* 108(7), 3047-3052.

Drago, L., De Vecchi, E., Gabrieli, A., De Grandi, R., and Toscano, M. (2015). Immunomodulatory Effects of Lactobacillus salivarius LS01 and Bifidobacterium breve BR03, Alone and in Combination, on Peripheral Blood Mononuclear Cells of Allergic Asthmatics. *Allergy Asthma Immunol Res,* 7(4), 409-413.

Du Plessis, P. H. C. and Erasmus, J. (1972). The relationship between egg production, egg weight and body weight in laying hens. *Worlds Poult Sci J,* 28(3), 301-310.

Ehrmann, M. A., Kurzak, P., Bauer, J., and Vogel, R. F. (2002). Characterization of lactobacilli towards their use as probiotic adjuncts in poultry. *J Appl Microbiol,* 92(5), 966-975.

Fuentes, C., Orozco, L., Vicente, J., Velasco, X., Menconi, A., Kuttappan, V., Kallapura, G., Latorre, J., Layton, S., Hargis, B., and Téllez, G. (2013). Effect of a lactic acid bacterium based probiotic, Floramax-B11Â®, on performance, bone qualities, and morphometric analysis of broiler chickens: an economic analysis. *Int J Poult Sci,* 2, 113.

Fulton, R. M., Nersessian, B. N., and Reed, W. M. (2002). Prevention of Salmonella enteritidis infection in commercial ducklings by oral chicken egg-derived antibody alone or in combination with probiotics. *Poult Sci,* 81(1), 34-40.

Gallazzi, D., Giardini, A., Mangiagalli, G. M., Marelli, S., Ferrazzi, V., Orsi, C., and Cavalchini, G. L. (2008). Effects of Lactobacillus acidophilus D2/CSL on laying hen performance. *Ital J Anim Sci,* 7(1), 27-37.

Garcia, V. G., Knoll, L. R., Longo, M., Novaes, V. C., Assem, N. Z., Ervolino, E., de Toledo, B. E., and Theodoro, L. H. (2016). Effect of the probiotic Saccharomyces cerevisiae on ligature-induced periodontitis in rats. *J Periodontal Res,* 51(1), 26-37.

Giannenas, I., Papadopoulos, E., Tsalie, E., Triantafillou, E., Henikl, S., Teichmann, K., and Tontis, D. (2012). Assessment of dietary supplementation with probiotics on performance, intestinal morphology and microflora of chickens infected with Eimeria tenella. *Vet Parasitol,* 188(1-2), 31-40.

Haddadin, M. S., Abdulrahim, S. M., Hashlamoun, E. A., and Robinson, R. K. (1996). The effect of Lactobacillus acidophilus on the production and chemical composition of hen's eggs. *Poult Sci,* 75(4), 491-494.

Hancock, R. D. and Viola, R. (2001). The use of micro-organisms for L-ascorbic acid production: current status and future perspectives. *Appl Microbiol Biotechnol,* 56(5-6), 567-576.

Hansen, K. K. (2002). *Aging and the role of estrogen in calcium mobilization in the laying hen*. ProQuest Dissertations and Theses.

Hayirli, A., Esenbuga, N., Macit, M., Yoruk, M. A., Yildiz, A., and Karaca, H. (2005). Nutrition practice to alleviate the adverse effects of stress on laying performance, metabolic profile and egg quality in peak producing hens: II. The probiotic supplementation. *Asian Austral J Anim,* 18(12), 1752-1760.

Hester, P. Y., Enneking, S. A., Haley, B. K., Cheng, H. W., Einstein, M. E., and Rubin, D. A. (2013). The effect of perch availability during pullet rearing and egg laying on musculoskeletal health of caged White Leghorn hens. *Poult Sci*, 92(8), 1972-1980.

Hou, J. W., Yu, R. C., and Chou, C. C. (2000). Changes in some components of soymilk during fermentation with bifidobacteria. *Food Res Int*, 33(5), 393-397.

Houshmand, M., Azhar, K., Zulkifli, I., Bejo, M. H., Meimandipour, A., and Kamyab, A. (2011). Effects of non-antibiotic feed additives on performance, tibial dyschondroplasia incidence and tibia characteristics of broilers fed low-calcium diets. *J Anim Physiol Anim Nutr (Berl)*, 95(3), 351-358.

Iwami, K. and Moriyama, T. (1993). Effects of short chain fatty acid, sodium butyrate, on osteoblastic cells and osteoclastic cells. *Int J Biochem*, 25(11), 1631-1635.

Jadamus, A., Vahjen, W., and Simon, O. (2001). Growth behaviour of a spore forming probiotic strain in the gastrointestinal tract of broiler chicken and piglets. *Arch Tierernahr*, 54(1), 1-17.

Jendral, M. J., Korver, D. R., Church, J. S., and Feddes, J. J. (2008). Bone mineral density and breaking strength of White Leghorns housed in conventional, modified, and commercially available colony battery cages. *Poult Sci*, 87(5), 828-837.

Jia, D., O'Brien, C. A., Stewart, S. A., Manolagas, S. C., and Weinstein, R. S. (2006). Glucocorticoids act directly on osteoclasts to increase their life span and reduce bone density. *Endocrinology*, 147(12), 5592-5599.

Jin, L. Z., Ho, Y. W., Abdullah, N., and Jalaludin, S. (1996). Influence of dried Bacillus subtilis and lactobacilli cultures on intestinal microflora and performance in broilers. *Asian Austral J Anim*, 9, 397-404.

Jin, L. Z., Ho, Y. W., Abdullah, N., and Jalaludin, S. (2000). Digestive and bacterial enzyme activities in broilers fed diets supplemented with Lactobacillus cultures. *Poult Sci*, 79(6), 886-891.

Kim, Y. S. and Ho, S. B. (2010). Intestinal goblet cells and mucins in health and disease: recent insights and progress. *Curr Gastroenterol Rep*, 12(5), 319-330.

Klingensmith, P. M. and Hester, P. Y. (1985). Phosphorus phase feeding and uterine and isthmus mucosal enzymes and minerals in relation to soft-shelled and shell-less egg production. *Poult Sci,* 64(11), 2180-2188.

Kurtoglu, V., Kurtoglu, F., Seker, E., Coskun, B., Balevi, T., and Polat, E. S. (2004). Effect of probiotic supplementation on laying hen diets on yield performance and serum and egg yolk cholesterol. *Food Addit Contam,* 21(9), 817-823.

Legette, L. L., Lee, W., Martin, B. R., Story, J. A., Campbell, J. K., and Weaver, C. M. (2012). Prebiotics enhance magnesium absorption and inulin-based fibers exert chronic effects on calcium utilization in a postmenopausal rodent model. *J Food Sci,* 77(4), H88-94.

Lei, K., Li, Y. L., Yu, D. Y., Rajput, I. R., and Li, W. F. (2013). Influence of dietary inclusion of Bacillus licheniformis on laying performance, egg quality, antioxidant enzyme activities, and intestinal barrier function of laying hens. *Poult Sci,* 92(9), 2389-2395.

Li, L., Xu, C. L., Ji, C., Ma, Q., Hao, K., Jin, Z. Y., and Li, K. (2006). Effects of a dried Bacillus subtilis culture on egg quality. *Poult Sci,* 85(2), 364-368.

Liu, Y. W., Liu, W. H., Wu, C. C., Juan, Y. C., Wu, Y. C., Tsai, H. P., Wang, S., and Tsai, Y. C. (2016). Psychotropic effects of Lactobacillus plantarum PS128 in early life-stressed and naive adult mice. *Brain Res,* 1631, 1-12.

Lorenzo, J., Horowitz, M., and Choi, Y. (2008). Osteoimmunology: interactions of the bone and immune system. *Endocr Rev,* 29(4), 403-440.

Maekawa, T. and Hajishengallis, G. (2014). Topical treatment with probiotic Lactobacillus brevis CD2 inhibits experimental periodontal inflammation and bone loss. *J Periodontal Res,* 49(6), 785-791.

Mahdavi, A. H., Rahmani, H. R., and Pourreza, J. (2005). Effect of probiotic supplements on egg quality and laying hen's performance. *Int J Poult Sci,* 4(4), 488-492.

Mansouri-Tehrani, H. A., Rabbani-Khorasgani, M., Hosseini, S. M., Mokarian, F., Mahdavi, H., and Roayaei, M. (2015). Effect of supplements: Probiotics and probiotic plus honey on blood cell counts and serum IgA in patients receiving pelvic radiotherapy. *J Res Med Sci,* 20(7), 679-683.

McCabe, L., Britton, R. A., and Parameswaran, N. (2015). Prebiotic and Probiotic Regulation of Bone Health: Role of the Intestine and its Microbiome. *Curr Osteoporos Rep,* 13(6), 363-371.

McCabe, L. R., Irwin, R., Schaefer, L., and Britton, R. A. (2013). Probiotic use decreases intestinal inflammation and increases bone density in healthy male but not female mice. *J Cell Physiol,* 228(8), 1793-1798.

Messora, M. R., Oliveira, L. F., Foureaux, R. C., Taba, M., Jr., Zangeronimo, M. G., Furlaneto, F. A., and Pereira, L. J. (2013). Probiotic therapy reduces periodontal tissue destruction and improves the intestinal morphology in rats with ligature-induced periodontitis. *J Periodontol,* 84(12), 1818-1826.

Mikulski, D., Jankowski, J., Naczmanski, J., Mikulska, M., and Demey, V. (2012). Effects of dietary probiotic (Pediococcus acidilactici) supplementation on performance, nutrient digestibility, egg traits, egg yolk cholesterol, and fatty acid profile in laying hens. *Poult Sci,* 91(10), 2691-2700.

Mountzouris, K., Tsirtsikos, P., Kalamara, E., Nitsch, S., Schatzmayr, G., and Fegeros, K. (2007). Evaluation of the efficacy of a probiotic containing Lactobacillus, Bifidobacterium, Enterococcus, and Pediococcus strains in promoting broiler performance and modulating cecal microflora composition and metabolic activities. *Poult Sci,* 86(2), 309-317.

Mountzouris, K. C., Tsitrsikos, P., Palamidi, I., Arvaniti, A., Mohnl, M., Schatzmayr, G., and Fegeros, K. (2010). Effects of probiotic inclusion levels in broiler nutrition on growth performance, nutrient digestibility, plasma immunoglobulins, and cecal microflora composition. *Poult Sci,* 89(1), 58-67.

Mountzouris, K. C., Palamidi, I., Tsirtsikos, P., Mohnl, M., Schatzmayr, G., and Fegeros, K. (2015). Effect of dietary inclusion level of a multi-species probiotic on broiler performance and two biomarkers of their caecal ecology. *Anim Reprod Sci,* 55(4), 484-493.

Murugesan, G. R. and Persia, M. E. (2015). Influence of a direct-fed microbial and xylanase enzyme on the dietary energy uptake efficiency and performance of broiler chickens. *J Sci Food Agric,* 95(12), 2521-2527.

Mutus, R., Kocabagli, N., Alp, M., Acar, N., Eren, M., and Gezen, S. S. (2006). The effect of dietary probiotic supplementation on tibial bone characteristics and strength in broilers. *Poult Sci,* 85(9), 1621-1625.

Nahashon, S. N., Nakaue, H. S., and Mirosh, L. W. (1994). Production variables and nutrient retention in single comb White Leghorn laying pullets fed diets supplemented with direct-fed microbials. *Poult Sci,* 73(11), 1699-1711.

Nasr, M. A., Nicol, C. J., and Murrell, J. C. (2012). Do laying hens with keel bone fractures experience pain? *PLoS One,* 7(8), e42420.

Netherwood, T., Gilbert, H. J., Parker, D. S., and O'Donnell, A. G. (1999). Probiotics shown to change bacterial community structure in the avian gastrointestinal tract. *Appl Environ Microbiol,* 65(11), 5134-5138.

O'Brien, C. A., Jia, D., Plotkin, L. I., Bellido, T., Powers, C. C., Stewart, S. A., Manolagas, S. C., and Weinstein, R. S. (2004). Glucocorticoids act directly on osteoblasts and osteocytes to induce their apoptosis and reduce bone formation and strength. *Endocrinology,* 145(4), 1835-1841.

O'Mahony, S. M., Clarke, G., Borre, Y. E., Dinan, T. G., and Cryan, J. F. (2015). Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. *Behav Brain Res,* 277, 32-48.

Ohlsson, C., Engdahl, C., Fak, F., Andersson, A., Windahl, S. H., Farman, H. H., Moverare-Skrtic, S., Islander, U., and Sjogren, K. (2014). Probiotics protect mice from ovariectomy-induced cortical bone loss. *PLoS One,* 9(3), e92368.

Onyango, E., Hester, P., Stroshine, R., and Adeola, O. (2003). Bone densitometry as an indicator of percentage tibia ash in broiler chicks fed varying dietary calcium and phosphorus levels. *Poult Sci,* 82(11), 1787-1791.

Panda, A. K., Reddy, M. R., Rama Rao, S. V., and Praharaj, N. K. (2003). Production performance, serum/yolk cholesterol and immune competence of white leghorn layers as influenced by dietary supplementation with probiotic. *Trop Anim Health Prod,* 35(1), 85-94.

Panda, A. K., Rao, S. V. R., Raju, M. V., and Sharma, S. R. (2006). Dietary supplementation of Lactobacillus sporogenes on performance and serum biochemico-lipid profile of broiler chickens. *J Poult Sci,* 43(3), 235-240.

Panda, A. K., Rao, S. S. R., Raju, M. V., and Sharma, S. S. (2008). Effect of probiotic (Lactobacillus sporogenes) feeding on egg production and quality, yolk cholesterol and humoral immune response of White Leghorn layer breeders. *J Sci Food Agric*, 88(1), 43-47.

Poggenpoel, D. G. (1986). Correlated Response in Shell and Albumen Quality with Selection for Increased Egg Production. *Poult Sci*, 65(9), 1633-1641.

Praharaj, I., John, S. M., Bandyopadhyay, R., and Kang, G. (2015). Probiotics, antibiotics and the immune responses to vaccines. *Philos Trans R Soc Lond B Biol Sci*, 370(1671).

Ramasamy, K., Abdullah, N., Jalaludin, S., Wong, M., and Ho, Y. W. (2009). Effects of Lactobacillus cultures on performance of laying hens, and total cholesterol, lipid and fatty acid composition of egg yolk. *J Sci Food Agric*, 89(3), 482-486.

Rauch, A., Seitz, S., Baschant, U., Schilling, A. F., Illing, A., Stride, B., Kirilov, M., Mandic, V., Takacz, A., Schmidt-Ullrich, R., Ostermay, S., Schinke, T., Spanbroek, R., Zaiss, M. M., Angel, P. E., Lerner, U. H., David, J. P., Reichardt, H. M., Amling, M., Schutz, G., and Tuckermann, J. P. (2010). Glucocorticoids suppress bone formation by attenuating osteoblast differentiation via the monomeric glucocorticoid receptor. *Cell Metab*, 11(6), 517-531.

Remesy, C., Levrat, M. A., Gamet, L., and Demigne, C. (1993). Cecal fermentations in rats fed oligosaccharides (inulin) are modulated by dietary calcium level. *Am J Physiol*, 264(5 Pt 1), G855-862.

Ribeiro, V., Albino, L. F. T., Rostagno, H. S., Barreto, S. L. T., Hannas, M. I., Harrington, D., de Araujo, F. A., Ferreira, H. C., and Ferreira, M. A. (2014). Effects of the dietary supplementation of Bacillus subtilis levels on performance, egg quality and excreta moisture of layers. *Anim Feed Sci Tech*, 195, 142-146.

Roberts, J. R., Chousalkar, K., and Samiullah. (2013). Egg quality and age of laying hens: implications for product safety. *Anim Prod Sci*, 53(12), 1291-1297.

Sadeghi, A. A. (2014). Bone Mineralization of Broiler Chicks Challenged with Salmonella enteritidis Fed Diet Containing Probiotic (Bacillus subtilis). *Probiotics Antimicrob Proteins*, 6(3-4), 136-140.

Safaa, H. M., Serrano, M. P., Valencia, D. G., Frikha, M., Jimenez-Moreno, E., and Mateos, G. G. (2008). Productive performance and egg quality of brown egg-laying hens in the late phase of production as influenced by level and source of calcium in the diet. *Poult Sci*, 87(10), 2043-2051.

Salehimanesh, A., Mohammadi, M., and Roostaei-Ali Mehr, M. (2016). Effect of dietary probiotic, prebiotic and synbiotic supplementation on performance, immune responses, intestinal morphology and bacterial populations in broilers. *J Anim Physiol Anim Nutr (Berl)*.

Salim, H. M., Kang, H. K., Akter, N., Kim, D. W., Kim, J. H., Kim, M. J., Na, J. C., Jong, H. B., Choi, H. C., Suh, O. S., and Kim, W. K. (2013). Supplementation of direct-fed microbials as an alternative to antibiotic on growth performance, immune response, cecal microbial population, and ileal morphology of broiler chickens. *Poult Sci,* 92(8), 2084-2090.

Salma, U., Miah, A. G., Tsujii, H., Schellander, K., and Sudekum, K. H. (2012). Effect of dietary Rhodobacter capsulatus on lipid fractions and egg-yolk fatty acid composition in laying hens. *J Anim Physiol Anim Nutr (Berl),* 96(6), 1091-1100.

Schneitz, C., Kiiskinen, T., Toivonen, V., and Nasi, M. (1998). Effect of BROILACT on the physicochemical conditions and nutrient digestibility in the gastrointestinal tract of broilers. *Poult Sci,* 77(3), 426-432.

Scholz-Ahrens, K. E., Ade, P., Marten, B., Weber, P., Timm, W., Acil, Y., Gluer, C. C., and Schrezenmeir, J. (2007). Prebiotics, probiotics, and synbiotics affect mineral absorption, bone mineral content, and bone structure. *J Nutr,* 137(3 Suppl 2), 838S-846S.

Sjogren, K., Engdahl, C., Henning, P., Lerner, U. H., Tremaroli, V., Lagerquist, M. K., Backhed, F., and Ohlsson, C. (2012). The gut microbiota regulates bone mass in mice. *J Bone Miner Res,* 27(6), 1357-1367.

Sohail, M. U., Ijaz, A., Yousaf, M. S., Ashraf, K., Zaneb, H., Aleem, M., and Rehman, H. (2010). Alleviation of cyclic heat stress in broilers by dietary supplementation of mannan-oligosaccharide and Lactobacillus-based probiotic: dynamics of cortisol, thyroid hormones, cholesterol, C-reactive protein, and humoral immunity. *Poult Sci,* 89(9), 1934-1938.

Sohail, M. U., Hume, M. E., Byrd, J. A., Nisbet, D. J., Ijaz, A., Sohail, A., Shabbir, M. Z., and Rehman, H. (2012). Effect of supplementation of prebiotic mannan-oligosaccharides and probiotic mixture on growth performance of broilers subjected to chronic heat stress. *Poult Sci,* 91(9), 2235-2240.

Steel, R., Torrie, J., and Dickey, D. (1997). *Principles and Procedures of Statistics: A Biometrical Approach*. New York, NY: McGraw Hill Book Co.

Strong, R. A., Hester, P. Y., Eicher, S. D., Hu, J., and Cheng, H. W. (2015). The Effect of Cooled Perches on Immunological Parameters of Caged White Leghorn Hens during the Hot Summer Months. *PLoS One,* 10(10), e0141215.

Surono, I. S., Martono, P. D., Kameo, S., Suradji, E. W., and Koyama, H. (2014). Effect of probiotic L. plantarum IS-10506 and zinc supplementation on humoral immune response and zinc status of Indonesian pre-school children. *J Trace Elem Med Biol,* 28(4), 465-469.

UEP (2016). *Animal Husbandry Guidelines for U.S. Egg Laying Flocks*. Retrieved from http://uepcertified.com/wp-content/uploads/2015/08/UEP-Animal-Welfare-Guidelines-20141.pdf.

Wang, C., Shoji, H., Sato, H., Nagata, S., Ohtsuka, Y., Shimizu, T., and Yamashiro, Y. (2007). Effects of oral administration of bifidobacterium breve on fecal lactic acid and short-chain fatty acids in low birth weight infants. *J Pediatr Gastroenterol Nutr,* 44(2), 252-257.

Weber, P. (1999). The role of vitamins in the prevention of osteoporosis--a brief status report. *Int J Vitam Nutr Res,* 69(3), 194-197.

Wen, K., Tin, C., Wang, H., Yang, X., Li, G., Giri-Rachman, E., Kocher, J., Bui, T., Clark-Deener, S., and Yuan, L. (2014). Probiotic Lactobacillus rhamnosus GG enhanced Th1 cellular immunity but did not affect antibody responses in a human gut microbiota transplanted neonatal gnotobiotic pig model. *PLoS One,* 9(4), e94504.

Whitehead, C. C. and Fleming, R. H. (2000). Osteoporosis in cage layers. *Poult Sci,* 79(7), 1033-1041.

Whitehead, C. C. (2004). Overview of bone biology in the egg-laying hen. *Poult Sci,* 83(2), 193-199.

Wideman, R. F., Jr., Hamal, K. R., Stark, J. M., Blankenship, J., Lester, H., Mitchell, K. N., Lorenzoni, G., and Pevzner, I. (2012). A wire-flooring model for inducing lameness in broilers: evaluation of probiotics as a prophylactic treatment. *Poult Sci,* 91(4), 870-883.

Wistedt, A., Ridderstrale, Y., Wall, H., and Holm, L. (2014). Exogenous estradiol improves shell strength in laying hens at the end of the laying period. *Acta Vet Scand,* 56(1), 34.

Yadav, V. K., Oury, F., Suda, N., Liu, Z. W., Gao, X. B., Confavreux, C., Klemenhagen, K. C., Tanaka, K. F., Gingrich, J. A., Guo, X. E., Tecott, L. H., Mann, J. J., Hen, R., Horvath, T. L., and Karsenty, G. (2009). A serotonin-dependent mechanism explains the leptin regulation of bone mass, appetite, and energy expenditure. *Cell,* 138(5), 976-989.

Yadav, V. K., Balaji, S., Suresh, P. S., Liu, X. S., Lu, X., Li, Z., Guo, X. E., Mann, J. J., Balapure, A. K., Gershon, M. D., Medhamurthy, R., Vidal, M., Karsenty, G., and Ducy, P. (2010). Pharmacological inhibition of gut-derived serotonin synthesis is a potential bone anabolic treatment for osteoporosis. *Nat Med,* 16(3), 308-312.

Yalcin, S., Yalcin, S., Cakin, K., Eltan, O., and Dagasan, L. (2010). Effects of dietary yeast autolysate (Saccharomyces cerevisiae) on performance, egg traits, egg cholesterol content, egg yolk fatty acid composition and humoral immune response of laying hens. *J Sci Food Agric,* 90(10), 1695-1701.

Yang, H., Zhao, X., Tang, S., Huang, H., Zhao, X., Ning, Z., Fu, X., and Zhang, C. (2016). Probiotics reduce psychological stress in patients before laryngeal cancer surgery. *Asia Pac J Clin Oncol,* 12(1), e92-96.

Yonezawa, T., Kobayashi, Y., and Obara, Y. (2007). Short-chain fatty acids induce acute phosphorylation of the p38 mitogen-activated protein kinase/heat shock protein 27 pathway via GPR43 in the MCF-7 human breast cancer cell line. *Cell Signal,* 19(1), 185-193.

Yoruk, M. A., Gul, M., Hayirli, A., and Macit, M. (2004). The effects of supplementation of humate and probiotic on egg production and quality parameters during the late laying period in hens. *Poult Sci,* 83(1), 84-88.

Zeidler, G., 2002. Chapter 57: Shell eggs and their nutritional value. Page 1109-1128 in: Bell, D.D., Weaver Jr, W.D. (Eds.), Commercial Chicken Meat and Egg Production, fifth ed., Kluwer Academic Publishers, Norwell, Massachusetts, United States.

Zhang, J., Motyl, K. J., Irwin, R., MacDougald, O. A., Britton, R. A., and McCabe, L. R. (2015). Loss of Bone and Wnt10b Expression in Male Type 1 Diabetic Mice Is Blocked by the Probiotic Lactobacillus reuteri. *Endocrinology,* 156(9), 3169-3182.

Zhang, J. L., Xie, Q. M., Ji, J., Yang, W. H., Wu, Y. B., Li, C., Ma, J. Y., and Bi, Y. Z. (2012). Different combinations of probiotics improve the production performance, egg quality, and immune response of layer hens. *Poult Sci,* 91(11), 2755-2760.

Zhang, Z. F. and Kim, I. H. (2013). Effects of probiotic supplementation in different energy and nutrient density diets on performance, egg quality, excreta microflora, excreta noxious gas emission, and serum cholesterol concentrations in laying hens. *J Anim Sci,* 91(10), 4781-4787.

Ziaie, H., Bashtani, M., Torshizi, M. A. K., Naeeimipour, H., Farhangfar, H., and Zeinali, A. (2011). Effect of antibiotic and its alternatives on morphometric characteristics, mineral content and bone strength of tibia in Ross broiler chickens. *Glob Vet,* 7(4), 315-322.

Zita, L., Ledvinka, Z., Tumova, E., and Klesalova, L. (2012). Technological quality of eggs in relation to the age of laying hens and Japanese quails. *Rev Bras Zootecn,* 41(9), 2079-2084.

CHAPTER 3. EFFECTS OF A *BACILLUS SUBTILIS* BASED PROBIOTIC ON BONE HEALTH IN BROILERS

3.1 Abstract

The objective of this study was to determine the effect of dietary supplementation of probiotic, a *Bacillus subtilis* based probiotic, on broiler bone health. One hundred and twenty 1-d-old Ross 708 chicks were assigned to 24 floor pens based on their BW. The pens were evenly divided into 2 groups (n = 12). One group was fed a control diet, and another group was fed a diet mixed with a commercial probiotic (250 ppm, 1 x 10^6 cfu/g of feed) for 6 wk. Compared to controls, the tibias and femurs of probiotic fed broilers had greater bone mineralization, wall thickness, size, and weight at 43 d of age ($P < 0.05$). Concomitantly, serum Ca concentrations were increased by probiotic at 14 d of age; whereas a trend of lower serum CTX concentrations ($P = 0.08$), a bone resorption indicator, occurred in 43-d-old broilers consuming probiotic. In addition, 5-HT concentrations were increased in the raphe nuclei, whereas NE and DA concentrations were decreased in the hypothalamus of broilers fed probiotic at 43 d of age ($P = 0.04$, 0.03, and 0.02, respectively). No differences in plasma concentrations of pro-inflammatory cytokines (IL-6, TNF-α, and IFN-γ), anti-inflammatory cytokine (IL-10), 5-HT, and TRP were observed ($P > 0.05$). These results indicate that dietary supplementation of a *Bacillus subtilis* based probiotic improved broiler bone traits, most likely through increased Ca intestinal absorption and also perhaps through reduced bone resorption mediated by 5-HT induced reduction of sympathetic activity. Dietary probiotic supplementation may be a useful strategy for improving skeletal health and welfare in broilers.

3.2 Introduction

Skeletal disorders are common in domesticated poultry. Due to its negative effects on the locomotor system, resulting in impaired mobility or lameness, leg disorders in broilers and turkeys are a serious economic loss to the commercial poultry meat industry. Over 27.6% of broilers are estimated to exhibit poor locomotion in the United Kingdom

(Knowles et al., 2008), with a range from 14.1% to 30.1% in other European countries (Sanotra et al., 2001; Sanotra et al., 2003). Selection for rapid growth rates and greater breast meat deposition is a likely contributor to lameness (Talaty et al., 2009). The effects of an uneven load and overweight on developing bones results in a high incidence of leg abnormalities causing lameness (Kestin et al., 2001). When adjusted for BW, fast-growing broilers had lower tibia density and percentage of bone ash than slow-growing broilers (Shim et al., 2012) as a consequence of less mineralization and higher porosity (Williams et al., 2004).

The effect of serotonin (also known as 5-HT) on bone is dependent on its source. Serotonin is synthesized in the brain as well as peripherally. Brain serotonin, acting as a neurotransmitter, stimulates bone formation and inhibits bone resorption causing an increase in bone mass, whereas peripheral serotonin, acting as a hormone, has the opposite effect resulting in inhibition of bone formation (Ducy and Karsenty, 2010). The majority (95%) of serotonin is found in the periphery (El-Merahbi et al., 2015). Serotonin produced in the brain cannot cross over into the blood (Mann et al., 1992), creating 2 independent sources with independent functions. Gut-derived 5-HT that directly regulates bone metabolism is dependent on its receptors. Specifically, when the 5-HT_{1B} receptor is blocked, bone mass, the number of osteoblasts, and bone formation are increased. Yadav et al. (2008) revealed the inhibitory effect of 5-HT on osteoblast proliferation through 5-HT_{1B} receptor, as *5-HT$_{1B}$*-/- mice displayed increased bone mass and bone osteoblast numbers. Intracellular serotonin is essential in osteoclast differentiation (Battaglino et al., 2004). Bone resorption in *Tph1*-/- (the key 5-HT synthesis enzyme in the gut) mice were markedly decreased, whereas addition of serotonin rescued osteoclastogenesis in bone cultures from *Tph1*-/- mice (Chabbi-Achengli et al., 2012). These results may explain, at least partially, why patients treated with some antipsychotic drugs, e.g., serotonin reuptake inhibitors, have low bone mass of the hips and a high risk of osteoporotic fractures (Gebara et al., 2014).

The sympathetic nervous system has also been known to negatively regulate bone mass via β2-adrenergic receptors expressed on osteoblasts and osteocytes (Elefteriou et al., 2005; Bonnet et al., 2008a; Kajimura et al., 2011). Upon activation, sympathetic nerves release NE which binds to β2-adrenergic receptors. The stimulated β2-adrenergic

receptors on osteoblasts subsequently trigger a series of signaling pathways leading to inhibition of osteoblast proliferation (Fu et al., 2005) and an increase in osteoclast formation (Bonnet et al., 2005; Bonnet et al., 2007; Niedermair et al., 2014). Brain-derived 5-HT stimulates bone mass accrual through reduction in sympathetic activity by binding to 5-HT_{2C} receptors on the ventromedial hypothalamic neurons (Yadav et al., 2009).

Probiotics are live microorganisms which confer health benefits, including improvements in bone (Scholz-Ahrens et al., 2007; Parvaneh et al., 2014; mccabe et al., 2015), on the host when administered in appropriate amounts (FAO/WHO, 2001). Few studies have directly tested the possible mode of actions underlying bone remodeling of probiotics, although some mechanisms have been proposed including nutrient acquisition, immune regulation, and hormonal regulation (Charles et al., 2015; mccabe et al., 2015; Ohlsson and Sjogren, 2015; Weaver, 2015; Hernandez et al., 2016). Serotonin has also been proposed to be one of the possible mechanisms involved in probiotic-based improvement of bone (Charles et al., 2015; mccabe et al., 2015). However, no study has been conducted to determine the effect of probiotics on bone fitness and the possible role of serotonin in broilers. The objective of the present experiment was to investigate the effect and mechanism of a *Bacillus subtilis* based probiotic on broiler bone health. We hypothesized that probiotic supplementation would improve bone traits in broilers through regulating mineral bioavailability, synthesis and release of 5-HT, sympathetic activity, immune cytokines, or combinations thereof.

3.3 Materials and Methods

3.3.1 Birds, Management, and Sample Collection

A total of one hundred and twenty d-old Ross 708 male broiler chicks were obtained from a commercial hatchery (Miller Poultry, Orland, IN). Chicks were weighed and placed into 24 floor pens (243 × 51 cm) ensuring similar average BW across pens. There were 5 chicks per pen resulting in a stocking density of 2,478.6 cm^2/broiler. The litter source was wood shavings and each pen was equipped with 1 hanging feeder and drinker. Room temperature was gradually decreased from 35 ºc on d 1 to 21 ºc by 0.55

ºc/d, and maintained at 21 ºc for the rest of the experimental period. The lighting program was gradually decreased from 23 light:1 dark (0100-0200h) at 30 lux up to the first 7 d of age, then 20 light:4 dark (0100-0500h) at 10 lux until 44 d of age. Pens were assigned to 2 dietary treatments of 12 replicate floor pens per treatment: regular diets and the diets mixed with 250 ppm probiotic (Sporulin®, Pacific Vet Group-USA, Inc., Fayetteville, AR). The probiotic consisted of 3 strains of *Bacillus subtilis* resulting in 1.0×10^6 spores/g of feed. The level of probiotic was recommended by the company, and the regular diets were formulated using the recommendations for nutrients by Aviagen (2014). Birds were fed a starter, grower, or finisher diet from 1 to 14, 15 to 28, and 29 to 44 d of age, respectively (Table 3.1). Feed and water were provided *ad libitum*. Prior to the experiment, all the diets were prepared and sampled for bacterial analysis to ensure the diets were mixed properly. The husbandry and the following procedures were approved by the Purdue Animal Use and Care Committee (Number: 1111000262).

At 14 and 28 d of age, starting from 0900h 1 bird per pen was weighted then sedated using intravenous administration of sodium pentobarbital (30 mg/kg of BW) followed by blood collection via cardiac puncture. A total of 8 mL blood was collected from each bird; 5 mL were placed into ice cooled EDTA-coated plasma tube and 3 mL were placed into a serum tube. The bird was euthanized immediately after bleeding by cervical dislocation. At dissection, the left tibia and femur were removed from the chicken and placed in individual plastic bags and kept at -20° C until assayed. After the sample collection at 28 d of age, 24 broilers (13 from probiotic and 11 from control groups) were culled due to mal-development (unknown reason) and the remaining broilers were randomly regrouped within each treatment to ensure same group size of 3. The subsequent replicate was 7 for probiotic and 8 for control groups during finisher period. At 43 d of age, samples of plasma, serum, and bones were collected from 1 bird per pen as previously described. The hypothalamus and raphe nuclei were additionally collected and immediately frozen on dry ice and stored at -80° C until assayed.

As an indication of a broiler's desire and capability to stand during an uncomfortable situation, the 2 broilers remaining in each pen at the end of the study were used to perform the latency to lie test at 44 d of age following the procedure of Berg and Sanotra (2003). Briefly, each bird was individually placed into a tub filled with 3 cm

water at 28° C. The length of time it took for the bird to sit down and touch the water was recorded. If the bird flew away, it was not included in the data set. If the broiler was still standing after 600 s, the test was stopped and the data retained for statistical analysis.

3.3.2 Bone traits

The BMD, BMC, and bone area of the tibia and femur were measured using DEXA (Norland Medical Systems Inc., Fort Atkinson, WI) following a previously described procedure (Hester et al., 2013). After scanning, all the bones were boiled for 5 min followed by the removal of muscle, connective tissue, epiphyseal caps, and the fibula (Hall et al., 2003). The bones were air dried overnight at room temperature. The bones were weighed individually, and its length, width, and cortical bone thickness on the medial and lateral sides were determined using a digital micrometer (Coolant Proof Micrometer Series 293, Mitutoyo America Corp., Aurora, IL). Traditional bone density indicators of robusticity index and bone weight to length index were also calculated (Riesenfeld, 1972; Seedor et al., 1991). Higher bone density was indicated by higher weight to length index but lower robusticity index.

$$Robusticity\ index = \frac{bone\ length}{\sqrt[3]{bone\ weight}}$$

$$Weight\ to\ length\ index = \frac{bone\ weight}{bone\ length}$$

3.3.3 ELISA

Commercial ELISA kits (Mybiosource, San Diego, CA) were used for detecting serum concentrations of OC, a bone formation indicator, and CTX, a bone resorption indicator. The serum Ca and Pi concentrations were determined using quantichrom kits (Bioassay Systems, Hayward, CA) following manufacturer's instructions.

Plasma concentrations of 5-HT, its precursor TRP, and cytokines, IL-6, IL-10, TNF-α, and IFN-γ were measured using commercial ELISA kits (Mybiosource, San Diego, CA).

3.3.4 HPLC

Metabolites of the hypothalamus and raphe nuclei from the left hemisphere of the brain were analyzed using HPLC (Ultimate™ 3000 RSLCnano System, Thermo Fisher Scientific Inc., Waltham, MA). The brain regions were weighed and homogenized in ice-cold 0.2 M perchloric acid at a 10:1 ratio (µl of perchloric acid:mg of sample). The homogenized mixture was centrifuged at 18,187g for 15 min at 4° C. The resultant supernatant was drawn into microcentrifuge tube and diluted 1:1 with mobile phase (MD-TM, Thermo Fisher Scientific, Waltham, MA). The mixture was centrifuged again at 18,187g for 15 min at 4° C. The supernatant was draw off and filtered through a 0.2-µm polyvinylidene fluoride filter into an HPLC sample vial. The mobile phase flow rate was 0.8 ml/min. A MD-150 column (3.2mm x 150mm, 3 µm C18; Thermo Fisher Scientific, Waltham, MA) was used. The concentrations of NE, EP, DOPAC, DA, 5-HIAA, HVA, 5-HT, and TRP were calculated from a reference curve made by using relative standards. The DOPAC/DA and the HVA/DOPAC turnover ratios were calculated as an index of dopaminergic activities (Bast et al., 2002; Badruzzaman et al., 2013).

3.3.5 RIA

Plasma concentrations of CORT were measured using a commercial ^{125}I CORT RIA kit (MP Biomedicals, Orangeburg, NY) following a method described previously (Cheng et al., 2001).

3.3.6 Statistical Analysis

A one-way ANOVA of the mixed model procedure of SAS 9.4 software (SAS Institute Inc., Cary, NC) was used to analyze all of the data. The fixed factor was the probiotic treatment. BW was used as a covariate for measures of bone mineralization and bone size when necessary. Transformation of data was performed for normality when variances were not homogeneous (Steel et al., 1997). Statistical trends were similar for both transformed and untransformed data; therefore, the untransformed least square means were presented. Statistical significance was set at $P < 0.05$.

3.4 Results

The concentrations of probiotic microorganisms in the probiotic-supplemented diets at the start and end of the experiment were similar to the targeted levels (Table 3.2).

Broiler pen averaged BW (Wang et al., unpublished data) was improved by probiotic inclusion at 43 d of age (control vs probiotic: 2296 vs 2493 g, P = 0.0003), but not at 14 (control vs probiotic: 242 vs 242 g, P = 0.99) and 28 d of age (control vs probiotic: 906 vs 867 g, P = 0.57). After culling and regrouping at 28 d of age, BW between the 2 groups still did not differ (control vs probiotic: 1026 vs 974 g, P = 0.25).

Compared to controls, supplementing the diet with a *Bacillus subtilis* based probiotic dramatically improved all measured bone traits of 43-d-old broilers including mineralization, weight, and physical parameters such as area, length, and width (Table 3.5). The response of earlier aged broilers to the probiotic on bone traits was either minimal (14 d of age, Table 3.3) or there was no effect (28 d of age, Table 3.4). Latency to lie was not affected by the probiotic treatment (P = 0.58; Figure 3.1).

Dietary probiotic increased serum Ca concentrations in 14 d-old broilers (P = 0.05), but not at 28 and 43 d of age (P = 0.40 and 0.64, respectively; Figure 3.2a). Serum Pi concentrations were unaffected by treatment (P = 0.0.2, 0.49, and 0.47, respectively; Figure 3.2b). Probiotic supplementation tended to reduce bone resorption as indicated by the decrease in serum CTX concentrations (P = 0.08; Figure 3.3b), but OC, an indicator of bone formation, was unaffected by dietary treatment (P = 0.58; Figure 3.3a). Neither plasma pro-inflammatory (IFN-γ, Figure 3.4d, P = 0.43; TNF-α, Figure 3.4b, P = 0.15; and IL-6, Figure 3.4a, P = 0.50) nor anti-inflammatory (IL-10; P = 0.14 Figure 3.4c) immune cytokines were affected by probiotic. Plasma CORT concentrations (P = 0.23; Figure 3.5) as well as the peripheral concentrations of plasma 5-HT (P = 0.43; Figure 3.6), and its precursor plasma TRP (P = 0.76; Figure 3.6) were similar between treatments. However, concentrations of 5-HT were increased in the raphe nuclei which is a major location for 5-HT synthesis in the brain (P = 0.04; Table 3.6). This upregulation was not found in the hypothalamus (P = 0.24; Table 3.7), nor were there any changes in brain TRP concentrations (P = 0.59 and 0.53; Tables 3.6 and 3.7, respectively). Hypothalamic concentrations (Table 3.7) of the catecholamines of DA and NE were dramatically reduced (P = 0.02 and 0.03, respectively) accompanied by an increase in the

turnovers of DA (expressed as DOPA/DA, P = 0.004) and DOPAC (expressed as HVA/DOPAC, P = 0.001). The concentrations of catecholamine, their metabolites, as well as turnover indices of the raphe nuclei were not affected by the probiotic treatment (Table 3.6, P = 0.24 or greater).

3.5 Discussion

There are several studies conducted in broilers indicating the positive effects of probiotics on bone health. These studies focused on measurements of the tibia, most likely due to its proneness to deformities such as valgus/varus angulations, osteodystrophy, and dyschondroplasia that can cause poor walking ability. The improved tibia traits as a result of providing probiotic supplementations included weight, size, wall thickness, tibiotarsal index, ash content, ash Ca and P percentage, and breaking strength (Mutus et al., 2006; Panda et al., 2006; Houshmand et al., 2011; Ziaie et al., 2011; Fuentes et al., 2013; Sadeghi, 2014). In line with previous findings, the supplementation of probiotic improved both tibia and femur bone traits in broilers of the current study, but the effect was not profoundly evident until market age of 43 d. Due to the short life cycle of broilers, providing probiotics at hatch or possibly even earlier through *in ovo* feeding prior to hatch is essential to allow establishment and growth of beneficial microbiota, providing the necessary time for the favorable physiological effects to take effect. The improved bone traits in the probiotic group, however, did not contribute to longer latency to lie in water, a test designed to assess lameness in broilers (Weeks et al., 2002; Berg and Sanotra, 2003). The chickens used in the latency to lie test were not evaluated for their ability to walk (e.g., gait score) or for the presence of leg deformities such as angular deviations. Any further studies evaluating the effect of probiotics using this test should first evaluate individual gait score and then select and compare broilers with similar gait scores. Probiotics show promise in improving walking ability as broilers purposely reared on a wired floor to induce bacterial chondronecrosis with osteomyelitis and fed probiotics beginning at 1 d of age had a lower incidence of lameness (Wideman et al., 2012).

Probiotics alter the composition and metabolic activity of the gut microbiota (Ohlsson et al., 2014). Intestinal integrity, as indicated by increased villi height,

absorptive area, and the secretion of the lubricant, mucin, is enhanced by probiotics (Thanh et al., 2009). A more favorable intestinal environment with optimum ph improves nutrient digestibility, retention, and absorption. For example, the organic matrix of bone would benefit from increased absorption of amino acids as building blocks for collagen and non-collagenous proteins. Likewise, bone mineralization would gain from the increased availability of minerals such as Ca and P as well as vitamins such as cholecalciferol. Osteoblasts synthesize and release OC to facilitate bone building and mineralization. Another role for OC is to assist in maintaining Ca homeostasis. Although circulating concentrations of Ca increased only in 14-d-old broilers and not in 28- and 43-d-old chickens and OC concentrations at 43 d of age were unaffected, it is hypothesized that bone sequestered the increased availability of intestinal Ca and P for placement in its hydroxyapatite matrix as evidenced by the dramatic increase in BMD in 43-d-old broilers consuming probiotics (Table 3.4). As a result of increased bone mineral accrual, circulating concentrations of Ca and Pi remained at consistent concentrations even though probiotics most likely allowed for more mineral absorption at the level of the intestines. Similar concentrations of OC between treatment groups may have contributed to maintaining concentrations of circulating Ca in probiotic fed broilers similar to controls. Increased solubility and absorption of minerals have been associated with probiotic-induced bone health benefits (Scholz-Ahrens et al., 2007). *Bacillus subtilis* enhanced utilization of Ca (Anderson et al., 2013), most likely due to the increase in lactic acid production from proliferating *Lactobacilli*.

Besides increased availability of nutrients needed for bone formation, other pathways, such as reduced bone resorption, may also be involved in increasing bone mass in probiotic fed broilers. Clinicians use serum CTX as a biomarker to evaluate bone turnover in patients as circulating concentrations of CTX are positively correlated to osteoclastic activity. The assay for CTX determines the concentrations of c-terminal telopeptide of type I collagen. This specific peptide sequence is cleaved from collagen by osteoclasts during bone resorption, so lower concentrations of CTX suggest reduced osteoclast activity. Bone resorption through the modulation of osteoclasts may have been reduced in 43 d-old broilers consuming probiotic as suggested by lower serum CTX concentrations as compared to controls (Figure 3.3, $P = 0.08$).

In rodents, one of the proposed mechanisms of gut microbiota regulating bone mass is through the down regulation of pro-inflammatory cytokines via the gut-blood-bone axis (Sjogren et al., 2012), as pro-inflammatory cytokines support osteoclast formation (Yokota et al., 2014; de Vries et al., 2015). For example, reduced TNF-α mRNA levels in the jejunum and ileum were accompanied by increased trabecular bone mass in healthy male mice after supplementation of *Lactobacillus reuteri* for 4 wk (Mccabe et al., 2013). On the other hand, cytokines such as INF-γ and IL-10 inhibit osteoclastogenesis (Takayanagi et al., 2000; Pappalardo and Thompson, 2013; Zhang et al., 2014). In the current study, plasma concentrations of IFN-γ, IL-10, IL-6, and TNF-α in the probiotic fed broilers were similar to those in the control group (Figure 3.4), indicating that the bone promoting effect of probiotic may not be through the regulation of systemic inflammation. Limited studies have been conducted on the effect of *Bacillus subtilis* on systemic immune cytokine levels with some studies focusing on gut and spleen cytokine expression. Dietary inclusion of 3 *Bacillus subtilis* strains (Enviva Pro, Danisco Animal Nutrition, UK) for 28 d did not affect mRNA expression of IFN-γ and IL-10 in the gut (pooled jejunal and ileal samples) of Ross 708 broilers (Lee et al., 2014). In contrast, reduced ileal IL-6 as well as splenic IL-6 and IL-10 transcripts in Ross 308 broilers were found after using the same probiotic product for 22 d suggesting a role for probiotics in suppressing inflammation (Waititu et al., 2014). Ducks also showed cytokine responses after consuming a single-strain of *Bacillus subtilis* for 63 d. Specifically, jejunal IFN-γ expression was higher, and ileal IL-10 expression was lower (Xing et al., 2015). Unfortunately, bone traits were not measured in these studies.

Corticosterone is the major avian glucocorticoid, which has biphasic effects on bone (Mak et al., 2009). Normal concentrations of endogenous glucocorticoid promote the differentiation of mesenchymal progenitor cells to the osteoblast lineage rather than to the adipocyte and chondrocyte lineages through regulating Wnt signaling (Zhou et al., 2008). In contrast, excessive glucocorticoid, especially when administered exogenously, profoundly suppresses bone formation by inhibiting osteoblast differentiation and inducing osteoblast apoptosis. High concentrations of glucocorticoids transiently promote bone resorption, probably by stimulating the production of RANKL but inhibits osteoclastogenesis in the long-term (Henneicke et al., 2014). In the current study, plasma

CORT concentrations between the probiotic and the control groups were similar with concentrations (2.28 ng/ml) indicative of unstressed broilers (Quinteiro-Filho et al., 2010). The broilers were raised under normal management and were not subjected to stressors, so it is not surprising that plasma CORT concentrations were unaffected by the probiotic treatment. It is hypothesized that under stressful conditions, the long bones of broilers consuming probiotic as compared to controls would show improved mineralization and strength because of reduced circulating concentrations of CORT.

With respect to the role of serotonin in bone remodeling, only concentrations in the raphe nuclei, and not in the hypothalamus or the blood, of broilers consuming probiotic were affected. The increased concentrations of 5-HT of the raphe nuclei of probiotic fed broilers (Table 3.5) suggest that brain serotonin may have played a role in bone mass accural by stimulating bone formation and inhibiting resorption. Located in the brain stem, the main function of this cluster of nuclei is to synthesize and release serotonin to the remaining part of the brain. Most of the brain 5-HT is synthesized in the raphe nuclei (Tork, 1990). The serotonergic projections arising from the raphe nuclei target many brain areas, among which the hypothalamus receive extremely dense serotonergic inputs (Heym and Gladfelter, 1982; Martin et al., 1985).

With respect to the catecholamines, as long as normal feed intake and BW were maintained, blocking sympathetic activty in mice increased bone mass (Gordeladze and Reseland, 2003). Both NE and DA concentrations of the hypothalamus (Table 3.6), but not the raphe nuclei, were reduced by supplementing the broiler diet with probiotics. The hypothalamus is the site where 5-HT is proposed to play an important role in bone regulation via binding to its specific 5-HT_{2C} receptor to modulate sympathetic tone (Yadav et al., 2009). The NE releasing neurons are located in the locus coeruleus, a nucleus in the pons which is an area of the brain stem. Both NE and 5-HT neurons terminate densely in the hypothalamus, suggesting an interaction between the 5-HT and NE neurons (Tian et al., 1993). The reduction of hypothalamic NE and DA, with a trend for lowered hypothalamic EP ($P = 0.08$, Table 3.6), in probiotic fed broilers suggests that serotonergic neurons topically inhibit central noradrenergic neuron activities in the hypothalamus (Tian et al., 1993). The reduced NE bioactivity through increased brain 5-

HT concentrations in probiotic fed broilers could be related to improved bone traits in the femur and tibia. However, the hypothesis needs to be further examined.

In the current study, higher DA turnover in probiotic fed broilers was also observed as indicated by increased DOPAC/DA and HVA/DOPAC ratios in the hypothalamus (Table 3.6) but not in the raphe nuclei. The hypothalamus is an integrated sensing system, receiving dense inputs from the mesocorticolimbic DA system (Quarta and Smolders, 2014) and contains dopaminergic neurons in its periventricular nucleus and the arcuate nucleus (Lerant et al., 1996). Our results suggest an enhanced activation of catecholaminergic neurons in the hypothalamus. Metabolism of DA involves several pathways. It could be degraded into inactive metabolites, such as DA to DOPAC via monoamine oxidase, and then to HVA via catechol-O-methyltransferase or be synthesized to NE by the enzyme, DA beta-hydroxylase (Meiser et al., 2013; Saylor et al., 2015). Our results suggest that DA metabolism in the hypothalamus of probiotic fed broilers was shifted away from NE production to degradation. The main neurotransmitter in the sympathetic nervous system is NE with its neurons innervating numerous organs including bone (Elefteriou et al., 2014). Sympathetic nerve fibers as well as adrenergic β2 receptors are present in bone (Duncan and Shim, 1977; Fan et al., 2010), suggesting that sympathetic neurons that release NE could regulate bone homeostasis through binding to its β2 receptor (Bonnet et al., 2008b). When stimulated, the β2 receptors expressed on osteoblasts enhance the production of bone remodeling regulatory factors, such as IL-6, RANKL, and prostaglandin E2 (Kondo and Togari, 2003; Elefteriou et al., 2005; Wang et al., 2015), which subsequently inhibit bone formation and promote bone resorption. Altogether, the possibility exists that dietary inclusion of probiotic up-regulates the synthesis of 5-HT in the raphe nuclei (Table 3.5), which is then released in the terminal areas of the hypothalamus leading to decreased NE synthesis (Table 3.6). This reduced sympathetic outflow in turn could possibly contribute to reduced bone resorption.

3.6 Conclusions

Dietary supplementation of *Bacillus subtilis* based probiotic conferred an improvement in broiler bone traits perhaps due to increased intestinal absorption of nutrients such as Ca. Another possible mechanism involved in bone mass accrual in

probiotic fed broilers is the increased brain 5-HT, which may function to reduce sympathetic activity, and thus inhibit bone resorption. Dietary probiotic supplementation has potential as a management strategy for improving skeletal health and welfare in broilers.

Table 3.1 The composition of the starter, grower, and finisher diets

	Starter	Grower	Finisher
Ingredient, %			
Corn	52.0	52.3	62.8
Soybean meal, 48 % crude protein	40.0	39.1	29.7
Soybean oil	3.59	4.97	4.11
Sodium chloride	0.51	0.46	0.43
DL Methionine	0.30	0.24	0.23
L-Lysine HCl	0.13	- - -	0.07
Threonine	0.06	- - -	- - -
Limestone	1.29	1.15	1.12
Monocalcium phoshate	1.75	1.48	1.17
Vitamin/mineral premix[1]	0.35	0.35	0.35
Calculated analyses			
Crude protein %	23.4	22.8	19.2
ME kcal/kg	3050	3151	3200
Ca %	0.95	0.85	0.75
Available P %	0.50	0.44	0.36
Methionine %	0.66	0.59	0.53
Methionine + cystine %	1.04	0.97	0.86
Lysine %	1.42	1.29	1.09
Threonine %	0.97	0.89	0.74
Na %	0.22	0.20	0.19

[1]Provided per kilogram of diet: vitamin A, 13,233 IU; vitamin D3, 6,636 IU; vitamin E, 44.1 IU; vitamin K, 4.5 mg; thiamine, 2.21 mg; riboflavin, 6.6 mg; pantothenic acid, 24.3 mg; niacin, 88.2 mg; pyridoxine, 3.31 mg; folic acid, 1.10 mg; biotin, 0.33 mg; vitamin B12, 24.8 µg; choline, 669.8 mg; iron from ferrous sulfate, 50.1 mg; copper from copper sulfate, 7.7 mg; manganese from manganese oxide, 125.1 mg; zinc from zinc oxide, 125.1 mg; iodine from ethylene diamine dihydroiodide, 2.10 mg; selenium from sodium selenite, 0.30 mg.

Table 3.2 The targeted concentrations of probiotic microorganisms and the actual concentrations in feed samples collected at the beginning of the experiment

Treatment	Target (cfu/g diet)	Starter (cfu/g diet)	Grower (cfu/g diet)	Finisher (cfu/g diet)
Control	0	0.027×10^6	0.013×10^6	0.006×10^6
Probiotic	1.0×10^6	1.1×10^6	1.3×10^6	0.84×10^6

Table 3.3 The effects of *Basillus subtilis* based probiotic on bone traits of 14-d-old broilers

	Control[2]	Probiotic[2]	SEM	P
Tibia				
BMD (g/cm^2) [1]	0.061	0.069	0.001	0.49
BMC (g) [1]	0.22	0.29	0.007	0.71
Area (cm^2)	3.68	4.12	0.099	0.38
Weight (g)	0.57	0.68	0.009	**0.01**
Relative weight (g/kg)	2.25	2.19	0.014	0.38
Length (mm) [1]	44.88	45.47	0.147	0.10
Width (mm) [1]	3.27	3.71	0.032	0.51
Weight/length index (mg/mm)	12.56	14.97	0.171	**0.009**
Robusticity index (g,cm)	5.45	5.19	0.016	**0.004**
Femur				
BMD (g/cm^2) [1]	0.058	0.064	0.001	0.30
BMC (g) [1]	0.16	0.20	0.005	0.87
Area (cm^2)	2.79	3.10	0.085	0.47
Weight (g)	0.41	0.48	0.007	**0.04**
Relative weight (g/kg)	1.63	1.54	0.010	0.10
Length (mm) [1]	34.33	34.57	0.147	0.15
Width (mm) [1]	3.31	3.81	0.030	0.17
Weight/length index (mg/mm)	11.93	13.95	0.180	**0.03**
Robusticity index (g,cm)	4.64	4.44	0.020	**0.05**

[1]BW was used as a covariate.
[2]The number of observations per least square mean is 12.

Table 3.4 The effects of *Basillus subtilis* based probiotic on bone traits of 28-d-old broilers

	Control[2]	Probiotic[2]	SEM	P
Tibia				
BMD (g/cm^2) [1]	0.150	0.161	0.003	0.33
BMC (g) [1]	1.27	1.33	0.029	0.58
Area (cm^2) [1]	8.40	8.14	0.078	0.29
Weight (g)	3.66	4.01	0.055	0.21
Relative weight (g/kg)	3.25	3.64	0.068	0.26
Length (mm)	70.21	71.50	0.277	0.35
Width (mm)	6.93	7.10	0.061	0.57
Weight/length index (mg/mm)	51.73	56.10	0.686	0.21
Robusticity index (g,cm)	4.59	4.51	0.018	0.35
Femur				
BMD (g/cm^2) [1]	0.129	0.128	0.001	0.90
BMC (g) [1]	0.83	0.82	0.014	0.76
Area (cm^2) [1]	6.39	6.29	0.062	0.55
Weight (g)	2.73	2.94	0.049	0.39
Relative weight (g/kg)	2.43	2.67	0.057	0.40
Length (mm)	54.46	56.25	0.326	0.27
Width (mm)	6.65	7.00	0.059	0.24
Weight/length index (mg/mm)	49.67	52.35	0.767	0.48
Robusticity index (g,cm)	3.94	3.94	0.021	0.94

[1]BW was used as a covariate.
[2]The number of observations per least square mean is 12.

Table 3.5 The effects of *Basillus subtilis* based probiotic on bone traits of 43-d-old broilers

	Control[2]	Probiotic[3]	SEM	P
Tibia				
BMD (g/cm^2)	0.168	0.190	0.001	**0.001**
BMC (g)	2.03	2.78	0.05	**0.002**
Area (cm^2)	12.07	14.57	0.20	**0.006**
Weight (g)	6.52	8.80	0.21	**0.01**
Relative weight (g/kg)	2.92	3.51	0.07	**0.05**
Length (mm)	89.88	94.86	0.56	**0.04**
Width (mm)[1]	8.52	10.36	0.11	**0.02**
Medial thickness (mm)	1.27	1.41	0.04	0.38
Lateral thickness (mm)	1.60	1.86	0.03	**0.05**
Weight/length index (mg/mm)	72.49	92.12	1.89	**0.02**
Robusticity index (g,cm)	4.82	4.63	0.03	0.09
Femur				
BMD (g/cm^2)	0.138	0.157	0.001	**0.002**
BMC (g)	1.27	1.71	0.03	**0.001**
Area (cm^2)	9.16	10.86	0.15	**0.01**
Weight (g)	4.55	6.55	0.16	**0.006**
Relative weight (g/kg)	2.03	2.61	0.06	**0.02**
Length (mm)	68.00	71.71	0.44	**0.05**
Width (mm)	8.21	9.85	0.09	**0.0005**
Medial thickness (mm)	1.27	1.53	0.03	**0.04**
Lateral thickness (mm)	1.50	1.59	0.03	0.45
Weight/length index (mg/mm)	66.91	90.72	1.89	**0.006**
Robusticity index (g,cm)	4.12	3.87	0.03	**0.02**

[1]BW was used as a covariate.
[2]The number of observations per least square mean is 8.
[3]The number of observations per least square mean is 7.

Table 3.6 The effects of *Basillus subtilis* based probiotic on catecholamines, 5-HT, and respective metabolites in the raphe nuclei of 43-d-old broilers

	Control[1]	Probiotic[2]	SEM	P
Catecholamine system				
DA (ng/g)	118.16	111.44	1.79	0.35
NE (ng/g)	1097.12	1123.97	22.82	0.77
EP (ng/g)	182.36	296.88	24.24	0.24
DOPAC (ng/g)	58.54	58.19	1.14	0.94
HVA (ng/g)	154.83	151.14	1.29	0.47
DOPAC/DA	0.50	0.52	0.007	0.42
HVA/DOPAC	1.32	1.37	0.02	0.53
5-HT system				
TRP (ng/g)	5299.45	5423.97	58.68	0.59
5HT (ng/g)	452.60	523.97	8.19	**0.04**
5HIAA (ng/g)	362.31	387.34	8.42	0.46
5HIAA/5HT	0.82	0.74	0.02	0.42

[1]The number of observations per least square mean is 8.
[2]The number of observations per least square mean is 7.

Table 3.7 The effects of *Basillus subtilis* based probiotic on catecholamines, 5-HT, and respective metabolites in the hypothalamus of 43-d-old broilers

	Control[1]	Probiotic[2]	SEM	P
Catecholamine system				
DA (ng/g)	354.70	267.35	8.17	**0.02**
NE (ng/g)	2138.45	1541.95	60.96	**0.03**
EP (ng/g)	362.30	268.76	12.95	0.08
DOPAC (ng/g)	95.55	83.72	1.81	0.11
HVA (ng/g)	233.09	212.51	4.92	0.30
DOPAC/DA	0.27	0.32	0.003	**0.004**
HVA/DOPAC	0.66	0.81	0.009	**0.001**
5-HT system				
TRP (ng/g)	4463.87	4235.27	92.14	0.53
5HT (ng/g)	1055.00	934.25	25.35	0.24
5HIAA (ng/g)	234.43	216.04	4.50	0.31
5HIAA/5HT	0.22	0.24	0.004	0.41

[1]The number of observations per least square mean is 8.
[2]The number of observations per least square mean is 7.

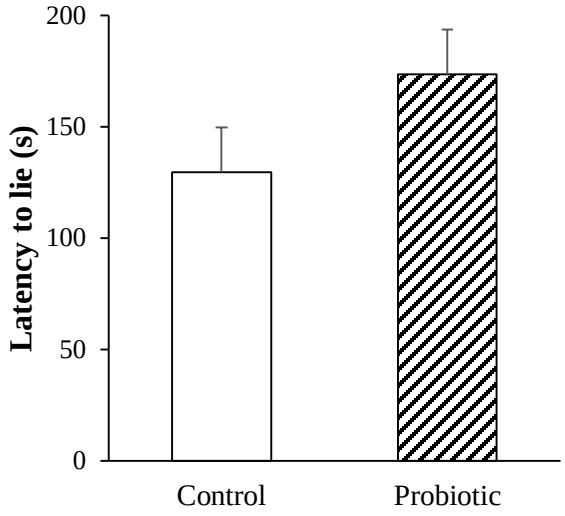
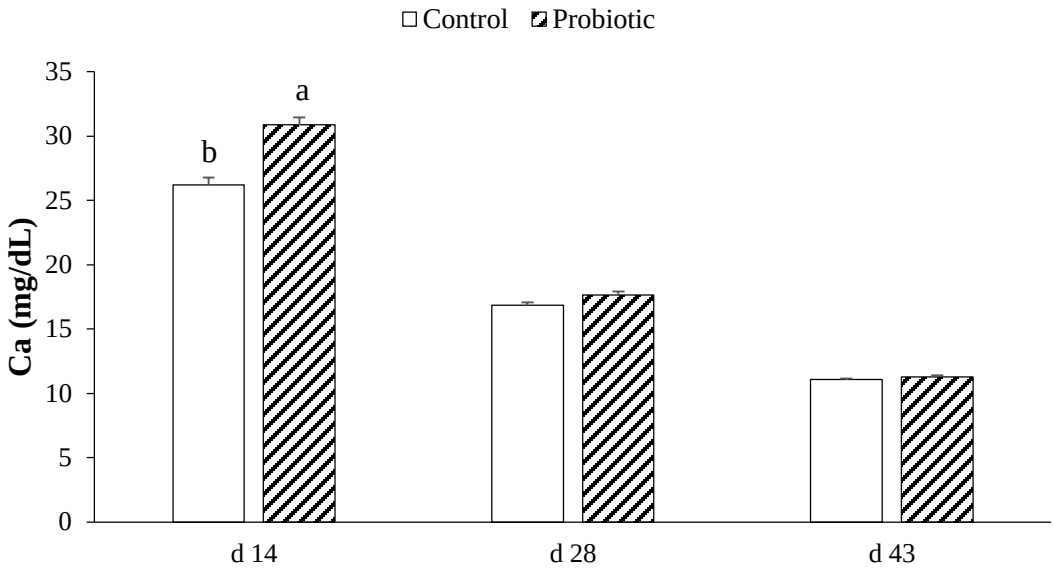


Figure 3.1 The effects of *Basillus subtilis* based probiotic on the latency to lie test in 44-d-old broilers.

The number of observations per least square mean is 16 for control and 14 for probiotic groups

a)



b)

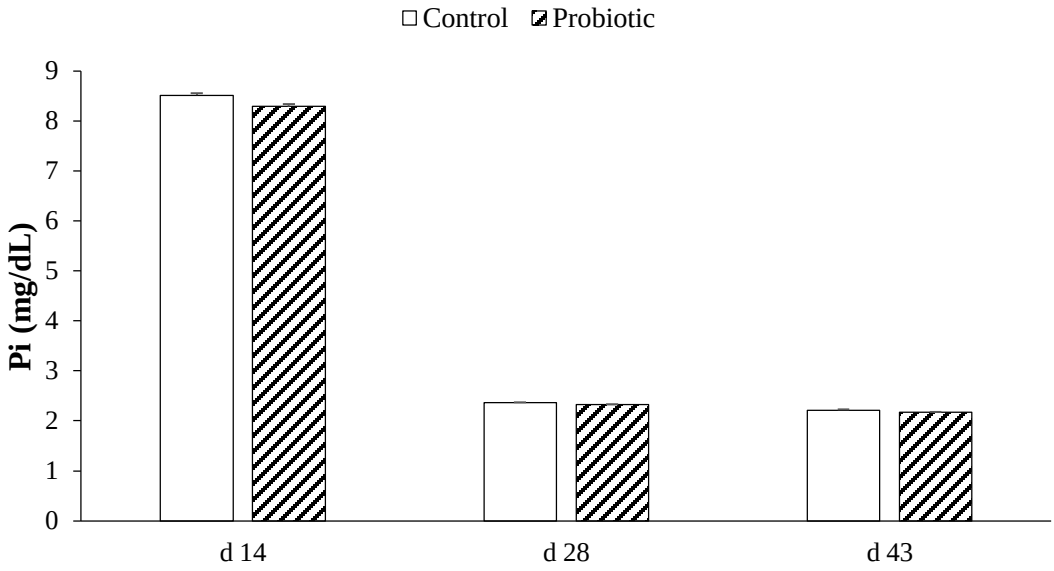


Figure 3.2 The effects of *Basillus subtilis* based probiotic on serum Ca (a) and Pi (b) concentrations in broilers at 14, 28, and 43 d of age

The number of observations per least square mean is 8 for control and 7 for probiotic groups. Significant treatment differences ($P < 0.05$) are indicted by letters (a,b).

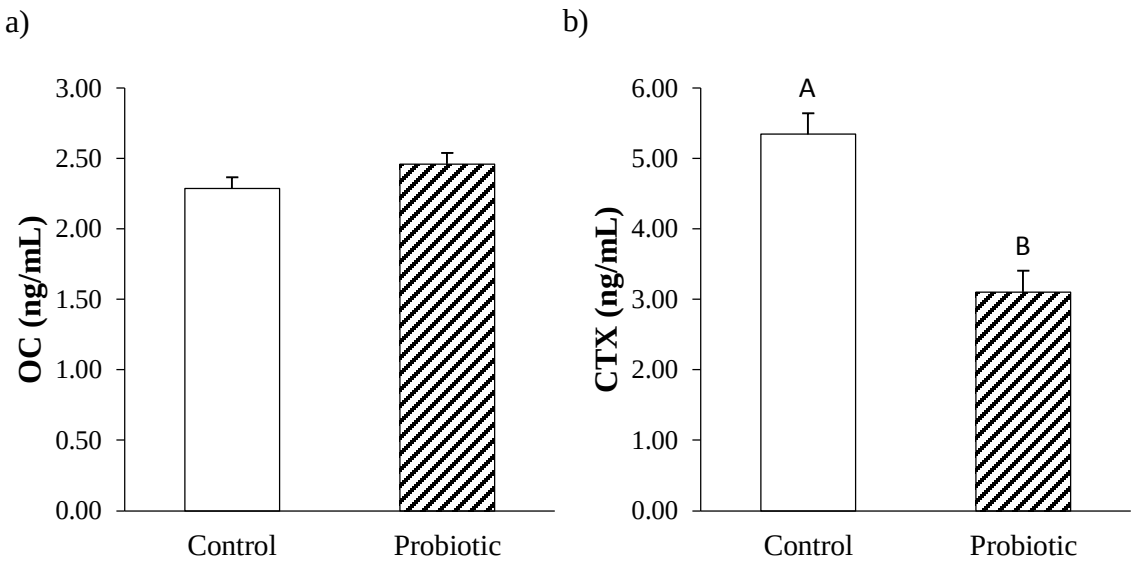


Figure 3.3 The effects of *Basillus subtilis* based probiotic on OC (a) and CTX (b) concentrations in broilers at 43 d of age

The number of observations per least square mean is 8 for control and 7 for probiotic groups. Trend treatment differences ($P < 0.10$) are indicted by letters (A,B).

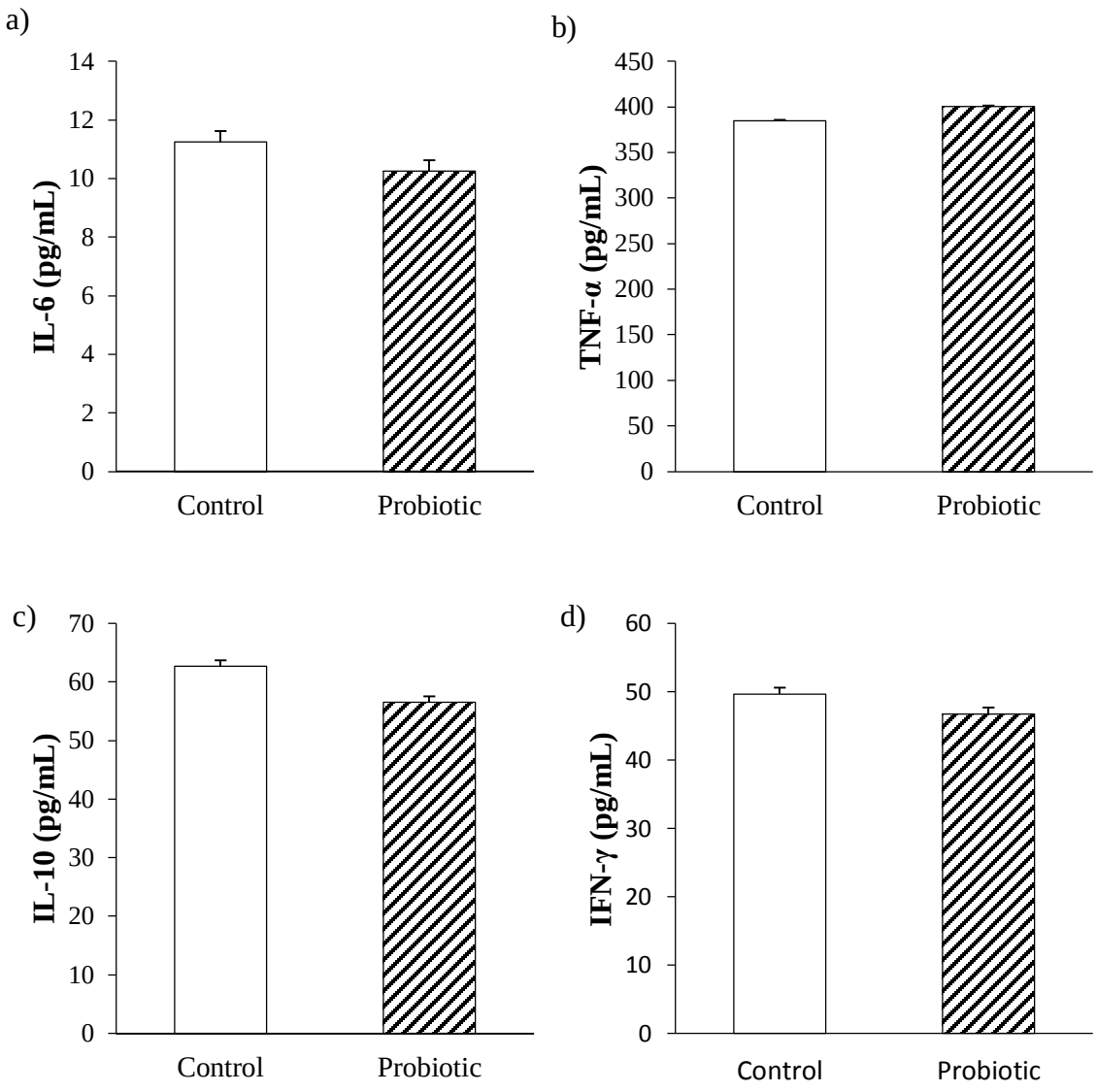


Figure 3.4 The effects of *Basillus subtilis* based probiotic on systemic immune cytokines in broilers at 43 d of age

The number of observations per least square mean is 8 for control and 7 for probiotic groups.

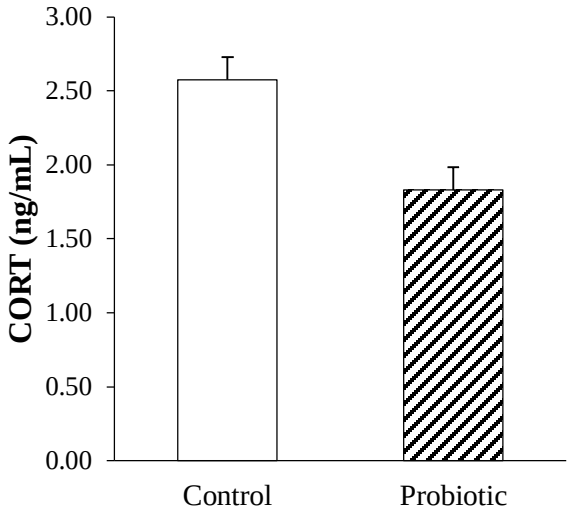


Figure 3.5 The effects of *Basillus subtilis* based probiotic on plasma CORT concentrations in 43-d-old broilers

The number of observations per least square mean is 8 for control and 7 for probiotic groups.

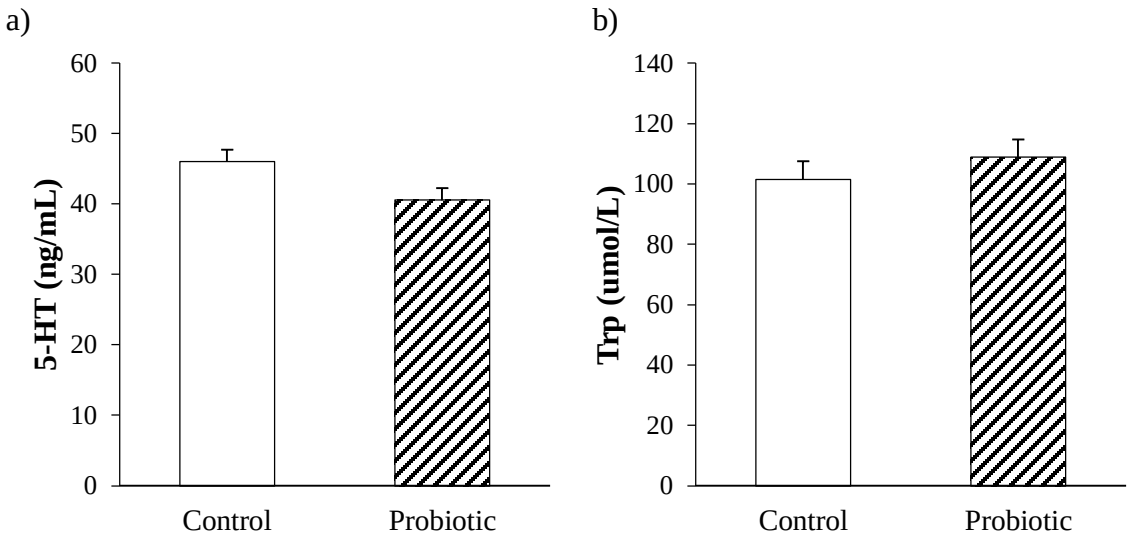


Figure 3.6 The effects of *Basillus subtilis* based probiotic on plasma 5-HT (a) and TRP (b) concentrations in 43-d-old broilers

The number of observations per least square mean is 8 for control and 7 for probiotic groups.

3.7 References

Anderson, G., Kubera, M., Duda, W., Lason, W., Berk, M., and Maes, M. (2013). Increased IL-6 trans-signaling in depression: focus on the tryptophan catabolite pathway, melatonin and neuroprogression. *Pharmacol Rep,* 65(6), 1647-1654.

Aviagen. (2014). *Ross 708 broiler nutrition specifications*. Retrieved from http://en.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross708BroilerNutritionSpecs2014-EN.pdf.

Badruzzaman, M., Bapary, M. A., and Takemura, A. (2013). Possible roles of photoperiod and melatonin in reproductive activity via changes in dopaminergic activity in the brain of a tropical damselfish, Chrysiptera cyanea. *Gen Comp Endocrinol,* 194, 240-247.

Bast, T., Diekamp, B., Thiel, C., Schwarting, R. K., and Gunturkun, O. (2002). Functional aspects of dopamine metabolism in the putative prefrontal cortex analogue and striatum of pigeons (Columba livia). *J Comp Neurol,* 446(1), 58-67.

Battaglino, R., Fu, J., Spate, U., Ersoy, U., Joe, M., Sedaghat, L., and Stashenko, P. (2004). Serotonin regulates osteoclast differentiation through its transporter. *J Bone Miner Res,* 19(9), 1420-1431.

Berg, C. and Sanotra, G. (2003). Can a modified latency-to-lie test be used to validate gait-scoring results in commercial broiler flocks? *Anim Welfare* 12(4), 655-659.

Bonnet, N., Benhamou, C. L., Brunet-Imbault, B., Arlettaz, A., Horcajada, M. N., Richard, O., Vico, L., Collomp, K., and Courteix, D. (2005). Severe bone alterations under beta2 agonist treatments: bone mass, microarchitecture and strength analyses in female rats. *Bone,* 37(5), 622-633.

Bonnet, N., Benhamou, C. L., Beaupied, H., Laroche, N., Vico, L., Dolleans, E., and Courteix, D. (2007). Doping dose of salbutamol and exercise: deleterious effect on cancellous and cortical bones in adult rats. *J Appl Physiol (1985),* 102(4), 1502-1509.

Bonnet, N., Benhamou, C. L., Malaval, L., Goncalves, C., Vico, L., Eder, V., Pichon, C., and Courteix, D. (2008a). Low dose beta-blocker prevents ovariectomy-induced bone loss in rats without affecting heart functions. *J Cell Physiol,* 217(3), 819-827.

Bonnet, N., Pierroz, D. D., and Ferrari, S. L. (2008b). Adrenergic control of bone remodeling and its implications for the treatment of osteoporosis. *J Musculoskelet Neuronal Interact,* 8(2), 94-104.

Chabbi-Achengli, Y., Coudert, A. E., Callebert, J., Geoffroy, V., Cote, F., Collet, C., and de Vernejoul, M. C. (2012). Decreased osteoclastogenesis in serotonin-deficient mice. *Proc Natl Acad Sci U S A,* 109(7), 2567-2572.

Charles, J. F., Ermann, J., and Aliprantis, A. O. (2015). The intestinal microbiome and skeletal fitness: Connecting bugs and bones. *Clin Immunol,* 159(2), 163-169.

Cheng, H. W., Dillworth, G., Singleton, P., Chen, Y., and Muirt, W. M. (2001). Effects of group selection for productivity and longevity on blood concentrations of serotonin, catecholamines, and corticosterone of laying hens. *Poult Sci,* 80(9), 1278-1285.

de Vries, T. J., Yousovich, J., Schoenmaker, T., Scheres, N., and Everts, V. (2016). Tumor necrosis factor-alpha antagonist infliximab inhibits osteoclast formation of peripheral blood mononuclear cells but does not affect periodontal ligament fibroblast-mediated osteoclast formation. *J Periodontal Res,* 51(2):186-195.

Ducy, P. and Karsenty, G. (2010). The two faces of serotonin in bone biology. *J Cell Biol,* 191(1), 7-13.

Duncan, C. P. and Shim, S. S. (1977). J. Edouard Samson Address: the autonomic nerve supply of bone. An experimental study of the intraosseous adrenergic nervi vasorum in the rabbit. *J Bone Joint Surg Br,* 59(3), 323-330.

El-Merahbi, R., Loffler, M., Mayer, A., and Sumara, G. (2015). The roles of peripheral serotonin in metabolic homeostasis. *FEBS Lett,* 589(15), 1728-1734.

Elefteriou, F., Ahn, J. D., Takeda, S., Starbuck, M., Yang, X., Liu, X., Kondo, H., Richards, W. G., Bannon, T. W., Noda, M., Clement, K., Vaisse, C., and Karsenty, G. (2005). Leptin regulation of bone resorption by the sympathetic nervous system and CART. *Nature,* 434(7032), 514-520.

Elefteriou, F., Campbell, P., and Ma, Y. (2014). Control of bone remodeling by the peripheral sympathetic nervous system. *Calcif Tissue Int,* 94(1), 140-151.

Fan, W., Bouwense, S. A., Crawford, R., and Xiao, Y. (2010). Structural and cellular features in metaphyseal and diaphyseal periosteum of osteoporotic rats. *J Mol Histol,* 41(1), 51-60.

FAO/WHO. (2001). *Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria*. Paper presented at the Joint FAO/WHO Expert Consultation.

Fu, L., Patel, M. S., Bradley, A., Wagner, E. F., and Karsenty, G. (2005). The molecular clock mediates leptin-regulated bone formation. *Cell,* 122(5), 803-815.

Fuentes, C., Orozco, L., Vicente, J., Velasco, X., Menconi, A., Kuttappan, V., Kallapura, G., Latorre, J., Layton, S., Hargis, B., and Téllez, G. (2013). Effect of a lactic acid bacterium based probiotic, Floramax-B11Â®, on performance, bone qualities, and morphometric analysis of broiler chickens: an economic analysis. *Biological systems, open access,* 2, 113.

Gebara, M. A., Shea, M. L., Lipsey, K. L., Teitelbaum, S. L., Civitelli, R., Muller, D. J., Reynolds, C. F., 3rd, Mulsant, B. H., and Lenze, E. J. (2014). Depression, antidepressants, and bone health in older adults: a systematic review. *J Am Geriatr Soc,* 62(8), 1434-1441.

Gordeladze, J. O. and Reseland, J. E. (2003). A unified model for the action of leptin on bone turnover. *J Cell Biochem,* 88(4), 706-712.

Hall, L. E., Shirley, R. B., Bakalli, R. I., Aggrey, S. E., Pesti, G. M., and Edwards, H. M., Jr. (2003). Power of two methods for the estimation of bone ash of broilers. *Poult Sci,* 82(3), 414-418.

Henneicke, H., Gasparini, S. J., Brennan-Speranza, T. C., Zhou, H., and Seibel, M. J. (2014). Glucocorticoids and bone: local effects and systemic implications. *Trends Endocrinol Metab,* 25(4), 197-211.

Hernandez, C. J., Guss, J. D., Luna, M., and Goldring, S. R. (2016). Links Between the Microbiome and Bone. *J Bone Miner Res*.

Hester, P. Y., Enneking, S. A., Haley, B. K., Cheng, H. W., Einstein, M. E., and Rubin, D. A. (2013). The effect of perch availability during pullet rearing and egg laying on musculoskeletal health of caged White Leghorn hens. *Poult Sci,* 92(8), 1972-1980.

Heym, J. and Gladfelter, W. E. (1982). Serotonergic innervation of the lateral hypothalamus: evidence from synaptosomal uptake studies. *Brain Res Bull*, 8(2), 131-137.

Houshmand, M., Azhar, K., Zulkifli, I., Bejo, M. H., Meimandipour, A., and Kamyab, A. (2011). Effects of non-antibiotic feed additives on performance, tibial dyschondroplasia incidence and tibia characteristics of broilers fed low-calcium diets. *J Anim Physiol Anim Nutr (Berl)*, 95(3), 351-358.

Kajimura, D., Hinoi, E., Ferron, M., Kode, A., Riley, K. J., Zhou, B., Guo, X. E., and Karsenty, G. (2011). Genetic determination of the cellular basis of the sympathetic regulation of bone mass accrual. *J Exp Med*, 208(4), 841-851.

Kestin, S. C., Gordon, S., Su, G., and Sorensen, P. (2001). Relationships in broiler chickens between lameness, liveweight, growth rate and age. *Vet Rec*, 148(7), 195-197.

Knowles, T. G., Kestin, S. C., Haslam, S. M., Brown, S. N., Green, L. E., Butterworth, A., Pope, S. J., Pfeiffer, D., and Nicol, C. J. (2008). Leg disorders in broiler chickens: prevalence, risk factors and prevention. *PLoS One*, 3(2), e1545.

Kondo, A. and Togari, A. (2003). In vivo stimulation of sympathetic nervous system modulates osteoblastic activity in mouse calvaria. *Am J Physiol Endocrinol Metab*, 285(3), E661-667.

Lee, K. W., Lillehoj, H. S., Jang, S. I., and Lee, S. H. (2014). Effects of salinomycin and Bacillus subtilis on growth performance and immune responses in broiler chickens. *Res Vet Sci*, 97(2), 304-308.

Lerant, A., Herman, M. E., and Freeman, M. E. (1996). Dopaminergic neurons of periventricular and arcuate nuclei of pseudopregnant rats: semicircadian rhythm in Fos-related antigens immunoreactivities and in dopamine concentration. *Endocrinology*, 137(9), 3621-3628.

Mak, W., Shao, X., Dunstan, C. R., Seibel, M. J., and Zhou, H. (2009). Biphasic glucocorticoid-dependent regulation of Wnt expression and its inhibitors in mature osteoblastic cells. *Calcif Tissue Int*, 85(6), 538-545.

Mann, J. J., McBride, P. A., Brown, R. P., Linnoila, M., Leon, A. C., DeMeo, M., Mieczkowski, T., Myers, J. E., and Stanley, M. (1992). Relationship between central and peripheral serotonin indexes in depressed and suicidal psychiatric inpatients. *Arch Gen Psychiatry,* 49(6), 442-446.

Martin, G. F., DeLorenzo, G., Ho, R. H., Humbertson, A. O., Jr., and Waltzer, R. (1985). Serotonergic innervation of the forebrain in the North American opossum. *Brain Behav Evol,* 26(3-4), 196-228.

McCabe, L., Britton, R. A., and Parameswaran, N. (2015). Prebiotic and Probiotic Regulation of Bone Health: Role of the Intestine and its Microbiome. *Curr Osteoporos Rep,* 13(6), 363-371.

McCabe, L. R., Irwin, R., Schaefer, L., and Britton, R. A. (2013). Probiotic use decreases intestinal inflammation and increases bone density in healthy male but not female mice. *J Cell Physiol,* 228(8), 1793-1798.

Meiser, J., Weindl, D., and Hiller, K. (2013). Complexity of dopamine metabolism. *Cell Commun Signal,* 11(1), 34.

Mutus, R., Kocabagli, N., Alp, M., Acar, N., Eren, M., and Gezen, S. S. (2006). The effect of dietary probiotic supplementation on tibial bone characteristics and strength in broilers. *Poult Sci,* 85(9), 1621-1625.

Niedermair, T., Kuhn, V., Doranehgard, F., Stange, R., Wieskotter, B., Beckmann, J., Salmen, P., Springorum, H. R., Straub, R. H., Zimmer, A., Grifka, J., and Grassel, S. (2014). Absence of substance P and the sympathetic nervous system impact on bone structure and chondrocyte differentiation in an adult model of endochondral ossification. *Matrix Biol,* 38, 22-35.

Ohlsson, C., Engdahl, C., Fak, F., Andersson, A., Windahl, S. H., Farman, H. H., Moverare-Skrtic, S., Islander, U., and Sjogren, K. (2014). Probiotics protect mice from ovariectomy-induced cortical bone loss. *PLoS One,* 9(3), e92368.

Ohlsson, C. and Sjogren, K. (2015). Effects of the gut microbiota on bone mass. *Trends Endocrinol Metab,* 26(2), 69-74.

Panda, A. K., Rao, S. V. R., Raju, M. V., and Sharma, S. R. (2006). Dietary supplementation of Lactobacillus sporogenes on performance and serum biochemico-lipid profile of broiler chickens. *J Poult Sci,* 43(3), 235-240.

Pappalardo, A. and Thompson, K. (2013). Activated gammadelta T cells inhibit osteoclast differentiation and resorptive activity in vitro. *Clin Exp Immunol,* 174(2), 281-291.

Parvaneh, K., Jamaluddin, R., Karimi, G., and Erfani, R. (2014). Effect of probiotics supplementation on bone mineral content and bone mass density. *ScientificWorldJournal,* 2014, 595962.

Quarta, D. and Smolders, I. (2014). Rewarding, reinforcing and incentive salient events involve orexigenic hypothalamic neuropeptides regulating mesolimbic dopaminergic neurotransmission. *Eur J Pharm Sci,* 57, 2-10.

Quinteiro-Filho, W. M., Ribeiro, A., Ferraz-de-Paula, V., Pinheiro, M. L., Sakai, M., Sa, L. R., Ferreira, A. J., and Palermo-Neto, J. (2010). Heat stress impairs performance parameters, induces intestinal injury, and decreases macrophage activity in broiler chickens. *Poult Sci,* 89(9), 1905-1914.

Riesenfeld, A. (1972). Metatarsal robusticity in bipedal rats. *Am J Phys Anthropol,* 36(2), 229-233.

Sadeghi, A. A. (2014). Bone Mineralization of Broiler Chicks Challenged with Salmonella enteritidis Fed Diet Containing Probiotic (Bacillus subtilis). *Probiotics Antimicrob Proteins,* 6(3-4), 136-140.

Sanotra, G. S., Lund, J. D., Ersboll, A. K., Petersen, J. S., and Vestergaard, K. S. (2001). Monitoring leg problems in broilers: a survey of commercial broiler production in Denmark. *World Poultry Sci J* 57(1), 55-69.

Sanotra, G. S., Berg, C., and Lund, J. D. (2003). A comparison between leg problems in Danish and Swedish broiler production. *Anim Welfare* 12(4), 677-683.

Saylor, R. A., Reid, E. A., and Lunte, S. M. (2015). Microchip electrophoresis with electrochemical detection for the determination of analytes in the dopamine metabolic pathway. *Electrophoresis,* 36(16), 1912-1919.

Scholz-Ahrens, K. E., Ade, P., Marten, B., Weber, P., Timm, W., Acil, Y., Gluer, C. C., and Schrezenmeir, J. (2007). Prebiotics, probiotics, and synbiotics affect mineral absorption, bone mineral content, and bone structure. *J Nutr,* 137(3 Suppl 2), 838S-846S.

Seedor, J. G., Quartuccio, H. A., and Thompson, D. D. (1991). The bisphosphonate alendronate (MK-217) inhibits bone loss due to ovariectomy in rats. *J Bone Miner Res,* 6(4), 339-346.

Shim, M. Y., Karnuah, A. B., Mitchell, A. D., Anthony, N. B., Pesti, G. M., and Aggrey, S. E. (2012). The effects of growth rate on leg morphology and tibia breaking strength, mineral density, mineral content, and bone ash in broilers. *Poult Sci,* 91(8), 1790-1795.

Sjogren, K., Engdahl, C., Henning, P., Lerner, U. H., Tremaroli, V., Lagerquist, M. K., Backhed, F., and Ohlsson, C. (2012). The gut microbiota regulates bone mass in mice. *J Bone Miner Res,* 27(6), 1357-1367.

Steel, R. G., Torrie, J. H., and Dickey, D. A. (1997). *Principles and Procedures of Statistics: A Biometrical Approach. McGraw Hill Book Co., New York, NY.*

Takayanagi, H., Ogasawara, K., Hida, S., Chiba, T., Murata, S., Sato, K., Takaoka, A., Yokochi, T., Oda, H., Tanaka, K., Nakamura, K., and Taniguchi, T. (2000). T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-gamma. *Nature,* 408(6812), 600-605.

Talaty, P. N., Katanbaf, M. N., and Hester, P. Y. (2009). Variability in bone mineralization among purebred lines of meat-type chickens. *Poult Sci,* 88(9), 1963-1974.

Tian, Y., Eaton, M. J., Goudreau, J. L., Lookingland, K. J., and Moore, K. E. (1993). Neurochemical evidence that 5-hydroxytryptaminergic neurons tonically inhibit noradrenergic neurons terminating in the hypothalamus. *Brain Res,* 607(1-2), 215-221.

Tork, I. (1990). Anatomy of the serotonergic system. *Ann N Y Acad Sci,* 600, 9-34; discussion 34-35.

Waititu, S. M., Yitbarek, A., Matini, E., Echeverry, H., Kiarie, E., Rodriguez-Lecompte, J. C., and Nyachoti, C. M. (2014). Effect of supplementing direct-fed microbials on broiler performance, nutrient digestibilities, and immune responses. *Poult Sci,* 93(3), 625-635.

Wang, J., Yu, L., Jiang, C., Fu, X., Liu, X., Wang, M., Ou, C., Cui, X., Zhou, C., and Wang, J. (2015). Cerebral ischemia increases bone marrow CD4+CD25+FoxP3+ regulatory T cells in mice via signals from sympathetic nervous system. *Brain Behav Immun,* 43, 172-183.

Weaver, C. M. (2015). Diet, gut microbiome, and bone health. *Curr Osteoporos Rep,* 13(2), 125-130.

Weeks, C. A., Knowles, T. G., Gordon, R. G., Kerr, A. E., Peyton, S. T., and Tilbrook, N. T. (2002). New method for objectively assessing lameness in broiler chickens. *Vet Rec,* 151(25), 762-764.

Wideman, R. F., Hamal, K. R., Stark, J. M., Blankenship, J., Lester, H., Mitchell, K. N., Lorenzoni, G., and Pevzner, I. (2012). A wire-flooring model for inducing lameness in broilers: Evaluation of probiotics as a prophylactic treatment. *Poult Sci,* 91(4), 870-883.

Williams, B., Waddington, D., Murray, D. H., and Farquharson, C. (2004). Bone strength during growth: influence of growth rate on cortical porosity and mineralization. *Calcif Tissue Int,* 74(3), 236-245.

Xing, Y., Wang, S., Fan, J., Oso, A. O., Kim, S. W., Xiao, D., Yang, T., Liu, G., Jiang, G., Li, Z., Li, L., and Zhang, B. (2015). Effects of dietary supplementation with lysine-yielding Bacillus subtilis on gut morphology, cecal microflora, and intestinal immune response of Linwu ducks. *J Anim Sci,* 93(7), 3449-3457.

Yadav, V. K., Ryu, J. H., Suda, N., Tanaka, K. F., Gingrich, J. A., Schutz, G., Glorieux, F. H., Chiang, C. Y., Zajac, J. D., Insogna, K. L., Mann, J. J., Hen, R., Ducy, P., and Karsenty, G. (2008). Lrp5 controls bone formation by inhibiting serotonin synthesis in the duodenum. *Cell,* 135(5), 825-837.

Yadav, V. K., Oury, F., Suda, N., Liu, Z. W., Gao, X. B., Confavreux, C., Klemenhagen, K. C., Tanaka, K. F., Gingrich, J. A., Guo, X. E., Tecott, L. H., Mann, J. J., Hen, R., Horvath, T. L., and Karsenty, G. (2009). A serotonin-dependent mechanism explains the leptin regulation of bone mass, appetite, and energy expenditure. *Cell,* 138(5), 976-989.

Yokota, K., Sato, K., Miyazaki, T., Kitaura, H., Kayama, H., Miyoshi, F., Araki, Y., Akiyama, Y., Takeda, K., and Mimura, T. (2014). Combination of tumor necrosis factor alpha and interleukin-6 induces mouse osteoclast-like cells with bone resorption activity both in vitro and in vivo. *Arthritis Rheumatol,* 66(1), 121-129.

Zhang, Q., Chen, B., Yan, F., Guo, J., Zhu, X., Ma, S., and Yang, W. (2014). Interleukin-10 inhibits bone resorption: a potential therapeutic strategy in periodontitis and other bone loss diseases. *Biomed Res Int,* 2014, 284836.

Zhou, H., Mak, W., Zheng, Y., Dunstan, C. R., and Seibel, M. J. (2008). Osteoblasts directly control lineage commitment of mesenchymal progenitor cells through Wnt signaling. *J Biol Chem,* 283(4), 1936-1945.

Ziaie, H., Bashtani, M., Torshizi, M. A. K., Naeeimipour, H., Farhangfar, H., and Zeinali, A. (2011). Effect of antibiotic and its alternatives on morphometric characteristics, mineral content and bone strength of tibia in Ross broiler chickens. *Glob Vet,* 7(4), 315-322.

CHAPTER 4. EFFECTS OF A *BACILLUS SUBTILIS* BASED PROBIOTIC ON BONE HEALTH IN BROILERS SUBJECTED TO CYCLIC HEATING EPISODES

4.1 Abstract

Heat stress as an environmental stressor causes abnormalities and architectural deterioration of bone tissue in animals including chickens. The objective of this study was to determine whether dietary supplementation of *Bacillus subtilis* can improve bone health in heat stressed broilers. One hundred and twenty 1-d-old Ross 708 broilers were assigned to 24 floor pens based on their BW. The pens were evenly divided into 2 groups (n=12). One group was fed a regular diet and the other group was fed the diet mixed with a commercial probiotic (250 ppm, 1 x 10^6 cfu/g of feed; consisting of 3 types of *Bacillus subtilis*) for 6 wk. Room temperature was gradually decreased from 35° C on d 1 by 0.55° C /d for the first 14 d. When broilers were 15 d of age, ambient temperature was increased from 28 to 32° C for 10 h (0700h to 1700h). These cycling heating episodes were imposed on the broilers daily until termination of the study at 44 d of age. Compared with the control fed broilers, probiotic supplementation increased BMC, weight, size, bone weight to length index, and reduced robusticity index in both the tibia and femur ($P < 0.05$) of 43-d-old broilers but had no effect on BMD or cortical thickness. The increase in bone size traits were observed in broilers subjected to elevated temperatures at 43 d of age and not at 28 d of age. Serum concentrations of Ca and Pi were not affected by probiotic, whereas the concentrations of serum CTX were reduced by the probiotic supplementation ($P = 0.02$). Pro-inflammatory TNF-α was decreased ($P = 0.003$) with no changes in plasma IL-6, IL-10, IFN-γ, and CORT concentrations as a result of including probiotic into the diet of broilers subjected to a cyclic heating episode. Moreover, both peripheral 5-HT and central 5-HT and catecholamines (NE, EP and DA) as well as their metabolites were not affected by probiotic ($P > 0.05$). These results indicate that dietary supplementation of *Bacillus subtilis* based probiotic probiotic increased bone growth and bone size of broilers under cyclic heating episodes via inhibition of bone resorption resulting from down-regulation of circulating pro-inflammatory TNF-α concentrations. Dietary probiotic supplementation may be a

management strategy for increasing skeletal growth in broilers under conditions of elevated ambient temperatures.

4.2 Introduction

Poultry is one of the most popular animals used as food sources world-wide. Global poultry meat production has increased remarkably over the last 40 years surpassing beef and veal production beginning in 1995 (Windhorst, 2006; Scanes, 2007; USDA-FAS, 2016). Another notable change is the rapid increase in broiler production in developing regions, especially in South America and Asia, which includes many tropical regions of the world (Windhorst, 2006). High ambient temperature, especially combined with high humidity, imposes severe stress on broilers, due to their limited ability to regulate heat loss by feathering and their rapid growth with heavy BW at market age (Geraert et al., 1996). Subsequently, reduced growth, carcass quality, and even death not only induce economic loss for poultry producers but also lead to welfare concerns for chickens (St-Pierre et al., 2003; Lara and Rostagno, 2013).

Leg disorders are common in broilers that cause chronic pain and lameness (Danbury et al., 2000; Caplen et al., 2013a; Caplen et al., 2013b). The situation is even worse when birds suffer from heat stress. Reduced bone mass, such as ash content and bone volume, occurs in both broilers and turkeys exposed to high temperatures (Jankowski et al., 2015; Hosseini-Vashan et al., 2016). Heat stress-associated mechanisms are still under investigation, but Ca bioavailability, hormones, and immunity are all directly impacted.

Heat stress induced behavioral changes include decreased feeding, increased water consumption, panting, and wing spreading (Syafwan et al., 2012; Mack et al., 2013). Whereas a depressed appetite decreases metabolic heat production, it also reduces the availability of nutrients for intestinal absorption like amino acids and Ca required for bone and body growth. Panting is the major method used by birds to dissipate excess heat through evaporation of moisture in the upper respiratory tract, but excessive rapid breathing causes respiratory alkalosis and upsets electrolyte balance. For example, in laying hens, acute heat stress caused an increase in blood pH due to exhalation of excess carbon dioxide. It was also noted that lactate and pyruvate increased and ionized Ca

decreased in the blood as the hens were experiencing respiratory alkalosis. Although excretion of Ca in urine or through heat-stressed induced diarrhea may be involved as well, the reduced concentrations of circulating ionized Ca could be due to the binding of Ca to these organic acids reducing the pool of freely available ionized Ca needed for shell formation (Odom et al., 1986) and skeletal health. In addition, chronic heat stress in broilers induces intestinal injury such as reduced height of villi, thinner gut mucosa, and reduced alkaline phosphatase activity (Hu et al., 2016) that can hamper Ca intestinal absorption. Therefore, a decrease in the bioavailability of circulating Ca concentrations may be a contributing factor towards reducing bone mineralization, strength, ash, and other indicators of skeletal health in heat stressed meat-type fowl (Jankowski et al., 2015; Hosseini-Vashan et al., 2016).

Heat stress induces over activation of the HPA axis and elevates blood CORT concentrations (Garriga et al., 2006; Star et al., 2008; Quinteiro-Filho et al., 2010, 2012; Manhiani et al., 2011). Excessive CORT negatively affects bone mass through inhibiting osteoblastogenesis, increasing osteoblast and osteocyte apoptosis, and promoting osteoclast survival (O'Brien et al., 2004; Jia et al., 2006; Rauch et al., 2010). In addition, many studies have demonstrated that heat stress induces immunosuppression in broilers (Bartlett and Smith, 2003; Niu et al., 2009; Jahanian and Rasouli, 2015) including mucosal immunity of the intestines (Hu et al., 2016), accompanied by the changes of cytokines concentrations such as increased spleen levels of TNF-α and IL-4 but decreased levels of IFN-γ and IL-2 (Xu et al., 2014). The pro-inflammatory cytokine of TNF-α that is locally produced in bone induces bone resorption by directly enhancing osteoclastic activity (Schett, 2011), or an indirect mode of action may be through downregulation of OPG or other bone metabolites that reduce bone resorption (Boyce et al., 2005). As a check and balance system and in contrast to TNF-α, the release of IFN-γ by mesenchymal stem cells and cells of immune origin in the bone microenvironment promotes bone formation in mice (Duque et al., 2011) and are exceptionally strong inhibitors of osteoclast differentiation (Schett, 2011). Considering the vital role of cytokines on bone cells (Inada and Miyaura, 2010; Schett, 2011), an upset in bone homeostasis may be caused by changes in immune cytokines under heat stress.

As discussed in chapter 3 of this dissertation, serotonin and the sympathetic nervous system may contribute to probiotic-based improvement of bone (Charles et al., 2015; mccabe et al., 2015). Specifically, our results showed that the inclusion of a dietary *Bacillus subtilis* based probiotic promoted bone mass in broilers raised under a standard management regimen. The mechanism of action was most likely through increased intestinal Ca absorption and a reduction in bone resorption, perhaps mediated by 5-HT induced reduction of sympathetic activity. Similar bone promoting results were also reported using other types of probiotics (Mutus et al., 2006; Panda et al., 2006; Houshmand et al., 2011; Ziaie et al., 2011; Fuentes et al., 2013; Sadeghi, 2014). However, little information is available regarding the effects of probiotic supplementation on bone health of broilers subjected to elevated ambient temperatures. The objective of the present study was to investigate the effects of a *Bacillus subtilis* based probiotic on bone health of broilers subjected to cycling high environmental temperatures. We hypothesized that probiotic supplementation would protect bone mass in broilers under elevated temperatures by increasing mineral bioavailability and the synthesis of brain 5-HT in the raphe nuclei, and diminishing the release of pro-inflammatory immune cytokines and the adrenal stress hormone of CORT.

4.3 Materials and Methods

4.3.1 Birds, Management, and Sample Collection

A total of one hundred and twenty d-old Ross 708 male broiler chicks were obtained from a commercial hatchery (Miller Poultry, Orland, IN). Chicks were weighed and placed into 24 floor pens (243 × 51 cm) ensuring similar average BW across pens. There were 5 chicks per pen resulting in a stocking density of 2,478.6 cm^2/broiler. The litter source was wood shavings and each pen was equipped with 1 hanging feeder and drinker. Room temperature was gradually decreased from 35° C on d 1 by 0.55° C /d until 15 d of age at which time ambient temperature was increased from 28 to 32° C for 10 h (0700h to 1700h). The cyclic increase in ambient temperature to 32° C was done daily by turning on a furnace until the end of the experiment at 44 d of age. The study was conducted in the summer months of June and July. The furnace was turned off at

1700 h allowing ambient temperatures to return to normal during evening and early morning hours. Data loggers (HOBO®, Onset Computer Corporation, MA) were used in recording of the room temperature and humidity (Table 4.1).

The lighting program was gradually decreased from 23 light:1 dark (0100-0200h) at 30 lux up to the first 7 d of age, then 20 light:4 dark (0100-0500h) at 10 lux until 44 d of age. Pens were assigned to 2 dietary treatments of 12 replicate floor pens per treatment: regular diets and the diets mixed with 250 ppm probiotic (Sporulin®, Pacific Vet Group-USA, Inc., Fayetteville, AR). The probiotic consisted of 3 strains of *Bacillus subtilis* resulting in 1.0×10^6 spores/g of feed. The level of probiotic was recommended by the company, and the regular diets were formulated using the recommendations for nutrients by Aviagen (2014). Birds were fed a starter, grower, or finisher diet from 1 to 14, 15 to 28, and 29 to 44 d of age, respectively (Table 4.2). Feed and water were provided *ad libitum*. Prior to the experiment, all the diets were prepared and sampled for bacterial analysis to ensure the diets were mixed properly. The husbandry and the following procedures were approved by the Purdue Animal Use and Care Committee (Number: 1111000262).

At 14 and 28 d of age starting from 0900h, 1 bird per pen was weighted then sedated using intravenous administration of sodium pentobarbital (30 mg/kg of BW) followed by blood collection via cardiac puncture. Sampling began 2 h into the heating episode for 28-d and 43-d old broilers. A total of 8 ml blood was collected from each bird; 5 ml were placed into ice cooled EDTA-coated plasma tube and 3 ml were placed into a serum tube. The bird was euthanized immediately after bleeding by cervical dislocation. At dissection, the left tibia and femur were removed from the chicken and placed in individual plastic bags and kept at -20° C until assayed. After the sample collection at 28 d of age, 24 broilers (16 from probiotic and 8 from control groups) were culled due to mal-development (unknown reason) and the remaining broilers were randomly regrouped within each treatment to ensure same group size of 3. The subsequent replicate was 6 for probiotic and 9 for control groups during finisher period. At 43 d of age, samples of plasma, serum, and bones were collected from 1 bird per pen as previously described. The hypothalamus and raphe nuclei were additionally collected and immediately frozen on dry ice and stored at -80° C until assayed.

The 2 broilers remaining in each pen at the end of the study were used to perform the latency to lie test at 44 d of age following the procedure described previously (Berg and Sanotra, 2003). Briefly, each chicken was individually placed into a tub previously filled with 3 cm water at 28° C. The time for chicken to sit down and touch the water was recorded. If the chicken flied or still stood after 600s the test was interrupted. The latency to lie test was conducted between 1000 and 1600 h when broilers were subjected to an elevated temperature. The broilers were removed from their pens one at a time and taken to the tub which was located in an adjacent room where normal ambient temperature was maintained.

4.3.2 Bone traits

Tibia and femur were measured for BMD, BMC, and bone area using DEXA (Norland Medical Systems Inc., Fort Atkinson, WI) following a previously described procedure (Hester et al., 2013). The entire bone was scanned. The area of the scanned bone was determined and expressed as cm^2. The BMD was calculated as BMC (measured in g) divided by the area of the bone and expressed as g/cm^2. After scanning, all the bones were broiled for 5 min and then meat, connective tissue and the fibula bone were completely removed (Hall et al., 2003). The bones were air dried overnight at room temperature and determined for weight, length, width, as well as cortical bone thickness (only determined in 43-d old broilers) using a digital micrometer (Coolant Proof Micrometer Series 293, Mitutoyo America Corp., Aurora, IL). Traditional bone density indicators, bone weight to length index and robusticity index, were also calculated (Riesenfeld, 1972; Seedor et al., 1991). Higher bone density was indicated by higher weight to length index but lower robusticity index.

$$Robusticity\ index = \frac{bone\ length}{\sqrt[3]{bone\ weight}}$$

$$Weight\ to\ length\ index = \frac{bone\ weight}{bone\ length}$$

4.3.3 ELISA

Commercial ELISA kits (Mybiosource, San Diego, CA) were used for detecting serum concentrations of OC, a bone formation indicator, and CTX, a bone resorption

indicator. The serum Ca and Pi concentrations were determined using quantichrom kits (Bioassay Systems, Hayward, CA) following manufacturer's instructions.

Plasma concentrations of 5-HT, its precursor TRP, and cytokines, IL-6, IL-10, TNF-α, and IFN-γ were measured using commercial ELISA kits (Mybiosource, San Diego, CA).

4.3.4 HPLC

Metabolites of the hypothalamus and raphe nuclei from the left hemisphere of the brain were analyzed using HPLC (UltiMate™ 3000 RSLCnano System, Thermo Fisher Scientific Inc., Waltham, MA). The brain regions were weighed and homogenized with ice-cold 0.2 M perchloric acid, at a 10:1 ratio (for µl of perchloric acid:mg of sample). The homogenized mixture was centrifuged at 18,187g for 15 min at 4° C. The resultant supernatant was drawn into a microcentrifuge tube and diluted 1:1 with mobile phase. The mixture was then centrifuged again at 18,187g for 10 min at 4° C. The supernatant was filtered through a 0.2-µm polyvinylidene fluoride filter into a HPLC sample vial. The mobile phase flow rate was 0.8 mL/min. The concentrations of NE, EP, DOPAC, DA, 5-HIAA, HVA, 5-HT, and TRP were calculated from a reference curve made using relative standards.

4.3.5 RIA

Plasma concentrations of CORT were measured using a commercial ^{125}I CORT RIA kit (MP Biomedicals, Orangeburg, NY) following the method described previously (Cheng et al., 2001).

4.3.6 Statistical analysis

A one-way ANOVA of the mixed model procedure of SAS 9.4 software (SAS Institute Inc., Cary, NC) was used to analyze all the data. The fixed factor was the probiotic treatment. Transformation of data was performed for normality when variances were not homogeneous (Steel et al., 1997). Statistical trends were similar for both transformed and untransformed data; therefore, the untransformed least square means were presented. The Tukey-Kramer test was used to partition differences among means. Statistical significance was set at $P < 0.05$.

4.4 Results

The concentrations of probiotic microorganisms in the probiotic-supplemented diets at the start and end of the experiment were similar to the targeted levels (Table 4.3).

Broilers of the current study were demonstrating signs of distress during the heating episodes as indicated by panting, wing spreading, and squatting close to the ground (Wang et al., unpublished data).

Broiler pen averaged BW (Wang et al., unpublished data) was improved by probiotic inclusion at 43 d of age (Control vs Probiotic: 1921 vs 2177 g, P = 0.0001), but not at 14 (Control vs Probiotic: 232 vs 219 g, P = 0.34) and 28 d of age (Control vs Probiotic: 918 vs 748 g, P = 0.01). After culling and regrouping at 28 d of age, BW between the 2 groups still differed (Control vs Probiotic: 975 vs 933 g, P = 0.03).

Bone traits of 14-d-old broilers 1 d before implementing the cyclic heating episode showed early benefits due to the dietary inclusion of the probiotic probiotic. In particular, the BMC of the tibia (P = 0.03) and femur (P = 0.04) increased as did tibial area (P = 0.04) and weight (P = 0.05, Table 4.4). However, 13 d after initiating the cyclic heating episode, any probiotic induced increases in bone traits observed prior to increasing ambient temperature had dissipated. More specifically, all bone traits in 28-d-old broilers were similar between the probiotic and control fed broilers (Table 4.5). After 28 d of elevated cycling temperatures, the probiotic induced increase in bone traits once again became evident in 43-d-old broilers. Both the tibia and femur of broilers responded similarly to the probiotics under an elevated temperature; in particular, traits associated with size, such as BMC, area, weight, and the weight/length index, all increased when compared to control-fed broilers also subjected to high ambient temperature. However, BMD and cortical thickness of bones were unaffected by probiotic feeding in 43-d-old broilers (Table 4.6). Latency to lie in 44-d-old broilers was similar between the probiotic fed and the control broilers (P = 0.85; Figure 4.1).

Serum concentrations of Ca and Pi at 14 (normal ambient temperature), 28, and 43 d of age (Figure 4.2) and OC at 43 d of age (Figure 4.3a) were similar between the probiotic fed and control broilers. Probiotic supplementation reduced serum CTX concentrations in 43-d-old broilers exposed to elevated temperature (P = 0.02, Figure 4.3b).

Peripheral plasma concentrations of 5-HT (Figure 4.4a, P = 0.50) and its precursor, TRP (Figure 4.4b, P = 0.93), were not affected by probiotic supplementation in 43-d-old broilers subjected to elevated temperatures. Similarly, concentrations of 5-HT, its precursor TRP, and metabolite 5-HIAA, as well as the catecholamines (NE, EP, and DA) and their metabolites (DOPAC and HVA) were not affected by probiotic supplementation in both the raphe nuclei and hypothalamus (Tables 4.7 and 4.8, respectively). Under conditions of elevated temperatures, plasma pro-inflammatory cytokine concentrations of TNF-α were decreased in 43-d-old broilers supplemented with probiotic (Fig. 4.5b, P = 0.003) with no effect on plasma concentrations of IL-6 (Figure 4.5a, P = 0.22), IL-10 (Figure 4.5c, P = 0.31), IFN-γ (Figure 4.5d, P = 0.62) and CORT (P = 0.42; Figure 4.6) as compared to control broilers.

4.5 Discussion

Probiotic supplementation improved bone mass in poultry (Mutus et al., 2006; Panda et al., 2006; Houshmand et al., 2011; Ziaie et al., 2011; Abdelqader et al., 2013; Fuentes et al., 2013; Sadeghi, 2014) and rodents (Rodrigues et al., 2012; Foureaux Rde et al., 2014; Zhang et al., 2015; Li et al., 2016). Under conditions of an elevated cycling temperature, our results showed that dietary inclusion of a probiotic increased the size and weight of the bones in 43-d-old broilers, but did not improve BMD or cortical thickness of bones (Table 4.4) as it did in Yan et al. (2016) using the same probiotic probiotic at an identical dosage. The strain and age of the broilers were the same between the current study and that of Yan et al. (2016) as well as the management of the chickens except for room temperature. Apparently, the elevated cycling temperature of the current study impaired the effectiveness of the probiotic in its ability to absorb intestinal Ca and deposit it along with P into bone to increase BMD. The latency to lie test also suggests that probiotic under conditions of elevated temperatures did not improve the leg health of broilers. The broilers subjected to elevated temperature may have sat down in the water more quickly than normal to cool themselves making it more difficult to show treatment effects due to diet. Gait score was not accessed in the current study, but lameness was reduced in wire-reared broilers fed probiotics (Wideman et al., 2012).

The increase in bone growth and bone size traits of broilers fed a probiotic as compared to controls was most likely due to increased nutrient absorption as a result of improved intestinal integrity, as bone traits were not influenced by increased intake of nutrients because feed consumption was not affected by probiotics (Wang et al., 2016). Although the current study was not able to compare the bone traits of broilers in the heated with the control environment due to the lack of replication, it has been reported by others that the inclusion of various probiotics ameliorates the negative effect of heat on gut health in broilers (Sohail et al., 2012; Song et al., 2014) and laying hens (Deng et al., 2012).

Calcium is a nutrient of interest that was most likely made more bioavailable in the probiotic fed broilers because of increased intestinal absorption. Other nutrients, such as P and amino acids used to build hydroxyapatite and the osteoid matrix of bone, respectively, also likely benefited from enhanced intestinal absorption due to probiotic feeding. If increased intestinal absorption of Ca and P occurred as a result of feeding probiotics to broilers, it was not reflected in the concentrations measured in the sera, which were similar between dietary treatments (Fig. 2). Calcium circulating in the blood is either unbound as an ionized salt or complexed to anions such as bicarbonate, phosphate, pyruvate, citrate, or lactate or to proteins like albumin. About 40% of the total Ca is in bound form. When circulating ionized Ca is utilized by tissues causing a drop in circulating concentrations, the Ca bound to protein is released to replenish the ionized Ca so as to maintain consistent concentrations. The protein-bound Ca in circulation is physiologically inactive because it cannot cross capillary membranes. It is the ionized pool of Ca that is available for use by animal tissues. Although Ca along with P are major constituents of hydroxyapatite, playing important roles in bone remodeling, Ca is also critical for other biological functions, such as blood clotting, muscular contraction, and release of synaptic neurotransmitters. Therefore, a possibility for the lack in difference in serum ionized Ca between the probiotic and control dietary treatments is that the concentrations in circulation are tightly regulated by Ca regulating hormones such as parathyroid hormone, calcitonin, and 1,25-dihydroxycholecalciferol (Stanford, 2006; de Matos, 2008). Even though heat stress reduces Ca and P bioavailability because of damaged intestinal integrity (Quinteiro-Filho et al., 2012; Santos et al., 2015),

concentrations in circulation may either remained constant or quickly stabilized so as to return to homeostasis if heat stress was not too severe. To support the concept of hormones tightly regulating blood Ca concentrations, no changes in circulating Ca concentrations were reported in laying hens acclimated to high temperature as compared to those subjected to a control ambient temperatures (Samara et al., 1996). Concentrations of serum Ca measured in broilers of the current study under elevated temperatures were normal as concentrations were similar to broilers raised under control temperatures that were sampled 2 h earlier (see Fig. 2 of Chapter 3).

The lack of difference in serum Ca concentrations between the probiotic fed and control broilers at 28 and 42 d of age under conditions of elevated temperatures could also be due to the fact that the broilers were not panting excessively and as a result, they were not experiencing respiratory alkalosis. In the study of Odom et al. (1986), laying hens were exposed to acute rather than chronic heat stress at a much higher temperature of 35° C than the 32° C used in the current study. A drop in ionized Ca occurred during acute heat stress as blood pH increased, proving that the hens were in a state of respiratory alkalosis. Total or bound Ca was not measured, but because lactate and pyruvate increased during respiratory alkalosis, the authors hypothesized that the bound Ca was most likely increasing in these acutely heat stressed laying hens (Odom et al., 1986).

Osteoblasts synthesize and release OC to facilitate bone building and mineralization. Another role for OC is to assist in maintaining Ca homeostasis along with the classical Ca regulating hormones described previously. Similar concentrations of serum OC between dietary treatment groups under conditions of cycling elevated temperature (Figure 4.3a) may have contributed to maintaining concentrations of circulating Ca in probiotic fed broilers similar to controls.

Besides increased availability of nutrients needed for bone formation, other pathways, such as reduced bone resorption, may also be involved in stimulating bone growth and bone size traits in probiotic fed broilers under conditions of elevated temperatures. Serum CTX is often used as a biomarker to evaluate bone turnover in humans because circulating concentrations of CTX are positively correlated to osteoclastic activity. The lower concentration of serum CTX as compared to controls

(Figure 4.3b, P =0.02) indicates that bone resorption was reduced in 43 d-old broilers consuming probiotic as compared to control fed chickens under conditions of elevated temperatures which may have facilitated bone growth, but did not result in bone with denser minerals (see BMD values for the tibia and femur at 43 d of age, Table 4.4).

The effect of 5-HT on bone is dependent on its source. Serotonin is synthesized in the brain as well as peripherally. Brain serotonin, acting as a neurotransmitter, stimulates bone formation and inhibits bone resorption causing an increase in bone mass, whereas peripheral serotonin, acting as a hormone, has the opposite effect resulting in inhibition of bone formation (Yadav et al., 2009; Yadav et al., 2010). Results with broilers consuming the same probiotic without heat stress provided evidence suggesting that reduced bone resorption was perhaps mediated by 5-HT induced reduction of sympathetic activity (see Chapter 3). More specifically, dietary probiotic up-regulated the synthesis of 5-HT in the raphe nuclei of the brain of 43-d-old broilers. The serotonin was then perhaps released in the terminal areas of the hypothalamus leading to decreased NE synthesis. The reduced sympathetic outflow in turn could possibly contribute to reduced bone resorption. However, this proposed mechanism was not upheld under conditions of cycling heating episodes as there was no effect of probiotic on both peripheral 5-HT and its precursor of TRP and central 5-HT and catecholamines (NE, EP and DA) as well as their metabolites or ratios. Perhaps if the serotonin response had been elicited in the probiotic fed broilers subjected to a cyclic heating episode, the BMD and cortical thickness of bone would have responded favorably as it did in the study of Yan et al. (2016).

Although both 5-HT and DA are involved in the regulation of behaviors such as locomotion, eating, and drinking (Fuller, 1984; Muller et al., 2003; King, 2006), the similar concentrations between treatments measured in the serum and brain suggest that these metabolites did not play an important role in the modified behavior noted in the probiotic fed broilers. The behavior of broilers consuming probiotic as compared to control fed chickens during periods of elevated temperature showed reduced drinking, sleeping, and sitting but increased feeding and standing ($P < 0.05$, unpublished data). The increase in feeding behavior, however, did not affect feed intake as inclusion of probiotic had no effect on feed consumption (Wang et al., 2016). Serotonin is known as an appetite

suppressant (Voigt and Fink, 2015), but because peripheral and brain 5-HT concentrations were similar between treatment groups under conditions of elevated temperature, similar feed intake levels would be expected (Wang et al., 2016).

The immune system and bone health are tightly linked (Criscitiello et al., 2015). Osteoblasts are derived from pluripotent mesenchymal stromal cells, whereas osteoclasts are derived from hematopoietic stem cell that also generate immune cells (Ohlsson and Sjogren, 2015). The RANKL is the direct regulator of osteoclast formation and bone turnover; which is expressed by the mesenchymal originated cells inside bones as well as T and B cells upon activation (Guerrini and Takayanagi, 2014). The binding of RANKL to its receptor RANK on osteoclast precursor cells activates the intracellular signaling cascades that leads to osteoclastogenesis (Kanazawa and Kudo, 2005). The RANKL/RANK axis is regulated by a variety of cytokines. For instance, IFN-γ, the main Th1 cytokine, functions to inhibit osteoclastogenesis (Takayanagi et al., 2000; Pappalardo and Thompson, 2013). IL-10 also shows the function to inhibit osteoclastic bone resorption and regulate osteoblastic bone formation (Zhang et al., 2014; Fujioka et al., 2015). In contrast, some cytokines support osteoclast formation, including the pro-inflammatory cytokines such as IL-6 (Axmann et al., 2009; Yokota et al., 2014) and TNF-α (Kitaura et al., 2013; de Vries et al., 2016).

Heat stress has been shown to suppresses immunity (Bartlett and Smith, 2003; Niu et al., 2009; Jahanian and Rasouli, 2015), including a rapid change of circulating cytokines. For instance, a study reported increased TNF-α and IL-4 concentrations but decreased IFN-γ and IL-2 concentrations in the spleen of chickens exposed to cycling heat for 16 h daily (4 h of 23.9 to 37° C, 8 h of 37° C, and 4 h of 37 to 23.9° C) for 4 wk (Xu et al., 2014). Elevated expressions of TNF-like, IFN-γ, and IL-1β genes occurred in chicken hepatocytes incubated at 40° C in vitro (Oskoueian et al., 2014). Our results showed that probiotic supplementation as compared to control fed broilers reduced the plasma concentrations of TNF-α but not IL-6, IL-10, and IFN-γ in broilers under heat conditions. The lowered concentrations of TNF-α may in turn decrease osteoclast formation, which agrees with the finding of reduced serum concentrations of CTX, a bone resorption indicator, in the current study. These lowered concentrations of plasma TNF-α may have facilitated greater bone growth and size in probiotic fed 43-d-old

broilers as compared to control-fed chickens. In line with our results, reduced pro-inflammatory cytokine TNF-α concentrations or gene expression were also considered as the main cause of improved bone mass in GF mice or mice fed probiotic supplementation (Sjogren et al., 2012; mccabe et al., 2013). In addition, probiotics, such as *Bacillus licheniformis*, reduce heat stress-induced elevation of serum concentrations TNF-α (Deng et al., 2012). Serum concentrations of CORT were also reduced by probiotic *Bacillus licheniformis* in the study of Deng et al. (2012). However, similar CORT concentrations occurred in broilers with and without probiotic supplementation subjected to an elevated cycling temperature in the current study suggesting that the probiotic may not regulate bone traits through adrenal glucocorticoids, but through down-regulation of the circulating pro-inflammatory TNF-α cytokine.

4.6 Conclusions

Dietary supplementation of *Bacillus subtilis* based probiotic caused larger bones under cycling elevated temperatures, but did not improve BMD or cortical thickness. Reduced circulating concentrations of the pro-inflammatory cytokine of TNF-α may have facilitated bone growth in broilers consuming the probiotic under conditions of elevated temperatures. Dietary probiotic supplementation may be a management strategy for increasing skeletal growth in broilers under heat stress.

Table 4.1 Ambient temperature and humidity recorded from d 15 to 44

Age	Temperature (° C)		Relative Humidity (%)	
	0700 to 1700 h	1700 to 0700 h	0700 to 1700 h	1700 to 0700 h
d 15-28	31.9 ± 0.6	26.4 ± 0.7	52.2 ± 1.7	55.0 ± 1.8
d 29-44	32.1 ± 0.5	26.7 ± 0.8	56.8 ± 1.4	59.2 ± 1.7

Values represent the least square means ± SEM.

Table 4.2 The ration formulation

	Starter	Grower	Finisher
Ingredient, %			
Corn	52	52.3	62.8
Soybean meal, 48 % crude protein	40	39.1	29.7
Soybean oil	3.59	4.97	4.11
Sodium chloride	0.51	0.46	0.43
DL Methionine	0.3	0.24	0.23
L-Lysine HCl	0.13	- - -	0.07
Threonine	0.06	- - -	- - -
Limestone	1.29	1.15	1.12
Monocalcium phoshate	1.75	1.48	1.17
Vitamin/mineral premix[1]	0.35	0.35	0.35
Calculated analyses			
Crude protein %	23.4	22.8	19.2
ME kcal/kg	3050	3151	3200
Ca %	0.95	0.85	0.75
Available P %	0.5	0.44	0.36
Methionine %	0.66	0.59	0.53
Methionine + cystine %	1.04	0.97	0.86
Lysine %	1.42	1.29	1.09
Threonine %	0.97	0.89	0.74
Na %	0.22	0.20	0.19

[1]Provided per kilogram of diet: vitamin A, 13,233 IU; vitamin D3, 6,636 IU; vitamin E, 44.1 IU; vitamin K, 4.5 mg; thiamine, 2.21 mg; riboflavin, 6.6 mg; pantothenic acid, 24.3 mg; niacin, 88.2 mg; pyridoxine, 3.31 mg; folic acid, 1.10 mg; biotin, 0.33 mg; vitamin B12, 24.8 µg; choline, 669.8 mg; iron from ferrous sulfate, 50.1 mg; copper from copper sulfate, 7.7 mg; manganese from manganese oxide, 125.1 mg; zinc from zinc oxide, 125.1 mg; iodine from ethylene diamine dihydroiodide, 2.10 mg; selenium from sodium selenite, 0.30 mg.

Table 4.3 The targeted concentrations of probiotic microorganisms and the actual concentrations in feed samples collected at the beginning of the experiment

Treatment	Target (cfu/g diet)	Starter (cfu/g diet)	Grower (cfu/g diet)	Finisher (cfu/g diet)
Control	0	0.027×10^6	0.013×10^6	0.006×10^6
Probiotic	1.0×10^6	1.1×10^6	1.3×10^6	0.84×10^6

Table 4.4 The effects of *Basillus subtilis* based probiotic on bone traits of 14-d-old broilers

	Control[1]	Probiotic[1]	SEM	P
Tibia				
BMD (g/cm^2)	0.060	0.066	0.001	0.07
BMC (g)	0.25	0.32	0.006	**0.03**
Area (cm^2)	4.07	4.73	0.06	**0.04**
Weight (g)	0.55	0.65	0.01	**0.05**
Relative weight (g/kg)	2.24	2.23	0.02	0.90
Length (mm)	43.00	44.63	0.19	0.10
Width (mm)	3.38	3.59	0.03	0.21
Weight/length index (mg/mm)	12.71	14.42	0.18	0.06
Robusticity index (g,cm)	5.29	5.19	0.02	0.26
Femur				
BMD (g/cm^2)	0.055	0.060	0.001	0.08
BMC (g)	0.17	0.22	0.004	**0.04**
Area (cm^2)	3.12	3.61	0.05	0.06
Weight (g)	0.39	0.46	0.01	0.09
Relative weight (g/kg)	1.58	1.58	0.01	0.93
Length (mm)	33.21	34.17	0.14	0.18
Width (mm)	3.40	3.67	0.03	0.12
Weight/length index (mg/mm)	11.71	13.41	0.18	0.07
Robusticity index (g,cm)	4.58	4.45	0.01	0.08

[1]The number of observations per least square mean is 12.

Table 4.5 The effects of *Basillus subtilis* based probiotic on bone traits of 28-d-old broilers subjected to daily cycling heating episodes

	Control[1]	Probiotic[1]	SEM	P
Tibia				
BMD (g/cm^2)	0.141	0.133	0.002	0.43
BMC (g)	1.17	1.03	0.02	0.25
Area (cm^2)	8.12	7.67	0.07	0.23
Weight (g)	3.41	2.99	0.08	0.30
Relative weight (g/kg)	3.13	3.10	0.06	0.90
Length (mm)	69.92	69.17	0.31	0.63
Width (mm)	6.77	6.56	0.06	0.52
Weight/length index (mg/mm)	48.26	43.07	0.95	0.28
Robusticity index (g,cm)	4.71	4.83	0.02	0.27
Femur				
BMD (g/cm^2)	0.127	0.120	0.001	0.32
BMC (g)	0.79	0.71	0.01	0.26
Area (cm^2)	6.18	5.88	0.06	0.28
Weight (g)	2.39	2.19	0.04	0.33
Relative weight (g/kg)	2.24	2.26	0.04	0.88
Length (mm)	53.92	52.42	0.19	0.11
Width (mm)	6.48	6.25	0.05	0.37
Weight/length index (mg/mm)	44.12	41.69	0.65	0.45
Robusticity index (g,cm)	4.06	4.07	0.02	0.89

[1]The number of observations per least square mean is 12.

Table 4.6 The effects of *Basillus subtilis* based probiotic on bone traits of 43-d-old broilers subjected to daily cycling heating episodes

	Control[1]	Probiotic[2]	SEM	P
Tibia				
BMD (g/cm^2)	0.167	0.176	0.001	0.074
BMC (g)	2.05	2.44	0.02	**0.001**
Area (cm^2)	12.23	13.91	0.12	**0.003**
Weight (g)	6.50	9.04	0.22	**0.01**
Relative weight (g/kg)	3.37	4.09	0.09	0.07
Length (mm)	92.11	93.50	0.44	0.44
Width (mm)	8.17	9.31	0.11	**0.02**
Medial thickness (mm)	1.07	1.14	0.01	0.15
Lateral thickness (mm)	1.61	1.60	0.02	0.91
Weight/length index (mg/mm)	70.29	96.87	2.37	**0.01**
Robusticity index (g,cm)	5.00	4.52	0.04	**0.02**
Femur				
BMD (g/cm^2)	0.142	0.145	0.001	0.56
BMC (g)	1.33	1.55	0.02	**0.01**
Area (cm^2)	9.38	10.75	0.11	**0.01**
Weight (g)	5.42	7.45	0.22	**0.03**
Relative weight (g/kg)	2.80	3.38	0.09	0.13
Length (mm)	69.33	71.33	0.37	0.20
Width (mm)	8.51	9.19	0.09	0.07
Medial thickness (mm)	1.41	1.27	0.03	0.25
Lateral thickness (mm)	1.41	1.50	0.02	0.38
Weight/length index (mg/mm)	77.85	103.74	2.66	**0.03**
Robusticity index (g,cm)	3.99	3.68	0.03	**0.03**

[1]The number of observations per least square mean is 9.
[2]The number of observations per least square mean is 6.

Table 4.7 The effects of *Basillus subtilis* based probiotic on catecholamines, 5-HT, and respective metabolites in the raphe nuclei of 43-d-old broilers subjected to daily cycling heating episodes

	Control[1]	Probiotic[2]	SEM	P
Catecholamine system				
DA (ng/g)	106.69	103.08	2.00	0.79
NE (ng/g)	921.93	946.96	23.34	0.83
EP (ng/g)	156.46	161.41	5.71	0.66
DOPAC (ng/g)	52.32	53.29	0.71	0.74
HVA (ng/g)	156.39	155.82	2.40	0.95
DOPAC/DA	0.49	0.53	0.01	0.3
HVA/DOPAC	1.48	1.56	0.04	0.64
5-HT system				
TRP (ng/g)	5753.77	5887.75	141.30	0.81
5HT (ng/g)	472.39	448.69	10.97	0.6
5HIAA (ng/g)	376.39	411.81	11.69	0.46
5HIAA/5HT	0.84	0.95	0.04	0.51

[1]The number of observations per least square mean is 9.
[2]The number of observations per least square mean is 6.

Table 4.8 The effects of *Basillus subtilis* based probiotic on catecholamines, 5-HT, and respective metabolites in the hypothalamus of 43-d-old broilers subjected to daily cycling heating episodes

	Control[1]	Probiotic[2]	SEM	P
Catecholamine system				
DA (ng/g)	301.77	295.84	6.76	0.37
NE (ng/g)	1673.75	1780.36	28.71	0.11
EP (ng/g)	273.22	322.92	7.39	0.83
DOPAC (ng/g)	88.25	97.36	1.31	0.1
HVA (ng/g)	235.71	268.94	4.27	0.07
DOPAC/DA	0.30	0.33	0.01	0.23
HVA/DOPAC	0.80	0.92	0.02	0.19
5-HT system				
TRP (ng/g)	5041.90	5609.00	107.84	0.21
5HT (ng/g)	966.84	954.74	13.43	0.82
5HIAA (ng/g)	275.33	328.64	7.21	0.08
5HIAA/5HT	0.28	0.35	0.01	0.07

[1]The number of observations per least square mean is 9.
[2]The number of observations per least square mean is 6.

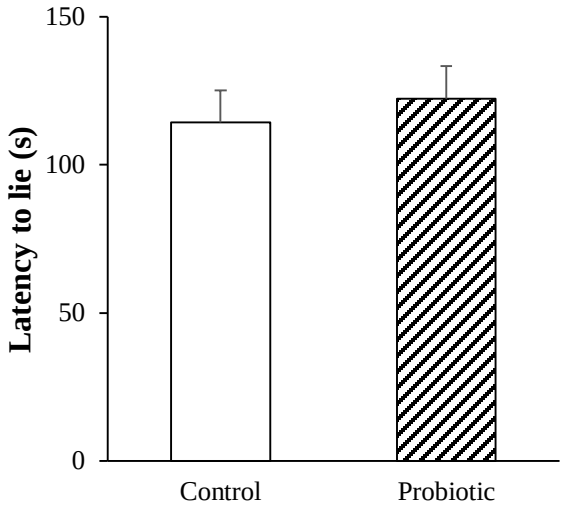


Figure 4.1 The effects of *Basillus subtilis* based probiotic on the latency to lie test in 44-d-old broilers subjected to daily cycling heating episodes

The number of observations per least square mean is 18 for control and 12 for probiotic groups.

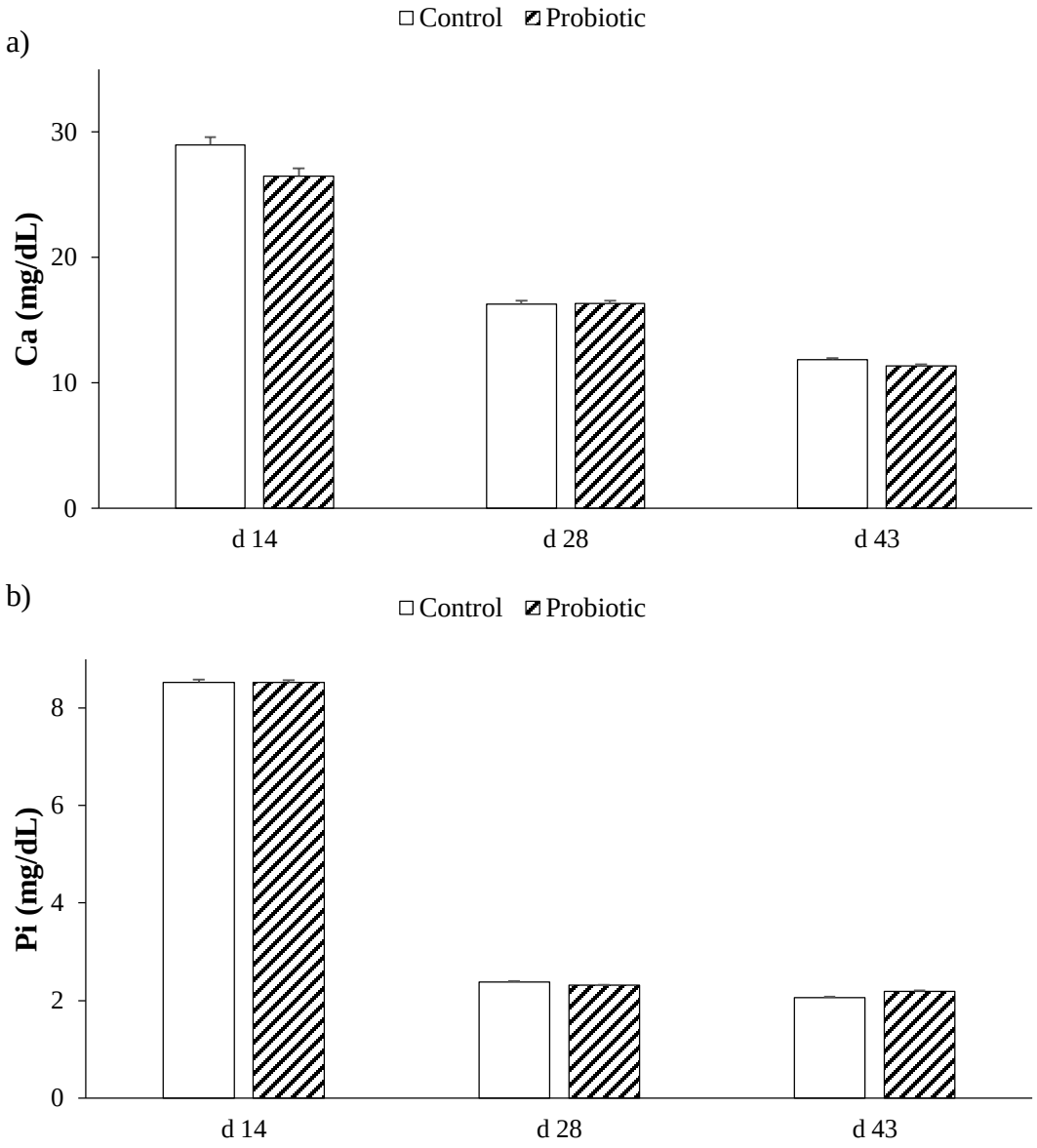


Figure 4.2 The effects of *Basillus subtilis* based probiotic on serum Ca (a) and Pi (b) concentrations in broilers at 14, 28, and 43 d of age

Broilers subjected to daily cycling heating episodes during 14 to 43 d of age. The number of observations per least square mean is 9 for control and 6 for probiotic groups.

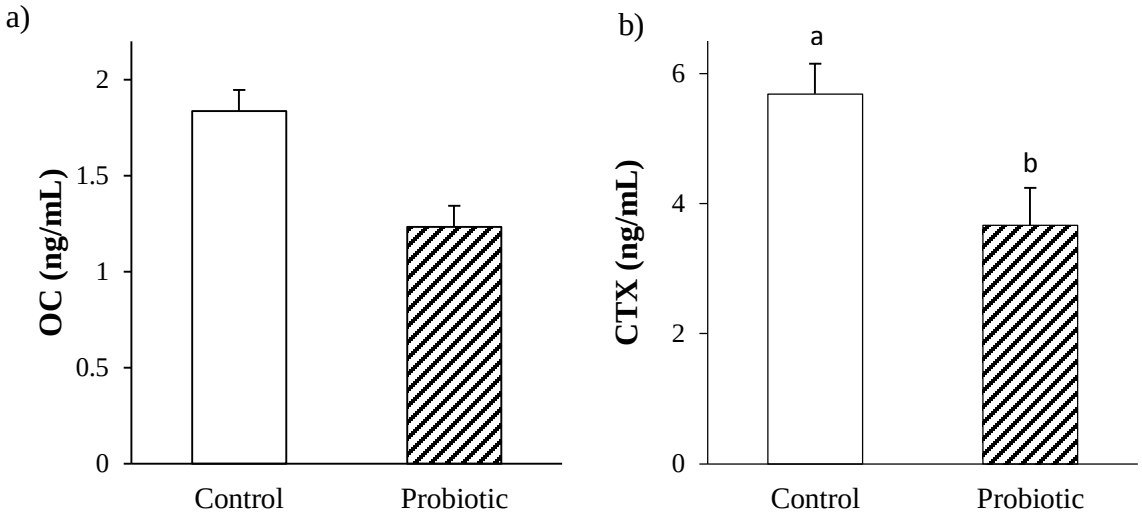


Figure 4.3 The effects of *Basillus subtilis* based probiotic on OC (a) and CTX (b) concentrations in 43-d-old broilers subjected to daily cycling heating episodes

The number of observations per least square mean is 9 for control and 6 for probiotic groups. Significant treatment differences ($P < 0.05$) are indicted by letters (a,b).

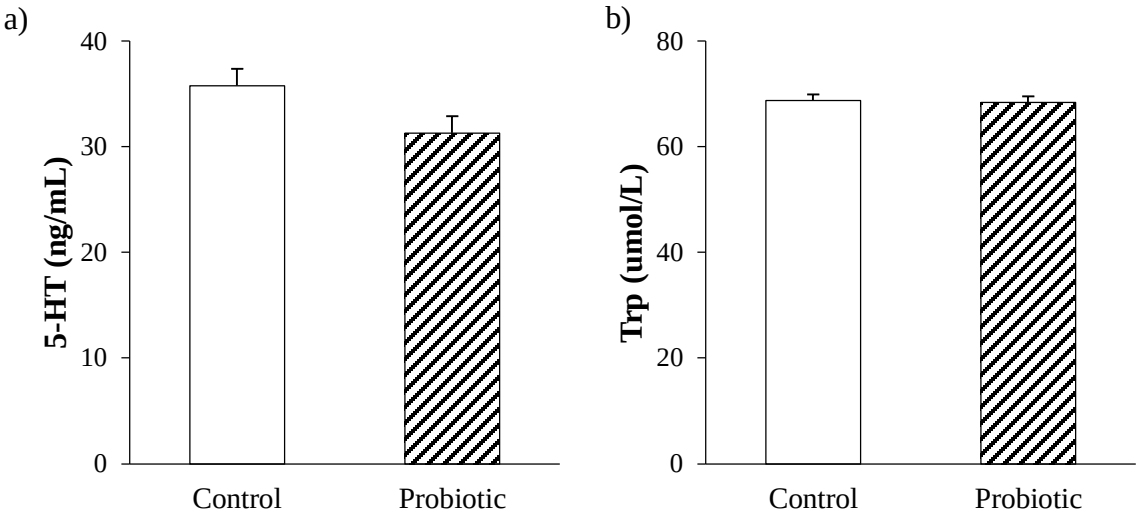


Figure 4.4 The effects of *Basillus subtilis* based probiotic on plasma 5-HT (a) and TRP (b) concentrations in 43-d-old broilers subjected to daily cycling heating episodes

The number of observations per least square mean is 9 for control and 6 for probiotic groups.

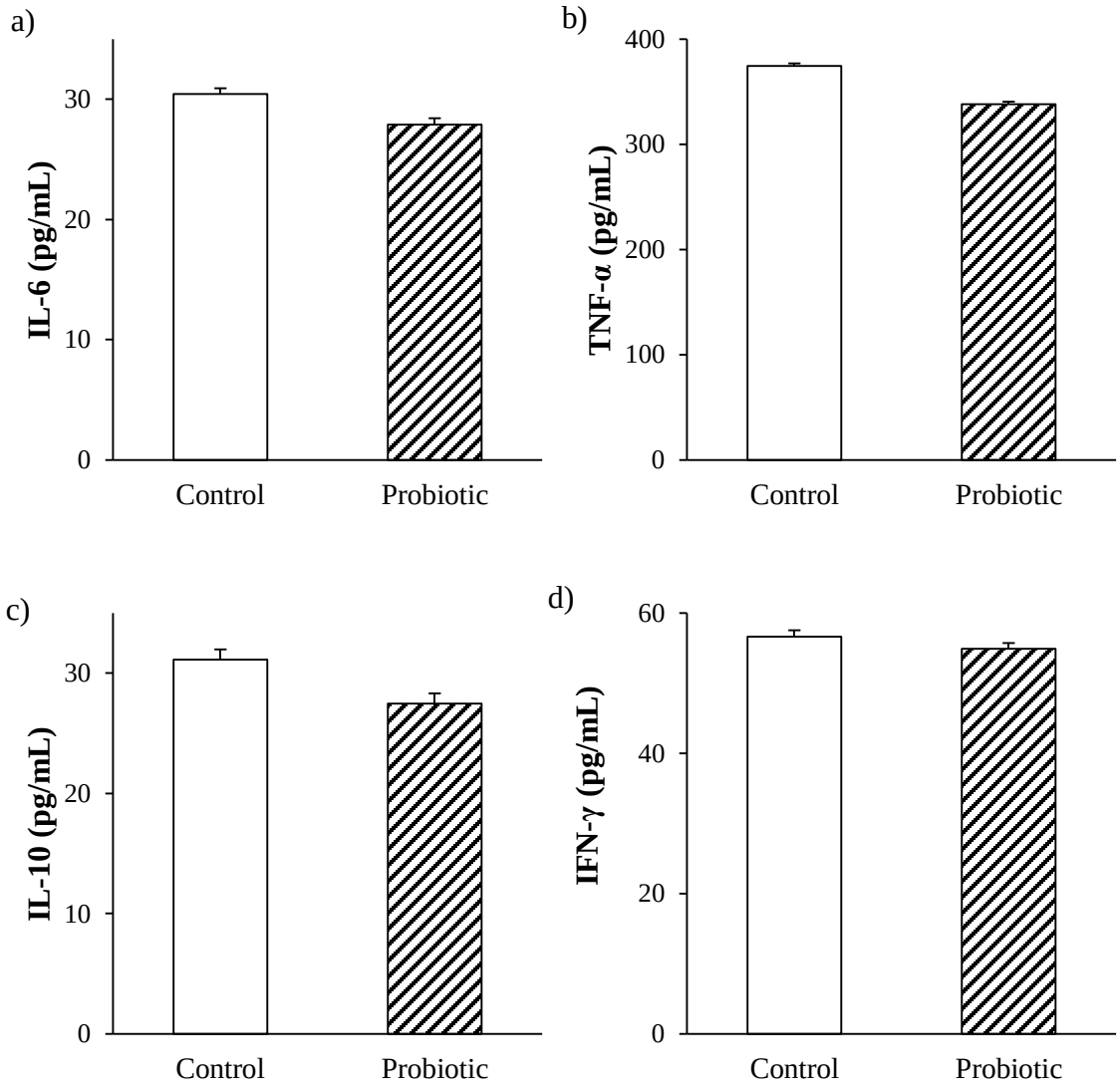


Figure 4.5 The effects of *Basillus subtilis* based probiotic on systemic immune cytokines in 43-d-old broilers subjected to daily cycling heating episodes

The number of observations per least square mean is 9 for control and 6 for probiotic groups. Significant treatment differences ($P < 0.05$) are indicted by letters (a,b).

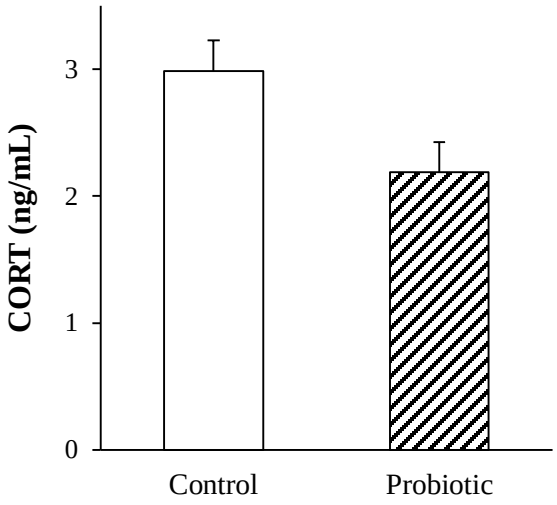


Figure 4.6 The effects of *Basillus subtilis* based probiotic on plasma CORT concentrations in 43-d-old broilers subjected to daily cycling heating episodes

The number of observations per least square mean is 9 for control and 6 for probiotic groups.

4.7 References

Abdelqader, A., Irshaid, R., and Al-Fataftah, A. R. (2013). Effects of dietary probiotic inclusion on performance, eggshell quality, cecal microflora composition, and tibia traits of laying hens in the late phase of production. *Trop Anim Health Prod, 45*(4), 1017-1024.

Aviagen. (2014). *Ross 708 broiler nutrition specifications*. Retrieved from http://en.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross708BroilerNutritionSpecs2014-EN.pdf.

Axmann, R., Bohm, C., Kronke, G., Zwerina, J., Smolen, J., and Schett, G. (2009). Inhibition of interleukin-6 receptor directly blocks osteoclast formation in vitro and in vivo. *Arthritis And Rheumatism, 60*(9), 2747-2756.

Bartlett, J. R., and Smith, M. O. (2003). Effects of different levels of zinc on the performance and immunocompetence of broilers under heat stress. *Poult Sci, 82*(10), 1580-1588.

Berg, C., and Sanotra, G. (2003). Can a modified latency-to-lie test be used to validate gait-scoring results in commercial broiler flocks? *Anim Welfare 12*(4), 655-659.

Boyce, B. F., Li, P., Yao, Z., Zhang, Q., Badell, I. R., Schwarz, E. M., O'Keefe, R. J., and Xing, L. (2005). TNF-alpha and pathologic bone resorption. *Keio J Med, 54*(3), 127-131.

Caplen, G., Baker, L., Hothersall, B., McKeegan, D. E., Sandilands, V., Sparks, N. H., Waterman-Pearson, A. E., and Murrell, J. C. (2013a). Thermal nociception as a measure of non-steroidal anti-inflammatory drug effectiveness in broiler chickens with articular pain. *Vet J, 198*(3), 616-619.

Caplen, G., Colborne, G. R., Hothersall, B., Nicol, C. J., Waterman-Pearson, A. E., Weeks, C. A., and Murrell, J. C. (2013b). Lame broiler chickens respond to non-steroidal anti-inflammatory drugs with objective changes in gait function: a controlled clinical trial. *Vet J, 196*(3), 477-482.

Charles, J. F., Ermann, J., and Aliprantis, A. O. (2015). The intestinal microbiome and skeletal fitness: Connecting bugs and bones. *Clinical Immunology, 159*(2), 163-169.

Cheng, H. W., Dillworth, G., Singleton, P., Chen, Y., and Muirt, W. M. (2001). Effects of group selection for productivity and longevity on blood concentrations of serotonin, catecholamines, and corticosterone of laying hens. *Poult Sci, 80*(9), 1278-1285.

Criscitiello, C., Viale, G., Gelao, L., Esposito, A., De Laurentiis, M., De Placido, S., Santangelo, M., Goldhirsch, A., and Curigliano, G. (2015). Crosstalk between bone niche and immune system: osteoimmunology signaling as a potential target for cancer treatment. *Cancer Treat Rev, 41*(2), 61-68.

Danbury, T. C., Weeks, C. A., Chambers, J. P., Waterman-Pearson, A. E., and Kestin, S. C. (2000). Self-selection of the analgesic drug carprofen by lame broiler chickens. *Vet Rec, 146*(11), 307-311.

de Matos, R. (2008). Calcium metabolism in birds. *Vet Clin North Am Exot Anim Pract, 11*(1), 59-82, vi.

de Vries, T. J., Yousovich, J., Schoenmaker, T., Scheres, N., and Everts, V. (2016). Tumor necrosis factor-alpha antagonist infliximab inhibits osteoclast formation of peripheral blood mononuclear cells but does not affect periodontal ligament fibroblast-mediated osteoclast formation. *J Periodontal Res, 51*(2), 186-195.

Deng, W., Dong, X. F., Tong, J. M., and Zhang, Q. (2012). The probiotic Bacillus licheniformis ameliorates heat stress-induced impairment of egg production, gut morphology, and intestinal mucosal immunity in laying hens. *Poult Sci, 91*(3), 575-582.

Duque, G., Huang, D. C., Dion, N., Macoritto, M., Rivas, D., Li, W., Yang, X. F., Li, J., Lian, J., Marino, F. T., Barralet, J., Lascau, V., Deschenes, C., Ste-Marie, L. G., and Kremer, R. (2011). Interferon-gamma plays a role in bone formation in vivo and rescues osteoporosis in ovariectomized mice. *J Bone Miner Res, 26*(7), 1472-1483.

Foureaux Rde, C., Messora, M. R., de Oliveira, L. F., Napimoga, M. H., Pereira, A. N., Ferreira, M. S., and Pereira, L. J. (2014). Effects of probiotic therapy on metabolic and inflammatory parameters of rats with ligature-induced periodontitis associated with restraint stress. *J Periodontal Res, 85*(7), 975-983.

Fuentes, C., Orozco, L., Vicente, J., Velasco, X., Menconi, A., Kuttappan, V., Kallapura, G., Latorre, J., Layton, S., Hargis, B., and Téllez, G. (2013). Effect of a lactic acid bacterium based probiotic, Floramax-B11Â®, on performance, bone qualities, and morphometric analysis of broiler chickens: an economic analysis. *Biol Syst Open Access, 2*, 113.

Fujioka, K., Kishida, T., Ejima, A., Yamamoto, K., Fujii, W., Murakami, K., Seno, T., Yamamoto, A., Kohno, M., Oda, R., Yamamoto, T., Fujiwara, H., Kawahito, Y., and Mazda, O. (2015). Inhibition of osteoclastogenesis by osteoblast-like cells genetically engineered to produce interleukin-10. *Biochem Biophys Res Commun, 456*(3), 785-791.

Fuller, L. M. (1984). The pharmacology of drinking behavior. *Pharmacol Ther, 24*(2), 179-206.

Garriga, C., Hunter, R. R., Amat, C., Planas, J. M., Mitchell, M. A., and Moreto, M. (2006). Heat stress increases apical glucose transport in the chicken jejunum. *Am J Physiol Regul Integr Comp Physiol, 290*(1), R195-201.

Geraert, P. A., Padilha, J. C., and Guillaumin, S. (1996). Metabolic and endocrine changes induced by chronic heat exposure in broiler chickens: growth performance, body composition and energy retention. *Br J Nutr, 75*(2), 195-204.

Guerrini, M. M., and Takayanagi, H. (2014). The immune system, bone and RANKL. *Arch Biochem Biophys, 561*, 118-123.

Hall, L. E., Shirley, R. B., Bakalli, R. I., Aggrey, S. E., Pesti, G. M., and Edwards, H. M., Jr. (2003). Power of two methods for the estimation of bone ash of broilers. *Poult Sci, 82*(3), 414-418.

Hester, P. Y., Enneking, S. A., Haley, B. K., Cheng, H. W., Einstein, M. E., and Rubin, D. A. (2013). The effect of perch availability during pullet rearing and egg laying on musculoskeletal health of caged White Leghorn hens. *Poult Sci, 92*(8), 1972-1980.

Hosseini-Vashan, S. J., Golian, A., and Yaghobfar, A. (2016). Growth, immune, antioxidant, and bone responses of heat stress-exposed broilers fed diets supplemented with tomato pomace. *Int J Biometeorol, 60*(8), 1183-1192.

Houshmand, M., Azhar, K., Zulkifli, I., Bejo, M. H., Meimandipour, A., and Kamyab, A. (2011). Effects of non-antibiotic feed additives on performance, tibial dyschondroplasia incidence and tibia characteristics of broilers fed low-calcium diets. *J Anim Physiol Anim Nutr (Berl), 95*(3), 351-358.

Hu, F., Gao, X., She, R., Chen, J., Mao, J., Xiao, P., and Shi, R. (2016). Effects of antimicrobial peptides on growth performance and small intestinal function in broilers under chronic heat stress. *Poult Sci*, doi: 10.3382/ps/pew3379.

Inada, M., and Miyaura, C. (2010). Cytokines in bone diseases. Cytokine and postmenopausal osteoporosis. *Clin Calcium, 20*(10), 1467-1472.

Jahanian, R., and Rasouli, E. (2015). Dietary chromium methionine supplementation could alleviate immunosuppressive effects of heat stress in broiler chicks. *J Anim Sci, 93*(7), 3355-3363.

Jankowski, J., Mikulski, D., Tatara, M. R., and Krupski, W. (2015). Effects of increased stocking density and heat stress on growth, performance, carcase characteristics and skeletal properties in turkeys. *Vet Rec, 176*(1), 21.

Jia, D., O'Brien, C. A., Stewart, S. A., Manolagas, S. C., and Weinstein, R. S. (2006). Glucocorticoids act directly on osteoclasts to increase their life span and reduce bone density. *Endocrinology, 147*(12), 5592-5599.

Kanazawa, K., and Kudo, A. (2005). Self-assembled RANK induces osteoclastogenesis ligand-independently. *J Bone Miner Res, 20*(11), 2053-2060.

King, B. M. (2006). The rise, fall, and resurrection of the ventromedial hypothalamus in the regulation of feeding behavior and body weight. *Physiol Behav, 87*(2), 221-244.

Kitaura, H., Kimura, K., Ishida, M., Kohara, H., Yoshimatsu, M., and Takano-Yamamoto, T. (2013). Immunological reaction in TNF-alpha-mediated osteoclast formation and bone resorption in vitro and in vivo. *Clin Dev Immunol, 2013*, 181849.

Lara, L. J., and Rostagno, M. H. (2013). Impact of Heat Stress on Poultry Production. *Animals (Basel), 3*(2), 356-369.

Li, J. Y., Chassaing, B., Tyagi, A. M., Vaccaro, C., Luo, T., Adams, J., Darby, T. M., Weitzmann, M. N., Mulle, J. G., Gewirtz, A. T., Jones, R. M., and Pacifici, R. (2016). Sex steroid deficiency-associated bone loss is microbiota dependent and prevented by probiotics. *J Clin Invest, 126*(6), 2049-2063.

Mack, L. A., Felver-Gant, J. N., Dennis, R. L., and Cheng, H. W. (2013). Genetic variations alter production and behavioral responses following heat stress in 2 strains of laying hens. *Poult Sci, 92*(2), 285-294.

Manhiani, P. S., Northcutt, J. K., Han, I., Bridges, W. C., Scott, T. R., and Dawson, P. L. (2011). Effect of stress on carnosine levels in brain, breast, and thigh of broilers. *Poult Sci, 90*(10), 2348-2354.

McCabe, L., Britton, R. A., and Parameswaran, N. (2015). Prebiotic and Probiotic Regulation of Bone Health: Role of the Intestine and its Microbiome. *Curr Osteoporos Rep, 13*(6), 363-371.

McCabe, L. R., Irwin, R., Schaefer, L., and Britton, R. A. (2013). Probiotic use decreases intestinal inflammation and increases bone density in healthy male but not female mice. *J Cell Physiol, 228*(8), 1793-1798.

Muller, C. P., Carey, R. J., and Huston, J. P. (2003). Serotonin as an important mediator of cocaine's behavioral effects. *Drugs Today (Barc), 39*(7), 497-511.

Mutus, R., Kocabagli, N., Alp, M., Acar, N., Eren, M., and Gezen, S. S. (2006). The effect of dietary probiotic supplementation on tibial bone characteristics and strength in broilers. *Poult Sci, 85*(9), 1621-1625.

Niu, Z. Y., Liu, F. Z., Yan, Q. L., and Li, W. C. (2009). Effects of different levels of vitamin E on growth performance and immune responses of broilers under heat stress. *Poult Sci, 88*(10), 2101-2107.

O'Brien, C. A., Jia, D., Plotkin, L. I., Bellido, T., Powers, C. C., Stewart, S. A., Manolagas, S. C., and Weinstein, R. S. (2004). Glucocorticoids act directly on osteoblasts and osteocytes to induce their apoptosis and reduce bone formation and strength. *Endocrinology, 145*(4), 1835-1841.

Odom, T. W., Harrison, P. C., and Bottje, W. G. (1986). Effects of thermal-induced respiratory alkalosis on blood ionized calcium levels in the domestic hen. *Poult Sci, 65*(3), 570-573.

Ohlsson, C., and Sjogren, K. (2015). Effects of the gut microbiota on bone mass. *Trends Endocrinol Metab, 26*(2), 69-74.

Oskoueian, E., Abdullah, N., Idrus, Z., Ebrahimi, M., Goh, Y. M., Shakeri, M., and Oskoueian, A. (2014). Palm kernel cake extract exerts hepatoprotective activity in heat-induced oxidative stress in chicken hepatocytes. *BMC Complement Altern Med, 14*, 368.

Panda, A. K., Rao, S. V. R., Raju, M. V., and Sharma, S. R. (2006). Dietary supplementation of Lactobacillus sporogenes on performance and serum biochemico-lipid profile of broiler chickens. *J Poult Sci, 43*(3), 235-240.

Pappalardo, A., and Thompson, K. (2013). Activated gammadelta T cells inhibit osteoclast differentiation and resorptive activity in vitro. *Clin Exp Immunol, 174*(2), 281-291.

Quinteiro-Filho, W. M., Ribeiro, A., Ferraz-de-Paula, V., Pinheiro, M. L., Sakai, M., Sa, L. R., Ferreira, A. J., and Palermo-Neto, J. (2010). Heat stress impairs performance parameters, induces intestinal injury, and decreases macrophage activity in broiler chickens. *Poult Sci, 89*(9), 1905-1914.

Quinteiro-Filho, W. M., Gomes, A. V., Pinheiro, M. L., Ribeiro, A., Ferraz-de-Paula, V., Astolfi-Ferreira, C. S., Ferreira, A. J., and Palermo-Neto, J. (2012). Heat stress impairs performance and induces intestinal inflammation in broiler chickens infected with Salmonella Enteritidis. *Avian Pathol, 41*(5), 421-427.

Rauch, A., Seitz, S., Baschant, U., Schilling, A. F., Illing, A., Stride, B., Kirilov, M., Mandic, V., Takacz, A., Schmidt-Ullrich, R., Ostermay, S., Schinke, T., Spanbroek, R., Zaiss, M. M., Angel, P. E., Lerner, U. H., David, J. P., Reichardt, H. M., Amling, M., Schutz, G., and Tuckermann, J. P. (2010). Glucocorticoids suppress bone formation by attenuating osteoblast differentiation via the monomeric glucocorticoid receptor. *Cell Metab, 11*(6), 517-531.

Riesenfeld, A. (1972). Metatarsal robusticity in bipedal rats. *Am J Phys Anthropol, 36*(2), 229-233.

Rodrigues, F. C., Castro, A. S., Rodrigues, V. C., Fernandes, S. A., Fontes, E. A., de Oliveira, T. T., Martino, H. S., and de Luces Fortes Ferreira, C. L. (2012). Yacon flour and Bifidobacterium longum modulate bone health in rats. *J Med Food, 15*(7), 664-670.

Sadeghi, A. A. (2014). Bone Mineralization of Broiler Chicks Challenged with Salmonella enteritidis Fed Diet Containing Probiotic (Bacillus subtilis). *Probiotics Antimicrob Proteins, 6*(3-4), 136-140.

Samara, M. H., Robbins, K. R., and Smith, M. O. (1996). Environmental heat stress does not reduce blood ionized calcium concentration in hens acclimated to elevated temperatures. *Poult Sci, 75*(2), 197-200.

Santos, R. R., Awati, A., Roubos-van den Hil, P. J., Tersteeg-Zijderveld, M. H., Koolmees, P. A., and Fink-Gremmels, J. (2015). Quantitative histo-morphometric analysis of heat-stress-related damage in the small intestines of broiler chickens. *Avian Pathol, 44*(1), 19-22.

Scanes, C. G. (2007). The global importance of poultry. *Poult Sci, 86*(6), 1057-1058.

Schett, G. (2011). Effects of inflammatory and anti-inflammatory cytokines on the bone. *Eur J Clin Invest, 41*(12), 1361-1366.

Seedor, J. G., Quartuccio, H. A., and Thompson, D. D. (1991). The bisphosphonate alendronate (MK-217) inhibits bone loss due to ovariectomy in rats. *J Bone Miner Res, 6*(4), 339-346.

Sjogren, K., Engdahl, C., Henning, P., Lerner, U. H., Tremaroli, V., Lagerquist, M. K., Backhed, F., and Ohlsson, C. (2012). The gut microbiota regulates bone mass in mice. *J Bone Miner Res, 27*(6), 1357-1367.

Sohail, M. U., Hume, M. E., Byrd, J. A., Nisbet, D. J., Ijaz, A., Sohail, A., Shabbir, M. Z., and Rehman, H. (2012). Effect of supplementation of prebiotic mannan-oligosaccharides and probiotic mixture on growth performance of broilers subjected to chronic heat stress. *Poult Sci, 91*(9), 2235-2240.

Song, J., Xiao, K., Ke, Y. L., Jiao, L. F., Hu, C. H., Diao, Q. Y., Shi, B., and Zou, X. T. (2014). Effect of a probiotic mixture on intestinal microflora, morphology, and barrier integrity of broilers subjected to heat stress. *Poult Sci, 93*(3), 581-588.

St-Pierre, N. R., Cobanov, B., and Schnitkey, G. (2003). Economic Losses from Heat Stress by US Livestock Industries1. *J Dairy Sci, 86, Supplement*, E52-E77.

Stanford, M. (2006). Calcium metabolism. Page 141-151 in: G. J. Harrison, Lightfoot, T. L. (Ed.), *Clinical Avian Medicine* . Palm Beach, Florida: Spix Publishing.

Star, L., Decuypere, E., Parmentier, H. K., and Kemp, B. (2008). Effect of single or combined climatic and hygienic stress in four layer lines: 2. Endocrine and oxidative stress responses. *Poult Sci, 87*(6), 1031-1038.

Steel, R. G., Torrie, J. H., and Dickey, D. A. (1997). *Principles And Procedures of Statistics: A Biometrical Approach.* McGraw Hill Book Co., New York, NY.

Syafwan, S., Wermink, G. J., Kwakkel, R. P., and Verstegen, M. W. (2012). Dietary self-selection by broilers at normal and high temperature changes feed intake behavior, nutrient intake, and performance. *Poult Sci, 91*(3), 537-549.

Takayanagi, H., Ogasawara, K., Hida, S., Chiba, T., Murata, S., Sato, K., Takaoka, A., Yokochi, T., Oda, H., Tanaka, K., Nakamura, K., and Taniguchi, T. (2000). T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-gamma. *Nature, 408*(6812), 600-605.

USDA-FAS. (2016). *Livestock and Poultry: World Markets and Trade*. Retrieved from http://usda.mannlib.cornell.edu/usda/current/livestock-poultry-ma/livestock-poultry-ma-10-12-2016.pdf.

Voigt, J. P., and Fink, H. (2015). Serotonin controlling feeding and satiety. *Behav Brain Res, 277*, 14-31.

Wang, W. C., Yan, F. F., Hu, J. Y., Zhang, C. Y., and Cheng, H. W. (2016). Effect of dietary supplementation of probiotic, Bacillus subtilis, on performance and immune parameters in the brain of broiler chickens under heat stress. *Poult Sci, 95(E-Suppl. 1)*, 40.

Wideman, R. F., Hamal, K. R., Stark, J. M., Blankenship, J., Lester, H., Mitchell, K. N., Lorenzoni, G., and Pevzner, I. (2012). A wire-flooring model for inducing lameness in broilers: Evaluation of probiotics as a prophylactic treatment. *Poult Sci, 91*(4), 870-883.

Windhorst, H. W. (2006). Changes in poultry production and trade worldwide. *World Poultry Sci J, 62*(4), 585-602.

Xu, D., Li, W., Huang, Y., He, J., and Tian, Y. (2014). The effect of selenium and polysaccharide of Atractylodes macrocephala Koidz. (PAMK) on immune response in chicken spleen under heat stress. *Biol Trace Elem Res, 160*(2), 232-237.

Yadav, V. K., Oury, F., Suda, N., Liu, Z. W., Gao, X. B., Confavreux, C., Klemenhagen, K. C., Tanaka, K. F., Gingrich, J. A., Guo, X. E., Tecott, L. H., Mann, J. J., Hen, R., Horvath, T. L., and Karsenty, G. (2009). A serotonin-dependent mechanism explains the leptin regulation of bone mass, appetite, and energy expenditure. *Cell, 138*(5), 976-989.

Yadav, V. K., Balaji, S., Suresh, P. S., Liu, X. S., Lu, X., Li, Z., Guo, X. E., Mann, J. J., Balapure, A. K., Gershon, M. D., Medhamurthy, R., Vidal, M., Karsenty, G., and Ducy, P. (2010). Pharmacological inhibition of gut-derived serotonin synthesis is a potential bone anabolic treatment for osteoporosis. *Nature Medicine, 16*(3), 308-312.

Yan, F. F., Wang, W. C., R., W., and Cheng, H. W. (2016). The effect of Bacillus subtilis based probiotic on bone health in broiler chickens. *Poult Sci, 95* (E-suppl. 1), 40.

Yokota, K., Sato, K., Miyazaki, T., Kitaura, H., Kayama, H., Miyoshi, F., Araki, Y., Akiyama, Y., Takeda, K., and Mimura, T. (2014). Combination of tumor necrosis factor alpha and interleukin-6 induces mouse osteoclast-like cells with bone resorption activity both in vitro and in vivo. *Arthritis Rheumatol, 66*(1), 121-129.

Zhang, J., Motyl, K. J., Irwin, R., MacDougald, O. A., Britton, R. A., and McCabe, L. R. (2015). Loss of Bone and Wnt10b Expression in Male Type 1 Diabetic Mice Is Blocked by the Probiotic Lactobacillus reuteri. *Endocrinology, 156*(9), 3169-3182.

Zhang, Q., Chen, B., Yan, F., Guo, J., Zhu, X., Ma, S., and Yang, W. (2014). Interleukin-10 inhibits bone resorption: a potential therapeutic strategy in periodontitis and other bone loss diseases. *Biomed Res Int*, doi:10.1155/2014/284836.

Ziaie, H., Bashtani, M., Torshizi, M. A. K., Naeeimipour, H., Farhangfar, H., and Zeinali, A. (2011). Effect of antibiotic and its alternatives on morphometric characteristics, mineral content and bone strength of tibia in Ross broiler chickens. *Glob Vet, 7*(4), 315-322.

CHAPTER 5. EFFECTS OF A MULTIPLE SPECIES PROBIOTIC ON BONE HEALTH IN BROILERS SUBJECTED TO CYCLIC HEATING EPISODES

5.1 Abstract

The objective of this study was to determine the effect of a multi-species probiotic on broiler bone health under daily cycling heating episodes. Three hundred and sixty Ross 708 broiler straight-run hatchlings were randomly assigned to 3 dietary treatments (n = 8): basal diet (control) and the basal diet mixed with a commercial probiotic product at 0.5 (0.5X) or 1.0 (1.0X) g/kg of feed. Room temperature was gradually decreased from 35 ºC on d 1 by 0.55 ºC/d for the first 14 d. When broilers were 15 d of age, ambient temperature was increased from 28 to 32 ºC for 9 h (0800 h to 1700 h) and then dropped to 25 to 26° C for the remainder of the 24 h period. The cyclic heating episodes were instigated daily from 15 d of age until termination of the experiment at 6 wk of age. Gait score and the latency to lie test were conducted when broilers were 40 and 41 d of age, respectively. The tibia, femur, and humerus were collected for measuring bone parameters at 42 d of age. The BMD, BMC, and bone area increased and the gait score decreased in the 1.0X group compared to the controls ($P < 0.05$), whereas the 0.5X group was similar to controls. The proportions of broilers showing signs of lameness were in the order of 1.0X < 0.5X < control dietary treatments or 25, 45, and 54%, respectively. For the latency to lie test, adding probiotic to the feed caused birds to stand longer in water as compared to controls ($P = 0.03$), but there was no additional benefit to doubling the dosage of the probiotic from 0.5X to 1.0X. In conclusion, dietary supplementation of probiotic improved bone health of broilers subjected to daily cycling heating episodes resulting in an improvement in walking ability.

5.2 Introduction

Leg disorders are a serious welfare and economic problem of the poultry meat industry as it affects the musculoskeletal system causing lameness and impairing mobility (Bokkers and Koene, 2003; Reiter and Bessei, 2009). Multiple factors influence the

incidence of leg disorders in broilers such as genetics, age, sex, growth rate, nutrition, housing, environment factors, and management (Kestin et al., 1999; Sorensen et al., 2000; Bercik et al., 2010; Toghyani et al., 2011; Schwean-Lardner et al., 2013). Fast growth rate is a key factor affecting lameness in commercial broiler flocks (Talaty et al., 2009; Toscano et al., 2013). For example, rapidly growing broilers have lower tibia density and percentage of bone ash than slow-growing broilers (Shim et al., 2012) with lower bone mineralization causing high bone porosity (Williams et al., 2004). Broilers with a poor gait score spend more time lying (86%) than non-lame (76%) chickens with no leg problems (Weeks et al., 2000). The lack of activity further exacerbates lameness as mechanical loading is essential for normal bone formation. For example, broilers in cages as compared to those in large enclosures where the chickens could walk freely had lower leg bone mineralization which the authors attributed to inactivity (Aguado et al., 2015).

Heat stress reduces growth because of reduced feed intake and impaired intestinal function (Lott, 1991; Belay and Teeter, 1993). High ambient temperatures also cause mortality, immunosuppression (Jahanian and Rasouli, 2015), acid-base imbalance (Borges et al., 2004), and tissue damage as indicated by increases in plasma lactate dehydrogenase, glutamic-oxaloacetic transaminase, and creatine kinase (Xie et al., 2015).

Heat stress induces bone loss in broilers (Hosseini-Vashan et al., 2016), laying hens (Koelkebeck et al., 1993), and turkey (Jankowski et al., 2015). Although lameness was not triggered with repeated episodes of heat (33° C, 3 d/wk from 4 to 6 wk of age), the subclinical incidence of tibial head necrosis was substantially greater at 28 and 35 d of age in heat-stressed broilers (Wideman and Pevzner, 2012). Elevated temperatures increase circulating CORT (Henneicke et al., 2014) and reactive oxygen substances in mitochondria leading to oxidative stress (Huang et al., 2015), perhaps contributing to impaired skeletal health. Excessive CORT negatively affects bone mass through inhibiting osteoblastogenesis, increasing osteoblast and osteocyte apoptosis, and promoting osteoclast survival (O'Brien et al., 2004; Jia et al., 2006; Rauch et al., 2010).

Femoral health status was improved in laying hens fed a multi-species based probiotic (Yan et al., 2015). The objective of this study was to determine the effects of the commercial probiotic product on broiler bone health when subjected to cycling

heating episodes. We hypothesized that probiotic would improve bone health and reduce lameness in broilers under conditions of cycling elevated temperatures.

5.3 Materials and Methods

5.3.1 Birds, Management, Diets

The experimental protocol was approved by the Purdue Animal Use and Care Committee (Number 1111000262). Three hundred and sixty d-old straight-run Ross 708 broiler chicks were obtained from a commercial hatchery (Miller Poultry, Orland, IN). Birds were weighed in groups of 15 chicks each and placed into 24 floor pens (243 x 152 cm), ensuring each pen had similar average BW. Stocking density was 2,462 cm^2/broiler. One hanging feeder and Plasson bell drinker were provided per pen. Feed and water were provided for *ad libitum* consumption.

Pens were randomly assigned to 3 dietary treatments of 8 replicate littered floor pens each for 42 d: control diet (Table 5.1) and the control diet mixed with a multi-species probiotic (PoultryStar®, BIOMIN America, Inc., San Antonio, TX)) at 0.5g/kg of feed (0.5X) or 1.0g/kg of feed (1.0X). The composition of the probiotic included 4 microbial strains of *Enterococcus faecium, Pediococcus acidilactici, Bifidobacterium animalis,* and *Lactobacillus reuteri*. Starter, grower, and finisher diets were fed from 1 to 14, 15 to 28, and 29 to 42 d of age (Table 5.1) using the nutrient recommendations of Aviagen (2014b). Room temperature was gradually decreased from 35 ºC on d 1 by 0.5 ºC/d for the first 14 d. When broilers were 15 d of age, ambient temperature was increased from 28 to 32 ºC for 9 h (0800h to 1700h) and then dropped to 25 to 26° C for the remainder of the 24 h period (Table 5.3). The cyclic heating episodes were instigated daily from 15 d of age until termination of the experiment. Data loggers (HOBO®, Onset Computer Corporation, Bourne, MA) were used to record room temperature and humidity when boilers were 3 to 6 wk of age (Table 5.2). The lighting program was 23light:1dark at 30 lux until 3 d of age, then 20light:4dark at 10 lux until 42 d of age.

5.3.2 Data Collection

At 40 d of age, 7 broilers per pen were randomly selected and individually evaluated for walking ability using a 3-point gait score system (Webster et al., 2008).

Using the individual gait scores collected from 7 broilers per pen, the proportion of broilers within a gait score for each dietary treatment was calculated and expressed as a percentage. The gait scores from the same pen were averaged and used in the statistical analysis rather than using individual data.

At 41 d of age, 2 broilers per pen were randomly selected and used to perform the latency to lie test using the procedure of (Berg and Sanotra, 2003). This test is an indicator of a broiler's desire and capability to stand in an uncomfortable situation. Briefly, an individual bird was placed into a tub previously filled with 3 cm of water at 28° C. The test was conducted between 1000 and 1300 h when broilers were experiencing the heat. The chicken was removed from its pen and taken to the tub located in an adjacent room where ambient temperature was normal. The time it took for the chicken to sit down and touch the water was recorded. If the bird flew away, it was not included in the data set. If the broiler was still standing after 600 s, the test was stopped and the observation of 600 s was recorded.

At 42 d of age, 2 broilers per pen were randomly selected. The chickens were sedated using sodium pentobarbital injected intravenously in the brachial vein (30 mg/kg of BW) followed by BW determination and cervical dislocation. The left wing, thigh, and drum were collected from each broiler, placed in a labelled plastic bag, and frozen (-20° C) for later analysis.

5.3.3 DEXA

Carcass samples were thawed and scanned using DEXA (Norland Medical Systems, Inc., Fort Atkinson, WI) with muscle, skin, and feathers intact to quantify BMD, BMC, and bone area of the humerus, femur, and tibia with fibula (Hester et al., 2013).

5.3.4 Statistical Analysis

A one-way ANOVA of the mixed model procedure of SAS 9.4 software (SAS Institute, Inc., Cary, NC) was used to analyze data. Linear and quadratic effects were tested for using regression analysis. The fixed factor was the probiotic treatment. The BW was used as a covariate for measures of bone mineralization and bone area when

necessary. Transformation of data was performed for normality when variances were not homogeneous (Steel et al., 1997). Statistical trends were similar for both transformed and untransformed data; therefore, the untransformed least square means and SEM were presented. The Tukey-Kramer test was used to partition differences among means. Statistical significance was set at $P < 0.05$. A Pearson correlation analysis was performed on bone traits and BW.

5.4 Results

Broilers of the current study were demonstrating signs of distress during the heating episodes as indicated by panting and wing spreading (Mohammed et al., unpublished data).

The 42-d-old BW of broilers increased linearly with increasing dosages of probiotic (P = 0.01, Figure 5.1), whereas gait score of 40-d-old broilers decreased linearly with increasing dosages of the combined product (P = 0.05; Figure 5.2a). Most of the broilers of the current study were categorized with a gait score of 0 (normal gait) or 1 (awkward gait) with only a small proportion (< 2%) identified with a poor gait score of 2 (Figure 5.2b). With respect to distribution of birds of the 3 dietary treatments within a gait score, the proportion of broilers with a normal gait (score of 0) increased linearly as the dosages of probiotic increased. Specifically, 46, 55, and 75% of the broilers consuming the control, 0.5X, and 1.0X diets, respectively, had a normal gait of 0 (P = 0.03, Figure 5.2b). Concomitantly, the awkward gait score of 1 showed the opposite effect, whereby the proportion of broilers among diets decreased linearly as dosages of probiotic increased. Specifically, 52, 41, and 23% of the broilers consuming the control, 0.5X, and 1.0X diets, respectively, had an awkward gait (score of 1). No differences in distribution were found among dietary treatments for the poor gait score of 2 (P = 0.77). For the latency to lie test, broilers consuming the 0.5X and 1.0X diets spent a greater length of time standing in water than the controls (P = 0.03; Figure 5.3), but the 1.0X group did not differ from the controls.

Mineralization (BMD and BMC) and area of the tibia 42-d-old broilers increased linearly as the dosage of probiotic increased in the diet (see individual P values in Table 5.4). The mineralization of the femur and humerus (BMD and BMC) did not respond

favorably to the probiotic treatment until a dosage level of 1.0X was used. The area of the humerus was unaffected by the dietary supplementation (P= 0.09, Table 5.4).

Positive correlations (P < 0.05 or lower) were found between BW and the bone traits of BMD, BMC, and bone area for the tibia, femur, and humerus (Table 5.5). Using BW as a covariate resulted in no differences among dietary treatments for tibia and femur bone traits (see adjusted P values in Table 5.4). An analysis of covariance was not used for traits of the humerus, because BW was not significant as a covariate.

5.5 Discussion

During the 9 h of elevated temperature of 32 ºC, broilers were panting. According the performance standards of the Ross 708 broiler, straight run chickens raised under normal management should have an average BW of 2,678 g at 42 d of age (Aviagen, 2014a). The controls of the current study were slightly below this performance standard with an average 42-d-old BW of 2,600 g, suggesting that the elevated temperature slowed growth, even when using very low stocking densities.

Use of a multi-species probiotic had a profound effect in countering the detrimental effects of elevated ambient temperatures on broilers. Market BW and skeletal health of the supplement fed-broilers were remarkably improved as compared to controls given no probiotic that were also subjected to daily cycling heating episodes. All measures of skeletal health used in the current study, including bone mineralization of leg (unadjusted for BW) and wing bones, gait score, and latency to lie test, showed benefits when the probiotic supplement was added to the diet. Of the 2 dosages of probiotic used in the current study, the higher dose (1.0X) is recommended under conditions of elevated temperatures as the response of broilers to the lower dosage of 0.5X was intermediate and in many instances did not differ from controls. Broilers raised under commercial conditions experience more crowded conditions than the chickens of the current study, therefore using the 1.0X as compared to 0.5X dosage provides a safeguard against competition at the feeders.

There are several other studies supporting improved skeletal health in poultry as a result of consuming probiotics. For example, probiotics reduced lameness in broilers diagnosed with BCO. Broilers purposely reared on wire floor to induce this bone disease

showed a reduction in lameness using the same probiotic and similar dosage (probiotic beginning at 1 d of age with the dose 0.55 g/kg of feed) of the lower dosage (0.50 g/kg of feed) used in the current study (Wideman et al., 2012). Utilizing another commercial product, broilers raised on wire and consuming BacPack 2X (a mannan oligosaccharide beta-glucan yeast cell wall prebiotic product plus a probiotic containing *Bacillus subtilis*) experienced a delay in age of onset as well as a lower incidence of BCO (Wideman et al., 2015). Additionally, feeding a single species probiotic of *Enterococcus faecium* (0.55 g/kg of feed) beginning at 1 d of age to broilers reared on wire resulted in a low incidence of femoral head transitional degeneration and tibial head necrosis. Dietary supplementation of a *Bacillus subtilis* based probiotic beginning at 1 d of age improved mineralization and cortical thickness of leg bones of 43-d-old broilers raised under a normal temperature regimen (see Chapter 3 of dissertation), but only bone size traits and growth were improved with the same supplementation for broilers exposed to cycling elevated temperatures (see Chapter 4 of dissertation). Other studies with poultry have also shown that probiotic supplementation improved bone mass (Abdelqader et al., 2013; Sadeghi, 2014). Collectively, these studies provide strong evidence that probiotics improve skeletal health in poultry.

Probiotics improve intestinal integrity allowing for increased absorption and bioavailability of minerals such as Ca and P for bone mineralization. Under conditions of high ambient temperatures, probiotics may be even more effective in enhancing intestinal absorption as it has been reported that the inclusion of various probiotics ameliorates the negative effect of heat on gut health in broilers (Sohail et al., 2012; Song et al., 2014) and laying hens (Deng et al., 2012). Chronic heat stress in broilers induces intestinal injury such as reduced height of villi, thinner gut mucosa, and reduced alkaline phosphatase activity (Hu et al., 2016) that can hamper Ca and P intestinal absorption.

The positive correlation between BW and bone mineralization as measured through DEXA (Table 5.5) is in agreement with previous studies (Talaty et al., 2010; Gonzalez-Ceron et al., 2015). Larger birds have higher bone mineralization than smaller birds. As an example, Leghorn and Cobb broiler females were raised together using standard management procedures. The mean BMD of the tibia of 55-wk-old Leghorn hens with an average BW of 1.53 kg was 0.186 g/cm^2. The same age broiler female had

over double the BW and BMD. Specifically, the mean BMD of the tibia of 55-wk-old broiler females with an average BW of 4.40 kg was 0.389 g/cm^2 (Schreiweis et al., 2005). Because of the strong association between BW and bone mineralization, an analysis of covariance using BW as a covariate is often conducted on bone traits (Lang et al., 2005; Schreiweis et al., 2005; Talaty et al., 2009, 2010; Hester et al., 2011; Hester et al., 2013) which was done in the current study. However, there are numerous studies using different animal models demonstrating the bone enhancing effect of probiotics that use only unadjusted skeletal data (Ziaie et al., 2011; Abdelqader et al., 2013; Li et al., 2016; Messora et al., 2016). With respect to the current study, the probiotic effect on the bone mineralization of leg bones, but not the wing bone (humerus), dissipated as a result of using BW as a covariate suggesting that the dietary supplement's main influence on weight bearing limbs was through stimulation of growth. The 17% increase in BW of broilers consuming the 1.0X probiotic as compared to controls (Figure 5.1) under conditions of cycling elevated temperatures exemplifies the profound effect that this supplement had in promoting growth. Nevertheless, because BMD and BMC of the humerus, gait score and the latency to lie test showed improvements in broilers consuming probiotics, it can still be concluded that skeletal health benefited from the dietary supplement under conditions of cycling elevated temperatures.

It is important to point out that the probiotic induced improvement in gait score and latency to lie results occurred without detrimentally affecting growth. Gait score and BW are positively and strongly correlated with heavier broilers having poorer walking ability (Sorensen et al., 1999; Su et al., 1999; Sorensen et al., 2000; Venalainen et al., 2006; Brickett et al., 2007), but this was not the case in the current study as BW was actually increased in 42-d-old broilers of the 1.0X fed group as compared to the controls (Fig. 5.1) without deleteriously affecting gait score.

5.6 Conclusion

The inclusion of a multi-species probiotic in the diets of broilers exposed to daily cycling heating episodes, that began at 15 d of age, increased market BW and bone mineralization and improved walking ability as compared to control fed broilers. Dietary

use of the multi-species probiotic is effective in improving broiler welfare as well as performance during hot weather.

Table 5.1 The ration formulation

	Starter	Grower	Finisher
Ingredient, %			
Corn	52	52.3	62.8
Soybean meal, 48 % crude protein	40	39.1	29.7
Soybean oil	3.59	4.97	4.11
Sodium chloride	0.51	0.46	0.43
DL Methionine	0.3	0.24	0.23
L-Lysine HCl	0.13	- - -	0.07
Threonine	0.06	- - -	- - -
Limestone	1.29	1.15	1.12
Monocalcium phoshate	1.75	1.48	1.17
Vitamin/mineral premix[1]	0.35	0.35	0.35
Calculated analyses			
Crude protein %	23.4	22.8	19.2
ME kcal/kg	3050	3151	3200
Ca %	0.95	0.85	0.75
Available P %	0.5	0.44	0.36
Methionine %	0.66	0.59	0.53
Methionine + cystine %	1.04	0.97	0.86
Lysine %	1.42	1.29	1.09
Threonine %	0.97	0.89	0.74
Na %	0.22	0.20	0.19

[1]Provided per kilogram of diet: vitamin A, 13,233 IU; vitamin D3, 6,636 IU; vitamin E, 44.1 IU; vitamin K, 4.5 mg; thiamine, 2.21 mg; riboflavin, 6.6 mg; pantothenic acid, 24.3 mg; niacin, 88.2 mg; pyridoxine, 3.31 mg; folic acid, 1.10 mg; biotin, 0.33 mg; vitamin B12, 24.8 µg; choline, 669.8 mg; iron from ferrous sulfate, 50.1 mg; copper from copper sulfate, 7.7 mg; manganese from manganese oxide, 125.1 mg; zinc from zinc oxide, 125.1 mg; iodine from ethylene diamine dihydroiodide, 2.10 mg; selenium from sodium selenite, 0.30 mg.

Table 5.2 Ambient temperature and humidity recorded during the third through the sixth wk of the experiment

Age	Temperature (° C)		Relative Humidity (%)	
	0800 to 1700 h	1700 to 0800 h	0800 to 1700 h	1700 to 0800 h
Third wk	31.9 ± 0.3	25.9 ± 0.4	49.2 ± 1.5	55.1 ± 1.6
Fourth wk	31.6 ± 0.3	25.4 ± 0.2	50.9 ± 0.8	52.7 ± 1.5
Fifth wk	31.7 ± 0.5	25.5 ± 0.3	60.0 ± 1.7	61.4 ± 1.4
Sixth wk	31.6 ± 0.3	25.4 ± 0.1	57.5 ± 1.2	53.7 ± 1.3

Values represent the least square means ± SEM.

Table 5.3 Description of a 3-point gait-scoring system

Score	Lameness	Walking ability
0	None	Bird can walk at least 1.5 m with a balanced gait. Bird may appear ungainly but with little effect on function.
1	Obvious signs	Bird can walk at least 1.5 m but with a clear limp or decidedly awkward gait.
2	Severe signs	Bird will not walk 1.5 m. May shuffle on shanks or hocks with assistance of wings.

Table 5.4 The effect of a multi-species probiotic on bone mineralization and area of 42-d-old broilers subjected to cyclic heating episodes

	Treatment[1]					
Parameter	Control	0.5X	1.0X	SEM	P	Adjusted P[2]
Tibia						
BMD (g/cm^2)	0.209^{b}	0.216^{ab}	0.226^{a}	0.004	**0.03**	0.97
BMC (g)	2.82^{b}	3.02^{ab}	3.30^{a}	0.09	**0.002**	0.76
Area (cm^2)	13.46^{b}	13.98^{ab}	14.62^{a}	0.25	**0.008**	0.59
Femur						
BMD (g/cm^2)	0.180^{b}	0.185^{b}	0.199^{a}	0.004	**0.006**	0.81
BMC (g)	1.80^{b}	1.92^{b}	2.21^{a}	0.08	**0.002**	0.81
Area (cm^2)	9.99^{b}	10.39^{ab}	11.08^{a}	0.25	**0.01**	0.82
Humerus						
BMD (g/cm^2)	0.215^{b}	0.214^{b}	0.234^{a}	0.006	**0.05**	NA[3]
BMC (g)	1.60^{b}	1.62^{b}	1.87^{a}	0.05	**0.0003**	NA
Area (cm^2)	7.47	7.62	8.00	0.17	0.09	NA

[a,b]Least square means within a row lacking a common superscript differ ($P < 0.05$). The average number of observations per least square means was 16.
[1]The probiotic dosage was 0 (Control), 0.5 (0.5X), or 1.0 (1.0X) g/kg of feed.
[2]BW was used as a covariate.
[3]NA: not applied.

Table 5.5 Correlation values for bone mineralization, bone area, and BW

	Tibia			Femur			Humerus		
	BMD	BMC	Area	BMD	BMC	Area	BMD	BMC	Area
BW	0.52**	0.63**	0.51**	0.63**	0.67**	0.55**	0.29*	0.52**	0.35*

*The r values are significant at $P < 0.05$.

**The r values are significant at $P < 0.001$.

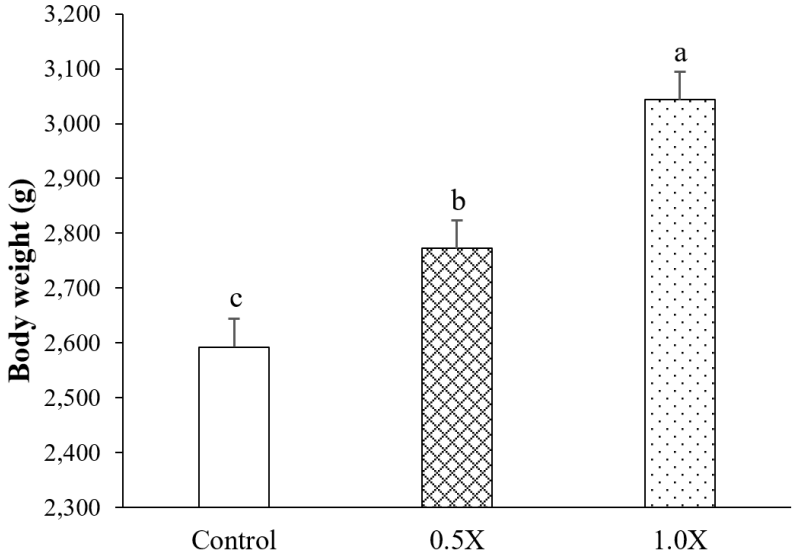


Figure 5.1 The effect of a multi-species probiotic on the BW of 42-d-old broilers subjected to cyclic heating episodes

The probiotic dosage was 0 (Control), 0.5 (0.5X), or 1.0 (1.0X) g/kg of feed. Least square means lacking a common superscript differ ($P < 0.05$). The average number of observations per least square means was 16.

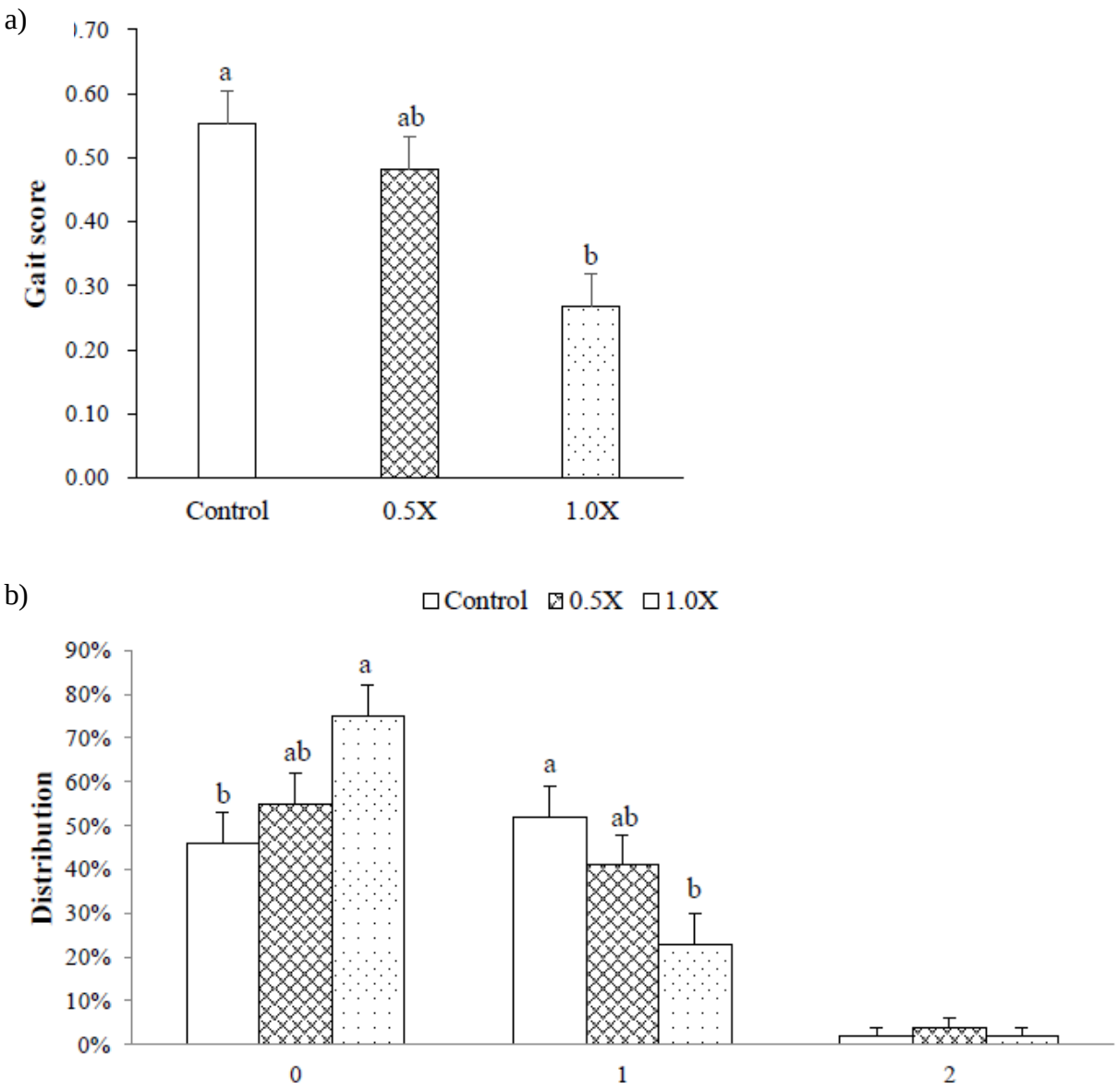


Figure 5.2 The effect of a multi-species probiotic on gait score (a) and its distribution (b) of 40-d-old broilers subjected to cyclic heating episodes

The probiotic dosage was 0 (Control), 0.5 (0.5X), or 1.0 (1.0X) g/kg of feed. Least square means lacking a common superscript differ ($P < 0.05$). The average number of observations per least square mean was 8.

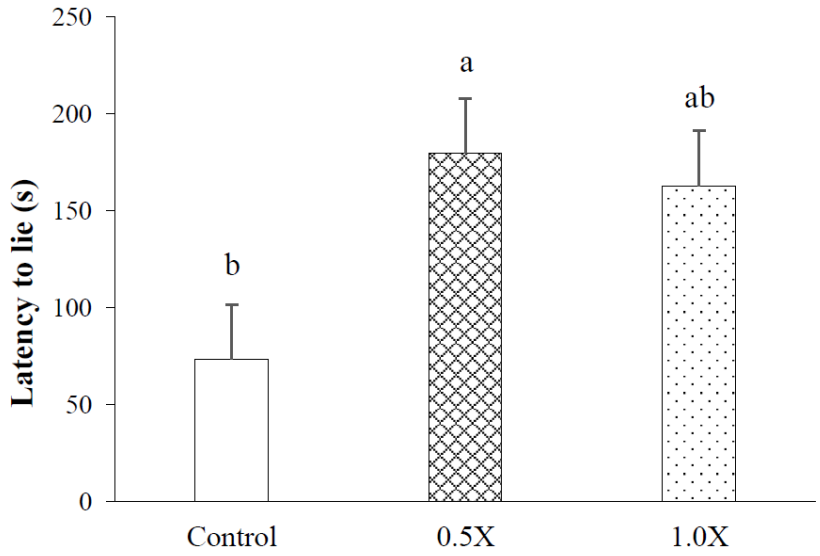


Figure 5.3 The effect of a multi-species probiotic on latency to lie test of 41-d-old broilers subjected to cyclic heating episodes

The probiotic dosage was 0 (Control), 0.5 (0.5X), or 1.0 (1.0X) g/kg of feed. Least square means lacking a common superscript differ ($P < 0.05$). The average number of observations per least square mean was 16.

5.7 References

Abdelqader, A., Irshaid, R., and Al-Fataftah, A. R. (2013). Effects of dietary probiotic inclusion on performance, eggshell quality, cecal microflora composition, and tibia traits of laying hens in the late phase of production. *Trop Anim Health Prod,* 45(4), 1017-1024.

Aguado, E., Pascaretti-Grizon, F., Goyenvalle, E., Audran, M., and Chappard, D. (2015). Bone mass and bone quality are altered by hypoactivity in the chicken. *PLoS One,* 10(1), e0116763.

Aviagen. (2014a). Broiler Performance Objectives. Retrieved from http://en.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross-708-Broiler-PO-2014-EN.pdf

Aviagen. (2014b). *Ross 708 broiler nutrition specifications*. Retrieved from http://en.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross708BroilerNutritionSpecs2014-EN.pdf.

Belay, T. and Teeter, R. G. (1993). Broiler Water Balance and Thermobalance During Thermoneutral and High Ambient Temperature Exposure. *Poult Sci,* 72(1), 116-124.

Bercik, P., Verdu, E. F., Foster, J. A., Macri, J., Potter, M., Huang, X., Malinowski, P., Jackson, W., Blennerhassett, P., Neufeld, K. A., Lu, J., Khan, W. I., Corthesy-Theulaz, I., Cherbut, C., Bergonzelli, G. E., and Collins, S. M. (2010). Chronic gastrointestinal inflammation induces anxiety-like behavior and alters central nervous system biochemistry in mice. *Gastroenterology,* 139(6), 2102-2112 e2101.

Berg, C. and Sanotra, G. (2003). Can a modified latency-to-lie test be used to validate gait-scoring results in commercial broiler flocks? *Anim Welfare* 12(4), 655-659.

Bokkers, E. A. M. and Koene, P. (2003). Behaviour of fast- and slow growing broilers to 12 weeks of age and the physical consequences. *Appl Anim Behav Sci* 81(1), 59-72.

Borges, S. A., Fischer da Silva, A. V., Majorka, A., Hooge, D. M., and Cummings, K. R. (2004). Physiological responses of broiler chickens to heat stress and dietary electrolyte balance (sodium plus potassium minus chloride, milliequivalents per kilogram). *Poult Sci,* 83(9), 1551-1558.

Brickett, K. E., Dahiya, J. P., Classen, H. L., Annett, C. B., and Gomis, S. (2007). The impact of nutrient density, feed form, and photoperiod on the walking ability and skeletal quality of broiler chickens. *Poult Sci,* 86(10), 2117-2125.

Deng, W., Dong, X. F., Tong, J. M., and Zhang, Q. (2012). The probiotic Bacillus licheniformis ameliorates heat stress-induced impairment of egg production, gut morphology, and intestinal mucosal immunity in laying hens. *Poult Sci,* 91(3), 575-582.

Gonzalez-Ceron, F., Rekaya, R., and Aggrey, S. E. (2015). Genetic analysis of bone quality traits and growth in a random mating broiler population. *Poult Sci,* 94(5), 883-889.

Henneicke, H., Gasparini, S. J., Brennan-Speranza, T. C., Zhou, H., and Seibel, M. J. (2014). Glucocorticoids and bone: local effects and systemic implications. *Trends Endocrinol Metab,* 25(4), 197-211.

Hester, P. Y., Wilson, D. A., Settar, P., Arango, J. A., and O'Sullivan, N. P. (2011). Effect of lighting programs during the pullet phase on skeletal integrity of egg-laying strains of chickens. *Poult Sci,* 90(8), 1645-1651.

Hester, P. Y., Enneking, S. A., Haley, B. K., Cheng, H. W., Einstein, M. E., and Rubin, D. A. (2013). The effect of perch availability during pullet rearing and egg laying on musculoskeletal health of caged White Leghorn hens. *Poult Sci,* 92(8), 1972-1980.

Hosseini-Vashan, S. J., Golian, A., and Yaghobfar, A. (2016). Growth, immune, antioxidant, and bone responses of heat stress-exposed broilers fed diets supplemented with tomato pomace. *Int J Biometeorol,* 60(8), 1183-1192.

Hu, F., Gao, X., She, R., Chen, J., Mao, J., Xiao, P., and Shi, R. (2016). Effects of antimicrobial peptides on growth performance and small intestinal function in broilers under chronic heat stress. *Poult Sci,* doi: 10.3382/ps/pew3379.

Huang, C., Jiao, H., Song, Z., Zhao, J., Wang, X., and Lin, H. (2015). Heat stress impairs mitochondria functions and induces oxidative injury in broiler chickens. *J Anim Sci,* 93(5), 2144-2153.

Jahanian, R. and Rasouli, E. (2015). Dietary chromium methionine supplementation could alleviate immunosuppressive effects of heat stress in broiler chicks. *J Anim Sci,* 93(7), 3355-3363.

Jankowski, J., Mikulski, D., Tatara, M. R., and Krupski, W. (2015). Effects of increased stocking density and heat stress on growth, performance, carcase characteristics and skeletal properties in turkeys. *Vet Rec,* 176(1), 21.

Jia, D., O'Brien, C. A., Stewart, S. A., Manolagas, S. C., and Weinstein, R. S. (2006). Glucocorticoids act directly on osteoclasts to increase their life span and reduce bone density. *Endocrinology,* 147(12), 5592-5599.

Kestin, S. C., Su, G., and Sorensen, P. (1999). Different commercial broiler crosses have different susceptibilities to leg weakness. *Poult Sci,* 78(8), 1085-1090.

Koelkebeck, K. W., Harrison, P. C., and Madindou, T. (1993). Research note: effect of carbonated drinking water on production performance and bone characteristics of laying hens exposed to high environmental temperatures. *Poult Sci,* 72(9), 1800-1803.

Lang, D. H., Sharkey, N. A., Lionikas, A., Mack, H. A., Larsson, L., Vogler, G. P., Vandenbergh, D. J., Blizard, D. A., Stout, J. T., Stitt, J. P., and McClearn, G. E. (2005). Adjusting data to body size: a comparison of methods as applied to quantitative trait loci analysis of musculoskeletal phenotypes. *J Bone Miner Res,* 20(5), 748-757.

Li, J. Y., Chassaing, B., Tyagi, A. M., Vaccaro, C., Luo, T., Adams, J., Darby, T. M., Weitzmann, M. N., Mulle, J. G., Gewirtz, A. T., Jones, R. M., and Pacifici, R. (2016). Sex steroid deficiency-associated bone loss is microbiota dependent and prevented by probiotics. *J Clin Invest,* 126(6), 2049-2063.

Lott, B. D. (1991). The effect of feed intake on body temperature and water consumption of male broilers during heat exposure. *Poult Sci,* 70(4), 756-759.

Messora, M. R., Pereira, L. J., Foureaux, R., Oliveira, L. F., Sordi, C. G., Alves, A. J., Napimoga, M. H., Nagata, M. J., Ervolino, E., and Furlaneto, F. A. (2016). Favourable effects of Bacillus subtilis and Bacillus licheniformis on experimental periodontitis in rats. *Arch Oral Biol,* 66, 108-119.

O'Brien, C. A., Jia, D., Plotkin, L. I., Bellido, T., Powers, C. C., Stewart, S. A., Manolagas, S. C., and Weinstein, R. S. (2004). Glucocorticoids act directly on osteoblasts and osteocytes to induce their apoptosis and reduce bone formation and strength. *Endocrinology,* 145(4), 1835-1841.

Rauch, A., Seitz, S., Baschant, U., Schilling, A. F., Illing, A., Stride, B., Kirilov, M., Mandic, V., Takacz, A., Schmidt-Ullrich, R., Ostermay, S., Schinke, T., Spanbroek, R., Zaiss, M. M., Angel, P. E., Lerner, U. H., David, J. P., Reichardt, H. M., Amling, M., Schutz, G., and Tuckermann, J. P. (2010). Glucocorticoids suppress bone formation by attenuating osteoblast differentiation via the monomeric glucocorticoid receptor. *Cell Metab,* 11(6), 517-531.

Reiter, K. and Bessei, W. (2009). Effect of locomotor activity on leg disorder in fattening chicken. *Berl Munch Tierarztl Wochenschr,* 122(7-8), 264-270.

Sadeghi, A. A. (2014). Bone Mineralization of Broiler Chicks Challenged with Salmonella enteritidis Fed Diet Containing Probiotic (Bacillus subtilis). *Probiotics Antimicrob Proteins,* 6(3-4), 136-140.

Schreiweis, M. A., Hester, P. Y., and Moody, D. E. (2005). Identification of quantitative trait loci associated with bone traits and body weight in an F2 resource population of chickens. *Genet Sel Evol,* 37(6), 677-698.

Schwean-Lardner, K., Fancher, B. I., Gomis, S., Van Kessel, A., Dalal, S., and Classen, H. L. (2013). Effect of day length on cause of mortality, leg health, and ocular health in broilers. *Poult Sci,* 92(1), 1-11.

Shim, M. Y., Karnuah, A. B., Mitchell, A. D., Anthony, N. B., Pesti, G. M., and Aggrey, S. E. (2012). The effects of growth rate on leg morphology and tibia breaking strength, mineral density, mineral content, and bone ash in broilers. *Poult Sci,* 91(8), 1790-1795.

Sohail, M. U., Hume, M. E., Byrd, J. A., Nisbet, D. J., Ijaz, A., Sohail, A., Shabbir, M. Z., and Rehman, H. (2012). Effect of supplementation of prebiotic mannan-oligosaccharides and probiotic mixture on growth performance of broilers subjected to chronic heat stress. *Poult Sci,* 91(9), 2235-2240.

Song, J., Xiao, K., Ke, Y. L., Jiao, L. F., Hu, C. H., Diao, Q. Y., Shi, B., and Zou, X. T. (2014). Effect of a probiotic mixture on intestinal microflora, morphology, and barrier integrity of broilers subjected to heat stress. *Poult Sci,* 93(3), 581-588.

Sorensen, P., Su, G., and Kestin, S. C. (1999). The effect of photoperiod:scotoperiod on leg weakness in broiler chickens. *Poult Sci,* 78(3), 336-342.

Sorensen, P., Su, G., and Kestin, S. C. (2000). Effects of age and stocking density on leg weakness in broiler chickens. *Poult Sci,* 79(6), 864-870.

Steel, R. G., Torrie, J. H., and Dickey, D. A. (1997). *Principles and Procedures of Statistics: A Biometrical Approach.* McGraw Hill Book Co., New York, NY.

Su, G., Sorensen, P., and Kestin, S. C. (1999). Meal feeding is more effective than early feed restriction at reducing the prevalence of leg weakness in broiler chickens. *Poult Sci,* 78(7), 949-955.

Talaty, P. N., Katanbaf, M. N., and Hester, P. Y. (2009). Variability in bone mineralization among purebred lines of meat-type chickens. *Poult Sci,* 88(9), 1963-1974.

Talaty, P. N., Katanbaf, M. N., and Hester, P. Y. (2010). Bone mineralization in male commercial broilers and its relationship to gait score. *Poult Sci,* 89(2), 342-348.

Toghyani, M., Toghyani, M., Shahryar, H. A., and Zamanizad, M. (2011). Assessment of growth performance, immune responses, serum metabolites, and prevalence of leg weakness in broiler chicks submitted to early-age water restriction. *Trop Anim Health Prod,* 43(6), 1183-1189.

Toscano, M. J., Nasr, M. A., and Hothersall, B. (2013). Correlation between broiler lameness and anatomical measurements of bone using radiographical projections with assessments of consistency across and within radiographs. *Poult Sci,* 92(9), 2251-2258.

Venalainen, E., Valaja, J., and Jalava, T. (2006). Effects of dietary metabolisable energy, calcium and phosphorus on bone mineralisation, leg weakness and performance of broiler chickens. *Br Poult Sci,* 47(3), 301-310.

Webster, A. B., Fairchild, B. D., Cummings, T. S., and Stayer, P. A. (2008). Validation of a three-point gait-scoring system for field assessment of walking ability of commercial broilers. *J Appl Poultry Res,* 17(4), 529-539.

Weeks, C. A., Danbury, T. D., Davies, H. C., Hunt, P., and Kestin, S. C. (2000). The behaviour of broiler chickens and its modification by lameness. *Appl Anim Behav Sci,* 67(1-2), 111-125.

Wideman, R. F., Hamal, K. R., Stark, J. M., Blankenship, J., Lester, H., Mitchell, K. N., Lorenzoni, G., and Pevzner, I. (2012). A wire-flooring model for inducing lameness in broilers: Evaluation of probiotics as a prophylactic treatment. *Poult Sci,* 91(4), 870-883.

Wideman, R. F., Al-Rubaye, A., Kwon, Y. M., Blankenship, J., Lester, H., Mitchell, K. N., Pevzner, I. Y., Lohrmann, T., and Schleifer, J. (2015). Prophylactic administration of a combined prebiotic and probiotic, or therapeutic administration of enrofloxacin, to reduce the incidence of bacterial chondronecrosis with osteomyelitis in broilers. *Poult Sci,* 94(1), 25-36.

Wideman, R. F., Jr. and Pevzner, I. (2012). Dexamethasone triggers lameness associated with necrosis of the proximal tibial head and proximal femoral head in broilers. *Poult Sci,* 91(10), 2464-2474.

Williams, B., Waddington, D., Murray, D. H., and Farquharson, C. (2004). Bone strength during growth: influence of growth rate on cortical porosity and mineralization. *Calcif Tissue Int,* 74(3), 236-245.

Xie, J., Tang, L., Lu, L., Zhang, L., Lin, X., Liu, H. C., Odle, J., and Luo, X. (2015). Effects of acute and chronic heat stress on plasma metabolites, hormones and oxidant status in restrictedly fed broiler breeders. *Poult Sci,* 94(7), 1635-1644.

Yan, F. F., Murugesan, G. R., and Cheng, H. W. (2015). The effects of dietary supplementation of probiotics on performance, eggshell quality, cecal microflora composition, and skeletal health of White Leghorn hens. *Poult Sci,* 94(E-Suppl. 1).

Ziaie, H., Bashtani, M., Torshizi, M. A. K., Naeeimipour, H., Farhangfar, H., and Zeinali, A. (2011). Effect of antibiotic and its alternatives on morphometric characteristics, mineral content and bone strength of tibia in Ross broiler chickens. *Glob Vet, 7*(4), 315-322.

SUMMARY

Skeletal disorders are a global welfare issue in the poultry industry. Bone fracture in laying hens and lameness in broilers commonly occur under routine management causing chickens to experience chronic pain and mortality. Probiotics are live microorganisms with health benefits for the host when administered in appropriate amounts. Studies conducted to date provide evidence that probiotics improve skeletal health in poultry. Probiotics improve gut health allowing for increased intestinal absorption and bioavailability of minerals such as Ca and P for bone mineralization. Besides enhanced bioavailability of nutrients, other modes of action of probiotics that may include neuroendocrine mechanisms have not been investigated. In this study, the effects of probiotics on skeletal health and underlying cellular mechanisms were examined under different circumstances, including using single or multiple species based probiotics at different dosages, laying hens or broilers, as well as thermoneutral or elevated temperatures.

Skeletal health was improved as a result of dietary probiotic supplementation regardless of the probiotic species, type of poultry (egg laying or meat-type fowl), and environmental temperature. Laying hens consuming a multi-species based probiotic PoultryStar® experienced an increase in tibial and femoral bone mineral density as well as a reduction in shell-less egg production. PoultryStar® increased bone mineralization of the tibia, femur, and humerus in heat stressed broilers and reduced lameness as indicated by a lower gait score and longer latency to lie. Similarly, a single-species based probiotic consisting of *Bacillus subtilis* with the trade name of Sporulin®, improved bone mineralization and bone size traits in broilers at 43 d of age. Moreover, Sporulin® reduced the concentration of systemic inflammatory cytokine TNF-α thereby inhibiting the promoting effect of TNF-α on bone resorption, ultimately stimulating bone growth and bone size traits of 43-d-old broilers subjected to daily cycling elevated temperatures. In addition to improved bioavailability of minerals from the gut, probiotic induced improvement of bone mineralization and other traits indicative of skeletal health may be related to low sympathetic activity mediated by central serotonin.

In conclusion, the current results support our hypothesis that dietary probiotic supplementation improves skeletal health and well-being in poultry. Dietary inclusion of probiotics is a management strategy for the poultry industry to use to improve skeletal health in chickens, especially during hot weather.

VITA

EDUCATION

- **Doctor of Philosophy, Animal Welfare, May 2013 – December 2016 (Expected)**

  Purdue University, West Lafayette, Indiana, USA

  Advisor: Hengwei Cheng, Patricia Y Hester

  Project: "Effect of dietary supplementation of probiotics on skeletal health of poultry".

- **Master of Science, Animal Welfare, January 2011 - May 2013**

  Purdue University, West Lafayette, Indiana, USA

  Advisor: Hengwei Cheng, Patricia Y Hester

  Project: "Effects of modifying cages with perches on physiological parameters of White Leghorn hens"

- **Bachelor of Science, Veterinary Medicine, September 2006 - June 2010**

  Zhejiang University, Hangzhou, Zhejiang, China

  Advisor: Caiqiao Zhang

  Project: "Promoting effect of IGF-1 on prehierarchical follicle development in laying hens"

SCHOLARSHIPS AND AWARDS

- Travel award, Center for Animal Welfare Science, Purdue University, 2016
- W. R. Featherston Outstanding Ph.D. Award, Purdue University, 2016.
- Certificates of Excellence for oral presentation, Poultry Science Annual Meeting, 2015.
- 1/2 Research assistant scholarship, January 2015 - December 2016.
- Travel award, Center for Animal Welfare Science, Purdue University, 2015
- Travel award, BIOMIN America Inc., 2015
- Graduate student team 3rd place, Intercollegiate Animal Welfare Judging/Assessment Contest, 2013.
- Travel award, Center for Animal Welfare Science, Purdue University, 2013
- G. W. Friars international graduate student fellowship, Purdue University, 2013.
- W. R. Featherston Outstanding M.S. Award, Purdue University, 2013.

- 1/4 Research assistant scholarship, January 2011 – December 2014.
- State Scholarship Fund from China Scholarship Council, January 2011 – December 2014.
- Top 100 Graduate Thesis, Zhejiang University, 2010.
- Outstanding Graduate, Zhejiang University, 2010.
- University Scholarships for 4 consecutive years, Zhejiang University, 2006-2010
- Merit Student for 4 consecutive years, Zhejiang University, 2006-2010

EXTENSION ACTIVITY

- Poultry Science Association annual meeting, July 2016
  - Student poster and oral presentation on Behavior and Well-Being
- Purdue Center for Animal Welfare Sciences Spring Symposium, May 2016
  - Student posters on Animal Welfare
- Indiana State Poultry Association /Purdue Poultry Day, September 2015
  - Student poster on Animal Welfare
- Poultry Science Association annual meeting, July 2015
  - Student oral presentation on Metabolism and Nutrition
- Animal Welfare Judging Contest, November 2013
  - Graduate student team
- Indiana State Poultry Association /Purdue Poultry Day, August 2013
  - Student poster on Animal Welfare
- Poultry Science Association annual meeting, July 2013
  - Student poster on Behavior and Well-Being
- Indiana State Poultry Association /Purdue Poultry Day, August 2012
  - Student poster on Animal Welfare
- Poultry Science Association annual meeting, July 2012
  - Student poster on Behavior and Well-Being

PROFESSIONAL MEMBERSHIP

- Poultry Science Association – graduate student member, 2012-present
- USA Branch of the World's Poultry Science Association- student member, 2012-present
- Graduate Student Association to Purdue University Department of Animal Sciences-Seminar Committee Representative, 2011-2012

PUBLICATIONS

A. Peer reviewed journals

1. 贾玉东，**颜菲菲**，曾卫东，张才乔. 胰岛素样生长因子-I 对鸡等级前卵泡发育的促进作用.中国农业科学. 2011, 44(20):4295-4301.
2. Jia Y.D., **F.F. Yan**, W.D. Zeng, C.Q. Zhang. 2011. The effects of IGF-I on avian prehierarchal follicles development. Scientia Agricultural Sinica. 44(20):4295-4301.
3. **Yan, F. F.**, P. Y. Hester, S. A. Enneking, and H. W. Cheng. 2013. Effects of perch access and age on physiological measures of stress in caged White Leghorn pullets. Poultry Science. 92:2853-2859.
4. **Yan, F. F.**, P. Y. Hester, and H. W. Cheng. 2014. The effect of perch access during pullet rearing and egg laying on physiological measures of stress in White Leghorns at 71 weeks of age. Poultry Science. 93:1318-1326.
5. Jiang, S., P. Y. Hester, J. Y. Hu, **F. F. Yan**, R. L. Dennis, and H. W. Cheng. 2014. Effect of perches on liver health of hen. Poultry Science. 93: 1618-1622.
6. Kim, H. W., **F. F. Yan**, J.Y. Hu, H. W. Cheng, and Y. H. B. Kim. 2016. Effects of probiotics feeding on meat quality of chicken breast during postmortem storage. Poultry Science. Poultry Science. 95(6):1457-64.
7. Kim, H. W., D. K. Miller, **F. F. Yan**, W. C. Wang, H. W. Cheng, and Y. H. B. Kim. 2017. Probiotic supplementation and fast freezing to improve quality attributes and oxidation stability of frozen chicken breast muscle. Food Chemistry. 75:34-41.
8. Wu, Y.N., **F. F. Yan**, J. Y. Hu, H. Chen, C. M. Tucker, A.R. Green, and H. W. Cheng. 2016. The effect of chronic ammonia exposure on physiological parameters of plasma and cytokine levels in laying hens. Poultry Science. (Accepted)

B. Abstracts

1. **Yan, F. F.**, P. Y. Hester, S. A. Enneking, and H. W. Cheng. 2012. Effects of modifying cages with perches on neuroendocrine homeostasis of White Leghorn pullets. Poultry Science. 91 (E-suppl. 1): 92
2. **Yan, F. F.,** P. Y. Hester, S. A. Enneking, and H. W. Cheng. 2013. The effect of perch access during pullet rearing and egg laying on physiological parameters of caged White Leghorn hens. Poultry Science. 92 (E-suppl. 1): 92
3. **Yan, F. F.,** G. R. Murugesan, and H. W. Cheng. 2015. The effects of dietary supplementation of probiotics on performance, eggshell quality, cecal microflora composition, and skeletal health of White Leghorn hens. Poultry Science. 94 (E-suppl. 1): 46
4. **Yan, F. F.**, W. C. Wang, R. Wolfenden, and H. W. Cheng. 2016. The effect of *Bacillus subtilis* based probiotic on bone health in broiler chickens. Poultry Science. 95 (E-Suppl. 1): 40
5. **Yan, F. F.**, W. C. Wang, R. Wolfenden, and H. W. Cheng. 2016. Probiotic, *Bacillus subtilis*, effects on bone health in heat stressed broiler chickens. Poultry Science. 95 (E-Suppl. 1):111
6. Wang, W. C., **F. F. Yan,** J. Y. Hu, C. Y. Zhang, and H. W. Cheng. 2016. Effect of dietary supplementation of probiotic, *Bacillus subtilis*, on performance and immune parameters in the brain of broiler chickens under heat stress. Poultry Science. 95 (E-Suppl. 1): 40
7. Wu, Y. N., **F. F. Yan**, C. M. Tucker, A. R. Green, and H. W. Cheng. 2016. The effect of chronic ammonia exposure on plasma physiological parameters of laying hens. Poultry Science. 95 (E-Suppl. 1): 30
8. Cramer, T., H. W. Kim, D. Setyabrata, **F. F. Yan**, H. W.Cheng, and Y. H. B. Kim. 2016. Effect of probiotic supplementation on meat quality attributes of broilers exposed to chronic heat stress. Poultry Science. 95 (E-Suppl. 1):170
9. Miller, D. K., H. W. Kim, W. C. Wang, **F. F. Yan**, H. W. Cheng, and Y. H. B. Kim. 2016. Effects of probiotic supplementation and fast freezing on quality attributes of chicken breast muscle. 69th Annual Reciprocal Meat Conference.