The effects of a single strain *Bacillus subtilis* DFM on pullet performance, laying hen performance, and egg quality when fed from day of hatch until 70 weeks

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Abstract for the General Public

Due to the decreased use of antibiotics across the poultry industry, producers and allied companies are developing new methods to increase bird health and performance when antibiotics are removed from or limited in the diet. One such method is through the use of "good" bacteria or technically called Direct-Fed Microbials (DFM). Direct-fed microbials are defined as live microorganisms that when fed, confer a health benefit to the host. The goals of this thesis were to determine the effects of a *Bacillus subtilis* based DFM on pullet performance, laying hen performance, and egg quality when fed from day of hatch until 70 weeks of age. In the pullet phase, DFM-fed pullets had reduced feed intake and improved feed conversion ratio (the ratio of feed intake to body weight gain), with no differences in body weights or body composition at the end of the 17-week growth period. In the laying phase, DFM fed hens had significantly improved egg production, but lighter eggs compared to the control fed hens. Addition of the DFM significantly improved feed conversion ratio per dozen eggs by 5.3 points and reduced feed conversion ratio on a g/g basis by 7.1 points in the 52-week laying phase. Small improvements were noted in specific gravity, but no differences were reported in shell weight or shell breaking strength. When fed from day of hatch until 70 weeks of age, the *Bacillus subtilis* DFM allowed the pullets to reduce their feed intake, but maintain body development for egg production resulting in an improved feed conversion ratio (FCR) in the growth phase before egg production and once hens initiated egg production resulted in smaller eggs, but increased egg production and

FCR in laying hens with minimal differences in egg quality. Therefore, the DFM may be able to be used in place of growth promoting antibiotics to help support efficient protein production.

The effects of a single strain *Bacillus subtilis* DFM on pullet performance, laying hen performance, and egg quality when fed from day of hatch until 70 weeks

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Scholarly Abstract

Direct-fed microbials (DFM) are a method to increase bird performance and health when antibiotics are removed or limited in the diet. The objective of this experiment was to determine the effects of a single strain *Bacillus subtilis* DFM on pullet performance, layer performance, and egg quality when fed from day of hatch until 70 weeks of age. In total, 576 Hy-Line W-36 laying hen pullets were randomly divided between two treatments (Control and DFM) on day of hatch across 12 cages for each of the treatments and maintained on those respective treatments for 70-weeks. The DFM was added at a target dose of 300,000 cfu/kg of feed. At the end of the 17-week pullet phase, DFM fed pullets resulted in reduced feed intake, improved FCRm with both similar body weights ($P = 0.98$) and body composition ($P > 0.05$) to the control fed pullets. At 17 weeks, 72 birds were randomly selected from each treatment and moved to 12 laying hen cages. At the end of the 52-week laying hen phase, DFM-fed hens had significantly higher hen-day egg production ($P = 0.02$), but decreased egg weights. No differences were reported in egg mass or feed intake, but FCR was significantly improved ($P = 0.02$) on a per dozen eggs basis, and improved ($P = 0.06$) on a g/g basis by 7.1 points with DFM-fed hens having an FCR of 2.016 and control fed hens having and FCR of 2.087. Supplementation of diets with DFM resulted in a trend in egg specific gravity, but no differences were reported in shell breaking strength or shell weight. Overall, supplementation of the DFM lowered feed intake and improved FCR without affecting body composition in the pullet phase, and increased production and improved FCR during the egg laying phase.

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Chapter 1

General Introduction

Concern over the use of growth promoting antibiotics in food animal production has prompted the government to issue regulatory policies aimed at reducing antibiotic use. (EC Regulation No. 1881/2003; FDA's Veterinary Feed Directive and Guidance #213). In response, producers and allied industries are developing alternatives for growth promoting antibiotics that help to maintain bird performance. One such alternative is Direct-fed microbial (DFM) or live microorganisms that when fed, confer a health benefit to the host (FAO, WHO 2002). Previous work done in broilers demonstrated potential DFM benefits including increased nutrient absorption (Murugesan *et al.,* 2014), regulation of gastrointestinal microbiota (Choi *et al.,* 2016), and improved performance in broilers (Gao, 2017). The positive responses noted in fast growing broiler chicken models suggest that positive responses in other poultry for DFM utilization should be investigated and warrant additional research in pullet and laying hens.

There are two main types of DFM that have been evaluated in the laying hen industry; lactic-acid producing and *Bacillus*-based organisms. The primary lactic-acid producing based DFM are species of *Lactobacillus,* a gram-positive bacterium that are naturally found in the microbiota of many species (Mead, 1997). *Lactobacillus* uses competitive exclusion to limit the growth of pathogens and can attach to the epithelial wall of the intestine (Heravi *et al.,* 2019). *Bacillus*-based direct-fed microbials are environmentally tolerant, spore forming microorganisms. The spores formed by *Bacillus* are coated in a protective layer and can stay dormant until environmental conditions are optimal for germination (Errington, 2003). The dormant spores of *Bacillus* species can survive in temperatures ranging from 30-121ºC and pH

ranging from 2-12 (Sharma *et al*., 2018). Once ingested, *Bacillus* spores are able to germinate in the chicken intestine (Casula and Cutting, 2002). Once active in the intestine, *Bacillus* can limit colonization of pathogens by binding to attachment sites, a process called competitive exclusion (Knap *et al.,* 2011). *Bacillus* bacteria can also secrete bacteriocins which can disrupt the cell membranes of competing bacteria (Sharma *et al.,* 2018) and create an anaerobic environment which aids in the balancing of intestinal microbiota (Mahmoud *et al.,* 2017; Sharma *et al.,* 2018). *Bacillus subtilis* also secrete enzymes which can include α-amylase, β-glucanase, lipases, proteases, and levansucrase (Ferrari *et al.,* 1993; Eggert *et al.,* 2000; Eggert *et al.,* 2001; Eggert *et al.,* 2002). *Bacillus* species can also increase the amount of volatile fatty acids produced by the host animal, which aids in the overall health and integrity of the intestinal barrier (Oladukun *et al.,* 2021). Although all of these activities have been associated with various *Bacillus* subspecies, they are not inherent to all subspecies, requiring further development for commercial use of specific organisms.

The efficacy of DFM use in pullet and laying hen production is inconsistent. The use of DFM in pullet production has resulted in contrasting results in feed intake (Neijat *et al.,* 2019; Upadhaya *et al.,* 2019) body weights (Li et *al.,* 2018, Updadhaya *et al.,* 2019) and feed conversion ratio (Neijat *et al.,* 2019; Upadhaya *et al.,* 2019). Neijat and colleagues (2019) fed three concentrations (1.1×10^8 g/kg (low), 2.2×10^8 g/kg (medium), and 1.1×10^9 g/kg (high)) of *Bacillus subtilis* to Hy-Line Brown pullets from 5-16 weeks of age and reported a decrease in feed intake, increase in body weights, and improvement in feed conversion ratio from 5-10 weeks. Another report fed 8.0×10^5 CFU of either *Bacillus subtilis* or *Bacillus licheformis* and reported a decrease in feed intake in the *Bacillus subtilis* supplemented group, but no differences in body weights or feed conversion ratio regardless of DFM (Upadhaya et al., 2019). Similar

results were reported in another study which fed a combination of antibiotic growth promotors (bacitracin zinc and colistin sulfate) and *Bacillus* subtilis (1.6×10^9 cfu/g) resulted in no difference in body weight, feed intake, or feed conversion ratio in Hy-Line Brown pullets (Li et al., 2018).

As seen with pullet production, laying hen egg production (Abdulrahim *et al.,* 1996; Nahashon *et al.,* 1996; Forte *et al.,* 2016; Zhang and Kim, 2013), egg weights (Ribeiro Jr, 2014; Nishiyama *et al.*, 2021), and feed conversion ratio (Wang *et al.,* 2021) have all been inconsistent. Different sub-strains of *Bacillus subtilis* have been shown to have individual responses on bird performance and effectiveness (Zhang and Kim, 2013; Forte *et al.,* 2016; Upadhaya *et al.,* 2019). Additionally, age and breed of laying hen could contribute to inconsistent reports.

Minimal differences in internal egg quality have been reported when feeding *Bacillus subtilis*. Direct fed microbial treatment of laying resulted in darker yolks, but no difference in Haugh unit (Upadhaya et al., 2019). Some reports have found no differences in Haugh units between control hens and DFM supplemented hens (Zou *et al.,* 2021). However, increases in eggshell weight and eggshell thickness have been found with supplementation of DFM, specifically *Bacillus subtilis* (Abdelqader *et al.,* 2013; Wang *et al.,* 2021).

Previous research using DFM treatment has focused on broiler models (Gao *et al.,* 2017; Wang *et al.*, 2018; Hernandez-Patlan *et al.,* 2019) and laying hen models starting at peak to post peak production (Tang *et al.,* 2015; Abd El-Hack *et al.,* 2016) or in the later stages of egg production (Abdelqader *et al.,* 2013; Wang *et al.,* 2021). Little research has been completed on the long-term effects of a DFM from the pullet rearing period to late-stage egg production. The purpose of the pullet phase is to reach growth and reproductive maturation targets to allow efficient egg production for the duration of the egg laying period. When chicks are fed DFM

starting as pullets, results are much more consistent compared to when hens were introduced to DFMs in the laying phase (Patterson and Burkholder, 2003). Hy-Line Brown pullets fed either a *Bacillus subtilis* or a *Bacillus lichenformis* DFM beginning at 12 weeks reported lower feed intakes with similar body weights during the 6-week experimental period. The pullets treated with DFM continued DFM treatment in the laying phase (25-45 weeks) and reported significantly higher egg production (Upadhaya *et al.,* 2019). Similar results were reported when feeding a single strain *Bacillus subtilis* to Shaver White pullets from 5-16 weeks of age, followed by a laying hen experiment from 19-48 weeks of age. Feed conversion ratio was improved in both the pullet and laying hen phases (Neijat *et al.,* 2019a; Neijat *et al.,* 2019b).

The first objective of this thesis was to determine the effects of a single-strain *Bacillus subtilis* (Q-Biotic™) on pullet performance including body weights, feed intake, mortality-corrected feed conversion ratio (FCRm), and body composition when fed from day of hatch until 17 weeks of age. The second objective was to determine the effects of the same strain of *Bacillus subtilis* on laying hen performance and egg quality from 18 to 70 weeks.Hens were kept on either the control of DFM treatment from the pullet phase.

Therefore, the hypothesis for this thesis is;

- At an inclusion rate of 300,000 cfu/g *Bacillus subtilis,* DFM-fed pullets will be more efficient in their growth.
- At an inclusion rate of 300,000 cfu/g *Bacillus subtilis,* DFM-fed hens will have increased production performance and egg quality.

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Chapter II

Review of the Literature

Overview and Reasons to Use Direct-Fed Microbials

Direct-Fed Microbials (DFMs), also called probiotics, are defined as a live microorganism that when provided in the diet, confers a health benefit to the host (FAO, WHO, 2002). With the decreased use of antibiotics within the industry due to public perception (Norberg *et al.,* 2005), increased bacterial resistance from continued use of antibiotics (van Boeckel *et al.,* 2015), and the growing demand for a No Antibiotics Ever (NAE) market, DFMs have been increasing in popularity across the poultry industry. Direct-fed Microbials were first used in the broiler industry because of the need to decrease the risk of coccidiosis, necrotic enteritis, and increase bird growth and performance when antibiotic growth promoters were removed from the diet (Teo and Tan; 2007; Wang *et al*., 2021). Probiotics are accepted as alternatives to antibiotics for use as growth promoters and in some cases, control of pathogens (Anadón *et al.,* 2006; Boyle *et al.,* 2007; Vilá *et al*, 2009). Many of the positive effects of increased nutrient absorption (Murugesan *et al.,* 2014), regulating gastrointestinal microbiota concentrations (Choi *et al.,* 2016), and improvement on performance parameters (Qui *et al.,* 2021) seen in broilers have translated well into the laying hen sector. Direct-Fed Microbials are now being researched and used more widely in the laying hens because of the benefits to limit food-borne pathogens (Lee *et al.,* 2015; Kamada *et al.,* 2013), increase egg production and quality parameters (Nahashon 1994; Abdularahim *et al.,* 1996; Li *et al.,* 2006; Panda *et al.,* 2008; Zhang and Kim, 2013), and increase the ability of laying hens to utilize nutrients more efficiently due to a more diverse and healthy gastrointestinal system (Choi *et al.,* 2016; Guo *et al.,* 2018;

Murugensen *et al.,* 2014). Previous research has focused on the peak to post peak egg production phase (Tang *et al.,* 2015; Abd El-Hack *et al.,* 2016), or in the late stages of egg production (Abdelqader *et al.,* 2013; Wang *et al.,* 2021). However, little research has been done on feeding a DFM across both the pullet and laying phases of hens. Direct-Fed Microbials are a way to potentially increase performance and increase bird health as well as meet governmental regulations over the ban of growth promoting antibiotics (EC Regulation No. 1881/2003; FDA's Veterinary Feed Directive and Guidance #213).

Previous Direct-Fed Microbial Research

Mixed results have been reported over the efficacy of feeding DFMs to both pullets and laying hens. There are many possible reasons results have been contrasting over the use of DFMs. Environmental conditions, strains, dosage, concentration, and viability of the DFM could be an answer as to why researchers have had differences in results. Differences have been reported in pullet performance (Li *et al.,* 2018; Upadhaya *et al.,* 2019), laying hen performance (Nahashon *et al.,* 1996; Tang *et al.,* 2018; Guo *et al.,* 2018; Wang *et al.,* 2021), and egg quality (Panda *et al.,* 2008; Gallazi *et al.,* 2009; Fathi *et al.,* 2017). Few of these studies supplied the same strain of DFM at the same concentration, and management practices were different between some experiments. Well managed birds will be at less of a risk for reduced performance and increased pathogen inhabitation, which limits the potential effects of the DFM. Additionally, there will be variations in gastrointestinal microflora in hens based on their geographic location. Forte *et al*., (2016) designed an experiment that studied the effects of probiotics on organically raised laying hens in Italy, and did not observe any performance differences, whereas Abd El Hack (2016) observed differences in traditionally commercial raised layers with higher stocking density and no access to the outside environment. In all chickens, diversity and stability of the

GIT microbes increase the fastest within the first few weeks of life, but environmental factors, housing, and feed access cause variability in the intestinal microbiota composition (Kers *et al*., 2018).

Impact of DFM in Pullet Growth and Performance

The pullet growth period is relatively short compared to the rest of the laying hen cycle, so it is important to start chicks correctly from day 1 to avoid potentially detrimental effects in their short growing period. It is now being recommended to begin probiotic supplementation starting at day of hatch, and researchers are even finding out new ways to begin DFM supplementation in ovo (Roto, 2016). There is limited research about the efficacy of DFMs in laying hen pullets due to the high cost and high labor associated with raising both layers and pullets. While broilers are much faster growing, the comparisons in young bird gastrointestinal systems and factors associated with pullet body growth and composition are similar. Broiler studies may provide the best model when pullet research is restricted.

Like any young animal, newly hatched pullets have a very weak and underdeveloped gastrointestinal microbiota population (Ballou *et al*., 2016). Therefore, it is important in early stages of the pullet phase to provide chicks a clean environment and begin to diversify the gastrointestinal microbiota population. Previous literature shows the benefits of early introduction of DFMs to pullets to prevent diseases and potential economic losses (Bansal *et al.,* 2011). Interruption of the gastrointestinal system can weaken the immune system in the pullet phase and can potentially affect the laying cycle. It is vitally important in both young broilers and young pullets to establish a diverse gastrointestinal community while birds are still young. Feeding DFMs could lead to a more diverse microbial community that limits the risk of harmful

pathogens to proliferate and could possibly affect production factors such as body weights, feed intakes, and FCRm (Li *et al*., 2018; Upadhaya *et al*., 2019).

During the pullet phase, many physiological changes take place to set pullets up for a productive life as layers. Interactions between the environment and intestinal microbes play an important role in preventing harmful pathogens from colonization (Kohl, 2012), metabolism of nutrients and production of SCFA (Dunkley *et al.,* 2007), and the development and diversity of the gastrointestinal immune system (Rakoff-Nahoum *et al*., 2004). A study completed discovered that the intestinal microbiota of white Lohmann laying hen pullets matured at day 50. In that experiment, they discovered that beneficial bacteria *Lactobacillus* was the dominant type of bacteria in the small intestine which matured around 2 weeks of age (Xiao *et al.,* 2021). Li and colleagues (2018) looked at the effects of introduction of *Bacillus subtilis* at different bird ages. Control pullets were fed an antibiotic growth promoter (AGP) as a control, and then supplemented with a combination of AGP (colistin sulfate and bacitracin zinc) and *Bacillus subtilis* lasting for 3, 6, 12, or 16 weeks at a concentration of 500 mg/kg *Bacillus subtilis*, 20 mg/kg bacitracin zinc, and 4 mg/kg colistin sulfate. No differences in body weights or feed intakes were observed throughout the experiment. Pullets fed *Bacillus subtilis* had a higher, more stable population of beneficial bacteria in the ceca and intestinal mucosa populations compared to pullets just fed AGP. At 13-16 weeks of age, sucrase expression was upregulated in the *Bacillus* subtilis/AGP supplemented pullets and the content of sIgA was increased, which could represent improved digestion, nutrient retention, and increased mucosal immunity. (Knap *et al*., 2011; Cui, 2017). Similarly, no differences were reported in body weight in a study determining the effects of *Bacillus subtilis* on Hy-Line Brown pullets. In the same experiment, pullets fed *Bacillus subtilis* consumed significantly less feed (Upadhaya *et al.,* 2019). Probiotics, such as

Bacillus subtilis, secrete certain carbohydrases, lipases, and amylases that can break down the more complex bonds of carbohydrates, which in turn gives the host animal access to those nutrients (Ferrari *et al.,* 1993; Eggert *et al.,* 2000; Eggert *et al.,* 2001; Eggert *et al.,* 2002). Contrary to the previous study, Hatab and colleagues (2016) found an increase in body weight and an improvement in FCR in young pullets treated with a combination of *Bacillus subtilis* and *Enterococcus faecium* across a 10-week period. Though the DFM-fed pullets weighed more, they consumed consistently less feed throughout the duration of the experiment. Neijat and colleagues (2019) performed an experiment on feeding three different levels (1.1×10^8 g/kg (low), 2.2×10^8 g/kg (medium), and 1.1×10^9 g/kg (high)) of *Bacillus subtilis* to Hy-Line Brown pullets from 5-16 weeks of age. This experiment consisted of two phases; grower (5-10 weeks) and developer (11-16 weeks). Neijat reported an increase in body weights and improvement in FCR in the grower phase in all supplemented groups. Neijat and collaborators also reported a decrease in feed intake in the developer phase.

In broiler studies, researchers observed a difference in body weight and an improved FCR in *Bacillus subtilis* supplemented birds compared with broilers fed the control diet. Supplementation also significantly increased the counts of *lactobacillus* in the ileum and decreased the counts of *E. coli* (Molnar *et al.,* 2011). In another broiler study, broilers fed 2.9×10^9 cfu/g had improved average daily gain and average daily feed intakes compared to control-fed broilers. DFM fed broilers consumed an average of 95.4 grams from day 1-42, compared to that of the control fed broilers that consumed 92.34 grams. The increased feed intake resulted in improved body weight gain in DFM fed broilers, although no differences were reported in feed intake (Qui *et al.,* 2021).

The purpose of the pullet phase is to set birds up for a productive life as laying hens. Any disruption in the pullet phase could potentially cause detrimental effects in laying production. Effects of early introduction to direct-fed microbials could make more of an impact in the underdeveloped gastrointestinal tract of pullets compared to the more developed and diverse microbiota population in laying hens. When chicks are fed DFMs starting as pullets, results are much more consistent compared to when hens were introduced to DFMs in the laying phase (Patterson and Burkholder, 2003). The benefits of early supplementation of a DFM to newly hatched pullets may lead to a higher productivity in the laying hen phase.

Impact of DFM on Laying Hen Performance

Laying hens go through an egg production cycle that peaks when hens are young and gradually decrease over time. The challenge for poultry producers is to ensure that egg production is maintained as hens age. Ribeiro (2014), Tang (2018), and Upadhaya (2019) saw significant differences in egg production, egg mass, feed intakes, and body weights. Tang (2018) observed that egg production was improved with *Bacillus amyloliquefaciens* supplementation when fed at a concentration of 1.0×10^7 cfu/kg. Guo and colleagues (2018) reported a significant difference in feed/egg ratio when Hy-Line Brown hens were fed *Bacillus subtilis* at 1.0×10^8 CFU/g. DFM-fed hens averaged an FCR of 1.94 over the 22-week experimental period, compared to 2.04 of the control fed hens. Similarly, Abdulrahim and colleagues (1996) also noted an increase in egg production and an improved FCR when hens were supplemented with 100 or 150 mg/kg of *lactobacillus acidophilus* and bacitracin. An effect of probiotic supplementation on differing levels of corn dried distillers grain solubles (DDGS) was also reported. Hi-sex brown hens aged 22-34 weeks were given 4 different levels of DDGS and supplemented with 1000mg/kg of *Bacillus* subtilis at a concentration of 1.5×10^8 CFU. Feed

conversion ratio, egg weight, and egg mass improved by 6.45%, 3.27% and 7.60% respectively. The interaction between DFM and DDGS was significant in egg weight, egg mass, and eggshell thickness (Abd El-Hack *et al.,* 2016). *Bacillus subtilis* secretes enzymes that can break down the more complex bonds of carbohydrates (Ferrari *et al.,* 1993), which could explain why hens supplemented with up to 20% DDGS and *Bacillus subtilis* in the diet showed better production parameters compared to hens given no supplementation of DFM. In late-stage production hens, a significant improvement in FCR was reported when older hens were supplemented with *Bacillus subtilis* (Wang *et al.,* 2021). This agrees with the findings of Abdelqader and colleagues (2013) who saw an improvement in egg production in late cycle Lohmann white hens when supplemented with 1g/kg of *Bacillus subtilis* at a concentration of $2.3x10^8$ CFU. Egg weight and egg mass (g/hen/day), and feed conversion were also improved. Nahashon (1994) fed viable dried forms of *lactobacillus* at 2 concentrations of 4.4×10^7 cfu/mg and 8.8×10^7 cfu/mg and reported a significant increase in egg production, with hens fed 4.4×10^7 cfu/mg averaging 90.7% production compared to control hens averaging 88.9% production. Haddadin (1996) fed hens three different concentrations of liquid cultured *lactobacillus*, at concentrations of 0.67×10^6 cfu/g, 2.0×10^6 cfu/g, and 4.0×10^6 cfu/g, respectively, and reported a significant increase in egg production in DFM supplemented groups.

Facing similar contrasting results, Mahadavi (2005) fed hens a double strain *Bacillus subtilis* and *Bacillus licheniformis* DFM at concentrations of 1.28×10^6 cfu/g, 3.2×10^6 cfu/g and 4.6×10^6 cfu/g, and noted no significant differences across egg weight, egg production, egg mass, or feed conversion ratio. Davis and Anderson (2002) and Tortuero and Fernandez (1995), saw an increase in egg weight with supplementation of a DFM in laying hen diets. Davis and colleagues (2002) achieved a significant increase of 0.6 g/egg with supplementation of a multi

strain lactic acid producing based DFM containing a minimum of 1.0×10^8 cfu/g of the organisms *Lactobacillus acidophilus, Lactobacillus casei, Enterococcus faecium,* and *Bifidobacterium thermophium*. Tortuero and Fernandez (1995) supplied a combination of *Lactobacillus acidophilus* and *Lactobacillus casei* at a concentration of 2.3 x 10^8 cfu/g, which significantly improved hen-day egg production, egg weights, and feed conversion ratio. Forte and colleagues (2016) fed hens a diet containing either *lactobacillus* or *Bacillus subtilis* at concentrations of 0.1% and 0.05% respectively. No differences were noted in egg production, egg weight, or feed conversion ratio. Forte (2016) supplied both DFMs at lower concentrations than Abdularahim (1996) and Xu (2006). Additionally, Forte's study was completed in an organic, outdoor, rearing system in the rural regions of Italy and was conducted using Hy-line Layer Hybrids.

Impact of DFM on Egg Quality

Production parameters such as FCR worsen as hens get older, eggs get larger, shells get thinner, and egg quality worsens with age. Hens are extremely efficient in peak production, often reaching production numbers as high as 95%. High volume egg production takes a toll on hens and effects can be seen in the later stages of production. As a result of this normal biological cycle, researchers have been looking at the effects of feeding probiotics to laying hens in the later stages of production, especially to enhance shell quality and thickness.

DFMs could not only affect production but add to the quality of production. Abdelqader (2013), Tang (2017), Sobczack and Koslowski (2016), Mohan (1995), and Upadhaya (2019) observed an increase in shell strength and quality. Upadhaya (2019), and Sobczack and Koslowski (2015) observed a positive increase in interior egg quality. The goal of DFMs is to enhance host animals' growth and performance while strengthing the production of VFAs from microbes. (Endt *et al*., 2010). Those VFAs are then used to provide energy to enhance

production. (Callaway *et al*., 2017). With those basic biological functions covered, hens can divert energy towards the production of higher quality eggs. Egg quality becomes critically important as hens age. The gastrointestinal mucosal system becomes weaker and unable to protect from harmful pathogens, which increases the likelihood of diseases to populate with the hen, which could lead to food borne illnesses, such as *salmonella* or *campylobacter.* Additionally, eggs become larger as hens get older, but eggshells become thinner (Jones 2002; Messens 2007). This is because the amount of calcium deposited in the shell remains the same, no matter the size of the egg. (Etches, 1996). This is an issue in the commercial industry as weak or broken shells account for an estimated 6-10% of all eggs produced worldwide (Roland, 1988). Laying hens pull an estimated 30-40% of calcium deposited in the eggshell from their bones, which causes osteoporosis in hens at the later stages of production (Sandilands, 2011). Many studies have tried to solve this problem of poor quality eggshells by increasing the calcium content fed in the diet, but the more effective approach has been increasing the amount of available calcium at the time of which the shell is formed, in the later hours of the day (Skrivan *et al*., 2010). Probiotics, such as *Bacillus subtilis,* do well in the low pH of the lower GIT (Scholz-Arens *et al*., 2007). Subsequently, minerals, such as calcium and phosphorus, also solubilize better in a lower pH environment. (Ashmead *et al*., 1985). *Bacillus subtilis* potentially increases the amount of lactic acid producing bacteria, such as *lactobacillus,* which decreases the pH of the lower GIT in the chicken (Wu *et al.,* 2011) The increased nutrient retention of hens supplemented with DFMs may help hens absorb more calcium from the diets, and thus increase eggshell strength and thickness (Wang *et al.,* 2021). Abelqader and colleagues (2012) observed the effects of *Bacillus subtilis* in laying hens aged 64-75 weeks. They found that calcium retention was improved in the probiotic supplemented group, and eggshell quality subsequently

increased. The DFMs favor the lower pH of the lower gastrointestinal tract and secrete more enzymes which helps solubilize for calcium and phosphorus that can then be used to create a stronger shell. This agrees with the findings of Zou *et al.,* (2021) that hens supplemented with *Bacillus subtilis* had stronger, thicker shells and stronger bones. Abdulrahaim and colleagues (2012) also reported increased shell weight, shell thickness, and shell strength. Hashemipour (2011) reported an increase in egg specific gravity, which is an indirect measure of eggshell thickness. Wang and colleagues (2021) saw an increase in breaking strength, shell thickness, calcium retention, and serum calcium content of 72-79 week old Hy-Line Brown laying hens when fed at a concentration of 2.9×10^9 cfu/kg.

Overview and Mode of Action of *Bacillus subtilis*

Bacillus bacteria are some of the toughest forms of life on earth and can survive in temperatures ranging from 30-121 degrees Celsius and a pH ranging from 2-12. (Sharma *et al*., 2018) They have gained more attention as a DFM because of their tolerance to many types of environments. They are extremely shelf stable, and spores have been shown to germinate within the gastrointestinal tract of laying hens (Casula and Cutting, 2002). *Bacillus* is a rod shaped, gram positive, spore forming bacteria that secretes bacteriocins, lipopeptides, and other pathogen inhibitory substances (Sharma *et al*., 2018). There are many different strains, including *amyloliqufians, lichenformas*, and *subtilis, Bacillus subtilis* is the most common strain of *Bacillus* used as a DFM because of its ability to secrete bacteriocins, upregulate genes associated with nutrient uptake, secrete enzymes, and have a strong relationship with the normal gut microbiota in chickens. *Bacillus subtilis* works in 3 main ways; competitive exclusion (Burton *et al*., 2013), secreting bacteriocins (Sharma *et al*., 2018) and altering the intestinal microbiota and mucosal genes (Choi *et al*., 2016; Guo *et al*., 2018).

In any environment, there is a finite amount of space and resources that provide nutrients for life to grow. When a host is supplemented with *Bacillus*, spores germinate and take up space along the epithelial wall of the intestine and take nutrients from otherwise harmful pathogens (Casula and Cutting, 2002). This is very well documented in studies where laying hens or broilers were challenged with diseases and then given *Bacillus* as a treatment. In a study done by Thirabunyanon and Thongwittaya (2011), *Bacillus subtilis* isolates were found to be highly effective in growth inhibition of *Salmonella* enteritidis, *Salmonella* Typhimurium, and *Escherichia coli.* Many studies such as Oh *et al.,* (2017), Vilà *et al.,* (2009), and Callaway *et al.,* (2017) observed lower counts of *salmonella* with and higher counts of good bacteria such as *lactobacillus* which shows the competitive exclusion properties of *Bacillus*. In broilers, Molnar (2011) also observed an increase in *lactobacillus* in the ileum and a decreased population of *E. coli* compared to the control fed birds. *Bacillus subtilis* also produce bacteriocins in two classes. Class I bacteriocins include antibiotic like bacteria that can undergo certain post translational modifications to kill specific pathogens and Class II bacteriocins are pH and heat tolerant ribosomal peptides. These bacteriocins work by forming pores along the cell membrane or wall of the pathogen and forcing the interior contents outside the cell. *Bacillus* also regulates the environment of the intestine and promotes the survival of epithelial cells, enhancing the function of tight-junction barriers, and stimulates the protective response of the intestine to enhance innate immunity (Sharma *et al*., 2018). *Bacillus subtilis* secrete lipopeptide surfaces which limit PLA2, which then downregulates the inflammatory cytokines and upregulates the anti-inflammatory cytokines, making for a healthier gastrointestinal system. The inflammatory cytokines include Il-6 and TNF-alpha which are associated with tissue damage (Selvam *et al*., 2009).

Impact of DFM on Intestinal Microbiota Concentrations

DFMs can be used to enhance the gastrointestinal microbes to limit potentially harmful bacteria and can enter an establish gastrointestinal system and improve the diversity of the microbe population (Callaway, 2017). DFMs can block the effects of pathogens by producing substances that are toxic to pathogens, known as bacteriocins, so they are unable to adhere to the epithelial wall. They may also regulate the immune system and stimulate protective responses (Selvam *et al*., 2009).

The gastrointestinal tract of any animal is a complex ecosystem. The function of the gastrointestinal system is not only the digestion and absorption of nutrients, but also directly correlates with the health of the animal. Many types of bacteria are found in the gastrointestinal tract, which make up the microbiota. Microbiota are defined as the commensal, symbiotic, and pathogenic communities that are twice as populous as somatic and germinal cells of the host animal (Sender *et al*., 2016). DFMs may potentially maintain the homeostasis of this system (Thaiss *et al.*, 2014). At day 0, the diversity of microbiota in the GIT of laying hen chicks is the highest in the duodenum, but diversity increases in the lower GIT (ileum and ceca) as birds get older, with particularly higher counts of anaerobic bacteria, such as *Bacteroides and Rumminococcaceae* (Xiao *et al*., 2021). Diet, environment, and resident microbiota are some factors that can affect the environment of the gastrointestinal system (Lan *et al.,* 2016; Ding *et al.,* 2017). Resident microbiota are the most important microorganisms because of their roles of digesting feed, protecting against infection, and maintaining homeostasis. Supplementation of direct-fed microbials aim to add to the numbers of good bacteria (*Firmicutes*, *Lactobacillus*, *Bifidobacterium*) to aid in absorption of nutrients, prevent pathogen invasion, and produce energy-rich substances through the main mode of action of competitive exclusion (CE). DFMs also aid to diversify the bacteria of the lower gastrointestinal system. The microbiome of poultry

has been directly related to immune system promotion and development (Jinmei *et al.,* 2017). Furthermore, diversity has been directly related to stability of the microbiome, which leads to sustained egg production and higher egg quality (Janczyk *et al.,* 2009).

Direct-Fed Microbials have their mode of action in the gastrointestinal tract of the chicken and use properties such as competitive exclusion to take up space and nutrients in the epithelial wall of the intestine to promote the growth of good bacteria and limit the colonization of harmful pathogens (Sharma *et al.,* 2018). Normal intestinal microbes are competitive by nature, and supplementation of a DFM enhances the effects of the good bacteria to limit pathogen colonization. Probiotics create an anaerobic environment that is favorable to beneficial bacteria and harmful to pathogens (Mahmoud *et al,* 2017; Sharma *et al.,* 2018). Probiotics compete for attachment sites along the epithelial wall, furthering their CE properties (Sharma *et al.,* 2018).

The ceca are the primary fermentation sites in the chicken and are home to the most diverse microbial community, which are mainly comprised of the phylum *firmicutes*, *bacteroidetes*, *proteobacteria*, and *actinobacteria* (Wei *et al*., 2013) The microbiota in the ceca aid in recycling nitrogen, and help digest non-starch polysaccharides, which in turn stimulate the production of short chain fatty acids (Marounek *et al*., 1999, Jamroz *et al*., 2002). Neijat and colleagues (2019) studied the effects of different concentrations of *Bacillus subtilis* on ceca microbiota concentrations. The main phyla of bacteria in the ceca, no matter the treatment were *Firmucutes* and *Bacteroidetes.* Though there were no main differences at the phyla level, more differences were noted at the class and family level. Notably, hens fed at a high concentration of *Bacillus subtilis* (1.1×10^9 cfu/kg) had more enriched members of the *Firmicute* phylum, including *Lachnospiraceae* and *Faecalibacterium*, which are associated with improving the

efficiency of the metabolism and lower counts of harmful pathogens. Also, the genus of *Alistipes* was positively correlated with the supplementation of *Bacillus subtilis*. *Alistipes* produce propionate, succinic acid, and isobutryric acid. (Rautio *et al.,* 2003; Polansky, 2016). Control fed pullets in Neijat's study had higher counts of undesirable bacteria, such as *Erysipelotrichaceae*, associated with inflammation, and *Bilophelia,* recognized as a mucin degrader in comparision to birds supplemented with probiotics. Yang and colleagues (2016) observed increased counts of *Lactobacillus and Bifodobacterium,* and lower counts of *Salmonella* and *E. Coli* in Huanin partridge chickens treated with *Bacillus subtilis* at a concentration of 1.0×10^9 cfu/g. Adams and Hall (1988) observed higher counts of lactic acid producing bacteria in the ceca of chickens fed *Bacillus subtilis.* In turn, decreased amounts of harmful pathogens lead to increased space and nutrients for a variety of helpful bacteria to populate. Kitano and Oda (2006) recommend the host animal to have a broader range of bacteria in the GIT to cope with potential environmental stressors. A study completed by Zhang and collaborators (2017) studied the effects of probiotic supplementation in heat stressed laying hens. The probiotic was comprised of 5.0×10^5 cfu/g *enterococcus faecium* and 4.0×10^6 cfu/g *Bacillus* subtilis. Significantly higher counts of *lactobacilli* in the ileum and ceca were reported in heat stressed hens supplemented with the probiotic compared to heat stressed hens receiving no probiotic (Zhang *et al.,* 2017). Guo and colleagues (2018) also revealed a significantly higher Shannon index, a method to determine alpha microbiota diversity, in birds treated with *Bacillus subtilis*, indicating a higher amount of diversity within the microbiota population. At the genus level, *Bacillus* treated hens had a higher abundance of *Alistipes, Bacillus, Lactobacillus,* and *Rumonococcaceae UG-10*. There was also lowered counts of *Campylobacter* and *Clostridium sense strico 1.* Relative abundance of *Tenericutes* were increased and abundance of *Fusobacteria* were decreased. This data

demonstrates the ability of DFMs to increase the diversity and therefore stability of the microbial community, which allows hens to have an improved immune system and divert more energy to production (Haghigi *et al.,* 2005). Guo was able to locate 942 genes the intestinal mucosa population of hens supplemented with *Bacillus subtilis.* Of these genes, 400 were upregulated. The upregulated genes were involved in the PPAR pathway, starch and sucrose metabolism, and amino acid metabolism including amino acids glycine and threonine. Park and colleagues (2020) observed a difference in 72 total metabolites with two different strains of *Bacillus subtilis.* Of the affected metabolites, 30 were increased and 42 were decreased. Dipeptides such as alanyleucine, phenyalaninealanine, and guanine were also altered, along with fatty acids valerylglycine and fructose.

Conclusion

Direct-fed microbials, specifically *Bacillus subtilis,* are a potential way to increase bird health and performance. They help improve gastrointestinal health through the properties of competitive exclusion (Burton *et al*., 2013; Callaway *et al*., 2017; Oh *et al*., 2017; Vilà *et al*., 2009) bacteriocin properties (Selvem *et al*., 2009; Sharma *et al*., 2018), and secreting enzymes that can break down the more complex bonds of feedstuffs and aid nutrient absorption. (Santoso *et al*., 2001; Guo *et al*., 2018). Though there have been contrasting results over the efficacy of probiotics in laying hens, factors such as environment, management, bird age, dosage, and strain are factors that could have caused the contrasting results between experiments. Nonetheless, researchers Nahashon (1994), Abdularahim et al., (1996), Xu et al., (2006), and Zhang and Kim (2013) reported statistically significant differences in the production performance of laying hens, contrary to the findings of Gooding (1987), Tortuero and Fernandez (1996), Mikulski (2012), and Forte et al., (2013). *Bacillus subtilis* may lower the pH of the lower GIT which helps

solubilize more calcium (Abedelqader *et al*., 2012) which in turn can lead to stronger eggshells as hens get age. Direct-fed microbials, specifically *Bacillus subtilis* increase the counts of beneficial bacteria such as *lactobacillus* and *bifodobacterium*. (Adams and Hall, 1988; Guo *et al.,* 2018; Neijat *et al.,* 2019) It can also aid in increasing the diversity of the GIT microbiome and upregulate mucosal genes associated with increased absorption of nutrients. (Guo *et al.,* 2018; Park *et al.,* 2020). *Bacillus subtilis* is becoming more popular as a DFM due to its ability to withstand harsh environments, long shelf-life stability, and its ability to germinate within the intestinal walls of laying hens (Sharma *et al.,* 2018; Casula & Cutting, 2002). Each strain of probiotic has different modes of action and can be selected for specific traits, such as increased enzymatic activity or increased bacteriocin production. Previous research has focused on broilers (Chen *et al.,* 2009; Molnar *et al.,* 2011; Qui *et al.,* 2021), laying hens in peak to post peak production (Tang *et al.,* 2015; Abd El-Hack *et al.,* 2016), or in late-stage production (Wang *et al.,* 2021). Further improvements in laying hen production may be seen when pullets are introduced to DFMs early and continued throughout the entire production cycle.

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Chapter III

Effects of a *Bacillus* subtilis DFM on Pullet and Laying Hen Performance when fed from Day 0 to Week 70
(Formatted for Publication in Journal of Applied Poultry Research)

Summary

Direct fed microbials (DFM) have been used to improve animal health and performance with the reduction or removal of antibiotics from the diet. This experiment was conducted to determine the effects of a DFM on pullet performance, laying hen performance, and egg quality when fed from day 0 to 70 weeks. In total, 576 Hy-Line W-36 chicks were housed in high rise three tier pullet cages with 24 chicks per cage to across 12 replicate cages for each of the two treatments (Control and DFM). Chicks were provided *ad libitum* access to water throughout the experiment and ad libitum access to feed for the first three weeks, followed by controlled daily feeding according to the Hy-Line performance objectives. At six and 12 weeks of age, 6 birds per cage were removed to generate more commercial relevant stocking density, resulting in a stocking density of 36 in^2 from 6-12 weeks and 48 in^2 from 12-17 weeks. At 17 weeks of age, three birds per cage were euthanized to determine lean and fatty tissue content via DXA analysis. Six pullets per EU (72 birds per treatment) were randomly selected and transferred to laying hen cages at a stocking density of 72 in^2. Hens were provided *ad libitum* access to water and controlled daily feeding according to breeder recommendations based on egg production. In the pullet phase, supplementation of DFM reduced feed intake over the length of the experiment ($P \leq 0.01$), with no effect on body weight ($P = 0.98$) or body composition ($P > 0.10$). Similar pullet body weight with reduced feed intake resulted in improved FCRm when repeated measures were used ($P = 0.03$) with an overall 17-week FCRm of 4.232 and 4.259 for DFM and control fed birds,

respectively. In the laying hen phase, supplementation of the DFM increased hen-day egg production ($P = 0.02$), resulting in 86.0% and 84.1% production for DFM and Con fed hens, respectively. No differences were reported in feed intake ($P = 0.56$). FCR was improved on a kg/dozen basis, with DFM-fed hens having a value of 1.378 kg/dozen (3.032 lbs/dozen) compared to 1.431 kg/dozen (3.148 lbs/dozen) in the control fed hens. Measured on a g feed/g egg basis, a 7.1 pt improvement ($P = 0.06$) was generated in FCR, with a value of 2.016 for the DFM-fed hens and 2.087 in the control hens. Egg shell quality responses were mixed as DFM eggs showed a trend to increase specific gravity ($P = 0.07$), but no differences were observed in eggshell breaking strength ($P = 0.14$) or shell weight ($P = 0.91$). Overall, DFM allowed for more efficient use of the feed consumed without altering the accretion of body tissue over the 17-week pullet growth period and increased HDEP and improved FCRm in 22- to 70-week-old laying hens.

Description of the Problem

Direct-fed microbials (DFM) are defined as live microorganisms that when fed, confer a health benefit to the host (FAO, 2001). Due to decreased growth promoting antibiotic use within the poultry industry, producers and allied companies are investigating new opportunities to increase poultry performance efficiency when antibiotics are removed or limited in the diet. Although antibiotic use is limited in egg production, there is still the potential for DFM to improve the efficiency of pullet growth or hen egg production. In broilers, DFM supplementation has increased body weight (Ahmad, 2006; Mountzouris *et al.,* 2017; Rhayat *et al.,* 2017), and improved feed efficiency (Gao *et al.,* 2017; Park *et al.,* 2020). The positive growth responses noted for fast growing broilers warrant additional research using slower growing laying hen

pullets to better understand if DFM are able to improve performance when growth is slower and body maturation and composition are key considerations for long term egg production.

Bacillus-based DFMs have tremendous potential as a stable feed additive due to their endospore forming capabilities. The dormant spores of *Bacillus* species can survive in temperatures ranging from 30-121ºC and pH ranging from 2-12 (Sharma *et al*., 2018). Once ingested, spores germinate in the chicken intestine (Casula and Cutting, 2002), where they use properties such as competitive exclusion to limit colonization of pathogens (Knap et al., 2011). *Bacillus* bacteria can secrete bacteriocins which can disrupt the cell membrane of harmful bacteria (Sharma *et al.,* 2018) and create an anaerobic environment which decrease the amount of pathogens and increase the amount of beneficial bacteria in the intestinal microbiota (Mahmoud *et al.,* 2017; Sharma *et al.,* 2018). *Bacillus subtilis* can also secrete enzymes which include α-amylase, β-glucanase, lipases, proteases, and levansucrase (Ferrari *et al.,* 1993; Eggert *et al.,* 2000; Eggert *et al.,* 2001; Eggert *et al.,* 2002). *Bacillus* species can also increase the amount of volatile fatty acids produced by the host animal, which aids in the overall health and integrity of the intestinal barrier (Oladukun *et al.,* 2021).

It is important to verify the responses of individual DFM on pullet and laying hen performance over the duration of the egg production period. When chicks are fed DFM starting as pullets, results are much more consistent in the laying hen phase (Patterson and Burkholder, 2003). Studies that have fed DFM across both pullet and laying hen phases have reported consistent results of improving performance over both of these time period (Neijat *et al.,* 2019a; Neijat *et al.,* 2019b; Upadhaya *et al.,* 2019). Other reports have shown contrasting results when feeding a DFM beginning in the laying hen phase (Nahashon *et al.,* 1994; Zhang and Kim, 2013; Forte *et al.,* 2016). Therefore, the objective of this experiment was to determine the effects of a

single-strain *Bacillus* subtilis (Q-Biotic) on pullet performance, laying performance, and egg quality when fed from day 0 to 70 weeks of age.

Materials and Methods

Animals, Housing and Diets

All animal procedures were approved by the Institutional Animal Care and Use Committee at Virginia Tech. Hy-Line W-36 laying hen chicks were transported to Virginia Tech from a commercial hatchery in Georgia. Chicks received a Marek's vaccine at the hatchery, and a killed *Salmonella enteritis* vaccine at 12 and 16 weeks of age. Chicks were selected, individually weighed, and allotted to groups of 24 chicks that were placed into pullet cages. Experimental treatments (Control and DFM) were randomly assigned to pullet cages. Pullets were housed in two high rise three tier cages with one cage left empty between pens to limit potential cross contamination between DFM and control birds. Stocking density began with 24 chicks per cage and 24 in^2 per bird. Six birds per cage were euthanized at six and 12 weeks of age resulting in 32 in^2 and 48 in^2 per bird, respectively. Feed was provided *ad libitum* in the first three weeks of the experiment, and a controlled feeding approach was used from 3 to 17 weeks of age. Feed was stored in individual buckets mirroring the pens, and separate scales and feed cups were used between the diets. Pullets were phase fed diets according to breeder recommendations. Diets were formulated to meet or exceed breeder requirements (Appendix 1). The DFM was provided at a concentration of 300,000 cfu/kg of feed via a calcium carbonate carrier. To account for the additional calcium supplemented with the DFM, small particle limestone was added to the control diet after common basal diets were mixed. Water was provided *ad libitum.* Pullets began at 20 hours of light for the first week, followed by a decrease

of one hour of light until eight weeks of age, and a final decrease of 30 minutes for nine and ten weeks of age. At 11 weeks of age, pullets received 12 hours of light until the end of the pullet phase. Chicks were checked at least twice daily for overall health, morality and cull removals, room temperature, and feed and water availability. If mortality were discovered that chick was immediately removed from the cage and weighed and recorded, the same process occurred for any chick culled. In the laying phase, 72 birds per treatment were selected from pullet cages and moved to randomly selected laying hen cages resulting in 144 total hens for the experiment. Hens were housed in two of three tiers in a three-tier battery cage system with three birds per cage resulting in a stocking density of 72 in^2. Each treatment was comprised of 12 replicates, with two consecutive cages of three hens constituting a replicate. Cages were blocked by replicate, with two empty cages between replicates to limit potential cross contamination of the DFM. Hens were lighted according to Hy-Line management guides (W-36 Commercial Layer Management Guide, Hy-Line North America). At 17 weeks of age, hens received 13 hours of light. Light was increased by 15 minutes every week, resulting in 16 hours of light at 32 weeks and for the duration of the experiment. Hens were phase fed receiving pre-lay diets from 17-22 weeks, Layer I diets from 22-38 weeks, Layer II diets from 38 to 52 weeks, and Layer III diets from 52-70 weeks (Appendix 3). Diets were formulated to meet or exceed breeder recommendations; complete diet formulation and nutrient specification are in appendices 1, 2, 3, and 4.

Pullet Performance

Body weights, feed intake, and feed conversion ratio were measured in three-week intervals from 0-15 weeks. Pullets were moved to laying hen cages at 17 weeks, resulting in a two-week interval for the final weeks of the experiment. Birds were weighed individually, but data were

analyzed on a cage basis and reported on a g/bird basis. Feed intake was calculated on a pen basis using the amount of feed offered over the three-week period, and the amount of feed refused at the end of each period. Mortality corrected feed conversion ratio (FCRm) was calculated using the total cage body weight gain plus any mortality body weight gain in each pen divided by the total amount of feed consumed by the birds within two consecutives cages (Sarsour *et al.,* 2022).

Dual-Energy X-Ray Absorptiometry (DXA)

At 17 weeks of age, three birds per cage were randomly selected and euthanized for dual-energy X-ray absorptiometry (DXA) analysis. Euthanized birds were scalded and defeathered and immediately frozen until analysis. Birds were thawed overnight, weighed, and placed on an x-ray table to determine total lean mass and total fat mass content (GE Lunar Prodigy Model 8743, GE Healthcare, Boston, MA). Analysis was completed with all three birds to maintain a cage level analysis (Schallier *et al.,* 2019).

Hen Production and Egg Quality Parameters

Body weights (BW), feed intake (FI), and feed conversion ratio (FCR) were calculated on a four-week basis to correspond with diet generation. Hens were weighed by pen and expressed on a kg/hen body weight experimental unit. Feed was stored in individual buckets labeled with both treatment and replicate number for each experimental unit. Individual treatment-based feed cups and scales were used to limit potential DFM contamination between treatments. Amounts fed ranged from 95 g/d at 30 weeks, to 99 g/d at 36 weeks, and a final increase to 102 g/d at 56 weeks. Feed intake was calculated on a per pen basis using the amount of feed offered over the 4-week period, and the amount of feed refused at the end of the period. Feed conversion ratio was calculated on a per dozen basis and on a g feed/g egg basis. The formula used to calculate

FCR per dozen is as follows; kg of feed x 12/total eggs produced (Araújo *et al.,* 2015). Feed conversion ratio on a g feed/g egg basis was calculated using the formula; total grams of feed consumed/ egg mass. Eggs were collected and weighed daily on a per pen basis. All intact eggs were counted and weighed to generate an egg weight and count for the pen. Egg mass was calculated by dividing egg production by egg weight (Barrett *et al.,* 2019) Egg quality analysis was completed every eight weeks from 26 to 50 weeks, every four weeks from 50 to 66 weeks, and every two weeks from 66 to 70 weeks. Egg quality parameters included yolk color (YC) measured on a Roche yolk color fan, yolk weight (YW), albumen weight (AW), albumen height (mm) to calculate Haugh unit (Egg Quality Micrometer, Model S-6428, B.C. Ames Co, Waltham, MA) shell weight (SW), shell thickness (ST) using a digital caliper (Visbrite, Guangdong, China), specific gravity (SG), and shell breaking strength (SS) using a TA.XT plus texture analyzer (Stable Micro Systems, Goldaming, UK). The equation used to calculate Haugh unit from albumen height and egg weight was as follows: $HU = 100 \log10 (h - 1.7w^{0.37} + 7.6)$, where HU is the Haugh unit, h is the albumen height in millimeters, and w is the unbroken egg weight in grams (Haugh, 1937). Eggs for egg quality analysis were collected over a four-day period, labeled by pen number, and refrigerated for one week before analysis. One egg per pen per day was then used for egg quality analysis, and one egg per pen per day was used for shell breaking strength. All leftover eggs were subjected to specific gravity measurements, which measuring the density of the eggshell relative to the concentration of salt in water (Barrett *et al.,* 2019).

Statistical Analysis

Laying hen performance data, and egg quality parameters were analyzed as a repeated measurement to generate a value over time. Pullet data were analyzed using ANOVA in the

MIXED procedure in SAS 9.4 (SAS Institute Inc, Cary, NC) from 0-3 weeks, 0-6 weeks, 0-9 weeks, 0-12 weeks, 0-15 weeks, and 0-17 weeks. All pullet performance data were also subject to repeated measure analysis. Dual-energy X-ray Absorptiometry data were analyzed using ANOVA at 17 weeks of age. Data for the first four weeks of laying hen production was analyzed using ANOVA in SAS 9.4 (SAS Institute, Cary, NC). After consistent egg production at was achieved at 22 weeks, all remaining data were analyzed as a repeated measure. Production parameters included body weights, hen-housed egg production, hen-day egg production to account for mortality, egg weight, egg mass, feed intake, and feed conversion ratio. Egg quality parameters included Haugh units, yolk weight, albumen weight, shell weight, shell thickness, shell breaking strength, and specific gravity. If ANOVA data were significant, LSMEANS were separated using Fishers least significant difference test. Tukey's honestly significant difference test was used to separate means (Barrett *et al.,* 2019). Data were analyzed in JMP Pro 16 (SAS Institute, Cary, NC) with significance accepted at $P \leq 0.05$.

Results and Discussion

Pullet Performance

No difference was noted in initial body weight between the two treatment groups with both groups averaging 38.9 grams per chick at placement ($P = 0.92$). However, at three weeks of age, DFM fed chicks were smaller than control fed chicks ($P = 0.03$) as chicks potentially adjusted to the DFM provided in the diet. Previous work done in broilers has shown an initial lag phase in bird performance when DFM are introduced (Jayaraman *et al.,* 2017). In the present experiment, no differences in body weight were noted at 6, 9, 12, 15, or 17 weeks of age between control and DFM fed pullets ($P > 0.05$; Table 1). At 17 weeks, body weights for pullets were 1.23 kg, regardless of treatment ($P = 0.99$), in accordance with breeder expectations (Hy-Line W-36

Management Guide, 2020). When repeated measurements were used to increase replication over time, no difference was noted ($P = 0.98$). These results agree with the findings of Upadhaya *et al.,* (2019), who reported no differences in body weight. Other researchers reported increased body weights and body weight gains in the grower phase (5-10 weeks) of Shaver White laying hen pullets that were supplemented with three concentrations of *Bacillus subitlis* (Neijat *et al.,* 2019). A controlled feeding approach was used in the present experiment, in contrast with the two previous experiments noted above. Previous literature has reported that pullets fed *ad libitum* consumed more feed and reached sexual maturity earlier than control fed pullets (Robbins *et al.,* 1987). The differences in feeding approach could be a potential reason why factors such as body weights and feed intakes have conflicting results between previous pullet studies (Neijat *et al.,* 2019) Though these researchers all used *Bacillus subtilis*, each DFM has specific properties that can affect birds in different ways, which could be a potential reason for differing results in body weights. Strains of DFM can be selected for specific properties, such as enzyme activity or increased bacteriocin production, and can react differently within the host animal (Burkard *et al.,* 2007; Larsen *et al.,* 2014), leading to further inconsistent results.

Throughout the experiment and when repeated measures were used, DFM-fed pullets consumed significantly less feed than control fed birds. Feed intake was expressed on a kg per pullet basis, and at the end of the 17-week period, DFM-fed pullets consumed 5.549 kg per pullet compared to 5.626 kg per pullet ($P = 0.05$; Table 1). Both values are in accordance with breeder guidelines (Hy-Line Management Guide, 2020). The similar body weights and reduced feeding resulted in numeric differences in FCRm when each individual period was considered, however, repeated measurement analysis resulted in significant differences over the entire growth period (Table 1). These results agree with the findings of Neijat *et al.,* (2019) who also reported

improvements in FCR when pullets were fed a single strain *Bacillus subtilis* at three concentrations. Though improvements in FCR in pullet studies have been well documented, limited pullet studies warrant further comparisons on improvement in FCR when feeding a DFM. Broiler studies reported an improvement in FCR when feeding two multi-strain *Bacillus* cultures at three different (3.67×10^8 (low), 7.35×10^8 (med), 1.10×10^9 (high) cfu/kg) concentrations (Johnson *et al.,* 2020). Another report confirms a significant 2-point improvement in FCR when feeding a blend of xylanase and a multi-strain *Bacillus* at a concentration of 1×10^5 cfu/g (Nusairant *et al.,* 2022). Similarly, a broiler study also noted a significant improvement in FCR when feeding *Bacillus amyloliquefaciens* to male Arbor Acres broilers at a concentration of 2.5×10^9 cfu/g (Lei *et al.,* 2015). Though broilers are faster growing than pullets, the similarities between improvement in FCR demonstrate the beneficial effects of DFM supplementation. Broiler studies can more likely show performance differences with DFM supplementation as they are faster growing, but improvements in FCR are consistent and support the results of pullet growth and efficiency.

In the pullet rearing period, it is important for pullets to not only reach target body weights, but also have the proper body composition and reproductive maturation which will lead to a productive life as a laying hen (Patterson and Burkholder, 2003). Although the importance of body composition is known, little research has been reported on the effects of DFM supplementation on pullet body composition over the growing phase. No differences were noted in total mass, lean mass, or fat mass between the control and DFM fed pullets (Table 2). Although the pullets fed the DFM consumed less feed, they reached the same body weight with the same body composition potentially suggesting the DFM allowed the pullets to more efficiently utilize the feed and nutrients. Differences in apparent retention of dietary components

were increased in pullets in the developer phase (16 weeks of age), which had significantly higher retention of calcium ($P < 0.01$), phosphorus ($P < 0.01$), crude protein ($P < 0.01$), and apparent metabolizable energy (AME); ($P < 0.01$) (Neijat *et al.,* 2019). The increased nutrient utilization seen with DFM supplementation could allow pullets to reduce feed intake, but maintain body weight and composition resulting in more efficient growth. Mortality for the pullet phase was 2.1% and 2.8% for control and DFM pullets, respectively.

Laying Hen Production Performance

Pullets transferred to the laying phase were re-weighed at the beginning of the laying hen experiment to ensure no differences in initial body weight ($P = 0.56$), resulting in 1.24 and 1.22 kg/pullets in the control and DFM fed birds, respectively. No difference in body weight were noted over the length of the experiment, with the control hens weighing 1.59 kg/hen and the DFM hens weighing 1.60 kg/hen when repeated measurement analysis was used (Table 4; $P = 0.14$). Although not analyzed statistically, mortality from 18 to 70 weeks was low with 2.8 (2 of 72 hens) and 4.2% (3 of 72 hens) for control and DFM fed hens, respectively.

Laying hen performance in the present experiment is reported in Tables 3 and 4. When looking at raw egg production data, control hens initiated egg production earlier than DFM fed hens. Once egg production was consistently initiated, repeated measurement analysis was undertaken from 22-70 weeks of age. Hen-day egg production was increased in hens fed DFM in comparison to hens fed control diets ($P = 0.02$). Previous reports document increased egg production when feeding different types of DFMs, including *Bacillus* and *Lactobacillus* at concentrations of 4.4×10^7 cfu/mg (Nahashon *et al.,* 1994), 8.8×10^7 cfu/mg *Lactobacillus* (Abdularahim *et al.,* 1996), a 4.0×10^6 cfu/g (Xu *et al.,* 2006), and 500 mg/kg of *Bacillus subtilis*

(Zhang and Kim, 2013). In contrast, no performance differences were reported in hens fed 4.4 x 10^7 mg/kg *Lactobacillus* (Nahashon *et al.,* 1996).

DFM fed hens had lower egg weights compared to control-fed hens ($P \leq 0.01$). DFM fed hens reported an egg weight of 60.1 g compared to 60.7 g of the control fed hens. Previous literature supports a negative relationship between egg weight and egg production (Du Pleiss and Erasmus, Review, 1972). Significant increases in egg weight have been reported with DFM supplementation. Previous studies report significantly heavier eggs with supplementation of a multi strain lactic acid producing based DFM containing a minimum of 1.0×10^8 cfu/g of the organisms *Lactobacillus acidophilus, Lactobacillus casei, Enterococcus faecium,* and *Bifidobacterium thermophium,* with no differences in egg production. (Davis and Anderson, 2002). No differences were reported in egg mass in the present experiment ($P = 0.14$), although DFM-fed hens had numerically higher EM, due to the increase in egg production.

No difference was noted in laying hen feed intake, with Con and DFM birds consuming an average of 98.1 g/d over the experimental period ($P = 0.56$). Hens fed the DFM had an FCR 1.375 kg/dozen eggs compared to 1.431 kg/dozen in the control fed hens ($P = 0.02$). A similar trend was noted when calculating FCR on a g feed/g egg basis. DFM fed hens had an FCR of 2.016 compared to 2.087 in control hens ($P = 0.06$). Reports have shown significant improvements in FCR when 72–79-week-old Hy-line Brown laying hens were supplemented with *Bacillus subtilis* at a concentration of 2.0 x 10^9 cfu/kg (Wang *et al.,* 2021). Another study also reported an improvement in FCR in late cycle Lohmann white hens when supplemented with 2.3 x 10^8 cfu/kg of *Bacillus subtilis (*Abdelqader *et al.,* 2012). The improvement in FCR in the egg production phase follows the trend of improved FCR from the pullet phase. DFM-fed

hens continued this trend of efficiency through significantly improving HDEP and FCR per dozen eggs and improving FCR on a g feed/g egg basis by 7.1 points.

Egg Quality

The use of DFMs to increase internal egg quality have shown contrasting reports (Panda et al., 2008; Mikulski et al., 2012; Abdelqader et al., 2013; Neijat et al., 2019). In the present experiment, as expected with the decreased egg weights, the DFM-fed hens also had significantly lighter egg yolk weights in comparison to the control-fed hens. Control hens reported a yolk weight of 17.1 g compared to 16.9-g yolk weights in DFM fed hens (Table 5). No differences were observed in albumen weight, with control hens having a weight of 35.1 g compared to the DFM fed hens' weight of 35.2 g ($P = 0.68$). Feeding DFM to the hens decreased Haugh unit, generating a value of 87.7 compared to 88.7 of the control fed hens. However, both Haugh unit values are above the threshold of grade A eggs, which need to have a Haugh unit of 72 or higher (Jones, 2012). Previous experiments reported a significant increase in Haugh units in hens fed 1.0 x 10^8 cfu/kg *Bacillus subtilis* from 18-42 weeks of age (Sobzack and Kowlaski, 2015). However, other studies reported no significant differences in Haugh units in when feeding 500 mg/kg *Bacillus subtilis* to 25–56-week-old Lohmann Brown laying hens (Xu *et al.,* 2006).

Measured shell quality parameters showed mixed results (Table 6). Specific gravity tended to be increased in the DFM fed hens in comparison to Con fed hens ($P = 0.07$). A significant interaction between treatment and time was noted in shell thickness (Figure 8) decreasing the shell thickness of the DFM hens. Shell thickness measurements began at 54 weeks. During this time, hens were subjected to higher temperatures in the summer months which accounted for the decrease in all shell quality parameters. At 58 weeks, both treatments had an increase in shell thickness. However, control fed hens had thicker shells at 58 weeks

which potentially resulted the interaction. No differences were noted in shell breaking strength ($P = 0.14$) or shell weight ($P = 0.91$). DFM fed hens had a shell breaking strength of 4048 g, compared to 3991 g of the Con fed hens. Control fed hens produced eggs with a shell weight of 6.03 g, compared to the 6.02 g of the DFM fed hens.

Positive results in shell quality have been reported with DFM supplementation. *Bacillus subtilis* potentially increases the amount of lactic acid producing bacteria, such as *Lactobacillus,* which decreases the pH of the lower GIT in the chicken (Chateau *et al.,* 1993) The increased nutrient retention of hens supplemented with DFMs may help hens absorb more calcium from the diets, and thus increase eggshell strength and thickness (Abdelqader *et al.,* 2013). In late stage production hens, significant increases in shell breaking strength were reported in 79-week-old Hy-Line Brown laying hens fed *Bacillus subtilis* at 2.9 x 10^9 cfu/kg *Bacillus subtilis* (Wang *et al.,* 2021). Similarly, an increase in eggshell weight and eggshell thickness was reported when feeding Lohmann White hens *Bacillus subtilis* at 2.3 x 10^8 cfu/kg (Abdelqader *et al.,* 2021). The results from this study were inconclusive compared to previous literature depicting the increase in eggshell quality with DFM supplementation.

Conclusion and Applications

1. Addition of a *Bacillus*-based DFM consistently lowered feed intake and improved FCRm with no effects on body weight or body composition in Hy-Line W-36 0- to 17-week-old pullets.
2. Continued supplementation of a *Bacillus*-based DFM to laying hens that had previously been fed the same DFM increased hen-day egg production in the laying hen phase.

3. Supplementation with DFM significantly improved FCR when calculated on an egg basis, and tended to improve FCR over the laying phase and when egg mass was also considered due to decreased egg weights.
4. Internal and external egg quality responses were mixed with the supplementation of DFM to laying hens.

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Tables and Figures

Table 1. Production Parameters of 0- to 17-week-old W-36 Laying Hen Pullets fed Diets with or without a direct fed microbial (DFM) [1,2]

Treatment	Control (C)	C+DFM	SEM	P-Value
Body Weight (g)				
0-3 Week	184.9[a]	180.3[b]	1.57	**0.05**
0-6 Week	434.1	428.1	2.58	0.11
0-9 Week	704.4	699.7	4.62	0.48
0-12 Week	945.9	940.2	5.37	0.46
0-15 Week	1141	1131	8.50	0.39
0-17 Week	1232	1232	9.90	0.99
Repeated Measure			1.58	0.98
Feed Intake (kg per bird)				
0-3 Week	0.382[a]	0.366[b]	0.0050	**0.04**
0-6 Week	1.126[a]	1.098[b]	0.0069	**≤ 0.01**
0-9 Week	2.159[a]	2.118[b]	0.0096	**≤ 0.01**
0-12 Week	3.357[a]	3.315[b]	0.0133	**0.04**
0-15 Week	4.684[a]	4.619[b]	0.0191	**0.02**
0-17 Week	5.626[a]	5.549[b]	0.0259	**0.05**
Repeated Measure			0.0035	**≤ 0.01**
FCRm[3] (kg/kg)				
0-3 Week	2.630	2.615	0.0377	0.78
0-6 Week	2.855	2.830	0.0219	0.44
0-9 Week	3.182	3.158	0.0227	0.47
0-12 Week	3.597	3.572	0.0274	0.53
0-15 Week	3.941	3.941	0.0309	0.99
0-17 Week	4.259	4.232	0.0324	0.57
Repeated Measure			0.0692	**0.03**

[1] Data are means of 12 groups of Hy-Line W-36 laying hen pullets. Each cage started with 24 chicks and enough birds (up to 6 per cage) were sampled on weeks 6 and 12 to result in 18 and 12 pullets remaining on those weeks, respectively. Body weights, feed intake, and FCRm was subjected to repeated measures analysis.

[2] No differences were reported in initial body weight, with each group averaging control pullets averaging 38.93 grams per pullet and DFM pullets averaging 38.88 grams per pullet (P = 0.22, SEM = 0.22).

[3] Mortality corrected feed conversion ratio

Table 2. Dual-energy X-ray Absorptiometry (DXA) of 17-week-old laying hen pullets fed diets with or without a direct fed microbial [1]

Treatment	Control (C)	C+DFM	SEM	P-Value
Total Mass[2] (g/hen)	1016	1003	13.2	0.52
Lean Mass (g/hen)	869.4	858.7	22.9	0.74
Lean Mass (%)	77.8	77.3	NA	0.88
Fat Mass (g/hen)	148.9	144.6	19.6	0.88
Fat Mass (%)	13.3	12.9	NA	0.86

[1] Data are means of 12 groups of 3 Hy-Line W-36 laying hen pullets.
[2] Total mass without feathers.

Table 3. Production Parameters of 18- to 22-week-old Hy-Line W-36 laying hens fed diets with or without a direct-fed microbial (DFM).[1,2]

Treatment	Control (C)	C+DFM	Pooled SEM	P Value
HHEP (%)	19.4	16.6	1.97	0.31
HDEP (%)	19.4	16.6	1.97	0.31
Egg Weight (g)	49.5	48.4	0.42	0.08
Egg Mass (g/d)	9.6	8.0	0.95	0.24
Feed Intake (g/d)	86.4	85.7	1.36	0.72
FCR (g/g)	9.703	14.514	2.1289	0.12
Body Weight (kg)[3]	1.520	1.532	0.0137	0.55

[1] Data are means of 12 groups of six Hy-Line W-36 Laying Hens. Initial 17-week body weight = 1.23 kg (P = 0.59).
[2] HHEP = hen housed egg production, HDEP = hen day egg production (corrected for mortality), FCR = feed conversion ratio.
[3] Body Weights were measured at 22 weeks.

Table 4. Repeated measurement analysis of 22- to 70-week-old Hy-Line W-36 laying hen production parameters when fed diets with or without a direct-fed microbial (DFM)[1,2]

Treatment	Control (C)	C+DFM	Pooled SEM	Treatment	Time	Trt x Time
				(P-Value)	(P-Value)	(P-Value)
HHEP (%)[3]	84.0	84.0	0.59	0.99	**≤ 0.01**	0.99
HDEP (%)[4]	84.1[a]	86.0[b]	0.55	**0.02**	**≤ 0.01**	0.99
Egg Weight (g)	60.7[a]	60.1[b]	0.10	**≤ 0.01**	**≤ 0.01**	0.99
Egg Mass (g/d)	50.9	51.6	0.33	0.12	**≤ 0.01**	0.96
Feed Intake (g/d)	98.1	98.1	0.08	0.56	**≤ 0.01**	0.66
FCR (kg/dz)[5]	1.437[a]	1.375[b]	0.017	**0.02**	**≤ 0.01**	0.95
FCR (g/g)	2.087	2.016	0.0268	0.06	**≤ 0.01**	0.88
Body Weight (kg)	1.585	1.596	0.0048	0.14	**≤ 0.01**	0.98

[1] Data are means of 12 groups of six Hy-Line W-36 Laying Hens.
[2] Values are a repeated measure over time, boldface indicates a significant *P* value.
[3] HHEP = hen housed egg production.
[4] HDEP = hen day egg production.
[5] FCR = feed conversion ratio.
[a-b] Means within a column that do not share a common superscript differ ($P \leq 0.05$).

Table 5. Repeated Measurement analysis of 22- to 70-week-old laying hen internal egg quality when fed diets with or without a direct-fed microbial (DFM)[1,2]

Treatment	Control (C)	C+DFM	Pooled SEM	Treatment	Time	Trt x Time
				(P-Value)	(P-Value)	(P-Value)
Egg Weight (g)[3]	62.1	61.6	0.22	0.15	**≤ 0.01**	0.24
Yolk Color	6.31	6.40	0.05	0.14	**≤ 0.01**	0.57
Yolk Weight (g)	17.4[a]	16.9[b]	0.13	**≤ 0.01**	**≤ 0.01**	0.95
Albumin Weight (g)	35.1	35.2	0.20	0.68	**≤ 0.01**	0.16
Haugh Unit	88.7[a]	87.7[b]	0.31	**0.02**	**≤ 0.01**	0.78

[1] Data are means of 12 groups of six Hy-Line W-36 Laying Hens.
[2] Values are repeated measurements over time, boldface indicates a significant *P* value.
[3] Egg weights from eggs collected for egg quality only.
[a-b] Means that do not share a common superscript differ ($P \leq 0.05$).

Table 6. Repeated measurement analysis of 22- to 70-week-old laying hen shell quality parameters when fed diet with or without a direct-fed microbial (DFM)[1]

Treatment	Control (C)	C+DFM	Pooled SEM	Treatment	Time	Trt x Time
				(P-Value)	(P-Value)	(P-Value)
Shell Weight (g)	6.03	6.02	0.027	0.91	**≤ 0.01**	0.11
Shell Thickness (mm)	0.336	0.330	0.0021	0.07	**≤ 0.01**	**≤ 0.01**
Shell Breaking Strength (g)	3991	4048	44.6	0.14	**≤ 0.01**	0.37
Specific Gravity (sgu)	1.0817	1.0822	0.00020	0.07	**≤ 0.01**	0.54

[1] Data are means of 12 groups of six Hy-Line W-36 Laying Hens.
[2] Values are a repeated measure over time, boldface indicates a significant *P* value.
[a-b] Means that do not share a common superscript differ ($P \leq 0.05$).

Chapter IV

Conclusion

The objective of this thesis was to determine the effects of a *Bacillus* subtilis-based DFM on pullet performance, laying hen performance, and egg quality when fed from day of hatch until week 70. In the pullet phase, supplementation of the DFM resulted in lower feed intake and an improvement in FCRm when repeated measurements were used. Despite the reduced feed intake, similar body weights were reported over the 17-week pullet phase ($P = 0.98$), with no differences in body composition ($P > 0.05$). In the laying phase, addition of a DFM increased ($P = 0.02$) hen-day egg production by 1.9 percentage points, significantly improved FCR per dozen eggs ($P = 0.02$) by 5.3 points and improved FCR on a g/g basis ($P = 0.06$) by 7.1 points. Differences were noted in internal egg quality and shell quality, with DFM supplemented hens having lighter egg yolks ($P \leq 0.01$), lower Haugh units ($P \leq 0.01$), and a trend in improved specific gravity ($P = 0.07$).

In the first three weeks of the pullet experiment, DFM-fed pullets experienced a decrease in body weights (P = 0.03). Previous research has found that the microbiome of pullets matured around day 50 (Xiao *et al.,* 2021). Introduction of a new microbial population before the microbiome was mature could have led to a decrease in body weights at 3 weeks of age. Additionally, previous work done in broilers have shown a reduction in early body weights (Jayaraman *et al.,* 2017), but no differences in later body weights as chicks adjust to the DFM. As time progresses, host animals have the ability to adapt to the DFM and the beneficial effects of DFM supplementation can be noticed. This was seen in the present experiment as the differences in 3-week body weight between control and DFM-fed pullets was lost at 6, 9, 12, 15,

or 17 weeks of age. Although the pullets had similar body weights between control and DFM-fed birds, feed intake was consistently reduced with DFM supplementation ($P \leq 0.05$). These data suggest that the DFM had some sort of positive effects on nutrient digestibility, intestinal health or overall maintenance requirements as carcass lean mass percentage ($P = 0.87$) and fat mass percentage ($P = 0.86$) were unaltered. *Bacillus* based probiotics have been shown to increase the number of beneficial bacteria in the lower gastrointestinal system (Molnar, 2011; Guo, 2018). These beneficial bacteria including *Lactobacillus* and *Bifidobacterium can* lower the pH of the intestines and create an anaerobic environment that reduces pathogenic bacteria ability to thrive. The potential reduced pathogen exposure could reduce immune system stimulation and allow pullets to allocate additional nutrients to production instead of maintenance. Additionally, these beneficial bacteria have been shown to aid in the production of volatile fatty acid production in the ceca which can be used as an energy source by the host animal (Bergman, 1990).

In the laying phase of this experiment, similar results were seen in terms of improvement. DFM-fed hens had consistently higher egg production from 22-70 weeks. The increase in egg production resulted in a significant improvement in FCR per dozen eggs ($P = 0.02$). Supplementation of the DFM resulted in a decrease ($P \leq 0.01$) of egg weights during the length of the experiment, though the relationship between higher egg production and lower egg weights has been documented in previous research (Du Pleiss and Erasmus, Review, 1972). The reduction in egg weights and improvement in egg production resulted in no differences for egg mass. When egg mass was taken into consideration, FCR on an egg mass basis was reduced, but not as consistently. Although not statistically significant, the improvement in FCR shows the ability of DFM supplementation to increase efficiency of production. According to Hy-Line W-36 performance objectives, Hy-Line W-36 hens generally produce a FCR of 1.93 – 2.08 from 20-

100 weeks (Hy-Line Management Guide, W-36 Commercial Layers, Hy-Line North America). Control fed hens had a slightly increased FCR that what was set forth by breeder guidelines (2.087), whereas DFM-fed hens were in accordance with recommendations (2.016).

Egg quality analysis values were not expected as no significant differences were reported in shell quality parameters, in contrast with previous studies (Abdelqader *et al.,* 2013; Wang *et al.,* 2021). A trend was noted in specific gravity, but the biological relevance of that trend is questionable as the numerical improvement in specific gravity is minimal. No differences were noted in shell breaking strength ($P = 0.14$). Specific gravity is an indirect measure of shell quality that measures density, whereas specific gravity is a more direct measurement of shell quality that measures the strength of the eggshell and helps to account for the flexibility of the shell membrane. In previous reports that have shown improvements in shell quality, supplementation of the DFM began at later stages and at higher doses. While minimal shell quality differences were noted in this thesis, previous research done in late cycle laying hens demonstrates improvement in shell quality when feeding a *Bacillus*-based DFM (Abdelqader *et al.,* 2013; Wang *et al.,* 2021). One time x treatment interaction was noted in shell thickness. Analysis of shell thickness began at 54 weeks. During this time, hens were subjected to higher temperatures in the summer months. Control fed hens had slightly thicker shells 54 weeks, which could potentially explain the interaction. Control-fed hens had heavier egg weights which resulted in heavier egg yolks. However, DFM-fed hens had lower Haugh units, which was unexpected. Both Haugh unit values (88.4 for control hens, 87.4 for DFM hens) were above the requirement of 72 Haugh units set forth by the FDA (USDA, 2000).

Bacillus probiotics are a transient type of DFM, meaning that they need to be fed continuously in order to achieve optimal effects and germinate in the lower small intestine.

Supplementation of DFM began at day 0 and continued until 70 weeks of age. This amount of time likely affected the microbiome of both pullets and laying hens, which aided in the overall efficiency of production. Potential next steps could include a microbiome diversity analysis in both the pullet and layer phases to determine types of bacteria present, and define if different types of beneficial bacteria aid in the stability of the microbiota and overall health of the hen. Additional research could determine apparent retention of specific nutrients to understand how the DFM supplementation is reducing FCR.

Results from this thesis suggest that supplementation of a *Bacillus*-based DFM offer beneficial effects to the efficiency of pullet rearing and the egg production cycle in laying hens. The pullet phase of production is often overlooked by producers as no direct marketing of products are possible, however, improvements seen in the pullet phase could lead to more consistent improvements as hens age. Various DFM feed additives are in widespread use and these DFM can potentially have different modes of action, highlighting the importance of individual determination of DFM effect poultry. Supplementation of the DFM used in this experiment improved FCR across both the pullet and laying hen phases and improved HDEP in 22-70 week old laying hens.

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Appendices

Appendix 1: Formulations of Diets to Hy-Line W-36 Pullets fed with or without a DFM[1] from 0-17 weeks of age.

Ingredient	Starter 1 0-3 wk	Starter 2 3-6 wk	Grower 6-12 wk	Developer 12-15 wk	Pre-Lay 15-17 wk
	%	%	%	%	%
Corn	63.56	63.49	63.49	65.11	63.55
Soybean meal 48 % CP	25.90	22.48	17.26	10.97	14.97
Dried Distillers Grain with Solubles	3.00	4.00	5.00	8.00	8.00
Poultry Biproduct meal	3.00	4.00	5.00	5.00	4.50
Wheat Bran	0.00	1.76	4.54	7.00	0.00
Soy oil	0.21	0.25	0.40	0.00	1.11
Salt (NaCl)	0.26	0.22	0.20	0.20	0.22
Sodium Bicarbonate	0.15	0.15	0.15	0.15	0.15
DL-Methionine	0.16	0.17	0.12	0.07	0.15
L-Lysine•HCl	0.15	0.12	0.10	0.23	0.08
Limestone, Small particle	1.01	1.04	1.12	1.24	2.53
Limestone, Large particle	0.00	0.00	0.00	0.00	2.53
Dicalcium Phosphate	1.87	1.72	1.51	1.35	1.61
Choline chloride (60%)	0.10	0.10	0.10	0.10	0.10
Vitamin and Mineral premix[2]	0.63	0.50	0.50	0.50	0.50

[1]DFM was provided at a concentration of 300,000 cfu/g. Control pullets received an equal part of small particle limestone to account for additional calcium supplied by DFM.

[2]Provided per kg of diet: vitamin A, 6600.00 IU; vitamin D3, 2200.00; vitamin E 14.30 IU; vitamin B12, 9.50 µg; menadione, 0.88 mg; biotin, 33 µg; choline, 357.50 mg; Folic acid, 1.10 mg; niacin, 33.00 mg; pantothenic acid, 9.00 mg; pyridoxine, 0.88 mg; riboflavin, 4.40 mg;

thiamine, 1.10 mg; Fe, 225.00 mg; Mg, 100.00 mg; Mn, 220.00 mg; Zn, 220.00 mg; Cu, 22.00 mg; I, 0.68 m; Se, 0.20 mg.

Appendix 2: Nutrient Specification of Diets to Hy-Line W-36 Pullets fed with or without a DFM from 0-17 weeks of age.[1]

Nutrient	Starter 1 %	Starter 2 %	Grower %	Developer %	Pre-Lay %
Crude protein	19.97 (19.30)	19.46 (18.90)	18.27 (18.20)	16.62 (18.30)	16.94 (17.30)
Dry Mater	87.2	87.3	87.2	87.1	88.1
ME kcal/kg	2977	2977	2977	2936	2940
Calcium	1.00	1.00	1.00	1.00	2.50
Phosphorus	0.80	0.78	0.76	0.73	0.73
Avail Phosphorus	0.50	0.49	0.47	0.45	0.48
Ash	(6.1)	(6.2)	(5.6)	(5.3)	(10.2)
Fat	3.71 (2.00)	4.00 (2.20)	4.43 (2.90)	4.39 (2.70)	5.09 (3.60)
Fiber	2.66 (2.60)	2.76 (2.50)	2.88 (2.60)	3.08 (4.30)	2.66 (2.50)
D Met + Cys	0.74	0.74	0.67	0.59	0.66
D Lys	1.05	0.98	0.88	0.84	0.78
D His	0.49	0.48	0.45	0.40	0.41
D Trp	0.20	0.19	0.17	0.15	0.15
D Thr	0.69	0.67	0.62	0.55	0.57
D Arg	1.18	1.13	1.04	0.90	0.93
D Iso	0.74	0.71	0.65	0.57	0.61
D Leu	1.54	1.50	1.41	1.29	1.33
D Val	0.84	0.82	0.77	0.69	0.71
Na	0.18	0.17	0.17	0.18	0.18
Cl	0.24	0.22	0.21	0.21	0.22
K	0.80	0.76	0.69	0.60	0.62
Linoleic acid	1.46	1.54	1.70	1.65	1.93

[1]Values in ( ) are analyzed values

Appendix 3: Formulation of Diets to Hy-Line W-36 Laying hens fed with or without a DFM[1] from 18-70 weeks of age

Ingredient	Layer I (22 to 38 wk) (%)	Layer II (38 to 52 wk) (%)	Layer III (52 to 70 wk) (%)
Corn	52.67	54.60	55.09
Soybean Meal (48% CP)	14.85	11.89	9.25
Dried Distillers Grain with Solubles	12.00	12.00	15.00
Poultry Byproduct Meal	5.00	6.00	6.00
Soy Oil	3.52	3.06	2.25
Salt	0.22	0.21	0.14
Sodium Bicarbonate	0.15	0.15	0.20
DL-Methionine	0.26	0.19	0.17
L-Lysine•HCL	0.14	0.11	0.12
Limestone, Small Particle	4.46	3.94	3.96
Limestone, Large Particle	4.46	5.91	5.94
Dicalcium Phosphate	1.68	1.36	1.28
Choline Chloride (60%)	0.10	0.10	0.10
Vitamin and Mineral Premix[2]	0.50	0.50	0.50

[1]DFM was provided at a concentration of 300,000 cfu/kg. Control hens received the same amount of small particle limestone to account for additional calcium supplied by DFM.
[2] Provided per kg of diet: vitamin A, 6600.00 IU; vitamin D3, 2200.00; vitamin E 14.30 IU; vitamin B12, 9.50 µg; menadione, 0.88 mg; biotin, 33 µg; choline, 357.50 mg; Folic acid, 1.10 mg; niacin, 33.00 mg; pantothenic acid, 9.00 mg; pyridoxine, 0.88 mg; riboflavin, 4.40 mg; thiamine, 1.10 mg; Fe, 225.00 mg; Mg, 100.00 mg; Mn, 220.00 mg; Zn, 220.00 mg; Cu, 22.00 mg; I, 0.68 m; Se, 0.20 mg.

Appendix 4: Nutrient Specification of Diets fed to Hy-Line W-36 Laying hens fed with or without a DFM[1] from 18-70 weeks of age

Nutrient	Layer I (22 to 38 wk)	Layer II (38 to 52 wk)	Layer III (52 to 70 wk)
	(%)	(%)	(%)
Crude Protein	17.7 (16.3)	16.9 (16.8)	16.5 (17.6)
Dry Matter	(90.9)	(90.6)	(89.3)
ME (kcal/kg)[2]	2900	2875	2825
Calcium	4.00	4.30	4.30
Phosphorus	0.75	0.69	0.68
Available Phosphorus	0.51	0.47	0.46
Ash	(12.1)[3]	(14.4)	(12.6)
Crude Fat	7.37 (5.7)	7.10 (5.2)	6.60 (4.3)
Crude Fiber	2.68 (3.2)	2.64 (3.0)	2.78 (4.6)
D Met	0.50	0.44	0.42
D Cys	0.26	0.25	0.25
D Met + Cys	0.77	0.69	0.67
D Lys	0.84	0.77	0.73
D His	0.41	0.40	0.38
D Trp	0.16	0.15	0.14
D Thr	0.59	0.56	0.55
D Arg	0.95	0.90	0.85
D Ile	0.63	0.60	0.58
D Leu	1.35	1.31	1.28
D Val	0.74	0.71	0.69
Na	0.19	0.19	0.18
Cl	0.24	0.23	0.20
K	0.61	0.56	0.53
Linoleic Acid	2.77	2.64	2.39

[1]DFM was provided at a concentration of 300,000 cfu/g. Control hens received the same amount of small particle limestone to account for additional calcium supplied by DFM.
[2] ME = Metabolizable Energy
[3]Values in ( ) are analyzed values.

Appendix 5: Figures of Laying Hen Performance

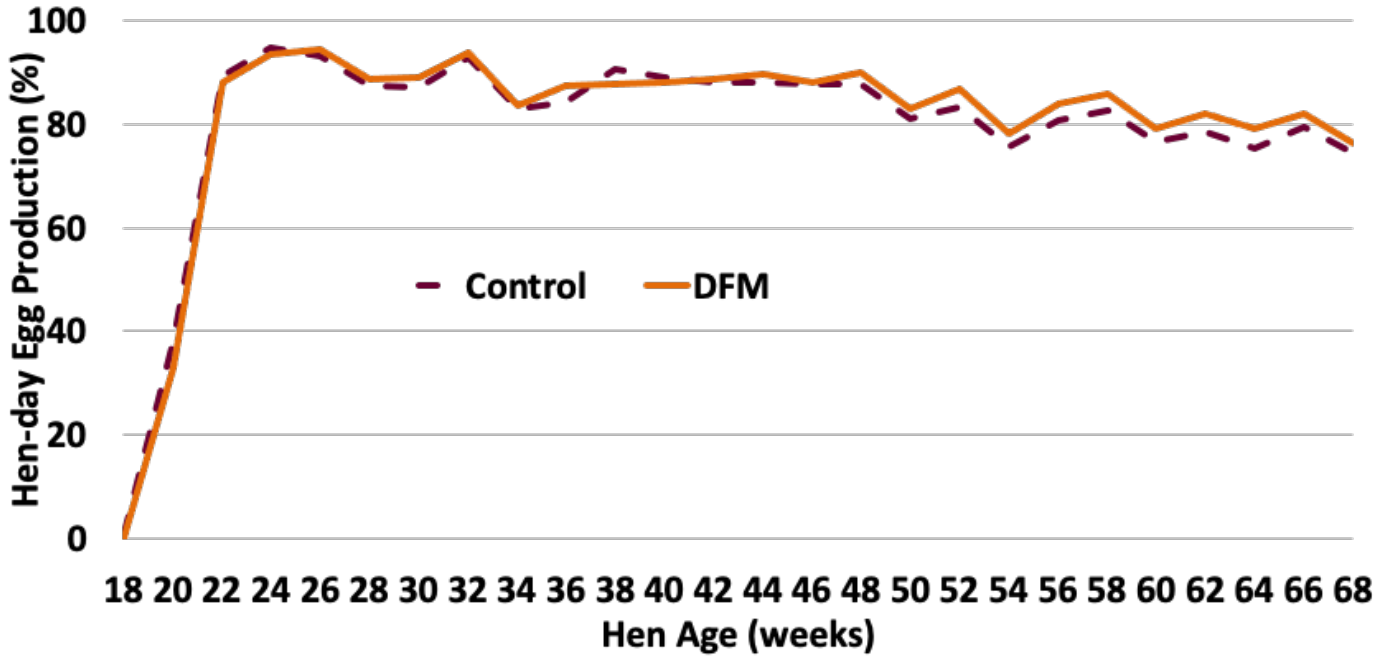

Figure 1. Hen-Day Egg Production of 18–70-week-old Hy-Line W-36 Laying hens fed diet with or without a Direct-Fed Microbial (***P* = 0.02**; SEM = 0.55).

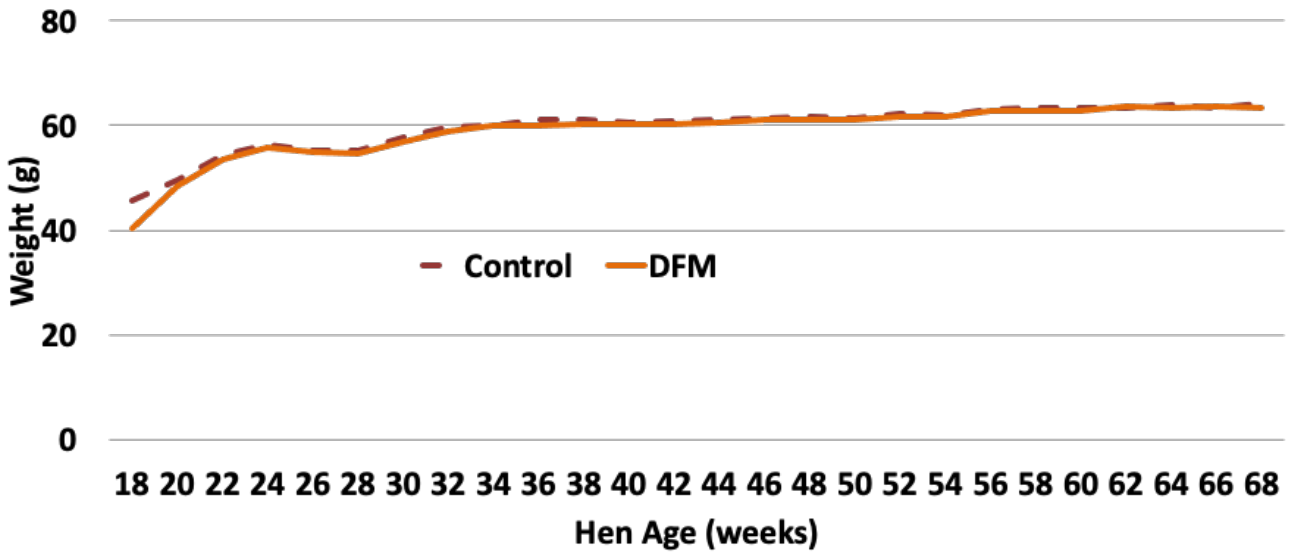

Figure 2. Egg weight of 18–70-week-old Hy-Line W-36 laying hens fed diet with or without a Direct-Fed Microbial. (***P* ≤ 0.01**; SEM = 0.10).

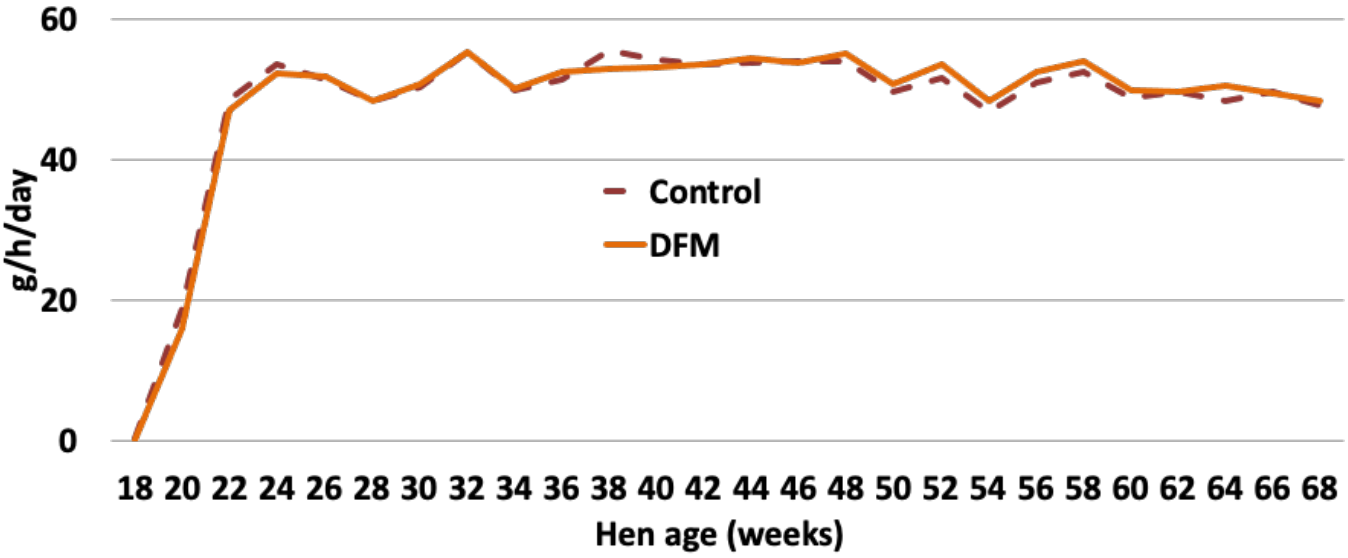

Figure 3. Egg mass of 18–70-week-old Hy-Line W-36 Laying hens fed diet with or without a Direct-Fed Microbial (P = 0.12; SEM = 0.33).

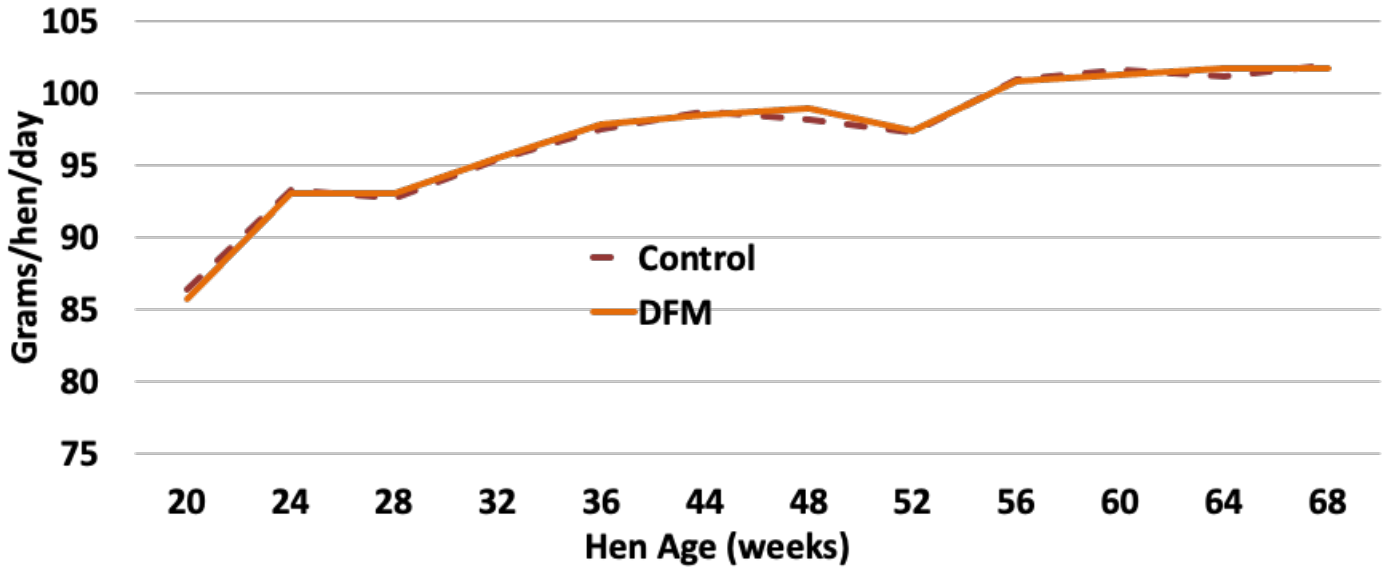

Figure 4. Feed Intake of 18–70-week-old Hy-Line W-36 Laying hens fed diet with or without a Direct-Fed Microbial (P = 0.56; SEM = 0.08).

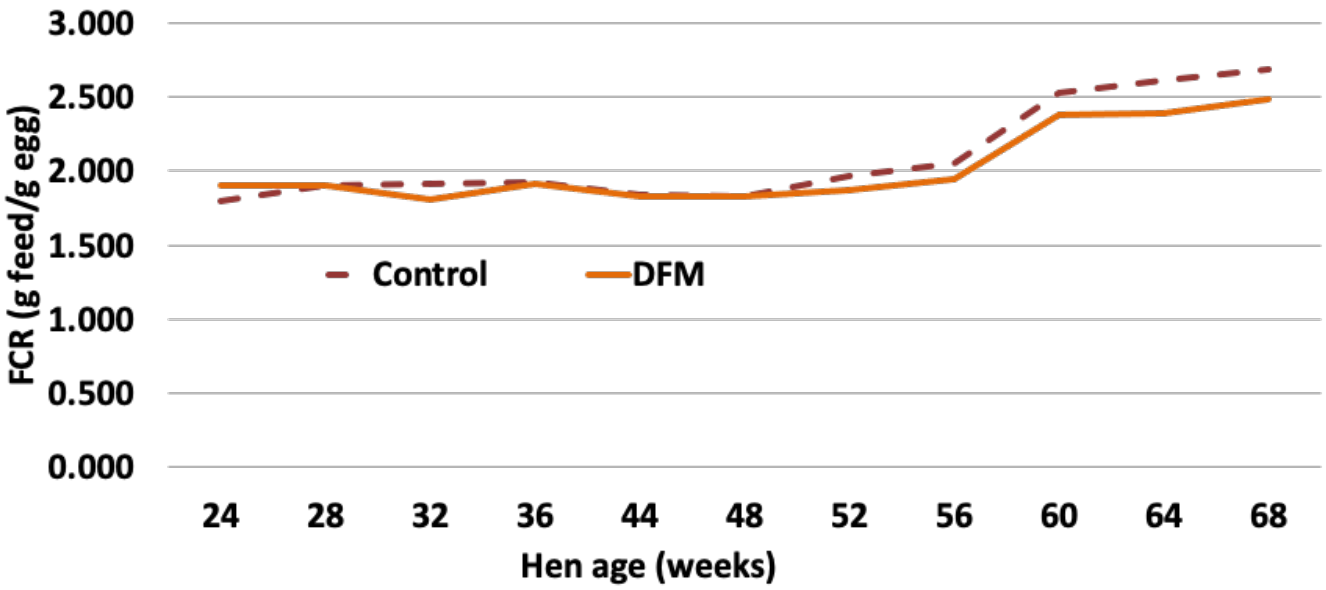

Figure 5. Feed Conversion Ratio of 18–70-week-old Hy-Line W-36 Laying hens fed diet with or without a Direct-Fed Microbial. (P = 0.06; SEM = 0.0268).

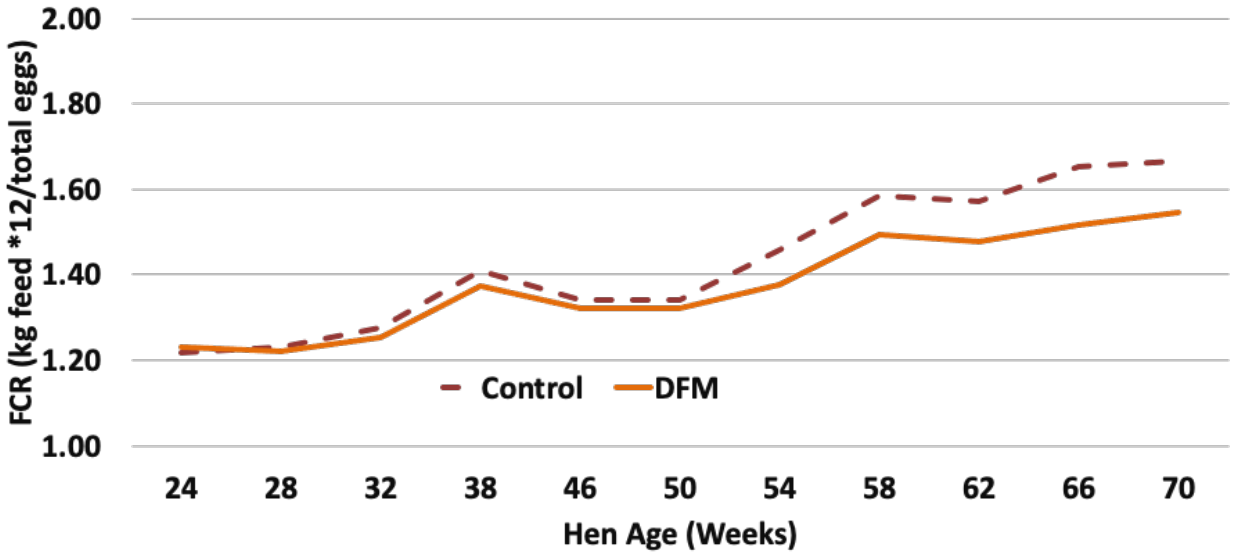

Figure 6. Feed Conversion Ratio (kg/dozen) of 18–70-week-old Hy-Line W-36 Laying hens fed diet with or without a Direct-Fed Microbial. (*P* = **0.02**; SEM = 0.017).

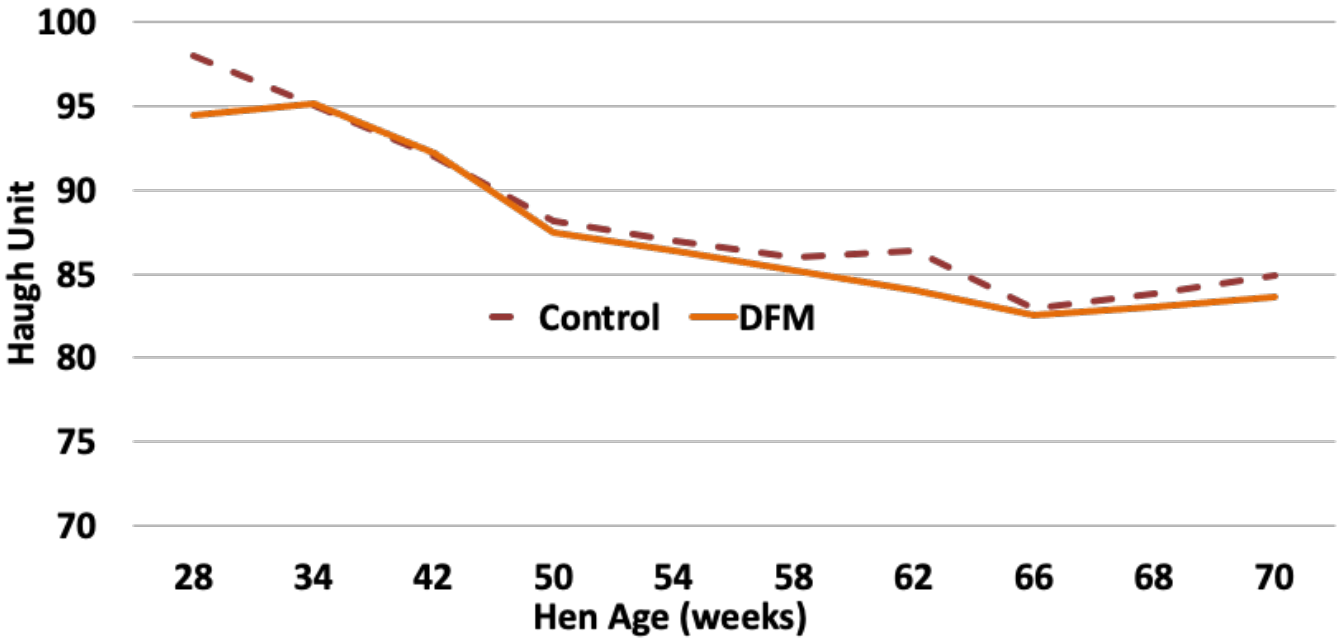

Figure 7. Haugh Units of 18–70-week-old Hy-line W-36 Laying hens fed diet with or without a Direct-Fed Microbial. (***P* = 0.02**; SEM = 0.31).

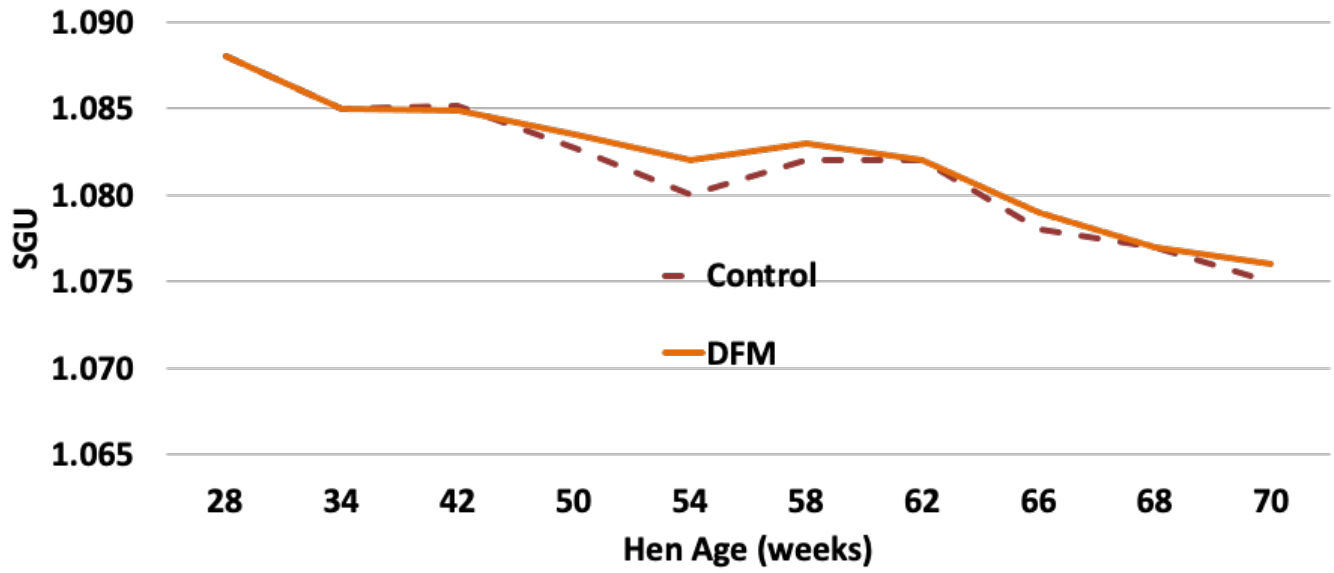

Figure 8. Specific Gravity of 18–70-week-old Hy-line W-36 Laying Hens fed diet with or without a Direct-Fed Microbial. (P = 0.07; SEM = 0.00020)

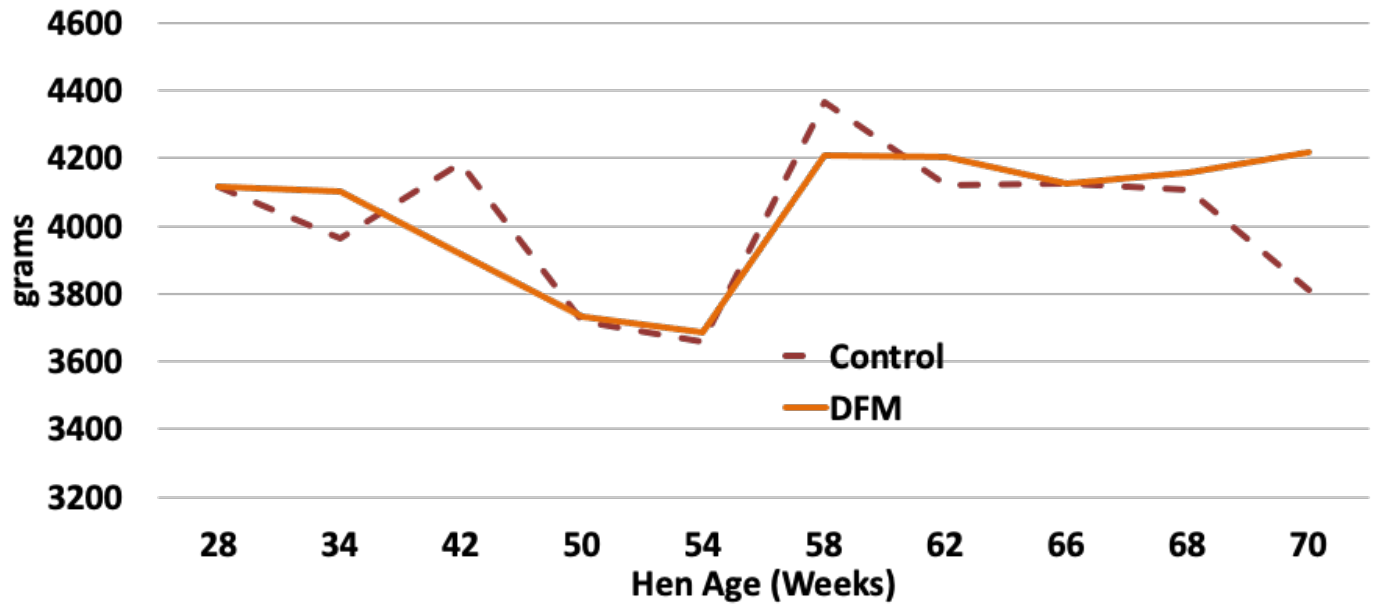

Figure 9. Shell Breaking Strength of 18–70-week-old Hy-line W-36 Laying Hens fed diet with or without a Direct-Fed Microbial. (P = 0.14; SEM = 44.6)

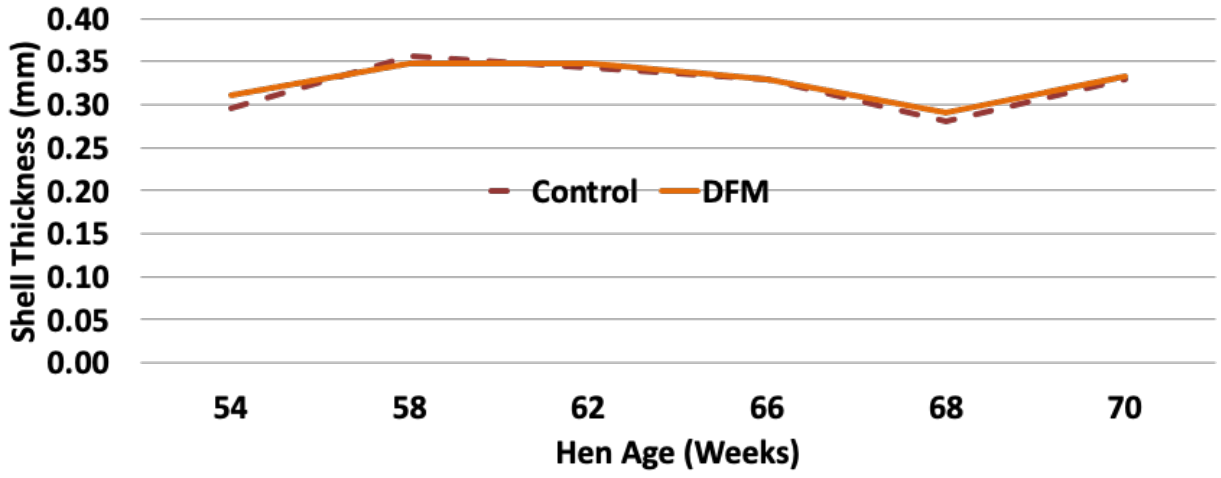

Figure 10. Shell Thickness of 18–70-week-old Hy-Line W-36 laying hens fed diet with or without a Direct-Fed Microbial (***P* ≤ 0.01;** SEM = 0.0021)