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The Effects of Butyric Acid on Performance Parameters, Egg Quality and Nutrient Utilization in Young White Leghorn Hens

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THE EFFECTS OF BUTYRIC ACID ON PERFORMANCE PARAMETERS, EGG QUALITY AND NUTRIENT UTILIZATION IN YOUNG WHITE LEGHORN HENS

By

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THE EFFECT OF BUTYRIC ACID ON PERFORMANCE PARAMETERS, EGG QUALITY AND NUTRIENT UTILIZATION IN YOUNG WHITE LEGHORN HENS

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The objective of this study was to evaluate the effects of Prophorce butyric acid on the performance of White Leghorn laying hens. Hens (180) were placed in 60 conventional cages (3 hens/cage) using a completely randomized design with 30 replicate cages per treatment allowing 722.58cm^2/hen. Hens were given the control or butyric acid. The trial spanned 40 weeks (35 weeks old at the start). Water was given ad libitum and hens were fed 110g/day. Egg production and mortality were recorded daily. Egg shell strength, weight, and mass were recorded biweekly; hen weights and egg components were measured monthly; calcium (Ca) / phosphorus (P) digestibility and apparent metabolizable energy (AME) were calculated at 55 and 65 weeks of age. Villi height and crypt depth measurements were taken from the jejunum and ileum at 35 (baseline), 55, and 75 weeks of age. Data were analyzed using the Proc GLIMMIX procedure in SAS. There was no significant treatment effect on average egg production, egg weight, egg mass, mortality, feed intake, egg components or hen weights. There was a significant treatment effect on egg shell strength ($p=0.0493$) in favor of butyric acid. There was significant improvement in AME ($p<0.0001$) for hens fed butyric acid and a significant diet*age interaction ($p=0.0002$). There were no significant differences in Ca digestibility. Phosphorus digestibility showed no significant differences however there was a significant diet*age interaction ($p=0.0410$). There was a significant age effect on both Ca and P digestibility (<0.0001 and 0.0004 respectively).

There was no treatment effect on villi height or crypt depth in the jejunum, but a significant improvement in villi height to crypt depth ratio for the control compared to butyric acid ($p=0.0148$). There was a significant age effect on villi height and crypt depth ($p=0.0091$ and 0.0122 respectively). In the ileum, there was a significant treatment effect on crypt depth in favor of the control ($p=0.0057$). These results show that butyric acid only improved the eggshell strength and AME.

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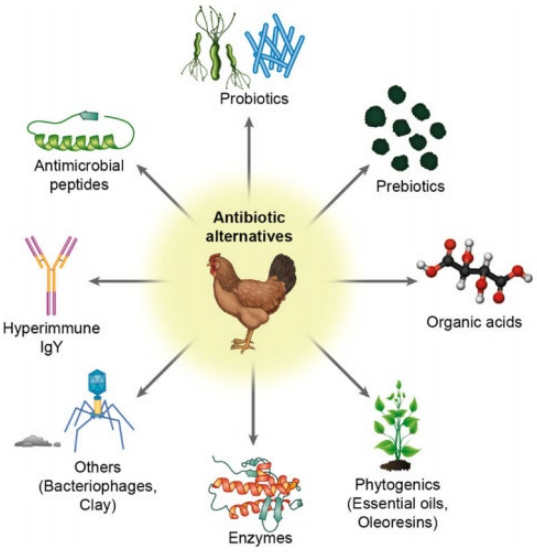
TABLE OF CONTENTS

Chapter 1: Literature Review

Introduction

In areas of the world such as Europe and the United States, antibiotics/ antibiotic growth promoters are no longer being added to poultry diets for various reasons (Ricke, 2003). These reasons include the high concentration of antibiotic residues found in meat and meat products, undesired changes in the microbial communities of the gastrointestinal (GI) tact (Kulshreshtha et al., 2014), and an increase in antibiotic resistance in pathogenic bacteria (Ricke, 2003). Consequently, the European Union has banned antibiotic use in animal feed (Kulshreshtha et al., 2014). Due to the increasing controversy of antibiotic use, researchers are actively searching for alternative supplements to add to the diet (Biggs and Parsons, 2008).

Figure 3. Antibiotic Alternatives



Gadde, U., W. Kim, S. Oh, and H. Lillehoj. 2017.Alternatives to antibiotics for maximizing growth performance and feed efficiency in poultry: a review. Anim. health res. Rev. 18.1 (2017): 26-45.

Ideally these alternatives would improve growth performance by acting to improve feed efficiency and nutrient absorption/ utilization as well as regulate harmful microbial populations and promote beneficial microbial growth in the gastrointestinal tract (Biggs and Parsons, 2008). Organic acids, specifically short chain fatty acids (SCFA), are thought to be such an alternative. Most member states of the European Union generally regard organic acids and their salts as safe and have approved them for use as feed additives for livestock and poultry production (Adil et al., 2011). SCFA can also be described as saturated straight-chain monocarboxylic acids, fatty acids, volatile fatty acids, and weak or carboxylic acids. Originally SCFA's were added to animal feed to acts as fungistats. Propionate and formic acids (and various combinations) have been shown to have bactericidal activity in feeds infected with foodborne pathogens such as *Salmonella* spp. (Ricke, 2003). Butyric acid is now increasingly researched as a feed additive for poultry due to its proposed effectiveness to improve feed conversion efficiency, gut health and growth performance. (Ricke, 2003)

Sources of Butyric Acid

Butyric Acid, along with other SCFAs, is produced in the GI tract in millimolar amounts in both humans and animals in areas that predominantly contain strictly anaerobic microflora (Ricke, 2003). For poultry, this area is the ceca which is the major site of microbial fermentation of unabsorbed starch (Liu et al., 2017), non-starch polysaccharides, (Levy et al., 2015) and proteins (Kulshreshtha et al., 2014). Butyric acid, propionic acid, and acetic acid are the major byproducts of these processes (Liu et al., 2017). Supplementation of butyric acid is important in poultry diets because although they can produce butyric acid by microbial fermentation in the ceca, there is very little

production in the small intestine (Levy et al., 2015). Currently researchers have focused on supplementing butyric acid to study its effects as an alternative to antibiotics as well as its ability to inhibit pathogens such as salmonella (Levy et al., 2015). Butyric acid is most effective in its undissociated (non-ionized, more lipophilic) form (Leeson et al., 2005) but is often supplemented as butyrate in the diet because of butyric acid's volatile nature (Liu et al., 2017) and pungent smell (Kaczmarek at al., 2016). Adil et al. (2011) suggests that reduced feed intake can be observed due to reduced palatability of the feed when SCFAs are supplemented in their acid form. Another advantage of butyric acid being supplemented in salt form is that it is less corrosive and more water soluble (Kahn and Iqbal, 2016).

Butyric acid is quickly absorbed and metabolized by mucosa cells. Absorption and metabolism of butyric acid begins in the mucosa of the crop and this process continues throughout the GI tract. This limits the amount of the butyric acid that will arrive and have effect on the small intestine. Butyrate can be microencapsulated for protection to help avoid this issue and improve its efficiency by allowing it to stay intact until it arrives in the small intestine. A common method of encapsulation is stearin or vegetable fat; it has been found that this method has had positive effects on gut morphology and reduction of pathogen colonization in the intestine (Liu et al., 2017). In a study by Liu et. al (2017), researchers created an assay to determine the optimal time for butyric acid release on the GI tract for broilers. It was found that encapsulated butyric acid aiming to stimulate epithelial cell development and improve digestibility should release at 30 minutes to 2.5 hours post ingestion; to focus on hind gut control, release should be at 2.5 to 4 hours post ingestion (Liu et al., 2017). Butyric acid needs to be in its

undissociated form before it arrives at the hindgut to have its antimicrobial effect while release in the small intestine should affect villi development and nutrient digestibility (Liu et al., 2017). Butyrate in its free form is used mostly as a feed sanitizer rather than as a supplement because it will be quickly absorbed in the crop (Leeson et al., 2005).

Several studies have shown that providing low doses of butyrate can improve performance in livestock including broilers and pigs (Kaczmarek et al., 2016), however, it is still unclear what optimal inclusion rate is needed to maximize efficiency for improving gut health and growth performance (Levy et al., 2015). Butyrate's mode of action is not entirely understood, and the performance response has been inconsistent across studies due to varying forms of butyrate used in the diet as well as the type of diet used (Moquet et al., 2017). It has been suggested by mammalian research that butyrate in the lumen can change endocrine regulation of digestion. Therefore, enzymatic activity, gut morphology and digesta transit time can all be affected. Subsets of enteroendocrine cells are localized in different parts of the GI tract (GIT) and elicit responses can be observed in the presence of butyrate. It is thought that different forms of butyrate are active in different parts of the GIT. Unprotected butyrate is active in the crop, proventriculus and the gizzard. Tributyrin (triglyceride of butyrate) is active in the small intestine and fat coated/ encapsulated butyrate is active the ceca and colon (Moquet et al., 2017). Butyrate supplemented as a glyceride is easier to handle than the acid form and does not have a pungent smell. It also facilitates passage to the lower GI tract where butyrate is then released by lipase activity (Moquet et al., 2017).

In previous studies, both monobutyrin and tributyrin have been used to potentially improve growth performance in broilers while the majority have been found

to use blends of mono-, di-, and triglycerides (Bedford et al., 2017). A recent study by Bedford et al. (2017) found that supplementing solely tributyrin had no significant effect on growth performance in broilers while mixtures of mainly mono- and tributyrin with some dibutyrin had positive effects on growth performance with 500ppm being the most effective dosage for tributyrin. Based on this study, another study by Bedford et al. (2017) was conducted with broilers to test varying combination levels of monobutyrin and tributyrin at varying intervals throughout the growth period. It was found that the most effective dosage combination was 500ppm of monobutyrin and 500ppm of tributyrin supplemented throughout the entire growth period (Bedford et al., 2017). Bedford et al. (2017) noted that variation in results found by other studies shows that the effects of butyrate glycerides are variable for broilers.

Sodium butyrate promotes water absorption and proliferation of epithelial cells, provides energy, stimulates synthesis of gastrointestinal hormones and stimulates intestinal blood flow in broiler chicks. (Hu and Guo, 2017)

In a study by Moquet et al. (2017), three forms of butyrate were tested in a diet with a poorly digestible protein source to investigate the effect of butyrate on various parts of the GI tract. It was reported that the presence of butyrate beyond the gizzard had an anorexic (appetite reducing) effect which was considered unusual for 1g/kg of supplemented butyrate (Moquet et al., 2017). Studies have found that this anorexic effect caused by butyrate (and other SCFA) is modulated by colonic L-cells that produce glucagon-like peptide 1 (GLP-1) and peptide YY (PYY). GLP-1 is released in the presence of digested protein as well as free fatty acids. PYY has an orexigenic (appetite stimulating) effect in chickens while it has an anorexic effect in rodents. PYY acts

directly on the hypothalamus and triggers CCK, which promotes the satiety effect via the vagus nerve reducing the appetite in rodents. The mechanism by which an orexigenic effect occurs in poultry is unclear (Furness et al., 2013).

In poultry, L-cells are located all along the distal small intestine, but the colon is the main site for anorexic effects (Moquet et al., 2017). L-cells are enteroendocrine cells that function by stimulating carbohydrate uptake, releasing insulin, and slow intestine transit (Furness et al., 2013). In the study by Moquet et al. (2017), anorexic effects were reduced when butyrate was delivered to the crop, gizzard and proventriculus in the unprotected form. It was also found that butyrate in the colon and ceca in the protected form increased total tract retention times thereby allowing more time for absorption and improved feed efficiency. There are very few studies that have demonstrated a link between colon motility and the effect of butyrate (Moquet et al., 2017). Short chain fatty acids have also been found to increase ileal proglucagon mRNA, protein and glucose transporter (GLUT-2) expression which can potentially improve gut epithelial cell proliferation (Adil et al., 2010).

In addition to finding which butyrate source is most effective, researchers have begun to examine which diet structure is best suited to maximize the effects of butyric acid and their salts when added to different diets. A portion of a study carried out by Qaisrani et. al. (2015) looked at how the relationship between diet structure (coarse or fine) and butyric acid supplementation (with or without) affected growth performance and gut morphology in broilers. It was found that feeding a course diet supplemented with butyric acid had a positive effect on performance and decreased crypt depth when added to a poorly digestible protein source. (Qaisrani et al., 2015).

Gut Health

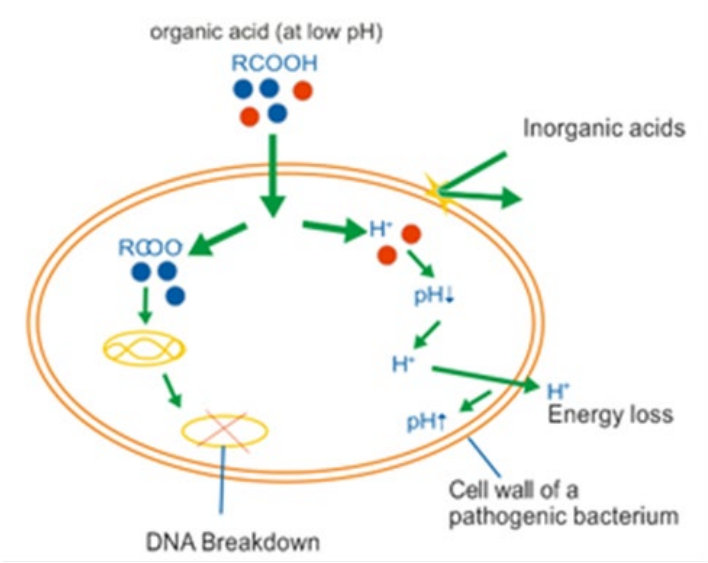
Gut health can be affected by nutrition, environment, and infectious agents such as pathogens. There is a direct relationship between gut health and animal performance and many researchers have attempted to create a gut health scoring index that can be applied to poultry diets (Kraieski et al., 2016). Researchers are spending increasing amounts of time studying gut health because it is proving to be a major factor in the performance of both broilers and layers (Grashorn et al., 2013). Optimal gut health is characterized in several ways. One of which is the villi height to crypt depth ratio. A high ratio indicates mature and well-functioning villi with a shallow crypt that is constantly providing cell renewal (Kaczmarek et al., 2016). Improved gut health has also been attributed to increased length of the GI tract allowing increased absorption (Adil et al., 2011).

Butyric acid can improve epithelial cell development (Levy et al., 2015). Supplemented butyric acid provides carbons which acts as an energy source for gut epithelial cells and promotes their proliferation, differentiation and multiplication (Qaisrani et al., 2015). Butyric acid is also thought to be effective at preserving cell viability and therefore enhancing enterocyte turnover which may improve intestinal recovery. It has been observed that butyrate supplementation can increase villi height and crypt depth in poultry and other nonruminant animals thereby increasing the absorptive surface. (Qaisrani et al., 2015).

SCFA's are theorized to have several mechanisms of antimicrobial activity. One of the most widely accepted mechanisms is that the acid changes the internal pH of the microbe (depolarization) and therefore disrupts nutrient synthesis and transport as well as

energy metabolism of that microbe (Adil et al., 2011). Organic acids can penetrate the surrounding membrane of the bacteria. Once inside of the membrane they will dissociate, forming H^+ ions as a result of the neutral pH releasing excess protons that will lower the pH. The microbe will then attempt to maintain a neutral pH by transporting excess protons out using adenosine triphosphate via active transport thereby depleting its cellular energy (Biggs and Parsons, 2008). At the same time, the disassociated $RCOO^-$ ions disrupt DNA and depresses its protein synthesis causing stress on the microbe.

Figure 4. Organic acid proposed antimicrobial mechanisms



Tran-Ngoc, K., S. Huynh, T. Nguyen, A. Roem, J. Schrama, J. Verreth. 2016. Oranic acids work best in Nile tilapia (Oreochromis niloticus) under suboptimal conditions. Aquaculture Nutrition 25: 132-144

The microbe is then no longer able to multiply efficiently (Adil et al., 2011). Butyric acid can have antimicrobial effects by decreasing the luminal pH and reducing bacterial colonization in the intestinal wall. With decreased toxic compound production, there is less damage to epithelial cells. (Qaisrani et al., 2015). Damaging epithelial cells

can disrupt the barrier between the internal and external environment of the lumen allowing toxins to enter circulation and increase the susceptibility of the intestine to pathogenic bacteria colonization (Abdelqader and Al-Fataftah, 2016). Butyric acid functions by inhibiting salmonella colonization in the ceca due to improvement of the intestinal barrier function (Abdelqader and Al-Fataftah, 2016) and down regulates Salmonella gene expression (Liu et al., 2017). Decreasing luminal pH is also beneficial because it stimulates growth of beneficial bacteria and hampers the growth of the pathogenic bacteria (Adil et al., 2010). Commonly, pathogen growth is likely to occur in the GIT when the lumen of the small intestine and ceca exceed a pH of 5.8-6.0 and a large intestine that exceeds pH 6.2 (Brzóska, 2013).

In a study by Adil et al. (2010), villus height was significantly different in the duodenum and jejunum when chicks were fed organic acids with the greatest height in chicks consuming the 3% butyric acid diet. It was suggested that the significant growth of the villi is due to a reduction in growth of both pathogenic and non- pathogenic bacteria allowing decreased colonization resulting in decreased inflammatory responses of the intestinal mucosa (Adil et al., 2010). Inflammation of intestinal mucosa due to increased pathogenic activity can lead to necrosis of intestinal epithelium (Brzóska et al., 2013). Villus height was therefore increased. In this study, crypt depth was not affected when compared broilers fed the control diet. (Adil et al., 2010)

A study by Hu and Guo (2017) found that supplemented sodium butyrate at 2000mg/kg in broilers had no effect on jejunal villi height and crypt depth and increased the villi height to crypt depth ratio ($p<0.01$) when compared to the control.

Performance Parameters

Increased efficiency of absorption due to improved gut health has led to various effects on performance parameters in broilers and laying hens depending on the form of butyric acid used and the inclusion rate. Researchers have found that butyric acid supplementation had positive effects on body weight gain (BWG) and feed conversion rate (FCR) in broilers (Liu et al., 2017). Several studies have showed that butyric acid does not significantly affect feed consumption. A study by Adil et. al (2010) found that there was no significant difference in feed consumption in broiler chicks between organic acids including butyric acid fed at 2% and 3% inclusion rates compared to the control diet. In this study there was a significant difference in FCR and BWG seen in favor of the organic acids suggesting that there was better absorption and nutrient utilization compared to birds on the control diet. It was also found in this study that there were higher serum calcium and phosphorus concentrations when compared to the control. (Adil, 2010)

Contradiction across experiments is common when researching butyric acid due to the type of diet and the form of butyrate (calcium salt, sodium salt, glyceride, etc.) (Kaczmarek et al., 2016).

In a study by Kaczmarek et al. (2016) with broiler chickens, researchers attempted to find a "matrix value" for butyrate in poultry diets to maximize its efficacy. In one of the experiments, 0.2, 0.3 and 0.4g/kg of protected calcium butyrate was added to the diet and compared to a control diet examining its effects on growth performance and nutrient digestibility. They found that overall butyric acid positively affected FCR and BWG, 0.2 g/kg of butyrate improved FCR, 0.3 g/kg improved FCR regardless of the birds age;

while 0.4g/kg decreased feed intake (FI) and significantly increased FCR. This study indicated the providing 0.3g/kg produced the most positive effects when compared to the control and other doses of butyrate. In a study by Leeson et. al (2005), they found contradictory results for the 0.2g/kg results when butyrate was supplemented as a glyceride. It was also found that butyrate in the glyceride form caused feed intake depression similar to that of the 0.4g/kg in the Kaczmarek et al. (2016) experiment. Abdelqader et al. (2016) found that 0.5g of butyric acid/ kg diet recovered intestinal epithelia and improved integrity in heat stressed broilers compared to the controls.

A study by Taherpour et. al. (2009) showed that broilers supplemented with butyric acid glycerides showed improved body weight gain when compared to the control. In this study the contradiction of these results when compared to other studies was attributed to differences in preparation of the diet, sex of the bird, and experimental conditions. Similar to aforementioned studies, FCR improved while feed intake was increased in favor of butyric acid glyceride. There was no significant difference in mortality for this study.

The ban on the use of antibiotics in poultry feed has increased mortality rates, specifically in birds younger than 21 days old. Modern farming standards suggest that a mortality rate of 4% or less is acceptable. Brzoska et al. (2013) suggests that the use of organic acids in the diet promote the production of prebiotics and probiotic lactic acid bacteria in young birds. Bzroska et al. (2013) noted that in several studies, organic acids including butyric acid significantly reduced mortality when compared to their control diets.

Metabolizable Energy and Nutrient Utilization

Organic acids have been found to increase the digestibility of calcium, phosphorus, magnesium, zinc and proteins (Adil et al., 2010). Adil et al. (2010) found hens supplemented with organic acids had higher serum calcium and phosphorus concentrations when compared to the control. These results were credited to the notion that acidic ions have a tendency to form a complex with minerals such as calcium and phosphorus thereby increasing their digestibility.

Organic acids also act as substrates for intermediary metabolism (Adil et al., 2011). Butyric acid can increase the solubility of feed, digestion and absorption of nutrients (Rahman et al., 2008). With fewer pathogenic bacteria due to the presence of organic acids, there is reduced microbial metabolic need thereby allowing more nutrients to be available for absorption by the host. The decrease in the toxins produced by harmful bacteria can also cause an increase in energy availability and protein digestibility (Adil et al., 2010). Adil et al. (2011) suggests that there is increased protein digestibility because feeding organic acids in the diet reduces gastric pH resulting in increased pepsin activity. Pepsin proteolyzes proteins releasing peptides that trigger hormones such as cholecystokinin (CCK) and gastrin to be released. These hormones play a large role in the digestion and absorption of proteins (Adil et al., 2011).

A study by Goodrazi et al. (2014) noted that the effects of organic acids on digestibility is debatable due to multiple factors affecting the results. There was no significant effect found for nutrient digestibility in this study when compared to the control for broilers.

Kaczmarek et al. (2016) found that overall, 0.2, 0.3, and 0.4g/kg of butyrate increased the apparent metabolizable energy (AME) compared to the control diet for broilers which can be explained partially by the significant increase of villi height and numerically increased mucosal thickness observed in this study. The villi height to crypt depth ratio was only numerically improved which was noted as signifying excellent experimental conditions. (Kaczmarek et al., 2016)

Calcium

Calcium is a major component of the eggshell (Sengor et al., 2007). Researchers have therefore spent a great deal of time looking for ways to both provide a calcium sufficient diet for the various phases of egg production as well as increase the efficiency of calcium absorption. In a study by Mraz (1972), diets containing calcium at 2.25% or higher in the diet had higher dry and ash weights of the tibia suggesting that increasing the calcium in the diet allowed greater absorption of calcium as well as decreased resorption from the structural bone decreasing the chances for osteoporosis. However, simply adding more calcium in the diet can lead to a reduction in feed consumption which can affect the level of other nutrients that the hen consumes and benefits from (Sengor et al., 2007). Calcium requirements may vary for layers depending on the level of egg production, daily feed intake and the age of the bird. Supplements can be added to counteract bone loss early in egg production (Webster, 2004). Calcium requirements can also vary depending on whether an eggshell is being formed that day or not. On days when the eggshell is being formed, hens fed complete diets tend to have increased feed consumption (Clunies et al., 1992).

In laying hen diets, calcium is typically provided as either limestone or oyster shell which both provide calcium in the carbonate form ($CaCO_3$). A study performed by Saunders-Blades et al. (2009) focused heavily on observing the effects of various particle sizes of limestone and how that compared to the use of oyster shell as a calcium source. The efficacy of these sources was determined by observing the results of various performance parameters such as bone integrity and egg shell breaking strength. The results of this study indicated that mixed sizes of limestone provided the best results in terms of maintaining bone health while large particle limestone seemed to increase bone health and shell strength. The industry is using limestone in increasing amounts as it has been found to be an easier obtained and less expensive calcium source when compared to oyster shell. Other studies have suggested that large particle limestone might be most effective because it is solubilized more slowly than finer particles in the gizzard allowing longer periods of use for absorption during the periods when hens do not have access to feed. It was also found that using this source of calcium increased the breaking strength of the tibia thereby decreasing the likelihood of osteoporosis (Safaa et al., 2008).

High producing laying hens have a high demand for ionic calcium (Ca^{+}) and therefore heavily depend on calcium homeostasis (Bar, 2008). The mechanisms involved are highly regulated by parathyroid hormone (PTH) and 1,25 dihydroxy-vitamin D_3 ($1,25(OH)_2D_3$). PTH acts mainly on the kidney and bone while $1,25(OH)_2D_3$ affects the absorption from the small intestine. These hormones have not been found to directly affect the shell gland, but it has been suggested that there are many Vitamin D dependent proteins that are located and modified in the eggshell gland (Bar, 2008). Vitamin D is thought to be the main factor in modulating calcium absorption in laying hens. Other

factors affect calcium through their effect on Vitamin D metabolism including maturation and gonadal activity, calcium and phosphorus levels in the diet, and egg laying and shell calcification (Bar, 2008).

Shell formation requires several times more calcium than is available in extracellular pools. The intestinal lumen becomes almost void of calcium about 4 to 5 hours after the feeding stage suggesting that intense shell calcification occurs when there is a lack of available dietary calcium, usually during the night hours of the hens' photoperiod. This results in increased efficiency of absorption of the remaining calcium as well as bone resorption from the medullary bone (Bar, 2008). Calcium and phosphorus are both stored in bone as hydroxyapatite in the medullary bone. Consequently, both calcium and phosphorus are mobilized from the bone thereby increasing serum calcium and phosphorus to provide calcium for the egg. Serum phosphorus levels become elevated due to the high calcium to phosphorus ratio of the egg shell compared to that of hydroxyapatite. Fecal phosphorus levels are increased because phosphorous is used in minute quantities on the egg shell (Clunies et al., 1992). Calcium is deposited onto the egg shell via shell mineralization as calcium carbonate in the form of calcite (Nys et al., 1999). This process takes approximately 17-20 hours as the egg passes through the oviduct while most of the calcium is deposited to the egg in the shell gland. During this time about 5 to 6g of calcium carbonate ($CaCO_3$) is deposited. (Lavelin et al., 2000). As the egg shell is calcified, serum calcium declines stimulating the release of PTH which binds to the receptors on osteoclasts initiating resorption (Bar, 2008). This change in serum calcium concentration occurs quickly as Ca^{2+} has a very short half-life (Pelicia et

al., 2009). Loss of calcium from the bone should be restored during the daytime period. Osteoblasts and osteoclasts form and reabsorb bone respectively (Bar, 2008).

Calcium is absorbed in all segments of the small intestine. This is especially true in the duodenum and jejunum. Pelicia et al. (2009) notes that animals fed a calcium deficient diet will have increased absorption whereas animals who have a calcium sufficient diet will have decreased absorption in comparison.

Egg Quality

In laying hens, it is important that hens consume the correct ratio of manganese, vitamin D, calcium and phosphorus to produce a strong egg shell. As the hen ages, mucosal cells in the duodenum weaken and villi begin to shorten and absorption in the small intestine decreases. Egg shell quality is reduced as a result (Sengor et al., 2007). Sengor et al. (2007) suggests that butyrate can function in maintenance of the mucosa and epithelial cells. In this study, improvement in egg shell strength and increased egg production were observed and attributed to healing of damaged epithelial cells in addition to increased growth of villi (Sengor et al., 2007). Maintaining a high egg shell breaking strength is needed as protection for the egg from penetration by pathogenic bacteria. Broken shells are a significant source of economic losses for producers (Świątkiewicz et al., 2010).

Formation of a normal (not misshapen) eggshell requires minerals to be released from the shell gland in the right proportions at the right time to ensure good egg shell quality. There must be adequate absorption and metabolism of nutrients to achieve this (Sengor et al., 2007). Butyrate has been found to improve calcium metabolism and

absorption by increasing villi growth. (Rahman et al., 2008). The study by Sengor et al. (2007) suggests that weakness in the egg shell in older hens can be altered with the use of butyrate if it is supplemented at 285mg/kg resulting in increased egg shell strength and decreased misshapen eggs.

Świątkiewicz et al. (2010) attributes reduced egg shell quality to hens increasing the weight of the egg while the calcium carbonate being added to the egg is not increasing proportionately. It was also determined that lowering the pH of the diet can benefit egg shell quality.

Butyric acid and its salts have shown variable results for egg quality and egg production. This variability has been attributed to the source of butyric acid, the inclusion rate, the environmental conditions and composition of the diet (Soltan, 2008). Rahman et al. (2008) reported, for hens 67-74 weeks of age, a significant increase in egg production for hens supplemented with various concentrations of organic acids (including calcium butyrate) compared to the control diet. The organic mixture showed no effect on egg weight while egg size was found to increase. There were no significant changes in shell percent, a decrease in yolk percent and a significant increase in albumen percent. This study also found that organic acids significantly increase the egg shell thickness compared to the control (Rahman et al., 2008). These findings are in agreement with Soltan (2008) but in disagreement with the study performed by Yesilbag and Colpan (2006) using an organic acid mixture that did not include butyric acid or its salt (Rahman et al., 2008).

Osteoporosis

Osteoporosis is a major bone related disease that can occur when there is increased demand of calcium from the medullary bone for the formation of the eggshell and maintenance of eggshell quality. Osteoporosis can be described as an increased porosity and reduced thickness of bone. This reduced thickness can result in bone breakage. In hens, osteoporosis is manifested as cage layer fatigue (Webster, 2004). Cage layer fatigue can be identified by a hen's inability to stand or walk. These hens tend to still have a willingness to eat or drink. The hen will die if cage layer fatigue is not treated. Cage layer fatigue can be classified as peracute and acute. Peracute being where the hen dies suddenly with no visible symptoms and acute being where the hen experiences leg paralysis and can potentially recover with assistance (Bell and Siller, 1962). Young hens that are in peak production are most likely to develop osteoporosis. Bell and Siller (1962) also concluded that some genetic lines of layers were more susceptible to osteoporosis than others.

Medullary bone development and the end of structural bone remodeling occurs simultaneously with the beginning of hen sexual maturity. The medullary bone stores large amounts of calcium which is released later for the formation of the egg shell when calcium is not present or cannot be readily absorbed from the digestive tract. Osteoporosis will occur if there is not enough calcium being absorbed from the intestine to remodel the structural bone after it provides calcium to the medullary bone (Webster, 2004). Studies done in ovariectomized rats suggest that organic acids can prevent osteoporosis by reducing the amount of bone turnover due to increased calcium absorption and solubility (Kamal and Ragaa, 2014). It has also been suggested that

osteoporosis cannot be avoided in the caged modern hybrid laying hen due to confinement and its high egg production (Webster, 2004).

Summary

The poultry feed industry is becoming increasingly interested in understanding the effects of organic acids such as butyric acid so that it can be used to replace antibiotics in livestock production settings. Butyric acid has the potential to become the ideal alternative because of its ability to promote a healthy gut manifested by improved villi growth and repair as well as its ability to improve nutrient digestion.

The efficacy of supplemental butyric acid in poultry diets is influenced by several factors including the age of the bird, the environmental conditions, the composition of the diet, the particle size of the calcium source, the inclusion rate, and the form of butyric acid supplemented. Although many researchers have found positive effects on the gut health and thereby nutrient absorption, there have also been conflicting results seen when considering its effects on growth performance and nutrient/energy utilization. Further research is needed.

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Chapter 2: The effects of butyric acid on performance parameters, egg quality and nutrient utilization in young White Leghorn hens

INTRODUCTION

In recent years, the poultry industry has been focused on finding new ways to improve the performance of layers and broilers through improved nutrient and energy utilization. The goal is to improve these parameters while maintaining and potentially improving the health of hens. For layers, egg production and egg quality are of great economic concern. On average, 65 to 75% of the cost of producing eggs is the feeding cost; therefore, producers want to improve egg production and egg quality while lowering feed costs (Shim et al., 2013). Improved egg quality can be identified as improving eggshell strength while maintaining a good egg size. Researchers have identified increasing calcium deposition into the eggshell as a means of increasing egg quality and shell integrity. Calcium is a major component of the layer diet and is incorporated into both eggshell and bone (Clunies et al., 1992). Calcium is transferred from the bone to the eggshell in the form of calcium carbonate ($CaCO_3$). Calcium used to restructure the bone is absorbed from the small intestine (Saunders-Blades et al., 2009). As the hen ages, its ability to absorb nutrients including calcium declines resulting in decreased eggshell thickness and breaking strength. This leads to increased economic losses due to broken eggs (Molnár et al., 2017). The National Research Council (NRC, 1994) recommends 3.25% calcium in the diet for White Leghorn laying hens eating approximately 100g per day. The calcium requirement for White Leghorn laying hens may increase if there are high levels of phytate phosphorus in the diet (NRC, 1994).

Several strategies to improve calcium absorption and utilization have been researched including increasing the amount of calcium in the diet, testing the effects of adding different amounts of phosphorus in the diet on the absorption of calcium, and adding different sources of calcium to the diet. Some studies have found that increasing calcium in the diet can increase egg production and egg mass (Safaa et al., 2008) while others have found that increasing calcium can decrease average daily feed intake and had no effect on egg production and egg weights (Roland and Bryant, 1994).

The use of organic acids for the improvement of performance parameters in both layers and broilers have been increasingly explored to replace the use of antibiotics as growth promoters. The European Union has banned the use of antibiotics in animal feed and countries such as the United States have begun to follow this trend as consumer awareness grows (Nguyen et al., 2018). Organic acids including butyric acid and its salt have shown variable effects on egg production and egg quality due to the source of the acid, diet composition and environment (Soltan, 2008).

Organic acids, such as butyric acid, are thought to have positive effects on gut health by providing carbons for villi growth, promoting the growth of beneficial bacteria, and decreasing harmful bacteria by decreasing luminal pH. Improved gut health is theorized to allow increased absorption resulting in increased nutrient and energy utilization in poultry thereby improving performance (Qaisrani et al.,2015). Butyric acid in its unprotected form is rapidly absorbed in the upper GIT suggesting that protection is needed to positively affect the small intestine (Kaczmarek et al., 2016). Previous studies show variable results likely due to factors including age, nutrition, diet structure, experimental conditions, flock health, source of butyric acid and inclusion rate

(Taherpour et al., 2009; Kaczmarek et al., 2016; Qaisrani et al.,2015; Levy et al., 2015). A study by Kacmarek et al. (2016) with broilers found that protected butyrate of various doses significantly improved villi height and apparent metabolizable energy ($p < 0.05$). Encapsulated butyric acid in a study by Levy et al. (2015) was found to show no significant effect on villi height when compared to the control in broilers. This variation in findings suggests a need for further research to determine the optimal source and inclusion rate of butyric acid to potentially overcome the variation seen due to other factors.

There are many studies conducted on how butyric acid and its salt for various doses affect growth performance and gut health in broilers, but more research is needed to further examine their effects on performance in layers. The objective of this study was to examine the effects of Prophorce butyric acid on young White Leghorn hens on nutrient/ energy utilization, gut health, performance parameters and eggshell quality. Prophorce butyric acid is produced by the company, Perstorp (Malmo, Sweden) and is in the form of tributyrin containing 55% butyric acid. It is hypothesized in this study that the high stability of tributyrin in the feed and stomach should increase the efficacy of butyric acid thereby improving efficiency of gut health and absorption of nutrients leading to improved performance.

MATERIALS AND METHODS

Birds and Housing

The conditions of this experiment were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Nebraska- Lincoln. White Leghorn

hens (180) were used in this 40-week trial from 35 to 75 weeks of age. The trial began in June, 2017 and concluded March, 2018. The hens were housed in 60 cages of a Choretime manure belt housing system (Choretime, a division of CTB, Inc. Milford Indiana) at 3 hens per cage. The cages were 40.64 cm wide, 53.34 cm deep, 46.99 cm tall at the front and 40.64 cm tall at the back providing 722.58 cm^2/ hen. Hens were given water ad libitum via nipple drinkers and fed 110g per day. The photoperiod 16 hours light: 8 hours dark was maintained throughout the trial with exception of the last week where lighting issues were experienced. Each cage was randomly assigned 1 of 2 diets with 30 replicate cages per treatment using a completely randomized design.

Diet

Diet formulation for this trial is shown in Table 1. Diet was formulated based on NRC (1994) recommendations. The independent variable for this trial was the diet (Control or Butyric Acid). Prophorce Butyric Acid was added to basal diet at a 0.055% inclusion rate. The butyric acid used in this study was in the powder form of Tributyrin (glycerol ester of butyric acid) containing silica and was manufactured by the company Perstorp (Malmo, Sweden).

Measurements

Measurements included daily egg production (EP), feed intake (FI), and mortality. Eggshell breaking strength (BS), and egg weights (EW) were taken biweekly. Hen weights (HW) and egg components (shell %, yolk %, albumen %) were measured monthly. For egg components, eggshell breaking strength, and egg weights, 2-3 eggs were collected per cage depending on the number of hens in each cage. Manure samples

were taken at 55 and 65 weeks of age and used with feed samples to calculate metabolizable energy (ME), calcium digestibility, and phosphorus digestibility. Samples of the jejunum and ileum were taken for villi measurement at 35 (start), 55, and 75 weeks of age.

Eggs were collected daily and recorded to calculate average weekly egg production by dividing the number of eggs collected per cage by expected number of eggs. Eggs were collected once every other week to record egg weights. Egg mass was calculated by multiplying percent egg production by egg weights every other week. Egg components were calculated by the following process: The whole egg was weighed on a tared scale. The egg was then cracked and broken over a yolk separator allowing the albumen to be removed. The eggshell was weighed after all of its contents were scraped out. The yolk was weighed separately. The albumen was calculated by subtracting the yolk and eggshell from the weight of the whole egg.

Feed intake was calculated by weighing back the feed left over from a pre-measured bucket every other week and was subtracted by the total feed given to each cage. Average feed intake per hen per day was calculated by dividing this value by the number of hens in each cage.

Hens were weighed individually in a tared box on a scale to 1-gram accuracy. Average hen weight was calculated by dividing the total of the hen weights per cage and divided by the number of hens per cage.

Eggshell breaking strength was measured using a texture analyzer (TA.XTPlus, Texture Technologies Corporation, Scarsdale, NY). The force used to crack an eggshell

was graphed and measured using an exponent software (Stable Micro Systems LTD., Surrey, UK) in conjunction with the texture analyzer. Force is reported as newtons to break the eggshell.

For the purpose of calculating calcium digestibility, phosphorous digestibility, and metabolizable energy, titanium dioxide (TiO_2) was added to the feed at a rate of 0.25% as an inert marker and fed to the hens for 3 days. At the end of the third day manure samples were then scraped from the manure belt beneath each cage and placed into separate Whirl Pak bags. Fecal samples were dried in a drying oven at 100°C for 3 days then ground with a grinder and sifted to remove feathers. Feed and ground fecal samples were sent to Midwest labs (Midwest Laboratories, Omaha, NE) for calcium and phosphorous content analysis.

Dry Matter: 0.5 g of each feed and fecal sample were weighed (wet sample) into individual foil tins in duplicate. Each tin was previously dried in an oven overnight and the initial weights of the tins after drying were recorded. Feed and fecal samples were heated in a drying oven overnight. The final weight of the dried tin and sample were recorded. Tin weight was subtracted from the final weight to obtain the dry sample weight. The duplicate dry sample weights were averaged. Dry matter was calculated by the equation: (dry sample weight ÷ wet sample weight) x 100.

Titanium dioxide analysis: 0.5g of each feed and 0.3g of each fecal sample were weighed and poured into individual 16 x 25 pyrex screw cap test tubes. Each sample was analyzed in duplicate. Tubes were labeled with high temperature paint markers to avoid ink being burned during ashing. Tubes were placed in a metal test rack and ashed at 580°C for 10 hours in a furnace. 0.8g of Na_2SO_4 was added to the ash after each tube was

cooled to room temperature. 5.0ml of concentrated H_2SO_4 was added to each tube and each tube was capped and vortexed gently. Tubes were placed in a heating block at 120°C for 72 hours and vortexed at 24-hour intervals. Heating blocks were turned off and allowed to cool to room temperature. Each tube was transferred to individual 50ml volumetric flasks that contained 10ml of millique water using a funnel. Tubes were inverted to mix and allowed to cool to room temperature. Each sample was poured into individual 50ml falcon tubes and allowed to sit overnight.

For titanium analysis, 96-well plates were utilized. One row of 3 wells were used for standard curve solutions of TiO_2 in duplicate. Standard curve solutions included: (0) 0mg TiO_2/ml, (1) 0.0100mg TiO_2/ml. (2) 0.0200 mg TiO_2/ml. (3) 0.0300mg TiO_2/ml, (4) 0.0400mg TiO_2/ml, (5) 0.0600mg TiO_2/ml, (6) 0.0800mg TiO_2/ml, (7) 0.1000mg TiO_2/ml. Standard curve solutions were tested against an 8-points standard curve. Each diet sample (300µL) was added to 1 row of 3 wells. Fecal sample (100µL) was added with 1.8M sulfuric acid (200µL) in 1 row of 3 wells per sample. H_2O_2 (15µL) was added to each well. Each plate was mixed on a plate shaker for 30 minutes. Plates were placed into a microplate reader (Fluostar Optima, BMG Labtech Inc., Cary, NC) and absorption measured at 410nm with a 5-level standard using analysis software (MARS 3.01R2, BMG Labtech Inc., Cary, NC). The TiO_2 template within the software was used to calculate titanium values. TiO_2 values were corrected for dry matter using the equation (TiO_2 x %DM)/ 100.

Gross energy (GE) was determined on 0.5g per fecal sample and 0.4g of feed sample in duplicate. Each sample was pressed into pellets and placed into individual crucibles**.** A bomb calorimeter (model No. 6400, Parr Instrument Co., Moline, IL) was

used to determine gross energy. Gross energy was averaged for each duplicated sample. Gross energy was corrected for dry matter by using the equation: (Gross energy x %DM)/ 100.

Digestibility and apparent metabolizable energy calculations were as follows:

% Ca digestibility= 1- [((% dietary Ti/% excreta Ti) x (excreta Ca/dietary Ca)]

% P digestibility= 1- [((% dietary Ti/% excreta Ti) x (excreta P/dietary P))]

AME=1- [(dietary Ti/excreta Ti) x (excreta GE/dietary GE)]* dietary GE

Histology: Intestinal samples of the jejunum and ileum were collected from six hens (3 per treatment). Hens were euthanized by cervical dislocation. Each hen was cut open at the abdomen and the jejunum and ileum were identified by locating the ceca and Meckel's Diverticulum. Each collected sample was rinsed with chilled PBS to remove digesta and excess fat was removed with a scalpel. Each sample was placed in a labeled tube with 10% buffered formalin. After 24 hours the formalin was decanted into a waste bottle and the tissue was submerged in 70% ethanol. After another 24 hours the 70% ethanol was decanted and replaced with fresh 70% ethanol for storage.

Dehydration: Each sample was then cut evenly so that the length was similar to the height of a labeled plastic cassette. The aim was to retrieve a circular cut of the sample (without bending). The cut tissue was placed between the sponges in the cassette and stored in 70% ethanol while the remaining samples were cut. Once all of the tissues were in cassettes, the cassettes were immersed in 100% ethanol for one hour. This immersion process was repeated 3 times with fresh ethanol utilized for the third immersion. The cassettes were then placed in CitriSolv (Fisher, Cat. No. 22-143975) for

an hour and the process was repeated 3 times with fresh CitriSolv being used for the third immersion.

Embedding Tissue: Cassettes were placed in warm parafilm overnight in an embedding machine (Tissue-Tek Tec III Embedding Center Model 4584, GMI, Ramsey, MN). Each cassette was taken out of the parafilm and the top broken off (revealing the sample). A metal cassette of similar size was half filled with warm parafilm and the sample was placed into the middle of the cassette. The parafilm was allowed to slightly solidify and then was completely filled with parafilm. The original plastic cassette was placed onto the metal cassette and placed onto the cooling plate for 1 to 2 hours. This process was completed for all of the samples followed by sectioning.

Sectioning: Each sample was sectioned using a microtome (AO Spencer Model No. 820, Microtome Service Company, Liverpool, NY) into 5µm sections. Sections were transferred to a warm bath at 35°C using 2 paint brushes to help remove wrinkles in the sample. Sections (4-5) were transferred to each slide by dipping the slides into the water at an angle. Slides (8-10) were made per sample. Slides were dried on a warm plate at 35°C overnight.

Staining: Each staining chemical was stored in wheaton glass rectangular staining jars. Steps for staining were: (1) Slides were deparaffinized using the clearing agent 100% xylene (Thermo Scientific Cat. No. BPX54) 3 times (3 separate jars) at 3-minute intervals; (2) 50:50 100% ethanol and 100% xylene for 3 minutes; (3) 100% ethanol for 3 minutes; (4) 95% ethanol for 3 minutes; (5) 95% ethanol of 3 minutes; (6) 95% ethanol for 3 minutes; (7) distilled water for 2 minutes; (8) Hemotaxylin stain (Fisher Cat. No. 353516) for 2 minutes; (9) 1% PBS without calcium (Hyclone, Cat. No. SH30256) for 1

minute; (10) distilled water for 2 minutes; (11) 1 Dip in Eosin stain (VWR Cat. No. 3801619) (12) 70% ethanol for 30 seconds (13); 95% ethanol for 30 seconds; (14) 100% Ethanol for 30 seconds; (15) 100% xylene for 2 minutes. After the staining process, cover slips were mounted onto the slide covering the sample sections using permount mounting medium (Fisher, Cat. No. FLSP15100). Slides were pressed to remove air bubbles.

Villi Measurement: Sections of the jejunum and ileum were examined under a light microscope with a color and monochrome camera (Model No. DP74, Olympus Corporation, Shinjuku, Tokyo, Japan). This camera was used in conjunction with the computer program, Cell Sens (Olympus Corporation, Shinjuku, Tokyo, Japan) to measure villi height and crypt depth in micrometers(µm). Healthy (undamaged) villi and crypt (5 each) were measured per section. Villi height was measured from the opening of the crypt to the tip of the villi. The crypt depth was measured from the opening of the crypt to the bottom of the crypt. Villi: crypt ratios were calculated from each villus measured. Examples of these measurements can be found below in figures 1 and 2.

Figure 1. Villi height measurement

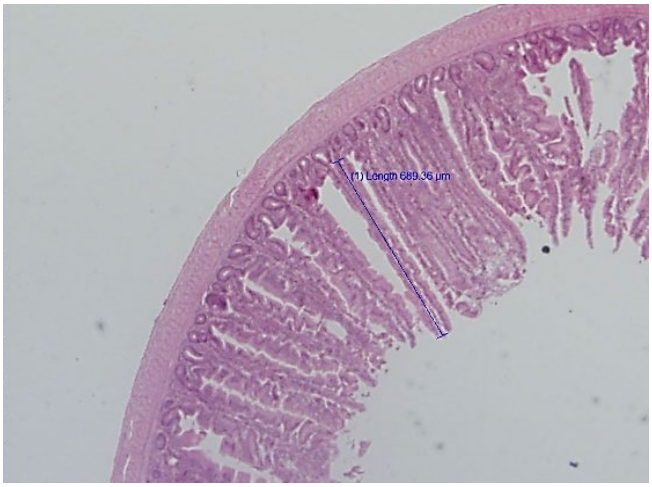
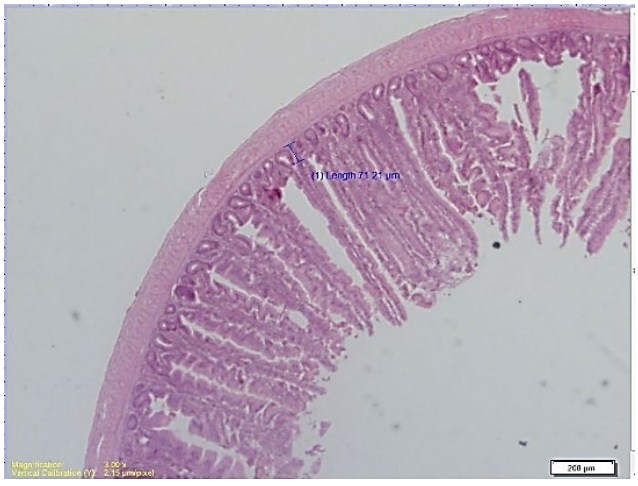


Figure 2. Crypt depth measurement



Data Analysis

Data were analyzed using the Proc Glimmix procedure in SAS (SAS Inc., Cary, NC) for a completely randomized design. Analysis were performed for the factors diet, age, and diet x age interactions. Linear mixed models were used for all variables. In each case, a Kenward-Roger adjustment was used to find the denominator degrees of freedom. Repeated measures were used to evaluate effects of the treatments over time. Correlation was assumed to be correlated under a heterogenous autoregressive 1 (ARH(1)) structure for all measurements. Treatment effects were considered significant at $P < 0.05$.

Egg production was analyzed assuming the *Poisson* distribution using the following model:

$$y|C_{l(i)} \sim Poisson(\exp(\eta_{ijkl})),$$

$$\eta_{ijkl} = \mu + H_l + D_i + (HD)_{ij} + A_k + (DA)_{ik} + C_{l(i)},$$

where η_{ijkl} is the linear predictor; μ is the overall mean; H_l is the number of hens in the l^{th} cage; D_i is the effect of the i^{th} diet; $(HD)_{ij}$ is the diet and number of hens interaction; A_k is the effect of the k^{th} age; $(DA)_{ik}$ is the diet and age interaction effect; and $C_{l(j)}$ is the cage within diet effect

Egg weight, egg mass, feed intake, hen weight, metabolizable energy, egg components, shell strength, calcium digestibility, phosphorous digestibility, villi height, crypt depth, and villi: crypt ratio were analyzed assuming a *normal* distribution with the following model:

$$y_{ijkl} = \mu + D_i + A_j + (DA)_{ij} + C_{k(i)} + \epsilon_l$$

where y_{ijkl} is the linear predictor; μ is the overall mean; D_i is the effect of the i^{th} diet; A_j is the j^{th} age effect; $(DA)_{ij}$ is the effect of the diet and age interaction; C_k is the cage within treatment

effect; and ϵ_l is the residual. For these variables, both the cage and residual were considered to be random.

RESULTS

There was no significant treatment effect observed for mortality. The were 7 and 4 deaths for the control and diet containing butyric acid respectively.

There was no significant treatment effect seen for egg production (Table 2) (P= 0.8130). A significant age effect (P = 0.0013) (Figure 5b). It was observed as both treatments showed increased egg production from about 90% at the start of trial until approximately week 43 and then it gradually declined (Figure 5b). The control and butyric acid showed similar averages of 91.57% and 90.87% respectively (Table 2).

There was no significant treatment effect for average daily feed intake (P = 0.6462) (Table 2) however there was a significant age effect observed (P < 0.0001) as feed intake gradually increased from about 95g per day to about 108g per day (Figure 6). Both treatments averaged about 104g per day overall (Table 2).

No significant treatment effects were observed for average egg weight (P = 0.6629) (Table 3). A significant effect was seen for age (P < 0.0001) as both treatments showed steady increases in egg weight as the trial progressed and both showed slight decline after 69 weeks of age (Figure 7). Control and butyric acid showed averages of 60.71g and 60.70g respectively (Table 3).

There were no significant treatment effects on average egg mass (Table 3). There was also a significant age effect (P < 0.0001) as both treatments generally increased until hens were 69 weeks of age where a decline was observed (Figure 8). There was a

diet*age (P<0.0001) interaction effect observed for egg mass (Table 3). At week 59, there was a sudden decrease in egg production and therefore egg mass for both treatments due to a temporary variation in the care staff. For week 67-71, both treatments recovered and steadily increased. Control and butyric acid showed averages of 55.23g and 54.74g respectively.

There were no significant treatment effects observed on average hen weight (P = 0.7836) (Table 3). Hen weights averaged 1716.10 g and 1720.51 g for the control and butyric acid treatments, respectively. The age effect was observed to be significant (P <0.0001) as hens gradually increased in weight after 49 weeks of age and slightly decrease after 71 weeks of age for both treatments (Figure 9).

There was a significant treatment effect on average eggshell strength (P=0.0493) in favor of the butyric acid treatment (Table 3). There was a significant age effect also seen (P<0.0001). Both treatments showed peaks at week 45 and 59 weeks of age and decreased breaking strength toward the end of the trial (Figure 10).

Egg components showed no significant treatment effect on shell percentage (P=0.7388), albumen percentage (P=0.8468), or yolk percentage (P=0.9275)/ Percent shell and percent albumen showed a significant age effect (P<0.0001) but there was no age effect observed for percent yolk (Table 4). Percent shell remained consistent until week 71 where both treatments showed a small decline (Figure 11). Percent albumen was greatest at the start of the trail and declined after 45 weeks of age after which it remained relatively consistent (Figure 12). A similar increase and decline were observed for percent yolk (Figure 13). Control and butyric acid treatments showed averages of 14.19%

and 14.29% respectively for shell percent; 59.85% and 60.14% for percent albumen respectively; and 28.54% and 28.7% respectively for percent yolk (Table 4).

For calcium digestibility, dietary treatment effect approached significance with the control group at P=0.065 having overall higher digestibility when compared to butyric acid. There was a significant age effect observed (P<0.0001) (Table 5). Both treatments showed improvement in digestibility from the collections at 55 to 65 weeks of age.

No significant treatment effect was observed for phosphorus digestibility but there was a significant age effect (P=0.0004) and diet*age interaction (P=0.0410) (Table 5). The phosphorus digestibility for both treatments showed improvement from the collection at 55 versus 65 weeks of age. The phosphorus digestibility was higher in hens fed butyric acid for the collections at 55 weeks but observed to be lower compared to the control in the 65 weeks collection accounting for the interaction effect.

There was a significant treatment effect observed for AME (P<0.0001) (Table 6). There was also significant age (P<0.0001) and diet*age interaction effects (P=0.0002) observed for AME. For both collections, hens fed butyric acid showed improved AME when compared to the control at 55 to 65 weeks.

There were no significant treatment effects observed for villi height (P=0.2695) in the jejunum but there was a significant age effect (P=0.0091) (Table 7). Both treatments showed an increased villus height from the collection at 55 to 75 weeks. There was no significant treatment effect observed for jejunum crypt depth (P=0.1568). There was a significant age effect observed (P= 0.0122) (Table 7). A higher crypt depth was observed

for butyric acid when compared to the control. Both treatments showed increased crypt depths from the 55 week to 75 week collection. There was a significant treatment effect observed for the villi: crypt ratio (P=0.0148) in favor of control in the jejunum (Table 7). The ratios remained relatively consistent for both treatments from the 55 to 75 week collection.

There was no significant treatment effect seen for ileal villus height (P=0.3755) (Table 8). Both treatments showed a decrease in ileal villus height from the 55 to 75 week collection. There was a significant treatment effect observed for ileal crypt depth (P=0.0057) in favor of the control (Table 8). There were no significant effects seen for the villi: crypt ratio in the ileum (P=0.0808) (Table 8).

DISCUSSION

In this study, a diet supplemented with butyric acid was compared to a control diet for young laying hens. The hypothesis was that including butyric acid in the diet should improve gut health and thereby nutrient absorption. These changes were thought to potentially improve production and performance parameters including egg production, egg weight, eggshell breaking strength, feed intake, hen weight, nutrient digestibility and energy utilization.

Mortality was not found to be significantly affected by the treatments. Similarly, Taherpour et al. (2009) reported that supplementing butyric acid glyceride had no significant effect on mortality when compared to the control. These results are in also agreement with Park et al. (2009) and Soltan (2008) who reported that organic acids showed no significant effect on mortality when compared to a control diet. Soltan (2008)

noted that increases in mortality are more often observed due to environmental conditions rather than treatment effects.

In this study, egg production and egg weight were not significantly different between treatments. The study by Rahman et al. (2008) was in partial agreement with these findings showing that organic acids significantly increased egg production but had no significant effect on egg weights. Yesilbag and Colpan (2006) reported that the use of organic acids also had no significant effect on egg weight and the overall egg production was not statistically different for organic acids compared to the control. Soltan (2008) observed that supplementing an organic acid mixture that included calcium butyrate helped to maintain steady egg production toward the end of the trial when compared to the control and lower doses of the organic acids. That study also found that there was not significant effect on egg weight for any of the doses of calcium butyrate (Soltan, 2008). Sengor et al. (2007) suggested that supplementing SCFA's, particularly butyrate, can increase egg production due to increased absorption of nutrients into the gut wall attributed to healing damaged villi in the small intestine. For that study, a mixture of SCFA's were used including calcium butyrate which might explain why using solely tributyrin in the current study was not as effective in improving egg production.

Feed intake and body weight for the current study was not found to be statistically different between treatments. Similarly, Adil et al. (2010) reported that for broiler chicks, feed intake was not significantly different for those consuming butyric acid at 2 or 3% compared to the control. Contrary to the current study, Adil et al. (2010) also reported increased weight gain for the organic acids when compared to the control. This was attributed to the previously proposed effects of organic acids in improving gut health

(Adil et al., 2010). Adil et al. (2011) reported that there was significantly decreased feed intake found for broiler chicks fed formic acid, lactic acid, or butyric acid at 2% or 3%. This decrease in feed intake was attributed to a decreased palatability of the organic acids. Our current study used tributyrin which is in the form of a triglyceride instead of the acid form. This may partially explain why feed consumption was not reduced compared to the control. Contrary to the current study, Leeson et al. (2005) reported reduced feed intake for broilers supplemented with 0.4% butyrate but showed no significant difference in weight gain when compared to the control suggesting improved absorption. Kaczmarek et al. (2015) found that 0.4g/kg of calcium butyrate supplementation led to a significant decrease in feed intake during the starter period but had no effect overall. These studies show that the form of butyric acid used as well as the inclusion rate can result in variable effects on feed intake and weight gain.

Eggshell breaking strength was found to be improved for the butyric acid compared to the control but with both treatments showing a decline as the trial proceeded. These results are in agreement with a study by Sengor et al. (2007) that report that an organic acid premix containing calcium butyrate significantly improved egg shell breaking strength when compared to the control. There was no significant effect on shell percent, yolk percent, and albumen percent observed in the current study. It is unclear the mechanism by which butyric acid increases breaking strength while shell percentage doesn't seem to be significantly affected. Further research into how butyric acid and its salt affects egg shell quality and the mechanisms involved is needed.

This study found that hens fed the diet containing butyric acid experienced significantly improved apparent metabolizable energy. Kaczmarek et al. (2016) also

reported significant increases in metabolizable energy for broilers fed protected calcium butyrate when compared to the control. Butyric acid supplementation causes pH reduction in the intestine leading to antimicrobial effects. These effects can reduce metabolic need as a result of inhibition of harmful bacteria which can allow increased availability of energy and nutrients as well as an increase in villi health (Adil et al., 2010).

The current study found no significant treatment effect for calcium and phosphorous digestibility. Contrarily, Khong et al. (2014) reported significantly higher calcium and phosphorous digestibility/ retention for laying hens supplemented with sodium butyrate. The results of our current study suggest that hens may have been in a state of osteoporosis as more calcium and thereby phosphorus was released from the bone during egg shell formation and excreted in the waste. It is thought that butyric acid may have caused the release of calcium and phosphorous from the bone by acidifying the blood (reducing its pH) resulting in less absorption of calcium and phosphorous. This also could have led to an increased amount of calcium and phosphorus in the feces. Differences could also be observed due to the efficacy of sodium butyrate versus tributyrin. Further research is needed to discern the effect of butyric acid and its salt on calcium and phosphorus digestibility in the laying hen and possibly its potentially effects in reducing the occurrence/ severity of osteoporosis.

In this study it was observed that, in the jejunum, there was no significant treatment effect for villus height or crypt depth but there was a significant effect for villus height to crypt depth ratio in favor of the control. A study by Liu et al. (2017) with broilers found similar results in that the addition of protected sodium butyrate had no

significant effect on the villus height and crypt depth, or the villus height to crypt depth ratio in the jejunum. In the current study it was observed that, in the ileum, there was no significant treatment effect observed for villus height and villus height to crypt depth ratio but there was found to be a significant improvement in the crypt depth in favor of the control. In the study by Liu et al. (2017), there were no significant treatment effects for the villus height and crypt depth but there was a significant effect on the villus height to crypt depth ratio for the ileum. It was noted in that study that significant effects on intestinal morphology may not have been observed in older birds because the intestine was already developed by that time. We find results of the villi measurements of our current study to be inconclusive perhaps due to the limited number of samples tested.

In summary, parameters including egg production, egg weight, feed intake, hen weight, and calcium/ phosphorous digestibility were not significantly affected by the supplementation of butyric acid. The literature discussed previously show that variability is expected for these parameters as researchers are still attempting to find the right supplementation that can provide consistent positive results across experiments. The greatest variability can to be due to inclusion rate, age of the bird, diet composition, and the form of butyric acid (Soltan, 2008). The form of butyric acid also effects where in the gut butyric acid will have its effect. Tributyrin is known to be active in the small intestine (Moquet et al., 2018). However, butyric acid in its salt form has been found to show positive effects on feed intake compared to the acids form due to reduced palatability. It was expected in this study that villi growth would be significantly improved. Limited sampling may have thwarted efforts to measure such effects.

Organic acid mixes have been found to show more consistent results in some studies when compared to a single type of organic acid, especially when butyric acid or its salt is included in the mix (Bedford et al., 2017). The increased performance of butyric acid in the organic mixes was explained by its positive effects on the growth and repair of villi in the small intestine (Qaisrani et al., 2015).

Results that have been found to be consistent across studies include improvement apparent metabolizable energy, eggshell breaking strength, and mortality with supplementation of butyric acid. Although studies suggest that mortality has not been statistically affected; it can be numerically improved as with the current study. With the ban of antibiotics in animal feed in 2006 by the European Union, producers have been concerned about an increase in mortality rates, especially in younger birds (Brzóska, et al., 2013). The aforementioned studies show organic acids to be a suitable alternative for that purpose. Increased energy availability due to changes in the microbial population of the intestine may have partially contributed to the significant increase in metabolizable energy. Calcium and phosphorus digestibility were not significantly affected. Eggshell breaking strength results improved in this trial as well as a previous study done at UNL in older hens (86-100 weeks of age) (Foley et al., 2017). This is a very important outcome that could result in less eggshell damage and more eggs packed.

Further research is needed to better ascertain the optimal supplementation needed to produce the best and most consistent results for performance, especially in the laying hen. Glycerides of butyric acid as well as protected butyric acid salts have the potential to be very effective because of their action in the small intestine. It would be beneficial to

study the effects of tributyrin at various inclusion rates to further examine its efficacy in young laying hens.

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Table 1. Composition of Diets

Ingredient	Diet 1	Diet 2
Fine Ground Corn	52.35	52.35
DDGS	10.00	10.00
Soybean Meal	21.91	21.91
Oil	3.42	3.42
Dicalcium Phosphate	1.10	1.10
Shell & Bone Builder	5.18	5.18
Limestone	5.18	5.18
Lysine	0.10	0.10
Salt, White	0.38	0.38
Methionine	0.19	0.19
Pinnacle Premix	0.20	0.20
Prophorce Butyric Acid*	0.00	0.055
Nutrient Analysis		
Protein	17.20	17.20
Lysine	0.90	0.90
Methionine	0.47	0.47
Calcium	4.25	4.25
Total Sulfur Amino Acid	0.80	0.80
Available Phosphorus	0.35	0.35
Sodium	0.19	0.19
Kcal/kg	2820	2820

*Prophorce Butyric Acid Source: Perstorp Holding AB Neptunigatan 1, 211 20 Malmo Sweden

Table 2. Effect of diet and age on egg production and feed intake

Diet	Egg Production (%)	Feed Intake (g/hen/day)
Control	91.57	104.27
Butyric Acid[1]	90.87	104.97
SEM[2]	0.01	2.72
Main Effects		
Diet		
P-value	0.8130	0.6424
Age		
P-value	0.0013	<0.0001
Interaction		
Diet x Age	0.9030	0.8056

[1]Prophorce Butyric Acid; Perstorp (Malmo,Sweden)
[2]Standard Error of Means

Table 3. Effect of diet and age on hen weight, egg weight, and eggshell breaking strength.

Diet	Hen Weight (g)	Egg Weight (g)	Egg Mass (g)	Eggshell Breaking Strength (N)
Control	1716.10	60.71	55.23	48.13
Butyric Acid[1]	1720.51	60.70	54.74	50.16
SEM[2]	14.30	0.17	0.16	0.33
Main Effects				
Diet				
P-value	0.7386	0.6649	0.7637	0.0493
Age				
P-value	<0.0001	<0.0001	<0.0001	<0.0001
Interaction				
Diet x Age	0.3364	0.9893	<0.0001	0.7583

[1]Prophorce Butyric Acid; Perstorp (Malmo,Sweden)

[2]Standard Error of Means

Table 4. Effect of diet and age on egg components (Percent shell, percent yolk, and percent albumen).

Diet	Shell (%)	Yolk (%)	Albumen (%)
Control	14.19	28.54	59.85
Butyric Acid[1]	14.29	28.70	60.14
SEM[2]	0.13	0.13	0.23
Main Effects			
Diet			
P-value	0.7388	.8468	0.9275
Age			
P-value	<0.0001	0.1211	<0.0001
Interaction			
Diet x Age	0.9629	0.2839	0.7773

[1]Prophorce Butyric Acid; Perstorp (Malmo,Sweden)
[2]Standard Error of Means

Table 5. Effect of diet and age on calcium and phosphorus digestibility.

Diet	Calcium digestibility		Phosphorous digestibility	
	55 weeks	65 weeks	55 weeks	65 weeks
Control	26.32	38.65	-10.10	6.07
Butyric Acid[1]	23.02	33.90	-5.19	-0.63
SEM[2]	2.13	1.63	3.09	2.40
Main Effects				
Diet				
P-value	0.0648		0.7475	
Age				
P-value	<0.0001		0.0004	
Interaction				
Diet x Age	0.6544		0.0410	

[1]Prophorce Butyric Acid; Perstorp (Malmo,Sweden)
[2]Standard Error of Means

Table 6. Effect of diet and age on metabolizable energy.

Diet	AME at 55 weeks (Kcal/kg)	AME at 65 weeks (Kcal/kg)
Control	1882.27	2193.78
Butyric Acid[1]	2154.20	2281.59
SEM[2]	22.66	22.66
Main Effects		
Diet		
P-value	<0.0001	
Age		
P-value	<0.0001	
Interaction		
Diet x Age	0.0002	

[1]Prophorce Butyric Acid; Perstorp (Malmo,Sweden)
[2]Standard Error of Means

Table 7. Effect of diet and age on villi height, crypt depth, and villi height- crypt depth ratio in the Jejunum

Diet	Villi Height(µm)		Crypt Depth(µm)		Villi height: Crypt Depth	
	55 weeks	75 weeks	55 weeks	75 weeks	55 weeks	75 weeks
Control	1029.22	1195.74	144.36	169.53	7.40	7.24
Butyric Acid[1]	940.63	1133.38	160.65	186.37	6.26	6.26
SEM[2]	48.18	54.69	12.50	7.96	0.4461	0.4543
Main Effects						
Diet P-value	0.2695		0.1568		0.0148	
Age P-value	0.0091		0.0122		0.8755	
Interaction Diet x Age	0.8402		0.9768		0.8794	

[1]Prophorce Butyric Acid; Perstorp (Malmo,Sweden)
[2]Standard Error of Means

Table 8. Effect of diet and age on villi Height, crypt depth, and villi height- crypt depth ratio in the Ileum.

Diet	Villi Height (µm)		Crypt Depth(µm)		Villi height: Crypt Depth	
	55 weeks	75 weeks	55 weeks	75 weeks	55 weeks	75 weeks
Control	920.35	900.77	188.31	174.06	5.52	5.25
Butyric Acid[1]	902.12	824.82	144.50	148.09	6.27	5.78
SEM[2]	44.66	43.3	13.68	8.86	0.44	0.32
Main Effects						
Diet P-value	0.3755		0.0057		0.0808	
Age P-value	0.3732		0.6436		0.3595	
Interaction Diet x Age	0.5942		0.4402		0.7886	

[1]Prophorce Butyric Acid; Perstorp (Malmo,Sweden)
[2]Standard Error of Means

Figure 5a. Effect of diet and age on percent egg production.

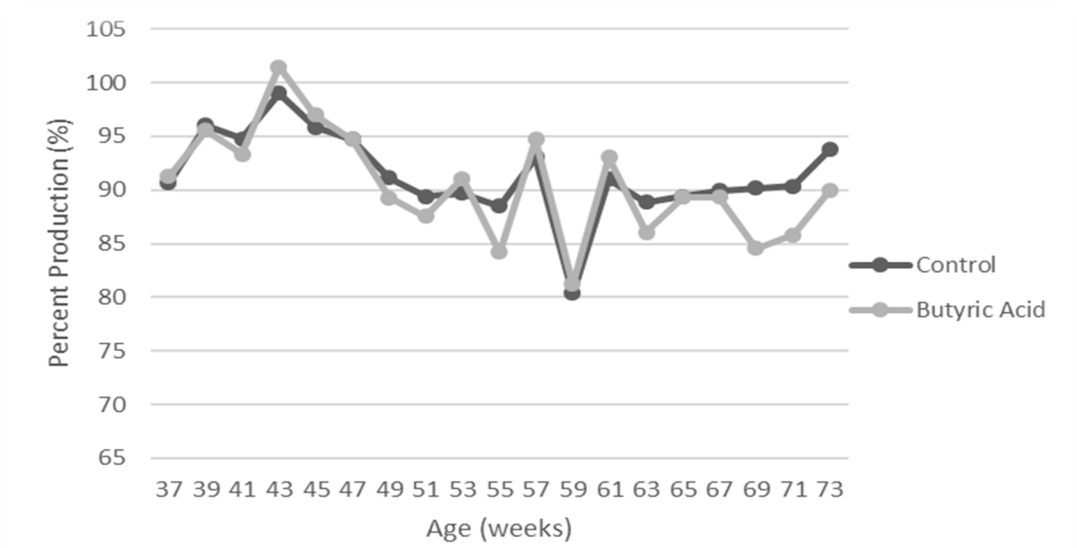

Figure 5b. LS means for the effect of diet and age eggs produced per hen.

Diet: P= 0.8130; Age: P= 0.0013; Diet x Age: P= 0.9030
Trt 1: Control; Trt 2: Butyric Acid
Time 1 corresponds to 35 weeks of age (Month 1)

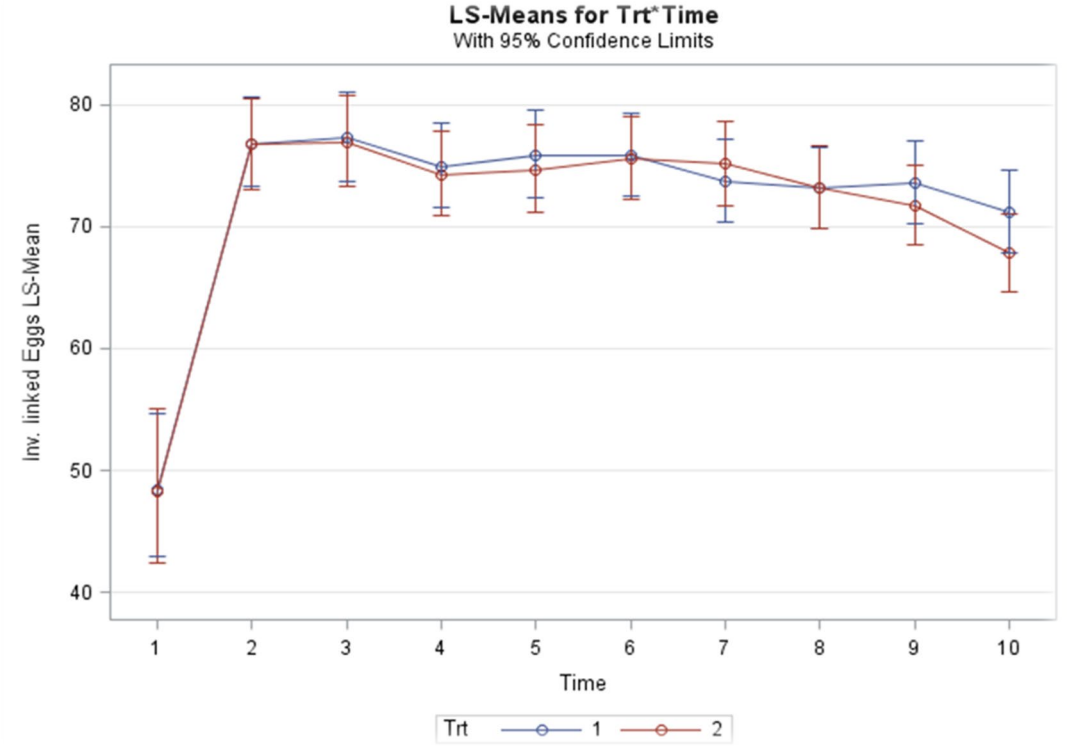

Figure 6. Effect of diet and age on feed intake.

Diet: P= 0.6424; Age: P <0.0001; Diet x Age: P= 0.8056

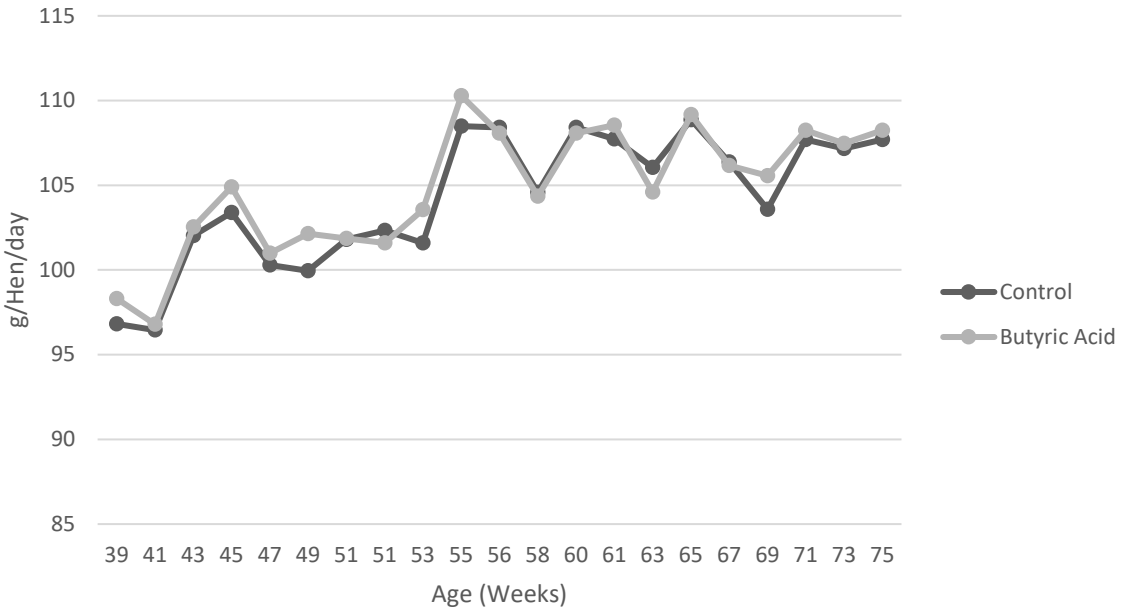

Figure 7. LS means for the effect of diet and age on egg weight.

Diet: P= 0.6649; Age: P<0.0001; Diet x Age: P= 0.9893
Trt 1: Control; Trt 2: Butyric Acid
Time 3 corresponds to 39 weeks of age

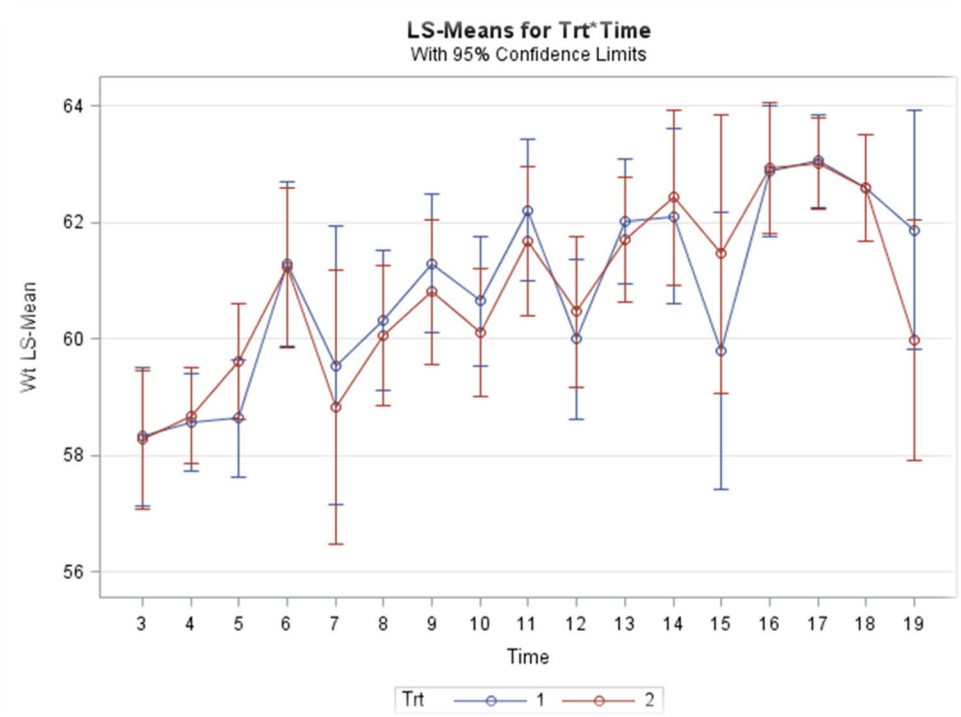

Figure 8. LS means for the effect of diet and age on egg mass.

Diet: P= 0.7637; Age: P< 0.0001; Diet x Age: P= <0.0001
Trt 1: Control; Trt 2: Butyric Acid
Time 3 corresponds to 39 weeks of age

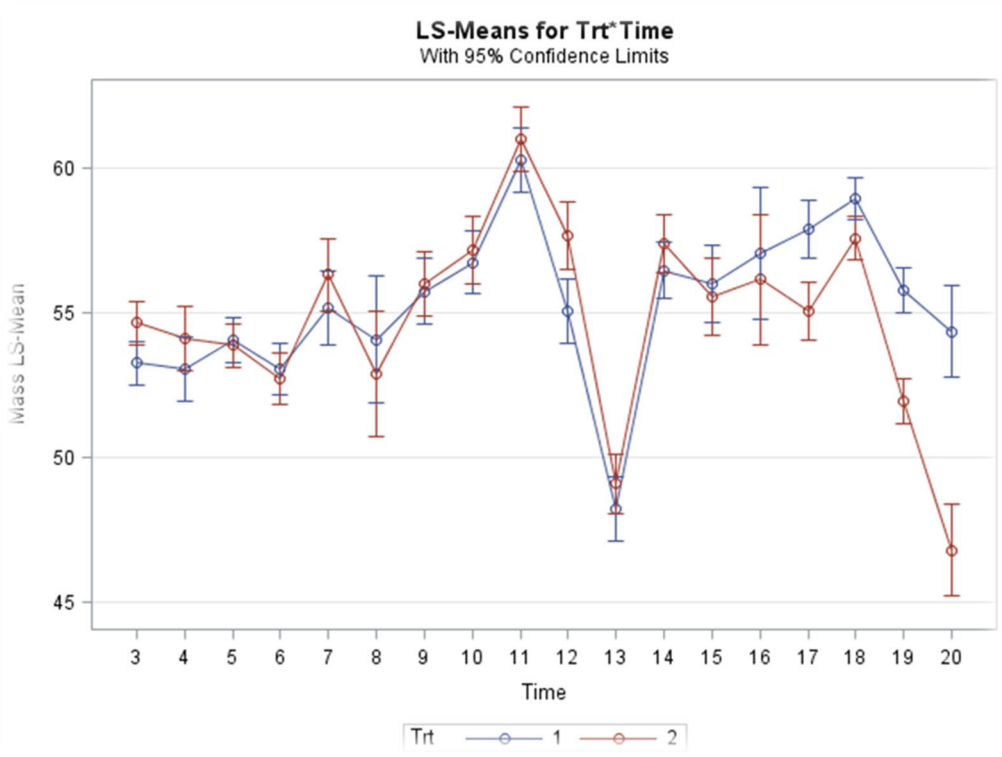

Figure 9. LS means for the effect of diet and age on hen weight

Diet: P= 0.7386; Age: P<0.0001; Diet x Age: P= 0.3364
Trt 1: Control; Trt 2: Butyric Acid
Time 1 corresponds to 35 weeks of age

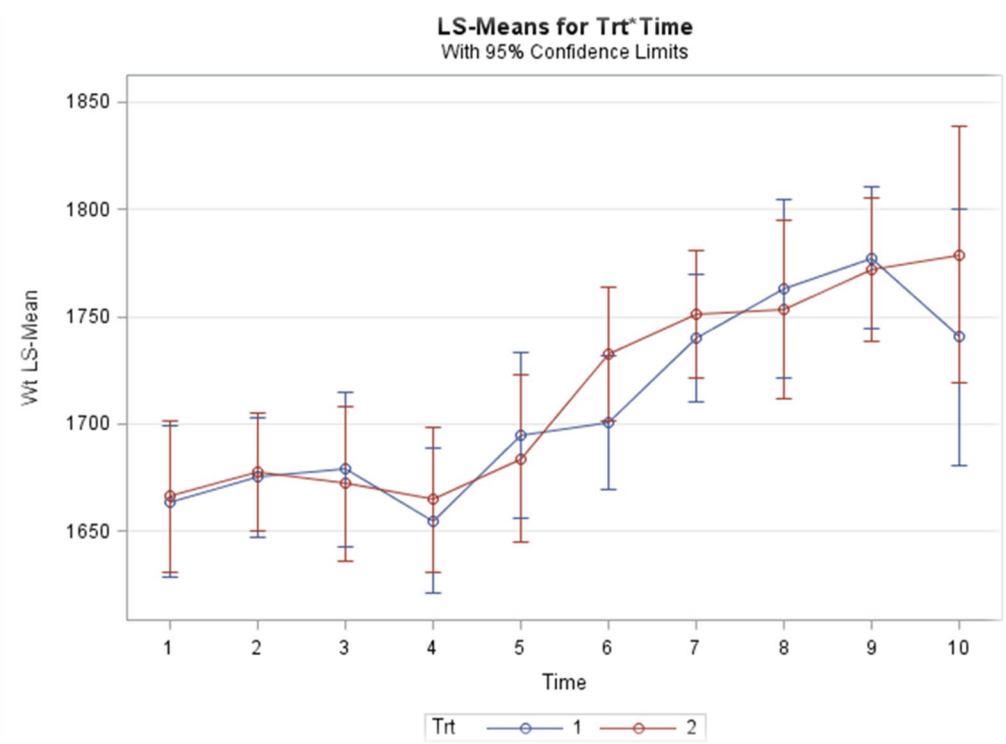

Figure 10. LS means for the effect of diet and age on eggshell breaking strength

Diet: P= 0.0493; Age: P<0.0001; Diet x Age: P= 0.7583
Trt 1: Control; Trt 2: Butyric Acid
Time 1 corresponds to 35 weeks of age

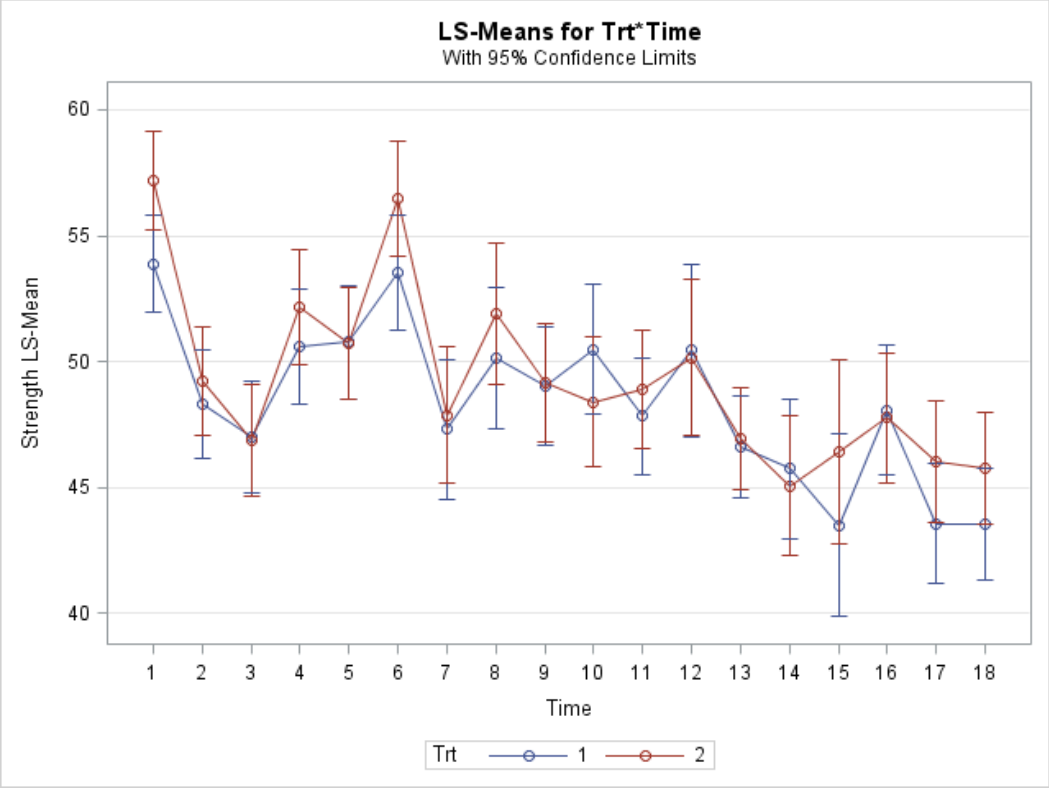

Figure 11. Effect of diet and age on eggshell percentage.

Diet: P= 0.7388; Age: P<0.0001; Diet x Age: P= 0.9629

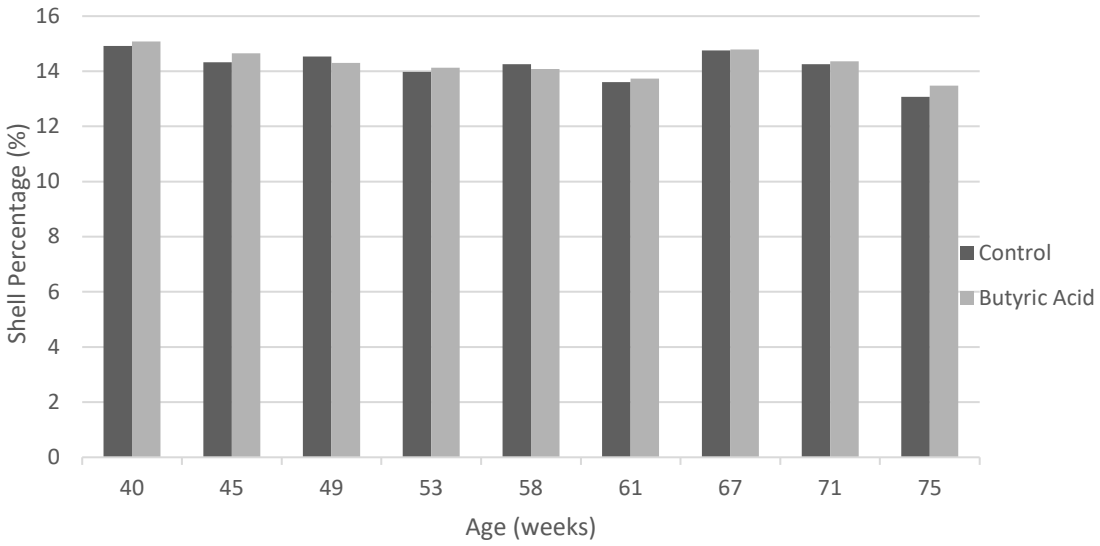

Figure 12. Effect of diet and age on albumen percentage.

Diet: P= 0.9275; Age: P<0.0001; Diet x Age: P= 0.7773

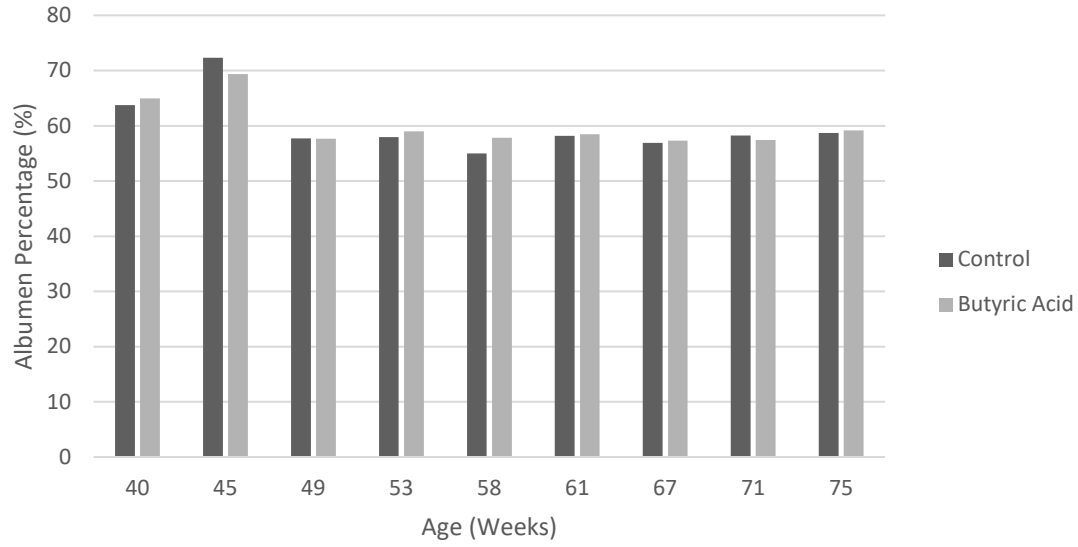

Figure 13. Effect of diet and age on yolk percentage.

Diet: P= 0.8468; Age: P= 0.1211; Diet x Age: P= 0.2839