DETERMINING THE EFFECTS OF ENCAPSULATED SODIUM BUTYRATE WITH VARYING RELEASING TIMES IN BROILERS

by

JUNDI LIU

(Under the Direction of Justin Fowler)

ABSTRACT

Sodium butyrate (Na-B) has been shown to impact growth performance and gut health in chickens raised without antibiotics. In Experiment 1, the feed rate-of-passage of 3-week-old broilers was determined via a computed tomography assay. The results suggested that 30 minutes to 2.5 hour post-ingestion is the appropriate time for the release of encapsulated Na-B aimed at stimulating intestinal epithelial development and improving nutrient digestibility. For those products that focus on hindgut bacterial control, 2.5 to 4 hour would be an optimal range for the releasing time. Experiment 2 evaluated two encapsulated Na-B products with different releasing times over a range of dosages (250, 500, 750, 1000, and 1500 ppm). The 2 h releasing time product at 1000 ppm significantly improved the BW on d 21, and showed the highest villus height in the jejunum when added at 750 ppm. Experiment 3 evaluated three Na-B products with varying releasing times on broiler performance with a *Salmonella* typhimurium challenge. The 2 h and 3-4 h releasing time Na-B products both showed the potential to improve villus growth and ileal digestible energy (IDE). However, both Experiment 2 and 3 did not show significant effect on *Salmonella* colonization. In Experiment 4, two releasing time Na-B products were evaluated for a full grow-out period in broilers. Adding 2 h and 3-4 h releasing time Na-B products at 500

and 1000 ppm improved the IDE, but did not show significant effect on growth without an experimental challenge. Experiment 5 evaluated two Na-B products with targeted releasing times on growth and mitigating necrotic enteritis. The 2 h releasing time product at 500 ppm showed significantly higher BW compared to the challenge control on d 21. Both the 2 h and 3-4 h releasing time products showed a mitigation impact on the lesions associated with necrotic enteritis. In conclusion, encapsulated Na-B has the potential to improve growth, IDE, villus development, and showed the ability to mitigate the impact of necrotic enteritis in challenged broilers. The beneficial effects on growth performance and gut health are affected not only by the dosage level, but also by the product's releasing time.

INDEX WORDS: sodium butyrate, growth, gut health, *Salmonella*, necrotic enteritis, broiler

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DEDICATION

I would like to dedicate this dissertation to my loving wife and parents. Without your support, accompany and love, I could not have accomplished this achievement.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

With the new changing consumer demands for antibiotic-free raised poultry, an increasing amount of research has focused on feed additive products, that can act as antibiotic replacements. Sodium butyrate, one of the short chain fatty acids, has been considered as a potential alternative product to antibiotics for improving growth performance and maintaining gut health. The objective of this project was to evaluate an encapsulated sodium butyrate with varying targeted releasing times on growth performance, energy digestibility, gut morphology and bacterial inhibition in broiler chickens. The central hypothesis was that the ability of encapsulated sodium butyrate to improve growth and bacterial control is not only affected by dosage, but also influenced by the product's releasing time.

In order to accomplish the research objective, five experiments were conducted to evaluate the efficacy of encapsulated sodium butyrate. As the first approach, we aimed to find the releasing time standard for the encapsulated sodium butyrate product that targeted its releasing at different intestinal segment:

Experiment 1. Computed tomographic precision rate-of-passage assay without a fasting period in broilers: More precise foundation for targeting the releasing time of encapsulated products

Next, an experiment was conducted to find the optimal dosage range for adding different targeted releasing time encapsulated sodium butyrate products:

Experiment 2. Encapsulate sodium butyrate with multiple adding dosage on broiler performance under a nalidixic acid resistant *Salmonella* typhimurium challenge

Afterwards, three experiments were conducted to evaluate the efficacy of encapsulated sodium butyrate with varying targeted releasing time under a *Salmonella* challenge, a non-challenged full grow-out, and a *Clostridium perfringes*-induced necrotic enteritis challenge condition:

Experiment 3 and 4. Evaluation of encapsulated sodium butyrate on growth performance, energy digestibility, gut development and *Salmonella* colonization in broilers

Experiment 5. Evaluation of encapsulated sodium butyrate with varying releasing times on growth performance and necrotic enteritis mitigation in broilers

LITERATURE REVIEW

Avian Digestive Tract and Digesta Rate of Passage

The digestive tract (Figure 1.1), also known as the gastro intestinal (GI) tract, is the place to breakdown the complex organic and inorganic molecules inside feed to the enable absorption by the chicken (Lesson and Summers, 2001). Chickens have a relative shorter GI tract compared with humans (Whittow, 2000). Starting at the mouth (pH = 7.0 to 7.5), birds use the beak to ingest and swallow the whole feed particles for lack of teeth and heavy jaw muscles (Kaiser, 2007). The saliva lubricates and softens the feed, as well as contains small quantities of the amylase enzyme to initiate starch digestion to a small degree (Osman, 1982; Lesson and Summers, 2001). Feed then passes through the esophagus into the crop (pH = 4.5). The crop is used as a storage organ for undigested feed and has some moistening and softening functions for feed (Soedarmo et al., 1961; Svihus et al., 2010). The feed then enters the proventriculus and ventriculus (gizzard). The proventriculus secrets hydrochloric acid, which creates a low pH (~2.5) environment. The secreted enzymes in the proventriculus will break down the protein and triglycerides into polypeptides and fatty acids (Lesson and Summers, 2001; Svihus, 2014). The ventriculus is a large mass of muscle tissue, which exerts mechanical force for feed breakdown from large particles into a smaller size (Svihus, 2011). Liver, gall-bladder and pancreas are organs closely related to the digestive process. Birds deliver bile to the duodenum via two ducts. The gall bladder stores the bile and releases it into the duodenum through another connected duct (Dibner and Richards, 2004). The main function of the pancreas is to provide exocrine and endocrine secretion of digestive enzymes and hormones such as insulin and glucagon (Lesson and Summers, 2001; Pinheiro et al., 2004).

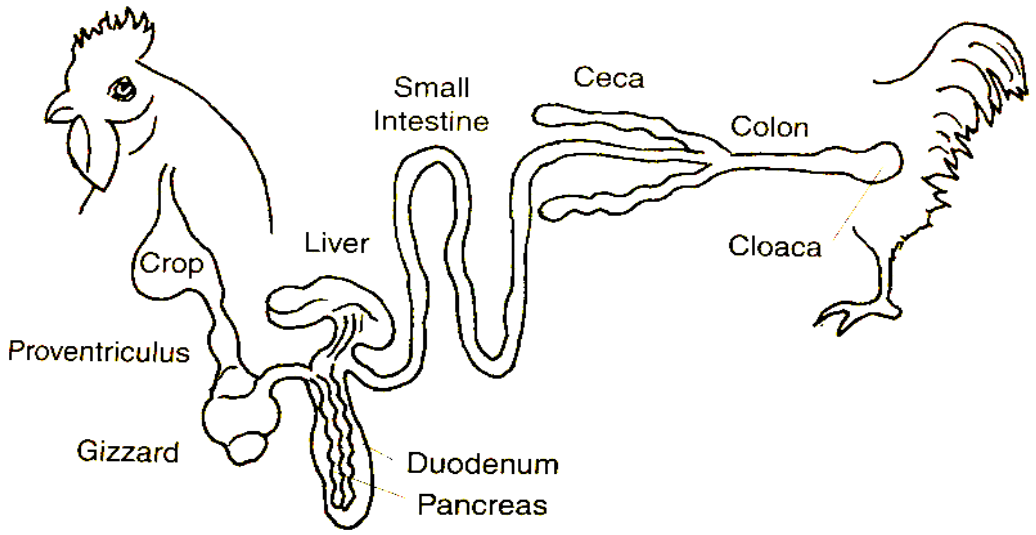

Figure 1.1 Digestive system of the chicken. Source: Leeson and Summers, 2001.

The small intestine is the major section of the GI tract for nutrient digestion and absorption. There are three segments that constitute the small intestine: duodenum, jejunum and ileum. Duodenum (pH = 6.0 to 6.8) is the first segment of the small intestine, which presents as a "U"-shaped loop (Lesson and Summers, 2001; Svihus, 2014). The duodenum secretes various enzymes (amylase, trypsin, chymotrypsin, lipase, carboxypeptidase and collagenase) to degrade the starch, dextrin, protein and fat into smaller molecules (Whittow, 2000; Lesson and Summers, 2001; Ren et al., 2012). The jejunum (pH = 5.8 to 6.8) is an extended segment of intestine for further nutrient digestion and absorption. In general, a majority of the nutrients are digested and absorbed by at the end of jejunum (Svihus, 2014). Meckel's diverticulum (the residual yolk sack) is normally used to separate the jejunum and ileum (Branton et al., 1988; Kim et al., 2011). The ileum (pH = 5.7 to 5.9) is the end part of the small intestine. Researchers have shown that it has beneficial effects on water and vitamin absorption (Svihus, 2014), as well as contributes to starch

digestion and absorption (Zimonja and Svihus. 2009). The large intestine of chickens is relatively short compared with other animal species. The colon has some water absorption function (Svihus, 2014). Birds also have the special organ, called ceca. The pair of ceca are located at the junction between small and large intestine (Clench and Mathias, 1995). This is the major place for bacterial growth and fermentation, as well as some water and electrolyte absorption (Klasing, 2005). The GI tract ends at the cloaca, where the feces and urine (uric acid) are mixed before excreting through the vent (Krogdahl and Dalsgard, 1981).

Traditionally, ferric oxide and chromic oxide are two indigestible markers that have been used for detecting the digesta rate of passage in birds (Hillerman et al, 1953; Almirall and Esteve-Garcia, 1994; Sales and Janssens, 2003): birds are orally gavaged with a certain percentage of the indigestible marker after a certain fasting period. Digesta samples are then collected from each intestinal segment for analyzing the concentration of the indigestible marker at different time points post-gavage. The average digesta rate of passage depends on the concentration of the indigestible marker in each segment compared with the total concentration in the initial feed used during gavage (Liu et al., 2017b). Svihus et al. (2002) showed that indigestible markers pass into the gizzard 30 minutes post-feeding. The marker was first found in the excreta around 2 to 2.5 hours after feeding (Tuckey et al., 1958). However, there are some disadvantages when detecting the digesta rate of passage using the traditional method. Studies have found that the fasting period can change the physiologic conditions in birds, which may lead to a faster digesta rate of passage than under full-fed conditions (Gonzales et al., 2003). Also, from the efficiency aspect, it will take more time and efforts for the sampling and measurement if collecting duodenum, jejunum, ileum and colon segments from multiple birds at multiple time points post-gavage.

Antibiotic and Potential Alternatives

The first chemical compound with antibiotic properties, penicillin, was identified by Alexander Fleming in 1928 (Davies and Davies, 2010). Since then, antibiotics have been widely used in human clinical and research areas and are well known for the prevention and treatment of diseases (Phillips et al., 2004). Antibiotics are also used by animal producers as a part of their comprehensive animal management: adding into water and feed to prevent and treat diseases. In the 1940s, when people first fed chicken with dried mycelia of *Streptomyces aureofaciens*, which contained chlortetracycline, it showed improved weight gain and feed efficiency in birds (Moore et al., 1946; Castanon, 2007). Later, antibiotics was approved to be used as a growth promoter in both U.S. and European countries (Jones and Ricke, 2003; Dibner and Richards, 2005).

However, an irresponsible use of antibiotics can lead to antibiotic resistant bacteria, which has been recognized as a global issue related to human health (Marshall and Levy, 2011). The Centers for Disease Control (CDC) (2013) reported that each year in United States, at least 2 million people become infected with antibiotic resistant bacteria and at least 23,000 people died as a direct result of the related infections (http://www.cdc.gov/drugresistance). It has been shown by the National Chicken Council (http://www.nationalchickencouncil.org) that only 2 (*Campylobacter* and non-typhoidal *Salmonella*) out of the 18 bacteria-specific resistance threats reported by CDC have a potential source related to the livestock industry. The overlap of antibiotics used between human and livestock animals is rare, and adequate cooking can also destroy the potential resistant bacterias contaminated animal-derived food (Phillips et al., 2004). In practice, there is also a certain withdrawal period to ensure that the antibiotic has been metabolized and is no longer present in the animals before entering the market (U.S. Food and Drug Administration).

The debate over using antibiotics in livestock animal and its safety related to human health is continuing (Falkow and Kennedy, 2001; Landers et al., 2012). Meanwhile, an increasing number of fast-food chains have begun marketing that they only use antibiotic-free chicken in their products. The major chicken producers and retail food chains are also changing their marketing strategies towards this new demand from customers (Ajuwon, 2016). From January 2017, the U.S. Food and Drug Administration started the Veterinary Feed Directive Final Rule, which is aimed at eliminating the use of sub-therapeutic dosages for antibiotics for "growth promoting" (weight gain and feed efficiency) purposes (https://www.fda.gov).

With the public pressure of potential antimicrobial resistance (Olonitola et al., 2015) and the rise in consumer demand for products labeled as "Raised Without Antibiotics" or "No Antibiotics Ever" (Gadde et al., 2017), an increasing amount of poultry research has been aimed at evaluating antibiotic alternatives, mainly prebiotics, probiotics (also known as direct-fed microbials), plant extracts, and organic acids (Yang et al., 2009; Hume, 2011; Huyghebaert et al., 2011; Roberts et al., 2015):

Prebiotics are a non-digestible diet component that can benefit the growth or activity of microorganisms in the host (Gibson and Roberfroid, 1995). They mainly include fructo-oligosaccharides (FOS, oligofructose, inulin), gluco-oligosaccharides, trans-galacto-oligosaccharides, glyco-oligosaccharides, lactulose, lactitol, xylo-oligosaccharides, malto-oligosaccharides, raffinose, stachyose, sucrose thermal oligosaccharides and mannan-oligosaccharides (MOS) (Patterson and Burkholder, 2003; Hume, 2011; Swiatkiewicz and Arczewska-Wlosek, 2012; Ganguly, 2013; Sugiharto, 2016). In a review article, Griggs and Jacob (2005) demonstrated that the application of FOS can improve the growth performance and feed efficiency, but the results are inconsistent within different studies: some studies reported

that adding 0.4% FOS product significantly improve the average daily gain and feed efficiency; but other researchers were unable to show consistent effects on growth and feed efficiency when adding 0.375% FOS products in broilers. In addition, adding MOS products at 0.1% may benefit the growth performance. The beneficial effects of prebiotics may be related to the improved growth and metabolic action of useful bacteria (bifido and lactic acid producing bacterias). It will help the beneficial bacteria compete with pathogenic bacteria for nutrients and binging sites on the intestinal epithelium, which overall improves the nutrient digestion/absorption, increase the volatile fatty acids concentration in GI tract, inhibit the pathogenic bacteria proliferation, as well as reduce the inflammatory reaction in birds (Simmering and Blaut, 2001; Patterson and Burkholder, 2003; Ganguly, 2013; Ajuwon, 2016).

Probiotics are live micro-organisms which benefit the host after consumption (Fuller, 1989). In poultry, mainly the yeasts and bacterial species, like *Lactobacillus, Bifidobacterium, Streptococcus, Bacillus,* and *Saccharomyces* are fed as probiotics (Applegate et al., 2010). Vila et al. (2010) also mentioned that the *Lactobacillus species* and *S. faecium* are normally present in the GI tract and *Bacillus* species and yeasts are only sporadically present in gut microbiota. Adding probiotics into the feed improved growth, feed conversion ratio, intestinal morphology, maintained the normal intestinal microbiota population balance (competitive exclusion and antagonism effects), and reduced the mortality (Griggs and Jacob, 2005; Kabir, 2009; Vila et al., 2010). Song et al. (2014) showed an improved feed to gain ratio but no temperature × diet interaction effect when adding 1.5 g/kg of probiotics under a heat stress environment. La Ragione et al. (2001) and La Ragione and Woodward (2003) also mentioned that probiotics can mediate immune system activity (increase antibody production, improve immune organ development), bacterial colonization resistance, and pathogen inhibition (in birds challenged

with *Escherichia coli* and *Salmonella* Enteritis).

Phytogenics, also known as botanicals or plant extracts come from a broad range of plants and contain multiple active compounds, such as thyme, anise, ginger, turmeric, cinnamon, essential oil and oleoresins (Duke and Beckstrom-Sternberg, 1994; Guo et al., 2003; Applegate et al., 2010; Gheisar et al., 2015). Jamroz et al. (2003) found that adding a blend of plant extracts containing capsaicin, carvacrol and cinnamic at 150 and 300 ppm both improved the BW and feed conversion ratio in broilers. Other researchers also found adding an oregano, clove and anise mixed essential oil product at 200 ppm improved BW gain, with no significant effect on feed intake (Ertas et al., 2005). Giannenas et al. (2003) added 300 mg/kg of an oregano essential oil product in the diet and found higher BW gain and better feed conversion ratio than the *E. tenella* challenged treatments two weeks after the challenge. Brenes and Roura (2010) summarized that the phytogenics have the potential to increase the growth, intestinal morphology development, digestive enzyme secretion, nutrient digestibility, reduce inflammation, and exert beneficial functions as an antimicrobial. The potential explanation for the beneficial effects of the phytogenic products is related to its effects on endogenous enzymes activities, intestinal integrity, GI tract and microbiota environment, antioxidative and antibacterial functions (Applegate et al., 2010; Hume, 2011; Mahmood et al., 2014).

Organic acids, including formic acid, acetic acid, propionic acid, butyric acid, lactic acid, citric acid, sorbic acid, fumaric acid, and malonic acid, are normally used as acidifiers in the water to regulate the intestinal pH and exert beneficial antimicrobial activity (Van Immerseel et al., 2006; Swiatkiewicz and Arczewska-Wlosek, 2012; Mahmood et al., 2014). Emami et al. (2017) found adding mixed organic acid products can significantly improve the growth performance, ileal morphology and immune response under an *E. coli* K88 challenge. The

similar beneficial effects on growth performance and pathogenic inhibition were also reviewed in swine studies (Partanen and Mroz, 1999). However, Goodarzi Boroojeni et al. (2014) found that adding a blend of formic and propionic acid in the diets did not have positive or negative effects on nutrient digestibility and growth performance in broilers. Biggs and Parsons (2008) also mentioned that feeding gluconic acid, citric acid and malic acid in the corn-soybean meal diet did not have consistent effects on growth, metabolizable energy, and amino acid digestibility. Studies showed that the organic acids can also relieve the effect of *Clostridium perfringens* induced necrotic enteritis (Timbermont et al., 2010). Several studies have found that organic acids can reduce the feed buffering capacity and maintain an optimal pH in the GI tract environment, which will inhibit the pathogenic bacteria populations (Van Immerseel et al., 2006; Swiatkiewicz and Arczewska-Wlosek. 2012).

Salmonella challenge and Clostridium Perfringens-induced Necrotic Enteritis Challenge

With changing consumer mindsets, poultry research is in the process of exploring this new feed additive environment. One important change includes the increasing number of antibiotic alternative products being evaluated via a pathogenic challenge model. *Salmonella* spp., coccidia, and *Clostridium perfringens* are commonly used in evaluation studies for the efficacy of such products.

Salmonella spp. are gram negative, rod-shaped, facultative anaerobic bacteria belonging to the family *Enterobacteriaceae* (D'Aoust and Maurer, 2007). *Salmonella enterica* subspecies serovar Typhrimurium and Enteritidis are the two of the most common serotypes, which are also recognized as a primary source of foodborne gastroenteritis in humans (Ricke et al., 2013). The *Salmonella* infection normally goes from infected hen to chick through the egg, or via the fecal-oral route. Adhikari et al. (2017) compared the colonization of *Salmonella* Enteritidis through

oral or intracloacal inoculation in laying hens. The authors used 0.1 mL of 10^8 cfu/mL *Salmonella* Enteritidis and collected the ceca, spleen, liver with gall bladder at 7 and 14 d post the challenge. There was no significant difference on *Salmonella* recovery 7 d post the challenge between the two infection routs. In addition, the fecal shedding was 100% positive 3 d after the challenge. But there was no *Salmonella* shedding observed in feces 2 weeks of the challenge. Cox et al. (1994) and Fernández-Rubio et al. (2009) found adding butyrate products in the diet significantly decreases the *Salmonella* colonization in different organs. In another broiler study, Spring et al. (2000) orally challenged the 3 d old birds with 10^4 *Salmonella* typhimurium and found adding a prebiotic product reduced the cecal *Salmonella* typhimurium concentration 7 d post challenge.

Coccidiosis and necrotic enteritis (NE) are common diseases in global poultry industry, which cause high economic losses (Williams, 2005). The coccidiosis-caused intestinal damage, together with *Clostridium perfringens*-induced NE, has grown to be a great concern under antibiotic-free raising programs. Coccidiosis is caused by the host-specific protozoan parasite, from the genus *Eimeria* (Chapman, 2014). There are seven common species of coccidia in poultry researches: *E. tenella, E. acervulina, E. maxima, E. necatrix, E. brunetti, E. mitis, E. praecox, (E. hagani and E. mivati* are not frequently mentioned and reviewed) (Chapman et al., 2010). The various coccidia will infect specific segments of the GI tract and cause different pathological signs (Conway and McKenzie, 2007), including decreased growth rate, poor feathering, diarrhea (even bloody droppings), different degrees of lesion and increased mortality. The coccidia infection is via the fecal-oral route, which is characterized by parasite replication in the host cells. The sporozoites will invade the intestinal epithelial cell line and damage the intestinal mucosa. The damaged gut will initiate villus fusion, increased mucus production, and

immune reaction caused inflammation, which leads more nutrients being released into the lumen, and these can be used as a growth-substrate by *Clostridium perfringes* (Williams, 2005; Timbermont et al., 2011). The whole process from the oocyst ingestion to release may range from 4 to 7 days (Price, 2012). Other studies have showed that coccidial vaccines were able to prevent *Clostridium perfringens*-associated NE (Dahiya et al., 2006).

NE is induced by the pathogenic strain of *Clostridium perfringens*, which is characterized by severe intestinal mucosa necrosis. *Clostridium perfringes* is a gram-positive, spore-forming, anaerobic bacteria (McDevitt et al., 2006). *Clostridial* diseases are related to the *Clostridium perfringens* toxin which results in the intestinal mucosa damage. The clinical signs of NE include decreased growth performance, dark/blood-stained feces, and gross lesions (Yegani and Korver, 2008; Timbermont et al., 2011). Research has shown the interaction between NE and coccidiosis. Subclinical coccidiosis can increase the incidence of NE because mucosal damage facilitates the proliferation of *Clostridium perfringens* (Yegani and Korver, 2008). Jerzsele et al. (2012) evaluated the effect of a combination of sodium butyrate and essential oil products via a NE challenge model. In that study, birds were treated with bursal disease vaccine on d 16 to cause immunosuppression. The challenge treatment birds were orally gavaged with 2 mL 6 - 8 $\times$ 10^8 cfu *Clostridium perfringens* on d 18, 19, 20 and 21 three times a day. On d 19, a 10-fold live attenuated vaccine was presented to the animal to mimic the detrimental effect of coccidiosis. Growth performance, intestinal morphology and lesion score was obtained on d 25. Song et al. (2017) evaluated the encapsulated sodium butyrate for mitigation of necrotic enteritis using coccidia in the challenge model. The birds were orally gavaged with *Eimeria* mixed strains at 12 d of age. On d 16, 17, 18 the NE birds were given a broth culture of 10^8 cfu *Clostridium perfringens*. Growth performance were assessed at d 12, 21 and 35. The intestinal gross lesion

was scored 3 d and 9 d after the *Clostridium perfringens* infection.

Sodium Butyrate and Targeted Releasing Time

Butyric acid is one of the important organic acids or short chain fatty acids (SCFAs), which are the end fermentation product of non-starch polysaccharide and unabsorbed starch in animals (Scheppach, 1994). Researchers (Lesson et al., 2005) have demonstrated that SCFAs can be considered as a potential alternative to antibiotics in the feed. They also mentioned that butyric acid plays an important role in growth performance, intestinal epithelium development, and may have some antimicrobial effects. In practice, because of the volatile and pungent nature of butyric acid, most butyric acid products are used as a salt form (Kaczmarek et al., 2016). Sodium butyrate is the most commonly and frequently used in both practice and in research (Figure 1.2).

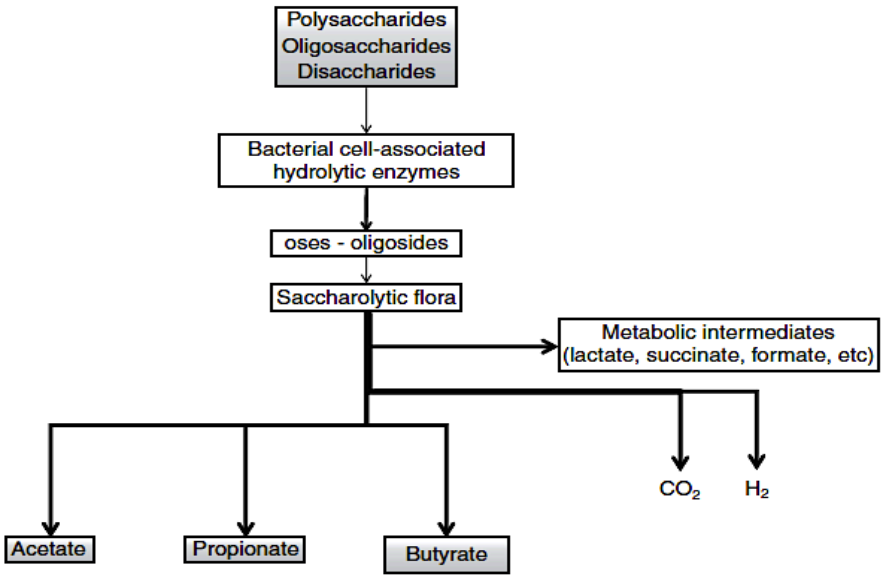

Figure 1.2 Overview of butyrate production in animals. Source: Guilloteau et al., 2010.

(1) Effect on Growth Performance and Energy Digestibility

The sodium butyrate molecule has four carbons (Figure 1.3), which can serve as the

energy source for the enterocytes (Mahdavi and Torki, 2009). It can increase villus growth and the overall absorptive surface area in the intestine (Guilloteau et al., 2010). Previous research has shown the positive effect of adding butyric acid on both BW gain and feed conversion ratio in broilers (Levy et al., 2015). Other researchers showed a significant increase effect in BW gain, but no effect on feed conversion ratio when adding sodium butyrate in the diet through day 21 (Hu and Guo, 2007). In their opinion, butyrate can increase the average daily body mass and feed intake at the same time, which overall leads a non-significant change in feed conversion ratio. Sikandar et al. (2017) added 500 ppm and 1000 ppm sodium butyrate in the diet and found significantly higher BW, BW gain and lower feed conversion ration compared with control treatment. Liu et al. (2017a) showed that sodium butyrate can improve the BW and BW gain at an early age in broilers with a *Salmonella* challenge, which may be because birds at a younger age may be more sensitive to butyrate's effects and the endogenous short chain fatty acid levels are low in the intestine of younger birds (Van der Wielen et al., 2000). However, under a non-challenge/full grow-out period, there was no significant effect on growth performance were observed.

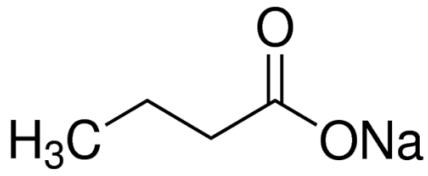

Figure 1.3 The chemical structure of sodium butyrate.

Sodium butyrate products can significantly increase the villus height and nutrient absorption surface area, which can benefit the ileal digestible energy in broilers (Guilloteau et al.,

2010; Liu et al., 2017a). Kaczmarek et al. (2016) conducted a similar study using a protected calcium butyrate, in which the product increased apparent total tract crude fat digestibility and AMEn. The authors thought that the butyrate salt may improve the secretion of pancreatic fluid which can affect the digestibility of crude fat and AMEn.

(2) Effect on Gut Development

The butyrate provides carbons that can serve as an energy source for epithelial cells in the intestine (Jozefiak et al., 2004; Mahdavi and Torki, 2009). Friedel and Levine (1992) showed sodium butyrate can induce water and sodium absorption. In addition, sodium butyrate improved the villus growth by increasing the epithelial cell proliferation and differentiation (Guilloteau et al., 2010). Hu and Guo (2007) also showed the villus height to crypt depth ratio increased linearly with the increase of sodium butyrate from 500 ppm, 1000 ppm to 2000 ppm. Liu et al. (2017a) raised broiler chickens on used litter and found that sodium butyrate can significantly improve the villus height to crypt depth ratio of broilers at 21 days of age. However, there were no significant differences for intestinal morphology results on d 42, which may be because the intestine is fully developed at the older age (Levy et al., 2015). With broilers raised on fresh litter, Levy et al. (2015) also found no difference in duodenum and jejunum morphology results when adding butyric acid (at 300 ppm) in the diet through d 42.

(3) Effect on Intestinal Microbiota and Antimicrobial Function

Butyrate is the end fermentation product of fiber in the large intestine, which is very important for the cecal microbiota population in chickens. It is commonly assumed that undissociated forms of butyric acid can easily penetrate the lipid membrane of the bacterial cell (Van Immerseel et al., 2004). Once internalized into the neutral pH of the cell cytoplasm, the proton will dissociate, resulting in a reduction of intracellular pH (Van der Wielen et al., 2000).

But most bacteria must maintain a neutral cytoplasm to sustain functional macromolecules. Further, the export of excess protons requires the consumption of cellular ATP by the bacteria and leads to depletion of cellular energy (Ricke, 2003). So, the butyric acid is able to change the micro-pH environment and lead inhibition effects on bacterial growth, which will control the pathogenic *Salmonella* and *Clostridium perfringens* bacteria and modulate the *Lactobacillus* populations (Bortoluzzi et al., 2017). Butyric acid has been shown to inhibit *Salmonella* colonization in the ceca (Cox et al., 1994). Fernández-Rubio et al. (2009) also showed that adding a butyric acid product significantly decreased the infection in different organs (crop, cecum and liver) when birds were challenged with *Salmonella* Enteritidis. Van Immerseel et al. (2006) demonstrated that butyric acid can down-regulate the gene expression and invasion of some bacteria. Feeding 0.1% butyrate significantly increased the host defense peptides in the intestinal tract of chickens (Sunkara et al., 2011). In addition, researchers found that butyrate can decrease the effects of NE related to *Clostridium perfringens* infection (Timbernont et al., 2010). The author did not find a direct antimicrobial effect against *Clostridium perfringens* and claimed that the beneficial effects of sodium butyrate may be secondary, related to its multiple effects on the gut mucosa. Butyrate is believed to stimulate villus growth and improve the function of intestinal mucosa, which could also be helpful for intestinal barrier integrity and the prevention or regeneration of the epithelia lesions (Kien et al., 2007; Sunkara et al., 2011).

(4) The Importance of Product Releasing Time

Chickens are unique for having a relatively short GI tract, faster passage rate (Tuckey et al., 1958) and a special digestive organ (ventriculus) when compared with other species. The unique digestive system has an intimate correlation with the efficacy of the various antibiotic alternative products inside the birds. Studies have shown that butyrate is easily absorbed in the

upper GI tract when fed in the free salt form (Bolton and Dewar, 1965; Van der Wielen, 2002). However, the butyric acid salt needs to be in an undissociated state before reaching the middle and hind gut to exert its functional effects (Warnecke and Gill, 2005). Normally, the products used in poultry diets are protected by an encapsulated layer (a vegetable or palm oil) to ensure the active component passes the anterior GI tract and makes it to the intestine where they have the beneficial effects (Huyghebaert et al., 2011). For example, the encapsulation process of butyrate starts with the product purification process, then the butyrate product will be expanded with certain carriers. Later, products will go through embedded granulation process before coated with certain vegetable fat to produce the encapsulated final products (King Techina Technology Co., Ltd., Hangzhou, China). The releasing time of the encapsulated products are examined through an *in-vitro* assay. In the assay, the encapsulated products are exposed to pH and enzyme mixtures that imitate the GI tract environment of the bird, while the concentration of the chemical compound in solution is determined at various time points as it releases. The products with different releasing time within the GI tract can have various biological responses based on where they release (Liu et al., 2017b). The small intestine is the main site for nutrient digestion and absorption, especially the duodenum and jejunum. The ceca are also a unique organ in poultry, which are not only important for water/electrolytes absorption, but also the main site for bacterial fermentation in the GI tract (Svihus, 2014).

Liu et al. (2017b) conducted a rate-of-passage study via CT technology, which showed that feed entered the gizzard less than 15 minutes after ingestion. Then, feed was shown in both the duodenum and jejunum after 30 minutes. After 1.5 to 2 h, feed reached the ileum and was found in ceca after 4 h. The enteric cavity was virtually cleared of the iodinated contrast between the 4 to 6 h time points, except for a few spots in the gizzard and ceca. The feed is digested in a

"digesta reflux" way, but we get an average feed digestion timeline from the CT result. So, when adding butyrate into the diet, the targeted releasing time of the product through the GI tract is very important for its proposed functional effects. Research has shown that partially protected sodium butyrate provides a unique balance of free and protected active substances that are effective throughout the GI tract, which also means the earlier releasing products may stimulate villus growth and nutrient digestibility in the small intestine, while later releasing products may have more of an inhibitory effect on pathogenic bacterial development in the ceca (Fernández-Rubio et at., 2009; Guilloteau et al., 2010). The real beneficial effects of butyrate products need to be closely correlated with its releasing time in the GI tract, which is controlled by how the product is encapsulated.

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CHAPTER 2

COMPUTED TOMOGRAPHIC PRECISION RATE-OF-PASSAGE ASSAY WITHOUT A FASTING PERIOD IN BROILERS: MORE PRECISE FOUNDATION FOR TARGETING THE RELEASING TIME OF ENCAPSULATED PRODUCTS[1]

[1]J. D. Liu, S. A. Secrest, and J. Fowler. 2017. *Livestock Science*. 200:60-63.

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ABSTRACT

The objective of this study was to develop a precision-fed rate-of-passage assay using iodinated contrast as an indigestible marker in broiler chickens. In this experiment, twenty-two Cobb-Cobb male broilers were obtained on the day of hatch and fed a standard corn-soybean meal starter diet until day 21. All birds were then orally gavaged 3 g of feed mixed with 2 ml of iodinated contrast. Two birds were selected for collection of the gastrointestinal tract (gizzard, duodenum, jejunum, ileum, ceca, and colon) at 0:15, 0:30, 0:45, 1:00, 1:30, 2:00, 2:30, 3:00, 4:00, 5:00, 6:00 h post-gavage. A computed tomographic exam of the intestinal tract was conducted to determine the location and percentage of each intestinal segment which contained the admixed contrast and feed. Results indicated that feed entered the gizzard extremely fast after the gavage (less than 0:15 h). The marked feed left the gizzard between the 0:15 and 0:30 h time points and was shown in both the duodenum and jejunum after 0:30 h. We found 67.3% of the duodenum and 48.4% of the jejunum containing the iodinated contrast at 0:30 h time point. After 1:30 to 2:00 h, feed reached the ileum. We found 63.5% of the colon length was occupied with iodinated contrast after 2:30 h, and the contrast was found in ceca 4:00 h after the gavage. The enteric cavity was virtually cleared of the iodinated contrast between the 4:00 to 6:00 h time points, except for a few spots still in the gizzard and ceca. These results indicate that the rate-of-passage can be easily determined in young broilers by using iodinated contrast as a marker without fasting the birds. The digestive time for feed passing through the anterior digestive tract in broiler chickens is less than 2:30 h, with feed arriving at the ceca at 3:00 to 4:00 h. Most of the feed is digested 4:00 to 5:00 h after consumption.

Key words: precision feeding, iodinated contrast, rate of passage, broiler

INTRODUCTION

Antibiotics have been used as feed additives for decades to prevent disease and stimulate growth (Moore et al., 1946). However, with a strong public concern of possible antimicrobial resistance (Olonitola et al., 2015), an increasing amount of research has been dedicated to antibiotic alternatives, such as prebiotics, direct-fed microbials, plant extracts, enzymes, and organic acids (Roberts et al., 2015; Yang et al., 2009). Most of antibiotic alternatives used in the poultry industry are protected by an encapsulated layer to ensure the active components pass the anterior gastrointestinal tract (GIT) without being broken down in the gizzard, and make it into the intestine where they have the effects (Huyghebaert et al., 2011). Furthermore, these products with different releasing time within the GIT, can have various biological responses based on when they release. Research has shown that earlier releasing products stimulate villi growth and nutrient digestibility in the small intestine, and later releasing products have a more inhibitory effect on bacterial development in the ceca (Fernández-Rubio et at., 2009; Guilloteau et al., 2010). The real active function of each product needs to correlate with its releasing site in the GIT, which is controlled by how the product is encapsulated.

The releasing time of most encapsulated products are examined through *in vitro* assays. In a typical assay, the encapsulated products are exposed to a certain pH and enzyme mixtures that imitate to the GIT environment of the bird, while the concentration of the chemical compound in solution is determined at various time points as it releases. The standard used for assaying the rate-of-passage time in chickens is based on results obtained from experiments that had a fasting period. After a fasting period, birds are orally gavaged with a certain indigestible marker, such as ferric oxide, chromic oxide, or titanium dioxide. Digesta samples are collected from different segments for analyzing the concentration of the marker at each time point post-

gavage (Svihus et al., 2002; Teeter et al., 1985). Average digestive time of the bird depends on the concentration of the indigestible marker in each segment of the GIT compared with the total amount in the initial feed used during gavage. However, studies have found that the fasting condition changes physiologic conditions in birds (Gonzales et al., 2003). In other words, the birds with a fasting period may have a much different passage rate when gavaged than under their normal, full-fed conditions. Thus, it is important to develop a more accurate assay to compute bird digestive times which more closely resembles normal physiologic conditions.

Computed tomography (CT) is a diagnostic imaging modality which uses x-rays to generate multi-planar images of an area of interest. This multiplanar imaging technique is used in a variety of species including humans, dogs and cats (Balthazar, 1991; Bouma et al., 2003; Secrest et al., 2012), to assess soft tissue and osseous structures. Iodine-based contrast agents are commonly administered either during or prior to the computed tomographic exam to help improve identification of soft tissue structures and areas of disease.

The objective of this study was to develop a precision-fed digestive rate-of-passage assay through CT methods in young broiler chickens. This assay would provide an accurate digestive time estimate within the bird in a non-fasted state, and support research conducted with encapsulated products that are evaluating releasing times using the *in vitro* technology.

MATERIALS AND METHODS

This experiment was conducted at the Poultry Research Center of the University of Georgia. The protocol was approved by the University's Institutional Care and Use Committee.

Animal, housing, and feeding

Birds were housed in thermostatically controlled floor pen units having negative-pressure ventilation with evaporative cooling pads. A total of 30 Cobb-Cobb male broilers were obtained on the day of hatch and fed a standard corn-soybean meal starter diet (Table 2.1) until 21 days of age.

Sampling and Measurements

At 21 days of age, 22 birds were randomly selected and orally gavaged with 3 g feed mixed with 2 ml iohexol (Omnipaque, GE Healthcare, Princeton, NJ), an iodinated contrast agent. The intubation equipment consisted of a 75 mm diameter funnel connected to a rubber tube measuring 14.5 cm in length, with a diameter of 9.7 mm. The tube was placed into the esophagus and the admixed feed and contrast were placed into the tube and pushed into the crop with a stainless-steel rod. Birds were placed into an adjacent pen which still had an *ad libitum* access to feed and water after the gavage.

Two birds per time point were then selected and euthanized with pentobarbital sodium for the collection of the GIT (gizzard, duodenum, jejunum, ileum, ceca, and colon) at 0:15, 0:30, 0:45, 1:00, 1:30, 2:00, 2:30, 3:00, 4:00, 5:00, 6:00 h post-gavage. The intestinal samples were placed on ice and stored under 4 °C before analysis.

All intestinal tracts from the 11 time points were placed on the table of a 64 slice helical CT scanner (Siemens Somatom Sensation, Siemens Medical, Muenchen, Germany). The intestinal tracts were oriented with the duodenum extending cranial to the gizzard (as “U-loop”), the jejunum running cranial-to-caudal and the ileum, ceca, and colon oriented caudal-to-cranial. The duodenum was recognized as the duodenal loop with Meckel’s diverticulum used to mark the separation between jejunum and ileum. Helical CT scans of the GIT were acquired with the following technical factors: slice thickness of 0.64 mm, a pitch of 0.8, 90 mAs and 120 kV. Post

processing viewing software (Osirix, Pixmo SARL, Bernex, Switzlerland) was used to view reconstructed dorsal plane maximum intensity projection images of the gastrointestinal tract in a soft tissue window (window width = 300, window level = 40). A board-certified veterinary radiologist (SS) recorded the location(s) of the contrast mixed feed in each of the intestinal tracts off the dorsal plane images.

Statistical analysis

At each time point, calibrated electronic calipers were used to measure the length of each segment of the GIT in each sample, with the final length recorded as an average value of the two birds (JMP 11 Software, SAS Institute Inc., Cary, NC, USA). Cecal measurements were calculated by adding the length of each cecum before averaging. The percent of contrast mixed feed within each segment was calculated by the following equation:

Percentage (%) = (Length of contrast coated feed within each intestinal tract segment / Length of each intestinal tract segment) × 100.

RESULTS

Reconstructed dorsal plane maximum intensity projection CT images of the GIT's at each time point are shown in Figure 2.1. The liquid iodinated contrast agent used in this study was easily mixed with the feed and allowed for easy identification of its location on the CT. Iodine, which has a high atomic number, readily attenuates to the x-rays, making it appear bright white on the CT images. Tables 2.2 show the average length of each intestinal tract segment. Then, the percentages of each segment of the intestinal tract occupied by contrast mixed feed were showed in Table 2.3.

Results indicated that feed entered the gizzard extremely fast after the gavage (less than 0:15 h). The marked feed left the gizzard between the 0:15 and 0:30 h time points, and was shown in both the duodenum and jejunum after 0:30 h. After 1:30 to 2:00 h, feed reached the ileum and was found in ceca after 4:00 h. The enteric cavity was virtually cleared of the iodinated contrast between the 4:00 to 6:00 h time points, except for a few spots still in the gizzard and ceca.

DISCUSSION

From the results, it is evident that feed passed the crop and proventriculus extremely fast, entering the gizzard before the 0:15 h time point. None of the contrast was found in duodenum, jejunum, ileum, ceca, and colon at that time. A marked amount of contrast mixed feed persisted in gizzard between 0:30 h to 2:00 h post gavage. This may be explained by the fact that feed stays in the gizzard until reaching a certain particle size (Clemens et al., 1975), as well as antiperistaltic refluxes of duodenal contents back into the gizzard during digestion (Duke, 1968).

At the 0:30 h time point, the contrast mixed feed was shown in both the duodenum and jejunum. Svihus et al. (2002) showed that indigestible markers pass into the gizzard 30 minutes post-feeding. We found the similar results with 67.3% of the duodenum and 48.4% of the jejunum containing the iodinated contrast at the 0:30 h time point.

By 2:30 h, 100% of the ileum was filled with iohexol coated feed. Tuckey et al. (1958) showed that the markers was first found in the excreta around 2:00 to 2:30 h after feeding. This current study found that none of the contrast mixed feed was present in the ceca or colon at the 2:00 h time point in ceca and colon. However, 63.5% of the colon length was occupied with

iodinated contrast 2:30 h after the gavage, suggesting that the first feces from the contrast mixed feed would be voided at this time.

The first occurrence of iodinated contrast in the ceca was between the 3:00 and 4:00 h time points. The enteric cavity was almost cleared of the contrast between 4:00 to 6:00 h after the gavage, except for a few stagnated spots in the gizzard and ceca. After 5:00 to 6:00 h, 59.2 and 72.1% of the ceca still contained iodinated contrast. Duke et al. (1968) showed that indigestible markers can still be found in cecal contents 72 h after feeding. In another study, Kim et al. (2011) showed that excreta no longer contained markers 8 h post feeding.

The results of this study showed that the digestive rate-of-passage can be easily determined in chickens via gavage feeding and CT. This assay provides a simple and efficient method of tracking feed digestion, and can work in concert with the current encapsulation *in vitro* assays of encapsulated feed additives. In this assay, the birds had no need to be fasted and always received *ad libitum* feed and water before and after gavage, which will better mimic typical conditions for the chicken. Researchers found a decreasing villus surface area, crypt depth, and mucin secretion in the small intestine during periods of feed withdrawal in broilers (Thompson and Applegate, 2006). Another benefit of this method is that only 3 g of feed mixed with 2 ml of iodinated contrast is needed, with the overall experimental period completed within a day. This greatly increases the efficiency of such studies, in which researchers normally fast the birds overnight and then collect the intestinal contents over the next day or two to determine the rate-of-passage. It should be noted that the access and expense of CT is variable from location to location and should be taken into consideration. In addition, while a board certified radiologist was used to validate the technique, other less experienced personal can be trained to interpret the images, which can provide both qualitative and quantitative information. Above all,

the assay presented here can provide a more accurate digestive time data for the manufacturers of encapsulated products when evaluating their releasing times using the *in vitro* techniques.

CONCLUSION

The results indicate that the rate-of-passage through the GIT of 3-week-old broiler chickens can be easily determined using CT methods without the need to fast the birds. For this assay, the birds need only be gavaged with 3 g of feed mixed with 2 ml of iohexol. We found that 0:30 to 2:30 h post-ingestion is the appropriate time for the release of encapsulated products aimed at stimulating intestinal epithelial development and improving nutrient digestibility. For those products that focus on hindgut bacterial control, 2:30 to 4:00 h would be an optimal range for releasing time.

CONFLICT OF INTEREST

The authors declare that no conflict of interest exists with the work presented in this report.

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Figure. 2.1. Reconstructed dorsal plane maximum intensity projection images of the intestinal tracts at 0:15 h (A), 0:30 h (B), 0:45 h (C), 1:00 h (D), 1:30 h (E), 2:00 h (F), 2:30 h (G), 3:00 h (H), 4:00 h (I), 5:00 h (J), and 6:00 h (K) post gavage.

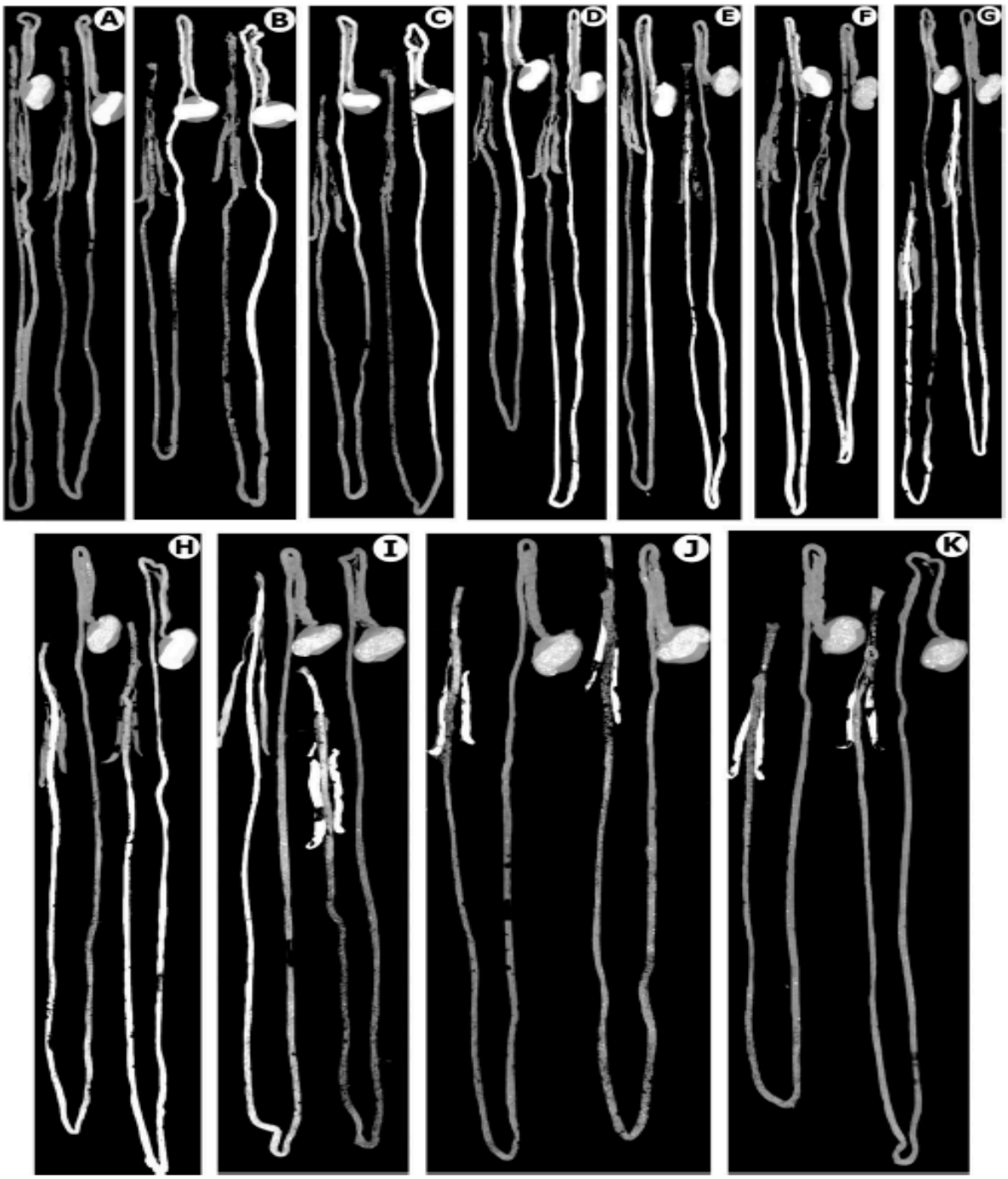

Table 2.1. Ingredient and nutrient composition of the diets (as-fed basis).

Item	Amount
Ingredient (% of diet)	
Corn, grain	56.12
Soybean meal, 48% CP	37.50
Soybean oil	3.07
Limestone	0.73
Defluorinated phosphate	1.75
Salt	0.30
DL-methionine	0.20
Vitamin premix[1]	0.25
Mineral premix[2]	0.08
BMD-50	0.05
Calculated composition	
ME, kcal/kg	3091
CP, %	22.24
Crude fat, %	5.26
Ca, %	0.98
Available P, %	0.47
Lys, %	1.27
Met, %	0.56

[1]Supplied per kilogram of diet: vitamin A, 5,511 IU; vitamin D3, 1,102 ICU; Vitamin E, 11.02 IU; vitamin B12, 0.01 mg; Biotin, 0.11 mg; Menadione, 1.1 mg; Thiamine, 2.21 mg; Riboflavin, 4.41 mg; d-Pantothenic Acid, 11.02 mg; Vitamin B6, 2.21 mg; Niacin, 44.09 mg; Folic Acid, 0.55 mg; Choline, 191.36 mg.

[2]Supplied per kilogram of diet: Mn, 107.2 mg; Zn, 85.6 mg; Mg, 21.44 mg; Fe, 21.04; Cu, 3.2 mg; I, 0.8 mg; Se, 0.32 mg.

Table 2.2. Length of each intestinal tract segment at 21 days of age.

Item	Length[1] (cm)				
	Duodenum	Jejunum	Ileum	Ceca	Colon
Time post gavage (hour : minute)					
0:15	21.9	62.9	56.9	16.5	12.3
0:30	22.6	55.8	52.2	18.3	11.2
0:45	21.3	64.6	59.5	23.1	10.6
1:00	22.9	66.9	64.2	22.7	7.9
1:30	22.6	71.6	64.5	18.3	9.4
2:00	19.4	70.9	60.3	19.3	10.6
2:30	23.8	72.6	51.4	18.6	9.6
3:00	22.4	75.8	64.5	17.8	10.1
4:00	20.0	66.6	59.5	27.0	7.8
5:00	21.1	60.7	60.2	19.1	8.6
6:00	21.9	66.1	58.4	17.9	10.0

[1]All values are represented as mean of the intestinal samples.

Table 2.3. Percentage of each segment of the intestinal tract occupied by contrast coated feed.

Item	Percentage[1] (%)				
	Duodenum	Jejunum	Ileum	Ceca	Colon
Time post gavage (hour : minute)					
0:15	0.0	0.0	0.0	0.0	0.0
0:30	67.3	48.4	0.0	0.0	0.0
0:45	79.3	50.9	0.0	0.0	0.0
1:00	49.3	72.3	25.1	0.0	0.0
1:30	17.7	59.2	47.6	0.0	0.0
2:00	45.9	65.3	47.1	0.0	0.0
2:30	2.5	45.6	100.0	0.0	63.5
3:00	2.2	52.5	82.0	0.0	30.7
4:00	0.5	24.8	65.7	74.1	84.6
5:00	0.5	0.0	0.0	59.2	5.8
6:00	0.0	0.0	1.0	72.1	3.0

[1]Percentage (%) = (Length of contrast coated feed within each intestinal tract segment / Length of each intestinal tract segment) × 100.

CHAPTER 3

ENCAPSULATED SODIUM BUTYRATE WITH MULTIPLE ADDING DOSAGES ON BROILER PERFORMANCE UNDER A NALIDIXIC ACID RESISTANT *SALMONELLA* TYPHIMURIUM CHALLENGE[1]

[1] J. D. Liu, D. E. Cosby, N. A. Cox, S. M. Williams, and J. Fowler. To be submitted to *Poultry Science.*

ABSTRACT

This study evaluated an encapsulated sodium butyrate (Na-B) with varying releasing time on broiler performance, energy digestibility and intestinal development with a nalidixic acid resistant *Salmonella* typhimurium (ST^{NAR}) challenge. A total of 792 Cobb-Cobb male broilers were placed 11 birds per pen with 6 replicates for each treatment into battery cages. Na-B was encapsulated and the varying releasing times were verified by an *in-vitro* assay (King Techina Group), targeting a 2 h (CMA) and 3-4 h (CMP) releasing time product. The study consisted of 12 treatments: non-challenged control, challenged control, CMA (250, 500, 750, 1000 and 1500 ppm) and CMP (250, 500, 750, 1000 and 1500 ppm). On d 4, Birds were orally gavaged with 0.1 mL of a 10^7 cfu/mL ST^{NAR}. BW and feed intake were recorded on d 4, 14 and 21. Ceca and liver/gall bladder were collected from 2 birds per pen on d 11 and 21 and analyzed for the presence of ST^{NAR}. On d 14 and 21, ileal segments and digesta were collected for histology and digestibility. Growth performance showed no significant difference among treatments on d 4 and 14. But there was a significant difference in BW on d 21, with CMA at 1000 ppm having the highest values ($P < 0.05$). CMP at 750 ppm showed significant higher ileal digestible energy ($P < 0.05$) on d 14 and 21 when compared to the challenged control. Results from the cecal ST^{NAR} colonization indicate a significant difference in log CFU ($P < 0.05$) between the non-challenged and challenged control. However, there was no differences for recovery of ST^{NAR} between the challenged control and Na-B treatments. This study demonstrates that Na-B has the potential to improve BW, BW gain and energy digestibility, with an earlier releasing product increasing the growth performance. However, no significant influence on *Salmonella* control was evident in this experiment. Therefore, it appears that the beneficial effect of Na-B in broilers is affected not only by dosage, but also by the product's targeted releasing time.

Key words: sodium butyrate, digestibility, gut health, *Salmonella*, broiler

INTRODUCTION

Butyric acid is one of the short chain fatty acids, which is considered as a potential alternative to antibiotics in the feed with the antibiotic-free raising program (Scheppach, 1994; Lesson et al., 2015). The butyrate molecule has four carbons, which can serve as the energy source for the epithelial cells of the intestine (Jozefiak et al., 2004; Dalmasso et al., 2008; Mahdavi and Torki, 2009). Guilloteau et al. (2010) demonstrated that butyrate increases the villus growth and overall intestinal absorptive surface area, which yield positive effects on both BW gain and feed conversion ratio (FCR) in broilers (Levy et al., 2015).

Salmonella spp. are gram negative, rod-shaped, facultative anaerobic bacteria belonging to the family *Enterobacteriaceae* (D'Aoust and Maurer, 2007). *Salmonella* typhimurium is one common serotypes, which is recognized as primary source of foodborne gastroenteritis for public health concerns (Ricke et al., 2013). Cox et al. (1994) and Fernández-Rubio et al. (2009) found adding butyrate products in the diet can significantly decrease the *Salmonella* colonization in ceca and different organs. The butyrate product can change the internal micro-pH and down-regulate key genes involved in the invasion of the gastrointestinal tract (GI) by *Salmonella* (Van der Wielen et al., 2000; Van Immerseel et al., 2006).

Chickens have a relatively short GI tract, faster passage rate and a special digestive organ (gizzard) (Tuckey et al., 1958; Liu et al., 2017b), which has an intimate correlation with the efficacy of the butyrate product. Bolton and Dewar (1965) and Van der Wielen (2002) also demonstrated that the free butyrate salt products will easily be absorbed in the upper GI tract. So, the active butyrate components need to be protected by an encapsulated layer (Huyghebaert et al., 2011) and remain in an undissociated form before reaching the middle and hind gut to exert certain beneficial effects (Warnecke and Gill, 2005). Studies have shown the beneficial dosage

for adding butyrate products can range from 100 to 2000 ppm (Hu and Guo, 2007; Timbermont et al., 2010; Levy et al., 2015). In addition, Fernández-Rubio et at. (2009) and Liu et al. (2017a) demonstrated that the releasing time of the butyrate product is closely correlated with its effects on growth performance and pathogenic bacterial inhibition.

The objective of this study was to evaluate two encapsulated sodium butyrate (Na-B), which differed in their targeted releasing time, over a range of doses on broiler performance with a nalidixic acid resistant *Salmonella* typhimurium (ST^{NAR}) challenge.

MATERIALS AND METHODS

This experiment was conducted at the University of Georgia Poultry Research Center, with protocol approved by the University Animal Care and Use Committee, and Biosafety Committee. Birds were obtained on the day of hatch from Cobb-Vantress hatchery in Cleveland, GA.

Experimental Design and Animal Husbandry

A total 792 Cobb-Cobb male broilers were distributed among 3 Petersime battery brooder units (72 pens; 11 birds per pen) in a thermostatically controlled room. A pathogen stress model was used to evaluate two encapsulated Na-B products: CMA (2 h releasing time) and CMP (3-4 h releasing time) added at 250, 500, 750, 1000 or 1500 ppm, respectively. The varying releasing times were verified by an *in-vitro* assessment (King Techina Technology Co., Ltd, China). The 10 product treatments and 2 control (non-challenged control and challenged control) treatments were randomly assigned to pens, with 6 replicates per treatment. At initiation 11 birds were weighed and randomly allocated to each pen, with pen weight controlled to within ± 10 grams of the entire mean flock weight of 11 birds. All birds were allowed *ad libitum* access to feed and

water with 24 h light. Birds were observed twice per day with regards to general flock condition, unanticipated events, and mortality was recorded daily for each pen.

Dietary Treatments

An industry-type, basal diet was used in this experiment (Table 3.1). The basal diet was then divided into 11 equally sized portions and further mixed for the 11 dietary experimental treatments. The titanium dioxide was added at 0.2% as an indigestible marker for ileal digestible energy (IDE).

Growth Performance and Sampling

Total pen and feed weight were recorded on d 1, 4, 14 and 21 for determination of BW gain and mortality-adjusted FCR. On d 4, all birds from the challenge treatments were orally gavaged with 0.1 mL of a 10^7 cfu/mL ST^{NAR}. Ceca and liver/gall bladder were collected from 2 birds per pen on d 11 and 21 and analyzed for the presence of ST^{NAR}. On d 14 and 21, intestinal segments and ileal digesta were collected for histology and energy digestibility.

Salmonella Colonization

On d 11 and 21, 2 birds per pen were euthanized, with pairs of ceca, and liver/gall bladder collected and placed in stomacher bags for ST^{NAR} colonization. Individual sample were weighed and diluted 1:3 with buffered peptone water, stomached for 60 s and spread plated with three swabs onto BGS-Nal plates (Blanchfield et al., 1984). Samples were then incubated at 37 ºC for overnight. All plates from the restreaked plates were read for negative samples and colonizing factor (CF).

Energy Digestibility

On d 14 and 21, ileal digesta were collected for IDE. Digesta samples were dried at 100 °C for 24 h and ground. CP and GE from feed and digesta samples were analyzed using AOAC

methods (2006). TiO_2 concentration was determined using a modified procedure (Short et al., 1996) at the University of Georgia Agricultural and Environmental Services Laboratories (Feed and Environmental Water Laboratory, Athens, GA). The IDE and IEDC were calculated by the following equations (Scott et al., 1982):

$$\text{IDE} = \text{Gross } E_f - \text{Excreta } E_i$$

where Excreta $E_i = GE \times (Ti_f/Ti_i)$

$$\text{IEDC} = [(NT/Ti)_d - (NT/Ti)_i]/[(NT/Ti)_d]$$

where NT represents kcal in sample, Ti represents the percentage of titanium, with the subscript "i" representing the ileal contents and subscripts "d" representing the diet.

Intestinal Histology

On d 14 and 21, the duodenum, jejunum and ileum tissues were collected from 1 bird per pen. Tissues were placed in 10% neutral buffered formalin immediately after the sample collection.

After fixation in the formalin, tissues were cut into cassettes and routinely processed overnight. Samples were then embedded in paraffin, sectioned at 4 microns, placed on slides and then stained with hematoxylin and eosin and cover slipped. Villus height and crypt depth from the duodenum, jejunum and ileum tissues were measured (Kik et al., 1990) via the ImageJ software (National Institutes of Health) at the Poultry Diagnostic and Research Center Histology Laboratory (Athens, GA).

Statistical Analysis

Data were analyzed by one-way ANOVA using GLM procedure via the JMP 13.0 (SAS Institute; Cary, NC, USA). The standard error of the mean was adopted as the measure of error

for the *Salmonella* colonization. Means from all results were deemed significant at $P \leq 0.05$ and were separated by Tukey's HSD test.

RESULTS

Table 3.2 shows the performance results of this experiment. On d 4 and 14, we did not find significant effects on BW, BW gain or FCR when adding Na-B product in the diet. Upon completion of the study at 21 days of age (17 days post challenge), adding CMA (2 h releasing time) product at 1000 ppm had a significant ($P < 0.05$) higher BW than both non-challenged control and challenged control treatments. The same trend but not significant effect ($P = 0.07$) was found on BW gain. But there was no significate effect on FCR after the challenge on d 21.

For the IDE results (Table 3.3). At 10 d post-challenge, adding CMA at 250 ppm and CMP (3-4 h releasing time) at 750 ppm showed significantly higher ($P < 0.05$) IDE compared with the challenge control treatment. On d 21, CMP at 750 ppm treatment still had the highest ($P < 0.05$) IDE. Meanwhile, CMA product added at lower dosages ($P < 0.05$) showed significantly higher IDE than the challenge control treatment.

Intestinal morphology results are shown in Table 3.4. There was no significant effect on morphology results after adding Na-B products in the diet on d 14. On d 21 (two weeks post-challenge), adding CMA at 750 ppm significantly increased the villus height ($P < 0.05$) in the jejunum compared to the challenge control treatment. We did not find any significant effect on the villus height to crypt depth ratio in the duodenum, jejunum or ileum.

Table 3.5 shows the *Salmonella* colonization results from the ceca samples. On d 11, the challenge caused a significant increase ($P < 0.05$) in log CFU between challenge control and non-challenge control treatments (3.35 vs 0.43). However, neither of the Na-B products at any

dose showed a beneficial effect on the *Salmonella* colonization compared to the challenge control treatment. Similar results were found on d 21, with non-challenge control treatment having the lowest value of the *Salmonella* colonization ($P < 0.05$).

DISCUSSION

From the current study, we found adding CMA (2 h releasing time) at 1000 ppm significantly ($P < 0.05$) improved the BW on d 21. Hu and Guo (2007) added sodium butyrate at 500, 1000 and 2000 ppm in the diet for a broiler study. They found Na-B treatments significantly increased the BW gain during the first 21 d period. However, the FCR showed a positive quadratic response and significantly increased when adding Na-B product at 2000 ppm when fed until d 42. In another study, researchers showed a linear increase on FCR when adding butyric acid at 100, 200 and 300 ppm for a 42 d period (Levy et al., 2015). For the current study, we also used the orthogonal contrast to separately determine the linear, quadratic and cubic effects on growth performance variables for both CMA and CMP products (only use the challenge control treatment plus five product treatment of CMA or CMP). There were no linear, quadratic or cubic effects for growth variables on d 4 and 14 for both products. However, we found a significant quadratic effect ($P = 0.03$) on BW for CMA product on d 21, with the optimal adding dosage at 830 ppm. For the CMP product, there was a significant linear increase ($P = 0.02$) in FCR from d 0 to 21 d. Sikandar et al. (2017) added 500 ppm and 1000 ppm Na-B in the diet and found significant higher BW, BW gain and lower FCR compared with control treatment. However, the beneficial effects of Na-B on growth performance under a non-challenge environment has not been showed consistently (Levy et al., 2015; Liu et al., 2017a).

For the IDE and morphology results, adding CMP at 750 ppm showed significantly higher ($P < 0.05$) IDE than other challenge treatments. Meanwhile, CMP with 1500 ppm showed significantly lower IDE than the other doses. At 17 d post-challenge, the difference in IDE between the two control treatments was significant ($P < 0.05$). The response of both products peaked at the dose range between 500 and 750 ppm. This result was also found by a previous 11 d *Salmonella* challenge study, where adding CMP product at both 500 and 1000 ppm increased the IDE compared with the challenge control treatment (Liu et al., 2017a). Adding CMA at 750 ppm had the highest ($P < 0.05$) villus height in the jejunum. Na-B served as energy source and improved the villus growth of the epithelia cells (Guilloteau et al., 2010), which increased the absorption surface area and overall digestibility of the nutrients. In addition, Friedel and Levine (1992) showed Na-B can induce water and sodium absorption. Liu et al. (2017a) found the Na-B can significantly improve the villus height to crypt depth ratio of the broilers on d 21. However, there were no significant differences for intestinal morphology results on d 42, which may be because the intestine is fully developed at the older age (Levy et al., 2015). Kaczmarek et al. (2016) conducted a 42 d broiler study using a protected calcium butyrate and showed an increased AMEn value after adding the product in the diet.

The *Salmonella* challenge was sufficient to increase the average log CFU recovered from the ceca between the challenged and unchallenged treatment. However, neither releasing time product showed significant influence on *Salmonella* colonization. The undissociated forms of butyric acid can easily penetrate the lipid membrane of the bacterial cell and dissociate the proton into the neutral pH of the cell cytoplasm, which will decrease the intracellular pH (Van der Wielen et al., 2000; Van Immerseel et al., 2004). So, the pH sensitive bacteria may not tolerate with the changed micro-pH environment and will export the excess protons to maintain a

near neutral pH cytoplasm to sustain functional macromolecules. This process will consume the cellular ATP and lead to a depletion of cellular energy or stop growth/death of the bacteria (Ricke, 2003). Butyrate also improves the function of intestinal mucosa, which could also be helpful for intestinal barrier integrity and the prevention or regeneration of the epithelia lesions (Kien et al., 2007; Sunkara et al., 2011). Butyric acid has been shown to inhibit the *Salmonella* colonization in the ceca (Cox et al., 1994). Fernández-Rubio et al. (2009) found adding butyrate acid significantly reduced the infection in the crop, cecum and liver with a challenge of *Salmonella* Enteritidis. However, Liu et al. (2017a) did not found significant effects on *Salmonella* colonization when adding Na-B within a 11 d *Salmonella* challenge. One possible explanation may be that short chain fatty acid are weak acids (pKa < 4.8). So, the short chain fatty acid (like butyric acid) would present as deprotonated in the nearly neutral gastrointestinal tract environment, which will be directly related to its anti-bacterial effect. As a feed additive, most butyric acid products are used as a sodium or calcium salt. The salt form products are solid and have less odor, which make them easier to handle in practice. However, the salt form products may also show less effective influence on the bacterial inhibition when compared with a butyric acid product.

In conclusion, this study demonstrated that Na-B products have the potential to improve BW, BW gain, and IDE. We found the 2 h releasing time Na-B product at 1000 ppm had the highest BW, significantly higher than all the 3-4 h releasing time dosages on d 21. The highest IDE values were seen in the 2 h releasing time product between 250 to 750 ppm, but not until 750 to 1000 ppm for the 3-4 h releasing time product. Therefore, the beneficial effect of Na-B in broilers is affected not only by dosage, but also by the product's targeted releasing time.

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Table 3.1. Ingredient and nutrient composition of the diets (as-fed basis).

Item	Amount
Ingredient (% of diet)	
Corn, grain	54.08
Soybean meal, 48% CP	38.47
Soybean oil	3.30
Limestone	1.55
Mono-dicalcium phosphate	1.51
Salt	0.51
DL-methionine	0.21
L-Lysine·HCl	0.04
Vitamin premix[1]	0.25
Mineral premix[2]	0.08
Calculated composition	
ME, kcal/kg	3050
CP, %	22.00
Crude fat, %	5.57
Ca, %	0.95
Available P, %	0.45
Lys, %	1.31
Met, %	0.56

[1]Supplied per kilogram of diet: vitamin A, 5,511 IU; vitamin D3, 1,102 ICU; Vitamin E, 11.02 IU; vitamin B12, 0.01 mg; Biotin, 0.11 mg; Menadione, 1.1 mg; Thiamine, 2.21 mg; Riboflavin, 4.41 mg; d-Pantothenic Acid, 11.02 mg; Vitamin B6, 2.21 mg; Niacin, 44.09 mg; Folic Acid, 0.55 mg; Choline, 191.36 mg.

[2]Supplied per kilogram of diet: Mn, 107.2 mg; Zn, 85.6 mg; Mg, 21.44 mg; Fe, 21.04; Cu, 3.2 mg; I, 0.8 mg; Se, 0.32 mg

Table 3.2. Evaluation of encapsulated sodium butyrate on broiler growth performance following challenge with a nalidixic acid resistant *Salmonella* typhimurium[1].

	Cont[2]		CMA					CMP						
Item	NC-Cont	C-Cont	250	500	750	1000	1500	250	500	750	1000	1500	SEM	*P*-value
d 1 to 4														
BW (g/bird)	110.9	119.9	116.5	119.2	117.8	120.5	117.8	110.0	121.4	119.4	120.3	116.9	0.84	0.09
BWG (g/bird)	68.8	77.7	74.3	76.9	75.9	78.4	75.7	67.8	79.1	77.1	78.0	74.4	0.83	0.08
FCR (Feed:Gain)	1.00	0.95	0.98	0.96	0.93	0.93	0.95	1.00	0.93	0.97	0.94	0.99	0.07	0.41
d 5 to 14														
BW (g/bird)	442.5	448.0	445.8	447.3	442.0	467.5	456.4	453.4	450.3	450.0	464.6	441.4	2.55	0.59
BWG (g/bird)	333.7	328.1	329.3	328.1	324.2	347.0	338.7	339.0	328.9	330.6	342.2	324.6	2.23	0.57
FCR (Feed:Gain)	1.31	1.33	1.33	1.34	1.33	1.34	1.31	1.27	1.33	1.30	1.29	1.33	0.01	0.60
d 15 to 21														
BW (g/bird)	871.4^{c}	920.6^{bc}	949.0^{ab}	949.0^{ab}	904.0^{bc}	997.4^{a}	912.9^{bc}	934.4^{b}	908.5^{bc}	935.2^{b}	906.5^{bc}	933.0^{b}	5.94	< 0.05
BWG (g/bird)	457.3	472.6	503.2	503.7	453.5	521.6	456.4	490.2	458.2	474.8	440.8	484.1	5.26	0.07
FCR (Feed:Gain)	1.18	1.25	1.22	1.24	1.28	1.18	1.27	1.17	1.22	1.24	1.31	1.30	0.01	0.45
Mortality (%)	0	0	0	0	0	0	0	3.03	0	0	1.52	1.52	0.25	0.19

[1]Day 1 to 4 was pre-challenge; day 5 to 21 was post-challenge.

[2]NC-Cont = non-challenged control; C-Cont = challenged control.

N = 66 birds/treatment

Table 3.3. Evaluation of encapsulated sodium butyrate on broiler ileal energy digestibility following challenge with a nalidixic acid resistant *Salmonella* typhimurium.

		Energy Digestibility			
		d 14		d 21	
Item	Dose	IDE[1]	IEDC[2]	IDE	IEDC
Treatment[3]					
NC-Cont	-	2775^{ab}	0.69^{ab}	2662^{b}	0.66^{c}
C-Cont	-	2686^{bc}	0.67^{c}	2555de	0.64^{e}
	250	2849^{a}	0.70^{a}	2623^{bc}	0.65^{d}
	500	2789^{ab}	0.70^{a}	2659^{b}	0.66^{c}
CMA	750	2770^{ab}	0.69^{ab}	2640^{bc}	0.66^{c}
	1000	2715^{b}	0.68^{bc}	2439^{f}	0.61^{f}
	1500	2686^{bc}	0.67^{c}	2435^{f}	0.61^{f}
	250	2728^{b}	0.69^{abc}	2526^{e}	0.63^{e}
	500	2721^{b}	0.68^{bc}	2734^{a}	0.68^{b}
CMP	750	2837^{a}	0.70^{a}	2778^{a}	0.69^{a}
	1000	2786^{ab}	0.70^{a}	2582^{cde}	0.65^{d}
	1500	2590^{c}	0.65^{d}	2595^{bcd}	0.65^{d}
SEM	-	12.78	0.003	11.74	0.003
P-value	-	< 0.05	< 0.05	< 0.05	< 0.05

[a-f]Means with different superscripts within a column differ significantly ($P \leq 0.05$).

[1]IDE, ileal digestible energy.

[2]IEDC, ileal energy digestible coefficient.

[3]NC-Cont = non-challenged control; C-Cont = challenged control.

N = 18 birds/treatment

Table 3.4. Evaluation of encapsulated sodium butyrate on broiler intestinal morphology following challenge with a nalidixic acid resistant *Salmonella* typhimurium on d 21.

Item	Control[1]		CMA					CMP					SEM	*P*-value
	NC-Cont	C-Cont	250	500	750	1000	1500	250	500	750	1000	1500		
Duodenum														
Villus height	1858.9	1780.3	1729.5	1724.3	1705.9	1729.0	1688.1	1661.0	1736.9	1770.3	1735.9	1719.6	16.73	0.64
Crypt depth	156.5	162.7	157.0	158.8	144.4	162.8	162.3	165.4	156.5	156.7	174.6	165.1	2.92	0.91
Ratio	12.9	10.9	11.4	11.2	12.1	10.9	11.0	10.7	11.7	12.1	10.8	10.9	0.24	0.75
Jejunum														
Villus height	996.4^{ab}	962.6^{b}	1099.6^{ab}	1003.8^{ab}	1118.9^{a}	1087.3^{ab}	1007.9^{ab}	1039.7^{ab}	1084.4^{ab}	969.8^{b}	953.4^{b}	987.0^{ab}	9.63	< 0.05
Crypt depth	129.2	127.6	129.9	117.7	120.7	131.7	123.9	132.7	137.7	117.1	125.3	132.2	1.81	0.48
Ratio	8.1	7.6	8.9	9.3	9.5	8.5	8.3	8.0	8.2	8.5	8.0	7.9	0.15	0.20
Ileum														
Villus height	876.4	858.3	849.5	837.8	915.9	892.2	925.0	921.6	886.5	838.6	870.1	912.3	7.57	0.15
Crypt depth	139.8^{a}	145.4^{a}	122.6^{ab}	109.1^{b}	127.2^{ab}	125.8^{ab}	141.1^{a}	134.8^{ab}	128.8^{ab}	121.6^{ab}	125.9^{ab}	133.9^{ab}	1.88	< 0.05
Ratio	6.4	6.1	7.2	7.9	7.3	7.7	6.7	7.1	7.2	7.0	7.1	7.1	0.11	0.08

[a-b]Means with different superscripts within a column differ significantly ($P \leq 0.05$).

[1]NC-Cont = non-challenged control; C-Cont = challenged control.

N = 18 birds/treatment

Table 3.5. Mean colonization factor (CF)[1] for adding encapsulated sodium butyrate after challenged with a nalidixic acid resistant *Salmonella* typhimurium (log_{10} cfu/g cecal material).

		CF[2]	
Item	Dose	d 11	d 21
Treatment[3]			
NC-Cont	-	$0.43^{c} \pm 0.78$	$0.50^{e} \pm 0.74$
C-Cont	-	$3.35^{b} \pm 1.97$	$2.68^{abcd} \pm 1.73$
	250	$3.16^{b} \pm 2.17$	$3.05^{abc} \pm 1.72$
	500	$3.39^{b} \pm 2.16$	$2.84^{abc} \pm 1.42$
CMA	750	$2.68^{b} \pm 1.93$	$1.58^{d} \pm 0.57$
	1000	$2.96^{b} \pm 2.22$	$2.56^{abcd} \pm 1.49$
	1500	$3.50^{b} \pm 2.05$	$2.84^{abc} \pm 1.17$
	250	$3.64^{b} \pm 1.65$	$1.97^{cd} \pm 0.84$
	500	$3.95^{ab} \pm 2.48$	$2.05^{bcd} \pm 0.99$
CMP	750	$2.44^{b} \pm 1.96$	$2.01^{bcd} \pm 1.32$
	1000	$2.69^{b} \pm 2.44$	$3.23^{ab} \pm 1.80$
	1500	$5.43^{a} \pm 1.36$	$3.31^{a} \pm 1.28$
SEM	-	0.18	0.12
P-value	-	< 0.05	< 0.05

[a-e]Means with different superscripts within a row differ significantly ($P \leq 0.05$).

[1]CF = mean log_{10} *Salmonella* typhimurium count per gram of cecal material in samples within one treatment.

[2]CF results are represented by mean ± standard deviation.

[3]NC-Cont = non-challenged control; C-Cont = challenged control.

N = 12 birds/treatment

CHAPTER 4

EVALUATION OF ENCAPSULATED SODIUM BUTYRATE ON GROWTH PERFORMANCE, ENERGY DIGESTIBILITY, GUT DEVELOPMENT AND *SALMONELLA* COLONIZATION IN BROILERS.[1]

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[1]J. D. Liu, H. O. Bayir, D. E. Cosby, N. A. Cox, S. M. Williams, and J. Fowler. 2017. *Poultry Science*. 96:3638-3644.

ABSTRACT

Two experiments were conducted to determine the effect of an encapsulated sodium butyrate (Na-B) with targeted releasing times on broiler performance, energy digestibility, intestinal morphology and ceca *Salmonella* colonization. In experiment 1, three different Na-B products (CMA, CMP and CMS) were evaluated following a challenge with a nalidixic acid resistant *Salmonella* typhimurium (ST^{NAR}). Cobb-Cobb male birds were placed 8 per pen into 6 replicates for each treatment. Treatments included six Na-B treatments (500 and 1000 ppm of each product) plus two control (non-challenged and challenged). Birds were orally gavaged with 0.1 mL of 10^7 cfu/mL ST^{NAR} on d 4. Ceca and ileal samples were collected on d 11. In experiment 2, CMA and CMP products were evaluated for a full grow-out period without an external challenge. Cobb-Cobb male birds were distributed among 45 floor pens with 24 birds per pen. Treatments included four product treatments (500 and 1000 ppm of each product) plus one control. Feed intake and pen weight were obtained on d 14, 28 and 42. Experiment 1 showed that CMP at 1000 ppm had the highest value for BW and BWG on d 4 ($P = 0.07$). Adding CMA and CMP at 500 ppm increased ileal digestibility energy (IDE) compared to the challenged control ($P \leq 0.05$). The *Salmonella* recovery data indicated that the challenge had a significant, but mild impact since it did not affect the performance variables but did result in a significant increase in $\log_{10}$ cfu/g cecal material between the non-challenged and challenged control (1.42 vs 3.72). Experiment 2 showed that both products improved the villus height in duodenum on d 21 ($P = 0.08$) and IDE on d 42 relative to the control ($P \leq 0.05$). This study demonstrates that Na-B has the potential to improve growth in broilers at an early age. The beneficial effects on intestinal morphology and IDE are affected not only by dosage level, but also by the product's releasing time.

Key words: sodium butyrate, growth, gut health, *Salmonella*, broiler

INTRODUCTION

Short chain fatty acids (SCFAs) are considered a potential alternative to antibiotics in the feed (Leeson et al., 2005). In animals, SCFAs are the end product of the fermentation of non-starch polysaccharide and unabsorbed starch by anaerobic bacteria (Scheppach, 1994), with acetic acid, propionic acid, and butyric acid as the major constituents (Bugaut, 1987). With respect to butyric acid, it has been shown that it has an important role in growth performance, intestinal epithelium development, and antimicrobial effects in poultry (Leeson et al., 2005). Previous research has shown the positive effect of adding butyric acid on both body weight gain (BWG) and feed convention ratio (FCR) in broilers (Levy et al., 2015). Butyrate may also serve as an energy source for enterocytes (Mahdavi and Torki, 2009), which can increase villus growth and overall absorptive surface area in the intestine (Guilloteau et al., 2010). In addition, butyric acid also inhibits *Salmonella* colonization in the ceca (Cox et al., 1994) and downregulates the gene expression and invasion of *Salmonella* (Van Immerseel et al., 2006).

In practice, most butyric acid products are used as a sodium or calcium salt because of the volatile and pungent nature of butyric acid (Kaczmarek et al., 2016). Studies have shown that butyrate is easily absorbed in the upper gastrointestinal (GI) tract when fed in the free salt form (Bolton and Dewar, 1965; Van der Wielen, 2002). The butyric acid salt needs to be in an undissociated state before reaching the hind gut to exert its antimicrobial effect (Warnecke and Gill, 2005). The common method to protect sodium butyrate (Na-B) from early GI tract absorption is to use palm stearin or other vegetable fat to encapsulate the Na-B and control its releasing time in the GI tract.

The small intestine plays an important role in nutrient digestion and absorption (Svihus, 2014). Ceca is the major site for unabsorbed nutrient fermentation and exert essential effects to

maintain gut microbiota balance (Svihus et al., 2013). Studies have shown that earlier releasing of products in small intestine can stimulate villi development and nutrient digestibility, and later releasing time in the ceca has an inhibitory effect on gut bacteria (Fernández-Rubio et at., 2009; Guilloteau et al., 2010). Liu et al. (2017) used a computed tomography technology and developed a precision-fed digestive rate-of-passage assay in broilers under non-fasted state. The assay suggested that 0:30 to 2:30 h post-ingestion releasing is the appropriate time for the encapsulated products aimed at stimulating epithelial cell development and improving nutrient digestibility in small intestine, and 2:30 to 4:00 h releasing would be an optimal range for products that focus on hindgut bacterial control.

The aim of this study was to determine the effects of adding an encapsulated Na-B with targeted releasing times on broiler performance, energy digestibility, intestinal morphology and ceca *Salmonella* colonization.

MATERIALS AND METHODS

The protocol used in this study was approved by the University of Georgia Animal Care and Use Committee, and Biosafety Committee. Both experiments were conducted at the University of Georgia Poultry Research Center, with birds obtained on the day of hatch from Cobb-Vantress hatchery in Cleveland, GA.

Experimental Design and Animal Husbandry

In Experiment 1 (EXP 1), a total of 384 Cobb-Cobb male broilers were housed in two Petersime battery brooder units in a thermostatically controlled room. Three Na-B products (CMA, CMP and CMS) were encapsulated and the varying releasing times were verified by an *in-vitro* assessment (King Techina Technology Co., Ltd, China), targeting a 2 h releasing

(CMA), 3-4 h releasing (CMP) and over 5 h releasing (CMS) time. The experimental design consisted of 8 treatments: non-challenged control, challenged control, CMA (500 ppm and 1000 ppm), CMP (500 ppm and 1000 ppm), and CMS (500 ppm and 1000 ppm) with 6 replicate pens for each dietary treatment. At study initiation 8 birds were randomly allocated to each treatment pen. Initial pen weights were controlled to be within ± 10 grams of the entire mean flock weight of 8 birds. All birds were allowed *ad libitum* access to feed and water with 24 h light.

In Experiment 2 (EXP 2), a total of 1080 Cobb-Cobb male broilers were allotted into 45 floor pens (1.22 × 1.83 m^2 each) with used pine shaving litter for bedding. Only CMA (2h releasing time) and CMP (3-4 h releasing time) products were included in EXP 2. The experimental design consisted of 5 treatments: control, CMA (500 ppm and 1000 ppm), and CMP (500 ppm and 1000 ppm) with 9 replicates for each dietary treatment. Initially, 24 birds were randomly allocated to each treatment pen and pen weights were controlled to be within ± 25 grams of the entire mean flock weight of 24 birds. The broilers were given a 3-phase feeding program including starter (1 to 14 d), grower (15 to 28 d), and finisher (29 to 42 d) phases. Feed was provided as mash in the starter phase and as pellets during the grower and finisher phases. All birds were allowed ad libitum access to feed and water.

For both experiments, birds were observed twice per day with regards to general flock condition, unanticipated events for the house, and mortality for each pen.

Dietary Treatments

A basal, industry-type broiler starter diet was used in EXP 1 (Table 4.1). The diet was divided into 8 equally sized portions creating a total 8 dietary treatments as outlined under the experimental design. Titanium dioxide (TiO_2, 0.2%) was used as an indigestible marker.

In EXP 2, the basal diets were formulated with 3080 kcal ME/kg, 22 % CP; 3100 kcal ME/kg, 20 % CP; and 3150 kcal ME/kg, 18.5 % CP for starter, grower, and finisher phases, respectively (Table 4.2). TiO2 (0.2%) was used as an indigestible marker for the last five days of the experiment.

Growth Data and Sample Collection

For EXP 1, total pen and feed weights were recorded at the start of the experiment. On d 4, all challenge treatment birds were orally gavaged with 0.1 mL $\times 10^7$ cfu/mL nalidixic acid resistant *Salmonella* typhimurium (ST^{NAR}). Diets were fed for a period of 11 days, with pen BW and feed intake (FI) measured on d 4 and 11 for determination of BWG and FCR adjusted for mortality. Five birds per pen were randomly selected for ileal digesta content, ileal tissue and ceca samples for ST^{NAR} colonization.

For EXP 2, pen BW and FI were recorded at day 14, 28 and 42 d of the experimental period. Duodenum, jejunum and ileum tissue samples from random 6 birds per treatment (1 bird per random pen) were collected on d 21 and 42 for intestinal morphology. On d 42, one bird per pen was randomly selected for ileal digesta collection.

Salmonella Recovery

In EXP 1, the ceca of one bird per pen from the non-challenged control and challenged control treatments were aseptically sampled on d 7 to confirm *Salmonella* colonization. On d 11, all birds were euthanized by carbon dioxide and 5 pairs of ceca per pen were collected and placed in stomacher bags for ST^{NAR} colonization. Individual ceca were weighed and diluted 1:3 with buffered peptone water, stomached for 60 s and spread plated with three swabs onto BGS-Nal plates (Blanchfield et al., 1984). All plates and ceca were incubated overnight at 37 ºC.

Then, the plates from the restreaked plates were read for negative samples and colonizing factor (CF).

Digestibility and Ileal Histology

Ileal digesta were collected for energy digestibility. All samples were dried at 100 °C for 24 h and ground for analysis (Campasino, 2015). Feed and digesta were evaluated for CP and GE using AOAC methods (2006). TiO2 concentration was determined using a modified procedure outlined by Short et al. (1996) at the University of Georgia Agricultural and Environmental Services Laboratories (Feed and Environmental Water Laboratory, Athens, GA). The following equations were used to calculated IDE and IEDC (Scott et al., 1982):

$$IDE = \text{Gross } E_f - \text{Excreta } E_i$$

where $\text{Excreta } E_i = GE \times (Ti_f/Ti_i)$

$$IEDC = [(NT/Ti)_d - (NT/Ti)_i]/[(NT/Ti)_d]$$

where NT represents kcal in sample, Ti represents the percentage of titanium, with the subscript "i" representing the ileal contents and subscripts "d" representing the diet.

In EXP 1, the ileal tissues were collected on d 11. In EXP 2, the duodenum, jejunum and ileum tissues were collected on both 21 and 42 d for quantifying intestinal histology. After collection, all tissues were immediately placed in 10% neutral buffered formalin. After fixation, tissues were cut into cassettes and routinely processed overnight. Samples were then embedded in paraffin, sectioned at 4 microns, placed on slides and then stained with hematoxylin and eosin and cover slipped. Villus height and crypt depth were measured using methods by Kik et al. (1990) and the ImageJ software (National Institutes of Health) at the Poultry Diagnostic and Research Center Histology Laboratory (Athens, GA).

Statistical Analysis

All data from EXP 1 and 2 were analyzed by one-way ANOVA using GLM producer via the JMP 13.0 (SAS Institute; Cary, NC, USA). Means were deemed significant at $P \leq 0.05$ and were separated by Tukey's HSD test. The standard error of the mean was adopted as the measure of error.

RESULTS

In EXP 1, the performance results (Table 4.3) showed an increasing trend on growth when adding Na-B in the diet compared with the control treatment, where CMP at 1000 ppm had the highest BW and BWG value ($P = 0.07$) on d 4. On d 11, the same general trends were seen, but there was not a significant effect on BW, BWG or FCR among any of the treatments after the *Salmonella* challenge. Table 4.4 shows the IDE results for birds post-ST^{NAR} challenge. There was a significant 10% decrease in IDE once the birds were challenged with *Salmonella.* Both CMA and CMP products improved the energy digestibility compared with the challenged control treatment. CMA and CMP at 500 ppm resulted in a significantly higher digestibility ($P \leq 0.05$) than the other treatments. For ileal morphology results (Table 4.5), adding CMA at 500 ppm and CMP at 1000 ppm resulted in a significantly higher ($P \leq 0.05$) villus height than any other treatment. However, there was no significant effect on the villus height to crypt depth ratio among different treatments. On d 7, we had approximately $\log_{10}$ 2.8 cfu/g of cecal material in the challenge control treatment. Based on this, we considered that the challenge was successful. The *Salmonella* colonization results from the ceca were shown in Table 4.6. The challenge had a significant effect ($P \leq 0.05$), with $\log_{10}$ cfu/g cecal material showing an increase between the non-challenged and challenged control treatments (1.42 vs 3.72). However, between the different products treatments, there were no significant differences in *Salmonella* colonization.

In EXP 2, there was no significant effect on growth performance when adding Na-B in the absence of an experimental challenge environment on d 14, 28 and 42. Table 4.7 shows the IDE and IEDC for broilers reared to 42 d of age. The CMA at 500 ppm and CMP products had significantly higher ($P \leq 0.01$) IDE than the control treatment. The morphology results from d 21 were shown in Table 4.8. On d 21, both CMA and CMP products treatments had a higher ($P = 0.08$) villus height in duodenum than the control treatment. CMA at 500 ppm showed the highest ($P = 0.06$) villus height in the jejunum compared with other treatments. For the villus height to crypt depth ratio in ileum, the control treatment showed the lowest value ($P \leq 0.05$), compared to the other treatments. However, there were no significant difference on villus height, crypt depth and villus height to crypt depth ratio on d 42 among all the treatments.

DISCUSSION

The SCFAs are the fatty acids with an aliphatic tail of less than six carbon atoms. Butyrate products (including the acid and salt forms) are considered as one of the AGP alternative feed additives (Leeson et al., 2005). From EXP 1, we found the CMP (3-4 h releasing time) added at 1000 ppm can improve the BW and BWG at an early age. Birds at younger age may be more sensitive to Na-B effects, because the SCFA levels are low in the intestine of the young birds (Van der Wielen et al., 2000). Levy et al. (2015) did not find significant increase in BWG when adding butyric acid only at 100 ppm, which illustrates the importance of dosage level for Na-B products. However, in current study, there were no significant differences for FCR between treatments after the *Salmonella* challenge. Hu and Guo (2007) showed a significant increase effect in BWG, but no effect on FCR when adding Na-B in the diet through 21 d, because Na-B can increase the average daily body mass and FI at the same time. Other

researchers found that butyrate improved the FCR in pigs (Manzanilla et al., 2006). Sodium butyrate improved the animals' health status and increased the nutrient use efficiency. The butyrate supplementations delayed the gastric emptying time, slowed down the digesta flow rate and optimized pH of the gastric digesta in the GI tract (Guilloteau et al., 2010).

EXP 1 also found that CMA (2 h releasing time) and CMP added at 500 ppm significantly increased the IDE when compared to the other challenged treatments. The same results on improved energy digestibility were found in EXP 2, that both CMA and CMP products treatments significantly improved the IDE compared with the control. The IDE results from EXP 1 and 2 were closed to a similar study using a protected calcium butyrate (Kaczmarek et al., 2016), in which the product increased apparent total tract crude fat digestibility and AMEn. The authors thought that the butyrate salt may improve the secretion of pancreatic fluid which can affect the digestibility of crude fat and AMEn. Fernández-Rubio et al. (2009) showed that partially protected Na-B provides a unique balance of free and protected active substances that are effective throughout the GI tract. The CMS product had lower IDE and IEDC compared with other Na-B treatments, which proved that > 5 h releasing time is too long for the encapsulated Na-B to have effect in the broiler's GI tract (Liu et al., 2017).

According to the intestinal morphology results in EXP 1, CMA at 500 ppm and CMP at 1000 ppm had the highest villus height ($P \leq 0.05$). In EXP 2, all product treatments had a higher ($P = 0.08$) villus height in duodenum and CMA at 500 ppm showed the highest ($P = 0.06$) villus height in the jejunum. Also, both products treatments significantly improved the villus height to crypt depth ratio ($P \leq 0.05$) in ileum on d 21. The beneficial effects on intestinal morphology is explained by Biagi et al. (2007). The SCFA provides carbons that can serve as an energy source for epithelial cells in the intestine (Mahdavi and Torki, 2009). It improved the villus growth,

increased the nutrient absorption surface area, which overall affected the nutrient digestibility and BW. However, there were no significant differences for intestinal morphology results on d 42, which may be because the intestine is fully developed at the older age (Levy et al., 2015). In addition, even the birds in EXP 2 were raised with used litter, but without any external challenge. Levy et al. (2015) also found no difference in duodenum and jejunum morphology results when adding butyric acid in the diet on d 42, due to the birds were also not involved in an external challenge.

For the *Salmonella* colonization results, under this mild challenge condition within a 11 d experimental period of EXP 1, all different releasing time products showed no significant influence on *Salmonella* colonization. Cox et al. (1994) found a significant decrease ($P \leq 0.05$) on ceca *Salmonella* colonization with adding butyric acid on d 21. Other researchers (Fernández-Rubio et al., 2009) have also showed a significant decrease ($P \leq 0.05$) on the infection in different organs (crop, cecum and liver) when birds were challenged with *Salmonella* Enteritidis. Butyrate is the end fermentation product of non-starch polysaccharide, which is very important for the ceca microbiota population and balance in chickens. The undissociated forms of butyrate acid can penetrate the cell membrane of the bacteria (Van Immerseel et al., 2004). It can dissociate into anions and protons, change the intracellular micro-pH environment, and downregulate the gene expression and invasion of *Salmonella* (Van Immerseel et al., 2006), which leads to an inhibition effect on the bacteria growth (Ricke, 2003). Na-B also promote the microbiota diversity and induce the competitive exclusion effect between the beneficial and pathogenic bacteria (Manzanilla et al., 2006; Jerzsele, 2012) Based on the results from EXP 1, it may be that a short experimental time period and mild challenge were not able to lead to a significant influence on the *Salmonella* colonization results.

In conclusion, this study demonstrates that Na-B has the potential to improve BW and BWG in broilers at the early age. An earlier releasing product can increase the villus growth and IDE. However, a later releasing product, targeted to control hind gut microbial population, didn't show significant influence on *Salmonella* control under the current experimental condition. The beneficial effects of Na-B on intestinal morphology and IDE from both studies, indicate that Na-B can positively influences the intestinal absorptive surface area, which can be affected not only by dosage, but also by the product's targeted releasing time.

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Table 4.1. Ingredient and nutrient composition of the diets in Experiment 1 (as-fed basis).

Item	Amount
Ingredient (% of diet)	
Corn, grain	59.00
Soybean meal, 48% CP	33.99
Soybean oil	2.60
Limestone	1.22
Dicalcium phosphate	1.79
Salt	0.50
DL-methionine	0.23
L-Lysine·HCl	0.20
Vitamin premix[1]	0.25
Mineral premix[2]	0.02
Titanium dioxide	0.20
Calculated composition	
ME, kcal/kg	3050
CP, %	22.00
Crude fat, %	5.18
Ca, %	0.95
Available P, %	0.48
Lys, %	1.31
Met, %	0.56

[1]Supplied per kilogram of diet: vitamin A, 5,511 IU; vitamin D3, 1,102 ICU; Vitamin E, 11.02 IU; vitamin B12, 0.01 mg; Biotin, 0.11 mg; Menadione, 1.1 mg; Thiamine, 2.21 mg; Riboflavin, 4.41 mg; d-Pantothenic Acid, 11.02 mg; Vitamin B6, 2.21 mg; Niacin, 44.09 mg; Folic Acid, 0.55 mg; Choline, 191.36 mg.

[2]Supplied per kilogram of diet: Mn, 107.2 mg; Zn, 85.6 mg; Mg, 21.44 mg; Fe, 21.04; Cu, 3.2 mg; I, 0.8 mg; Se, 0.32 mg.

Table 4.2. Ingredient and nutrient composition of the diets in Experiment 2 (as-fed basis).

Item	Starter (d 1 to 14)	Grower (d 15 to 28)	Finisher (d 29 to 42)
Ingredient (% of diet)			
Corn, grain	55.93	61.70	65.47
Soybean meal, 48% CP	36.84	31.90	28.28
Soybean oil	3.15	2.58	2.75
Limestone	1.53	1.48	1.42
Dicalcium phosphate	1.50	1.41	1.31
Salt	0.44	0.39	0.32
DL-methionine	0.20	0.21	0.12
L-Lysine·HCl	0.08	-	-
Vitamin premix[1]	0.25	0.25	0.25
Mineral premix[2]	0.08	0.08	0.08
Calculated composition			
ME, kcal/kg	3080	3100	3150
CP, %	22.00	20.00	18.50
Crude fat, %	5.31	4.93	5.19
Ca, %	0.95	0.90	0.85
Available P, %	0.48	0.45	0.43
Lys, %	1.31	1.11	1.01
Met, %	0.56	0.55	0.44

[1]Supplied per kilogram of diet: vitamin A, 5,511 IU; vitamin D3, 1,102 ICU; Vitamin E, 11.02 IU; vitamin B12, 0.01 mg; Biotin, 0.11 mg; Menadione, 1.1 mg; Thiamine, 2.21 mg; Riboflavin, 4.41 mg; d-Pantothenic Acid, 11.02 mg; Vitamin B6, 2.21 mg; Niacin, 44.09 mg; Folic Acid, 0.55 mg; Choline, 191.36 mg.

[2]Supplied per kilogram of diet: Mn, 107.2 mg; Zn, 85.6 mg; Mg, 21.44 mg; Fe, 21.04; Cu, 3.2 mg; I, 0.8 mg; Se, 0.32 mg.

Table 4.3. Effect of encapsulated sodium butyrate on performance of broilers challenged with a nalidixic acid resistant *Salmonella* typhimurium[1] (Experiment 1).

	Cont[2]		CMA		CMP		CMS			
Item	NC-Cont	C-Cont	500	1000	500	1000	500	1000	SEM	*P*-value
d 1 to 4										
BW (g/bird)	76.7	80.3	88.8	88.2	90.6	94.1	80.3	86.2	1.58	0.07
BWG (g/bird)	39.9	43.1	51.2	51.2	53.1	56.8	43.4	49.2	1.56	0.07
FCR (Feed:Gain)	2.06	1.75	1.35	1.47	1.47	1.38	1.46	1.56	0.06	0.34
d 5 to 11										
BW (g/bird)	251.9	253.4	287.2	265.4	288.9	299.1	259.7	279.7	5.02	0.21
BWG (g/bird)	175.2	173.1	198.5	177.2	198.3	205.0	179.4	193.5	3.70	0.34
FCR (Feed:Gain)	1.74	1.49	1.34	1.44	1.41	1.32	1.38	1.45	0.04	0.46
Mortality (%)	8.33	10.42	4.17	0.00	8.33	4.17	8.33	10.42	1.65	0.78

[1]Day 1 to 4 was pre-challenge; day 5 to 11 was post-challenge.

[2]NC-Cont = non-challenged control; C-Cont = challenged control.

Table 4.4. Effect of encapsulated sodium butyrate on ileal energy digestibility after challenged with a nalidixic acid resistant *Salmonella* typhimurium (Experiment 1).

		Energy Digestibility	
Item	Dose	IDE[1]	IEDC[2]
Treatment[3]			
NC-Cont	-	2755[a]	0.69[a]
C-Cont	-	2479[c]	0.62[c]
CMA	500	2693[ab]	0.68[b]
	1000	2456[c]	0.62[c]
CMP	500	2733[ab]	0.69[a]
	1000	2669[b]	0.67[b]
CMS	500	2245[d]	0.57[d]
	1000	2242[d]	0.57[d]
SEM	-	29.39	0.007
P-value	-	< 0.01	< 0.01

[a-d]Means with different superscripts within a column differ significantly ($P \leq 0.05$).

[1]IDE, ileal digestible energy.

[2]IEDC, ileal energy digestible coefficient.

[3]NC-Cont = non-challenged control; C-Cont = challenged control.

Table 4.5. Effect of encapsulated sodium butyrate on ileal morphology after challenged with a nalidixic acid resistant *Salmonella* typhimurium (Experiment 1).

Item	Dose	Villus height (μm)	Crypt depth (μm)	Villus height to crypt depth (μm: μm)
Treatment[1]				
NC-Cont	-	706.82^{cd}	133.01^{abc}	5.43
C-Cont	-	563.27^{d}	92.76^{c}	5.92
CMA	500	888.49^{a}	162.48^{a}	5.77
	1000	730.99^{bc}	110.91^{bc}	6.62
CMP	500	690.37^{cd}	125.53^{abc}	5.93
	1000	869.10^{a}	148.19^{ab}	6.11
CMS	500	598.47^{cd}	108.41^{bc}	5.69
	1000	729.37^{bcd}	120.54^{abc}	6.14
SEM	-	18.10	2.20	0.08
P-value	-	< 0.01	< 0.01	0.70

[a-d]Means with different superscripts within a column differ significantly ($P \leq 0.05$).

[1]NC-Cont = non-challenged control; C-Cont = challenged control.

Table 4.6. Mean colonization factor (CF)[1] for adding encapsulated sodium butyrate after challenged with a nalidixic acid resistant *Salmonella* typhimurium (log_{10} cfu/g cecal material) (Experiment 1).

	Cont[2]		CMA		CMP		CMS			
Item	NC-Cont	C-Cont	500	1000	500	1000	500	1000	SEM	*P*-value
CF[3]	$1.42^{b} \pm 0.26$	$3.72^{a} \pm 0.59$	$3.14^{ab} \pm 1.14$	$4.80^{a} \pm 0.81$	$4.34^{a} \pm 2.09$	$3.76^{a} \pm 1.07$	$4.08^{a} \pm 0.84$	$3.72^{a} \pm 0.41$	0.21	< 0.01

[a-b]Means with different superscripts within a row differ significantly for colonization factor ($P \leq 0.05$).

[1]CF = mean log_{10} *Salmonella* typhimurium count per gram of cecal material in samples within one treatment.

[2]NC-Cont = non-challenged control; C-Cont = challenged control.

[3]CF results are represented by mean ± standard deviation.

Table 4.7. Effect of encapsulated sodium butyrate on ileal energy digestibility reared to 42 d of age (Experiment 2).

		Energy Digestibility	
Item	Dose	IDE[1]	IEDC[2]
Treatment			
Cont	-	2713[c]	0.67[d]
CMA	500	2915[ab]	0.72[b]
	1000	2802[b]	0.69[c]
CMP	500	2998[a]	0.74[a]
	1000	2996[a]	0.74[a]
SEM	-	17.94	0.04
P-value	-	< 0.01	< 0.01

[a-d]Means with different superscripts within a column differ significantly ($P \leq 0.05$).

[1]IDE, ileal digestible energy.

[2]IEDC, ileal energy digestible coefficient.

Table 4.8. Effect of encapsulated sodium butyrate on intestinal morphology on d 21 (Experiment 2).

		CMA		CMP			
Item	Cont	500	1000	500	1000	SEM	*P*-value
Duodenum							
Villus height (μm)	1669.18	1775.93	1785.87	1728.52	1769.52	14.51	0.08
Crypt depth (μm)	144.19	147.11	147.45	142.74	148.69	2.67	0.96
Villus height to crypt depth (μm: μm)	12.02	12.34	12.36	12.37	12.11	0.19	0.97
Jejunum							
Villus height (μm)	1213.74	1296.10	1175.57	1180.73	1112.80	19.53	0.06
Crypt depth (μm)	118.32	115.98	115.88	100.19	101.71	2.50	0.06
Villus height to crypt depth (μm: μm)	10.55	11.64	10.57	12.12	11.23	0.27	0.33
Ileum							
Villus height (μm)	860.37	867.84	890.85	877.62	861.49	10.81	0.92
Crypt depth (μm)	136.00[a]	117.67[b]	126.01[ab]	117.84[b]	118.19[b]	2.15	0.06
Villus height to crypt depth (μm: μm)	6.43[b]	7.49[a]	7.21[ab]	7.59[a]	7.35[a]	0.12	< 0.05

[a-b]Means with different superscripts within a column differ significantly ($P \leq 0.05$).

CHAPTER 5

EVALUATION OF ENCAPSULATED SODIUM BUTYRATE WITH VARYING RELEASING TIMES ON GROWTH PERFORMANCE AND NECROTIC ENTERITIS MITIGATION IN BROILERS.[1]

[1]J. D. Liu, B. Lumpkins, G. Mathis, and J. Fowler. To be submitted to *Poultry Science*.

ABSTRACT

This study was conducted to evaluate the effect of an encapsulated sodium butyrate (Na-B) with targeted releasing times on growth performance and mitigating the impact of necrotic enteritis in broilers. Two Na-B products CMA (2 h releasing time) and CMP (3-4 h releasing time) were evaluated following a necrotic enteritis challenge model. The experiment consisted of 4 Na-B treatments (500 and 1000 ppm of each product) plus 2 control (non-challenged and challenged). A total of 336 Cobb-Cobb male broilers were placed 8 birds per pen into 7 replicate battery cages. On d 14, birds from challenge treatments were orally gavaged with ~5,000 oocysts of *Eimeria maxima*. On d 19, 20 and 21, the challenged birds received 1 mL of 10^8 cfu/mL *Clostridium perfringens*. Total pen and feed weights were assessed on d 14, 21 and 28 for weight gain and mortality-adjusted FCR. On d 21, 3 birds were randomly selected per pen and scored for intestinal lesions. Results showed no significant effect of Na-B on growth performance before the challenge on d 14. CMA at 500 ppm showed significantly higher BW and BW gain ($P < 0.05$) compared to the challenge control at d 21. Adding CMA at 500 ppm also improved the cumulative FCR to a level that was comparable to the non-challenged control. CMA treatments showed equivalent BW, BW gain and FCR after an additional seven days post-challenge on d 28. Both products at 500 or 1000 ppm had the significantly ($P < 0.05$) lower lesion scores compared to the challenged control. However, among the different Na-B treatments, there was no difference in lesion scores. Adding encapsulated Na-B showed a mitigation impact on necrotic enteritis in broilers. The Na-B product targeted to release in the anterior intestinal tract showed beneficial effects on growth and feed utilization efficiency in challenged broilers.

Key words: sodium butyrate, growth, coccidiosis, necrotic enteritis, broiler

INTRODUCTION

With the new changing consumer mindsets, one important challenge that poultry producers face is to maintain gut health and prevent necrotic enteritis (NE) under the antibiotic free raising program (Smith, 2011; Cervantes, 2015; Gaucher et al, 2015).

NE is induced by the pathogenic strain of *Clostridium perfringes* (CP), which is characterized by severe intestinal mucosa necrosis in broiler chickens (Jerzsele et al., 2012). It is a common disease in the global broiler industry and causes over $2 billion in economic losses annually (Williams, 2005; Timbermont et al., 2011). It is more prevalent to see the subclinical NE, where CP toxins damage the structure and function of the epithelial cell, which causes gut inflammation, reduced nutrient digestion/absorption, and decreased BW gain and feed utilization efficiency (Yegani and Korver, 2008). Researchers have demonstrated that coccidiosis induced intestinal lesions, together with CP induced NE is a great concern in broiler industry (Yegani and Korver, 2008; Timbermont et al., 2011). Coccidiosis is caused by the host-specific protozoan parasite, from the genus *Eimeria* (Chapman, 2014). The sporozoites will invade the intestinal epithelial cell lining and damage the intestinal mucosa. The damaged gut will initiate villus fusion, increase mucus production and initiate an immune response via inflammation, which leads to nutrients being released into the gut. Those effects together provide an environment favorable to the proliferation of CP and contribute to the incidence of the NE (Williams, 2005; Timbermont et al., 2011).

Butyric acid has been shown to play an important role in impacting growth performance, intestinal epithelium development, and reducing the shedding and number of bacteria in gut (Hirshfield et al., 2003; Leeson et al., 2005; Levy et al., 2015) in birds raised without antibiotics. The undissociated butyric acid can penetrate the bacterial cell wall, disassociate the H^+, alter

internal pH and cause osmotic problems which can reduce the incidence of NE (Timbermont, 2009). Butyrate can also serve as an energy source for enterocytes, which increase villus development and nutrient absorption (Mahdavi and Torki, 2009; Guilloteau et al., 2010). Because of both its pungent odor and that in an unencapsulated form is readily absorbed before reaching the lower intestinal tract, most butyric acid products are used as a salt of either sodium or calcium and encapsulated with the plant triglycerides (Kaczmarek et al., 2016).

The beneficial effects of encapsulated sodium butyrate (Na-B) on growth performance and gut health has been shown not only to be affected by dosage, but also by the product's targeted releasing time (Bortoluzzi et al., 2017; Liu et al., 2017). Therefore, the objective of this study was to evaluate the effect of an encapsulated Na-B with two targeted releasing times on growth performance and on the mitigation of necrotic enteritis in broilers.

MATERIALS AND METHODS

The experiment was conducted at Southern Poultry Research Inc. research facility (Athens, GA) and in accordance with the principles and guidelines by the Federation of Animal Science Societies (FASS, 2010) and Southern Poultry Research Inc. Animal Care and Use Committee.

Experimental Design, Dietary Treatments, and Animal Husbandry

Birds were obtained on the day of hatch from Cobb-Vantress hatchery in Cleveland, GA. A total of 336 Cobb-Cobb male broilers were randomly allocated into 6 equal groups and housed in the battery brooder units. Two Na-B products (CMA and CMP) were encapsulated and the varying releasing times were verified by the *in-vitro* assessment (King Techina Technology Co., Ltd, China), targeting a 2 h releasing (CMA) and 3-4 h releasing (CMP) time. The experimental

design consisted of 6 treatments: non-challenged control (NC-Cont), challenged control (C-Cont), CMA (500 ppm and 1000 ppm), and CMP (500 ppm and 1000 ppm) with 8 birds per pen and 7 replicate pens per treatment. A basal, industry-type broiler starter diet was used in this experiment (Table 5.1). All birds were allowed *ad libitum* access to feed (mash form) and water. Birds and housing facilities were inspected twice daily with regards to general health status, feed, water, temperature, mortality and any unanticipated events.

Coccidia and Necrotic Enteritis Challenge

On d 14, birds from all challenged treatments were orally gavaged with 1 mL coccidial inoculum, containing ~5,000 *Eimeria maxima* oocysts. On d 19, 20 and 21, those same birds were given a 1 mL of broth culture containing 10^8 cfu/mL *Clostridium perfringens* each morning.

Growth Performance

Total pen and feed weights were recorded at the beginning of the experiment. Diets were fed for a 28 d period, with pen BW and feed retain measured on d 14, 21 and 28 for determination of BW gain and mortality adjusted feed conversion ratio (FCR).

Lesion Scoring

On d 21, 3 birds per pen were randomly selected and euthanized by cervical dislocation. The middle regions of the intestine were scored for the presence and degree of lesions characteristic of NE, based on a 0 to 3 score: 0 for normal, 1 for slight mucus covering and loss of tone, 2 for severe necrotizing enteritis, and 3 for extreme necrotizing enteritis with presence of blood in the lumen.

Statistical Analysis

Data were analyzed by ANOVA using the GLM producer via JMP 13.0 (SAS Institute; Cary, NC, USA). Means were deemed significant at $P \leq 0.05$ and were separated by Tukey's HSD test.

RESULTS

The growth performance results are shown in Table 5.2 and 5.3. Before the birds received the coccidia challenge, there was no significant differences in growth performance among any of the treatments.

The coccidiosis and NE challenge greatly suppressed the growth of the birds, with the C-Cont treatment showing significantly decreased ($P < 0.05$) BW (39%) and BW gain (58%) compared with the NC-Cont. Both CMA (2 h releasing time) and CMP (3-4 h releasing time) products significantly increased the BW compared to the C-Cont on d 21. Adding CMA at 500 ppm had the numerically highest BW (451 g) and BW gain (184 g) than the other Na-B treatments. However, there were no significant effects on BW gain and FCR from d 15 to 21 between the products and the C-Cont treatments. Birds from the NC-Cont treatment showed lowest FCR ($P < 0.05$) compared to all other challenged treatments. From d 1 to 21, both products significantly ($P < 0.05$) increased the BW, but only CMA added at 500 ppm showed higher BW gain than the C-Cont treatment. Adding CMA at 500 ppm also showed an improved cumulative FCR, which had no significant difference compared with the NC-Cont through the 21 d period. In addition, the CP challenge significantly ($P < 0.05$) increased the total mortality for C-Cont treatment (12.5%) compared with NC-Cont treatment (0%). The CMA product showed significantly lower ($P < 0.05$) total mortality compared with C-Cont treatment on d 21.

Table 5.3 shows the growth performance after seven days post intestinal sampling. On d 28, CMA added at both doses and CMP added at 1000 ppm showed no significant differences on BW compared to the NC-Cont. For the overall 28 d experimental period, Na-B treatments had numerically higher BW and BW gain than the C-Cont treatment. CMA (2 h releasing time product) treatments showed equivalent growth performance for BW, BW gain and FCR from d 1 to 28. There was no beneficial effect on cumulative mortality from d 1 to 28 seen among challenged treatments.

Intestinal lesions were scored on d 21 (Table 5.4). The CP challenge damaged the intestine, with lesion scores showing a significant increase ($P < 0.05$) between the C-Cont and NC-Cont treatments (1.52 vs 0.00). Both CMA and CMP products at 500 ppm or 1000 ppm had significantly ($P < 0.05$) lower lesion score compared with the C-Cont. However, among the different Na-B product treatments, there were no significant difference on the average lesion score.

DISCUSSION

Coccidiosis and NE are common diseases in the global poultry industry (Williams, 2005; Abdelrahman et al., 2014). The coccidiosis caused intestinal lesion, together with *Clostridium perfringens*-induced NE has continues to be a great concern under an antibiotic-free raising program. Different *Eimeria* species will infect specific segments of the intestine (Conway and McKenzie, 2007), and the sporozoites will invade the intestinal epithelial cell line and damage the intestinal mucosa. Timbermont et al. (2011) demonstrated that the coccidial oocyst will cause gut damage, which leads to more nutrients being released into the lumen, greatly aiding the growth and proliferation of CP (Williams, 2005).

In this current study, we did not find significant effects on growth performance before the birds received the coccidial challenge on d 14. Liu et al. (2017) likewise showed no significant effects on growth results when adding Na-B in the absence of an experimental challenge through 42 d. NE causing a decreased in growth performance and inducing the intestinal lesions has been well demonstrated (Yegani and Korver, 2008; Timbermont et al., 2011). This explains the significant decrease in both BW and BW gain in the challenge control treatment. The small intestine is the major place for nutrient digestion and absorption (Svihus, 2014). CMA (2 h releasing time) product release Na-B along the anterior intestinal tract (Liu et al., 2017) and they serve as an energy source for intestinal epithelial cells. The increased villus height and nutrient absorption surface area will contribute to an overall increase in the BW of the birds. Researchers have shown a significant increase in BW gain when adding Na-B in the diet through a 21 d period (Hu and Guo, 2007). However, Jerzsele et al. (2012) evaluated the effect of a combination of Na-B and essential oil products via a NE challenge model. The authors did not find a significant effect on BW when Na-B was added alone at either d 16 or 25. Song et al. (2017) evaluated an encapsulated Na-B for the mitigation of necrotic enteritis for a 35 d period. The birds were orally gavaged with *Eimeria* mixed strains at 12 d of age. On d 16, 17, 18, the birds were given a broth culture of 10^8 cfu CP. They found significant heavier BW and improved FCR in the NE-infected birds that were fed a Na-B supplemented diet, compared to the challenged control.

Researchers have demonstrated the beneficial effect of Na-B as an anti-microbial and for immune regulation and anti-inflammatory effects (Guilloteau et al., 2010; Meijer et al., 2010; Jerzsele et al. 2012). In this study, we found both CMA and CMP products added at 500 ppm or 1000 ppm had significantly ($P < 0.05$) lower lesion scores when compared with the challenged

control treatment. Timbernont et al. (2010) found butyrate decrease the necrotic enteritis related to CP infection. The author did not found a directly antimicrobial effect against CP, and claimed that the beneficial effects of Na-B may be related to its multiple effects on gut mucosa itself. Butyrate is considered to stimulate villus growth and improve the function of intestinal mucosa, which could also be helpful for intestinal barrier integrity and the prevention or regeneration of the epithelia lesions (Kien et al., 2007; Sunkara et al., 2011). Sunkara et al. (2011) added 1000 ppm butyrate product in the diet and found a significant increase of the host defense peptides in the intestinal tract. In addition, butyrate will alter the micro-pH in the lumen, which has been shown to modulate the *Lactobacillus* population and inhibit pathogenic *Salmonella* and *Clostridium perfringens* populations (Fernández-Rubio et al., 2009; Namkung et al, 2011; Bortoluzzi et al., 2017).

In conclusion, adding encapsulated Na-B showed the ability to mitigate the impact of NE in challenged broilers. The Na-B product targeted to release in the anterior intestinal tract showed beneficial effects on growth and feed utilization efficiency in broiler chickens.

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Table 5.1. Ingredient and nutrient composition of the diets (as-fed basis).

Item	Amount
Ingredient (% of diet)	
Corn, grain	52.99
Soybean meal, 48% CP	39.84
Soybean oil	3.14
Limestone	1.12
Dicalcium phosphate	2.03
Salt	0.38
Methionine-MHA	0.33
L-Lysine·HCl	0.02
Vitamin premix[1]	0.07
Mineral premix[2]	0.08
Calculated composition	
ME, kcal/kg	3000
CP, %	23.30
Crude fat, %	5.35
Ca, %	0.92
Available P, %	0.45
Lys, %	1.34
Met, %	0.65

[1]Supplied per kilogram of diet: vitamin A, 12,346 IU; vitamin D3, 3,472 ICU; 25-hydroxyvitamin D3, 0.10 IU; Vitamin E, 49.38 IU; vitamin B12, 0.02 mg; Biotin, 0.23 mg; Menadione, 2.78 mg; Thiamine, 2.62 mg; Riboflavin, 10.80 mg; d-Pantothenic Acid, 18.52 mg; Vitamin B6, 4.63 mg; Niacin, 61.73 mg; Folic Acid, 1.54 mg.

[2]Supplied per kilogram of diet: Mn, 107.2 mg; Zn, 85.6 mg; Mg, 21.44 mg; Fe, 21.04; Cu, 3.2 mg; I, 0.8 mg; Se, 0.32 mg.

Table 5.2. Effect of encapsulated sodium butyrate on performance of broilers challenged with necrotic enteritis on d 14 and 21.

	Cont[1]		CMA		CMP			
Item	NC-Cont	C-Cont	500	1000	500	1000	SEM	*P*-value
d 1 to 14								
BW (g/bird)	248.4	221.3	266.9	215.6	222.4	232.4	6.3	0.14
BWG (g/bird)	204.5	177.7	222.8	172.4	179.3	188.7	6.2	0.15
FCR (Feed:Gain)	1.82	2.28	1.86	2.28	2.28	1.99	0.06	0.14
Mortality (%)	0.00	2.08	0.00	0.00	1.79	0.00	0.44	0.53
d 15 to 21								
BW (g/bird)	577.9^{a}	351.6^{c}	450.7^{b}	355.3^{bc}	369.3^{bc}	388.0^{bc}	15.3	< 0.05
BWG (g/bird)	329.5^{a}	137.6^{b}	183.8^{b}	140.0^{b}	146.9^{b}	155.6^{b}	11.8	< 0.05
FCR (Feed:Gain)	1.43^{b}	2.72^{a}	2.25^{a}	2.50^{a}	2.74^{a}	2.62^{a}	0.09	< 0.05
Mortality (%)	0.00^{b}	13.35^{a}	3.57^{b}	2.38^{b}	5.61^{ab}	0.00^{b}	1.07	< 0.05
d 1 to 21								
BW (g/bird)	577.9^{a}	351.6^{c}	450.7^{b}	355.3^{bc}	369.3^{bc}	388.0^{bc}	15.3	< 0.05
BWG (g/bird)	534.0^{a}	308.0^{c}	406.6^{b}	312.1^{bc}	326.2^{bc}	344.3^{bc}	15.3	< 0.05
FCR (Feed:Gain)	1.57^{b}	2.51^{a}	2.02^{ab}	2.36^{a}	2.48^{a}	2.26^{a}	0.07	< 0.05
Mortality (%)	0.00^{b}	12.50^{a}	3.57^{b}	2.08^{b}	5.36^{ab}	0.00^{b}	1.02	< 0.05

[1]NC-Cont = non-challenged control; C-Cont = challenged control.

[a-c]Means within a row with different superscript letters differ ($P \leq 0.05$).

N = 56 birds/treatment

Table 5.3. Effect of encapsulated sodium butyrate on performance of broilers challenged with necrotic enteritis on d 28.

	Cont[1]		CMA		CMP			
Item	NC-Cont	C-Cont	500	1000	500	1000	SEM	*P*-value
d 22 to 28								
BW (g/bird)	943.4[a]	619.5[b]	828.9[ab]	733.3[ab]	638.0[b]	740.8[ab]	26.2	< 0.05
BWG (g/bird)	365.6	267.9	378.2	378.1	268.7	352.8	16.5	0.12
FCR (Feed:Gain)	1.63	2.57	2.13	2.22	2.71	2.17	0.14	0.28
Mortality (%)	0.00	8.50	7.66	14.88	6.80	9.69	1.63	0.22
d 1 to 28								
BW (g/bird)	943.4[a]	619.5[b]	828.9[ab]	733.3[ab]	638.0[b]	740.8[ab]	26.2	< 0.05
BWG (g/bird)	899.6[a]	575.8[b]	784.8[ab]	690.1[ab]	594.9[b]	697.1[ab]	26.1	< 0.05
FCR (Feed:Gain)	1.51[b]	2.49[a]	1.94[ab]	2.22[a]	2.49[a]	2.20[a]	0.07	< 0.05
Mortality (%)	0.00	7.14	7.14	14.58	5.36	8.93	1.52	0.16

[1]NC-Cont = non-challenged control; C-Cont = challenged control.

[a-b]Means within a row with different superscript letters differ ($P \leq 0.05$).

N = 56 birds/treatment

Table 5.4. Effect of encapsulated sodium butyrate on intestinal average lesion score of broilers challenged with necrotic enteritis.

	Cont[1]		CMA		CMP			
Item	NC-Cont	C-Cont	500	1000	500	1000	SEM	*P*-value
Lesion Score[2]	$0.00^{c} \pm 0.00$	$1.52^{a} \pm 0.18$	$0.95^{b} \pm 0.30$	$0.95^{b} \pm 0.25$	$0.86^{b} \pm 0.18$	$0.67^{b} \pm 0.27$	0.08	< 0.01

[1]NC-Cont = non-challenged control; C-Cont = challenged control.

[2]Lesion score results are represented by mean ± standard deviation.

[a-c]Means within a row with different superscript letters differ ($P \leq 0.05$).

N = 21 birds/treatment

CHAPTER 6

CONCLUSIONS

CONCLUSION

Sodium butyrate has been considered as one of the antibiotic alternative products in recent years. Researchers have demonstrated its beneficial effects on growth performance and maintaining gut health in an antibiotic-free rearing environment. Besides the dosage at which sodium butyrate is added, another important factor directly related to the efficacy of these products in chickens is its releasing time in the GI tract.

The current research used an iodine marked feed evaluated with CT technology to find that 30 minutes to 2.5 h post-ingestion is the appropriate time for the release of encapsulated products aimed at stimulating intestinal epithelial development and improving nutrient digestibility in the small intestine. For those products that focus on hindgut bacterial control, 2.5 to 4 h would be an optimal range for the releasing time (Experiment 1). Then, a *Salmonella* challenge model was used to determine the efficacy of sodium butyrate with two releasing times (2 h or 3-4 h releasing time) added at a range of doses (250, 500, 750, 1000, and 1500 ppm). For the 2 h releasing time product, there was increased BW when added at 1000 ppm (Experiment 2). However, the results from both Experiment 2 and 3 did not show any significant effect on *Salmonella* colonization with adding sodium butyrate in the diet. The encapsulated sodium butyrate showed the potential to improve the intestinal villus development and increase the ileal energy digestibility through a full-grow out period (Experiment 4). However, lack of evidence

was found on growth performance when adding sodium butyrate in the absence of any experimental challenge. In addition, sodium butyrate showed the ability to mitigate the impact of necrotic enteritis (Experiment 5). The product targeted to release in the anterior intestinal tract showed beneficial effects on both growth and feed utilization efficiency in broiler chickens.

With the current trends of raising birds without antibiotics, this research is beneficial to the broiler industry in showing that encapsulated sodium butyrate can impact broiler performance and gut health in the absence of antibiotics in the diet. Sodium butyrate has the potential to improve BW gain, energy digestibility, villus development, and mitigate the impact of necrotic enteritis in broilers. The beneficial effects on growth and gut health are affected not only by the dosage level, but also by the product's releasing time. The 2 h releasing time sodium butyrate added at 750 to 1000 ppm has the beneficial effects on BW, villus development, ileal energy digestibility, and mitigation of necrotic enteritis in broilers. Further research should focus on a method to test the releasing time of encapsulated sodium butyrate via an *in-vivo* assay, which will provide a more accurate model for determining the actual site of release in the GI tract.