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Bile Salt Hydrolase: A Microbiome Target for Enhanced Animal Health

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I am submitting herewith a thesis written by Rekek Negga entitled "Bile Salt Hydrolase: A Microbiome Target for Enhanced Animal Health." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

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Bile Salt Hydrolase: A Microbiome Target for Enhanced Animal Health

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DEDICATION

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ABSTRACT

Antibiotic growth promoters (**AGP**) use has been associated with the emergence of antibiotic-resistant human pathogens of animal origins. The global trend of restricting AGP necessitates the need to develop effective alternatives that will maintain safety and sustainability of food animals. Bile salt hydrolase (**BSH**) is an intestinal enzyme that is produced by diverse gut bacterial species and involved in host lipid metabolism. Recent studies suggest that BSH inhibitors are promising alternatives to AGP for enhanced growth performance and animal health. Using a high-purity BSH from a chicken *Lactobacillus salivarius*, a panel of BSH inhibitors has been identified. However, it is still unknown if these inhibitors also inhibit the function of the BSH from other bacterial species with significant sequence variation and substrate spectrum. In this study, we compared the BSH from *L. salivarius* to that from *L. acidophilus* BSH. Sequence alignment and structure modeling indicated the two BSH enzymes contain conserved catalytically important amino acid residues and domain. Using a high-purity BSH from *L. acidophilus*, we demonstrated that the previously identified BSH inhibitors also exhibited potent inhibitory effects on the *L. acidophilus* BSH.

A large scale chicken experiment was conducted to examine the effect of dietary supplementation of riboflavin, a potent BSH inhibitor, on growth performance of broilers. Briefly, 300 one-day-old Hubbard broiler chicks were randomly assigned into three treatment groups (10 pens per group, 10 birds per pen) that received one of following diets: 1) a basal diet with no riboflavin added (control); 2) a basal diet + low dose of riboflavin (20 mg/kg); and 3) a basal diet + high dose of riboflavin (200 mg/kg). Dietary supplementation of riboflavin, regardless of dose, significantly increased BW gain by day 21 ($P < 0.0053$). Significantly improved FCR was only

observed for the chickens that received the low dose of riboflavin on day 21 ($P < 0.030$). High performance liquid chromatography was used to determine the levels of riboflavin in various diets. The concentrations of total bile acids in the blood and ileal samples collected on day 14, 33, and 42 were not significantly changed in response to riboflavin treatment.

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CHAPTER I

LITERATURE REVIEW

Antibiotic Growth Promoters

The therapeutic nature of penicillin was discovered in the 1920s and utilized in the 1940s, which lead to the discovery of other antibiotics from the 1930s to the 1960s (Cantas, *et al.* 2013). In the early 1950's, during the study of hepatic toxicity of alkaline hydrolyzed aureomycin scientists observed that a tetracycline antibiotic enhanced weight gain and altered the intestinal flora of rats (Gyorgy, *et al.* 1951). Subsequently, a group of subtherapeutic doses of antibiotics, referred as antibiotic growth promoters (**AGP**), were used to enhance feed efficiency and weight gain in food animals. Antibiotic growth promoters have been primarily used in the cattle, poultry, and swine industries (Laxminarayan, *et al.* 2015). Effective AGPs used in food animals have low drug substitution and include a panel of diverse antibiotics, such as bacitracin (a polypeptide), avoparcin (a glycopeptide), bambermycin (a phosphoglycolipid), virginiamycin (a streptogramin) and tylosin (a macrolide) (Marshall, *et al.* 2011). AGPs such as avoparcin, zinc bacitracin, virginiamycin, tylosin, and spiramycin have shown a 1.5 - 5% increase in weight gain and a 3-7% increase in feed efficiency in chickens and pigs (Buttery, *et al.* 1986; Hao, *et al.* 2014); while ionophores have shown an increased in feed efficiency of 1.6 - 7.5% in cattle (Jouany, *et al.* 2007). To date, many antimicrobials with different mechanisms of action have been found capable of improving weight gain and efficiency of feed utilization and have been widely used in food animal industry (Gaskins et al., 2002). Usually a combination of different AGPs is used in feeds during a complete production cycle to maximize the efficacy of AGPs.

At present, the precise mechanisms of growth promoting effect of AGPs are still unknown. However, it is widely accepted that the growth promoting effect of AGPs is mediated by the interaction between AGPs and the intestinal microbiota (Buttery, *et al.* 1986; Gaskins, *et al.* 2002; Dibner, *et al.* 2005). The use of AGPs may change the diversity and structure of microbial communities in the animal intestine and ultimately result in an optimal and balanced microbiota for increased energy harvest and better growth performance of food animals. The proposed modes of action of AGPs include reduction of growth-depressing microbial products, defensive function against pathogens conferred by certain normal flora, and production of nutrients by microbiota for host utilization (Buttery, *et al.* 1986; Gaskins, *et al.* 2002; Dibner, *et al.* 2005).

Precipitous AGP use caused the emergence of multiple drug resistant *Escherichia coli*, *Enterococcus* species, *Salmonella enterica*, as well as methicillin/ oxacillin-resistant *staphylococcus aureus* (MRSA) in the 1950s and 1960s (Cantas, *et al.* 2013). Concerns of the impact of AGP usage on public health were documented early on by England's Swann report to the British Parliament in 1967 and by USA's two notices to the Food and Drug Administration (**FDA**) in 1977 (Kux, et al. 2011). Epidemiological studies have linked AGP application to the emergence of antibiotic resistant bacteria (Bager, *et al.* 1997; Aarestrup, *et al.* 1999). Antibiotic-resistant bacteria as well as resistance genes can therefore spread from animals to humans, which compromises the effectiveness of antibiotics for the treatment of human infections and poses a serious threat to public health (Levy, *et al.* 1976; Bager, *et al.* 1997; Dibner, *et al.* 2005; Akwar, *et al.* 2007; Khanna, *et al.* 2008; Ogata, *et al.* 2012; Ramos, *et al.* 2013). For this reason, there is a worldwide trend to reduce and even stop AGP use in animal production systems as described in the following section.

In the United States the FDA (2009) reported an increase in agricultural production and purchases of fifteen drug classes that have broad bactericidal effects and structural compatibility to other antibiotic classes. These AGPs such as cephalosporins, macrolides, penicillins, and tetracyclines are some medically relevant classes that are used to enhance the weight gain of food animals (FDA 2012). As of 2012, the FDA began encouraging the phasing out of sub-therapeutic antibiotics for weight gain efficiency, but it has not resubmitted an additional notice since 1977 that aimed at prohibiting tetracycline and penicillins as AGPs (Dibner, *et al.* 2005; 2012; Cully 2014).

Current status of AGP

The findings that linked AGP avoparcin usage to the emergence of the pathogens resistant to vancomycin, an analog of avoparcin for human therapeutic treatment, led to the ban of AGPs in Denmark in 1998. Altogether, EU initially banned polypeptide antibiotics (e.g. avoparcin and bacitracin), macrolides (e.g. spiramycin and tylosin), and virginiamycin combination. In 2006, the EU completely reformed its agricultural practices and banned all AGP usage that led to a decrease in AGP usage. The impact of AGP limited usage is still tangible.

For instance, Sweden and Denmark abstained from AGP use prior to the EU's 2006 ban and has demonstrated a decrease in AGP. Overall, Sweden's drug market has sold 10% less AGPs than in 1986 (Sweden 2012). However, in 2002, Sweden can still detect the presence of vancomycin resistance and reported incomplete recovery of swine production after their ban (Casewell, *et al.* 2003; Hao, *et al.* 2014; Jensen, *et al.* 2014). Denmark reported a decrease of avoparcin, macrolides and virginiamycin resistance after AGP ban. However, they have been

contending with an increase in morbidity (2.7- 3.5%) and mortality (11%) in their swine industry as well as resistance to tetracycline, vancomycin, and sulphonamide since the ban (Casewell, *et al.* 2003; Hao, *et al.* 2014).

The international antimicrobial market increased in value from $8.65 billion to 20.1 billion dollars, from 1992 to 2010 and a $42.9 billion dollars increase is predicted by year 2018. The EU's Veterinary Medicines Directory reported an increase of 383 tons to 437 tons in therapeutic drug sold from 1999 to 2000. Predominately, tetracycline (36 ton), sulphonamide (12 ton) and macrolide (12 ton) were sold to the swine and poultry industries, suggesting that these industries are most vulnerable to the ban (Casewell, *et al.* 2003). Thus, Demark monitors veterinary prescription allocation, oversight, and a depreciation of medication by using VETSTAT that censors over use and resistance to antibiotics (Jensen, *et al.* 2014). With an increase in the antibacterial market a strict control of antibiotics may become a global trend.

Meanwhile, some countries, such as China, that is the user of four times the AGPs than that used in the United States (U.S.) and Spain, which is the predominate AGP user in Europe, have detected all drugs in the manure and waste water of livestock (Hao, *et al.* 2014). In the U.S., cattle and swine have tetracycline and ampicillin (beta-lactam) resistant genes while steers have more tetracycline resistant genes in fecal flora than non-AGPs treated animals (Harvey, *et al.* 2009). Resistant genes in the fecal flora is a major concern because the AGPs belonging to tetracyclines, lincosamides, macrolides, beta-lactam, sulfas, and aminoglycosides have similar medically relevant drugs that are used as therapeutics in animals and humans. According to the FDA's total report on antibiotic sales in the United States in 2012 the two most produced and

purchased antibiotic are tetracycline (41%) and ionophores (31%) in the food animal industry (FDA 2012).

In December of 2013, US FDA issued Guidance 209 and 213 that outline the voluntary phasing out of medically important antibiotics, which are used in healthy animals. In response to this trend, Tyson and Perdue, the two major poultry producers, announced removal of human-related antibiotics from its broiler chicken feed in fall 2014 (Teillant, *et al.* 2015). In September of 2014, President Barack Obama signed an executive order that promotes the cooperation of industry, academia, government, the general public, and agriculture community. Aims of the executive order are to generate novel stewardship approaches, to regulate antibiotic use, and to monitor general public and agricultural community use of antibiotics (Jooma, 2015).

Development of alternatives to AGP

Successful AGP removal will depend on effective alternatives that are practical, cost effective, and capable to maintain enhanced weight gain and feed efficiency. Developing effective alternatives to AGP is urgently needed of maintaining current animal production level without threatening public health. Several classes of alternatives to AGPs, such as probiotics, prebiotics, and organic acids, are briefly summarized below (Jouany, *et al.* 2007; Cheng, *et al.* 2014).

Probiotics, generally consisting of *Bacillus*, *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Bifidobacterium*, and *Bacteriodes*, have been observed to improve growth performance of monogastrics and ruminates (Cheng, *et al.* 2014). Live microbe supplementation in ruminants is hypothesized to promote a lowered pH that decrease pathogenic bacteria and in monogastrics the

microbes are postulated to produce bacteriocins and acids that decrease level pathogenic bacteria (Jouany, *et al.* 2007; Cheng, *et al.* 2014). The probiotic supplementations have led to enhanced weight gain in post weaning caves (Cruywagen, *et al.* 1996; Sun, *et al.* 2010). Unfortunately, these results have been inconsistent. For instance, supplementation of probiotics in growing ruminants did not enhance weight gain (Morrill, *et al.* 1977; Schwab, *et al.* 1980; Zhang, *et al.* 2015). The poultry industry has also observed inconsistency with the use of probiotics. They have observed improved weight gain (Mountzouris, *et al.* 2007), stimulated immune response (Zhang, *et al.* 2012), and improved food safety (Gaggìa, *et al.* 2010); however due to complicated interactions and low numbers of birds for statistical power more studies are urgently needed (Alloui, *et al.* 2013). Probiotic use in swine has shown an increase in weight gain that is comparable to AGP usage (Kyriakis, *et al.* 2003; Kritas, *et al.* 2005). Conversely, data from swine studies exist to suggest that probiotic do not improve weight gain nor feed efficiency (Harper, *et al.* 1983; Lähteinen, *et al.* 2014). Altogether, inconsistent results on growth performance of food animals have been observed following probiotic administration, thus warranting rationale design and development of effective probiotic products.

Prebiotics are nondigestible oligosaccharides that sustain some beneficial bacterial populations, such as *Bifidobacterium* and *Lactobacillus*, in the gut (Pharmaceutiques 1995). Probiotics have been shown to bolster the microbiota, to prime the immune system of the host, and to portray antiviral traits (Cheng, *et al.* 2014). Combination of probiotic and prebiotics has shown to increase weight gain and decrease feed cost in broilers (Saiyed, *et al.* 2015). Prebiotic supplementation in heat stressed broilers resulted in greater weight gain than those without prebiotics (Hasan, *et al.* 2014). Additionally, probiotic with prebiotic (mannanoligosaccharides)

decreased the *E. coli* load by adhering to *E. coli* and merging with the fecal mass (Baurhoo, *et al.* 2007). Even with these benefits, prebiotics are not permitted in the European Union as they may cause bloating and diarrhea in food animals. Additionally, the symbiotic mixture of prebiotic and probiotics are still not well understood and need further perusal for use of them as weight enhancers (Cheng, *et al.* 2014).

Acids, such as acetic, propionic, butyric and formic acids (Partanen, *et al.* 1999) are enticing AGPs alternatives. The acids can generate a low pH environment that enhance pepsin proteolytic activity and likely reduce the population of pathogenic bacteria in the gastrointestinal tract (Knarreborg, *et al.* 2002; van der Eijk 2002). Benzoic acid and some essential oils (extracts of rosemary, sage, thyme, oregano and tea) provide improved weight gain in poultry and an increase in lactic acid and coliform bacteria (Giannenas, *et al.* 2014). Acids have improved feed conversion ratio in piglets and, in adult pigs, weight gain and digestibility of feed (Partanen, *et al.* 1999). However, fumaric acid supplementation in young pigs diet did not show improvements in weight gain and ileal digestibility (Giesting, *et al.* 1991). Studies have shown weight enhancement due to fumaric acid supplementation, but no improvements were reported with citric acid supplementation (Radecki, *et al.* 1988). However, other studies have shown that pure citric acid or acidifiers blend did not cause weight gain but improved immunity in piglets infected with *Salmonella*, (Ahmed, *et al.* 2014). More studies are needed to elucidate how specific acid treatment could enhance weight gain and immune response.

Natural extracts such as saponins, tannins, and essential oils appear to be another class of alternatives to replace AGPs. Saponins are bactericidal in that they bind to membrane steroidal units and compromise the bacteria's cell membrane in monogastrics. In ruminants, the use of

saponins has lowered protein degradation by protozoa which has resulted in increase in weight gain and feed efficiency. Unfortunately, protozoa impediment has not been observed with use of saponins (Milgate, *et al.* 1995; Cheeke 1996). Tannins provide protection from helminthes and some antibacterial effects when placed inside the rumen; in non-ruminants they reduce iron available for bacterial metabolic purposes (Aerts, *et al.* 1999; Barry, *et al.* 1999; Jouany, *et al.* 2007). Essential oils have shown to be bactericidal; however, like the other natural extracts, the mechanisms, toxicity effects, upper level concentration, and particulars of harvesting period are not well understood (Jouany, *et al.* 2007; Cheng, *et al.* 2014).

Enzyme supplementation is being explored as a potential effective alternative to AGPs. The addition of enzymes in feedstuff of ruminants and monogastrics has increased foodstuff digestibility as well as bioavailability of minerals. However, the potential of the enzyme supplementation to increase nutrient for gut microbiota make them less desirable (Jouany, *et al.* 2007; Cheng, *et al.* 2014). The combination of probiotics with phytases has shown an increase in weight gain in broilers when compared to those given adequate phosphate diet. Although enzyme supplementation is a promising non-antibiotic alternative approach, more studies are needed to better justify and understand the mechanism of this strategy (Askelson, *et al.* 2014).

Altogether, there are significant challenges for developing effective non-antibiotic alternatives to AGPs. Therefore, examination of gut microbiota in response to AGP treatment would provide science-based information into the modes of action of AGP and facilitate the development of novel alternatives to AGP (Collier, *et al.* 2003; Knarreborg, *et al.* 2004; Kim, *et al.* 2012; Lin, *et al.* 2013).

The effect of AGP usage on intestinal microbiota and microbiota-derived factors

The microbiota includes bacteria, archaea, and fungi populations that work symbiotically with the host. In the last sixty-four years, subtherapeutic doses of antibiotics or AGPs have exhibited improved weight gain in food animals (Feighner, *et al.* 1987; Dumonceaux, *et al.* 2006; Cully 2014; Hao, *et al.* 2014). With the aid of culture-independent molecular approaches, the investigations of the effect of AGP on intestinal microbiota have been initiated in different food animals, including poultry, and swine. For example, the 16S rRNA sequencing of two pig herds at separate farms identified *Firmicutes* and *Bacteroidetes* as dominate bacterial groups, with *E. coli* population having a relatively low concentration of 0.12% (Kim, *et al.* 2011). Experimentation of the pig's intestines identified the following bacteria: *Streptococcus, Lactobacillus, Eubacterium, Fusobacterium, Bacteriodes, Peptostreptococcus, Bifidobacterium, Selenomonas, Clostridium, Butyrivibrio*, and *Escherichia*, with the order showing rate of occurrence (Gaskins, *et al.* 2002). A 16S rRNA clone library sequencing system showed *Lactobacillus* and *Streptococcus* vulnerability to AGP treatment (Gaskins, *et al.* 2002; Dibner, *et al.* 2005). Another swine study illustrated a preferential dominance of *Lactobacillus, Clostridiaceae,* and *Turicibacter* spp. in the ileum of control and a decrease in *L. johnsonii* and *L. amylovorus* in chlortetracycline treatment (Rettedal, *et al.* 2009). A multiple AGP formulations such as, chlortetracycline, sulfamethazine, and penicillin in swine feed, has shown a decrease in *Bacteroidetes* in comparison to *Firmicutes* and an increase in *Proteobacteria* for instance, *E. coli*, and *Salmonella* (Looft, *et al.* 2012). Bacterial vulnerability during weeks of multiple AGP treatment, such as week one exposure: chlortetracycline, sulfathiazole, penicillin; week two exposure: tylosin, bacitracin, and roxarsone;

and third through the fifth week exposures: lincomyocin, carbadox and virginiamycin respectfully have shown diminish of *Lactobacillus, Streptococcus,* and *Bacillus* spp. (Collier, *et al.* 2003).

In regards to birds, bacterial colonization occurs in the crop, gizzard, duodenum, jejunum, ileum and cecum (Buttery, *et al.* 1986; Walter 2008). *L. furmenta* and *L. salivarius* populate the crop and *L. salivarius* dominates the jejunum and ileum (Fuller, *et al.* 1974; Buttery, *et al.* 1986; Gong, *et al.* 2007). Tylosin and penicillin AGPs have caused decreases in *Lactobacillus* spp. in the ileum of fowls while penicillin treatments can also decrease *Bacteroidetes, Bacteriaceae, Enterococcus, Streptococcus* spp. (Lin 2011; Singh, *et al.* 2013). Multiple combinations of AGPs, such as virginiamycin and bacitracin methylene, cause an increase in *Enterococcus* spp. and a decrease in the *L. salivarius* in the ileum (Zhou, *et al.* 2007). Interestingly, preparation of either non-AGP wheat or medium chain fatty acids diets have improved feed efficiency and caused a shift in the microbiota in broilers. The microbiota shift suppressed gram positive *Firmicutes* such as *Lactobacillus, Enterococcaceae* and *Micrococcaceae* and promoted gram negative bacteria such as *Enterobacteriaceae* (van der Hoeven-Hangoor, van der Vossen et al. 2013).

Lactobacillus and *Bacteroidetes* vulnerability to AGP underscore these species and related derived factors as targets for developing alternatives to AGP. Some microbiota-derived proteins in the intestinal tract may be implicated in growth promotion, such as bile salt hydrolase (**BSH**). Both bacterial population produce BSH that hydrolyzes conjugated bile salts, the strong emulsifiers of lipids and play an important role in lipid metabolism. An inverse relationship with BSH activity and AGPs have been observed in chickens as early as in 1987 (Feighner and Dashkevicz 1987). Together, these findings indicate that the body weight gain in food animals is inversely related to the activity of BSH enzymes as well as the abundance of corresponding

bacterial producers, which provides a strong rationale for us to examine if BSH inhibitors are promising alternatives to AGP as described in our recent studies (Wang et al, 2012; Smith et al, 2014; Lin et al. 2014) and in this project.

Molecular and biochemical features of bile salt hydrolase

Bile salt hydrolase enzyme is specific to many commensal bacteria species such as *Bacteriodes*, *Clostridium*, *Enterococcus*, *Bifidobacterium*, and *Lactobacillus* in the intestines (Gilliland, *et al.* 1977; Ferrari, *et al.* 1980; Gopal-Srivastava, *et al.* 1988; Coleman, *et al.* 1995; Grill, *et al.* 2000; Elkins, *et al.* 2001; Franz, *et al.* 2001; Knarreborg, *et al.* 2002; Begley, *et al.* 2006). A single bacterium can have more than one BSH gene in its genome. Studies have elucidated three BSH (BSHA/1, BSHB/2, and BSHC/3) genes in *Bifidobacterium* spp. and two (*bsh*A/1 and *bshB*/2) for *Lactobacillus acidophilus* (Kim, *et al.* 2004; Begley, *et al.* 2006). Interestingly, the homology of the two BSH genes from *L. acidophilus* is very low, likely attributed to horizontal gene transfer during evolution (McAuliffe, *et al.* 2005; Begley, *et al.* 2006).

Bile salt hydrolase, penicillin G acylases, and penicillin V acylases are N terminal nucleophiles that have a cysteine residue, visible post transcriptional modifications at the N-terminus (Suresh, *et al.* 1999). The N-terminal can begin with either cysteine, threonine, or serine and is the beginning of the BSH's catalytic site that has key amino acid residues such as Cys-2, Arg-16, Asp-19, Asn-79, Asn-171, and Arg 224 (Begley, *et al.* 2006). These conserved amino acid residues can play a role in providing a universal target within the BSH's catalytic site.

Bile salt hydrolase is thought to benefit the bacteria's nutrition and membrane integrity (Begley, *et al.* 2006). *Clostridium* isolates from rats demonstrated colony growth after unconjugation of taurine's sulfur containing bile salts, while sulfur compounds and sulfhydryl containing amino acid did not stimulate growth (Huijghebaert, *et al.* 1982). Membrane integrity can be bolstered as low pH can cause precipitation of the unconjugated cholesterol that may initiate host's cholesterol integration into bacteria membrane; the cholesterol integration can decrease the host's immune-recognition of the bacterial cell (Taranto, *et al.* 1997; Taranto, *et al.* 2003).

Though BSH appears to be an adaptation mechanism of bacteria in the presence of detergent like bile salts they can have advantageous and harmful effects on the host. Post unconjugation, additional modification such as dehydroxylation and dehydrogenation of bile salt by BSH have been associated with an increase in gall stone formation and colon cancer in humans (Marcus, *et al.* 1986; Begley, *et al.* 2006). However, some bacteria can only hydrolyze the conjugated bile salt and have not evolved the capabilities to further modify the acid, thus these BSH producers can be critical in preventing cholesterolemia (Kim, *et al.* 2004).

Role of BSH in lipid metabolism and host energy harvest

The BSH enzyme is produced by gut bacteria and catalyzes the deconjugation of conjugated bile acids, an essential gateway reaction in the metabolism of bile acids (Begley, *et al.* 2006). The intestinal BSH activity, the widely distributed function of the gut microbiota, has been demonstrated to play an important role in host fat metabolism, energy harvest, and body weight gain. Previous studies have shown that oral administration of BSH-producing *lactobacilli* could

affect lipid metabolism, which consequently lowered cholesterol level in humans. The cholesterol-lowering efficacy of a microencapsulated bile salt hydrolase-active *Lactobacillus reuteri* NCIMB 30242 in yoghurt formulation was reported in hypercholesterolaemic adults (Jones, *et al.* 2012). Hypocholesterolaemic effect of dietary inclusion of two putative probiotic bile salt hydrolase-producing *Lactobacillus plantarum* strains in Sprague-Dawley rats (Kumar, *et al.* 2011); and in pigs (Smet, *et al.* 1998) showed cholesterol lowering effects through enhanced bacterial bile salt hydrolase activity, which is likely mediated through BSH activity of the tested probiotics. Recently, Joyce et al analyzed the impact of *in situ* BSH activity on host metabolism and weight gain, and provided compelling direct evidence that showed the critical role of intestinal BSH in regulating host weight gain and lipid metabolism. Colonization of germ-free mice or conventional mice with a BSH-producing *E. coli* strain elevated intestinal BSH activity, consequently reducing body weight, reducing serum cholesterol level, changing bile acid profiles in feces and plasma, and influencing both local and systemic gene-expression profiles in pathways governing lipid metabolism in this animals (Joyce, *et al.* 2014). This work clearly identifies BSH as a mechanism through which the microbiota modulates host lipid metabolism and demonstrates that BSH represents a key target for developing novel alternatives to AGPs for enhanced animal production and feed efficiency.

As described above, population of intestine *Lactobacillus* species, the major intestinal BSH-producer, was significantly reduced in response to AGP use (Knarreborg, *et al.* 2002; Dumonceaux, *et al.* 2006; Guban, *et al.* 2006). Consistent with this finding, growth-promoting effect of AGP was highly correlated with the decreased activity of BSH enzyme in the chicken intestine (Feighner, *et al.* 1987).

Bile salt hydrolase catabolizes the bile salt into amino acid and cholesterol moieties that produces a primary bile acid. This structure is not readily reabsorbed and can stimulate nuclear receptor farnesoid X receptor (FXR) in the host's intestinal lumen. The FXR stimulation causes the up regulation of Cyp7A1 that promote *de novo* synthesis of bile salts by the removal of cholesterol from the blood (Lu, *et al.* 2000; Watanabe, *et al.* 2006; Joyce, *et al.* 2014). Further modifications of primary bile acids into secondary and tertiary structure are associated with colon cancer and gallstone formation. However, the *Lactobacillus* and *Bifidobacterium* BSH enzymes cannot modify primary bile acids, this consequentially lowers cholesterolemia and the occurrence of colon cancer in humans (Choi, *et al.* 2014). In summary, hydrolysis of conjugated bile salts leads to less amphiphilic molecules (unconjugated bile acids), which compromises fat digestibly and absorption. The primary structures stimulate FXR that ultimately leads to the liver sequestering cholesterol from the blood to synthesize bile salts *de novo*. When the primary bile salt is modified into secondary bile salt it can accumulate and cause cancer of epithelial tissue due to DNA damage. However, if additional modifications do not occur, the primary molecule merges with the fecal mass and is excreted, and this potentiates a promising therapeutic measure in obesity and hypercholesterolemia therapy.

CHAPTER II

INTRODUCTION

The food animal industry has manipulated the gut microbiota to increase body weight and feed efficiency through the use of sub-therapeutic level of antibiotics, called antibiotic growth promoters (**AGP**), as feed additives for more than 60 years (Dibner, *et al.* 2005). However, AGP usage has been linked to the emergence of antibiotic resistant bacteria (Wegener 2003). Thus, a worldwide trend to limit AGP use in food animals in order to protect public health and improve food safety has created challenges for the animal industries (Wegener 2003; Turnidge 2004). Effective alternatives to AGP are urgently needed to maintain current animal production levels without threatening public health.

The examination of AGP effects on intestinal microbiota in food animals could enhance understanding of the mode of action of AGP and facilitate the development of novel alternatives to AGP. Although reduction of gut pathogens due to AGP usage is potentially a mechanism contributing to growth promotion, it has been widely accepted that use of AGP would restructure the complex gut microbial environment for optimal host growth performance from a nutrition standpoint. Recent independent food animal studies (Feighner, *et al.* 1987; Knarreborg, *et al.* 2002; Knarreborg, *et al.* 2004; Guban, *et al.* 2006) have shown that growth-promoting effects of AGP were highly correlated with the decreased activity of bile salt hydrolase (**BSH**), a gut bacterial enzyme that has negative impact on host fat digestion and energy harvest (Dibner, *et al.* 2005; Begley, *et al.* 2006). Notably, using both gnotobiotic and conventionally raised mice, Joyce et al recently obtained direct supporting evidence that BSH activity, the widely distributed function of

the gut microbiota, significantly influences host lipid metabolism and weight gain. Based on these extensive supporting evidence, we have proposed that BSH is a promising microbiome target for developing novel alternatives to AGP; specifically, BSH inhibitors are promising feed additives that may replace AGP for enhanced host lipid metabolism and growth performance (Lin 2014).

The BSH enzyme produced by gut bacteria catalyzes deconjugation of conjugated bile acids, an essential gateway reaction in the metabolism of bile acids. The natural functions of this BSH-mediated metabolic activity in the producing bacteria are still not clear despite various theories with contradictory findings (Begley, *et al.* 2006). However, it has been increasingly recognized that intestinal BSH plays an important role in host metabolism and energy harvest (Begley, *et al.* 2006; Jones, *et al.* 2008; Joyce, *et al.* 2014; Lin 2014). Because conjugated bile acids function as a more efficient "biological detergent" than unconjugated bile acids to emulsify and solubilize lipids for fat digestion (Begley, *et al.* 2006), BSH activity has significant impact on host physiology by reducing fat digestion and lipid metabolism, consequently affecting body weight gain (Begley, *et al.* 2006; Jones, *et al.* 2008; Joyce, *et al.* 2014). Recently, we have identified and characterized a powerful BSH enzyme with broad substrate specificity from a *Lactobacillus salivarius* strain that was isolated from a chicken (Wang, *et al.* 2012). In addition, with the aid of the purified *L. salivarius* BSH, we have identified a panel of BSH inhibitors through targeted screening (Wang, *et al.* 2012) as well as high-throughput screening procedures (Smith, *et al.* 2014). The *L. salivarius* BSH displayed potent hydrolysis activity towards both glycoconjugated and tauroconjugated bile salts; the broad substrate specificity nature of this BSH may make it an ideal candidate for screening desired BSH inhibitors that can targeting various BSH enzymes (Wang, *et al.* 2012; Smith, *et al.* 2014).

However, given different types of BSH enzymes present in the intestine (Begley, *et al.* 2006; Jones, *et al.* 2008), a significant question is raised: can these identified inhibitors also effectively inhibit the function of the BSH from other bacterial species with significant sequence variation and substrate spectrum? Addressing this issue is critical for us to identify desired BSH inhibitors using the established *L. salivarius* BSH-based high-throughput screening system (Smith, *et al.* 2014). In this study, comparative genomic, structural, and biochemical analysis of a BSH from a different strain *L. acidophilus* were performed. The inhibitory effect of previously identified BSH inhibitors on the purified BSH from different species was determined.

In addition, using a well-established HTS system, a panel of novel BSH inhibitors with potential as alternative to AGP was successfully identified. Riboflavin is of particular interest because it has potent inhibitory effect on BSH (Smith, *et al.* 2014). Riboflavin, a yellow compound harvested from milk, eggs, and cereals as free or bound compound and in most other foods found as coenzyme flavin adenine dinucleotide or flavin mononucleotide. It can become hydrogenated and hydroxylated among other forms (Massey 2000). Notably, riboflavin is a vitamin that has been used as feed additive in poultry to treat the hypovitaminosis B2. However, long-term dietary supplementation of riboflavin for growth promotion in broilers has never been explored. While our *in vitro* studies support the feasibility of using riboflavin as an alternative to AGPs, a large animal trial is needed to determine the effect of dietary supplementation of riboflavin on growth performance and host lipid metabolism. Notably, a recent swine study has shown that the dietary supplementation of high-level riboflavin (20 mg/kg feed) significantly increased feed efficiency and body weight gain in the pigs with high lean growth although underlying mechanisms are still not clear (Stahly, *et al.* 2007). Therefore, in this study, a large chicken nutritional trial was

conducted that determined the effects of dietary supplementation of riboflavin on chicken growth performance. Weekly body weight gain, feed intake, and feed conversion ratio were assessed. Furthermore, total bile acids in serum and ileal content were measured in response to riboflavin treatment.

CHAPTER III

MATERIALS AND METHODS

Bioinformatics analysis of BSH

A BSH gene from *L. acidophilus* PF01 has been identified and characterized Oh, *et al.* (2008); the nucleotide sequence of this BSH gene was deposited in the GenBank database (Accession No. EF536029). The BSH gene from the *L. acidophilus* PF01 strain (Oh, *et al.* 2008) was compared to those identified from *L. salivarius* NRRL B-30514 (Wang, *et al.* 2012) and to other diverse bacteria using the BLASTP program from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). To reveal the phylogenetic relationship, multiple sequence alignment of BSH sequences from different bacterial species and penicillin V acylase from *Bacillus sphaericus* (BPVA) were performed with the use of the ClustalW program in MEGA 6.0 (Tamura, *et al.* 2013). The dendrogram was constructed by neighbor-joining methods. To identify the conserved amino acid motifs that potentially is involved in BSH activity, multiple sequence alignment of BSH enzymes were performed with the use of the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

The modeling of BSH was performed in the Molecular Operating Environment (MOE), version 2008.10 (Chemical Computing Group, Montreal, QC). The BSH from *Clostridium perfringens* (Rossocha, *et al.* 2005) was chosen as template. Total 10 models were generated in the MOE homology module, using the AMBER99 force field. The one with highest packing score was chosen to superimpose with the *C. perfringens* BSH (Rossocha, *et al.* 2005), by way of the substitution matrix blosum62.

Purification of recombinant BSH (rBSH)

A pET-21b (+) vector-derived recombinant plasmid encoding recombinant *L. acidophilus* BSH (Oh, *et al.* 2008) was kindly provided by Dr. Dae-Kyung Kang (Dankook University, Korea). This recombinant plasmid bears a histidine-tagged rBSH gene with a full-length of BSH gene that was cloned from *Lactobacillus acidophilus* PF01, a commensal strain isolate from swine intestine (Oh, *et al.* 2008). In this study, this recombinant plasmid was introduced into the *E. coli* BL21 (DE3) host strain via transformation. The desired transformants were selected after an overnight incubation at 37 ^{o}C with the use of Luria-Bertani (LB) agar plates that were supplemented with ampicillin (100 μg/ml). The recombinant plasmid in one transformant, designated as JL1139, was extracted and subsequently sequenced; no mutations in the coding sequence of the BSH gene were detected. Expression and purification of the His-tagged rBSH from JL1139 were performed by the procedure described in previous publications (Lin, *et al.* 2005; Oh, *et al.* 2008; Wang, *et al.* 2012). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 12% (wt/vol) polyacrylamide separating gel was performed to monitor production and purification of the rBSH. The purified rBSH was finally dialyzed against PBS buffer with 10% glycerol (pH 7.0) and stored in -80 ^{o}C freezer prior to use. Protein concentration was measured by BCA protein assay kit (Pierce).

Effect of identified BSH inhibitors on the activity of BSH

The following three groups of compounds that have been identified as inhibitors for the *L. salivarius* BSH (Wang, *et al.* 2012; Smith, *et al.* 2014) were used in standard BSH assay in this study: 1) the approved feed additives used in food animals including $CuCl_2$, $CuSO_4$, $ZnCl_2$, $ZnSO_4$,

$NaHIO_3$, KIO_3 and $NaIO_4$; 2) the novel BSH inhibitors identified using high-throughput screening, which include caffeic acid phenethyl ester, riboflavin, epicatechin monogallate, gossypetin, menadione, and purpurogallin (Smith, *et al.* 2014); 3) the antibiotics that can inhibit BSH activity including oxytetracycline, demeclocycline hydrochloride, methacycline hydrochloride, doxycycline hydrochloride, roxarsone, and lincomyocin (Smith, *et al.* 2014).

A modified two-step standard BSH assay (Wang, *et al.* 2012) was performed to determine the inhibitory effect of the selected BSH inhibitors on the activity of the rBSH from *L. acidophilus*. Briefly, 10 µl of specific inhibitor, 10 µl of rBSH (1.20 µg/ul), 168 µl of reaction buffer (0.1 M sodium-phosphate, pH 6.0) and 2 µl of 1 *M* DTT were mixed gently and incubated at 37°C for 30 min. Then 10 µl of glycocholic acid (100 mM) was added in the 190 µl of reaction mix and the final reaction mix (total volume of 200 µl) was incubated at 37 °C for another 30 min. A 50-µl aliquot of the reaction mixture was then immediately mixed with 50 µl of 15% (w/v) trichloroacetic acid for stopping the reaction, followed by centrifugation for 5 min at 12,000 × *g* at room temperature to remove the precipitate. The supernatant was used in the second step, in which 50 µl of supernatant was thoroughly mixed with 950 µl of ninhydrin reagent mix (0.25 ml of 1% [wt/vol] ninhydrin in 0.5*M* sodium-citrate buffer, pH 5.5; 0.6 ml of glycerol; and 0.1 ml of 0.5 *M* sodium-citrate buffer, pH 5.5). A positive control (with BSH enzyme, without BSH inhibitor) and a negative control without BSH added were set up in each independent experiment. All assays were performed in triplicate. Percentage inhibition was calculated by dividing the inhibited activity (mean activity of positive control – mean residual activity of presence of a compound) relative to the mean activity of positive control and then multiplied by 100.

Chicken experiment: husbandry, diets, growth measurements and sample collection

Three hundred mixed sex day old broilers from the hatchery at Hubbard in Pikeville, TN were brought to Joseph E. Johnson Animal Research and Teaching Unit (JARTU). Ten broilers were randomly selected and placed in each of thirty floor pens with approximately12.5 square feet floor space per pen. Shredded papers were used as litter material. The chickens were randomly assigned into three treatment groups (10 pens per group) that received one of following diets: 1) a basal diet with no riboflavin added (control); 2) a basal diet + low dose of riboflavin (20 mg/kg of diet); and 3) a basal diet + high dose of riboflavin (200 mg/kg of diet). The riboflavin was purchased from http://www.BulkSupplements.com (99% purity). The diet formulations for a common starter, grower, and finisher corn-soybean diet were described in Table 1. Water and feed were available for *ad libitum* consumption during the experiment. Weights of birds, feed consumption per pen and feed conversion ratio (gain/intake) were determined at age of 7, 14, 21, 33, and 42 days and recorded. On days 14, 33, and 42, one average sized bird per pen was further selected for sample collection. Following blood collection from the wing vein, the birds were euthanized with carbon dioxide gas and ileal content collected. Each ileal sample was transferred into 1.5 ml microcentrifuge tubes and then put on ice, while at JARTU and during transportation. The blood samples were centrifuged at 11,337 × *g* for 5 min and serum was pipetted into sterile microcentrifuge tube. The collected serum and ileal samples were stored in -20 °C freezer.

Riboflavin analysis in feed using high performance liquid chromatography (HPLC)

The level of riboflavin in the formulated feeds was analyzed with HPLC technique. Feed samples from each growth phase (starter, grower, and finisher) and treatment (control, low dose

of riboflavin, and high dose of riboflavin) were collected immediately following on site preparation in JARTU and stored in -20 °C freezer prior to analysis. Initially, the feed samples were dried at 55 °C for 24 hr with in an oven (Thermo Fisher Scientific Inc.). Each sample was grounded using a Wiley Mill grinder that was thoroughly cleaned between samples. The grounded sample was mixed with 0.1 *M* HCl, vortexed for 1 min followed by incubation at room temperature for 30 min. The homogenized solution was then centrifuged at room temperature at 2,504 × *g* for 15 min. Subsequently, 1 ml of the supernatant was pipetted into 3 ml of 25 mM NaH_2PO_4 (pH 2.5). This mixture was vortexed and centrifuged at room temperature for 15 min at 2,504 × *g*. The supernatant was filtered using 0.2 µm filter, and stored in 4°C (up to 24 hr) for chromatographic analysis. Riboflavin standards (1 ppm, 2 ppm, and 5 ppm) were prepared using 25 mM NaH_2PO_4 (pH 2.5) and the riboflavin was purchased from SIGMA (catalogue number PHR1054). Riboflavin levels from each of the treatments in the starter, grower, and finisher paradigms were tested in two independent HPLC experiments with single sample used in each independent experiment.

The HPLC procedure was performed with the use of an Agilent Technologies liquid chromatography equipped with an auto sampler (model: G1329A). The analytical column was a 5 µm Zorbax Eclipse Pluse C18 in 4.5 mm x 150 mm column. A Quatemary pump (model: G1311A), diode array detector (model: G1315P), and a multiple wavelength detector (model: G1315D) were used. The mobile phase was 25 mM of NaH_2PO_4 (pH 2.5). The samples were injected by the auto sampler by way of a mobile phase with delivery rate of 1 ml/min. UV was detected at 280 nm.

Determination of total bile salts concentration

Total bile salt levels in the collected serum and ileal samples were detected with Diazyme® kit (San Diego, California). Principally, the enzyme, 3-α-hydroxysteroid dehyrogrenase, oxidizes bile salts with the coenzyme Thio-NAD that was converted into Thio-NADH. The Thio-NADH is a colorimetric label that can be read at the absorbance of 405 nm. Briefly, 3.6 ul of collected sample or the standard along with 243 ul of Reagent 1 were added into each well of a 96 well plate then incubated for 3 min at 37 °C. Subsequently, 81 ul of Reagent 2 was added to each well and immediately placed in the plate reader, BioTek Instrucments ®ELX808. Readings were performed at one minute interval for a total of two minutes with the Gen 5® version 2.03.1 software. The opacity difference between the two readings was used to determine the total bile salt concentration (µmol/L) with the use of a Diazyme kit equation. Total bile salt levels were detected in the serum and ileum samples that were collected from ten chickens per treatment on sample collection days (14, 33, and 42).

Statistical Analysis

Feed intake, bird weight gain, feed conversion ratio, and total bile salt levels from serum and ileal samples have been analyzed by mix model analysis of variance with statistical program SAS 3.9. The large scale chicken experiment's independent variables were riboflavin concentrations and dependent variables were weight gain, feed intake, and feed conversion ratio. All variance were detected by least square means that compared least significant difference at 5% level of confidence.

CHAPTER IV

RESULTS

Phylogenetic and structural analysis of BSH

The complete BSH genes from diverse bacteria species were retrieved from database for analysis. As shown in Figure 1A, the BSH produced by *L. acidophilus* PF01 (LaciP) shared high homology (93% aa identity) to a BSH from *L. gasseri* (Lgass) but is phylogenetically distant from the BSH that was identified in many other bacteria, such as the BSH from *L. salivarius* NRRL B-30514 (LsalN1). Although the BSH enzymes from various bacterial species showed significant sequence variation (Figure 1A), multiple sequence alignment indicated that these BSH enzymes contain all conserved catalytically important amino acid residues in the proposed active site of BSH (Cys-2, Arg-16, Asp-19, Asn-79, Asn-171, and Arg 224) (Begley, *et al.* 2006) (Data not shown). This conservativeness of catalytically important motifs suggests that previously identified BSH inhibitors may effectively inhibit diverse BSH enzymes.

Structural modelling of the *L. acidophilus* BSH (LaciP) and the *L. salivarius* BSH (LsalN1) was performed with the use of the only known crystal structure of the *Clostridium perfringens*-produced BSH (Rossocha, *et al.* 2005) (Cperf in Figure 1A). The models indicated that the *L. acidophilus* BSH and the *L. salivarius* BSH shared similar structure with the evidence of the typical canonic αββα-folding pattern (Figure 1B). Consistent with the structural similarity between the two different BSH enzymes (Figure 1B), the critical amino acids are also superimposed very well, particularly with respect to the typical Cys2, which served as an N-terminal nucleophile, and the Arg16, which plays a potentially essential role in catalytic functioning of the enzyme

(Rossocha, *et al.* 2005). This structure modeling provides further evidence that supports the feasibility of using the *L. salivarius* BSH for screening desired BSH inhibitors.

Expression and purification of *L. acidophilus* recombinant BSH (rBSH)

A pET-21b(+) vector that was bearing the full length of a *L. acidophilus* BSH gene, was transformed to an *E. coli* BL21 (DE3) host strain for production of recombinant BSH (rBSH). Upon induction by 0.5 mM of IPTG for as short as 1 hr, the recombinant *E. coli* construct significantly produced an additional protein with approximate molecular mass of 32 kDa on SDS-PAGE, which was consistent with the calculated molecular mass from the deduced amino acid sequence of the rBSH (Figure 2). The high-purity of the C-terminal His-tagged rBSH was subsequently obtained from the *E. coli* culture with the use of a one-step Ni-NTA agarose affinity chromatography. As shown in Figure 2, the high-purity of rBSH was predominantly present in the eluted fractions number 3 to 6. Interestingly, a band with slightly smaller molecular mass was co-present with the rBSH; this band likely represents a partially degraded rBSH (Figure 2). Approximately 25 mg of the rBSH was consistently purified from 1 liter of induced culture.

The identified BSH inhibitors also inhibited the activity of the *L. acidophilus* BSH

As shown in Table 2, almost all of previously identified BSH inhibitors using the *L. salivarius* BSH (Wang, *et al.* 2012; Smith, *et al.* 2014) also exerted potent inhibitory effect on the phylogenetically distant BSH from *L. acidophilus*, which strongly supported the hypothesis that the *L. salivarius* BSH is an ideal candidate for screening desired BSH inhibitors and can target various BSH enzymes in the intestine. Only limited BSH inhibitors, such as $ZnSO_4$ and roxarsone,

displayed weaker inhibitory effect for the *L. acidophilus* BSH (Table 2) when compared to the *L. salivarius* BSH. Based on these findings, the *L. salivarius* BSH in conjunction with an efficient high-throughput screening system (Smith, *et al.* 2014) would serve as a solid platform for us to identify desired BSH inhibitors with potential to replace AGP for enhanced host lipid metabolism and growth performance.

A recent study (Smith, *et al.* 2014) has suggested that two novel BSH inhibitors, riboflavin and caffeic acid phenethyl ester (CAPE), have high potential as novel alternative to AGP. In the current study, potent inhibitory effect of these two compounds on the *L. acidophilus* BSH (Table 2) was also observed. Since we are particularly interested in CAPE and riboflavin as AGP alternatives, subsequent dosing experiments were conducted to examine BSH activity at lower concentrations. As shown in Figure 3, CAPE still inhibited rBSH activity by more than 50% at a final concentration of 0.625 mM (Fig. 3A) and riboflavin by more than 50% at a final concentration as low as 0.03125 mM (Fig. 3B).

Growth performance of broilers in response to riboflavin treatment

As shown in Table 3, the BW did not differ significantly ($P > 0.05$) in response to dietary treatment of riboflavin regardless of dosage. However, BW gain significantly increased ($P = 0.0053$) for the birds receiving riboflavin (either low or high level) at 21 days of age. In addition, the birds on the 20 ppm of riboflavin treatment had lower feed intake than those in control group. However, dietary supplementation of high level of riboflavin (200 ppm) led to inconsistent feed intake pattern compared to the control group. For example, feed intake at day 14 in high-level riboflavin group (0.3759 kg/bird per week) was significantly lower than control group (0.4101

kg/bird per week) (P = 0.0393); however, feed intake significantly increased (P = 0.0001) in the 200 ppm riboflavin treatment when compared to control group on day 21 (Table 3). In terms of feed conversion efficiency (BW gain over feed intake), only low riboflavin treatment led to significantly increased feed conversion efficiency on day 21 (Table 3).

HPLC determination of riboflavin in feed

In this study, all chicken feed were prepared on site in JARTU. Given small amount of riboflavin supplemented in various feeds (Table 1), it is important to ensure that riboflavin was mixed within the feed evenly so that desired level was achieved. To determine the level of riboflavin in the feed, a HPLC method was established for this project. As shown in Figure 4A, a sharp peak was observed for the standard riboflavin. The riboflavin that was extracted from the feed, was successfully detected at similar eluent phase (Figure 4B). Based on comparison of peak intensity, the riboflavin level was determined in various feeds. Due to the trace presence of riboflavin in basal feed, it is not surprising that approximately 26-34 ppm of riboflavin were determined in the Control feed. The addition of low level riboflavin (20 ppm) led to the increase of riboflavin in all starter, grower and finisher feeds (Table 4). As expected, the riboflavin level in High group (200 ppm riboflavin supplementation) was higher than those in Control and Low group (20 ppm riboflavin supplementation).

The level of bile salts in serum and intestine

A totally of 90 serum samples and 90 ileal samples from individual chickens were subjected to determination of bile salt levels. As expected, the level of total bile in blood is much

lower than that in ileum (Table 5). However, there is no significant difference between the control and treatment ($P > 0.05$) (Table 5), mainly due to significant variation among individual chickens.

CHAPTER V

DISCUSSION

The development of alternative feed additives is urgently needed to maintain enhanced weight gain and feed conversion ratio levels while phasing out the use of AGPs. The understanding of the AGP mechanism is essential to the development of an effective and sustainable alternatives to AGP. It is widely accepted that the use of AGPs changes the ecology of gut microbiota, which leads to enhanced weight gain and feed efficiency (Collier, *et al.* 2003; Knarreborg, *et al.* 2004; Dumonceaux, *et al.* 2006; Guban, *et al.* 2006; Kim, *et al.* 2012; Lin, *et al.* 2013). Our lab recently found the correlation between AGP-mediated growth promotion and the decreased *L. salivarius* population, a major BSH-producer, in the chicken intestine (Lin, *et al.* 2013; Lin 2014). Given that the usage of AGPs is also strongly associated with the decreased BSH activity (Feighner, *et al.* 1987; Knarreborg, *et al.* 2004; Guban, *et al.* 2006), we hypothesized that BSH is an attractive microbiome target for developing alternatives to AGP and have made significant progress in obtaining additional compelling evidence to support this hypothesis (Wang et al, 2012; Lin et al., 2013; Smith et al, 2014: Lin 2014). In particular, we recently have developed an efficient High Throughput Screening (HTS) system that identifies BSH inhibitors using a BSH from a chicken *L. salivarius* strain (Smith et al, 2014). In this project, with the use of a *L. acidophilus* BSH, we obtained more evidence that demonstrated the feasibility of this HTS system.

Bile salt hydrolase is part of the choloylglycine hydrolase enzyme family that has a conserved cysteine N-terminal residue. It is produced by various commensal bacteria, such as *Lactobacillus*, *Bacteriodes*, *Clostridium*, *Enterococcus*, and *Bifidobacterium* (Begley, *et al.* 2006).

The natural function of BSH for these commensal bacteria is still controversial and not clear; however, the BSH enzyme plays an important role in host physiology by affecting host lipid metabolism and energy harvest (Coleman, *et al.* 1995; Jones, *et al.* 2008; Chae, *et al.* 2013). Although the research in BSH is still in its infancy (Patel, *et al.* 2010; Joyce, *et al.* 2014), previous BSH studies have shown that intestinal BSH displayed significant sequence variation and specificity for substrate choice (Begley, *et al.* 2006; Jones, *et al.* 2008; Chae, *et al.* 2013). Notably, a recent functional metagenomics work (Jones et al, 2008) identified functional BSH in all major bacterial divisions and archaeal species in the gut and demonstrated the BSH enrichment in the gut microbiome. Phylogenetic analysis illustrates that selective pressure in the form of conjugated bile acid has driven the evolution of members of the Ntn_CGH-like family of proteins toward BSH activity in gut-associated species.

In this study, we chose the BSH from *L. acidophilus* PF01 (Oh, *et al.* 2008) for validation work because of the following several reasons. First, the BSH enzyme from *L. salivarius* that was used for screening BSH inhibitors (Wang, *et al.* 2012; Smith, *et al.* 2014), is comparatively different than the BSH produced by different bacterial species. Second, the BSH-producing *L. acidophilus* PF01 and *L. salivarius* NRRL B-30514 strains were originally isolated from the intestine of two different food animals, swine and chicken, respectively. Finally, the *L. acidophilus* BSH (316 amino acids, aa) and the *L. salivarius* BSH (324 aa) displayed significant sequence variation (only 35% aa identity) and different substrate specificity (Oh, *et al.* 2008; Wang, *et al.* 2012; Smith, *et al.* 2014). Therefore, the *L. acidophilus* BSH makes an appropriate candidate enzyme that can be used to determine if previously identified BSH inhibitors (Wang, *et al.* 2012;

Smith, *et al.* 2014), which is based on the *L. salivarius* BSH, could effectively inhibit the activity of diverse BSH enzymes in the intestine.

Our laboratory's recent work (Smith, *et al.* 2014) indicated that riboflavin and caffeic acid phenethyl ester (CAPE), two BSH inhibitors, are promising alternative to AGP. In this study, using a BSH from a different bacterial species, we further demonstrated that riboflavin and CAPE are effective BSH inhibitors (Figure 3 and Table 2). Riboflavin is a vitamin that has been used as feed additive in poultry feed stuff to treat the hypovitaminosis of B2. However, long-term dietary supplementation of riboflavin for growth promotion in broilers has never been explored. Notably, a recent swine study has showed that the dietary supplementation of high-level riboflavin (20 mg/kg feed) significantly increased feed efficiency and body weight gain in the pigs with high lean growth, however underlying mechanisms are still not clear (Stahly, *et al.* 2007). CAPE has antioxidant/anti-inflammatory effects and is an emerging natural food additive that recently has drawn extensive attention for human and animal application. Therefore, a standard large pen trial is highly warranted to determine if dietary supplementation of such BSH inhibitors could enhance feed efficiency and growth performance in chickens.

In this study, riboflavin was formulated in the feed at low (20 ppm) and high (200 ppm) doses for a large chicken experiment (Table 3). However, we only observed significant body weight gain due to riboflavin treatment by day 21. This finding is consistent with an early report by Olkowski et al (1998) in which enhanced growth was observed in broilers receiving riboflavin-supplemented feed (5 mg/kg of fee) during the first 21 days. In addition, in this study the significantly enhanced feed efficiency ($P < 0.05$) was only observed for the birds receiving low level of riboflavin at 21 days of age (Table 3). However, in general, dietary supplementation of

riboflavin, a BSH inhibitor, did not significantly improve broiler growth performance in this project as expected, which may be attributed to following several factors. First, we incorporated male and female Hubbard broilers in this chicken study. Statistically significantly higher weight gain in male than female broilers have been observed in previous studies (Lowe, *et al.* 1986; López, *et al.* 2011; Salim, *et al.* 2012). In the future, we may address this issue by the use of same sex broilers, such as male Cobb broilers. Using male broilers will eliminate any confounding factors that involve hormones such as estrogen, which has shown to decrease weight enhancement (Mohammadrezaei, *et al.* 2014). In future chicken trial, we may also remove the broilers with significantly impaired growth (the outlier chicken) within the first week for proper statistical analysis. Second, intestinal bioavailability of riboflavin may have been low because it is moderately water soluble 0.07 g/L in water. In addition, it has been reported that there are three different transporters present in rat's intestines and kidneys to maintain homeostasis. Riboflavin does not have an upper toxicity level as the kidneys could filter excess riboflavin (Yonezawa, *et al.* 2008; Yonezawa, *et al.* 2013). Thus, similar transporters in chickens may make riboflavin be quickly taken up by gut epithelial cells, which can lead to undesired low concentration of riboflavin in the intestine. To address this potential problem, encapsulation of riboflavin may achieve a targeted and steady release of riboflavin that would increase intestinal lumen exposure time as well as the probability of interaction with the microbiota (Khansari, *et al.* 2013; Bou, *et al.* 2014; O'Neill, *et al.* 2015).

We hypothesized that riboflavin, a BSH inhibitor, would inhibit BSH activity and consequently change the relative proportion of conjugated bile salts. However, determination of profiles of different bile salts in the intestine and blood is technically demanding (Joyce et al.

2014). It has been reported that BSH activity may also influence total bile salt concentration in addition to directly reducing conjugated bile salts (Joyce et al. 2014). Thus, in this study, using a commercially available kit, we determined total bile acid concentration in ileum and blood; however, no significant difference was observed among treatment groups (Table 5). This is likely due to the significant variation among chickens and because of complex bile metabolism in chicken host. In the future, a more accurate technology, such as ultra-performance liquid chromatography mass spectronomy (UPLCMS) (Joyce et al. 2014), should be used to measure specific conjugated and unconjugated bile salts in the intestine in conjunction with chicken growth performance evaluation.

Though the pilot HTS process has identified some potent BSH inhibitors (Smith, *et al.* 2014), a larger scale HTS with an extensive compound library is still needed to identify BSH inhibitors with potential as alternatives to AGP. To identify desired BSH inhibitors, in conjunction with HTS screening and *in vitro* assay, we must perform extensive review of relevant material safety data sheet and literature for the hits with emphasis on availability, stability, intestinal absorption, toxicity, cost, and environmental impact. In addition, the FDA Food Additive Listings (http://www.fda.gov/Food/) will be used to choose priority candidates that may be quickly approved for practical application. More importantly, a single BSH inhibitor such as riboflavin, as in this project, may have multiple impacts on animal host physiology and complex interaction with gut microbiota (Burgess, *et al.* 2006; Arena, *et al.* 2014; Russo, *et al.* 2014). Thus, comprehensive animal trials are always highly warranted for promising BSH inhibitors.

Notably, BSH inhibitors may be used to optimize current probiotics (the BSH producers). In animal industry, probiotics have been used to enhance growth and over all animal welfare

(Fajardo, *et al.* 2012; Roodposhti, *et al.* 2012; Amerah, *et al.* 2013; Sadrasaniya, *et al.* 2013; Lähteinen, *et al.* 2014). Despite multiple beneficial effects of probiotics, probiotics can have side effects in the animal host, such as decreased fat digestibility due to production of BSH (Dibner, *et al.* 2005). Therefore, not surprisingly, the results on animal growth performance following probiotic administration have been inconsistent. For instance, supplementation of probiotics in growing ruminants did not enhance weight gain (Morrill, *et al.* 1977; Schwab, *et al.* 1980; Zhang, *et al.* 2015). In poultry, probiotics administration have been reported to improve weight gain (Mountzouris, *et al.* 2007), to stimulate immune response (Zhang, *et al.* 2012), and to improve food safety (Gaggìa, *et al.* 2010). However, due to complicated interactions and low numbers of birds for statistical power more studies are urgently needed (Alloui, *et al.* 2013). Probiotic use in swine has also suggested that probiotics do not improve weight gain nor feed efficiency (Harper, *et al.* 1983; Lähteinen, *et al.* 2014). Taken together, probiotics could negatively impact host growth performance due to the production of BSH enzymes. BSH inhibitor together with probiotics could mitigate negative features of probiotics and optimize probiotic products for enhanced growth performance of food animals and profitability of feed additive industry.

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APPENDIX

Figure 1. Sequence and structural analysis of BSH.

(**A**) Phylogenetic relationship of BSH from different bacteria. The amino acid-based dendrogram was constructed in MEGA 6.0 by using neighbor-joining methods. LaciP, *L. acidophilus* PF01 (ABQ01980.1)*;* LsalN1, *L. salivarius* NRRL B-30514 BSH1 (JX120368); LsalN2, *L. salivarius* NRRL B-30514 BSH2 (JX120369); LsalU1, *L. salivarius* UCC118 BSH1(ACL98201.1); LsalU2, *L. salivarius* UCC118 BSH2 (ABD99327.1); LsalJ, *L. salivarius* JCM1046 BSH1 (ACL98203.1); LaciN, *L. acidophilus* NCFM (AAV42923.1)*;* Lgass, *Lactobacillus gasseri* (EFQ47028.1); Ljohn, *L. johnsonii* (EGP12391.1); Lplan, *L. plantarum* (AAA25233.1); Bbifi, *B. bifidum* (AAR39435.1); Blong, *B. longum* (AAF67801.1); Efaec, *Enterococcus faecium* (AAP20760.1); LmonM7, *Listeria monocytogenes* M7(AEH93162.); Cperf, *Clostridium perfringens* (AAC43454.1); BPVA, *Bacillus Sphaericus* PVA (YP_001698896). The number in parentheses is GenBank accession number. Furthermore, the following sequences of BSH homologs were extracted from IMG database (https://img.jgi.doe.gov/cgi-bin/w/main.cgi) based on similarity (>30%): Elimo, *Eubacterium limosum* KIST612; Bprod, *Blautia producta* ATCC 27340; Sbovis, *Streptococcus bovis* SN033; Panta, *Planococcus antarcticus* DSM 14505; Mbark, *Microbacterium barkeri* 2011-R4; Prumi, *Pseudobutyrivibrio ruminis* HUN009; Cmalt, *Carnobacterium maltaromaticum* MX5; Molle, *Methanobrevibacter olleyae* DSM 16632. (**B**) Structural modeling of BSH. Using *C. perfringens* BSH as a template, the structures of *L. acidophilus* BSH (green backbone) and *L. salivarius* BSH (red backbone) were predicted and superimposed. The RMSD value is 2.749 A. The side chains of critical residues C2 and R16 were denoted.

(A)

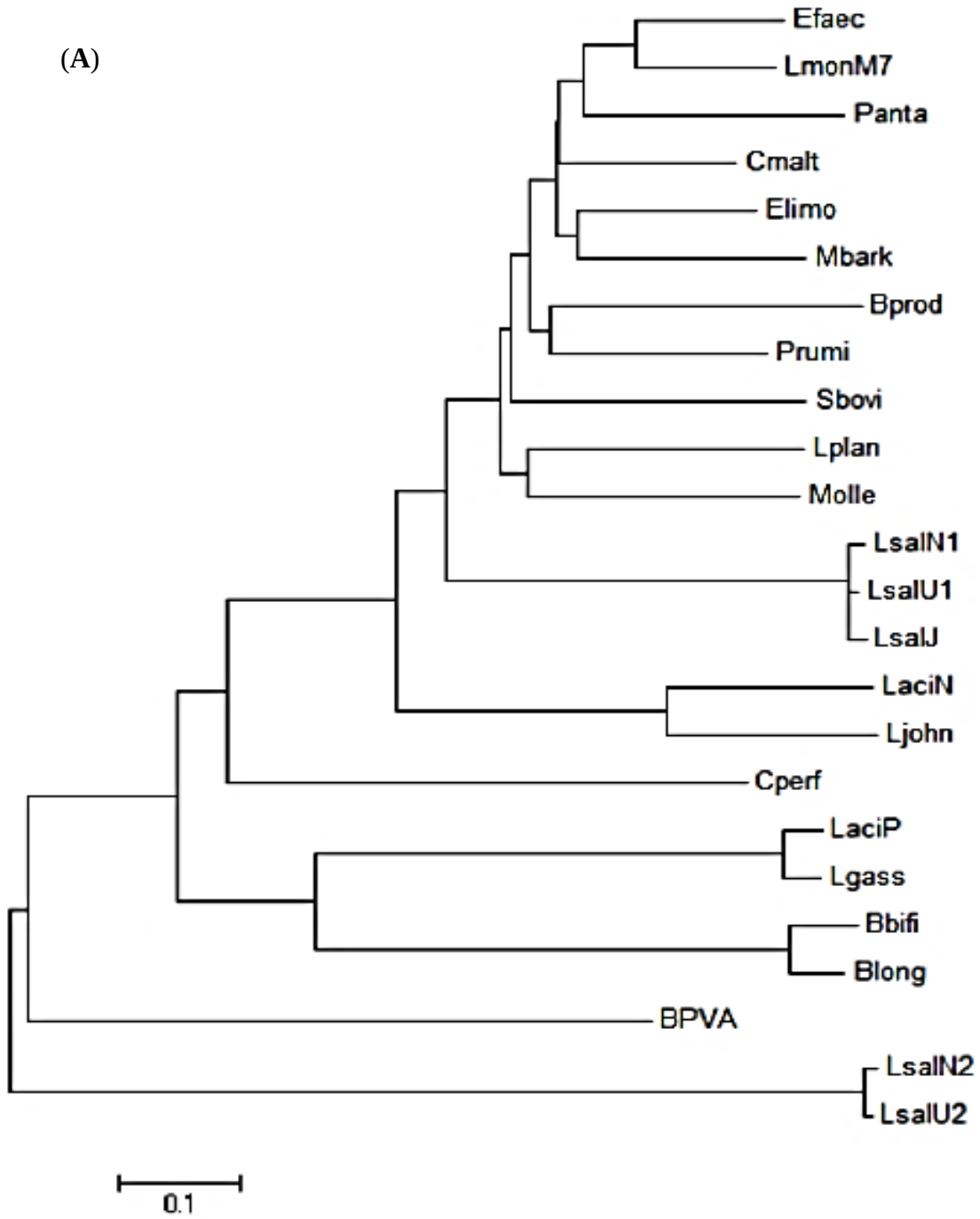


Figure 1. Continued

(**B**)

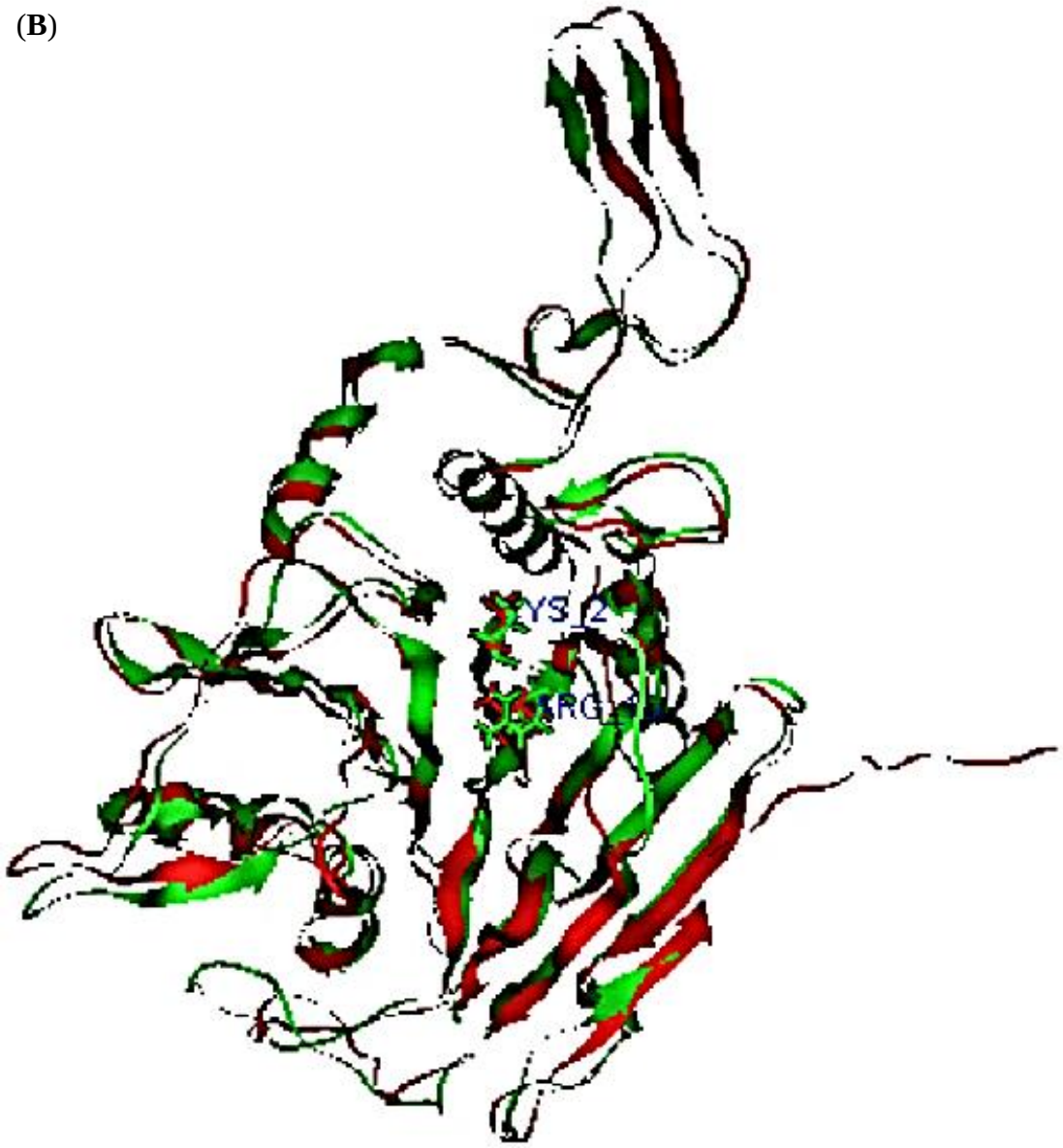


Figure 1. Continued

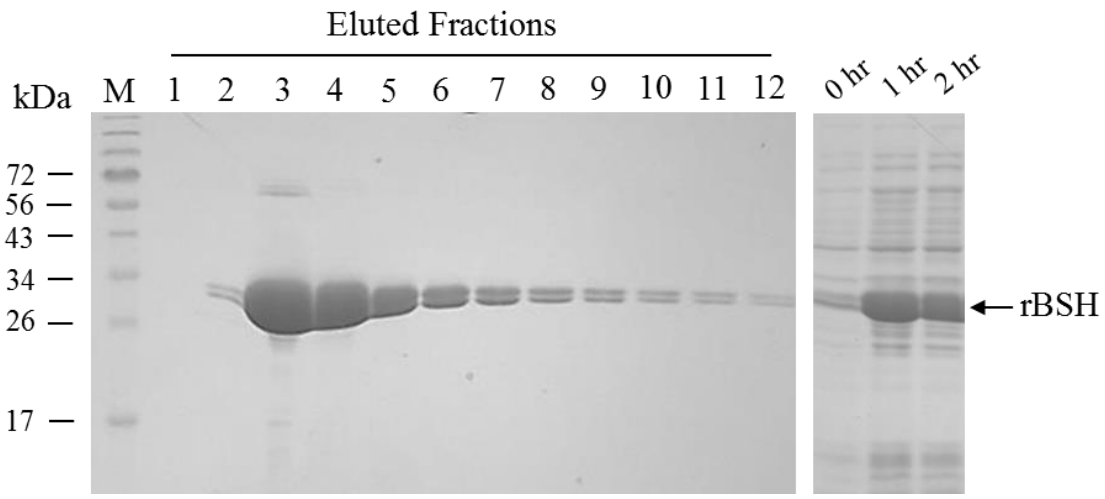
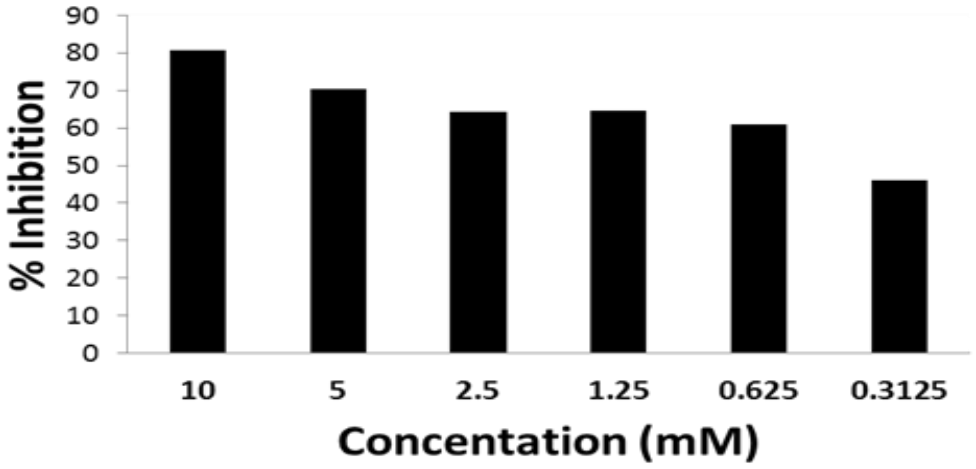


Figure 2. Production of purification of the *L. acidophilus* BSH enzyme.

Lane M, EZ Run™ prestained molecular mass marker (Fisher Bioreagent); Lane 1-12, eluted fractions during Ni-nitrilotriacetic acid affinity chromatography purification; 0 hr, whole-cell lysate of noninduced *E. coli.*; 1 hr, whole-cell lysate of *E. coli* induced with 0.5m*M* IPTG for 1 hr; 2 hr, whole-cell lysate of *E. coli* induced with 0.5m*M* IPTG for 2 hr.

(**A**)



(**B**)

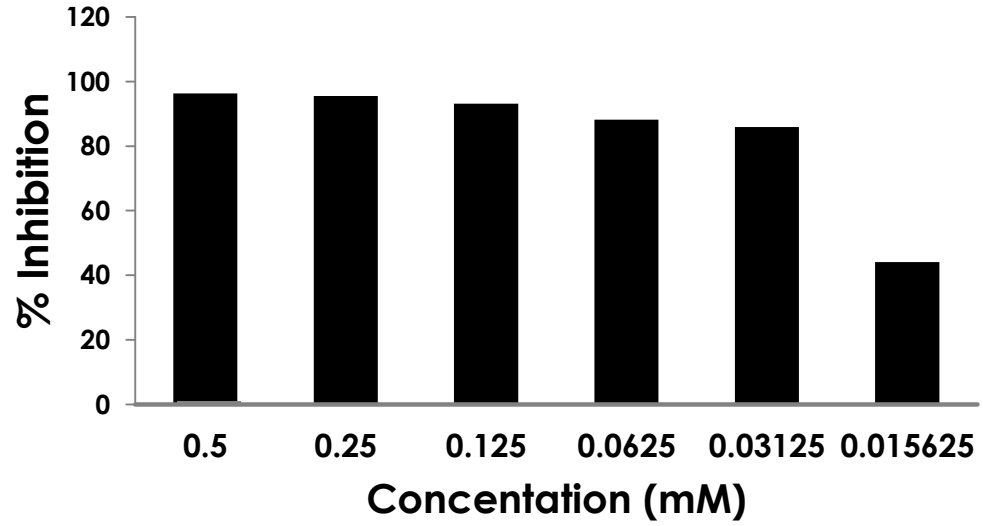
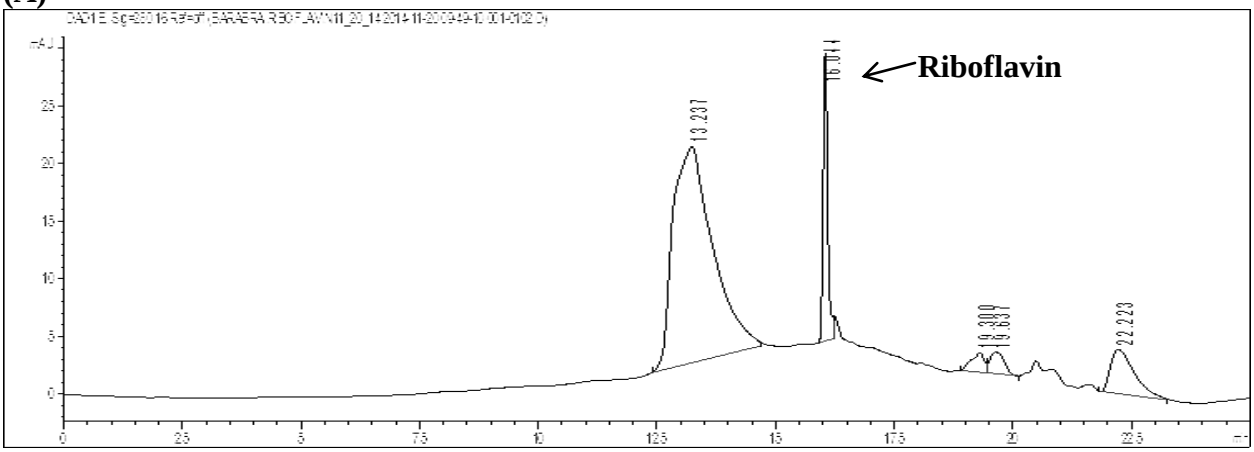


Figure 3. Dose-dependent effects of selected BSH inhibitors on the activity of the *L. acidophilus* BSH.

(**A**) Inhibition of BSH activity by CAPE. (**B**) Inhibition of BSH activity by riboflavin.

(A)



(B)

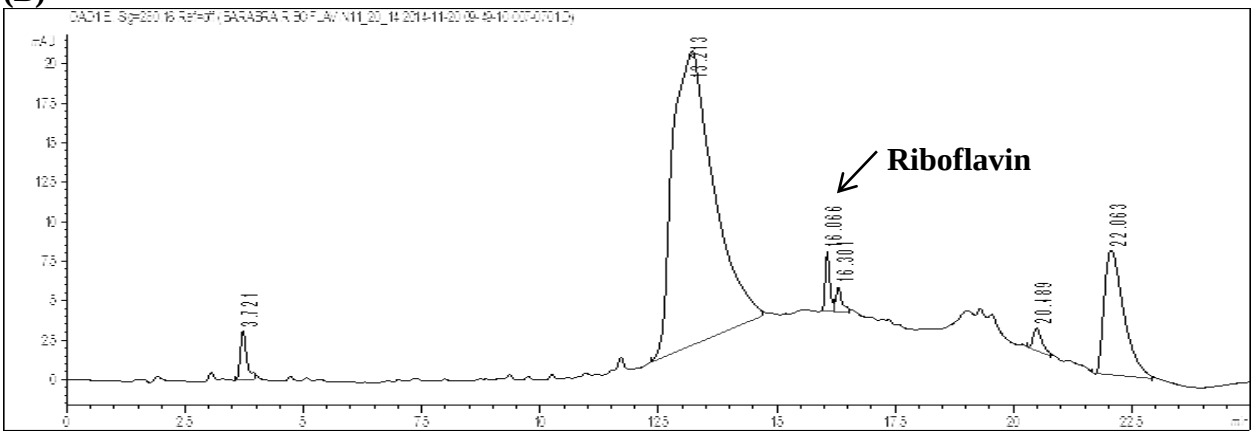


Figure 4. High-performance liquid chromatography analysis of riboflavin compound in standard solution and in feed sample.

(**A**) Standard riboflavin (5 ppm) detected by HPLC (Peak 16.044: Riboflavin). (**B**) The riboflavin detected in the treated Starter feed supplemented with high level of riboflavin (200 ppm) (Peak 16.066: Riboflavin)

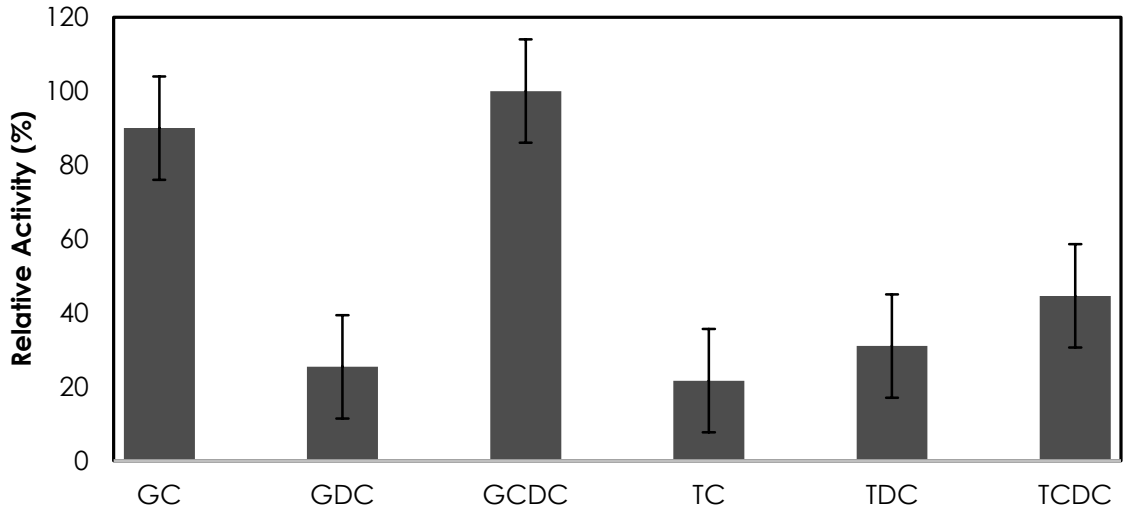


Figure 5. *L. acidophilus* BSH Substrate Preference.

Relative rBSH activity for various substrates when compared to the activity for glycochenodeoxycholate (GCDC). GC: Glycocholate, GDC: Glycodeoxycholate, GCDC: Glycochenodeoxycholate, TC: Taurocholate, TDC: Taurodeoxycholate, and TCDC: Taurochenodeoxycholate

Table 1. Composition of basal feed (Control) and the feed supplemented with low level of riboflavin (20 ppm, Low) or high level of riboflavin (200 ppm, High) during the starter, grower, and finisher phases.

	Starter 0 to 14 d (as fed basis)			Grower 15 to 32 d			Finisher 33 to 42 d		
	(%)								
	Control	Low	High	Control	Low	High	Control	Low	High
Corn, grain	53.700	53.700	53.700	61.2	61.2	61.2	65.5	65.5	65.5
Soybean meal	33.300	33.300	33.300	25.8	25.8	25.8	21.5	21.5	21.5
Alfalfa meal	1.960	1.960	1.960	1.960	1.960	1.960	1.960	1.960	1.960
Fish meal	3.000	3.000	3.000	3.000	3.000	3.000	3.000	3.000	3.000
Vitamin Premix[1]	0.027	0.027	0.027	0.027	0.027	0.027	0.027	0.027	0.027
Sand*	0.020	0.018	0.000	0.020	0.018	0.000	0.020	0.018	0.000
DL Methionine	0.150	0.150	0.150	0.150	0.150	0.150	0.150	0.150	0.150
Salt	0.350	0.350	0.350	0.350	0.350	0.350	0.350	0.350	0.350
Limestone	0.900	0.900	0.900	0.900	0.900	0.900	0.900	0.900	0.900
Dicalcium phosphate	1.500	1.500	1.500	1.500	1.500	1.500	1.500	1.500	1.500
Trace Mineral Premix[2]	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100
Fat, animal	5.000	5.000	5.000	5.000	5.000	5.000	5.000	5.000	5.000
Riboflavin*	0.000	0.002	0.020	0.000	0.002	0.020	0.000	0.002	0.020
Calculated Nutrition Composition									
ME, kcal/kg	3,133	3,133	3,133	3,201	3,201	3,201	3,240	3,240	3,240
Crude protein	22.990	22.990	22.990	19.990	19.990	19.990	18.270	18.270	18.270

The corn-soybean formulation (reported in percentage) was used in the starter, grower, and finisher diets for the 42 day large scale chicken experiment. Metabolizable energy increased as crude protein decreased from the starter diet to the finisher.

*Riboflavin was supplemented at low (20 ppm) or high (200 ppm) levels at the expense of sand**.**

[1]Supplied per kilogram of Starter diet: Vitamin A, 3,751 IU; Vitamin D, 1,191 ICU; Vitamin E 9 IU; Vitamin B_{12}, 0.0048 mg; Menadione, 0.595 mg; Vitamin B_2, 2.98 mg; Pantothenic Acid, 5 mg; Niacin, 15 mg; Folic Acid, 0.298 mg; Vitamin B_6, 0.893 mg; Thiamine, 0.417 mg.

[2]Supplied per kilogram of Starter diet: Cobalt, 0.020 mg; Iodine, 2 mg; Selenium, 0.6 mg.

Table 2. Effect of identified BSH inhibitors on the activity of the *L. acidophilus* BSH enzyme.

Compound Category	BSH Inhibitor	% Inhibition
[a]The approved feed additives used in food animal industry (Wang, *et al.* 2012)	KIO_3	99.1
	$NaHIO_3$	99.3
	$NaIO_4$	99.0
	$CuSO_4$	94.7
	$CuCl_2$	97.2
	$ZnSO_4$	27.4
	$ZnCl_2$	38.4
[b]The novel BSH inhibitors identified using high-throughput screening (Smith, *et al.* 2014)	Menadione	97.9
	Riboflavin[c]	96.5
	Gossypetin	96.1
	Caffeic Acid Phenethyl Ester (CAPE)	71.8
	Epicatechin monogallate	52.8
	Purpurogallin	36.1
[d]The antibiotics that can inhibit BSH activity (Smith, *et al.* 2014)	Oxytetracycline	99.6
	Demeclocycline Hydrochloride	99.6
	Methacycline Hydrochloride	99.2
	Doxycycline Hydrochloride	98.3
	Roxarsone	48.6
	Lincomycin	26.8

[a] The final concentration of dietary compound in the reaction mix was 5mM to achieve optimal resolution with the quantitative BSH activity assay.

[b] Unless specified, the final concentration of specific BSH inhibitor was 2.5 mM.

[c] The final concentration of riboflavin in reaction mix was 0.5 mM.

[d] The final concentration of specific antibiotic was 2.5 mM.

Table 3. Summary of the growth performance of broiler in response to riboflavin treatment.

BW Gain (kg/bird per week)					
Chicken Age (Day)	Control	Low	High	Standard Error	*P* value
14	0.3369	0.3381	0.3099	±0.010	0.1137
21	0.4605 [a]	0.4966 [b]	0.5103 [b]	±0.010	0.0053
28	0.6847	0.6592	0.6443	±0.020	0.1825
33	0.4588	0.4490	0.4199	±0.020	0.1893
42	0.7943	0.8053	0.8274	±0.030	0.7786
Feed Intake (kg/bird per week)					
Chicken Age (Day)	Control	Low	High	Standard Error	*P* value
14	0.4101 [a]	0.3964 [ab]	0.3759 [b]	±0.010	0.0393
21	0.7799 [a]	0.7601 [a]	0.8847 [b]	±0.020	0.0001
28	1.0536	1.0448	1.0384	±0.020	0.7765
33	0.8196	0.8067	0.7810	±0.170	0.2701
42	1.7548	1.7505	1.7946	±0.050	0.7965
Feed Conversion Ratio (kg of gain/ kg of feed per bird)					
Chicken Age (Day)	Control	Low	High	Standard Error	*P* value
14	0.8217	0.8560	0.8261	±0.020	0.5638
21	0.5925 [a]	0.6546 [b]	0.5789 [a]	±0.010	0.0030
28	0.6504	0.6313	0.6206	±0.010	0.2744
33	0.5591	0.5569	0.5364	±0.010	0.4039
42	0.4519	0.4614	0.4657	±0.020	0.8774
Average BW (kg/bird)					
Chicken Age (Day)	Control	Low	High	Standard Error	*P* value
07	0.1753	0.1739	0.1695	±0.002	0.2431
14	0.5122	0.5062	0.4794	±0.010	0.0842
21	0.9727	1.0027	0.9897	±0.010	0.1849
28	1.6813	1.6867	1.6340	±0.010	0.0968
33	2.1204	2.1357	2.0539	±0.030	0.0655
42	2.9147	2.9409	2.8861	±0.040	0.6225

[ab] Effects means within columns with no common superscript differ significantly ($P < 0.05$)

Table 4. Determination of riboflavin level in feed using high performance liquid chromatography.

Feed	Concentration of Riboflavin (mg/kg of feed) ±SD		
	Control	Low	High
Starter	39.15 ±20.11	51.55 ±21.42	128.87 ±37.90
Grower	34.06 ±6.20	40.57 ±11.12	144.55 ±28.27
Finisher	26.79 ±4.31	31.86 ±15.74	116.44 ±15.74

Riboflavin levels were detected in each of the starter, grower, and finisher treatment paradigms in two independent HPLC experiments.

Table 5. Total bile salt levels in serum and ileal samples of chickens.

Sample Type	Age (Day)	Levels of Total Bile			Pooled Standard Error	*P* value
		Control	Low	High		
Serum (umole/L)	14	27.01	27.24	31.23	±3.64	0.6810
	33	17.16	15.00	16.88	±2.06	0.7268
	42	16.98	21.43	15.71	±2.77	0.3248
Ileal (umole/g)	14	256.64	113.39	208.50	±104.55	0.6202
	33	87.90	108.60	140.85	±32.21	0.4638
	42	149. 15	361.81	334.54	±126.05	0.4481

VITA

Rekek Negga was born in Ethiopia on December 25, 1985. She was raised in Grundy, VA and graduated from Mountain Mission School in 2004. In 2009 she received a B.S. in biology from King University and in 2013 began working on a M.S. in animal science at the University of Tennessee, Knoxville. Beginning of August 2015, Rekek will matriculate at the University of Tennessee College of Veterinary Medicine to pursue a DVM.