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Identification of bile salt hydrolase inhibitors, the promising alternative to antibiotic growth promoters to enhance animal production

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I am submitting herewith a thesis written by Katie Rose Smith entitled "Identification of bile salt hydrolase inhibitors, the promising alternative to antibiotic growth promoters to enhance animal production." 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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Identification of bile salt hydrolase inhibitors, the promising alternative to antibiotic growth promoters to enhance animal production

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DEDICATION

This thesis is dedicated to Dr. Joanna Badara, who persistently muffled the alluring beckons of the department of English. You, ma'am, are a staunch character. Thanks for everything.

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ABSTRACT

Currently there is a global trend to eliminate the use of antibiotic growth promoters (AGP) in animal production due to the risk of creating reservoirs of antibiotic resistant bacteria. Previous studies have shown that the ability of AGP to promote growth is highly correlated with a decrease in the activity of bile salt hydrolase (BSH), an enzyme produced by commensal bacteria and whose actions may compromise host lipid metabolism; thus, BSH inhibitors could be a novel AGP alternative. In this study, a recombinant bile salt hydrolase (rBSH) from *Lactobacillus salivarius* was produced in an *E. coli* expression system, purified, and enzymatically characterized. Purification by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography consistently yielded approximately 15 mg of rBSH per liter of induced culture. A standard 2-step BSH activity assay which estimates the amount of amino acids liberated from conjugated bile acids was used to determine substrate specificity of the BSH and the effect of pH, temperature, and dietary compounds on BSH activity. The rBSH displayed its highest hydrolysis activity for glycochenodeoxycholic acid although there was no preference among other substrates tested. Optimal activity was observed between pH of 5.0 and 6.0 and between temperatures of 35°C to 55°C. Preliminary assays identified different dietary compounds that were potent inhibitors, including copper and zinc compounds which have previously been shown to boost feed efficiency and promote growth of poultry and swine. Furthermore, a high-throughput screening system (HTS) was developed for fast and efficient identification of potent BSH inhibitors. This HTS system was utilized to screen a compound library comprised of 2,240 biologically active and structurally diverse compounds. The pilot screen led to 107 hits and a preliminary review of biochemical information of the corresponding compounds further narrowed down those of interest. Several lead compounds have been validated by the standard 2-step BSH activity assay and will be subjected to future *in vivo* analysis in a large-scale animal

study. Overall, this study characterized a BSH with broad substrate specificity and developed and validated different strategies for identification of BSH inhibitors, the promising alternatives to AGP for enhancing the productivity and sustainability of food animals.

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CHAPTER I
LITERATURE REVIEW

Antibiotic growth promoters: history and mode of action

The animal production industry is one that measures success in terms of quality. In order to maximize efficiency, a balance must exist between growth and health. One of the primary means that producers seek to ensure such quality in food animals is via antibiotic growth promoters (**AGP**). Antibiotic growth promoters are subtherapeutic quantities of antibiotics, such as tetracycline or bacitracin, that are added to animal feed for enhancement of growth and feed conversion ratio (Marshall & Levy 2011). Unlike antibiotics that are used for therapeutic treatment for sick animals or prophylactically for infection prevention, AGP are aimed at enriching feed utilization and early indications of improvement of production efficiency occurred as early as 1946 (Moore *et al*.). However, AGP generally started being promoted in the mid-1950s after it was found that they could improve the feed-to-weight ratio in poultry, swine, and beef cattle (Stokestad & Jukes 1950). Since then, AGP have been widely used in the United States and elsewhere in agriculture as a means of propagating healthy and robust food animals. Other ancillary values of AGP may include environmental gains because of less nitrogen and phosphorous excretion, improved animal welfare, and even enhanced milk, wool, and egg production as well as fertility (Barton 2000). Equally important, from an economic standpoint, AGP are attractive to producers because they can improve financial gains due to improved feed efficiency and reduced morbidity and mortality. Recent estimates are difficult to gauge; however, a1999 book compiled by the Committee on Drug use in Food Animals *et al*. states that during some period of their lives nearly 100% of poultry, 90% of swine and veal calves, and 60% of beef cattle are fed diets incorporating AGP (Animals, Panel on Animal Health *et al*.

1999), so clearly producers rely on these products to benefit their production systems. Based on the wide-ranging effects that AGP confer to both animals and farmers, recent suggestions to curb their usage (as will be later discussed) are undoubtedly a cause for great concern among producers and consumers alike.

Although precise mechanisms have yet to be elucidated, it is widely accepted that AGP achieve growth promoting effects because of their interactions with intestinal microflora. This is supported by the fact that oral antibiotics fail to produce any growth promoting effects in germ free animals (Coates *et al*. 1963), and in fact, AGP tend to create intestinal conditions which approach those of germ free animals (Commission on Antimicrobial Feed Additives 1997). The gastrointestinal (GI) tract of vertebrate animals is rife with diverse microflora, much of which is gram-positive bacteria, and as such are extremely influential to GI physiology, immunity, and nutrition (Dibner & Richards 2005). Although commensal bacteria perform critical roles in nutrition and development, advantages may be countered by certain detrimental effects to the host. Microflora provide benefits in the form of nutrients through the production and secretion of volatile fatty acids and vitamins, competitively exclude pathogenic bacteria from colonization, and are important in stimulating host intestinal immune defenses (Dibner & Richards 2005). Negative impacts are the result of competition for nutrients, secretion of toxic compounds, and undesired immunological responses in the GI tract (Gaskins *et al*. 2002). Antibiotics, therefore, are important modulators of gut microflora and are implicated to improve host interactions with normal microflora by limiting nutrient competition and reducing harmful secondary metabolites that may weaken growth (Dibner & Richards 2005).

AGP and antibiotic resistance in zoonotic pathogens

Any use of antibiotics, whether it is for therapeutic or subtherapeutic purposes, will create selection pressures for drug resistant bacteria. Particularly as is the case for AGP, the large number of animals that receive a low-dosage of antibiotic in combination with a steady and prolonged delivery into the feed system generates selection pressure for emergence, persistence, and transmission of antibiotic resistant bacteria (Marshall & Levy 2011). If and when these resistant bacteria proliferate, modes of transmission from animal to humans can occur through direct contact with the colonized or infected animal or indirectly through the food chain (van den Bogaard & Stobberingh 1999). The major concerns regarding animal derived antibiotic resistant zoonotic pathogens are aimed at enteric bacteria like *E. coli, Salmonella, Campylobacter,* and *Enterococcus*. All could be commensals of food animals but have the potential to become dangerous human pathogens and evidence exists for isolates of antibiotic resistant pathogens after AGP treatment. Not long after AGP became common additives to feed, tetracycline resistant *E. coli* isolates were detected from chickens and pigs (Smith 1967); soon after, antibiotic resistant *Salmonella* was reported (Anderson 1968). Emerging multi-drug resistant *Salmonella* in the 1960s prompted the establishment of the Swann Committee to address the concerns of transferable animal derived human pathogens. Their report recommended that AGP in a class also used for human therapy should be discontinued to prohibit drug-resistant infections (Swann 1969), yet these practices continued in the U.S. and Europe. Both *E. coli* and *Salmonella* resistance is a continuing phenomenon (Folster *et al*. 2012; Ramos *et al*. 2012), and in the 1990s different concerns arose when *Campylobacter* isolates of poultry and swine were found to be resistant to erythromycin, ampicillin, streptomycin, and ciprofloxacin, among others (Lucey *et al*. 2000; Moore *et al*. 1996; Saenz *et al*. 2000). In 1995 the Danish Veterinary

Laboratory documented vancomycin-resistant *Enterococcus faecium* in pigs and poultry receiving avoparcin laced feed (Barton 2000); Aarestrup *et al*. (1999) later found a high prevalence for *E. faecium* resistance to tylosin and virginiamycin in Norwegian swine and poultry. Additionally, methicillin resistant *Staphylococcus aureus* (MRSA) colonization in food animals and their farmers has been implicated as a reservoir of animal derived MRSA that could contribute to human infection and for community acquired MRSA infections as a result of contaminated meat consumption (Khanna *et al*. 2008; Ogata *et al*. 2012; Oppliger *et al*. 2012).

The last few decades have unveiled selective pressures of AGP on commensal and pathogenic flora of food animals (Akwar *et al*. 2007; Bager *et al*. 1997; Hummel *et al*. 1986; Levy 1976) and knowing that they can easily cross the human barrier is a formidable realization. A 2011 review published in Clinical Microbiology by Marshall and Levy lists crucial evidence of just such resistance transfer and it cites multiple studies confirming human colonization and infection of animal derived resistant bacteria. Furthermore, estimates of AGP excretion back into the environment range from 75-90% (Costa *et al*. 2010; Kumar *et al*. 2005), constituting exposure of innumerable environmental bacteria to minute doses of antibiotics and generating the potential for widespread resistance selection, which may be further complicated by horizontal gene transfer as a means of resistance transmission. Clearly, efforts need to be made to curtail this phenomenon, and discontinuing the use of AGP is likely a good means of doing so.

The current status of AGP

Because of the looming threat that using antibiotics as growth promoters in animals has aroused valid suspicion that human health could negatively be impacted, efforts have been made to generate awareness about the potentially dangerous practice as well as to limit and terminate

altogether the use of AGP. The previously mentioned Swann Report resulted in changes in England, Canada, and some European countries, but failed to make an impact elsewhere. Other councils and committees have drafted similar guidelines as to how AGP usage should be monitored or curtailed for preservation of public health, including the Institute of Medicine, the Council for Agricultural Science and Technology, the Committee on Drug Use in Food Animals, and the Poultry Science Association (Dibner & Richards 2005); the World Health Organization (WHO) has also published several reports regarding antimicrobial usage in food animals in an ongoing effort to reduce their need in agriculture and aquaculture (WHO 1997; 2000; 2004; 2006). Leaders in the front to revolutionize safety practices in animal production, European Union (EU) countries were among the first to implement restrictive policies and create a standard by which agricultural practices may be modeled throughout other areas of the world. Sweden was the first to ban AGP in 1986, followed by a ban on avoparcin in Denmark in 1995 and in 1997 by all EU member states; Denmark further banned all AGP for swine at the weaning and finishing levels on January 1, 2001 (Dibner & Richards 2005). Following bans on individual AGP in 1998 and 1999, the EU later banned all others as of January 1, 2006.

With increased concern from public health officials, it is likely that the trend of phasing out or eliminating AGP will continue in the United States and elsewhere. In 2000, the World Health Organization issued a report urging the need for national governments to "adopt a proactive approach to reduce the need for antimicrobials in animals and their contribution to antimicrobial resistance and to ensure their prudent use (including reducing overuse and misuse), as elements of a national strategy for the containment of antimicrobial resistance" (WHO 2000). The report further suggested that food producers voluntarily, but by legislation if necessary, terminate or speedily phase out AGP that are in a class used by humans and avoid the

prophylactic use of antimicrobials in animal management. In 2010, the FDA issued a draft guidance in support of phasing out antimicrobials in food animals to reduce selection pressures and protect public health (Sharfstein 2010). This was followed by a court ruling in March of 2012 that the FDA should begin retracting the allowance for the use of certain antibiotics used for production purposes in food animals except under veterinary supervision (Veterinary Record 2012). Clearly the impetus exists to discontinue using AGP, and sooner or later their removal will be dictated by both public demand and economic pressures to maintain export markets with countries that have already banned AGP as an agricultural practice. While eliminating AGP is a good idea to prevent widespread resistance, it is clear that alternative strategies must be implemented to guarantee that animal health and nutrition are not compromised and to offset economic setbacks for animal producers.

Although eliminating AGP usage is a noble goal, there is obvious concern over the potentially negative consequences that may ensue from so suddenly ceasing to include these subtherapeutic antibiotics in animal feed. Shortly after the early EU AGP ban there was a consequent rise in the use of therapeutic antibiotics to treat infection, generating alarm because they were those also used in human medicine (Casewell *et al*. 2003). Likewise, after Denmark's 2001 ban of AGP there was an increase in tetracycline and sulphonamide resistance in *S. typhimurium* in both pigs and humans and more *Campylobacter* resistance to tetracycline and fluoroquinolones in humans than in animals (DANMAP 2001). Animal welfare also seemed to suffer immediately following Denmark's ban: increased reports of morbidity and mortality were identified in pigs and attributed largely to enteric infections (Casewell *et al*. 2003) and clostridial necrotic enteritis emerged as well (Tornee 2002). These immediate pitfalls were somewhat expected, but what enduring effects does AGP banning really introduce?

Denmark's early ban of AGP even before the comprehensive ban in the entire EU presents us with a unique opportunity to gauge how large-scale bans in other countries may play out. One of the most comprehensive studies was conducted by Aarestrup *et al*. (2010) and examined production, disease, and antibiotic use on swine farms from 1992 to 2008. As to overall antimicrobial consumption per animal, there was a decrease by over 50% and long-term productivity actually improved despite an initial increase in diarrhea in weaning pigs and a consequent increase in therapeutic antibiotics. A 2002 executive summary published by WHO also mentioned loss in productivity in weaner pigs and decreased feed efficiency in poultry production, but noted that in Denmark the termination of AGP led to a drastically reduced food animal pool of resistant enterococci and concluded that necrotic enteritis in broilers was only a minor problem since ionophores were still implemented for prophylaxis of coccidiosis. The WHO further determined that discontinuing AGP is largely beneficial in reducing the total amount of antimicrobials given to food animals and consequently reducing antimicrobial resistance that threatens both animal and human health. Additionally, they estimate that economic impacts are relatively minor and in some cases offset by cost savings associated with not having to buy AGP, although they suggest that costs may have been substantial for some producers.

Though the consensus in Denmark seems to favor the ban on AGP, Sweden has experienced more problems. Sixteen years after their 1986 ban of AGP, the loss in swine production had not been fully recovered (Casewell *et al*. 2003) and total antimicrobial consumption was at the same level as before the ban (Aarestrup *et al*. 2010). Considering this and the early difficulties experienced in Denmark, results seem mixed as to whether the ban is largely beneficial to animal production or compromising of animal welfare. Therefore, how the

United States would respond to a total ban of AGP is unclear since antimicrobial consumption here is much higher than the EU mean value (Aarestrup *et al*. 2010). Moving away from a reliance on AGP in animal production is in our best interest and may even benefit production long-term, but due to initial compromises in animal health, it would still be beneficial to institute alternatives to AGP that facilitate growth enhancement in their absence. It is fair to assume that, at least initially, safe AGP alternatives would be a logical first step in the transition.

Response of the intestinal microbiota to AGP

In order to implement alternatives, we must reexamine the mechanism of action of AGP. As previously discussed, it is proposed that AGP promote growth because of their effects on the GI microflora. Specifically, the small intestine is a prime target of effect because 1) it is where most nutrients are absorbed and 2) here there is a high density of bacterial numbers (Rettedal *et al*. 2009). Studies performed on swine and poultry to demonstrate the effects of antibiotics on intestinal microflora note interesting bacterial shifts (Collier *et al*. 2003; Dumonceaux *et al*. 2006; Rettedal *et al*. 2009). What seems to happen is that the abundance of some bacterial species increase in numbers while others decrease, and particularly *Lactobacilli* are affected. Collier *et al*. (2003) found that antibiotic rotations selected against three *Lactobacilli*, one *Bacillus*, and one *Streptococcus* species in pig ileal microbiota, while treatment with tylosin alone tended to increase lactobacilli concentration as a percentage of total bacteria here. Also observed was an antibiotic-induced homogenization of the ileal microbiota, which could explain uniform growth enhancement seen in animals given AGP. Similarly, Retedal *et al*. (2009) reported that inclusion of chlortetracycline in the diet influenced porcine ileal bacteria in such a way as to decrease the abundance of *L. johnsonii* and increase that of *L. amylovorus*. Regarding

poultry, the observation has been made that dietary AGP improved weight gain and feed conversion ratios while diminishing ileal *L. salivarius* populations, indicating their potential detrimental effect on poultry nutrition (Dumonceaux *et al*. 2006; Guban *et al*. 2006). By focusing on these small intestine microbes that are heavily influenced by AGP, beneficial commensals may be noted, but more importantly the opportunity arises to understand why certain species are deleterious to animal growth.

Response of intestinal bile salt hydrolase activity to AGP

Clearly a connection has been made between AGP, growth promotion, and intestinal bacterial populations, but the precise mechanism is yet to be delineated as to how these all coincide. Strides have been made in uncovering this mystery, however, and it has been shown that the growth promoting effect of low-dose antibiotics does coincide with a decrease in bile salt hydrolase activity (**BSH**) in the gut (Feighner & Dashkevicz 1987; Guban *et al.* 2006; Hunkapillar *et al*. 2009; Knarreborg *et al*. 2004). BSH is an enzyme produced by commensal bacteria in the host's intestine whose main function is to transform conjugated bile salts into unconjugated bile salts. Bile salts (also referred to as bile acids) are one of several components of bile and their amphipathic nature, which is facilitated by amino acid conjugation with glycine or taurine, significantly contributes to the emulsification of dietary lipids and micelle formation. BSH liberates this glycine or taurine moiety by hydrolysis of the amide bond and transforms a conjugated bile salt that is effective at solubilizing lipids into a deconjugated bile salt that is inefficient in lipid emulsification and undesirable for lipid digestion and absorption. Because this results in altered lipid metabolism and micelle formation, normal host digestive functions may be impaired. Indeed, because unconjugated bile acids are less water soluble, they are

readily excreted in the feces. The potential for BSH producing bacteria to lower serum cholesterol by reducing its solubility and increasing the amount of free bile acids lost in the feces is debatable as a desirable trait of probiotics (Begley *et al.* 2006). However, secondary bile acids that are produced from the multistep process of 7 α-dehydroxylation (alpha-dehydroxylation), subsequent to deconjugation, can accumulate and have been linked in humans to gallstones, colon cancer, and other gastrointestinal illnesses (Pavlović *et al*. 2012). Regardless of whether or not humans may benefit from a BSH-mediated hypocholesterolemic effect, malabsorption of lipids may deter growth in production animals, particularly in poultry which derive a substantial percentage of energy from dietary lipids and thus rely on efficient fat digestion (Knarreborg *et al.*, 2004).

Feighner and Dashkevicz (1987) provided early evidence that antibiotic feed additives affect the transformation potential and hydrolysis activity of BSHs from intestinal contents of poultry. By keying in on a more specific aspect of lipid metabolism, Knarreborg *et al*. (2004) demonstrated an enhanced bioavailability of α-tocopheryl (alpha-tocopherol) acetate in broilers given AGP, and this was attributed to a reduced concentration of unconjugated bile salts. Furthermore, Guban and colleagues (2006) correlated dietary supplementation of AGP to improved weight gain and fat digestibility in broilers, decreased population levels of *Lactobacillus salivarius*, and a reduced pool of deconjugated bile salts. Based on these discoveries, the growth promoting effect of AGP likely is attributed to the reduced BSH activity, and thus the improvement of host lipid metabolism.

Functions and characteristics of bile salt hydrolase

Microbial BSH is a member of the choloylglycine hydrolase family of enzymes and is predominantly associated with gastrointestinal bacteria of both humans and animals. Additionally, it is classified as an N-terminal nucleophilic hydrolase and can recognize substrate at both the amino acid conjugate or steroid nucleus (Patel *et al.* 2010). Bile salt hydrolase is particularly abundant in lactic acid fermenting probiotic stains like lactobacilli and bifidobacteria although certain pathogenic strains possess *bsh* homologs in their genomes (Begley *et al.* 2006). From studies which have purified and characterized BSH from different microorganisms, it is generally found that the enzyme is located intracellularly, oxygen insensitive, and optimally active at a slightly acidic pH (Pavlović *et al*. 2012).

As previously stated, the enzymatic function of BSH is to catalyze deconjugation of bile salts, but there are several explanations as to how this benefits the bacteria that produce it. One reason may be that BSH-producing strains derive nutritional advantages in the form of liberated amino acids released from the deconjugation reaction that could be used as carbon, nitrogen, and energy sources (Begley *et al.* 2006). Another suggestion is that BSH facilitates cholesterol or bile incorporation into the bacterial membrane to improve its structural integrity against host immune defenses (Patel *et al.* 2010). The most likely role for BSH activity, however, is that it aids in bile detoxification and gastrointestinal persistence (Kim & Lee 2005). Bile is a natural detergent with antimicrobial activity which is largely aimed at dissolution of bacterial membranes (Begley *et al*. 2005) so naturally GI bacteria must have some mechanism to combat this threat and persist in their natural environment. Bustos *et al.* (2012) investigated the role of BSH positive strains to withstand bile toxicity and proposed that by deconjugating primary bile acids, lactic acid bacteria were able to avoid intracellular acidification associated with

protonated, conjugated bile acids and subsequently avoid collapse of the proton motive force. Furthermore, in agreement with the previous work of Grill *et al.* (2000), it was found that BSH negative strains experienced significant cell death by cytoplasmic acidification after being exposed to both glyco- and tauro-conjugated bile acids whereas the BSH positive strains were only affected by high concentrations of glyco-conjugated bile acids, which are more toxic at acidic pH values. This is not surprising since most BSH producing microbes prefer hydrolyzing glycine rather than taurine conjugates (Pavlović *et al.* 2012); however, some exceptions have been characterized (Hae-Keun *et al.* 2008; Chae *et al.* 2012).

In a metagenomic analysis of BSH activity in the human gut, Jones *et al*. (2008) demonstrate that conjugated bile acid has been the selective pressure driving the gut-associated microbiome toward BSH activity. Interestingly, to further improve their ability to thrive in the harsh conditions of the GI tract by mechanisms just described and perhaps those which are not fully understood, many bacterial strains possess more than one *bsh* homolog in their genomes (Pavlović *et al*. 2012). Research has shown that there are five amino acid sites that are highly conserved in the catalytic site of BSH (Begley *et al.* 2006). Even with such strict conservation, among distinct functional BSHs in the same strain, there is variation in their relative activity and substrate preference (Coleman & Hudson 1995; Hae-Keun *et al.* 2008; Lambert *et al*. 2008; Ren *et al.* 2011; Chae *et al.* 2012). In some cases multiple *bsh* genes from one strain show greater similarity to those from other strains or species than with each other, indicating that horizontal gene transfer may play a role in *bsh* acquisition (Lambert *et al.* 2008). Ren and colleagues (2011) noted that differences in *bsh* gene expression and protein function may be the cause of regulation factors and that substrate specificity could be the result of currently unrecognized binding or catalytic sites.

CHAPTER II
INTRODUCTION

Antibiotic growth promoters (AGP) are defined as a group of antibiotics delivered in animal feed at subtherapeutic levels to increase feed efficiency and average daily weight gain in food animals (Dibner & Richards 2005). For more than fifty years this has been a standard practice of animal husbandry and measurable benefits to animal production are still apparent. Although these benefits were once believed to come at no appreciable risk, epidemiological studies have suggested that antibiotic resistant bacteria are associated with the use of AGP, so what was once regarded as a benign approach to produce robust food animals is now considered a hazard to public health (Marshall & Levy 2011). Indeed, such resistant bacteria can be transferred to humans as a result of direct contact or through more complicated, indirect means (van den Bogaard & Stobberingh 1999). Recognizing that zoonotic reservoirs of drug-resistant bacteria constitute definite concern for animal as well as human safety, the European Union banned all AGP in 2006 and this approach has now become a worldwide trend (Marshall & Levy 2011). The transition to AGP-free production systems was in many regards successful, yet it proved to be challenging for some countries where animal health was compromised as a result (Casewell *et al*. 2003). Completely eradicating AGP will present obstacles on the level of farmer to feed industry; thus, in order to maintain high-quality food animals and current production levels, it is essential that valid alternatives to AGP be discovered which do not pose a threat to public health, are able to promote feed efficiency, and are economically viable.

Understanding how AGP exert growth promotion is critical to developing effective replacements. Although the exact mode of action is unclear, it is commonly accepted that AGP modulate the host intestinal microflora, as their antibacterial nature would suggest, and thereby

generate optimal conditions for animal growth to occur (Marshall & Levy 2011; Dibner & Richards 2005; Barton 2000). Recent studies using swine and poultry have helped us to understand the relationships between AGP supplementation and gastrointestinal bacterial composition (Knarreborg *et al.* 2002; Collier *et al.* 2003; Dumonceaux *et al*. 2006; Wise & Siragusa *et al.* 2007; Rettedal *et al*. 2009; Danzeisen *et al.* 2011; La-ongkhum *et al.* 2011; Kim *et al.* 2012; Lin *et al.* 2013). The results of such studies prove that AGP create bacterial shifts and alter the microbial diversity of the intestine, indicating that certain populations may be more favorable to animal growth than others. Simultaneously, these findings suggest that populations reduced by AGP are potentially harmful to animal performance and could be targeted by non-antibiotic therapy.

Although the definitive gut microbial community required for AGP-mediated optimal growth promotion is still largely unknown, previous studies have shown that the ability of AGP to promote growth is highly correlated with a decrease in activity of bile salt hydrolase (BSH) (Feighner & Dashkevicz 1987; Knarreborg *et al*. 2004; Guban *et al*. 2006; Hunkapillar *et al*. 2009). BSH is an enzyme produced by commensal bacteria in the intestine whose main function is to convert conjugated bile salts into unconjugated bile salts. The exact purpose for which microbes utilize this enzyme is still uncertain, but several theories exist to explain its importance; these include roles in bile detoxification, gastrointestinal persistence, microbial nutrition, and alterations to bacterial membrane characteristics (Begley *et al*. 2006). Regardless of the natural function of BSH for its bacterial producers, the impact of BSH on the host is clear. Unconjugated bile acids are amphipathic and able to solubilize lipids for micelle formation, however, when the amide bond is hydrolyzed by BSH, the resulting unconjugated form is much less efficient at doing so. Thus, BSH effectively transforms bile salts that promote efficient lipid

solubilization into those that adversely affect lipid metabolism and subsequent energy harvest. Therefore, the probable mechanism in which AGP promote growth is to inhibit the activity of BSH that are produced by gut microflora and consequently confer positive conditions for lipid metabolism and utilization. Lactobacilli are the primary inhabitants of the chicken intestine and are primary BSH producers in the gut (Begley *et al.* 2006; Guban *et al.* 2006). Notably, *L. salivarius*, the dominant *Lactobacillus* species present in the chicken intestine, was reduced in response to AGP treatment (Engberg *et al.* 2000; Knarreborg *et al.* 2002; Guban *et al.* 2006; Dumonceaux *et al.* 2006; Zhou *et al.* 2007). Based on these findings, inhibition of BSH activity using specific inhibitors is likely a promising approach to improve growth performance of food animals.

Recently, Wang *et al.* (2012) identified and characterized a *bsh* gene from *L. salivarius* B30514, a BSH producer isolated from chicken intestine (Stern *et al.* 2006). Characterization of this BSH strongly supported our hypothesis for developing BSH inhibitor-based feed additives as alternatives to AGP and established a solid platform for us to discover novel BSH inhibitors. In this project, the following three specific objectives were pursued:

1. Purify recombinant BSH and perform enzymatic characterization.
2. Determine the inhibitory effects of various dietary factors on BSH activity.
3. Develop and perform a rapid, convenient, and high-throughput assay for discovery of BSH inhibitors.

CHAPTER III
MATERIALS AND METHODS

Preparation of lysates for His-tagged BSH purification

The recombinant BSH (rBSH) used in this study was purified from an *E. coli* construct (JL885) expressing a *bsh* gene originally from *L. salivarius* B-30514(Wang et al., 2012). To prepare cell lysate for rBSH purification, the JL885 stock was streaked on a Luria-Bertani (LB) agar plate supplemented with ampicillin (100 µg/ml [microgram/milliliter]). After overnight growth, a single colony was picked and inoculated into 50 ml of LB broth containing 100 µg /ml of ampicillin. The culture was grown in a rotary shaker at 37°C overnight. All of the 50-ml overnight culture was transferred to 1L of LB broth containing 100 µg/ml of ampicillin and grown in a rotary shaker (250 rpm) at 37°C until the optical density at 600 nm ($O.D._{600}$) reached 0.5-0.6 (~2 hr). To induce the production of rBSH, isopropyl-beta-D-thiogalactopyranoside (IPTG) (100 mM) was added to a final concentration of 0.5mM to the remaining early-log phase culture. After 3 hours of induction, the cells were harvested by centrifugation at 5,000 × g at 4°C (Avanti J-26 XP Centrifuge, Beckman Coulter) for 20 minutes, and the pellets were washed with cold phosphate buffered saline (PBS) (pH 7.0). The PBS-washed cell pellets were resuspended in 30 ml of ice cold lysis buffer (50 mM sodium phosphate buffer, 300 mM NaCl, 10 mM imidazole, pH 7.0) supplemented with 2 mM beta-mercaptoethanol (β-ME), 5 mM of adenosine triphosphate (ATP), 5 mM of $MgCl_2$ (magnesium chloride), and 1 mg/ml of lysozyme. The resuspension was transferred into a clean polystyrene tube and put on ice for 1 hour. The cell suspension was disrupted by sonication for 5 cycles (alternating between 30 s on and 60 s off; 50% duty) on ice/NaCl mixture. The lysate underwent centrifugation at 12,000 rpm for 30

minutes at 4°C using a Beckman centrifuge equipped with a JLA rotor. The supernatant (soluble fraction), containing rBSH, was transferred into a clean polystyrene tube for further purification.

Purification of His-tagged rBSH by Ni-NTA affinity chromotagraphy

Approximately 2.5 ml of settled nickel-nitrilotriacetic acid (Ni^{2+} -NTA) agarose resin (Qiagen) was equilibrated with the same lysis buffer used for cell pellet resuspension by washing three times with 5 ml of lysis buffer and centrifugation at 3,000 rpm for 1 minute after each wash. The above bacterial lysate was mixed with the lysis buffer-treated Ni-NTA agarose resin with gentle rocking for 60 minutes at 4°C. The mixture was loaded into a column and the flow through was collected. The column was washed with 5 bed volumes of wash buffer (50 mM sodium phosphate buffer, 300 mM NaCl, 60 mM imidazole, 10% glycerol, pH 7.0) supplemented with 2 mM β-ME, 5 mM of ATP, and 5 mM of $MgCl_2$ with flow through collected. The proteins bound to the Ni-NTA were then eluted from the column with 5 bed volumes of elution buffer (50 mM sodium phosphate buffer, 300 mM NaCl, 300mM imidazole, 10% glycerol, pH 7.0) supplemented with 2 mM β-ME (2 µl/100 ml buffer) and approximately 1 ml of eluent was collected per tube. 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 rBSH-containing fractions were pooled and dialyzed against PBS buffer (50 mM, pH 7.0) containing 10% glycerol. β-ME was then added to a final concentration of 2 mM. The dialyzed rBSH was aliquoted into clean sterile tubes and stored at -80°C prior to use. Protein concentration was measured by a bicinchoninic acid (BCA) protein assay kit (Pierce).

BSH activity assay

Determination of BSH activity was performed by following a two-step procedure (Tanaka *et al*.,2000) to determine the amount of liberated amino acids from glycoconjugated and tauroconjugated bile salts (purchased from Sigma). For the first step, the following components were combined in clean 1.5 ml plastic microcentrifuge tube to a final volume of 200 µl: 178 µl of reaction buffer (0.1 M sodium-phosphate, pH 6.0), 10 µl of purified rBSH (diluted to 1 µg/µl with reaction buffer), 10 µl of a particular conjugated bile salt (100 mM), and 2 µl 1M dithiothreitol (DTT). This mixture was vortexed gently and incubated in a 37°C water bath for 30 minutes. Following incubation, 50 µl of reaction mix was immediately transferred to a clean microcentrifuge tube containing 50 µl of 15% (wt/vol) trichloroacetic acid (TCA) to stop the reaction. After centrifugation at 12,000×g for 5 minutes to remove precipitate, 50 µl of supernatant was transferred to a clean 1.5 ml microcentrifuge tube for the second step. To this, 950 µl of ninhydrin reaction 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]) was added and vortexed to mix thoroughly. The resulting reaction mix was incubated in a boiling water bath for 14 minutes and cooled on ice for 3 minutes to stop the reaction. The absorbance at 570 nm (A_{570}) was measured with a Smart Spec Plus spectrophotometer (Bio-Rad). A control without BSH was set up simultaneously in each independent experiment. A standard curve using glycine or taurine was performed for each independent assay. All experiments were performed in triplicate. The BSH activity was expressed as 1 µmol (micromole) of amino acids liberated from the substrate per minute per mg of BSH.

Effect of pH and temperature on the activity of rBSH

To determine the temperature dependence of rBSH activity, the assay was carried out as described above in 0.1 M sodium phosphate buffer (pH 6.0) at various temperatures ranging from 20 to 75°C. Likewise, the optimal pH for rBSH activity was determined by performing the assay at pH values ranging from 3.0 to 8.0 using 0.1 M sodium citrate-citrate acid buffer (pH 3.0 to 5.4) or 0.1 M sodium phosphate buffer (pH 6.0 to 8.0). Both pH and temperature assays were performed using the standard procedure with glycocholic acid as the substrate. All experiments were run in triplicate.

Effect of dietary compounds on the activity of BSH

A panel of dietary compounds, many of which have been used as feed additives for food animals, was used in a BSH activity assay at a final concentration of 5 mM to determine if they were able to inhibit enzyme activity. The following compounds were tested: $CuCl_2$ (copper chloride), $ZnCl_2$ (zinc chloride), $MnCl_2$ (manganese chloride), $FeCl_3$ (ferric chloride), $CaCl_2$ (calcium chloride), $MgCl_2$ (magnesium chloride), $CoCl_2$ (cobalt chloride), KCl (potassium chloride), NaCl (sodium chloride), KI (potassium iodide), NaI (sodium iodide), Na_2SeO_3 (sodium selenite), $CuSO_4$ (cupric sulfate), $FeSO_4$ (ferrous sulfate), $MnSO_4$ (manganese sulfate), $ZnSO_4$ (zinc sulfate), $NaSO_4$ (sodium sulfate), $MgSO_4$ (magnesium sulfate), KIO_3 (potassium iodate), $NaIO_3$ (sodium iodate), $NaIO_4$ (sodium periodate), $NaHCO_3$ (sodium bicarbonate), citric acid, ascorbic acid (vitamin C), cholecalciferol (vitamin D_3), α-tocopherol (vitamin E), retinol (vitamin A), stearic acid, linolenic acid, linoleic acid, sodium acetate, sodium propionate, sodium lactate, and sodium butyrate. We also tested the effect of several different switchgrass extracts on BSH activity. Switchgrass (*Panicum virgatum*) is considered to be a potential feedstock used

to bioprocess liquid fuels like ethanol and is suggested as such under the UT Biofuels Initiative (Garland, UT Extension 2010). Since cellulosic biomass is used directly for biofuel production, up to 20% of the extractives are left over, which could potentially be used in other applications to add value to the product as a whole. The switchgrass extracts, which were previously dried and prepared using pressurized liquid extraction with water or 95% ethanol, were kindly provided by Dr. Niki Labbé of the UT Center for Renewable Carbon for use in our enzymatic assays.

Prior to the addition of sodium glycocholate to the reaction mix, the rBSH was incubated with or without a particular compound for 30 minutes in a 37°C water bath. Subsequently, the standard enzyme assay was performed as described above. A control with no added compound was set up in each independent assay. All assays were run in triplicate. The percentage of inhibition was calculated by dividing the inhibited activity (calculated by subtracting the mean residual activity in the presence of a compound from the mean activity of the control) relative to the mean activity of the control and then multiplied by 100.

Development of a high-throughput BSH assay

In order to move towards creating a rapid method of screening for BSH inhibitors, our standard BSH activity assay was adapted for use in a 96-well plate, similar to a procedure described by Tanaka *et al*. (2000) but with modifications. This is a precipitation assay which is based on the fact that hydrolysis of conjugated bile acid substrates will produce deconjugated bile acids which are insoluble at the reaction pH and can easily be visualized as a white precipitate; this feature provides a great advantage to develop a rapid and convenient HTS system to identify potent BSH inhibitors. For initial assays, rBSH (10 µl [1µg/1µl]) was added to the bottom of a clear 96-well microtiter plate with a round bottom. To this, 190 µl of reaction

mix (178 µl of reaction buffer [0.1 M sodium-phosphate, pH 6.0], 10 µl of a particular conjugated bile salt [100 mM], and 2 µl 1M DTT) was added for a total reaction volume of 200 µl. Plates were incubated at 37°C for up to 6 hours. Precipitation of insoluble unconjugated bile salts was monitored every 30 minutes by visual observation concomitant to absorbance measurement at 600 nm (A_{600}) using a microplate reader (model Multiskan EX; Thermo Fisher Scientific, Vantaa, Finland). For subsequent assays, different parameters were altered to determine the optimal assay conditions. BSH was added in 10 µl amounts diluted from stock concentration (8 µg/µl) from 2 to 300-fold; pH values of 6.0 or 6.5 were tested using 0.1 M sodium phosphate buffer (pH 6.0) or 0.05 M sodium phosphate buffer (pH 6.5); incubation was tested at room temperature (20°C), 37°C, or 40°C; glyco- and tauro-conjugated substrates were tested at final concentrations ranging from 0 mM to 50 mM.

After the assay conditions were optimized for the 200 µl reaction in a 96-well plate, we confirmed the ability of this procedure to screen compounds for BSH inhibition by a proof of concept assay using known BSH inhibitors. Before adding the reaction mix, 10 µl of known inhibitor was added to the BSH in the bottom of each well and mixed gently by pipetting. Inhibitors were added at an initial 10 mM concentration and subjected to twofold serial dilutions up to 9 times through column 10 of the plate. As a control, two columns on each plate served as controls: a positive control with BSH and reaction mix only and a negative control with no added BSH or inhibitor. Assays were performed in duplicate. After all conditions were optimized, including use of inhibitors, the entire reaction was scaled down to a 50 µl total volume and modified accordingly for a future preliminary HTS library screen at Vanderbilt University. An additional assay was performed using dimethyl sulfoxide (DMSO), the library

compound solvent, as a control to ensure that it did not negatively affect the ability of BSH to effectively hydrolyze substrate.

High-throughput screening of BSH inhibitors

High-throughput screening (HTS) of BSH inhibitors was performed at the HTS facility at the Vanderbilt Institute of Chemical Biology (Nashville, TN). The library (2,240 compounds in 7 source plates from Spectrum) includes biologically active and structurally diverse compounds of known drugs, experimental bioactives, and pure natural products. All compounds tested were dissolved in DMSO at a concentration of 10 mM. Briefly, 0.25 µl of a specific compound from the Spectrum Collection library was shot into the bottom of a clear 384-well microplate (Greiner cat # 781182) with flat bottom using the Echo 550/555 (Labcyte). Next, 12.5 µl of BSH (diluted 50x from a stock concentration of 8 µg/µl) was added using the Multidrop Combi reagent dispenser (Thermo Scientific) and shaken for 5 minutes. Finally, 37.5 µl of reaction mix (32 µl reaction buffer [0.1 M sodium-phosphate, pH 6.0], 2.5 µl of taurodeoxycholic acid [200 mM], and 0.5 µl of 1M DTT) was added using the Multidrop Combi for a total reaction volume of 50 µl. The plates were subsequently shaken for 5 minutes to insure thorough mixing and spun to pull any reaction mixture back into the bottom of the well before incubation. Plates were incubated at 37°C with humidity and 5% CO_2 and absorbance measured every hour at 600 nm (A_{600}) for 4 hours using a SpectraMax M5 (Molecular Devices) with temperature controlled at 37°C; visual observations were concomitantly documented for precipitation. The reader on the system is connected via intranet to the facility's network, enabling automated data acquisition, analysis, visualization, and archival using a powerful management and chemiinformatics tool, Accelrys Pipeline Pilot; all data are stored in an Oracle database. Each plate contained three

controls manually added with a multichannel pipette in a predetermined pattern to the side wells. Control 1 consisted of BSH, DMSO, and reaction mix; control 2 contained a known inhibitor, BSH, and reaction mix; control 3 contained DMSO and reaction mix only. Validation plates were also tested using known inhibitors prior to HTS of the compound libraries to ensure reproducibility of the assay in a new setting.

Selection and *in vitro* validation of identified BSH inhibitors.

A preliminary list of BSH inhibitor hits was generated after the above HTS based on the dynamics and magnitude of precipitation formation in each well (reflected by OD_{600}). Extensive review of relevant material safety data sheet (**MSDS**) and literature were performed for the hits with emphasis on availability, stability, toxicity, cost, and environmental impact. Notably, the hit compounds belonging to an antimicrobial family were eliminated and will not be considered for further evaluation because of the well-known food safety issue related to antimicrobial growth promoters.

The selected BSH inhibitor candidates were subjected to further *in vitro* validation using the 96-well microplate assay and the standard 2-step BSH assay as described above. The standard BSH assay is essential to confirm if the identified compounds are indeed real BSH inhibitors because we cannot completely rule out a possibility that certain compounds may cause less or no precipitation in reaction mix in a BSH activity-independent manner.

CHAPTER IV
RESULTS

Expression and purification of rBSH from *E. coli*

To obtain sufficient rBSH for enzymatic characterization and screening of BSH inhibitors for this project, large quantities of rBSH were produced and purified. As early as one hour after IPTG induction, a significant amount of rBSH was produced, reflected by the presence of additional protein band with a molecular mass of 37 kDa on SDS-PAGE (Fig. 1, lane 3). Using 1-step Ni-NTA affinity chromatography, large quantities of high purity C-terminal His-tagged rBSH were obtained, as seen in different eluent fractions (Fig. 2, lanes 6-10). Fractions displaying the highest BSH quantities (e.g lanes 6-8 in Fig. 2) were pooled together for dialysis against PBS. From 1 liter of induced culture, the purification procedure yielded approximately 15 mg of rBSH.

Substrate preference of rBSH

Using the standard BSH activity assay, six different bile salts were tested to determine the substrate specificity (Table 1). Overall, the rBSH displayed a broad substrate affinity and was able to hydrolyze both the glycine and taurine conjugates. The highest hydrolysis activity occurred when glycochenodeoxycholic acid (defined as 100% activity) was used as the substrate. However, in examining the activities given other substrates, there was no clear preference for the other glycoconjugated bile salts compared to the three tauroconjugated bile salts tested. The relative activities of the remaining bile salts ranged from 22.3% to 47.9% (Table 1). Furthermore, among the different substrates, the BSH showed no obvious preference among conjugated cholic, deoxycholic, or chenodeoxycholic acids.

Effect of pH and temperature on rBSH activity

The effect of pH on rBSH activity was determined by conducting the standard activity assay at different pH values ranging from 3.0 to 8.0 using either 0.1 M sodium citrate-citrate acid buffer or 0.1 M sodium phosphate buffer (Fig. 3). Optimal activity was observed between pH values of 5.0 and 6.0, with maximal activity occurring at a pH of 5.4. Moderate activity was still evident at pH limits of 4.4 and 6.4, but as pH declined or increased beyond these measures, BSH activity greatly declined and was almost negligible at a pH of 8.0. We also tested BSH activity at temperatures ranging from 25°C to 75°C and found that peak activity occurs at 41°C (Fig. 4). Activity was stable from 35°C to 55°C but experienced a dramatic drop when the temperature exceeded 55°C. Following the 30 minute incubation, at our lowest temperature tested (22°C), BSH lost approximately 52% of its activity, whereas, at the upper extreme of 75°C the activity had diminished by nearly 90% of original activity.

Identification of dietary compounds inhibitory to the activity of rBSH.

Various compounds that are used as dietary supplements in animal feeds were selected for determination of their ability to inhibit BSH activity. As shown in Table 2, it is clear that several compounds, such as $CuCl_2$, $CuSO_4$, $FeSO_4$, $CoCl_2$, $NaSeO_3$, $NaIO_4$, KIO_3, retinol, and linolenic acid, were potent inhibitors, having greater than 90% inhibition. In contrast, some compounds tested actually improved the catalytic activity of the rBSH, such as citric acid (Table 2). In addition, several switchgrass extracts (*Panicum virgatum*) representing collection at different growth stages and location were screened. All samples tested, which included both ethanol and water extracted components, enhanced BSH activity by 10.5 to 35.1% when tested at a 50-fold dilution (data not shown).

Optimization of a HTS system to determine BSH inhibitors

To determine optimal conditions for a HTS system, the standard BSH activity procedure was performed in a 96-well plate. pH, temperature, substrate concentration, and incubation length were altered in separate assays for assessment. As expected, a pH of 6.0 and an incubation temperature of 37°C, both of which are used in the standard 2-step assay, were conducive to efficient BSH hydrolysis. However, regarding substrate choice, it was found that taurodeoxycholic acid rather than glycocholic acid was much better at eliciting an observable activity response; additionally, a final substrate concentration of 10 mM produced ideal results. An incubation length of 2 to 4 hours was targeted in order to get several progressive measurements during the development of precipitation; using 10 µl of undiluted BSH (8 µg/µl) caused the reaction to proceed too quickly so it was diluted by factors of 5 up to 300. It was determined that a 50-fold dilution (10 µl) was sufficient to elicit adequate hydrolysis of substrate with maximal precipitation occurring after approximately 2 hours of incubation in a 200 µl total reaction volume. Changes were easily able to be observed visually, as insoluble deconjugates precipitated and results were able to be corroborated by additional measurements using a microplate reader. The control wells without the addition of BSH further confirmed our assay validity by remaining clear and consistently producing only a background spectrophotometric measurement.

With the above parameters optimized, a proof-of-concept experiment using known BSH inhibitors was performed. As shown in Fig. 5, in the presence of high levels of specific potent BSH inhibitor (e.g. A1-A5 & B1-B5 for $NaIO_4$, E1-E4 & F1-F4 for $NaIO_3$), the activity of BSH was inhibited, which was reflected by displaying clear, transparent wells. In the presence of low concentrations of the BSH inhibitor and in the absence of the BSH inhibitor (right section except

the far right negative control wells, Fig. 5), the reaction mix became turbid due to the precipitation of unconjugated bile salts due to BSH activity. The other two iodide compounds, NaI and KI, displayed lower inhibitory effect than $NaIO_4$ and $NaIO_3$ (Fig. 5), which is consistent with the results using quantitative 2-step assay (Table 2). Although the copper and zinc compounds were potent inhibitors at higher concentrations in our 2-step assay (Table 2), they tended to form aggregates at high concentrations after prolonged incubation in the plate assay; furthermore, they were less inhibitive at lower concentrations than were the iodate compounds (Fig. 6, A-D rows). Similarly, Na_2SeO_3 aggregated even at a starting concentration of 10 mM and 5 mM (Fig. 6). After several stepwise dilutions this effect was diminished and Na_2SeO_3 still clearly inhibited BSH activity at low concentrations, but the aggregation potential negates its usefulness as a control compound (Fig. 6). KIO_3 appears to be an ideal control inhibitor for HTS due to its ability to inhibit BSH activity at low concentrations and consistently show a dose dependent response with high clarity (Fig. 6). After modifying the dilution factor, it was subsequently concluded that a starting compound (KIO_3) concentration of 10 mM (10 µl per well) was adequate to inhibit activity and 2-fold dilutions in nine successive wells were used to show a dose dependent response.

The assay was further scaled down the assay to a 50 µl total reaction volume, which is required for HTS, using 384-well plates. A prolonged incubation length (3-4 hours) was needed for precipitation to fully develop. Additionally, an experiment was done to insure that DMSO, the solvent used for HTS library compounds, was itself not inhibitory to BSH. It was found that there was negligible interference of DMSO (final concentration of 50 mM) on precipitation formation (data not shown).

HTS discovery of BSH inhibitors

At the HTS facility at the Vanderbilt Institute of Chemical Biology, all assays were performed in a scaled down 50 µl total reaction volume for use in a 384-well plate, the format of which their compound libraries are stored and routinely used for other HTS assays. One day prior to screening of the compound libraries, validation assays containing different controls were carried out to ensure the system was optimal for actual inhibitor screening and that the optimized conditions translated well to this HTS format. Several plate layouts were designed and included in selected controls were either KIO_3 or $NaIO_4$, inhibitors routinely used in the 96-well assays, and DMSO. The results of these test plates indicated that the assay could clearly distinguish between wells with or without precipitation as a result of BSH hydrolysis. Both negative controls (no BSH) and those using $NaIO_4$ and KIO_3 remained clear in stark contrast to positive controls (no inhibitor) that displayed obvious precipitation (also confirmed by reading on the Spectramax). The simple checkerboard layout of one such validation plate (Fig. 7), shows that the assay could clearly distinguish between wells with or without precipitation as a result of BSH hydrolysis and indicate that DMSO has no negative impact of the ability of BSH to hydrolyze a bile acid substrate. Thus, we were able to undergo a pilot screening of a 2,240-compound library composed of structurally and functionally diverse compounds. It should be noted that for the actual HTS screen, BSH was diluted with buffer containing 3 mM of DTT to prevent oxidation of BSH enzyme during a prolonged incubation period between setup of assays; this modification improved enzyme stability and visible hydrolysis. A total of 7 plates were used for this screening, which was very successful and has led to 107 hits that were considered potent inhibitors. Hits were confirmed based on visual observation (Fig. 8) and corroborated via spectrophotometric measurement.

Preliminary review of biochemical information of the corresponding hits eliminated most of compounds and led to a 10-compound list (Table 3). These 10 compounds were subjected to further validation using the plate assay as well as the 2-step activity assay to ascertain the quantitative inhibitory effect; these included phenethyl caffeate (also called 'caffeic acid phenethyl ester', CAPE), carnosic acid, chrysophanol, epicatechin monogallate, gossypetin, purpurogallin, riboflavin, theaflavanin, folic acid, and menadione. The ten compounds selected for further testing were purchased directly from Vanderbilt, which have been solubilized in DMSO; compounds were stored at -20°C after arrival. Ninety-six-well plate assays were first conducted to test repeatability of results from the original HTS screen. Each compound was tested at an initial concentration of 10mM. Although most compounds displayed inhibitory activity even after the first dilution, several did not display inhibitory effect as expected (folic acid, chrysophanol, CAPE, epicatechin monogallate, and theaflavanin), particularly since the library compounds were initially screened using 0.25 µl (10mM) rather than the 2.5 µl (10mM) we routinely use in our laboratory. Plate assays were repeated to confirm these findings, and it was consistently noted that chrysophanol and folic acid had no inhibitory effect on BSH and CAPE, epicatechin monogallate, and theaflavanin appeared only to inhibit at the highest concentrations tested (final concentrations of 0.5, 0.25 mM). Compounds showing greater inhibition included carnosic acid, menadione, gossypetin, and riboflavin (Fig. 9).

In addition to the above precipitation-based microplate assay, each compound was subsequently tested in the 2-step assay to determine a quantitative inhibitive response when used at a higher concentration; $CuCl_2$ was included as a known inhibitor control, as demonstrated in the preliminary screen of dietary feed additives. It was exciting to note that several of the compounds, despite a lower capacity to offer visual proof of inhibition using the plate method,

actually were able to inhibit BSH activity at relatively high percentages (Table 4). Notably, CAPE and epicatechin monogallate consistently inhibited activity by over 90%, indicating they are potent BSH inhibitors. Riboflavin, gossypetin, menadione, and carnosic acid, also displayed high ranges of inhibition, consistent with the plate assay. Several of these compounds of particular interest were also tested at higher dilutions. CAPE still inhibited rBSH activity by more than 85% at a final concentration of 0.625 mM; carnosic acid and riboflavin each inhibited at rates over 90% at a final concentration of 0.625 mM and 0.05 mM, respectively. However, chrysophanol was very poor at inhibiting BSH hydrolysis and folic acid actually enhanced activity. This indicates that while the HTS plate method is mostly accurate at revealing genuine BSH inhibitors, it is best to clarify these results using the quantitative BSH assay.

CHAPTER V
DISCUSSION

Antibiotic growth promoters (AGP) have undoubtedly had a positive influence on animal production since their early facilitation in the 1950s; however, the current concern over antibiotic resistance of zoonotic origin is the driving impetus for their discontinuation. Different products have been proposed as alternatives for AGP, including essential oils, prebiotics, probiotics, enzymes, and organic acids, among others, to change gut microbiota for enhanced animal health and growth performance (Huyghebaert *et al.* 2011); however, very limited data is available to justify the choice of specific bacterial species or products for such microbiota manipulation.

Exogenous enzymes have garnered much attention as one alternative to AGP. Generally, these target non-starch polysaccharides like hemicellulose and β[beta]-glucans and have been noted to improve performance via enhanced diet digestibility and may also beneficially manipulate host microflora and immunity (Bedford 2000; Huyghebaert *et al.* 2011). Although some recent studies do suggest improvements in poultry and swine health and performance (Jo *et al.* 2012; Zou *et al*. 2013), there are other studies which note no significant effect of enzyme treatments and even negative impacts on performance (Yegani & Korver 2013; Karimi *et al*. 2013). This lack of consistency is a notable drawback for dietary enzyme inclusion, and although it may improve nutrient digestibility, results will be highly variable depending on enzyme source, dietary form and quality, as well as dosage, intestinal uptake and availability, and animal age and breed. Furthermore, many performance studies do not address how exogenous enzymes affect the intestinal microflora; improved digestion rates because of enzyme supplementation undoubtedly reduce the available substrate available for gastrointestinal bacteria, which could target harmful bacteria, but also negatively impact beneficial populations.

Probiotics, the live bacterial organisms, have also been extensively evaluated as potential AGP alternatives, particularly for poultry, and improvements in performance, gut mucosal immunity, and nutrient digestibility have been noted (Mountzouris *et al.*, 2010). However, introducing live microorganisms into the gut environment could potentially have unforeseen consequences. Indeed, AGP generally target intestinal populations of gram-positive bacteria, including lactobacilli which have been commonly used as probiotic strains (Barton 2000; Gaskins e*t al*. 2002; Sharifi *et al*., 2012); thus, there seems to be some disparity in the ultimate efficacy increased numbers may have on gastrointestinal health. Certainly many considerations must be weighed, and administration level and frequency could be a point of contention. For example, Mountzouris *et al.* (2010) tested the growth-promoting efficacy of a 5-bacterial strain probiotic and concluded that treatment at the lowest inclusion level tested resulted in the best performance, while higher levels actually lowered ileal digestibility coefficients. Additionally, bird age and diet likely will pose challenges in developing probiotic strategies. In a study conducted to evaluate differences between flavomycin and probiotics on dietary fat utilization, Sharifi *et al.* (2012) observed that "probiotics caused significant growth-depressing effects, inferior feed conversion, and reduced fat and GE [gross energy] digestibility irrespective of the source of dietary fat." They proposed that increased lactobacilli numbers greatly enhanced the deconjugation of bile salts caused by BSH activity, thereby reducing the ability to digest fat; furthermore, the young age of the birds meant they had limited pancreatic lipase secretion which rendered the effects of the probiotics disadvantageous. Therefore, the overall beneficial effects associated with specific probiotics should be carefully evaluated. Understanding the science of potential negative traits of probiotics can help us develop 'negative-traits-mitigation' strategies

to optimize probiotic products for enhanced growth performance of food animals and profitability of the feed additive industry.

Various studies link AGP induced growth promotion with altered gastrointestinal (GI) microflora, but data are limited to provide compelling justification for which bacterial species should be modulated (Marshall & Levy 2011; Dibner & Richards 2005; Barton 2000). Perhaps, then, it would be feasible to approach this issue at the cellular level by targeting general enzymatic activities and metabolite production influenced by AGP usage. One such avenue worth pursuing is examination of bile salt hydrolase (BSH). In 1987, Feighner and Dashkevicz observed an inverse relationship between BSH activity and growth performance in poultry supplemented with subtherapeutic doses of antibiotics; they further suggested that specific enzyme inhibitors could enhance growth and feed conversion in livestock and negate the need for antibiotic feed additives. Although other studies have since corroborated these findings and agree that AGP usage decreases gut BSH activity (Guban *et al*. 2006; Knarreborg *et al*. 2004), there is a lack of published information regarding relevant attempts to identify BSH inhibitors. Alternatively, there is mounting evidence that AGP use greatly influences intestinal populations of *Lactobacillus*, prime BSH-producing commensals, by reduction of overall numbers or modulation of strain abundance (Knarreborg *et al.* 2002; Dumonceaux *et al*. 2006; Rettedal *et al*. 2009); specifically, AGP use in broilers is associated with decreased populations of *L. salivarius* (Engberg *et al.* 2000; Knarreborg *et al.* 2002; Guban *et al.* 2006; Dumonceaux *et al.* 2006; Zhou *et al.* 2007). Furthermore, Guban *et al*. (2006) proposed that because of its capacity for deconjugating bile salts and promoting decreased weight gain, *L. salivarius* would be a prime target to eliminate from the broiler intestine. Building off of these published findings, in this study, a recombinant BSH (rBSH) from a chicken *L. salivarius* strain (Wang *et al.* 2012) was

used to perform preliminary enzyme characterization and initiate the discovery of BSH inhibitors via a standard quantitative assay as well as an efficient high-throughput screening (HTS) procedure.

Biochemical characterization of the rBSH from *L. salivarius* NRRL B-30514 revealed unique functional qualities. Six different bile salts were tested to determine substrate preference. Although the enzyme showed the highest activity for glycochenodeoxycholic acid, the relative activities of the other glycine and taurine-conjugated bile salts were similar when compared to one another. Additionally, there was no major difference in activity against cholic, deoxycholic, or chenodeoxycholic acids. Bile salt hydrolase enzymes produced by commensal bacteria tend to either have a broad or narrow substrate range and most often they have higher efficiencies at hydrolyzing glycoconjugated bile salts (Begley *et al.* 2006). The rBSH used in this study displayed a broad substrate range and was able to efficiently hydrolyze both glyco- and tauroconjugated bile salts. Its maximal activity occurred at a pH of 5.4, falling under the slightly acidic pH optima to which most BSH enzymes conform (Pavlović *et al.* 2012). Interestingly, BSHs from different strains of the same species can have surprisingly different biochemical preferences. Specifically, two BSH1 enzymes from *L. salivarius* UCC118 and JCM1046 had variable specific activities on a range of glycine and taurine conjugated bile salts (Fang *et al.* 2009). Although the $rBSH_{NRRL\ B\text{-}30514}$ in this study effectively hydrolyzed a wide range of substrates, $Bsh1_{UCC118}$ preferred glycoconjugated bile acids and showed limited activity for tauroconjugated bile acids whereas $Bsh1_{JCM1046}$ had higher catalytic activities when the substrate was tauroconjugated. Both BSH1 enzymes had broad pH optima, but maximum activity occurred at a pH of 5.5 or 6.5 for $Bsh1_{JCM1046}$ and $Bsh1_{UCC118}$, respectively. These differences in substrate specificity and other hydrolysis kinetics may be linked to the internal deletion of 8

amino acid residues of $Bsh1_{UCC118}$, considered to be in the conserved active site (Fang *et al.* 2009). Thus, our understanding of differences in phenotypic functionality of BSH enzymes from *L. salivarius*, or from any species in general, would likely benefit from future structural and comparative sequence analyses that may reveal key residues in the active site or other binding sites which may help to explain biochemical preferences.

As shown in this study, the rBSH of *L. salivarius* was able to efficiently hydrolyze a broad range of substrates. This particular enzyme trait makes it an ideal candidate when screening for inhibitors, since any potential inhibitor would have to target a wide range of BSH enzymes, each with different substrate preferences and hydrolyzing capabilities. From our initial screen using different feed additives and dietary trace minerals, we discovered several inhibitors with moderate or potent inhibition. Of particular interest among these are the copper and zinc compounds. Research has shown that adding high concentrations of dietary copper and zinc can improve growth performance and feed efficiency of poultry (Ewing *et al*. 1998; Miles *et al*. 1998; Arias & Koutsos 2006; Liu *et al*. 2011) and swine (Smith *et al*. 1997; Armstrong *et al*. 2004; Jacela *et al.* 2010; Shelton *et al.* 2011); in addition, supplementary zinc can improve the carcass traits and meat quality of broilers (Liu *et al.* 2011). As of now, there is a lack of compelling scientific evidence used to explain a specific mechanism of action for how copper and zinc act as growth promoters, although it is loosely assumed to arise from their antimicrobial properties (Jacela *et al.* 2010). However, there is an additive effect of adding high concentrations of copper along with AGP in the diet (Jacela *et al.* 2010), which suggests that there are other mechanisms at work outside of its antimicrobial effect. Potentially the reason that copper and zinc can mimic the effects of AGP when given in high doses is that they are acting as intestinal BSH inhibitors and improving lipid digestion through maintenance of the pool of

conjugated bile salts. While it is tempting to assume that high concentrations of copper and zinc would make adequate AGP replacements, there are several concerns about high levels of these metals being used long-term in animal feed. The obvious problem is potential copper/zinc toxicosis due to prolonged exposure; likely the threshold for effective metabolism and absorption would depend on animal type and age as well as the dietary source. Additionally, higher nutrient composition in the feed will be reflected in an increased amount excreted in the feces. This can have undesirable repercussions on the environment, particularly for soil where manure with accumulated levels of copper and zinc are applied (Jacela *et al.* 2010), and it has been suggested that plants could develop toxicities if bulk quantities of manure were applied to a limited land area for an extended duration (Singh 2005). Furthermore, metals that are frequently added in animal feed may co-select for antibiotic resistant bacteria, as Cavaco *et al*. (2011) observed that zinc resistance of zoonotic derived *Staphylococcus aureus* has a strong association with methicillin resistance. For these reasons, careful consideration should be given when choosing novel inhibitors, such that negative impact to both animal and environment is minimized.

Besides copper and zinc, various other compounds in the preliminary screen also exhibited a large inhibitory effect on rBSH hydrolysis. Retinol, a fat-soluble vitamin important for growth, vision, and immunity, was found to be a potent inhibitor, but it can potentially accumulate to toxic levels in many animal species so prolonged supplementation would not be a good option (National Academic Press, 1987). A panel of iodate compounds also showed great inhibition (Table 2), but again, these warrant cautious justification for use as AGP alternatives. It is possible that high dietary concentrations of iodine may have multiple host effects, some of which may be undesirable. As constituents of thyroid hormones, prolonged administration of iodate compounds at high levels could increase basal metabolic rate and consequently lead to

weight loss, negating the effect of inhibiting BSH activity. One additive with potential from the preliminary screen is linolenic acid, which inhibited rBSH activity by approximately 90%. Linolenic acid (or α-linolenic acid) is one of several omega-3 polyunsaturated fatty acids which, collectively, are precursors of immune and inflammation mediators and play important roles in regulating membrane fluidity and in disease prevention and intervention (Connor 2000). From a human perspective, supplementing chickens with α-linolenic acid may benefit poultry consumers since it can enhance omega-3 long chain polyunsaturated fatty acid levels in chicken tissues (Kartikasari et al. 2012; Haug et al. 2012). In addition, linolenic acid can affect bone strength as Tarleton *et al.* (2013) found that supplementing free-range hens with α-linolenic acid improved skeletal health and reduced keel fractures. However, this evidence needs corroboration since a previous, similar study found no effect of omega-3 supplementation on bone health as well as increased mortality compared to birds in a control group (Toscano *et al.* 2012). Moreover, although there is little published information regarding solely how linolenic acid affects growth performance, it has been shown that feeding diets rich in fish oil omega-3 polyunsaturated fatty acids improves body weight gain in poultry (Geier *et al*. 2009). Research regarding other omega-3 supplementation may be warranted.

The preliminary screen also identified several compounds which actually enhance BSH activity. While these are not necessarily of interest relative to this study, compounds that improve the ability of BSH to hydrolyze conjugated bile salts could have potential human applications. The capacity to hydrolyze bile salts has frequently been a criterion for probiotic strain selection because of the potential to lower blood cholesterol levels (Begley *et al.* 2006). As previously stated, deconjugated bile salts are less soluble and consequently less likely to be reabsorbed. This lowers cholesterol solubility and absorption, and excretion in the feces will

necessitate an increased demand for cholesterol for *de novo* synthesis of more bile salts. Additionally, conjugated bile salts help expedite lipid metabolism and act as signaling molecules to regulate endocrine function and glucose homeostasis (Jones *et al*. 2008; Pavlović *et al.* 2012), so BSH has the potential to significantly impact host physiology and thereby reduce the risk of metabolic disorders such as diabetes and obesity. The BSH enhancing compounds, therefore, could be used as a novel food additive to increase the activity of BSH in the intestine and facilitate health improvements in persons who are hypercholesterolemic or overweight.

Because of the potentially confounding effects associated with several of the inhibitors discovered in the preliminary screen of dietary factors, the best way to identify powerful, safe, and cost-effective BSH inhibitors should involve large scale screening of diverse compounds including emerging feed additives. An understanding of what contributes to successful enzyme inhibition, for example, competitive binding to the active site or allosteric hindrance, may lead to the utilization of computational techniques to identify these. However, homology modeling and molecular docking would both require an accessible three-dimensional structure of important BSH enzymes, and many of these are currently unavailable, including one for the enzyme in this study. Therefore, high-throughput screening (HTS) such as that used in drug discovery is currently the most promising avenue to pursue regarding BSH inhibitor identification. Additionally, HTS is beneficial because automating the BSH assay can greatly improve time efficiency and save money due to a lesser quantity of reagents needed. For example, scaling the reaction down from a total volume of 200 µl to 50 µl reduces reagent use by 75%, and although the precipitation-based method requires four hours of incubation, setup and preparation times are comparable to the 2-step method. The end result is that thousands of compounds are screened

simultaneously compared to only a handful; it would take weeks, if not months, to achieve these results using the standard assay, which relies on manual pipetting vs. a bulk reagent dispenser.

Capitalizing on this idea, we successfully developed and validated such a HTS system in this project. Although conjugated bile salts are soluble in aqueous solutions, upon hydrolysis via BSH, the resulting deconjugated version is insoluble and leads to significant precipitation that is evident to the naked eye. This unique feature allowed us to modify our quantitative 2-step screen for a scaled down version that was amenable for use in multiple settings. Specifically, the convenience of this precipitation-based screen allowed us to travel to the nearest HTS center to the University of Tennessee which is located at Vanderbilt University's Institute of Chemical Biology. There we were able to test out this new system by screening a diverse compound library for novel BSH inhibitors.

Overall, the ability of the HTS system to detect rBSH inhibitors proved to be a reliable means of inhibitor detection. The concept adapted well to Vanderbilt's 384-well plate format in which their libraries are routinely screened for multiple purposes, and the opportunity to utilize some of their advanced instrumentation, namely the Echo 550/555, contributed greatly to the convenience and rapidity with which this assay could be carried out. There are some minor complications that need to be addressed to improve efficacy of future screens. Notably, not all of our featured compounds reacted as expected once validation assays were attempted. Chrysophanol and folic acid, both recognized as inhibitors in the library screen, subsequently only inhibited activity to a very small extent or even improved hydrolysis of substrate, respectively. It is possible that these were false positives since we only had the time and resources to do one all-encompassing screen. A second re-screen, including only a subset of compounds of interest, would have been beneficial in confirming our suspected compounds as

legitimate inhibitors. Another possibility is that binding affinity of chrysophanol and folic acid to BSH may be lower than that of other compounds, and the extended shaking step at Vanderbilt could have improved the compound-enzyme interaction, whereas we rely only on manual pipetting to mix the two in our 96-well plate version; the additional large surface area to volume ratio of the wells of the 384-well plate could have contributed in this sense as well.

Finally, it must be resolved why some of the compounds were positive HTS hits with further validation by standard 2-step BSH assay but produce confounding results when using the 96-well plate assay in our laboratory. For example, the cloudy appearance of wells including CAPE and epicatechin monogallate belied their potency as tested in the 2-step assay. Since this effect was clearly not noted at Vanderbilt during the initial library screen, likely the solution stability or chemical properties may have been altered in transit or as a result of the freeze/thaw process which affected the way it behaved during the reaction. Organic compounds dissolved in DMSO can dissolute or precipitate out after repeated freeze/thaw cycles or water uptake (Oldenburg *et al.* 2005). Should CAPE, for example, have experienced partial dissolution, upon introduction into the aqueous buffer a precipitate may have formed which would be confused as precipitation as a result of bile salt deconjugation. Although it may be a far-reaching conjecture, large-scale screening could be amenable to the discovery of compounds that behave in this fashion and create downstream confusion. More consideration should be given to these and other possibilities that would ultimately lead to contradictions in assay results interpretation.

The Spectrum Collection library that was screened contained a wide range of structurally diverse compounds, including drug components, natural products, and other bioactive constituents. Although the library did contain known antibiotics which are obviously not of interest to us, the fact that many of them did inhibit rBSH activity is in support of our belief that

AGP contribute to decreased BSH activity and improved animal growth performance. We further investigated and validated ten promising hits (Table 3). Out of these ten compounds, several were strong inhibitors that possess intriguing beneficial qualities. Carnosic acid and epicatechin monogallate are both nutraceuticals with noted antioxidant and neuroprotective effects (Terrao *et al.* 1994; Azad *et al*. 2011; Kelsey *et al.* 2010; Morán *et al.* 2012; Xiang *et al*. 2012). Additionally, carnosic acid is an anti-inflammatory (Kuo *et al.* 2011) and epicatechin monogallate displays some chemopreventive (Du *et al*. 2012) and antimicrobial properties (Hamilton-Miller & Shah 2000). Similarly, gossypetin was among the powerful inhibitors and also is noted to be both anti-inflammatory (Trendafilova *et al*. 2011) and an antioxidant (Mounnissamy *et al.* 2002). Each of these compounds warrants further animal research, and should availability permit, growth performance studies could reveal more as to their potential use. However, two particular compounds stood out as potent inhibitors with definite novel potential as feed additives that could replace AGP: riboflavin and phenethyl caffeate (CAPE). Riboflavin, or vitamin B2, plays a key role in energy metabolism and is a conenzyme in numerous redox reactions (Combs 2012). Aside from contributions to animal physiology, it is water-soluble and causes no known toxicities from supplementation at upper limits (National Academic Press 1987), although even at the lowest concentrations tested, riboflavin was still an extremely potent BSH inhibitor. Because it is an FDA-approved feed additive with well-established metabolic function and would be readily available to incorporate into feed, riboflavin likely may be an acceptable candidate to improve growth performance for food animals. CAPE, on the other hand, is an emerging plant bioactive studied because it has antioxidant (Altuğ *et al.* 2008; Lee (b) *et al.* 2008; Dos Santos & Monte-Alto-Costa 2013), anticarcinogenic (Chung *et al.* 2004; Chan *et al.* 2012;), anti-inflammatory (da Cunha *et al.* 2004; Yilmaz *et al.* 2005; Dos

Santos & Monte-Alto-Costa 2013), immunomodulatory (Chan *et al.* 2012;), and antimicrobial (Yilmaz *et al.* 2005) effects. It is a phenolic component of propolis and can be directly extracted or artificially synthesized by several methods (Akyol *et al.* 2012). Although much research has been done to characterize the aforementioned effects of CAPE, no published data exist concerning its effects on growth performance of food animals. Because CAPE can inhibit BSH at low concentrations (0.625 mM), a trial of this natural product as a feed supplement would certainly be highly warranted.

This initial 2,240 compound screen was undoubtedly a step in the right direction towards identifying AGP replacements. The HTS protocol may have some limitations, but nevertheless proved that it could successfully identify compounds that were potent BSH inhibitors. Future chemical screens will be performed on libraries of over 50,000 small molecule candidates which may reveal more novel BSH inhibitors. Having a greater number of applicable compounds will allow us to choose those with the most redeemable qualities that would include animal and environmental safety, cost, and availability. A comparison of BSH inhibitors (riboflavin and CAPE) versus AGP will be useful for assessing performance improvements relative to the cost of supplementation. Likely it will be challenging to compete with the low price of AGP supplementation. One study reported that the cost of AGP ranged from $1.25 to $3.00 per ton of feed, and for chickens specifically the cost of AGP per bird was $0.0093 (Graham *et al*. 2007). However, despite that low price, the authors noted that the improved production performance was still not enough to offset the cost of AGP use. Potent BSH inhibitors, therefore, may be able to compensate for price via larger improvements to animal health and performance. Based on the *in vitro* data, it is estimated that riboflavin and CAPE could be delivered in the feed at 10 mg/kg and 20 mg/kg, respectively, to exert efficient inhibition of BSH activity. This is

comparable to AGP dosages that range from one up to 100 mg/kg in the feed depending on compound and animal type (van den Bogaard & Stobberingh 1999). Particularly, riboflavin, a very potent BSH inhibitor, is a widely available product and if purchased in bulk, may come closer to approaching the cost of AGP supplementation. Future HTS screens could also reveal BSH inhibitors that are broadly recognized compounds that would be especially cost effective. Furthermore, consumers may be willing to pay slightly more for products coming from animals reared without the use of AGP, which is stigmatized in today's market.

The long term goal of this study is, of course, to undertake a large-scale animal study using poultry as our model to test some of these novel inhibitors, including riboflavin and CAPE. While *in vitro* studies are absolutely useful for gathering preliminary data, intestinal *in vivo* conditions are certainly more complex. Particularly, to be an effective inhibitor of intracellular BSH enzymes, our chemical compounds would need to traverse the cell envelope effectively by a means comparable to many clinical antibiotics. Additionally, *in vivo* stability and bioavailability must be addressed in order to establish the validity and practicality of utilizing such inhibitors as a legitimate alternative to AGP. Moreover, growth performance parameters like body weight gain, feed intake, and feed conversion ratio will need to be measured as well as morphological characteristics of the intestinal tract and meat and carcass quality. This will help to rule out or identify any negative physiological consequences associated with prolonged use of a particular BSH inhibitor. For example, because the BSH inhibitors should improve lipid metabolism, it is important to determine that energy harvest and weight gain is partitioned adequately and not skewed toward excess fat deposition, which would be undesirable for consumers. If potent inhibitors are identified which do result in improved performance and carcass characteristics, it may also be beneficial to do an additional study assessing the sensory

evaluation of meat to ensure that there is no negative effect on meat flavor, odor, or tenderness. It is hoped that subsequent studies will be able to address the above concerns and corroborate the performance-boosting benefits of novel BSH inhibitors.

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APPENDIX

Table 1. Activity of rBSH for different bile salts.

Substrate[a]	BSH activity (μmol/min/mg)	Relative activity (%)
GCDCA	17.7 ± 1.18	100
GDCA	4.0 ± 0.54	22.3
GCA	7.7 ± 0.48	41.9
TCDCA	8.0 ± 2.37	45.1
TDCA	8.5 ± 2.26	47.9
TCA	5.6 ± 0.33	31.4

[a] The following bile salts were used to determine the hydrolysis activity of rBSH: glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), and taurocholic acid (TCA)

Table 2. Effect of different compounds on rBSH activity.[a]

Compound	% Inhibition
$CuCl_2$	98.1
KIO_3	96.5
$NaIO_4$	96.4
$FeSO_4$	96.1
$CoCl_2$	95.9
Na_2SeO_3	93.1
Retinol	93.1
$CuSO_4$	91.7
Linolenic acid	90.1
$ZnSO_4$	89.5
$NaIO_3$	86.4
Linoleic acid	84.7
$MnSO_4$	83.1
$CaCl_2$	22.4
$FeCl_3$	73.0
$ZnCl_2$	68.3
$MnCl_2$	68.1
KI	36.8
Cholecalciferol	36.0
Sodium acetate	32.7
NaI	31.8
Sodium proprionate	31.4
$MgSO_4$	31.3
Sodium butyrate	29.0
$NaSO_4$	27.7
NaCl	27.7
KCl	25.9
$MgCl_2$	25.7
CaCl2	22.4
Ascorbic acid	21.8
$NaHCO_3$	20.6
Sodium lactate	15.4
α-tocopherol	-1.0
Stearic acid	-9.4
Citric acid	-38.9

[a] The final concentration of all compounds in the reaction mix is 5 mM.

Table 3. The ten promising hits of BSH inhibitors discovered from HTS using a small compound library (2,240 compounds from Spectrum).

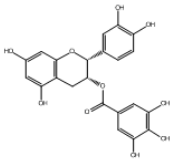
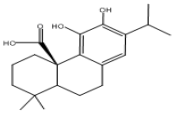
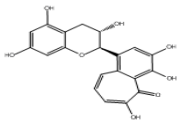
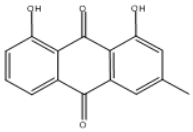
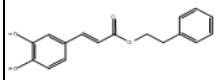
Compound Name	Structure	Formula (MW)	Source	Features	References
Epicatechin monogallate		$C_{22}H_{18}O_{10}$ (442.38)	Tea pigment	antioxidant; chemopreventive;	Du *et al.* 2012; Hamilton-Miller *et al.* 2000; Terao *et al.* 1994
Carnosic acid		$C_{20}H_{28}O_4$ (332.44)	Salvia spp, *Rosmarinus officinalis*	Potent antioxidant; anti-inflammatory; neuroprotective	Morán *et al.* 2012; Xiang *et al.* 2012; Azad *et al.* 2011; Kuo *et al*. 2011
Theaflavanin		$C_{20}H_{16}O_8$ (384.34)	Semi-synthetic analog of theaflavin	Anti-inflammatory; antiviral and antibacterial	Zu *et al.* 2012; Betts *et al.* 2011; Aneja *et al.* 2011
Chrysophanol		$C_{15}H_{10}O_4$ (254.24)	Cassia and Rumex spp.	Anti-inflammatory; anticarcinogenic; anti-diabetic properties	Lee *et al.* 2011; Kim *et al.* 2010; Lee *et al.* 2008
Phenethyl caffeate (CAPE)		$C_{17}H_{16}O_4$ (284.31)	Natural component of propolis from honeybee hives	Antioxidant; anticarcinogenic; anti-inflammatory; immuno-modulatory; antimicrobial	Chan *et al.* 2012; Altuğ *et al.* 2008; Lee(b) *et al.* 2008; Yilmaz *et al.* 2005; Chung *et al.* 2004; da Cunha *et al.* 2004

Table 3. Continued

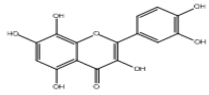
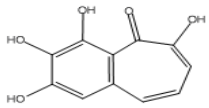
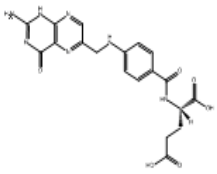
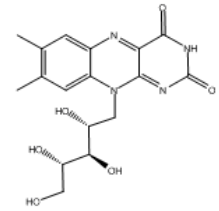
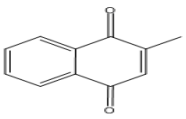
Compound Name	Structure	Formula (MW)	Source	Features	References
Gossypetin		$C_{15}H_{10}O_8$ (318.24)	Widespread in plants	Antioxidant; anti-inflammatory	Trendafilova *et al.* 2011; Mounnissamy *et al.* 2002
Purpurogallin		$C_{11}H_8O_5$ (220.18)	Gall of *Dryophanta divisa*	Antioxidant; anti-inflammatory; hepatoprotective; cardioprotective; antibacterial activity	Kim (b) *et al.* 2012; Inamori *et al.* 1997; Wu *et al.* 1996; Prasad *et al.* 1994; Wu *et al.* 1994; Wu *et al.* 1991
Folic acid		$C_{19}H_{19}N_7O_6$ (441.40)	Liver, kidney, green plants and fungi	Vitamin used for nucleotide biosynthesis; upper limits considered nontoxic; no environmental risks associated with use in animal nutrition	EFSA 2012; National Academic Press 1987
Riboflavin		$C_{17}H_{20}N_4O_6$ (376.37)	Retina, whey and urine	Vitamin that has a role in energy metabolism; coenzyme in numerous redox reactions; upper limits considered nontoxic	Combs & Gerald 2012; National Academic Press 1987
Menadione		$C_{11}H_8O_2$ (172.18)	Asplenium and Juglans spp.	Vitamin important in process of blood clotting and calcium metabolism; used as a nutritional supplement in chicken and turkey feed; can be added as high as 1000 times the dietary requirement without resulting in adverse effects	FDA 2012; Pillai *et al.* 2008

Table 4. Validation of the selected BSH inhibitors determined by HTS.[*]

Compound	% Inhibition
CAPE	96.4
Carnosic Acid	96.8
Chrysophanol[a]	3.7
Epicatechin monogallate	98.6
Folic Acid[b]	-10.8
Gossypetin	97.3
Menadione	87.2
Purpurogallin	83.0
Riboflavin[c]	94.5
Theaflavanin	18.1

[*] Unless specified, the final concentration of compound in the reaction mix was 5mM.
[a]The final concentration of chrysophanol in reaction mix was 1.25 mM.
[b]The final concentration of folic acid in reaction mix was 1.5 mM.
[c] The final concentration of riboflavin in reaction mix was 1 mM.

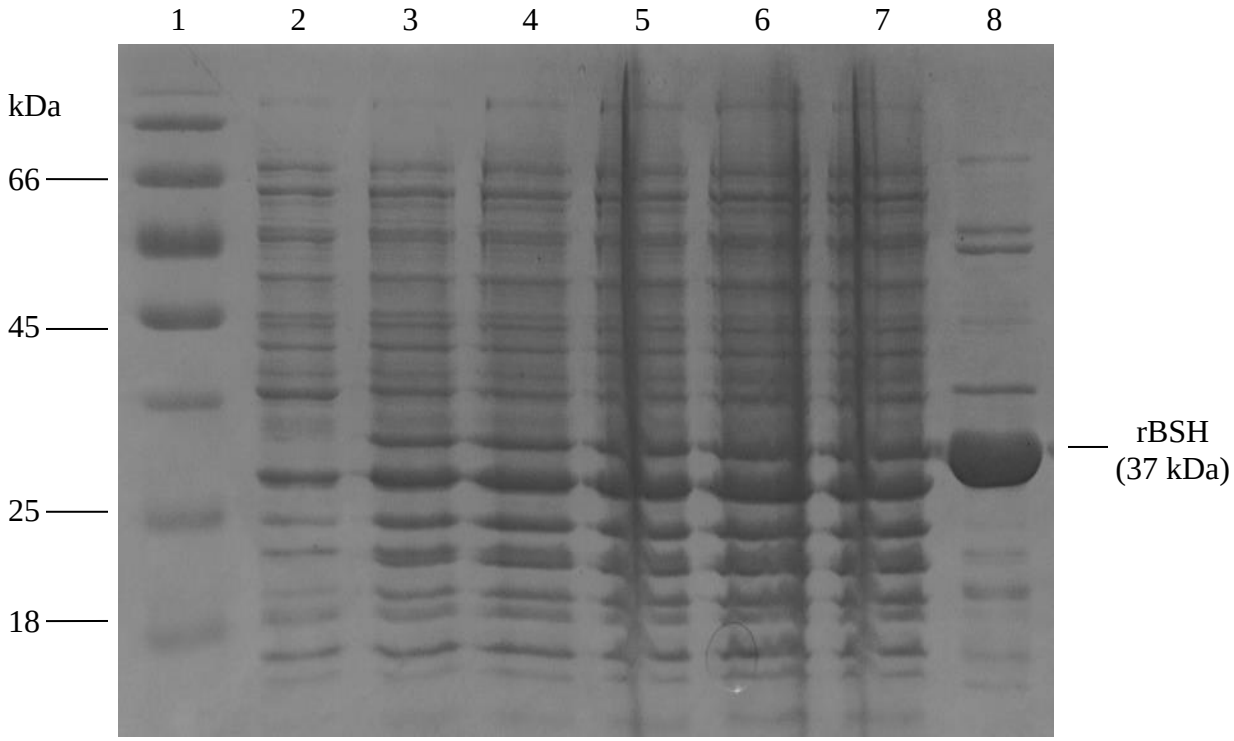


Figure 1. Production and purification of rBSH (SDS-PAGE analysis). Lane 1 is a prestained molecular marker. Lane 2 is whole-cell lysate of noninduced *E. coli*, followed by whole-cell lysate of *E. coli* induced with 0.5 mM IPTG after 1 hr (Lane 3), 1.5 hrs (Lane 4), 2 hrs (Lane 5), 2.5 hrs (Lane 6), and 3 hrs (Lane 7). Lane 8 is rBSH purified by Ni-NTA affinity chromatography.

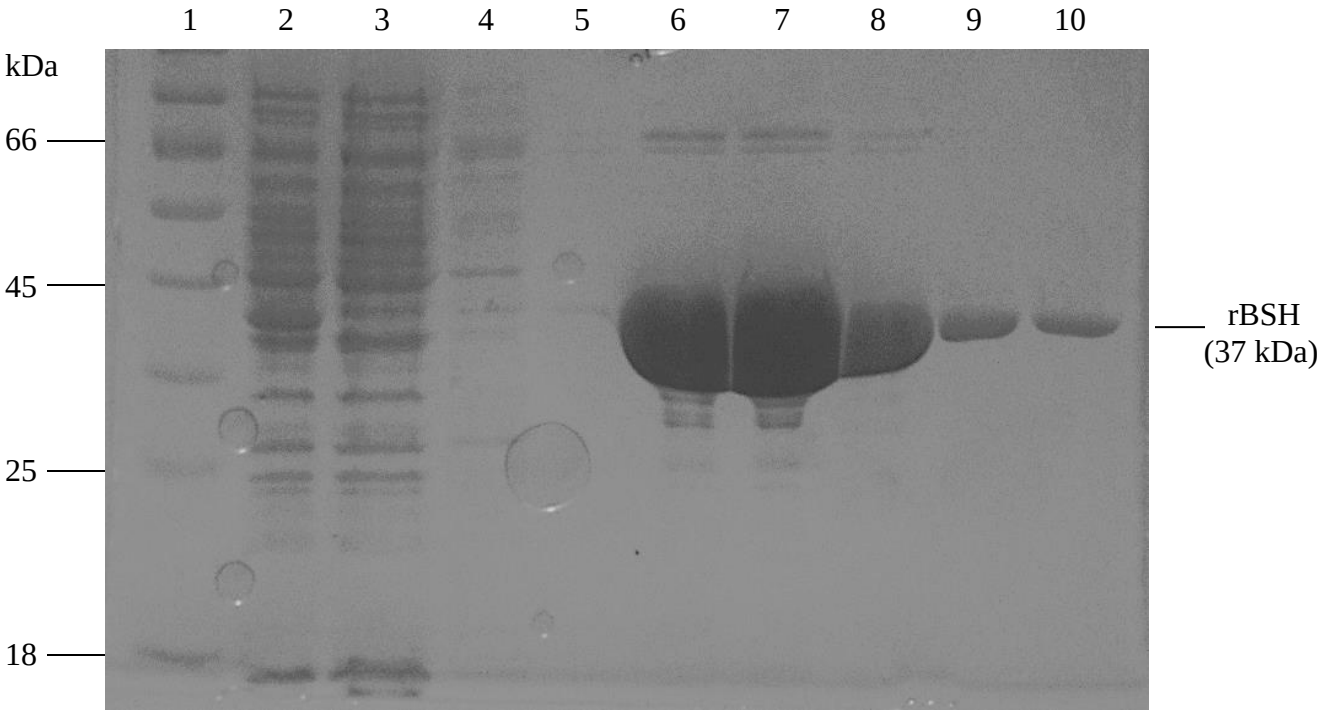


Figure 2. SDS-PAGE analysis of stepwise purification of the rBSH. Lane 1, a prestained molecular marker; lane 2, induced whole-cell lysate of *E. coli*; lane 3, flow-through fraction; lane 4, wash fraction. Lanes 5-10 indicate eluents 1 through 6, respectively.

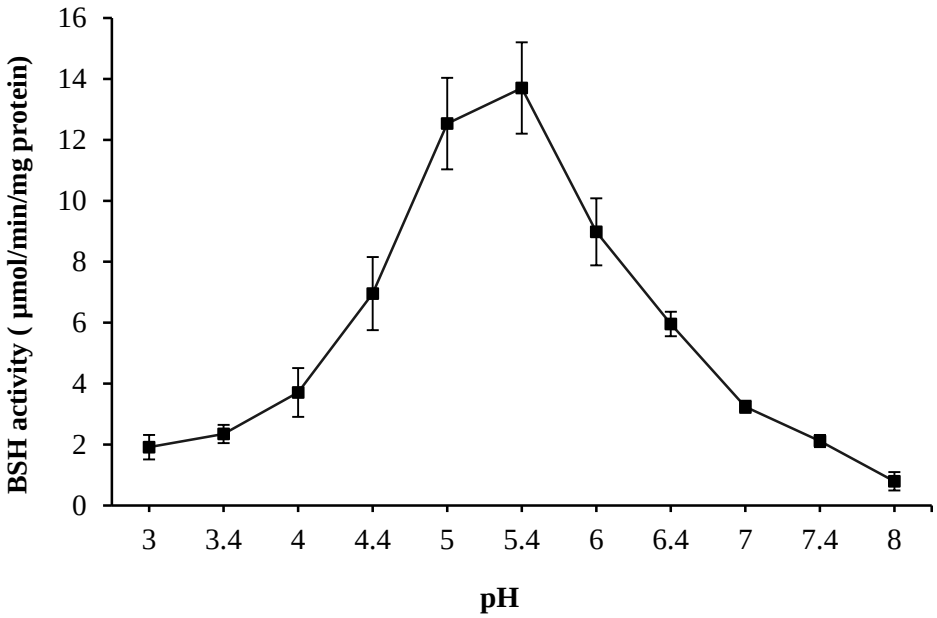


Figure 3. The effect of pH on rBSH activity. rBSH activity was measured in 0.1 M sodium citrate-citrate acid buffer for pH values ranging from 3.0 to 5.4 and in 0.1 M sodium phosphate buffer for pH values ranging from 6.0 to 8.0. Each bar represents the mean BSH activity ± standard deviation at each pH tested.

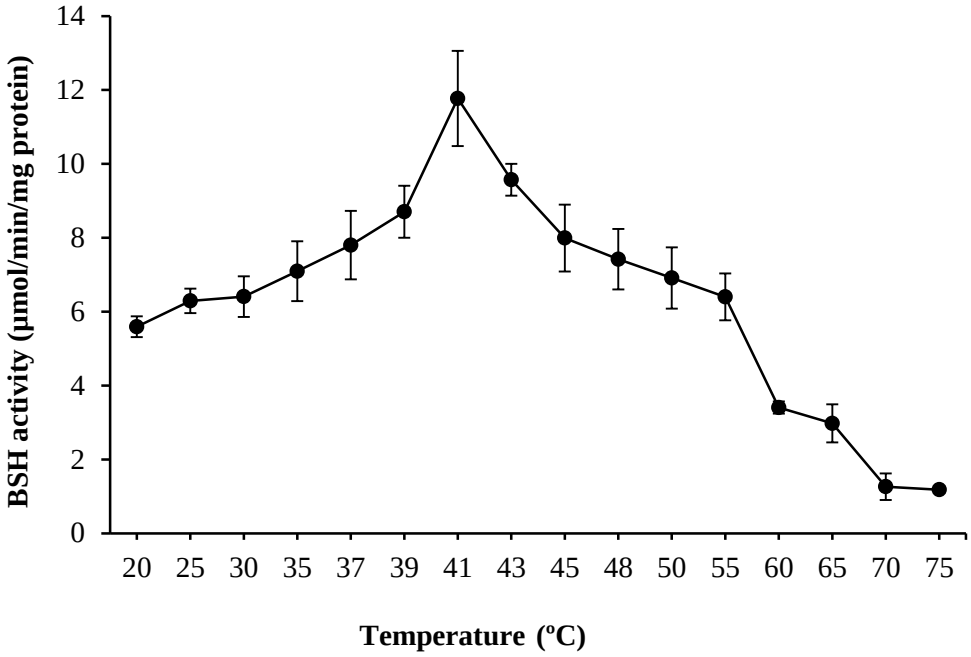


Figure 4. The effect of temperature on rBSH activity. rBSH activity was measured over a range of temperatures (20°C-75°C) in 0.1 M sodium phosphate buffer (pH 6.0). Each bar represents the mean BSH activity ± standard deviation of each temperature.

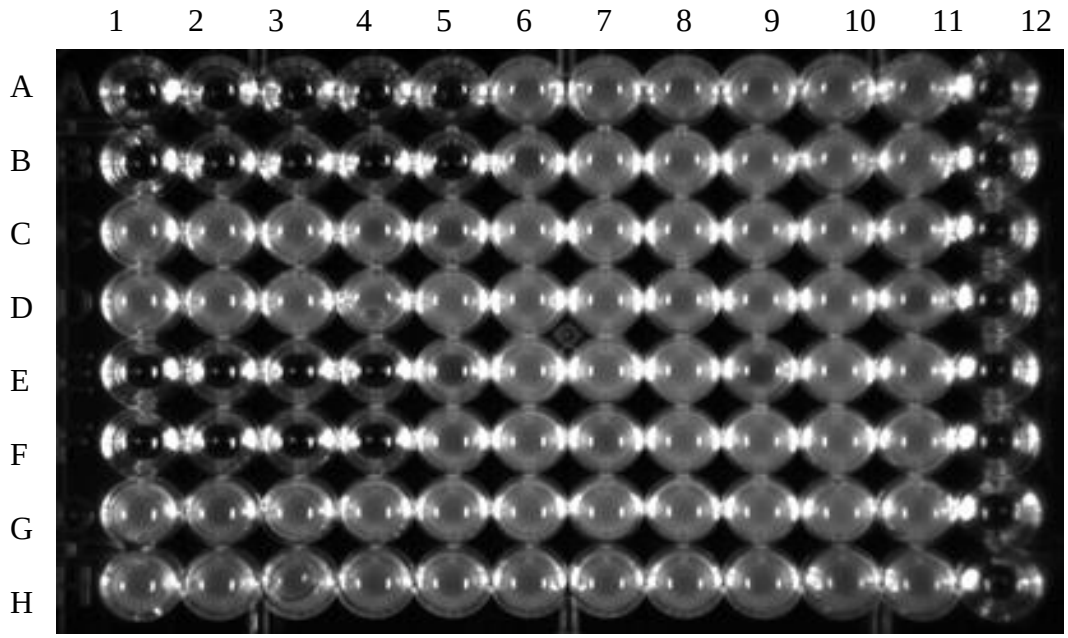


Figure 5. Validation of microplate method for screening BSH inhibitors using HTS. A panel of iodine compounds were used to validate the microplate precipitation-based assay using a 200 µl of total reaction volume. All compounds were added at a final concentration of 0.5 mM in column 1 with 2-fold serial dilution through column 10. Rows A and B: $NaIO_4$; C and D: NaI; E and F: $NaIO_3$; G and H: KI. A 12.5 µl volume of BSH (50-fold diluted from a stock concentration of 8 µg/µl) was added in column wells 1-11. Column 12 served as a negative control with no enzyme and compound added.

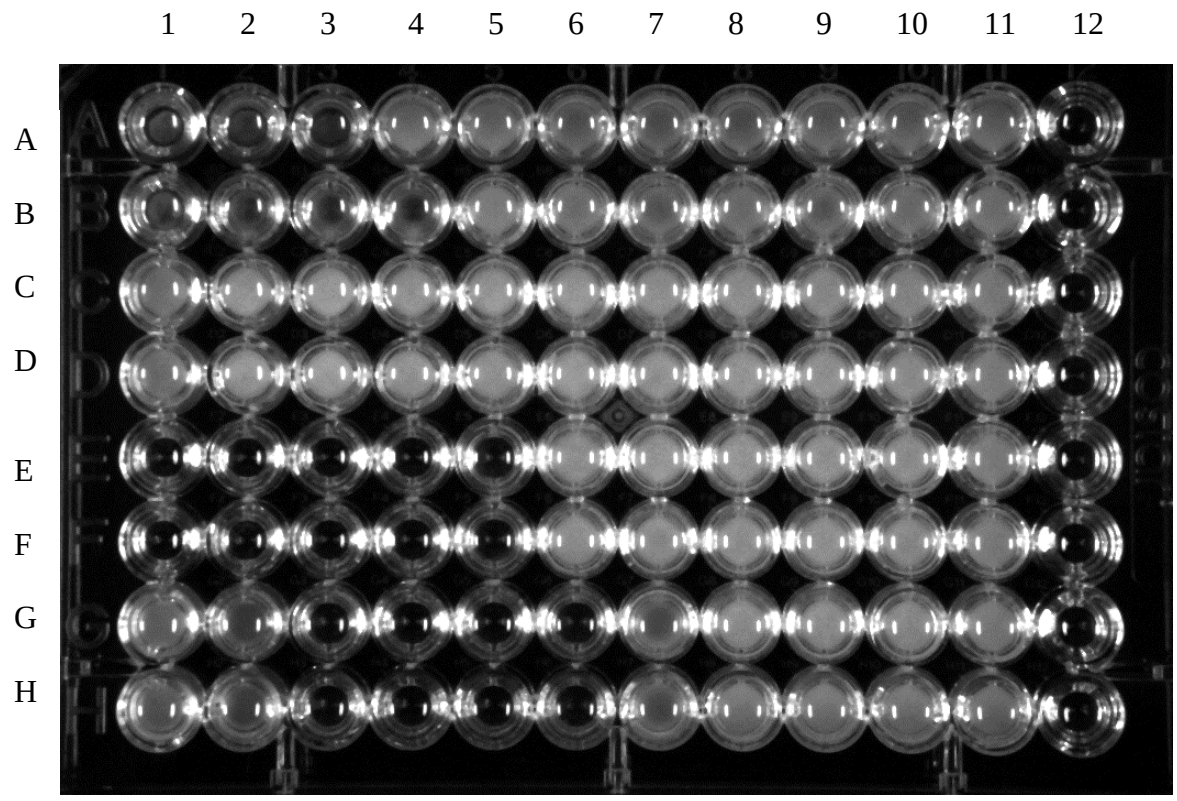
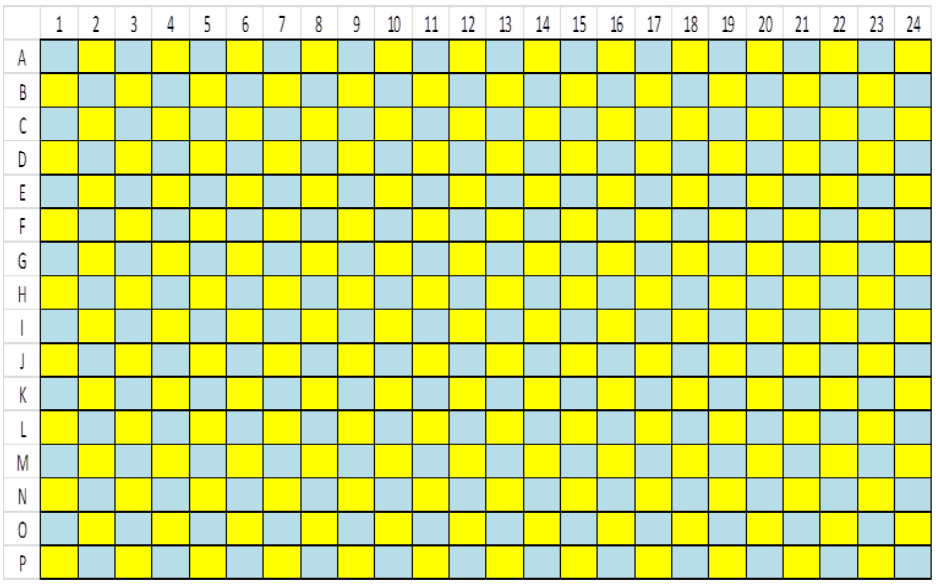


Figure 6. Microplate precipitation-based assay for four dietary compounds that display potent inhibitory effect on BSH using the standard BSH assay. Using 200 µl of total reaction volume, all compounds were added at a final concentration of 0.5 mM in column 1 with 2-fold serial dilution through column 10. Rows A and B: $CuCl_2$; C and D: $ZnSO_4$; E and F: KIO_3; G and H: Na_2SeO_3. A 12.5 µl volume of BSH (50-fold diluted from a stock concentration of 8 µg/µl) was added in column wells 1-11. Column 12 served as a negative control with no enzyme and compound added.

A



B

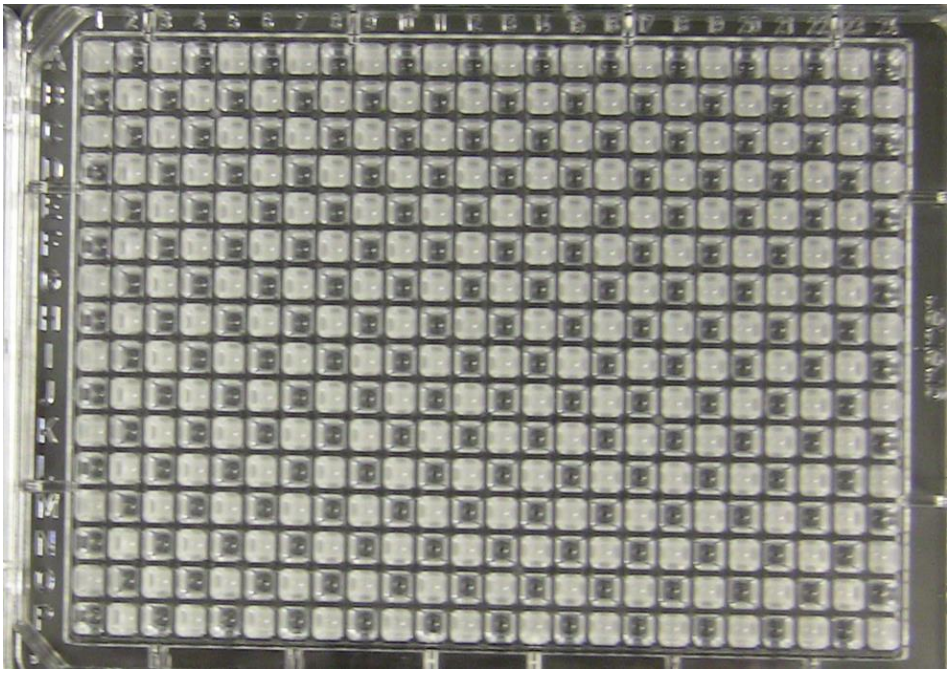


Figure 7. Validation of HTS protocol for screening BSH inhibitors. (A) Design of validation plate: blue boxes are activity controls (BSH, reaction mix containing substrate, and solvent DMSO) and yellow boxes are inhibition controls (BSH, reaction mix containing substrate, and $NaIO_3$). (B) Results of the validation assay indicate that DMSO had no adverse effects on BSH activity (cloudy wells with precipitate) when compared to the wells containing a known inhibitor $NaIO_3$ (clear wells).

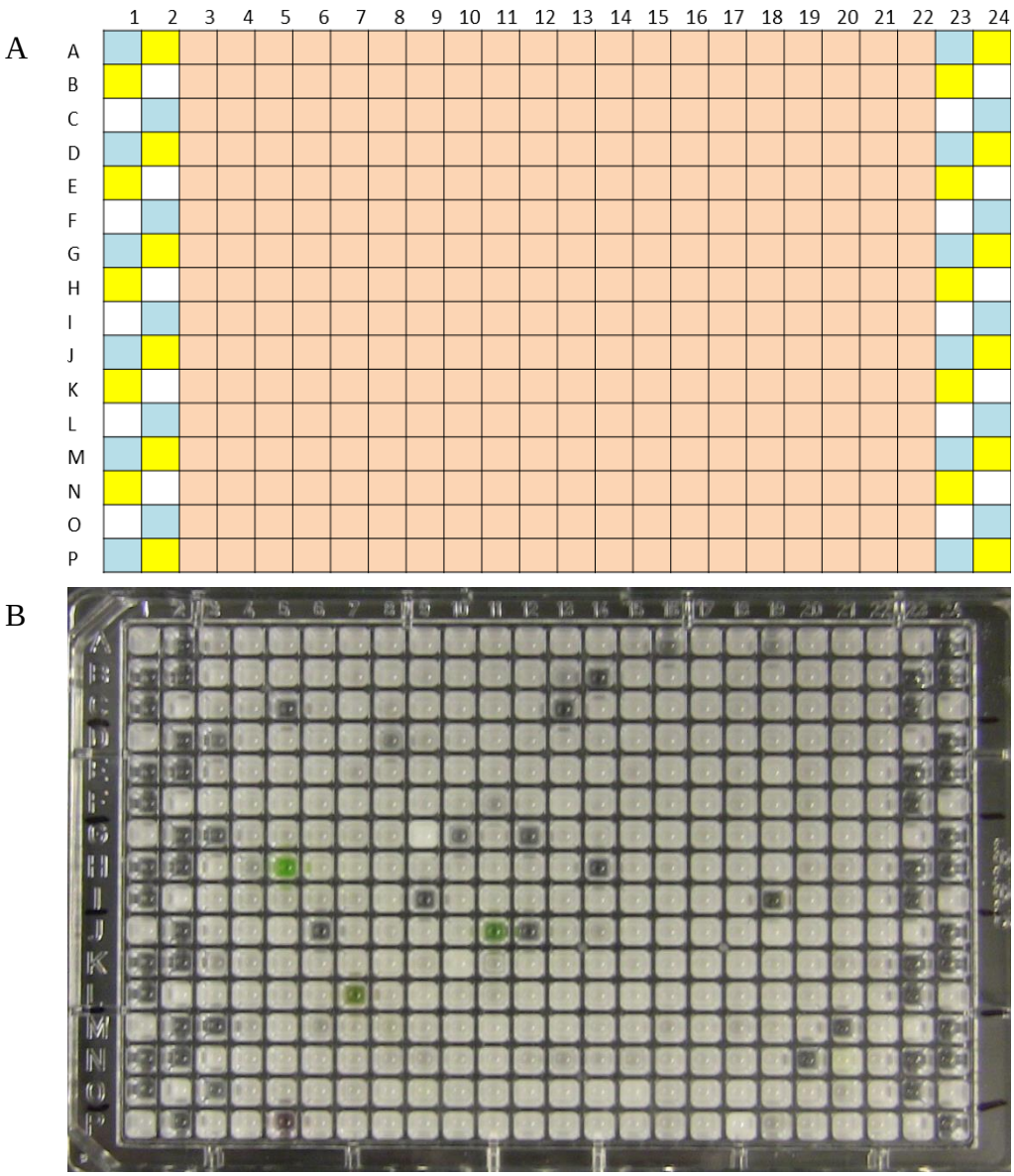


Figure 8. Representative result of one plate from HTS of BSH inhibitors. (A) Plate layout for HTS inhibitor screening. Pink boxes indicate test wells that contain a library compound of interest, BSH, and reaction mix containing substrate. Library compounds were shot into the well bottom using Echo 550/555 and enzyme and reaction mix were added using Multidrop Combi. Controls were added manually to the side wells: blue boxes indicate activity controls (BSH, reaction mix containing substrate, and solvent DMSO), yellow boxes correspond to inhibition controls (BSH, reaction mix containing substrate, and $NaIO_3$), and white boxes are negative controls with no BSH added but include reaction mix with substrate and DMSO. (B) The HTS results represented by one 384-well plate. Control wells indicate the assay proceeded normally. The wells in columns 3-22 that appeared clear, regardless of alternative color due to compound, and had low absorbance readings were considered hits (putative BSH inhibitors).

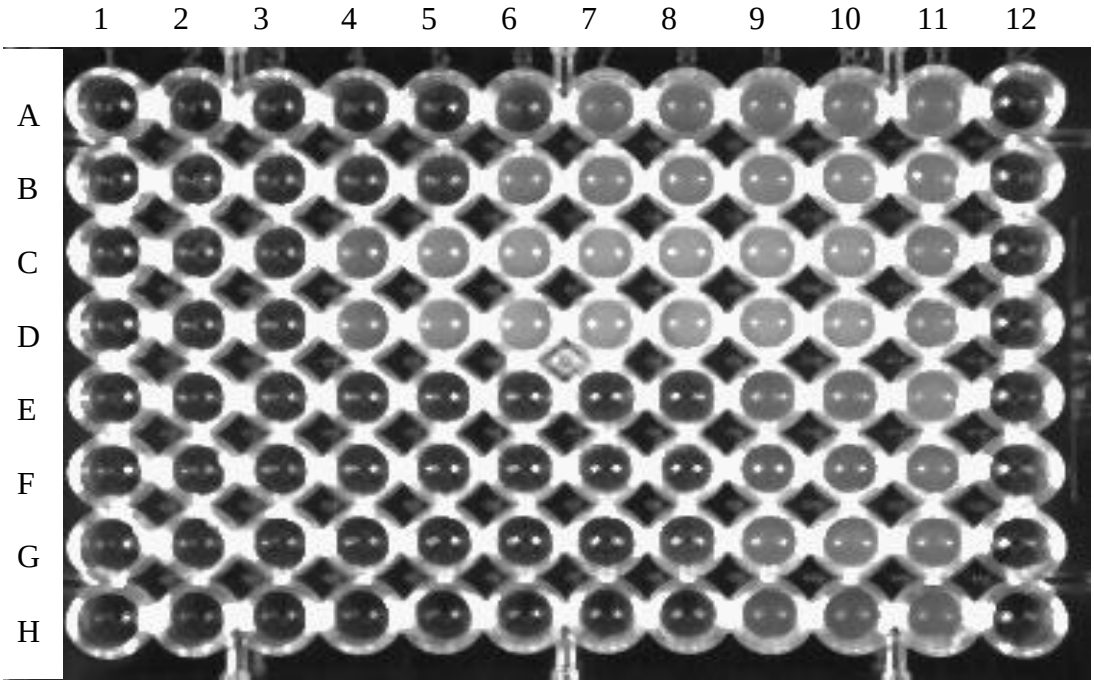


Figure 9. Validation of four rBSH inhibitors identified by HTS using the 96-well plate assay. Using 50 µl of total reaction volume, all compounds were added at a final concentration of 0.5 mM in column 1 with 2-fold serial dilution through column 10. Rows A and B: carnosic acid; C and D: menadione; E and F: gossypetin; G and H: riboflavin. BSH was added at a fixed concentration in column wells 1-11. Column 12 served as a negative control with no enzyme and compound added. After 4 hours of incubation, gossypetin and riboflavin display the greatest inhibition, through well 8. Menadione and carnosic acid inhibited activity at the three and five highest concentrations tested, respectively.

VITA

Katie Rose Smith was born in Knoxville, TN on January 30, 1988. She was raised in Tazewell, TN and graduated from Claiborne High School in 2006. In 2010 she received a B.S. in biology from Lincoln Memorial University and in 2011 began working on a M.S. in animal science at the University of Tennessee, Knoxville. Beginning in August 2013, Katie will matriculate at the University of Tennessee College of Veterinary Medicine to pursue a D.V.M.