Bile Salt Hydrolase: From Basic Science to Translational Innovation

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Dedication

This dissertation is dedicated to my family and my friends who have encouraged and supported me to pursue my Ph.D. degree.

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Abstract

Bile salt hydrolase (**BSH**) is an intestinal bacterial enzyme that catalyzes deconjugation of conjugated bile acids, an essential gateway reaction in the metabolism of bile acids. Therefore, BSH is a key mechanism through which the microbiota modulates host lipid metabolism and energy harvest. Recent studies have shown that BSH is a promising microbiome target for developing novel alternatives to antibiotic growth promoters (**AGPs**) for animal industry and new measures to control obesity in humans. However, research on BSH is still in its infancy in terms of both basic science and translational innovation. In this dissertation study, multidisciplinary approaches in conjunction with two animal model systems (rat and chicken) were used to examine the impact of BSH on host physiology, to understand the BSH structure-function relationship, and to evaluate *in vivo* efficacy of three identified BSH inhibitors with potential as novel alternatives to AGPs. Large quantities of recombinant BSH were purified and encapsulated using a novel encapsulation system. Oral administration of rats with the encapsulated BSH did not significantly affect host growth performance, lipid metabolism, and energy harvest although intestinal bile profile was significantly changed in response to BSH treatment. The crystal structure of the BSH from *Lactobacillus salivarius* NRRL B-30514 was elucidated. Comparative structural analysis identified the residues and domains that are critical for catalysis and substrate specificity of BSH, which was subsequently validated by site-directed mutagenesis as well as comparative BSH activity examination. The *in vivo* efficacies of three BSH inhibitors caffeic acid phenethylester, riboflavin, and carnosic acid were evaluated using chicken model. Dietary supplementation of the BSH inhibitors in broilers (25 mg/kg body weight/day) enhanced body weight gain/feed efficiency, and significantly changed host bile acid and transcriptome profiles at both local and systemic levels, which provided physiological,

metabolomics, and molecular evidence demonstrating *in vivo* efficacies of the tested BSH inhibitors.

Keywords: Bile salt hydrolase; lipid metabolism; structure; BSH inhibitors; non-antibiotic feed additive

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Introduction

Overweight and obesity are worldwide challenges. The latest data provided by the World Health Organization (WHO) showed that around 39% of adults aged 18 and over were overweight and 13% were obese in 2014 all over the world (WHO, 2017). Although factors, such as genetic background and diet, contribute significantly to the prevalence of obesity, recent research has linked obesity development to the composition and function of gut microbiota (Clarke *et al.*, 2012; Everard *et al.*, 2013; Turnbaugh *et al.*, 2009a). Therefore, manipulation of gut microbiota might be a promising strategy to control obesity (Ley *et al.*, 2005; Turnbaugh *et al.*, 2009b). However, key microorganisms as well as their derived factors that affect host energy harvest, remain to be elucidated (Boulangé *et al.*, 2016; Hullar *et al.*, 2012; John *et al.*, 2016; Lin, 2011).

Interestingly, despite recent extensive efforts to solve the overweight and obesity problems in humans by targeting gut microbiota, the animal industry has manipulated gut microbiota of food animals for a long time to improve production and growth performance (Dibner *et al.*, 2005). Specifically, a panel of antibiotics called antibiotics growth promoters (AGPs), have been used at sub-therapeutic levels for more than sixty years to increase feed efficiency, energy harvest, and body weight gain of farm animals (Moore *et al.*, 1946). It has been widely recognized that the growth-promoting effect of AGPs is primarily mediated through altered microbiota in the animal intestine. With the aid of recent 16S rDNA-based, culture-independent technologies, significant progresses in understanding how AGPs reshape intestinal microbial population have been made. Specifically, recent microbiota work has shown that the growth-promoting effect of AGPs was highly correlated with the decreased activity of bile salt hydrolase (BSH), an intestinal enzyme

that is involved in lipid metabolism and produced by many commensal bacteria, such as *lactobacillus* species (Engberg *et al.*, 2000; Lin, 2014).

The BSH protein catalyzes deconjugation of conjugated bile acids, an essential gateway reaction for bile acid metabolism in the small intestine (Begley et al., 2006). The bile acids have dual digestive and signaling roles in the host; therefore, it has been increasingly recognized that intestinal BSH plays an important role in host lipid metabolism and energy harvest (Begley *et al.*, 2006; Joyce *et al.*, 2014b). In a recent study, Joyce *et al.* (Joyce *et al.*, 2014a) used a controlled and reductionist approach to examine the impact of bacterial BSH activity on host physiology and metabolism, which is the first direct evidence demonstrating that manipulation of *in situ* BSH activity significantly affects host lipid metabolism, signaling functions and weight gain. Recently, our preliminary rat study showed that oral administration of Zucker fatty rats with a purified BSH protein significantly increased respiratory exchange ratio (RER), suggesting reduction of host lipid digestion or absorption (Spratt *et al.*, 2014). This finding promoted us to perform a multi-disciplinary study in Chapter II by taking advantage of our well-characterized BSH protein in conjunction with an established rat model as well as encapsulation technology; the findings from the studies described in Chapter II are expected to provide more direct evidence showing BSH protein's critical role in host energy harvest and lipid metabolism.

To date, although a number of BSHs have been identified from different commensal bacteria, our understanding of the BSH structure-function relationship is still limited (Kumar *et al.*, 2006; Rossocha *et al.*, 2005). Characterization of the residues and domains that are critical for the function of the BSH protein will facilitate us to discover and optimize BSH inhibitors using a

computational structure-based approach. To achieve this goal, a unique BSH protein, which was identified and characterized in our previous study (Wang *et al.*, 2012b), was successfully crystalized in this project (Chapter III). More importantly, comparative structural analysis of the *L. salivarius* BSH identified the residues critical for catalysis and substrate specificity, which were further validated by site-directed mutagenesis approach in Chapter III.

In light of the findings from recent functional microbiome studies, we hypothesize that dietary supplementation of BSH inhibitors may inhibit intestinal BSH activity, consequently promoting host lipid metabolism and enhancing feed efficiency and body weight gain. Recently, we identified and characterized a group of promising BSH inhibitors using an effective High-Throughput Screening system (Lin *et al.*, 2014; Smith *et al.*, 2014). In particular, three novel BSH inhibitors—riboflavin, caffeic acid phenethyl ester (CAPE), and carnosic acid—have shown potential as non-antibiotic growth promoters in poultry (Lin *et al.*, 2014; Smith *et al.*, 2014). However, it is unknown if these three BSH inhibitors could exert inhibitory effect on BSHs in the intestine and then affect host bile profile as well as lipid metabolism. Therefore, *in vivo* evaluation of these three promising BSH inhibitors is essential from both translational development and basic mechanistic research standpoints. In Chapter IV, a pilot chicken study was performed to evaluate *in vivo* efficacy of BSH inhibitors and understand the mechanisms that underpin chickens physiology through analysis of bile acid profiles and host responses, utilizing state-of-the-art metabolomics and genomics approaches.

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Chapter I. Review of literature

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Bile acids

Primary bile acids are *de novo* synthesized from cholesterol in the liver and are conjugated to either glycine or taurine to form conjugated bile acids (Appleby *et al.*, 2014; Camilleri *et al.*, 2015; Schaap *et al.*, 2014). The amphipathic characteristic of conjugated bile acid helps dietary lipids or fat-soluble vitamins form micelles, which facilitate their metabolism by pancreatic enzymes prior to their absorption (de Aguiar Vallim *et al.*, 2013). Thus, conjugated bile acids are more efficient than unconjugated bile acids for emulsification and digestion of dietary lipids or lipid soluble nutrients (Hofmann *et al.*, 1992; Ridlon *et al.*, 2006). Following synthesis, bile salts are stored and concentrated in the gallbladder. Upon food consumption, chyme from partly digested food is expelled from stomach into the duodenum, acids and partially digested fat would stimulate the secretion of secretin and cholecystokinin (CCK) (Begley *et al.*, 2005). Subsequently, CCK stimulates the contraction of the gallbladder and leads to the release of bile salts from the gallbladder into the small intestine for lipid digestion (Johnson, 1998). In the animals without a gallbladder, such as horses and rats, bile salts continuously flow directly from the liver into the duodenum via the bile duct.

After reaching the ileum, bile salts are taken up into enterocytes via efficient membrane transporters and further absorbed into the portal vein to get back to the liver and finally get re-secreted into bile; this process is called enterohepatic circulation (Begley *et al.*, 2006; Ridlon *et al.*, 2006; Roberts *et al.*, 2002; Russell, 2009; Vlahcevic *et al.*, 1996). In humans, 200-600 mg are synthesized daily which consist 5% of the total bile acid pool of about 3 g; approximately 400-800 mg of bile salts are subjected to microbial transformations in the intestine (Vlahcevic *et al.*, 1996). Among the various bile salts transformations, deconjugation of conjugated bile salts

is the gateway reaction for bile alteration and is a prerequisite for all sterol transformation (Batta *et al.*, 1990; Begley *et al.*, 2006; Kim *et al.*, 2005b). Notably, in addition to direct digestive role in the emulsification of dietary fats in the intestine, bile acids can act as signaling molecules to affect energy metabolism, bile acids enterohepatic circulation, host cholesterol level, and triglyceride and glucose homeostasis (Joyce *et al.*, 2014b). In particular, unconjugated bile acids have been shown to specifically interact with orphan nuclear hormone receptors such as farnesoid X receptor (FXR) and G-protein-coupled receptor TGR 5 (Evans *et al.*, 2009; Gupta *et al.*, 2001; Houten *et al.*, 2006; Inagaki *et al.*, 2006; Qiao *et al.*, 2003).

Bile acids signaling

Bile acids has been recognized to be important signaling molecules by their interaction with the nuclear receptor FXR, membrane G-protein-coupled receptor TGR 5 and G protein coupled receptor S1PR2 in the gastrointestinal system (Evans *et al.*, 2009; Gupta *et al.*, 2001; Houten *et al.*, 2006; Inagaki *et al.*, 2006; Li *et al.*, 2014; Qiao *et al.*, 2003). The signaling pathway through FXR is one of the most important one.

FXR is expressed in the liver, intestine and adipose tissues. In the liver, chenodeoxycholic acid activates FXR and consequently induce the negative nuclear receptor small heterodimer partner (Jayashree *et al.*, 2014) which activates c-Jun N-terminal kinase thus inhibits the transcription of CYP7A1, the enzyme catalyzes the rate limiting step for bile acid synthesis (Chiang, 2017; Holt *et al.*, 2003). The FXR/SHP mechanism is more likely to be activated under the circumstance of high liver bile acid concentration to inhibit bile acid synthesis to prevent liver injury. The mechanism of FXR anti-lipogenic action is that activation of FXR/SHP inhibits liver X receptor

(LXR) induced activation of SREBP1c which in turn inhibit lipogenic genes (Ma *et al.*, 2006). The activation of FXR can also increase fatty acid oxidation by the induction of peroxisome proliferator-activated receptor alpha (PPARα) (Pineda Torra *et al.*, 2003). Very low density lipoprotein secretion is also regulated by FXR. The activation of FXR/SHP suppresses the hepatocyte nuclear factor 4α (HNF4α) activity which is the master regulator of microsomal triglyceride transfer protein (MTP) and Apolipoprotein B (apoB) expression and this important for VLDL secretion (Hirokane *et al.*, 2004).

Activation of FXR can also decrease host glucose concentration by increasing glycogen synthesis and decreasing gluconeogenesis and glycogenolysis (Ma *et al.*, 2006). The key gene involved in these process is the fibroblast growth factor 19 (FGF19). The activation of FXR promotes FGF 19 expression which directly promotes glycogen synthesis in the liver (Kir *et al.*, 2011). When glucose level is high, FXR activation reduces liver pyruvate kinase (LPK) transcription, which leads glucose metabolites towards glycogen synthesis (Duran-Sandoval *et al.*, 2005). Besides, inducing of FGF 19 inhibits gluconeogenesis by reducing hepatic expression of peroxisome proliferator-activated receptor gamma coactivator alpha (PGC1α) and cyclic AMP response element binding protein (CREB) (Potthoff *et al.*, 2011). The expression of PGC1α and CREB positive regulate phosphoenolpyruvate carboxykinase (PEPCK) activity which increases gluconeogenic (Potthoff *et al.*, 2011).

In addition to nutrient metabolism, FXR also plays important role on anti-inflammatory activity by inhibition NF-κB transcriptional activation of liver pro-inflammatory genes (Wang *et al.*, 2008). Host immunology process can be modulated by FXR as well via autophagy and cell

proliferation through an interaction with energy-sensitive transcriptional coregulators (Massafra *et al.*, 2017; Jayashree *et al.*, 2014).

Bile Salt Hydrolase (BSH)

The BSH protein produced by intestinal bacteria catalyzes deconjugation of conjugated bile acids by hydrolyzing the amide bond and producing free amino acids and unconjugated bile acids; this is an essential gateway reaction in the metabolism of bile acids in the small intestine (Begley *et al.*, 2006). The BSH protein belongs to the choloylglycine hydrolase (EC 3.5.1.24) family. Phylogenetic analysis indicated that BSH was derived from a wider Ntn_CGH-like family of proteins, specifically penicillin V acylase (Jones *et al.*, 2008; Kumar *et al.*, 2006). BSHs from various sources differ in activity, substrate specificity, and optimal temperature and pH for enzymatic activity (Begley *et al.*, 2006). Molecular weight of the BSH subunit ranges from 28 kDa to 50 kDa, and optimal pH for BSH activity is slightly acidic with range from 3.5 to 6. Most identified BSHs still display activity at temperatures up to 60^{o}C. Many identified BSHs have a narrow substrates spectrum and display much higher activity in hydrolyzing glycine-conjugated bile salts than tauroconjugated bile salts (Coleman *et al.*, 1995; Kim *et al.*, 2004; Liong *et al.*, 2005; Pavlović *et al.*, 2012; Smet *et al.*, 1995; Tanaka *et al.*, 2000). However, some BSHs show a preference for tauroconjugated bile salts, such as two BSHs in *Lactobacillus jonsonii* PF01 (Chae *et al.*, 2013) and the BSHs from five lactobacilli strains (Jiang *et al.*, 2010). Recently, a potent BSH protein was identified and characterized from a chicken *Lactobacillus salivarius* strain; this BSH displayed potent hydrolysis activity towards both glycoconjugated and tauroconjugated bile salts (Wang *et al.*, 2012b). It has been proposed that BSHs recognize conjugated bile acids on both amino acid moieties and the cholate steroid nucleus (Begley *et al.*,

2006). Not surprisingly, substrates preferences of BSH may differ under different pH, likely due to pH-mediated structural changes (Corzo *et al.*, 1999).

To date, the structural basis of the BSH function is still largely unknown. Crystal structures of the BSHs from only three specific species, *Bifidobacterium longum, Clostridium perfrigens*, and *L. salivarius* have been reported (Kumar *et al.*, 2006; Rossocha *et al.*, 2005; Xu *et al.*, 2016). The 1.90 Å crystal structure of the *L. salivarius* BSH was recently determined by molecular replacement using the starting model of *C. perfringens* BSH (Xu *et al.*, 2016). Comparative structural analysis of the *L. salivarius* BSH also identified potential residues contributing to catalysis and substrate specificity. Together, unlike the binding pocket in other BSH such as the *C. perfrigens* BSH that shows an open entrance with shallow bottom, a panel of unique residues in the *L. salivarius* BSH make this BSH display narrow entrance of the binding pocket and the increased inner capacity of the binding pocket, which may enable substrates to sit deeply in the pocket with different conformation and lead to the broad spectrum of specificity (Wang *et al.*, 2012b; Xu *et al.*, 2016). Previous comparative genomics and structural studies have identified some conserved, catalytically important residues in the active sites of BSH (Cys2, Arg 16, Asp19, Asn79, Asn171, and Arg224); however, this conclusion was primarily based on the comparison of BSH structure to penicillin V acylase (Begley *et al.*, 2006; Kumar *et al.*, 2006; Wang *et al.*, 2012). To date, Cys2 is the only residue that has been subjected to site-directed mutagenesis and validated for its essential role in BSH activity (Kumar *et al.*, 2006). Therefore, future in-depth structural analysis of the unique *L. salivarius* BSH (e.g. in complex with specific substrate) in conjunction with comprehensive amino acid substitution mutagenesis would help to discover residues critical in catalysis and understand why this BSH displayed potent catalytic

activity toward a broad spectrum of substrates including both glycoconjugated and tauroconjugated bile salts.

BSH-producing bacteria in the intestine

BSHs have been identified in diverse bacterial species from different sources (Summarized in Table 1). Among the BSH-producing organisms, most are Gram-positive bacteria, except two from Gram-negative genus *Bacteroides* (Lambert *et al.*, 2008; Masuda, 1981; Stellwag *et al.*, 1976). Jones *et al.* (2008) performed a functional and comparative metagenomics analysis of BSH activity in the human intestinal microbiome and showed a high level of redundancy of BSH distribution in the human intestine ecosystem; most BSH activity was distributed in all major phyla within intestinal microbiota (primarily *Firmicutes*, followed by *Bacterioidetes* and *Actinobacteria*) and across two domains of life (Bacteria and Archaea in the intestine) (Jones *et al.*, 2008).

BSH genes are particularly abundant in lactic acid fermenting probiotics, such as lactobacilli and bifidobacteria, which are the species most commonly used as probiotics due to their health-promoting activities (Reviewed by Begley *et al.*, 2006). As is shown in Table 1, BSH activity and corresponding enzymes have been identified primarily in lactic acid bacteria isolated from gastrointestinal tract, which include but are not limited to *L. salivarius*, *L. acidophilus*, *L. johnsonii*, *L. plantarum*, *B. longum*, *B. bifidum*, *B. adolescentis*, and *B. animalis.* BSH genes are either located in the chromosome or in the mobile element, such as the megaplasmid identified in *L. salivarius* UCC118 (Claesson *et al.*, 2006). It is not unusual that multiple BSH homologs, which are not identical, could be present in a single intestinal bacterial strain (Begley *et al*.,

2006; Wang *et al*., 2012). It has been speculated that BSH genes may be acquired horizontally among intestinal microorganisms (Begley *et al.*, 2006). However, there is no compelling evidence demonstrating horizontal transfer of BSH genes in intestinal microorganisms.

Jones *et al*. (2008) also have determined that active BSHs are restricted to intestinal microorganisms, suggesting that BSH activity plays a role in *in vivo* adaptation of intestinal microorganisms in gastrointestinal environment and in the mutualism between intestinal microbiota and animal host (Jones *et al.*, 2008). Physiological advantages of BSH for bacterial producers themselves are still not well understood. One popular opinion is that BSH activity contributes to the resistance of commensal bacteria towards bile salts, a natural antimicrobial presents in the intestine (Begley *et al.*, 2006). For example, it has been demonstrated that BSH activity plays an important role in the bile resistance and intestinal colonization of *Listeria innocua* in a mice model (Jones *et al.*, 2008). However, the unconjugated bile salts resulting from BSH hydrolysis could still display antimicrobial activity; thus, there are contradictory findings about the contribution of BSH activity to bile tolerance in intestinal probiotic bacteria (Begley *et al.*, 2006). At present, there is no convincing *in vivo* evidence demonstrating that BSH protein contributes to bile resistance in probiotic bacteria, such as lactobacilli. Fang *et al*. (2009) demonstrated that the production of BSH does not determine the bile resistance level in *L. salivarius*, the dominant *Lactobacillus* species present in animal intestine (Fang *et al.*, 2009). In addition to this popular hypothesis, there are some other opinions about the roles of BSH in bacterial physiology based on some evidence in certain commensal bacteria. For example, it has been proposed that hydrolysis of conjugated bile acids by BSH can provide cellular carbon, nitrogen, and sulfur, as well as energy source for some bacteria species (Ridlon *et al.*, 2006;

Tanaka *et al.*, 2000; Vlahcevic *et al.*, 1996). BSH may also trigger the influx of cholesterol or bile into bacterial cells and increase membrane electrochemical characteristics, which may facilitate some microorganisms to inhabit the gastrointestinal epithelium in the host *via* immune evasion (Jones *et al.*, 2008; Mukherji *et al.*, 2015).

The impact of bacterial BSH activity on host physiology

Despite the lack of understanding of the benefits of BSH for BSH-producing bacteria, it has been recognized that intestinal BSH plays an important role in host lipid metabolism, dietary energy harvest and body weight gain, because BSH catalyzes the gateway reaction in the metabolism of bile acids in the intestine (Begley *et al.*, 2006; Jones *et al.*, 2008; Joyce *et al.*, 2014b). To date, functional research on the relationship between bacterial BSH and host physiology and health has been primarily focused on human probiotics using laboratory animal model systems. There are very limited efforts to determine the impact of intestinal bacterial BSH activity on growth and health in food animals (Du Toit *et al.*, 1998; Feighner *et al.*, 1988; Guban *et al.*, 2006; Knarreborg *et al.*, 2004; Lin, 2011; Lin *et al.*, 2014). The following paragraphs summarize the findings from laboratory animal studies and human trials, which would shed light on food animal health research in the future.

Host lipid metabolism, cholesterol, and body weight

Obesity associated diseases are increasing as children and adults are increasingly becoming overweight and obese (Kahn *et al.*, 2006; Van Gaal *et al.*, 2006). Recent studies have indicated that intestinal microbiota are implicated in obesity in humans (Tremaroli *et al.*, 2012); however,

the key microbial functions influencing host energy harvest remain to be clearly elucidated. The BSH protein has been increasingly recognized as a critical intestinal microbiome target for developing an intervention strategy to control obesity.

Given that the bile acids have dual digestive and signaling roles in the host, intestinal BSH plays an important role in host metabolism and energy harvest; BSH activity has a significant impact on host physiology by disturbing conjugated bile acids-mediated fat metabolism and endocrine functions (Begley *et al.*, 2006; Jones *et al.*, 2014; Joyce *et al.*, 2014b; Patel *et al.*, 2010). Recent probiotics studies have already shown that oral administration of BSH-producing lactobacilli could affect lipid metabolism, consequently reducing body weight and/or cholesterol level in humans (Jones *et al.*, 2013), rats (Kumar *et al.*, 2011; Pato *et al.*, 2004), mice (Miyoshi *et al.*, 2014; Park *et al.*, 2013; Park *et al.*, 2014), and pigs (De Smet *et al.*, 1998).

Molecular and cellular studies also have provided insights into the underlying mechanisms of the effect of BSHs on host lipid metabolism and energy harvest. Clearly, unconjugated bile acids, directly resulting from BSH activity, are less effective than conjugated bile acids in the emulsification of dietary fat, and consequently affect lipid absorption and metabolism. However, unconjugated bile acids could exert more profound impacts on host energy harvest both locally and systemically. Farnesoid X receptor (FXR), which is preferentially stimulated by unconjugated bile acids, can not only regulate lipogenesis and triglyceride synthesis (Li *et al.*, 2013; Watanabe *et al.*, 2004), but also regulates glucose homeostasis by increasing glycogen synthesis (Caron *et al.*, 2013; Zhang *et al.*, 2006) or decreasing glycolysis (Caron *et al.*, 2013). Using a pig model, Pereira-Fantini *et al.* (2014) examined the impact of BSH-mediated bile acid

dysmetabolism on FXR signaling pathways and clinical outcomes, and showed that alterations in bile acid composition may have contributed to the observed disturbance in FXR-mediated signaling pathways (Pereira-Fantini *et al.*, 2014).

Notably, obesity development is a complex physiological issue. The BSH-mediated bile salt metabolism is only one of several potential mechanisms by which microbiota affect host energy harvest and weight gain (Walker *et al.*, 2013). The studies described above only provide indirect evidence supporting the role of BSH-producing probiotics or BSH-mediated bile metabolism in host lipid metabolism and energy harvest. Direct and controlled approaches are required in order to obtain complete understanding of BSH-mediated regulation of host weight gain and lipid metabolism.

Recently, using a controlled system in conjunction with a mouse model, Joyce *et al*. (2014a) obtained the first direct evidence demonstrating that manipulation of *in situ* BSH activity alone significantly influenced lipid metabolism, signaling functions, and weight gain (Joyce *et al.*, 2014a). Briefly, two well characterized *L. salivarius* BSHs were cloned into an *E. coli* host strain (MG1655). The recombinant *E. coli* constructs could effectively colonize the gastrointestinal tract of mice with expression of high level of BSH activity. Colonization of germ-free mice with such BSH-producing *E. coli* strain elevated intestinal BSH activity and resulted in local bile acids deconjugation with concomitant reduced levels in body weight and cholesterol, alternations in lipid metabolism, signaling functions, local and systemic transcriptome profiles in the pathways governing lipid metabolism (Joyce *et al.*, 2014a). Notably, in conventionally raised mice, enhanced *in situ* BSH activity also caused local bile acid

deconjugation, reduced mouse weight gain, lowered serum cholesterol level, and reduced liver triglyceride level, which further demonstrates that BSH is a key mechanism through which the microbiota modulates host lipid metabolism and dietary energy harvest (Joyce *et al.*, 2014a). In addition to its ability to alter local (gastrointestinal) functions, BSH activity could systemically affect host physiology such that the BSH activity-mediated bile acids can interact with transporters (e.g. *Abcg5/8*) and regulators (e.g. FXR regulon, *Fiaf*) which lead to change in body mass (Joyce *et al.*, 2014a).

Other physiological processes

The BSH-mediated unconjugated bile acids also affect immune homeostasis because of their ability to modulate a panel of effectors in the intestine, such as inducible NO synthase (iNOS) (Inagaki *et al.*, 2006), the antimicrobial peptide RegIIIγ produced by intestinal paneth cells (Joyce *et al.*, 2014a), and dendritic cell differentiation (Ichikawa *et al.*, 2012; Joyce *et al.*, 2014b). In addition to the pathway via intestinal FXR, unconjugated bile acids also affect TGR5-mediated adipose tissue development and weight loss (Svensson *et al.*, 2013; Watanabe *et al.*, 2006). Interestingly, Joyce *et al.* (2014a) also observed that enhanced *in situ* BSH activity reversed the expression pattern of genes responsible for regulating circadian rhythm (e.g., *Dbp*) and other genes central to circadian clock (Joyce *et al.*, 2014a). Finally, unconjugated bile acids can also alter intestinal microbiota, and consequently may exert more complex impacts on the host (Inagaki *et al.*, 2006; Islam *et al.*, 2011).

Potential adverse effects due to high-level BSH activity in the intestine

High-level BSH activity would result in a large proportion of unconjugated bile acids, which can lead to malabsorption of lipids and may cause steatorrhea in the host (Kim *et al.*, 2005b). Recent research also indicates that deconjugation of bile salts by BSH-producing lactobacilli is an important factor leading to short bowel syndrome due to abnormal lipid metabolism and a disrupted bile acid profile (Bongaerts *et al.*, 2000; Choi *et al.*, 2014).

BSH mediated deconjugation of bile salts can increase bile recovery from passive absorption across the colonic epithelium by making bile salts more hydrophobic, which may also cause some adverse effects. For example, a high concentration of secondary bile acids in the blood and feces, produced by a multistep 7α-dehydroxylation reaction from unconjugated bile acids, are proposed to be related to the pathogenesis of cholesterol gallstone diseases as well as colon cancer (Färkkilä *et al.*, 1990; Marteau *et al.*, 1993; McGarr *et al.*, 2005; Ou *et al.*, 2013; van Faassen *et al.*, 1987; Venneman *et al.*, 2010). Secondary bile acids may increase the risk of cancer by increasing oxidative stress and associated DNA damage (Bernstein *et al.*, 2005; Cooke *et al.*, 2003). The sulfonic acid moiety in unconjugated bile acids could be reduced and dissimilated to hydrogen sulfide, which is highly toxic and can increase colon cell turnover (Christl *et al.*, 1996; Corzo *et al.*, 1999; Laue *et al.*, 2001; Lie *et al.*, 1999; Ridlon *et al.*, 2006). Hydrogen sulfide is a potent inhibitor of colonic butyrate metabolism, which is a key nutrient and a regulator of cell turnover (Christl *et al.*, 1996; Van Eldere *et al.*, 1996). Hydrogen sulfide can also reduce apoptosis in colon cancer cells by preventing the function of the chemo-preventative agent β-phenylethyl isothiocyanate (PEITC) (Rose *et al.*, 2005).

Target BSH for enhanced animal production and health

In contrast to the significant progress on BSH research for human health described above, little information exists concerning BSH and BSH-producing bacteria in food animals. Some early studies evaluated direct usage of bile salts as a feed additive to improve feed efficiency due to the well-recognized role of bile salts in fat digestion (Kussaibati *et al.*, 1982; Reinhart *et al.*, 1988). In chickens, supplementation of bile salts in the diet increased the absorption of fatty acids, but had no influence on chickens with fat-free diet (Kussaibati *et al.*, 1982). Presence of bile salts in the diet also increased fat digestibility in swine after the weaning period (Reinhart *et al.*, 1988). Although the findings from these studies are encouraging, bile salts have not been adopted by the feed industry as feed additives to improve growth performance of food animals, likely due to the issues of cost, availability, and complex biotransformation of bile salts in the gastrointestinal tract.

AGPs are defined as a group of antibiotics used in feed at sub-therapeutic level to improve average daily weight gain and feed efficiency in food animals. This husbandry technique has been practiced since the 1950s. However, use of AGPs has been associated with the emergence of antibiotic-resistant human pathogens of animal origins. Therefore, ending the use of AGPs is a worldwide trend to protect public health. Effective alternatives to AGPs are urgently needed to maintain current animal production levels without threatening public health. Recent animal studies on the effect of AGP usage on intestinal microbiome indicate that the enhanced feed efficiency and body weight gain in food animals due to AGP usage is inversely related to the BSH activity as well as the abundance of potent BSH-producers in the intestine (Lin, 2014).

As early as the 1980s, Feighner and Dashkevicz (1987) reported that use of AGP reduced intestinal BSH activity in poultry and proposed that inhibition of BSH activity would promote feed efficiency and weight gain in food animals. In this early study, a radiochemical method was successfully developed to directly determine BSH activity in intestinal contents; however, the method used in this study was technically challenging and time consuming (Feighner *et al.*, 1987). Notably, the standard BSH activity assay widely used is not feasible for examining fecal BSH activity because of the high levels of background caused by free amino acids in intestinal contents. To date, fecal bile acid profile is an acceptable indicator for evaluating BSH activity in the intestinal contents. Consistent with the finding by Feighner and Dashkevicz (1987), Knarreborg *et al*. (2004) also observed that AGP usage reduced concentration of unconjugated bile salts in the intestine of broilers by using reversed-phase HLPC method, which led to an enhanced bioavailability of α-tocopheryl (alpha-tocopherol) acetate. In multiple pen trials, Guban *et al.* (2006) further confirmed that AGP treatment improved weight gain and fat digestibility in broilers, decreased population levels of *L. salivarius*, and significantly reduced BSH activity in the intestine, which was reflected by a decreased pool of deconjugated bile salts in ileal contents using the HPLC method. In pigs, De Smet *et al.* (1998) observed that oral administration of *L. reuteri* with BSH activity influenced host lipid metabolism and decreased total and LDL-cholesterol concentrations. Du Toit *et al.* (1998) also had a similar finding in a minipig feeding trials using BSH-positive probiotic mix. However, both of these pig studies (De Smet *et al*, 1998; Du Toit *et al.*, 1998) lack determination of intestinal BSH activity, which is needed to rule out potential pleotropic effects resulting from the treatment with BSH-producing probiotics.

Regarding the response of intestinal microbiota to AGPs, a key issue for us to understand the mode of action of AGP, culture-independent molecular approaches have been used to examine the effect of AGPs on intestinal microbiota in poultry and swine; to date, more than ten papers have been published in this filed (Lin, 2014). Not surprisingly, long-term supplementation of diet with AGPs significantly affected the microbial ecology in the intestine in all reported studies. However, the specific bacteria or environmental niches changes that are meaningful and are linked to the desired phenotype of enhanced growth performance need to be clarified. In-depth comparative analysis of these animal microbiome studies led to an interesting finding: in most chicken and swine studies, use of AGPs reduced the population of *Lactobacillus* species, the major BSH-producing bacteria in the animal intestine (Begley *et al.*, 2006; Lin, 2014). The independent findings from these food animal studies together with those from human BSH research summarized above are like jigsaw pieces, which seem to be scattered but are in fact tightly interrelated. Therefore, it was proposed that BSH is a key mechanistic microbiome target for developing novel alternatives to AGPs and this hypothesis prompted us to identify and characterize a potent BSH protein from a chicken *L. salivarius* probiotic strain (Wang *et al.*, 2012b). Interestingly, copper and zinc compounds displayed potent inhibitory effect on BSH activity in this study, which not only provides scientific evidence to understand the mode of action of high dietary concentrations of copper/zinc for growth promotion, but also strongly supports our hypothesis that BSH inhibitors may serve as promising alternatives to AGPs (Wang *et al.*, 2012b). Subsequently, by taking advantage of the unique feature of the *L. salivarius* BSH (Wang *et al.*, 2012b), an efficient high-throughput screening system was successfully developed and used to discover BSH inhibitors (Smith *et al.*, 2014). Unlike many BSHs from other bacteria that have narrow substrate spectrum, the *L. salivarius* BSH displayed potent hydrolysis activity

towards both glycoconjugated and tauroconjugated bile salts. The broad substrate specificity nature of this BSH makes it an ideal candidate for screening desired BSH inhibitors. This hypothesis is further tested by our recent study showing the identified BSH inhibitors also exhibited potent inhibitory effects on a phylogenetically distant BSH from *L. acidophilus* (Lin *et al.*, 2014).

Despite the recognized AMR issues associated with antibiotic usage in food animals, animal industries still heavily rely on antibiotics due to the lack of practical and consistent antibiotic alternative approaches. Solely limiting antibiotics without providing effective alternatives would compromise animal production and health. BSH inhibitors are promising alternatives to AGPs for enhanced feed efficiency and growth performance. Successful development of effective non-antibiotic BSH inhibitor feed additives could reduce the dependence on in-feed antibiotics for growth promotion, consequently mitigating AMR pressure in agriculture ecosystems, which is a significant and timely issue impacting animal health and food safety.

Other types of antibiotic-alternative products, such as probiotics, prebiotics, and organic acids, have drawn wide attention and have been developed and used to alter intestinal microbiota for improving animal health and production (Dibner *et al.*, 2005; Lin, 2014). However, very limited data is available to scientifically justify the choice of specific bacterial species or products for growth promotion and results are inconsistent from independent studies (Dibner *et al.*, 2005). For example, although probiotics containing *Lactobacillus* are well recognized for their beneficial effects on boosting host immunity, these probiotics could have a negative impact on host lipid metabolism due to BSH production. For example, in a large pen trial, (Mountzouris *et*

al., 2010; Sharifi *et al.*, 2012) it was observed that supplementation of a 7-bacterial species probiotic (Protexin) to fat-rich diets significantly reduced body weight gain, fat digestibility, and feed conversion in broilers (Sharifi *et al.*, 2012). Using a different 5-bacterial species competitive exclusion probiotic product, Mountzouris *et al*. also observed similar inferior feed conversion efficiency and reduced fat digestibility in response to probiotic treatment in broilers (Mountzouris *et al.*, 2010). These investigators have proposed that the enrichment of the intestinal microflora, particularly lactobacilli, due to probiotic supplementation caused enhanced BSH activity in the intestine, leading to detrimental effects on lipid metabolism and growth performance in broilers. Therefore, improved knowledge in the role of BSH and BSH-producing bacteria will help design rationally tailored probiotics that will enhance animal health and performance. For example, the BSH inhibitors could also be used together with certain BSH-producing probiotics to maximize the beneficial effects of the probiotics by mitigating their potential negative impact on host fat digestion. This approach may further help animal production industries optimize existing probiotic and prebiotic additives for enhanced feed efficiency, growth performance and profitability.

Conclusions and research gaps

Antibiotics have been heavily used for animal farming to maintain animal production and health. However, farm usage of antibiotics is a driving force to enrich AMR genes (called the 'resistome') in various niches and to promote pools of resistant pathogenic bacteria, raising food safety and public health concerns (Davies, 2014; Perry *et al.*, 2014). To effectively mitigate AMR in agricultural systems, a reduction in the use of antibiotics in farming is imperative. Thus, intensive efforts are critically needed to develop effective non-antibiotic growth promotion

strategies that can be practically implemented by animal producers. Recent microbiome studies have provided compelling evidence that BSH is a key mechanistic microbiome target for developing novel alternatives to AGPs. Development of BSH inhibitor-based non-antibiotic feed additives directly addresses the nutrition concern (feed efficiency/growth rate) that prevents animal industries from reducing antibiotic usage. In addition to benefitting healthy animals under routine management, the weight-enhancing BSH inhibitors may also help sick animals better harvest dietary energy while combatting infectious diseases or environmental/production stresses.

Despite the significant role of bacterial BSH activity in host lipid metabolism and energy harvest, research on BSH is still in its infancy. In particular, little effort has been placed on characterization of BSHs and/or BSH-producing bacteria in food animals. Several significant gaps remain in knowledge associated with BSH in food animal production and health. Filling these gaps will not only directly benefit animal health but also provide insights and likely new model systems for human health research, leading to novel "One Health" measures for enhanced animal production, food safety, and human nutrition.

- Ecology of BSHs and BSH-producing bacteria in the intestine. To date, very limited BSHs were identified in the intestinal bacteria isolated from food animals (Table 1-1). With the aids of new generation of sequencing technologies and bioinformatics tools, functional and comparative metagenomics analysis of intestinal BSH in food animals is warranted and will provide a better picture for the diversity and function of BSH in the intestine. Information in conjunction with other phenotypic examinations would improve

our understanding on the role of BSH in the symbiotic relationship between the gastrointestinal microbiome and animal host. Given that specific BSH protein(s) and corresponding BSH-producing bacteria may serve as biomarker for health status of animal host, understanding the ecology of BSHs and BSH-producing bacteria in the intestine would facilitate the development of diagnostics to evaluate the health status of animals and humans.

- Comprehensive evaluation using a controlled system together with a new model system is still critically needed to provide new mechanistic information for the role of BSH in host energy harvest and weight gain. Given the increasing awareness of the important roles of microbiota in intestine health, development of specifically tailored probiotics is a logical strategy for practical application, although this approach needs an in-depth understanding of the molecular, physiological, and ecological features of probiotic organisms in order to select and design probiotics for safe, effective administration for specific purposes. To date, there are not any studies using BSH negative and BSH-overproducing probiotic organisms to definitively link BSH activity to the specific phenotype and their impacts on the host animals and native microbiome. This is likely due to the challenge of manipulating BSH activity in commensal organisms for specific laboratory animal host and unacceptability of using generally modified organisms (GMOs) in human trials. While this concern has been partly addressed by recent *E. coli* knock-in model (Joyce *et al.*, 2014a), manipulating the BSH activity of a natural intestinal commensal organism in an animal model would be a better approach. Recent characterization of *L. salivarius* as a potent BSH producer (Wang *et al.*, 2012b) provides an excellent opportunity to address

this issue using food animal model system because genetic tools to manipulate *L. salivarius* have been well established. Such research efforts would enable us to better manage body weight by manipulating microbiota in humans and animals.

- Developing alternatives to AGPs by inhibiting BSH activity in the intestine. In addition to discovering more novel BSH inhibitors, comprehensive animal trials are essential to further evaluate and select desired BSH inhibitors. It is likely that prolonged use of a particular BSH inhibitor could lead to negative physiological consequences due to pleotropic effects of specific inhibitor and complexity of host physiology. For example, because the BSH inhibitors are expected to improve lipid metabolism, it is important to examine if energy harvest and weight gain is partitioned adequately and not skewed toward excess fat deposition, which would be undesirable for both animal producers and consumers. In addition, it is also warranted to examine how inhibition of BSH activity affects the bile profile, as well as the gastrointestinal microbial community and all the implications that these changes hold for animal health and productivity.

- Structural basis of BSH function. Given the ecological diversity of BSH in intestinal microbiome, structure analyses of BSHs from various species are highly warranted, which would discover critical residues in catalysis and provide key information on the substrate selectivity of BSHs. Clearly, such basic studies also will directly facilitate future translational research, such as using molecular docking to develop desired BSH inhibitors for growth promotion in food animals.

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Appendix

Table 1- 1. The BSHs identified in bacteria from various sources.

Source	Host Strain[a]	Molecular Mass (kDa)[b]	*Km*[d]						pH Optimum[b]	Temperature Optimum[b] (℃)	Reference
			GCA	GDCA	GCDCA	TCA	TDCA	TCDCA			
Human intestine											
	Bacteroides fragilis ATCC 25285	32.5	0.35	0.2	0.26	0.45	0.17	0.29	4.2-4.5	ND	(Stellwag *et al.*, 1976)
	Bacteroides fragilis NCTC 9343	ND	ND	ND	+	ND	ND	+	5.0-6.0	ND	(Aries *et al.*, 1970)
	Bacteroides fragilis 2536	ND	ND	+	+	+	+	+	4.5-5.0	ND	(Masuda, 1981)
	Bacteroides vulgatus I-1	ND	ND	-	-	-	-	+	4.5-5.0	ND	(Masuda, 1981)
	Bacteroides vulgatus VI 31	36	-	-	-	+	+	+	5.6-6.4	ND	(Kawamoto *et al.*, 1989)
	Bifidobacterium longum BB536	40	1.33	1.37	1.4	0.875	0.516	1.61	5.5-6.5	35-40	(Grill *et al.*, 1995)
	Bifidobacterium longum SBT2928	35	0.16	0.28	0.13	1.12	0.79	0.33	5.0-7.0	40-45	(Tanaka *et al.*, 2000)

Table 1- 1. Continued.

Source	Host Strain[a]	Molecular Mass (kDa)[b]	*Km*[d]	pH Optimum[b]	Temperature Optimum[b] (℃)	Reference	Source	Host Strain[a]	Molecular Mass (kDa)[b]	*Km*[d]	pH Optimum[b]
	Bifidobacterium bifidum ATCC 11863	35	+	+	+	+	+	+	ND	ND	(Kim *et al.*, 2004)
	Bifidobacterium adolescentis ATCC 15705	35	ND	ND	ND	ND	ND	ND	ND	ND	(Kim *et al.*, 2005a)
	Clostridium perfringens ATCC 19574	ND	ND	ND	ND	ND	ND	ND	5.6-5.8	ND	(Nair *et al.*, 1967)
	Clostridium perfringens PB 6K	ND	+	+	+	+	+	+	4.5-5.0	ND	(Masuda, 1981)
	Clostridium sordellii 4709	ND	+	+	+	+	+	+	4.5-5.0	ND	(Masuda, 1981)
	Lactobacillus acidophilus L1	126[c]	+	+	+	+	+	+	3.5-5.5	ND	(Corzo *et al.*, 1999)
	Lactobacillus acidophilus O16	126[c]	+	+	+	+	+	+	3.5-6.0	ND	(Corzo *et al.*, 1999)
	Lactobacillus acidophilus NCFM	ND	+	+	+	+	+	+	ND	ND	(McAuliffe *et al.*, 2005)

Table 1- 1. Continued.

Source	Host Strain[a]	Molecular Mass (kDa)[b]	*Km*[d]	pH Optimum[b]	Temperature Optimum[b] (℃)	Reference	Source	Host Strain[a]	Molecular Mass (kDa)[b]	*Km*[d]	pH Optimum[b]
	Lactobacillus sp. strain100-12	ND	ND	ND	ND	+	ND	ND	ND	ND	(Lundeen *et al.*, 1990)
	Listeria monocytogenes	ND	ND	ND	ND	ND	ND	ND	ND	ND	(Dussurget *et al.*, 2002)
Murine intestine											
	Lactobacillus sp. strain100-100	42	ND	ND	ND	+	ND	ND	3.8-4.5	ND	(Lundeen *et al.*, 1990)
	Lactobacillus sp. strain100-16	ND	ND	ND	ND	+	ND	ND	ND	ND	(Lundeen *et al.*, 1990)
	Lactobacillus sp. strain RI	ND	ND	ND	ND	+	ND	ND	ND	ND	(Lundeen *et al.*, 1990)
Pig intestine											
	Lactobacillus acidophilus ATCC 43121	126[c]	+	+	+	+	+	+	3.5-5.5	ND	(Corzo *et al.*, 1999)

Table 1- 1. Continued.

Source	Host Strain[a]	Molecular Mass (kDa)[b]	*Km*[d]	pH Optimum[b]	Temperature Optimum[b] (℃)	Reference	Source	Host Strain[a]	Molecular Mass (kDa)[b]	*Km*[d]	pH Optimum[b]
	Lactobacillus acidophilus PF01	35	-	-	-	+	+	+	6	40	(Oh *et al.*, 2008)
	Lactobacillus johnsonii PF01	36 &37	+	+	+	+	+	+	5.0	55 (BSH A) &70 (BSH C)	(Chae *et al.*, 2013)
	Lactobacillus sp. strain 100-33	ND	ND	ND	ND	+	ND	ND	ND	ND	(Lundeen *et al.*, 1990)
Chicken intestine											
	Lactobacillus salivarius NRRL B-30514	37	1.71	1.15	2.48	3.21	3.19	2.53	5.0-6.0	35-55	(Wang *et al.*, 2012b)
Other											
Fermented milk	*Bifidobacteriu. animalis* DN 173010	ND	ND	ND	ND	ND	ND	ND	ND	ND	(Lepercq *et al.*, 2004)
Springs	*Brevibacullus sp.*	28	ND	ND	ND	ND	ND	ND	9	60	(Sridevi *et al.*, 2009)

Table 1- 1. Continued.

Source	Host Strain[a]	Molecular Mass (kDa)[b]	*Km*[d]	pH Optimum[b]	Temperature Optimum[b] (℃)	Reference	Source	Host Strain[a]	Molecular Mass (kDa)[b]	*Km*[d]	pH Optimum[b]
Fermented milk	*Clostridium perfringens* MCV 815	56.0	+	+	+	+	+	+	5.8-6.4	ND	(Gopal-Srivastava *et al.*, 1988)
Fermented finger millet	*Pediococcus pentosaceus* KID7	ND	ND	ND	ND	ND	ND	ND	ND	ND	(Damodharan *et al.*, 2015)
Fermented milk	*Lactobacillus acidophilus* sp.	ND	ND	ND	ND	ND	ND	ND	ND	ND	(Pinto *et al.*, 2006)
Parakeet	*Lactobacillus salivarius* LMG 14476	140 - 142[c]	+	+	+	+	+	+	5.5-7.0	ND	(Bi *et al.*, 2013; Li *et al.*, 2006)
Raw milk	*Lactobacillus plantarum*	ND	ND	ND	ND	ND	ND	ND	ND	ND	(Sieladie *et al.*, 2011)
Silage	*Lactobacillus plantarum* CGMCC 8198	35-39	ND	ND	+	-	ND	+	ND	ND	(Gu *et al.*, 2014)
Silage	*Lactobacillus plantarum* Lp09 AND Lp45	ND	ND	ND	ND	ND	ND	ND	ND	ND	(Huang *et al.*, 2013)

Table 1- 1. Continued.

Source	Host Strain[a]	Molecular Mass (kDa)[b]	*Km*[d]	pH Optimum[b]	Temperature Optimum[b] (℃)	Reference	Source	Host Strain[a]	Molecular Mass (kDa)[b]	*Km*[d]	pH Optimum[b]
Kefir grains	*Lactobacillus plantarum* BBE7	43	ND	ND	+	ND	ND	ND	ND	ND	(Dong *et al.*, 2012)
Soil	*Xanthomonas maltophilia* CBS 827.97	52	1.1	ND	ND	ND	ND	ND	7.9-8.5	25-40	(Dean *et al.*, 2002)

[a]ATCC, American Type Culture Collection; JCM, Japanese Collection of Microorganisms; CGMCC, China General Microbiological Culture Collection Center; NRRL, Northern Regional Research Laboratory, the Agricultural Research Service Culture Collection.
[b]ND: Not Determined.
[c]Molecular mass of tetramer.
[d]*Km*, Michaelis constant in Michaelis-Menten kinetics.

Chapter II. Effects of oral administration of bile salt hydrolase on host lipid and energy metabolism using a rat model

Abstract

Bile salt hydrolase (**BSH**), a widely distributed enzyme of the gut microbiota, has recently been proposed to play a critical role in host lipid metabolism and energy harvest. Specifically, BSH catalyzes an essential gateway reaction in the metabolism of bile salts, leading to profound impact on host lipid digestion. Extensive animal and human studies have suggested that BSH is a promising microbiome target for developing novel feed additives to replace antibiotic growth promoters and for developing new measures to control obesity in humans. However, direct evidence supporting the significant role of intestinal BSH in host lipid metabolism, energy harvest, as well as body weight change is still very limited. In this study, the effect of oral administration of encapsulated BSH on lipid and energy metabolism was determined using a rat model system. Large quantities of recombinant BSH were purified from *Escherichia. coli* and encapsulated using a novel system for controlled release in the gut. Three-week old male SD rats (8 rats per group) were fed with encapsulated BSH (treatment) and BSA (control) at the equivalent amount of 2.7 mg/kg of rat body weight daily by gavage for 9 weeks. During each week, rats were placed in separate cages in a Comprehensive Lab Animal Monitoring System (CLAMS) to monitor individual rat respiratory exchange ratio (RER), an indicator of host energy harvest status. Data were analyzed using proc mixed command in SAS v9.4 with the model fitted with BSH treatment, time, and animal. The rats from two groups did not show significant differences ($P > 0.05$) in body weight, feed efficiency, weekly RER, nor concentrations of plasma glucose, triglycerides, and total cholesterol. However, bile salt profile, determined by UPLC-MS, showed a dramatic difference in response to BSH treatment, especially in fecal samples.

Keywords: Lipid metabolism, bile salt hydrolase, encapsulation, bile salt profile

Introduction

Bacterial bile salt hydrolase (BSH) enzymes in the gut catalyze an essential gateway reaction in the metabolism of bile acid, which plays an important role in direct digestive role in the emulsification of dietary fats in the intestine and also serves as signaling molecules to affect energy metabolism, host cholesterol level, and energy homeostasis (Begley *et al.*, 2006; Joyce *et al.*, 2014b). Although studies have provided indirect evidence to support the critical role of intestinal BSHs in host lipid metabolism and body weight gain, direct supporting evidence is still very limited. Recently, Joyce *et al.* (Joyce *et al.*, 2014a) obtained the first direct evidence demonstrating that manipulation of *in situ* BSH activity significantly affected host lipid metabolism, signaling functions, and weight gain by using a controlled and reductionist approach. In this study, colonization of germ-free mice or conventional mice with a BSH-producing *E. coli* strain elevated intestinal BSH activity and resulted in local bile acid deconjugation with concomitant reduced levels in body weight and cholesterol, alternations in lipid metabolism, signaling functions, local and systemic transcriptome profiles in the pathways governing lipid metabolism (Joyce *et al*., 2014a). This mice work clearly identified BSH as a key mechanism through which the microbiota modulates host lipid metabolism and energy harvest. However, this finding should be further confirmed using other controlled approaches with a different animal model.

The hypothesis of this study is that oral administration of purified BSH would enhance intestinal BSH activity, consequently changing host bile profile, decreasing host lipid metabolism, energy harvest, and body weight gain. To maintain maximum BSH activity in the intestine, a novel encapsulation approach was used to protect BSH activity through long time storage and when it

passes gastrointestinal tract. To test our hypothesis, large quantities of recombinant BSH (rBSH) enzymes were purified and then subjected to encapsulation. After oral administration of encapsulated rBSH (treatment) or BSA (control) to rats, various parameters, such as body weight, weekly body weight gain, feed efficiency, blood parameters (plasma glucose, triglycerides and total cholesterol), weekly respiratory exchange ratio (RER), and bile profile in fecal and plasma samples were measured.

Materials and methods

Bacterial strain, plasmid, and compound

The *E. coli* JL1139 that contains a recombinant plasmid bearing a histidine-tagged BSH gene (Lin *et al.*, 2014) was used for rBSH expression and purification in this study. The key background information for the strain was summarized in Table 2-1. Glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDCA), taurocholic acid (TCA), taurodeoxycholic acid (TDCA), taurochenodeoxycholic acid (TCDCA) and ampicillin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Expression and Purification of rBSH

The procedures of protein expression and purification were as described in previous publications with slight modification (Lin *et al.*, 2005; Wang *et al.*, 2012b). Stored *E. coli* JL1139 was streaked on Luria-Bertani (LB) agar plate with ampicillin (100 μg/ml) and cultivated overnight at 37 ℃. Two to three single colonies were picked from the LB agar plate and cultured in 50 mL LB broth with ampicillin (100 μg/ml) at 37 ℃ overnight (>16 h) in a rotary shaker. The

overnight culture (10 mL) was then inoculated into 200 mL Luria-Bertani (LB) broth with ampicillin (100 μg/ml) and grown at 37 ℃ in a rotary shaker (300 rpm) to the mid-log phase (OD_{600nm}=0.4-0.6). One milliliter of bacterial culture was transferred to microcentrifuge tube to prepare "pre-induction" whole cell sample by centrifuging for 1 min at 13,000 rpm (accuSpin Micro 7, Fisher Scientific, Pittsburgh, PA, USA). After removing all supernatant, the pellet was re-suspended in 50 μL 2 x loading buffer, pre-induction whole cell sample was stored at -20 ℃ freezer prior to Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Subsequently, isopropyl β-D-1-thiogalactopyranoside was added to the remaining culture at final concentration of 0.5 mM to induce the production of rBSH. The culture was incubated at 37 ℃ for 3 hours. At the time point of one and three hours, "post-induction" samples were prepared with the same procedure as "pre-induction" sample. All of the rest cultures were centrifuged at 4 ℃ at 5,000 *g* for 20 min (Avanti J-26 XP Centrifuge, Beckman Counter, Indianapolis, IN, USA). The pellet was washed with 20 mL PBS for three times. The pellet was finally suspended in 20 mL of ice-cold Lysis buffer (50 mM sodium phosphate buffer, 300 mM NaCl, 10 mM imidazole, pH 7.0) supplemented with 10 mM β-mercaptoethanol (β-ME), 5 mM of ATP, 1 mg/ml lysozyme and 5 mM of $MgCl_2$. The cell solution was processed for sonication and the cell suspension was centrifuged at 10,000 rpm for 30 min. After centrifugation, the supernatant (lysate) was used for rBSH purification as described below.

The lysate was mixed with the nickel-nitrilotriacetic acid (Ni-NTA) agarose resin (Qiagen) that had been equilibrated with the same lysis buffer; the mixture was gently rocked for 60 min at 4 ℃. Then, the mixture was loaded into a 15 mL polypropylene column (Qiagen) and the flow-through was collected. The column was then washed with 5-bed volumes of Wash buffer (50

mM sodium phosphate buffer, 300 mM NaCl, 20 mM imidazole, 10% glycerol, pH 7.0) supplemented with 5 mM β-ME, 5 mM of ATP, and 5 mM of $MgCl_2$. The flow-through samples were collected for further SDS-PAGE analysis. Five-bed volumes of Elution buffer (50 mM sodium phosphate buffer, 300 mM NaCl, 300 mM imidazole, 10% glycerol, pH 7.0) supplemented with 5 mM β-ME were used to wash the column in order to elute the protein bounded with Ni-NTA. The fractions containing rBSH, which were determined by SDS-PAGE as detailed below, were pooled and dialyzed against Dialysis buffer (50 mM sodium phosphate buffer, 10% glycerol, pH 7.0) containing 5 mM L-glutathione. The dialyzed rBSH was collected and stored at -80 ℃ prior to use. For the BSH that was used for encapsulation purpose, glycerol was not added in the Dialysis buffer. The activity of purified rBSH was tested by a modified two-step BSH activity assay (Tanaka *et al.*, 2000; Wang *et al.*, 2012b) and protein concentration was measured using bicinchoninic acid (BCA) protein assay kit (Pierce).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

A 5 mL 12% resolving gel was prepared by mixing 1.6 mL dd H_2O, 2.0 mL 30% acrylamide, 1.3 mL 1.5 M Tris (pH6.8), 50 μL 10% SDS, 50 μL 10% ammonium persulfate (APS) and 2 μL TEMED. The mixture was loaded into hand casting plates (Bio-Rad). The top of the gel was sealed with water saturated isobutanol. After polymerization was complete (30 min), the overlay was poured off and the resolving gel was covered by stacking gel (1.4 mL dd H_2O, 330 μL 30% acrylamide mix, 250 μL of 1.5 M Tris (pH8.8), 20 μL 10% SDS, 20 μL 10% APS and 2 μL TEMED for 2 mL stacking gel). A clean Teflon comb was immediately inserted into the stacking solution until the gel is polymerized. Five microliters of the whole cell lysate suspension or purified protein sample was mixed with 5 μL 2 x protein loading butter and protein

was degenerated by incubating in boiling water for 10 min; degenerated samples were then loaded in each lane and separated by SDS-PAGE at 80V for 25 min followed by 160V for 40 minutes by electrophoresis. The gel was stained with Coomassie blue G-250.

BSH activity assay

The two step BSH activity assay was performed based on the standard procedures described previously (Tanaka *et al.*, 2000; Wang *et al.*, 2012b). In the first step, the reaction mix was composed of 178 µL reaction buffer (0.1 M sodium phosphate, pH 6.0), 10 µL of 1 µg/ µL BSH, 10 µL of 100 mM sodium glycocholate and 2 µL of 1 M DTT. The reaction mix was incubated in 37 °C water bath (or heat block) for 30 min. Following the reaction, 50 µL reaction mix was mixed immediately with 50 µL of 15% trichloroacetic acid (TCA) by vortexing. In the second step, the 50 µL supernatant from last step after centrifugation (12,000 *g* for 5 min) was mixed thoroughly with 950 µL ninhydrin reaction mix (5 mL of 1% w/v ninhydrin, 0.2 mL of 0.5 M sodium citrate buffer [pH 5.5], 1.2 mL glycerol) by vortexing. The mixture was then incubated on boiling water for 14 min and the reaction was stopped by putting the reaction mix tube on ice for 3 min. After cooling down, the reaction reagents was transferred into 1 mL cuvette and the absorbance of the reagents was measured with a Smart Spec Plus spectrophotometer (Bio-Rad) at 570 nm. The absorbance at 570 nm was used to present relative BSH activity.

Protein encapsulation

The purified rBSH, as well as the control protein bovine serum album (BSA, Fisher Scientific), were encapsulated using an established coating system (Zhang *et al.*, 2016). Briefly, 650 mL of

dialyzed rBSH (6.15 mg/mL) was spray dried to produce micro particles with a mini spray-dryer (model B-290 mini spray-dryer, BÜCHI Labortechnik AG, Flawil, Switzerland) at inlet temperature of 105 ℃ and outlet temperature of 60 ℃ with the airflow rate of 35 m^3/h and feed rate at 10%-15%. Then 2.18 g of micro particles were suspended in 22 mL soybean oil in a beaker by stirring for 30 min. The suspension was subsequently emulsified with 176 mL sugar-beat pectin at O : W phase volume ratio of 1:8 using a high-speed homogenizer (12,000 rpm for 3 min) (model Cyclone I.Q.^{2}, The VirTis Co., Inc., Gardiner, NY, USA). Finally, the obtained emulsion was vacuum freeze-dried to produce powdered sample that was stored in a -80 ℃ freezer prior to oral gavage for rats. The procedures for BSA encapsulation were the same as rBSH encapsulation procedures. The encapsulated particles for encapsulation measurement purpose was subjected to spray drying instead of freeze-drying to produce high resolution image through scanning electron microscopy.

Scanning electron microscopy (Akbarian et al., 2016)

LEO 1525 SEM microscope (SEM/FIB Zeiss Auriga, Oberkochen, Germany) was used for particle images at the Advanced Microscopy and Imaging Center at the University of Tennessee, Knoxville. After encapsulation, the emulsion was spray-dried and the produced powdered samples were glued onto an adhesive tape mounted on the specimen stub and sputter-coated with a gold layer of about 5 nm in thickness as described by Zhang *et al* (Zhang *et al.*, 2015a).

Encapsulation efficiency

Encapsulation efficiency was determined by measuring protein amount in the aqueous phase after encapsulation and encapsulation efficiency were measured in duplicate. Specifically, 40 mL emulsions were centrifuged at 13,000 *g* for 2 min and the supernatant was collected for protein quantification using BCA assay (Pierce). Calculation formula was as follows.

$$\text{Encapsulation efficiency (\%)} = 100 * \frac{\text{Total protein(g)} - \text{Free protein(g)}}{\text{Total protein (g)}}$$

Zeta potential

The stability of encapsulation dispersion was reflected by zeta potential. To measure Zeta potential, 1 mL of encapsulation emulsion was transferred to a 1.5 mL Eppendorf tube and centrifuged at 13,000 *g* for 2 min to remove free molecules in the supernatant. After removing supernatant, the precipitate was dissolved in 1 mL distilled water (pH 7.0) and was further diluted 100-fold using distilled water (pH 7.0) for measuring zeta potential with Delsa Nano instrument (Beckman Coulter Inc., Fullerton, CA, USA). Zeta potential was measured in triplicate.

Rat experiment

The process of rat experiment followed the University of Tennessee Institutional Animal Care and Use Committee protocol (IACUC # 2123). Newly born Sprague Dawley (SD) male rats were kept with original mother rats for 3-week of lactation period. Subsequently, 16 male SD rats at weaning were randomly allocated into two groups (eight rats per group) and each group of rats was randomly allocated into four rat stocks (two rats per stock). During 9-week experiment

period, each rat was fed with water dissolved with encapsulated rBSH or BSA (2.7 mg/kg body weight) daily by gavage in the evening (at 6:00 pm) when rodents begin to eat. Along the experimental process, rat body weight (BW) was determined weekly in conjunction with dorsal blood glucose concentration measurement using glucometer (Bayer). Each rat was housed in one chamber in a comprehensive laboratory animal monitoring (CLAMS) system for two times each week and for each time, rat respiratory exchange ratio (RER) was constantly measured for 24 hours. RER is the ratio between amounts of carbon dioxide (CO_2) produced and oxygen (O_2) consumed in metabolism; the value of RER gives an indication of predominant fuel sources for the host. Rat feed intake was measured during the time rats were kept in CLAMS. All rats were deprived of food for 12 hour and euthanized by CO_2 upon termination of the experiment. Upon euthanizing, rats were dissected and 5 mL rat vena cava blood, intestine and fecal samples (triplicate) were collected. Blood samples were stored in vacuum blood collection tubes at 4 °C immediately and centrifuged at 4,000 *g* for 15 min, while the upper phase of plasma was collected and aliquoted into Eppendorf tubes and stored in -80 °C freezer prior to use. The collected tissue samples were stored in a -80 °C freezer prior to analysis.

Plasma biochemical analyses

The concentrations of plasma glucose, triglycerides, and total cholesterol were determined using commercial kits (Stanbio Laboratory Inc., Boerne, TX, USA) following standard protocols. Analyses were conducted in triplicate.

Bile profiles analyses

Chicken fecal samples (about 300 μL) and plasma samples (500-800 μL) from all sixteen rats were packed with dry ice and shipped to Dr. Susan Joyce (APC Microbiome Institute, University College Cork, Ireland) for bile acid profile analysis. The metabolomics analysis was performed by Ultra Performance Liquid Chromatography–Tandem Mass Spectrometry (UPLC-MS); the detailed procedures for bile acids extraction, characterization, and quantification were described in a previous publication (Joyce *et al.*, 2014a). The metabolomics data were subjected to statistical analysis as described below.

Statistical analysis

The concentrations of plasma glucose, total cholesterol and triglycerides; body weight and weekly blood glucose concentrations were analyzed using mixed model analysis of variance procedure; RER data were analyzed using mixed model analysis of variance procedure with rat age as repeated measure and rBSH treatment as fixed effect. All of these analyses were conducted with SAS v9.4 (SAS Institute, Cary, NC, USA) and least square means were compared at the significance level of *P*-value less than 5% (0.05).

Results

Expression and purification of rBSH

As is shown in Figure 2-1, upon induction by IPTG for 1 hr, there was a dramatic increase in the production of protein with the molecular size of 32 kDa on SDS-PAGE, which was consistent with the estimated molecular size of *L. acidophilus* rBSH. High purity of the C-terminal His-

tagged rBSH was subsequently detected in eluted fractions (lane1-8) of *E. coli* culture usingNi-NTA agarose affinity chromatography. Approximately 250 mg of rBSH were routinely purified from 1 liter of induced *E.coli* culture. A total 7.24 g of rBSH was purified for this study. Detailed information about stock rBSH is presented in Table 2-2 and the purified rBSH showed comparable activity based on BSH two-step activity assays (data not shown).

Protein recovery rate during encapsulation and encapsulation efficiency

After the very first step of rBSH encapsulation-spray drying, the activity of enzyme powder was also measured by BSH activity assay and showed that the activity of spray-dried BSH powder was dramatically decreased (>70% reduction). However, it was not applicable to measure encapsulated rBSH activity before oral gavage due to technique limitation. The recovery rate for rBSH in the spray drying step was 27% (1.109 g/4 g). The recovery rate for BSA in the spray drying step was 23.6% (0.3776 g/1.6 g). Encapsulation efficiency of BSA was 25% based on duplicate experiments.

Morphology of spray-dried encapsulated protein

SEM images of spray-dried BSA emulsions are shown in Figure 2-2. Particles from different scopes showed that most particles had consistent size and shape; most spray-dried encapsulated BSA were ball-shaped particles with a dimension of around 10 μm. Since only spray-dried encapsulated protein can be tested with SEM microscope, spray-drying had a very low recovery rate and a dramatic enzyme activity loss, the morphology of encapsulated rBSH was not verified.

Zeta potential of reconstituted emulsions

Zeta potential of reconstituted emulsions of BSA is reported in Table 2-3. Zeta potential was measured at pH 7.0 where emulsions showed most stable emulsions based on previous publication (Zhang *et al.*, 2015b). The mean of zeta potential measurements of triplicate was 43.6±7.27 mV.

Growth performance in response rBSH treatment

During the nine weeks of the experiment, rats showed normal growth behavior, except that one rat in the treatment group stopped growing at five-week-old all through it showed abnormal behavior. Data from this specific rat were excluded from later analysis. Due to the uncertainty of encapsulated BSH and potential low efficacy of BSH in the intestine, the treatment plan was changed by the end of week 6 of the experiment time. Instead of feeding rats with encapsulated rBSH or BSA, rats started to be fed with freshly prepared rBSH and BSA in the treatment group and the control group respectively at an equivalent amount of 2.7 mg/kg relative to rat body weight. Rat growth performance data (body weight, body weight gain, feed intake, and feed conversion ratio) did not show statistically significant difference ($P > 0.05$) between rBSH and BSA treatment group (Table 2-4).

Respiratory exchange ratio

RER is an indicator of predominant fuel sources for animals. In this study, RER in both light cycle (7:00 -19:30) and dark cycle (19:30 -7:00) between encapsulated BSH and BSA treatment group during nine weeks of the treatment period were measured (Table 2-5). The RER value in

the light cycle and dark cycle ranging from 0.9279 to 1.0019, which indicated that carbohydrate was the predominant fuel sources for rats when they were set in the CLAMS chambers.

Biochemical analysis of plasma

Weekly dorsal blood glucose concentration of rats tested by glucometers from rBSH and BSA treatment group did not show a statistical difference for nine weeks of the treatment period ($P > 0.05$) (Table 2-6). Besides, compared to BSA treatment, BSH treatment did not affect the concentration of glucose ($P = 0.3902$), triglycerides ($P = 0.7813$), and total cholesterol ($P = 0.6161$) in rat plasma (Table 2-7).

Bile salts profile alterations in rat plasma and fecal samples by rBSH

Plasma samples and fecal samples from each rat in the two treatment groups (encapsulated rBSH and BSA) were processed for bile salts profile test by UPLC-MS. Twenty-two bile acids and amino acids related to bile metabolism were qualified and quantified (Table 2-8). The variation for some bile salts was large especially for those bile salts with low concentration; in fecal glyhyodeoxycholic acid (GHCA), the mean square error was 0.4605 which was almost half for the mean concentration of fecal GHCA in the treatment group and one third of the mean concentration of fecal GHCA in the control group.

PCA plot of bile acids (Figure 2-3) showed that rBSH treatment did not cause well separation of bile acid profile from BCA control group based on PC1, PC2, and PC3 for fecal samples (Figure 2-3A) and plasma samples (Figure 2-3B), which indicated that rBSH treatment caused bile acid

signature difference in neither fecal nor plasma samples. When checking each individual bile acids (Table 2-8) concentration in fecal samples, rBSH treatment caused significant difference in most of the measured bile acids ($P < 0.05$), namely taurine, TCA, TLCA, TUDCA,TDCA, total TMCA, LCA, HCA, α-MCA, β-MCA, ω-MCA, GCA ,GCDCA, GUDCA, GHCA, and total secondary and tertiary bile acids. While in the plasma, three of secondary bile salts had a significantly higher level in rBSH treated group than in the control group; the three bile salts were LCA ($P = 0.0033$), DCA ($P = 0.0423$) and GHCA ($P = 0.0027$).

Discussion

For this project, we intended to encapsulate BSH using the novel technology (Zhang *et al.*, 2016) for enhanced *in vivo* delivery and availability of BSHs. However, multiple steps of encapsulation have led to dramatic reduction in both yield and activity of rBSH. Specifically, the recovery rate for rBSH in the spray-drying step was only 27% and the activity of the rBSH following spray-drying decreased to about 33% of freshly prepared rBSH. In addition, during the encapsulation step, the method used in this project only led to about 25% encapsulation rate (estimated by the encapsulation efficiency of BSA). Therefore, approximately 98% of the BSH activity was lost after multiple steps of encapsulation in this study, which raised a significant challenge for encapsulation of BSHs for the proposed rat study.

Due to the challenge of dramatic reduction in the quality and activity of rBSH, an alternative approach to oral delivery of protein is likely a promising method. Oral administration of genetically modified BSH-producing *Lactobacillus* will be considered. First of all, genetic modification of *Lactobacillus* has already been established (Law *et al.*, 1995; Van Gaal *et al.*,

2006) and *Lactococcus* has been developed as a vehicle to deliver therapeutic agents to the gastrointestinal (GI) tract in the past decades (Steidler *et al.*, 2006; Wells *et al.*, 2008). Bao *et al.* (Bao *et al.*, 2013) tested the effectiveness of oral administration of *Lactobacillus*. They found that after 24 h of oral feeding of *Lactobacillus* to neonatal SD rats, about 3% of the bacteria were retained in the GI tract and more bacteria were found in the stomach and small intestine than cecum and colon. *Lactobacillus* as an oral delivery vehicle also showed dramatically lower mortality than using *E. coli* as an oral delivery vehicle to neonates (Bao *et al.*, 2013). In addition, preparation of sufficient *Lactobacillus* culture requires much less work than the preparation of protein. Notably, to enhance bacterial colonization in the small intestine and decrease cell death under harsh conditions in the GI tract, a novel encapsulation system has been established in emulsion of *Lactobacillus* with multiple lipid-protein-pectin layers (Zhang *et al.*, 2016).

Respiratory exchange ratio was used in this project to indicate the fuel source and energy expenditure of rats. However, there was no significant difference between the rBSH and BSA treatment groups, primarily due to the low activity of encapsulated BSH. This also could be due to lower energy requirement when the rats were staying in the CLAMS chambers. In the process, it was observed that rats had lower activity when individual rats were kept in separate chambers than two rats kept in the regular chambers. In a recent study (Paes *et al.*, 2016), researchers looked into RER change of Wistar rats during activities with different intensities. In a moderate-intensity continuous excise, the RER of Wistar rats was significantly lower than a high-intensity continuous excise. Even though they used a different animal model, their results

implied that the RER data from this study from the CLAMS system may not represent the RER level when rats were in normal natural activity.

In the future, other animal models, such as mouse and chicken, may be used to validate the physiological role of BSH. However, the several factors need to be taken into consideration. First and foremost, bile profile for different animals varies. In rats, the predominant conjugated bile salts are glycine conjugated bile salts, while in mouse and chicken, predominant conjugated bile salts are taurine conjugated bile salts. While the proposed mechanism of BSH effects on host physiology is mediated through deconjugation of bile salts in the intestine, the downstream changes in bile profile resulting from bile decongujation can further affect host physiology through complex signaling pathways (Joyce *et al.*, 2014b). In this case, even if other factors are well controlled, the animals fed with the same BSH may show different physiological response due to differences in bile profile and substrates preference of BSH. To address this issue, we may use the BSH with wide substrates spectrum such as the BSH from *Lactobacillus salivarius* NRRL B-30514 (Wang *et al.*, 2012b). Additionally, different animal models also have different characteristics of lipid metabolism (Suckling *et al.* (1993). Take fatty acid synthesis process for example, chicken use the liver as the major tissue for fatty acid synthesis while for mouse, both liver and adipose tissues are used for fatty acid synthesis. When testing plasma parameters (especially lipoproteins) as indicators of host lipid metabolism, the choice of a specific animal model is also a factor that needs to be considered (Suckling *et al.*, 1993).

In conclusion, though sufficient high-quality rBSH (7.24 g) was purified in this study and subsequently encapsulated for rat oral administration, solid direct evidence related to BSH

function towards host lipid metabolism and energy harvest was not obtained. Dramatic fecal bile profile change in response to BSH treatment gave us confidence of BSH physiological roles. The above approaches need to be considered to get direct evidence of BSH function.

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Appendix

Table 2- 1. Key background information for the BSH produced by the recombinant *E. coli* JL1139.

Parameters	Corresponding information
Source organism	*Lactobacillus acidophilus* PF01
DNA source	Genomic DNA
Cloning vector	pET-21b
Expression vector	pET-21b
Expression host	*E. coli* BL-21(DE3)
Complete amino acid sequence of the construct produced [a]	MCTGLRFTDDQGNLYFGRNLDVGQDYGEGVIITPGNYPLPYKF LDNTTTKKAVIGMGIVVDGYPSYFDCYNEDGLGIAGLNFPHFA KFSDGPIDGKINLASYEIMLWVTQNFTHVSEVKEALKNVNLVN EAINTSFAVAPLHWIISDSDEAIIVEVSKQYGMKVFDDKVGVLT NSPDFNWHLTNLGNYTGLNPHDATAQSWNGQKVAPWGVGTG SLGLPGDSIPADRFVKAAYLNVNYPTAKGEKANVAKFFNILKS VAMIKGSVVNDQGKDEYTVYTACYSSGSKTYYCNFEDDFELK TYKLDDHTMNSTSLVTYLERHHHHHH

[a] The His-tag site is underlined.
The key information of BSH was originally introduced in a previous publication(Oh *et al.*, 2008).

Table 2- 2. Summary of mass production of rBSH.

Experiment stock	Culture volume (mL)	rBSH concentration (mg/mL)	Purified rBSH volume (mL)	rBSH amount (Paes *et al.*, 2016)
1/7/2017	500	13.6	3.5	47.6
		7.0	8	56
1/9/2015	2000	15.9	13	206.7
		5.3	33	174.9
1/21/2015	1500	16.8	16	268.8
		3.8	34	129.2
2/10/2015	2500	16.0	32.5	520
		2.4	50	120
3/23/2015	2000	9.0	42.5	382.5
		2.3	50	115
3/24/2015	2000	10.4	41	426.4
		2.5	53	132.5
4/22/2015	3000	11.7	55	643.5
		2.6	62	161.2
4/23/2015	3000	11.8	57	672.6
		1.5	82	123
4/28/2015	3000	7.7	40	308
		7.6	45	342
5/7/2015	3000	9.2	45	414
		8.9	41	364.9
5/16/2015	3000	10.6	45	477
		10.0	46.5	465
5/27/2015	3000	6.8	34	231.2
		8.6	27	232.2
		6.8	35	239

Table 2- 3. Zeta potential of reconstituted emulsions at pH 7.0.

	Temperature (℃)	Zeta potential (mV)
Mean	25	-43.6
Standard Deviation	0.1	7.27
RSD %[a]	0.28	16.7
Minimum	24.9	-58.5

[a] RSD stands for relative standard deviation.

Table 2- 4. Growth performance of rats in response to rBSH and BSA treatment.

Body weight (g/rat)			
Treatment time (week)	**BSA**	**rBSH**	***P* value**
1	54.73±5.30	54.9±5.70	0.9016
2	91.07±6.84	89.12±8.05	0.9281
3	143±8.38	136.81±10.06	0.5895
4	185.35±7.72	176.53±11.5	0.5217
5	232.86±11.0	222.81±14.01	0.8450
6	265.65±11.38	253.98±16.06	0.9485
7	296.12±11.21	280.39±17.74	0.8224
8	315.65±10.97	302.97±19.45	0.4704
9	336.03±11.6	339.04±12.81	0.5931
Body weight gain (g/rat/day)			
Treatment time (week)	**BSA**	**rBSH**	***P* value**
1	5.19±0.53	5.21±0.56	0.9764
2	6.49± 0.51	6.21± 0.97	0.8073
3	7.06± 0.46	6.97± 1.19	0.9495
4	7.92± 0.81	8.29± 2.20	0.8798
5	5.47± 0.17	5.68± 0.42	0.6524
6	4.35± 0.14	4.15± 0.15	0.3677
7	2.79± 0.19	3.46± 0.20	0.0529
8	2.91± 0.19	2.78± 0.20	0.6517
Feed intake (g of feed/rat/day)			
Treatment time (week)	**BSA**	**rBSH**	***P* value**
1	17.18±2.35	13.91±5.86	0.6253
2	14.55±1.14	13.87±1.21	0.6991
3	22.64±2.96	15.10±7.57	0.3895
4	19.24±0.87	18.94±0.93	0.8202
5	24.53±0.86	25.39±2.22	0.7286
6	25.17±0.87	25.19±1.80	0.9933
7	24.14±1.05	25.16±2.30	0.7019

Table 2- 4. Continued.

Feed intake (g of feed/rat/day)			
Treatment time (week)	**BSA**	**rBSH**	***P* value**
8	25.06±1.00	24.95±1.08	0.9469
Feed Conversion Ratio (g of feed/ g of weight gain)			
Treatment time (week)	**BSA**	**rBSH**	***P* value**
1	4.03±1.22	2.86±3.30	0.7524
2	2.33±0.20	2.25±0.22	0.7844
3	3.27±0.48	2.19±1.23	0.4418
4	2.74±0.46	2.30±1.24	0.7497
5	4.50±0.15	4.48±0.29	0.9566
6	5.82±0.30	6.16±0.33	0.4649
7	8.80±0.53	7.45±0.96	0.2661
8	8.65±0.48	9.32±0.52	0.3875

Growth performance data (body weight, body weight gain, feed intake and feed conversion ratio) are represented as mean of corresponding parameters with standard error. Statistical significant level of difference is defined by $P<0.05$.

Table 2- 5. Respiratory exchange ratio for rats in response to encapsulated rBSH and BSA treatment.

Respiratory exchange ratio at Light cycle				
Treatment time (week)	**BSA**	**rBSH**	**MSE**	***P* value**
1	0.9297	0.9320	0.006450	0.8071
2	0.9426	0.9481	0.007441	0.6104
3	0.9612	0.9699	0.007509	0.2659
4	0.9491	0.9579	0.007623	0.2676
5	0.9685	0.9810	0.01003	0.2328
6	0.9663	0.9901	0.01556	0.1472
7	0.9728	0.9486	0.02092	0.2670
8	0.9764	0.9717	0.008247	0.5801
9	0.9715	0.9600	0.01152	0.3575
Respiratory exchange ratio at Dark cycle				
Treatment time (week)	**BSA**	**rBSH**	**MSE**	***P* value**
1	0.9753	0.9663	0.008618	0.4689
2	0.9919	0.9884	0.007666	0.6598
3	1.0019	0.9955	0.003775	0.2569
4	0.9854	0.9883	0.003676	0.5791
5	0.9944	0.9972	0.005567	0.7242
6	0.9873	0.9798	0.004367	0.2483
7	0.9912	0.9853	0.005181	0.4370
8	0.9908	0.9891	0.003125	0.7043
9	0.9831	0.9795	0.005307	0.6432

Mean of RER was expressed as least square means and mean square error [MSE]. Statistical significant level of difference is defined by $P<0.05$.

Table 2- 6. Weekly blood glucose concentration (mg/dL) for rats in response to encapsulated rBSH and BSA treatment.

Experimental Time (Week)	Control	Treatment	MSE	*P* value
1	155	141.75	10.844	0.4162
3	119.75	131.00	8.663	0.2507
4	129.5	116.17	10.58	0.2967
5	133.88	124.86	10.103	0.4067
6	122.25	130.5	8.1761	0.3466
7	115.63	115.25	5.939	0.9514
8	117.29	106	8.8579	0.2586

Mean of weekly blood glucose concentration was expressed as least square means and mean square error [MSE]. Statistical significant level of difference is defined by $P<0.05$.

Table 2- 7. Summary of rats' vena cava plasm biochemical analyses in response to encapsulated rBSH and BSA treatment.

Plasma parameters (mg/dL)	Control	Treatment	MSE	*P* value
Glucose	462.37	441.49	9.7722	0.3902
Triglycerides	104.78	100.79	7.9383	0.7813
Total Cholesterol	105.59	100.47	7.6389	0.6161

Plasma glucose, triglycerides and total cholesterol concentrations were expressed as least square means and mean square error [MSE]. Statistical significant level of difference is defined by $P<0.05$.

Table 2- 8. Fecal bile acid (ug/g) profile analyzed by UPLC-MS.

Bile salts	Full name of bile salts	Control	Treatment	MSE	*P* value
Taurine	Taurine	120.38	148.64	9.8794	0.0515
TCA	Taurocholic acid	10.5798	6.6377	1.0495	0.0149
TCDCA	Taurochenodeoxycholic acid	6.0094	5.1918	1.8417	0.7430
TLCA	Taurolithocholic acid	1.3550	1.0168	0.1270	0.0671
TUDCA	Tauroursodeoxycholic acid	2.9235	4.4414	0.2618	0.0009
THCA	Taurohyodeoxycholic acid	4.7950	6.1966	1.8979	0.5869
TDCA	Taurodeoxycholic acid	9.2947	4.5432	1.2712	0.0153
Total TMCA	Total Tauromuricholic acid	6.8778	2.4340	0.7090	0.0005
LCA	Lithocholic acid	245.45	770.26	95.7859	0.0014
DCA	Deoxycholic acid	2260.62	2908.53	265.06	0.0894
UDCA	Ursodeoxycholic acid	3961.67	5735.64	644.03	0.0594
CDCA	Chenodeoxycholic acid	5.5065	2.6832	1.9783	0.3019
CA	Cholic acid	44.3177	61.8340	11.0836	0.2553
HCA	Hyodeoxycholic acid	13.7126	24.9600	2.2431	0.0026
β _MCA	β-Muricholic acid	903.56	3343.24	252.76	<.0001
ω _MCA	ω-Muricholic acid	1744.25	3314.10	328.48	0.0036
α _MCA	α-Muricholic acid	1458.80	2737.73	331.24	0.0129
GCA	Glycocholic acid	5.3912	28.2321	4.0795	0.0012
GCDCA	Glycochenodeoxycholic acid	1712.03	5415.42	552.84	0.0003
GUDCA	Glycoursodeoxycholic acid	339.18	854.93	113.43	0.0049
GDCA	Glydeoxycholic acid	2.6854	3.9589	0.4267	0.0435
GHCA	Glyhyodeoxycholic acid	1.6064	1.1561	0.4605	0.4740
PBA	Primary bile salts	49.8242	64.5172	10.6524	0.3177
STBA	Total secondary and tertiary bile salts	6467.74	9414.43	842.73	0.0214

Mean of each bile acids was expressed as least square means and mean square error [MSE]. Statistical significant level of difference is defined by $P<0.05$.

Table 2- 9. Plasma bile acid (ug/mL) profile analyzed by UPLC-MS.

Bile salts	Full name of bile salts	Control	Treatment	MSE	*P* value
Taurine	Taurine	13.5814	17.4414	1.6963	0.1299
TCA	Taurocholic acid	3.7112	4.1470	1.0001	0.7625
TCDCA	Taurochenodeoxycholic acid	0.9345	0.9103	0.1338	0.9002
TLCA	Taurolithocholic acid	0.08604	0.1794	0.05488	0.2489
TUDCA	Tauroursodeoxycholic acid	0.4873	0.5627	0.07624	0.4958
THCA	Taurohyodeoxycholic acid	1.7714	2.2943	0.3138	0.2583
TDCA	Taurodeoxycholic acid	0.8528	1.0904	0.1637	0.3222
Total TMCA	Total Tauromuricholic acid	0.7660	1.0493	0.2001	0.3339
LCA	Lithocholic acid	0.2654	0.6737	0.08185	0.0033
DCA	Deoxycholic acid	1.8694	3.4227	0.4917	0.0423
UDCA	Ursodeoxycholic acid	7.7613	24.1660	7.5709	0.1478
CDCA	Chenodeoxycholic acid	6.1267	12.1510	2.5302	0.1144
CA	Cholic acid	47.5023	116.87	29.8691	0.1228
HCA	Hyodeoxycholic acid	0.1175	0.09284	0.02424	0.4837
β _MCA	β-Muricholic acid	5.3919	14.3672	4.5837	0.1879
ω _MCA	ω-Muricholic acid	5.6672	20.0781	7.2193	0.1799
α _MCA	α-Muricholic acid	3.7615	14.2457	5.4299	0.1937
GCA	Glycocholic acid	5.8857	13.1302	3.4670	0.1617
GCDCA	Glycochenodeoxycholic acid	947.96	1765.69	464.70	0.2338
GUDCA	Glycoursodeoxycholic acid	211.68	583.19	180.92	0.1685
GDCA	Glydeoxycholic acid	1.1492	2.4109	0.4379	0.0610
GHCA	Glyhyodeoxycholic acid	1.0377	0.1966	0.1639	0.0027
PBA	Primary bile salts	53.6289	129.02	32.1905	0.1200
STBA	Total secondary and tertiary bile salts	9.8961	28.2625	7.9712	0.1255

Mean of each bile acids was expressed as least square means and mean square error [MSE]. Statistical significant level of difference is defined by $P<0.05$.

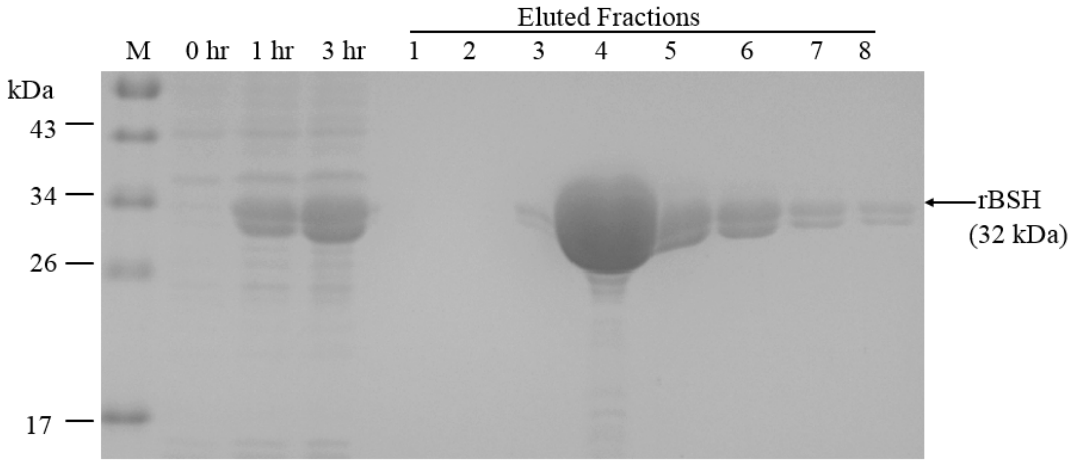

Figure 2- 1. Purification of *L. acidophilus* BSH enzyme.
Lane M, EZ Run™ prestained molecular mass marker; 0 hr, whole cell lysate of non-induced *E.coli*; 1 hr, whole cell lysate of *E. coli* induced with 0.5 mM IPTG for 1 hr; 3 hr, whole cell lysate of *E. coli* induced with 0.5 mM IPTG for 3 hr; lane 1-8, eluted fractions during protein purification.

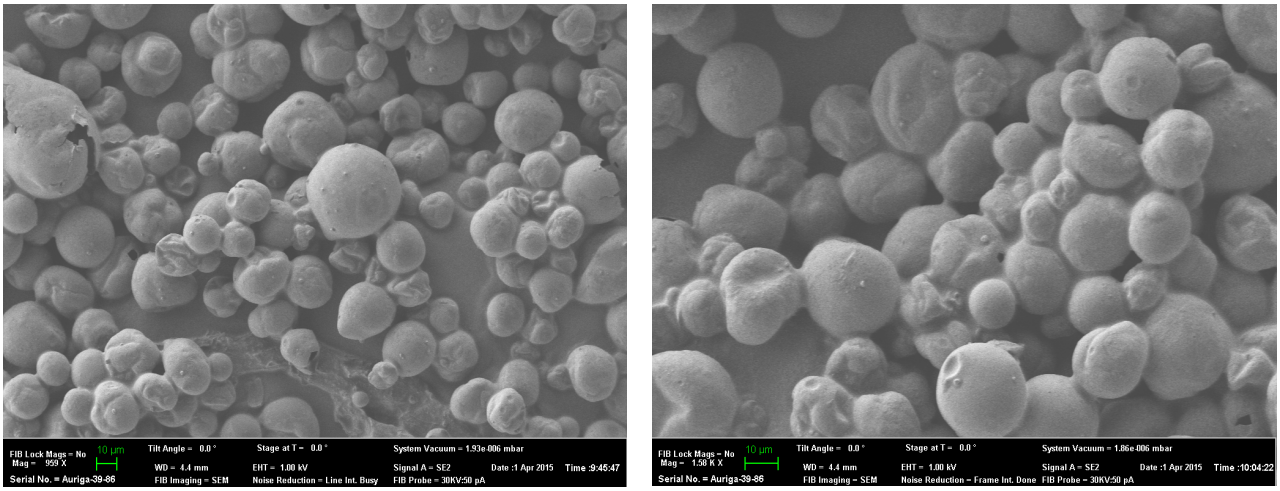

Figure 2- 2. Morphology of spray-dried encapsulated powder with BSA. Both figures are for spray-dried encapsulated BSA from different scope. Scale bar equals to 10 µm.

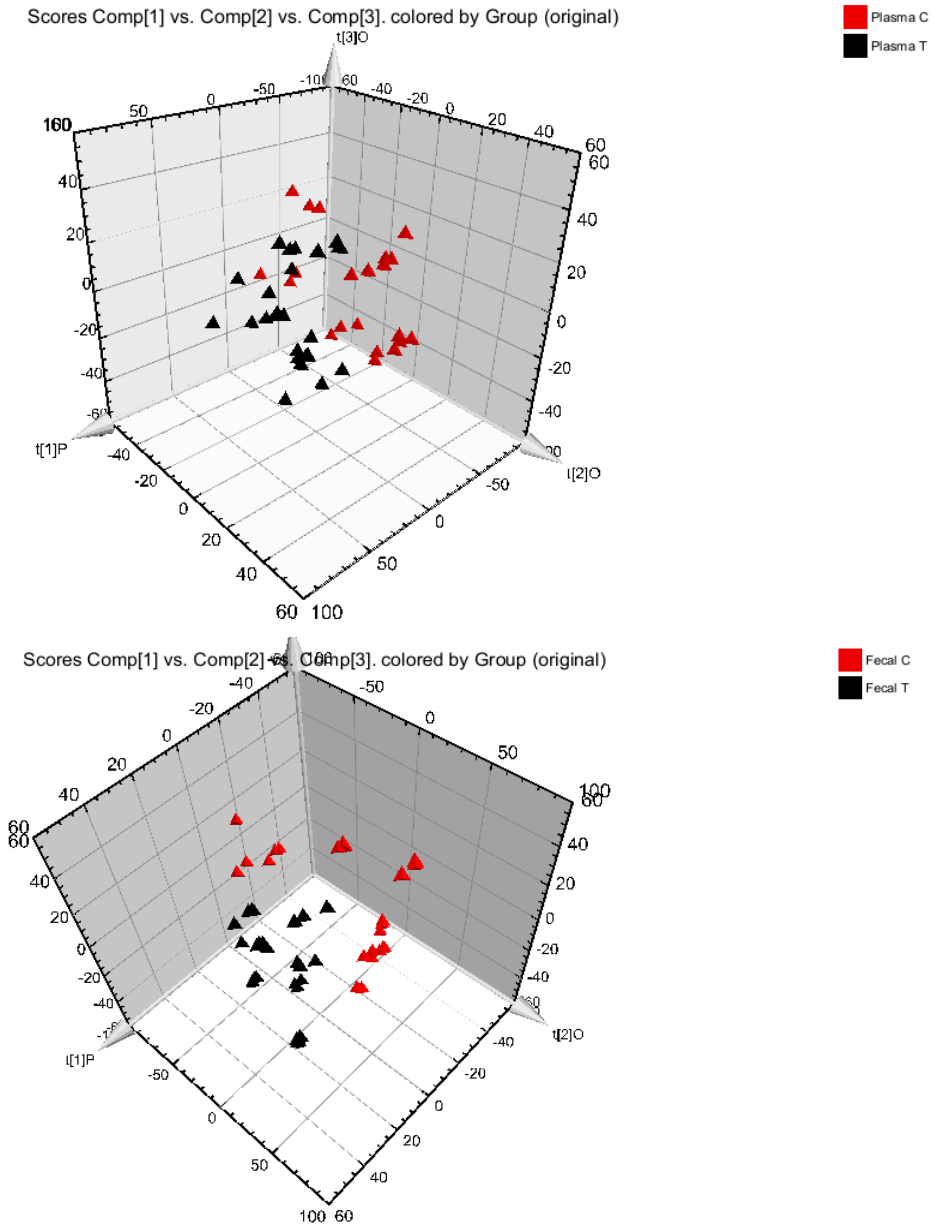

Figure 2- 3. PCA plot of bile acid signatures for rBSH and BSA treated rats groups in (A) fecal and (B) plasma.

Chapter III. Structural and functional studies of the bile salt hydrolase from *Lactobacillus salivarius*

Abstract

The bile salt hydrolase (BSH) is a gut bacterial enzyme that negatively influences host fat digestion and energy harvest. Bile salt hydrolase activity functions as a gateway reaction in the small intestine by deconjugation of conjugated bile acids. Extensive gut microbiota studies have suggested that BSH is a key mechanistic microbiome target for developing novel non-antibiotic feed additives to improve food animal production, and for designing new measures to control obesity in humans. However, research on BSH is still in its infancy, particularly with respect to the structural basis of BSH function, which has hampered the development of BSH-based strategies for improving human and animal health. Based on previous protein crystallization work of BSH from *Lactobacillus salivarius* NRRL B-30514 and comparative structural analysis of *L. salivarius* BSH with other published BSH structure, several residues critical for catalysis and substrate specificity were identified. Replacement of the residue Cys2 with serine abolished the activity of the *L. salivarius* BSH, confirming that Cys2 plays an essential role in BSH catalysis. In addition, the Tyr24, Phe65, Asn171, Gln257, Glu270 amino acids and the motif from 164 to 171, were verified to be important for BSH activity and/or substrates specificity using site-directed mutagenesis approach. Together, based on the first crystal structure of the BSH from *Lactobacillus* species, which are the major BSH producer in the intestine. Our findings also provided insights into the structural basis of BSH activity, which is important for future translational research by identifying compounds to modulate *in situ* BSH activity.

Keywords: Bile salt hydrolase, crystal structure, critical residues

Introduction

Recent studies indicated that gut microbiota affects host energy metabolism and obesity development (Gérard, 2016; Krznarić *et al.*, 2012). Thus, manipulating gut microbiota can be a promising way to control obesity and obesity-associated diseases. The understanding of the working mechanism of antibiotic growth promoters (AGPs) also related animal energy harvest and growth promotion effects to intestinal microbiota (Begley *et al.*, 2006). Those findings suggested that gut bile salt hydrolase (BSH) is a key microbial target to control host lipid metabolism and energy harvest, thus to develop alternatives to AGPs and serve as new measures to control obesity in human. The BSH catalyzes gateway reaction in bile acids metabolism in the small intestine and the change of bile profile will affect host physiology locally and systemically (Begley *et al.*, 2006; Jones *et al.*, 2014; Joyce *et al.*, 2014a).

Even though critical role of BSH function has been realized, research on the structural basis of BSH function is limited. To date, the structure of BSH has only been revealed from *Bifidobacterium longum* (Kumar *et al*., 2006) and *Clostridium perfrigens* (Rossocha *et al*., 2005). A BSH protein with broad substrate spectrum from *Lactobacillus salivarius* NRRL B-30514 was identified previously in our lab (Wang *et al.*, 2012). This *L. salivarius* BSH (*ls*BSH) hydrolyzes glycoconjugated and tauroconjugated bile salts effectively, whereas *B. longum* BSH and *C. perfrigens* BSH only have narrow substrate spectrum. Therefore, *ls*BSH is an ideal candidate for structure-function analyses of BSH. The structure of *ls*BSH was successfully in our previous study (Xu *et al.*, 2016). In this study, according to structure analysis of *ls*BSH, several residues were predicted to be important for enzyme activity or substrate specificity; those residues were subjected to amino acid substitution mutagenesis. The BSH mutants were purified and their

activities were measured to examine the role of these residues in BSH activity and/or substrate specificity.

Structural analyses of BSHs from various species would not only discover critical residues in catalysis and provide key information on the substrate specificity of BSHs, but also will directly facilitate future translational research, such as using molecular docking to develop BSH inhibitors-based alternatives to AGPs or BSH enhancers-based weight-reducing aids.

Materials and methods

Bacterial strain, plasmid, and compound

The bacterial strains and plasmids used in this study and their sources are listed in Table 3-1. A plasmid with *bsh* gene from *L. salivarius* NRRL B-30514 inserting in vector pET21b constructed in our previous work was used for aa substitution mutagenesis; the *Escherichia coli* recombinant strain JL885 constructed in our previous study, which harbors pBSH was used for wild-type *ls*BSH purification (Wang *et al.*, 2012b). The major information for the wild-type *ls*BSH and the *ls*BSH-producing strain JL885 is summarized in Table 3-2. Ampicillin, as well as Glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDCA), taurocholic acid (TCA), taurodeoxycholic acid (TDCA), and taurochenodeoxycholic acid (TCDCA) were purchased from Sigma-Aldrich (USA).

Site-directed amino acid (aa) substitution mutagenesis

Based on structure analysis of *ls*BSH, amino acids sites that are important for *ls*BSH activity and substrate specificity (Xu *et al.*, 2016). The predicted amino acids in *ls*BSH that may be important for catalysis and substrate specificity were replaced with different amino acids using QuickChange II site-directed mutagenesis kit (Agilent Technologies, USA). Briefly, the partial overlapping primer pairs containing the desired mutations (Table 3-3) were used for polymerase chain reaction (PCR) amplification with the pBSH plasmid. The cycling conditions were as follows: 95°C for 30 seconds, followed by 12 (for single nuclide mutation), 16 (for single amino acid mutation), or 18 (for multiple amino acids deletion or insertion) cycles of 95 °C for 30 seconds, 55 °C for 1 min, and 68 °C for as time needed (1 min/kb for extension), and then stayed at 4 °C until the next step. The PCR products were treated with Dpn I restriction enzyme at 37 °C for 1 hour to digest the methylated, non-mutated parental DNA template. The digested products were transformed into XL1-Blue super competent cells and cell cultures were grown on LB agar plates with ampicillin (100 μg/mL). Plates were incubated at 37 °C in an incubator overnight. In the following steps, single colonies were incubated and plasmids were extracted. A plasmid with a specific aa substitution in pBSH was verified by sequencing (University of Tennessee Genomic Core). Eight plasmids with different amino acids substitutions or deletion (Table 3-1) were generated and then transferred into *E. coli* BL21(DE3) competent cells, creating the constructs JL1189, JL1191, JL1193, JL1196, JL1198, JL1201, JL1203, and JL1205 (Table 3-1). These constructs and control strain JL885 were used for protein expression, purification, and two-step BSH activity assay to determine the roles of specific amino acids in *ls*BSH.

BSH activity assay

The wild-type *ls*BSH and corresponding mutants were expressed and purified following the procedures detailed in the Materials and Methods section in Chapter II of this dissertation. Modified two-step standard activity assays were performed to determine enzyme activity and substrate specificity (Tanaka *et al.*, 2000; Wang *et al.*, 2012b). Activity was expressed by the amount of free amino acids liberated from conjugated bile salts as substrates. Ten microliters of purified enzymes (1 μg/μL), 10 μL of conjugated bile salts (GCA, GDCA, GCDCA, TCA, TDCA, TCDCA), 178 μL of reaction buffer (0.1 M sodium-phosphate, pH 6.0), and 2 μL of 1 M DTT were mixed gently and incubated at 37 °C for 30 min. Following the incubation, reaction tubes were put on ice to stop the reaction. Then, 50 μL aliquot of the reaction mixture was mixed with 50 μL15% trichloroacetic acid (w/v) to fully stop the reaction, and the sample was centrifuged at 12,000 *g* for 5 min to remove the precipitate. The supernatant was mixed thoroughly with 950 μL ninhydrin reaction mix (0.25 mL of 1% ninhydrin (w/v), 0.1 mL sodium-citrate buffer (pH5.5), 0.6 mL glycerol) and incubated in boiling water for 14 min. The reactions were stopped by putting reaction tubs on ice for 3 min and the absorbance of reaction mix at 570 nm wavelength was read with a Smart Spec Plus spectrophotometer (Bio-Rad). Standard curves using glycine or taurine were determined for each independent assay. All assays were performed in triplicate. Enzyme activity was expressed as 1 μmol of amino acids released from substrates per minute per mg of BSH (Wang *et al.*, 2012b) and mutants relative activity compared to wild-type *ls*BSH was also calculated.

Results

Structural analysis of *ls*BSH

In a previous study, a 1.90 Å resolution crystal structure of *ls*BSH was obtained and it belongs to the N-terminal nucleophile hydrolase superfamily (Xu *et al.*, 2016). Based on the comparison of the crystal structure of *ls*BSH with previously published structure from *Clostridium perfrigens* and *Bifidobacterium longum* (Kumar *et al.*, 2006; Rossocha *et al.*, 2005), these three BSHs shared similar conserved catalytic active center at Cys2 and its coordinated neighbor amino acids (Xu *et al.*, 2016). However, several other surrounding amino acids were significantly different which may contribute to enzyme activity and specificity towards glycine and taurine conjugated bile salts (Table 3-4). It was speculated that in loop I, Tyr24 along with Phe65 at the bottom of the binding pocket force the substrates to sit deeply in the binding pocket, which may contribute to the increase of *ls*BSH activity. Leu18 and Phe22 in loop I may contribute to different enzyme-substrate interaction which probably is the reason that *ls*BSH has a broader substrates spectrum. On loop II, Leu134 and Phe130 residues may contribute to restraint of the spatial configuration by condensing the substrate-binding pocket entrance. Two residues, Ile56 and Leu63, at the bottom of the binding pocket may determine the different substrate specificities. Asn171 coordinates to Cys2 and helps to stabilize Cys2 or transfer electron in catalysis. Substrate-enzyme interaction revealed that Gln257 and Glu270 may play an important role in stabilizing the reactant which makes *ls*BSH be able to digest taurine-conjugated bile salts.

Together with BSH gene sequencing results from different bacteria sources, it was also speculated that the amino acid residues 164-171 play a role in maintaining the environment

around Cys2 and this residue may also play a significant role in substrate specificity (Fang *et al.*, 2009).

Site-directed mutagenesis and protein purification

Sequences of mutated plasmids were confirmed by DNA sequencing and a sequence chromatogram was performed in Chromas Lite 2.3 (Figure 3-4). Mutated codons corresponding to designed amino acid sites were marked in black frame and mutated codons were marked with asterisks. Sequencing results revealed that there were no additional mutations occurring on the genes except for the anticipated ones. The SDS-PAGE gel also revealed that *ls*BSH and its mutants were properly expressed and purified with satisfactory purity (Figure 3-5).

BSH enzyme activity of various BSH mutants for different bile salts

The enzyme activity of eight purified BSH mutants was measured for six different conjugated bile salts and the activity of wild-type *ls*BSH was used as control (Table 3-5). Site-directed mutagenesis of Cys2 in *ls*BSH almost abolished its activity towards all six tested bile salts. The *ls*BSH with Cys2 mutation only displayed marginal BSH activity for TCA when compared to wild-type *ls*BSH. Site mutation on Tyr24 led to dramatic activity decrease towards GCA and GDCA; enzyme activity towards tauroconjugated bile salts did not show dramatic difference relative to *ls*BSH. The mutation of amino acid site of Phe65, which was predicted to force substrates to sit deeply in the binding pocket with Tyr24, showed similar enzyme activity with Tyr24 mutated BSH. Mutation on Phe65 totally abolished the BSH activity for GCA and GDCA, leaving a marginal activity for GCDCA. The enzyme activity for TCA and TDCA

decreased by 40%-50% compared to wild *ls*BSH, and enzyme activity for TCDCA was not affected.

In the mutant with Asn171, which was speculated to be important for Cys2 function, mutation caused a dramatic decrease in BSH activity for all six bile salts; enzyme activity for GCA, GDCA, GCDCA, and TDCA was marginal and only 16.41% activity for TCDCA and 10.75% activity for TCA compared to wild-type *ls*BSH was left. The deletion of the motif of amino acid residues of 164-171 caused a total cessation of BSH activity for all six bile salts.

Mutation at Gln257 did not show a dramatic difference in tauroconjugated bile salts (TCA, TDCA, TCDCA), but the activity for glycoconjugated bile salts was decreased by 90% to 75%. Mutation on Glu270 did not cause a big difference in BSH activity towards all six substrates. However, the double mutation of Gln257 and Glu270 caused a conspicuous enzyme activity decrease for all six substrates, the decrease ranging from 65% to 95% relative to wild-type *ls*BSH.

Discussion

Based on the structure of *ls*BSH, several residues that may either be important for BSH activity or substrate specificity were predicted. The major amino acid residues predicted that are involved in *ls*BSH catalysis and substrate specificity (Table 3-4) were revealed for the first time other than the six predicted BSH conservative sites (Cys-2, Arg-16, Asp-19, Asn-79, Asn-171, and Arg-224). Within these predicted residues, Asn171 and motif 164-171 showed the strongest effect on BSH activity, which was consistent with our prediction that they help Cys2

stabilization or electron transfer and manipulation of the environment around Cys2. Together with the finding that Cys2 mutation totally abolished BSH activity, it was also confirmed that BSH, the member in Ntn hydrolase family, has Cys2 in its active center for catalysis. Residues interacting with Cys2 might have a profound effect on enzyme activity. However, the exact mechanism for the effects needs further confirmation by structure-function analysis.

The *ls*BSH has a broader substrates spectrum and can efficiently hydrolyze both glycoconjugated and tauroconjugated bile salts relative to BSH from other bacterial sources which have a higher activity towards glycoconjugated bile salts (Kumar *et al.*, 2006; Rossocha *et al.*, 2005). Our structure analysis predicted that Gln257 and Glu270 may play a role in stabilizing the taurine group, which contributed to the potent *ls*BSH activity towards tauroconjugated bile salts. However, the mutagenesis and enzyme activity assay did not show dramatic activity decrease specific towards taucoconjugated bile salts. This mis-predication was probably caused by the technical difficulty of structure-substrates analysis. Our prediction was based on the interaction between taurine and *ls*BSH. The size of glycine is too small for structure-substrates analysis. The residues predicted to be important for tauroconjugated bile salts may also be important for glycoconjugated bile salts.

The structure analysis on the broad substrates spectrum *ls*BSH provided a solid foundation for BSH structure analysis, which is helpful for future transnational research. Due to the significant role of BSH in host physiology, BSH can be a novel target to control host body weight and lipid metabolism (Geng *et al.*, 2016; Joyce *et al.*, 2014a). With increasing over-weight and obesity rates in the world, BSH enhancers would be promising in controlling host lipid metabolism and

body weight. In agriculture, BSH inhibitors would be potential antibiotic growth promoters alternative to promote the growth of farm animals. Future research in screening BSH inhibitors and enhancers would be really beneficial for human and animal health.

These BSH inhibitors and enhancers can be developed by molecule docking, which would dramatically increase hits rather than using standard chemical library (Taylor *et al.*, 2002; Yuriev *et al.*, 2015; Yuriev *et al.*, 2013). Current development in machine learning also improved the docking accuracy (Ain *et al.*, 2015). With the successful expression and purification of *ls*BSH mutants in this study, the future crystallization of mutants would also provide new aspects of BSH interaction between its substrates or enzyme inhibitors and enhancers. Those hits by molecule docking can be validated with our established high-throughput screening system (Smith *et al.*, 2014) and those chemicals with outstanding enhancing or inhibiting effects on BSH would be used for further *in vivo* and *in vitro* experiments to validate their physiological roles.

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Appendix

Table 3- 1. Key bacterial plasmids and strains used in this study.

Plasmids or strains	Description	Source or Reference
Plasmids		
pBSH	pET21b containing *bsh* gene from *Lactobacillus salivarius* NRRL B-30514, Amp^r	(Wang *et al.*, 2012b)
pBSH (C2S)	pBSH derivative with C2S mutation in BSH	This study
pBSH (Y24F)	pBSH derivative with Y24F mutation in BSH	This study
pBSH (F65A)	pBSH derivative with F65A mutation in BSH	This study
pBSH (N171A)	pBSH derivative with N171A mutation in BSH	This study
pBSH (164-171)	pBSH derivative with motif 164 to 171 amino acid deletion in BSH	This study
pBSH (Q257A)	pBSH derivative with Q257A mutation in BSH	This study
pBSH (E270A)	pBSH derivative with E270A mutation in BSH	This study
pBSH (Q257A E270A)	pBSH derivative with double mutations (Q257A and E270A) in BSH	This study
Strains		
E.coli BL21(DE3)	F– ompT gal dcm lon hsdSB(rB- mB-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	Novagen
E.coli XL1-Blue	EndA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 $proAB^+$ $lacI^q$ Δ(lacZ)M15] hsdR17(r_K^- m_K^+)	Agilent Technologies
JL885	*E.coli* BL21(DE3) containing the plasmid pBSH	(Wang *et al.*, 2012b)
JL1189	*E.coli* BL21(DE3) containing the plasmid pBSH (C2S)	This study
JL1191	*E.coli* BL21(DE3) containing the plasmid pBSH (Y24F)	This study
JL1193	*E.coli* BL21(DE3) containing the plasmid pBSH (F65A)	This study
JL1196	*E.coli* BL21(DE3) containing the plasmid pBSH (N171A)	This study
JL1198	*E.coli* BL21(DE3) containing the plasmid pBSH (164-171)	This study
JL1201	*E.coli* BL21(DE3) containing the plasmid pBSH (Q257A)	This study
JL1203	*E.coli* BL21(DE3) containing the plasmid pBSH (E270A)	This study
JL1205	*E.coli* BL21(DE3) containing the plasmid pBSH (Q257A E270A)	This study

Table 3- 2. Key background information for the *ls*BSH produced by the recombinant *E. coli* JL885.

Parameters	Corresponding information
Source organism	*Lactobacillus salivarius* NRRL B-30514
DNA source	Genomic DNA
Forward primer [a]	5'-CGCGGATCCATGTGTACAGCAATTACTTT-3' (BamHI)
Reverse primer [a]	5'-CCGCTCGAGATTCAACTTATTTATTATTTGT-3' (XhoI)
Cloning vector	pET-21b
Expression vector	pET-21b
Expression host	*E. coli* BL-21(DE3)
Complete amino acid sequence of the construct produced [b]	MCTAITLNGNSNYFGRNLDLDFSYGEEVIITPAEYEFKFRKEKAI KNHKSLIGVGIVANDYPLYFDAINEDGLGMAGLNFPGNAYYSD ALENDKDNITPFEFIPWILGQCSDVNEARNLVEKINLINLSFSEQL PLAGLHWLIADREKSIVVEVTKSGVHIYDNPIGILTNNPEFNYQ MYNLNKYRNLSISTPQNTFSDSVDLKVDGTGFGGIGLPGDVSPE SRFVRATFSKLNSSKGMTVEEDITQFFHILGTVEQIKGVNKTESG KEEYTVYSNCYDLDNKTLYYTTYENRQIVAVTLNKDKDGNRL VTYPFERKQIINKLNLERHHHHHH

[a] Restriction sites in the forward and reverse primers sequences are underlined, and the restriction enzymes are indicted in parentheses.
[b] The His-tag site is underlined.
The original information of *ls*BSH was introduced in a previous publication (Wang *et al.*, 2012b).

Table 3- 3. The primers used for site-directed aa substitution mutagenesis in this study.

Primer Name	Sequence 5`-3` [a]
BSHMT1-F	CCATTTAAAGTAATTGCTGT**AGA**CATGGATCCCGACCCATTT
BSHMT1-R	AAATGGGTCGGGATCCATG**TCT**ACAGCAATTACTTTAAATGG
BSHMT2-F	GATTACCTCCTCACC**AAA**TGAAAAATCTAAATCTAAGTTTCTTCCA A
BSHMT2-R	TTGGAAGAAACTTAGA**TTT**AGATTTTTCATTTGGTGAGGAGGTAA TC
BSHMT3-F	TCCATCCTCATTAATAGCATC**TGC**ATACAATGGGTAATCGTTAGCG ACAATTCC
BSHMT3-R	GGAATTGTCGCTAACGATTACCCATTGTAT**GCA**GATGCTATTAATG AGGATGGA
BSHMT4-F	CAGATTGTACATCTGATAATTAAATTCTGGATT**TGC**AGTCAATATT CCAATTGG
BSHMT4-R	CCAATTGGAATATTGACT**GCA**AATCCAGAATTTAATTATCAGATG TACAATCTG
BSHMT5-F	GTACATCTGATAATTAAATTCTGGATTATCATAAATATGTACTCCA GATTTAGTTACTTC
BSHMT5-R	GAAGTAACTAAATCTGGAGTACATATTTATGATAATCCAGAATTT AATTATCAGATGTAC
BSHMT6_Q257A-F	CCATATACTAGGGACAGTAGAA**GCA**ATAAAGGGCGTTAATAAGA CAG
BSHMT6_Q257A-R	CTGTCTTATTAACGCCCTTTAT**TGC**TTCTACTGTCCCTAGTATATGG
BSHMT7_E270A-F	GACAGAATCAGGAAAAGAA**GCA**TACACTGTATATTCGAATTGC
BSHMT7_E270A-R	GCAATTCGAATATACAGTGTA**TGC**TTCTTTTCCTGATTCTGTC

[a] Bolded letters indicate the nucleotides designed for aa substitution mutagenesis.

Table 3- 4. Major amino acid residues of *ls*BSH that are potentially involved in catalysis and substrate specificity based on comparative structural analysis.

Residue	Specific location	Speculation
Tyr24	Loop I	Along with Phe65, may force the substrate to sit deeply in the binding pocket
Leu18		Given their differences when compared with the structures of cpBSH and blBSH, they may contribute to different enzyme–substrate interactions
Phe22		
Leu134	Loop II	Both residues contribute to restraint of the spatial configuration by condensing the substrate-binding pocket entrance
Phe130		
Ile56	Bottom of the binding pocket	Compared with the structures of cpBSH and blBSH, these two residues may determine the differing substrate specificities
Leu63		
Phe65		Along with Tyr24, may force the substrate to sit deeply in the binding pocket
Asn171		Coordinate to Cys2 and help to stabilize Cys2 or transfer electron in catalysis
Gln257		These two sites plays an important role in stabilizing the reactant
Glu270		
164-171		This residue may play a role in substrate specificity and rBSH activity by manipulation environment around Cys2

Table 3- 5. Comparison of BSH enzyme activity of various BSH mutants for different bile salts.

Exp No.[b]	BSH	BSH activity[a]					
		GCA	GDCA	GCDCA	TCA	TDCA	TCDCA
1	WT	2.4±0.04 (100)	1.4±0.07 (100)	5.4±0.27 (100)	5.3±0.15 (100)	5.5±0.15 (100)	5.3±0.07 (100)
	Cys2Ser	–	–	–	0.16±0.02 (3.02)	–	–
	Tyr24Phe	1.9±0.09 (79.17)	1.0±0.00 (71.43)	6.4±0.22 (118.59)	5.3±0.03 (100)	5.5±0.02 (100)	5.3±0.08 (100)
	Phe65Ala	–	–	0.2±0.01 (3.70)	3.4±0.07 (64.15)	2.9±0.11 (52.73)	5.4±0.07 (101.89)
	Asn171Ala	0.17±0.05 (7.08)	0.05±0.00 (3.57)	0.22±0.01 (4.07)	0.57±0.03 (10.75)	0.26±0.01 (4.73)	0.87±0.05 (16.41)
	Δ164-171	–	–	–	–	–	–
2	WT	1.47±0.17 (100)	0.97±0.11 (100)	2.74±0.03 (100)	1.28±0.11 (100)	1.32±0.10 (100)	1.31±0.09 (100)
	Gln257Ala	0.37±0.014 (25.17)	0.16±0.02 (16.49)	0.38±0.05 (13.87)	1.26±0.014 (98.44)	1.27±0.09 (96.21)	1.22±0.06 (93.13)
	Glu270Ala	2.35±0.09 (159.86)	0.77±0.02 (79.38)	2.84±0.05 (103.65)	1.29±0.011 (100.78)	1.38±0.19 (104.55)	1.34±0.01 (102.29)
	Gln257Ala Glu270Ala	0.4±0.02 (27.21)	0.11±0.02 (11.34)	0.19±0.02 (6.93)	0.45±0.13 (35.16)	0.15±0.06 (11.36)	0.22±0.004 (16.79)

[a] The BSH activity was expressed as μmol of amino acids released from the substrate per minute per mg of BSH (μmol/min/mg). The activity of following six bile salt substrates were tested: glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDCA), taurocholic acid (TCA), taurodeoxycholic acid (TDCA), taurochenodeoxycholic acid (TCDCA). Experiments were carried out in triplicate. The numbers in parentheses indicate the relative activity (%) of the activity of specific BSH mutant when comparing with that of wild-type (Ou *et al.*, 2013) BSH enzyme. "–" represents no BSH activity detected (no significant difference when comparing with negative control)

[b] Two independent experiments were performed. For each independent experiment, all BSHs were purified in parallel under same condition and the freshly purified enzymes were subjected to BSH activity assay.

Figure 3- 1. Sequence chromatogram of confirmed site directed mutagenesis of BSH.

Sequence chromatogram of confirmed site directed mutagenesis of BSH. Black frame marked the mutations in plasmids and mutated codons were marked with asterisks in corresponding sites. (a) Sequence chromatogram of plasmid pBSH (C2S) and pBSH (Y24F) compared to wild type pBSH. (b) Sequence chromatogram of plasmid pBSH (F65A) compared to wild type pBSH. (c) Sequence chromatogram of plasmid pBSH (N171A) and pBSH (164-171) compared to wild type pBSH. (d) Sequence chromatogram of plasmid pBSH (Q257A) and pBSH (E270A) compared to wild type pBSH. Sequences presented were the reverse compliment of original sequence. Amino acid site 257 mutation from CTG to TGC represents Gln (CAG) to Ala (GCA). Amino acid sit 270 mutation from TTC to TGC represents Glu (GAA) to Ala (GCA). (e) Sequence chromatogram of double sites mutated plasmid pBSH (Q357A E270A). Sequences presented were the reverse compliment of original sequence. Amino acid site 257 mutation from CTG to TGC represents Gln (CAG) to Ala (GCA). Amino acid sit 270 mutation from TTC to TGC represents Glu (GAA) to Ala (GCA).

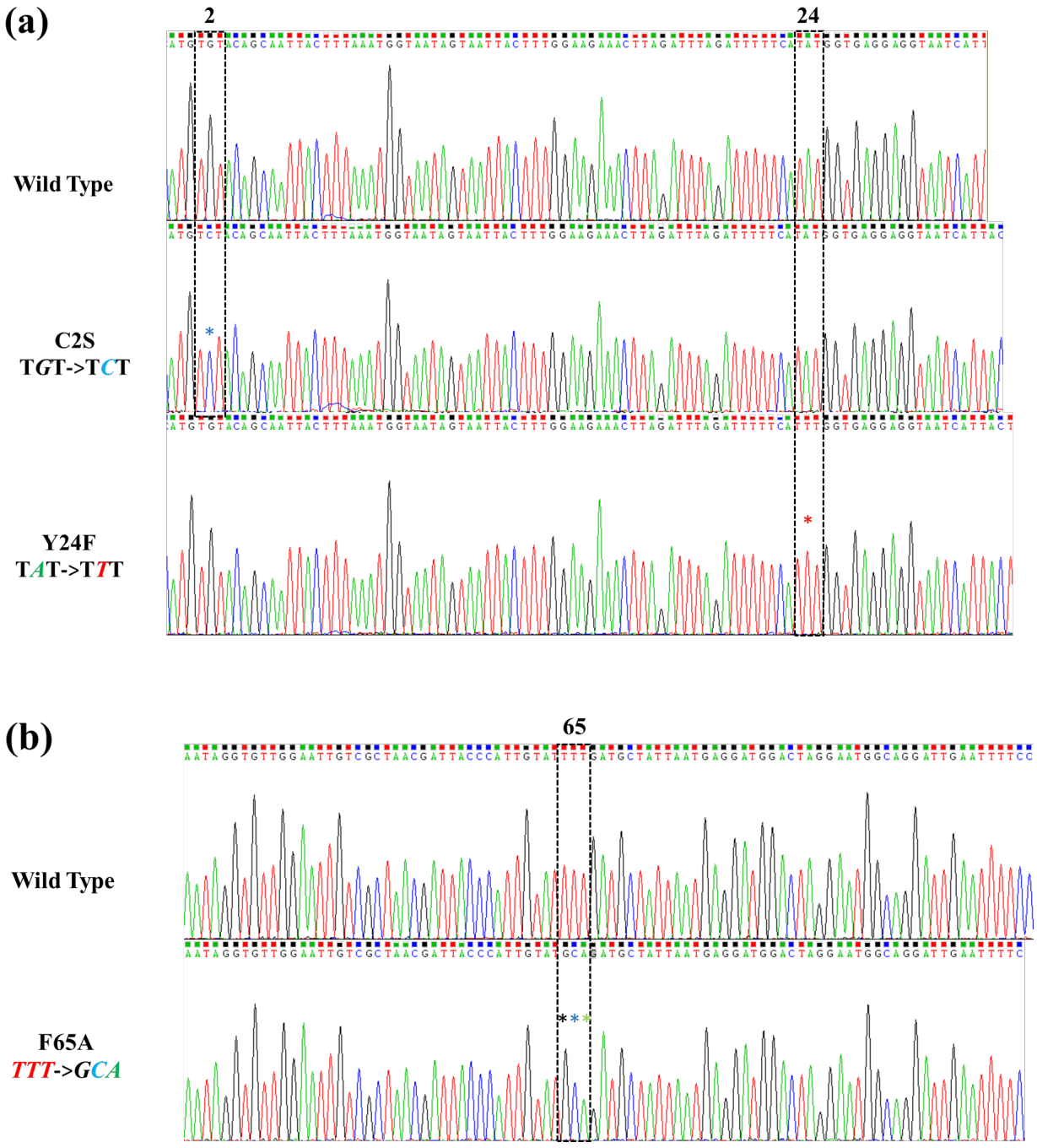

Figure 3- 1. Continued.

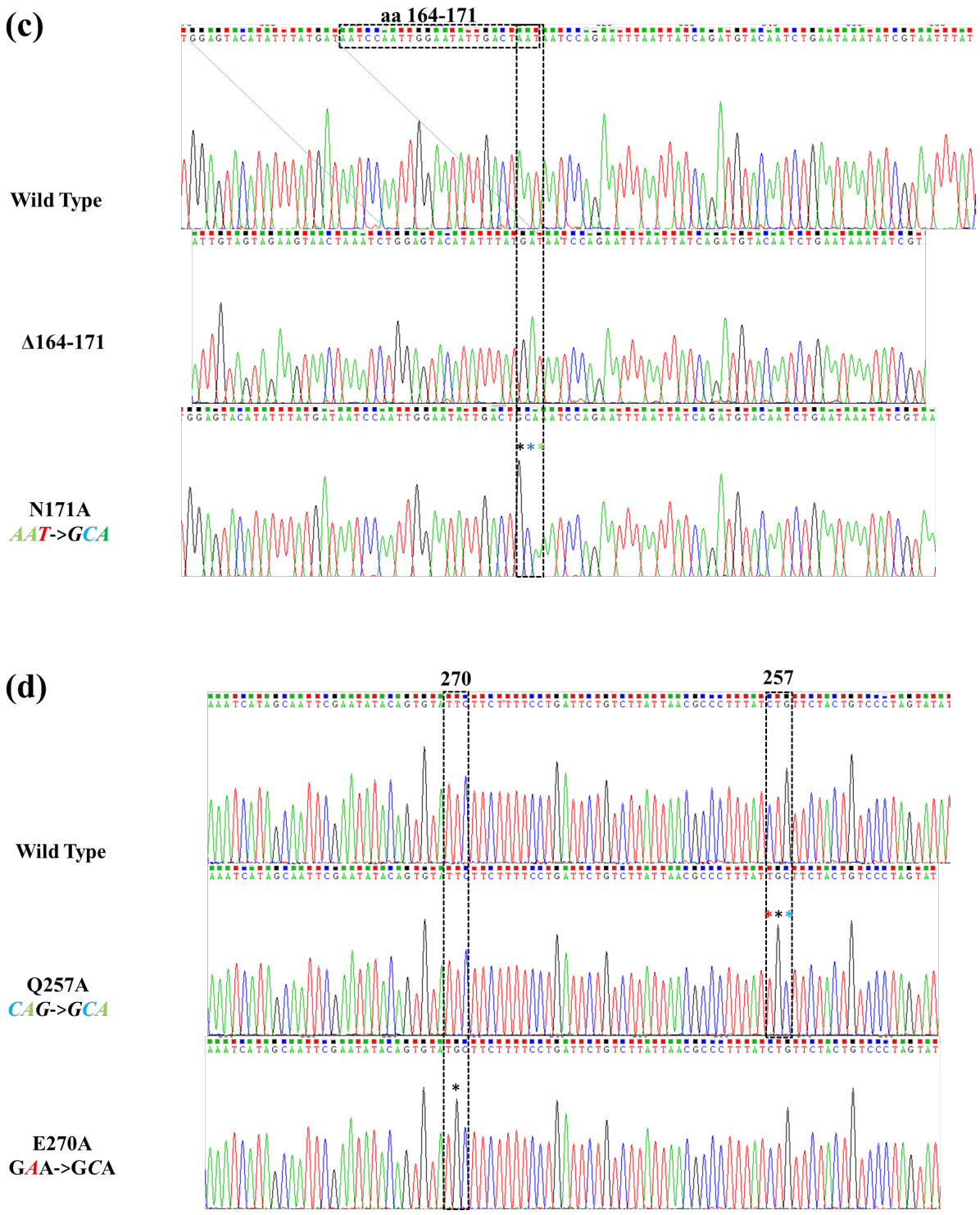

Figure 3- 1. Continued.

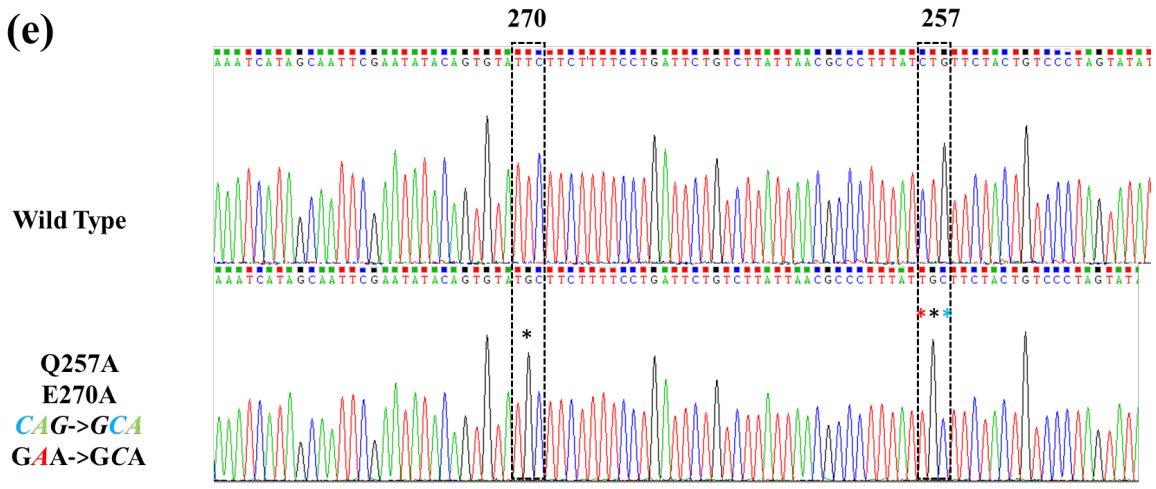

Figure 3- 1. Continued.

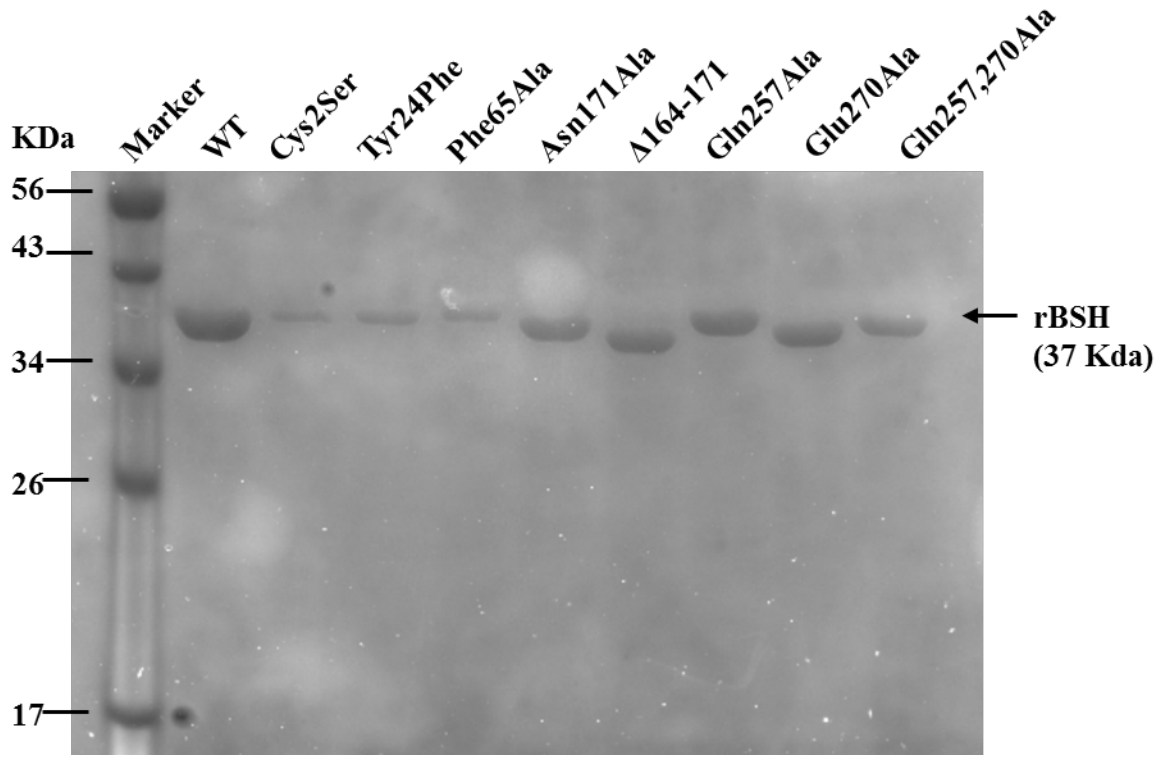

Figure 3-2. Expression and purification of wild type *ls*BSH and its mutants.

Chapter IV. *In vivo* evaluation of bile salt hydrolase inhibitors physiological roles using broiler chicken

Abstract

Recent studies strongly suggest that bile salt hydrolase (BSH) inhibitors may serve as innovative alternatives to antibiotic growth promoters (AGPs) for sustainable animal production. A panel of promising BSH inhibitors also have been identified in our recent study. However, it is still unknown if dietary supplementation of the identified BSH inhibitors can really inhibit the activity of the BSH enzyme in the intestine, and lead to significant changes in bile profile as well as host physiology. Understanding of the *in vivo* efficacy of the BSH inhibitor is critically needed for us to develop effective non-antibiotic feed additives for commercial applications. In this study, the effects of dietary supplementation of three BSH inhibitors, caffeic acid phenethylester, riboflavin, and carnosic acid, were evaluated in broilers. Forty newly hatched Hubbard broiler chickens received water and standard basal chicken feed *ad libitum* throughout the study. From 7 days of age, the chicks were assigned into 4 groups (10 chickens per group) that one group received control solution and the each of the other three groups received three different BSH inhibitors (25 mg/kg body weight) *via* oral gavage for 17 days. Despite significant body weight variation among individual birds in each group, the broilers in all three treatment groups showed consistently higher body weight and body weight gain compared to chicken from the control group, but the difference was not statistically significant. Metabolomics analysis of intestinal samples using UPLC-MS showed significant changes in bile acid (BA) profiles in response to BSH inhibitor treatment, demonstrating *in vivo* efficacy of the BSH inhibitors. There was no significant difference among the groups in terms of the levels of serum biochemical parameters. However, transcriptome analyses of liver and intestine samples indicated that carnosic acid treatment led to significant differential expression of those genes involved in lipid metabolism and bile acid metabolism. Together, the findings from this study

demonstrated *in vivo* efficacy of the identified BSH inhibitors, which is a significant step toward our long-term goal of developing effective BSH inhibitor-based alternatives to AGPs.

Keywords: Bile salt hydrolase inhibitor, *in vivo* efficacy, bile profile, RNA-Seq

Introduction

Antibiotic growth promoters (AGPs), a group of low-dose antibiotics, have been widely used as feed additives for more than six decades to increase feed efficiency, energy harvest and body weight gain of food animals (Moore *et al.*, 1946). However, due to a potential link bewteen AGP usage and antimicrobial resistance (AMR), there is a worldwide trend to limit the use of AGPs to mitigate AMR and enhance food safety (Dibner *et al.*, 2005; Turnidge, 2004). To maintain current animal production without threatening the public health, it is urgent to develop effective alternatives to AGPs.

In turn, developing effective alternatives to AGPs makes it essential to understand the mechanisms of AGPs. Of various proposed such mechanisms, the AGPs mediated modulation of intestinal bacteria is a major one (Brown *et al.*, 2017). In particular, recent microbiome studies provided compelling evidence that intestinal bacterial bile salt hydrolase (BSH), which is affected by AGP usage and negatively correlated to host energy harvest and lipid metabolism, is a promising microbiome target for developing novel alternatives to AGPs (Lin, 2014). Specifically, the small compounds that can inhibit BSH activity may function similarly as AGPs for enhanced feed efficiency and growth performance.

Recently, we identified and characterized a BSH enzyme with broad substrate specificity isolated from a *Lactobacillus salivarius* strain from chicken intestine (Wang *et al.*, 2012b). With this ideal candidate, we identified and characterized a group of promising BSH inhibitors using an effective High-Throughput Screening (HTS) system (Smith *et al.*, 2014). Among these BSH inhibitors, riboflavin is a vitamin participating in a range of redox reactions in the host (Massey,

2000; Powers, 2003). In a recent study, the riboflavin level in feed was observed to influence animal behavior and growth (Qi *et al.*, 2017). In addition, dietary supplementation of riboflavin was shown to increase feed efficiency and body weight gain in pigs (Stahly *et al.*, 2007). The caffeic acid phenethylester (CAPE), a potent BSH inhibitor, was originally discovered as a bioactive component in propolis from honeybee hives (Bankova, 2005). The CAPE has shown multiple bioactivities, such as anti-tumor, anti-oxidation, anti-inflammatory, and immune regulation (Zhang *et al.*, 2014a); however, its effect on host lipid metabolism or growth performance was limited. The other BSH inhibitor carnosic acid is a phenolic terpene extracted from rosemary and sage which was claimed to have anti-oxidant, anti-inflammatory, and anti-carcinogenic activities (Bahri *et al.*, 2016; de Oliveira, 2016; Naimi *et al.*, 2017; Tolba *et al.*, 2013). Both *in vitro* and *in vivo* experiments showed that carnosic acid is involved in glucose and lipid metabolism (Ninomiya *et al.*, 2004).

It is hypothesized that oral administration of these three promising BSH inhibitors will exert inhibitory effect on intestinal BSH activity, thus leading to significant changes in bile profiles as well as host physiology. Therefore, this study investigated the effects of oral administration of the three BSH inhibitors on host physiology using broilers. Chicken bile salt profile, lipid metabolism, and growth performance were analyzed. Transcriptome analyses using different pipelines and statistical models were also conducted to reveal molecular mechanisms in response to carnosic acid treatment.

Materials and methods

Animal experiment

The process of chicken experiment followed the University of Tennessee Institutional Animal Care and Use Committee protocol (IACUC # 2187). Forty newly hatched male broilers from Hubbard LLC in Pikeville, TN were raised in the Joseph E. Johnson Animal Research and Teaching Unit (JARTU, Knoxville, TN, USA). After one week, chicks were randomly allocated into four groups (10 birds per group). During the 28 days of experiment period, one group (control) received 50% propylene glycol; the second group received riboflavin (Bulk Supplements, Henderson, NV, USA); the third group received carnosic acid (Purify, Chengdu, Sichuan, China) and the fourth group received caffeic acid phenethyl ester (Wuhan Yuancheng Gongchuang Tech, Wuhan, Hubei, China). The administration method was daily oral gavage. Solutions of riboflavin, carnosic acid and CAPE were prepared by dissolving 700 mg compound into 10 mL solvent (water: propylene glycol = 1:1, v/v). Gavage dosage was equivalent to 25 mg compounds per kilogram of chicken and dosage was adjusted by chicken body weight on days 7, 10, 14, 17, 21, and 24. The chicken feed used in this project was All Natural Meat Bird Start Feed purchased from Co-op Feeds (#91107). Water and feed were available *ad libitum* during the project. On days 7, 10, 14, 17, 21, 24, and 28, the bird body weights were measured. Feed dropped on the pans of the cages was collected as much as possible to provide accurate feed intake value and feed conversion ratio data. Due to an unrecorded chicken feed intake at 9-day of chicken age, we started recording chicken feed in and out from day 10; feed intake and body weight data were recorded twice a week. On days 10, 14, 17, 21, 24 and 28 chicken feed consumption per cage was measured and feed conversion ratio (feed intake/body weight gain) was also determined. On day 28, all birds were sized bird per pen was further selected for sample

collection. Following blood collection from the wing vein, the birds were euthanized with carbon dioxide and about 1 mL chicken blood from wing vein was collected from each chicken; chicken feces were also collected as duplicates in 1.5 mL Eppendorf tubes and placed on ice; about 5 cm of chicken ileum from each chicken was collected and rinsed with saline (0.9% sodium chloride, w/v); chicken liver samples were also collected. After sample collection, liver and ileum were wrapped in aluminum foil and then saved in liquid nitrogen immediately; blood samples were centrifuged at 4,000 *g* for 15 min; and the supernatant (serum) was transfer into sterile 1.5 mL Eppendorf tubes. All collected samples were stored in -80 °C freezer before further analysis.

Serum biochemical analyses

Serum concentrations of glucose, triglycerides, total cholesterol, and very low-density lipoprotein were determined with corresponding kits following standard protocol (glucose and triglyceride, Cayman Chemical, Ann Arbor, MI, USA; total cholesterol, Bioassay System, Hayward, CA, USA; VLDL, Novatein Biosciences, Woburn, MA, USA).

Bile profiles analyses

Chicken fecal samples (about 0.5 g) and serum samples (200 μL) from all four treatment groups (10 chickens/group) were packed with dry ice and shipped to Dr. Susan Joyce (APC Microbiome Institute, University College Cork, Ireland) for bile acid profile analysis. The metabolomics analysis was performed by Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS); the detailed procedures for bile acids extraction, characterization and quantification were described in a previous publication (Joyce *et al.*, 2014a). Notably, our

collaborators did not know the specific treatment for each group; therefore, the 'blinded' metabolomics measurements ensure the quality of this collaborative work.

RNA extraction

Total RNA from liver and ileum samples of four chickens from the control group and the carnosic acid group was extracted with Qiagen RNeasy mini kit (Cat#: 74104, Qiagen, California, USA) following the standard protocol. Then, the RNA integrity number (RIN), concentration and the 28S/18S ratio of RNA were evaluated with Agilent RNA 600 Nano kit using Agilent 2100 Bioanalyzer (genomics Hub, UTIA). The total RNA was stored at -80 ℃ prior to use.

Transcriptome sequencing

Ten microliters of total samples extracted from chicken liver and ileum were packed in a dry ice box, which was shipped to the DNA facility of the Iowa State University Office of Biotechnology for mRNA-Seq libraries construction and transcriptome sequencing. The concentrations of each total RNA samples are presented in Table 4-6. The mRNA-Seq libraries were constructed based on high sample protocol in the TruSeq® RNA sample preparation v2 guide. Overall, sixteen sequencing libraries were constructed from RNA samples. All mRNA-Seq libraries were loaded into a single lane of the Illumina Hiseq 3000 system. However, samples LC1, LC4, and IC2 showed really low reads and mapping rates after the first run. Therefore, these three samples were subjected to RNA-Seq again using the same procedure

except that the mRNA-Seq libraries were constructed based on low sample protocol in the TruSeq® RNA sample preparation v2 guide.

RNA-Seq data process and analyses

The RNA-Seq analyses were performed using two different pipelines. The overview of RNA-Seq data processing pipeline is summarized in Figure 4-1. Regarding the RNA-Seq analysis performed in the Office of Advanced Research Computing at Rutgers University (NJ, USA), the raw reads were quality trimmed using Trimmomatic-0.33 with leading and trailing Q score 25, minimum length 25 bp, and minimum length 50 bp. The cleaned reads were mapped to *Gallus_gallus* genome - 5.0 version, using Tophat v.2.0.13. The reference genome sequence and annotation files were downloaded from ENSEMBLE, release.87 (Gallus_gallus.Gallus_gallus-5.0.dna.toplevel.fa and Gallus_gallus.Gallus_gallus-5.0.87.gtf). The aligned read counts were obtained using htseq-count as part of the package HTSeq-0.6.1.

The RNA-Seq analysis performed at The University of Tennessee, Knoxville (UTK) is described below. Short raw reads were performed for quality assessment with FastQC v 0.11.5. Then the raw reads were trimmed using Skewer and the trimmed data were processed for quality assessment again with MultiQc v 1.2. The reads that passed filtering were aligned to *Gallus_gallus* NCBI build 5.0 reference genome with STAR v 2.3.0. The alignment process produced a SAM (Sequence Alignment/Map) format file, which need to be processed with SAM tools and a table of gene read counts was generated using HTSeq v 0.9.1. Raw reads quality assessment was performed on the Newton High Performance Computer (HPC) at UTK. The

mapping and gene reads counting were performed on the Advanced Computing Facility (ACF) at UTK.

The Bioconductor packages edgeR_3.8.6 with limma_3.22.7 and DESeq2 v 1.18.0 were used to perform the differential gene expression analysis in R Studio v 1.0.143. For the differentially expressed genes (DEGs), corresponding *P*-values and fold changes were uploaded to DAVID Bioinformatics Resources (https://david.ncifcrf.gov/) online bioinformatics analysis system for functional annotation and canonical pathway analyses. The detailed statistic model used in two Bioconductor packages were described in the Statistical Analyses section in this chapter.

Reverse transcription and quantitative PCR assay

The DEGs involved in immune response, bile acids metabolism, glucose metabolism, and lipid metabolism, as identified by the DAVID-BR analysis were also examined for the expression by performing qPCR analysis. These genes include those encoding aldo-keto reductase family1 (AKR1D1), cytochrome P450, family 3, subfamily A, polypeptide 80 (CYP3A80), glutathione S-transferase alpha 3 (GSTA3) in the liver and glutathione S-transferase alpha 3 (GSTA3), and G0/G1 switch 2 (G0S2) in the ileum. Ileum and liver RNA samples from four individual birds of each group were used. Two hundred micrograms of total RNA from each sample were used for cDNA synthesis using Thermo Scientific RevertAid First Strand cDNA synthesis kit (Cat#: K1621) according to the manufacturer instructions. The qPCR assays were performed using iTaq^{TM} universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Briefly, cDNA was diluted 100-fold, and 1 µL of each diluted sample was added to 10 µL volume reaction, also containing 1 µL of each forward and reverse primers of the target genes (50 ng/µL)

and 5 μL PCR master mix. The primers of the target genes were designed using NCBI primer blast with corresponding mRNA sequences and the sequencing for primers are summarized in Table 4-1. The β-actin gene was used as an internal reference to normalize target gene transcript levels since this is a commonly used house-keeping gene as an internal control for gene expression (De Boever *et al.*, 2008). The qRT-PCR was conducted in triplicate and the mean values were used for analysis. Fluorescence measurements were collected after each annealing step. The threshold cycle (C_T) values for genes of interest were normalized to an average C_T value of the house-keeping genes and the relative expression of replicate was calculated by the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Chicken body weight, body weight gain, feed conversion ratio; serum levels of glucose, total cholesterol and triglycerides, and bile salts profile were compared among groups using mixed model analysis of variance procedure. The statistical analysis of bile salts profile was conducted independently by our collaborator at University College Cork and by our laboratory. These analyses were conducted with SAS v9.4 (SAS Institute, Cary, NC, USA) and least square means were compared at the significance level of *P* less than 5% (0.05). For DEGs analysis, animal tissue and treatment were fixed effects fit in design model and individual animal was used as replicates when using the edgeR package. Two different statistical design models were used when using the Deseq2 package. In the first design, animal tissue and treatment were used as fixed effects in the design model and individual animal was used as replicates. In the second design animal, animal tissue, and treatment were used as fixed effects to fit in the design model.

Results

Growth performance in response to BSH inhibitors treatment

All chickens exhibited normal growth behavior and there was no mortality during the 28 days of the experiment period. As shown in Table 4-2, there was no statistical difference of chicken body weight and weekly body weight gain among four different groups ($P > 0.05$). As for feed conversion ratio (FCR), from the experiment day 10 to 14, chickens in the riboflavin treatment group had a significantly higher FCR than chicken in the CAPE treatment group and carnosic acid treatment group ($P = 0.0297$); the difference in FCR among control group, CAPE treatment group, and carnosic acid treatment group was not statistically different ($P > 0.05$). FCR from day 14-17, 17-21, 21-24 and 24-28 did not show a significant difference among all groups ($P > 0.05$).

Biochemical analyses of serum samples

The effects of BSH inhibitors on chicken serum metabolites are shown in Table 4-3. BSH inhibitors treatment did not cause significant difference of chicken serum glucose ($P = 0.08$), total cholesterol ($P = 0.33$), triglycerides ($P = 0.85$) and VLDL ($P = 0.18$) compared to the control group.

Bile salts profile alteration in chicken fecal and serum samples in response to BSH inhibitor treatment

The statistical analysis of the bile salts profile conducted by our collaborator at University College Cork and by our lab showed the same results.

Ten chicken fecal samples from each of the four groups were subjected to bile profile analyses by UPLC-MS. Twenty-six bile acids and amino acids related to bile metabolism were qualified and quantified (Table 4-4). Representative bile profiles obtained from chicken fecal samples in response to BSH inhibitors treatment are presented in Figure 4-2. BSH inhibitors treatment in all three groups had lower murocholic acid concentration than the control group, but only murocholic acid concentration in chicken fecal samples from the riboflavin treatment group was statistically significant lower than the control group ($P = 0.04$). Similarly, chicken fecal lithocholic acid (LCA), glycohyodeoxycholic acid (GHCA), and glycodeoxycholic acid (GCDCA) concentrations decreased corresponding to BSH inhibitor treatments, but riboflavin treatment also caused the largest change ($P < 0.05$). The concentration of glycoursodeoxycholic acid (GUDCA) decreased in response to BSH inhibitors treatment, but it was at a marginally significant level ($P = 0.06$). The concentration of secondary and tertiary bile salts also decreased in BSH inhibitors treatment groups compared to the control group, and only riboflavin treatment group showed the significant decrease ($P = 0.03$). Notably, taurine concentration, an indication of bile salts deconjugation, was lower in all three BSH inhibitors treatment groups than in the control group, however, the difference was not significant. There was no statistical difference shown among the other bile salts ($P > 0.05$).

Using the same process, all ten chicken serum samples from each of the four groups were subjected to bile profile analyses by UPLC-MS. Twenty-six bile acids and amino acids related to bile metabolism were determined and quantified (Table 4-5). Of the tested bile salts, riboflavin treatment caused a significantly low concentration of glycohyocholic acid (GHCA) in

serum compared to the control group and carnosic acid treatment group ($P = 0.01$). However, there was not a statistical difference among four groups for other bile salts ($P > 0.05$).

General analysis of gene expression profiles among different samples

RNA-Seq data analysis was successfully performed by our collaborator Dr. Janet Chang (Rutgers University) using the established software and package in the core facility at her institution. However, due to the supercomputer system's transformation from HPC to ACF at UTK, some technical issues arose and we were not able to process RNA-Seq mapping and gene reads counting process using UTK's ACF supercomputer system. Instead, we used the gene count data generated by our collaborator and processed the DEGs analysis with DESeq2 package in R as shown in Figure 4-1.

The concentration and RIN (Table 4-6) implied high quality of extracted RNA. More than 685 million raw reads in pairs were obtained from RNA-Seq, ranging from 9.2 to 162.7 M raw reads per sample; both the numbers of raw reads and trimmed reads are presented in Table 4-6. The mapping rate of RNA samples from liver samples and ileum samples ranged from 61.2% to 88.2%. The number of DEGs caused by carnosic acid treatment in liver and ileum samples were analyzed by edgeR and DeSeq2 package, the number of DEGs from three different methods presented in Table 4-7 and the Venn diagram (Figure 4-3) revealed the different number of DEGs determined by three different methods. The number of DEGs in the liver (up regulated and down regulated) were more than the DEGs in the ileum (up regulated and down regulated). The representative DEGs in response to carnosic acid were summarized in Table 4-8.

Validation of DEGs by quantitative PT-PCR (qRT-PCR)

The expression of DEGs involved in bile acid metabolism and lipid metabolism in the liver or ileum, as identified by DAVID-BR, was further validated using qRT-PCR. All the tested genes showed the same pattern in their expression levels in both qPCR and RNA-Seq analysis (Table 4-9), indicating successful completion of RNA-Seq analysis.

Discussion

BSH takes part in the gateway reaction of bile acid metabolism by the deconjugation of conjugated bile acid and production of unconjugated bile acid and free amino acids (taurine or glycine) (Begley *et al.*, 2006). The hypothesis of this study is that BSH inhibitors treatment would act on chicken intestinal bacterial BSH, which would further change the host bile profile. In this study, the oral administration of BSH inhibitors treatment caused chicken fecal bile profile changes, while the bile profile change in the serum samples was not as dramatic as in the fecal samples. The decrease of taurine and increase of total conjugated bile acid (except in CAPE treatment group, Table 4-4) in fecal samples implied decreased BSH activity in the intestine. Besides, the concentration of secondary and tertiary bile acids in fecal samples (Figure 4-2) from all three BSH inhibitors treatment groups were lower compared to fecal samples from the control group. This was due to the decreased unconjugated bile acids which are precursors of secondary and tertiary bile acids (Begley *et al.*, 2006; Russell, 2009; Vlahcevic *et al.*, 1996). The concentrations of murocholic acid (McAuliffe *et al.*, 2005) and lithocholic acid (LCA) were also decreased significantly in the fecal samples from the BSH inhibitors treatment group (Table 4-4, Figure 4-2). Notably, LCA has long been recognized as a toxic component for different animals such as chickens and rabbits (Bernstein *et al.*, 2005; Fischer *et al.*, 1974; Hofmann,

1999; Leveille *et al.*, 1964; Leveille *et al.*, 1966). The LCA was considered an implication of colon carcinogenesis (Bernstein *et al.*, 2005) and highly hepatotoxic for experimental animals (Hofmann, 1999). In previous studies, it was shown that when adding LCA to chicken feed, animal plasma lipid levels and liver size were increased and liver fat was decreased; the biliary hyperplasia in chicks was also induced (Leveille *et al.*, 1964; Leveille *et al.*, 1966). The decreased LCA concentration in the fecal samples from the BSH inhibitor groups indicated a liver protective effect. As the liver is the major organ for fatty acid synthesis and bile synthesis, lower level of LCA concentration may be one of the factors that lead to host body weight gain.

The chicken growth performance data showed that from day 10 to 28, oral administration of BSH inhibitors to chickens did not cause statistical difference in body weight, even though chicken body weight from the BSH inhibitor groups had consistently higher body weight than in the control group. A similar trend of advantages of chickens from BSH inhibitor treatment groups had superior feed conversion ratio than chickens from control group from day 10 to day 24, the difference was only statistically significant during day 10 to14 of the treatment period. Power analysis showed that the power for chicken body weight data for each data point was higher than 0.8 which suggested that chicken body weight data had enough statistic power and low type II errors. However, power analysis for chicken feed conversion data ranged from 0.076 to 0.706. To decrease type II error of the experiment, a larger animal trial (250 birds per treatment group) is needed to maintain a statistical power more than 0.8 for the BSH inhibitors effects validation.

The two most commonly used bioconductor packages, edgeR and Deseq2, were used for DEGs analysis in this study. Both methods were used when count data had a negative binomial distribution (Anders *et al.*, 2010; Kvam *et al.*, 2012; Love *et al.*, 2014; Robinson *et al.*, 2010). These two methods normally give a higher power estimation, especially when the number of samples is less than five. They provide the highest sensitivity of the algorithms that controll type-I error in the sense that actual False discovery rate (FDR) is below or at 0.1 (Ching *et al.*, 2014; Love *et al.*, 2014). However, with the same gene counts data, the revealed number of DEGs were different with the two different methods but the results have been inconsistent regarding which method is a better approach (Ching *et al.*, 2014; Rajkumar *et al.*, 2015). The edgeR moderates the variance estimate for each gene toward a common estimate across all genes using a weighted conditional likelihood. The DESeq2 approach corrects variance that is too low through modeling of the dependence of the variance on the average expression strength over all samples (Ching *et al.*, 2014; Love *et al.*, 2014). In future studies, DEGs lists from both method can be compared to generate a new DEGs list which contains the genes from both methods for qRT-PCR validation, functional annotation, and canonical pathway analyses.

To understand how BSH inhibitors impact host metabolic processes, we used RNA-Seq analysis of chicken liver and ileum tissues from the carnosic acid and control groups and identified several interesting genes. The expression of the AKR1D1 gene in the liver was up-regulated by in carnosic acid treated chickens. AKR1D1 belongs to the aldo-keto reductase family and it involves in significant steps in bile acid synthesis. Multiple reactions happen from 7-α-hydroxycholesterol to primary bile acids (cholic acid and chenodeoxycholic acid), and AKR1D1 takes part in two different reduction steps in this process (Russell, 2003). This is consistent with

the bile profile change in chicken fecal samples, where the concentration of cholic acid in the carnosic acid treatment group increased 17% compared to the control group; however, the serum concentration of cholic ancid and chenodeoxycholic acid between treatment group and control group were not statistically different.

The CYP3A80 gene in chicken liver samples was also up-regulated by oral administration of carnosic acid. DAVID-BR analysis revealed that CYP3A80 is related to secondary metabolites biosynthesis. The CYP3A80 is predominantly expressed in the liver (Watanabe *et al.*, 2013). In chicken, CYP3A37 and CYP3A80 are presented in birds as CYP3A family genes (Watanabe *et al.*, 2013). Chicken CYP3A37 and CYP3A80 are 51% and 59% identical to human CYP3A4 (Shang *et al.*, 2013). Even though the literature about CYP3A80 function is limited, the function of CYP3A4 is well understood. CYP3A4 catalyzes the 6-hydroxylation of LCA, which is consistent with our funding that LCA in the chicken fecal samples from the carnosic acid treatment group is much lower than the samples from treatment group (Araya *et al.*, 1999; Staudinger *et al.*, 2001).

The G0S2 gene was also up-regulated in the ileum samples from the chicken oral administrated with carnosic acid compared to the chicken from the control group. The G0S2 gene is a highly conserved in vertebrates and it is a master regulator of tissue-specific balance of triglyceride storage and mobilization, partitioning of metabolic fuels between the adipose tissue, the liver and the whole body adaptive energy response (Zhang *et al.*, 2017b). G0S2 is a lipolysis inhibitor by inhibiting adipose triglyceride lipase (ATGL) which is responsible for catalyzing the first step of three step-wise reactions that define lipolysis (Zhang *et al*., 2017b; Zimmermann *et al*., 2004).

Multiple physiological roles of BSH inhibitors may confound the specifically desired feature of BSH inhibition, which was also verified by RNA-Seq analysis. For example, carnosic acid is an anti-oxidant molecule and possesses anti-inflammatory and anti-carcinogenic properties (Aruoma *et al.*, 1992; de Oliveira, 2016; Erkan *et al.*, 2008). This is verified by a list of DEGs related to immune and inflammation response in both liver and ileum tissues (Table 4-8). Carnosic acid exerts its anti-oxidant effects by the activation of the PI3K/Akt/Nrf2 signaling pathway, which would lead to the expression of anti-oxidant enzymes and neuronal differentiation (de Oliveira, 2016). The activation of Nrf2 can induce the Nrf2-targeted genes such as GSTA3, which is confirmed by RNA-Seq analysis and qRT-PCR (Zhang *et al.*, 2017a; Zhao *et al.*, 2007). In mouse a study, GST3 levels increased dramatically during adipose conversion process and the expression of GSTA3 is associated with acquisition of the adipocyte phenotype (Jowsey *et al.*, 2003). This may also contribute to the increased body weight (not statistically significant) of the broiler chickens receiving oral administration of carnosic acid.

Due to the global trend of removing AGPs from food producing animals, alternatives to AGPs are urgently needed. When developing effective non-antibiotic feed additives, development of effective and practical delivery approach is also highly warranted (Bento *et al.*, 2013; Kailasapathy, 2009; Zhang *et al.*, 2014b). Leading encapsulation methods, such as microparticles and nanoparticles approaches, have shed light on improving the bioactivity and bioavailability of feed additives (Bale *et al.*, 2016; Gangadoo *et al.*, 2016). In a recent study by Zhang *et.al* (Zhang *et al.*, 2014b), the encapsulation of an essential oil carvacrol with alginate-whey protein into dry microparticles showed high encapsulation efficiency. Notably, the *in vivo* experiment also showed that carvacrol in microparticle form had good gastric resistance and

rapid intestinal release compared to unencapsulated carvarol during oral administration to chicken (Zhang *et al.*, 2014b). In another study, citral was proposed to control necrotic enteritis caused by *Clostridium perfringens*; oral administration of encapsulated citral showed a superior effect in reducing intestinal necrotic enteritis in broiler chickens compared to unencapsulated citral (Yang *et al.*, 2016). Besides, nanoparticles also enables feed additives to target organs or systems while avoiding fast degradation when passing through the GI tract, which would be promising to apply in our future study of BSH inhibitors *in vivo* effects, due to the fact that BSH inhibitors exert inhibition effects on bacterial BSHs in the host intestine (Gangadoo *et al.*, 2016). The three compounds used in this study have characteristics that may weaken their bioactivity and bioavailability. Riboflavin is a yellow powder which has good solubility in water. However, the rate of riboflavin destruction increases dramatically in oxygen permeable container during storage which is a challenge for riboflavin storage either separately or after mixing with food (Dennison *et al.*, 1977). The carnosic acid and CAPE have low water solubility, which is a challenge for delivery efficiency. Therefore, to incorporate advanced encapsulation technology into BSH inhibitor-based non-antibiotic feed additive development is a promising approach for future study.

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Appendix

Table 4- 1. Primers for qRT-PCR used in this study.

Genes	Primer Sequence (5`-3`)	Length (bp)	Annealing Temperature (°C)	Gene bank accession no.
Liver				
AKR1D1	F: TCCACCAGAGCTGGTACGTC	319	53	NM_001277393.1
	R: ATAGGGATGGCATTCAACCTGG			
CYP3A80	F: AATGGGACTCCTTCCAGACCT	235	53	NM_001329508.1
	R: CCTGCCATCATAAATCCCCC			
GSTA3	F: AATTTCCCCTCTTGCAGAGTT	194	51	NM_001001777.1
	R: CACTCCGCTTATCAGCAAAC			
Ileum				
G0S2	F: CGGCCTCAGAACGGAGC	113	54	NM_001190924.3
	R: TCACCATCTTCCTGTTGGGC			
GSTA3	F: AATTTCCCCTCTTGCAGAGTT	194	51	NM_001001777.1
	R: CACTCCGCTTATCAGCAAAC			
Internal reference				
β-actin	F: CTCTGACTGACCGCGTTACT	172	53	NM_205518.1
	R: TACCAACCATCACACCCTGAT			

Table 4- 2. Body weight, feed conversion ratio and body weight gain in chickens treated with different BSH inhibitors.

Chicken Body Weight (g/bird)						
Chicken Age	Control	CAPE	Riboflavin	Carnosic Acid	MSE	*P* value
Day 7	163.56	162.57	164.19	162.76	3.79	0.99
Day 10	249.42	244.91	258.46	251.58	5.55	0.43
Day 14	421.16	421.37	431.98	436.48	8.73	0.54
Day 17	580.32	585.68	611.68	601.10	13.61	0.40
Day 21	813.11	829.46	869.59	859.72	20.39	0.25
Day 24	967.95	1022.36	1053.10	1053.98	28.20	0.19
Day 28	1234.26	1294.11	1304.03	1293.40	36.93	0.56
Feed Conversion Ratio (g of feed/ g of weight gain)						
Chicken Age	Control	CAPE	Riboflavin	Carnosic Acid	MSE	*P* value
Day 10-14	1.64 [ab]	1.56 [b]	1.81 [a]	1.53 [b]	0.06	0.03
Day 14-17	2.03	1.94	1.92	1.91	0.10	0.84
Day 17-21	1.93	1.82	1.83	1.66	0.08	0.21
Day 21-24	2.49	1.98	2.15	1.93	0.19	0.22
Day 24-28	2.21	2.15	2.36	2.31	0.18	0.84
Chicken Body Weight Gain (g/bird/day)						
Chicken Age	Control	CAPE	Riboflavin	Carnosic Acid	MSE	*P* value
Day 7-10	28.68	27.45	31.48	29.58	1.02	0.11
Day 10-14	42.99	44.15	43.33	46.11	1.47	0.48
Day 14-17	53.05	54.77	59.90	54.87	2.67	0.36
Day 17-21	58.20	60.95	64.48	64.66	2.33	0.23
Day 21-24	51.61	64.30	61.17	64.75	4.23	0.18
Day 24-28	66.67	68.06	62.78	59.90	4.12	0.52

Growth performance data (body weight, body weight gain, and feed conversion ratio) is represented as least square means and mean square error [MSE]. Different letters in the same row represent significant difference between treatments (Tukey, $P < 0.05$).

Table 4- 3. Summary of chicken serum biochemical analyses in response to three different BSH inhibitors.

Biochemical Parameters	Control	CAPE	Riboflavin	Carnosic Acid	MSE	*P* value
Glucose (mg/dL)	224.49	189.96	186.32	186.58	9.77	0.08
Total Cholesterol (mg/dL)	96.94	91.84	105.26	110.61	7.94	0.33
Triglycerides (mg/dL)	42.69	40.20	39.35	41.86	3.95	0.85
VLDL (mmol/L)	11.53	14.35	16.43	14.38	3.56	0.18

Serum glucose, triglycerides and total cholesterol concentrations were expressed as least square means and mean square error [MSE]. Statistical significant level of difference is defined by $P<0.05$.

Table 4- 4. Bile salts profile alteration in chicken fecal samples (ng/g) in response to BSH inhibitors treatment.

Bile Salts	Control	CAPE	Riboflavin	Carnosic Acid	MSE	*P* value
Primary BA	105159	90700	108314	102713	31492	0.98
All primary BA	106935	93581	111070	106105	31822	0.98
Secondary/Tertiary BA	660.55 [a]	391.94 [ab]	277.63 [b]	377.54 [ab]	72.11	0.03
Unconjugated BA	107596	93974	111347	106484	31867	0.98
Tauroconjugated BA	585103	462953	831280	741745	152232	0.38
Glycoconjugated BA	1911.53	1219.22	2566.67	2375.07	566.92	0.40
Total conjugated BA	586952	464147	833837	744145	152534	0.38
Total BA	695328	560087	945207	851290	166556	0.42
Taurine	32157	21386	17501	19894	5627.11	0.33
Cholic Acid	18346	20973	20952	21497	6857.69	0.99
Chenodeoxycholic Acid	86840	69913	87528	81231	25860	0.96
Hyocholic Acid	128.7	207.72	157.03	284.59	60.1217	0.34
Hyodeoxycholic Acid	30	20.21	23.17	28.48	4.9952	0.51
Murocholic Acid	31.6172 [a]	14.5006 [ab]	10.5949 [b]	15.451 [ab]	4.3342	0.04
a,w-Muricholic Acid	1250.77	2035.6	1919.35	2408.9	621.05	0.63
β-Muricholic Acid	233.53	437.71	631.33	612.78	162.04	0.33
Ursodeoxycholic Acid	111.03	80.5341	81.276	77.1654	18.5616	0.56
Deoxycholic Acid	92.7548	106.04	68.409	79.3409	39.1108	0.91
Lithocholic Acid	457.21 [a]	204.1 [b]	128.11 [b]	219.31 [b]	43.32	0.01
Taurocholic Acid	176790	188016	337641	298439	73680	0.38
Taurohyocholic Acid	906.6	914.5	1648.41	1843.62	339.57	0.18
Taurochenodeoxycholic Acid	393385	261451	462722	420166	89789	0.47
Tauroursodeoxycholic Acid	904.21	622.09	1348.56	1038.45	235.52	0.26
Taurodeoxycholic Acid	231.21	158.14	301.33	411.91	88.07	0.29
Taurohyodeoxycholic Acid	1301.23	1328.43	3334.59	1679.47	574.52	0.11
Taurolithocholic Acid	135.51	87.88	215.82	174.94	41.12	0.23

Table 4- 4. Continued.

Bile Salts	Control	CAPE	Riboflavin	Carnosic Acid	MSE	*P* value
Tauromucholic Acid	11616	10153	24077	18021	4311.96	0.17
Glycocholic Acid	885.30	586.53	1652.44	1289.44	446.58	0.41
Glycohyocholic Acid	61.18	47.97	41.31	48.24	7.01	0.31
Glycohyodeoxycholic Acid	125.34 [a]	80.67 [ab]	55.42 [b]	95.13 [ab]	12.70	0.03
Glycochenodeoxycholic Acid	572.46	327.53	691.44	749.93	150.20	0.28
Glycoursodeoxycholic Acid	127.72 [a]	88.46 [ab]	73.82 [b]	92.14 [ab]	11.81	0.06
Glycodeoxycholic Acid	126.12 [a]	79.70 [ab]	46.33 [b]	87.48 [ab]	14.63	0.03
Glycolithocholic Acid	14.24	9.27	6.28	10.49	2.28	0.18

Bile salt concentration was expressed as least square means and mean square error [MSE]. Different letters in the same row represent significant difference between treatments (Tukey, $P < 0.05$).

Table 4- 5. Bile salts profile alteration in chicken serum samples (ng/mL) in response to BSH inhibitors treatment.

Bile Salts	Control	CAPE	Riboflavin	Carnosic Acid	MSE	*P* value
Primary BA	367.62	394.89	648.51	369.17	168.15	0.61
All primary BA	412.36	431.83	709.90	411.81	180.94	0.61
Secondary/Tertiary BA	106.74	113.13	125.12	109.60	18.43	0.90
Unconjugated BA	517.72	547.06	832.49	521.62	174.82	0.55
Tauroconjugated BA	13249	11837	13689	13870	1911.94	0.87
Glycoconjugated BA	168.08	159.61	158.6	163.52	15.29	0.97
Total conjugated BA	13413	11997	13847	14032	1915.15	0.87
Total BA	13931	12538	14684	14560	2031.31	0.87
Taurine	5158.07	6303.62	5128.02	5486.30	462.94	0.32
Cholic Acid	48.88	44.37	137.26	43.90	48.88	0.41
Chenodeoxycholic Acid	319.07	349.76	510.66	325.09	129.01	0.70
Hyocholic Acid	7.83	7.19	7.14	6.90	1.61	0.98
Hyodeoxycholic Acid	5.61	4.85	3.60	5.10	1.64	0.85
Murocholic Acid	5.92	3.83	6.34	6.24	1.13	0.41
a,w-Muricholic Acid	19.57	16.75	39.17	18.05	13.60	0.63
β-Muricholic Acid	6.04	4.09	5.35	6.35	2.02	0.86
Ursodeoxycholic Acid	22.89	18.30	16.19	22.72	5.62	0.80
Deoxycholic Acid	26.88	24.97	42.06	25.80	12.88	0.76
Lithocholic Acid	57.50	68.99	67.81	60.74	6.01	0.51
Taurocholic Acid	1800.90	1716.03	2416.99	2202.29	445.02	0.66
Taurohyocholic Acid	16.40	16.24	20.23	21.94	4.37	0.75
Taurochenodeoxycholic Acid	11004	9670.62	10664	11251	1500.11	0.89
Tauroursodeoxycholic Acid	31.08	33.62	34.76	31.56	4.41	0.93
Taurodeoxycholic Acid	13.79	14.01	12.97	19.31	2.62	0.37
Taurohyodeoxycholic Acid	70.37	76.08	80.75	56.70	12.36	0.57
Taurolithocholic Acid	15.25	18.07	25.14	21.44	3.68	0.34
Tauromucholic Acid	296.93	304.84	450.66	261.07	114.68	0.67
Glycocholic Acid	20.20	19.38	26.76	20.39	5.55	0.77
Glycohyocholic Acid	16.23 [a]	11.65 [ab]	9.64 [b]	15.04 [a]	1.14	0.01
Glycohyodeoxycholic Acid	26.03	25.21	24.55	24.98	3.64	0.99

Table 4- 5. Continued.

Bile Salts	Control	CAPE	Riboflavin	Carnosic Acid	MSE	*P* value
Glycochenodeoxycholic Acid	50.13	47.62	51	52.44	4.29	0.88
Glycoursodeoxycholic Acid	22.99	20.57	18.60	21.99	2.03	0.50
Glycodeoxycholic Acid	28.11	31.74	24.48	25.09	3.41	0.46
Glycolithocholic Acid	4.14	3.19	2.84	3.29	1.04	0.85

Bile salt concentration was expressed as least square means and mean square error [MSE]. Different letters in the same row represent significant difference between treatments (Tukey, $P < 0.05$).

Table 4- 6. RNA Sample description and mapping statistics to reference genome.

Tag name	Treatment [a]	Concentration (ng/µL)	RIN	28S/18S	Number of reads in pair (raw)	Number of reads in pair (Q trimmed)	Mapping Rate (%)	Non-specific mapping of mapped (%)
Liver								
LT1	T	320	8.6	1.9	26343666	24990680	86.4	3.4
LT2	T	2664	9.4	2.1	37647864	35569752	86.7	3.6
LT3	T	4328	9.2	1.9	9188235	8510958	85.0	3.3
LT4	T	848	9.2	1.9	90545141	84666914	68.9	3.4
LC1	C	3576	9.2	2.1	27499176	25823234	88.2	3.5
LC2	C	3216	9.1	2.0	14620336	13514440	86.4	3.5
LC3	C	4760	9.1	2.1	36029297	34089068	85.8	4.1
LC4	C	4912	9.3	2.2	134362985	128352800	64.8	3.8
Ileum								
IT1	T	2088	9.5	2.0	14406611	13461675	82.8	4.18
IT2	T	3424	9.6	2.2	14867215	14004769	83.4	4.0
IT3	T	2320	9.4	2.0	20300211	18961626	84.1	4.3
IT4	T	1460	8.9	2.0	29154179	27535357	82.6	5.5
IC1	C	2068	9.0	1.8	18346963	17380507	83.7	7.9
IC2	C	1524	9.3	1.9	162762428	155661706	61.2	4.0
IC3	C	1476	8.6	1.8	35944368	33371893	82.8	4.6
IC4	C	320	8.9	1.8	13162436	12258294	82.2	4.0

[a] T stands for treatment; C stands for control.

Table 4- 7. The number of differentially expressed genes detected by three different methods.

Tissue	DEGs method	Number of up regulated gene	Number of down regulated gene	Total number of DEGs
Liver	edgeR	495	112	607
	DeSeq_1	360	248	608
	DeSeq_2	617	296	913
Ileum	edgeR	116	99	215
	DeSeq_1	246	230	476
	DeSeq_2	289	234	523

DeSeq_1 means analysis conducted under R environment with DeSeq package, in the statistic model animal tissue and treatment were fixed effects and individual animal were used as repeated measure; DeSeq_2 means analysis conducted under R environment with DeSeq package, in the statistic model individual animal, animal tissue and treatment were fixed effects. edgeR means analysis conducted under R environment with DeSeq package, in the statistic model animal tissue and treatment were fixed effects and individual animal were used as repeated measure.

Table 4- 8. Representative differentially expressed genes in response to carnosic acid treatment based on DAVID bioinformatics resources[a].

Tissue	Gene name	Potential function	References
Liver			
Up regulated	CD3e molecule(CD3E)	Immune	(Ueda *et al.*, 2017)
	2'-5'-oligoadenylate synthetase-like(OASL)		
	Major histocompatibility complex class II beta chain BLB1(BLB1)		(Wang *et al.*, 2012a)
	aldo-keto reductase family 1, member D1 (delta 4-3-ketosteroid-5-beta-reductase)(AKR1D1)	Bile acid metabolism	(Russell, 2003)
	phosphoglycerate dehydrogenase(PHGDH)	Taurine metabolism	(Xie *et al.*, 2016)
	apolipoprotein D(APOD)	Lipid metabolism	(Perdomo *et al.*, 2009)
	lipase, member I(LIPI)		
	adenylate kinase 1(AK1)	Energy metabolism	(Janssen *et al.*, 2003)
	protein kinase, AMP-activated, gamma 3 non-catalytic subunit(PRKAG3)	Glucose metabolism	
	cytochrome P450, family 2, subfamily C, polypeptide 23a(CYP2C23a)	Secondary metabolites biosynthesis	
	cytochrome P450, family 3, subfamily A, polypeptide 80(CYP3A80)		(Staudinger *et al.*, 2001)
	cytochrome P450, family 4, subfamily B, polypeptide 7(CYP4B7)		
	glutamine synthetase-like(LOC417253)	Carnosic acid metabolism related	(Tong *et al.*, 2017; Zhang *et al.*, 2017a)
	glutathione S-transferase alpha 3(GSTA3)		
	glutathione S-transferase omega 1(GSTO1)		
	glutathione S-transferase-like(LOC100859645)		

Table 4- 8. Continued.

Tissue	Gene name	Potential function	References
Liver	insulin like growth factor binding protein 5(IGFBP5)	Insulin regulation	
Down regulated	S100 calcium binding protein A9(S100A9)	Immune	
	T-cell immunoglobulin and mucin domain containing 4(TIMD4)		
	avian beta-defensin 1(AvBD1)		(Cuperus *et al.*, 2013)
	avian beta-defensin 2(AvBD2)		
	avian beta-defensin 6(AvBD6)		
	cathelicidin-1(CATH1)		(Cuperus *et al.*, 2013)
	cathelicidin-2(CATH2)		
	cytochrome P450 family 39 subfamily A member 1(CYP39A1)	Bile acid metabolism and liver health	(Khenjanta *et al.*, 2014; Russell, 2003)
	mitogen-activated protein kinase kinase kinase 15(MAP3K15)		(Noel *et al.*, 2016)
	hexokinase domain containing 1(HKDC1)	Carbohydrate metabolism	
	solute carrier family 7 (amino acid transporter light chain, L system), member 5(SLC7A5)	Amino metabolism	

Table 4- 8. Continued.

Tissue	Gene name	Potential function	References
Ileum			
Up regulated	2'-5'-oligoadenylate synthetase-like(OASL)	Immune	
	eomesodermin(EOMES)		(Šahmatova *et al.*, 2017)
	radical S-adenosyl methionine domain containing 2(RSAD2)		(De Wit *et al.*, 2008)
	ATP-binding cassette, sub-family C (CFTR/MRP), member 2(ABCC2)	Carnosic acid metabolism related	(Yan *et al.*, 2015)
	aldehyde dehydrogenase 8 family member A1(ALDH8A1)		(Wu *et al.*, 2012)
	glutathione S-transferase alpha 3(GSTA3)		
	prostaglandin reductase 1(PTGR1)		(Wu *et al.*, 2011)
	G0/G1 switch 2(G0S2)	Lipolysis and energy harvest	(Kitareewan *et al.*, 2008; Zhang *et al.*, 2017b)
	hydroxyacid oxidase (glycolate oxidase) 1(HAO1)	Fatty acid alpha-oxidation	
	solute carrier family 2 (facilitated glucose/fructose transporter), member 5(SLC2A5)	Fructose absorption	(Barone *et al.*, 2009)
	aldehyde dehydrogenase 8 family member A1(ALDH8A1)		
	cytochrome P450, family 2, subfamily C, polypeptide 23a(CYP2C23a)	Secondary metabolites biosynthesis	
	glucagon receptor(GCGR)	Bile acid metabolism	(Ikegami *et al.*, 2006)
	eomesodermin(EOMES)		

Table 4- 8. Continued.

Tissue	Gene name	Potential function	References
Ileum			
Down regulated	ATP-binding cassette, sub-family G (WHITE), member 1(ABCG1)	Lipid metabolism	(Schmitz *et al.*, 2001)
	alkaline ceramidase 2(ACER2)	Lipid metabolism	(Jiang *et al.*, 2015)
	C-C motif chemokine receptor 10(CCR10)	Immune and inflammation	(Homey *et al.*, 2002)
	CD247 molecule(CD247)		
	transcription factor 7 (T-cell specific, HMG-box)(TCF7)		
	NADPH oxidase 1(NOX1)	Carnosic acid metabolism related	(Buendia *et al.*, 2016)
	NADPH oxidase organizer 1(NOXO1)		
	family with sequence similarity 132 member A(FAM132A)	Insulin regulation	
	hydroxyacid oxidase 2 (long chain)(HAO2)	Fatty acid oxidation	
	putative short-chain dehydrogenase/reductase family 42E member 2-like(LOC771638)	Steroid biosynthesis	

[a] Determined by DAVID bioinformatics resources (https://david.ncifcrf.gov/).
The genes denoted as up-regulated indicated that those genes have higher expression in carnosic acid treatment group than in the control group; genes denoted as down-regulated indicated that those genes have lower expression in carnosic acid treatment group than in the control group. All genes showing a significant difference in expression had the cut-off of *P*-value ≤ 0.05.

Table 4- 9. Comparison of RNA-Seq and qPCR analyses of the selected genes involved in bile acid metabolism and lipid metabolism in response to oral administration of carnosic acid in broilers.

Tissue	Gene	RNA-Seq			qPCR		
		Log_2 fold -change	fold-change	*P* value	Log_2 fold -change	fold-change	*P* value
Liver	AKR1D1	-1.43	2.69	0.02	-1.59	3.02	0.43
	CYP3A80	-0.81	1.75	0.05	-0.73	1.66	0.06
	GSTA3	-1.95	3.85	<0.001	-1.13	2.19	0.28
Ileum	G0S2	-2.04	4.12	0.02	-1.63	3.10	0.05
	GSTA3	-1.29	2.44	0.03	-1.15	2.22	0.09

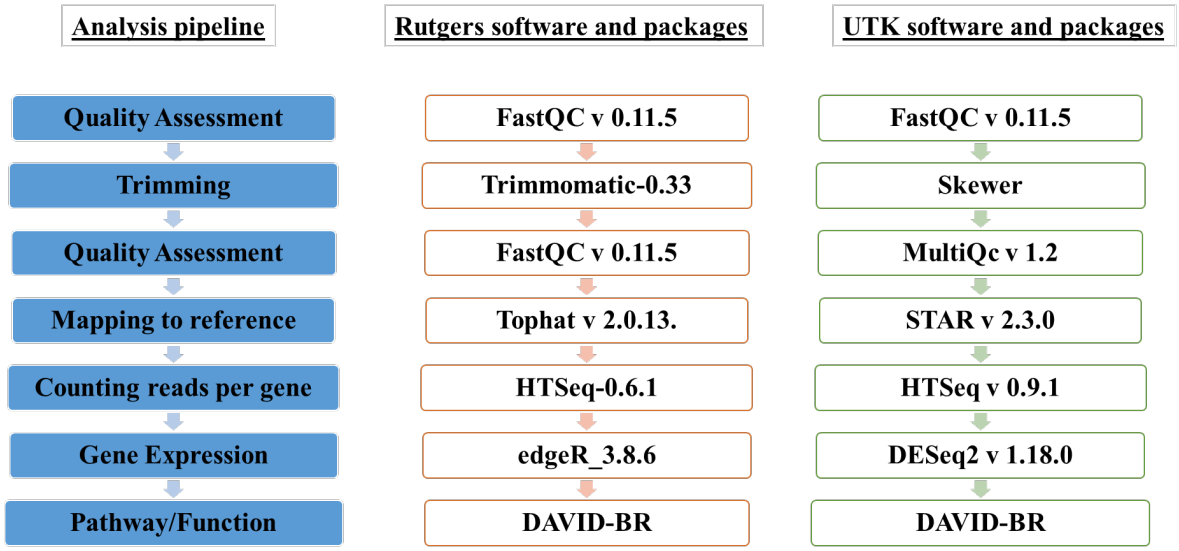

Figure 4- 1. The RNA-Seq pipeline and comparison of the software used for RNA-Seq data analysis and software/packages at Rutgers University (collaborator) and The University of Tennessee, Knoxville.

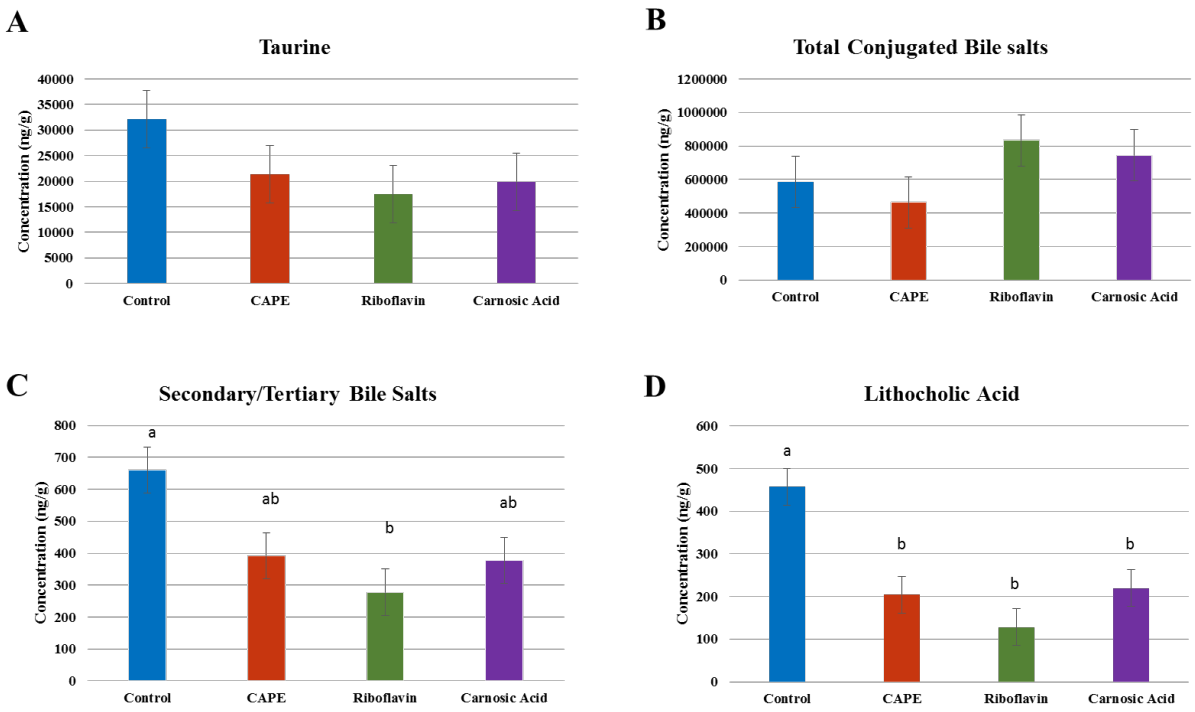

Figure 4- 2. Representative bile acids profile in chicken fecal samples by UPLC-MS. (A) Concentration of taurine in chicken fecal samples from BSH inhibitors groups and treatment group. (B) Concentration of conjugated bile salts in chicken fecal samples from BSH inhibitors groups and treatment group. (C) Concentration of secondary/tertiary bile salts in chicken fecal samples from BSH inhibitors group and treatment group. (D) Concentration of lithocholic acid in chicken fecal samples from BSH inhibitors groups and treatment group.

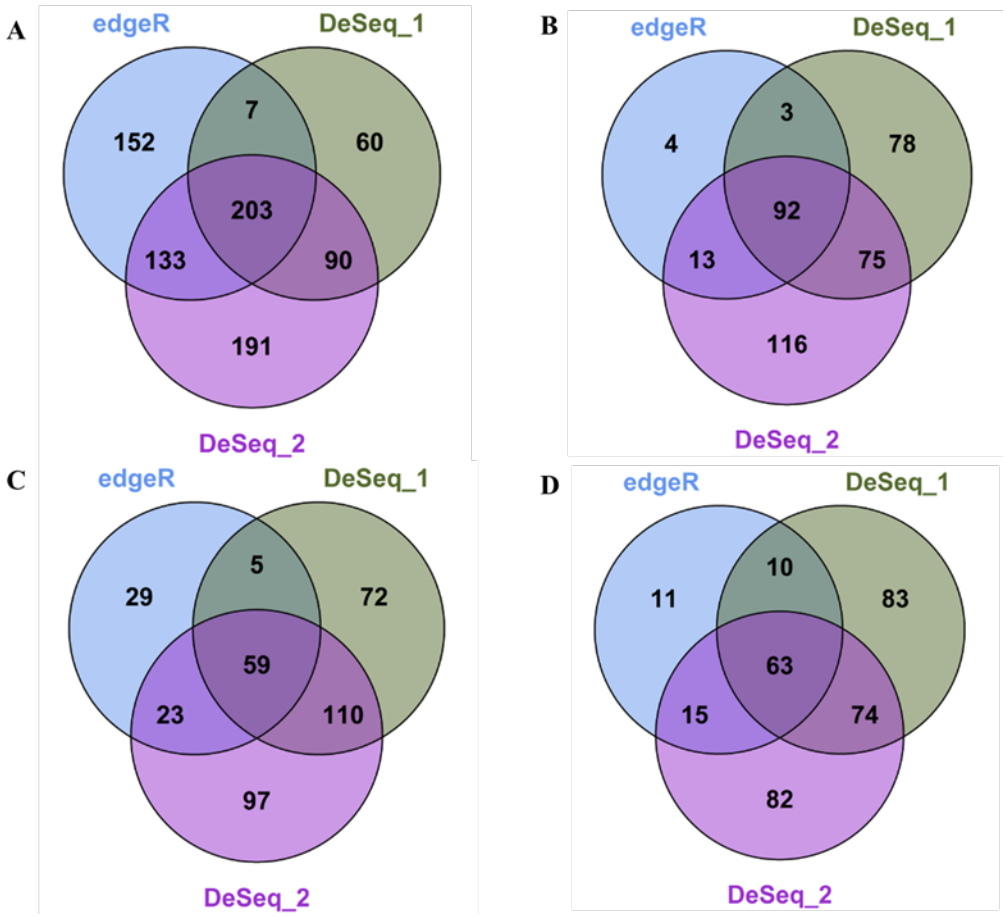

Figure 4- 3. Venn diagram of the number of differentially expressed genes detected by three different methods. DeSeq_1 means analysis conducted under R environment with DeSeq package, in the statistic model animal tissue and treatment were fixed effects and individual animal were used as repeated measure; DeSeq_2 means analysis conducted under R environment with DeSeq package, in the statistic model individual animal, animal tissue and treatment were fixed effects. edgeR means analysis conducted under R environment with DeSeq package, in the statistic model animal tissue and treatment were fixed effects and individual animal were used as repeated measure. (A) Comparison of number of DEGs up regulated in the liver with three methods. (B) Comparison of number of DEGs down regulated in the liver with three methods. (C) Comparison of number of DEGs up regulated in the ileum with three methods. (D) Comparison of number of DEGs down regulated in the ileum with three methods.

Conclusions and perspectives

Bile salt hydrolase (BSH) catalyzes the essential gateway reaction for bile acid metabolism by deconjugates the conjugated bile salts into unconjugated bile salts and taurine or glycine (Begley *et al.*, 2006). Bile salts plays dual role in host lipid absorption and signaling *via* some key signaling molecules such as FXR and TGR5, thus exerts effects on host lipid metabolism and energy harvest (Joyce *et al.*, 2014b). Animal studies also provided direct and indirect evidence of BSH function on host lipid metabolism and energy harvest (Begley *et al.*, 2006; Joyce *et al.*, 2014a; Lin, 2014). These evidence make BSH as a promising target of developing alternatives for antibiotic growth promoters and as new measures for control obesity. However, research on BSH is still in its infancy regarding to its basic science and translation innovation.

Our preliminary rat study showed that oral administration of Zucker fatty rats with purified BSH enzyme significantly increased rat respiratory exchange ratio, suggesting reduction of host lipid digestion or absorption. In the study presented in Chapter II, sufficient high quality BSH was purified and subsequently encapsulated for rat oral administration. In rat study, solid direct evidence related to BSH function towards host lipid metabolism and energy harvest was not obtained, despite dramatic fecal bile profile change in response to BSH treatment. In the future, direct evidence of *in vivo* BSH function is needed in different systems, such as using different an animal model or using a more efficient protein encapsulation system.

In a previous study, the crystal structure of *Lactobacillus salivarius* BSH (*ls*BSH) was revealed (Xu *et al.*, 2016). In Chapter III, based on structure comparison of *ls*BSH and previous published BSH structure from other bacteria resources, key amino acid residues of *ls*BSH for enzyme activity and substrate specificity were revealed. These residues were verified by site-directed mutagenesis and mutants' activity were tested by two step enzyme activity assay. These structure analysis on *ls*BSH provided a solid foundation for BSH structural analysis. Due to the significant role of BSH in host physiology, in the future, structural based BSH inhibitors (potential alternatives for AGPs) and BSH enhancers (potential lead drug to control obesity) need to be developed by molecule docking. Those hits with outstanding enhancing or inhibiting effects on BSH could be further validated with *in vivo* and *in vitro* experiment.

Due to BSH negative relation to host lipid metabolism, BSH inhibitors could be potential alternatives for AGPs (Lin, 2014). A group of promising BSH inhibitors were characterized using an effective High-Throughput Screening (HTS) system (Smith *et al.*, 2014). In chapter VI, three BSH inhibitors verified by HTS on host bile acid metabolism and lipid metabolism were validated in a chicken experiment. Growth performance data did not showed significant difference among three BSH inhibitors group and control group. Metabolomics analysis of intestinal samples using UPLC-MS showed some significant changes in bile acid profile in response to BSH inhibitors treatment suggesting *in vivo* efficacy of the BSH inhibitors. Transcriptome analysis of liver and intestine samples indicated that carnosic acid treatment caused differential expression of genes involved in lipid metabolism, bile acid metabolism and host immunology. Future larger

animal trial to validate the efficiency of BSH inhibitors on farm animal growth promotion. Some leading encapsulation methods can also be used for developing effective and practical delivery approach to apply promising BSH inhibitors to food animal industry.

Vita

Wenjing Geng was born on September 20, 1990 in Shanxi, P. R. China. She attended Shanxi Medical University to pursue a bachelor degree in Pharmacy in 2008. After that, she continued a master program in the Department of Pharmacy at Wuhan University and graduated in 2012. Driven by the enthusiasm for animal and human health research, she was enrolled in the doctoral program in the Department of Animal Science at the University of Tennessee, Knoxville in Fall 2014.