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Review

Bacterial bile salt hydrolase: an intestinal microbiome target for enhanced animal health

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Abstract

To effectively mitigate antimicrobial resistance in the agricultural ecosystem, there is an increasing pressure to reduce and eliminate the use of in-feed antibiotics for growth promotion and disease prevention in food animals. However, limiting antibiotic use could compromise animal production efficiency and health. Thus, there is an urgent need to develop effective alternatives to antibiotic growth promoters (AGPs). Increasing evidence has shown that the growth-promoting effect of AGPs was highly correlated with the reduced activity of bile salt hydrolase (BSH), an intestinal bacterial enzyme that has a negative impact on host fat digestion and energy harvest; consistent with this finding, the population of *Lactobacillus* species, the major intestinal BSH-producer, was significantly reduced in response to AGP use. Thus, BSH is a key mechanistic microbiome target for developing novel alternatives to AGPs. Despite recent significant progress in the characterization of diverse BSH enzymes, research on BSH is still in its infancy. This review is focused on the function of BSH and its significant impacts on host physiology in human beings, laboratory animals and food animals. The gaps in BSH-based translational microbiome research for enhanced animal health are also identified and discussed.

Keywords: bile salts, bile salt hydrolase, lipid metabolism, antibiotic growth promoters, non-antibiotic feed additives, intestinal microbiome.

Introduction

Antibiotic use clearly serves as a selective driving force to enrich antimicrobial resistance (AMR) genes and promote the emergence of antibiotic-resistant bacterial pathogens (Davies, 2014). Thus, reducing or eliminating the use of in-feed antibiotics in healthy animals has been a worldwide trend to effectively mitigate AMR and protect food safety. US Food and Drug Administration recently implemented a new policy to recommend a voluntary withdrawal of medically important antibiotic from routine animal production practices by December 2016. Therefore, there is an urgent need to develop effective strategies to maintain animal productivity and health without relying on in-feed antibiotics.

Food animal producers have manipulated intestinal microbiota for more than 60 years to increase feed efficiency and body weight gain through the routine use of low-dose antibiotics as feed additives, called antibiotic growth promoters (AGPs). With the aid of culture-independent molecular approaches, investigations of the effect of AGPs on intestinal microbiota have been initiated in different food animals, including poultry and swine (Lin, 2014). These microbiome studies have shed light on the mechanism of mode of action of AGPs and on the development of novel alternatives to AGPs. Specifically, data indicate that the body weight gain in food animals is inversely related to the activity of bile salt hydrolase (BSH) as well as the abundance of potent BSH-producing bacteria in the intestine (Lin, 2014). Because the BSH enzymes produced by intestinal bacteria catalyze deconjugation of conjugated bile acids, an essential gateway reaction in the metabolism of bile acids which play an important role in host fat metabolism, energy harvest and body weight gain (Begley *et al.*, 2006; Joyce *et al.*, 2014b), we propose that BSH is a key mechanistic microbiome target for developing novel alternatives to AGPs, such as BSH inhibitors for enhanced animal production and health. This

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article reviews recent progress on BSH research, with emphasis on BSH functions and its impact on host physiology.

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 and Walters, 2014; Schaap *et al.*, 2014; Camilleri and Gores, 2015). 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 and Mysels, 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 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 animals without a gallbladder, such as horses and rats, bile salts continuously flow directly from the liver to the duodenum via the bile duct.

After reaching the ileum, bile salts are taken up into enterocytes via efficient membrane transporters, further absorbed into the portal vein to get back to the liver and finally re-secreted into bile; this process is called enterohepatic circulation (Vlahcevic *et al.*, 1996; Roberts *et al.*, 2002; Begley *et al.*, 2006; Ridlon *et al.*, 2006; Russell, 2009). In human beings, approximately 400–800 mg of bile salts daily are subjected to microbial transformations in the intestine (Vlahcevic *et al.*, 1996). Among various bile salt 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; Kim and Lee, 2005). Notably, in addition to a 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 TGR5 (Gupta *et al.*, 2001; Qiao *et al.*, 2003; Houten *et al.*, 2006; Inagaki *et al.*, 2006; Evans *et al.*, 2009).

Bile salt hydrolase

The BSH enzyme 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). BSH enzyme belongs to the choloylglycine hydrolase (EC 3.5.1.24) family. Phylogenetic analysis indicated that BSH was derived from the wider Ntn_CGH-like family of proteins, specifically penicillin V acylase (Kumar *et al.*, 2006; Jones *et al.*, 2008).

BSH enzymes from various sources differ in activity, substrate specificity, and optimal temperature and pH for enzymatic activity (Begley *et al.*, 2006). Molecular weights of the BSH subunit range from 28 to 50 kDa, and optimal pH for BSH activity is slightly acidic, ranging from 3.5 to 6. Most identified BSH enzymes still display activity at temperatures up to 60°C. Many identified BSH enzymes have a narrow substrate spectrum and display much higher activity in hydrolyzing glycine-conjugated bile salts than taurine-conjugated bile salts (Coleman and Hudson, 1995; Smet *et al.*, 1995; Tanaka *et al.*, 2000; Kim *et al.*, 2004; Liong and Shah, 2005; Pavlović *et al.*, 2012). However, some BSH enzymes show a preference for taurine-conjugated bile salts, such as two BSH enzymes in *Lactobacillus jonsonii* PF01 (Chae *et al.*, 2013) and the BSH enzymes from five lactobacilli strains (Jiang *et al.*, 2010). Recently, a potent BSH enzyme was identified and characterized from a chicken *Lactobacillus salivarius* strain; this BSH displayed potent hydrolysis activity towards both glycol-conjugated and taurine-conjugated bile salts (Wang *et al.*, 2012). It has been proposed that BSH enzymes recognize conjugated bile acids on both amino acid moieties and the cholate steroid nucleus (Begley *et al.*, 2006). Not surprisingly, substrate preferences of BSH may differ under different pH, likely due to pH-mediated structural changes (Corzo and Gilliland, 1999).

To date, structural basis of BSH function is still largely unknown. Crystal structures of the BSH enzymes from only three specific species, *Bifidobacterium longum, Clostridium perfrigens*, and *L. salivarius* have been reported (Rossocha *et al.*, 2005; Kumar *et al.*, 2006; 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 BSHs 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.*, 2012; Xu *et al.*, 2016). Previous comparative genomics and structural studies have identified some conserved, catalytically important residues in the active site of BSH (Cys2, Arg 16, Asp19, Asn79, Asn171, and Arg224); however, this conclusion was primarily based on the comparison of BSH structure with 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

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residues critical in catalysis and understand why this BSH displayed potent catalytic activity toward a broad spectrum of substrates including both glycol-conjugated and taurine-conjugated bile salts.

BSH-producing bacteria in the intestine

BSH enzymes have been identified in diverse bacterial species from different sources (Summarized in Table 1). Among the BSH-producing organisms, most of them are Gram-positive bacteria, except two from the Gram-negative genus, *Bacteroides* (Stellwag and Hylemon, 1976; Masuda, 1981; Lambert *et al.*, 2008). Jones *et al.* (2008) performed a functional and comparative metagenomic 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 shown in Table 1, BSH activity and corresponding enzymes have been identified primarily in lactic acid bacteria isolated from the gastrointestinal tract, which include but are not limited to *L. salivarius, Lactobacillus acidophilus, Lactobacillus johnsonii, Lactobacillus plantarum, Bifidobacterium longum, Bifidobacterium bifidum, Bifidobacterium adolescentis*, and *Bifidobacterium animalis*. BSH genes are either located in the chromosome or in 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 BSH enzymes are restricted to intestinal microorganisms, suggesting that BSH activity plays a role in *in vivo* adaptation of intestinal microorganisms in the gastrointestinal environment and in the mutualism between intestinal microbiota and animal hosts (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 present 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 mouse 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 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 enzyme contributes to bile resistance in probiotic bacteria, such as lactobacilli. Fang *et al.* (2009) demonstrated that 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, sulfur as well as energy source for some bacteria species (Vlahcevic *et al.*, 1996; Tanaka *et al.*, 2000; Ridlon *et al.*, 2006). 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 in the gastrointestinal epithelium in the host via immune evasion (Jones *et al.*, 2008; Mukherji and Prabhune, 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 well 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/health have 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 (Feighner and Dashkevicz, 1988; Knarreborg *et al.*, 2004; Guban *et al.*, 2006; Lin, 2011). The following paragraphs summarize findings from laboratory animal studies and human trials, which shed light on future directions for food animal health research.

Host lipid metabolism, cholesterol, and body weight

As children and adults are increasingly becoming overweight and obese, obesity-associated diseases will increase (Kahn *et al.*, 2006; Van Gaal *et al.*, 2006). Recent studies have indicated that intestinal microbiota are implicated in obesity in people (Tremaroli and Bäckhed, 2012); however, key microbial functions influencing host energy harvest remain to be clearly elucidated. The BSH enzyme has been increasingly recognized as a critical intestinal microbiome target for developing 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 significant impacts on host physiology by disturbing conjugated bile acid-mediated fat metabolism and endocrine functions (Begley *et al.*, 2006; Patel *et al.*, 2010; Jones *et al.*, 2014; Joyce *et al.*,

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Table 1. The BSH enzymes identified in bacteria from various sources

Source	Host strain	Molecular mass (kDa)	pH optimum	Temperature optimum (°C)	Reference
Human intestine					
	Bacteroides fragilis ATCC 25285	32.5	4.2–4.5	ND	Stellwag and Hylemon (1976)
	Bacteroides fragilis NCTC 9343	ND	5.0–6.0	ND	Aries and Hill (1970)
	Bacteroides fragilis 2536	ND	4.5–5.0	ND	Masuda (1981)
	Bacteroides vulgatus I-1	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	5.5–6.5	35–40	Grill *et al.* (1995)
	Bifidobacterium longum SBT2928	35	5.0–7.0	40–45	Tanaka *et al.* (2000)
	Bifidobacterium bifidum ATCC 11863	35	ND	ND	Kim *et al.* (2004)
	Bifidobacterium adolescentis ATCC 15705	35	ND	ND	Kim *et al.* (2005)
	Clostridium perfringens ATCC 19574	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[a]	3.5–5.5	ND	Corzo and Gilliland (1999)
	Lactobacillus acidophilus O16	126[a]	3.5–6.0	ND	Corzo and Gilliland (1999)
	Lactobacillus acidophilus NCFM	ND	ND	ND	McAuliffe *et al.* (2005)
	Lactobacillus sp. strain 100–12	ND	ND	ND	Lundeen and Savage (1990)
	Listeria monocytogenes	ND	ND	ND	Dussurget *et al.* (2002)
Murine intestine					
	Lactobacillus sp. strain 100–100	42	3.8–4.5	ND	Lundeen and Savage (1990)
	Lactobacillus sp. strain 100–16	ND	ND	ND	Lundeen and Savage (1990)
	Lactobacillus sp. strain RI	ND	ND	ND	Lundeen and Savage (1990)
Pig intestine					
	Lactobacillus acidophilus ATCC 43121	126[a]	3.5–5.5	ND	Corzo and Gilliland (1999)
	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	Lundeen and Savage (1990)
Chicken intestine					
	Lactobacillus salivarius NRRL B-30514	37	5.0–6.0	35–55	Wang *et al.* (2012)
Other					
Fermented milk	*Bifidobacteriu. animalis* DN 173010	ND	ND	ND	Lepercq *et al.* (2004)
Springs	*Brevibacullus* sp.	28	9	60	Sridevi *et al.* (2009)
Fermented milk	*Clostridium perfringens* MCV 815	56.0	5.8–6.4	ND	Gopal-Srivastava and Hylemon (1988)
Fermented finger millet	*Pediococcus pentosaceus* KID7	ND	ND	ND	Damodharan *et al.* (2015)
Fermented milk	*Lactobacillus acidophilus* sp.	ND	ND	ND	Pinto *et al.* (2006)
Parakeet	*Lactobacillus salivarius* LMG 14476	140–142[a]	5.5–7.0	ND	Bi *et al.* (2013), Li *et al.* (2006)
Raw milk	*Lactobacillus plantarum*	ND	ND	ND	Sieladie *et al.* (2011)
Silage	*Lactobacillus plantarum* CGMCC 8198	35–39	ND	ND	Gu *et al.* (2014)
Silage	*Lactobacillus plantarum* Lp09 AND Lp45	ND	ND	ND	Huang *et al.* (2013)
Kefir grains	*Lactobacillus plantarum* BBE7	43	ND	ND	Dong *et al.* (2012)
Soil	*Xanthomonas maltophilia* CBS 827.97	52	7.9–8.5	25–40	Dean *et al.* (2002)

[a]Molecular mass of tetramer.

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, ND = not determined.

2014b). 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 human beings (Jones *et al.*, 2013), rats (Pato *et al.*, 2004; Kumar *et al.*, 2011), mice (Park *et al.*, 2013, 2014; Miyoshi *et al.*, 2014), and pigs (De Smet *et al.*, 1998).

Molecular and cellular studies also provided new insights into underlying mechanisms of the effect of BSH enzyme 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, not only regulate lipogenesis and triglyceride synthesis (Watanabe *et al.*, 2004; Li *et al.*, 2013), but also regulate glucose homeostasis by increasing glycogen synthesis (Zhang *et al.*, 2006; Caron *et al.*, 2013) 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 the microbiota affect host energy harvest and weight gain (Walker and Parkhill, 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* BSH enzymes were cloned into an *E. coli* host strain (MG1655). The recombinant *Escherichia 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*) that lead to change in body mass (Joyce *et al.*, 2014a).

Other physiological process

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 nitric oxide 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 (Watanabe *et al.*, 2006; Svensson *et al.*, 2013). 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, consequently may exert more complex impacts on 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 lipid and may cause steatorrhea in the host (Kim and Lee, 2005). Recent research also indicated 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 blood and feces, that are produced by a multistep of 7α-dehydroxylation reaction from unconjugated bile acids, are proposed to be related to the pathogenesis of cholesterol gallstone diseases as well as colon cancer (van Faassen *et al.*, 1987; Färkkilä and Miettinen, 1990; Marteau and Rambaud, 1993; McGarr *et al.*, 2005; Venneman and van Erpecum, 2010; Ou *et al.*, 2013). Secondary bile acids may increase the risk of cancer by increasing oxidative stress and associated DNA damage (Cooke *et al.*, 2003; Bernstein *et al.*, 2005). 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 and Gilliland, 1999; Lie *et al.*, 1999; Laue *et al.*, 2001; Ridlon *et al.*, 2006). Hydrogen sulfide is a potent inhibitor of colonic butyrate metabolism, which is a key nutrient and regulator of cell turnover (Christl *et al.*, 1996; Van Eldere *et al.*, 1996).

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Hydrogen sulfide can also reduce apoptosis in colon cancer cells by preventing the function of a 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 in 1980s, Feighner and Dashkevicz (1987) reported that use of AGP reduced intestinal BSH activity in poultry and they 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 and Dashkevicz, 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 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 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 a HPLC method. In pigs, De Smet *et al.* (1998) observed that oral administration of the *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 trial 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 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 field (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 niche 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 AGP 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 enzyme from a chicken *L. salivarius* probiotic strain (Wang *et al.*, 2012). Interestingly, copper and zinc compounds displayed a potent inhibitory effect on BSH enzyme 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.*, 2012). Subsequently, by taking advantage of the unique feature of the *L. salivarius* BSH enzyme (Wang *et al.*, 2012), an efficient high-throughput screening system was successfully developed and used to discover BSH inhibitors (Smith *et al.*, 2014). Unlike many BSH enzymes from other bacteria that have narrow substrate spectrum, the *L. salivarius* BSH displayed a potent hydrolysis activity towards both glycol-conjugated and taurine-conjugated bile salts. The broad substrates 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

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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, 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 and Richards, 2005; Lin, 2014). However, very limited data are available to scientifically justify the choice of specific bacterial species or products for growth promotion and results are inconsistent from independent studies (Dibner and Richards, 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. Specifically, in a large pen trial, Sharifi *et al.* (2012) 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. Moreover, using a different 5-bacterial species competitive exclusion probiotic product, Mountzouris *et al.* (2010) also observed similar inferior feed conversion efficiency and reduced fat digestibility in response to probiotic treatment in broilers. 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 of 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 effect 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 use 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 stress.

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 BSH enzymes 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 BSH enzymes and BSH-producing bacteria in the intestine. To date, only a limited number of BSH enzymes have been identified in the intestinal bacteria isolated from food animals (Table 1). With the aid of next generation sequencing technologies and bioinformatics tools, functional and comparative metagenomic analyses of intestinal BSH in food animals are warranted and will provide a better picture of 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 enzyme(s) and corresponding BSH-producing bacteria may serve as biomarkers for health statuses of animal hosts, understanding the ecology of BSH enzymes and BSH-producing bacteria in the intestine would facilitate the development of diagnostics to evaluate the health status of animals and people.
- 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 important roles of microbiota in intestine health, development of specifically tailored probiotics is a logical strategy for practical application, but 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 host animals and native microbiomes. This is likely due to the challenge for manipulating BSH activity in commensal organisms for specific laboratory animal hosts and to the lack of public acceptance of using genetically modified organisms (GMOs) in human trials. While this concern has been partly addressed with a recent *E. coli* knock-in model (Joyce *et al.*,

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2014a), manipulating 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.*, 2012) provides an excellent opportunity to address this issue using a 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 people 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 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 ecological diversity of BSH in the intestinal microbiome, structure analyses of BSH enzymes from various species are highly warranted, which would reveal critical residues in catalysis and provide key information on the substrates selectivity of BSH enzymes. 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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