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HIJACKING HOST METABOLISM WITH *LACTOBACILLUS*—UNDERSTANDING THE IMPLICATIONS OF BILE SALT HYDROLASE DIVERSITY

A Dissertation in

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Abstract

The tremendous bacterial community which inhabits the human gastrointestinal tract is a newly appreciated intermediary for nutrient uptake and processing. Compositional variations in this bacterial community are associated with obesity, and recent evidence suggests that bacterial modification of bile acids secreted in the small intestine contributes to the regulation of fat storage. Bile salt hydrolase (BSH) activity against the bile acid tauro-beta-muricholic acid (T-β-MCA) in particular has been suggested as a critical mediator of host bile acid, glucose, and lipid homeostasis via the farnesoid X receptor (FXR) signaling pathway. *Lactobacillus* species are key players in this feedback loop, and their history of use as probiotic bacteria for promoting gastrointestinal health in humans makes them ideally suited for applications that exploit the bile acid regulatory feedback mechanism to control metabolism. BSH activity is widely associated with *Lactobacillus* species, but BSH substrate specificity for T-β-MCA had not been characterized in *Lactobacillus* prior to this work.

Here, a strain of *L. johnsonii* with robust BSH activity against T-β-MCA *in vitro* was identified from the mouse gut microbiota. A screening assay performed on a collection of 14 probiotic strains from nine species of *Lactobacillus* identified BSH substrate specificity for T-β-MCA only in two of three *L. johnsonii* strains. Genomic analysis of these *L. johnsonii* strains revealed the presence of three *bsh* genes which are homologous to *bsh* genes in the human associated strain *L. johnsonii* NCC533. Further analysis revealed broad differences in substrate specificity even among the closely related *bsh* homologs, and suggests that the phylogeny of these enzymes does not closely correlate with substrate specificity. Predictive modeling was able to explain the difference in BSH activity for T-β-MCA in these homologs, and may be a useful tool for identifying additional BSHs from within the gut microbial community with the potential to affect host metabolism.

This research also demonstrated the capability of transgenic BSH active bacteria to alter bile acid composition and affect FXR signaling in monocolonized germ free mice. However, these changes did not affect weight gain in the host, indicating the need for future research to understand the factors governing FXR mediated metabolic control. Additionally, studies of *Lactobacillus* administration to conventional and germ free mice revealed a disconnect between *in vitro* BSH activity and the ability of a probiotic to affect host bile acid composition. In particular, colonization of germ free mice with a strain of *L. johnsonii* did not result in reduced cecal T-β-MCA concentrations. Moreover, background BSH activity endogenous to the gut microbiota of conventional mice was found to almost completely reduce intestinal concentrations of T-β-MCA at baseline, leaving little potential for probiotic intervention. Follow up studies focusing on how BSH active probiotics affect the total BSH capacity of the gut microbiota are therefore needed.

Finally, the biological role of BSHs in *Lactobacillus* is not entirely clear. BSH activity has been associated with increased colonization and intestinal persistence in several bacterial species, and is thought to provide some protection against bile acid toxicity. However, several studies have suggested BSHs comprise part of a global response to bile and acid stress, which may not be entirely consistent among strains. Isotopic Radio Outlier Analysis™ (IROA) was used to study the metabolic response of several probiotic *Lactobacillus* strains to bile acid stress. This approach improves metabolite identification from background noise compared to traditional metabolomics methods. Comparison of *L. acidophilus* NCFM, *L. plantarum* WCFS1, *L. johnsonii* LB1, and *L. johnsonii* NCK88 revealed differences in bile acid response among the strains, and suggests a role for amino acid metabolism in *L. acidophilus* NCFM. Further optimization of the IROA experimental design may uncover new roles for BSHs in *Lactobacillus*, and will inform improved probiotic strain selection.

Ultimately this research discovered a strain of *Lactobacillus* autochthonous to the mouse intestine which exhibits clear BSH activity against T-β-MCA and offers promise for controlling host metabolism as a probiotic. These findings will guide ongoing efforts to optimize next generation probiotics for weight control in humans and agricultural animals.

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List of Abbreviations

°C	degrees Celsius
µg	micrograms
µL	microliters
A	adenine
Å	Ångstroms
ABCB11 (aka BSEP)	ATP binding cassette transporter 11 (aka bile salt exporting pump)
ABCB4	ATP binding cassette transporter 4
ala	alanine
arg	arginine
ASBT	apical sodium dependent transporter
asn	asparagine
asp	aspartic acid
ATCC	American Type Culture Collection
BBL LBS	*Lactobacillus* selection agar
BIC	Bayesian Information Criterion
BLAST	Basic Local Alignment Search Tool
bp	basepairs
BSH	bile salt hydrolase
C	cytosine
CA	cholic acid
CDCA	chenodeoxycholic acid
CFU	colony forming units
CYP7A1	7α-hydroxylase
CYP8B1	sterol 12α-hydroxylase
cys	cysteine
DCA	deoxycholic acid
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotides
EBSS	Earle's Balanced Salt Solution
FGF15	fibroblast growth factor 15
FGF19	fibroblast growth factor 19
FWD	forward
FXR	farnesoid X receptor
g	grams
G	guanine
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCA	glycocholic acid
GCDCA	glycochenodeoxycholic acid
GDCA	glycodeoxycholic acid
h	hours

I-BABP (aka FABP6)	intestinal bile acid-binding protein (aka fatty acid-binding protein subclass 6)
ile	isoleucine
IROA	Isotopic radio outlier analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes
L	liters
LAB	lactic acid bacteria
LB	lysogeny broth
LCA	lithocholic acid
LC-MS	liquid chromatography mass spectrometry
LDL	low-density lipoprotein
leu	leucine
LRH-1	liver related homolog-1
Lsp	large surface protein
LXRα	liver X receptor α
m/z	mass-to-charge ratio
MapA	mucus adhesion promoting protein A
Mb	megabases
MCA	muricholic acid
mg	milligrams
mL	milliliters
mM	millimolar
mm	millimeters
MRS	de Man, Rogosa, Sharpe
ms	mass spectrometry
MsrB	methionine sulfoxide reductase
Mub	mucus binding protein
MUMs	Maximum Unique Matches
nM	nanomolar
NTCP	sodium-taurocholate cotransporting polypeptide
Ntn	N-terminal nucleophilic
OATPs	organic anion-transporting peptides
OD	optical density
ORF	open reading frame
OSTα	organic solute transporter α
OSTβ	organic solute transporter β
PBS	phosphate buffered saline
PCR	polymerase chain reaction
phe	phenylalanine
pro	proline
PVA	penicillin V acylase
qPCR	quantitative polymerase chain reaction
REV	reverse

RPM	revolutions per minute
rRNA	ribosomal ribonucleic acid
SDM	site directed mutagenesis
ser	serine
SHP	small heterodimer partner
T	thymine
TCA	taurocholic acid
TCA	citric acid cycle
TCDCA	taurochenodeoxycholic acid
TDCA	taurodeoxycholic acid
thr	threonine
tyr	tyrosine
T-β-MCA	tauro-beta-muricholic acid
UPLC-ESI-QTOFMS	ultra-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry
V	volts
val	valine

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Chapter 1
Statement of the Problem

Obesity is a major worldwide health concern which is associated with increased risks for cardiovascular disease, diabetes, and cancer. In the US, prevalence of obesity is steadily increasing and economic forecasts predict obesity associated health care costs to account for 16-18% of total US health care costs by 2030 (1). Over the last decade, the human gut microbiota has received increasing attention for its role in the development of obesity and is considered a promising target for novel treatments. A mechanistic understanding of the microbiota-host relationship is only just beginning to emerge, and suggests a role for *Lactobacillus*.

Bile acids are a diverse group of amphipathic steroids which are synthesized from cholesterol in the liver, conjugated to either taurine or glycine, then secreted into the small intestine to aid in fat digestion, and finally recycled in a process known as enterohepatic circulation. Growing evidence suggests that bile acids also act as important signaling molecules which regulate their own synthesis and mediate regulation of glucose, lipid, and energy metabolism through interactions with the Farnesoid X Receptor (FXR). Intestinal BAs are subject to modification and degradation by the gut microbiota, and microbial interactions with the bile acid pool have been found to influence FXR signaling (2). Recent research suggests that bacterial bile salt hydrolase (BSH) activity in the gut mediates host metabolism as a result of deconjugation of the potent FXR antagonist tauro-β-muricholic acid (T-β-MCA), and correlates with *Lactobacillus* populations (3, 4).

The recently elucidated link between gut microbiota BSH capacity and host metabolism has important implications for the development of probiotics for weight control in humans and animals. Many strains representing a wide range of *Lactobacillus* species are commonly marketed as probiotics for human and animal consumption, and BSH activity has historically been considered an advantageous trait for colonization and maintenance of host cholesterol. Oral

administration of probiotic *Lactobacillus* strains has been reported to improve growth and feed efficiency in agricultural animals, and human clinical trials aimed at improving overall gut health have resulted in a perplexing mix of outcomes—including both weight gain and weight loss—which are strain and host dependent (5, 6). Differences in bile acid composition, FXR signaling, and metabolism among host agricultural animals and humans may contribute to divergent clinical outcomes, and are not fully understood. Moreover, BSH activity has been shown to vary widely among probiotic strains of *Lactobacillus*, particularly with respect to bile acid substrate specificity for T-β-MCA (7). The contribution of differences in BSH activity to reports of weight gain or loss associated with certain *Lactobacillus* probiotic strains is not clear.

Overall, manipulation of the gut microbiota through administration of BSH active probiotics is a potentially promising strategy for controlling obesity and offering alternatives to controversial antibiotic growth promoters used in animal agriculture (8). However, administration of BSH active probiotics has never been demonstrated to directly affect FXR signaling and metabolism in a host. This dissertation will consider T-β-MCA substrate specificity in *Lactobacillus* BSHs and its effect on FXR mediated weight gain in order to lay the groundwork for future development of probiotic based clinical interventions.

Chapter 2
Literature Review

2.1 Introduction

Over the course of the past decade, an improved understanding of the vast microbial ecosystem inhabiting the gastrointestinal tracts of humans and animals has illuminated a new scientific perspective of obesity. The gut microbiota, as it is known, consists of approximately 10 trillion bacteria representing hundreds to thousands of species, and has been shown to be a causative factor influencing body mass (9, 10). Gut microbial communities in obese individuals are characterized by a reduced bacterial richness and increased capacity for energy harvest (10–12). Broad scale shifts in the relative ratio of Firmicutes to Bacteriodetes as well as changes in individual genus and species level populations of *Lactobacillus, Staphylococcus, Escherichia coli,* and *Methanobrevibacter smithii* have been found to differentiate obese and lean gut microbiota (11–16). In addition to directly altering dietary caloric availability and intake, altered gut microbiota compositions may also promote obesity by increasing bacterial lipopolysaccharide (LPS) concentrations in blood plasma, triggering metabolic endotoxemia (17). More recently, bacterial modification of host secreted bile acids has been shown to effect lipid and glucose metabolism via the FXR signaling pathway (2–4). Collectively, these findings have stoked interest in the development of probiotic based therapeutics for preventing and treating obesity and facilitating growth promotion in agricultural animals.

Achieving a working understanding of the gut microbiota that can be effectively channeled to develop probiotics for the purpose of controlling weight is an inherently complex and multidisciplinary endeavor. As such, this review will focus on *Lactobacillus,* as they include some of the most well characterized species autochthonous to the gut microbiota of humans and animals. More importantly, there is growing evidence some *Lactobacillus* are capable of affecting host metabolism, and their extensive history of use as probiotics in humans and animals

makes them ideally suited for development into next generation probiotics for metabolic control. **This review will discuss the role of *Lactobacillus* within the gut microbiota as it relates to the regulation of weight. In particular, its aim is to consider the relevant factors influencing the selection and development of *Lactobacillus* probiotics for controlling host metabolism as well as to identify the limits to our understanding.**

2.2 An overview of *Lactobacillus*

Lactobacillus is a phylogenetically diverse genus including hundreds of different species commonly used in industrial fermentation processes and associated with carbohydrate rich environments including milk, plants, and the intestinal tracts of a diverse range of hosts (18). The genus belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, and family *Lactobacillacaea* (18). They are non-spore forming gram positive rods and are members of the broadly defined lactic acid bacteria (LAB) known for fermenting hexoses into primarily lactic acid. Biochemically, *Lactobacillus* can be homofermentative (i.e. produce lactic acid via the Embden-Meyerhof pathway), heterofermentative (i.e. produce lactic acid, acetic acid, formic acid, carbon dioxide, and ethanol via the phosphoketolase pathway), or facultative heterofermentative (i.e. ferment hexoses via the Embden-Meyerhof pathway and pentoses via the phosphoketolase pathway) (19).

Generally, *Lactobacillus* are characterized by genomes ranging from just under 2.0 Mb to 3.3 Mb containing a relatively low GC percentage (20). Genomic analysis and phyletic reconstruction of several representative *Lactobacillus* strains suggests that these organisms have undergone reductive evolution during adaptation to nutrient rich environments (20, 21). One particularly striking example is *L. bulgaricus*, which is used extensively for dairy fermentation. The genome of *L. bulgaricus* encodes several incomplete carboyhydrate metabolic pathways and shows a distinct preference for growth in lactose (20). The genome of *L. bulgaricus* also does not

encode mucus binding proteins or BSHs which are commonly found in species adapted to gastrointestinal habitats (20). Genetic acquisition by horizontal gene transfer also appears to have shaped niche adaptation within the genus. For instance, the relatively large genome of *L. plantarum* WCFS1 contains a large, lower GC content region encoding genes for carbohydrate transport and metabolism, reflecting its ability to adapt to a wide range of environmental niches (22). A differential blast analysis of putative proteins encoded in *L. gasseri* ATCC33323, *L. acidophilus* NCFM, *L. johnsonii* NCC533, and *L. plantarum* WCFS1 revealed common mucus- and fibrinogen-binding proteins which distinguish these from other LAB and were later found to contribute to cell-host interactions in the gastrointestinal tract (20). Adaptive evolution also appears to drive diversity of strains within a single *Lactobacillus* species, as strains of *L. reuteri* isolated from mice were shown to possess an essential genetic inventory distinct from strains of *L. reuteri* isolated from other vertebrate hosts (23).

The availability of genome sequences for *Lactobacillus* strains has grown tremendously since the publication of *L. plantarum* WCFS1 in 2003, and to date representative genomes are publicly available for over 200 *Lactobacillus* species (22). An improved understanding of how phenotypic differences among these *Lactobacillus* species and strains contribute to colonization and community assembly within the gut microbiota is certain to develop as comparative genomic methods improve. These studies are essential in order to reveal new insights into the evolution and niche adaptation of *Lactobacillus* and inform their development as effective probiotics in humans and animals.

2.3 *Lactobacillus* associations with obesity

Comparisons of gut microbiota compositions in lean and obese humans and rodent models have consistently identified differences in *Lactobacillus* populations. In the first published study to directly consider compositional changes beyond characteristic phylum level

shifts in Firmicutes and Bacteriodetes, Armougom *et al.* identified significantly increased populations of *Lactobacillus* in obese adults compared to healthy controls (13). The same research group followed up on their findings with two larger studies which analyzed *Lactobacillus* species composition and discovered that *L. reuteri* in particular was strongly associated with obesity (14, 24). Interestingly, subsequent mechanistic studies have shown that obesity related inflammation appears to select for and increase the population of *L. reuteri* in the Peyer's patches of the small intestine, resulting in a decreased anti-inflammatory response (25, 26). *L. gasseri* and *L. taiwanensis* populations have also been shown to drastically increase in response to weight gain induced by high fat diet feeding in a mouse obesity model (27). These changes were associated with reduced lipid droplet size in the small intestine, which the authors propose may be related to altered intestinal bile acid composition. Another group associated increased *Lactobacillus* populations with the production of volatile organic compounds in stool samples of obese patients with non-alcoholic fatty liver disease (28). Fecal *Lactobacillus* populations also reduce significantly in the months following Roux-en-Y gastric bypass surgery in patients who were morbidly obese prior to the procedure, nicely demonstrating that *Lactobacillus* populations correlate with weight changes in the same individuals over time (29). Additionally, cross sectional and comparative studies of children have associated elevated *Lactobacillus* populations with obesity, but it is unclear if the common underlying mechanisms driving gut microbiota mediated metabolic changes are similar in adults and children (30, 31). Paradoxically, there are also reports of decreases in *Lactobacillus* populations in response to the onset of obesity in rodents. In a diet induced rat obesity model, Lecomte *et al.* identified a negative correlation between *L. intestinalis* and change in body weight and fat mass (32). Similarly, Lam *et al.* reported significantly reduced *Lactobacillus* populations in the colons of mice fed a high fat diet compared to control mice (33).

Overall the evidence suggests that *Lactobacillus* populations change in association with changes in body mass, but species and strain level compositional changes are not consistent across studies and remain under characterized.

2.4 Effects of *Lactobacillus* probiotics on weight in humans and animals

Probiotics, by definition, provide health benefits to their host beyond basic nutrition (34). *Lactobacillus* species have been administered as probiotics to humans and animals dating back to the early twentieth century, when Elie Metchnikoff first reported improved health and longevity associated with drinking milk fermented with *Lactobacillus* (35). Health benefits associated with consumption of *Lactobacillus* probiotics are now well documented, and include pathogen exclusion, treatment of inflammatory bowel diseases, and weight control (36–38). Accordingly, the literature encompassing studies of *Lactobacillus* as probiotics is exceedingly vast. Recently though, in response to many of the aforementioned studies associating intestinal *Lactobacillus* populations with weight changes, animal and clinical trials of probiotics have monitored weight change as an outcome. Predictably, the results of these studies are mixed and difficult to compare due to confounding differences in experimental design. Objective and statistically valid conclusions about the efficacy of *Lactobacillus* probiotics for controlling weight are therefore better drawn from meta-analyses designed to make these comparisons by analyzing a broad range of studies together and identifying common factors influencing their outcomes.

One recent meta-analysis focusing on randomized controlled trials studying the effects of *Lactobacillus* probiotics on weight identified significant weight loss in obese adults, and significant weight gain in children relative to controls in response to daily supplementation (39). Notably, there was significant heterogeneity in the results among the included clinical trials, which may be explained in part by a lack of regard for species and strain specific effects in the analysis. Similarly, in a less rigorous analysis which included studies with agricultural animals,

Lahtinen *et al.* argue that *Lactobacillus* supplementation increases weight gain in children and developing livestock, but has a reductive effect on weight in adult humans (40).

Table 2.1: *Lactobacillus* species associated with significant weight changes as a result of controlled feeding trials in humans and animals

species associated with weight gain	species associated with weight loss
L. acidophilus	*L. gasseri*
L. ingluviei	*L. plantarum*
L. fermentum	*L. rhamnosus*

*This summary is based on conclusions from meta-analyses performed by Million *et al.* (5) and Mekkes *et al* (37).

Another more extensive meta-analysis performed by Million *et al.* included a broader range of studies in both humans and animals, which allowed for the analysis of species specific effects (5). *L. acidophilus*, *L. ingluviei*, and *L. fermentum* were associated with weight gain, whereas *L. gasseri*, and *L. plantarum* were associated with weight loss or an anti-obesity effect. Additional species including *L. reuteri*, *L. casei*, *L. delbreuckii*, and *L. sporogenes* were not associated with any significant weight changes. Again, the results displayed statistical heterogeneity, emphasizing the importance of factors such as host species, age, initial body mass, and length of study for influencing experimental outcomes. Interestingly, the same group followed up their meta-analysis with a comparative genomic analysis of the *Lactobacillus* species that were found to associate with weight changes (41). Genes corresponding to lipid metabolism and bacteriocin production were associated with species found to cause weight gain, while genes corresponding to fructose catabolism and defense against oxidative stress were associated with species found to protect against weight gain. Strain specific effects on weight changes were identified in an analysis of data from human clinical trials conducted by Mekkes *et al.* From their study, the authors specifically identified *L. gasseri* SBT 2055 and *L. rhamnosus* ATCC 53103 as having a reductive effect on adiposity and body mass (37).

Despite some differences in the species and strains associated with weight changes as well as the directionality of the weight changes, all of these analyses agree that *Lactobacillus* probiotics are capable of inducing significant weight changes in humans and animals.

2.5 Colonization of *Lactobacillus* probiotics in the host gastrointestinal tract

Regardless of the intended health benefits to the host, the efficacy of a given probiotic is inherently associated with its ability to survive passage through the upper gastrointestinal tract and reach its intended target site (34). Early studies of microbial ecology in the intestinal tracts of animals identified *Lactobacillus* associated with both mucosal and epithelial surfaces, and provided the first clues for identifying potentially successful probiotic strains (42–47). *In vitro* screening paradigms were developed from these pioneering findings, and were used to identify probiotic strains able to colonize the intestinal tracts of animals and humans. For instance, three probiotic strains which colonized and persisted in human stool samples were identified from an initial pool of 47 candidate *Lactobacillus* strains based on their resistance to low pH and bile, adhesion to heterogeneous human epithelial colorectal adenocarcinoma (Caco-2) cells, and antimicrobial activity (48). Likewise, Yu *et al.* found that attachment to cultured epithelial cells, bile tolerance, and ability to survive in low pH gastric juice predicted efficacy of colonization for *L. acidophilus, L. casei,* and *L. fermentum* probiotic strains in rabbits, and similar screening studies identified additional *Lactobacillus* probiotic strains in chickens and horses (49–51). The assays informing these screens are now widely considered to encompass the criteria for essential probiotic traits in a host dependent manner (34). Additionally, probiotics must elicit a targeted and predictable host response such as relief of lactose intolerance, modulation of immunity, or regulation of metabolism (34). These principles were nicely demonstrated in a study conducted by the Probiotic Research Group based at University College Cork that identified the probiotic *L. salivarius* UCC118 from 1500 bacterial strains isolated from resected human terminal ilea and verified its efficacy in mouse studies and a human clinical trial (52).

An immense amount of research has gone into characterizing the bacterial traits responsible for mediating the attachment, bile tolerance, acid tolerance, and antimicrobial

properties sought after in effective probiotics. Cell surface carbohydrates and proteins that mediate adherence to intestinal epithelial cells and mucus are some of the most extensively characterized factors affecting probiotic colonization, and have been shown to be highly strain dependent (53, 54). Many of the proteins known to directly mediate adhesion to epithelial cells and gastric mucus were originally characterized in *L. reuteri*. These include MapA, Mub, CmbA, Lsp, and MsrB, all of which are cell surface associated (55–59). Genomic analysis has subsequently identified homologs of many of these proteins in other probiotic strains. For instance, closely related variants of Mub have also been identified in strains of *L. reuteri, L. acidophilus, L. gasseri, L. johnsonii, L. fermentum* and *L. plantarum* (56, 60, 61). A mannose dependent mechanism which complements less specific surface level carbohydrate and protein interactions affects the binding of many *L. plantarum* strains to colonic epithelial cells (47, 48). Cell surface associated lipoteichoic acid and exopolysaccharides have also been found to mediate and enhance attachment to instestinal epithelial cells (62, 63). Another common protein mediated mode of probiotic attachment to the intestinal epithelium involves fibronectin binding proteins, which have been identified in *L. acidophilus, L. gasseri, L. johnsonii,* and *L. plantarum* (60, 64). Surface layer proteins have also been found to mediate attachment to mucosal antigens in *L. brevis,* and cell surface glyceraldehyde-3-phosphate dehydrogenase has been shown to affect mucosal attachment in *L. plantarum* LA 318 (65, 66).

Before attaching to and colonizing mucosal and epithelial surfaces in the intestines, probiotics must remain viable as they encounter acidic conditions in the stomach and bile in the small intestine. Bile is a complex fluid consisting of bile acids, cholesterol, phospholipids, and bile pigments synthesized in the liver mainly for the purpose of enhancing the solubility of dietary fats (67). The amphipathic nature of bile acids in particular confers bile with antimicrobial properties due to its ability to destabilize the lipid bilayer of bacterial cell membranes (68). Additionally, bile acids can impose stresses resulting from intracellular pH changes, protein misfolding and denaturation, and the generation of oxidative free radicals (69).

Naturally, *Lactobacillus* have evolved defense mechanisms against these stressors, although our understanding of physiological responses *in vivo* is only just beginning to emerge since effective probiotic strains commonly display multifaceted responses to a given stressor which also provide cross resistance against other stressors. For instance, acid and bile tolerance are often associated traits (70). It is also apparent that significant variability in the nature and extent of these stress responses exists among strains, as is manifest in the results of numerous probiotic screening studies (48–52). Moreover, environmental factors can even affect stress tolerance, as exposure to various levels of bile, pH, temperature, and atmospheric conditions has been shown to alter subsequent tolerance to chemical and physical stressors in many probiotic strains (69).

Global profiling studies have helped to reveal the complex physiological responses probiotics have upon exposure to gastrointestinal conditions. Early bioinformatics studies nicely demonstrated changes in the synthesis of proteins known to mediate compositional changes in cellular membrane structure and protection against oxidative damage to proteins and DNA (71–75). One particularly thorough study by Pfeiler *et al.* characterized a bile inducible operon encoding a two component regulatory system as well as four transporters, presumably involved in bile acid efflux, in *L. acidophilus* NCFM (76). Bile acid efflux systems have also been described in *L. johnsonii* and *L. reuteri* (77, 78). Pfeiler *et al.* also reported a broad scale increase in cellular carbohydrate metabolism associated with bile tolerance (76). Subsequent studies have discovered similar metabolic changes in response to bile exposure in *L. reuteri* and *L. delbreuckii* subsp. *lactis* (79, 80). Genes encoding the stress-related proteins GroEL, DnaK and ClpP, as well as adhesion-related genes encoding mucin and fibronectin binding proteins have also been implicated in the *L. acidophilus* NCFM response to simulated gastrointestinal conditions, suggesting the possibility that stress response is coupled to additional cellular adaptations to the gastrointestinal environment (81). Another seminal study of bile tolerance in *L. plantarum* identified a multifaceted response including two glutathione reductases involved in protection against oxidative injury caused by bile salts, a cyclopropane-fatty-acyl-phospholipid synthase

implicated in maintenance of cell envelope integrity, a bile salt hydrolase, an ABC transporter, and an F0F1-ATP synthase which participates in the active removal of bile-related stress factors (82). Increased expression of bile salt hydrolase genes has also been shown in response to bile exposure in *L. johnsonii*, and BSHs were previously shown to directly mediate bile tolerance in mutants of *L. amylovorous* and *L. plantarum* (83–86). More recently, transcriptomic and proteomic characterizations of *L. rhamnosus* GG and *L. johnsonii* PF01 exposed to simulated gut conditions implicated altered exopolysaccharide production and increased phosphotransferase activity in the *Lactobacillus* stress response in addition to widely recognized alterations in cell membrane fatty acid composition (86, 87). Cumulatively, these findings suggest that probiotic *Lactobacillus* undergo a broad scale adaptive physiological response to the stresses associated with the gastrointestinal environment. This includes altered cell envelope construction and expression of cell surface carbohydrates and proteins, increased carbohydrate metabolism, and induction of proteins involved in protection against oxidative damage, bile acid efflux, and degradation of bile acids.

In addition to surviving host defenses and attaching to surfaces of the gastrointestinal tract, colonization of probiotics necessitates integration into a highly developed microbial ecosystem. Effective probiotics typically show a propensity for displacing other bacteria via aggregation, competitive exclusion, and production of antimicrobial compounds. Surface layer proteins in *L. crispatus, L. gasseri, L. johnsonii, L. helveticus* and *L. brevis* have been found to mediate an aggregative phenotype which improves colonization, persistence, and pathogen exclusion (65, 88–92). A bacteriocin has been implicated in the antiinfective activity of *L. salivarius* UCC118, and a similar bacteriocin in *L. plantarum* has been found to favorably modulate host immune response (93). Finally, organic acid production is thought to mediate competitive exclusion in *L. acidophilus, L. gallinarium, L. crispatus,* and *L. helveticus* (94, 95).

Mechanistic explanations for the observed differences in survival and colonization among *Lactobacillus* strains are still incomplete, and have also been found to depend on complex

interactions between the probiotic, the gut microbiota, and the host. For example, niche specific differences in the physical and chemical environment throughout the gastrointestinal tract have important consequences for probiotic colonization. A study of *L. casei* Shirota identified significant differences in growth rates among probiotic cells in the duodenum, jejunum, ileum, and colon of mice which affected colonization and persistence (96). Alander *et al.* also noted significant differences in fecal and colonic mucosal populations of *L. rhamnosus* GG in humans, particularly after cessation of daily administration (97). These findings are supported by a host of ecological studies documenting differences in native *Lactobacillus* composition throughout the gastrointestinal tract reflective of progressively changing host cell defenses, nutrient availability, and rate of passage (9, 45, 98, 99). The composition of the gut microbiota prior to introduction of a probiotic has also been shown to have an effect on colonization. In a human trial of *L. casei* subsp. *rhamnosus* Lcr35, patients harboring the highest indigenous populations of *Lactobacillus* at the start of the trial responded with the greatest increases in *Lactobacillus* populations in response to probiotic treatment (100). Likewise, a human trial of *L. rhamnosus* DR20 reported a relationship between probiotic populations and the presence or absence of a stable indigenous *Lactobacillus* population prior to the start of treatment (101). Similarly, mice with high initial titers of *Lactobacillus* were more efficiently colonized by a commensal strain of *L. reuteri* (102). Presumably, these effects are related to both cell to cell interactions within the gut microbiota as well as between the microbiota and the host. The potential elegance and complexity of these interactions was cleverly revealed in a study by Sonnenburg *et al.* which analyzed the transcriptome of the gut commensal *Bacteroides thetaiotaomicron* monocolonized and cocolonized with *L. casei*. In this study, *B. thetaiotaomicron* was found to expand the range of polysaccharides it degrades in the presence of *L. casei*, which in turn induces host genes involved in innate immunity (103). In another revealing experiment comparing colonization of *L. johnsonii* NCC 533 and *L. paracasei* NCC 2461, two strains with similar *in vitro* growth, adherence, and survival characteristics, Ibnou-Zekri *et al.* showed that these strains elicit

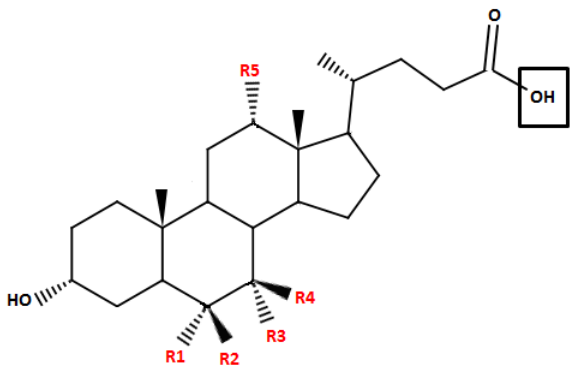
divergent host immune responses which result in comparatively poorer colonization for *L. paracasei* NCC 2461 (104).

Lactobacillus have necessarily evolved adaptive physiological responses to the harsh gastrointestinal environment. These include broad changes in cellular structure and metabolism that protect against low pH and bile as well as more targeted responses such as the production of adhesive factors, bile salt hydrolases, and bile acid efflux pumps. Many *Lactobacillus* also produce antimicrobial compounds that provide a competitive advantage against other bacteria in the gut microbiota, and successful integration of a new strain can have far reaching effects on both gut microbiota composition and host response. Ultimately, **colonization of probiotics in the intestinal tract enables cross talk with the host resulting from the interaction of the bacterial physiological response with host signaling and defenses. Consequently, differences in adaptive responses among probiotic strains in addition to differences in host physiology influence clinical outcomes, and must be considered for effective probiotic selection and development.**

2.6 The gut microbiota, bile acid homeostasis, and host metabolism

The gut microbiota plays a well-documented role in the synthesis and recycling of bile (67). Bile acids in particular are the focus of an elegant signaling system in the host that retains nearly 95% of the bile acids initially synthesized in the liver and released into the small intestine to aid in the solubilization of dietary fats (67). In a process known as the enterohepatic circulation, bile acids are reabsorbed into the portal circulation by active transport in the ileum and passive transport throughout the length of the gastrointestinal tract (67). Bile acids escaping reabsorption are resynthesized from cholesterol in the liver, a process which is regulated by a bile acid controlled feedback mechanism (105). Importantly, bile acids undergo extensive chemical modifications as they pass through the intestines as a result of bacterial mediated enzymatic

activity, affecting new bile acid synthesis in the liver and providing a direct link between the gut microbiota and a host signaling pathway (106). A number of recent studies have implicated this symbiotic network in metabolic changes in the host, and have brought forth a new perspective on probiotic interactions with bile (2–4).



	R1	R2	R3	R4	R5
β-muricholic acid (β-MCA)	H	OH	H	OH	H
cholic acid (CA)	H	H	OH	H	OH
chenodeoxycholic acid (CDCA)	H	H	OH	H	H
deoxycholic acid (DCA)	H	H	H	H	OH
lithocholic acid (LCA)	H	H	H	H	H

Figure 2.1: Chemical structures of common bile acids. After initial synthesis from cholesterol in the liver, bile acids are conjugated to either taurine or glycine in place of the boxed hydroxyl group.

Bile acids consist of a hydrophobic steroid nucleus conjugated to either glycine or taurine, which makes them amphipathic and increases their solubility (106). The multistep synthesis of bile acids starts with cholesterol and typically results in the production of two primary bile acids, and production of additional bile acids is possible through modifications of the steroid nucleus (106). Ultimately, the bile acid pool of an organism consists of over one hundred different bile acids, many of which are secondary and tertiary products of bacterial metabolism in the intestinal tract and influence host signaling pathways (106, 107). In humans and rats, for instance, cholic acid (CA) and chenodeoxycholic acid (CDCA) are primary bile acids, while CA and β-muricholic acid (β-MCA) predominate in mice. Deoxycholic acid (DCA) and lithocholic acid (LCA) are secondary bile acids in humans, resulting from 7 alpha-dehydroxylation of CA

and CDCA, respectively. Additional bacterial mediated biotransformations result in oxidation of hydroxy groups at positions C-3, C-7 and C-12 and subsequent reduction of these groups to either alpha- or beta-configuration (108, 109). All bile acids in the enterohepatic circulation are conjugated to either glycine or taurine in the liver, and cleavage of the peptide bond to release a "free" bile acid is the gateway to subsequent bacterial modifications (108, 109). This deconjugation reaction is facilitated by microbial bile salt hydrolases (108, 109).

New bile acid synthesis in the liver is regulated by the expression of two rate limiting enzymes in the biosynthetic pathway, cholesterol 7α-hydroxylase (CYP7A1) and sterol 12α-hydroxylase (CYP8B1) (110). An accumulation of bile acids in the enterohepatic circulation induces a negative feedback mechanism mediated by FXR resulting in decreased expression of CYP7A1 and CYP8B1 (106). Consequently, FXR is the predominant signaling molecule linking the enzymatic activity of the gut microbiota to bile acid synthesis in the host. A complementary mechanism also exists whereby cholesterol accumulation will increase bile acid synthesis via a liver X receptor α (LXRα) mediated increase in CYP7A1 and CYP8B1 expression (106).

FXR is a transcription factor which is expressed throughout the enterohepatic system and regulates genes associated with bile acid metabolism as well as glucose and lipid metabolism (105). FXR activation in the small intestine results in regulation of the expression of a series of bile acid transporters which move bile acids from the intestine into the portal circulation. Apical sodium dependent transporter (ASBT) pulls bile acids into the ileal enterocyte brush border membrane, and its expression is decreased upon bile acid mediated FXR activation via the small heterodimer partner (SHP) (111). Bile acids are shuttled from the apical to the basolateral membrane of enterocytes by intestinal bile acid-binding protein (I-BABP), also known as fatty acid-binding protein subclass 6 (FABP6), and transported into blood vessels by the organic solute transporters (OSTα and OSTβ), all of which are positively regulated by FXR (112, 113). Intestinal enterocytes can directly suppress hepatic bile acid synthesis via fibroblast growth factor 19 (FGF19) in humans, or its homolog FGF15 in mice, which is secreted and circulated to the

liver in response to bile acid activation of FXR, ultimately reducing expression of the gene encoding CYP7A1 (114, 115). FGF19 has also been shown to activate hepatic glycogen synthesis and inhibit hepatic gluconeogenesis independent of insulin signaling, directly linking FXR to the regulation of carbohydrate metabolism (116). Bile acid synthesis in the liver is additionally controlled by an FXR-dependent small heterodimer partner (SHP) signaling pathway which regulates the expression of CYP7A1 and CYP8B1 via liver related homolog-1 (LRH-1) (117). FXR mediated control of bile acid homeostasis in hepatocytes also occurs through regulation of transporters for bile acid uptake and export. These include sodium-taurocholate cotransporting polypeptide (NTCP) and organic anion-transporting peptides (OATPs), which are responsible for the uptake of conjugated and unconjugated bile acids into the hepatocellular basolateral membrane, respectively, and the ATP binding cassette transporters bile salt exporting pump (BSEP, or ABCB11) and ABCB4 (118–121). Notably, intestinal and hepatic bile acid transporters also mediate absorption of dietary lipids, and so altered FXR signaling effects lipid homeostasis (122).

In one of the seminal publications identifying bile acids as the endogenous ligands for FXR, Parks *et al.* showed a striking diversity in FXR activation among bile acid species (123). The presence of hydroxyl groups in the steroid ring of bile acids greatly influences their hydrophobicity, which in turn affects their binding affinity for FXR, such that the potency of bile acids to activate FXR is CDCA > DCA > LCA > CA (123). These differences serve to fine tune host regulation of host bile acid homeostasis (110, 124). Importantly, MCA's did not activate FXR in the Parks *et al.* study, and have since been shown to uniquely antagonize and act as a natural counterbalance for regulating FXR activity (2, 123). T-β-MCA in particular is now thought to be a critical modulator of FXR signaling in the ileum, and is predominantly affected by gut microbial bile salt hydrolase activity (2, 3). As conjugated and free bile acids are similarly capable of binding FXR *in vitro*, it is still unclear why deconjugation of bile acids has such pronounced *in vivo* effects on FXR signaling. One possibility relates to active transport likely

contributing to a predominance of conjugated bile acids in enterocytes. The reduced solubility of free bile acids also makes them prone to precipitation onto solids bound for excretion and in turn reduces their likelihood for enterohepatic recycling (110, 125). In fact, precipitation of free bile acids is the basis for a common assay to identify BSH activity in *Lactobacillus* (126).

The newly appreciated interplay between bile acid modification by the gut microbiota and FXR signaling in the enterohepatic system places an increased emphasis on BSH activity in *Lactobacillus* probiotics. Changes to the host bile acid pool can affect dietary fat absorption and uptake, cholesterol metabolism, and glucose metabolism both directly and indirectly by altering FXR signaling in the host. Recent findings, particularly with respect to the role of T-β-MCA as a critical regulator of this symbiotic response network raise important questions about the consequences of probiotic administration**.**

2.7 Bile salt hydrolase activity in *Lactobacillus* probiotics

To date, BSH genes have been identified in over 200 strains of bacteria and archaea mostly associated with the gastrointestinal tract, and BSH activity is thought to represent a functional adaptation of the gut microbiota to the gut environment (127). *Lactobacillus* in particular account for 85% of the total BSH activity in the mouse ileum, and the trait is strongly associated with strains isolated from intestinal environments (128–132). For this reason, *Lactobacillus* probiotics have historically been selected to exhibit strong BSH activity, and evidence that BSH activity can help to regulate host cholesterol levels has reinforced its desirability as a probiotic trait (7, 133). The potential for BSH activity to regulate host metabolism has only recently gained appreciation and demands a careful review of the literature surrounding probiotic modification of the host bile pool as well as the physiological role of BSHs in the bacteria.

BSHs are classified as N-terminal nucleophilic (Ntn) hydrolases and belong to the choloylglycine hydrolase family of enzymes. Ntn hydrolases have a characteristic N-terminal

cysteine residue which becomes a catalytic center after autoproteolytic removal of the initiation formyl methionine (134). The thiol group of the N-terminal cysteine in particular is essential for catalysis of conjugated bile acids, and its oxidation has been shown to result in loss of enzymatic activity (135–138). Crystal structure determinations of BSHs from *Clostridium perfringens* and *Bifidobacterium longum* provided insight into the evolution of these enzymes from closely related penicillin V acylase (PVA) and identified the key residues of the active site and substrate binding pocket (139, 140). Five aspartic acid and asparagine residues were shown to form a hydrogen bonding network with the amino acid moiety of the bile acid in the active site of the enzyme, and a much less specific series of hydrophobic interactions was shown to stabilize the steroid moiety within the substrate binding site (139). This description nicely fits with data from early kinetic studies which suggested BSHs exhibit substrate specificity based primarily on the amino acid moeity (141–145). However, several subsequent BSH characterizations have reported activity differences related to the steroid moiety, indicating that hydrophobic interactions in the substrate binding pocket contribute to BSH substrate specificity more than initially thought and possibly in a BSH dependent manner (136, 146–153).

These studies invariably describe BSH activity with respect to glyco- and tauro-conjugates of CA, DCA, and CDCA. The total bile acid pool in humans and other animals is exceedingly complex though, and it is still unclear how differences in BSH substrate specificity affect bile acid composition in the host, particularly with respect to T-β-MCA (125, 154–157). A series of experiments conducted in Japan in the late 1980's and early 1990's provided some of the only evidence to date that the human microbiota is capable of deconjugating T-β-MCA. Sacquet *et al.* were the first to demonstrate this using ^{14}C labeled T-β-MCA that they synthesized in rats and administered to human subjects (158). Additional studies using gnotobiotic mice identified human associated strains of *Bacteroides vulgatus*, *C. ramosum*, *B. longum*, *Peptostreptococcus productus* and *L. gasseri* as having BSH activity against T-β-MCA (159, 160). A mixed population of clostridia isolated from a rat has also been shown to exhibit activity against T-β-

MCA (161). Unfortunately though, the BSHs associated with these strains were never characterized further. Two more recent mouse studies have considered the effect of BSH activity on host bile acid composition. Roager *et al.* administered *L. acidophilus* NCFM to germ free mice and observed a significant increase in free bile acids compared to controls which affected vitamin E acetate metabolism (162). Interestingly, the difference appeared to be driven in large part by BSH activity against TCA, with no difference in T-β-MCA concentrations (162). In fact, Joyce *et al.* have published the only study to date to describe probiotic mediated BSH activity against T-β-MCA. In their study, the authors independently cloned two BSH genes from *L. salivarius* JCM1046 into *E. coli* MG1655, which lacks BSH activity, and observed broad differences in their ability to modify the host bile acid pool and affect weight gain (4). **These results suggest that differences in BSH activity among probiotic *Lactobacillus* with respect to individual bile acids can have important consequences for host physiology, and need to be reconsidered.**

The incontrovertible association of BSH activity with the gastrointestinal environment informs its purported role in bile tolerance. Indeed, this has been directly demonstrated by comparing bile sensitivity in wild type and BSH mutant strains of *L. amylovorous, L. plantrum,* and *Listeria monocytogenes* (83, 84, 163, 164). In the latter case, the presence of the BSH mutant strain of *L. monocytogenes* in guinea pig feces was nearly five logs less than that of the wild type strain 48 hours after inoculation (164). In addition to inducing increased membrane permeability due to their detergent properties, bile acids also can contribute to intracellular acidification, and so BSH activity might protect the cell by generating the weaker unconjugated bile acid (84). Aforementioned profiling studies identified BSHs as part of a global cellular response to bile and acid stress, and lend support to the idea that BSHs contribute to bile tolerance (82, 86). However, other studies have failed to identify a link between BSH activity and bile tolerance (147, 165–169). In a comparison of plant and intestinal isolates of *L. casei,* for instance, Kimoto-Nira *et al.* reported bile tolerance in significantly more of the intestinal isolates,

as expected (169). Surprisingly though, none of the bile tolerant isolates exhibited BSH activity, and the authors discovered that bile tolerance in these strains was related to differences in cell membrane fatty acid composition (169). Interestingly, BSH activity has been shown to mediate changes in the fatty acid composition of the cell membrane in *L. reuteri* which complex with precipitated bile acids and cholesterol to maintain membrane fluidity and provide bile tolerance (170, 171). It is possible then that BSH activity is part of a broader strategy to alter cell membrane composition in response to bile stress. Recently, a study of BSH mediated resistance to bile acid stress in *Lactococcus lactis* revealed the formation of micelles as a result of bile acid deconjugation within the cytoplasm of the cell which were eventually exported to the surface of the cell membrane (172). Modest BSH activity was found to improve bile tolerance, but increased activity resulted in excessive micelle production and inhibition of cell growth (172). While it is unclear how well conserved this bile acid disposal mechanism is among BSH active bacteria, these results do offer a potential explanation for conflicting results in previous studies by suggesting that the benefits of BSHs for bile tolerance are not necessarily linearly dependent on the strength of the enzymatic activity.

Importantly, the relationship between BSH activity and bile tolerance has often been examined without regard for differences in BSH substrate specificity. The toxicity of glyco-conjugated bile acids has been shown to be far greater than that of tauro-conjugated bile acids, and might explain reports of biased BSH activity towards the former (84, 141–145, 163). In that case, it has been suggested that tauro-conjugated BSH activity serves the purpose of freeing taurine for use as an electron acceptor. This has been demonstrated in a mixed culture of *Clostridium* and *Bacteriodes,* where free taurine was produced by the *Clostridium*, subsequently reduced by the *Bacteriodes*, and finally utilized again in the *Clostridium* (173). Notably, release of taurine also increased the rate of 7α-dehydroxylation in the culture, alleviating the toxicity of the free bile acid (173). Similar findings have never been reported in *Lactobacillus,* but it is likely that this simply reflects the challenges associated with uncovering mutualistic relationships

within the gut microbiota. A characterization of four BSHs encoded by *L. plantarum* WCFS1 nicely highlights how differences in BSH substrate specificity might be contributing to the confusion surrounding their function. BSH1 was found to predominantly contribute to BSH activity and confer resistance to bile salts, while the other BSHs were suggested to actually be penicillin acylases which are involved in the assimilation of phenyl and phenoxyacetylated compounds commonly associated with plant cell degradation (152, 174). Intriguingly, despite their evolutionary divergence, BSHs and penicillin acylases have been found to exhibit activity against a broad range of common substrates, raising the possibility that differences in BSH substrate specificity reflect their adaptation to multiple physiological functions (140). Indeed, multiple BSH enzymes have also been identified in probiotic strains of *L. acidophilus, L. johnsonii,* and *L. salivarius* (4, 60, 146).

Bsh genes are highly diverse, and several groups have suggested that they are acquired horizontally. Elkins *et al.* found very little synteny flanking *bsh* loci in closely related strains of *L. acidophilus* and *L. johnsonii*, and discovered group II intron proteins, which are associated with mobile genetic elements, downstream of these genes (130). An analysis of two BSH protein sequences in *L. acidophilus* NCFM revealed a higher similarity to BSHs from other *Lactobacillus* species than to each other, implying the potential for horizontal acquisition (146). On a broader scale, BSH genes are also thought to flow horizontally throughout the gut microbiota, and numerous comparative genomic analyses have implicated a strong role for horizontal gene transfer in the evolution and niche adaptation of *Lactobacillus* (21, 127, 175–178). Nonetheless, a more careful analysis of a collection of *L. plantarum* strains revealed that nearly all of the strains encoded homologs of the same four *bsh* genes (152). Another study of closely related species within the *L. acidophilus* group revealed a stepwise decrease in genomic and protein similarity among members of the group, suggesting that vertical evolution more strongly shaped the evolution of these species (179). These results call into question the role of horizontal gene

transfer in shaping the phenotypic diversity of BSH activity among closely related strains, particularly as it relates to niche adaptation.

Overall, the relationship between BSH function and bile acid substrate specificity is not well understood. Alternative roles for BSHs, particularly BSHs targeting tauro-conjugated bile acids have the potential to shape the microbial ecology of the gut microbiota and ultimately affect host physiology. For instance, mutualistic partners likely benefit from BSH activity, and it is possible that changes in the populations of these bacteria have additional consequences (e.g. increased LPS production). More specifically, **an improved understanding of how BSH activity influences niche adaptation in probiotic *Lactobacillus* would help to inform the selection and development of new strains with improved colonization efficacy and persistence in the host.**

2.8 Manipulating bile salt hydrolase activity to control host metabolism

Animal agriculture offers some of the strongest evidence for the role of BSHs in regulating host metabolism. Growth promotion by dietary supplementation with sub therapeutic levels of antibiotics has been practiced in poultry production since the 1950s and has long been attributed to changes in the gut microbiota, particularly *Lactobacillus* species (180–184). Several studies provide evidence that microbial alterations of bile acid composition in the small intestine are at least partly responsible for differences in weight gain and feed efficiency in animals fed antibiotics, although T-β-MCA was never considered (185–187). For example, Guban *et al.* identified an inverse relationship between ileal bile acid deconjugation by *L. salivarius* and weight gain mediated by antibiotic growth promoters (188). More recent research has focused on identifying and utilizing BSH inhibitors to achieve changes in intestinal bile acid composition in the absence of antibiotic growth promotors, but the biology underlying how BSH inhibitors affect host metabolism remains largely uncharacterized, and has never been shown to directly involve

FXR (150, 189). Notably, the directionality of this relationship appears to contradict similar findings linking BSH activity and weight gain in rodent studies, suggesting that host specific signaling responses to altered bile acid composition likely differ.

Additional evidence for BSH mediated regulation of FXR signaling stems from the historical use of probiotics for cholesterol control. BSH activity has been linked to cholesterol control dating back to the first comparisons of cholesterol accumulation in conventional versus germ free mice (190, 191). De Smet *et al.* were the first to explicitly demonstrate the efficacy of a BSH active probiotic for lowering LDL cholesterol levels in an animal model by altering fecal bile acid output (192). Subsequent trials in humans and mice have reported similar effects from administration of BSH active *L. reuteri* and *L. plantarum* strains (193–195). Importantly, administration of BSH active *L. plantarum* to mice offered the first clues that altered cholesterol metabolism was related to increased hepatic bile acid synthesis via upregulation of CYP7A1 (195). More recently, a follow up trial using *L. reuteri* reported changes in bile acid composition and cholesterol synthesis which correlated with FGF19, a key mediator of FXR signaling (196). Together, these results indicate that BSH active probiotics are capable of altering FXR signaling in the host, although their effect on host metabolism is unclear.

Interestingly, BSH activity in *Lactobacillus* was found to have no effect on weight gain in mice in a study conducted by Bateup *et al.* in the mid 1990's (167). The authors administered five strains of *Lactobacillus* isolated from mouse intestines with varying levels of BSH activity to mice harboring a modified microbiota lacking any other *Lactobacillus* for a period of two weeks. However, the authors only considered release of CA in their BSH assay, so differences in BSH substrate specificity were not considered, particularly with respect to T-β-MCA. It is possible that these results discouraged additional research on the subject in murine models even in spite of the excitement surrounding the role of BSHs in growth promotion for agricultural animals. One ensuing publication suggested a role for differences in BSH activity corresponding to altered cholesterol metabolism and feed efficiency in rats fed three strains of *Lactobacillus*, but the

strains also exhibited differences in antimicrobial and amylolytic properties (197). Moreover, the authors did not analyze corresponding changes in host bile acid composition or FXR signaling, making the results difficult to interpret. Only recently has interest in the subject been revived in light of several seminal publications. Sayin *et al.* finally linked altered cholesterol and bile acid metabolism in germ free mice to differences in FXR signaling mediated specifically by T-β-MCA (2). More recently, the same group demonstrated that the gut microbiota promotes obesity through FXR, and provided evidence that FXR directly contributes to shaping gut microbiota composition (199). Li *et al.* published the first study to definitively show that reduced BSH activity for T-β-MCA in the gut microbiota limits weight gain through attenuated FXR signaling in mice fed a high fat diet (3). Changes in the composition of the gut microbiota and its overall BSH activity were specifically linked to changes in the population of *Lactobacillus*. A follow up study conducted by many of the same researchers has since implicated bile acid mediated FXR signaling in the pathogenesis of nonalcoholic fatty liver disease (198).

Table 2.2: Summary of results from studies relating altered intestinal BSH capacity to weight changes

↓ *L. salivarius*	↑ CBAs*	**?** FXR signaling	↑ weight gain	Guban *et al.*[188] †
↑ *Lactobacillus*	**?** T-β-MCA	**?** FXR signaling	no weight change	Bateup *et al.*[167]
+ microbiota	↓ T-β-MCA	↑ FXR signaling	↑ weight gain	Sayin *et al.*[2], Parseus *et al.*[199]
↓ BSH activity	↑ T-β-MCA	↓ FXR signaling	↓ weight gain	Li *et al.*[3]
↑ BSH activity	↓ T-β-MCA	**?** FXR signaling	↓ weight gain	Joyce *et al.*[4]

*conjugated bile acids (CBAs); †Study conducted in chickens, all other studies were conducted in mice

These studies provide exciting evidence that the gut microbiota is capable of regulating host metabolism by modifying the bile acid pool, and have inspired new questions surrounding the use of probiotics for weight control. In particular, these findings were each based on large scale shifts in gut microbial ecology, and so it is unclear if the introduction of a single defined probiotic strain is capable of manifesting similar changes in host metabolism. A definitive report of probiotic mediated control of host metabolism via altered FXR signaling has still not been

published, however Joyce *et al.* did successfully affect weight gain in mice administered transgenic *E. coli* strains expressing *bsh* genes from *L. salivarius* (4).

2.9 Conclusions and research objectives

An understanding of the mechanistic relationship between the gut microbiota and host weight regulation is only just beginning to emerge, and many essential questions remain unanswered. Of particular relevance to this dissertation is the question of whether changes in the population of a single bacterial strain within the vast community of the gut microbiota can affect host response. Li *et al.* broadly implicated *Lactobacillus* as the likely mediator of their observed changes in BSH activity against T-β-MCA in the mouse (3). However, the authors also observed differences in populations of a number of additional microorganisms which are also associated with BSH activity, including members of *Clostridium* (139). Therefore, **it is not clear if *Lactobacillus* are truly capable of controlling host metabolism or if they are simply correlating with broader changes in the composition of the gut microbiota.**

Only three published reports have directly considered the effects of administering BSH active bacteria on weight gain in rodents, and each them arrived at different conclusions (4, 167, 197). Bateup *et al.* identified no effect of treatment, while Guo *et al.* reported differences in feed efficiency among strains of *Lactobacillus* (167, 197). Both of these studies focused on total BSH activity, but paid little regard to BSH substrate specificity. Joyce *et al.* are the only group so far to have linked weight changes with BSH activity specifically for T-β-MCA, but they stopped short of attributing these changes with altered FXR signaling (4). At this point, **there is still no definitive report linking administration of a probiotic with BSH activity against T-β-MCA to altered FXR signaling and metabolism in the host.** In fact, despite the breadth of published literature characterizing BSH activity and substrate specificity in probiotic *Lactobacillus*, only one probiotic strain, *L. salivarius* JCM1046, has ever been shown to exhibit activity against T-β-

MCA (4, 136, 141–153). In light of its newly discovered role in regulating metabolism, it is important to determine if any other common *Lactobacillus* probiotics exhibit BSH activity against T-β-MCA.

The first objective of this research is to identify the predominant *Lactobacillus* strain in the mouse intestine and characterize its BSH activity against T-β-MCA. Additionally, the aim is to screen a broader collection of probiotic *Lactobacillus* strains for activity against T-β-MCA in order to understand how substrate specificity for T-β-MCA relates to well characterized substrate specificity for glyco- and tauro-conjugates of CA, DCA, and CDCA. More importantly, this work will consider the *in vivo* effects of differences in BSH substrate specificity for T-β-MCA on FXR signaling and weight gain.

In order to develop next generation probiotics for metabolic control it is important to understand how differences in BSH activity and substrate specificity among strains affects their probiotic efficacy. This review outlined a multitude of factors involved in the physiological stress response of probiotic *Lactobacillus*, and the literature overall suggests that BSHs at least confer an advantage to bacteria in the intestinal environment. However, the physiological importance of differences in BSH substrate specificity among *Lactobacillus* strains has never been directly considered from the perspective of the bacteria. Therefore, **the final objective of this work is to characterize the metabolic response of *Lactobacillus* strains with different BSH activities and substrate specificities in response to bile acid exposure.** The idea is to generate new hypotheses about the role for BSHs in these strains and ultimately set the table for future research aimed at improving the design and selection of BSH active probiotics for host specific metabolic control.

Chapter 3
Identification of two *L. johnsonii* strains with potential for metabolic control based on bile salt hydrolase activity against the potent FXR antagonist T-β-MCA

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3.1 Introduction

The gut microbiota has received increasing attention throughout the last decade for its role in the development of obesity (200). Metagenomic studies have consistently identified increases in the relative abundance of Firmicutes to Bacteriodetes in obese individuals (10–12), and have stoked interest in the development of probiotics as therapeutics (8). *Lactobacillus* species have a long history of use as probiotics for improving gastrointestinal health, and are leading candidates for weight control applications in humans and animals (8, 201). However, a mechanistic understanding of bacterial mediated metabolic regulation is only just beginning to emerge, and extensive diversity within the *Lactobacillus* genus is a potential obstacle to targeted therapeutic development (202).

Several recent studies have elucidated a metabolic control system in the intestine based on interactions between the gut microbiota, bile acids, and FXR (2, 3). Bile acids are conjugated

to either taurine or glycine in the liver, and microbial BSHs facilitate the deconjugation of the amino acid from the steroid core of the bile acid, effectively removing it from enterohepatic recirculation (7). FXR is a ligand activated transcription factor which primarily regulates bile acid synthesis from cholesterol in the liver and also affects glucose and lipid metabolism (105). Most endogenous bile acids bind and activate FXR, however, tauro-beta-muricholic acid (T-β-MCA) has been shown to uniquely antagonize FXR, making it an important counterbalance for maintaining metabolic homeostasis (2, 3). Interactions between the gut microbiota and the host bile acid pool, particularly T-β-MCA, influence FXR signaling and ultimately host metabolism, and appear to be driven in large part by *Lactobacillus* (2–4). In fact, Li *et al.* have reported significantly lower rates of weight gain in mice with reduced *Lactobacillus* populations and corresponding increases in T-β-MCA concentrations, but *Lactobacillus* were never directly shown to exhibit activity against T-β-MCA (3). Likewise, Joyce *et al.* showed that BSH activity can mediate weight loss in mice, but a clear link to T-β-MCA mediated regulation of FXR was never established (4). Despite the promise of these findings as well as an extensive knowledge of BSH activity in *Lactobacillus* based probiotics, little information exists regarding BSH activity specifically against T-β-MCA (7). In particular, it is unclear how BSH activity in *Lactobacillus* strains autochthonous to the mouse intestine compares to well characterized BSH activity in commercially available probiotic strains.

Here, we set out to identify the predominant *Lactobacillus* species in the mouse intestine and determine if they are indeed capable of altering T-β-MCA concentrations. As interactions between *Lactobacillus* strains and T-β-MCA are thought to be essential to developing probiotics for metabolic control, we also screened a wider collection of *Lactobacillus* probiotics for BSH activity against T-β-MCA. Moreover, in an effort to understand the factors affecting BSH substrate specificity and ultimately identify new probiotic strains for weight regulation, we modeled critical interactions within the substrate binding pocket of BSHs exhibiting activity against T-β-MCA. Our work suggests that *Lactobacillus* strains in the mouse are important

mediators of metabolism through their interactions with the potent FXR antagonist T-β-MCA, and identifies two *L. johnsonii* strains with potential for further development as next generation probiotics.

3.2 Results

3.2.1 Isolation of mouse intestinal *Lactobacillus.*

Li et al. observed a reduction in the relative proportion of *Lactobacillus* within the small intestinal microbiota in leaner mice, but did not determine the predominant species or strains affected by their treatment. Since BSH activity has been shown to vary considerably among probiotic *Lactobacillus* strains (7), we aimed to isolate and identify the predominant *Lactobacillus* species inhabiting the mouse intestine. *Lactobacillus* populations ranged from 10^7 to 10^9 CFU/g cecal contents, and all of the isolates were identified as *L. johnsonii* based on 16s rRNA sequencing using universal bacterial primers (203). Comparison of growth rates and acidification of de Man, Rogosa, Sharpe (MRS) media revealed no noticeable phenotypic differences among the isolates (data not shown), and a single isolate, designated LB1, was selected for further characterization.

3.2.2 *Lactobacillus* BSH activity against T-β-MCA.

BSH activity against the potent FXR antagonist T-β-MCA has been shown to regulate metabolism, and is likely dependent on the strain specific composition of the gut microbiota (2–4). Therefore, we determined if LB1 is capable of deconjugating T-β-MCA *in vitro*. Cultures and cell lysates of LB1 spiked with T-β-MCA were analyzed by ultra-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOFMS) and found to have undergone nearly complete reductions in the chromatographic peak area associated with T-β-MCA (m/z 514.2844) with corresponding increases in the peak area associated with β-MCA (m/z 407.2803). These results indicate that

LB1 exhibits BSH activity towards T-β-MCA and suggest that changes in intestinal LB1 populations have the potential to mediate FXR signaling.

Next, we extended our assay to a collection of common probiotic *Lactobacillus* strains in order to identify additional strains with BSH activity against T-β-MCA. We selected *Lactobacillus* strains which are fully sequenced, encode at least one BSH gene, and are publically available through the American Type Culture Collection (ATCC). Each strain was incubated in both MRS and MRS containing 0.1% porcine bile in order to identify inducible BSH activity. To our surprise, only LB1 and NCK88, both strains of *L. johnsonii*, were capable of deconjugating T-β-MCA (Table 3.1). *L. johnsonii* ATCC33200 did not exhibit any activity against T-β-MCA, a finding that reinforces an extensive body of evidence indicating that BSH activity is strain dependent in *Lactobacillus* (7). None of the additional 11 *Lactobacillus* strains we screened exhibited any detectable activity against T-β-MCA regardless of whether they were pre-incubated in media containing bile. These results led us to further investigate BSH activity in *L. johnsonii*.

Table 3.1: BSH activity against T-β-MCA in *Lactobacillus* strains

Strain ID	MRS	MRS + 0.1% porcine bile
L. acidophilus NCFM	-	-
L. acidophilus La5	-	-
L. plantarum WCFS1	-	-
L. plantarum Lp 39 (ATCC 14937)	-	-
L. reuteri SD2112 (ATCC 55730)	-	-
L. reuteri MM4-1A	-	-
L. gasseri DSM 20243 (ATCC 33323)	-	-
L. johnsonii VPI 7960 (ATCC 33200)	-	-
L. johnsonii LB1	+	+
L. johnsonii NCK88 (ATCC 11506)	+	+
L. brevis 118-8 (ATCC 367)	-	-
L. fermentum B1 28 (ATCC 14931)	-	-
L. salivarius subsp. *salivarius* HO66 (ATCC 11741)	-	-
L. rhamnosus GG (ATCC 53103)	-	-

*Positive activity is defined as a mean 20% reduction in T-β-MCA concentration over the course of three independent assays.

3.2.3 Genomic comparison of *L. johnsonii* strains.

Since little is known about either *L. johnsonii* LB1 or NCK88, we sequenced and assembled draft genomes of each strain in order to compare them to *L. johnsonii* NCC533, the

first fully sequenced *L. johnsonii* strain isolated from a human (60). A visual comparison of the ordered contigs indicated that all three strains have a similar genome architecture, size, and GC% (Figure 3.1A). Dot plot comparisons of translated open reading frames also revealed strong conservation and relatedness among the strains, with little evidence of large scale inversions or translocations (Figure 3.1B,C).

Next, we used BLAST to identify BSH loci within the draft genomes of LB1 and NCK88 and compare them to BSH genes from the other strains in our *Lactobacillus* collection. LB1 and NCK88 each contain three BSH loci in the same relative genomic locations, designated BSHA, BSHB, and BSHC, which share 98.5, 99.1, and 98.2% amino acid identity and are homologous to LJ1412, LJ0056, and LJ1147 in NCC533, respectively (Figures S3.1-S3.3). A subsequent BLAST comparison of all three *L. johnsonii* BSH homologs did not identify any of them in other species. Hierarchical clustering and phylogenetic analysis of the BSHs within our strain collection reflects the homology of each BSH identified in *L. johnsonii* and also illustrates the diversity of BSHs in our strain collection (Figure 3.2). BSHA in LB1 and NCK88 are nearly identical and share a distinct branch with the BSHA homolog identified in *L. johnsonii* ATCC33200. BSHB in LB1 and NCK88 are closely related to the BSH identified in *L. gasseri* ATCC33323, and BSHC from both strains are related to *L. acidophilus* BSHB. Notably, closely related BSHs likely exhibit differences in substrate specificity for T-β-MCA, since *L. acidophilus* NCFM, *L. gasseri* ATCC33323, and *L. johnsonii* ATCC33200 did not display any activity against T-β-MCA in our *in vitro* assay (Table 3.1).

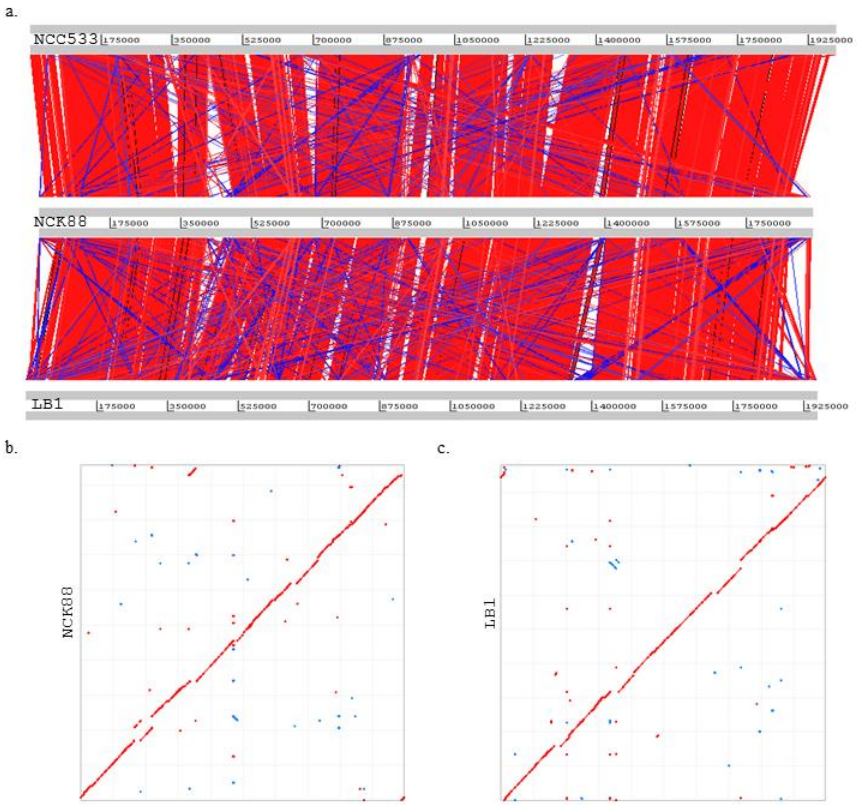

Figure 3.1 Visual comparisons of *L. johnsonii* NCK88 and LB1 draft genomes to the reference strain NCC533 (a) WebACT visualization of genome similarity of NCK88 and LB1 to NCC533 based on BlastN analysis with a filter cutoff of 100. Red bars indicate matches in the same orientation, and blue bars indicate matches in the reverse orientation. (b and c) Dot plots representing the maximum unique matches (MUMs) of the six frame amino acid translations of LB1(b) and NCK88 (c) draft genomes relative to NCC533. Forward MUMs are plotted as red lines/dots while reverse MUMs are plotted as blue lines/dots. A line of dots with slope of 1 represents an undisturbed segment of conservation between the two sequences and a line with a slope of -1 represents an inverted segment of conservation between the two sequences.

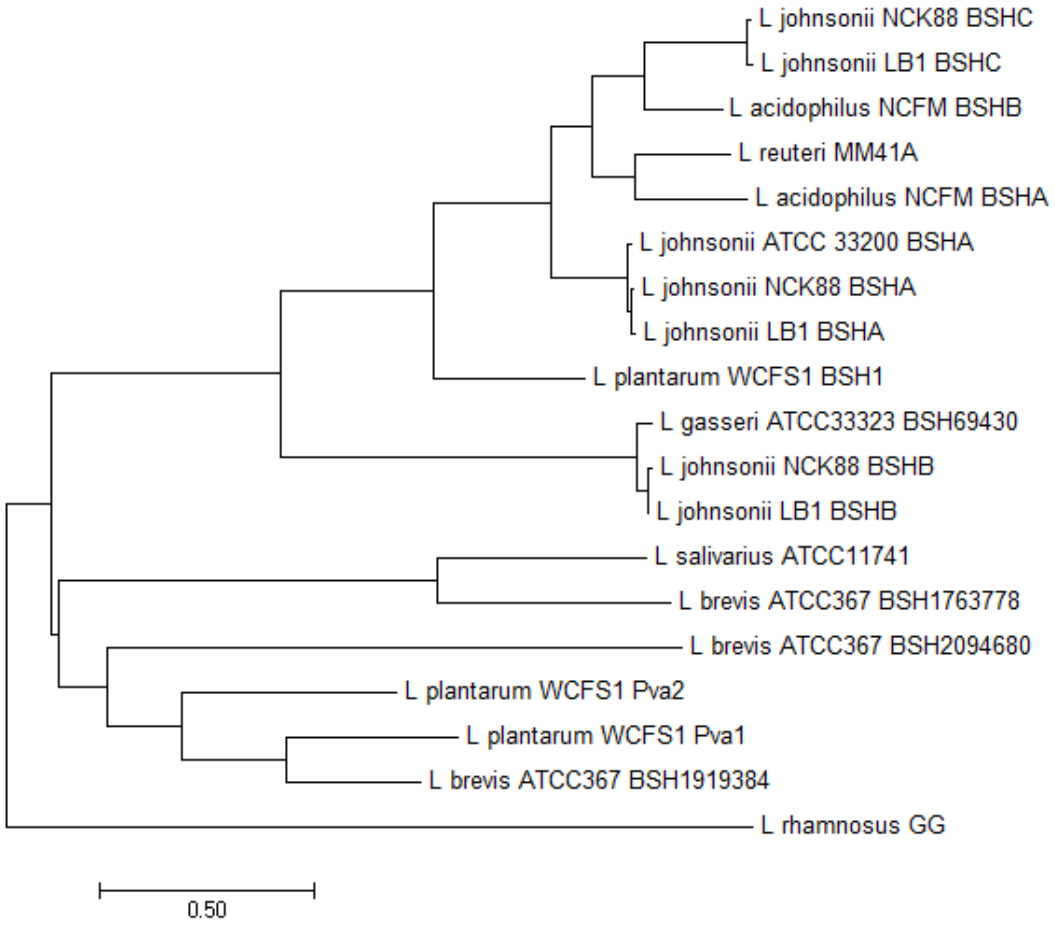

Figure 3.2 Phylogenetic analysis of *Lactobacillus* BSH sequences by Maximum Likelihood method The evolutionary history of BSHs encoded by *Lactobacillus* strains screened in our *in vitro* assay was inferred by using the Maximum Likelihood method based on the Le Gascuel 2008 model (223). The tree with the highest log likelihood (-8040.5390) is shown. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (3 categories (+*G*, parameter = 2.2994)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 4.7893% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 19 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 297 positions in the final dataset. Evolutionary analyses were conducted in MEGA6(222).

3.2.4 Comparison of BSH substrate specificity.

In order to effectively identify the BSHs exhibiting substrate specificity towards T-β-MCA in *L. johnsonii*, we cloned each *bsh* gene from strains LB1, NCK88, and ATCC33200 into a constitutive *E. coli* expression vector. As expected based on the results of our *Lactobacillus* BSH activity screen, BSHA from *L. johnsonii* ATCC33200 and LB1 did not exhibit any activity against T-β-MCA (Table 3.2). BSHB from LB1 and NCK88, which are 98.7% identical (Figure S3.2), completely deconjugated the full range of substrates. Interestingly, LB1 BSHC also effectively deconjugated all of the bile acids, but NCK88 BSHC was not active against any tauro-conjugated substrates, including T-β-MCA—these enzymes only differ by six amino acids (Figure S3.3).

Table 3.2: BSH substrate specificity of BSHs in *L. johnsonii*

	T-β-MCA	TCA	TCDCA	TDCA	GCA	GCDCA
L. johnsonii LB1 BSHA	-	-	-	-	-	-
L. johnsonii LB1 BSHB	+	+	+	+	+	+
L. johnsonii LB1 BSHC	+	+	+	+	+	+
L. johnsonii NCK88 BSHB	+	+	+	+	+	+
L. johnsonii NCK88 BSHC	-	-	-	-	+	+
L. johnsonii ATCC 33200 BSHA	-	-	-	-	-	-

*Positive activity is defined as a mean 20% reduction in substrate concentration over the course of three independent assays.

3.2.5 Modeling BSH substrate interactions with T-β-MCA

The active site of BSHs is comprised of two regions which interact with the conjugated bile acid and influence substrate specificity (139, 140). The first is a snug hydrophobic pocket lined by a number of nonpolar residues that stabilizes the steroid moeity of the substrate through van der Waals interactions and the second is a positively charged surface where the negatively charged SO3 modification of the taurine or glycine anchors. We utilized predictive modeling to computationally identify and compare relevant structural differences among *L. johnsonii* BSHs which might contribute to determining substrate specificity, particularly for T-β-MCA. Our models are in agreement with crystal structural determinations of the well characterized

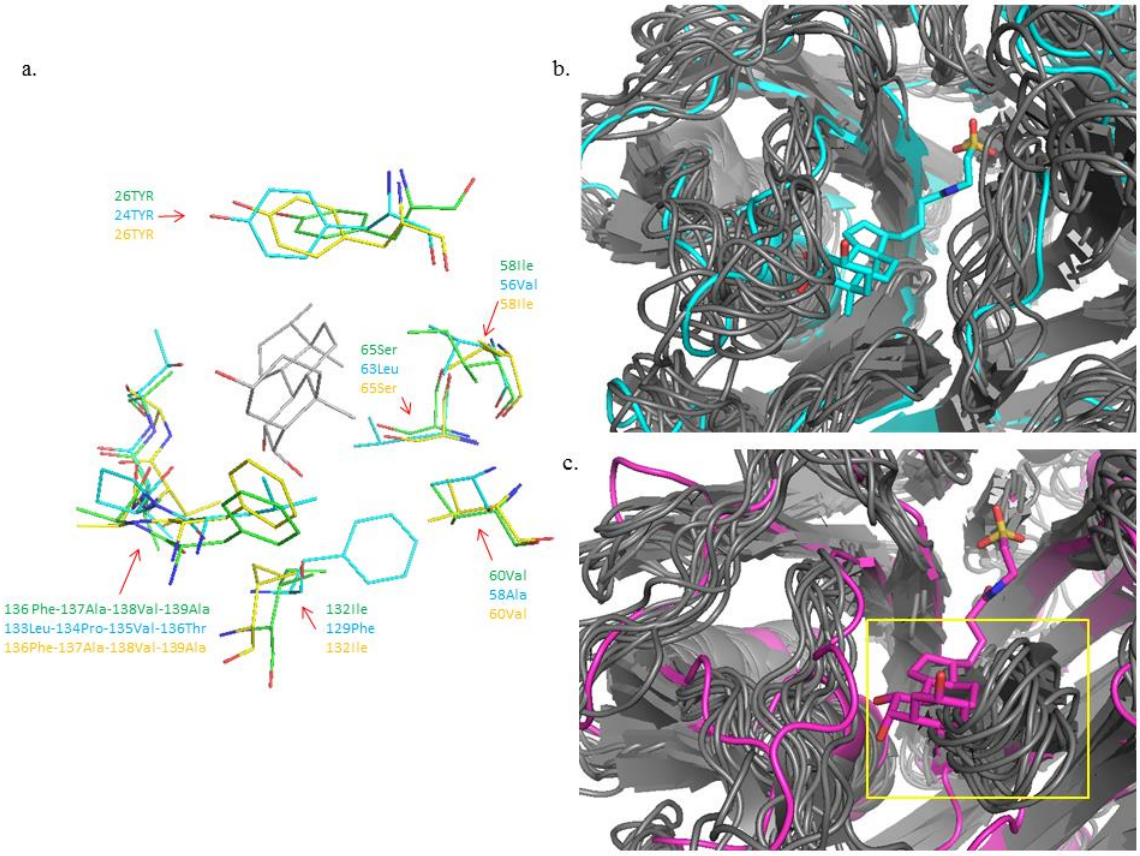

Figure 3.3 Visualizations of model differences in BSH substrate interactions with T-β-MCA (a) Depiction of residue side chains within a 4Å radius of the T-β-MCA ring structure in the substrate binding pocket of enzymes with activity against T-β-MCA. LB1 BSHC is in blue, LB1 BSHB is in green, NCK88 BSHB is in yellow, and T-β-MCA is in grey. Stick depictions of T-β-MCA and amino acid side chains indicate oxygen atoms in red, and nitrogen atoms in dark blue. Hydrogens have been removed to improve visual clarity. (b and c) Cartoon comparisons of LB1 BSHC (b) and NCK88 BSHC (c) with T-β-MCA in the binding pocket. YASSARA models energetically minimized to T-β-MCA are depicted in blue (LB1 BSHC) and magenta (NCK88 BSHC), and the range of loop movements for each model based on CABS-flex simulations is depicted in grey. Occlusion of the substrate binding pocket by the loop structure in NCK88 BSHC is indicated in a yellow box in (c).

conjugated bile acid hydrolase from *Clostridium perfingens* (139), and are energetically consistent among the full range of experimental bile acids we screened for each BSH. An analysis of distance measurements suggests that critical arginine, asparagine, and N-terminal cysteine residues are capable of hydrogen bonding with taurine in the active site in all of the *L. johnsonii* BSHs (Table S3.1) (139). Hydrophobic interactions between amino acid residues in the

substrate binding pocket and the ring structure of T-β-MCA are generally well conserved among the BSHs exhibiting activity against T-β-MCA (Figure 3.3A, Table S3.1). BSHA from strains LB1 and ATCC33200 both have unresolvable steric clashes between the aromatic side chain of Phe_{66} and the steroid moiety of all of the bile acid substrates, providing a possible explanation for their lack of experimental activity (Table 3.2). Interestingly, BSHC from LB1 and NCK88, which share 98.2% amino acid identity but differ in their substrate specificity for T-β-MCA, exhibit noticeable differences in the positions of the loop structures defining the opening to the substrate binding pocket (Figure 3.3B, C). This pocket spans 20.1 Å in NCK88 BSHC, and only 15.2 Å in BSHB based on the position of the loops in our original energy minimized models. To further investigate the potential relevance of the loop structures lining the substrate binding pocket in these enzymes, we used CABS-flex to simulate protein structure fluctuations and assess the range of motion for these loops (204). The new models revealed that the range of loop movement in NCK88 BSHC occludes the steroid binding pocket and likely contributes to its more limited substrate specificity (Figure 3.3B, C)

3.3 Discussion

Li *et al.* reported an association between *Lactobacillus* populations, T-β-MCA concentrations, FXR signaling, and weight gain, but did not directly attribute BSH activity against T-β-MCA to any *Lactobacillus* strain (3). Importantly, the observed changes in *Lactobacillus* populations were part of a broader shift in the overall composition of the gut microbiota, and BSHs have been identified in every representative lineage throughout the microbial community (127). Therefore, it remained unclear if more specific changes in *Lactobacillus* populations were capable of influencing compositional changes in host bile acid composition with respect to T-β-MCA. Our results provide the first evidence that a *L. johnsonii* strain, LB1, autochthonous to the mouse intestine exhibits BSH activity against T-β-MCA, a critical mediator of FXR signaling.

Genomic comparison of *L. johnsonii* LB1 and NCK88, which has an unknown origin, to the human strain NCC533 revealed no large scale inversions indicative of host specific adaptation as described by Guinane *et al.* for other strains of *L. johnsonii* (205–209). We identified three independent BSH homologs in all three strains, and one of the three homologs was also identified in *L. johnsonii* ATCC33200 (Figures S3.1-S3.3). Phylogenetic comparison of these BSHs among a broader collection of BSHs on an amino acid level revealed close evolutionary relationships among *L. johnsonii* BSHB and the BSH found in *L. gasseri* ATCC33323 as well as *L. johnsonii* BSHC and *L. acidophilus* NCFM BSHB (Figure 3.2). *L. johnsonii* BSHA meanwhile appears more distantly related to BSHs from other strains, which perhaps reflects its lack of BSH activity (Figure 3.2). These results largely agree with a report of predicted protein similarity among these species and suggest that BSHs are likely following the proposed stepwise vertical evolution within the *L. acidophilus* group (179). Indeed, none of the *L. johnsonii* BSH homologs were identified in any other species. While useful for identifying homology and delineating evolutionary relationships among BSHs, it is also apparent from our screening results that hierarchical clustering and phylogenetic analysis of BSH amino acid sequences does not closely parallel the associated bile acid substrate specificities of these enzymes. For example, despite encoding BSHs closely related to those found in *L. johnsonii*, *L. acidophilus* NCFM and *L. gasseri* ATCC33323 did not exhibit any activity against T-β-MCA (Table 3.1). More clearly, BSHC in *L. johnsonii* LB1 and NCK88 are nearly indistinguishable phylogenetically, but exhibit broad differences in substrate specificity (Table 3.2).

Conjugated muricholic acids are capable of acting as FXR antagonists, making them important potential targets for BSHs in the intestine (2, 123). T-β-MCA's role in the regulation of FXR signaling is now firmly established in mice, and it has been identified as part of a complex pool of bile acids found in humans (2, 3, 125, 154, 155, 157). BSH activity has historically been considered a desirable trait in *Lactobacillus* probiotics for bile tolerance as well as maintenance of host cholesterol levels, raising the question of whether any commercially

available probiotics are capable of altering intestinal T-β-MCA concentrations (7, 202). Previous characterizations of BSH activity in probiotics have revealed considerable diversity in substrate specificity for glyco- and tauro-conjugates of CA, DCA, and CDCA, but activity against T-β-MCA has rarely been studied (136, 141, 146, 152). Several bacterial strains isolated from a healthy human microbiota have been reported to deconjugate T-βMCA, including *Bacteroides vulgatus*, *Clostridium ramosum*, *Bifidobacterium longum*, *Peptostreptococcus productus* and *L. gasseri*, but the genes encoding these enzymes were never identified (159–161). Likewise, the complete mouse gut microbiota has been shown to exhibit BSH activity against T-β-MCA, and recently, Joyce *et al.* identified a BSH from the probiotic strain *L. salivarious* JCM1046 with activity against a broad range of mouse bile acids, including T-β-MCA (2, 4). Since *L. johnsonii* is closely related to many *Lactobacillus* probiotics with well characterized BSH activity against tauro-conjugates of CA, DCA, and CDCA, we were surprised that none of the other strains we considered exhibited detectable activity against T-β-MCA (Table 3.1). The prevalence of BSHs with activity against T-β-MCA is not clear from our limited study, but our results raise an important ecological question regarding why closely related bacterial species have acquired BSHs with distinct substrate specificities against T-β-MCA. These results also suggest that species and strain level compositional changes within the gut microbiota may have important consequences for host bile acid metabolism.

A large number of BSH genes have been identified through metagenomics and have never been characterized *in vitro*, making it difficult to understand how shifts in the gut microbial community might affect host bile acid composition (127). This would be particularly useful for mining clinical data relating to probiotics and obesity for added insights into the underlying role of BSHs, and would ultimately inform the development of next generation probiotics for controlling metabolism (5, 210). Data from our predictive modeling experiments generally aligns with the results of our *in vitro* screening assay, and provides new insights into the basis for BSH substrate specificity. Substrate specificity seems to be linked to how well the two active site

regions are favorably interacting, and thus activity is likely lower if either of the surfaces is not optimally matched. Ideally, the hydrophobic pocket should contour the steroid rings with no steric hindrance from protein residues and also should not be too large to have favorable van der Waals interactions. Interactions at the amino end also need to be suitable for hydrogen bonding with the SO3 group and the protonation state of the cysteine side chain must be optimal to facilitate cleavage of the bond. Indeed, our models implicated unfavorable side chain positioning in the hydrophobic pocket in the lack of activity we observed for *L. johnsonii* BHSA, but we were unable to identify differences in critical hydrogen bond interactions at the amino end among the active enzymes (Table 3.2). Although both enzymes appear capable of forming critical hydrogen bonds between $Cys_{2,}$ Asp_{19}, and Asn_{79} residues and taurine, NCK88 BSHC did not exhibit activity against any tauro-conjugated bile acids in our assay, so it is likely that differences in the configuration of substrate binding pocket of the active site compared to LB1 BSHC are also contributing to its narrower substrate specificity (Table S3.1). Notably, a crystal structure depicting the intact bile acid in the binding pocket of a BSH is not available, and taurine is thought to only partially occupy the space, so our ability to effectively model these interactions is limited (139). Mutated residues Ser/Asn_{108}, Thr/Pro_{122} and Thr/Ala_{289} (residues in NCK88/LB1) lie in a 12 Å sphere of the active site and appear to perturb the substrate binding dynamics in the active site region (Figure S3.3). We suggest that the relative position of the loop structures defining the edge of the substrate binding pocket and their inherent flexibility may contribute to to differences in substrate specificity, as an increased range of loop movement in NCK88 BSHC hinders substrate binding (Figure 3.3B,C). Such long range interactions are quite common in evolutionary related proteins as seen by a statistical coupling analysis of residue networks (211).

Our UPLC-ESI-QTOFMS based screening assay for activity against T-β-MCA clearly and effectively identified two strains of *L. johnsonii* with high potential for metabolic control, and improves on existing BSH substrate specificity screening methods such as the costly and subjective agar plate precipitation assay or an assay based on incubation in extracted mouse bile

(4, 7). Importantly, our assay utilizes only 500 nM of T-β-MCA, which allows for cost effective, high volume screening of potential probiotics without concerns about growth inhibition. Admittedly, quantification of specific enzymatic activity is not possible with our experimental design, as we aimed to identify strains of *Lactobacillus* with clear potential to alter T-β-MCA concentrations *in vivo*. We were careful to consider the potential for inducible BSH expression in our screen, but it is still possible that factors such as growth state, bile acid transport, and culture conditions affect apparent BSH activity and have caused us to overlook strains with low levels of T-β-MCA activity. However, we contend that robust activity will be necessary to achieve metabolic control in the harsh and competitive environment of the intestine.

This work was the first to directly characterize T-β-MCA substrate specificity in probiotic strains of *Lactobacillus* using a clear and cost effective screening assay, and our results indicate that activity against T-β-MCA cannot be easily inferred from previously characterized activity against common tauro-conjugated bile acids. Our data suggests that differences in BSH substrate specificity may manifest even among highly homologous BSHs. Ultimately, these results lay the groundwork for future *in vivo* experiments with *L. johnsonii* LB1 and NCK88 aimed at exploiting FXR mediated metabolic signaling by altering intestinal T-β-MCA concentrations.

3.4 Materials and Methods

3.4.1 Bacterial strains and growth conditions

L. johnsonii NCK88, *L. acidophilus* NCFM and *L. plantarum* WCFS1 were generously provided by Dr. Todd Klaenhammer, *L. johnsonii* LB1 was isolated directly from mouse cecal contents, and all other *Lactobacillus* strains were purchased from the American Type Culture Collection (ATCC). Cultures were inoculated in sterile MRS broth from 10% frozen glycerol stocks and incubated anaerobically in an atmosphere composed of 85% N, 10% CO_2, and 5% H_2 at 37°C for two consecutive passages. *E. coli* C600 and *E. coli* DH5α were inoculated from 10%

frozen glycerol stocks in sterile Lysogeny Broth (LB) and incubated aerobically at 37°C with 300 RPM shaking agitation. Transgenic *E. coli* C600 strains harboring pSF-OXB12:BSH plasmids were inoculated in sterile LB supplemented with 50 μg/mL kanamycin and incubated at 37°C with 300 RPM shaking agitation for two consecutive passages.

3.4.2 Enumeration and isolation of *Lactobacillus*

Cecal contents were harvested from a healthy 6 week old male C57BL/6J mouse and diluted in sterile PBS, pH 7.4. Dilutions were plated in duplicate on *Lactobacillus* selection (BBL LBS) agar (BD Difco) and incubated anaerobically for 48 hours at 37°C for colony counting (212). Twenty individual colonies from the highest dilution plates were randomly selected and streaked onto MRS agar for additional purification and designated as strains LB1-LB20 (213). Isolated colonies were inoculated in MRS broth, incubated anaerobically at 37°C overnight, and stored for future work at -80°C in 10% glycerol.

3.4.3 Identification and characterization of *Lactobacillus*

Isolated colonies were scraped into a PCR tube and microwaved for 5 mins to rupture the cells. PCR was performed using primers specific to *Lactobacillus* 16s rRNA using conditions described by Byun *et al*. (203). Reaction products were visualized on a 2.0% agarose gel with SYBR-SAFE dye (Applied Biosystems) and treated with 10 units of exonuclease I (New England Biolabs), and 1 unit of antarctic phosphatase (New England Biolabs) at 37°C for 45 mins to remove unincorporated primers and dNTPs, then at 85°C for 15 mins to inactivate the enzymes. Purified PCR amplicons were sequenced by the Penn State Genomics Core Facility. Individual sequences were assembled using the DNASTAR Lasergene 12 software suite (DNASTAR Inc.) and identified using NCBI's BLAST database (214). Each isolate was also characterized phenotypically by inoculation in sterile MRS broth and anaerobic incubation at 37°C for 24 hours as OD_{600} and media pH was monitored every four hours.

3.4.4 DNA sequencing

L. johnsonii strains LB1, which was isolated from mouse cecal contents, and NCK88, which was generously provided by Dr. Todd Klaenhammer, were each inoculated from frozen glycerol stocks in sterile MRS and incubated anaerobically at 37°C overnight. Cells were precipitated by centrifugation and genomic DNA was extracted using Promega's Wizard DNA kit. Whole genome sequencing was performed by the Penn State Genomics Core Facility using an illumina MiSeq instrument with 300 bp paired end reads. Sequencing data was assembled *de novo* with DNASTAR's SeqMan NGen (DNASTAR Inc.), and contigs were mapped against the reference *L. johnsonii* NCC533 genome with progressiveMauve (215, 216). Genomes were compared with WebACT and dotplots of translated open reading frames were generated with PROmer in Galaxy (217–221). Sequence data is available under BioProject ID PRJNA315676.

3.4.5 BSH sequence comparisons

BSH sequences from each strain in our *Lactobacillus* collection with publically available genomes were identified using NCBI's Gene database. The fully sequenced and annotated strain *L. johnsonii* NCC533 was used as a reference to identify *bsh* genes from *L. johnsonii* LB1 and *L. johnsonii* NCK88 contigs using BLASTp (60). All putative translated open reading frames were also screened to ensure no additional *bsh* genes were present in either LB1 or NCK88. Sequences were translated and aligned by ClustalW with MEGA6 (222). Maximum Likelihood phylogenic reconstruction of the alignment was performed by bootstraping with 100 replications based on the Le Gascuel 2008 model of amino acid substitution (223). The amino acid substitution model was selected based on the lowest Bayesian Information Criterion (BIC) score of maximum likelihood fit from 56 different amino acid substitution models. Active site amino acids were identified with NCBI's Conserved Domains Database search tool (224).

3.4.6 BSH cloning

BSH genes identified for sequence comparisons (see previous section) in the genomes of *L. johnsonii* LB1, *L. johnsonii* NCK88, *L. johnsonii* ATCC33200, and *L. acidophilus* NCFM

were amplified using primers containing the *Nco*I restriction recognition sequence and targeting the beginning and end of the coding sequences (Table S2). PCR amplicons were cloned into pSF-OXB12 (Oxford Genetics) using high efficiency DH5α competent cells (New England Biolabs) and proper insert orientation was confirmed by DNA sequencing. The *NcoI* cloning site positions the ORF downstream of a Shine-Delgarno site encoded by pSF-OXB12, and changes the second codon and active site residue from "TGT" to "GTG". The original ORF sequences were restored using New England Biolabs' site directed mutagenesis kit, and the final constructs were confirmed by DNA sequencing and transformed into *E. coli* C600.

3.4.7 BSH activity assay

For whole cell assays, cells were centrifuged and washed twice with sterile PBS, pH 7.4, then resuspended in fresh media. T-β-MCA (Steraloids) was added to a concentration 500 nM, and cultures were incubated at 37°C for 90 mins. Cells were precipitated by centrifugation and 100 μL of supernatant was added directly to 100 μL of ice cold methanol and stored at -80°C for analysis by UPLC-ESI-QTOFMS. Cell lysate assays were used to assess BSH substrate specificity in transgenic *E. coli* C600 strains and were performed by washing overnight cultures twice in sterile PBS, pH 7.4, and resuspending the cells in 3 mM sodium acetate buffer, pH 5.2. Cells were lysed by vortexing with 0.1 mm glass beads (Mo Bio Laboratories, Inc.) according to the manufacturer's instructions. Total protein was measured by Bradford assay and normalized to 0.100 mg/mL in 100 μL of buffer before adding a concentrated master solution of T-β-MCA, TCA, TDCA, TCDCA, GCA, and GCDCA (Sigma) to achieve a final concentration of 500 nM. Cell lysates were incubated at 37°C for 90 mins, quenched with 100 μL of ice cold methanol and stored at -80°C for analysis by UPLC-ESI-QTOFMS.

3.4.8 UPLC-ESI-QTOFMS analysis

UPLC-ESI-QTOFMS analysis was performed in positive and negative mode with a G2S QTOFMS (Waters Corp), which was operated in full-scan mode at m/z 100–1,000. The liquid

chromatography system was an ACQUITY UPLC (Waters Corp.) consisting of a reverse-phase 2.1x-50 mm ACQUITY UPLC BEH C18 1.7 μm column (Waters Corp.) with a gradient mobile phase comprising 0.1% formic-acid solution (A) and acetonitrile containing 0.1% formic acid solution (B). The gradient was maintained at 100% A for 0.5min, increased to 100% B over the next 7.5 min and returned to 100% A in last 2 min. Nitrogen was used as both cone gas (50 l h^{-1}) and desolvation gas (600 l h^{-1}). Source temperature and desolvation temperature were set at 120°C and 350°C, respectively. The capillary voltage and cone voltage were 3,000 and 20 V, respectively. Quantification of bile-acid composition was determined by comparison against bile acid standards, and positive activity was defined as a 20% reduction in substrate concentration over the course of the assay. Positive activity was confirmed by at least one additional independent repeat experiment. Data was analyzed with Peakview™ software version 1.1.0.0 (AB SCIEX).

3.4.9 Predictive modeling

BSH protein structures were predicted from amino acid sequences using I-TASSER. PDB coordinate files for each conjugated bile acid substrate were generated from Isomeric SMILES in the PubChem database using the National Cancer Institute's Online SMILES Translator and Structure File Generator. Bile acid and BSH PDB files were merged using the crystal structure of the *C. perfringens* BSH bound to TDCA as a guide in Coot (139, 225). Finally, each predicted BSH/bile acid complex was energetically minimized using the YASARA Energy Minimization Server (226). Distance measurements for hydrogen bonding and hydrophobic interactions as well as figures for publication were generated with PyMOL (Schrödinger). CABS-flex was used to simulate protein structure fluctuations and assess the range of motion of loop structures within BSH protein structures (204).

Table S3.1: Distance measurements between active site residues and T-β-MCA in models of *L. johnsonii* BSHs

NCK88 BSHB	β-MCA	Distance (Å)	LJ LB1 BSHB	β-MCA	Distance (Å)	NCK88 BSHC	β-MCA	Distance (Å)	LJ LB1 BSHC	β-MCA	Distance (Å)
Van der Waals Interactions (max 4.0A)											
C20LEU	C24	3.40	C58ILE	C11	3.65	C58ALA	C19	3.68	C24TYR	C2	3.78
C26TYR	C2	3.87	C58ILE	C12	3.93	C136THR	C15	3.78	C63LEU	C18	3.63
C136PHE	C3	3.65	C67PHE	C21	3.62	C65CYS	C21	3.52	C63LEU	C18	3.66
C136PHE	C3	3.98	C67PHE	C21	3.66	C65CYS	C21	3.54	C65CYS	C20	3.63
C136PHE	C4	3.86	C136PHE	C3	3.59	C134PRO	C4	3.83	C65CYS	C21	3.67
C136PHE	C4	3.99	C136PHE	C4	3.85	C136THR	C16	3.97	C65CYS	C21	3.6
C67PHE	C18	3.87	C136PHE	C5	3.89	C138LEU	C16	3.92	C65CYS	C22	3.9
C67PHE	C20	3.97	C139ALA	C14	3.91	C63LEU	C18	3.81	C136THR	C16	3.87
C67PHE	C20	3.90	C139ALA	C15	3.92	C63LEU	C19	3.96	C136THR	C15	3.8
C67PHE	C12	3.66	C66TYR	C21	3.92	C63LEU	C18	3.65	C102LEU	C21	3.87
C67PHE	C12	3.69	C141LEU	C23	3.91				C20TYR	N26	3.65
C67PHE	C21	3.72	C60VAL	C19	3.90				C133LEU	C4	3.98
C141LEU	C23	3.92							C138LEU	C16	3.86
									C56VAL	C11	4.00
									C58ALA	C19	3.80
NCK88 BSHB	**Taurine**	**Distance (Å)**	**LJ LB1 BSHB**	**Taurine**	**Distance (Å)**	**NCK88 BSHC**	**Taurine**	**Distance (Å)**	**LJ LB1 BSHC**	**Taurine**	**Distance (Å)**
H-Bonding (max 3.4A)											
O21ASN	O5	3.12	OH26TYR	N1	3.10	O19ASN	S4	3.18	O19ASN	O5	2.82
N21ASN	O5	2.94	N21ASP	O5	2.95	O19ASN	O5	2.85	S2CYS	O5	3.12
S2CYS	O6	3.09	N18ARG	O5	3.13	N19ASN	O5	3.17	N79ASN	O7	3.13
N81ASN	O6	3.05	S2CYS	O6	3.30	S2CYS	O5	2.91			
			N81ASN	O7	3.08	N79ASN	O6	3.03			
						S2CYS	O6	3.12			
						N2CYS	O7	3.17			

Table S3.2: Primer sequences for BSH cloning, site directed mutagenesis, and sequencing

Primers for BSH cloning into pSF-OXB12	
Primer Name/Target	**Sequence (5'-3')***
LB1 BSHA FWD	ATATACTCGAGATGTGTACCTCAATTGTTTATAGT
LB1 BSHA REV	ATATACTCGAGTGTTGAACAGGAAGAAGCTTTA
LB1 BSHB FWD	ATATACTCGAGATGTGTACTGGTTTAAGATTCACAG
LB1 BSHB REV	ATATACTCGAGATCGAGCGGTTGACGGTATT
LB1 BSHC FWD	ATATACTCGAGATGTGTACATCAATTTTATATAGTCCA
LB1 BSHC REV	ATATACTCGAGTGATCAAATCATGTCCAGTA
NCK88 BSHB FWD	ATATACTCGAGATGTGTACTGGTTTAAGATTCACAG
NCK88 BSHB REV	ATATACTCGAGCCGGTATTGCGATATCAGGCT
NCK88 BSHC FWD	ATATACTCGAGATGTGTACATCAATTTTATATAGTCCA
NCK88 BSHC REV	ATATACTCGAGGAATGATCAAATCATGTCCAGT
ATCC 33200 BSHA FWD	ATATACTCGAGATGTGTACCTCAATTGTTTAT
ATCC 33200 BSHA REV	ATATACTCGAGCAGATATCTTCTATCTGGCAA
Primers for Site Directed Mutagenesis	
Primer Name/Target	**Sequence (5'-3')**
SDM pSF-OXB12 (used for all constructs)	GGTGGGTACCTCCTTTGA
SDM LB1 BSHA	ATGTGTACCTCAATTGTTTATAGTTC
SDM LB1 BSHB	ATGTGTACTGGTTTAAGATTCAC
SDM LB1 BSHC	ATGTGTACATCAATTTTATATAGTCC
SDM NCK88 BSHB	ATGTGTACTGGTTTAAGATTCAC
SDM NCK88 BSHC	ATGTGTACATCAATTTTATATAGTCC
SDM ATCC 33200 BSHA	ATGTGTACCTCAATTGTTTATAGTTC
Sequencing primers	
Primer Name/Target	**Sequence (5'-3')**
pSF-OXB12 FWD	GATCTTTGTCGATCCTACCATCC
pSF-OXB12 REV	CGCTGTATCTCAGTCAGTCAAG

*The *XhoI* site is underlined.

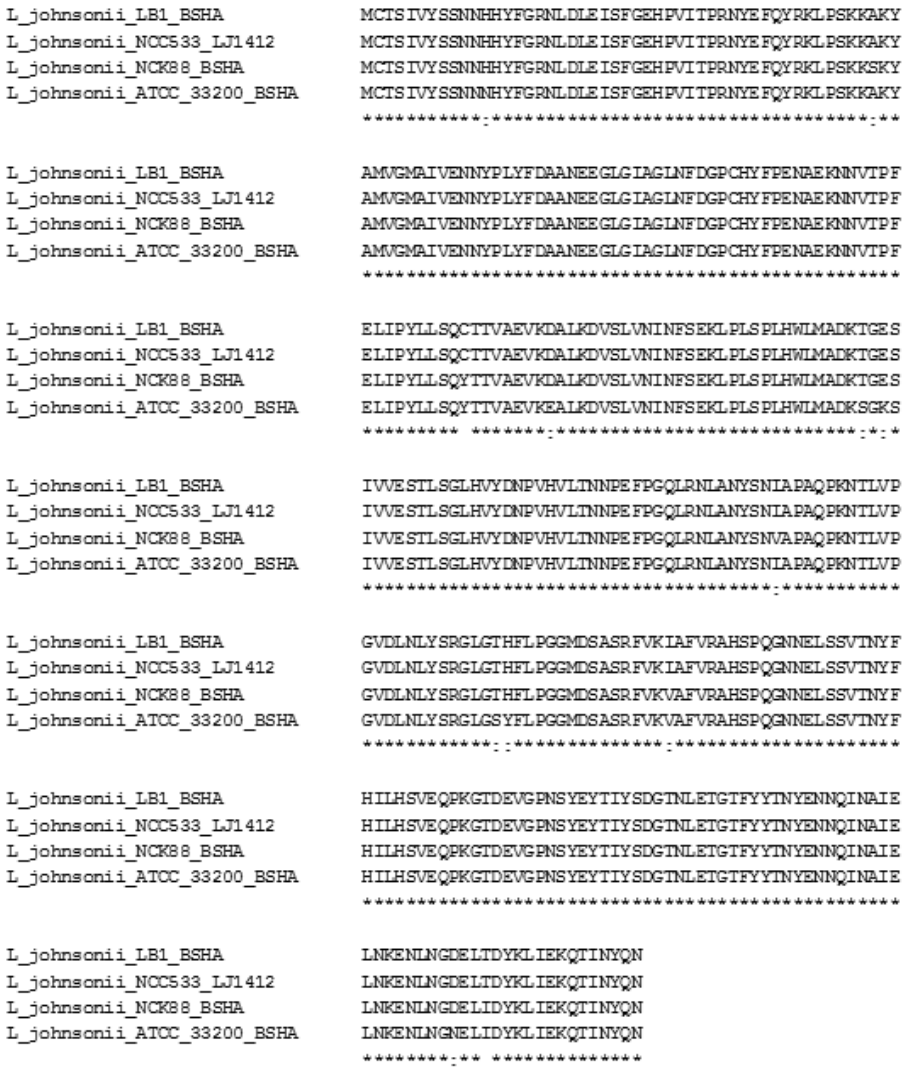

Figure S3.1 ClustalW multiple sequence alignment of BSHA homologs from *L. johnsonii* strains LB1, NCC533, NCK88, and ATCC 33200. Conservation of residues among the four strains is indicated with an asterisk (*), and conservation among three strains is indicated with a colon (:).

```
L_johnsonii_LB1_BSHB          MCTGLRFTDDQGNLYFGRNLDVGQDYGEGVIITPRNYPLPYKFLDNTTTK
L_johnsonii_NCC533_LJ0056     MCTGLRFTDDQGNLYFGRNLDVGQDYGEGVIITPRNYPLPYKFLDNTTTK
L_johnsonii_NCK88_BSHB        MCTGLRFTDNQGNLYFGRNLDVGQDYGEGVIITPRNYPLPYKFLDNTTTK
                              *********:****************************************

L_johnsonii_LB1_BSHB          KAVIGMGIVVDGYPSYFDCYNEDGLGIAGLNFPHFAKFSDGPIDGKINLA
L_johnsonii_NCC533_LJ0056     KAVIGMGIVVDGYPSYFDCYNEDGLGIAGLNFPHFAKFSDGPIDGKINLA
L_johnsonii_NCK88_BSHB        KAVIGMGIVVDGYPSYFDCYNEDGLGIAGLNFPHFAKFSDGPIDGKINLA
                              **************************************************

L_johnsonii_LB1_BSHB          SYEIMLWVTQNFTHVSEVKEALKNVNLVNEAINTSFAVAPLHWIISDSDE
L_johnsonii_NCC533_LJ0056     SYEIMLWVTQNFTHVSEVKEALKNVNLVNEAINTSFAVAPLHWIISDSDE
L_johnsonii_NCK88_BSHB        SYEIMLWVTQNFTHVSEVKEALKNVNLVNEAINTSFAVAPLHWIISDSDE
                              **************************************************

L_johnsonii_LB1_BSHB          AIIVEVSKQYGMKVFDDKVGVLTNSPDFNWHLTNLGNYTGLNPHDATAQS
L_johnsonii_NCC533_LJ0056     AIIVEVSKQYGMKVFDDKVGVLTNSPDFNWHLTNLGNYTGLNPHDATAQS
L_johnsonii_NCK88_BSHB        AIIVEVSKQYGMKVFDDKVGVLTNSPDFNWHLTNLGNYTGLNPHDATAQS
                              **************************************************

L_johnsonii_LB1_BSHB          WNGQKVAPWGVGTGSLGLPGDSIPADRFVKAAYLNVNYPTAKGEKANVAK
L_johnsonii_NCC533_LJ0056     WNGQKVAPWGVGTGSLGLPGDSIPADRFVKAAYLNVNYPTAKGEKANVAK
L_johnsonii_NCK88_BSHB        WNGQKVAPWGVGTGSLGLPGDSIPADRFVKAAYLNVNYPTATGEKANVAK
                              ****************************************:*********

L_johnsonii_LB1_BSHB          FFNILKSVAMIKGSVVNDQGKDEYTVYTACYSSKSKTYYCNFEDDFELKT
L_johnsonii_NCC533_LJ0056     FFNILKSVAMIKGSVVNDQGKDEYTVYTACYSSGSKTYYCNFEDDFELKT
L_johnsonii_NCK88_BSHB        FFNILKSVAMIKGSVVNDQGSDEYTVYTACYSSGSKTYYCNFEDDFELKT
                              ********************:************:*****************

L_johnsonii_LB1_BSHB          YKLDDHTMNSTSLVTY
L_johnsonii_NCC533_LJ0056     YKLDDHTMNSTSLVTY
L_johnsonii_NCK88_BSHB        YKLDDHTMNSTSLVTY
                              ****************
```

Figure S3.2 ClustalW multiple sequence alignment of BSHB homologs from *L. johnsonii* strains LB1, NCC533, and NCK88. Conservation of residues among the three strains is indicated with an asterisk (*) and conservation among two strains is indicated with a colon (:).

```
L_johnsonii_LB1_BSHC          MCTSILYSPKDNYFGRNLDYEIAYGQKVVITPRNYQLNYRHLPTQDTHYA
L_johnsonii_NCC533_LJ1147     MCTSILYSPKDNYFGRNLDYEIAYGQKVVITPRNYQLNYRHLPTQDTHYA
L_johnsonii_NCK88_BSHC        MCTSILYSPKDNYFGRNLDYEIAYGQKVVITPRNYQLNYRHLPTQDTHYA
                              **************************************************

L_johnsonii_LB1_BSHC          MIGVSVVANDYPLYCDAINEKGLGIAGLNFTGPGKYFAVDESKKNVTSFE
L_johnsonii_NCC533_LJ1147     MIGVSVVANDYPLYCDAINEKGLGIAGLNFTGPGKYFSVDESKKNVTSFE
L_johnsonii_NCK88_BSHC        MIGVSVVANDYPLYCDAINEKGLGIAGLNFTGPGKYFAVDESKKNVTSFE
                              ************************************:*************

L_johnsonii_LB1_BSHC          LIPYLLSSCETIEDVKKLLSETNITDESFSKDLPVTTLHWLMGDKSGKSI
L_johnsonii_NCC533_LJ1147     LIPYLLSNCETIEDVKKLLSETNITDESFSKDLPVTTLHWLMGDKSGKSI
L_johnsonii_NCK88_BSHC        LIPYLLSNCETIEDVKKLLSEPNITDESFSKDLPVTTLHWLMGDKSGKSI
                              *******:*************:****************************

L_johnsonii_LB1_BSHC          VIESTETGLHVYDNPVNTLTNNPVFPAQVETLANFASVSPAQPKNTLVPN
L_johnsonii_NCC533_LJ1147     VIESTETGLHVYDNPVNTLTNNPVFPAQVETLANFASVSPAQPKNTLVPN
L_johnsonii_NCK88_BSHC        VIESTETGLHVYDNPVNTLTNNPVFPAQVETLANFASVSPAQPKNTLVPN
                              **************************************************

L_johnsonii_LB1_BSHC          ADINLYSRGLGTHHLPGGTDSNSRFIKASFVLAHSPKGNDEVENVTNFFH
L_johnsonii_NCC533_LJ1147     ADINLYSRGLGTHHLPGGTDSNSRFIKASFVLAHSPKGNDEVENVTNFFH
L_johnsonii_NCK88_BSHC        ADINLYSRGLGTHHLPGGTDSNSRFIKASFVLAHSPEGNDEVENVTNFFH
                              ************************************:*************

L_johnsonii_LB1_BSHC          ILHSVEQAKGTDEVEDNVFEFTMYSDCMNLDKGILYFTTYDNNQINAVDM
L_johnsonii_NCC533_LJ1147     VLHSVEQAKGTDEVEDNVFEFTMYSDCMNLDKGILYFTTYDNNQINAVDM
L_johnsonii_NCK88_BSHC        ILHSVEQAKGADEVEDNVFEFTMYSDCMNLDKGILYFTAYDNNQINAVDM
                              :*********:****************************:************

L_johnsonii_LB1_BSHC          NNENLDTSDLITYELFKDQAIKFEN
L_johnsonii_NCC533_LJ1147     NNEDLGTSDLITYELFKDQAIKFEN
L_johnsonii_NCK88_BSHC        NNEDLDTSDLITYELFKDQAIKFEN
                              ***:*:*******************
```

Figure S3.3 ClustalW multiple sequence alignment of BSHC homologs from *L. johnsonii* strains LB1, NCC533, and NCK88. Conservation of residues among the three strains is indicated with an asterisk (*) and conservation among two strains is indicated with a colon (:).

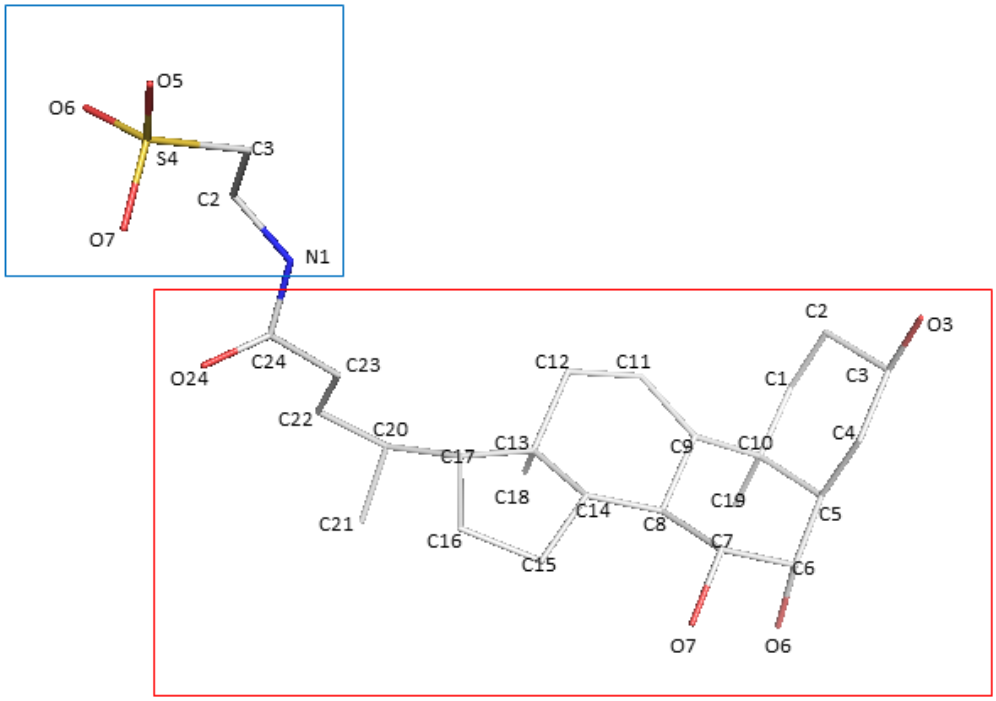

Figure S3.4 Labeling designations for T-β-MCA

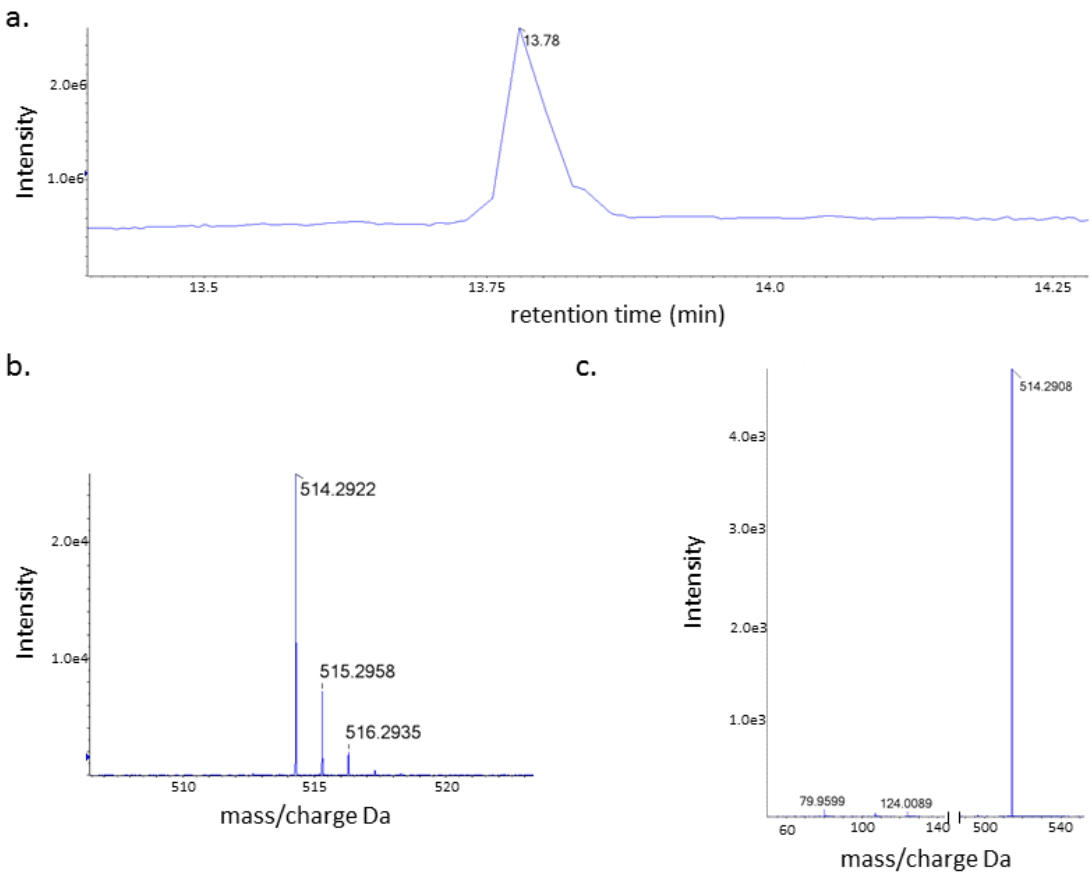

Figure S3.5 UPLC-ESI-QTOFMS identification of T-β-MCA. A) The extracted ion chromatograph at m/z 514.2844 +/- 0.0025 Da with the corresponding retention time for T-β-MCA. B) MS peaks for 13.755 to 13.831 mins. C) MS-MS peaks for 13.765 mins. Images were generated with Peakview™ software version 1.1.0.0 (AB SCIEX).

Chapter 4
Effects of bile salt hydrolase producing bacteria on bile acid composition, FXR signaling, and weight gain *in vivo*

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4.1 Introduction

The gut microbiota is a vast microbial community consisting of nearly 100 trillion bacteria representing hundreds to thousands of species (9). Compositional changes in this ecosystem are a causative factor in obesity, and are characterized by increases in the relative population of Firmicutes to Bacteriodetes (10, 11). *Lactobacillus,* members of the Firmicutes phylum, have been particularly associated with weight gain in humans and animals as part of broader shifts in microbiota composition (13, 14, 24, 29). Indeed, dietary supplementation with a number of *Lactobacillus* probiotics has been linked to weight changes in humans and animals (5).

Recent evidence suggests that bacterial modification of bile acids affects host lipid and glucose metabolism via the FXR signaling pathway (2–4). FXR is a transcription factor and nuclear receptor which regulates the expression of a network of genes in ileal enterocytes and hepatocytes involved in bile acid circulation and synthesis (105). Bile acids are conjugated to either taurine or glycine in the liver prior to secretion into the small intestine, where microbial BSHs cleave the peptide bond and reduce recirculation of the resultant free bile acids. The bile acid T-β-MCA has been shown to represent a critical target for mediating FXR signaling, as it is an FXR antagonist (2, 3, 123).

Lactobacillus species are widely associated with intestinal BSH activity, and Li *et al.* recently linked decreased *Lactobacillus* populations to weight loss in mice. A strain of *L.*

johnsonii isolated from mouse cecal contents was subsequently shown to exhibit BSH activity against T-β-MCA *in vitro*, but changes in the population of a single *Lactobacillus* strain have yet to be associated with FXR mediated weight change *in vivo* (Chapter 3). The aim of this study was to demonstrate the capacity of a probiotic with BSH activity against T-β-MCA to affect FXR signaling and weight gain in mice. Ultimately, our results reinforce previous studies and suggest that bacterial BSH activity against T-β-MCA affects FXR signaling in the intestine, although we were not able to make a link to metabolic changes in the host. Furthermore, this study demonstrates the challenges associated with studying host bacteria interactions in mouse models and suggests future considerations which may improve experimental design.

4.2 Results

4.2.1 Developing and assessing the efficacy of a delivery system for administration of *L. johnsonii* LB1 to conventional mice

Evidence for BSH activity against T-β-MCA in *L. johnsonii* LB1, a *Lactobacillus* strain autochthonous to the mouse intestine, led us to hypothesize that changes in its intestinal population would influence metabolism by affecting FXR signaling. Li *et al.* showed that reductions in intestinal *Lactobacillus* populations corresponded with increased T-β-MCA concentrations and reduced weight gain in mice (3). Conversely, we aimed to consider if increased LB1 populations would effectively reduce T-β-MCA concentrations, antagonize FXR signaling, and promote weight gain.

A delivery system was developed for administering *L. johnsonii* LB1 to the mice in order to establish and increase its population in the intestine. Cell pellets from *L. johnsonii* LB1 cultures were incorporated into a bacon flavored, edible dough matrix and formed into standardized 100 mg pills. The maximum number of cells possible to incorporate into the pills was determined empirically, and *L. johnsonii* LB1 was found to consistently incorporate and

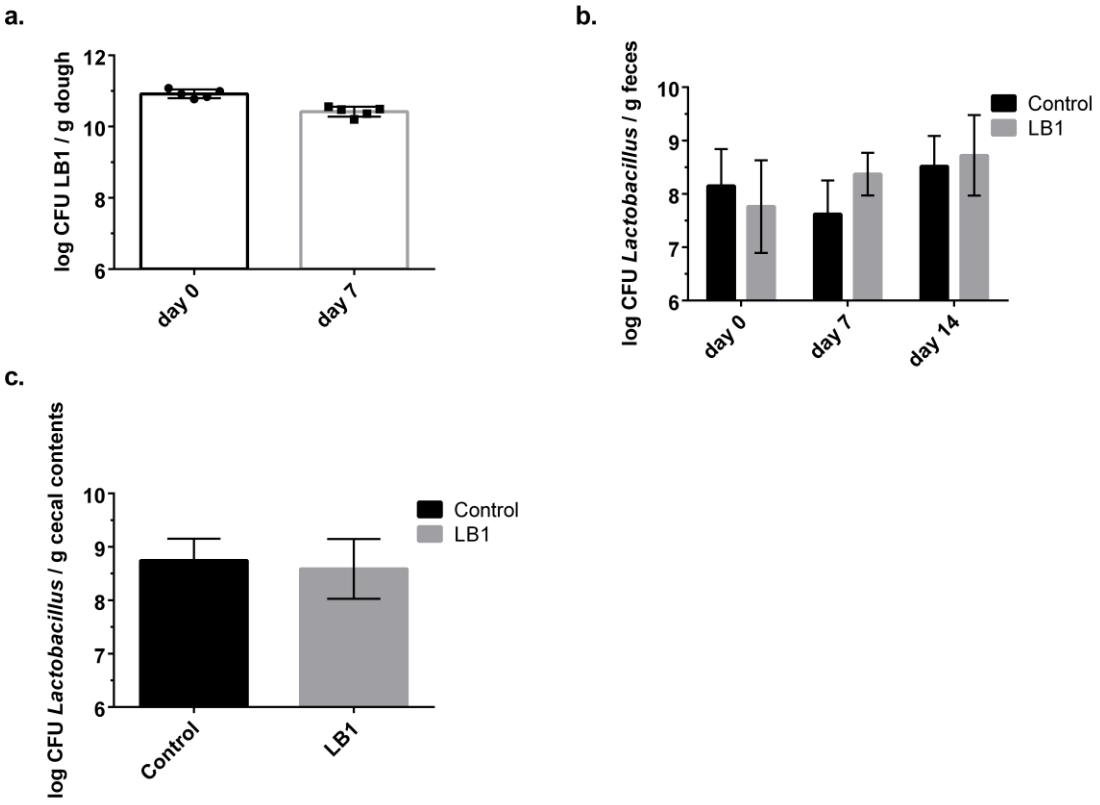

Figure 4.1: Effects of feeding control or *L. johnsonii* LB1 containing dough pills on *Lactobacillus* populations in conventional mice. A) LB1 populations in dough pills throughout the course of storage. B) *Lactobacillus* populations in feces throughout the experiment and (C) in cecal contents of mice administered control or LB1 containing dough pills. All values represent means +/- standard deviations and were compared by multiple student's t-tests corrected for multiple comparisons using the Holm-Sidak method (B) or student's t-test (C). * $p < 0.05$.

survive at a greater than 10 log CFU/g when pills were stored at 4°C for seven days (Figure 4.1A).

Dough pills were fed to mice under supervision once daily for a period of 56 days, and fecal *Lactobacillus* populations remained consistently in the range of 7 to 9 log CFU/g over the first two weeks of the experiment for mice in both the treatment and control groups and did not differ between the groups (Figure 4.1B). Similarly, *Lactobacillus* populations in cecal contents harvested on day 56 were not significantly different among mice receiving treatment or control pills and ranged from 8 to 9 log CFU/g (Figure 4.1C). Strain specific selective markers have not

been developed for *L. johnsonii* LB1, so it is not clear if the treatment altered the relative strain composition within the intestinal *Lactobacillus* community structure. The population of *L. johnsonii* LB1 delivered via the dough pills was at least one to two orders of magnitude higher than the population of *Lactobacillus* in mouse feces or cecal contents, suggesting that exposure to increased numbers of *L. johnsonii* LB1 cells alone is not sufficient to alter intestinal *Lactobacillus* population levels.

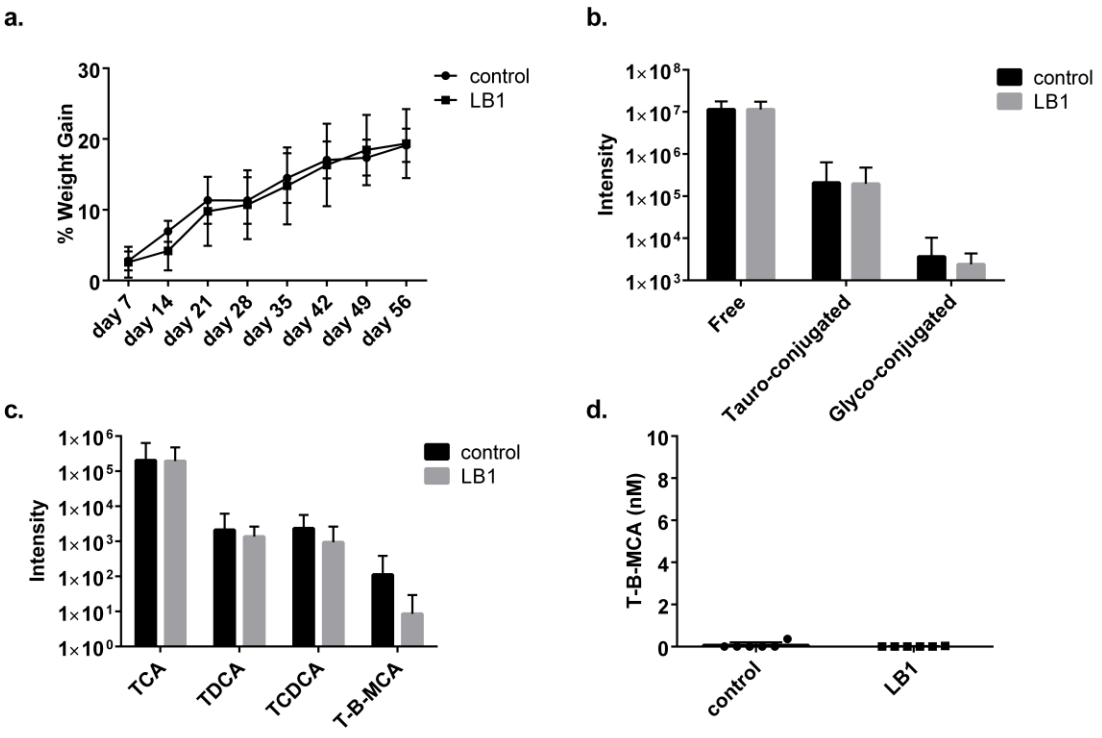

Figure 4.2: Weight gain and bile acid composition in cecal contents of mice treated with control or *L. johnsonii* LB1 (LB1) containing dough pills. A) Weight change relative to day 0. B) Total chromatographic peak intensities of Free (CA, DCA, CDCA), Tauro-conjugated (TCA, TDCA, TCDCA, T-β-MCA), and Glyco-conjugated (GCA, GDCA, GCDCA) bile acids. C) Chromatographic peak intensities of the most abundant Tauro-conjugated bile acids. D) Concentration of T-β-MCA (in nM). All values represent means +/- standard deviations and were compared by multiple student's t-tests corrected for multiple comparisons using the Holm-Sidak method (A, B, and C) or student's t-test (D). * $p < 0.05$.

4.2.2 Effects of *L. johnsonii* LB1 administration on weight gain and bile acid composition of conventional mice

The effect of *L. johnsonii* LB1 administration on metabolism was measured based on the relative weight change in each mouse over the course of the experiment. All of the mice gained nearly 20% of their original body mass over the course of the experiment, an expected outcome for mice aging from 6 to 14 weeks (Figure 4.2A). However, there was no difference in weight gain among mice administered control or *L. johnsonii* LB1 containing dough pills at any time point throughout the experiment.

Li *et al.* described broad changes in the relative levels of tauro-conjugated bile acids and their corresponding free bile acid derivatives coincident with reduced populations of *Lactobacillus* (3). UPLC-ESI-QTOFMS analysis of cecal contents harvested from the mice on day 56 revealed no significant changes in bile acid composition among mice receiving control or *L. johnsonii* LB1 containing dough pills (Figure 4.2B-D). Total chromatographic peak intensities of free bile acids (i.e. CA, DCA, CDCA), tauro-conjugated bile acids (i.e. TCA, TDCA, TCDCA, T-β-MCA), and glyco-conjugated bile acids (i.e. GCA, GDCA, GCDCA) were similar among all of the mice and reflected a predominance of free bile acids with relatively low levels of glyco-conjugated bile acids (Figure 4.2B). Tauro-conjugated bile acids were predominantly TCA, with low levels of TDCA and TCDCA, and near zero concentrations of T-β-MCA (Figure 4.2C, D). These results lead us to conclude that it is not possible to achieve any meaningful further reduction in intestinal T-β-MCA concentrations in conventional mice due to a saturation of BSH activity endogenous to the native gut microbiota.

4.2.3 Assessing colonization of *Lactobacillus* strains in germ free mice

Germ free mice do not harbor a gut microbiota, and consequently offer an alternative model for studying the effect of BSH activity on host bile acid composition without interference from the high levels of BSH activity endogenous to the conventional mouse microbiota.

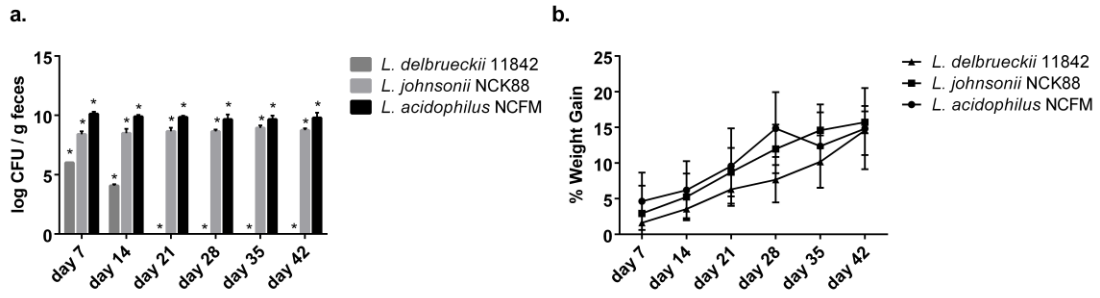

Figure 4.3: Colonization and weight gain in mice monocolonized with *L. delbrueckii* 11842, *L. johnsonii* NCK88, and *L. acidophilus* NCFM. A) Populations of each strain in feces. B) Weight change relative to day 0. All values represent means +/- standard deviations and were compared by two-way ANOVA with Tukey's multiple comparison test. * $p < 0.05$.

Additionally, colonization of bacterial strains in a germ free intestine does not require integration into a complex microbial community, and monitoring the population of a strain in the intestines no longer depends on selective markers. Therefore, a monocolonized germ free mouse model was used to assess the effects of BSH activity differences among *Lactobacillus* strains on the host. *L. delbrueckii* subsp. *bulgaricus* 11482, hereafter referred to simply as *L. delbrueckii* 11842, does not encode any *bsh* genes, and was used as a control for the experiment. Additionally, *L. acidophilus* NCFM, which exhibits BSH activity against a broad range of conjugated bile acid substrates, but no activity against T-β-MCA *in vitro*, and *L. johnsonii* NCK88, which exhibits BSH activity against T-β-MCA *in vitro*, were included (Figure S1, Chapter 3) (2). *L. johnsonii* NCK88 was included instead of *L. johnsonii* LB1 because genetic manipulation is possible in this strain, allowing for the possibility of follow up experiments with *bsh* gene knockouts (206).

All three *Lactobacillus* strains colonized the mice at different population levels and were isolated from fecal samples seven days after inoculation (Figure 4.3A). *L. acidophilus* NCFM colonized at near 10 log CFU/g feces, *L. johnsonii* NCK88 colonized at closer to 9 log CFU/g feces, and *L. delbrueckii* 11842 colonized at 6 log CFU/g feces, reflecting strain specific differences in colonization efficiency (Figure 4.3A). *L. delbrueckii* 11842 was present at only 4

log CFU/g feces after two weeks, and was no longer detectable after three weeks, indicating poor adaptation and survival within the gastrointestinal environment. However, the populations of *L. acidophilus* NCFM and *L. johnsonii* NCK88 remained consistent throughout the course of the experiment. These results suggest that widely characterized strain specific differences in colonization and survival in the intestine still manifest in the absence of the gut microbiota, and emphasize the importance of direct host bacterial interactions (48, 52, 147).

4.2.4 Strain specific effects of *Lactobacillus* colonization on weight gain in germ free mice

All of the mice consistently gained weight throughout the course of the 42 day experiment, and treatment groups showed no differences in relative weight gain at any time point (Figure 4.3B). All of the mice appeared healthy and produced normal stools throughout the course of the experiment, and liver mass relative to body mass was not significantly different among treatment groups on day 42 (data not shown). Interestingly, weight gain in the *L. delbrueckii* 11842 treatment group did not differ from the other treatment groups even after the bacteria had been eliminated from the intestine. This provides evidence that colonization of the intestine with *L. acidophilus* NCFM and *L. johnsonii* NCK88 does not influence energy harvest in a comparable way to colonization with a complex gut microbiota (227).

4.2.5 Strain specific effects of *Lactobacillus* colonization on bile acid composition in germ free mice

L. acidophilus NCFM and *L. johnsonii* NCK88 both exhibit clear BSH activity *in vitro,* and we expected mice colonized with high populations of these strains to have obvious differences in cecal bile acid composition compared to control mice originally colonized with *L. delbrueckii* 11842. However, UPLC-ESI-QTOFMS analysis of cecal contents harvested from the mice on day 42 revealed a surprising extent of similarity among treatment groups (Figure 4.4A-C). The total chromatographic intensity of peak areas associated with free bile acids appears slightly increased in mice colonized with *L. johnsonii* NCK88 compared with mice colonized

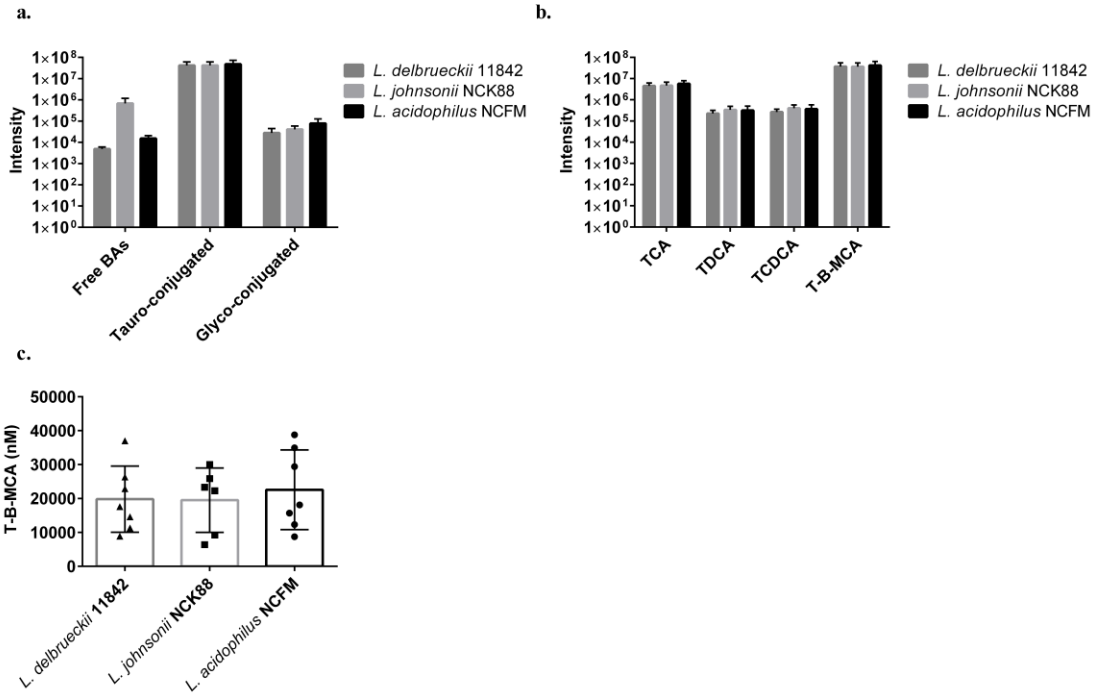

Figure 4.4: Bile acid composition in cecal contents of mice monocolonized with *L. delbrueckii* 11842, *L. johnsonii* NCK88, and *L. acidophilus* NCFM. A) Total intensities of Free (CA, DCA, and CDCA), Tauro-conjugated (TCA, TDCA, TCDCA, T-β-MCA), and Glyco-conjugated (GCA, GDCA, and GCDCA) bile acids. B) Individual intensities of Tauro-conjugated bile acids. C) Concentration of T-β-MCA (in nM). All values represent means +/- standard deviations and were compared by two-way ANOVA with Tukey's multiple comparison test. * $p < 0.05$.

with *L. delbrueckii* 11842 and *L. acidophilus* NCFM, but the difference was not statistically significant (Figure 4.4A). Intensities of TCA, TDCA, TCDCA, and T-β-MCA are remarkably consistent among the treatment groups (Figure 4.4B). T-β-MCA concentrations in all of the mice were clearly much higher than in conventional mice, and ranged from 10 to 30 μM, but did not differ among the treatment groups (Figures 4.2D, 4.4C). This data is not indicative of high levels of BSH activity in the intestines of these mice, and shows that we were not able to effectively alter intestinal T-β-MCA concentrations in our monocolonized germ free mouse model.

4.2.6 Strain specific effects of *Lactobacillus* colonization on FXR signaling in germ free mice

FXR signaling in the enterohepatic system is predominantly influenced by bile acid interactions with FXR as a result of active transport of bile acids into enterocytes in the ileum.

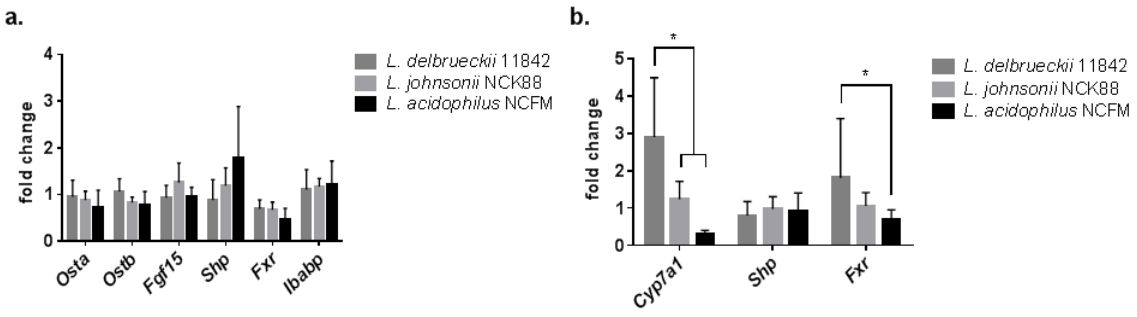

Figure 4.5: FXR signaling in mice monocolonized with *L. delbrueckii* 11842, *L. johnsonii* NCK88, and *L. acidophilus* NCFM. A) Gene expression in ileal tissue and B) liver tissue normalized to *Gapdh*. All values are means +/- standard deviation and were compared by Kruskal-Wallis with Dunn's multiple comparison test. *$p < 0.05$.

No differences were detected in the expression of *Ibabp*, *Ostα,* or *Ostβ,* genes involved in shuttling bile acids across the enterocyte and transporting them into blood vessels, respectively, among treatment groups (Figure 4.5A). Expression of *Fgf15* and *Shp*, genes involved in regulating bile acid synthesis in the liver and linked to carbohydrate and lipid metabolism was more variable, but not significantly different among treatment groups (Figure 4.5A). FXR is a nuclear receptor, and its expression typically remains consistent even as it induces changes in expression of genes under its control. Accordingly, no differences in *Fxr* expression were observed among treatment groups (Figure 4.5A). These results coincide with consistent bile acid compositions among the treatment groups, and are therefore not surprising. FXR mediated control of bile acid synthesis in the liver largely depends on the regulation of *Cyp7a1*, one of the rate limiting enzymes for conversion of cholesterol to bile acids. Expression of *Cyp7a1* in the liver was significantly increased in mice colonized with *L. delbrueckii* 11842 compared to mice colonized with *L. johnsonii* NCK88 and *L. acidophilus* NCFM. Unexpectedly, we also identified a significant difference in *Fxr* expression levels in the liver between mice colonized with *L. delbrueckii* 11842 and *L. acidophilus* NCFM. It is possible that these differences reflect uncharacterized changes in circulating bile acid compositions among the treatment groups, but in

the absence of such data and considering the lack of differences in weight gain among the treatment groups these results are difficult to interpret.

Table 4.1 BSH substrate specificity of transgenic *E. coli* C600 strains expressing *Lactobacillus* BSHs

	T-β-MCA	TCA	TCDCA	TDCA	GCA	GCDCA
control	-	-	-	-	-	-
L. acidophilus NCFM BSHA	-	+	+	+	+	+
L. johnsonii NCK88 BSHB	+	+	+	+	+	+

*Positive activity is defined as a minimum 80% reduction in substrate concentration over the course of the assay.

4.2.7 Assessing colonization of transgenic *E. coli* C600 strains in germ free mice

E. coli is one of the most well characterized species associated with the gastrointestinal tract, and does not naturally express BSHs. Transgenic strains of *E. coli* MG1655 have been utilized to deliver BSH activity to streptomycin treated mice (4). Previously, we constructed and characterized transgenic *E. coli* C600 strains expressing *bsh* genes from *L. johnsonii* NCK88 and *L. acidophilus* NCFM under the control of a constitutive promoter (Table 1, Figure S2) (Chapter 3). These strains represent a highly controlled delivery system for BSHs with different bile acid substrate specificities that is not confounded by background genomic differences or differences in *bsh* gene expression inherent to comparison of *Lactobacillus* strains. Therefore, we colonized germ free mice with transgenic *E. coli* C600 strains expressing *L. johnsonii* NCK88 BSHB, which exhibits activity against the complete range of conjugated bile acids tested in our *in vitro* assay, or *L. acidophilus* NCFM BSHA, which exhibits activity against the complete range of conjugated bile acids tested in our *in vitro* assay except T-β-MCA. A control group was colonized with transgenic *E. coli* C600 lacking any BSH activity.

4.2.8 Strain specific effects of transgenic *E. coli* C600 colonization on weight gain in germ free mice

All three transgenic *E. coli* C600 strains colonized the mice after initial inoculation and were detected in feces at 8 to 9 log CFU/g one week later (Figure 4.6A). The control strain

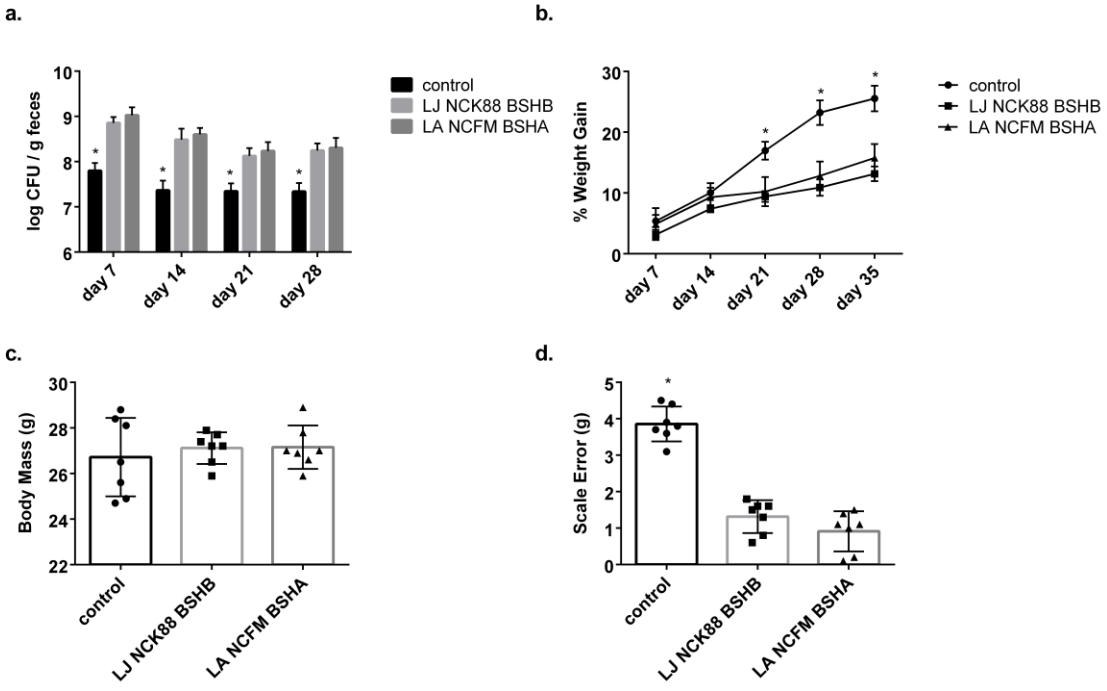

Figure 4.6: Colonization and weight gain in mice monocolonized with transgenic *E. coli* C600 harboring pSF-OXB12 (control), pSF-OXB12:*L. johnsonii* NCK88 BSHB (LJ NCK88 BSHB), or pSF-OXB12:*L. acidophilus* NCFM BSHA (LA NCFM BSHA). A) Populations of each strain in feces. B) Weight change relative to day 0. C) Body mass of mice on day 36. D) Difference in body mass measurements between the spring loaded scale used throughout the experiment and an analytical balance on days 35, and 36, respectively. All values represent means +/- standard deviations and were compared by two-way ANOVA (A and B) or one-way ANOVA (C and D) with Tukey's multiple comparison test. * $p < 0.05$.

lacking BSH activity was consistently present at nearly 1 log CFU/g lower levels than the strains encoding *bsh* genes (Figure 4.6A). Regardless of BSH activity, all of the strains exhibited slight reductions in population over the first two weeks of the experiment, and maintained a stable population level thereafter (Figure 4.6A).

Again, all of the mice gained weight over the course of the study similarly to mice monocolonized with strains of *Lactobacillus* (Figure 4.6B, 4.3B). Mice colonized with the control strain of transgenic *E. coli* C600 showed significantly increased relative weight gain compared to mice colonized with transgenic *E. coli* C600 strains expressing BSHs from *L. johnsonii* NCK88 and *L. acidophilus* NCFM (Figure 4.6B). Differences in weight gain among

the treatment groups clearly manifest by day 21 of the experiment, and continued to diverge until the end of the experiment (Figure 4.6B). However, these differences were not reflected in the body mass of the euthanized mice on day 36 and liver mass relative to body mass was not significantly different among treatment groups (Figure 4.6C). Measuring the weight of germ free mice accurately can be a challenge due to their containment in sterile isolators and a reliance on mechanical, spring based scales. There was no statistical difference in the age or weights of the mice at the start of the experiment, so it was surprising that the differences in relative weight gain did not correspond with differences in the final body masses of the mice determined with a more accurate analytical balance. Therefore, we calculated the differences in weight measurements taken in the isolators and by the analytical balance on the final day of the study to determine the scale error. Indeed, a comparison of the scale error among the treatment groups revealed a strikingly disproportionate error in the control group, explaining the discrepancy in the data (Figure 4.6D). Ultimately, these results suggest that colonization of mice with these transgenic strains of *E. coli* C600 expressing BSHs does not affect weight gain.

4.2.9 Strain specific effects of transgenic *E. coli* C600 colonization on bile acid composition in germ free mice

UPLC-ESI-QTOFMS analysis of cecal bile acid composition in the mice after 36 days of treatment revealed differences in free and tauro-conjugated bile acid levels among the treatment groups (Figure 4.7A). These differences appear to be driven largely by differences in T-β-MCA concentrations, although the pattern did not mirror observed *in vitro* substrate specificities for the transgenic *E. coli* strains (Figure 4.7B, C). Mice colonized with transgenic *E. coli* C600 expressing *L. acidophilus* NCFM BSHA had significantly reduced concentrations of T-β-MCA compared to mice colonized with transgenic *E. coli* C600 expressing *L. johnsonii* NCK88 BSHB, but not compared to mice colonized with the control strain (Figure 4.7C). Coincidentally, mice colonized with transgenic *E. coli* C600 expressing *L. acidophilus* NCFM BSHA also had significantly increased concentrations of MCAs, providing evidence that the differences in T-β-

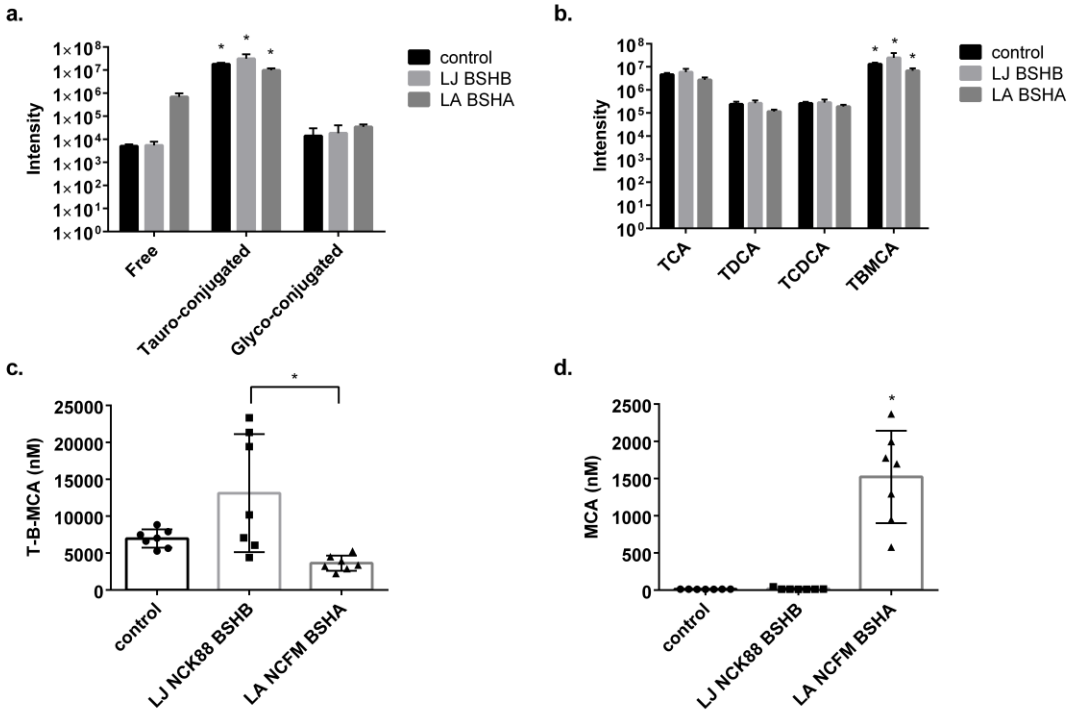

Figure 4.7: Bile acid composition in cecal contents of mice monocolonized with transgenic *E. coli* C600 harboring pSF-OXB12 (control), pSF-OXB12:*L. johnsonii* NCK88 BSHB (LJ NCK88 BSHB), or pSF-OXB12:*L. acidophilus* NCFM BSHA (LA NCFM BSHA). A) Total intensities of Free (CA, DCA, and CDCA), Tauro-conjugated (TCA, TDCA, TCDCA, T-β-MCA), and Glyco-conjugated (GCA, GDCA, and GCDCA) bile acids. B) Individual intensities of Tauro-conjugated bile acids. C) Concentration of T-β-MCA and (D) MCA. All values represent means +/- standard deviations and were compared by two-way ANOVA (A and B) or one-way ANOVA (C and D) with Tukey's multiple comparison test. * $p < 0.05$.

MCA concentrations were likely the result of BSH activity (Figure 4.7D). Notably, despite exhibiting BSH activity towards a broad range of conjugated bile acids, *E. coli* C600 strains expressing *L. johnsonii* NCK88 BSHB and *L. acidophilus* NCFM BSHA did not significantly alter the composition of any other glyco- or tauro-conjugated bile acids relative to the control. This observation, in conjunction with the unexpected BSH activity towards T-β-MCA observed in mice colonized with transgenic *E. coli* C600 expressing *L. acidophilus* NCFM BSHA is perplexing, and casts doubt on the efficacy of our *E. coli* based BSH delivery system for targeted modification of bile acid composition in the upper intestinal tract.

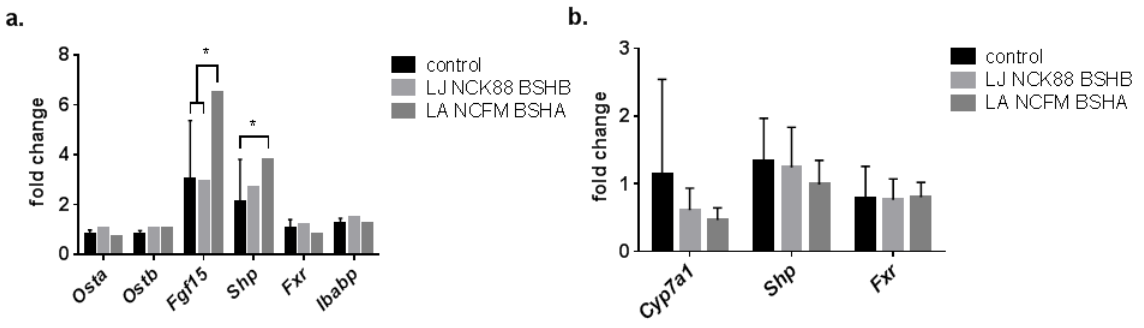

Figure 4.8: FXR signaling in mice monocolonized with transgenic *E. coli* C600 harboring pSF-OXB12 (control), pSF-OXB12:*L. johnsonii* NCK88 BSHB (LJ NCK88 BSHB), or pSF-OXB12:*L. acidophilus* NCFM BSHA (LA NCFM BSHA). A) Gene expression in ileal tissue and B) liver tissue normalized to *Gapdh*. All values are means +/- standard deviation and were compared by Kruskal-Wallis with Dunn's multiple comparison test. $*p < 0.05$.

4.2.10 Strain specific effects of transgenic *E. coli* C600 colonization on FXR signaling in germ free mice

FXR signaling in the ileum of mice treated with transgenic *E. coli* C600 expressing *L. acidophilus* NCFM BSHA is increased compared to control mice and mice treated with transgenic *E. coli* C600 expressing *L. johnsonii* NCK88 BSHB corresponding to reduced concentrations of the FXR antagonist T-β-MCA (Figures 4.8A, 4.7C). In particular, expression of *Fgf15* and *Shp* is elevated approximately 2-fold and 3-fold, respectively, while expression of other FXR signaling markers did not differ among the treatment groups (Figure 4.8A). No changes in FXR signaling were observed in the liver in agreement with the observations of Li *et al.* in mice with altered T-β-MCA concentrations (Figure 4.8B) (3). These results indicate that even relatively modest 10 μM differences in T-β-MCA concentrations affect FXR signaling in the lower small intestine, although these changes did not ultimately result in weight differences in the mice.

4.3 Discussion

The overarching goal of these studies was to definitively link probiotic BSH activity to altered T-β-MCA concentrations, FXR signaling, and weight gain. However, over the course of three different *in vivo* experiments we were unable to elicit a targeted and predictable change in T-β-MCA levels in the mouse intestine. Instead, our results highlight the challenges and limitations associated with studying a multifaceted host-bacterial relationship, and lay the groundwork for eventual success.

Utilization of monocolonized germ free mouse models allowed for improved control over modification of intestinal bile acid composition by eliminating BSH activity endogenous to a complex gut microbiota, which has been shown to affect T-β-MCA concentrations (2, 159–161). Indeed, we found a near complete absence of T-β-MCA in conventional mice without any probiotic intervention (Figure 4.2D). Curiously though, other characterizations of T-β-MCA concentrations in the intestines of conventional mice have detected T-β-MCA, albeit in relatively low concentrations compared to other conjugated bile acids. Li *et al.* and Sayin *et al.* each reported concentrations of approximately 100 μM in cecal contents of conventional mice, and analysis of authentic standards indicated the limit of detection in our system to be 10 nM, suggesting that we should have been able to identify similar concentrations of T-β-MCA if it were present (2, 3). The Li *et al.* study was based on C57BL/6J mice fed a high fat diet, and the Sayin *et al.* study used Swiss Webster mice, suggesting that mouse strain, diet, age, and even housing conditions might affect bile acid composition and present a considerable obstacle to effectively comparing results across studies. Notably, extraction of cecal contents and subsequent preparation for LC-MS analysis is also inherently plagued by variability in moisture content and sample transfer methods. We were able to detect micromolar concentrations of T-β-MCA from cecal contents of germ free mice, confirming the effectiveness of our extraction protocol and

offering a basis for altering baseline T-β-MCA concentrations with BSH active bacteria (Figures 4.4C, 4.7C).

Our *in vitro* assay for assessing BSH activity against T-β-MCA in *Lactobacillus* and transgenic *E. coli* C600 clearly identified differences among strains (Figures S4.1, S4.2). These results were based on standardization of cell density from OD_{600} measurements, and while not as reliable as plating, these measurements indicate that the cultures contained somewhere on the order of 8 log CFU/mL (228, 229). Populations of *Lactobacillus* and transgenic *E. coli* C600 strains in fecal contents of monocolonized germ free mice were of comparable orders of magnitude, raising the question as to why our *in vitro* assay did not effectively predict reductions of T-β-MCA concentrations *in vivo* (Figures 4.3A, 4.4C, 4.6A, 4.7C).

One potential explanation for this discrepancy is that fecal bacterial populations do not effectively predict bacterial populations in other niches of the gastrointestinal tract, and therefore our treatment strains may have poorly colonized the small intestine (9). However, Roager *et al.* reported upwards of 8 log CFU/g of *L. acidophilus* NCFM in the jejunum and cecum of monocolonized germ free mice, providing some reassurance that it can indeed colonize the upper gastrointestinal tract, although their study also characterized only modest changes in bile acid composition (162). Practical limitations related to mouse euthanasia and tissue collection precluded us from addressing this question directly, and in hindsight viable cell counts of each strain throughout the intestinal tract would have provided valuable data. Analysis of fecal bile acid composition would also have been informative if they proved to be significantly different than the bile acid composition in cecal contents.

Joyce *et al.* successfully manipulated plasma bile acid composition in a streptomycin treated mouse model after administering *E. coli* MG1655 expressing a *bsh* gene from *L. salivarius* JCM1046 (4). The changes in bile acid composition generally reflected those observed *in vitro* and corresponded with fecal populations of streptomycin resistant *E. coli* on the order of 6 log CFU/g. Similarly, we were able to elicit a reduction in T-β-MCA concentrations in cecal

contents of germ free mice colonized with transgenic *E. coli* C600 expressing *L. acidophilus* NCFM BSHA (Figure 4.7C, D). However, these results directly contrasted with the results from our *in vitro* assay, which showed no reduction in T-β-MCA concentration in cultures of transgenic *E. coli* C600 expressing *L. acidophilus* NCFM BSHA, and yet complete reduction after incubation with transgenic *E. coli* C600 expressing *L. johnsonii* NCK88 BSHB (Figure S4.2). Moreover, both of these transgenic *E. coli* C600 strains exhibited clear activity against TCA, TDCA, and TCDCA *in vitro* but did not correspondingly reduce concentrations of these conjugated bile acids in mice (Figure S4.2, 4.7B). Plasmids purified from fecal isolates taken from representatives of each treatment group on day 35 confirmed that the plasmids had not been lost, no mutations were present in the *bsh* genes, and the groups were identified correctly (data not shown). Cell surface permeability is known to reduce drastically after bile exposure in *Vibrio cholerae*, and bile has also been shown to induce changes to the cell membrane structures of LAB (230, 231). Thus, it may be that cellular uptake of conjugated bile acids in the harsh environment of the gastrointestinal tract is limited compared with uptake in nutrient rich media such as MRS or LB and account for differences in BSH activity *in vitro* and *in vivo*. Notably, we never characterized the pH optima, stability, and kinetics of these enzymes, so it is also possible that differences in the biochemistry of these enzymes under *in vitro* and *in vivo* conditions underlie the discrepancies in our results.

Expression of BSHs in *E. coli* C600 conferred an approximately 1 log CFU/g advantage in colonization, while no such difference was reported by Joyce *et al.* (Figure 4.6A). BSH activity has been shown to confer a colonization advantage in *Listeria monocytogenes,* providing indirect evidence that these strains were exhibiting BSH activity at some point in the gastrointestinal tract (163). Likewise, *L. delbrueckii* 11842 was present at only 4 log CFU/g feces after two weeks, and was no longer detectable after three weeks, indicating poor adaptation and survival within the gastrointestinal environment. This is consistent with the its niche in dairy fermentations and may also be related to a lack of BSH activity (147).

FXR signaling in our monocolonized germ free mouse models generally coincided with observed changes in bile acid composition. Mice colonized with *L. delbrueckii* 11842 at the start of our *Lactobacillus* study had a bile acid compositions consistent with those reported in germ free mice (2, 162). These mice also were distinguished by increased expression of *Cyp7a1* in the liver, which was also reported by Sayin *et al.* in conjunction with an increased bile acid pool size in germ free mice compared to conventional mice (Figure 4.5B) (2). Mice with reduced levels of T-β-MCA in the BSH active *E. coli* study had elevated expression of *Shp* and *Fgf15* in ileal tissue in agreement with other studies (2, 3). This signaling response was driven by changes in T-β-MCA concentrations of only 10 μM, suggesting that even modest improvements in our BSH delivery system have the potential to alter host metabolism (Figure 4.7C). These results encourage the translation of this work to controlling obesity in humans, where T-β-MCA concentrations tend to be two to five fold lower than in mice based on analysis of circulating bile acids in serum (154, 155).

Reduced T-β-MCA concentrations and increased FXR signaling in mice colonized with transgenic *E. coli* C600 expressing *L. acidophilus* NCFM BSHA did not result in increased weight gain (Figure 4.6C). Effects of FXR signaling on weight gain in the Li *et al.* study were based on mice fed a high fat diet, and together with our results it seems that dietary fat content may exacerbate FXR's effect on weight gain (3). Joyce *et al.* observed weight gain associated with altered bile acid composition in mice fed both high fat diets and conventional diets, but the authors did not explicitly characterize FXR signaling (4). They did however characterize sweeping changes in host gene expression throughout the gastrointestinal tract and liver related to lipid and cholesterol metabolism, immune function, and circadian rhythm. Therefore, it is likely that BSH mediated alterations of bile acid composition elicit additional host responses which might abrogate or exacerbate FXR associated metabolic changes. Our data also serves as a cautionary example of the inherent challenges associated with germ free mouse studies, and in consideration of the potential range of the host response to bile acid modification, we suggest that

additional MRI and NMR based analyses of body composition should be employed to reinforce weight data (232–234).

Although administration of *L. johnsonii* LB1 did not alter bile acid composition or weight in conventional mice, the results of our study raise some interesting ecological questions related to probiotic colonization (Figures 4.1, 4.2). Similar studies administering probiotic strains of *Lactobacillus* to conventional mice have resulted in high levels of colonization and persistence, but none of these strains were autochthonous to the mouse intestine (197, 235, 236). Our study was hampered by our inability to distinguish LB1 from the resident *Lactobacillus* in the mouse, which we have in fact shown to consist predominantly of *L. johnsonii* (Chapter 3). Regardless, we hypothesized that continuous administration of LB1 at higher levels than are present in the intestine would increase the overall population of *Lactobacillus*, and we were surprised to observe no change compared to controls after 56 days (Figure 4.2A). This suggests that *Lactobacillus* populations in the mouse gut microbiota may be at a carrying capacity. Our study was not designed to garner further insight into the ecology of this community, but our results hint at the importance of community composition for establishing probiotics.

Future experiments should focus on improving control of the germ free mouse models in order to effectively manipulate T-β-MCA concentrations in the small intestine. A sensible next step would be to conduct a short term experiment with the complete range of transgenic *E. coli* C600 strains constructed in chapter 3 in order to identify better candidates for manipulating bile acid compositions. For instance, in addition to the transgenic *E. coli* C600 strain expressing *L. johnsonii* NCK88 BSHB, strains expressing *L. johnsonii* LB1 BSHB and *L. johnsonii* NCK88 BSHC also exhibit activity against T-β-MCA (Chapter 3). Additionally, it would be invaluable to acquire *L. salivarius* JCM1046 in order to develop a control strain for comparing results directly to the Joyce *et al.* study. Notably, Joyce *et al.* used a transposon based system to insert a single copy of the *bsh* gene into the *E. coli* genome, while our study expressed *bsh* genes using a constitutive promoter on a high copy number plasmid (4). Theoretically, our transgenic *E. coli*

C600 constructs should express higher levels of BSH activity than those used in the Joyce *et al.* study, but it is possible that this difference in expression has some unforeseen biological consequences. The pSF plasmid system used in our study contains interchangeable promoters which drive various expression levels of protein, so another alternative would be to construct strains containing the same *bsh* gene under the control of different strength promoters in order to test how expression levels affect colonization and bile acid modification *in vivo*. Finally, it is worth considering the possibility that other members of the gut microbiota may be more effective at altering intestinal bile acid composition. We expected to restore a large percentage of the normal BSH activity by colonizing germ free mice with strains of *Lactobacillus*, as it has been suggested to contribute a majority of BSH activity in conventional mouse intestines (237). However, our results suggest that the strains we introduced are not nearly capable of such dramatic transformations. BSH activity in members of *Clostridia* has been shown to target T-β-MCA, and Li *et al.* also identified shifts in *Clostridia* as part of a broad microbial remodeling making it a worthwhile reservoir for future probiotic candidates to control host metabolism (3, 159, 161).

4.4 Materials and Methods

4.4.1 Bacterial strains and growth conditions

L. johnsonii NCK88 and *L. acidophilus* NCFM were generously provided by Dr. Todd Klaenhammer, *L. delbrueckii* 11842 was purchased from the ATCC, and *L. johnsonii* LB1 was isolated directly from mouse cecal contents as described in chapter 3. Prior to preparation of inoculums for mouse experiments, cultures were inoculated in sterile MRS broth from 10% frozen glycerol stocks and incubated anaerobically in an atmosphere composed of 85% N, 10% CO_2, and 5% H_2 at 37°C overnight for two consecutive passages. Transgenic strains of *E. coli* C600 expressing *L. acidophilus* NCFM BSHA and *L. johnsonii* NCK88 BSHB were constructed and screened for BSH activity as described in chapter 3. Cultures of transgenic *E. coli* C600

strains were inoculated from 10% frozen glycerol stocks in sterile LB and incubated aerobically at 37°C with 300 RPM shaking agitation overnight for two consecutive passages prior to preparation of inoculums for mouse experiments.

4.4.2 Animal studies

For conventional mouse studies, 4-week old C57BL/6J wild-type male mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). The mice were housed in polypropylene cages with corncob bedding in a controlled environment (temperature, 65−75 °F; relative humidity, 30%−70%; photoperiod, 12 h light/dark cycle) with water and regular chow supplied ad libitum. Mice were acclimatized for one week, and trained for an additional week to consume 100 mg dough pills (Transgenic Dough Diet – Sterile, Bioserve). For the experiment, dough pills were prepared by mixing either 2 mL of sterile PBS, pH 7.4 (control), or a washed cell pellet from 100 mL of *L. johnsonii* LB1 culture (treatment) into 6 g of dough, smearing the mixture onto a 50 x 100 mg tablet triturate mold (Medisca) with an autoclaved spatula, and ejecting pills into a sterile petri dish. Control pills were prepared with an equivalent volume of sterile PBS in order to replicated moisture consistency and caloric density compared to the treatment. Pills were prepared weekly and stored at 4°C prior to use, and contained > 1.0×10^9 CFU *L. johnsonii* LB1/pill. At the start of the experiment, mice were randomly grouped and administered control or treatment pills once daily in segregated treatment cages under supervision prior to returning to their permanent cages. Fecal samples were collected directly from each mouse and each mouse was weighed on a weekly basis prior to treatment. Mice were euthanized on day 56 and ileum, liver and cecal contents samples were collected immediately following CO_2 asphyxiation. A portion of all fecal and cecal samples were utilized immediately for bacterial enumeration, and all tissue samples were quickly placed in liquid nitrogen and stored at −80 °C until further analysis. For *Lactobacillus* enumeration, samples of fecal pellets and cecal contents were weighed and homogenized in sterile PBS, pH 7.4. Dilutions were plated in duplicate on

BBL LBS agar (BD Difco), and incubated anaerobically for 48 hours at 37°C for colony counting.

Germ-free, male, wild-type C57BL/6J mice were bred and maintained by the Pennsylvania State University Gnotobiotic Facility, housed in germ-free isolators, and fed an autoclaved diet. All materials and supplies were sterilized before transfer into the isolators. Germ-free status was monitored continuously and confirmed through a series of culture-based assays prior to treatment. Mice were five to six weeks old at the start of each monocolonized germ free experiment and were grouped randomly to minimize age differences across treatment groups. Concentrated bacterial cultures containing > 1.0×10^9 CFU/mL (*Lactobacillus*) or > 1.0×10^{10} CFU/mL (transgenic *E. coli* C600) in PBS, pH 7.4 were administered to mice by intragastric gavage at the start of the experiment. Fecal samples were collected directly from each mouse and each mouse was weighed on a weekly basis. Mice were euthanized on day 42 (*Lactobacillus* experiment) or day 35 (transgenic *E. coli* C600 experiment) and duodenum, jejunum, ileum, colon, liver, adipose, urine, blood serum, and cecal contents were collected immediately following CO_2 asphyxiation. A portion of all fecal samples were utilized immediately for bacterial enumeration, and all tissue samples were quickly placed in liquid nitrogen and stored at −80 °C until further analysis. For *Lactobacillus* enumeration, samples of fecal pellets and cecal contents were weighed and homogenized in sterile PBS, pH 7.4. Dilutions were plated in duplicate on MRS agar (BD Difco), and incubated anaerobically for 48 hours at 37°C for colony counting. Transgenic *E. coli* C600 strains were enumerated similarly on LB agar plates containing 50 μg/mL of kanamycin.

All procedures were performed in accordance with the Institute of Laboratory Animal Resources guidelines and approved by the Pennsylvania State University Institutional Animal Care and Use Committee.

4.4.3 qPCR analysis of gene expression

RNA was extracted from frozen tissue samples by homogenizing in TRIzol reagent (Invitrogen) with 1.0 mm silica beads using a tissue homogenizer at 4,000 RPM for two 20 s cycles. Complementary DNA was synthesized from 150 ng of total RNA using qScript™ cDNA SuperMix (Quanta Biosciences). Primers for qPCR were identified using qPrimerDepot and are listed in Table S1. qPCR was carried out using PerfeCTa® SYBR® Green FastMix® in an ABI Prism 7900HT sequence detection system (Applied Biosystems) or BioRad IQ5 system (BioRad). Measured expression of mRNA levels were normalized to those of *Gapdh* and expressed as fold change relative to a representative of the control group.

4.4.4 UPLC-ESI-QTOFMS analysis of bile acid composition

Samples containing 100 mg of cecal contents were resuspended and homogenized in 1 mL of 100% ethanol and incubated at 60 °C for 30 mins, then 100 °C for an additional 3 mins. The samples were centrifuged at 16,000 x g for 10 mins and the supernatants were transferred to low volume plastic autosampler vials (VWR) for analysis. UPLC-ESI-QTOFMS analysis was performed in positive and negative mode with a G2S QTOFMS (Waters Corp), which was operated in full-scan mode at m/z 100–1,000. The liquid chromatography system was an ACQUITY UPLC (Waters Corp.) consisting of a reverse-phase 2.1x-50 mm ACQUITY UPLC BEH C18 1.7 μm column (Waters Corp.) with a gradient mobile phase comprising 0.1% formic-acid solution (A) and acetonitrile containing 0.1% formic acid solution (B). The gradient was maintained at 100% A for 0.5min, increased to 100% B over the next 7.5 min and returned to 100% A in last 2 min. Nitrogen was used as both cone gas (50 l h^{-1}) and desolvation gas (600 l h^{-1}). Source temperature and desolvation temperature were set at 120 °C and 350 °C, respectively. The capillary voltage and cone voltage were 3,000 and 20 V, respectively. Quantification of bile-acid composition was determined by comparison against authentic bile acid standards.

4.4.5 Data analysis

All data represents means +/- standard deviations, and was considered statistically significant at an alpha of 0.05 using GraphPad Prism 6 (GraphPad Software, Inc.). Statistical tests used for data comparison are described in the corresponding figure legends.

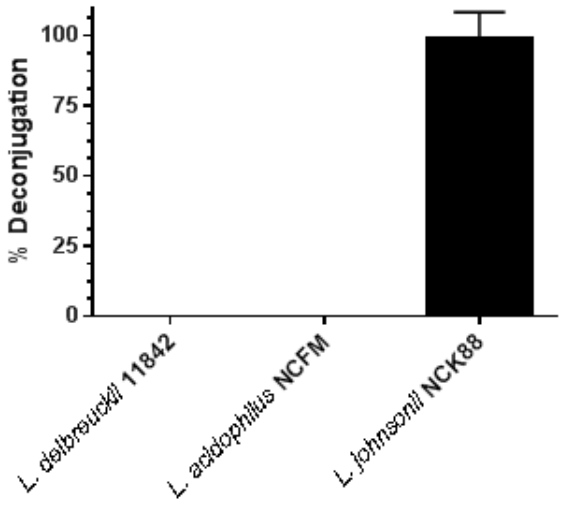

Figure S4.1: *In vitro* BSH activity against T-β-MCA in *L. delbrueckii* 11842, *L. acidophilus* NCFM, and *L. johnsonii* NCK88. Results are based on three replicates with cultures standardized to an OD_{600} of 1.0 in MRS and incubated anaerobically at 37°C with 500 nM of T-β-MCA for 90 mins.

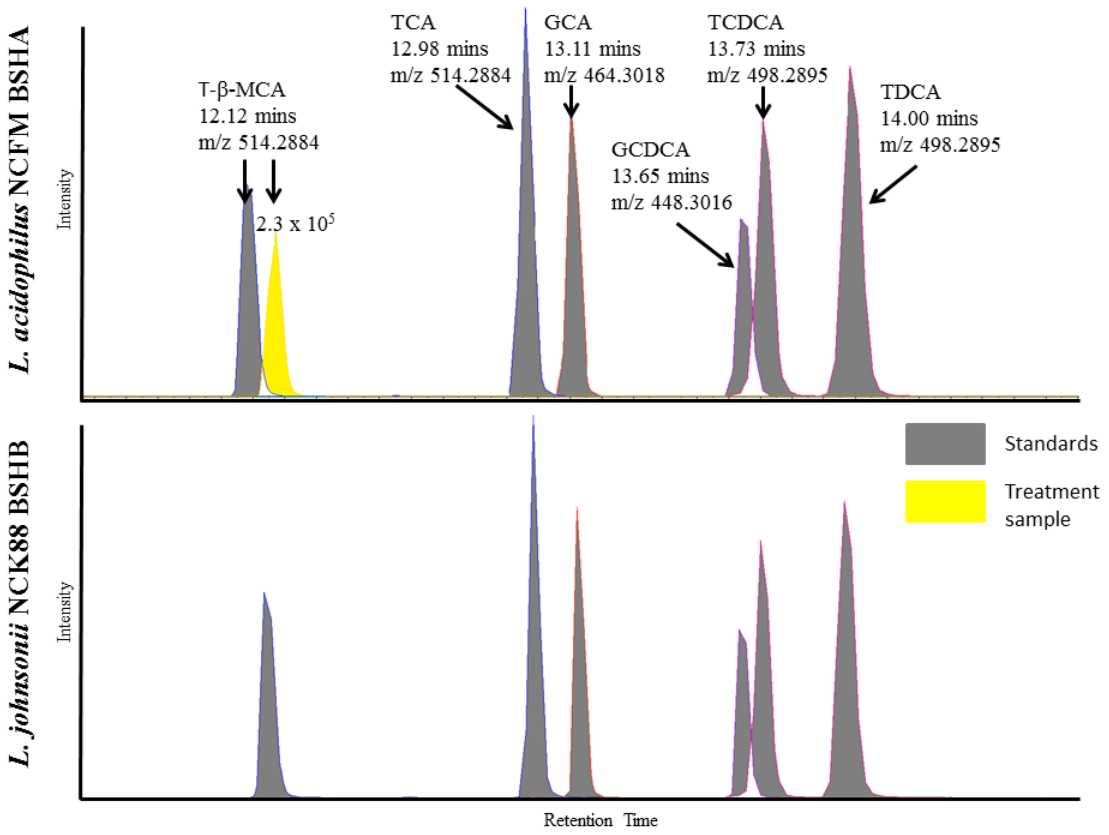

Figure S4.2: Chromatographic peak intensities of conjugated bile acids after incubation with cultures of transgenic *E. coli* C600 expressing BSHs from *L. acidophilus* NCFM and *L. johnsonii* NCK88. Results represent one of three experimental replicates with cultures standardized to an OD_{600} of 1.0 in LB and incubated aerobically at 37°C with 300 RPM shaking agitation with 500 nM of TCA, TDCA, TCDCA, T-β-MCA, GCA, and GDCA for 90 mins. Peaks from authentic standards are colored in grey and labeled with identification, retention time, and m/z and are identical in each chromatograph. Peaks from experimental samples are colored in yellow and labeled with total peak area.

Table S4.1: qPCR primer sequences

Target/Name	Sequence (5' to 3')
FABP6 FWD (IBABP)	CTTCCAGGAGACGTGATTGAAA
FABP6 REV (IBABP)	AACTTGTTGCTCATAATGTTGCC
CYP7A1 FWD	AACAACCTGCCAGTACTAGATAGC
CYP7A1 REV	GTGTAGAGTGAAGTCCTCCTTAGC
BSEP FWD	TCTGACTCAGTGATTCTTCGCA
BSEP REV	GTGTAGAGTGAAGTCCTCCTTAGC
GAPDH FWD	CCTCGTCCCGTAGACAAAATG
GAPDH REV	TGAAGGGGTCGTTGATGGC
OSTA FWD	TACAAGAACACCCTTTGCCC
OSTA REV	CGAGGAATCCAGAGACCAAA
OSTB FWD	GTATTTTCGTGCAGAAGATGCG
OSTB REV	TTTCTGTTTGCCAGGATGCTC
18S FWD	ATTGGAGCTGGAATTACCGC
18S REV	CGGCTACCACATCCAAGGAA
FGF15 FWD	GCCATCAAGGACGTCAGCA
FGF15 REV	CTTCCTCCGAGTAGCGAATCAG
SHP FWD	TCTGCAGGTCGTCCGACTATTC
SHP REV	AGGCAGTGGCTGTGAGATGC
FXR FWD	TGGGCTCCGAATCCTCTTAGA
FXR REV	TGGTCCTCAAATAAGATCCTTGG

Chapter 5
Optimization of Isotopic Radio Outlier Analysis™ for characterizing metabolic responses to bile acids in *Lactobacillus*

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5.1 Introduction

Commercially available *Lactobacillus* probiotics have been selected for their ability to withstand the harsh and competitive environment of the gastrointestinal tract (34). Obstacles to colonization include the acidic conditions of the stomach, competition for mucosal attachment, and bile. *Lactobacillus* have evolved a host of physiological adaptations to these stressors, and the bile response in particular is thought to be multifaceted. In addition to facilitating the permeabilization and dissolution of the cell membrane, bile causes intracellular acidification, disruption of protein folding, and DNA damage (75, 79, 238, 239). Consequently, a number of transcriptomics and proteomics studies of *Lactobacillus* exposure to bile have characterized a global response involving the production of stress response proteins, cell surface proteins and exopolysaccharides, altered carbohydrate, amino acid, and nucleotide metabolism, and changes in cell membrane composition (73, 74, 79, 81, 82, 86). BSHs have also been implicated as part of the bile response, although their exact physiological function remains unclear (82, 86).

Ultimately the bile response of a given strain contributes to its bile tolerance, which has been shown to vary widely even with species of *Lactobacillus* (48, 82, 166, 240). An improved understanding of the factors affecting strain to strain variability in bile tolerance is essential to identifying effective probiotics, especially considering the newly appreciated importance of bacterial interactions with bile for controlling host metabolism (2–4).

Gene and protein expression profiling are powerful tools for characterizing cellular response, but despite mapping changes in gene and protein expression to metabolic pathways for carbohydrate, amino acid, and nucleotide metabolism, these screening methods were not designed to study changes in important chemical intermediates of these pathways such as fatty acids and amino acids. Metabolomics offers a powerful complement to these methods, and has revealed new insights into bacterial physiology. For example, metabolomics uncovered a new role for the autoinducer-2 synthase (LuxS) in *L. reuteri*, and it helped researchers understand the response of *L. plantarum* WCFS1 to folate production (241, 242). Metabolomics studies are often limited by bottlenecks associated with analytical variability, ion suppression, and metabolite identification, but recently a new platform for metabolomics was introduced to address these challenges. Isotopic Radio Outlier Analysis™ (IROA) utilizes complete metabolic labelling of the metabolite pool with ^{13}C to create distinct chemical signatures in each molecule and facilitate identification. By utilizing an automated software program, ClusterFinder™, to identify the labeled metabolite library, IROA eliminates the large amounts of background noise typical for liquid chromatography mass spectrometry (LC-MS) data and reduces the need for chemical standards (243).

The aim of this work was to use the IROA metabolomics platform to characterize the metabolic response of four *Lactobacillus* strains to bile acid stress. Two strains of *L. johnsonii,* LB1 and NCK88, were included to assess strain specific differences within a species, and the well characterized probiotics *L. acidophilus* NCFM and *L. plantarum* WCFS1 were included to compare our results to those from published transcriptomics and proteomics studies. IROA has

not yet been utilized in a bacterial profiling experiment, so a secondary objective of this work was to explore its potential for further development in this area and identify its limitations.

5.2 Results and Discussion

5.2.1 Optimization of IROA media and experimental conditions

The IROA approach to metabolomics profiling depends on incorporation of ^{13}C into the metabolome of the cells under study, which is achieved by growing the cells in media containing ^{13}C labeled glucose. It is important that no other unlabeled carbon sources be available for growth, as they will dilute the labeled carbon pool and interfere with the analysis. Therefore, it is not possible to simply utilize complex media spiked with ^{13}C labeled glucose. IROA technologies has developed a series of pre-made labeled medias which aim to replicate the most common laboratory medias, but none of these medias closely resembles MRS media which is typically used for growing *Lactobacillus* (213). *Lactobacillus* has undergone metabolic simplification throughout its evolution as it has adapted to nutritionally rich environments which makes developing defined medias somewhat challenging (21). As such, the first objective of this study was to develop a suitable media for conducting an IROA based metabolomics experiment.

First, we screened *L. johnsonii* LB1, *L. johnsonii* NCK88, *L. acidophilus* NCFM, and *L. plantarum* WCFS1 for growth in IROA's mammalian cell culture medium supplemented with standard RPMI vitamins, which contains a complete complement of amino acids. OD_{600} measurements of cell density increased only negligibly after 48 hours of growth and were not indicative of the five cell divisions necessary for ^{13}C labelling had occurred in any of the cultures (Table 5.1). Further analysis of the IROA mammalian cell culture media composition revealed significantly lower concentrations of amino acids and glucose in the IROA media compared to a chemically defined media for growth of *L. johnsonii* (244). Additionally, inosine was found to be a critical component of the chemically defined media, and completely lacking in the IROA media (244). Adjustments were made, and a 2X concentrate of the IROA media with added glucose was

determined to support robust growth of *L. plantarum* WCFS1, although the modifications had little effect on the growth of the other strains (Table 5.1). These results demonstrated that *L. plantarum* WCFS1 could serve as a reference strain for a phenotypic IROA experiment if grown in a modified IROA mammalian cell culture media.

Table 5.1: Growth of *Lactobacillus* strains in various modifications of IROA mammalian cell culture media

		OD_{600}			
Media Composition	Strain	0 hrs	24 hrs	48 hrs	48 hr Difference
1X IROA	*L. johnsonii* LB1	0.100	0.230	0.255	0.155
	L. johnsonii NCK88	0.110	0.172	0.193	0.083
	L. acidophilus NCFM	0.120	0.179	0.164	0.044
	L. plantarum WCSF1	0.147	0.388	0.380	0.233
2X IROA	*L. johnsonii* LB1	0.086	0.231	0.160	0.074
	L. johnsonii NCK88	0.113	0.203	0.072	-0.041
	L. acidophilus NCFM	0.134	0.229	0.230	0.096
	L. plantarum WCSF1	0.151	0.501	0.467	0.316
2X IROA + 20g/L glucose	*L. johnsonii* LB1	0.088	0.186	0.197	0.109
	L. johnsonii NCK88	0.104	0.176	0.186	0.082
	L. acidophilus NCFM	0.125	0.144	0.131	0.006
	L. plantarum WCSF1	0.140	0.429	1.519	1.379
2X IROA + 20g/L glucose + 10g/L inosine	*L. johnsonii* LB1	0.079	0.172	0.182	0.103
	L. johnsonii NCK88	0.105	0.169	0.246	0.141
	L. acidophilus NCFM	0.121	0.165	0.149	0.028
	L. plantarum WCSF1	0.131	1.539	1.690	1.559

L. plantarum WCFS1 is characterized by a large, metabolically robust genome compared to the other strains of *Lactobacillus* we screened, so it was not surprising that it exhibited the strongest growth in the nutrient limiting modified IROA mammalian cell culture media (22). Initially, we hoped to achieve growth of all of the *Lactobacillus* strains in an IROA media in order to perform a traditional IROA experiment. However, none of the other strains showed even modest changes in growth after altering amino acid, glucose, and inosine concentrations as well as increasing the buffering capacity of the IROA mammalian media. This result indicated that developing a universal defined media for these strains would likely prove to be challenging, time consuming, and potentially even unfeasible with labeled ^{13}C components. Therefore, we elected

to adopt a phenotypic approach to IROA profiling based on labeled metabolites from *L. plantarum* WCFS1 (Figure 5.1). This approach was advantageous because it allowed for study of all of the strains in MRS media, but its potential for discovery was limited by the range of metabolites identified in *L. plantarum* WCFS1, as any metabolites unique to the other strains would not be identified.

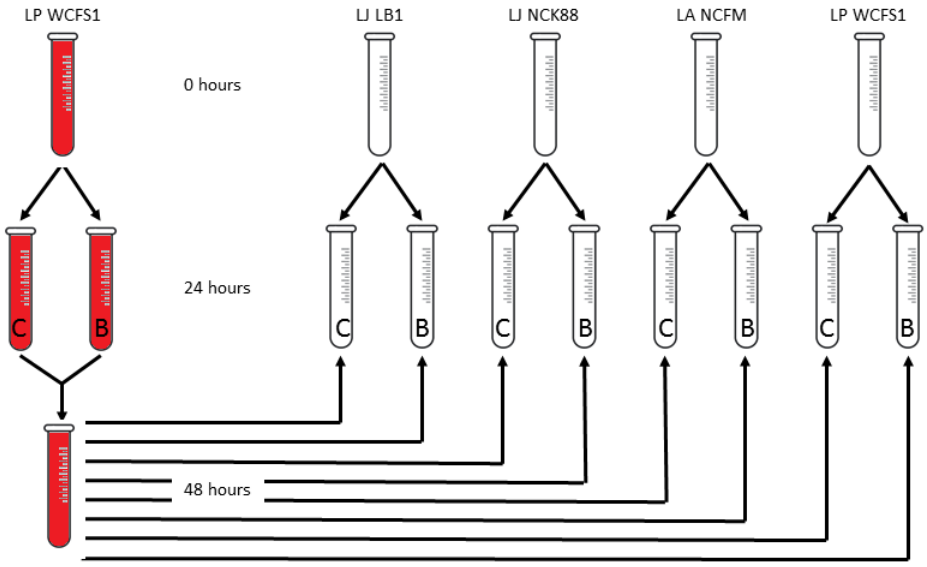

Figure 5.1: Schematic of IROA Phenotypic experimental design. Red tubes represent cultures of *L. plantarum* WCFS1 (LP WCFS1) incubated anaerobically in ^{13}C labeled modified IROA mammalian cell culture media, and white tubes represent cultures of LP WCFS1, *L. johnsonii* LB1 (LJ LB1), *L. johnsonii* NCK88 (LJ NCK88), and *L. acidophilus* NCFM (LA NCFM) incubated anaerobically in MRS. Tubes labeled with "C" represent controls, and tubes labeled with "B" represent samples treated with a bile acid mixture consisting of 10 µM TCA, TDCA, TCDCA, GCA, and GCDCA. The combined 48 hour LP WCFS1 control and treatment culture in ^{13}C labeled modified IROA media was extracted and used as an extraction media for each of the other cultures incubated in MRS.

The primary aim of IROA analysis was to study the global metabolic response of *Lactobacillus* strains to bile acid exposure. Notably, the exact concentration of bile acids in the intestines is difficult to quantify due to changes in bile acid composition and dilution effects as the bile acids interact with food, the gut microbiota, and active and passive transport mechanisms throughout their circulation (3, 155, 156). Generally though, predominant bile acid species are

present in micromolar amounts, and so an experiment was conducted to measure the effect of a mixture containing TCA, TDCA, TCDCA, GCA, and GCDCA on the growth of *L. plantarum* WCFS1 in the modified IROA mammalian cell culture media. Growth was not significantly inhibited compared to cells grown in the absence of bile acids when the bile acid mixture was added at a concentration of 1μM, and growth was progressively inhibited in a concentration dependent manner at higher concentrations (Table 2). Treatment with 10 μM of the bile acid mixture introduced an obvious stress to the cells without completely inhibiting growth, and therefore was considered the optimal condition for profiling experiments.

Table 5.2: Effect of bile acids on growth of *L. plantarum* WCFS1 in modified IROA mammalian cell culture media

Media Composition	0 hrs	24 hrs	48 hrs
modified IROA	0.116	1.080	2.51
modified IROA + 1 μM bile acids	--	1.080	2.46
modified IROA + 10 μM bile acids	--	1.080	2.15
modified IROA + 100 μM bile acids	--	1.080	1.36

*The bile acid mixture consisted of equimolar concentrations of TCA, TDCA, TCDCA, GCA, GCDCA and was added 24 hours after initial inoculation of the culture.

5.2.2 Comparison of the global metabolic response to bile acid treatment in four *Lactobacillus* strains

A total library of 132 compounds was identified in the experimental samples based on comparison to corresponding ^{13}C labeled compounds in the pooled reference sample. The compounds were clustered based on their relative intensities in each sample under each treatment condition, and a correlation plot of the 100 most significant compounds revealed differences in the global metabolic response of each strain to the bile acid treatment (Figure 5.2A-D). *L. acidophilus* NCFM exhibited the clearest response to treatment of the four strains, and noticeable changes in the correlation plot are also apparent for *L. johnsonii* NCK88 (Figure 5.2A, C).
In order to more directly consider the effect of bile acid treatment on each strain, the relative intensities of the compounds identified were compared among control and treated samples. Data was available from only three replicates for each strain under control and bile acid

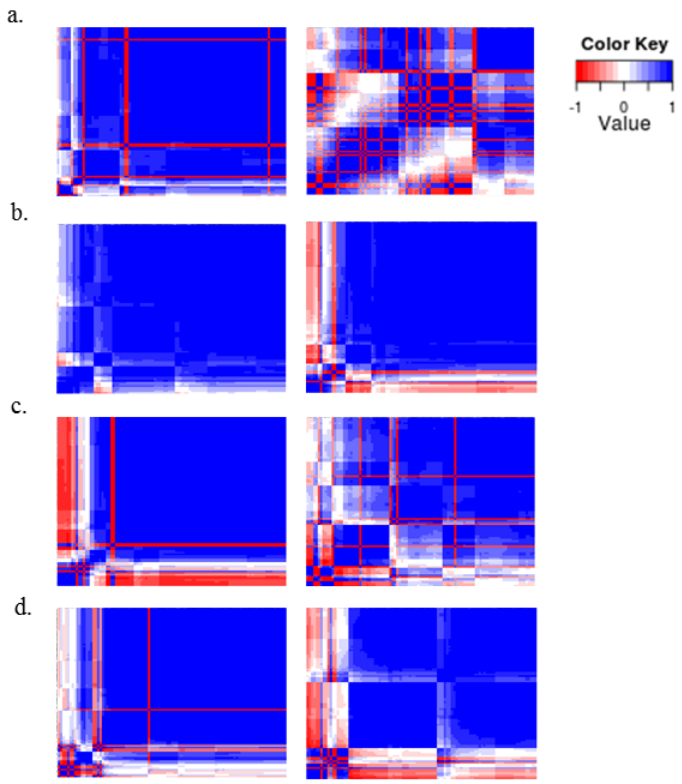

Figure 5.2: Correlation plots of the 100 most affected compounds in control (Left) and bile acid treated (right) cultures of *L. acidophilus* NCFM (a), *L. johnsonii* LB1 (b), *L. johnsonii* NCK88 (c), and *L. plantarum* WCFS1 (d).

treated conditions, which limited the power of the statistical comparison. Therefore, a p-value of 0.20 was used as a cutoff to compensate for the reduced statistical power and identify metabolites that may have been affected by the treatment in each strain. In total, 34 compounds were identified in *L. johnsonii* LB1, 32 in *L. acidophilus* NCFM, 9 in *L. johnsonii* NCK88, and 4 in *L. plantarum* WCFS1 (Table S5.1). Strikingly, the metabolites associated with bile acid treatment were almost entirely unique to each strain, and no metabolites were identified as part of a common response in all four strains (Figure 5.3). One compound, D-2-hydroxyisocaproate, was common to all but *L. plantarum* WCFS1. Three compounds, 5-hydroxypentanoate, N-acetyl-L-aspartate, and allantoate were common to *L. johnsonii* LB1 and *L. johnsonii* NCK88, two compounds, ethionamide, and mirasan, were common to *L. johnsonii* LB1 and *L. acidophilus* NCFM, and another two compounds, L-phenylalanine, and an unknown, were common to *L.*

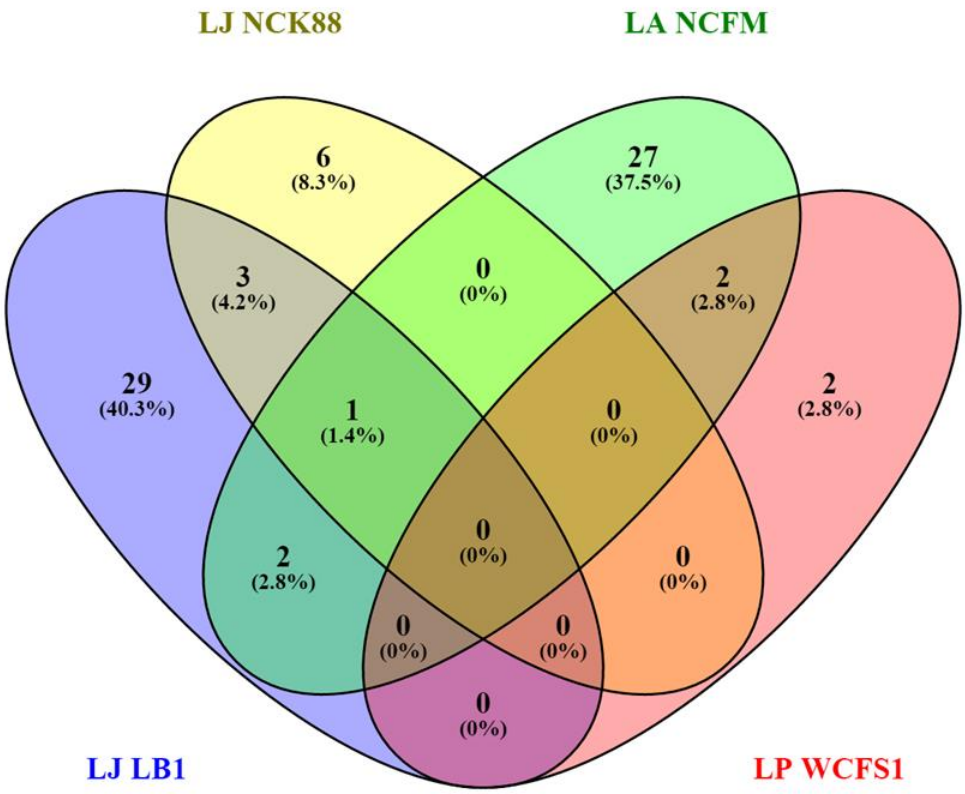

Figure 5.3: Venn diagram depicting overlap of compounds affected by bile acid treatment in *L. johnsonii* LB1, *L. johnsonii* NCK88, *L. acidophilus* NCFM, and *L. planatrum* WCFS1.

acidophilus NCFM and *L. plantarum* WCFS1. It is tempting to suggest that these results reflect poorly conserved, strain specific metabolic responses, but it is also possible that the differences we observed in metabolic response simply reflect different bile acid tolerances among the strains. While *L. plantarum* WCFS1 exhibited an expected growth inhibition in modified IROA mammalian cell culture media in the presence of bile acids, none of the strains were similarly inhibited in MRS (Table 5.3). Therefore, it would be interesting to repeat the experiment with progressively higher concentrations of added bile acids in order to understand how bile acid tolerances differ among the strains.

Unfortunately, many of the compounds implicated as part of the bile acid response in each strain could not be identified. It is likely that some of these are modified species of more well characterized compounds as well as fragments of larger molecules. For instance, such

Table 5.3: Growth of *Lactobacillus* cultures under control and treatment conditions

Strain	Growth Condition	OD_{600} 24 hours	OD_{600} 48 hours
L. plantarum WCFS1	modified IROA	2.260	2.550
	modified IROA + bile acids	--	2.229
L. plantarum WCFS1	MRS	6.147	6.640
	MRS + bile acids	--	6.760
L. acidophilus NCFM	MRS	5.240	5.400
	MRS + bile acids	--	5.450
L. johnsonii LB1	MRS	6.390	6.870
	MRS + bile acids	--	6.820
L. johnsonii NCK88	MRS	6.860	5.870
	MRS + bile acids	--	6.010

*OD_{600} values are taken from a pooled sample of three biological replicates for each growth condition

products are common with amino acids and peptides. However, it is also possible that the large percentage of unknown compounds reflects the relative lack of microbial standards used for metabolomics.

5.2.3 Identification of metabolic pathways affected by bile acid treatment

Next, the broader metabolic response to bile acid treatment was explored by considering the pathways impacted by the identified metabolites in each *Lactobacillus* strain. A MetaboAnalyst pathway analysis identified nine significantly affected pathways in *L. acidophilus* NCFM and two in *L. plantarum* WCFS1 based on KEGG reference libraries for *Bacillus subtilis* subsp. *subtilis* 168 (Table 5.4). No pathways were identified in *L. johnsonii* NCK88 or *L. johnsonii* LB1. Five of the pathways identified in *L. acidophilus* NCFM and both of the pathways identified in *L. plantarum* WCFS1 were related to amino acid metabolism and biosynthesis, and a closer look at several amino acids identified in *L. acidophilus* NCFM revealed noticeable reductions in the intensities of eight amino acids in the bile acid treated samples (Figure 5.4). Catabolism of amino acids in LAB differs from that of *B. subtilis*, particularly since many of these pathways intersect with the citric acid cycle (TCA) and LAB do not possess a complete TCA (245). Nevertheless, *B. subtilis* likely offers the best reference available for characterization

Table 5.4: Metabolic pathways impacted by bile acid treatment

Strain	Pathway	Hits / Total pathway compounds	p-value
L. plantarum WCFS1	Phenylalanine metabolism	1/3	0.006445
	Phenylalanine, tyrosine and tryptophan biosynthesis	1/22	0.046777
L. acidophilus NCFM	Aminoacyl-tRNA biosynthesis	7/66	4.55E-05
	Alanine, aspartate and glutamate metabolism	3/20	0.003991
	Phenylalanine, tyrosine and tryptophan biosynthesis	3/22	0.005278
	Valine, leucine and isoleucine biosynthesis	3/26	0.008541
	Tyrosine metabolism	2/11	0.013952
	Nitrogen metabolism	2/14	0.022398
	Citrate cycle (TCA cycle)	2/20	0.044033
	Pantothenate and CoA biosynthesis	2/21	0.048184
	Phenylalanine metabolism	1/3	0.050784
L. johnsonii LB1	none identified		
L. johnsonii NCK88	none identified		

*Based on MetaboAnalyst 3.0 Pathway Analysis of KEGG pathways in *Bacillus subtilis* subsp. *subtilis* 168

of these pathways in *Lactobacillus*, and pathway analysis identified a potential impact of bile acid treatment on the TCA cycle in *L. acidophilus* (Table 5.2).

Amino acid utilization is known to relate to physiological roles within LAB ranging from intracellular pH control, generation of metabolic energy, and resistance to stress (245). Exposure to bile acids has been shown to inhibit growth of *L. salivarius* by reducing internal pH levels and increasing membrane permeability, suggesting that exposure to bile acids is likely to induce a general acid stress response (68). Indeed, altered protein synthesis and amino acid metabolism has been observed in strains of *L. acidophilus* and *L. plantarum* specifically as part of an acid adaptation response (246, 247). Changes in amino acid metabolism have also been implicated in response to acid, salt, and ethanol stress in other species of *Lactobacillus* (248–251). Alternatively, it is possible that the relative reductions in amino acid intensities we observed arise from protein

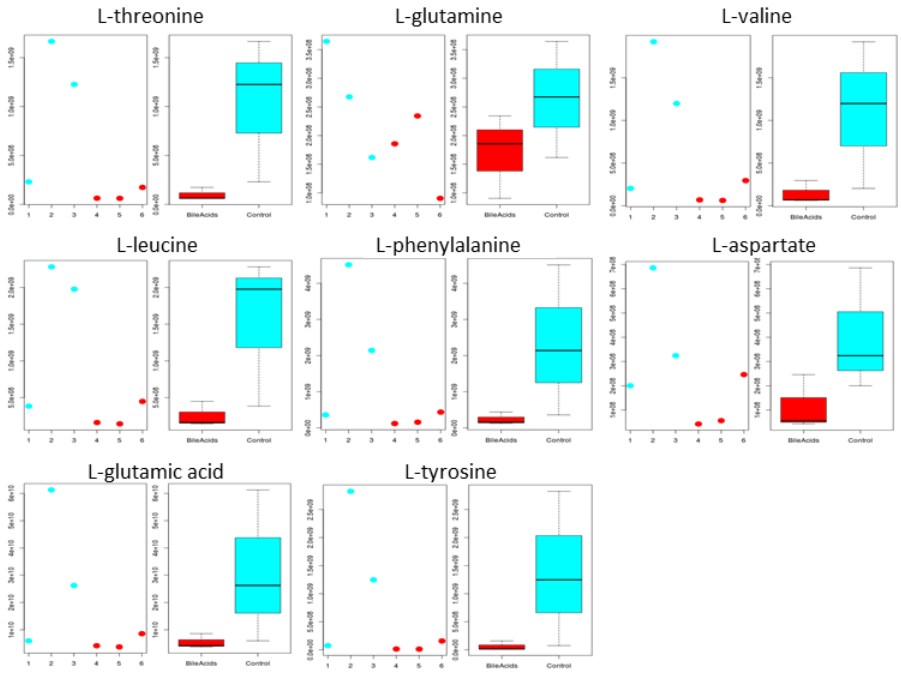

Figure 5.4: Quantification of amino acids significantly affected by bile acid treatment in *L. acidophilus* NCFM. Control samples are in light blue, and bile acid treated samples are in red. Graphs indicate individual sample peak areas with a single dot (left panel), the mean of the three replicates with a line (right panel), the standard deviation from the mean with the shaded box (right panel), and the range with the extended error bars (right panel).

denaturation and degradation as a consequence of altered intracellular pH levels, as has been reported in *L. reuteri* (75). Curiously, a number of global profiling studies of various *Lactobacillus* species exposed to bile stress have also identified altered amino acid metabolism as part of a much broader physiological response involving production of chaperone proteins, altered carbohydrate and nucleotide metabolism, and increased cell wall biosynthesis (73, 74, 79, 81, 82, 86). None of these additional pathways were identified in our study, which may be attributed in part to a relatively low stress induction in the cultures. It is also possible that the physiological response to bile acids alone is more limited than the response to bile, which includes additional cytotoxic components.

5.2.4 Conclusions and design considerations for future IROA experiments

Overall, the IROA approach effectively identified a core library of metabolites and yielded some interesting insights into the metabolic response of *Lactobacillus* to bile acid exposure. Our results suggest that amino acid metabolism is altered in *L. acidophilus* NCFM and to a lesser extent *L. plantarum* WCFS1, and does not appear to be part of a broader physiological response in these strains under the conditions tested. Conclusive evidence for this observation is lacking though, as our data is not statistically robust. Our results were inherently limited by the number of labeled compounds in the *L. plantarum* samples as well as the general lack of reference data for microbial metabolites. However, consideration of several experimental design factors in future experiments will help to reinforce this data and potentially even lead to new discoveries.

The most obvious factor contributing to the limited statistical power in our data is the number of replicates for each treatment group. For instance, wide variability in the standard deviations for the relative intensities of amino acids such as L-phenylalanine and L-aspartate is apparent in Figure 4, and appears to be mostly attributed to control sample 1. All of the samples were normalized to the same optical density prior to extraction, but in hindsight normalization to total protein might have helped to reduce sample to sample variability. The number of replicates was practically limited by the amount of labeled *L. plantarum* WCFS1 cells, but clearly an increase in the number of replicates would also reduce this variability as well as allow for outlier analysis. It also might be possible to supplement the current dataset by conducting the experiment without the labeled reference cells, since the data obtained here informed some targets for traditional analysis (e.g. amino acids).

Another outstanding question arising from the data is whether the limited responses observed in *L. johnsonii* LB1 and *L. johnsonii* NCK88 reflect increased bile acid tolerances in these strains. Likewise, it is possible that the limited pathway response identified in *L. acidophilus* NCFM and *L. plantarum* WCFS1 simply reflects a low level of bile acid stress.

Future experiments would benefit greatly from including an increasing range of bile acids as treatment groups in order to identify the progression of the physiological response in these strains and better uncover differences among them. Moreover, comparison of the data to similar data from the literature suggests that the response to bile acids may simply reflect a generalized response to intracellular pH changes. Therefore, it would be informative to include control treatments with reduced pH media. For instance, the pH of the media might be adjusted with hydrochloric acid to induce the traditional acid response in the absence of bile acids. This would allow bile acid specific effects to be separated from the general acid response. Similarly, the inclusion of a control group treated with dehydrated bile would separate the effects of bile acids from other bile constituents. In fact, if BSH activity were specifically quantified in each sample, it might help to compare the role BSHs play in bile acid versus complete bile detoxification. By extension, it would be informative to include treatment groups with different bile acid compositions, deliberately including bile acids which are not deconjugated by a given strain in order to observe how BSH activity affects metabolism. Finally, generation of BSH knockouts for use as controls would be the most effective way to study the role of these enzymes in the bile acid response, and was originally intended as part of this work. BSH knockouts have been successfully constructed in *L. plantarum* WCFS1 and *L. acidophilus* NCFM, and other gene manipulation has been achieved in *L. johnsonii* NCK88 (146, 152, 206). However, preliminary attempts at transforming plasmid DNA into *L. johnsonii* LB1 were not successful (data not shown).

Lastly, increased sensitivity might be gained by optimizing the sample preparation protocol. Several groups have demonstrated differences in compound identification related to quenching and extraction methods. Moreover, each group identified a different set of optimal conditions based on either cold methanol, which was used here, or cold glycerol saline (252–254). These methods were developed for different *Lactobacillus* species and LC-MS methods, indicating that optimization of the quenching and extraction protocol should be conducted

specifically for a given experimental design and workflow. It is certainly possible that optimization of the protocols used here would reduce variability and improve compound identification.

5.3 Materials and Methods

5.3.1 Bacterial strains and growth conditions

L. johnsonii NCK88, *L. acidophilus* NCFM and *L. plantarum* WCFS1 were generously provided by Dr. Todd Klaenhammer and *L. johnsonii* LB1 was isolated from a health mouse cecum as described previously (Chapter 3). For all experiments, cultures were inoculated in sterile MRS broth from frozen glycerol stocks and incubated anaerobically in an atmosphere composed of 85% N, 10% CO_2, and 5% H_2 at 37°C for two consecutive passages and washed in phosphate buffered saline (PBS), pH 7.4, prior to inoculation in either modified IROA mammalian cell culture media or MRS. Modified IROA mammalian media was prepared by dissolving IROA mammalian cell culture media (IROA technologies) in sterile deionized dH_2O, adding appropriate amounts of RPMI 1640 Vitamins Solution (100x) (Sigma-Aldrich), glucose, and/or inosine, and Earle's Balanced Salt Solution (EBSS) (Sigma-Aldrich) and filtering through a 0.22 μm membrane (VWR). The modified IROA media used for the metabolic profiling experiment consisted of a 2x concentrate of IROA mammalian cell culture media with 20 g/L of glucose and 2x EBSS.

5.3.2 IROA experimental design and sample preparation

The metabolomics profiling experiment followed the phenotypic IROA workflow (Figure 1) (19). A culture of *L. plantarum* WCFS1 was inoculated in 95% ^{13}C labeled modified IROA media and incubated anaerobically for 24 hours, the OD_{600} was measured, and the initial culture was split into two separate cultures. A concentrated 5 mM solution of TCA, TDCA, TCDCA, GCA, and GCDCA was added to the treatment sample to a final concentration of 10 μM, and

nothing was added to the control. Each culture was incubated anearobically for an additional 24 hours, the control sample was diluted to match the OD_{600} of the treatment sample, and an equal volume of the control and treatment cultures was combined. The combined culture was centrifuged at 5,000 x g for 5 mins at 4°C to precipitate the cells, and the cells were washed twice with PBS. The pellet was resuspended and extracted twice in ice cold 100% methanol by lysing with 0.1mm glass beads (MoBio, Inc.) in a tissue homogenizer at 4,000 RPM for two 20 s cycles. The ^{13}C labeled *L. plantarum* WCFS1 cell extract was used a an extraction solvent to harvest *L. plantarum* WCFS1, *L. acidophilus* NCFM, *L. johnsonii* LB1, and *L. johnsonii* NCK88 control and bile acid treated cells incubated anaerobically in MRS in parallel to the reference strain. Cell extracts were evaporated to dryness under vacuum at room temperature and resuspended in 100 µL of 3% methanol with sonication. Finally, the samples were centrifuged at 10,000 x g for 1 min and the supernatants were transferred to low volume plastic autosampler vials (VWR) and stored at -20°C until Orbitrap LC-MS analysis.

5.3.3 Orbitrap LC-MS analysis

Samples were analyzed by LC-MS using a modified version of an ion pairing reversed phase negative ion electrospray ionization method (255). Samples were separated on a Supelco (Bellefonte, PA) Titan C18 column (100 x 2.1 mm 1.9 µm particle size) using a water-methanol gradient with 10 mM tributylamine and 15 mM acetic acid added to the aqueous mobile phase. The LC-MS platform consisted of a Dionex Ultimate 3000 quaternary HPLC pump, a Dionex 3000 column compartment, a Dionex 3000 autosampler, and an Exactive plus orbitrap mass spectrometer controlled by Xcalibur 2.2 software (all from ThermoFisher Scientific, San Jose, CA). The HPLC column was maintained at 30 ºC and a flow rate of 200 uL/min. Solvent A was 3% aqueous methanol with 10 mM tributylamine and 15 mM acetic acid; solvent B was methanol. The gradient was 0 min., 0% B; 5 min., 20% B; 7.5 min., 20% B; 13 min., 55% B; 15.5 min., 95% B, 18.5 min., 95% B; 19 min., 0% B; 25 min 0% B. The Exactive plus was operated in negative ion mode at maximum resolution (140,000) and scanned from m/z 85 to m/z 1000.

5.3.4 Data analysis and statistics

Thermo Raw data files were centroided and converted to mzXML data files with MSConvertGUI (ProteoWizard). Compounds were identified with ClusterFinder™ software (IROA technologies) based on automated recognition of ^{13}C isotopic profiles with a minimum peaks intensity of 2,000,000 and ms noise level of 10,000 and manually curated prior to creation of the final library. Peak intensity data was statistically compared by multivariate clustering and correlation analysis with the IROA Portal (IROA technologies). Peak intensities were also compared directly by multiple student's t-test corrected for multiple comparisons by the Holm-Sidak method ($\alpha = 0.05$) with Graphpad Prism 6 (Graphpad Software, Inc.). Visualizations of unique matabolites associated with each strain were made with Venny 2.1 (Oliveros, J.C. 2007-2015. http://bioinfogp.cnb.csic.es/tools/venny/), and MetaboAnalyst 3.0 (256)was used for pathway analysis.

Table S5.1: List of metabolites affected by bile acid treatment in each *Lactobacillus* strain

***L. johnsonii* LB1**		***L. acidophilus* NCFM**	
Compounds	**p-values**	**Compounds**	**p-values**
ETHIONAMIDE	0.0100	GLYCEROL-3-PHOSPHATE	0.0509
5-HYDROXYPENTANOATE	0.0277	ETHIONAMIDE	0.0683
HOMOVANILLATE	0.0331	SUCROSE	0.0822
UNK MZ 270.1716 RT 19.10	0.0370	SUCCINATE	0.0822
N-ACETYL-L-ASPARTATE	0.0501	L-THREONINE	0.0918
ALLANTOATE	0.0511	L-LEUCINE	0.0954
Possibly C15H30N9S	0.0602	PHOSPHOENOLPYRUVATE	0.1094
UNK MZ 370.2605 RT 18.85	0.0632	(S)-MALATE	0.1166
UNK MZ 158.0824 RT 14.27	0.0707	UNK MZ 228.1605 RT 18.22	0.1213
UNK MZ 128.0112 RT 7.67	0.0766	4-OXOPROLINE	0.1247
Possibly C7H16N5O2S	0.1054	L-VALINE	0.1278
UNK MZ 229.1556 RT 17.94	0.1064	UNK MZ 370.2603 RT 18.85	0.1300
UNK MZ 214.1815 RT 19.58	0.1109	UNK MZ 322.2027 RT 18.56	0.1318
MIRASAN	0.1132	UNK MZ 306.1503 RT 1.29	0.1336
Possibly C15H30N8O3	0.1219	3-PHOSPHO-D-GLYCERATE	0.1347
UNK MZ 230.1759 RT 17.52	0.1285	UNK MZ 258.1710 RT 18.04	0.1348
UNK MZ 272.1870 RT 17.88	0.1321	Possibly C8H18N5S	0.1361
UNK MZ 158.1187 RT 16.91	0.1335	L-ASPARTATE	0.1453
GLYCERONE	0.1341	UNK MZ 226.1451 RT 18.57	0.1473
UNK MZ 340.2499 RT 19.19	0.1386	UNK MZ 272.1869 RT 18.28	0.1496
Possibly C17H31NS2	0.1408	UNK MZ 290.1977 RT 18.49	0.1499
UNK MZ 227.1290 RT 18.06	0.1416	L-PHENYLALANINE	0.1571
UNK MZ 274.2026 RT 18.02	0.1434	UNK MZ 175.0975 RT 15.25	0.1588
UNK MZ 359.2558 RT 18.72	0.1480	UNK MZ 288.1819 RT 18.51	0.1665
UNK MZ 258.1711 RT 18.03	0.1530	D-2-HYDROXYISOCAPROATE	0.1686
UNK MZ 275.1869 RT 18.57	0.1549	MIRASAN	0.1718
Possibly C18H23N3O	0.1555	L-TYROSINE	0.1734
UNK MZ 229.1443 RT 17.97	0.1559	UNK MZ 359.2556 RT 18.50	0.1744
UNK MZ 265.1925 RT 18.65	0.1731	UNK MZ 174.1135 RT 16.85	0.1812
Possibly C17H32P	0.1731	4-OXOPROLINE	0.1853
CARTEOLOL	0.1732	2-AMINO-3,7-DIDEOXY-D-THREO-HEPT-6-ULOSONIC ACID	0.1876
UNK MZ 270.2079 RT 18.69	0.1764	L-GLUTAMIC ACID	0.1889
Possibly C8H18N5O2	0.1867	UNK MZ 270.2078 RT 18.72	0.1891
Possibly C13H25N8O2	0.1990		
D-2-HYDROXYISOCAPROATE	0.1997		
***L. johnsonii* NCK88**		***L. plantarum* WCFS1**	
Compounds	**p-values**	**Compounds**	**p-values**
ALLANTOATE	0.0088	GAMMA-L-GLUTAMYL-D-ALANINE	0.1607
N-ACETYL-L-ASPARTATE	0.0135	Possibly C11H14N2PS	0.1809
5-HYDROXYPENTANOATE	0.0325	L-PHENYLALANINE	0.1920
Possibly C17H25N3	0.0352	UNK MZ 370.2603 RT 18.85	0.1974
D-2-HYDROXYISOCAPROATE	0.0899		
Possibly C15H32N2O6PS2	0.1720		
Possibly C14H25N8O4P2	0.1732		
Possibly C9H20N7O11	0.1740		
L-ALPHA-D-HEPP-(1->3)-L-ALPHA-D-HEPP	0.1740		
UNK MZ 270.1716 RT 19.11	0.1974		

*All compounds with a $p < 0.20$ are listed.

Chapter 6
Conclusions and Future Directions

At its core, this work identified two strains of *L. johnsonii* with BSH activity against T-β-MCA, a potent FXR antagonist, which offer potential as probiotics for controlling metabolism. Additionally, this research is the first to demonstrate that colonization with a BSH active strain of bacteria can alter intestinal bile acid composition and affect FXR signaling. These results ultimately suggest the possibility for one day using BSH expressing probiotics to control weight gain. However, several critical questions remain unanswered, and need to be addressed in future studies.

Phylogenetic relationships among BSHs do not reflect bile acid substrate specificity, making it difficult to identify probiotics with the capability to alter T-β-MCA concentrations (Chapter 3). A straightforward and effective *in vitro* screening assay was developed in Chapter 3 to address this limitation. Predictive modeling also proved effective at explaining BSH substrate specificity, but it is not a reliable alternative to experimental data and it does not consider the potential effects of genomic background on BSH expression and activity (Chapter 3). In fact, even our *in vitro* screening assay did not predict *in vivo* changes in bile acid composition in germ free mice colonized with *Lactobacillus* (Chapter 4). Therefore, additional work is needed to understand the factors limiting the efficacy of BSH expressing *Lactobacillus* strains *in vivo*. Differences in *bsh* gene expression, bile acid transport, and niche specific colonization should all be considered in addition to the potential for compensatory changes in bile acid synthesis and recycling in the host.

This research also demonstrated the importance of endogenous *Lactobacillus* and BSH activity in hosts harboring a gut microbiota, and suggests that controlling the background microbiota may present the most formidable obstacle to modifying host bile acid composition with *Lactobacillus* probiotics (Chapter 4). Future work should consider the factors contributing

to the assembly and total population of *Lactobacillus* in the gastrointestinal tract. The strain level composition of the *Lactobacillus* community has the potential to affect BSH activity and substrate specificity, and characterization of the diversity within this community may ultimately lead to the discovery of more effective BSH producing strains. Additionally, the relative contribution of the *Lactobacillus* community to the overall BSH activity of the gut microbiota, particularly with respect to T-β-MCA, is not known, and it may be that other members of the gut microbiota are simply better candidates for probiotics to control weight.

Importantly, this work generally considered weight changes as a direct measure of treatment effects on host metabolism. However, altered metabolism in a host does not necessarily affect body weight, and it is possible that BSH mediated changes to FXR are providing benefits to the host which were not characterized here. Additional consideration of changes in markers of glucose and lipid metabolism in the liver would help to address this possibility.

Finally, the IROA approach to understanding bile acid stress in *Lactobacillus* offers promise for understanding strain specific differences in the role of BSHs, but it needs more refinement (Chapter 5). Its greatest utility may ultimately be in comparing differences in BSH wild type and mutant strains of *Lactobacillus* to isolate the role of these enzymes in the bile stress response. The biological implications of differences in BSH substrate specificity are not clear, and it is possible that these differences factor into host specific colonization differences in *Lactobacillus* probiotics.

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Appendix

Mice with reduced populations of *Lactobacillus* in the Li *et al.* study were administered tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl), an antioxidant and radiation protectant drug. However, tempol was never shown to directly affect *Lactobacillus*. Prior to the work described in the body of this dissertation focusing on interactions between *Lactobacillus* and bile acids, an effort was made to directly characterize tempol's effect on *Lactobacillus*.

Lactobacillus populations were measured in the Li *et al.* study by high-throughput 16S ribosomal RNA (16S rRNA) gene sequencing, which is subject to experimental error due to its inability to distinguish live from dead cells. Therefore, two independent experiments were conducted to confirm tempol's effect on *Lactobacillus* populations by traditional plating. Adult male C57BL/6J mice were administered a single 250 $mgkg^{-1}$ dose of tempol or sterile PBS, pH 7.4, by intragastric gavage and euthanized seven days later for enumeration of cecal *Lactobacillus* populations as described in chapter 3. The mean population of *Lactobacillus* was 3.8×10^8 CFU/g cecal contents in the control group, and 9.6×10^7 CFU/g cecal contents in the tempol treated group (Figure A1). The difference was not statistically significant at an alpha of 0.05 ($p = 0.0651$) when compared by the non-parametric Mann Whitney test, but variability in the data suggests that a significant difference may manifest with increased statistical power.

In vivo characterization of cecal *Lactobacillus* populations cannot provide evidence for direct interactions between tempol and *Lactobacillus*, as it is possible that changes in *Lactobacillus* populations are the result of changes in the population of other microbial community members. Therefore, *L. johnsonii* LB1 was isolated from mouse cecal contents and used to directly characterize tempol's effect on growth. *L. johnsonii* LB1 was inoculated in MRS, MRS with 0.2% oxgall, and MRS with 0.4% oxgall with or without 100 mM tempol and incubated under anerobic conditions for 24 hours over the course of three independent experiments. Culture preparations and growth conditions were the same as those described in

chapter 3. Measurements of cell density by OD_{600} revealed significantly reduced growth in tempol treated cultures of *L. johnsonii* LB1 compared to controls in MRS after four and six hours, but no differences after eight or 24 hours (Figure A2). No differences were observed in tempol treated cultures with added bile compared to controls at any time points. These results suggest that tempol affects the lag time of a pure *L. johnsonii* LB1 culture *in vitro*, and offer preliminary evidence for future investigations into how tempol affects *Lactobacillus* populations in the mouse.

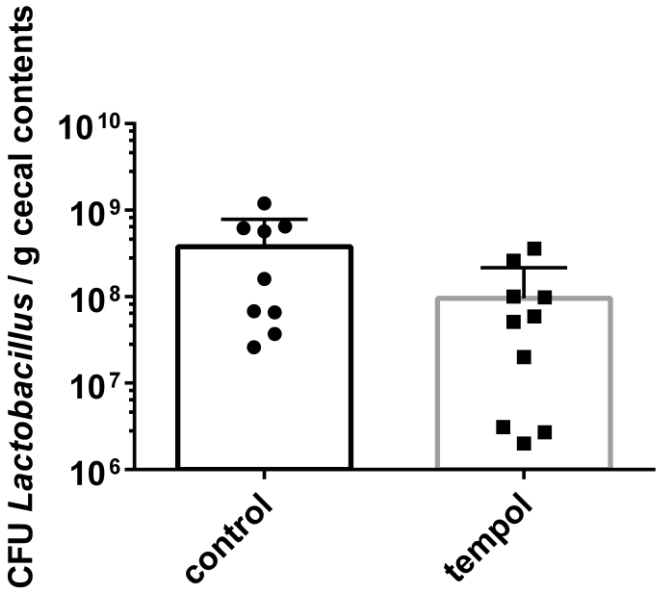

Figure A1: Populations of *Lactobacillus* in cecal contents of mice treated with control or tempol. Values represent means +/- standard deviations and were compared by Mann Whitney test ($p = 0.0651$).

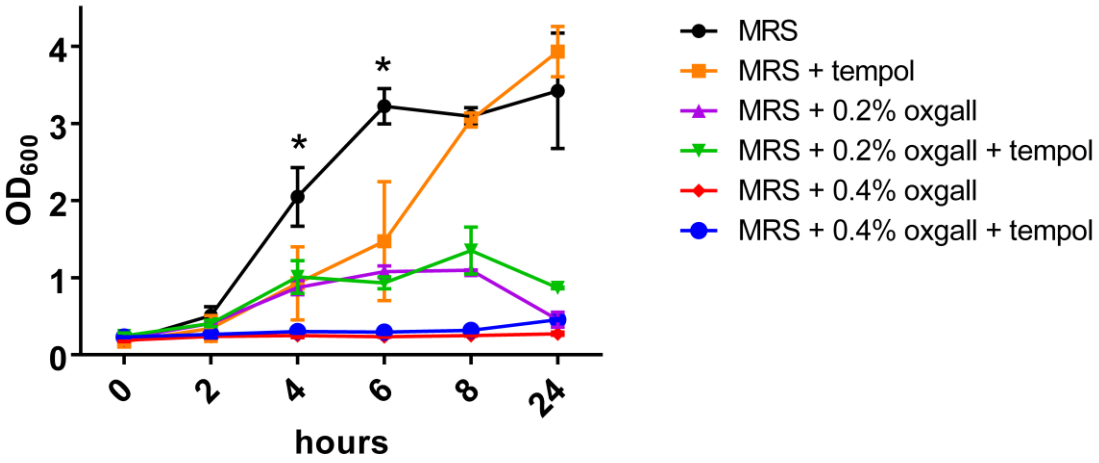

Figure A2: Growth of *L. johnsonii* LB1 in the presence of tempol. Values represent means +/- standard deviations from three independent experiments and were compared by multiple student's t-test corrected for multiple comparisons by the Holm-Sidak method ($\alpha = 0.05$) * $p < 0.05$.

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Featured Publications

DiMarzio M, Shariat N, Kariyawasam S, Barrangou R, Dudley EG. *Antibiotic resistance in Salmonella Typhimurium associates with CRISPR sequence type.* Antimicrobial Agents and Chemotherapy, 2013.

Shariat N, **DiMarzio MJ**, Yin S, Dettinger L, Sandt CH, Lute JR, Barrangou R, Dudley EG. *The combination of CRISPR-MVLST and PFGE provides increased discriminatory power for differentiating human clinical isolates of Salmonella enterica subsp. enterica serovar Enteritidis*. Food Microbiology, 2013.

Shariat N, Sandt CH, **DiMarzio MJ**, Barrangou R, Dudley EG. *CRISPR-MVLST subtyping of Salmonella enterica subsp. enterica serovars Typhimurium and Heidelberg and application in identifying outbreak isolates.* BMC Microbiology, 2013.

Mathes AL, Rice L, Affandi AJ, **DiMarzio M**, Rifkin IR, Stifano G, Christmann RB, Lafyatis R. *CpGB DNA activates dermal macrophages and specifically recruits inflammatory monocytes into the skin.* Expermental Dermatology, 2014.

DiMarzio M, Farina G, Lafyatis R. *Modeling innate immunity in murine skin: utilization of subcutaneous osmotic pumps for inflammatory and fibrotic skin disease.* Methods in Molecular Biology, 2012.

Farina GA, York MR, **DiMarzio MJ**, Collins CA, Meller S, Homey B, Rifkin IR, Marsahk-Rothstein A, Radstake TR, Lafyatis R. *Poly(I:C) drives type I IFN- and TGFβ-mediated inflammation and dermal fibrosis simulating altered gene expression in systemic sclerosis.* Journal of Investigative Dermatology, 2010.

Honors and Awards

USDA National Needs Fellowship
College of Agricultural Sciences Competitive Grant Award
Robert D. and Jeanne L. Mccarthy Memorial Graduate Teaching Award
William Rosskam II Memorial Scholarship in Food Science
Fred & Florence Jacobson Scholarship
White Star Kay Scholarship in Food Science